Regulation of type I collagen mRNA in lung fibroblasts by cystine availability

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The steady-state level of α1(I) collagen mRNA is regulated by amino acid availability in human lung fibroblasts. Depletion of amino acids decreases α1(I) collagen mRNA levels and repletion of amino acids induces rapid re-expression of α1(I) mRNA. In these studies, we examined the requirements for individual amino acids on the regulation of α1(I) collagen mRNA. We found that re-expression of α1(I) collagen mRNA was critically dependent on cystine but not on other amino acids. However, the addition of cystine alone did not result in re-expression of α1(I) collagen mRNA. Following amino acid depletion, the addition of cystine with selective amino acids increased α1(I) collagen mRNA levels. The combination of glutamine and cystine increased α1(I) collagen mRNA levels 6.3-fold. Methionine or a branch-chain amino acid (leucine, isoleucine or valine) also acted in combination with cystine to increase α1(I) collagen mRNA expression, whereas other amino acids were not effective. The prolonged absence of cystine lowered steady-state levels of α1(I) collagen mRNA through a mechanism involving decreases in both the rate of gene transcription as assessed by nuclear run-on experiments and mRNA stability as assessed by half-life determination in the presence of actinomycin D. The effect of cystine was not mediated via alterations in the level of glutathione, the major redox buffer in cells, as determined by the addition of buthionine sulphoximine, an inhibitor of γ-glutamylcysteine synthetase. These data suggest that cystine directly affects the regulation of α1(I) collagen mRNA.

INTRODUCTION

Interstitial fibroblasts within a biomatrix are exposed to varying concentrations of amino acids. We found previously that the steady-state level of α1(I) collagen mRNA in human lung fibroblasts was selectively regulated by alterations in amino acid availability [1]. Deprivation of amino acids resulted in large decreases in α1(I) collagen mRNA levels that were rapidly restored following re-exposure to amino acids. These effects were mediated by alterations in the rate of transcription of the α1(I) collagen gene and the stability of α1(I) collagen mRNA. In contrast, the steady-state levels of cyclooxygenase 1 or Gs, a GTP-binding protein, were not affected by amino acid deprivation.

The full complement of amino acids contained in standard tissue-culture medium contains both essential and non-essential amino acids. The amino acid content of media was formulated to provide optimal growth conditions for cells rather than to simulate conditions *in vivo*. The uptake of amino acids is mediated by several distinct transport systems whose activities are variably affected by Na^+ concentration, pH, amino acid levels and effector substances [2,3]. The differential regulation of transport systems suggests the possibility that individual amino acids exert distinct actions on intracellular systems, perhaps by changes in the level of aminoacylation of tRNA [4–6]. In this regard, the rate of protein metabolism in histidine-starved Chinese-hamster ovary cells is modified by the level of aminoacylation of tRNA [4].

Effector substances may alter α1(I) collagen mRNA levels, in part via changes in amino acid availability. Prostaglandin E_2 and retinoic acid decrease both amino acid uptake and collagen formation [7–9]. The decrease in amino acid uptake precedes the decrease in the steady-state level of α1(I) collagen mRNA. Interferon-γ decreases expression of collagenase and stromelysin by decreasing the intracellular concentration of tryptophan [10,11]. Interferon-γ also decreases the production of α1(I) collagen mRNA [11]. We found that the addition of tryptophan to amino acid-deficient medium did not restore α1(I) mRNA levels [1]. These results suggest that one or more amino acids other than tryptophan are required to re-express α1(I) mRNA levels following amino acid deprivation.

In these studies, we examined the action of specific amino acids on α1(I) collagen mRNA levels. We found that α1(I) collagen mRNA levels were critically dependent on cystine availability. The omission of cystine caused large decreases in the rate of transcription of the α1(I) collagen gene and decreases in the stability of the mRNA. Cystine interacted strongly with glutamine and branch-chain amino acids to regulate α1(I) collagen mRNA levels through a mechanism that did not involve alterations in the redox state of the cell as assessed by glutathione levels.

