Regulation of Internal Ribosome Entry Site-mediated Translation by Eukaryotic Initiation Factor-2α Phosphorylation and Translation of a Small Upstream Open Reading Frame*

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Adaptation to amino acid deficiency is critical for cell survival. In yeast, this adaptation involves phosphorylation of the translation eukaryotic initiation factor (eIF)2α by the kinase GCN2. This leads to the increased translation of the transcription factor GCN4, which in turn increases transcription of amino acid biosynthetic genes, at a time when expression of most genes decreases. Here it is shown that translation of the arginine/lysine transporter cat-1 mRNA increases during amino acid starvation of mammalian cells. This increase requires both GCN2 phosphorylation of eIF2α and the translation of a 48-amino acid upstream open reading frame (uORF) present within the 5′-leader of the transporter mRNA. When this 5′-leader was placed in a bicistronic mRNA expression vector, it functioned as an internal ribosomal entry sequence and its regulated activity was dependent on uORF translation. Amino acid starvation also induced translation of monocistronic mRNAs containing the cat-1 5′-leader, in a manner dependent on eIF2α phosphorylation and translation of the 48-amino acid uORF. This is the first example of mammalian regulation of internal ribosomal entry sequence-mediated translation by eIF2α phosphorylation during amino acid starvation, suggesting that the mechanism of induced Cat-1 protein synthesis is part of the adaptive response of cells to amino acid limitation.

Amino acid starvation of yeast and mammalian cells induces phosphorylation of the translation initiation factor eIF2α, altering the pattern of gene expression to remedy the stress and conserve resources for the starving cells (1). However, stress-response proteins are synthesized under conditions when global protein synthesis is inhibited (2). An adaptive response to amino acid starvation for any single amino acid has been extensively characterized in yeast; translation of the transcription factor GCN4 increases during total or single amino acid starvation, causing a transcriptional induction of the amino acid biosynthetic genes (2–5). Yeast have developed a sophisticated mechanism to increase translation of the GCN4 mRNA when eIF2α is phosphorylated and levels of eIF2-GTP-Met-tRNA<sup>Met</sup> ternary complexes decrease (6); initiation at the GCN4 ORF is inversely related to the concentration of ternary complexes in the cell (5). The regulatory system that mediates the response of yeast to amino acid starvation is known as a general amino acid control mechanism.

Mammalian cells have also developed an adaptive response to changes in amino acid availability (7). Given that essential amino acids have to be transported into cells via membrane-spanning amino acid transporter proteins, part of the adaptive response involves the increased expression of amino acid biosynthetic and transporter genes (8). A significant part of this adaptive response is the increased expression of the cationic amino acid transporter 1 gene (cat-1), the main transporter of the essential amino acids arginine and lysine (9). It has been shown previously that amino acid starvation induces the stability (10) and translation of the cat-1 mRNA (11). This report demonstrates that the mechanism of increased translation of the cat-1 mRNA by amino acid limitation shares features with the general amino acid control mechanism as described in yeast.

EXPERIMENTAL PROCEDURES

**Generation of Expression Vectors—** All the cat-1 5′-UTR-containing bicistronic mRNA expression vectors were generated by PCR amplification of the cat-1 5′-UTR cDNAs using the SalI/NcoI site of the pSV-CAT/ICS/LUC plasmid (12) by replacing the ICS DNA sequence. The plasmids containing the hairpin were constructed by replacing the ICS with the pSVhphCAT/ICS/LUC (12) vector, at the SalI/NcoI sites. The cat-1 5′-UTR<sup>mut</sup> mutants were generated by PCR-directed mutagenesis. The mut<sup>a</sup>-stop was generated by mutating the uORF stop codon (Fig. 1A, TGA to TTA within the cat-1 5′-UTR<sup>a</sup>) thus placing the uORF/ATG in frame with the LUC start codon. The mut<sup>a</sup>-start was generated by mutating the uORF/ATG from ATG to TTA within the cat-1 5′-UTR<sup>a</sup> thus placing the uORF/ATG in frame with the LUC start codon. The mut<sup>a</sup>-start was generated by mutating the uORF/ATG from ATG to TTA within the cat-1 5′-UTR<sup>a</sup> and cat-1 5′-UTR<sup>a</sup>DNAs. The cat-1 5′-UTR<sup>a</sup> and cat-1 5′-UTR<sup>a</sup>NcoI have an NcoI site at the 3′ end, which contains the start codon ATG for the LUC cistron. The context of the A<sup>5′</sup>-TG LUC/stop codon within the mut<sup>a</sup>-stop and mut<sup>a</sup>-start vectors is AGCGCCCA<sup>5′</sup>-TG (compare this sequence with Fig. 1A). The monocistronic expression vectors, cat-1 5′-UTR/LUC and cat-1 5′-UTRmut/LUC, were generated by cloning the SalI/XbaI cat-1 5′-UTR/LUC and cat-1 5′-UTRmut/LUC fragments from the bicistronic vectors into the EcoRI/XbaI site of the pUHD10–3 vector. In this vector, transcription is directed by the minimal cytomegalovirus 2050

