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The polyamine-derived amino acid hypusine: its post-translational formation in eIF-5A and its role in cell proliferation

Minireview Article

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Summary. The unusual amino acid hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] is a unique component of one cellular protein, eukaryotic translation initiation factor 5A (eIF-5A, old terminology, eIF-4D). It is formed posttranslationally and exclusively in this protein in two consecutive enzymatic reactions, (i) modification of a single lysine residue of the eIF-5A precursor protein by the transfer of the 4-aminobutyl moiety of the polyamine spermidine to its ε-amino group to form the intermediate, deoxyhypusine [N^{ε}-(4-aminobutyl)lysine] and (ii) subsequent hydroxylation of this intermediate to form hypusine. The amino acid sequences surrounding the hypusine residue are strictly conserved in all eukaryotic species examined, suggesting the fundamental importance of this amino acid throughout evolution. Hypusine is required for the activity of eIF-5A *in vitro*. There is strong evidence that hypusine and eIF-5A are vital for eukaryotic cell proliferation. Inactivation of both of the eIF-5A genes is lethal in yeast and the hypusine modification appears to be a requirement for yeast survival (Schnier et al., 1991 [Mol Cell Biol 11: 3105–3114]; Wöhl et al., 1993 [Mol Gen Genet 241: 305–311]). Furthermore, inhibitors of either of the hypusine biosynthetic enzymes, deoxyhypusine synthase or deoxyhypusine hydroxylase, exert strong anti-proliferative effects in mammalian cells, including many human cancer cell lines. These inhibitors hold potential as a new class of anticancer agents, targeting one specific eukaryotic cellular reaction, hypusine biosynthesis.

Keywords: Amino acids – Hypusine – Deoxyhypusine – eIF-5A – Polyamines – Posttranslational modification

Introduction

Hypusine [N^{ε}-(4-amino-2-hydroxybutyl)-L-lysine] is a unique amino acid which occurs in all eukaryotic cells as a single residue of one protein, eukaryotic protein synthesis initiation factor 5A (eIF-5A)[1] (for a review[2] see Park et al., 1993a). This unusually basic amino acid was discovered in bovine brain extracts by Shiba et al. (1971), who determined its structure and named it hypusine on the basis of its structural relationship to portions of two compounds, *hy*droxy*pu*trescine and ly*sine*. The stereochemistry was later established as (2S, 9R)-2,11-diamino-9-hydroxy-7-azaundecanoic acid (Shiba et al., 1982). Subsequently, it was found in various animal tissues (Nakajima et al., 1971; Imaoka and Nakajima, 1973), in diverse eukaryotic species (Park, 1993a), and in archaebacteria (Schümann and Klink, 1989), as a component of protein as well as the free amino acid. It is not found in eubacteria. The formation of hypusine through a specific posttranslational modification of the eIF-5A precursor protein is well established. No pathway for its biosynthesis as a free amino acid is known; free hypusine in cells or tissues most likely is derived from the proteolytic degradation of eIF-5A. Excretion of hypusine in the urine suggests that it is an endometabolite (Nakajima et al., 1971). In brain, which contains the highest concentration of free hypusine, γ-aminobutyrylhypusine and β-alanylhypusine were found and postulated to be involved in neurotransmission (Sano et al., 1986; Ueno et al., 1991). There is no substantial evidence, however, for the biological significance of hypusine as the free amino acid. Thus, the main physiological role for hypusine appears to be as an essential component of eIF-5A, a protein known to be vital for eukaryotic cell proliferation (Park et al., 1993a, 1993b).

eIF-5A, the hypusine-containing protein

The hypusine-containing protein was serendipitously detected in experiments designed to identify protein(s) into which polyamines were incorporated by culturing human peripheral lymphocytes in the presence of [^{3}H]putrescine or [^{3}H]spermidine (Park et al., 1981). One labeled protein (M_r 18,000; pI 5.3) was found (Fig. 1). The single radiolabeled component released upon acid hydrolysis was identified as hypusine. By proteolytic digestion of the labeled protein and Edman degradation of tryptic peptides, hypusine was shown to occur at a single position in this protein (Park et al., 1984a). The radiolabeling of the 18-kDa protein was markedly increased in mitogen-treated

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[1] In the recommended nomenclature of initiation factors, NC-IUB (Safer, 1989), the earlier notations for this factor, e.g. eIF-4D, were changed to eIF-5A (eukaryotic initiation factor 5A) to indicate its presumed site of action late in the initiation phase of protein synthesis.

