TWO HYPUSINE-CONTAINING PROTEINS IDENTIFIED BY METABOLIC LABELING IN CHICK EMBRYO FIBROBLASTS

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Incubation of various cultured mammalian cells under growth-stimulatory condition with [^{3}H]putrescine or [^{3}H]spermidine results in a labeling of an 18,000-dalton protein (Cell, **1982**, *29*, 791; BBA, **1983**, *756*, 395). The labeling is due to post-translational conversion of a lysine residue to hypusine residue, using the butylamino moiety derived from spermidine. In order to search for an abundant source for the purification of this protein, we have examined possible existence of this hypusine-containing 18,000-dalton protein (hyp-18k) in chick embryos by the metabolic labeling method. Metabolic labeling of chick embryo fibroblasts, prepared from the Day 11 embryos, by [^{3}H]putrescine resulted in two prominently labeled protein bands, *M*r=18,000 and 20,000, as shown by sodiun dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Two-dimensional gel analysis indicated that the labeled 20,000-dalton protein had a pI value of 5.5 and the labeled 18,000-dalton protein exhibited isoform structures with pI values ranging from 4.6 to 5.1. Peptide map analysis showed that these two proteins are similar but not identical. Both labeled proteins contained radioactive hypusine residue and exhibited strong binding to Cibacron Blue dye. The time course of the metabolic labeling of these two proteins, however, differed dramatically. The labeling of the 18,000-dalton protein appeared early and continued to increase 24 h after serum stimulation. In contrast, the labeling of the 20,000-dalton protein became prominent only after much longer period of incubation.

INTRODUCTION

Hypusine (N-(4-amino-2-hydroxybutyl) lysine), an unusual amino acid first discovered in bovine[1,2], was recently found to be present in an 18,000-dalton cellular protein due to a unique spermidine-dependent post-translational modification of one lysine residue on this protein[3,4]. This post-translational reaction is highly conserved among eukaryotes and the hypusine-containing 18,000-dalton protein appears to be universally present in eukaryotes[5-7]. Although the function of the modified protein is still not clear, the apparent ubiquity and the uniqueness of the spermidine dependent labeling reaction, together with the recognized importance of polyamines in growth regulation suggest that this protein may be fundamentally important in growth regulation. This notion is further underscored by the finding that the labeling is responsive to growth condition[3-7].

The interest in this 18,000-dalton hypusine-containing protein, hyp-18K, in eukaryotic protein synthesis is also enhanced by the finding that hyp-18K protein is identical to initiation factor 4D (eIF-4D)[8]. However, the function of eIF-4D has been obscure and remains to be elucidated[9]. Since hypusine formation on the 18,000-dalton protein involves at least two different enzymes, one for the transference of the butylamino moiety from spermidine to lysine residue and the other for the hydroxylation of resulted deoxyhypusine residue[3,10], and polyamines are abundant in cells, it is tempting to speculate that hypusine formation in living organisms may not be limited only to one cellular protein. In this report, we describe that, in addition to the 18,000-dalton protein, another 20,000-dalton protein in chick embryo fibroblast can be metabolically labelled by radioactive polyamine and that the label was identified as hypusine after acid hydrolysis.

EXPERIMENTAL PROCEDURES

Tissue Culture:

Dedicated to Professor K.T. Wang on the occasion of his 60th birthday.

Day 11 chick embryos were used to prepare fibroblast culture[11]. Primary fibroblast cultures grew to the confluency were expanded and used for metabolic labeling experiments.

Metabolic Labeling:

Chick embryo fibroblasts at confluency were diluted by a split ratio of 1:5 or 1:8, using trypsinization procedure, in fresh Dulbecco's medium supplemented with 10% of dialyzed fetal bovine serum. Metabolical labeling was initiated 16–24 h after plating by additions of aminoguanidine to a final concentration of 0.1 mM and [^{3}H]putrescine (final concentration 3 μCi per mL) to the culture. Metabolic labeling was carried out at 37°C in a Forma CO_2 incubator. At designated times, cells were harvested and processed for biochemical analysis as previously described[4]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out according to th procedure of Laemmli using a 7.5–15% acrylamide gradient[12]. For two-dimensional gel electrophoresis the method of O'Farrell[13] was followed. For V8 proteinase digestion analysis, the spots corresponding to the labeled 18,000-dalton and 20,000-dalton protein were excised from the isoelectric focusing-sodium dodecyl sulfate polyacrylamide two-dimensional gel, treated with *S. aureus* V8 and then analyzed on a 20% sodium dodecyl sulfate polyacrylamide gel as described by Cleveland *et al*[14].

