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Deoxyhypusine/hypusine formation on a 21 000-dalton cellular protein in a *Neurospora crassa* mutant in vivo and in vitro

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Hypusine formation on an 18 000-dalton cellular protein is a unique spermidine-dependent, post-translational modification that appears to be ubiquitous in mammalian cells. To determine whether this modification also exists in lower eukaryotes, we examined possible labeling in vitro and in vivo of cellular protein(s) by [³H]spermidine in a mutant strain of *Neurospora crassa* (*arge-12 ota aga*) in which ornithine and polyamine synthesis could be nutritionally manipulated. Because of poor uptake of polyamines in this organism, [³H]ornithine, the immediate precursor of polyamines, was used for the in vivo labeling experiment. Both in vitro and in vivo labeling resulted in a specific labeling of a 21 000-dalton protein. Radioactive hypusine was recovered from radiolabeled 21 000-dalton protein following acid hydrolysis. The in vitro labeling of the 21 000-dalton protein was dramatically stimulated by NAD⁺ and NADP⁺, but not by FMN or FAD, suggesting that an NAD⁺/NADP⁺-dependent oxidative cleavage of spermidine is involved in deoxyhypusine formation. Isoelectric focusing/sodium dodecyl sulfate two-dimensional gel analysis revealed three isoforms of the in vitro labeled 21 000-dalton protein, with pI values ranging from 5.2 to 6.5. In contrast, the 21 000-dalton protein metabolically labeled in vivo gave only one spot with a pI value of approx. 3.5.

Introduction

The essential role of polyamines (putrescine, spermidine and spermine) in growth regulation is well documented [1–3]. The precise functions and mechanism of their actions are still not very clear. The ability of these organic polycations to bind various biological macromolecules has made it difficult to identify specific, polyamine-dependent biochemical events both in vitro and in vivo.

Hypusine formation on an 18 000-dalton cellular protein in mammalian cells is a unique polyamine-dependent post-translational modification. In this reaction, a lysine residue of the 18 000-dalton protein is first converted to deoxyhypusine by addition of a butylamino group derived from spermidine, and the deoxyhypusine

is then hydroxylated to become hypusine [4,5]. The modified hypusine-containing protein has been shown to be identical to the eukaryotic initiation factor 4D (eIF-4D) isolated from rabbit reticulocytes [6]. The physiological function of eIF-4D, however, is poorly understood. Nevertheless, since hypusine formation represents one of the most specific polyamine-dependent processes, it is tempting to speculate that some of the important actions of polyamines in growth regulation may be mediated via hypusine formation. This notion is strengthened by observations that hypusine formation increases following growth stimulation in mammalian cells [4,7–9]. The physiological significance of hypusine formation is further suggested by its highly conserved nature as evidenced by its presence in yeast, insect [10] and human cells [10,11].

Due to our interest in the metabolism and function of polyamines in the fungus *Neurospora crassa* [12–15], we have begun to examine the possible existence of an 18 000-dalton, hypusine-containing protein in this organism. Initially, we have attempted to use [³H]spermidine to metabolically label cellular proteins in the cell-wall deficient slime variant of *N. crassa* [14]. However, the poor polyamine uptake [16] and high con-

Abbreviations: eIF-4D, eukaryotic initiation factor 4D, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF/SDS, isoelectric focusing/sodium dodecyl sulfate; DFMO, α -difluoromethyl ornithine.

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centration of internal polyamines [14] have made the labeling intensity too weak to pursue further biochemical studies. We therefore examined the possibility of metabolic labeling of a *N. crassa* triple mutant (strain IC51: *arg-12 ota aga*) whose polyamine content can be nutritionally manipulated [15,17]. We found that [^3H]ornithine specifically radiolabeled a cellular protein with an apparent molecular mass of 21 000 dalton. Taking advantage of the recent development of the *in vitro* assay system for deoxyhypusine/hypusine formation in mammalian cells [18,19], we also examined the possibility of labeling the 21 000-dalton protein by [^3H]spermidine *in vitro*. We found that [^3H]spermidine specifically labeled a 21 000-dalton protein in *N. crassa* extracts, and the labeling was dependent upon the presence of added NAD^+ or NADP^+ . Other nucleotides tested, including FAD, FMN, ATP and GTP, were all ineffective. We also found that, in addition to the apparent molecular mass, the *pI* values of the labeled hypusine-containing protein(s) in this *N. crassa* mutant differ significantly from that reported for mammalian cells [4–8], insect [10] and yeast [10]. Using the *in vitro* labeling system, we demonstrated that a significant amount of unmodified 21 000-dalton protein was present in this mutant when deprived of polyamines. This is in agreement with previous studies with mammalian cells which display unmodified 18 000-dalton protein when polyamine biosynthesis is inhibited by α -difluoromethyl ornithine [20,21].

