Chemistry of Hypusine Formation on Eukaryotic Initiation Factor 5A in Biological Systems

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Hypusine, an unusual amino acid, is derived from the hydrolysis of eukaryotic initiation factor 5A (eIF-5A), the only cellular protein known to contain hypusine residue. Hypusine residue is formed through a spermidine-dependent posttranslational modification of eIF-5A at a specific lysine residue. Each mature eIF-5A molecule contains only one hypusine. Hypusine formation on eIF-5A is essential for cell survival and proliferation. The biochemistry of hypusine formation has been actively investigated in a number of leading laboratories during the past decade. The precise functional role of eIF-5A remains a mystery. This paper reviews recent progress in the study of the biochemistry of hypusine formation and offers speculation on the possible function of eIF-5A.

INTRODUCTION

Eukaryotic initiation factor 5A (eIF-5A) exists in both eukaryotic cells and archaea, but not in eubacteria. The eIF-5A protein is the only cellular protein known to contain hypusine. The complete posttranslational modification pathway of hypusine formation on eIF-5A precursor is shown in Fig. 1. In this modification reaction, Nature has committed two enzymes, deoxyhypusine synthase and deoxyhypusine hydroxylase, to modify one unique lysine residue on a single polypeptide, eIF-5A, into hypusine. This commitment underscores the importance of this modification. All the evidence in the literature indicates that eIF-5A is indispensable for cell survival and proliferation. The conservation of the amino acid sequence of eIF-5A, from archaebacteria to human (Fig. 2), also testifies to the importance of this protein. This hypusine-containing protein is named as an initiation factor because it was originally isolated from ribosomal high salt wash fractions where other initiation factors were found. However, the name may be a misnomer since its role in translational initiation is tenuous. Indeed, what remains mysterious is that we do not know the cellular function of eIF-5A and hypusine modification.

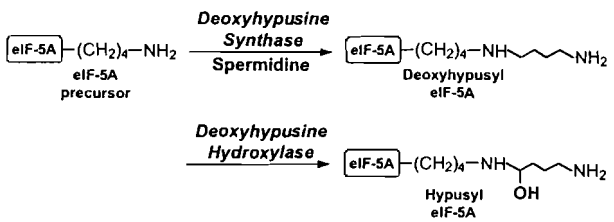


Fig. 1. Hypusine formation on the eIF-5A precursor. Two enzymes, deoxyhypusine synthase and deoxyhypusine hydroxylase, are involved in this modification. NAD^+ is the obligatory co-factor for deoxyhypusine synthase.

HISTORY

Free hypusine was isolated and identified as N^6-(4-amino-2-hydroxybutyl)-2,6-hexanoic acid in 1971.[1] The name "hypusine" was coined to indicate that it comprises moieties of hydroxyputrescine and lysine. Two groups reported the metabolic labeling of an 18,000-dalton cellular protein by radioactive spermidine or putrescine.[2,3] This labeling was found to be due to hypusine formation, and this unique posttranslational process involves two steps as shown in Fig. 1.[2,4] In 1983 Cooper et al. found that the 18,000-dalton hypusine-containing protein is identical to a known translation initiation factor eIF-4D,[5] originally isolated in 1976.[6] The protein eIF-4D was renamed eIF-5A in 1989.[7] Hypusine formation could be clearly demonstrated in the cell free system after the discovery of NAD^+ as the required co-factor for deoxyhypusine synthase.[8] The progress of hypusine research started to gain momentum after the molecular cloning of eIF-5A[7] and isolation and cloning of deoxyhypusine synthase.[9-12] The milestones of hypusine re-

Dedicated to Professor Kung-Tsung Wang on the occasion of his 70th birthday.

search are summarized in Table 1.

