Translation mediated by the internal ribosome entry site of the cat-1 mRNA is regulated by glucose availability in a PERK kinase-dependent manner

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SUMMARY

The cationic amino acid transporter, Cat-1, is a high affinity transporter of the essential amino acids, arginine and lysine. Expression of this gene is known to be regulated by amino acid availability. It is shown here that cat-1 gene expression is also induced by glucose (Glc) limitation, which causes a 7-fold increase in cat-1 mRNA, a 30-fold induction of Cat-1 protein levels, and a 4-fold stimulation of arginine uptake. Glc limitation is known to induce the unfolded protein response (UPR) by altering protein glycosylation in the endoplasmic reticulum (ER). The studies here demonstrate that synthesis of Cat-1 occurs during the UPR when global protein synthesis is inhibited. The 5'-UTR of the cat-1 mRNA contains an internal ribosomal entry site (IRES) that is activated by amino acid starvation by a mechanism that involves phosphorylation of the translation initiation factor, eIF2α, by the GCN2 kinase. It is shown here that translation from the cat-1/IRES is also induced by Glc deprivation in a manner dependent on phosphorylation of eIF2α by the transmembrane ER kinase, PERK. Because PERK is a key constituent of the UPR, it is concluded that induction of cat-1 gene expression is part of the adaptive response of cells to ER stress. These results also demonstrate that regulation of IRES activity in cellular mRNAs is part of the mechanism by which the UPR protects cells from unfolded proteins in the ER.
INTRODUCTION

Changes in the cellular nutrient supply cause changes in the transcriptional and translational regulation in many genes, including nutrient transporters, transcription factors and amino acid biosynthetic genes (1,2). One of the major nutrients involved in this regulation is glucose (Glc). Glc limitation has been shown to regulate gene expression by modulating transcription, mRNA stability, and translation initiation (3). Cells respond to Glc limitation by decreasing global protein synthesis (3) and increasing the synthesis of proteins essential for cell survival (4). Recent work has provided insights into the molecular mechanisms of this response (5-8). The most extensively studied genes regulated by nutrients are asparagine synthase(9) and the transcription factor, CHOP (10). Transcription of both genes is increased by Glc and amino acid limitation (9,10). DNA sequences within the promoter of these genes and trans-acting transcription factors have been identified (9,10). However, the molecular events that activate these transcription factors during amino acid or Glc limitation are not known.

We have previously shown that expression of the arginine/lysine transporter cat-1 gene is induced by amino acid availability (11). Amino acid starvation led to increased levels of cat-1 mRNA and protein, as well as increased high affinity transport of cationic amino acids (12,13). Because the Cat-1 protein is synthesized during amino acid starvation when global protein synthesis decreases, we recently investigated the mechanism by which cat-1 mRNA is translated (13). We showed that translation of cat-1 mRNA, during amino acid starvation occurs via a cap-independent mechanism involving an internal ribosomal entry site (IRES) in the 5’-UTR of
the mRNA (13). Translation from this IRES is activated during amino acid starvation in a process that requires phosphorylation of the translation initiation factor eIF2α by GCN2 kinase (13).

In this report, we demonstrate that deprivation of another crucial cellular nutrient, Glc, induces expression of the cat-1 gene. Levels of cat-1 mRNA and protein, as well as transport activity, were all increased by Glc limitation. Moreover, we demonstrate that translation from the cat-1/IRES is also increased in Glc-deprived cells.

The detailed mechanism by which Glc limitation induces gene expression is not known (14). One effect of Glc limitation is altered protein glycosylation, which causes improper folding of newly made glycoproteins (3) in the endoplasmic reticulum (ER). The accumulation of these proteins in the ER induces the unfolded protein response (UPR), a stress response that causes decreased protein synthesis and increased expression of genes that assist in protein folding in the ER. In yeast, Glc limitation induces the activation of the transmembrane serine/threonine protein kinase, Ire1p (5,8). Ire1p activation results in transcriptional up-regulation of UPR target genes. In the mammalian UPR pathway, at least three different ER transmembrane kinases, IRE1α, IRE1β, and PERK, sense changes in protein folding in the ER lumen (14). Activation of these kinases regulates transcription of UPR responsive genes. It also represses global protein synthesis and causes cell cycle arrest (8).
This study demonstrates that translation from the cat-1/IRES is stimulated by Glc limitation via a mechanism that involves phosphorylation of eIF2α by PERK. These findings suggest that Glc deprivation activates cat-1/IRES-mediated translation as part of the adaptive response to ER stress.