Material and Methods

Strain and *Neurospora* culture

The mutant strain IC51, carrying mutations in the *arg-12*, *ota* and *aga* genes (alleles CD3, UM 728 and UM906, respectively), is from the collection of R.H. Davis. This strain lacks ornithine transcarbamylase (*arg-12*), ornithine transaminase (*ota*) and arginase (*aga*) (see Fig. 1). It depends on arginine to grow, but because arginine feedback inhibits ornithine synthesis and cannot form it in the arginase reaction, the strain must also be given spermidine to grow optimally. Slow growth of this strain in arginine alone is due to the formation of the putrescine and spermidine analogs, cadaverine and aminopropylcadaverine, from lysine via elevated levels of ornithine decarboxylase [15,17]. The conidial inoculum was grown in the presence of 1 mM arginine and 1 mM spermidine as described [22]. Mycelia were grown exponentially from a conidial inoculum (about 10^6 conidia per ml) in a boiling flask (1 l) with forced air at 24°C [22]. Vogel's minimal medium [23] with 1.5% sucrose and 0.5 mM L-arginine HCl was used as the medium of growth. This regime leads to polyamine starvation during growth.

In vivo metabolic labeling

During the logarithmic growth of the culture, 20–30 ml of culture was harvested on a Whatman No. 540 filter and washed three times with fresh Vogel's minimal medium. The wet mycelial pad was transferred to a 50 ml culture tube and resuspended in 20–30 ml Vogel's minimal medium. Forced air was used to maintain the homogeneity of the cell suspension. Metabolic labeling was initiated by adding [^3H]ornithine or [^3H]spermidine to the culture. At designated times, aliquots of the culture were harvested by filtration, washed, and acetone-dried as described [22]. The dried pads were made into fine powder in a 1.5 ml Eppendorf tube by vortexing with 0.3–0.5 mm glass beads. The cell powder was extracted with 0.4 ml extraction buffer containing 50 mM potassium phosphate (pH 7.2), 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, by vortexing and centrifuging at $10\,000 \times g$ for 5 min. The supernatants were used for protein determination and gel electrophoresis.

In vitro labeling

Logarithmic cultures of mycelia were harvested by filtration and washed with water. The wet mycelial pads were suspended in 0.1 M glycine-NaOH (pH 9.5) and cells were disrupted by 4 mm glass beads as described by Cramer et al. [24]. The supernatants obtained after centrifugation were employed as lysates for *in vitro* labeling by addition of [^3H]spermidine (1–10 $\mu\text{Ci}/\text{ml}$). The reaction mixture was incubated at 37°C with shaking for various times and the reaction was terminated by adding 1/5 vol. of sodium dodecyl sulfate sample buffer (12% SDS, 0.5 M Tris-HCl (pH 9.0), 10% β -mercaptoethanol, 5 mM EDTA, 25% glycerol and 0.004% pyronin Y) and heated at 100°C for 3 min.

Gel electrophoresis and other procedures

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as previously described [8]. The procedure of O'Farrell [25] was adopted for performing isoelectric focusing/sodium dodecyl sulfate two-dimensional gel electrophoresis. A Pharmacia broad *pI* calibration kit which contains 11 polypeptides with *pI* values ranging from 3.5 to 9.3 was used to determine the *pI* values of labeled proteins revealed on the fluorograms. Protein amount was determined by the Pierce BCA protein assay method [26]. Fluorograms were prepared by presoaking gels in an Enhancer solution (New England Nuclear) before exposing gels to the X-ray film.