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‡‡‡ The abbreviations used are: eIF, eukaryotic initiation factor; LUC, luciferase; ORF, open reading frame; uORF, upstream open reading frame; UTR, untranslated region; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; CON, amino acid-containing; F, fed; S, starved; IRES, internal ribosome entry site; ITAF, IRES-specific trans-acting factor; hp, hairpin; ER, endoplasmic reticulum; CAT, chloramphenicol acetyltransferase.
Cloning and Translational Control by Amino Acid Starvation

**RESULTS**

**Cloning and Translational Control by Amino Acid Starvation of the cat-1 5′-UTR**—The previously described leader (5′-UTR) for the cat-1 mRNA was 224 bases (cat-1 5′-UTR), and it was incomplete (11). However, this truncated cat-1 5′-UTR functioned as an IRES and was regulated by amino acid starvation (11). To further study this regulation, a full-length 5′-UTR of the cat-1 mRNA was obtained by isolating a rat genomic DNA clone that contained the promoter region, the first exon, and the first intron of the cat-1 gene. The transcription start site was determined in C6 cells (data not shown). This full-length UTR (Fig. 1A, cat-1 5′-UTR) consists of exon 1 (154 nucleotides, in blue), exon 2 (98 nucleotides, in red), and the 5′ end of exon 3 (18 nucleotides, in red). An interesting feature of the cat-1 5′-UTR is the presence of a uORF. Both cat-1 5′-UTR and cat-1 5′-UTR contained a 48-amino acid uORF, starting 46 bases 3′ to the mRNA cap site (Fig. 1, A and B).

The ability of the cat-1 5′-UTR sequence to mediate internal translation initiation was tested in a bicistronic expression vector (12). In these vectors (Fig. 2A), the CAT enzyme is translated from the first cistron by a cap-dependent scanning mechanism (12). The second cistron, encoding the firefly LUC enzyme, is efficiently translated only if it is preceded by an IRES in the intercistronic spacer region (Fig. 2A). Translation of the second cistron should be independent of translation of the first cistron. A vector that contained a stable RNA hairpin (hp) upstream of the CAT cistron (Fig. 2A) was used to demonstrate that when translation of the CAT cistron is inhibited, translation of the LUC cistron is unaffected. Two bicistronic expression vectors were generated by introducing the cat-1 5′-UTR into the intercistronic space of the empty expression vector: CAT/cat-1 5′-UTR/LUC and hpCAT/cat-1 5′-UTR/LUC (Fig. 2A). The LUC/CAT ratio was 20-fold higher for the hpCAT/cat-1 5′-UTR/LUC when compared with the CAT/cat-1 5′-UTR/LUC. C6 cells were transiently transfected with these vectors, and LUC and CAT activities were analyzed. This increase was because of decreased CAT and sustained LUC activities in the hairpin-containing vector (data not shown). These data demonstrated that the full-length cat-1 5′-UTR leader has IRES activity. Transfection of these vectors into C6 cells demonstrated that translational control mediated by the full-length cat-1 5′-UTR was indistinguishable (data not shown) to the previously tested truncated cat-1 5′-UTR (11). Notice that both cat-1 5′-UTR and cat-1 5′-UTR contain the uORF (Fig. 1). The following studies have been performed using both the truncated (11) and the full-length (Fig. 2A) UTRs within the bicistronic expression vectors (12).
tion were different from the kinetics of induction of eIF2α phosphorylation. Phosphorylation of eIF2α transiently increased during the first hour of amino acid starvation (11), whereas cat-1 IRES-mediated translation increased between 6 and 12 h and declined thereafter (11). These studies suggested that eIF2α phosphorylation does not directly regulate cat-1 IRES activity. This is in contrast to the regulation of the GCN4 and other mammalian mRNAs (5, 20). In these cases, mRNA translation increases at the time when eIF2GTP Met-tRNAMet ternary complexes decrease (5).

In yeast, uncharged tRNAs, which accumulate in cells depleted by any single amino acid, bind to and activate the yeast GCN2 cellular kinase, leading to phosphorylation of eIF2α and translational control (21). To gain an insight on the involvement of eIF2α phosphorylation in the increased cat-1 mRNA translation during amino acid starvation, the regulation of cat-1 IRES-mediated translation by individual amino acids was studied in C6 cells. Depletion of any single essential amino acid increased cat-1 IRES activity with the same kinetics and to the same degree as described for total amino acid starvation (Fig. 2B and Table I). This is in agreement with translational control of the GCN4 mRNA by amino acid depletion (2) and supports the hypothesis that eIF2α phosphorylation is involved indirectly in cat-1 IRES translational control.