AMINO ACID SEQUENCE AND STRUCTURE OF eIF-5A

The biosynthesis of eIF-5A precursor and its post-translational modifications are tightly coupled.[13] Thus, in almost all eucaryotic cells, including red blood cells, eIF-5A exists in a hypusine-containing form (i.e., mature form). With the advent of various genome sequencing projects, more than 30 sequences of eIF-5A are now available for analysis. Sequence alignment as shown in Fig. 2 demonstrates that eIF-5A is an ancient protein, conserved from archaebacterium to human. Most of the conserved regions are clustered in the N-terminus half of the protein, especially around the hypusine site. The sequence STSKTGKHGHAK, where the bold K is the hypusination site, is absolutely conserved from yeast to humans. In addition to the hypusine residue, eIF-5A is also unique in that there is little sequence similarity between eIF-5A and other proteins. Recent X-ray diffraction data on two eIF-5A protein crystals obtained from *Methanococcus jannashii* and from *Pyrobaculum aerophilum*, however, suggest that the structure of eIF-5A may resemble some other proteins at a three dimensional level.[14,15]

Fig. 3 shows the tertiary structure of the unmodified recombinant MJ eIF-5A in a ribbon diagram.[14] The most striking feature is the clear separation between the N-terminal domain (first half of the eIF-5A) and C-terminal domain (second half of the protein). There are six β strands in the N-terminal domain and five β-strands in the C-terminal domain. The two domains are connected with a short flexible hinge. The N-terminal domain shares structural similarity