MATERIALS AND METHODS

Cells and tissue cultures

Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research, Camden, NJ, U.S.A.) were grown in Dulbecco's modified Eagle's medium with 0.37 g of sodium bicarbonate/100 ml, 10 % (v/v) fetal bovine serum, 100 U of penicillin/ml, 10 μg streptomycin/ml and 0.1 mM non-essential amino acids. After confluence was reached, the serum content of the medium was reduced to 0.4 %. Cell numbers were determined in triplicate with an electronic particle counter (Coulter Counter ZM).

Abbreviations used: BSO, buthionine sulphoximine; PAPS, adenosine 3′-phosphate 5′-phospho-sulphate; UTR, untranslated region.

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RNA isolation and Northern-blot analysis

Total cellular RNA was isolated by the single-step method employing guanidine thiocyanate/phenol/chloroform extraction, as described by Chomczynski and Sacchi [12]. RNA was quantified by absorbance at 260 nm. Purity was determined by absorbance at 280 and 320 nm. RNA (10 μg) was electrophoresed on a 1 % (w/v) agarose/6 % (v/v) formaldehyde gel and transferred to a nitrocellulose filter. RNA loading was assessed by ethidium bromide staining of ribosomal bands fractionated on agarose/formaldehyde gels and by co-hybridization with an oligonucleotide that encodes the 18 S ribosomal fragment. Hybridization was performed using $0.5–1.0 \times 10^6$ c.p.m./lane of labelled probe (specific activity, $4–10 \times 10^8$ c.p.m./μg), and the filter was washed according to methods described by Thomas [13]. The filter was exposed to X-ray film for autoradiography at several different times to ensure that the bands could be quantified by densitometry within the linear range. The probes utilized in these experiments were: rat α1(I) collagen [14], which specifically identifies corresponding human α1(I) mRNA; Gs, which encodes a GTP-binding protein (kindly provided by Dr. R. Reed, Johns Hopkins University School of Medicine, Baltimore, MA, U.S.A.) [15]; transforming growth factor-β receptor 1 [16]; and an oligonucleotide that specifically identifies the 18 S ribosomal subunit [17].

Assessment of protein synthesis

Quiescent fibroblast cultures were incubated at 37 °C with fresh medium with or without cystine and containing [^{35}S]methionine. Following the incubation, the cellular material was extracted with 1 ml of 10 % (v/v) trichloroacetic acid. The amount of trichloroacetic acid-insoluble [^{35}S]methionine was determined by scintillation counting.

Nuclear run-on assay

Medium was removed from 150 mm dishes, and the cells were washed twice with Puck's saline and scraped into a Nonidet P-40 lysis buffer. Following two low-speed spins, the pellet was reconstituted in a glycerol buffer. Labelling *in vitro* of nascent RNA and hybidization with cDNAs immobilized on nitrocellulose filters were performed according to the methods outlined by Greenberg and Ziff [18] and Groudine et al. [19]. No hybridization occurred to filters containing plasmids without inserts.

Glutathione assay

The fibroblasts were trypsinized, counted and resuspended. Proteins were precipitated by the addition of perchloric acid (0.3 %, final concentration), sonication and centrifugation at 3000 ***g*** for 20 min at 4 °C. The supernatants were adjusted to pH 7 with 1 M KOH/0.3 M Mops and centrifuged at 10000 ***g*** for 5 min at 4 °C. The level of glutathione was determined by a colorimetric assay using Tietze's method [20] with modifications described by Akerboom and Sies [21]. Each assay was calibrated with standard glutathione. Glutathione content was expressed as nmol per 10^6 cells.

Statistics

A Student's *t* test was used for means of unequal size [22]. Probability values < 0.05 were considered significant.

RESULTS

We determined the effect of omitting individual amino acids from the culture medium on the amino acid-induced re-expression of α1(I) collagen mRNA following amino acid depletion. The addition of complete medium resulted in dramatic increases in α1(I) mRNA levels confirming previous results [1]. This medium contains a full complement of 15 amino acids. The effect of each of these amino acids was tested in deletion experiments. The absence of cystine prevented the re-expression of α1(I) mRNA (Figure 1). In contrast, the absence of valine, histidine, tryptophan or lysine only minimally affected α1(I) mRNA re-expression. Equal loading of RNA was verified by reprobing the filter with an oligonucleotide directed against the 18 S ribosomal subunit.

