FEBS Letters 437 (1998) 34–38 FEBS 20950

Tissue transglutaminase expression affects hypusine metabolism in BALB/c 3T3 cells

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Received 27 July 1998; received in revised form 8 September 1998

Abstract Post-translational formation of hypusine in eukaryotic initiation factor 5A (eIF-5A) is essential for cell viability. Recently, we showed that hypusine protein is an in vitro substrate for transglutaminases (TGases). We report the effect of tissue TGase expression on the in vivo hypusine metabolic pathway. The stable expression of tTGase in BALB/c 3T3 cells induced a 100-fold reduction of hypusine levels and a 50% increase of γ-glutamyl-ϖ-hypusine formation. Such changes were paralleled by a consistent decrease in the free polyamine pool and an enhancement of their excretion and of the formation of their γ-glutamyl derivatives. These effects occurred together with a significant reduction of cell proliferation. In this report we suggest, for the first time, that tTGase affects hypusine metabolism, thus regulating the eIF-5A activity and cell proliferation.

Key words: Transglutaminase; Eukaryotic initiation factor 5A; Hypusine; Polyamine; Post-translational modification

1. Introduction

Hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] is an unusual basic amino acid found only in the eukaryotic translation initiation factor 5A (eIF-5A) [1]. Hypusine is formed by the transfer of the butylamine portion from spermidine to the ε-amino group of a specific lysine residue of eIF-5A precursor [2] and by the hydroxylation at carbon 2 of the incoming 4-aminobutyl moiety [3,4]. eIF-5A probably acts in the final stage of the initiation phase of protein synthesis by promoting the formation of the first peptide bond [5]. Hypusine plays a key role in the regulation of eIF-5A function because its precursors, which do not contain hypusine, have little, if any, activity [6]. The correlation between hypusine content and cell proliferation [7,8] suggests that hypusine might play a role in cell growth and differentiation [9]. In fact, eIF-5A and hypusine are vital for *Saccharomyces cerevisiae* growth [9]. Moreover, agents that reduce cell hypusine levels [10–12] inhibit the growth of mammalian cells, demonstrating that hypusine is crucial for the proliferation of eukaryotic cells.

Recently, an in vitro post-translational modification of eIF-5A catalyzed by transglutaminases (EC 2.3.2.13) has been evidenced [13]. Transglutaminases are calcium-dependent enzymes that catalyze post-translational modifications of proteins by the formation of covalent crosslinks between γ-carboxamido groups of glutamine endoresidues and ε-amino groups of lysine endoresidues [14]. Monoamines and polyamines can also act as amino group donors. Tissue transglutaminase (tTGase; type II TGase) is an ubiquitous member of the transglutaminase enzyme family. This protein, however, is expressed at very high levels in endothelial cells and chondrocytes and is localized mainly at cytoplasmic level [15]. Several studies show that the tTGase gene expression is regulated by retinoids and cAMP [16–18]. Moreover, growth factors (i.e. epidermal growth factor (EGF) and transforming growth factor β (TGFβ)) [19,20] and cytokines (i.e. interleukin-6 (IL-6)) [21] can regulate tTGase gene expression in cancer cells. Previous data showed that tTGase activity is directly involved in some cellular activities, i.e. receptor mediated endocytosis [22], programmed cell death or apoptosis [23], cell adhesion, cell growth and tumor cell proliferation [24–26].

Since we have found that tTGase catalyzes the in vitro formation of crosslinks between dimethylcasein and eIF-5A [13], we have investigated whether tTGase could regulate the in vivo activity of eIF-5A and, consequently, interfere with the biological behavior of eukaryotic cells. In detail, we studied the in vivo effect of tTGase expression and activity on hypusine synthesis, its metabolic precursors polyamines and γ-glutamyl derivatives formation in a BALB/c 3T3 derived cell line in which we stably transfected the gene for human tTGase. These cell lines were obtained by clonal selection and have been previously characterized for several biochemical and biological parameters (i.e. tTGase expression and activity, cell morphology and adhesion) [26]. Therefore, they represent a suitable tool to determine the in vivo relationship between tTGase and eIF-5A protein expression and activity.

2. Materials and methods

2.1. Establishment of stably transfected BALB/c 3T3 cell lines

BALB/c 3T3 stably transfected mouse cell lines were obtained as previously described by Gentile et al. [26]. pSG5-tTGase (Stratagene, La Jolla, CA, USA) and pSV2-Neo eukaryotic expression vectors were used to transfect BALB/c 3T3 cells by a calcium phosphate procedure [26]. Stably transfected cells were then selected and grown in alpha DME.

