Hypusine modification in eukaryotic initiation factor 5A in rodent cells selected for resistance to growth inhibition by ornithine decarboxylase-inhibiting drugs

Margaret E. TOME and Eugene W. GERNER*

Departments of Radiation Oncology and Biochemistry, Arizona Health Sciences Center, The University of Arizona, Tucson, AZ 85724, U.S.A.

INTRODUCTION

The polyamines are abundant cations that are required for cell growth and normal cell function [1,2]. Although growth can be sustained in the presence of a decreased polyamine pool in some cell types (e.g. L1210 cells containing only 15% of the control intracellular spermidine level) [3], depletion of polyamines generally reduces the growth rate and causes cytostasis [3–8], as seen in Chinese hamster ovary cell polyamine auxotrophs when spermidine pools reach 6% of control concentrations [4].

The requirement for polyamines during eukaryotic cell growth may be partly due to the need for spermidine as a substrate for eukaryotic initiation factor 5A (eIF-5A) modification [6,8–10]. The eIF-5A precursor protein is post-translationally modified by spermidine in a two-step process: (1) deoxyhypusine synthetase catalyses the transfer of the butylamine moiety from spermidine to the ε-amino group of a specific lysine residue in the eIF-5A precursor protein; and (2) this residue is hydroxylated in a reaction catalysed by deoxyhypusine hydroxylase to form mature eIF-5A, which contains hypusine [N'-(4-amino-2-hydroxybutyl)-lysine] [11–14]. Although the precise cellular function of eIF-5A is unclear, the necessity for hypusine-containing eIF-5A for proliferation is well established. Yeast with rapidly degraded eIF-5A are blocked in G1, and yeast knockout mutants produce non-viable spores [15,16]. Mammalian cells treated with inhibitors of deoxyhypusine synthetase or deoxyhypusine hydroxylase fail to proliferate [17–19].

Selection of cells using drugs that inhibit ornithine decarboxylase (ODC), the first and often the rate-limiting step in polyamine synthesis, have generally produced cell lines that overexpress ODC [20–25] due to an amplified ODC gene. In earlier studies, we and others have found that this selection can have consequences for cell growth [26,27]. In the studies described here, we have used HTC cells and two drug-resistant mutant cell lines, HMOA and DH23A/b, to examine the consequence(s) of selection using ODC-inhibiting drugs on eIF-5A modification, since this pathway is both spermidine-requiring and essential for proliferation. In addition, it has been possible to exploit differences in cellular modified eIF-5A to examine the role of eIF-5A in general protein synthesis in these cells.

MATERIALS AND METHODS

Cell culture and drug treatments

HTC and HMOA cells were obtained from Dr. Peter McCann (Marion Merrell Dow Pharmaceutical Co., Cincinnati, OH, U.S.A.). HMOA cells were isolated for their resistance to growth inhibition in α-methylornithine and characterized by Mamont [26]. DH23A and DH23b cells (DH23A/b), two variants selected for their ability to grow in the presence of α-difluoromethylornithine (DFMO), were donated by Dr. John L. A. Mitchell (Northern Illinois University, DeKalb, IL, U.S.A.). The development and characterization of these cells have been described [24]. HTC, HMOA and DH23A cells were grown as previously described [27]. DH23b cells were grown in similar medium with the addition of 25 mM Hepes (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.5 g/l sodium bicarbonate (Mallinkrodt, Inc. Paris, KY, U.S.A.). DH23A/b cells were maintained in medium containing 10 mM DFMO unless noted. Treatment of cells with DFMO was generally at 5 mM DFMO for HTC and HMOA cells and at 10 mM for DH23A/b cells in order to achieve the same level of polyamine depletion. DFMO was generously donated by the Marion Merrell Dow Company (Cincinnati, OH, U.S.A.).

Polyamine and protein measurements

Acid-soluble polyamines were extracted and cellular protein precipitated as previously described [27]. Polyamines were eluted and detected using the method of Seiler and Knodgen [28]. Peaks

Abbreviations used: eIF-5A, eukaryotic initiation factor 5A; ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine.

* To whom reprint requests should be addressed.
were identified and quantified by comparison with commercially available compounds used as external standards.

Acid-precipitable proteins were quantified using the BCA protein assay kit from Pierce (Rockford, IL, U.S.A.), as previously described [27].