We therefore tested whether eIF2α phosphorylation and mammalian GCN2 kinase activation are involved in cat-1 IRES activation during amino acid starvation. To test this hypothesis, C6 cells were transfected with the bicistronic expression vector containing the cat-1 5′-UTRt along with expression vectors expressing either the wild type mammalian GCN2 kinase (22, 23) or a mutant GCN2, which lacked kinase activity (23). The mutant kinase forms inactive heterodimers with the wild type GCN2 kinase. Therefore, activation of the endogenous wild type GCN2 (23) is inhibited, leading to inhibition of eIF2α phosphorylation by amino acid starvation. The transfected wild type and mutant GCN2 kinases contained the FLAG epitope (15), which allowed us to monitor expression of the mutant proteins by Western blot analysis (data not shown). Following 36 h of transfection, cells were incubated in either amino acid-containing (F) or amino acid-depleted (S) medium for 9 h.
described previously (11). As shown, expression of the mutant kinase inhibited cat-1 IRES activation by amino acid starvation (Fig. 2C, compare the -fold change of the ratio LUC/CAT). Induction in LUC activity was 9-fold for control, 15-fold for the GCN2 wild type, and 2-fold for GCN2 mutant-expressing cells (Table I). The increases in LUC/CAT ratios were 13-, 21-, and 2.5-fold respectively (Fig. 2C and Table I). The CAT activity decreased by 30% (Table I). When the bicistronic LUC/CAT ratios were measured, regulation of translation of the GCN2-mut mRNA was tested, regulation of translation of the GCN2 wild type, and 2-fold for GCN2 mutant-expressing cells (Table I). These data indicated that decreased cap-dependent translation of the GCN2-mut mRNA (11). We therefore conclude that eIF2α phosphorylation by the endogenous GCN2 kinase is activated (15) and phosphorylates eIF2α. 65 18 65 18 57 4 293 27 15 0.6 64 2 1061 39 16 1.0 59 3 1016 63 17 0.8 57 3 905 99 16 0.1 56 2 925 95 16 1.1 59 4 923 27 15 0.6 61 3 1036 107 17 1.7 -Thr 58 ± 1 943 ± 42 16 ± 0.6 -Tyr 64 ± 4 1120 ± 65 18 ± 1.0 -Val 55 ± 4 871 ± 95 16 ± 0.5 Exp. from Fig. 2C Control Fed 72 ± 3 158 ± 28 2.2 ± 0.4 GCN2 Fed 73 ± 9 119 ± 36 1.7 ± 0.5 Starved 50 ± 3 1950 ± 211 39 ± 2.5 GCN2-mut Fed 74 ± 11 86 ± 16 1.2 ± 0.1 Starved 57 ± 2 166 ± 33 2.9 ± 0.6 PERK Fed 69 ± 16 190 ± 12 2.8 ± 0.4 Starved 42 ± 2 1478 ± 371 34 ± 8.1 PERK-mut Fed 77 ± 6 103 ± 16 1.3 ± 0.2 Starved 59 ± 3 795 ± 136 13 ± 2.2 PKR+/− Fed 31 ± 3 247 ± 55 8.0 ± 0.7 Starved 22 ± 2 1048 ± 72 47 ± 1.8 PKR−/− Fed 32 ± 2 232 ± 27 7.2 ± 0.5 Starved 20 ± 1 925 ± 75 46 ± 4.5

Table I Absolute values of LUC and CAT activities from experiments described in Fig. 2

| Experiments     | CAT units/µg protein | LUC units/µg protein | LUC/CAT |
|-----------------|----------------------|----------------------|---------|
| Exp. from Fig. 2B |                      |                      |         |
| Control         | 97 ± 4               | 98 ± 19              | 1.0 ± 0.2 |
| -Arg            | 73 ± 2               | 995 ± 46             | 13 ± 0.3  |
| -Cys            | 56 ± 6               | 998 ± 80             | 17 ± 2.4  |
| -His            | 57 ± 6               | 974 ± 100            | 17 ± 2.0  |
| -Ile            | 64 ± 2               | 1061 ± 39            | 16 ± 1.0  |
| -Leu            | 59 ± 3               | 1016 ± 63            | 17 ± 0.8  |
| -Lys            | 57 ± 3               | 905 ± 99             | 16 ± 0.1  |
| -Met            | 56 ± 2               | 925 ± 95             | 16 ± 1.1  |
| -Phe            | 59 ± 4               | 923 ± 27             | 15 ± 0.6  |
| -Thr            | 61 ± 3               | 1036 ± 107           | 17 ± 1.7  |
| -Tyr            | 58 ± 1               | 943 ± 42             | 16 ± 0.6  |
| -Val            | 64 ± 4               | 1120 ± 65            | 18 ± 1.0  |
| -Met            | 55 ± 4               | 871 ± 95             | 16 ± 0.5  |