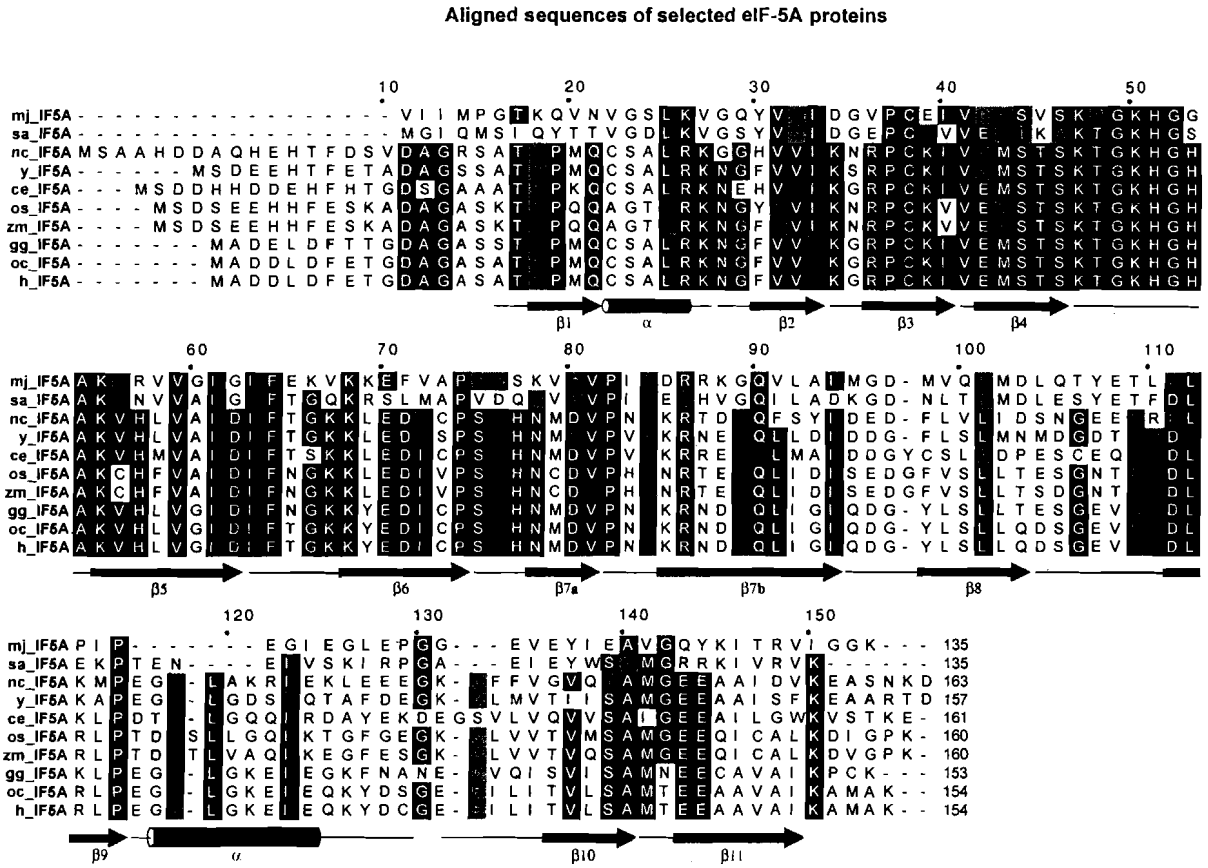


Fig. 2. Alignment of amino acid sequences of eIF-5A from 10 different species across the evolutionary spectrum. The position of α helices (cylinders) and β strands (arrows) are shown at the bottom of the aligned sequences. Identical amino acid residues are indicated in black solid blocks and similar amino acid residues are indicated in shaded blocks. Alignment was generated using CLUSTAL W program.[39] mj, *Methanococcus jannaschii*; sa, *Sulfolobus acidocaldarius*; nc, *Neurospora crassa*; y, yeast; ce, *C. elegans*; os, *Oryza sativa* (rice); zm, *Zea mays*; gg, *Gallus gallus* (chicken); oc, *Oryctolagus cuniculus* (rabbit); h, human.

Table 1. Milestones of Hypusine and eIF-5A Research

Date	Major Events	Reference
1971	Discovered free hypusine in bovine brain	1
1976	Purified eIF-4D, originally named M2Bα	6
1981	Hypusine formation on an 18kDa demonstrated *in vivo* by metabolic labeling using radioactive spermidine	2, 3
1982	Elucidation of hypusine formation pathway	4
1983	Identification of the 18kDa protein as eIF-4D	5
1988	Identification of NAD^+ as the necessary co-factor for hypusine formation in cell free system	8
1989	Cloning of human eIF-4D, renamed eIF-5A	7
1991	Essentiality for cell viability demonstrated in yeast	38
1995	Purification and cloning of deoxyhypusine synthase	9, 11

to part of dihydrofolate reductase, whereas the topology of C-terminal domain closely resembles bacterial cold shock proteins. Although cold shock proteins are known as RNA binding proteins,[15] it remains to be seen whether the structural similarity between eIF-5A and cold shock protein can be correlated with their functional similarity.

DEOXYHYPUSINE SYNTHASE

Deoxyhypusine synthase is the key enzyme for hypusine formation. The enzyme is a homotetramer[9] and the amino acid sequence of the enzyme also appears to be highly conserved, particularly at the C-terminal half (Fig. 4). The crystal structure of human deoxyhypusine synthase has been determined.[16] The enzymatic reaction can be divided into three separate steps:[17] (1) oxidative cleavage of spermidine, (2) formation of enzyme-imine intermediate, and (3) transference of the butylimino group from the enzyme-imine intermediate to the lysine residue on eIF-5A. The critical lysine residue that is involved in enzyme-substrate intermediate formation has been identified as K329 in human deoxyhypusine synthase.[18] Deletion of the deoxyhypusine synthase gene leads to lethal phenotype in yeast, again indicating that hypusine formation is essential for cell viability.[19]

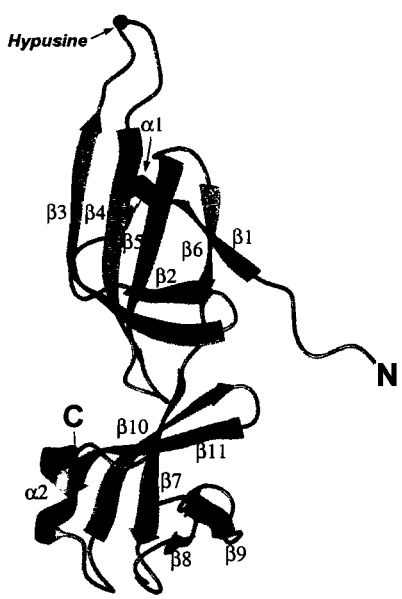


Fig. 3. Three dimensional ribbon structure of archaebacterial eIF-5A.[14,15] The arrows represent β-strands. The position of hypusine residue is indicated. The diagram was generated with the MOLMOL program.