**Modification of eIF-5A in intact cells**

Cells were incubated in the presence of terminal methylenes [1,8-$^3$H]spermidine; DuPont NEN, Boston, MA, U.S.A.) under the conditions described below. For analysis of modification rates, cells were incubated for 48 h in the presence of DFMO and subsequently incubated with 5 µM spermidine in the presence of 20 µCi/ml radiolabelled spermidine. Measurements of eIF-5A turnover were made by first treating cells with DFMO for 48 h, pulse-labelling the cells in the absence of DFMO with 3 µCi/ml [1,8-$^3$H]spermidine for 6 h and then incubating the cells in the presence of 5 µM spermidine for the indicated times.

The amounts of radiolabel in hypusine (in eIF-5A) were analysed by eluting the protein from an SDS/PAGE gel as described below (under ‘Protein gel electrophoresis’) or by hydrolysis of precipitated protein and elution of hypusine from the HPLC column. At specified times, cells were harvested by centrifugation (500 g for 5 min) and washed twice in PBS (136.9 mM NaCl, 27 mM KCl, 1.4 mM KH$_2$PO$_4$, 8.1 mM NaHPO$_4$), pH 7.4. For SDS/PAGE analysis, samples were boiled in sample buffer and separated by one-dimensional gel electrophoresis (see below). For analysis by HPLC, total cellular protein was precipitated, hydrolysed and separated as described [29].

Hypusine was identified as radiolabel eluting at a position corresponding to the elution position of free hypusine [29]. Hypusine was quantified by counting of radioactive activity in aliquots of column eluate on a liquid scintillation counter (LS 5000TD, HYPUSINE was quantified by counting of radioactivity in aliquots of column eluate on a liquid scintillation counter (LS 5000TD, DuPont–NEN) was added to each culture. Samples were removed from the culture at specified times and incubated in the presence of 20 µCi/ml [35$^S$]methionine for 3 min at 37 °C. Incorporation of [35$^S$]methionine into protein and acid-soluble label were quantified and normalized as described above.

**Protein synthesis measurements**

For continuous labelling experiments, cells were added to fresh medium minus methionine, and 50 µCi/ml [35$^S$]methionine (DuPont–NEN) was added to each culture. Samples were removed at specified times and diluted 30-fold into medium containing no label on ice. Cells were pelleted by centrifugation (500 g for 5 min) and the cell pellet was washed twice with PBS, pH 7.4. Cellular proteins were acid-precipitated and protein pellets dissolved as for polyamine measurements. Acid-precipitable counts were quantified by liquid scintillation counting as described above, and measurements normalized to cellular protein. For the rate measurements during the longer time course, aliquots were removed from the culture at specified times and incubated in the presence of 20 µCi/ml [35$^S$]methionine for 3 min at 37 °C. Incorporation of [35$^S$]methionine into protein and acid-soluble label were quantified and normalized as described above.

**Partial purification of modified and unmodified eIF-5A**

eIF-5A was partially purified for analysis and for use in deoxyhypusine synthetase assays using the method described by Park [31], except that a Mono Q HR 5/5 column connected to an FPLC system (Pharmacia LKB Biotechnology, Uppsalas, Sweden) was used for the separation. Preparation of the partially purified unmodified eIF-5A for use in the deoxyhypusine synthetase assays required the pooling of cell pellets (total cell number > 1.6 x 10$^6$) from HTC cells grown for 4 days in the presence of 5 mM DFMO. eIF-5A was eluted using the conditions described by Park [31]. Samples corresponding to the fractions containing trace radiolabelled eIF-5A were pooled and concentrated using Centricon concentrators (3000 Da cut-off) (Amicon, Inc., Beverly, MA, U.S.A.). Aliquots were analysed by SDS/PAGE to check for eIF-5A and for protein content as described above.

**Collection of samples for measurement of unmodified eIF-5A and deoxyhypusine synthetase**

Cells were harvested, and the precipitates from the 0-45% ammonium sulphate cut (containing deoxyhypusine synthetase activity) and the 45–75% ammonium sulphate cut (containing eIF-5A protein) were separated and prepared as described [14]. An aliquot of the final sample was assayed for protein content and the sample was stored at –80 °C until analysis. Before measurement of enzyme or substrate, an aliquot, normalized for protein, was diluted into 0.25 M glycine/NaOH buffer, pH 9.5.