Exp. from Fig. 2C

| Experiments | CAT units/µg protein | LUC units/µg protein | LUC/CAT |
|-------------|----------------------|----------------------|---------|
| Fed         | 72 ± 3               | 158 ± 28             | 2.2 ± 0.4 |
| Starved     | 49 ± 6               | 1341 ± 254           | 28 ± 6.8  |
| GCN2 Fed    | 73 ± 9               | 119 ± 36             | 1.7 ± 0.5 |
| Starved     | 50 ± 3               | 1950 ± 211           | 39 ± 2.5  |
| GCN2-mut Fed| 74 ± 11              | 86 ± 16              | 1.2 ± 0.1 |
| Starved     | 57 ± 2               | 166 ± 33             | 2.9 ± 0.6 |
| PERK Fed    | 69 ± 16              | 190 ± 12             | 2.8 ± 0.4 |
| Starved     | 42 ± 2               | 1478 ± 371           | 34 ± 8.1  |
| PERK-mut Fed| 77 ± 6               | 103 ± 16             | 1.3 ± 0.2 |
| Starved     | 59 ± 3               | 795 ± 136            | 13 ± 2.2  |
| PKR+/− Fed  | 31 ± 3               | 247 ± 55             | 8.0 ± 0.7 |
| Starved     | 22 ± 2               | 1048 ± 72            | 47 ± 1.8  |
| PKR−/− Fed  | 32 ± 2               | 232 ± 27             | 7.2 ± 0.5 |
| Starved     | 20 ± 1               | 925 ± 75             | 46 ± 4.5  |

Fig. 3. cat-1 IRES activation by amino acid starvation is inhibited in cells expressing a dominant negative GCN2 kinase. C6 cells were stably transfected with the pcDNA3 expression vector containing a mutant FLAG-epitope containing GCN2 kinase (15). Cells were selected in G418, and mass cultures (C6-GCN2-mut) were generated. A, total cell extracts (15 µg) from C6-GCN2-mut cells incubated for the times indicated in CON medium, S medium, or thapsigargin-containing medium (400 nM, Thaps) were probed for the GCN2 mutant protein (M2 anti-FLAG monoclonal), 4EBP-1 (rabbit anti-Phas 1), eIF2α total, and phospho-eIF2α. The γ (phosphorylated), β (semiphosphorylated), and α (unphosphorylated) species of 4E-BP-1 are indicated. Notice that cap-dependent translation decreases when eIF4E binds the α and β but not the γ species. B, C6-GCN2-mut cells were transiently transfected with the bicistronic expression vector containing the cat-1 5'-UTR. Following transfections (36 h), the medium was changed to either CON or S for the times indicated. Cell extracts were prepared, and LUC and CAT activities were measured. Results are average of three independent experiments and are presented as the -fold change of the ratio LUC/CAT in reference to the amino acid-containing medium value. The bars indicate standard error. The absolute LUC and CAT values are presented in Table II.

of amino acid starvation, thus decreasing cap-dependent translation by sequestering the cap-binding protein eIF4E (Fig. 3A). However, expression of the mutant kinase is critical the first hour of amino acid starvation when the endogenous GCN2 kinase is activated (15) and phosphorylates eIF2α. At 1 h of amino acid starvation, the mutant kinase was expressed at the same level as in amino acid-fed cells (Fig. 3A). These data further demonstrate that decreasing cap-dependent translation is not the cause for increased cat-1 IRES-activation. This is in agreement with our previous finding that treatment of cells with rapamycin did not alter cat-1 IRES-mediated translation (11). We therefore conclude that eIF2α phosphorylation by the GCN2 kinase is required for increased cat-1 IRES-mediated translation by amino acid starvation. This finding is in agreement with the regulation of translation of the yeast GCN4 mRNA by the yeast GCN2 kinase (5). However, in contrast to
the yeast paradigm, eIF2α phosphorylation regulates cat-1 IRES-mediated translation indirectly.

At least four kinases are known to phosphorylate eIF2α: GCN2, PERK, PKR, and HRI (24). To determine the specific requirement for GCN2, the ER stress-induced ER-resident transmembrane kinase PERK (16) was tested. We transfected C6 cells with the bicistronic mRNA expression vector that contained the cat-1 5′-UTR along with expression vectors expressing either the wild type PERK (14) or a mutant PERK, which lacked kinase activity (14). As with GCN2, overexpression of the mutant kinase forms dimers with the wild type PERK; however, this should not affect the activation of GCN2 by amino acid starvation. The expression of the mutant kinase was monitored by Western blot analysis of cell extracts prepared from transfected cells using an antibody against a Myc epitope (14) that was contained at the NH₂ terminus of the kinase (data not shown). The levels of the mutant PERK kinase were sustained at the same level for the first 6 h of amino acid starvation and gradually declined thereafter (data not shown).