[2] A more complete citation of the original literature is found in a recent review Park et al., (1993a). In the interest of brevity only selected references could be included in this minireview.

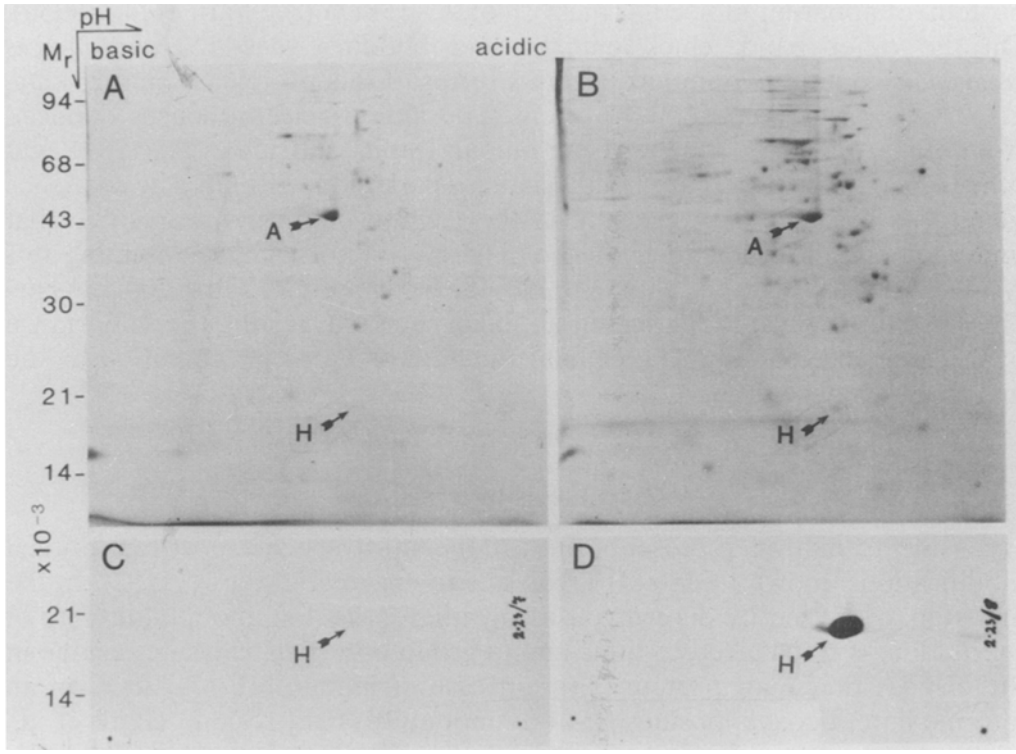

Fig. 1. [^{3}H]spermidine radiolabels a single protein in resting (**A,C**) and mitogen-stimulated (**B,D**) lymphocytes after culture for 48 h. Cell proteins were separated by two-dimensional polyacrylamide gel electrophoresis: **A,B** stained with Coomassie blue; **C,D** the lower portion of a fluorogram from the same gel. The hypusine-containing protein is designated by H, and actin, a reference marker, by A. Adapted from Cooper et al. (1982)

lymphocytes compared to resting cells, suggesting that this protein is important in cell proliferation (Cooper et al., 1982). Identification of the hypusine-containing protein as eIF-5A was based on the co-migration of the two proteins on two-dimensional gel electrophoresis, and the finding that eIF-5A isolated independently as an initiation factor was found to contain 1 mol/mol of hypusine (Cooper et al., 1983). eIF-5A was initially purified from ribosomes of rabbit reticulocytes as a factor that stimulates methionyl-puromycin synthesis in a model assay for translation initiation (Kemper et al., 1976). However, it did not enhance translation of a natural globin messenger RNA (Schreier et al., 1977). A recent study on the effects of eIF-5A depletion on yeast protein synthesis suggests that it is not a typical initiation factor involved in global protein synthesis (Kang and Hershey, 1994), but instead it may serve as an initiation factor selective for a subset of specific mRNAs.