EXPERIMENTAL PROCEDURES

Expression vectors. The bicistronic mRNA expression vectors have been described previously (13). pSVCAT/BiP/LUC encodes a bicistronic mRNA, containing the chloramphenicol acetyltransferase (CAT) cDNA at the 5’ end and the firefly luciferase (LUC) cDNA at the 3’ end (15). The spacer between these cistrons contains the 5’-UTR of BiP (15). In pSVCAT/cat-1 5’-UTR/LUC the BiP intercistronic spacer has been replaced with the 5’-UTR of cat-1 mRNA (13). Expression plasmids encoding flag-tagged wild-type and mutant murine GCN2 were obtained from R. Wek, Indiana University. Mutant GCN2 contains a Lys618 to Met mutation in the kinase domain. Plasmids encoding myc-tagged wild-type and mutant PERK were obtained from D. Ron, New York University School of Medicine. Mutant PERK (PERKΔC) is missing the C-terminal kinase domain. All these cDNAs were cloned into the XbaI/HindIII site of a derivative of pCDNA3 in which the neo resistance gene was replaced with a cDNA for the CD2 cell surface marker gene. In these expression vectors the cDNAs are transcribed from the cytomegalovirus promoter.
**Cells and cell culture.** C6 glioma and NIH 3T3 cells were maintained in DMEM/F12 medium supplemented with 10% FBS. NIH 3T3 cells stably expressing the PERKC mutant have been described (4). Cells were plated at 2x10^5 cells/35 mm dish and transfected the next day using the calcium phosphate technique (13). Cotransfections were performed with equimolar amounts of plasmid DNAs. Two days after transfection, cells were incubated in Glc-free MEM supplemented with 10% dialyzed FBS (11) for the indicated times. Control cells were incubated in the same medium containing 3.152 g/L Glc. Similar results were obtained when the effects of Glc deprivation were examined in DMEM or DMEM/F12 media. Cells were starved for amino acids as described earlier (13).

**Enzymatic assays, Northern, and Western blot analyses.** Cell extracts were assayed for LUC and CAT activities as described previously (13). The activities were normalized to the protein content of the cell extracts, which was measured using the Biorad DC assay. cat-1, AS mRNAs and 18S ribosomal RNA were detected by Northern blotting using \(^{32}\)P-labeled DNA hybridization probes. Western blot analysis of Cat-1 protein was carried out using a polyclonal antibody as previously described (13). eIF2\(\alpha\) and phospho-eIF2\(\alpha\) were detected using a mouse monoclonal and rabbit polyclonal antibodies respectively (Quality Controlled Biochemicals, Inc). Total and phosphorylated (Ser\(^{209}\)) eIF4E were detected using polyclonal antibodies (Cell Signaling). 4EBP1 was detected using an anti-PHAS-1 peptide antibody (Zymed Laboratories). PERK\(\Delta\)C and GCN2mut proteins were detected using anti-myc and anti-FLAG peptide antibodies (Santa Cruz).
[3H]arginine transport studies. Cells were plated at 2 x 10^5 cells/18 mm well, cultured in growth medium for 48 h and then incubated under Glc-fed or starved conditions prior to assay. Uptake of [3H]Arg was then measured as previously described (11).

RESULTS

cat-1 mRNA and protein levels are induced by Glc limitation

We have previously shown that amino acid starvation regulates cat-1 gene expression at several different levels, causing increases in the amounts of cat-1 mRNA, protein, and transport activity (11-13). To determine whether other nutrients that cause cellular stress induce cat-1 expression, we examined the effects of Glc deprivation. First, the effect of Glc withdrawal on cat-1 mRNA levels was examined (Fig. 1A). cat-1 mRNAs of 3.4 and 7.9 kb are found in cells, which result from the use of alternative polyadenylation sites (16). Levels of both cat-1 mRNAs remained constant for the first 3 h of deprivation and increased thereafter, with a 7-fold increase seen after 6 h. As controls, we examined the levels of two other RNAs. Asparagine synthase (AS) mRNA levels are known to increase during Glc starvation (2), a finding that is confirmed by our results (Fig. 1A). The level of 18S ribosomal RNA was measured to normalize for the amount of RNA analyzed. These results indicate that the level of cat-1 mRNA increases during Glc starvation as a response to this cellular stress.