Following amino acid depletion, the addition of selective amino acid-deficient culture medium revealed that cystine was the only amino acid that was critical for re-expression of α1(I) collagen mRNA (Figure 1 and results that are not shown). Densitometric analysis of three experiments revealed that the absence of cystine inhibited the re-expression of α1(I) mRNA by 88 ± 4 % (mean $\pm$ S.E.). The addition of cysteine effectively substituted for cystine. Cystine or cysteine appear to act in combination with certain other amino acids because the addition of cystine alone was insufficient for re-expression of α1(I) mRNA.

We examined the effect of omitting individual amino acids from the culture medium on basal α1(I) collagen mRNA expression (as compared with re-expression following amino acid depletion). The levels of α1(I) mRNA were determined following the omission of each amino acid from the usual complement of 15 amino acids. We found that the absence of cystine but not other amino acids resulted in large decreases in α1(I) mRNA levels. Densitometric analysis revealed that α1(I) mRNA levels were decreased by 72 % in fibroblasts maintained without cystine

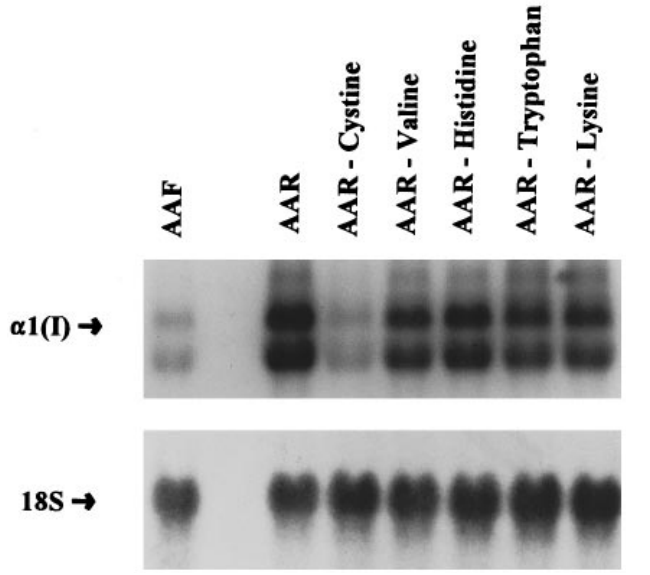

Figure 1 The effect of omitting individual amino acids from complete medium on α1(I) collagen mRNA levels

Quiescent fibroblast cultures were maintained in amino acid-free medium (AAF) for 48 h. The medium was replaced with AAF, amino acid-replete medium (AAR) or medium with selected amino acids omitted. After 24 h, the RNA was isolated and the expression of α1(I) mRNA assessed. After probing, an autoradiogram was obtained and densitometry performed.

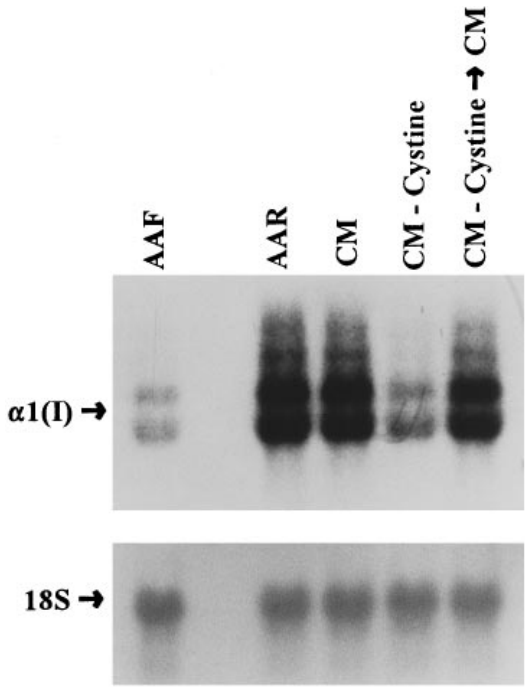

Figure 2 The effect of omitting cystine from complete medium on α1(I) collagen mRNA levels

