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Synthesis of homocysteine thiolactone by methionyl-tRNA synthetase in cultured mammalian cells

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Homocysteine thiolactone is a product of an error-editing reaction, catalyzed by *Escherichia coli* and *Saccharomyces cerevisiae* methionyl-tRNA synthetases, which prevents incorporation of homocysteine into tRNA and protein both in vitro and in vivo. Here, homocysteine thiolactone is also shown to be synthesized by cultured mammalian cells such as human cervical carcinoma (HeLa), mouse renal adenocarcinoma (RAG), and Chinese hamster ovary (CHO) cells labeled with [^{35}S]methionine, but not by normal human and mouse (Balb/c 3T3) fibroblasts. A temperature-sensitive methionyl-tRNA synthetase mutant of CHO cells, Met-1, does not make the thiolactone at the non-permissive temperature. The data indicate that methionyl-tRNA synthetase is involved in synthesis of homocysteine thiolactone in CHO cells, thereby extending this important proofreading mechanism to mammalian cells.

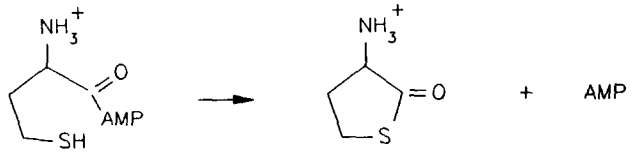
Methionyl-tRNA synthetase; Homocysteine thiolactone; Translational accuracy; Error-editing reaction

1. INTRODUCTION

Accurate reading of the genetic code is determined by two pivotal steps of protein synthesis. In the first step, each of the twenty aminoacyl-tRNA synthetases correctly matches a cognate amino acid selected from the twenty protein amino acids (and a few nonprotein ones like homocysteine, homoserine, ornithine, etc.) with a cognate tRNA selected from the twenty tRNA families. In the second step, a correct aminoacyl-tRNA is selected in the codon programmed ribosomal A site. High accuracy of aminoacyl-tRNA synthetases assures proper assignment of an amino acid to its cognate tRNA and is achieved by highly specific recognition of tRNA [1] and by proofreading or editing mechanisms which remove errors in amino acid selection [2,3].

The editing mechanism of methionyl-tRNA synthetase is directed against homocysteine, which is an obligatory intermediate in methionine biosynthesis in all organisms. As expected from in vitro studies [4,5], homocysteine can be misactivated in vivo at unacceptably high levels [6,7]. Misactivated homocysteine is very efficiently edited, both in vitro [5] and in vivo [6,7], which prevents its incorporation into cellular proteins in microorganisms.

A distinctive feature of homocysteine editing is that the enzyme-bound homocysteinyl adenylate (**I**) is cyclized to yield homocysteine thiolactone (**II**) (Equation 1):



I **II** **(1)**

This allowed editing to be studied in vivo and led to experimental measurements of the energy costs associated with editing of homocysteine by methionyl-tRNA synthetase in *E. coli* [6] and the yeast *S. cerevisiae* [7]. Here we report that homocysteine thiolactone is synthesized by mammalian cells in tissue culture. In Chinese hamster ovary (CHO) cells, the thiolactone synthesis is due to editing of homocysteine by methionyl-tRNA synthetase, thus establishing the existence of an error-editing mechanism in mammalian cells.

2. MATERIALS AND METHODS

2.1. *Cell lines*

Normal human fibroblasts (GM1374) were obtained from NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Human cervi-

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Abbreviations: CHO, Chinese hamster ovary; MEM, minimal essential medium; TLC, thin layer chromatography; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

cal carcinoma (HeLa) was from American Type Culture Collection (Rockville, MD). Normal embryonic mouse fibroblasts (Balb/c 3T3 A31) [8] and mouse renal adenocarcinoma (RAG) [9] cells were obtained from H. Ozer [10] and R. Athwal, respectively (New Jersey Medical School, Newark, NJ). Temperature-sensitive Chinese hamster ovary (CHO) mutants for methionyl-tRNA (Met-1) and arginyl-tRNA (Arg-1) synthetases as well as parental wild type (Gat$^+$; glycine, adenosine and thymidine requiring) were kindly supplied by Larry Thompson (Lawrence Livermore Laboratory, Livermore, CA) (11).