Next, the effect of Glc deprivation on the amount of Cat-1 protein was determined. Extracts from control and Glc-deprived cells were analyzed on Western blots using an anti-Cat-1
antiserum (Fig. 1B). The level of Cat-1 protein increased continuously during Glc deprivation; by 12 h, the level was 30-fold higher than in control cells (Fig 1B). Finally, to determine whether the Cat-1 protein synthesized during Glc deprivation was functional, transport activity was assessed by measuring the uptake of \[^3\text{H}\text{Arg}\] (Fig. 1C). Transport activity increased during Glc deprivation, showing a 4.5 fold increase by 12 h, the same increase caused by amino acid starvation (13). Together, these results demonstrate that Glc deprivation increases cat-1 gene expression. mRNA levels increase, and this mRNA is translated into functional protein on the cell surface that is able to carry out the uptake of cationic amino acids.

cat-1/IRES activity is induced by Glc limitation

The results in Fig 1 suggest that cat-1 mRNA is subject to translational control because the Cat-1 protein/mRNA ratio increased 4-fold during Glc deprivation. Moreover, the absolute level of Cat-1 protein levels increased during Glc limitation when global protein synthesis decreases. Translational control of cat-1 mRNA under these conditions is likely because we have previously shown that this mRNA contains an IRES sequence whose activity is stimulated by amino acid starvation, another type of cellular stress (13). To determine whether the cat-1/IRES is also regulated by Glc limitation, we used a vector encoding a bicistronic mRNA containing two reporter genes, chloramphenicol acetyltransferase (CAT) and luciferase (LUC) (15). The CAT open reading frame is at the 5’-end of the mRNA, followed by a spacer and the LUC reading frame (Fig 2). CAT translation is initiated from the 5’ cap of the mRNA. In contrast, LUC is translated only by initiation from the intercistronic spacer. Efficient LUC expression
occurs only if the spacer contains an IRES sequence. We have previously shown that the 5′-end of the cat-1 mRNA contains an IRES element that can drive the translation of LUC in this bicistronic mRNA (13). To test the regulation of the cat-1/IRES during Glc limitation, C6 rat glioma cells were transiently transfected with a vector containing the cat-1 5′-UTR as the intercistronic spacer (13). Cells were then grown in medium with or without Glc for various times and cell extracts were assayed for CAT and LUC activities (Fig. 2). LUC activity increased during Glc deprivation, reaching a peak at 9 h (4.5-fold increase) and then declining. Therefore, the activity of the cat-1/IRES increases during Glc deprivation. In contrast, CAT activity, which is due to cap-dependent translation of the bicistronic mRNA, decreased slowly during Glc deprivation. Fig. 2 also shows the effect of Glc deprivation on the LUC/CAT activity ratio. This ratio showed a time-dependent increase during Glc limitation, indicating that translation from the cat-1/IRES was stimulated during a time when global protein synthesis was declining. Interestingly stimulation of the cat-1/IRES by Glc deprivation was reversible. When cells were incubated in Glc-free medium for 6 h and then returned to Glc-containing medium, CAT and LUC activities, as well as the LUC/CAT ratio returned to control levels within 3 h (Fig. 2).

As a control for these studies of the cat-1/IRES, the regulation of the BiP IRES by Glc limitation was examined. BiP, also known as GRP78 (17), is a protein, whose expression increases during Glc limitation (18). Furthermore, BiP is the first mammalian mRNA shown to have an IRES in its 5′-UTR (15). Cells were transiently transfected with pSVCAT/BiP/LUC, which encodes a
bicistronic mRNA containing the BiP IRES in the intercistronic spacer between the CAT and LUC open reading frames. As expected, Glc deprivation decreased CAT expression (Fig. 2), consistent with the inhibition of global protein synthesis caused by Glc deprivation. In contrast, LUC activity remained constant during Glc limitation. The LUC/CAT ratio increased slightly during Glc deprivation, due to the decreased CAT activity. These results demonstrate that translation from the BiP IRES is maintained, but not increased, during Glc deprivation when global protein synthesis decreases. These findings are consistent with our previous study of the effects of amino acid starvation on IRES activity (13,19). Translation from the cat-1/IRES was stimulated by amino acid starvation, but the BiP IRES was not affected. Note that the small increase in LUC/CAT at 9 h of glucose deprivation with BiP is a result of decreased cap-dependent translation (CAT) and not an increase in IRES-mediated translation (LUC). These results demonstrate that the activity of the cat-1/IRES is regulated by cellular stress using a mechanism that is not shared by IRES sequences of other cellular mRNAs.

