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Regulation of Internal Ribosomal Entry Site-mediated Translation by Phosphorylation of the Translation Initiation Factor eIF2α*

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James Fernandez‡, Ibrahim Yaman‡, Peter Sarnow§, Martin D. Snider¶, and Maria Hatzoglou‡‖

From the Departments of ‡Nutrition and ¶Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio, 44106 and the §Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305

Initiation of translation from most cellular mRNAs occurs via scanning; the 40 S ribosomal subunit binds to the m^7G-cap and then moves along the mRNA until an initiation codon is encountered. Some cellular mRNAs contain internal ribosome entry sequences (IRESs) within their 5′-untranslated regions, which allow initiation independently of the 5′-cap. This study investigated the ability of cellular stress to regulate the activity of IRESs in cellular mRNAs. Three stresses were studied that cause the phosphorylation of the translation initiation factor, eIF2α, by activating specific kinases: (i) amino acid starvation, which activates GCN2; (ii) endoplasmic reticulum (ER) stress, which activates PKR-like ER kinase, PERK kinase; and (iii) double-stranded RNA, which activates double-stranded RNA-dependent protein kinase (PKR) by mimicking viral infection. Amino acid starvation and ER stress caused transient phosphorylation of eIF2α during the first hour of treatment, whereas double-stranded RNA caused a sustained phosphorylation of eIF2α after 2 h. The effects of these treatments on IRES-mediated initiation were investigated using bicistronic mRNA expression vectors. No effect was seen for the IRESs from the mRNAs for the chaperone BiP and the protein kinase Pim-1. In contrast, translation mediated by the IRESs from the cationic amino acid transporter, cat-1, and of the cricket paralysis virus intergenic region, were stimulated 3- to 10-fold by all three treatments. eIF2α phosphorylation was required for the response because inactivation of phosphorylation prevented the stimulation. It is concluded that cellular stress can stimulate translation from some cellular IRESs via a mechanism that requires the phosphorylation of eIF2α. Moreover, there are distinct regulatory patterns for different cellular mRNAs that contain IRESs within their 5′-untranslated regions.

The vast majority of eukaryotic mRNAs is translated via the scanning mechanism (1, 2). This mechanism involves the recognition of the 5′-end of the mRNA and its m^7G-cap structure by the translation initiator factor eIF4F, which is composed of eIF4A, eIF4G, and eIF4E. This is followed by binding of the 40 S ribosomal subunit/eIF2·GTP·Met-tRNA$_i$ ternary complex and scanning downstream to the initiation codon (1, 2). Following GTP hydrolysis, the 60 S ribosomal subunit joins the complex to form the 80 S ribosome (3).

Recently, it has been shown that translation of some mRNAs is initiated by cap-independent mechanisms (1, 4). Elements within the 5′-untranslated region (UTR)[1] of the mRNAs known as internal ribosome entry sequences (IRESs) can direct ribosome binding without the need for the eIF4F complex (1, 5). This mode of initiation has mainly been described for viral RNAs, which are translated in infected cells when cap-dependent translation is inhibited (6). Some cellular mRNAs also contain IRESs in their 5′-UTRs (7). It has been shown that translation of some of these IRESs is regulated by the cell cycle (8), developmental stage (9), apoptosis (10, 11), and cellular stress (12–14). Many important features of how IRESs in cellular mRNAs mediate translation initiation are poorly understood. It is not known how many different types of IRESs are found in cellular mRNAs. In addition, the mechanisms by which IRESs are regulated and the number of different control mechanisms are poorly understood.

We have recently shown that the mRNA for the Arg/Lys transporter, cat-1, contains an IRES sequence (12). This IRES is located in the 5′-UTR, which also contains a 48-residue open reading frame (14). Translation initiation from this IRES is increased during amino acid starvation when global and cap-dependent protein synthesis is decreased (15), allowing cat-1 protein expression when amino acids are limiting (12). The phosphorylation of translation initiation factors plays a key role in this regulation (14). During amino acid starvation, phosphorylation of eIF2α increases, which decreases its activity, causing reduced levels of ternary complexes (16). In addition, eIF4F activity is decreased due to dephosphorylation of eIF4E and the eIF4E-binding protein 4E-BP-1 (16). It was shown previously that phosphorylation of eIF2α by the kinase GCN2, whose activity is stimulated by uncharged tRNAs, is required for enhanced cat-1 IRES activity during amino acid deprivation (14).

In this report, we expand these studies of the regulation of IRES activity by eIF2α phosphorylation. It is shown that two other types of cellular stress that increase eIF2α phosphorylation also stimulate translation mediated by the cat-1 IRES.