Following 36 h of transfection, cells were incubated in either amino acid-fed or amino acid-starved medium for 9 h as described previously (11). Expression of the PERK kinases did not affect induction of cat-1 IRES activity by amino acid starvation (Fig. 2C, compare the -fold change of the ratio LUC/CAT in PERK and PERK-mut cells). Amino acid starvation increased the LUC activity by 8-fold and decreased the CAT activity by 35 and 25%, respectively, in PERK wild-type and PERK mutant-expressing cells (Table I). The LUC/CAT ratio increased 12-fold for the PERK and 11-fold for the PERK-mut cells (Fig. 2C). It should be noted that the expression of the mutant PERK kinase decreased basal cat-1 IRES activity. This was because of a decrease in basal eIF2α phosphorylation levels in these cells (data not shown).

The involvement of the PKR eIF2α kinase was tested next. PKR is activated by binding double-stranded RNA (25, 26). To address the role of the PKR kinase, mouse embryo fibroblasts that either expressed the endogenous PKR kinase (PKR+/−) or had the kinase inactivated (PKR−/−) by homologous recombination were used (27). cat-1 IRES-mediated translation was measured following 9 h of amino acid starvation. The basal activity for LUC and CAT were similar in PKR+/− and PKR−/− cells (Fig. 2C and Table I). Amino acid starvation of cells induced the LUC activity by 4-fold and decreased the CAT by 40% in both cell lines (Fig. 2C and Table I). The LUC/CAT ratio increased 6-fold in the PKR+/− and 5.5-fold in the PKR−/− cells (Fig. 2C). The increase of cat-1 IRES activity was lower than C6 cells (4-fold as compared with 7-fold in C6 cells). The lower induction in PKR cells may be because of cell type differences. The latter is supported by the fact that the basal LUC activity in PKR cells was 4-fold higher than the equivalent in C6 cells (Table I). These data suggest that eIF2α phosphorylation and cat-1 IRES translational induction during amino acid starvation depend on GCN2 activation.

To further demonstrate that phosphorylation of eIF2α is required for cat-1 IRES activation, we overexpressed an eIF2α that can either not be phosphorylated (Ser-51 changed to Ala) or mimics the phosphorylated protein (Ser-51 changed to Asp), and we assessed the translational control of the cat-1 IRES. Stress-induced phosphorylation of eIF2α occurs at Ser-51. It has been shown previously that overexpression of the variant Ser-51 to Ala eIF2α (S-A), in COS cells in vivo affects translation by converting the endogenous eIF2 complex to a variant form (19). This occurs because the variant eIF2α S-A freely exchanges into the endogenous eIF2 complexes (19). Expression of the eIF2α S-A impaired its phosphorylation in vitro and in vivo and inhibits translational control of the GCN4 mRNA by amino acid starvation (2, 4).

Furthermore, substitution of Ser-51 by Asp (S-D) mimics the phosphorylated eIF2α and if overexpressed in amino acid-fed cells, it would be expected to mimic amino acid starvation. C6 cells were transfected with the bicistronic expression vector that contained the cat-1 5′-UTR, along with expression vectors expressing either the eIF2α S-A or S-D. Efficient transfection of the eIF2α was monitored by detecting expression of the CD2 cell surface protein, which was included in the expression vector (data not shown). The expression of the eIF2α S-A and S-D proteins in C6 cells was determined as described under "Experimental Procedures." In the presence of amino acids, expression of the eIF2α S-A had no effect on translational activity of the IRES (Fig. 4A and Table III). By comparison, expression of the eIF2α S-D increased IRES-mediated translation to even higher levels (Fig. 4A, S-D, LUC/CAT: 16-fold) than observed by amino acid starvation (Fig. 2C, con, LUC/CAT: 13-fold). Amino acid starvation of cells expressing the eIF2α S-D did not increase IRES activity further (data not shown). Expression of the eIF2α S-A was tested next, to see whether it would impair induction of translation mediated by the cat-1 5′-UTR during amino acid starvation. As shown, overexpression of the eIF2α S-A impaired translational activation by amino acid starvation (Fig. 4B and Table III). We conclude that eIF2α phosphorylation regulates in an indirect manner cat-1 IRES-mediated translation.