To characterize the effect of Glc deprivation on the cat-1/IRES, we examined the Glc concentration dependence of the increased translation (Fig. 3). Decreasing the Glc concentration to 1 mM had no effect on translation during a 9 h incubation. Further decreases in concentration stimulated translation from the IRES. The midpoint of the increase occurred at 0.05 mM and the highest activity was seen in Glc-free medium. The concentration dependence supports the conclusion that the increase in translation from the cat-1/IRES is part of a physiological response to cellular stress.
PERK kinase is required for IRES-mediated translational regulation by Glc availability

How does Glc deprivation increase translation mediated by the cat-1/IRES? Cells respond to Glc deprivation by activating the UPR, which affects protein synthesis. This pathway increases the phosphorylation of eIF2α, causing a decrease in ternary complexes and global inhibition of translation initiation (14). At the same time, translation of certain mRNAs increases via a mechanism that involves phosphorylation of eIF2α (20). We therefore tested if eIF2α phosphorylation is involved in the increased cat-1/IRES-mediated translation by Glc limitation. At least 4 kinases are known to phosphorylate eIF2α: GCN2, PERK, PKR, and HRI (21). PERK is an ER protein that is regulated by unfolded proteins in the ER lumen. It has been suggested that Glc deprivation activates PERK by causing the accumulation of unfolded proteins in the ER.

It has also been suggested that Glc deprivation in yeast can activate GCN2 by causing the accumulation of uncharged tRNAs (22). To test whether these kinases are involved in the regulation of the cat-1/IRES by Glc deprivation, we examined the effects of dominant negative mutants of GCN2 and PERK. C6 glioma cells were transiently transfected with expression vectors for bicistronic mRNA containing the cat-1/IRES along with expression vectors encoding wild-type or dominant-negative mutant kinases (23). The dominant negative effect of the mutant kinases has been previously described (4,23). The kinases were epitope tagged and their expression was monitored by Western blot analysis.

In cells overexpressing wild-type PERK, Glc deprivation caused an increase in LUC translation from the cat-1/IRES (Fig. 4), similar to the response seen in cells that only contained
endogenous PERK (Fig. 2). In contrast, in cells overexpressing the PERKΔC mutant, Glc deprivation did not cause an increase in the LUC level, indicating that PERK regulates translation of the cat-1/IRES. This effect was specific for PERK, because neither wild-type nor mutant GCN2 affected the regulation of CAT or LUC expression by Glc deprivation.

We wished to examine the effectiveness of these mutant kinases on inhibiting phosphorylation of eIF2α by Glc deprivation. However, we could not use the C6 cells employed in Figs. 2 and 3 because only a fraction of the cells are transfected in this transient system. Consequently we evaluated the effects of the PERKΔC mutant on Glc deprivation-induced eIF2α phosphorylation in a stably-transfected NIH 3T3 cell line. Significantly, this cell line was used to demonstrate that PERK mediates the cell cycle inhibition that occurs during the UPR (4). We first showed that Glc deprivation of the parent NIH 3T3 cells induced translation mediated by the cat-1/IRES as observed in C6 cells (Fig. 5). This increase was not seen in cells overexpressing PERKΔC (Fig. 5). Cap-dependent translation of CAT mRNA showed the same decline during Glc starvation in controls and in cells overexpressing PERKΔC. These results confirm the importance of PERK kinase in regulation of the cat-1/IRES activity in Glc starvation.