Identification of hypusine and deoxyhypusine

The radiolabeled 21 000-dalton protein was eluted from the sodium dodecyl sulfate-polyacrylamide gel after electrophoresis. The eluted protein was then hydrolyzed in 6 M HCl at 110°C for 24 h under N_2 . The

hydrolysates were processed for dansylation according to procedures described previously [27]. The dansylated hypusine and deoxyhypusine were identified and quantitated by TLC analysis on silica gel using the solvent system $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{HOAc}$ (125/5/1, v/v).

Materials

[terminal methylenes- ^3H]Spermidine-3HCl (40 Ci/mmol), [2,3-n- ^3H]putrescine-2HCl (30 Ci/mmol) and L-[2,3- ^3H]ornithine (55 Ci/mmol) were purchased from DuPont NEN Research Products (Boston, MA). NAD^+ , NADP^+ , NADH , FAD , FMN , ATP and GTP were obtained from Sigma (St. Louis, MO). All other chemicals were of standard reagent grade. Synthetic hypusine was kindly provided by Dr. Tetsuo Shiba (Osaka University, Japan). α -Difluoromethyl ornithine (DFMO) was a generous gift from Merrell Dow Research Center (Cincinnati, OH).

Results

In the present study we used a mutant *N. crassa* strain to search for hypusine-containing proteins using the approach of metabolic labeling. This mutant has three enzyme deficiencies in the biosynthesis and utilization of ornithine, as shown in Fig. 1. Since ornithine is an immediate precursor of polyamine biosynthesis, these mutations ensure that exogenously added [^3H]ornithine will be channeled solely to the polyamine biosynthetic pathway. In addition, the low polyamine content in this mutant when grown in the presence of arginine [15,17] should allow an accumulation of [^3H]polyamines, derived from [^3H]ornithine, at high specific radioactivity (see Materials and Methods). With this rationale, we used [^3H]ornithine as the precursor for a metabolic labeling experiment. Fig. 2A shows that [^3H]ornithine specifically labeled a cellular protein with an apparent molecular mass of 21 000 daltons according to SDS-polyacrylamide gel electrophoresis. The labeled protein was found in the soluble fraction after cell homogenization (lanes 1 and 2 vs. lanes 3 and 4 in Fig.

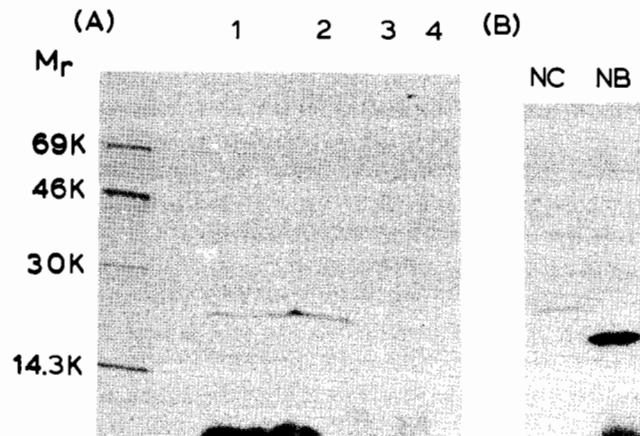


Fig. 2. (A) Time course of the metabolic labeling of *Neurospora* mutant by [^3H]ornithine. The *in vivo* metabolic labeling using [^3H]ornithine (1 $\mu\text{Ci}/\text{ml}$) was performed as described in Materials and Methods. After labeling for 3 (lanes 1 and 3) and 5 h (lanes 2 and 4), cells were extracted and separated into soluble and pellet fractions and were subjected to SDS-polyacrylamide gel electrophoresis (10–20% acrylamide gradient). Lanes 1 and 2, supernatant fraction; lanes 3 and 4, pellet fraction. Each lane contained 400 μg proteins. (B) Relative mobility of the 21 000-dalton protein metabolically labeled by [^3H]ornithine in *N. crassa* (NC) and the 18 000-dalton protein metabolically by [^3H]spermidine in NB-15 mouse neuroblastoma cells (NB) on a SDS-polyacrylamide gel (7.5–15%).