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‖ To whom correspondence should be addressed: 10900 Euclid Ave., Case Western Reserve University, Dept. of Nutrition, Cleveland, OH 44106-4906. Tel.: 216-368-3012; Fax: 216-368-6644; E-mail: mxh8@po.cwru.edu.

[1] The abbreviations used are: UTR, untranslated region; IGR, intergenic region; IRES, internal ribosome entry site; ICS, intercistronic spacer; ORF, open reading frame; CAT, chloramphenicol acetyltransferase; cat-1, cationic amino acid transporter-1; CrPV, cricket paralysis virus; dsRNA, double-stranded RNA; PKR, double-stranded RNA-dependent protein kinase; PERK, PKR-like ER kinase; ER, endoplasmic reticulum; LUC, luciferase; FLUC, firefly LUC; RLUC, renilla LUC; poly(IC), poly(I)·poly(C); DMEM, Dulbecco's modified Eagle's medium.

Agents that cause the accumulation of unfolded proteins within the endoplasmic reticulum (ER) trigger the unfolded protein response by activating the eIF2α kinase, PERK, in the ER membrane (17). We show that thapsigargin, which mobilizes sequestered Ca^{2+} from the ER, and tunicamycin, which disrupts protein glycosylation, increase cat-1 IRES-mediated translation by the activation of PERK. We also show that double-stranded RNA (dsRNA), which mimics viral infection (18), stimulates translation mediated by the cat-1 IRES by activating the eIF2α kinase, PKR. These results demonstrate that this IRES can be regulated by a variety of cellular stresses that stimulate eIF2α phosphorylation.

We also tested whether other cellular IRESs can be regulated by these cellular stresses. The IRESs from the BiP and Pim-1 mRNAs were studied. BiP is a chaperone protein that assists in protein folding within the ER (19). Transcription of the *BiP* gene is induced as part of the unfolded protein response (20). The BiP mRNA is translated under these conditions via an IRES element found within its 5′-UTR (21). Pim-1 is a serine-threonine kinase that functions with c-Myc in cellular transformation (22). This mRNA is translated in poliovirus-infected cells via an IRES element within its 5′-UTR (23). We show here that the activity of these IRESs is maintained but not increased by the cellular stresses that increase eIF2α phosphorylation. These results demonstrate that IRESs from cellular mRNAs are a diverse group because they are not all regulated by the same mechanism.

EXPERIMENTAL PROCEDURES

Expression Vectors—The following bicistronic mRNA expression vectors have been described previously: pSVCAT/cat1-5′-UTR^f/LUC, which encodes an mRNA containing CAT as the 5′-cistron and LUC as the 3′-cistron (14). The intercistronic spacer (ICS) is the 270-bp 5′-UTR of the cat-1 mRNA (14). pSVCAT/BiP/LUC encodes an mRNA with the 5′-UTR of the BiP mRNA as the ICS (21). Three bicistronic plasmids encoding renilla luciferase (RLUC) as the 5′-cistron and firefly luciferase (FLUC) as the 3′-cistron were used (23). The IGR construct (SV40/T7ΔEMCV/Fluc-IGR/CrPVORF2) contains 207 bp from the intergenic region (IGR) of cricket paralysis virus (CrPV) in the ICS (23, 24). The IGRmut construct contains the CrPV IGR with the CC residues corresponding to bases 6214 and 6215 of the CrPV sequence mutated to GG (23, 24). The Pim-1 construct contains the 5′-UTR of the human Pim-1 mRNA in the ICS in place of the CrPV sequence.

Expression vectors for PERK, PERK-mut, GCN2, GCN2-mut, and eIF2α S-A, were kindly provided by D. Ron (New York University School of Medicine). The cDNAs in all vectors were inserted at the *Xba*I/*Hin*dIII site of pCDNA3. In these vectors, transcription is directed by the cytomegalovirus promoter (25).

Cells and Cell Culture—All cells were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS). Plasmid DNAs were transfected into C6 rat glioma cells (5×10^5/35-mm dish) using the calcium phosphate technique (26). Cotransfections were performed with equimolar amounts of plasmid DNAs. Cells were cultured for 48 h in growth medium followed by incubation under test conditions for the indicated times. Control cells were incubated in DMEM/F12 supplemented with FBS dialyzed against phosphate-buffered saline (26). Cells were starved for amino acids by incubating in Krebs-Ringer buffer supplemented with dialyzed FBS (26). No difference in the regulation of the *cat-1* gene by amino acid starvation was observed when Krebs-Ringer buffer containing all amino acids was used in place of DMEM/F12 medium (26). Cells were also incubated with 2.5 μg/ml tunicamycin, 400 nM thapsigargin, or 100 μg/ml poly(I)·poly(C) (poly(IC)) for the appropriate times. To address the role of PKR kinase, wild-type mouse embryo fibroblasts (PKR$^{+/+}$) or fibroblasts with the kinase inactivated by homologous recombination (PKR$^{-/-}$) were used (27).