2.2. Cell culture and cell proliferation assays

BALB/c 3T3 mouse cells were grown in alpha DME supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 10% (v/v) Serum Plus (Hazleton Research Products, Lenexa, KS, USA), 50 U/ml penicillin and 50 μg/ml streptomycin. Transfected BALB/c 3T3 cells were grown in normal growth medium containing 400 μg/ml

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Abbreviations: eIF-5A, eukaryotic initiation factor 5A; TGase, transglutaminase; EGF, epidermal growth factor; TGFβ, transforming growth factor β; IL-6, interleukin-6; PBS, phosphate buffered saline; HPLC, high performance liquid chromatography; SDS-PAGE, sodium-dodecylsulfate polyacrylamide gel electrophoresis

PII: S0014-5793(98)01191-0

G418 (Geneticin; Gibco, Gaithersburg, MD, USA). For cell proliferation experiments 1.5×10^5 control or tTGase-transfected cells were seeded in 6 multiwell plates and incubated at 37°C. At the selected times the cell number was determined with a hemocytometric count after trypan blue dye.

2.3. Immunodetection of eIF-5A in BALB/c 3T3 cells

For eIF-5A immunodetection, cells growing in complete alpha DME medium (80% confluent) were washed three times with phosphate buffered saline (PBS). Five μg of total homogenate protein were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel. Proteins were then electroblotted to type HA 0.45-mm pore nitrocellulose paper (Bio-Rad, Richmond, CA, USA). The primary antibody, a rabbit anti-eIF-5A antibody kindly donated by Dr. K. Igarashi (Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan), was diluted 1:2000 with a blocking solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% FBS. The secondary antibody, mouse anti-rabbit IgG antiserum conjugated with peroxidase (Sigma, St. Louis, MO, USA) was diluted 1:3000 with the blocking solution previously described. The intensities of the bands associated to eIF-5A were determined by laser scanning using a common software (Gel-Pro Analyzer, Media Cybernetics, Silver Spring, MD, USA).

2.4. Isolation, purification and identification of hypusine and polyamines

BALB/c 3T3 cells were seeded in 100-mm dishes and 24 h before processing 8 μl [terminal methylenes-^{3}H]spermidine 3·HCl (15 Ci/mmol) were added to each dish. Cell lysates were prepared using cells from 10 dishes (0.1-ml volume of washed cells) by suspending the cells in 4 ml of PBS, sonicating (10 s at 70 W), and finally centrifuging for 30 min at $25\,000\times g$. The lysates were treated with solid ammonium sulfate (40–80% cut) and the precipitate was hydrolyzed in 6 N HCl at 110°C for 18 h. The hydrolysates were applied to 0.5×4-cm columns of AG 50×2 (H^+ form, 200–400 mesh) and eluted with 30 ml of 1 N HCl, 20 ml 3 N HCl and 30 ml 6 N HCl. Hypusine, contained in the 3 N HCl fraction, was determined by using a reversed-phase high performance liquid chromatography (HPLC) method following a published procedure [27]. Polyamines were determined by using an LKB Plus amino acid analyzer and a five-buffer system [13].

2.5. Isolation, purification and identification of γ-glutamyl derivatives

γ-Glutamyl-ω-hypusine, γ-glutamyl-ω-putrescine, γ-glutamyl-ω-spermidine and γ-glutamyl-ω-spermine were isolated from the incubation mixture by ion-exchange chromatography, employing the five-buffer system previously described [13].

3. Results

3.1. Hypusine levels and eIF-5A immunodetection in BALB/c 3T3 cell lines

Since we have previously reported that tTGase catalyzes the in vitro formation of crosslinks between dimethylcasein and eIF-5A [13], we have developed a cell model in which the in vivo effect of tTGase expression on the hypusine pathway could be studied. A BALB/c 3T3 derived cell line was stably transfected with the gene for the human tTGase by using a pSG5-tTGase expression vector (clone 5). The control counterpart was represented by BALB/c 3T3 cells transfected with the pSG5 vector alone (clone 1). We have indeed found a dramatic reduction (about 100–200-fold) of the intracellular hypusine content in the cells transfected with tTGase. In fact,