Determination of Acid Insoluble Radioactivity:

Cells harvested after metabolic labeling with [^{3}H]putrescine were homogenized by sonication as previously described[6]. Trichloroacetic acid insoluble radioactivity was determined by measuring the radioactivity associated with acid (10% trichloroacetic acid) precipitable cellular materials using the filter paper disc assay method as previously described.[6]

Hypusine Determination:

Radioactively labeled 18,000-dalton and 20,000-dalton protein were excised from sodium dodecyl sulfate polyacrylamide gel, electroeluted, dialyzed, and lyophilized. The dried protein samples were hydrolyzed in 6 N HCl under N_2 at 110°C for 24 h. Acid hydrolyzed samples were then processed for dansylation and tlc analysis as previously described[15].

Materials:

[2,3-^{3}H]Putrescine dihydrochloride (30 Ci/mmol) and [terminal methylenes-^{3}H]spermidine trichloride (40.1 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Tissue culture media and sera were obtained from Gibco, Grand Island, NY. V8 proteinase was from Miles Scientific, Naperville, IL. All other chemicals were of reagent grade.

RESULTS

Fig. 1 shows that under our metabolic labeling condition only one protein band was labeled in NB-15 mouse neuroblastoma cells (Fig. 1, lane A) as we previously reported[4]. In contrast, two labeled protein bands were observed in chick embryo fibroblasts (Fig. 1, lane B), one with an apparent molecular weight of 18,000 and the other 20,000. The labeling intensity of the 20,000-dalton protein in lane B was about 20% of that of the labeled 18,000-dalton protein. This ratio, however, varied from experiment to experiment depending on the length of incubation period and split ratio of fibroblast culture. Since it is known that hypusine-containing 18,000-dalton protein or (eIF-4D) can bind to Cibacron Blue dye[15,16], we then tested the binding affinity of the labeled 20,000-dalton protein to this dye. As shown in Fig. 1, lane C, one step Cibacron Blue dye column enriched both the 18,000- and the 20,000-dalton protein by about 5-fold, suggesting that these two proteins may share some common chemical properties.

Two dimensional gel electrophoresis of the cell homogenates of chick embryo fibroblasts obtained

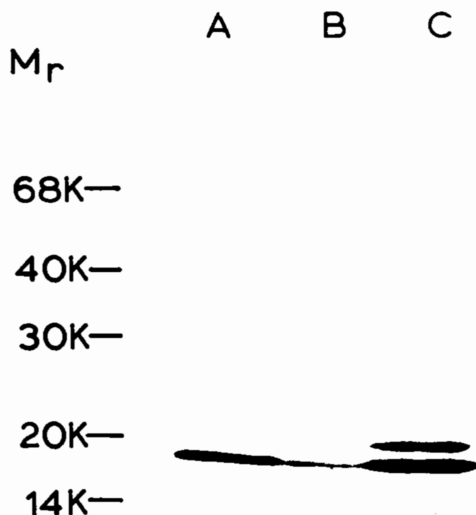

Fig. 1 Fluorogram of sodium dodecyl sulfate-polyacrylamide gel analysis of the radiolabeled hypusine-containing proteins in chick embryo fibroblasts, cells at confluent state were expanded by a split ratio of 1:5 in fresh Dulbecco's medium supplemented by 10% dialyzed fetal bovine serum. Metabolic labeling was initiated by adding [^{3}H]putrescine (5 μ Ci/mL) and amino-quanidine (final concentration 0.1 mM) into the culture. The cultures were incubated for 24 h at 37°C in a CO_2 incubator. NB-15 mouse neuroblastoma cells were metabolically labeled for 24 h as previously described[4,6]. After labeling, cells were harvested, sonicated, and supernatants prepared after a 1 h centrifugation at 12,000 xg were employed for gel analysis and column chromatography. Lane A, NB-15 mouse neuroblastoma cells; Lane B, chick embryo fibroblasts; Lane C, chick embryo fibroblasts after Cibacron Blue dye column chromatography. Each lane contained 50 μg proteins.

