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NAD^+ stimulated the spermidine-dependent hypusine formation on the 18 kDa protein in cytosolic lysates derived from NB-15 mouse neuroblastoma cells

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When incubated with cultured mouse neuroblastoma cells under growth stimulatory condition, [^{3}H]putrescine or [^{3}H]spermidine can metabolically label a cellular protein of apparent molecular mass 18 kDa. The labeling, which leads to hypusine formation, is due to a covalent linkage between a lysine residue and the butylamino group derived from spermidine. This reaction can be demonstrated in the cytosolic fractions obtained from cells whose spermidine pool was depleted by prior treatment with α-difluoromethylornithine. In an effort to characterize the enzyme system involved in this unique post-translational modification, we found that NAD^+ at 0.1 mM stimulated labeling more than 150-fold. Other nucleotides such as $NADP^+$, ATP and GTP were ineffective. The fact that NAD^+ dramatically stimulated labeling of the 18 kDa protein indicated that the enzyme involved in hypusine formation may be an NAD^+-requiring enzyme.

NAD^+; Hypusine formation; Post-translational modification; Eukaryotic initiation factor 4D; (Mouse neuroblastoma cell)

1. INTRODUCTION

[^{3}H]Spermidine can metabolically label a cellular protein with an apparent molecular mass of 18 kDa in various cultured mammalian cells [1,2]. The labeling represents a unique post-translational modification in which the aminobutyl moiety of spermidine is transferred to the ε-amino group of a lysine residue of the 18 kDa protein [3]. The resulted deoxyhypusine residue is further hydroxylated to become hypusine (N^{ε}-(4-amino-2-hydroxybutyl)lysine) [3,4]. This modification, ubiquitous in mammalian cells [1,2], has also been shown to be coupled to cell proliferation [1–6]. The precise functions of the 18 kDa protein and its modification, however, are unknown. Cooper et al. [7] have shown that the hypusine-containing 18 kDa protein (hyp-18K) in Chinese hamster ovary cells is identical chromatographically to eukaryotic initiation factor 4D (eIF-4D) in rabbit reticulocytes. Nevertheless, despite the fact that it is called initiation factor, the physiological role of eIF-4D is not clear and remains to be determined [8].

The enzyme system responsible for hypusine formation on the 18 kDa protein has yet to be defined and characterized. Recently, Murphy and Gerner [9] have found that [^{3}H]spermidine can label the 18 kDa protein in cell lysates provided that the pH of the reaction buffer is kept at 9.5. The rate of this in vitro labeling reaction, however, is slow; incubation for 6–24 h is needed for appreciable radioactivity to be incorporated into the 18 kDa protein [9]. We reasoned that a dilution or a loss of certain effector(s) in cell lysates may contribute to the slow rate of the in vitro labeling reaction. We therefore examined the effects of various potential effectors including coenzymes such as NAD^+ and $NADP^+$ on the labeling of the 18 kDa protein in the lysates prepared from NB-15 mouse neuroblastoma cells. We found that NAD^+ specifically and dramatically stimulated the label-

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ing of the 18 kDa protein by [^{3}H]spermidine. It is likely that NAD^+ is required for the oxidative cleavage of spermidine during hypusine formation.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[*terminal methylenes*-^{3}H]Spermidine trichloride (40.1 Ci/mmol) and Enhancer were purchased from DuPont NEN Research Products (Boston, MA). Tissue culture media and sera were purchased from Gibco (Grand Island, NY). NAD^+, $NADP^+$, FAD, FMN, ATP, GTP and Sephadex G-50 resins were obtained from Sigma (St. Louis, MO). All other chemicals were of standard reagent grade. α-Difluoromethyl ornithine (DFMO) was a generous gift from Merrell Dow Research Center (Cincinnati, OH). Synthetic hypusine was kindly provided by Dr Tetsuo Shiba (Osaka University, Japan).

2.2. *Cell culture and lysate preparation*

NB-15 mouse neuroblastoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as described [1]. At 80 of 90% confluence, the cultures were replenished with fresh growth medium containing 5 mM DFMO for another 48 h. Cells were washed with phosphate-buffered saline (pH 7.2) and then suspended in either 0.1 M glycine (pH 9.5) or Earle's balanced salt solution (pH 7.2). The cell suspension was homogenized by brief sonication (5–10 s) at 4°C and the cytosolic lysates were prepared by centrifugation at 18000 × *g* for 30 min. To remove small molecules from the lysates, the cytosolic lysates were further passed through a Sephadex G-50 spun column and the eluted fractions designated as G-50 lysates. The spun column was prepared as described by Maniatis et al. [10]. Protein was assayed according to Lowry et al. [11].

