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Interaction of Eukaryotic Initiation Factor 5A with the Human Immunodeficiency Virus Type 1 Rev Response Element RNA and U6 snRNA Requires Deoxyhypusine or Hypusine Modification

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Eukaryotic initiation factor 5A
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Hypusine
RNA
Rev

Abstract
Hypusine formation on the eukaryotic initiation factor 5A (eIF-5A) precursor represents a unique posttranslational modification that is ubiquitously present in eukaryotic cells and archaebacteria. Specific inhibition of deoxyhypusine synthase leads to growth arrest and cell death. The precise cellular function of eIF-5A and the physiological significance of hypusine modification are not clear. Although the methionyl-puromycin synthesis has been suggested to be the functional assay for eIF-5A activity in vitro, the role of eIF-5A in protein synthesis has not been established. Recent studies have suggested that eIF-5A may be the cellular target of the human immunodeficiency virus type 1 Rev and human T cell leukemia virus type 1 Rex proteins. Motif analysis suggested that eIF-5A resembles a bimodular RNA-binding protein in that it contains a stretch of basic amino acids clustered at the N-terminal region and a leucine-rich stretch at the C-terminal region. Using Rev target RNA, RRE, as a model, we tested the hypothesis that eIF-5A may be an RNA-binding protein. We found that both deoxyhypusine and hypusine-containing eIF-5A can bind to the 252-nt RRE RNA, as determined by a gel mobility shift assay. In contrast, the unmodified eIF-5A precursor cannot. Deoxyhypusine-containing eIF-5A, but not its precursor, could also cause supershift of the Rev stem-loop IIB RRE complex. Preliminary studies also indicated that eIF-5A can bind to RNA such as U6 snRNA and that deoxyhypusine modification appears to be required for the binding. The ability of eIF-5A to directly interact with RNA suggests that deoxyhypusine formation of eIF-5A may be related to its role in RNA processing and protein synthesis. Our study also suggests the possibility of using a gel mobility shift assay for eIF-5A-RNA binding as a functional assay for deoxyhypusine and hypusine formation.

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Introduction

The highly conserved nature of eukaryotic initiation factor 5A (eIF-5A) protein and the responsiveness to growth stimulation [1–3], together with the recognized importance of polyamines in growth regulation [4–6], suggest that hypusine has an important role in cell physiology. Disruption of the two eIF-5A genes or the deoxyhypusine synthase gene in yeast has been shown to be lethal [7, 8]. The notion that eIF-5A is an initiation factor comes from the earlier observations that it was isolated from the ribosomal high-salt wash fraction and that it can stimulate the synthesis of methionyl-puromycin [9–11]. However, lack of a strong correlation between eIF-5A and general protein synthesis has raised doubt on the role of eIF-5A in translation initiation [12–14]. Recently, Ruhl et al. [15] showed that eIF-5A can be cross-linked to a 19-amino-acid synthetic Rev effector domain (amino acids, aa, 75–93), suggesting that eIF-5A may be the cellular target of Rev. The human immunodeficiency virus type I (HIV-1) Rev protein is a positive post-transcriptional regulator of viral structural gene expression *(gag, pol* and *env)* and is the first protein that has been shown to regulate the nuclear export of unspliced or singly spliced pre-mRNA in a sequence-specific manner [16, 17]. Katahira et al. [18] have recently shown that an expression of eIF-5A in COS-7 cells restored the Rex function impaired by a *trans*-dominant Rex mutant, TAg-Rex, suggesting that eIF-5A may serve as the Rex target protein. If indeed Rev recruits (or hijacks) eIF-5A for viral RNA processing, this would imply that eIF-5A may be involved in nuclear RNA processing and transport. Dual nuclear and cytoplasmic functions are characteristic of many RNA-binding proteins like the mRNA 5′-cap-binding protein eIF-4E [19, 20]. Using Rev-Rev response element (RRE) binding as a model, we have examined the possible interaction of eIF-5A protein (precursor, deoxyhypusine form and hypusine form) with Rev-RRE and RRE alone. Our study demonstrated for the first time that eIF-5A can function as an RNA-binding protein and the binding depends on the presence of deoxyhypusine or hypusine modification.

Methods

Materials

[α-^{32}P]UTP and [γ-^{32}P]ATP (3,000 Ci/mmol) were from ICN Chemical Radioisotope Division, Irvine, Calif., USA. Restriction enzymes and other molecular biological supplies were from Promega, Madison, Wisc., USA or Pharmacia, Piscataway, N.J., USA. All other chemicals were of reagent grade.

