Structural Features of the eIF-5A Precursor Required for Posttranslational Synthesis of Deoxyhypusine*

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Eukaryotic translation initiation factor 5A (eIF-5A, older nomenclature, eIF-4D) is a highly conserved protein that contains the unusual amino acid hypusine (N'-\(4\)-amino-2-hydroxybutyl)lysine. The biosynthesis of hypusine occurs posttranslationally in only this protein by modification of a single lysine residue (Lys\(^{40}\)) in the human eIF-5A precursor. The basis for the specificity of this modification with respect to the substrate protein was investigated using fragments of eIF-5A precursor protein, each containing the NH\(_2\) terminus or of substrates for deoxyhypusine synthesis. A series of truncated forms of the eIF-5A precursor protein generated by expression in E. coli of recombinant deletion constructs from the human eIF-5A cDNA generated by specific cleavage by endoproteinases Arg-C, Asp-N, or Glu-C, did not act as substrates for deoxyhypusine synthesis. The nomenclature for initiation factors has been revised (IUB-NC 1989 Eur. J. Biochem. 186, 1–3). In early studies eIF-5A was termed eIF-4D. The abbreviations used are: eIF-5A, eukaryotic initiation factor 5A; ec-eIF-5A, the precursor form of eIF-5A produced in Escherichia coli by expression of the human eIF-5A cDNA; ec-eIF-5A (the precursor form of eIF-5A produced in Escherichia coli) by expression of the human eIF-5A cDNA generated by specific cleavage by endoproteinases Arg-C, Asp-N, or Glu-C, did not act as substrates for deoxyhypusine synthesis. The nomenclature for initiation factors has been revised (IUB-NC 1989 Eur. J. Biochem. 186, 1–3). In early studies eIF-5A was termed eIF-4D. The abbreviations used are: eIF-5A, eukaryotic initiation factor 5A; ec-eIF-5A, the precursor form of eIF-5A produced in Escherichia coli by expression of the human eIF-5A cDNA; ec-eIF-5A, the unmodified precursor form of eIF-5A, containing Lys\(^{40}\) of recombinant deletion constructs from the human eIF-5A cDNA were tested. Truncation of up to 9 amino acid residues (Met\(^{-}\)Thr\(^{9}\)) from the NH\(_{2}\) terminus or 64 amino acid residues (Leu\(^{-}\)Lys\(^{94}\)) from the COOH terminus did not significantly decrease the substrate reactivity, but removal of an additional 10 amino acids from either side did. Deletion of 34 amino acid residues (Met\(^{-}\)Lys\(^{40}\)) from the NH\(_{2}\) terminus or of 84 amino acid residues (Asp\(^{-}\)Lys\(^{184}\)) from the carboxyl terminus caused complete loss of substrate property. The results obtained thus far define the minimum domain of the eIF-5A precursor protein required for enzymatic deoxyhypusine synthesis as Phe\(^{-}\)Asp\(^{8}\), which corresponds to a region of high amino acid conservation in this protein throughout the eukaryotic kingdom.

Eukaryotic translation initiation factor 5A (eIF-5A, old nomenclature, eIF-4D\(^{1}\)) is a small acidic protein that has been suggested to participate in the first peptide bond formation during protein synthesis. The biosynthesis of hypusine involves a unique posttranslational modification of one specific lysine residue (Lys\(^{40}\)) in the human eIF-5A precursor to an unusual amino acid, hypusine (N'-\(4\)-amino-2-hydroxybutyl)lysine, which occurs exclusively in this protein (see Ref. 2 for a recent review). The biosynthesis of hypusine in a single cellular protein, the eIF-5A precursor, and of diphthamide in the eukaryotic elongation factor 2 (eEF-2) precursor, represent the two most specific protein modifications known to date (3).

Hypusine formation occurs by way of two enzyme-catalyzed steps (4): (i) transfer of the butylamine moiety of the polyamine spermidine to the \(\varepsilon\)-amino group of one specific lysyl residue of the eIF-5A precursor protein to form an intermediate deoxyhypusine (N'-\(4\)-aminobutyl)lysine (5–7) and (ii) hydroxylation of the deoxyhypusyl residue to form hypusine (8).