eIF-5A exists as one 18-kDa form in all mammalian cells. Other eukaryotes, *e.g. Drosophila melanogaster*, plants, *Neurospora crassa* and *Dictyostelium discoideum*, also appear to contain a single hypusine-containing

protein, of apparent molecular mass 18 or 20–21 kDa (see Park et al., 1993a). On the other hand, chick embryo and budding yeast, *Saccharomyces cerevisiae*, contain two molecular mass forms of this protein, 18 and 20 kDa. eIF-5A is a highly conserved protein. The amino acid sequences deduced from eIF-5A cDNAs or genes from human (Smit-McBride et al., 1989), and other eukaryotic species are highly similar (Park et al., 1993a). The sequence identity is remarkable, especially in the vicinity of the lysine residue that undergoes modification to hypusine. The 12 amino acids surrounding this lysine residue (marked with *), *i.e.* -Ser-Thr-Ser-Lys-Thr-Gly-Lys*-His-Gly-His-Ala-Lys-, are identical in all eukaryotes, suggesting the importance of this sequence for a crucial cellular function and/or for recognition by the modifying enzymes.

Biosynthesis of hypusine

Hypusine formation represents one of the most specific posttranslational modifications known to date. It involves two enzymatic steps (Fig. 2). In the first step, catalyzed by deoxyhypusine synthase, the 4-aminobutyl moiety of spermidine is transferred to the ε-amino group of a specific lysine residue in the eIF-5A precursor protein (Lys^{50} in case of human eIF-5A) to form an intermediate, deoxyhypusine [N^{ε}-(4-aminobutyl)lysine] residue (Park et al., 1982). In the second step, catalyzed by deoxyhypusine hydroxylase, the

