Identification of a substrate site for transglutaminases on the human protein synthesis initiation factor 5A

Simone BENINATI,*§ Laura NICOLINI,* Judit JAKUS,† Amelia PASSEGGIO‡ and Alberto ABBRUZZESE‡

*Department of Biology, II University of Rome 'Tor Vergata', Via della Ricerca Scientifica, 00133, Rome, Italy, †Laboratory of Cellular Development and Oncology, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892, U.S.A., and ‡Department of Biophysics and Biochemistry, University of Naples, Via Costantinopoli, 16, 80136, Naples, Italy

Protein synthesis initiation factor 5A (eIF-5A) from human erythrocytes was found to be a substrate for both plasma transglutaminase (Factor XIIIa) and guinea pig liver transglutaminase (GPLTG). When purified eIF-5A was incubated with GPLTG or Factor XIIIa in the presence of succinylated β -casein, a covalent complex was identified. By isolating and analysing the product of the transglutaminases (TGases) reaction, the site of modification on eIF-5A has been identified as the unique amino acid hypusine. The complex β -casein·eIF-5A was enzymically digested with proteinases and the predicted covalent cross-link of γ -glutamyl- ω -hypusine was isolated from the digests by ion-exchange chromatography and purified by reversed-phase h.p.l.c. Acid hydrolysis of the purified dipeptide

yielded equimolar amounts of hypusine and glutamic acid. Furthermore, fast atom bombardment m.s. analysis confirmed the isomer assignment to be γ -glutamyl- ω -hypusine. These data indicate that hypusine-50 of the eIF-5A chain functions as acyl acceptor substrate for TGases, and reveal that eIF-5A may be cross-linked to intracellular proteins by TGases. Because the precise function of eIF-5A is still unknown, our results appear particularly stimulating in the light of the recent finding of a new biological role for this protein as a cellular factor binding specifically to the human immunodeficiency virus-1 Rev activation domain [Ruhl, Himmelsbach, Bahr, Hammerschmid, Jaksche, Wolff, Auschauer, Farrington, Probst, Bevec and Hauber (1993) J. Cell Biol. 123, 1309–1320].

INTRODUCTION

Eukaryotic initiation factor 5A (eIF-5A, formerly known as eIF-4D) is a small protein that stimulates the synthesis of methionyl-puromycin in *in vitro* assay [1] and globin synthesis [2]. Its precise role in the mechanism of initiation of protein synthesis is not known, but its function has been localized to the ribosomal subunit joining step or at a later stage in the pathway of 80S initiation complex formation [3]. eIF-5A is the only cellular protein known to contain the unusual amino acid N^{ϵ} -(4-amino-2-hydroxybutyl)lysine (hypusine) [4]. The post-translational modification of eIF-5A leading to the formation of hypusine occurs in two steps: transfer of a butylamino group from spermidine to the ϵ -amino group of a specific lysine residue to form deoxyhypusine; and hydroxylation of the deoxyhypusine side chain to form a hypusine residue [5,6].

Transglutaminases (TGases) (EC 2.3.2.13) catalyse the aminolysis of the γ -carboxamide group of the glutamine side chain of peptide and protein substrate. The reaction proceeds via an acyl-transfer mechanism in which the γ -carboxamide group acts as acyl donor and the ϵ -amino groups of peptide-bound lysine residues or suitable primary amines act as acyl acceptors [7]. The number of glutamyl substrates is highly restricted, whereas the tolerance to structural differences in acyl acceptors varies, depending on the type of TGases. Because of the structural similarities between polyamines and hypusine, we investigated whether eIF-5A might be a substrate for TGases. In the present study we demonstrate that the unusual basic amino acid hypusine is a substrate site for guinea pig liver TGase (GPLTG) and Factor XIIIa. This property confers to eIF-5A the ability to inter-

molecularly cross-link and to bind to proteins with reactive glutamine residues.