Hypusine and eIF-5A occur in all eukaryotic species examined (2). The hypusine synthesis rate and/or hypusine content appear to correlate with cellular proliferation in various mammalian cells, suggesting an important role for this protein in cell metabolism (2). Hypusine was shown to be essential for the activity of eIF-5A (9, 10) in stimulating methionyl puromycin synthesis, an in vitro model assay for translation initiation. Although the precise physiological function of eIF-5A in eukaryotic cells is unknown, recent studies that involved the inactivation of the two eIF-5A genes in Saccharomyces cerevisiae provide strong evidence that eIF-5A and hypusine are vital for yeast growth (11, 12). The arrest of growth of mammalian cells by agents that cause the reduction of cellular hypusine, either by depletion of spermidine (13) or by inhibition of deoxyhypusine synthase (14), further supports a crucial role of hypusine in eukaryotes (15).

eIF-5A is a highly conserved protein (11, 16–21). There is a 99% amino acid identity between rabbit and human eIF-5A and 60–68% identity between human and yeast eIF-5A sequences (11, 17). Even in the regions of nonidentity, amino acid replacements are often conservative. Sequence similarity is high in the amino-terminal half of the protein and especially in the vicinity of the hypusyl residue. The sequence of 12 amino acids surrounding the Lys residue (Lys\(^{40}\)) that undergoes modification to hypusine, -Ser-Thr-Ser-Thr-Lys-Thr-Gly-Lys*-His-Gly-His-Ala-Lys*, is invariant in all eukaryotes. It suggests the importance of this region for a fundamental cellular function throughout eukaryotic evolution and/or for recognition of the precursor lysyl residue by the enzymes that catalyze the formation of hypusine. The occurrence of hypusine at a single locus of only this

\[\text{frag}\] that cleaves the peptide bond at the amino side of Asp-N residue; Glu-C, endopeptidase from S. aureus V8 that cleaves the peptide bond at the carboxyl side of Glu residue; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.

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cellular protein, together with the high conservation of amino acids that surround this residue, suggests a narrow specificity of deoxyhypusine synthase toward its protein substrate. In preliminary experiments, two synthetic peptides that correspond to the amino acid sequence surrounding Lys5 in the human eIF-5A precursor, a nanopeptide KTGK'OHGHAK and a hexa- decamer IYEMSTSKTGK'OHGHAK, were found neither to act as substrates for deoxyhypusine synthase nor to inhibit the synthesis of deoxyhypusine in the intact substrate protein eIF-5A. In an effort to explore the structural features of the eIF-5A precursor that confer specific recognition by deoxyhypusine synthase, we carried out a systematic study on the structure-substrate function relationships of deoxyhypusine synthesis. Evidence is presented that a substantial portion of the primary structure of the substrate protein is required for recognition and modification by deoxyhypusine synthase.

EXPERIMENTAL PROCEDURES

Materials—1,8-3H]Spermidine-3HCl ([terminal methylenes-3H]) (15 Ci/mmol) was purchased from DuPont NEN; pET-11a expression vector and the host Escherichia coli strain BL21/DE3 were purchased from Novagen. Oligonucleotide primers were synthesized by the Midland Certified reagent company (Midland, TX). Endoproteinasises Arg-C from mouse submaxillary gland, Asp-N from Pseudomonas fragi, and Glu-C from Staphylococcus aureus were from Calbiochem. The nanopeptide, Lys-Thr-Gly-Lys-His-Gly-His-Ala-Lys-His-Ala-Lys, was synthesized and kindly supplied by Dr. W. Huber (University of California, Davis, CA). Deoxyhypusine synthase was prepared from rat testis as described (7). Other reagents and chemicals were obtained as indicated in the text.

Proteolytic Digestion—The eIF-5A precursor protein, ec-eIF-5A, and its truncated fragments were digested with one of the three endoproteinasises, Arg-C or Asp-N in 100 mM Tris-Cl, pH 8.5, or Glu-C in 100 mM Tris Cl, pH 7.8, buffer as described in the legend of Fig. 1.