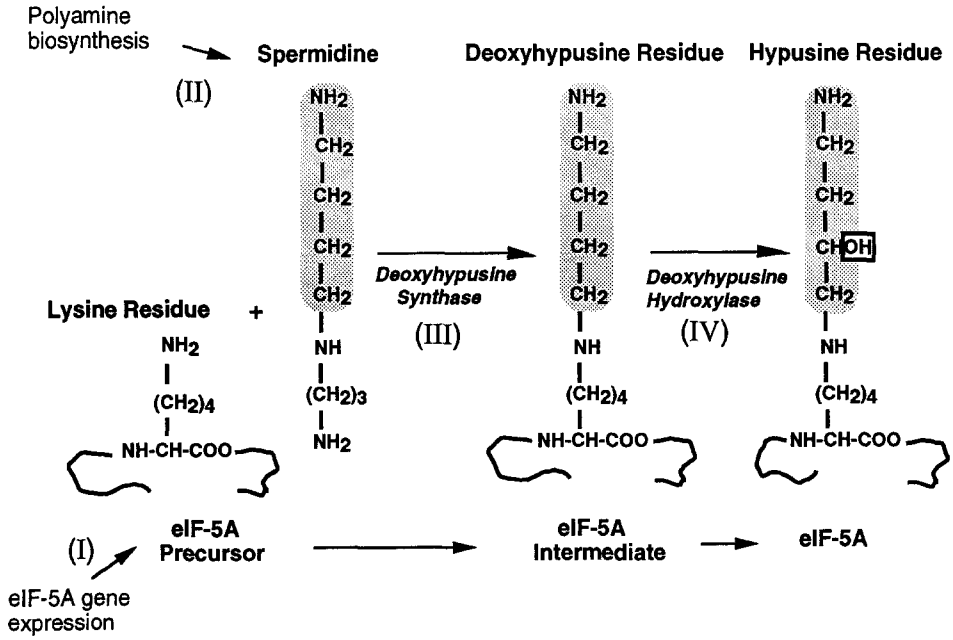

Fig. 2. Schematic representation of the posttranslational formation of eIF-5A. The 4-aminobutyl group in deoxyhypusine and hypusine that is transferred from spermidine is indicated by shading and the hydroxyl group of hypusine is outlined. Modified from Park et al. (1993b)

hydroxylation of the side chain of this intermediate completes hypusine synthesis and eIF-5A maturation (Abbruzzese et al., 1986). Unlike many other posttranslational modification reactions, hypusine synthesis is irreversible; no reversal pathway is known.

The precise structural contributions of spermidine and lysine to hypusine formation were determined in a series of experiments carried out in cultured CHO cells using precursors labeled with isotopes at specific positions, followed by identification of the location of the isotopes in hypusine isolated from acid hydrolysates of the cellular protein (Fig. 3) (Park et al., 1984b; Park and Folk, 1986). These experiments enabled us to determine the origin of the atoms of hypusine and provided definitive evidence for the precursor role of spermidine, and for the removal of one hydrogen from carbon 5 of spermidine during deoxyhypusine synthesis (Park and Folk, 1986).

The use of metal chelators, *e.g.* α,α-dipyridyl, led to the identification of deoxyhypusine [N^{ε}-(4-aminobutyl)lysine] as an intermediate in hypusine biosynthesis, Park et al. (1982). In CHO cells cultured with [^{3}H]putrescine or [^{3}H]spermidine in the presence of α,α-dipyridyl, a new radiolabeled component, identified as deoxyhypusine, accumulated in the 18 kDa protein in place of [^{3}H]hypusine. After removal of the metal chelator, conversion of the deoxyhypusine residue to the hypusine residue was observed in cells or in lysates of the cells, suggesting the existence of a metal-dependent enzyme responsible for deoxyhypusine hydroxylation.

In exponentially growing mammalian cells, hypusine synthesis appears to proceed efficiently following translation of the eIF-5A precursor protein. New synthesis of hypusine, measured by radiolabeling with [^{3}H]spermidine, is totally blocked in the presence of cycloheximide, suggesting that the steady state level of the eIF-5A precursor is quite low (Duncan and Hershey, 1986; Park 1987). Likewise, the deoxyhypusine-containing eIF-5A intermediate is not normally detectable in rapidly growing cells (Park et al., 1982). Thus the translation of eIF-5A mRNA, rather than the modification reactions, seems to be the rate limiting step in the biogenesis of eIF-5A. A substantial accumulation of eIF-5A precursor or its intermediate occurs only if one of the modification steps becomes blocked by inhibition of the enzymes (Park et al., 1982), or by depletion of spermidine (Park, 1987, 1988).

Deoxyhypusine synthase

Deoxyhypusine synthase catalyzes the first step in hypusine biosynthesis. *In vitro* this reaction requires NAD^+ (Chen and Dou, 1988), in addition to spermidine, and eIF-5A precursor. The pH optimum of the enzyme is near 9.5 (Murphey and Gerner, 1987). We have presented evidence that NAD^+ accepts a hydrogen from spermidine (Wolff et al., 1990), presumably generating a dehydrospermidine intermediate and/or an enzyme imine intermediate (Fig. 4). In the complete reaction, the transfer of the 4-aminobutyl moiety to the ε-amino group of the lysine residue of the precursor and the subsequent reduction of the imine intermediate generates deoxyhypusine. However, in