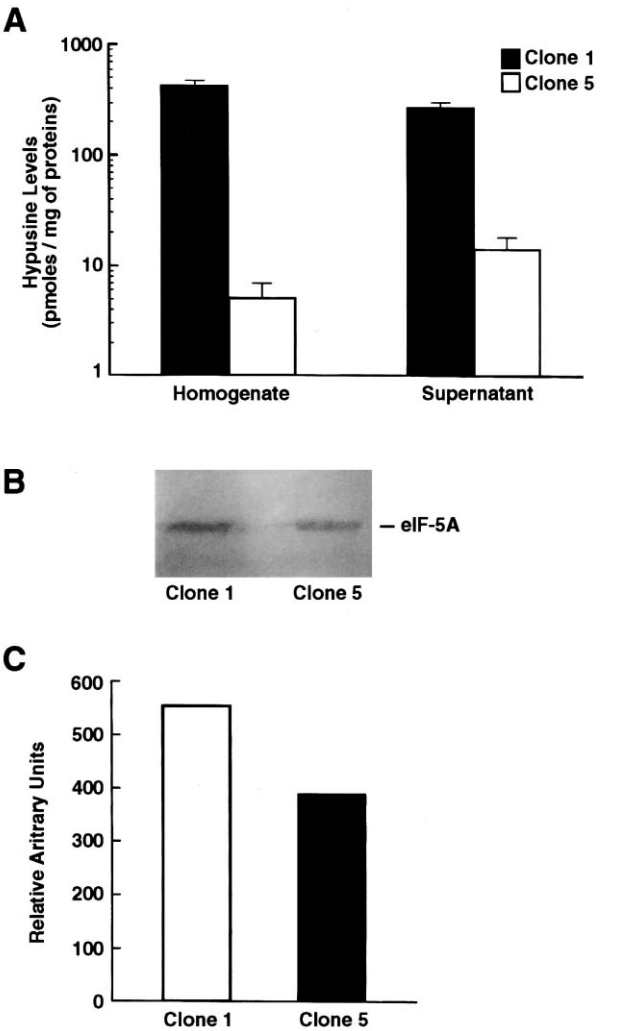

Fig. 1. (**A**) Hypusine levels in homogenates and supernatants in control and TGase-transfected BALB/c 3T3 cells. The values are the mean of 4 experiments and are expressed as pmol/mg of proteins. (■) Clone 1; (□) clone 5. Bars: standard deviations. B: Western blot analysis of eIF-5A in total cell homogenates derived from BALB/c 3T3 cell clones. Five μg of total proteins were fractionated on 10% SDS-PAGE, transferred to nitrocellulose and probed with antisera raised against eIF-5A. Further experimental details are reported in Section 2. Lane 1: clone 1; lane 2: clone 5. C: Laser scanning of the bands corresponding to eIF-5A in the Western blot experiment expressed as relative arbitrary units derived from a common software (Gel-Pro Analyzer, Media Cybernetics, Silver Spring, MD, USA).

Table 1
Polyamine levels in control and in tTGase transfected BALB/c 3T3 cells

Polyamines		Clone 1	Clone 5
Putrescine	Total homogenate	18.75 ± 0.98	N.D.
	Supernatant	8.74 ± 0.56	N.D.
Spermidine	Total homogenate	1212.80 ± 60.12	65.89 ± 4.25
	Supernatant	983.75 ± 41.26	206.62 ± 18.76
Spermine	Total homogenate	700.34 ± 38.46	26.77 ± 1.52
	Supernatant	590.12 ± 28.78	82.27+4.76

Values are expressed as pmol/mg of protein ± S.D. Results represent the means of 4 experiments.

Table 2
γ-Glutamyl derivatives in control and in tTGase transfected BALB/c 3T3 cells

γ-Glutamyl derivatives		Clone 1	Clone 5
γ-Glutamyl-ϖ-hypusine	Total homogenate	25.03 ± 5.07	37.06 ± 6.16
	Supernatant	N.D.	N.D.
γ-Glutamyl-putrescine	Total homogenate	6.25 ± 1.81	12.35 ± 0.76
	Supernatant	N.D.	N.D.
γ-Glutamyl-spermidine	Total homogenate	31.25 ± 1.51	24.71 ± 0.98
	Supernatant	N.D.	N.D.
γ-Glutamyl-spermine	Total homogenate	N.D.	18.53 ± 4.10
	Supernatant	N.D.	N.D.