Construction of Deletion Recombinant Subclones of Human eIF-5A cDNA—A series of different size constructs of human eIF-5A cDNA was prepared by PCR amplification from the full-length human eIF-5A cDNA (17) using synthetic oligonucleotide primers (Table I). These constructs were designed for insertion into pET 11a expression vector (Novagen). The PCR primers were synthesized with an extension of 16 nucleotides containing an NdeI site (CATATG) at the 5' end or a termination codon TGA followed by BamHI site (TCTCTA) at the 3' end, which is flanked by the desired coding or complementary sequences (Table I). The conditions for PCR were as follows: 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension reaction at 74°C for 2 min for 35 cycles, and the final extension reaction at 74°C for 6 min. Insertion of the NdeI site at the 5' end adds an additional methionine residue at the amino terminus of the clones except those with methionine as an NH2-terminal residue. The PCR products were cleaved with NdeI and BamHI and ligated to the insertion site of the vector pET-11a digested with NdeI and BamHI. The complete nucleotide sequences of the cDNA inserts were determined by the dideoxynucleotide termination method using a sequenase kit (U. S. Biochemical Corp.) to verify the fidelity of PCR amplification.

Expression and Purification of ec-eIF-5A and Its Deletion Fragments—E. coli B strain BL21/DE3 lacking the lon protease and the omp T outer membrane protease was used for expression of eIF-5A cDNA and its deletion fragments. The recombinant strains obtained by transformation of the competent host cells with pET-11a vectors containing the desired inserts were grown in LB medium, supplemented with 50 µg/ml of ampicillin, to an optical density of 0.6 at 600 nm. Expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and, after 3 h, cells were harvested by centrifugation. Pelleted cells (5 g) were resuspended in 25 ml of buffer A (50 mM Tris acetate, pH 8.5, 0.1 mM EDTA, 1 mM dithiothreitol), broken by sonication (60 s at 70 watts), and cell debris removed by centrifugation (30 min at 15,000 g). The clarified supernatant solution was applied to a column of Mono Q HR 10/10 (Pharmacia Biotech Inc.) equilibrated with buffer A. Adsorbed proteins were eluted with a linear gradient of 0-0.5 M KCl in buffer A. The Mono Q fractions containing ec-eIF-5A were pooled, concentrated, and dialyzed against buffer B (50 mM Tris acetate, pH 6.6, 0.1 mM EDTA, 1 mM dithiothreitol), then applied to a column of Mono S HR 5/5 (Pharmacia Biotech Inc.) equilibrated with buffer B. Highly pure ec-eIF-5A was obtained by elution with a linear gradient of 0-0.5 M KCl in buffer B. Truncated fragments of ec-eIF-5A expressed in the same manner as the intact protein were purified on a column of Mono S HR 5/5. Fractions containing the deletion fragments were identified by SDS-PAGE on a precast Tricine gel (16% acrylamide, Novex).

Deoxyhypusine Synthase Assay—The deoxyhypusine synthase assay is based on the incorporation of radioactivity from the aminobutyl side of [1,8-3H]perimidine into deoxyhypusine in the substrate protein eIF-5A precursor or its fragments. The enzyme assay was carried out according to the published method (7) with modification. A typical reaction mixture contained 50 mM Tris acetate, 0.5 mM MnCl2, 5 mM NAD+, 2 pCi of [1,8-3H]perimidine (15 Ci/mmol), 6-15 units of deoxyhypusine synthase from rat testis, and varying amounts of substrate protein, ec-eIF-5A, or its peptide fragments, either in a purified form or as the crude lysate of the E. coli cells that expressed them. The assay mixture was incubated at 37°C for 2 h. The radiolabeled product protein or peptides were separated by SDS-PAGE on Tricine gel (16% acrylamide, Novex, San Diego, CA) and visualized by fluorography. For quantitation of the reaction product, the assay mixtures were precipitated with trichloroacetic acid, the trichloroacetic acid precipitates were hydrolyzed in 6 N HCl, and the radiolabeled deoxyhypusine was measured after its separation by ion exchange chromatography as described previously (7, 22).

RESULTS

Because attempts to achieve deoxyhypusine synthesis using small synthetic peptides containing the sequence of amino acids surrounding Lys5 in human eIF-5A precursor were unsuccessful, we turned to larger peptide fragments generated by specific proteolytic cleavage of ec-eIF-5A. The three endoproteinasises, Arg-C, Glu-C, and Asp-N, which catalyze cleavages at the carboxyl side of Arg, at the carboxyl side of Glu, and at the amino side of Asp, respectively, were chosen on the basis of