Enzyme Assays—Cell extracts were prepared and analyzed for LUC (Tropix Luciferase Assay Kit) and CAT activities as described previously (28). The activities were normalized to the protein content of the cell extracts, which was measured using the Bio-Rad D_C assay. Cells transfected with dual luciferase plasmids were lysed, and RLUC and FLUC were measured using the Promega Dual Luciferase Analysis Kit.

Western Blot Analysis—The expression of eIF2α and eIF4E was analyzed by Western blotting. The expression of phospho-eIF2α and phospho-eIF4E was analyzed using antibodies specific for the phosphorylated forms of these proteins, all as described previously (13).

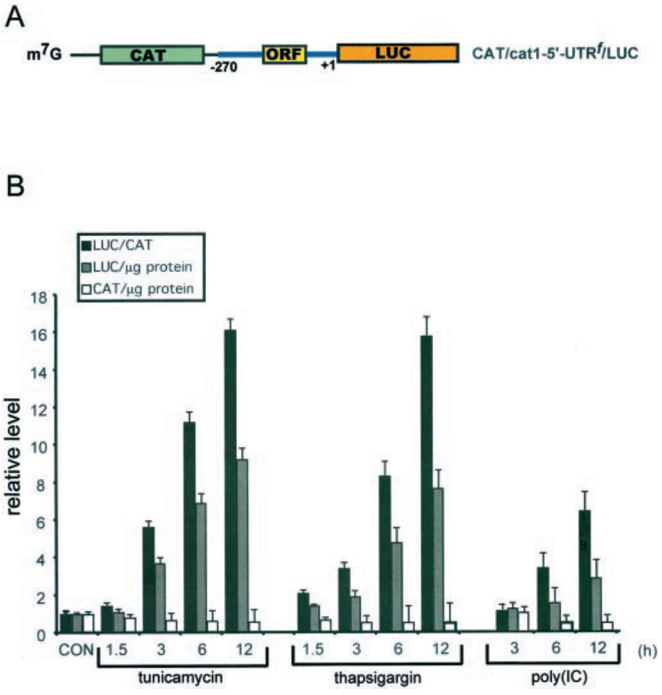

FIG. 1. **ER stress and dsRNA stimulate translation mediated by the cat-1 IRES.** *A*, diagram of the bicistronic mRNA transcribed from the CAT/cat-1-5′-UTR^f/LUC plasmid. This mRNA has ORFs for CAT and LUC with the entire 5′-UTR of the cat-1 mRNA in the intercistronic spacer. The 48-amino acid ORF in the cat-1 5′-UTR is shown. *B*, C6 cells transfected with CAT/cat-1-5′-UTR^f/LUC DNA treated with tunicamycin (2.5 μg/ml), thapsigargin (400 nM), or poly(IC) (100 μg/ml) for the indicated times. Cell extracts were prepared, and LUC and CAT activities were measured and normalized to protein content. Data are expressed relative to the values for untreated cells (*CON*). The average ± S.E. of three independent experiments is shown.

RESULTS

Translation from the cat-1 IRES Is Stimulated by ER Stress and by dsRNA—Our previous studies have shown that translation from the cat-1 IRES is stimulated by amino acid starvation via a mechanism that requires phosphorylation of eIF2α by GCN2 kinase (14). It is known that several other cellular stresses induce phosphorylation of eIF2α, including ER stress and the presence of dsRNA (18), which occurs during viral infection. Consequently, we investigated whether these stresses also stimulate translation from the cat-1 IRES.

Studying IRESs in the 5′-UTR of cellular mRNAs is difficult. It is believed that these mRNAs are translated by both cap-dependent and independent mechanisms under normal conditions. IRES-mediated translation may be activated under stress conditions when cap-dependent translation decreases (1). However, it is difficult to know how translation is initiated *in vivo* because cap-dependent and independent initiation cannot be readily distinguished. To study IRES-mediated translation exclusively, we used the bicistronic expression vector, CAT/cat1-5′-UTR^f/LUC, employed in our previous studies (14). The mRNA synthesized from this vector contains open reading frames for the CAT and LUC enzymes (Fig. 1*A*). The first cistron encodes CAT, which is translated by a cap-dependent mechanism. The second cistron encodes LUC, which is translated only if initiation occurs in the intercistronic spacer, which contains the entire 270-bp 5′-UTR of the cat-1 mRNA.