Quiescent fibroblast cultures were maintained in complete medium (CM), amino acid-free medium (AAF) or CM without cystine (CM-Cystine) for 48 h. The AAF was replaced with amino acid-replete medium (AAR). In some dishes, as indicated, the cystine-free medium was replaced with complete medium (CM-Cystine → CM). After 24 h, the RNA was isolated and the expression of α1(I) mRNA assessed.

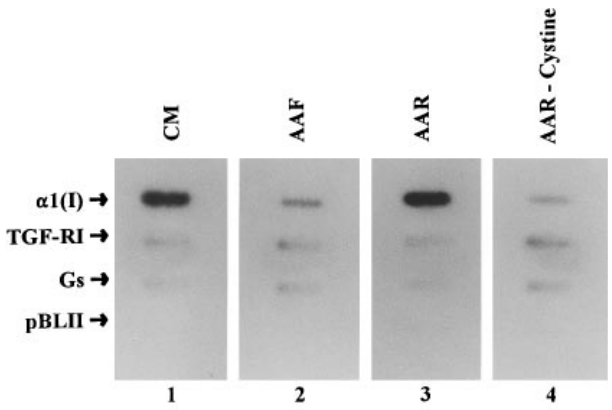

Figure 3 The rate of transcription of the α1(I) collagen gene

Fibroblast cultures were maintained in complete medium (CM; lane 1) or amino acid-free medium (AAF; lane 2). After 48 h, in selected dishes maintained in AAF, the medium was replaced with amino acid-replete medium (AAR; lane 3) or AAR without cystine (AAR-Cystine; lane 4). After 16 h, the nuclei were harvested and the levels of transcription for α1(I) collagen, transforming growth factor-β receptor I (TGF-RI) and Gs were assessed. No signal was detected for plasmids without insert (pBluescript II, pBLII).

and by 86% when maintained without any amino acids (Figure 2). The results of three similar experiments revealed that the absence of cystine resulted in a 66±6% (mean±S.E.) decrease in α1(I) mRNA level. The addition of medium containing cystine restored α1(I) mRNA levels. Cystine depletion caused only a minimal decrease in overall protein synthesis as assessed by [^{35}S]methionine labelling [(17.5±2.0)×10^3 c.p.m. in controls and (14.1±1.7)×10^3 c.p.m. in cystine-depleted cultures; mean±S.E., $n = 4$).

We examined the effect of cystine omission on amino acid-induced increases in α1(I) gene transcription and α1(I) mRNA stability. Nuclear run-on assays were performed to assess the effect of cystine depletion on the rate of transcription of the α1(I) collagen gene. Nuclei were isolated from fibroblasts maintained in medium with or without cystine for 48 h. The rate of transcription of the α1(I) collagen gene was reduced by 87% in nuclei obtained from fibroblasts maintained in amino acid-free medium (Figure 3). In contrast, the rates of transcription for transforming growth factor-β receptor I or Gs were unchanged. The addition of medium containing the full complement of amino acids restored the transcription rate. However, the rate of transcription was not restored when cystine was absent from the medium.

The stability of the α1(I) collagen mRNA was determined in fibroblasts maintained in serum-free medium containing the full complement of amino acids or a similar medium without cystine for 48 h. The stability of the α1(I) collagen mRNA was assessed by measuring the decay of the mRNA after the addition of actinomycin D. The stability of the mRNA was decreased in cystine-free medium (Figure 4). Linear regression analysis using the results of two experiments with varying periods of actinomycin D exposure revealed that amino acid depletion decreased the half-life of the α1(I) mRNA from 7.1 to 3.7 h.