2A). There was no significant difference in labeling intensity of the 21 000-dalton protein whether the labeling was carried out for 3 or 5 h (lane 2 vs. lane 1 in Fig. 2A). It is possible that [^3H]ornithine had mostly been consumed by the earlier time. Fig. 2B shows the position of labeled 21 000-dalton protein from *N. crassa* relative to that of labeled 18 000-dalton protein from mouse neuroblastoma cells on the same SDS-polyacrylamide gel. Acid hydrolysis of the labeled 21 000-dalton protein band excised from the gel resulted in a recovery of radioactive hypusine as indicated by TLC analysis using synthetic hypusine as a standard (data not shown).

We then examined whether the modification of the 21 000-dalton protein can be demonstrated in a cell-free system. Previous studies in mammalian cells [18,19] indicated that only lysates derived from DFMO-treated cells can support deoxyhypusine/hypusine formation *in vitro*, suggesting that in the presence of polyamines, synthesis of the 18 000-dalton protein is followed by immediate modification. Since growth of *N. crassa* mutant in arginine deprives cells of ornithine and thus polyamines, we anticipated that unmodified 21 000 dalton protein may accumulate in these cells too. Fig. 3 summarizes our *in vitro* labeling studies using cell extracts prepared from polyamine-starved *N. crassa*. We found that the labeling of 21 000-dalton protein could be detected *in vitro* only if NAD^+ or NADP^+ (1 mM) was added to the reaction mixture (Fig. 3, lanes 2 and 3 vs. lane 1). The stimulatory effect of NAD^+ was about 3 to 5-fold greater than that of NADP^+ . Other nucleo-

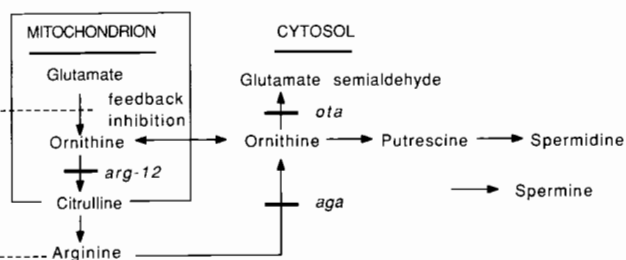


Fig. 1. Metabolic pathways of polyamine biosynthesis in the *N. crassa* mutant *arg-12 ota aga* strain. The *arg-12* mutation blocks ornithine carbamoyl transferase; the *ota* mutation blocks ornithine aminase and the *aga* mutation blocks arginase.

tides tested, including FMN, FAD, ATP and GTP, were all inactive (Fig. 3, lanes 4–7). These data support the notion that unmodified 21 000-dalton protein does accumulate in this *N. crassa* mutant where polyamine-starvation can be achieved by nutritional manipulation. Acid hydrolysis of the in vitro labeled 21 000-dalton protein band (lane 2 in Fig. 3) indicated that the radioactivity was recovered both as deoxyhypusine and hypusine with a ratio of 2 to 1.

Since the apparent molecular weight of the labeled protein in this *N. crassa* mutant is different from hypusine-containing proteins found in other organisms, it was of interest to examine whether the *pI* value of this protein also differs from that of other hypusine-containing proteins. Fig. 4A shows the fluorogram of the two-dimensional gel of the in vitro labeled sample. Three isoforms could be identified, with *pI* values ranging from 5.2 to 6.5. In contrast, the *pI* value of the in vivo labeled 21 000-dalton protein was extremely acidic, close to 3.5 as shown in Fig. 4B. To ensure that the dramatic difference in *pI* values of the in vivo and in vitro labeled 21 000-dalton protein in *N. crassa* mutant is not due to some artifact of sample preparation, both the in vivo and the in vitro labeled *Neurospora* cell extracts were mixed with radiolabeled 18 000-dalton protein partially purified from NB-15 cells and the mixture was co-chromatographed on an IEF/SDS two-dimensional polyacrylamide gel. Fig. 5 shows that