To test the effect of ER stress on translation from the cat-1 IRES, C6 glioma cells transiently transfected with CAT/cat1-

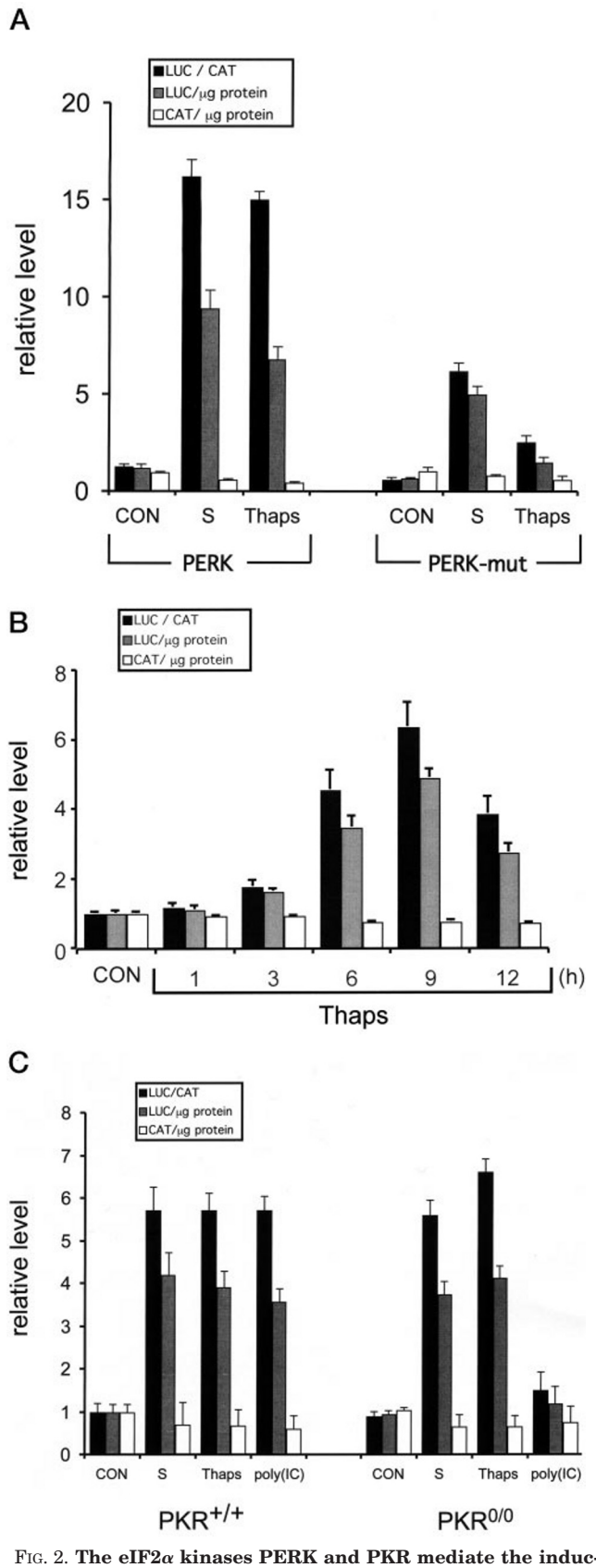

FIG. 2. **The eIF2α kinases PERK and PKR mediate the induction of translation from the cat-1 IRES by ER stress and dsRNA.** *A*, C6 cells cotransfected with CAT/cat-1 5′UTR^f/LUC and expression vectors for wild-type (*PERK*) or dominant-negative mutant (*PERK-mut*) PERK. The cells were cultured in DMEM/F12 (*CON*), in amino acid-free medium (*S*), or in the presence of 400 nM thapsigargin (*Thaps*) for 9 h. Cell extracts were prepared, and LUC and CAT activities were measured. Results were analyzed as described in the legend for Fig. 1 *B*, C6 cells stably expressing a dominant-negative GCN2 mutant (12) transiently transfected with pSVCAT/cat-1-5′-UTR^f/LUC. The cells were cultured without (*CON*) or with 400 nM thapsigargin (*Thaps*) for the times indicated, and LUC and CAT activities were measured. *C*, PKR$^{+/+}$ and PKR$^{-/-}$ mouse embryo fibroblasts transfected with pSV-CAT/cat-1-5′-UTR^f/LUC and incubated for 9 h in control conditions (*CON*) or amino acid-free medium (*S*) or treated with thapsigargin or poly(IC) as described in the legend for Fig. 1. LUC and CAT activities were then determined. Data were normalized to the values in control PKR$^{+/+}$ cells. The *bars* represent the average ± S.E. of three independent experiments.