**eIF2α phosphorylation by PERK increases transiently during Glc deprivation**

PERK is known to regulate translation initiation by phosphorylating the translation initiation factor, eIF2α (24). In addition, decreased phosphorylation of a second initiation factor, eIF4E is
thought to be important in the inhibition of cap-dependent translation during cellular stress (25). Furthermore, the availability of eIF4E is controlled by binding to 4EBP-1 (also known as PHAS-1); dephosphorylated 4EBP-1 binds eIF4E, thus reducing active eIF4F complexes (26). We examined the phosphorylation of these factors during Glc starvation to study the role they may play in translational regulation mediated by the cat-1/IRES. First, we examined the phosphorylation of eIF2α in C6 cells (Fig. 6A). The amount of phosphorylated eIF2α increased by 30 min of Glc deprivation, remained elevated at 1 h and declined to the control level by 4 h. The total amount of eIF2α did not change during this time (Fig 6A). These data were quantified and the ratio of phosphorylated to total eIF2α was calculated to estimate the change in the extent of eIF2α phosphorylation. eIF2α phosphorylation showed a 3-fold increase during the first hour of Glc deprivation and then declined to levels slightly below the control by 6 h (Fig. 6A). The phosphorylation of eIF2α during Glc deprivation should contribute to the decrease in global protein synthesis. However, this transient increase in phosphorylation cannot be directly responsible for the changes in translation from the cat-1/IRES during Glc deprivation because translation continues to increase after eIF2α phosphorylation has increased and then declined.

Next, the effect of Glc limitation on the phosphorylation of the cap-binding protein eIF4E was examined (Fig. 6B). As expected, the amount of phosphorylated eIF4E showed a slow transient decrease during Glc limitation. The amount of phosphorylated protein began to decline after 1 h, reached a minimum between 4 and 6 h and then returned to the control level by 12 h. The total amount of eIF4E did not change during this period, so the extent of eIF4E phosphorylation
showed a transient decrease. The time course of the changes in eIF4E phosphorylation does not match the changes in translation from the cat-1/IRES (Fig. 2). Translation from this IRES remained elevated during prolonged Glc deprivation after eIF4E phosphorylation had returned to the control level.

Finally, the effect of Glc deprivation on the phosphorylation of 4EBP-1 was analyzed. A Western blot probed with an antibody to 4EBP-1 revealed two bands, β and γ, which represent phosphorylated forms of 4EBP-1 (Fig. 6C). The γ form is inactive, whereas the β form is able to bind eIF4E (27). The unphosphorylated α form of 4EBP1 is not present. Glc deprivation for 1 h caused a decrease in the phosphorylation of 4EBP1, which is seen as a decrease in the γ form and an increase in the β form. This change was transient; the increase in the β form persisted for several hours, followed by a gradual increase in the γ form. The decreased phosphorylation of 4EBP1 is expected to increase binding of eIF4E, thereby decreasing the amount of active form of the latter protein. These data support that there is no co-ordinate regulation of cat-1/IRES activation and the activity of eIF4E.

The experiments in Fig. 6 demonstrate that Glc deprivation leads to transient changes in the phosphorylation of translation initiation factors. Moreover, we have shown that PERK kinase is required for the changes in translation from the cat-1/IRES (Figs. 4 and 5). To determine whether PERK mediates eIF2α phosphorylation during Glc deprivation, we studied the phosphorylation of eIF2α in NIH 3T3 cells expressing the dominant-negative mutant PERK.
The transient increase in eIF2α phosphorylation seen during the first hour of Glc deprivation of parent cells was not observed in cells expressing mutant PERK (Fig. 7A). Moreover, the amount of eIF2α protein and the amount of mutant PERK did not change during Glc deprivation. Consequently, this experiment demonstrates that PERK is the kinase responsible for the enhanced eIF2α phosphorylation in Glc-starved cells.

As a control for this experiment, two other treatments that induce cellular stress and eIF2α phosphorylation were studied in cells expressing the PERKΔC mutant. First, we examined the effects of thapsigargin, which triggers the UPR by blocking the uptake of Ca^{2+} into the ER (14). In cells expressing PERKΔC, increased eIF2α phosphorylation was not observed, confirming that PERK is responsible for eIF2α phosphorylation during the UPR (Fig. 7B). In contrast, thapsigargin caused increased eIF2α phosphorylation in the parent NIH3T3 cells (data not shown). Second, we examined the effects of amino acid starvation. In this case, the amount of phosphorylated eIF2α increased after 30 min and remained elevated throughout the 6 h course of the experiment (Fig. 7C), indicating that PERK is not required for this phosphorylation. This is the expected result because we and others have shown that GCN2 kinase phosphorylates eIF2α in response to amino acid starvation in both animal cells and yeast (23,28). Taken together, these results demonstrate that Glc starvation results in the specific activation of PERK kinase, which phosphorylates eIF2α.