**Translation of the uORF and eIF2α Phosphorylation Are Required for cat-1 IRES Activation by Amino Acid Starvation**—

What is the mechanism by which eIF2α phosphorylation leads to induction of cat-1 mRNA translation? Given the importance of upstream ORFs in the regulation of translation of the GCN4 expression in yeast (28), we hypothesized that the 48-amino acid uORF may play a role in cat-1 IRES activation by amino acid starvation. The presence of uORFs in mRNAs is believed to regulate translation of the downstream ORF (29). To determine the importance of the uORF in cat-1 IRES-mediated translation, the regulation of cat-1 IRES activity by amino acid starvation was tested in bicistronic mRNAs that either contained the uORF or a uORF having a mutated initiation codon (AUG was mutated to TTG, in both the cat-1 5′-UTR and the cat-1 5′-UTR bicistronic expression vectors). C6 cells were transfected independently with the vectors CAT/cat-1 5′-UTR/LUC (data not shown), CAT/cat-1 5′-UTRmut5′-start/LUC (data not shown), CAT/cat-1 5′-UTR/LUC, and CAT/cat-1 5′-UTRmut5′-start/LUC (Fig. 4C). The media were changed 36 h after transfection to either amino acid-containing (F) or amino acid-depleted (S) and analyzed at different times (Fig. 4C, the 9-h time point is shown). Mutation of the uORF abolished induction of the LUC activity, in both the full-length (Fig. 4C and Table III) or the cat-1 5′-UTR (data not shown). To further support the data that the uORF is important in cat-1 IRES-mediated translation, the TGA stop codon was mutated to TTA within the CAT/cat-1 5′-UTR/LUC vector (CAT/cat-1 5′-UTRmut5′-stop/LUC). In this vector translation initiating at the AUG of the uORF was in frame with the LUC/AUG (see "Experimental Procedures"), generating a fusion protein with LUC (Fig. 4C, inset). The presence of both
Translational Control by eIF2α Phosphorylation

Fig. 4. Activation of cat-1 IRES-mediated translation is mediated by eIF2α phosphorylation and translation of a small uORF. A, C6 cells were transiently transfected with bicistronic expression vectors containing the cat-1 5′-UTR or cat-1 5′-UTRmutf-start alone or with either expression vectors for eIF2α S-A (Ser-51 to Asp) or S-D (Ser-51 to Asp). Cell extracts were prepared 36 h after transfection, and LUC and CAT activities were measured. Results are presented as in Fig. 3 in reference to cat-1 5′-UTRf-CON. B, C6 cells were transiently transfected with bicistronic expression vectors containing the cat-1 5′-UTR alone or with an expression vector for the eIF2α S-A. The rest of the analysis was as in A. C, C6 cells were transiently transfected with the bicistronic expression vectors indicated on the bottom of the figure. Following transfections (36 h), the medium was changed to either F or S medium for 9 h. Cell extracts were prepared, and LUC and CAT activities were measured. Results are presented as in Fig. 3. The fold change of the ratio LUC/CAT is given in reference to F-cat-1 5′-UTR value. Top inset, Western blot analysis of protein extracts (15 μg) from C6 cells transfected with bicistronic DNAs containing either cat-1 5′-UTR or cat-1 5′-UTRmutf-stop DNAs, using an antibody for LUC (the LUC and fusion LUC proteins are indicated); bottom inset, Northern blot analysis of RNA isolated from the same cells shown in the top inset. The full-length bicistronic 3.4-kb CAT/LUC mRNAs are shown.

| Experiments          | CAT (units/μg protein) | LUC (units/μg protein) | LUC/CAT |
|----------------------|------------------------|------------------------|---------|
| Fed                  | 57 ± 9                 | 203 ± 16               | 3.6 ± 0.5 |
| Starved              | 67 ± 16                | 82 ± 9.0               | 1.2 ± 0.1 |
| mutf-start           | 57 ± 9                 | 203 ± 16               | 3.6 ± 0.5 |

LUC and fusion LUC proteins in mutf-stop cells indicates that translation initiates at both the uORF/AUG and the LUC/AUG (see “Discussion”). This mutation abolished induction by amino acid starvation (Fig. 4C and Table III). Therefore, the presence and translation of the 48-amino acid uORF appears to be required for induction of translation mediated by the cat-1 IRES. We have shown earlier that expression of the eIF2α S-D protein into C6 cells increased cat-1 IRES-mediated translation independent of amino acid availability (Fig. 4A).

We therefore tested if the eIF2α S-D-dependent induction required translation of the uORF. The cat-1 5′-UTRmutf-start vector was cotransfected with the eIF2α S-D expression vector, and the LUC and CAT activities were measured. The LUC/CAT ratio did not increase with overexpression of the variant eIF2α S-D (Fig. 4A, right panel) and Table III). Similar to cat-1 5′-UTR, the cat-1 5′-UTRmutf-mediated translation was not induced by overexpression of the eIF2α S-A (Fig. 4A, right panel). It is concluded that the eIF2α phosphorylation-dependent increase in IRES-mediated translation requires the translation of the uORF.

Amino Acid Starvation Induces Translation Mediated by the cat-1 5′-Leader in Monocistronic mRNAs via a Mechanism That Involves eIF2α Phosphorylation—The results described in Figs. 1–4 demonstrate that the IRES activity of the cat-1 5′-leader increases in amino acid-depleted cells in a manner dependent
on eIF2α phosphorylation and translation of the 48-amino acid uORF. Translation of the uORF within the bicistronic mRNA occurs via an IRES at the 5’ end of the leader (data not shown). Because the authentic cat-1 mRNA is monocistronic, we determined the eIF2α and uORF-dependent regulation of translation mediated by the full-length leader in monocistronic mRNAs. Previous studies using the truncated cat-1 5’-leader in monocistronic mRNAs suggested that translation of the uORF plays a role in translational induction by amino acid starvation (11). Expression vectors for monocistronic mRNAs were constructed that contained either the cat-1 5’-UTR/LUC or cat-1 5’-UTRmutLUC alone or with either expression vectors for eIF2α S-A or S-D. Following transfections (36 h), the medium was changed to either amino acid-containing or amino acid-depleted for 9 h (as indicated at the bottom of the figure). Cell extracts were prepared, and the LUC activities were determined. Results are presented as in Fig. 3 in reference to cat-1 5’-UTR/CON (first column). Bars indicate standard error.