Values are expressed as pmol/mg of protein ± S.D. Results represent the means of 4 experiments.

hypusine levels decreased from 425.05 to 5.15 pmol/mg of protein in cell total homogenates and from 273.12 to 14.25 pmol/mg of protein if measured in cell supernatants obtained after centrifugation of the cell lysates at 25 000 × *g* for 30 min (Fig. 1A). The latter fraction is known to contain mainly free polyamines while conjugated polyamines are mostly present in the precipitated fraction. The change in hypusine content was not due to differences in eIF-5A protein expression, as determined by a Western blot analysis (limit of sensitivity: 1–2 ng of purified antigen) performed on the total cell homogenates derived from the two clones. As shown in Fig. 1B, in both cell lines an immunoreactive signal corresponding to the eIF-5A protein was detected (Fig. 1C). The data showed an about 30% decrease of the intensity of the eIF-5A band in transfected cells that did not account for corresponding changes in hypusine synthesis. These results suggest that tTGase expression did not interfere with the synthesis of the protein but it rather caused a dramatic reduction of the levels of its mature form (hypusine-containing protein).

3.2. *Effect of tTGase expression on polyamine levels and γ-glutamyl derivatives formation in BALB/c 3T3 cells*

Since hypusine biosynthesis and polyamine levels are strictly correlated, we have studied the effect of tTGase expression on polyamine metabolism. Putrescine levels were relatively low in both the total homogenate and supernatant of control cells and were no more detectable after transfection. Spermidine was the most abundant polyamine, but its concentration dramatically decreased from 1212.80 to 65.89 pmol/mg of protein in the supernatant and from 983.75 to 206.62 if measured in the total homogenates. Likewise, spermine levels decreased from 700.34 to 26.77 and from 590.12 to 82.27, respectively, in the two fractions (Table 1). Interestingly, the strong decrease of the intracellular polyamines in tTGase-transfected cells was paralleled by the increase of their excretion in the culture medium. In fact, putrescine was not detectable in control cell medium while 623.5 pmol/mg of protein were found in transfected cell medium. In the same way, spermidine and spermine excretion increased from 115.3 to 555.1 and from 181 to 1226.2 in control and transfected cells, respectively. Therefore, we have found a decreased intracellular polyamine content that paralleled the increased tTGase expression and the low hypusine levels.

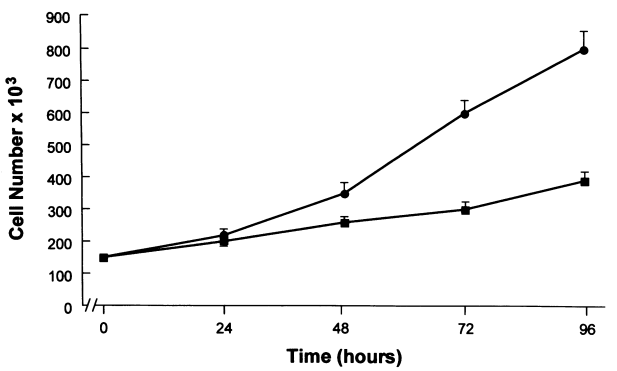

Fig. 2. Cell proliferation experiments performed on control (●) and tTGase-transfected (□) BALB/c 3T3 cells. Cell growth was evaluated at the selected times as described in Section 2. Points: means of three different experiments. Bars: SDs.

In order to investigate the hypusine and polyamine cross-link formation following tTGase transfection, the γ-glutamyl-conjugates levels were determined (Table 2). γ-Glutamyl-ϖ-hypusine homogenate content increased from 25.03 pmol/mg of protein in control cells to 37.06 in tTGase transfected cells. γ-Glutamyl-ϖ-hypusine was not detectable in both cell line supernatants (Table 2). Moreover, an about 2-fold increase of glutamyl-putrescine was found in transfected cell homogenates (Table 2). Glutamyl-spermine levels were 18.53 pmol/mg of protein in transfected cell homogenates while they were not detectable in control cells. On the other hand, a small decrease of glutamyl-spermidine content was found in tTGase transfected cell homogenates (Table 2). Notably, the polyamine derivatives were never found in supernatants of both clones (Table 2). We found that part of the intracellular polyamines of transfected cells was converted to a conjugated form, while the free polyamine pool was drastically depleted, likely as a consequence of the enhanced excretion.