RNA Preparation

The 252-nt RRE RNA was synthesized with T7 RNA polymerase using linearized pGEM-RRE as the template in the presence of [α-^{32}P]UTP and purified by electrophoresis as described [21]. The U6 snRNA was synthesized with SP6 RNA polymerase using pGEM1-U6 as the template in the presence of [α-^{32}P]UTP and purified as previously described [22]. The 29-nt RRE stem-loop IIB RNA was synthesized from synthetic DNA templates using the RiboMAX transcription system with T7 RNA polymerase, treated with shrimp alkaline phosphatase and then phosphorylated at the 5′ end with ^{32}P-ATP by T4 polynucleotide kinase [23].

Protein Preparation

The histidine-tagged recombinant human eIF-5A precursor, designated as 18K°, was purified with an Ni(II)-NTA column as previously described [24]. The deoxyhypusine modification was carried out in 0.1 *M* glycine buffer (pH 9.5) containing recombinant human deoxyhypusine synthase, spermidine (30 μ*M*), NAD^+ (1 m*M*) and 18K° (4.5 μ*M*) for 3–5 h at 37°C. The deoxyhypusine-modified eIF-5A, designated as $18K^{dh}$, was then purified from the reaction mixture using mono Q and mono S column chromatography. Wild-type human eIF-5A was purified from HeLa cells as described [25]. Recombinant Rev protein was generated from pT7-SC and purified by ion exchange and

heparin-Sepharose column chromatography as previously described [23].

RNA-Protein Binding

The binding reaction contained ^{32}P-labeled RNA (~ 1 n*M*) and protein at various concentrations in a Tris buffer (43 m*M*, pH 8.0) containing 50 m*M* KCl, 5 m*M* DTT, 8% glycerol, 1 μg *Escherichia coli* tRNA and 10 U RNasin (Promega) in a total volume of 20 μl. After an incubation at 4°C or room temperature for 30 min, the reaction was stopped by adding 4 μl dye solution. The samples were loaded on 4 or 6% nondenaturing polyacrylamide gel for gel electrophoresis in Tris-borate buffer (pH 8.3, 44.5 m*M* Trizma base, 44.5 m*M* boric acid, 0.1% Triton X-100) at 4°C.

Results

Motif Analysis of eIF-5A

RNA-binding proteins such as HIV-1 Rev (116 aa) or Influenza A NS1 protein (237 aa) have a modular structure similar to some transcriptional activator proteins in that they include: (1) an arginine-rich basic domain (aa 35–50 in Rev) required for RNA binding and (2) a leucine-rich effector domain (aa 75–84 in Rev) mediating protein-protein interaction. The RNA-binding domains in Rev or NS1 overlap with a nuclear localization signal (NLS). Three major types of NLS have been reported, each containing a cluster of basic amino acids [26]. Figure 1 shows that the human eIF-5A has a stretch of basic amino acid residues (aa 44–57) located at the N-terminal part of the protein. Within this 11-amino-acid stretch, there are 6 basic residues, including histidine, which may contain close to 6 positive charges (deoxyhypusine or hypusine carries two positive charges) under appropriate conditions. This charge density is comparable to that of the classical NLS in SV40 T antigen (KKKRKVEDPK), which contains 6/10 basic residues, with 4 net-positive charges. It is therefore tempting to speculate that this hypusine-containing sequence may have the potential to serve as an RNA-binding site, similar to NLS in Rev and other Rev-like proteins. The Rev effector domain (aa 75–84) contains a leucine-rich motif that is also present in several nuclear shuttle proteins, including Rex, NS1, protein kinase A inhibitor α and β and TFIIIA [27, 28]. Recent studies have convincingly shown that this motif represents the nuclear export sequence [27, 28]. A 14-amino-acid stretch (aa 89–102) in eIF-5A has a sequence similar to that of the effector domains in Rev (aa 71–84) and NS1 (aa 134–147) (fig. 1). There are 4 leucine and 2 isoleucine residues within this 14-amino-acid stretch which are conserved in eIF-5A from yeast to human. Interestingly, there are 4 leucine and 2 isoleucine residues in the effector domain (aa 134–147) of NS1, too. In addition, two other segments (112-LRLPEGDLG-KEI-113 and 133-ILITVLSAM-140) in eIF-5A resemble a leucine-rich repeat, a motif recently proposed to be involved in protein-protein interaction [29]. Again, most of the hydrophobic residues in these two sequences are conserved in eIF-5A from yeast to human. It is noteworthy that mutant eIF-5A with mutations within amino acids 133–140 can block Rev translocation [30], suggesting that this region may be involved in Rev-mediated nuclear export.