2.2. *Cell culture and ^{35}S-labeling conditions*

Cells were maintained as monolayer cultures in 10 cm dishes at 37°C or 34°C (CHO cells) in an atmosphere of 7.5% CO_2. The culture medium was α-MEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 10 μg/ml adenosine, and 10 μg/ml deoxythymidine. For ^{35}S-labeling, cells were plated in 3 cm dishes and grown to 70–80% confluency. The medium was then replaced with 0.45 ml methionine-free α-MEM supplemented with 5% fetal bovine serum, 10 μg/ml adenosine, 10 μg/ml deoxythymidine, and 10 μM [^{35}S]methionine at 0.1 mCi/ml (10^4 Ci/mol; 1 Ci = 37 GBq) (Amersham). Unless otherwise stated, cells were labeled at 37°C for 6 h.

2.3. *Preparation and analysis of ^{35}S-labeled extracts*

At specified time intervals, cell-free medium was removed and frozen at −80°C. Cells were extracted with 2 × 0.125 ml 1 M formic acid at 0°C for 30 min and frozen at −80°C. Incorporation of [^{35}S]methionine into protein was determined by trichloroacetic acid precipitation. Levels of homocysteine thiolactone in samples were determined by two-dimensional TLC. Prior to TLC, samples were thawed and clarified by centrifugation in an Eppendorf microcentrifuge for 5 min at 4°C. Two-dimensional TLC was carried out as described previously [7]. Radioactivity co-migrating on TLC plates with an authentic homocysteine thiolactone (Sigma) standard was visualized by autoradiography and quantitated by scintillation counting. In control experiments, the radioactivity associated with homocysteine thiolactone spots was shown to be sensitive to NaOH treatment. The method allows detection of homocysteine thiolactone at concentrations as low as 1 nM. Commercial preparations of [^{35}S]methionine were found to contain < 0.01% [^{35}S]homocysteine thiolactone.

3. RESULTS

Labeling protocols utilizing ^{35}S-labeled sulfate or cysteine which allowed detection of homocysteine thiolactone in microorganisms [6,7] cannot be used with mammalian cells due to different organization of relevant metabolic pathways [12]. In mammalian cells, the transsulfuration pathway converts methionine, an essential amino acid, into cysteine with homocysteine as an intermediate (Fig. 1). The pathway starts with formation of *S*-adenosylmethionine, which yields *S*-adenosylhomocysteine in subsequent transmethylation reactions. In addition to being transformed into cysteine, homocysteine is remethylated back to methionine. Thus, the conversion of methionine to homocysteine can be exploited in order to determine whether homocysteine thiolactone is formed in mammalian cells.

Several different mammalian cell lines growing in monolayer cultures were labeled with [^{35}S]methionine for 6 h. Aliquots of cell-free media as well as formic acid extracts of labeled cells were analyzed by two-dimensional TLC and the thiolactone spots were quantitated by scintillation counting. As shown in Table I, homocysteine thiolactone was present in cultures of HeLa

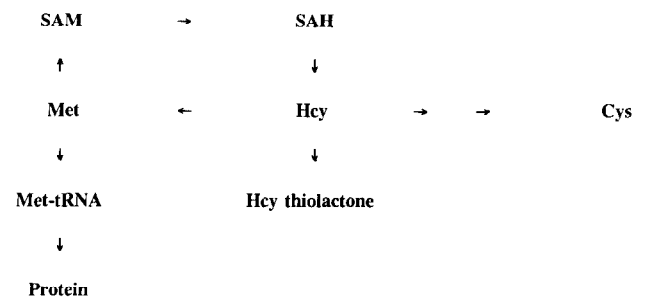

Fig. 1. Schematic representation of metabolism of homocysteine and methionine in mammalian cells [12]. Synthesis of homocysteine thiolactone by mammalian cells has been established in this work.

cells, mouse RAG cells, and CHO cells. Most (95%) of the thiolactone formed in cell cultures was found in cell-free media, which indicates that the thiolactone is secreted from cells, as found previously with microbial cells [6,7]. The highest level of homocysteine thiolactone of 17.4 pmol/10^7 cells, measured in RAG cell cultures, corresponds to a homocysteine thiolactone concentration of 20 nM in cell-free medium. Homocysteine thiolactone was not detectable in cell cultures of normal human (GM1374) and mouse (Balb/c 3T3) fibroblasts at any time of labeling up to 24 h (Table I). Control experiments showed that these cells incorporated [^{35}S]methionine into *S*-adenosylmethionine and protein as expected (not shown).