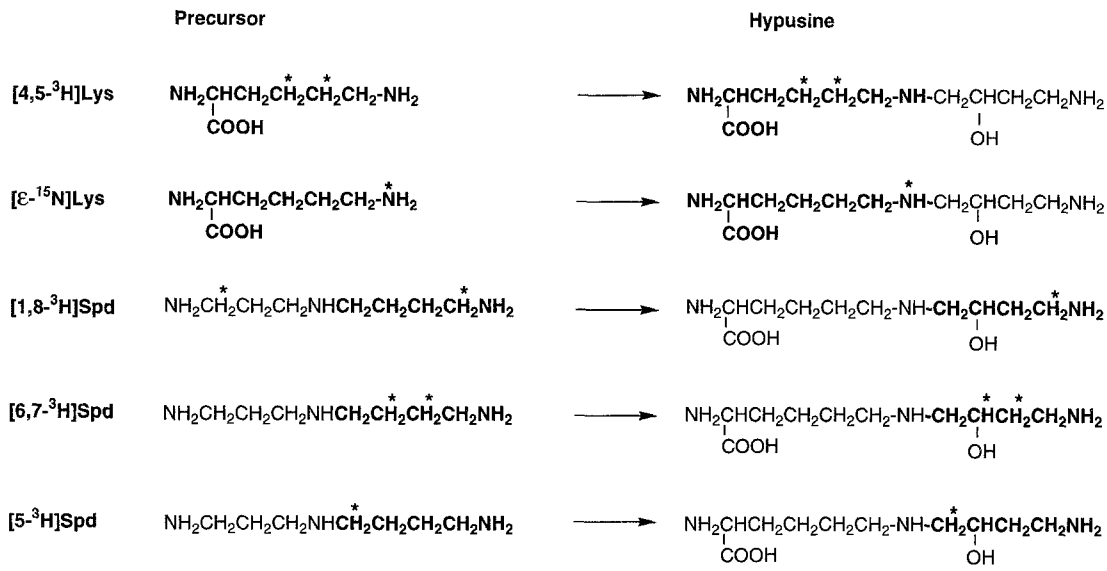

Fig. 3. The origin of the atoms of hypusine deduced by conversion of specifically labeled precursors to correspondingly labeled hypusine in CHO cells. The portion of each labeled molecule that is incorporated into hypusine is shown in boldface, and the labeled atoms are shown by asterisks (*). The locations of these atoms were determined by analysis of the labeled products after periodate/permanganate oxidation. Modified from Park et al. (1993a)

the absence of eIF-5A precursor protein, the enzyme catalyzes the NAD^+-dependent cleavage of spermidine to produce 1,3-diaminopropane and Δ^1-pyrroline (Wolff et al., 1990). The enzyme displays a quite narrow specificity for its substrates. Among many spermidine analogs tested, only (3-aminopropyl)cadaverine (Park et al., 1991), and unsaturated spermidines (Byers et al., 1992), act as amine substrate. Neither free lysine nor a synthetic 9- or 16-member peptide modeled on the amino acid sequence encompassing the lysine residue that undergoes hypusine modification functions as a substrate for deoxyhypusine synthase. Studies by Joe and Park (1994) with various truncated forms of the eIF-5A precursor protein showed that the minimum domain required for deoxyhypusine synthesis is Phe^{30}-Asp^{80}, which corresponds to a region of high amino acid conservation in this protein throughout the eukaryotic kingdom.

Deoxyhypusine synthase has been purified recently from three different species, *Neurospora crassa* (Tao and Chen, 1995), rat testis (Wolff et al., 1995) and HeLa cells (Klier et al., 1995). The human HeLa cDNA (Joe et al., 1995) and the yeast gene (Kang et al., 1995) were cloned and the recombinant enzymes were produced. The enzymes isolated from these species and the recombinant enzymes share many similar physical and catalytic properties (Joe et al., 1995).