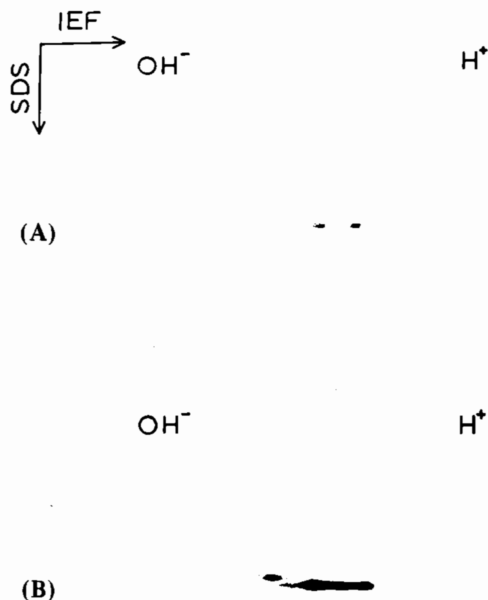

Fig. 2 Fluorograms of two dimensional gels of radio labeled hypusine-containing proteins in chick embryo fibroblasts and NB-15 mouse neuroblastoma cells. Cells were metabolically labeled by [^{3}H] putrescine for 24 h and harvested for two dimensional gel analysis as described in MATERIALS AND METHODS. (A) 100 μg of cytosolic proteins obtained from radiolabeled chick embryo fibroblasts; (B) a combination of labeled chick embryo fibroblasts and NB-15 mouse neuroblastoma cells, 250 μg of proteins each. Both fluorograms (A) and (B) were exposed for 35 days at −70°C.

after metabolic labeling that the 20,000-dalton polypeptide had a pI value of 5.5 and that the 18,000-dalton band existed in two isoforms with pI values of 4.6 and 5.1 (Fig. 2, A). We designated these two forms as hyp-18K-a (acidic form of the hypusine containing 18,000-dalton polypeptide) and hyp-18K-b (basic form of the hypusine containing 18,000-dalton polypeptide) respectively.

These two isoforms in chick embryo fibroblasts exhibited identical chromatographic behavior as that of NB-15 mouse neuroblastoma cells as shown in Fig. 2, B. In this fluorogram, there was another faintly labeled protein spot with a pI value of 5.0 and apparent molecular weight of 22,000 which we

Table 1 Hypusine Determination

Samples		Rf[a] Solvent 1	Solvent 2	Radioactivity recovered from hypusine band (cpm)
Hypusine[b]		0.16	0.38	
Hypusine[c]		0.16	0.38	
18 kDa	(NB[d])	0.16	0.38	210 (12%)[e]
18 kDa	(CEF)	0.16	0.38	860 (15%)
20 kDa	(CEF)	0.16		700 (30%)

a. The radioactively labeled 18,000 and 20,000 dalton bands were excised from the gel, electroeluted and acid hydrolyzed as described under MATERIALS AND METHODS. The acid hydrolysates were dansylated, extracted with toluene and separated on Silica G gel. Solvent 1 is $CHCl_3/CH_3OH/CH_3COOH$ (125:5:1) and Solvent 2 is $CHCl_3/CH_3OH/NH_4OH$ (90:15:4).

b. Authentic hypusine was dansylated directly.

c. Authentic hypusine was subjected to acid hydrolysis under identical conditions as described for proteins and then dansylated.

d. NB, NB-15 mouse neuroblastoma cells; CEF, chick embryo fibroblasts.

e. Number in the parenthesis indicates the percentage of recovery as calculated with respect to the radioactivity associated with the protein after electroelution.