By normalizing the amount of homocysteine thiolactone to incorporation of methionine into protein, one can estimate the fraction of methionine metabolized into the thiolactone in mammalian cells. The highest fraction (0.6%) of methionine was converted into homocysteine thiolactone in mouse RAG cells. This level

Table I

Homocysteine thiolactone levels and ratios of homocysteine thiolactone to incorporation of methionine into protein in mammalian tissue cultures

Cell line	Hcy thiolactone (pmol/10^7 cells)	Hcy thiolactone/ protein × 10^5
Human		
Normal fibroblasts (GM1374)	< 0.06[a]	< 2
HeLa	0.9	16
Mouse		
Balb/c 3T3 (A31N)	< 0.2[a]	< 10
RAG	17.4	600
Chinese hamster ovary (Gat)	2.0	240

The thiolactone levels were measured at 37°C (human and mouse cells) or 34°C (CHO cells) as described in section 2.

[a]Similar results were obtained after 3, 6, 12 and 24 h labeling.

of the thiolactone synthesis is comparable to that observed in microbial cells [6,7].

In order to test whether methionyl-tRNA synthetase is involved in synthesis of homocysteine thio-lactone, the thiolactone levels were measured in Met-1, a methionyl-tRNA synthetase mutant of CHO cells. The Met-1 mutant has a temperature-sensitive methionyl-tRNA synthetase and grows at 34°C but not at 39.5°C. Methionyl-tRNA synthetase activity is undetectable in crude extracts prepared from the Met-1 mutant cells at any temperature [11], which we have also confirmed. As shown in Table II, the Met-1 mutant produces less homocysteine thiolactone than wild type even at the permissive temperature (34°C). At a nonpermissive temperature (39.5°C), no thiolactone was detectable in cultures of the Met-1 mutant whereas wild type cells made 10 times more thiolactone at this temperature than at 34°C. In order to rule out the possibility of effects due to aminoacyl-tRNA synthetase mutations in general, or other non-specific effects, a control experiment was performed with a temperature-sensitive arginyl-tRNA synthetase mutant, Arg-1. This control experiment showed that the Arg-1 mutation did not diminish homocysteine thiolactone levels at 39.5°C and, for reasons not understood, actually had a 3- to 4-fold increase in the thiolactone levels at 34°C. It is not clear why homocysteine thiolactone levels increase at 39.5°C in wild type and Arg-1 cells. Perhaps relative affinity of homocysteine for methionyl-tRNA synthetase is greater at 39.5°C than at 34°C. More important, however, is the fact that the Met-1 mutant was partially deficient in homocysteine thiolactone synthesis at a permissive temperature and totally deficient at a nonpermissive temperature, which indicates that methionyl-tRNA synthetase is involved in homocysteine thiolactone synthesis in CHO cells.

Table II

Homocysteine thiolactone levels in cultures of wild type and temperature-sensitive aminoacyl-tRNA synthetase mutants of Chinese hamster ovary cells

Cell line		Homocysteine thiolactone (pmol/10^7 cells)	
		34°C	39.5°C
Wild type,	6 h	2.0	3.7
	12 h	1.6	16.4
Met-1,	6 h	0.3	< 0.1
	12 h	0.5	< 0.1
Arg-1,	6 h	3.0	5.8
	12 h	7.6	21.3

The thiolactone levels were measured at 34°C and 39.5°C after 6 h and 12 h of labeling with [^{35}S]methionine as described in section 2.