Binding of eIF-5A to RRE RNA

Based on motif analysis as described above, we decided to use Rev target RNA, RRE, as a model to examine whether eIF-5A had RNA-binding activity. If the result was positive, we wished to know whether deoxyhypusine or hypusine modification was required for the interaction. Histidine-tagged recombinant human eIF-5A precursor was used to prepare the deoxyhypusine form of eIF-5A, designated as 18K^{dh}. Since we have not yet developed a procedure to generate pure hypusine-modified recombinant eIF-5A

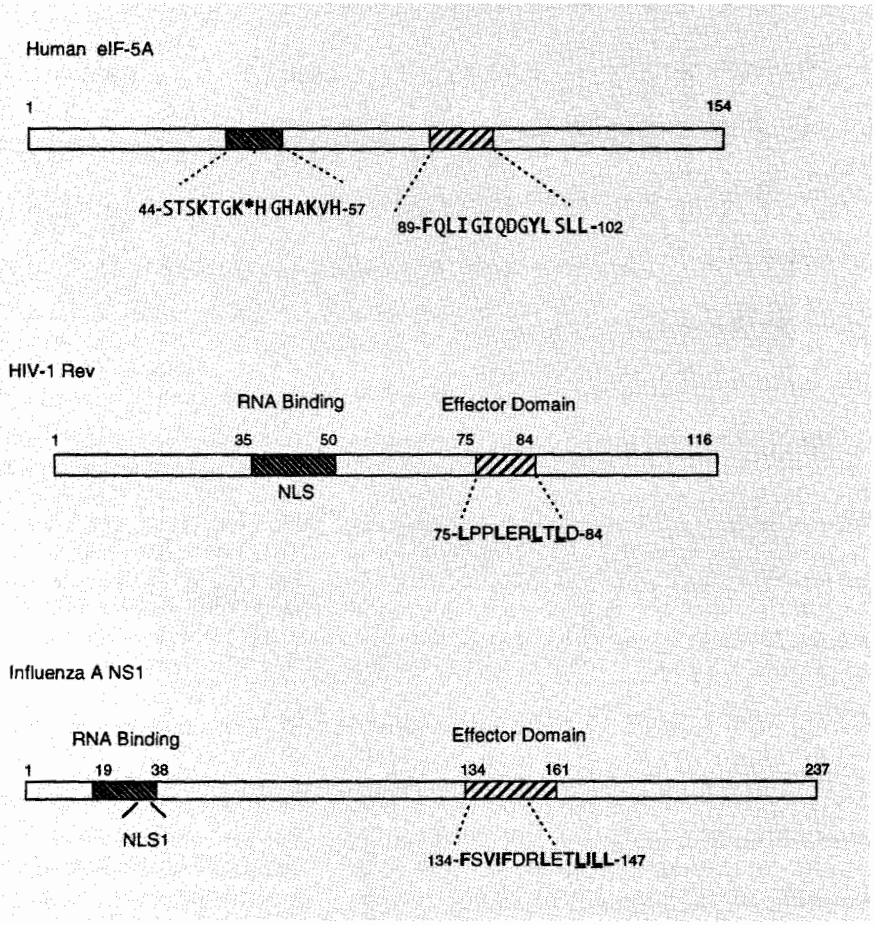

Fig. 1. Motif comparison of human eIF-5A with HIV-1 Rev and Influenza A NS1 protein. The hypusination site (K50) is marked with an asterisk. The basic amino acids in stretch 44–57 and the hydrophobic residues in stretch 89–102 are in boldface. The RNA-binding sites for Rev and NS1 are shown as hatched blocks. The hydrophobic amino acid residues in the effector domain in Rev and NS1 are shown with boldface, and the two critical leucine residues are underlined.