MATERIALS AND METHODS

All chemicals were reagent grade. GPLTG [8] and human erythrocyte eIF-5A [9] were purified as previously described. Human plasma coagulation Factor XIII was from Calbiochem (La Jolla, CA, U.S.A.). Factor XIII (100 mg) was activated to form Factor XIIIa by incubation with α -thrombin (100 NIH units/ml) for 25 min at 37 °C. Hypusine and deoxyhypusine were kindly supplied by Dr. M. H. Park (NIH, Bethesda, MD, U.S.A.), succinylated β -casein was a gift from Dr. S. I. Chung (NIH) and N^{α} -benzyloxycarbonyl-L-glutamyl-L-leucine (Z-L-Gln-L-Leu) was kindly provided by Dr. J. E. Folk (NIH).

Synthesis of γ -glutamyl- ω -deoxyhypusine and γ -glutamyl- ω -hypusine

Standards of γ -glutamyl- ω -deoxyhypusine and γ -glutamyl- ω -hypusine were prepared through *in vitro* GPLTG-catalysed incorporation of deoxyhypusine and hypusine respectively in place of ammonia at the carboxamide group of Z-L-Gln-L-Leu. The procedure was essentially as outlined for the preparation of γ -glutamylpolyamine [10], except with deoxyhypusine and hypusine as amine substrates. Treatment of the reaction mixtures with carboxypeptidase A catalysed removal of the L-leucine residue. Removal of the benzyloxycarbonyl group was accomplished by treatment of the dried reaction mixtures with 30 % (v/v) hydrogen bromide in acetic acid. The acids were removed under vacuum and the residues were dissolved in water.

Abbreviations used: eIF-5A, eukaryotic initiation factor 5A; f.a.b., fast atom bombardment; GPLTG, guinea pig liver TGase; HIV, human immunodeficiency virus; hypusine, N^{ϵ} -(4-amino-2-hydroxybutyl)lysine; TGase, transglutaminase; Z-L-Gln-L-Leu, N^{α} -benzyloxycarbonyl-L-glutamyl-L-leucine.

§ To whom correspondence and reprints request should be addressed.

GPLTG or Factor XIIIa reaction on hypusine, deoxyhypusine and eIF-5A

The reaction was carried out in 0.1 ml of buffer containing 50 mM Tris/HCl, pH 7.4, 10 mM dithiothreitol, 2.5 mM calcium chloride, 150 mM NaCl, either Factor XIIIa (10 mg/ml) or GPLTG (20 mg/ml), and the substrate to be assayed. The substrate concentrations used were: succinylated β -casein, 1 mg/ml; Z-L-Gln-L-Leu, 2 mg/ml; hypusine and deoxyhypusine, 0.2 mg/ml; and eIF-5A, 0.1 mg/ml. Reaction mixtures were incubated for 2 h at room temperature and stopped by the addition of 0.2 ml of 5% (w/v) trichloroacetic acid (final concentration).

Time-course experiments of TGase-catalysed incorporation of the synthetic dipeptide Z-L-Gln-L-Leu on purified eIF-5A were performed by incubating at room temperature for 0–90 min samples containing 0.5 mg of dipeptide in 0.25 ml of 50 mM Tris/HCl, pH 7.5, and 0.1 mg/ml of eIF-5A with Factor XIIIa or GPLTG (10 mg/ml). The amount of dipeptide incorporated was evaluated by measuring the concentration of γ -glutamyl- ω -hypusine formed.

Isolation, purification and identification of γ -glutamyl derivatives of hypusine and deoxyhypusine