Cystine alone did not result in the re-expression of α1(I) collagen mRNA, indicating that cystine acted in combination with certain other amino acids to affect both the rate of α1(I) gene transcription and the stability of α1(I) mRNA. These amino acids were identified by assessing the effect of individual amino acids in combination with cystine on the re-expression of α1(I) mRNA levels. We found that the addition of glutamine but not arginine or serine increased α1(I) mRNA expression (Figure 5). In contrast, the addition of glutamine or cystine alone had no effect (results not shown). The results of three experiments revealed that the combination of glutamine and cystine increased α1(I) mRNA levels 6.3-fold [this represented 59±10% (mean±S.E.) of the increase induced by the full complement of amino acids]. Additional experiments revealed that methionine

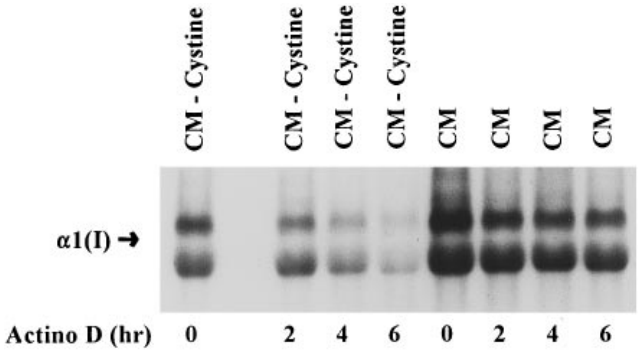

Figure 4 The stability of α1(I) collagen mRNA in fibroblasts maintained in complete medium or in cystine-free medium

Fibroblast cultures were left untreated in complete medium (CM) or cultured in cystine-deficient medium (CM-Cystine) for 48 h. Following the addition of 5 μM actinomycin D, the cells were harvested at various time intervals and the level of α1(I) collagen mRNA determined.

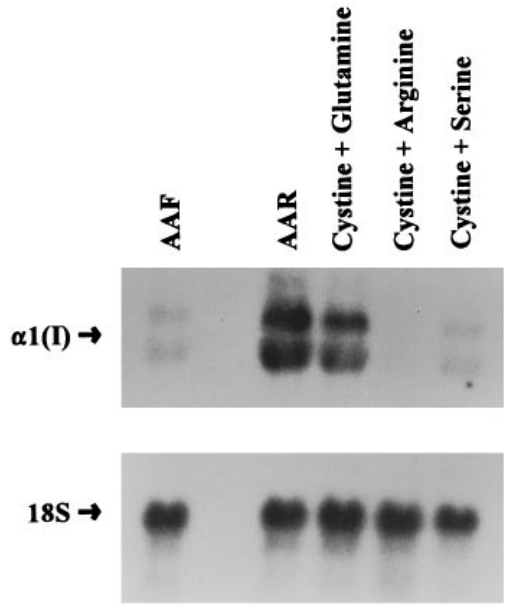

Figure 5 Glutamine and the amino acid-induced re-expression of α1(I) collagen mRNA

Quiescent fibroblast cultures were maintained in amino acid-free medium (AAF). After 48 h, the medium was replaced with AAF or amino acid-replete medium (AAR). Selected dishes received the combination of cystine (0.2 mM) with glutamine (4.0 mM), arginine (0.4 mM) or serine (0.4 mM). After 24 h, the RNA was isolated and the expression of α1(I) mRNA assessed.

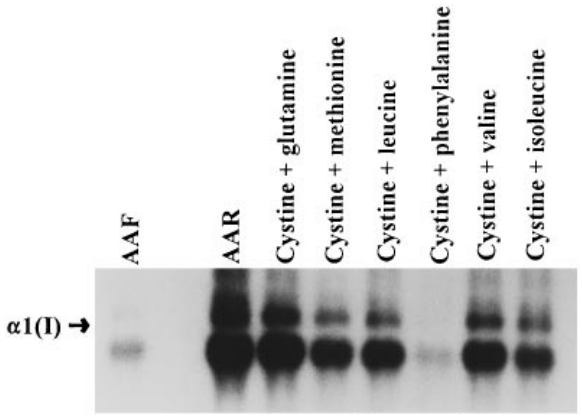

Figure 6 The effect of combining cystine with glutamine, methionine or a branch-chain amino acid on α1(I) collagen mRNA levels

Quiescent fibroblast cultures were maintained in amino acid-free medium (AAF). After 48 h, the medium was replaced with AAF, amino acid-replete medium (AAR) or cystine plus the indicated amino acid. After 24 h, the RNA was isolated and the expression of α1(I) mRNA assessed.

or the branch-chain amino acids leucine, isoleucine or valine also acted in combination with cystine to increase α1(I) mRNA levels following amino acid depletion, whereas other amino acids were not effective (Figure 6 and results that are not shown).