The native enzymes consist of four identical subunits of 41 kDa (human), 42 kDa (rat), 40 kDa (*Neurospora crassa*) or 43 kDa (yeast) polypeptides. The deduced amino acid sequences of the human and the yeast enzymes are 58% identical and 72% similar. Sequences of tryptic peptides isolated from the rat

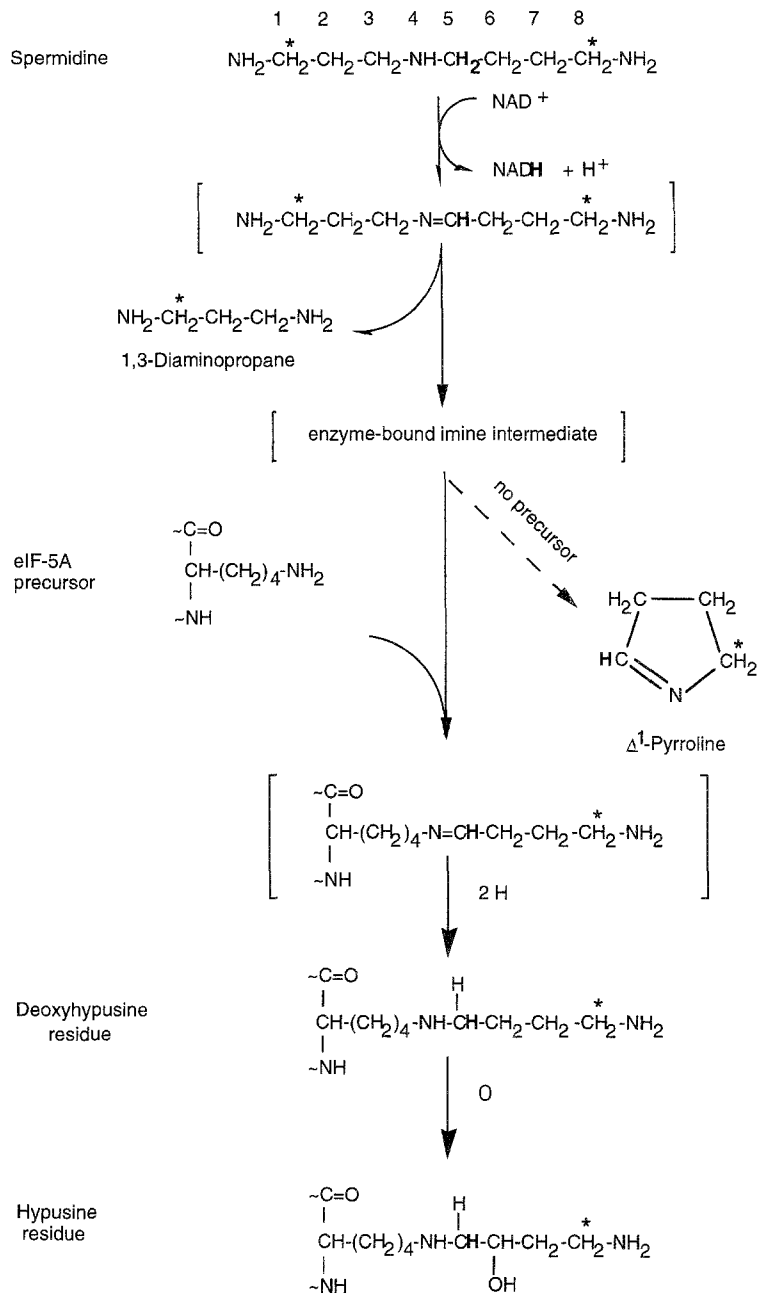

Fig. 4. The proposed enzymatic pathway of hypusine biosynthesis. The positions of the specific tritium labeling is indicated by asterisks for [1,8-^{3}H]spermidine and its metabolites, and by boldface type for [5-^{3}H]spermidine and its metabolites. Postulated transient imine intermediates are shown in brackets. Modified from Wolff et al. (1990)

testis enzyme (Wolff et al., 1995) and the *Neurospora crassa* enzyme (Tao and Chen, 1995) also show good matches with the sequences of the human and yeast enzymes (Joe et al., 1995). Furthermore, the enzyme of rat and the recombinant human and yeast enzymes all exhibit strict specificity toward NAD^+ as NADP, NADH, FAD, or FMN can not substitute for NAD^+ (Wolff