γ -Glutamyl- ω -deoxyhypusine and γ -glutamyl- ω -hypusine were isolated from the incubation mixture by ion-exchange chromatography, employing the five buffer system described previously [11] with some modifications. Briefly, aliquots of the incubation mixture were subjected to enzymic digestion at 37 °C with Pronase, aminopeptidase M and carboxypeptidases A, B and Y as reported [12]. At the completion of this procedure, digestion was continued for an additional 10 h period after addition of proteinase K at a level of 0.05 mg/mg of protein. Ion-exchange chromatographic separation of γ -glutamyl derivatives of hypusine and deoxyhypusine was performed using a Dionex D-400 analyser and a five-buffer system as described earlier [11]. In this system, the majority of neutral and acidic amino acids are eluted in the break-through volume. For the purification of the derivatives, effluent collection was made directly from the ion-exchange column without mixing with the fluorimetric reagent. γ -Glutamyl- ω -hypusine and γ -glutamyl- ω -deoxyhypusine eluted very closely to one another in the chromatographic system used, with elution times of 37.5–39.0 and 42.0–43.5 min respectively. Purification of the two derivatives was conducted with a Waters Associated h.p.l.c. system consisting of a Model 6000A solvent delivery system, a Model U6K sample injector, a Model 660 solvent programmer, and a Model 420E fluorescence detector. Data were collected and processed using a Hewlett-Packard Model 3390 integrator. Reversed-phase separations were conducted at room temperature in a 30 cm $\times$ 3.9 mm (internal diam.) Bondapak C₁₈ 10 μ m column (Waters Assoc.) employing an elution solvent of 45% (v/v) methanol in 10 mM ammonium bicarbonate over a period of 10 min at a flow rate of 2 ml/min. Progress was assessed by monitoring the A_{210} of the eluent. Measurement of glutamic acid and deoxyhypusine or hypusine released upon acid hydrolysis [13] of γ -glutamyl derivatives was a preliminary proof of the identity of these compounds [14]. In addition, mass spectra were recorded on a JEOL SX102 fast-atom-bombardment (f.a.b.) mass spectrometer. Samples were desorbed from a thioglycerol matrix using 6 keV Xe atoms.

SDS/PAGE analysis of eIF-5A incorporation into succinylated β -casein

SDS/PAGE analysis was performed on samples prepared as described for the TGase reaction on hypusine and deoxyhypusine

except that smaller amounts of Factor XIIIa (5 mg/ml), GPLTG (5 mg/ml) and β -casein (0.1 mg/ml) were used. Proteins were separated on 10% (w/v) polyacrylamide slab gels with a 4% (w/v) stacking gel according to the method of Laemmli [15]. Gels were stained with Coomassie Brilliant Blue. Protein molecular-mass standards used were: β -lactoglobulin (18.4 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (43.0 kDa), BSA (68.0 kDa), phosphorylase *b* (97.4 kDa) and myosin heavy chain (200.0 kDa). For amino acid analyses the Coomassie-Blue-stained bands were cut from gels and washed with 10% (v/v) methanol. Following hydrolysis with 6 M HCl at 110 °C for 14 h, the amount of amino acids was estimated with an automated amino acid analyser [16] using *o*-phthalaldehyde detection and for hypusine following an h.p.l.c. procedure [13].

RESULTS AND DISCUSSION

The possibility that eIF-5A acts as an acyl donor substrate of GPLTG and Factor XIIIa was explored by incubating the purified protein with TGases in the presence of labelled spermidine and by verifying the amount of radioactivity bound to the protein. Under these conditions a negligible portion of the

Figure 1 Time-course of TGase-catalysed Z-L-Gln-L-Leu incorporation into eIF-5A

Z-L-Gln-L-Leu (0.5 mg) in 0.25 ml of incubation buffer containing 0.1 mg/ml purified human erythrocyte eIF-5A and 10 mg/ml Factor XIIIa (●) or GPLTG (■) were incubated for 0–90 min at room temperature and then processed as described in the Materials and methods section. Data represent means $\pm$ S.E.M. ($n = 3$).

Figure 2 Analysis by SDS/PAGE of GPLTG- and Factor XIIIa-catalysed incorporation of eIF-5A into succinylated β -casein

The acrylamide concentration of the running gel was 10% (w/v). Samples for SDS/PAGE analysis were made 2% in SDS, 10% in glycerol and 1% in dithiothreitol, heated at 100 °C for 2 min, then applied to the gel. The concentration of acrylamide in the running gel was 10% (w/v). Gels were stained with Coomassie Brilliant Blue. Lane 1: Factor XIIIa + eIF-5A + β -casein + 10 mM EGTA; lane 2: Factor XIIIa + eIF-5A + β -casein; lane 3: GPLTG + eIF-5A + β -casein + 10 mM EGTA; lane 4: GPLTG + eIF-5A + β -casein. The letter A identifies the 'faster migrating protein' component of eIF-5A [9]; B succinylated β -casein; and C–F cross-linked products. The molecular masses (kDa) of marker proteins appear at the left-hand side of the Figure.