DEOXYHYPUSINE HYDROXYLASE

The enzyme deoxyhypusine hydroxylase has not been purified yet. One difficulty in developing a feasible purification scheme is due to a lack of convenient enzyme assay method to monitor the hydroxylation reaction. Recently, a relatively simple procedure, employing the oxidative cleavage of the labeled hypusyl residue and subsequent extraction of the generated aldehydes for radioactive counting, has been reported.[20] We are attempting to improve this method and to develop a feasible protocol for purification of this enzyme. Although some metal chelators have been reported to inhibit deoxyhypusine hydroxylase activity *in vivo*,[20] the specificity of these compounds is questionable, making it difficult to assess whether hydroxylation is absolutely required for the eIF-5A activity.

TISSUE DISTRIBUTION AND INTRACELLULAR LOCALIZATION

Hypusine formation is one of the most specific polyamine-dependent biochemical reactions. It is quite possible that some of the important physiological functions of polyamines are mediated through hypusination of eIF-5A. Tissue distribution of polyamines have been examined in several animal models and found to be variable from tissue to tissue.[21] To gain clues about possible physiological functions of eIF-5A, we have examined tissue distribution of eIF-5A and deoxyhypusine synthase activity in mice. As shown in Fig. 5, we did not observe any significant difference in tissue distribution of eIF-5A as measured by Western blot analysis. However, we noticed that deoxyhypusine synthase activity is much higher in brain and heart tissue. We are expanding this study to other animal models.

The cellular localization of a protein can also shed light on its possible biological function. In this regard, two groups, although using the same immunofluorescent staining method for detection, have reported conflicting results. One study found that eIF-5A is present in nucleus,[22] whereas the other claims that eIF-5A only exists in cytoplasm.[23] The cause for the discrepancy is intriguing, since both groups have done proper controls. Clearly further work needs to be done to resolve the issue of eIF-5A localization.

IS eIF-5A A BIMODULAR PROTEIN?

One good approach to understand the function of eIF-5A is to identify its interacting partners inside the cell. Possible interactions of eIF-5A with other proteins have been investigated. Using a yeast two hybrid system to probe a HeLa cell library, eIF-5A is found to interact with deoxyhy-