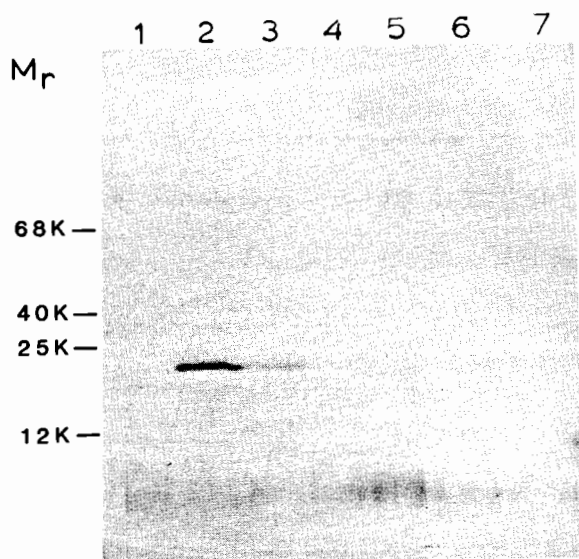


Fig. 3. Effects of various nucleotides on radiolabeling of the 21 000-dalton protein in cytosolic lysates derived from the *Neurospora* mutant cells. The preparation of lysates and in vitro labeling reaction were carried out as described in Materials and Methods. Lane 1, no addition; lane 2, 1 mM NAD^+ ; lane 3, 1 mM NADP^+ ; lane 4, 1 mM FMN; lane 5, 1 mM FAD; lane 6, 1 mM ATP; lane 7, 1 mM GTP. All the reaction mixtures were incubated with ^3H spermidine ($5 \mu\text{Ci}/500 \mu\text{g}$ protein) for 20 h at 37°C . Each lane contained $400 \mu\text{g}$ protein.

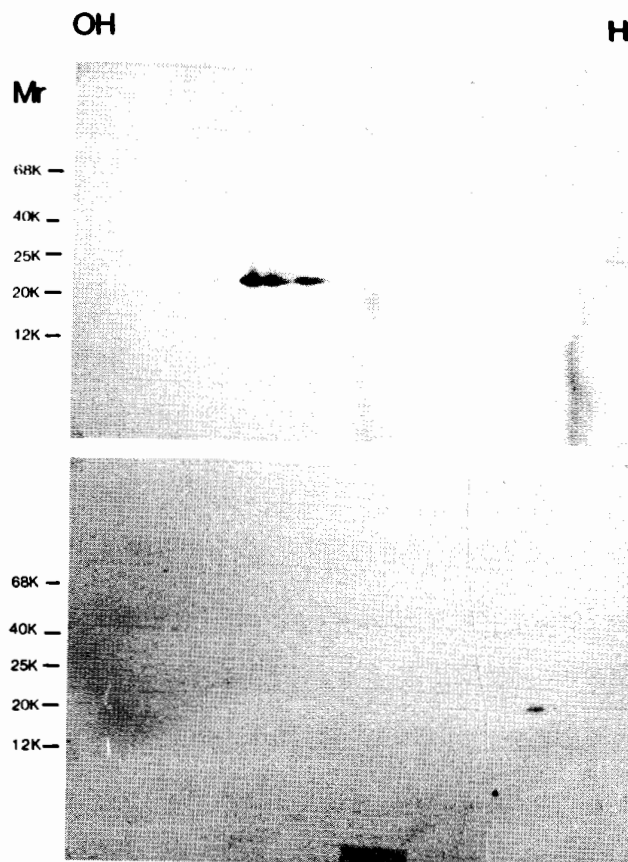


Fig. 4. Comparison of the electrophoretic behavior of radiolabeled 21 000-dalton protein (both in vivo and in vitro labeled) from *N. crassa* mutant. (Top) IEF/SDS PAGE of the in vitro labeled *N. crassa* mutant cells. The cell lysates were incubated with 1 mM NAD^+ and ^3H spermidine at $5 \mu\text{Ci}/500 \mu\text{g}$ proteins in a glycine-NaOH buffer (pH 9.5) for 3 h at 37°C . For the isoelectric focusing gel, $400 \mu\text{g}$ labeled lysates were used. The gel was exposed to the X-ray film for 72 h. (Bottom) IEF/SDS PAGE of in vivo labeled *N. crassa* mutant cells. Cells at log phase of growth were labeled metabolically by ^3H ornithine ($2 \mu\text{Ci}/\text{ml}$) for 3 h at 25°C . For isoelectric focusing, $460 \mu\text{g}$ proteins were used. The *pI* values of labeled proteins were determined by a Pharmacia *pI* standard calibration kit. The gel was exposed to the X-ray film for 4 weeks.