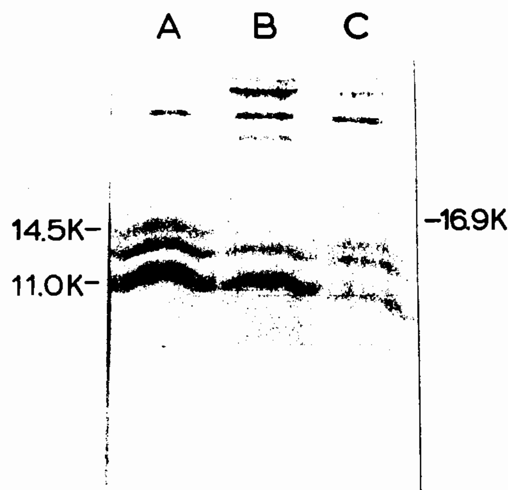

Fig. 3 V8 proteinase digestion peptide map for the hypusine-containing 20,000 and 18,000 dalton protein. V8 proteinase digestion of protein spots obtained from two-dimensional gel was carried out as described by Cleveland *et al*[14]. The digests were analyzed on a 20% sodium dodecyl sulfate-polyacrylamide gel. Lane A, hyp-18K-a; Lane B, hyp-18K-b; Lane C, 20,000 dalton protein. A faint 16.9 kDa band was seen in lane C but was missing in both lanes A and B.

have previously noted in NB-15 cells[15]. This faintly labeled protein did not exist in chick embryo fibroblasts and the chemical nature of the label has yet to be determined. The radioactive labeling in the 20,000-dalton band in chick embryo fibroblasts was intense enough for chemical analysis. Table 1 shows that radioactive hypusine can be recovered from the labeled 20,000-dalton band, indicating that 20,000-dalton polypeptide is a hypusine-containing protein. As a comparison, the labeled 18,000-dalton band from chick embryo fibroblasts and NB-15 cells were also subjected to acid hydrolysis and hypusine determination. Both labeled protein bands contained hypusine. In all three samples, we also noted that approximately 15–35% of radioactivity associated with the labeled bands was recovered as polyamines (data not shown). Whether polyamines are covalently or noncovalently linked to these proteins remain to be investigated.

To further investigate the relationship between the 20,000 and the 18,000-dalton protein, we compared the peptide map of the 20,000-dalton protein with that of the two isoforms of the 18,000-dalton protein and the eIF-4D. Results shown in Fig. 3 indicate that all these proteins showed similar but not identical V8 peptide map.

The time course of metabolic labeling of the 18,000- and 20,000-dalton bands is shown in Fig. 4A. While the labeling of 18,000-dalton protein band, containing both hyp-18K-a and hyp-18K-b, occurred almost immediately after growth stimulation, the labeling of the 20,000-dalton band occurred much