Our results show that Glc limitation increases translation of the cat-1/IRES and that this effect is
mediated by PERK. They also demonstrate that PERK is responsible for the phosphorylation of eIF2α induced by Glc deprivation. Because eIF2α phosphorylation occurs during the first hour of Glc deprivation, it is possible that this event is responsible for the subsequent induction of translation from the cat-1/IRES. To test this hypothesis, we studied the effects of overexpressing a mutant of eIF2α with Ser51 mutated to Ala (eIF2αS-A). Because this mutant is missing Ser51, it cannot be phosphorylated by PERK during Glc deprivation. Others have shown that eIF2αS-A acts as a dominant negative mutant when it is overexpressed in transiently transfected cells by exchanging with the endogenous eIF2α in the ternary complexes (29). We found that expression of eIF2αS-A abolished the stimulation of translation from the cat-1/IRES by Glc limitation (Fig. 8). These results demonstrate that phosphorylation of eIF2α is required for the enhanced translation from the cat-1/IRES during the UPR.

DISCUSSION

We have shown that Glc deprivation regulates cat-1 gene expression by increasing both mRNA and protein levels. The molecular mechanism involved in increased mRNA levels during glucose deprivation is currently under investigation. However it has been previously shown that the cat-1 mRNA levels increase by amino acid starvation via a mechanism that involves post-transcriptional mRNA stabilization (12). Our results also demonstrate that cat-1 expression during Glc deprivation is subject to translational control. Two lines of evidence support this conclusion. First, we have shown that the Cat-1 protein:mRNA ratio increases during Glc deprivation, consistent with enhanced translation of the mRNA during this period. Second,
translation mediated by the cat-1/IRES is regulated by Glc availability when it was studied in a bicistronic mRNA. This regulation results in a 5-fold increase in IRES-mediated translation during Glc-deprivation via a mechanism that requires the phosphorylation of eIF2α on Ser51 by PERK kinase.

How does Glc deprivation stimulate translation from the cat-1/IRES? Our demonstration that PERK kinase is required for this stimulation strongly suggests that Glc deprivation has its effects by triggering the UPR. The UPR is a stress response induced by the accumulation of unfolded secretory proteins within the ER. Glc deprivation triggers the UPR by interfering with the glycosylation of newly made glycoproteins (14). The central role of the UPR in the stimulation of translation from the cat-1/IRES is demonstrated by our finding that this IRES is also stimulated by two other compounds that trigger the UPR, thapsigargin and tunicamycin (data not shown).

The UPR is mediated by the ER transmembrane kinases: PERK, IRE1, and IRE2 in mammalian cells. These molecules have domains in the ER lumen that are thought to sense the accumulation of unfolded proteins and kinase domains, which face the cytoplasm. Activation of the kinases mediates the stress response. The importance of PERK kinase has been shown for translational control and cell survival during the UPR (6,8). Our studies extend the importance of PERK by showing that this kinase is required for stimulation of the cat-1/IRES.

The UPR involves several important responses to the presence of unfolded proteins in the ER.
First, activated PERK phosphorylates eIF2α, inhibiting global protein synthesis (24). This acts to decrease the load of proteins that must be folded in the ER. Second, there is increased expression of proteins involved in protein folding or degradation (3). Finally, expression of other proteins required for cell survival is modulated (5,8). Because Cat-1 is required for the uptake of the essential amino acids, Arg and Lys, we believe that the increased expression of this gene is part of this latter set of responses. Furthermore, functional Cat-1 protein is probably made via the UPR-induced synthesis of ER proteins that facilitate the synthesis of new membrane proteins (30). The regulation we have described allows the cells to maintain or increase the amount of Cat-1 protein, thus allowing a supply of Arg and Lys for protein synthesis and progression through the cell cycle.