2.3. *In vitro labeling with [^{3}H]spermidine*

For all labeling experiments, freshly prepared cytosolic or G-50 lysates were used. [^{3}H]Spermidine was added to lysates (200–500 μg/100 μl) to a final concentration of 20–100 μCi/ml in the absence or presence of various additives as indicated in the figure legends. The labeling reaction was carried out at 37°C for various periods of times. At the end of incubation, the reaction mixture was mixed with 0.2 vol. Laemmli's stop solution containing 12% SDS, 0.5 M Tris-HCl (pH 9.0), 10% β-mercaptoethanol, 5 mM EDTA, 25% glycerol and 0.005% pyronin Y and heated at 100°C for 3 min. Samples were then subjected to SDS-polyacrylamide gel electrophoresis and fluorography as in [1].

3. RESULTS AND DISCUSSION

The in vitro labeling of the 18 kDa protein by [^{3}H]spermidine can be demonstrated in the cytosol prepared from NB-15 mouse neuroblastoma cells whose polyamine pools have been partially depleted by prior treatment with DFMO (fig.1, lane 1). The pellet fraction obtained after a 30 min centrifugation at 18000 × *g* did not support the labeling (not shown). To determine whether low molecular mass effector(s) may be required for this labeling reaction, we prepared the G-50 lysates by passing cytosolic lysates through a Sephadex G-50 spun column at 1600 × *g* for 5 min. The labeling intensity using G-50 lysates was extremely low and almost undetectable (fig.1, lane 2), suggesting that some effector(s) may have been removed by the Sephadex G-50 spun column. Since the butylamino moiety for deoxyhypusine formation may be derived from spermidine via oxidative cleavage of a carbon–nitrogen bond, it is possible that some cofactor of redox enzymes may affect the labeling reaction. Among the four coenzymes examined, NAD^+ at 1 mM stimulated labeling of the 18 kDa protein in G-50 lysates more than 300-fold (fig.1, lane 6 vs lane 2). $NADP^+$ was completely ineffective (fig.1, lane 7). FAD and FMN stimulated labeling of the 18 kDa protein but the effect was much less dramatic than that of NAD^+ (fig.1, lanes 8,9). Additions of Ca^{2+}, ATP, and GTP had no effect on labeling (fig.1, lanes 3–5). That the

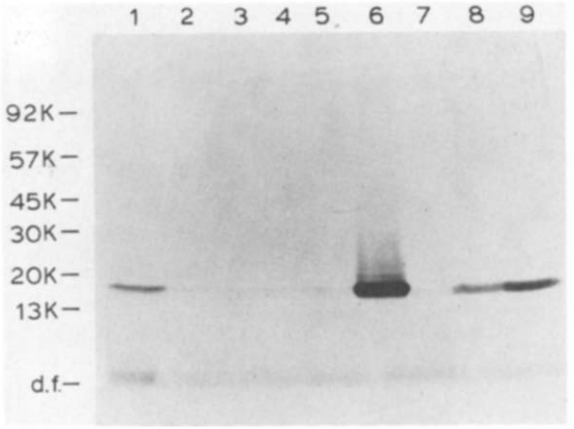

Fig.1. Effects of calcium and various nucleotides on radiolabeling of the 18 kDa protein in cytosolic lysates (lane 1) and G-50 lysates (lanes 2–9) derived from NB-15 neuroblastoma cells. NB-15 cells were treated with DFMO for 48 h and harvested. Cytosolic and G-50 lysates were prepared as described in section 2. Lanes: 1,2, no addition; 3, 0.4 mM Ca^{2+}; 4, 1 mM ATP; 5, 1 mM GTP; 6, 1 mM NAD^+; 7, 1 mM $NADP^+$; 8, 1 mM FAD; 9, 1 mM FMN. Reaction mixtures were incubated with 40 μCi/ml [^{3}H]spermidine for 4 h at 37°C. The fluorogram was exposed for 48 h. Each lane contained 150 μg protein. Radioactivities incorporated into the 18 kDa band in lanes 1,2,6 were 1900, 120, and 48000 cpm respectively. d.f., dye front.

NAD^+-stimulated labeling was due to hypusine formation was confirmed by TLC analysis of the labeled acid hydrolysates using authentic hypusine as standard (not shown).

The stimulatory effect of NAD^+ on labeling of the 18 kDa protein in G-50 lysates by [^{3}H]spermidine could be detected in the micromolar concentration ranges (fig.2, lanes 2–6). Maximal stimulation by NAD^+ occurred at 0.1–0.3 mM (fig.2, lanes 7,8), well within the range of K_d values for some dehydrogenase-NAD^+ complexes [11].