**Deoxyhypusine synthetase assays and measurement of unmodified eIF-5A in vitro**

Deoxyhypusine synthetase activity was measured by quantifying the incorporation of [1,8-$^3$H]spermidine in vitro into the unmodified eIF-5A precursor partially purified from HTC cells, as described above, using reaction conditions similar to those described by Wolff et al. [32]. Reaction mixtures contained 10 µCi of [1,8-$^3$H]spermidine (4.9 µM), 0.5 mM NAD$^+$, 1 mM diethiothreitol, 125 µg of BSA, 28 nM eIF-5A precursor protein and an aliquot of enzyme preparation in a total volume of 0.1 ml of 0.25 M glycine/NaOH buffer, pH 9.5. Reactions were incubated for 6 h at 37 °C and then 500 µg of BSA was added to each tube. The protein was immediately precipitated by adjusting the concentration of the solution to 0.2 M HClO$_4$/2 mM spermidine, and radiolabel incorporated into the protein was analysed by HPLC as described above. Activities were normalized to protein measurements.

Measurements of unmodified eIF-5A were obtained using reaction conditions similar to those for the measurement of enzyme activity, except the enzyme/substrate ratio was decreased and the reaction was allowed to run to completion. Reaction mixtures contained the components described for the synthetase assays with additional [1,8-$^3$H]spermidine (9.8 µM; 20 Ci), a known amount of deoxyhypusine synthetase from HTC cells and an aliquot of the substrate preparation. Samples were incubated for 6 h or 16 h at 37 °C, and radiolabel incorporated into hypusine was measured by HPLC or eluted from the excised eIF-5A band on an SDS/PAGE gel as described above. A similar measurement was used to determine the concentration of unmodified eIF-5A in the large substrate partial purification used for the enzyme assays. Amounts were normalized for protein content.

**Northern analyses**

A cDNA probe was constructed for Northern analyses by using PCR to synthesize cDNA with the human eIF-5A sequence.
PCR reactions contained the exact-match human primers (4 μM concentration) 5’ TGCGAGGCAGGATTACCT 3’ and 5’ CGAGTTGGAATGCGCCCT 3’ [33], and a human buccal mucosa DNA template. Buccal mucosa cells were harvested by scraping into saline solution, washed twice and the DNA isolated using standard methods [34]. A 30-cycle PCR amplification was run using the following parameters: 95 °C for 30 s; 50 °C for 30 s and 72 °C for 1 min, with reaction mixtures as recommended by the protocol accompanying the Taq polymerase (Gibco-BRL, Gaithersburg, MD, U.S.A.). A region of the agarose gel containing the predicted product size range (~ 500 bp) was excised, the DNA extracted using the ‘freeze-squeeze’ method [35] and the extracted DNA used as the template for a second PCR amplification as above. PCR yielded a single product of ~ 511 bp which was subcloned and sequenced according to standard protocols [34]. The resulting subclone used to synthesize the probe was 89.9% identical to the published human eIF-5A sequence [36] at the nucleic acid level.

For RNA isolation, 2 × 10⁶ cells were harvested and washed twice with PBS, pH 7.4. Cell pellets were sonicated into lysis solution and RNA isolated using the solutions and protocol from the RNaid kit (Bio 101, Inc., La Jolla, CA, U.S.A.). RNA was separated by size using standard RNA gel electrophoresis methods on a 1.5 % agarose gel run for 16 h at 20 V with 20 μg of RNA/lane [34]. Size determinations were made by comparison with a commercial 0.24–9.5 kb RNA ladder (Gibco-BRL). The RNA was transferred to a nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH, U.S.A.) [34] and UV-cross-linked to the membrane using the Stratalinker 1800 autocrosslink program (Stratagene, Inc., La Jolla, CA, U.S.A.).