et al., 1990, 1995; Kang et al., 1995; Joe et al., 1995). Despite the fact that the deoxyhypusine synthases from various species exclusively modify a single cellular protein, eIF-5A precursor, they display cross species reactivities with heterologous eIF-5A precursors (Kang et al., 1995). This may be due to the high conservation of amino acid sequences of eIF-5A precursors, as well as those of the enzymes. These enzymes also share very similar features in the catalysis of the partial reaction, spermidine cleavage, and the complete reaction, deoxyhypusine synthesis. The K_m values for spermidine (4–8μM) and the eIF-5A precursor protein (0.4–1μM) are similar for the rat, the human, and the yeast enzymes. The only marked difference noted is in the K_m values for NAD^+, 4.8μM, 40μM, and 720μM for the human, rat, and the yeast enzyme, respectively (Joe et al., 1995).

Deoxyhypusine hydroxylase

The final step in hypusine biosynthesis, hydroxylation at carbon 2 of the 4-aminobutyl portion of the deoxyhypusine residue, is catalyzed by deoxyhypusine hydroxylase. This enzymatic activity is found in all tissues, with a higher content in proliferative ones. The enzyme was partially purified from rat testis (Abbruzzese et al., 1986). Sulfhydryl compounds are required for activity. Dialysable low molecular weight fraction of tissues or cells stimulates the activity, but no specific cofactor has been identified as yet. Addition of α-ketoglutarate, ascorbic acid and Fe^{2+} did not enhance the reaction, suggesting that the mechanism of deoxyhypusine hydroxylase is distinct from that of the α-keto acid-dependent dioxygenases such as lysyl and prolyl hydroxylases. Inhibition of deoxyhypusine hydroxylase by certain metal chelating agents. *e.g.* α,α-dipyridyl (Park et al., 1982) and hydralazine (Paz et al., 1984) and by catechol peptides designed for binding to its active site (Abbruzzese et al., 1991) provide evidence for the role of a tightly-bound metal; this metal, however, has not been identified.

Role of hypusine in cell proliferation

Early observations of a correlation between hypusine formation and proliferation in various mammalian cells led to an initial suggestion of an important role of hypusine in growth (Cooper et al., 1982). Hypusine was shown to be essential for the *in vitro* activity of eIF-5A; mature eIF-5A (containing hypusine) caused a 3 to 4-fold stimulation of methionyl-puromycin synthesis, while the unmodified eIF-5A precursor, or eIF-5A variant proteins (containing arginine or homodeoxyhypusine in place of hypusine), were inactive (Park, 1989; Park et al., 1991; Smit-McBride et al., 1989b). The eIF-5A intermediate containing deoxyhypusine exerted only partial activity in this system, suggesting a stringent structural requirement for the interaction of eIF-5A with the protein synthetic machinery. Evaluation of the precise role of hypusine *in vivo* has been difficult because the true physiological function of eIF-5A is as yet unknown. However, there is strong evidence for the require-

ment of hypusine in eukaryotic cell proliferation. Intervention at any one of the four major steps involved in the biogenesis of eIF-5A (Fig. 2): *i.e.*, (I) *de novo* synthesis of the eIF-5A precursor protein, (II) synthesis of the polyamine spermidine, (III) formation of deoxyhypusine, or (IV) deoxyhypusine hydroxylation, leads to arrest of cell growth.

In yeast, inactivation of both of the two eIF-5A genes, *TIF51A* and *TIF51B* is lethal, whereas expression of either one of the two genes permits growth (Schnier et al., 1991). Transfection with a plasmid carrying the wild type gene (*TIF51A*) supported the growth of a null strain (*tif51A::tif51B*). On the other hand transfection with a plasmid carrying a mutated gene in which the codon for the lysine at the modification site was replaced with the arginine codon did not. These findings support the notion that hypusine synthesis is, in fact, vital for yeast growth.