The effect of NAD^+ on the time course of labeling of the 18 kDa protein band is shown in fig.3. Based on the radioactivity incorporated into the 18 kDa protein and the specific activity of [^{3}H]spermidine used, we estimated that the rate of hypusine formation in the in vitro labeling system in the presence of NAD^+ was about 8 pmol/mg protein per 30 min. In contrast, little or no labeling of the 18 kDa protein was observed in G-50 lysates in the absence of NAD^+ even after incubation for 20 h. The rate of labeling of the 18 kDa protein by spermidine in the cytosolic fraction was measurable but very low compared to that obtained in the presence of exogenously added NAD^+. These data indicated that the presence of NAD^+ in the in vitro labeling system significantly altered the kinetics of hypusine formation.

Here, we also found that the presence of NAD^+ in the incubation buffer effectively eliminated the high pH requirement for the in vitro labeling of the 18 kDa protein. At 0.1–0.5 mM NAD^+, the labeling intensity of the 18 kDa protein in G-50 lysates maintained in Earle's salt solution (pH 7.2) was comparable to that obtained at pH 9.5 (not shown). Thus, although deprotonation of spermidine may be an important first step in the post-translational modification of the 18 kDa protein, the presence of NAD^+ may shift the reaction equilibrium in such a way that even at pH 7, hypusine formation proceeds rapidly.

The finding that NAD^+ stimulated the in vitro labeling of the 18 kDa protein enables us to perform the assay at physiological pH within a short period of time. It also offers the possibility of using an NAD^+-affinity column to separate the enzyme system from the substrate protein. At present, the assay system has already allowed us to screen quickly various eukaryotic cells for their relative ability to label their own 18 kDa protein.

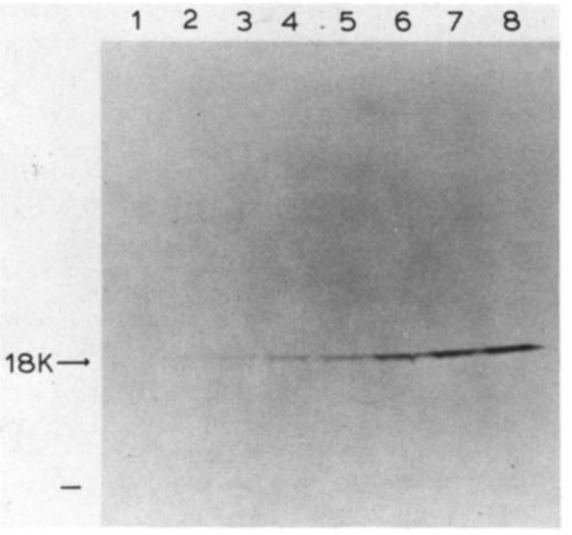

Fig.2. Dose-response curve of the effects of NAD^+. G-50 lysates were used here. Lanes: 1, no addition; NAD^+ was added at the following final concentrations – lanes: 2, 5 μM; 3, 10 μM; 4, 20 μM; 5, 40 μM; 6, 80 μM; 7, 160 μM; 8, 320 μM. Reaction mixtures were incubated at 37°C for 4 h in the presence of 50 μCi/ml [^{3}H]spermidine. The fluorogram was exposed for 48 h. Each lane contained 150 μg protein.

The specificity and magnitude of the stimulatory effect of NAD^+ on the in vitro labeling of the 18 kDa protein suggested that NAD^+ may either be the coenzyme for the deoxyhypusine-formation

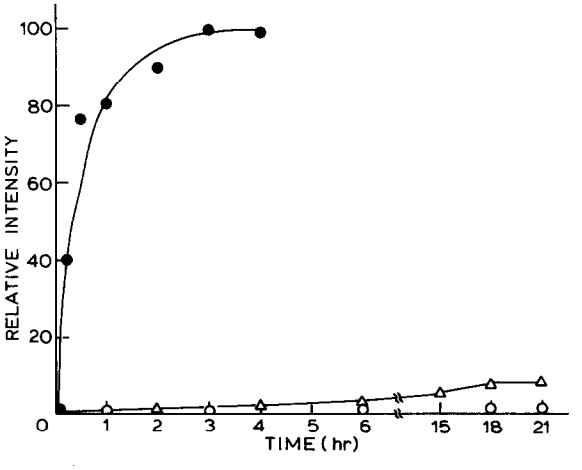

Fig.3. Effects of NAD^+ on the time course of labeling of the 18 kDa protein in G-50 lysates (○,●) or cytosolic lysates (△). Reaction was carried out at 37°C in 0.1 M glycine buffer (pH 9.5) containing 50 μCi/ml [^{3}H]spermidine in the absence (○, △) or presence (●) of 1 mM NAD^+.

enzyme or the physiological effector which regulates the post-translational modification of the 18 kDa protein.

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