Table 1 Cross-links of eIF-5A to β -casein catalysed by GPLTG, estimated by SDS/PAGE and amino acid analysis

The β -casein:eIF-5A ratio was calculated from comparison of the values for hypusine and lysine content of the SDS/PAGE protein bands. The amount of protein in the SDS/PAGE band was estimated from the amino acid composition of the SDS/PAGE protein bands (Figure 2, lane 4). Data represent means $\pm$ S.E.M. ($n = 3$). —, Not determined.

Molecular mass (kDa)	β -Casein:eIF-5A ratio	Amount of protein in SDS/PAGE band (% of total)
18	—	—
26	—	17.2 $\pm$ 2.0
60	1:2	3.4 $\pm$ 0.4
78	1:3	3.5 $\pm$ 0.5
95	1:4	68.9 $\pm$ 5.9
200	1:10	6.9 $\pm$ 0.7

labelled amine in the incubation mixture was incorporated into the eIF-5A protein (results not shown). Purified eIF-5A, in contrast, showed a large incorporation of Z-L-Gln-L-Leu, a synthetic dipeptide acting as acyl donor substrate for TGases [17] (Figure 1). This result indicates that acyl acceptor sites for GPLTG and Factor XIIIa are present on eIF-5A.

In order to obtain convincing evidence of TGase-catalysed cross-link formation through eIF-5A in a macromolecular system, it seemed necessary to choose as the glutamine substrate a protein molecule that was essentially devoid of ϵ -amino groups. In the presence of these groups it would have been difficult to determine whether cross-linking, if it occurred, was a consequence of ϵ -(γ -glutamyl)lysine bond formation or was attributable to the linkage under consideration. Succinylated β -casein was selected as a potential acyl donor substrate for eIF-5A, as β -casein is an

excellent substrate for both GPLTG and Factor XIIIa [17]. Succinylation of the primary ϵ -amino group of lysine residues of β -casein is a very useful way of avoiding polymerization of this protein because of ϵ -(γ -glutamyl)lysine cross-link formation catalysed by TGases.

Electrophoretic analysis by SDS/PAGE of the incubation mixture containing eIF-5A, succinylated β -casein and GPLTG or Factor XIIIa, showed six protein bands, with apparent molecular masses of 18, 26, 60, 78, 95 and 200 kDa (Figure 2). Our preparations of purified eIF-5A from human red blood cells were shown by SDS/PAGE to contain only the so-called 'faster migrating band' (Figure 2, band A) [9]. This was observed to be identical to that of the single band of eIF-5A isolated from Chinese hamster ovary (CHO) cells [14]. Hypusine was confirmed to be a component of the protein of the faster migrating band [9]. When the eIF-5A protein was incubated with Factor XIIIa or GPLTG in the presence of succinylated β -casein, several bands appeared, which must have resulted from TGase-mediated intermolecular cross-linking of eIF-5A with β -casein, as shown in Figure 2, lanes 2 and 4 (bands C–F). The distinct protein band observed at the top of the running gel (200 kDa) presumably arose as a result of the extensive covalent cross-linking of β -casein with eIF-5A (Figure 2, band F).

To confirm that the cross-linked proteins arose from TGase activity, EGTA was used as inhibitor of the reaction. It is evident that when eIF-5A (Figure 2, band A) and β -casein (Figure 2, band B) were incubated in the presence of 10 mM EGTA, the cross-linked protein bands were not visualized (Figure 2, lanes 1 and 3). Examination of each of the cross-linked protein bands for components released after acid hydrolysis, as described in the Materials and methods section, revealed that all of them contained hypusine in various amounts. Comparison of the values for hypusine and total amino acid content of the SDS/PAGE protein bands based upon the amino acid composition of β -casein [18] and eIF-5A [19] reveals the number of molecules of

Figure 3 F.a.b.-m.s. of the purified derivative isolated from the incubation of Z-L-Gln-L-Leu with hypusine in the presence of GPLTG

The mass spectrum of the h.p.l.c.-purified hypusine derivative was recorded on a JEOL SX102 Fast Atom Bombardment mass spectrometer. Samples were desorbed from a thioglycerol matrix using 6 keV Xe atoms. The chemical structure of γ -glutamyl- ω -hypusine depicted in the Figure corresponds to the parent ion at m/z 363 [$M+H$] $^+$.

eIF-5A cross-linked to one molecule of succinylated β -casein (Table 1).