The combination of cystine plus glutamine was particularly effective in increasing α1(I) collagen mRNA levels following amino acid depletion. Cysteine and glutamate, together with glycine, are used to synthesize glutathione. The intracellular ratio of reduced glutathione:disulphide glutathione determine to a large degree the redox state of the cell [23–25]. Fibroblasts

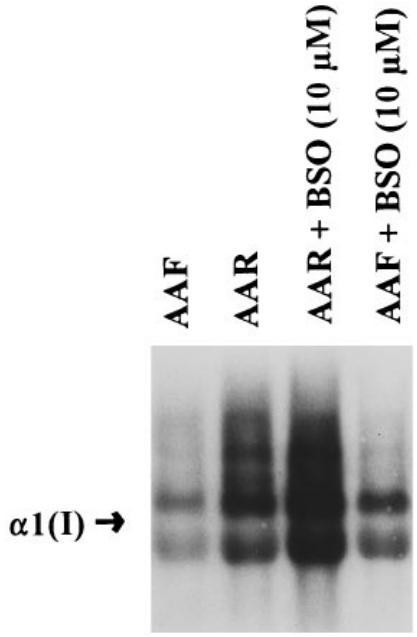

Figure 7 The effect of inhibition of γ-glutamylcysteine synthetase by BSO on α1(I) collagen mRNA levels

Quiescent fibroblast cultures were maintained in amino acid-free medium (AAF). After 48 h, the medium was replaced with AAF or amino acid-replete medium (AAR) with or without BSO at 10 μM. After 24 h, the RNA was isolated and the expression of α1(I) mRNA assessed.

maintained in amino acid-free culture medium for 72 h had a 98% decrease in glutathione levels (3.92 ± 0.47 nmol per 10^6 cells maintained in complete medium, and 0.06 ± 0.01 nmol per 10^6 cells maintained in amino acid-free medium, $n = 4$). To determine whether glutathione re-synthesis was required for re-expression of α1(I) mRNA, we employed buthionine sulphoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase. The presence of BSO at 10 μM decreased glutathione levels by 77% in cells maintained in complete medium. Pretreatment of amino acid-deficient fibroblasts with BSO, inhibited the resynthesis of glutathione by 89% following the addition of complete medium. In contrast, BSO did not inhibit the re-expression of α1(I) mRNA (Figure 7). The levels of α1(I) mRNA following the addition of complete medium were actually increased when BSO was present. These data suggest that the re-expression of α1(I) mRNA does not depend on redox status.

DISCUSSION

We previously found that expression of α1(I) collagen mRNA was directly dependent on amino acid availability [1]. To further define the mechanism whereby amino acid availability regulates α1(I) mRNA levels, we selectively omitted individual amino acids from the culture medium. We found that amino acid-induced re-expression of α1(I) mRNA was critically dependent on cystine but not on other amino acids. The effect of cystine was mediated by decreases in both α1(I) collagen gene transcription and mRNA stability. It is noteworthy that other effector substances, such as transforming growth factor-β, regulate α1(I) mRNA via co-ordinated effects on both gene transcription and mRNA stability [26,27].

Cystine availability is determined by several interacting metabolic systems that include: the uptake of cystine and cysteine by

specific amino acid-transport systems; the synthesis and degradation of glutathione; the utilization of cysteine for protein formation; and the metabolism of cysteine itself [28]. Lung fibroblasts primarily transport cysteine via the ASC transport system and cystine via the x_c^- system [29]. The ASC system is an Na^+-dependent transporter of neutral amino acids. In contrast to the A system, it does not accept *N*-methylated substrates and is unaffected by adaptive regulation or changes in extracellular pH. The x_c^- system transports cystine intracellularly in exchange for glutamate [29]. Interestingly, the activity of this system is affected by the partial pressure of oxygen [30].