($18K^{hy}$), we have used eIF-5A purified from HeLa cells (>95% in hypusine form) as $18K^{hy}$. The three different forms of eIF-5A (18K°, $19K^{dh}$ and $18K^{hy}$) were tested for their binding activity with radiolabeled RRE RNA by a gel mobility shift assay. Figure 2 shows that the unmodified eIF-5A (18K°) did not cause a mobility shift of the RNA probe. In contrast, both deoxyhypusine-modified ($18K^{dh}$) and wild type eIF-5A ($18K^{hy}$) clearly exhibited binding activity to RRE RNA. These results demonstrate that eIF-5A can bind to RNA and that such binding depends on the presence of deoxyhypusine/hypusine modification. It has been shown that tRNA has two defined binding sites for spermidine [31].

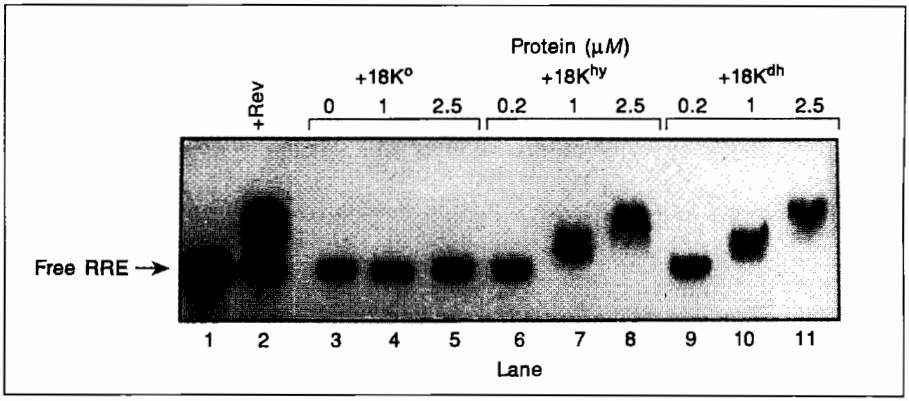

Fig. 2. RNA gel mobility shift assay showing interaction between eIF-5A and RRE. Radiolabeled RRE RNA (252 nt) was prepared as described in 'Methods'. Samples with a total volume of 20 μl containing 20,000 cpm of radiolabeled RRE RNA and unmodified (18K°), modified (18K^{dh}) or wild-type HeLa eIF-5A (18K^{hy}) protein were incubated at room temperature for 30 min in the presence of 1 μg *E. coli* tRNA and 20 units of RNasin. The binding mixture was analyzed by nondenaturing 4% polyacrylamide gel electrophoresis and autoradiography. Lane 1: free RRE RNA; lane 2: RRE with 0.1 μ*M* Rev; lane 3: RRE; lane 4: RRE with 1 μ*M* 18K°; lane 5: RRE with 2.5 μ*M* 18K°; lane 6: RRE with 0.2 μ*M* 18K^{hy}; lane 7: RRE with 1.0 μ*M* 18K^{hy}; lane 8: RRE with 2.5 μ*M* 18K^{hy}; lane 9: RRE with 0.2 μ*M* 18K^{dh}; lane 10: RRE with 1.0 μ*M* 18K^{dh}, and lane 11: RRE with 2.5 μ*M* 18K^{dh}.

Since the side chain of deoxyhypusine is homospermidine, it is possible that the deoxyhypusine (or hypusine) residue is directly involved in RNA binding. The binding affinity of 18K^{dh} or 18K^{hy} to RRE is about 10-fold less than that of Rev. This is not surprising, since clearly RRE is not the native RNA target of eIF-5A. Figure 2 also shows that 18K^{dh} and 18K^{hy} exhibited similar binding activity with respect to RRE RNA. Thus, at least in the case of RRE, the hydroxyl group on the hypusine residue may not be important in the binding.

Interaction of 18K^{dh} with the RRE-Rev Complex

Possible interaction of eIF-5A with the Rev-RRE complex was examined by a gel mobility supershift assay. For this series of experiments, we used the 29-nt stem-loop IIB, the RRE high-affinity-binding site for Rev, as the probe in the binding assay. Figure 3 shows that 18K^{dh}, but not 18K°, produced a clear supershift of the RRE-Rev complex. Bevec et al. [30] have shown that GST-fused eIF-5A precursor protein (GST-18K°) could produce a supershift of the RRE (252 nt)-Rev complex. However, in our binding assay, GST-18K° did not cause any detectable supershift of the 29-nt RRE-Rev complex (fig. 3, lanes 6–8). The apparent discrepancy could be due to differences in the RRE probes used. The 252-nt RRE (~83,000 dalton), but not the IIB RRE, could allow mixed multimer formation of Rev to occur along the long stem [32].