5′-UTR^f/LUC were treated with tunicamycin or thapsigargin (Fig. 1*B*). Tunicamycin interferes with protein folding in the ER by blocking the glycosylation of Asn residues of newly made proteins (25). Thapsigargin induces ER stress by depleting ER Ca^{2+} stores (29). Tunicamycin and thapsigargin decreased CAT activity after 1.5 h, consistent with the inhibition of cap-dependent translation by these agents. In contrast, both treatments caused slow increases in LUC activity. Increases were only seen after 3 h of treatment, and activity then increased throughout the 12-h course of the experiment. Changes in both CAT and LUC activities are reflected in the LUC/CAT ratio, which increased by 3 h of treatment and was 16 times the control level by 12 h (Fig. 1*B*). These results demonstrate that translation from the cat-1 IRES is increased by treatments that induce ER stress. Moreover, the long lag and slow increase in translation are similar to the kinetics of increased translation during amino acid starvation (12).

To test the effects of dsRNA, C6 cells transiently transfected with the CAT/cat1-5′-UTR^f/LUC vector were treated with poly(IC). This treatment had effects similar to tunicamycin and thapsigargin (Fig. 1*B*). LUC activity increased, but only after a lag. There was also a decrease in CAT activity. These changes are reflected in an increase in the LUC/CAT ratio. An increase was first seen after 6 h of treatment, and the highest activity was observed after 12 h, although dsRNA caused a smaller increase (7-fold) than the other treatments. These results indicate that dsRNA increases translation mediated by the cat-1 IRES with a long lag period and a persistent increase in activity.

Distinct eIF2α Kinases Mediate the Regulation of the cat-1 IRES by Cellular Stress—We have shown previously that amino acid starvation increases translation from the cat-1 IRES via a mechanism that involves phosphorylation of the translation initiation factor, eIF2α, by GCN2 kinase. GCN2, which is active when uncharged tRNAs are present, is one of at least four kinases known to phosphorylate eIF2α, regulating the activity of this factor in response to distinct upstream signals (27). eIF2α is also phosphorylated by PERK kinase, which is stimulated during ER stress, and by PKR kinase, which is activated by dsRNA (18). To determine whether the effects of ER stress and dsRNA on translation from the cat-1 IRES are mediated by PERK and PKR, the effects of overexpressing dominant-negative kinase mutants were studied (25). To examine the involvement of PERK, the effects of amino acid starvation and thapsigargin were studied in C6 cells cotransfected with CAT/cat1-5′-UTR^f/LUC and an expression plasmid encoding either wild-type or dominant-negative PERK (Fig. 2*A*). The stimulation of cat-1 IRES-mediated translation by amino acid starvation or thapsigargin was not affected by overexpression of wild-type PERK (Fig. 2*A*). The 15-fold increase in LUC/CAT ratio is similar to that observed in cells with no PERK overexpression (Fig. 1*B* and Ref. 13). Expression of the dominant-negative PERK mutant caused a decrease in LUC expression mediated by the cat-1 IRES, suggesting that basal PERK activity regulates IRES activity (Fig. 2*A*, compare control values). Amino acid starvation of these cells increased translation mediated by the IRES. In fact, the LUC/CAT ratio

increased by 15-fold as compared with untreated cells expressing mutant PERK, the same increase seen in cells overexpressing wild-type PERK (Fig. 2*A*). In contrast, thapsigargin treatment only caused a 4-fold increase in the LUC/CAT ratio in cells expressing mutant PERK. This experiment demonstrates that PERK is required for the control of IRES activity by ER stress but not by amino acid starvation.

To further support this finding, the effect of a dominant-negative mutant of GCN2 on the response of the cat-1 IRES to ER stress was studied. We have shown previously that overexpression of this mutant in C6 cells blocks the increase in cat-1 IRES activity by amino acid starvation (14). In contrast, thapsigargin caused a large increase in LUC expression in C6 cells overexpressing mutant GCN2 (Fig. 2*B*). These data support the idea that ER stress stimulates cat-1 IRES-mediated translation via PERK, whereas amino acid starvation stimulates translation via GCN2.