Radiolabelled probe was synthesized using the protocol accompanying the Taq polymerase (Gibco-BRL), except that 100 μCi of [α-32P]dATP (3000 Ci/mmol; ICN) was used for labelling instead of dCTP. Labelled probe was separated from unincorporated label using column chromatography [34]. Blots were prehybridized and then hybridized with 2 × 10⁶ c.p.m./ml at 65 °C overnight [34]. Membranes were washed at high stringency as in [36] and exposed to film (X-Omat; Kodak, Rochester, NY, U.S.A.) at ~ 80 °C. eIF-5A transcript levels were compared by calculation of band density using a phosphoimager (Imagequant Densitometer; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

Rate of formation of modified eIF-5A

The first indication that the eIF-5A modification pathway might be altered by drug selection came from a comparison of the formation of modified eIF-5A in the three cell lines (Figure 1A). Modified eIF-5A was apparently synthesized at different rates in the three cell lines after cellular spermidine had first been depleted by DFMO treatment and then re-added to the cell culture. HTC cells synthesized modified eIF-5A at a moderate rate, while the rate in HMOA cells was somewhat lower. DH23A cells synthesized modified eIF-5A much faster than either of the other two cell types. Under these conditions cellular spermidine levels were similar (Figure 1B), suggesting that this apparent rate difference was not due to spermidine concentration. No accumulation of the deoxyhypusine form of eIF-5A was found in any of these cell lines (results not shown).

Turnover of modified eIF-5A and comparison of eIF-5A protein

To test whether an apparent difference in synthesis was actually an alteration in degradation rate, the eIF-5A half-life was compared in the three cell lines (Figure 2). Pulse–chase experiments showed that the turnover of eIF-5A was similar in HTC, HMOA and DH23A cells. Modified eIF-5A is a very stable protein with a half-life of about 24 h, which corresponds to the culture doubling times of these cells.

Comparison of deoxyhypusine synthetase activities in the three cell lines

Deoxyhypusine synthetase activity measurements showed that the activity was similar in HTC cells (mean 145 c.p.m./h per μg of protein; range 123–164; n = 16), in HMOA cells (mean 136 c.p.m./h per mg of protein; range 126–156; n = 18) and in DH23A cells (mean 128 c.p.m./h per mg of protein; range 118–146; n = 18). The activity appears to be unaltered by growth phase or by growth in the presence of DFMO.

Analysis of the accumulation of unmodified eIF-5A

Unmodified eIF-5A accumulated only to a small degree under normal growth conditions in HTC cells; however, a significant amount accumulated when the cells were incubated in the
presence of DFMO (Figure 3). Cells continued to synthesize eIF-5A as cell growth continued into the plateau phase. Analysis of the accumulation of unmodified eIF-5A in the three cell lines showed that the DH23A cells can accumulate more unmodified eIF-5A than either of the other two cell lines when grown in the presence of DFMO (Figures 3B and 3C). A comparison of the accumulation of unmodified eIF-5A in HTC and HMOA cells by a more sensitive assay indicated that the HTC cells appear to accumulate slightly more unmodified eIF-5A than the HMOA cells on average (9.06 ± 1.29 compared with 6.82 ± 1.52 fmol/mg of protein) after 2 days' growth in the presence of 5 mM DFMO. In DH23A cells, accumulation of additional eIF-5A was not due to increased eIF-5A mRNA (Figure 4). eIF-5A transcript levels were similar in HTC and HMOA cells, and apparently not affected by treatment with DFMO for 48 h. DH23b cells showed slightly less eIF-5A mRNA, ~80% of that in HTC cells in the absence of DFMO. Growth in DFMO also slightly depressed eIF-5A mRNA in these cells; transcript levels in DH23b cells in the presence of DFMO were ~70% of those seen in its absence. eIF-5A mRNA in these cells also appeared to be unaltered by growth into the plateau phase, as shown for HTC cells (Figure 4A).

Effects of altered amounts of cellular eIF-5A on general protein synthesis

To test the hypothesis that eIF-5A is involved in general protein synthesis, protein synthesis rates in these cells were compared under conditions where the intracellular eIF-5A content should
deoxyadenosine, which blocks spermidine synthesis by inhibiting the enzymatic activity of S-adenosylmethionine decarboxylase [6,8,9]. In the absence of DFMO, little unmodified eIF-5A accumulates, suggesting that modification of eIF-5A occurs shortly after translation of the eIF-5A precursor [39–41]. Comparison of the components of the eIF-5A modification process has shown that this pathway is similar in the three cell lines. No alterations in deoxyhypusine synthetase activity, deoxyhypusine hydroxylase activity, turnover of modified eIF-5A protein or the eIF-5A protein itself were seen. This suggests that differences in precursor accumulation are probably the cause of the observed differences in modification. Slightly lower accumulation of unmodified eIF-5A in HMOA cells when compared with that in HTC cells after the cells were cultured in the presence of DFMO for 48 h is consistent with the slightly decreased modification rate observed upon spermidine depletion. The elevated rate observed in DH23A cells is due to increased accumulation of the unmodified substrate, as the cells are continuously grown under chronically limiting spermidine.