The data presented in Table 1 clearly establish that TGases can catalyse the formation of cross-links between β -casein and the eIF-5A. The cross-linking reaction appears to proceed by the attachment of several molecules of eIF-5A on to the glutamyl residues of β -casein. This finding is in agreement with the absence of reactive lysine residues on succinylated β -casein. Indeed, the amino acids analysis performed on the cross-linked proteins eluted from the SDS/PAGE bands (Figure 2) revealed a ratio of β -casein cross-linked to eIF-5A of: (a) 1:2 for the apparent molecular mass of 60 kDa; (b) 1:3 for the band of 78 kDa; (c) 1:4 for the band of 95 kDa; and (d) 1:10 for the high-molecular-mass polymer found on the top of the running gel (200 kDa). It is interesting to note that after treatment of eIF-5A with TGases in the presence of β -casein, the most abundant product of the cross-linking reaction is a protein with an apparent molecular mass of 95 kDa (Figure 2, lanes 2 and 4), which represents 68.9% of the total protein found in the incubation mixture (Table 1). Whether this particular conformation is of biological significance is under investigation. At present we have preliminary evidence to favour an increased affinity of the enzyme deoxyhypusine hydroxylase [20] for this 95 kDa protein obtained from deoxyhypusine and β -casein (S. Beninati and A. Abbruzzese, unpublished work).

When proteolytic digest of the products of the reaction catalysed by GPLTG or Factor XIIIa on eIF-5A and β -casein was tested by the ion-exchange chromatographic procedure employing the five-buffer system previously reported [11], a new γ -glutamyl-derivative appeared with a retention time very similar to that of standard γ -glutamyl- ω -hypusine prepared as outlined in the Materials and methods section. This product was isolated and analysed for amino acid content and showed equimolar amounts of glutamic acid and hypusine (results not shown). The γ -glutamyl derivative of hypusine was purified by h.p.l.c. as described in the Materials and methods section and examined by f.a.b.-m.s., which showed a parent ion at m/z 363 $[M+H]^+$ (Figure 3). The mass spectrum of the compounds obtained either from Z-L-Gln-L-Leu with hypusine or from eIF-5A and β -casein were similar. These data confirmed the identity of this compound and established the formation of a new type of post-translational modification of eIF-5A catalysed by TGases (Figure 3).

Whether TGase-catalysed modification of the eIF-5A is involved in biological processes remains an open question. Although it is not clear how the hypusine residue in eIF-5A functions during protein synthesis, it is of interest in this context that the modification induced by TGases may contribute to the mechanism of interaction with ribosomes. The evidence presented in this report on the potential association of eIF-5A with proteins should contribute to a better understanding of the mechanism of its interaction with ribosomes or the components of the cellular protein synthesizing machinery. Furthermore, there is an increasing interest in the importance of eIF-5A cross-linking to proteins, as recent studies have revealed that the successful binding of the host eIF-5A to a virus-encoded regulatory

phosphoprotein, Rev, plays a key role in the expression of the human immunodeficiency virus (HIV) type 1 structural proteins in infected cells [21]. The mechanism of this interaction is not known, but a direct cross-linking of eIF-5A to Rev appears to be an essential step required for the replication of HIV-1. Studies aimed at a detailed understanding of the molecular mechanisms of cross-links between eIF-5A and HIV-1 Rev protein, currently under way, should provide a basis for a better understanding of eIF-5A function in acquired immunodeficiency syndrome (AIDS).

The data presented here clearly establish that TGases can catalyse the formation of cross-links between peptide chains and the hypusine residue of eIF-5A; this cannot of course be construed as evidence for these linkages in nature. Nevertheless, this possibility appears solid enough to promote further investigation, particularly in the light of the potential for the development of anti-HIV agents.

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