Depletion of spermidine, the amine precursor of hypusine, also causes inhibition of growth in yeast (Tabor and Tabor, 1984), as is the case with mammalian cells (Pegg, 1988). It is well established from studies with inhibitors of polyamine biosynthesis and with mutant cells defective in the synthesis of putrescine and spermidine that polyamines, especially spermidine, are vital for eukaryotic cell replication. The role of spermidine as the direct precursor of hypusine may represent a key cellular function for this polyamine. The observed delay in the onset of growth inhibition after the virtual depletion of cellular spermidine suggested that the arrest in growth is not caused by loss of the polyamine *per se* but rather is a consequence of the exhaustion of a polyamine-derived mediator with a long half-life (Hölttä et al., 1979). Evidence for eIF-5A as this postulated mediator was obtained in L1210 cells depleted of spermidine by administration of AbeAdo, an irreversible inhibitor of S-adenosylmethionine decarboxylase (Byers et al., 1992). Cytostasis appeared to correlate with the depletion of mature eIF-5A due to prolonged suppression of hypusine synthesis. Reversal of the growth inhibition was observed with spermidine, spermine, and only those spermidine analogs that appear to serve as substrates for deoxy hypusine synthase.

Inhibitors of the hypusine synthetic enzymes offer a more direct approach in assessing the role of hypusine in cells. Several inhibitors of deoxyhypusine synthase that are structurally related to spermidine were developed (Jakus et al., 1993; Lee et al., 1995). Among these *mono*-guanyl derivatives of the two diamines, 1,7-diaminoheptane and 1,8-diaminooctane, were especially effective, N^1-guanyl-1,7-diaminoheptane being the most potent inhibitor with a K_i value ~450-fold less than the K_m for spermidine. These guanyl diamines caused effective inhibition of both hypusine formation and growth in CHO cells (Park et al., 1994) and in various human cancer cell lines (Lee et al., 1996). The potential utility of these compounds as novel anti-proliferative drugs is currently under investigation.

In other studies, a panel of metal chelating compounds was used to inhibit deoxyhypusine hydroxylase in mammalian cells and therefore to assess the possible role of deoxyhypusine hydroxylation (Abbruzzese et al., 1991). Certain metal-chelating inhibitors of deoxyhypusine hydroxylase, such as mimosine, caused inhibition of proliferation by arrest of cell cycle progression

at the boundary of the G_1/S transition (Hanauske-Abel et al., 1994). Inhibition of the enzyme and of growth displayed the same dose- and structure-activity dependencies. These findings led to speculation of a function of eIF-5A in cell cycle regulation, conceivably as an initiation factor selective for a subset of mRNAs encoding proteins that have a crucial function in the G_1/S transition.

Concluding remarks

The ubiquity of hypusine, the high conservation of the hypusine-containing protein and its modification enzyme, deoxyhypusine synthase, and the unique specificity of hypusine biosynthesis suggest an important fundamental function for this amino acid and for eIF-5A in eukaryotes. Although several potential functions of eIF-5A have been postulated to date, including that as a selective initiation factor involved in the G_1/S transition, mentioned above, or as a Rev binding protein required for HIV-1 replication (Ruhl et al., 1993), its precise celluar activity still remains an open question. Whatever the true *in vivo* function of eIF-5A may be, the studies discussed here make a convincing case that hypusine and eIF-5A are crucial for cell proliferation. Efforts are underway to identifiy cellular macromolecules to which eIF-5A binds in order to exert its activity, and to elucidate the interactions at the molecular level. The availability of the human and yeast deoxyhypusine synthase cDNA clones, and their recombinant enzymes has made possible attempts by X-ray crystallography to determine the structure of the active site of the enzyme and of the enzyme-substrate complexes. Characterization of the enzyme mechanism and the physical structure of deoxyhypusine synthase should aid in the design and development of more specific and potent inhibitors of the enzyme. Targeted inhibition of hypusine biosynthesis provides new prospects for exogenous control of cell proliferation.

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