the *pI* values of the labeled 18 000-dalton protein of NB-15 cells remain at 5.1 and 4.8 as we previously reported [28]. The *pI* values of both in vivo and in vitro labeled 21 000-dalton proteins of *Neurospora crassa* also remained unchanged. Thus, the significant shift in *pI* values between the in vitro labeled and the in vivo labeled 21 000-dalton protein appears to be a unique property of *N. crassa*. It is possible that an additional post-translational modification, in addition to deoxyhypusine/hypusine formation, of the 21 000-dalton has occurred in *N. crassa* in vivo but not in vitro.

Discussion

We have shown that deoxyhypusine/hypusine formation occurs in the fungus *N. crassa* by metabolically

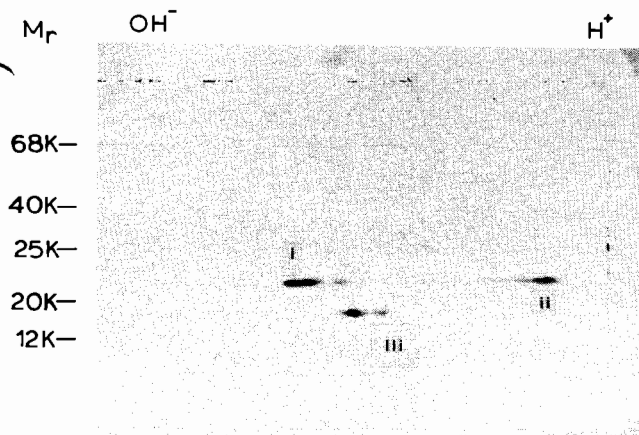


Fig. 5. Co-chromatograph of protein samples from *N. crassa* mutant cells and mouse neuroblastoma cells on an SDS/isoelectric focusing two-dimensional gel. Both in vivo and in vitro labeling of the *N. crassa* cells were performed as described in Materials and Methods. Mouse neuroblastoma cells were metabolically labeled by [^3H]spermidine as previously described [9,27]. Approx. 200 μg of the in vitro labeled *N. crassa* lysates and 460 μg of in vivo labeled *Neurospora* lysates were mixed with 50 μg of the labeled neuroblastoma lysates. The mixture was loaded on a tube gel for isoelectric focusing and two-dimensional gel analysis. The pI values of the labeled proteins were estimated by using a Pharmacia standard pI calibration kit. The gel was exposed to X-ray film for 3 days. (i) in vitro labeled *N. crassa* sample, (ii) in vivo labeled *N. crassa* sample, (iii) in vivo labeled mouse neuroblastoma sample.

Labeling the cells using polyamine precursor [^3H] ornithine and by direct labeling in a cell-free system using [^3H]spermidine (Figs. 2 and 3). In both cases, only one cellular protein with an apparent molecular mass of 21 000-dalton was prominently labeled, as revealed by SDS-PAGE analysis (Figs. 2 and 3). Radioactive hypusine was recovered from both in vivo and in vitro labeled 21 000-dalton protein after acid hydrolysis. However, radioactive deoxyhypusine was recovered only from the in vitro labeled 21 000-dalton protein. Although our results confirm the notion that hypusine formation is a ubiquitous and highly conserved reaction, our data also demonstrate the presence of a unique hypusine-containing protein in *N. crassa* mutant cells with apparent molecular mass and pI values different from those reported in all other eukaryotic cells [4,6,8] including yeast [10]. A difference in apparent molecular mass of proteins on an SDS-polyacrylamide gel could be due to (i) a true difference in the molecular size or (ii) certain protein modifications which give an abnormal mobility of the protein on the gel. It has been shown that phosphorylation and glycosylation of proteins may affect their apparent molecular masses on an SDS-polyacrylamide gel [29]. Whether the difference in the apparent molecular mass of the hypusine-containing proteins in *N. crassa* and other eukaryotic cells is due to a difference in size or post-translational modification remains to be investigated. The fact that the in vitro labeled 21 000-dalton protein has pI values more basic