| Table I The nucleotide sequences of primers used in PCR reactions |
|---------------------------------------------------------------|
| Primer | 5' | 3' |
|--------|----|----|
| Primer 1 (1-9) | gga gta cat tac atg gca gat cag | ctttcagagtctgatcata |
| Primer 2 (10-19) | gga gta cat tac atg gca gat cag | ctttcagagtctgatcata |
| Primer 3 (20-29) | cga ctc gga tca cgg tca ggc cgc acc ttc | ctttcagagtctgatcata |
| Primer 4 (30-39) | cga ctc gga tca cgg tca ggc cgc acc ttc | ctttcagagtctgatcata |
| Primer 5 (40-50) | gga gta cat tac atg gca gat cag | ctttcagagtctgatcata |
| Primer 6 (51-60) | gga gta cat tac atg gca gat cag | ctttcagagtctgatcata |
| Primer 7 (61-70) | gga gta cat tac atg gca gat cag | ctttcagagtctgatcata |
| Primer 8 (71-80) | gga gta cat tac atg gca gat cag | ctttcagagtctgatcata |
| Primer 9 (81-90) | cag gat acc tct gaa ggg ccc cgg cgt cag | ctttcagagtctgatcata |

3 E. C. Wolff and M. H. Park, unpublished results.
their specificity and because there are no preferred cleavage sites for these enzymes in the vicinity of Lys\(^\alpha\) in ec-eIF-5A. The pattern of proteolytic peptides produced varied with the amount of enzyme and the length of digestion. None of the peptides (3–6 kDa) generated by digestion with Arg-C, Asp-N, or Glu-C served as substrates for deoxyhypusine synthesis (data not shown), with the exception of those 8–17-kDa peptides in the partial digest with Asp-N. Fig. 1 shows the Coomassie Blue-stained pattern and the fluorogram of gels of deoxyhypusine synthase reaction mixtures containing ec-eIF-5A or its Lys\(^\alpha\)-containing peptides. Only in the 8–17-kDa peptide of the Asp-N partial digest (Fig. 1, lanes 5 and 6) was strong radiolabeling observed. No labeling was seen with other proteolytic peptides. Intact ec-eIF-5A was included in the reaction mixtures of the even numbered lanes to monitor the reaction. In each case ec-eIF-5A was effectively labeled, thus indicating that the reaction mixtures containing protease inhibitors or possibly the residual protease did not interfere with deoxyhypusine synthesis.

In an effort to define more precisely that portion of the ec-eIF-5A molecule required for the enzymatic formation of deoxyhypusine in the modification reaction, systematic deletions from the NH$_2$ terminus, from the COOH terminus, or from both were carried out by deletion recombinant subcloning of human ec-eIF-5A cDNA, and the resulting fragments were tested as deoxyhypusine synthase substrates (Figs. 2 and 3). Most transformants expressed the desired peptides. However, the level of expression varied with different deletion subclones and transformants. In general, the levels of recombinant peptides of low molecular mass (<6 kDa) in the transformants were low, presumably due to the rapid degradation of small peptides by E. coli. Peptides representing stepwise deletion from the COOH terminus of ec-eIF-5A, 5A(1–154), 5A(1–120), 5A(1–90), and 5A(1–80) (Fig. 2) were efficiently labeled when lysates of the transformants were tested in the deoxyhypusine synthase reaction. Although, in general, efficiency as a substrate declined progressively with deletions from the COOH terminus (Table II, Fig. 2), it appears that the carboxyl half (Val75–Lys154) of ec-eIF-5A is not an absolute requirement for deoxyhypusine synthesis. No radiolabeling was detected in peptides 5A(1–60) and 5A(1–70), indicating that the boundary of the minimum interaction domain on the carboxyl side of ec-eIF-5A lies between Glu75 and Asp80. In order to assess the requirement for the amino acid sequence on the NH$_2$ side of Lys\(^\alpha\), the effects of stepwise deletion from the NH$_2$ terminus were examined (Fig. 2). Deletion of 9 amino acids from the NH$_2$ terminus caused only a small reduction in substrate efficiency (Fig. 2, compare 1–90 and 10–90). Deletion of 19 or 29 NH$_2$-terminal amino acids, however, decreased significantly the substrate property (Table II, Fig. 2). Further deletions from the NH$_2$ terminus, as in the cases of 5A(Met35–154), and 5A(49–154) totally abolished the substrate capacity. These findings, together with the COOH-terminal deletion studies, establish Phe\(^\alpha\)-Asp\(^\omega\) as the minimum domain of ec-eIF-5A required for deoxyhypusine synthesis. Indeed, deoxyhypusine synthesis in the peptide fragment with this sequence, 5A(Met30–80), even when its expression level was low in the transformant, was clearly demonstrable (Fig. 2). The schematic diagram presented in Fig. 3 illustrates the relationship between the substrate potential of the peptides and the conservation of amino acid sequence of ec-eIF-5As from several eukaryotes, including human (17), alfalfa (18), tobacco (19), slime mold (20), yeast (11), and chick embryo (21).