5’-leader of the cat-1 mRNA is responsible for increased translation of the cat-1 mRNA during amino acid starvation and eIF2α phosphorylation.

DISCUSSION

It has been shown here that the activity of an IRES element within the mRNA 5’-leader sequence of the arginine/lysine transporter cat-1 increases during amino acid starvation. This increase depends both on eIF2α phosphorylation by GCN2 and translation of a 48-amino acid uORF within the leader.

The GCN2 kinase functions in the general amino acid control pathway of yeast Saccharomyces cerevisiae. Starvation for any single amino acid activates GCN2, which in turn phosphorylates eIF2α, causing a decrease in eIF2-GTP-Met-tRNAMet ternary complexes. Interestingly, translation of the GCN4 mRNA increases at the time ternary complexes decrease and global translation initiation is inhibited (5). The mechanism of induction involves translation of four small uORFs within the GCN4 leader RNA sequence, followed by reinitiating at the GCN4 ORF (5). The mammalian homologue of the GCN2 eIF2α kinase was cloned (15, 22) and was shown to be required for translational control of the mRNA for the transcription factor ATF4 during amino acid starvation (20). The induction of translation of the mammalian ATF4 mRNA occurs by a mechanism similar to that described for the yeast GCN4 (20). Both of these examples of translational control by amino acid starvation involving direct induction of translation by eIF2α phosphorylation.

The translational regulation of the cat-1 mRNA by amino acid starvation has some features similar to the translational control of the GCN4 and ATF4 mRNAs. We have clearly shown that GCN2-dependent phosphorylation of eIF2α is required for increased translation of the cat-1 mRNA during amino acid starvation.

An interesting finding of the current study was that amino acid limitation in the absence of eIF2α phosphorylation is ineffective in inducing cat-1 IRES-mediated translation. Two pieces of data support this conclusion: (i) induction of cat-1 IRES activity was abolished by the expression of the eIF2α S-A during amino acid starvation and (ii) amino acid starvation did not induce cat-1 IRES-mediated translation in the C6-GCN2mut cells. The importance of eIF2α phosphorylation on cat-1 IRES activation was further demonstrated by the finding that expression of the eIF2α S-D that mimics the phosphorylated eIF2α variant increases cat-1 IRES-mediated translation in the presence of amino acids. This increase was higher than the increase caused by amino acid starvation. We conclude that eIF2α phosphorylation is the key regulator for cat-1 IRES activation. Our data and our knowledge on amino acid control of mammalian cells (20) support the idea that eIF2α phosphorylation by GCN2 is a key regulator of the cell’s response to amino acid starvation. This phosphorylation protects cells by inhibiting global protein synthesis (20) and, as shown here, signals the induction of translation of mRNAs that encode proteins essential for survival. The importance of translational control as a signaling pathway in metabolic processes is supported by the recent work of Kauffman and co-workers (30), demonstrating that genetically engineered mice expressing an eIF2α that cannot be phosphorylated have fatal hypoglycemia at birth and pancreatic β-cell defects.

However, in contrast to the reinitiation mechanism used by ATF4, translation of the cat-1 mRNA is mediated via an IRES (11). Furthermore, in contrast to the ATF4 and GCN4 mRNAs that require eIF2α phosphorylation at the time of induction, translational induction of the cat-1 IRES activity occurs indirectly. Phosphorylation of eIF2α occurs during the first hour of amino acid starvation, whereas IRES-mediated translation increases at 6–12 h.
How is the cat-1 ORF translated within the bicistronic mRNA? To study exclusively regulation of IRES-mediated translation in this study, we constructed an artificial bicistronic mRNA expression vector where the cat-1 ORF was replaced with the LUC ORF. This bicistronic mRNA allowed us to study IRES-mediated translation independently of cap-dependent translation. The eIF2α-dependent increase in LUC activity caused by amino acid starvation in bicistronic mRNAs can be explained by either increased reinitiation following translation of the uORF or increased IRES-mediated translation.