Interaction of eIF-5A with U6 snRNA and the U6-NS1 Complex

The influenza A NS1 protein (237 aa) binds to the poly(A) sequence at the 3′ end of NS2 mRNA and of other mRNA containing

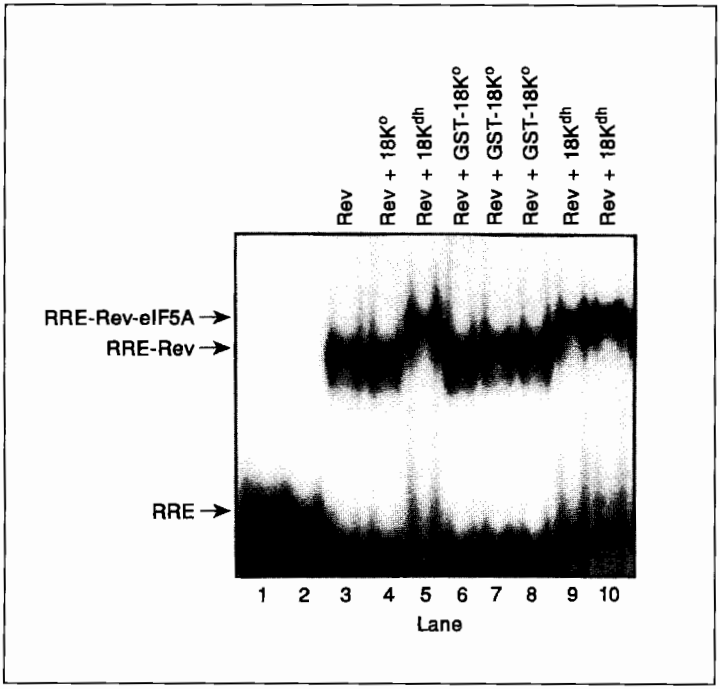

Fig. 3. RNA gel mobility supershift assay. The binding reaction was carried out in the binding buffer (25 m*M* Tris, pH 7.4, 40 m*M* NaCl, 0.05% Triton X-100, 1 m*M* DTT). ^{32}P-labeled 29-nt stem-loop IIB RRE and Rev (0.1 μ*M*) were added to the buffer and incubated for 30 min on ice. Where indicated, eIF-5A protein was then added to the mixture, and the incubation was carried out for another 30 min. The reaction mixture was analyzed on a 6% nondenaturing polyacrylamide gel after electrophoresis. Binding complexes were visualized by autoradiography. Lane 1: single-strand RNA; lane 2: duplex 29-nt RRE; lane 3: Rev-RRE; lane 4: Rev-RRE and 18K° (13 μ*M*); lane 5: Rev-RRE and 18K^{dh} (2 μ*M*); lanes 6–8: Rev-RRE and GST-18K° (1, 6, 12 μ*M*, respectively); lane 9: Rev-RRE and 18K^{dh} (1 μ*M*), and lane 10: Rev-RRE and 18K^{dh} (2 μ*M*).

the poly(A) 3′ end [33]. NS1 is a modular protein, with an arginine-rich RNA-binding domain and NLS, and an effector domain with a sequence (FDRLETLILL) similar to that in Rev and Rev-like proteins. However, unlike Rev that facilitates the nuclear export of viral mRNA, NS1 inhibits the nuclear export of poly(A)-containing mRNA [34]. Recently, the NS1 protein was found to bind spliceosomal U6 snRNA at a specific stem-bulge structure [22]. This binding leads to an inhibition of pre-mRNA splicing in vitro and in vivo [22].

As an initial approach to explore the specificity of eIF-5A-RNA interaction, we also examined whether eIF-5A can bind to U6 snRNA. Figure 4 shows that 18K^{dh}, but not 18K°, can bind to U6 RNA. In addition, 18K^{dh}, but not 18K°, produced a clear supershift of the NS1-U6 complex (fig. 4, lane 7 vs. lane 5), suggesting that there may also exist interaction between eIF-5A and NS1.