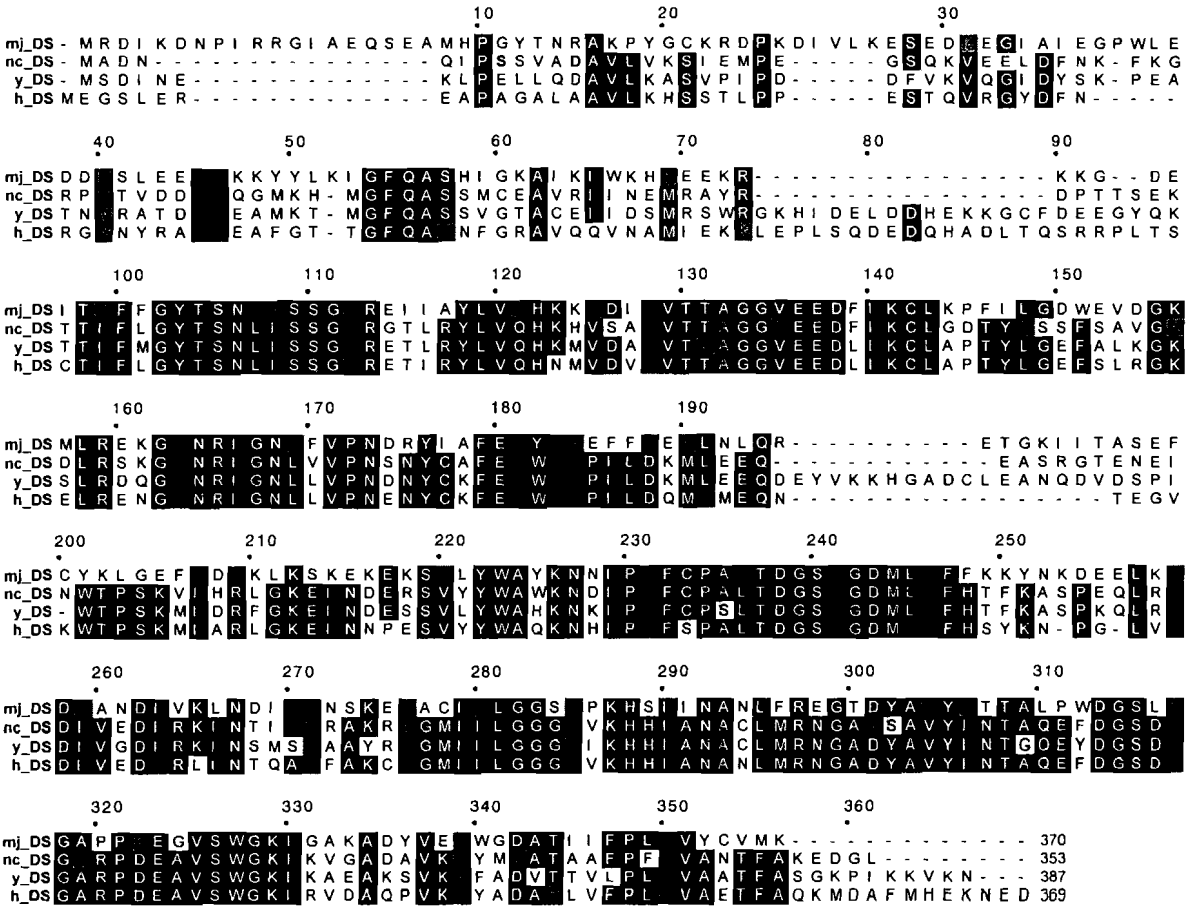


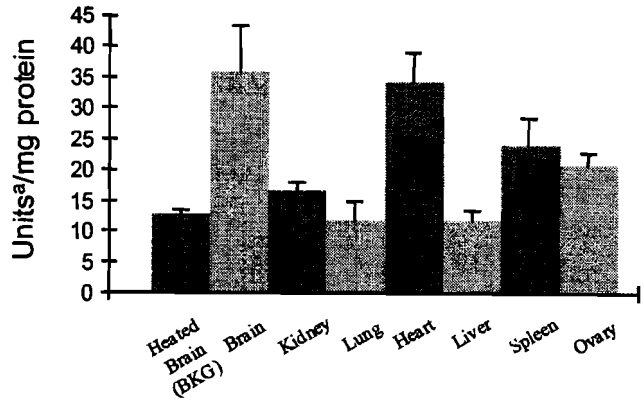
Fig. 4. Alignment of amino acid sequences of deoxyhypusine synthase from 4 different species across the evolutionary spectrum. Identical amino acid residues are indicated in black solid blocks and similar amino acid residues are indicated in shaded blocks. Alignment was generated using CLUSTAL W program.[39] mj, *Methanococcus jannaschii*; nc, *Neurospora crassa*; y, yeast; h, human.

pusine synthase in one study (Zhu, L. and Chen, K. Y. unpublished results) and with L5 ribosomal protein in another study.[24] Using traditional biochemical methods such as immunoprecipitation and photo-crosslinking, it has been reported that eIF-5A binds to transglutaminase II[25] or to Rev.[22] However, it is unclear whether any of these interacting proteins are truly the biological partners of eIF-5A. Although a yeast two hybrid system is a powerful means for searching interacting proteins,[26] its maximal potential can be limited by many factors, including the choice of a suitable library, the quality of the library, and the expression level of fusion proteins. We are currently expanding our search using a yeast two hybrid system with other libraries derived from human tissues.