3.3. *Cell proliferation of control and tTGase-transfected cells*

Since both polyamine and hypusine levels are related with cell growth [7,8,27,28], we measured the proliferative activity of control and tTGase-transfected BALB/c 3T3 cells. tTGase-transfected cells displayed a growth rate lower than 50% with respect to control cells as assessed with the evaluation of cell growth at different times from the seeding (Fig. 2). The absence of cytotoxic effects was demonstrated by trypan blue assay and by the observation that the growth of transfected cells was slowed down but not completely arrested. Therefore, the alteration of hypusine synthesis was paralleled by a significant decrease of the proliferative potential of transfected cells.

4. Discussion

It has been reported that polyamines can regulate in vivo and in vitro cell proliferation allowing the DNA binding of

transactivating proteins [28]. Moreover, polyamines, particularly spermidine, are involved in the eIF-5A post-translational modifications that lead to the formation of the basic amino acid hypusine [1–4]. Several findings suggest that the conversion of lysine to hypusine may play a role in the regulation of cell growth through the control of protein synthesis machinery and/or nuclear RNA export [7–12,29]. In particular, hypusine synthesis could be an additional mechanism by which polyamines regulate cell proliferation.

tTGase is a Ca^{2+}-dependent enzyme directly involved in several cell functions correlated with the control of eukaryotic cell growth [14,22–26]. The mechanisms by which tTGase induces such biological effects are still not clear. The formation of cross-links between cellular proteins that regulate cell proliferation could have a role in the growth inhibition induced by tTGase. Folk and associates [30] have defined the presence of polyamine-peptide conjugates in mammalian systems that are at least in part catalyzed by TGases and we have, recently, reported that eIF-5A is an in vitro substrate for TGases, demonstrating that the site of the catalytic modification is hypusine [13]. Moreover, Singh and coworkers [29] have reported that the GDP-bound form of tTGase associates with eIF-5A in HeLa cells and that this interaction is promoted by Ca^{2+}, Mg^{2+} and retinoic acid. On the basis of these considerations, we have determined if tTGase could in vivo interfere with hypusine metabolism in mammalian cells. We investigated whether the amount of intracellular hypusine and polyamines and of their γ-glutamyl derivatives was altered as a function of tTGase overexpression in a BALB/c-3T3 derived cell line. We found that part of the intracellular polyamines of transfected cells was converted to a conjugated form and the free polyamine pool was drastically depleted, likely as a consequence of an enhanced excretion of the cations, as previously reported by Melvin et al. [31]. The mechanism by which tTGase could interfere with polyamine excretion is not clear. It could be hypothesized that the enzyme could affect the function of the channels that regulate polyamine transport through the plasma membrane by the formation of cross-links with other cellular substrates. Moreover, we found a significant increase of the formation of γ-glutamyl polyamine conjugates in transfected cells. The modification of intracellular polyamine levels was paralleled by an about 100-fold reduction of the hypusine levels in the transfected cells, likely due both to the altered polyamine metabolism and to the formation of γ-glutamyl derivatives. Therefore, one of the effects of tTGase expression appeared to be on polyamine efflux and changes in hypusine levels could be, at least in part, considered as an indirect consequence of the reduction of spermidine levels. These biochemical intracellular changes induced by the tTGase transfection caused relevant biological effects. In fact, the biochemical modulation of the eIF-5A activity and of polyamine levels occurred together with significant change of BALB/c 3T3 cell proliferation. BALB/c 3T3 cells, stably transfected with the gene for the human tTGase, showed an about 50% retardation of their proliferation without cytotoxic effects.

Our findings suggest that tTGase modulates the activity of eIF-5A by affecting its post-translational modifications. This effect could be a mechanism by which tTGase regulates several biological functions such as protein synthesis, nuclear (RNA) export and cell proliferation. As a consequence, tTGase could represent an additional target for the blockade of hypusine formation in tumor cells. Therefore, the combination of tTGase activators and of hypusine synthesis inhibitors could be a useful tool for the development of new strategies in cancer therapy.

Acknowledgements: We are indebted to Dr. K. Igarashi, Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan and Dr. Stefano Del Duca, Department of Biology, University of Bologna, Italy for providing us the anti-eIF-5A monoclonal antibody. Michele Caraglia is a fellow of the Foundation for International Educational and Cultural Exchange (Delaware, USA). We thank Dr. Mario Pagano, Department of Biochemistry and Biophysics, II University of Naples for the cooperation in the elaboration of figures. This work was supported by an AIRC grant to V.G. and by MURST grants to A.A.

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