Comparison of eIF-5A mRNA levels in the three cell lines suggests that the increased accumulation of eIF-5A in DH23A cells is not due to increased transcription; in fact, eIF-5A mRNA is slightly decreased in DH23A cells compared with HTC cells. Expression of eIF-5A mRNA appears to be constitutive in HTC cells and in the other cell lines as well (results not shown); however, there is some evidence that eIF-5A expression is growth-related after long-term culture. For example, newly cultured IMR-90 fibroblasts show decreased eIF-5A mRNA during senescence that subsequently increases when serum is added to the medium [42]. This response is attenuated as the passage number of the cells increases. Further, a survey of eIF-5A expression in a number of tissue culture cell lines by another group found constitutively high expression [43]. Immortalization of the cells in tissue culture may select for cells with constitutively high eIF-5A expression. In the three cell lines used in the present work there is no evidence for major growth regulation of eIF-5A mRNA expression or for regulation by putrescine and spermidine depletion. eIF-5A mRNA is slightly decreased, however, in DH23A cells, especially in the presence of DFMO. This may be connected to the slightly decreased growth rate observed under these culture conditions in these cells [27].

Manipulation of eIF-5A levels, at least on this scale, does not affect general protein synthesis in this system. Decreased eIF-5A, due to DFMO treatment, did not decrease general protein synthesis; nor did increased time of incubation in DFMO alter the rate in DH23A cells. This suggests that eIF-5A may not be involved in general protein synthesis in mammalian systems; however, the possibility that eIF-5A is not limiting has not been ruled out. Although eIF-5A was initially characterized as a protein synthesis initiation factor because it stimulated the methionyl-puromycin assay [44], a role for eIF-5A in general protein synthesis has not been found. In yeast, when eIF-5A was decreased to non-detectable levels, general protein synthesis has not been found. In yeast, when eIF-5A was decreased to non-detectable levels, general protein synthesis decreased only by 30% [16]. Further, eIF-5A does not stimulate globin synthesis in vitro in the presence of all the other initiation factors [44,45]. The role of eIF-5A in protein synthesis may be more specific, e.g. stimulating the translation of a specific subset of mRNAs, as suggested by the data of Hanauke-Abel et al. [46]. Another possibility has been suggested by the results of Ruhl et al. [33], showing that eIF-5A is necessary for Rev function during viral infection. Rev has been implicated in the processing and/or transport of certain mRNAs, resulting in increased translation. eIF-5A may facilitate one of these processes. Selection in drugs that inhibit ODC does not cause alterations in the eIF-5A modification pathway, although modification of eIF-5A is both dependent upon the presence of spermidine and spermine. In DH23A cells cultured continuously in DFMO, where spermidine is limiting, cells accumulated unmodified eIF-5A (Figure 5), and yet proliferated, thereby decreasing the cellular eIF-5A content. Incorporation of [35S]methionine into cellular protein, however, was very similar in HTC and HMOA cells in the absence of DFMO to that in DH23A cells in the presence of 10 mM DFMO (Figure 5A). Further, general protein synthesis rates appeared to be unaffected by the content of intracellular deoxyhypusine, as suggested by the data of Hanauske-Abel et al. [46]. Another possibility has been suggested by the results of Ruhl et al. [33], showing that eIF-5A is necessary for Rev function during viral infection. Rev has been implicated in the processing and/or transport of certain mRNAs, resulting in increased translation. eIF-5A may facilitate one of these processes.

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critical for growth. DH23A/b cells, growing in the presence of DFMO, apparently respond to chronically decreased spermidine levels by funneling a portion of newly synthesized spermidine to eIF-5A modification to allow the cells to proliferate. Modified eIF-5A may be limiting, especially in DH23A cells, and that this is a critical factor in the cytostasis often observed after depletion of cellular polyamines.

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