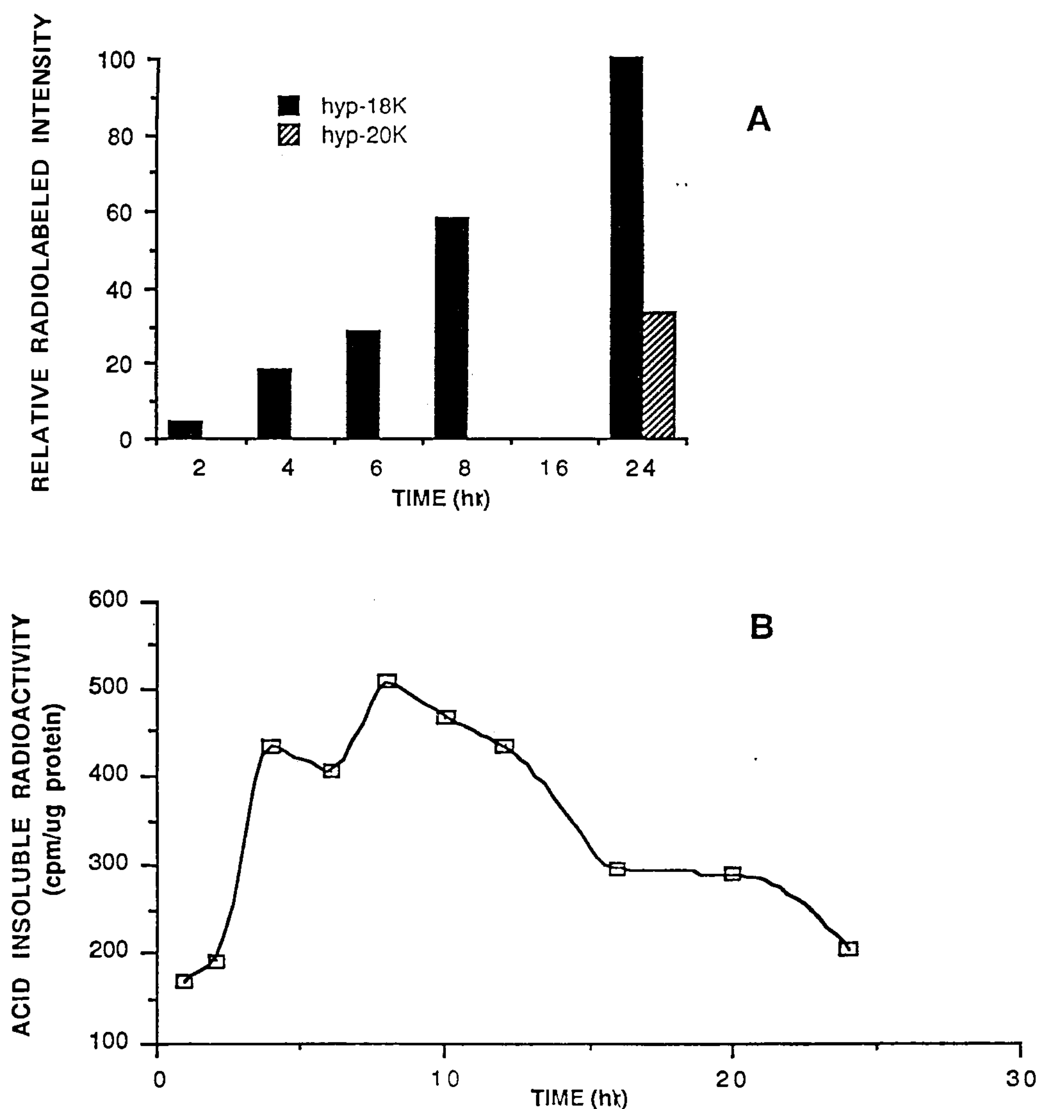

Fig. 4 Time courses of metabolic labeling of the 20,000- and the 18,000-dalton proteins and the acid-insoluble radioactivity in chick embryo fibroblasts during metabolic labeling with [^{3}H] putrescine. At designated times, cells were harvested, washed, and prepared for protein determination, trichloroacetic acid- precipitable radioactivity determination, and sodium dodecyl sulfate-polyacrylamide gel analysis. (A) Time course of the radiolabeling of the 18,000- and 20,000-dalton proteins. The relative intensity of radiolabeled protein bands on the flurogram was determined by densitometric tracing. The value 100 on the coordinate corresponds to 300 cpm as determined by counting the gel slice of the 18,000-dalton band at the 24 h time point using liquid scintillation counter. About 50 μg of total proteins were applied on each lane of the gel. (B) Time course of the acid-insoluble radioactivity in the homogenate during metabolic labeling of chick embryo fibroblasts. At 24 h time point, the radioactivity associated the 18,000-dalton protein was 300 cpm whereas the acid-insoluble radioactivity was 9,500 cpm in a sample of cell homogenates which contained 50 μg of proteins.