We have shown that induction of translation from the cat-1/IRES during Glc deprivation requires phosphorylation of eIF2α by PERK. However, the mechanism by which phosphorylation of eIF2α stimulates translation from this IRES is not clear. Despite the fact that eIF2α phosphorylation by PERK is required to stimulate translation from the cat-1/IRES, the phosphorylation returns to the control level before large increases in translation from the cat-1/IRES are seen. The transient changes in eIF4E and 4EBP-1 phosphorylation also did not correlate with the induced activity of the cat-1/IRES.

We propose that the stimulation of translation from the cat-1/IRES occurs by an indirect mechanism. Activation of PERK and phosphorylation of eIF2α at Ser^51 must be early events in
this process. However we do not know the nature of the later events in the control mechanism. Changes in the level of a protein that regulates the cat-1/IRES are a likely mechanism because the induction is a slow process. This could involve increases in the level of a translational enhancer or decreases in the level of a suppressor of IRES activity. This would explain the slow induction, and the persistence of the effect long after the increased eIF2α phosphorylation has returned to the control level. The regulation of the IRES-controlling factor could occur at the level of transcription or translation. In fact, translation of the transcription factor ATF4 has been shown to increase when eIF2α is phosphorylated (6). It is worth noting that the IRES in a second Glc-regulated cellular mRNA, BiP, does not show the same regulation as the cat-1/IRES; translation from the BiP/ IRES is maintained, but not stimulated, during Glc limitation. This suggests that the mechanism that regulates the cat-1/IRES may not be shared IRESs in other cellular mRNAs.

Our results support the idea that phosphorylation of eIF2α is a key mediator of positive and negative regulation of gene expression during nutritional stress. Induction of the UPR via Glc deprivation stimulates translation mediated by the cat-1/IRES. Translation from this IRES is also stimulated by amino acid starvation, a process that requires eIF2α phosphorylation by a second kinase, GCN2 (31). Moreover, it is likely that translational regulation and nutrient homeostasis are linked processes. This is supported by the recent findings that regulation of translation initiation via eIF2α phosphorylation plays an important role in Glc metabolism (6,8). Mice expressing the eIF2αS-A mutant had severe hypoglycemia and died within hours of birth. Animals with a PERK knockout were hyperglycemic and had symptoms of diabetes mellitus.
Our findings suggest that translational regulation via eIF2α phosphorylation is also important in amino acid regulation in response to cellular stress.

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Footnotes

1The abbreviations used are: CAT, chloramphenicol acetyltransferase; cat-1, cationic amino acid transporter; FBS, fetal bovine serum; Glc, glucose; IRES, internal ribosome entry site; LUC, firefly luciferase; ORF, open reading frame; 5’-UTR, 5’-untranslated region.
FIGURE LEGENDS

Fig 1. **Glc deprivation induces expression of the cat-1 gene.** C6 cells were incubated in fresh Glc-containing medium for 4 h and then incubated in Glc-free medium for the times indicated. (A) Northern blot analysis of RNA (15 µg) cells using probes for the cat-1 and AS mRNAs and 18S ribosomal RNA. (B) Western blot analysis of cell extracts probed with anti-cat-1 antibody. (C) y+ transport activity measured by [3H]Arg uptake. Values are the mean ± S.D. of five independent experiments.

Fig 2. **Stimulation of translation from the cat-1/IRES and not the BiP/IRES by Glc deprivation.** C6 cells transfected with bicistronic mRNA expression vectors containing either the cat-1/IRES (CAT/cat-1 5’UTR/LUC) or the BiP/IRES (CAT/BiP/LUC) were cultured in either Glc-containing (9 h) or Glc-free media for the indicated times. Cell extracts were prepared and LUC and CAT activities were measured. Results are the average of three independent experiments and are presented as the activities of LUC, CAT per µg protein or the LUC/CAT ratio. All values are expressed relative to the values for Glc-fed cells. The bars represent the average ± S.E. of three independent experiments. The inset shows the elements of the bicistronic mRNAs (31).

Fig 3. **Dependence of cat-1/IRES-mediated translation on Glc.** C6 cells transfected with CAT/cat-1 5’-UTR/LUC were incubated in media containing the indicated concentrations of
Glc for 9 h. Cell extracts were prepared and LUC and CAT activities were measured. Results were analyzed as described in the legend to Fig. 2. The bars represent the average ± S.E. of three independent experiments.