A similar experiment was performed to examine the importance of PKR kinase. These experiments took advantage of a well characterized mouse embryo fibroblast cell line in which PKR has been inactivated by homologous recombination (27). In wild-type cells ($PKR^{+/+}$), all three cellular stresses caused increased translation from the cat-1 IRES (Fig. 2*C*). The only difference between the results from these cells and C6 cells was the level of stimulation by dsRNA. In $PKR^{+/+}$ cells, all three stresses increased the LUC/CAT ratio by the same extent, whereas the increase caused by dsRNA in C6 cells was only half that caused by the other two stresses. In $PKR^{-/-}$ cells, translation from the cat-1 IRES was induced by both amino acid starvation and thapsigargin, consistent with the idea that PKR kinase does not mediate the effects of these cellular stresses (Fig. 2*C*). In contrast, stimulation of IRES-mediated translation by dsRNA was abolished in these mutant cells. Taken together, these results support our hypothesis that translation mediated by the cat-1 IRES is regulated by eIF2α phosphorylation. Moreover, they support the idea that this regulation involves several independent signaling pathways: GCN2 kinase mediates the effects of amino acid starvation, PERK mediates the effects of ER stress, and PKR mediates the effects of dsRNA.

Cellular Stress Causes Transient Changes in the Phosphorylation of Translation Initiation Factors—Our results suggest that phosphorylation of eIF2α by specific kinases is important in the regulation of the cat-1 IRES in response to cellular stress. To support this conclusion, we examined the effect of these cellular stresses on the phosphorylation of eIF2α. This was accomplished by Western blot analysis using antibodies specific for either total eIF2α or the phosphorylated form of this protein. Both amino acid starvation and thapsigargin treatment caused a rapid transient increase in phosphorylated eIF2α levels (Fig. 3, *A* and *B*). The amount was increased within 30–60 min of treatment, was maximal at 1 h, and returned to base-line levels by 2–6 h. The amount of total eIF2α protein did not significantly change during these treatments, indicating that there was a transient increase in the extent of eIF2α phosphorylation. poly(IC) treatment also caused induction of eIF2α phosphorylation (Fig. 3*C*). However, in this case, there was a sustained induction, which began after 2 h of treatment and was still evident after 24 h. Significantly, for both ER stress and dsRNA, the increases in eIF2α phosphorylation and translation mediated by the cat-1 IRES follow different time courses (Fig. 1). For ER stress, the increase in IRES-mediated translation did not occur until eIF2α phosphorylation had increased and then returned to base-line levels. These kinetics are similar to those observed previously for amino acid starvation (12). For dsRNA, IRES-mediated translation also increased several hours after the increase in phosphorylation of eIF2α.

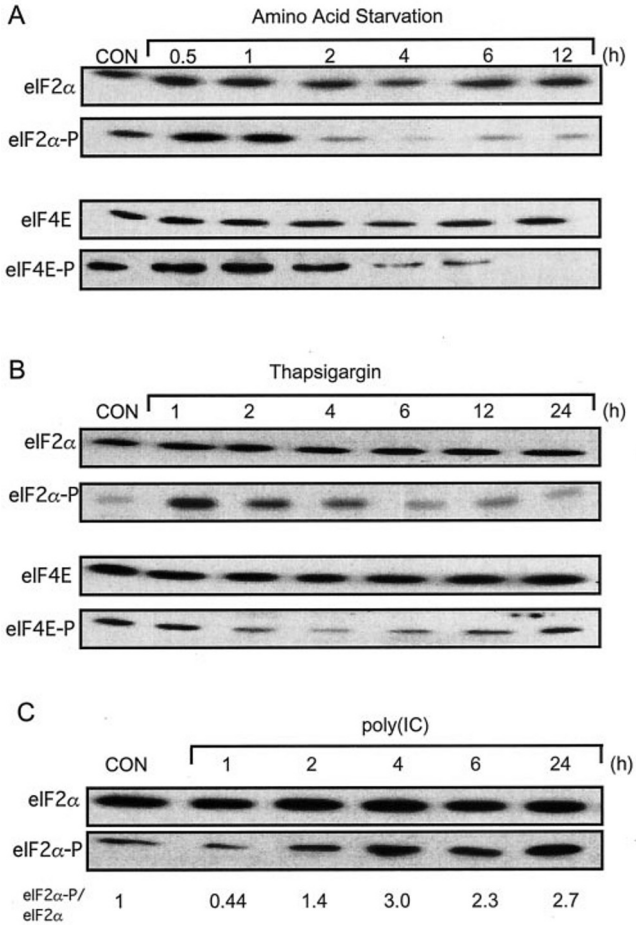

FIG. 3. **Amino acid starvation, ER stress, and dsRNA induce transient changes in the phosphorylation of translation initiation factors eIF2α and eIF4E.** *A* and *B*, Western blot analysis of cell lysates (15 μg) from C6 cells incubated in either amino acid-free medium (*A*) or thapsigargin (*B*) for the times indicated. Blots were probed with antibodies for total eIF2α, phospho-eIF2α, total eIF4E, and phospho-eIF4E. *CON*, cells incubated in DMEM/F12. *C*, Western blot analysis of cell lysates from $PKR^{+/+}$ cells incubated with poly(IC) for the times indicated using antibodies for eIF2α and phospho-eIF2α. Bands were visualized by chemiluminescence and quantified by densitometry. The ratio of phospho-eIF2α/total eIF2α is shown with the ratio normalized to 1 in untreated (*CON*) cells.