Is increased cat-1 IRES-mediated translation during starvation caused by translation of the uORF followed by increased reinitiation at the cat-1 ORF? This is possible, because translation of the cat-1 mRNA uORF is required for induction of IRES-mediated translation. Our data do not support this model. As discussed above and described earlier for the GCN4 (2) and mammalian ATF4 (20) mRNAs, increased reinitiation at the cat-1/ORF should occur in the first hour of amino acid starvation when eIF2-GTP-Met-tRNA\(^{\text{Met}}\) ternary complexes are low. However, cat-1 IRES-mediated translation of the LUC did not increase during the first 3 h of amino acid starvation. Moreover, by the time that IRES activity increased, eIF2α phosphorylation had fallen to base-line levels.

The mechanism by which eIF2α phosphorylation increases cat-1 IRES-mediated translation is not known. Our working hypothesis is that eIF2α phosphorylation induces the synthesis of a protein that interacts with the IRES, inducing its activity. This is possible, because eIF2α phosphorylation directly induces translation of mRNAs, such as ATF4 (20). The existence of such an IRES-specific trans-getting factor (ITAF), which mediates translation, has been demonstrated (31). ITAF\(^{45}\) has been shown to be involved in the cell type-specific expression of the IRES in the foot-and-mouth disease virus (31).

It has clearly been shown here that translation of the 48-amino acid uORF is essential for the eIF2α-mediated increase in cat-1 IRES activity during amino acid starvation in both monocistronic and bicistronic mRNAs. What is the mechanism of this requirement? Models that involve the translation of a small uORF have been described previously (29). Geballe and co-workers (32) have very elegantly demonstrated that peptide-specific attenuation within uORF 2 of the human cytomegalovirus gpUL4 mRNA (gp48) inhibits downstream translation by 10-fold. Interestingly, translation of these uORFs in the gp48 and cat-1 mRNAs is mediated by suboptimal AUGs (and Fig. 1A). Efficient initiation codons contain purines at positions −3 and +4 (with +1 being the A of A\(^{+1}\)UG). This can explain that, when the uORF/AUG was placed in frame with the LUC/AUG (mut\(^{-}\)-stop bicistronic mRNA), translation initiation occurred at the LUC/AUG as well as at the uORF/AUG (Fig. 4). However, in all known examples (29) of translation attenuation within a uORF, translation of the downstream cistron is inhibited. In contrast, it is shown here that translation of the uORF is required for increased IRES-mediated translation.

One possible mechanism of stimulation is that translation of the cat-1/uORF resolves a secondary structure at the 5′ end of the cat-1 mRNA leader, allowing a newly synthesized protein to interact with the RNA sequences and form an active IRES. It is shown in Fig. 1 that the 5′ end of the cat-1 leader is involved in a stable RNA structure (\(ΔG = -98.11\)). It is therefore possible that the 5′ end of the structure of the uORF RNA sequence has an inhibitory role on cat-1 IRES activation and translation of the uORF relieves this inhibition. Our model proposes the following translational control mechanism for the natural cat-1 mRNA. Translation of the uORF within the cat-1 mRNA leader in amino acid fed cells is in part cap-dependent (the GC-rich region at the 5′ end of the leader may inhibit cap-dependent translation, Fig. 1A) (33). Under these conditions the cat-1 ORF is translated via (i) leaky scanning of the uORF (the ribosomes do not recognize the uORF/AUG, initiating at the cat-1/ORF/AUG); (ii) reinitiation following translation of the uORF; or (iii) an IRES, acting independently of uORF translation. During amino acid starvation when cap-dependent protein synthesis decreases (11), our model predicts that IRES-mediated translation prevails in a manner dependent on translation of the uORF and eIF2α phosphorylation. This probably occurs via formation of a new IRES or activation of an existing one. Future studies will determine the validity of these translational control models.

The proposed model for regulation of translation mediated by the cat-1 IRES is paralleled by recent findings on regulation of IRES-mediated translation (34). Apoptotic stress induces IRES-mediated translation of the IAP proteins (35), which are potent inhibitors of apoptosis (25, 36). However, induction of the cat-1 IRES-mediated translation was not the result of induction of apoptosis (data not shown). Two IRES elements that specifically function during the G/M phase of the cell cycle were reported for the mRNAs for ornithine decarboxylase (37) and protein kinase PITSLRE (38). A striking finding of the first in vivo study of the fibroblast growth factor 2 IRES (39) was that, although the IRES was active in a few tissues during development, it was restricted to the brain in adult mice (39). This tropism of the fibroblast growth factor 2 IRES further supports the regulation of IRES activity by cell-type specific ITAFs.

We propose that some mammalian mRNAs use IRES-mediated translation to provide cells with proteins essential for survival when the nutrient supply is limited. Among these proteins is the Cat-1 arginine/lysine transporter, which also provides cells with the substrate for NO synthesis (40). Expression of the cat-1 gene has been shown to be regulated at the transcriptional and post-transcriptional level by hormones (41, 42), nutrients (9, 10) and growth factors (8, 43). The translational regulation described in this study further extends the complex regulatory mechanisms that control the cell's supply of arginine and lysine. Our finding, that arginine and lysine transport increases under nutritional stress, supports further the importance of these amino acids in cell survival.

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