slower. The specific radioactivity associated with the 18,000-dalton protein band continued to increase and remained high throughout the time period studied. In contrast, the labeling of the 20,000-dalton protein was not appreciable until 20 hours after the initiation of metabolic labeling. It should be noted that the radioactivity associated with the 18,000- and the 20,000-dalton protein band at 24 h time point only accounted for about 5–10% of total trichloroacetic acid insoluble radioactivity at that time (*e.g.* Fig. 4, A vs B). Acid insoluble radioactivity is generally considered a reliable indication of covalent incorporation of radioactive precursors into macromolecules. This is not the case if [^{3}H]putrescine is used as a precursor since less than 10% of acid insoluble counts were associated with radiolabeled proteins after gel electrophoresis in the presence of sodium dodecyl sulfate. We therefore concluded that the majority of acid insoluble counts shown in Fig. 4B was not due to covalent incorporation of [^{3}H]putrescine into the 18,000- and 20,000-dalton proteins, but due to strong ionic and/or hydrophobic interactions of polyamines with macromolecules. This also explains why the time course of changes of acid insoluble radioactivity in chick embryo fibroblasts (Fig. 4B) was very different from that of the labeling of both 18,000-dalton and 20,000-dalton proteins (Fig. 4A). The fact that the labeling of the 20,000-dalton protein occurred much later than that in the 18,000-dalton protein appears to preclude the possibility that hyp-18K proteins were derived from hyp-20K via proteolytic digestion.

DISCUSSION

Since the discovery that radioactive polyamine can metabolically label an 18,000-dalton protein[3,4] and that the label is due to hypusine formation[3,10], the presence of this 18,000-dalton hypusine-containing protein has been demonstrated in all eukaryotes examined[5-7]. Although the function of this protein and the significance of the modification are not known, the finding that this 18,000-dalton hypusine-containing protein is identical to eIF-4D has raised a possibility that it may be involved in regulating protein synthesis[8]. However, a definitive role for eIF-4D in protein synthesis has not been established yet[9]. Some recent studies have shown that hypusine formation is not correlated with translational repression, suggesting a lack of role of hypusine formation in protein synthesis[17,18]. So far, the 18,000-dalton protein appears to be the only hypusine-containing protein in the literature[5-7]. Whether there are other cellular proteins modified in the similar fashion has not been previously examined. In this report, we have provided evidence that, at least in chick embryo fibroblasts, in addition to the 18,000-dalton protein, another 20,000-dalton cellular protein could also be metabolically labeled by spermidine (Fig. 1) and that the radiolabeled 20,000-dalton protein contained hypusine (Table 1). The time course of the labeling (Fig. 4) suggests that it is unlikely that the labeled 20,000- and 18,000-dalton protein are interconvertible via proteolytic digestion or other modification. Since the labeling intensity of the 20,000-dalton protein band was significant as compared to that of the 18,000-dalton protein (Fig. 1), it is intriguing that such labeling was not observed in other cultured cells such as NB-15 cells (Fig. 1). It is possible that in other cells, hypusine formation on the 20,000-dalton protein is tightly coupled to the synthesis of the protein and thus makes it difficult to detect by radiolabeling. Alternatively, it is possible that the amount of hypusine-containing 20,000-dalton protein in other cells is too low to be detectable. On the other hand, it is of interest to note that the faintly labeled 22,000-dalton protein in NB-15 mouse neuroblastoma cells was not detected in chick embryo fibroblasts (Fig. 2, A vs B).

In spite of the difference in the time course of metabolic labeling of these two proteins (Fig. 4A), they gave similar V8 peptide maps (Fig. 3). In

addition, they exhibited similar binding affinity to Cibacron Blue dye column (Fig. 1, Lane C). Preliminary studies also indicated that both proteins copurify after a series of ion exchange column chromatography (Dou and Chen, unpublished data), suggesting that these two proteins, although not identical, may be closely realted. In view of the abundance of polyamines in eukaryotes and the uniqueness of the mechanism of hypusine formation, it is possible that there is a cluster of proteins that can be modified via the same mechanism. The amount of these proteins and the degree of modification may vary from cell to cell. In any event, the present findings indicate, for the first time, that hypusine formation is not limited only to the 18,000-dalton protein.

Although labeling of the 18,000- and 20,000-dalton protein represents a highly specific spermidine-dependent biochemical event, it should be noted that only less than 1% of total radioactive spermidine in the cell was recovered as hypusine from the labeled proteins. Further understanding of the regulation and function of the modification of these proteins relies on the availability of purified proteins and DNA probes, and the characterization of the enzyme system required for the modification. In this regard, we have recently demonstrated that NAD^+ may be a co-factor required for hypusine formation in cytosolic lysates[19] and succeeded in a reconstitution of hypusine formation system *in vitro*[20].

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