Fig 4. **Induction of cat-1/IRES-mediated translation by Glc deprivation is dependent on PERK kinase.** C6 cells were transfected with CAT/cat-1 5’-UTR/LUC alone, or with expression vectors for wild-type and mutant GCN2 (GCN2mut) and PERK (PERKΔC) eIF2α kinases. The cells were cultured in either Glc-free or Glc-containing medium for 9 h. Cell extracts were prepared and LUC and CAT activities were measured. Results were analyzed as described in the legend to Fig. 2. The bars represent the average ± S.E. of three independent experiments.

Fig 5. **Cells expressing a dominant-negative PERK (PERKΔC) block induction of cat-1/IRES-mediated translation in response to Glc deprivation.** Cells (NIH3T3 and NIH3T3-PERKΔC) transfected with the CAT/cat-1 5’-UTR/LUC expression vector were cultured in either Glc-containing (9 h) or Glc-free medium for the times indicated. Results are the average of three independent experiments and are presented as the activity relative to the values from Glc-fed cells. The bars represent the average ± S.E. of three independent experiments.

Fig 6. **Glc starvation induces transient changes in the phosphorylation of translation initiation factors eIF2α, eIF4E and 4EBP-1.** Western blot analysis of cell extracts (15 µg protein) from C6 cells incubated in Glc-free medium for the times indicated using antibodies for (A) eIF2a and
phospho-eIF2α, (B) eIF4E and phospho-eIF4E and (C) 4EBP-1. Bands were visualized by chemiluminescence and quantified by densitometry. The ratio of phospho-eIF2α/eIF2α is shown in A, with the ratio normalized to 1 in Glc-fed cells.

**Fig 7. Phosphorylation of eIF2α by Glc starvation is inhibited in cells expressing a dominant negative PERK.** PERKΔC cells were incubated for the times indicated in (A) Glc-free medium, (B) medium containing 400 nM thapsigargin (Thaps) and (C) amino acid-free medium. Cell lysates (15 µg protein) were analyzed using antibodies for eIF2α, phospho-eIF2α and myc-tagged PERKΔC protein.

**Fig 8. Phosphorylation of eIF2α during Glc-starvation is required for increased cat-1/IRES activity.** C6 cells transfected with the CAT/cat-1 5'-UTR/LUC alone or with an expression vector for eIF2αS-A. were incubated in either Glc-containing or Glc-free medium for the times indicated. Cell extracts were assayed for LUC and CAT activities. Results are the average of three independent experiments and are normalized to the values in Glc-fed cells transfected with CAT/cat-1 5'-UTR/LUC alone. The bars represent the average ± S.E. of three independent experiments.
FIGURE 1

A

- Glc 0 1 1.5 2 3 4 6 9 12 (h)

- Glc 0 1 2 4 6 12 (h)

- Glc 0 3 11 20 30 fold induction

B

- Glc 0 1 3 11 20 30 fold induction

C

[3H]Arginine Transport

nmol/mg protein/30s

- Glc

- Glc
FIGURE 3

- **LUC/CAT**
- **LUC/µg protein**
- **CAT/µg protein**

**Relative Level vs. Glc (mM)**

- **0**
- **0.005**
- **0.01**
- **0.05**
- **0.1**
- **0.5**
- **1**
- **17.5**
FIGURE 6

A

-Gr  0  0.5  1  2  4  6  12 (h)

| eF2α-P | 1  3.1  2.9  1.4  1.1  0.7  0.7 |
| eF2α  |  |
| eF2α-P/eF2α | 1  3.1  2.9  1.4  1.1  0.7  0.7 |

B

-Gr  0  1  2  4  6  12 (h)

| eF4E |  |
| eF4E-P |  |
| 4E-BP1α |  |
FIGURE 7

A

PERKΔC

-GLc  0  0.5  1  3  6  (h)

eF2α-P

eF2α

4E

4E-P

c-myc tag
FIGURE 8

|       | LUC/CAT | LUC/µg protein | CAT/µg protein |
|-------|---------|----------------|----------------|
| - GR  |         |                |                |
| 3     |         |                |                |
| 6     |         |                |                |
| 9     |         |                |                |

Relative level

- GR - 3 - 6 - 9

cat1-5 - UTR$^f$
cat1-5 - UTR$^f$ eF2αS-A
Translation mediated by the internal ribosome entry site of the cat-1 mRNA is regulated by glucose availability in a PERK kinase-dependent manner

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