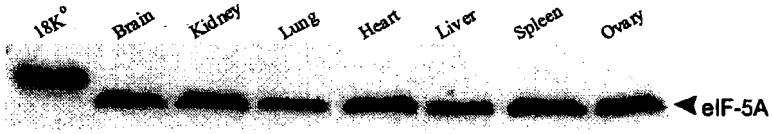
We have also examined the possibility of whether eIF-5A may directly interact with RNA.[27] Fig. 6 illustrates that eIF-5A can bind to RRE RNA and that hypusine modification on eIF-5A appears to be necessary for the binding between eIF-5A and RNA.[27] This finding raises the possibility that eIF-5A can serve as an RNA binding protein that may be involved in RNA processing. It is interesting that recently eIF-5A has been suggested to be involved in mRNA stabilization in yeast.[28] Currently, we are using the SELEX (systematic evolution of ligand exponential enrichment) method[29] to determine the RNA sequence specificity for eIF-5A recognition. If indeed eIF-5A binds to both protein and RNA *in vivo*, it can be viewed as a bimodular protein, similar to many other DNA and RNA binding proteins. As shown in Fig. 3, eIF-5A polypeptide can be divided into two parts, the N-terminal half and C-terminal half, which are connected by a short flexible hinge. It is very likely that each part of eIF-5A contains one functional domain. We have speculated that the N-terminal half may contain the RNA binding domain because of the presence of a cluster of basic amino acid residues around the hypusine site. On the other hand, it is also likely that the C-terminal half contains the RNA binding domain since it resemble structurally to cold shock protein. Future mutational analysis is needed to determine the location of the functional domains in eIF-5A.

POSSIBLE ROLE OF eIF-5A IN CANCER, AGING, AND AIDS

In an initial effort to investigate the physiological significance of eIF-5A, we have compared hyusine formation



(A)



(B)

Fig. 5. Tissue distribution of deoxyhypusine synthase activity and eIF-5A in mouse. (A) Deoxyhypusine synthase activity in tissue samples obtained from CD-1 female mouse. The enzyme activity was analyzed by using *in vitro* labeling assay. One unit of enzyme activity indicates the formation of 1 pmol of deoxyhypusine residue per hour. (B) Relative amount of eIF-5A in various CD-1 mouse as estimated by Western blot analysis.

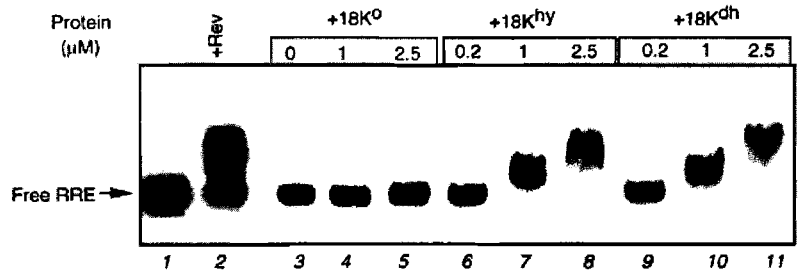


Fig. 6. RNA gel mobility shift assay indicating the interaction between RRE RNA and eIF-5A. Radiolabeled RRE RNA (252 nt) was incubated with Rev, unmodified eIF-5A (18K°), mature eIF-5A isolated from HeLa cells (18K^{hy}), or partially modified deoxyhypusine-containing eIF-5A (18K^{dh}). The binding mixture was analyzed by nondenaturing 4% polyacrylamide gel electrophoresis and autoradiography. Lane 1, free RRE RNA; lane 2, RRE with 0.1 μM Rev; lane 3, RRE; lane 4, RRE with 1 μM 18K°; lane 5, RRE with 2.5 μM 18K°; lane 6, RRE with 0.2 μM 18K^{hy}; lane 7, RRE with 1.0 μM 18K^{hy}; lane 8, RRE with 2.5 μM 18K^{hy}; lane 9, RRE with 0.2 μM 18K^{dh}; lane 10, RRE with 1.0 μM 18K^{dh}; lane 11, RRE with 2.5 μM 18K^{dh}. (Adapted from Ref. 27).