than that of the modified 18 000-dalton protein argues against phosphorylation being the cause of any difference in apparent molecular mass. Furthermore, we have recently found that the cell extracts of *N. crassa*, in addition to labeling of the 21 000-dalton protein, could also label the unmodified mouse neuroblastoma 18 000-dalton substrate protein without changing its apparent molecular mass on SDS-polyacrylamide gel (Yang, Y.C. and Chen, K.Y., unpublished data). This finding suggests that the *N. crassa* hypusine-containing protein may indeed differ in size from other hypusine-containing proteins. In addition to the apparent molecular mass, the most striking features of the labeled 21 000-dalton proteins are (i) a presence of three isoforms in the in vitro labeled sample (Fig. 3A) and (ii) a large difference in pI value between the in vitro and the in vivo labeled 21 000-dalton protein (Fig. 4). All three isoforms found in the *N. crassa* in vitro labeling reaction had pI values more basic than the labeled 18 000-dalton protein in yeast or mammalian cells [4,10,28]. It may be that these three isoforms differ in certain amino acid residues, and that such a difference became negligible following an additional post-translational modification which could only occur in vivo. Possible candidates for such additional post-translational modification may include phosphorylation, polyphosphorylation and deamidation.

Similar to our recent finding with the 18 000-dalton proteins in mammalian cells [18], NAD^+ specifically and dramatically stimulated the in vitro labeling of the 21 000-dalton protein in the *N. crassa* cell extracts (Fig. 3). In contrast to mammalian cells, however, we found that NADP^+ was also effective in stimulating deoxyhypusine/hypusine formation on the 21 000-dalton protein (Fig. 3, lane 3 vs. lane 1). It is known that some classes of dehydrogenases can have different affinities and requirement for NAD^+ and/or NADP^+ depending on source and species [30]. All other nucleotides tested, including ATP, GTP, FMN and FAD, were ineffective.

Previous studies [18,19] have shown that the synthesis of the 18 000-dalton protein and its subsequent modification by spermidine are tightly coupled in mammalian cells. Thus, in order to detect deoxyhypusine/hypusine formation in vitro, it is necessary to ensure the presence of a sufficient amount of 18 000-dalton substrate protein by prior treatment of the mammalian cells with DFMO in order to suppress spermidine formation [18,19]. In *N. crassa*, polyamine deprivation was imposed by ornithine starvation, and thus the in vitro labeling of the 21 000-dalton protein could be carried out using cells not subjected to prior DFMO treatment (Fig. 3). Moreover, treatment of polyamine-starved *N. crassa* cells with DFMO did not further enhance the in vitro labeling of the 21 000-dalton protein (data not shown). These data indicate that the biosynthesis of the 21 000-dalton substrate protein and its subsequent de-

oxyhypusine/hypusine formation in this mutant, grown in the presence of arginine, are either uncoupled or not tightly coupled. Based on the labeling intensity of the *in vitro* labeled 21 000-dalton protein and the specific radioactivity of [³H]spermidine used, we estimated that the concentration of unmodified 21 000-dalton in this *Neurospora* mutant was greater than 80 pmol/mg protein, assuming that only one lysine residue per 21 000-dalton protein molecule is modified. We consider this finding significant because the *Neurospora* mutant can be used as an abundant and convenient source for the isolation of unmodified 21 000-dalton protein, the substrate for both fungal and mammalian hypusine/deoxyhypusine-forming enzymes. Although the *N. crassa arg-12 ota aga* mutant grows slower than the wild type *N. crassa* in the absence of ornithine, its doubling time of 9–10 h still compares favorably to that of cultured mammalian cells, and thus gram quantities of *Neurospora* cells can be easily obtained in the laboratory as a starting material for the isolation of the unmodified 21 000-dalton protein. Moreover, the presence of a significant amount of unmodified 21 000-dalton substrate protein in this mutant may offer us a unique opportunity to investigate the physiological function of this protein and its post-translational modification.

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