4. DISCUSSION

The presence of homocysteine thiolactone in mammalian cell cultures and involvement of methionyl-tRNA synthetase in its synthesis document conservation of a basic error editing mechanism in selection of amino acids for protein synthesis in cells of organisms as diverse as bacteria and mammals. This editing reaction (Eq. 1) was discovered in vitro [5] and subsequently shown to exist in vivo in *E. coli* and *S. cerevisiae* (6,7). It is possible that homocysteine thiolactone can be made from methionine by another route in mammalian cells; however, the behavior of the Met-1 mutant of CHO cells indicated that the thiolactone is synthesized by methionyl-tRNA synthetase (Table II), and it seems unlikely that methionyl-tRNA synthetase would convert methionine into homocysteine thiolactone. Moreover, homocysteine thiolactone levels are positively correlated with Hcy/Met ratios: cells known to have higher Hcy/Met ratios (e.g. a variety of transformed cells; refs. [13,14]) also produce more homocysteine thiolactone (Table I). Thus, the mechanism of homocysteine thiolactone formation from methionine in mammalian cells most likely involves the route depicted in Fig. 1. Similar mechanism has also been shown to be responsible for homocysteine thiolactone formation from methionine in bacterial cells [15].

Homocysteine thiolactone has not previously been reported in cultured mammalian cells. However, there were several attempts to detect homocysteine thiolactone in mammalian tissues. Spindel and McCully [16] reported that intraperitoneally injected [^{14}C]methionine was metabolized into [^{14}C]homocysteine thiolactone in guinea pig liver, but the identification of the ^{14}C-radioactivity was questionable because the aqueous wash procedure, which removes authentic homocysteine thiolactone from chloroform-methanol extracts, failed to remove the ^{14}C-radioactivity from chloroform/methanol extracts of the liver. Homocysteine thiolactone has been reported to be absent in human serum and plasma [17–19] and in rabbit plasma and urine [20] but the detection limits (10–50 μM) of the methods used were poor. In this study, using a method allowing detection of homocysteine thiolactone at nanomolar concentrations, we have demonstrated that the thiolactone is a component of sulfur amino acid pools in mammalian cells in tissue culture. The levels of homocysteine thiolactone in tissue cultures of at least some mammalian cells can be as high as the thiolactone levels in cultures of microbial cells.

Our inability to detect the thiolactone in normal mammalian cells is reminiscent of the situation in wild type yeast cells in which homocysteine thiolactone is almost undetectable [7]. Apparently, in normal mammalian cells, as in yeast, the need for homocysteine editing is minimized due to the presence of a pathway that converts homocysteine into cysteine (Fig. 1) which

leads to relatively low Hcy/Met ratios. The Hcy/Met ratios for normal diploid cells are 0.06 [13], a value similar to that in wild type yeast cells [7]. In transformed human diploid cells the Hcy/Met ratio is 0.15 [13].

The observation that homocysteine thiolactone is present in transformed mammalian cells but undetectable in normal cells is consistent with the notion that transformation leads to imbalances in metabolism of homocysteine and methionine [13,14,21] and raises a possibility that the thiolactone may be a biochemical marker for transformed cells. It should be noted that methionyl-tRNA synthetase levels are similar in normal and transformed human fibroblasts [22], which indicates that increased homocysteine thiolactone synthesis in transformed cells is not due to increases in methionyl-tRNA synthetase levels. Altered metabolism of homocysteine and methionine in transformed cells is manifested phenotypically as an inability of the transformed cells to grow in methionine-free homocysteine medium [13,14,20,23]. The enhanced ability of transformed cells to synthesize homocysteine thiolactone described here may also contribute to this phenotype. Transformed cells incubated in homocysteine medium have Hcy/Met ratios at least 20 times higher than normal cells [13,14]. Such high Hcy/Met ratios would lead to excessive synthesis of homocysteine thiolactone by methionyl-tRNA synthetase which is inadvertently associated with expenditure of energy (ATP hydrolysis) and may be reflected in slower growth rate [7]. As shown in Table I, 0.6% of energy used for synthesis of Met-tRNA is expended on homocysteine editing in RAG cells. In homocysteine medium the energy expenditure would increase to over 12%. This level of the energy expended on editing is known to diminish growth rate in yeast [7].

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