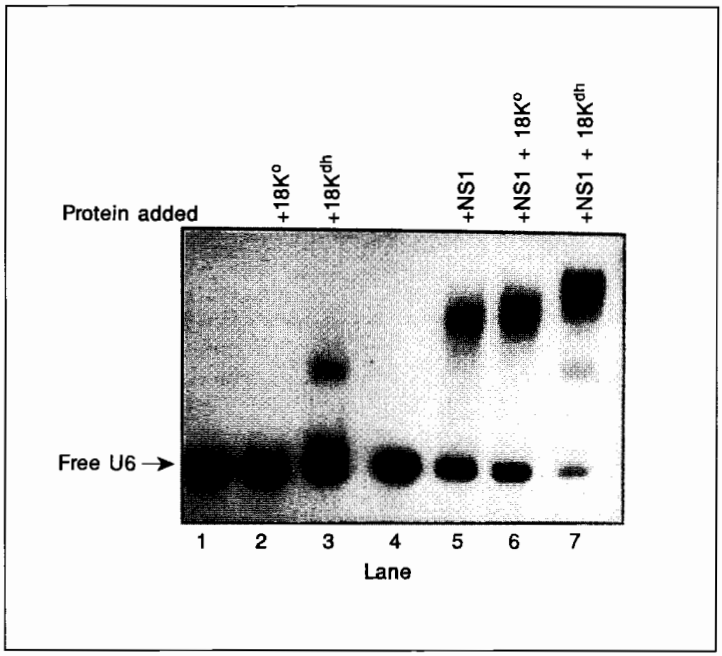

Fig. 4. Interaction of eIF-5A with U6 and the NS1-U6 complex. Each reaction mixture contained 10,000 cpm ^{32}P-labeled U6 RNA, 1 μg of *E. coli* tRNA and 20 units RNasin in a total volume of 20 μl, as described in 'Methods'. Lane 1: U6 alone; lane 2: U6 plus 18K° (1 μ*M*); lane 3: U6 plus 18K^{dh} (1 μ*M*); lane 4: U6; lane 5: U6 with NS1 protein (1 μ*M*); lane 6: U6 and NS1 (1 μ*M*) with 18K° (1 μ*M*), and lane 7: U6 and NS1 (1 μ*M*) with 18K^{dh} (1 μ*M*).

Discussion

The availability of recombinant eIF-5A precursor (18K°) allows us to modify this protein in a step-wise manner and examine possible differences in biological activities between unmodified and serially modified forms of eIF-5A (i.e. 18K° vs. 18K^{dh} or 18K^{hy}). Our studies showed that eIF-5A can bind to RRE RNA and U6 snRNA and that the binding requires the presence of deoxyhypusine or hypusine residues on eIF-5A (fig. 2, 4). Until now, the methionyl-puromycin assay was the only functional assay reported for deoxyhypusine/hypusine formation in vitro [10, 11]. The complexity of the methionyl-puromycin assay system, however, makes it cumbersome and difficult to use on a routine basis. Our present study suggests that the gel mobility shift assay for eIF-5A-RNA interaction can be used as a convenient functional assay for deoxyhypusine/hypusine formation. In addition, this assay system should allow us to further probe the structural basis for eIF-5A-RNA interaction. Our studies also indicate that deoxyhypusine-containing eIF-5A can cause supershift of Rev-RRE (IIB) and NS1-U6 complexes (fig. 3, 4). It is not clear whether the supershift is caused by an additional interaction of eIF-5A with the RNA or direct binding with Rev (or NS1), or both. Using protein affinity chromatography, we found that both 18K° and 18K^{dh} can bind Rev with equal affinity [Yan, Y.P. and Chen, K.Y., unpubl. results], suggesting that deoxyhypusine modification is not necessary for eIF-5A-Rev binding. The mode of interaction of eIF-5A with the Rev-RRE or NS1-U6 complexes is currently under investigation.

The long half-lives of eIF-5A [14] and lack of a mechanism to reverse hypusine formation suggest that the cellular eIF-5A activity

may not be regulated simply by hypusine formation alone. The regulation of eIF-5A activity can be achieved through one or a combination of the following means: (1) cellular deoxyhypusine synthase activity, including enzyme level, NAD^+, spermidine concentration, etc; (2) biosynthesis and degradation of eIF-5A; (3) compartmentalization of eIF-5A, and (4) interactions with its interacting partners. Our studies suggest that eIF-5A is capable of interacting with RNA and possibly with other RNA-binding proteins, too. It is conceivable that eIF-5A may interact with different RNA and proteins during the course of cell cycling or differentiation. If so, identification of eIF-5A interacting proteins and target RNA in cells will be essential for understanding the regulation and functional significance of eIF-5A.

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