It has been suggested that IRES-mediated translation may increase when the translation initiation factor eIF4F, which is important in cap-dependent translation initiation, is inactivated (30). The activity of the cap-binding protein eIF4E, an important constituent of eIF4F, is regulated by phosphorylation (30). Consequently, we measured the effects of amino acid starvation and ER stress on the phosphorylation of eIF4E. eIF4E activity is known to be independently regulated by phosphorylation of both the protein itself and the sequestering protein, 4EBP-1 (3). Each of these modifications results in inactivation of eIF4F. Amino acid starvation caused a decrease in the level of phosphorylated eIF4E in agreement with previous findings (13). Thapsigargin also caused a transient decrease in the level of phosphorylated protein. Decreased levels were first detected at 2 h of treatment, remained low for 4–6 h, and had returned to base-line levels by 24 h. The decrease of eIF4E phosphorylation by these treatments and the induction of translation mediated by the cat-1 IRES occurred with different kinetics. The increase in IRES activity occurred after the decrease in eIF4E phosphorylation.

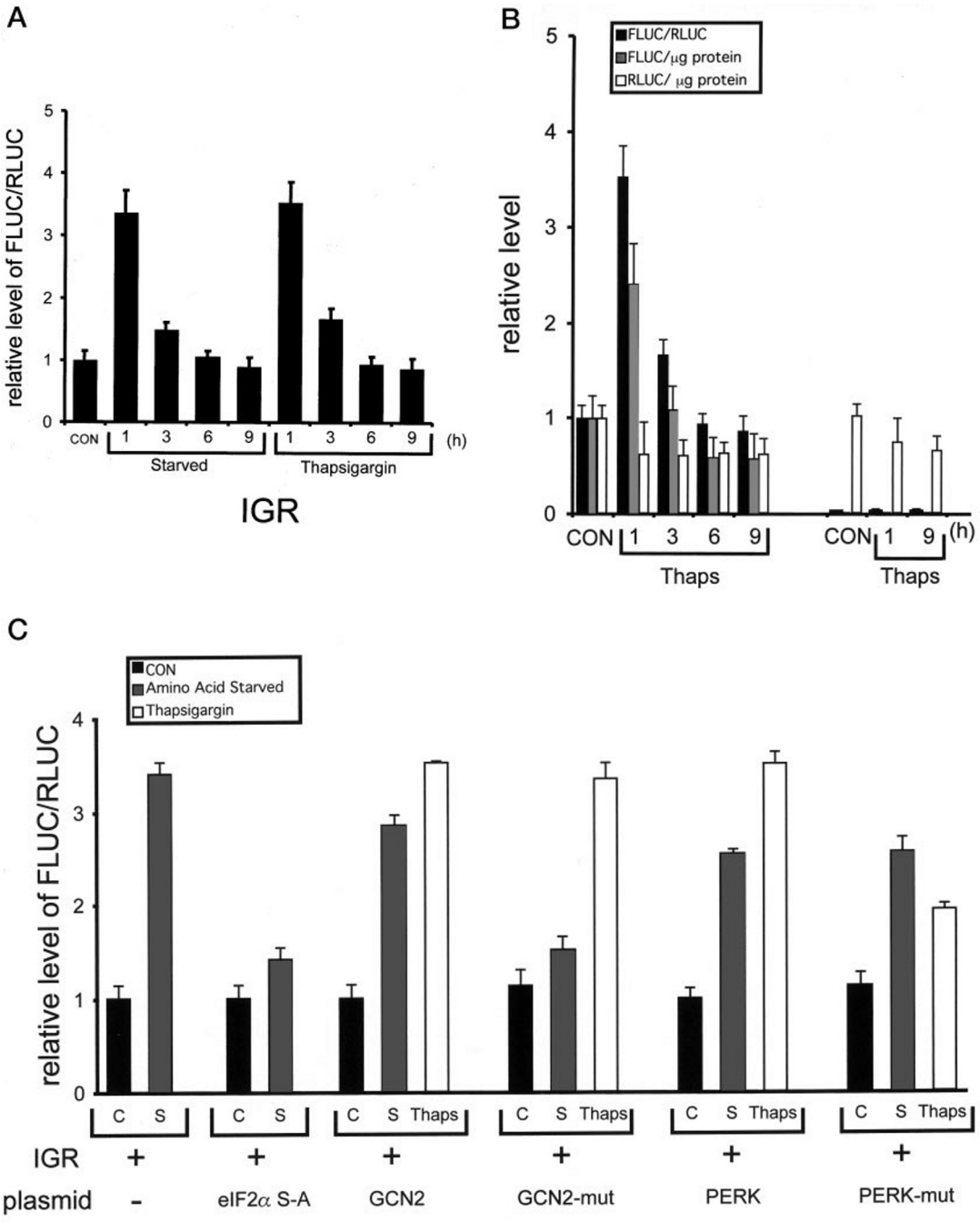

FIG. 4. **Amino acid starvation and ER stress induce IGR IRES-mediated translation in parallel with eIF2α phosphorylation.** *A*, C6 cells transfected with a bicistronic mRNA expression vector containing the IGR IRES in the intercistronic spacer. The cells were cultured in DMEM/F12 (*CON*), in amino acid-free medium (*Starved*), or with thapsigargin for the times indicated. FLUC and RLUC activities in the cell extracts were measured and expressed as described in the legend for Fig. 1. *B*, C6 cells transfected with either T7ΔEMCV/Fluc-IGR/CrPVORF2 (*IGR*) or an inactive mutant (*IGRmut*) and cultured with or without thapsigargin (*Thaps*) for the indicated times. FLUC and RLUC activities were then measured. *C*, C6 cells transfected with T7ΔEMCV/Fluc-IGR/CrPVORF2 and one of the following expression vectors: eIF2α S-A, S51A mutant of eIF2α; GCN2, wild-type GCN2; GCN2-mut, dominant-negative GCN2 mutant; PERK, wild-type PERK; PERK-mut, dominant-negative PERK mutant. The cells were cultured in DMEM/F12 (*CON*), amino acid-depleted (*S*), or thapsigargin-containing (*Thaps*) media for 1 h. Cell extracts were prepared, and RLUC and FLUC activities were measured and normalized against the control (*IGR*). In all cases, the *bars* represent the average ± S.E. of three independent experiments.