Following uptake, cystine is reduced to cysteine for intracellular use, particularly glutathione and protein synthesis [28]. Glutathione has a short intracellular half-life because of transport to the extracellular space where it is degraded by γ-glutamyl transferase on the extracellular membrane of epithelial cells [31]. The constituent amino acids of glutathione, including cysteine, become available for reuptake and utilization. Cysteine supply is rate limiting for intracellular glutathione synthesis which, in turn, influences the redox state of the cell [32]. Our results indicate that the mechanism whereby intracellular cysteine levels regulate α1(I) collagen mRNA does not appear to involve alterations in the levels of glutathione. The addition of BSO blocked the resynthesis of glutathione but did not inhibit re-expression of α1(I) mRNA levels. These data suggest that cystine was utilized by other cellular pathways that increased steady-state levels of α1(I) mRNA.

The catabolism of cysteine is complex. It utilizes several different pathways in different cell types for the production of a variety of thiols and other small molecules, some of which may be biologically active. Cultured cells vary in their ability to catabolize cysteine as a source of sulphate [33]. The primary pathway in the liver involves oxidation to cysteinesulphinate, which in turn is metabolized to taurine or pyruvate and sulphate [28]. The addition of cysteinesulphinate to the culture medium did not substitute for cystine, indicating that this metabolite and its derivatives were not involved in regulating collagen formation (D. C. Rishikof, unpublished work). However, other metabolic pathways are available, including transamination of cysteine to β-mercaptopyruvate or cleavage by cystathione γ-lyase. Potential products include cysteamine, cysteic acid and PAPS (adenosine 3′-phosphate 5′-phospho-sulphate, the primary sulphate donor). The culture medium used in our experiments contains high levels of inorganic sulphate (0.8 mM $MgSO_4$), indicating that PAPS and sulphate are not involved in the regulation of α1(I) collagen mRNA.

Cysteine may interact directly with a DNA-binding protein that alters the rate of transcription or with a RNA-binding protein that alters stability. Several nuclear and cytoplasmic proteins bind to the 3′ untranslated region (UTR) of the α1(I) collagen mRNA [34–37]. The binding of one such protein (α1-RBF) to the 3′ UTR of α1(I) collagen mRNA is sensitive to alterations in the redox status that involve the availability of free SH groups on cysteine [36]. However, it is not yet clear whether any of these proteins regulate the stability of the transcript. Stefanovic and associates identified a C-rich sequence in the 3′ UTR that regulates the stability of the α1(I) transcript in hepatic stellate cells [37]. Other regions of the transcript may also influence the stability of the mRNA. The decrease in stability induced by N-ras in rat cells was not mediated by elements in the 3′ UTR of the α1(I) collagen mRNA [38].

Activation of α1(I) collagen mRNA following amino acid depletion required cystine and at least one other amino acid. The combination of cystine and glutamine or cystine and a branch-chain amino acid induced large increases in α1(I) mRNA, although less than that induced by the full complement of amino acids. These results indicate that specific amino acids alter the rate of transcription and stability of specific mRNAs. Interestingly, glutamine is utilized for nucleotide synthesis [39] and it increases α1(I) collagen mRNA in dermal fibroblasts via increases in gene transcription [40]. The work of others also supports our finding that the availability of specific amino acids selectively alters the stability of specific mRNAs [41]. In contrast to α1(I) collagen mRNA, the stability of c-myc mRNA was increased by valine or leucine but not by cysteine deprivation. The regulation of specific mRNA by individual amino acids probably has physiological significance *in vivo*. It is noteworthy that low plasma cystine levels are associated with catabolic states associated with cancer, HIV infection and senescence [42]. In addition, administration of glutamine and branch-chain amino acids decrease protein catabolism during sepsis [43,44].

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