activity and eIF-5A level in NIH3T3 cells and in v-Ha-Ras-transformed 3T3 cells.[30] In addition, we have also examined various parameters related to hypusine formation activity in normal human diploid fibroblasts during cell senescence.[31] We found a marked increase of hypusine formation in transformed cells as compared to the normal counterparts[30] and a striking attenuation of hypusine formation activity in normal cells during senescence.[31] These studies substantiate the notion that hypusine formation is tightly coupled to cell proliferation and suggest that the enzymes involved in hypusine formation may be a good target for chemotherapeutic manipulation. Toward this goal several modified spermidine and diaminohydrocarbon analogs have been synthesized and tested as potential inhibitors for deoxyhypusine synthase.[32,33] Among them, N^1-guanyl-1,7-diaminoheptane (GC7) turns out to be the most potent one, with an apparent Ki value less than 10 nM.[33] Inhibition of deoxyhypusine synthase by GC7 causes growth arrest of mammalian cells.[33,34] However, within a narrow concentration range, GC7 could promote the differentiation of mouse neuroblastoma cells in the presence of a suboptimal amount of dibutyryl cAMP.[34] Thus modulation of hypusine formation clearly has profound effect on cell physiology.

One unexpected finding is the possible link of eIF-5A to the action of HIV-1 Rev protein.[22] Rev plays a key role in the complex regulation of HIV viral gene expression.[22] The finding that eIF-5A binds to Rev is significant not only because of the implication in AIDS therapy, but also because it suggests a new avenue to probe the action of eIF-5A. If eIF-5A is indeed involved in the action of Rev, one would expect to observe a co-localization of eIF-5A and Rev in nucleus. The conflicting results on the localization of eIF-5A[22,23] have cast some doubt on the validity of eIF-5A-Rev interaction. Nevertheless, using a different experimental approach, work from another lab suggests that eIF-5A may interact with HTLV-1 Rex protein.[35] Their study substantiates the notion that eIF-5A may be involved in the action of these retroviral proteins, since Rex and Rev are functionally interchangeable.

PERSPECTIVES

The current effort on hypusine research is focused on the following two fronts: (1) further characterization of the enzyme biochemistry involved in hypusine formation and (2) continued search for the function of eIF-5A in various biological systems. However, there are still areas of hypusine research which remain unexplored. For example, physiological roles and metabolism of hypusine formation have not been actively investigated in plants or in archaebacteria. The mechanism of the regulation of hypusine formation in cultured cells or in animals under various physiological states has not been carefully studied. Thus, it is unclear how eIF-5A is degraded *in vivo* and what regulatory pathway controls its biosynthesis and degradation. The potential of using deoxyhypusine synthase and eIF-5A as chemotherapeutic targets for cancers and AIDS have not been systematically explored. Patients with familial hyperlysinemia show dramatic increase in hypusine level in their urine.[36] The mechanism and significance of this finding have not been investigated. Free hypusine is present in various tissues and body fluid. It is unclear whether free hypusine may have its own biological activities. In this regard,

it is interesting that several hypusine derivatives, including GABA-hypusine, have been found in animal tissue and body fluid.[37] The predominent presence of GABA-hypusine in the brain prompts one to speculate that GABA-hypusine may function as a neurotransmitter.

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Key Words

Hypusine; eIF-5A; Deoxyhypusine synthase.

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