**DISCUSSION**

Among the many amino acids that are derived by posttranslational modification, hypusine is a most remarkable one, vital for eukaryotic cell proliferation, yet occurring at a single position in only one cellular protein. In order for deoxyhypusine synthase, the first enzyme in hypusine biosynthesis, to act on a
The Protein Substrate Specificity of Deoxyhypusine Synthase

Efficiency of deoxyhypusine synthesis in the eIF-5A precursor and its peptide fragments

Each substrate (50 pmol) purified from an overproducing transformant was incubated with rat testis deoxyhypusine synthase as described under "Experimental Procedures." The amount of product formed was determined by measuring the radioactivity in deoxyhypusine after ion exchange chromatographic separation of the hydrolysed protein.

| Substrate | Mass | Deoxyhypusine formed | Relative efficiency |
|-----------|------|----------------------|--------------------|
| ec-eIF-5A | 16,703 | 6.3 pmol/2 h | 100 % |
| 5A(1-120) | 15,188 | 4.8 pmol/2 h | 76 % |
| 5A(1-90) | 11,480 | 4.8 pmol/2 h | 76 % |
| 5A(1-80) | 10,069 | 1.0 pmol/2 h | 15 % |
| 5A(Met10-90) | 10,261 | 4.3 pmol/2 h | 63 % |
| 5A(20-90) | 9,207 | 1.1 pmol/2 h | 16 % |
| 5A(Met30-90) | 7,939 | 0.3 pmol/2 h | 4 % |

Fig. 2. Deoxyhypusine synthesis in ec-eIF-5A recombinant deletion peptides. Lysates of E. coli cells expressing the recombinant deletion peptides were used in place of ec-eIF-5A as the substrate fraction for the deoxyhypusine synthesis reaction. A, Coomassie Blue staining of proteins in the reaction mixture after SDS-PAGE on Tricine gel (16% in acrylamide) as in Fig. 1. B, Fluorogram of the same gel. The positions of the expressed peptides are indicated by arrowheads. pEP-11a, E. coli lysate transformed with the control plasmid containing no insert sequences. The peptides are designated by the corresponding amino acid residue numbers of ec-eIF-5A (see also Fig. 3).

4 A. Abbruzzese, personal communication.
Despite the well established essential role of hypusine and eIF-5A in eukaryotic cell proliferation, the specific action of eIF-5A in cell metabolism remains an open question. Although its role in general protein synthesis has been challenged (23), its possible function as an initiation factor selective for certain specific messages has not been excluded (2, 23). Recent studies suggest that eIF-5A is the host cellular factor required for the Rev function essential for the human immunodeficiency virus type 1 replication (24) and open a new possibility for the role of this putative initiation factor as a posttranscriptional regulator of specific gene expression. The current results provide a foundation for future studies directed toward determination of the structural features of eIF-5A required for its physiological role in eukaryotic cells.

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FIG. 3. Schematic representation of ece-eIF-5A, its recombinant deletion peptides, and the amino acid sequence conservation of eIF-5A in several eukaryotes. A, the peptides in group I (aa 1-n) represent stepwise truncation from the COOH terminus of ece-eIF-5A and the peptides in group II (aa n-90), group III (aa n-154), and group IV (aa n-80) from the NH2 terminus. Amino acid residue numbers and the position of the deoxyhypusine synthesis site (Lys5′) are indicated. Methionyl residues were introduced for the expression in E. coli as marked on the left side of the bars. The relative efficiency of each peptide needed for deoxyhypusine synthesis is indicated. B, the deduced amino acid sequences of eIF-5As from several eukaryotic species, including human (17), alfalfa (18), tobacco (19), slime mold (20), yeast (11), and chick embryo (21) were compared. Solid bars indicate identical sequences, dotted bars indicate conservative replacements, and open bars indicate nonconservative replacements.
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