Translation from the Cricket Paralysis Virus IGR IRES Is Also Stimulated by Cellular Stress—We have shown that translation from the cat-1 IRES is stimulated by several cellular stresses. Moreover, the stimulation is mediated by the phosphorylation of eIF2α via at least three distinct eIF2α kinases. Is this regulation specific to the cat-1 IRES, or is it a property of all IRESs? To address this question, the regulation of several other IRESs by cellular stresses was examined.

We wished to study the regulation of other IRESs-mediated translation by eIF2α phosphorylation and cellular stress. However, there are no other cellular IRESs known to be regulated in this fashion. Consequently, we studied the IGR IRES from cricket paralysis virus, which has been shown to be regulated by eIF2α phosphorylation (24). This is a very interesting IRES because it mediates translation initiation without the initiator Met-tRNA (24). The IRES initiates translation with a CCU triplet at the P site of the ribosome and the alanine-encoding GCU triplet at the A site. It has been suggested that the

activity of this IRES is stimulated when the availability of 40 S ribosomal subunits, depleted of ternary complexes, increases (24). Phosphorylation of eIF2α can cause such a scenario. We therefore hypothesized that IGR IRES-mediated translation should increase during cellular stress when eIF2α is phosphorylated.

To test the effect of cellular stresses on this IRES, a bicistronic mRNA expression vector containing the IGR IRES within the intercistronic region was used. The first cistron of the mRNA from this vector encodes RLUC, which is translated via a cap-dependent mechanism. The second cistron encodes FLUC, which is translated from the IGR IRES. C6 cells transfected with this vector were subjected to either amino acid starvation or ER stress, and enzyme activities were assayed in cell extracts. Translation from the IGR IRES was stimulated transiently by both stresses (Fig. 4*A*), and the FLUC/RLUC reached a maximum at 1 h and then declined rapidly, reaching control levels by 6–9 h. This time course parallels the transient phosphorylation of eIF2α, which reaches a peak at 1 h and then declines. However, it is quite different from the kinetics of stimulated translation from the cat-1 IRES, which did not begin to increase until 3 h of stress, long after eIF2α phosphorylation had returned to base-line levels. dsRNA also caused an increase in IGR IRES-mediated translation with kinetics that matched the increased phosphorylation of eIF2α caused by this treatment (not shown).

To demonstrate that the regulated translation from the IGR sequence is mediated by the IGR IRES, we tested the effects of cellular stress on a vector containing a mutation that has been shown previously to inactivate this IRES (23). The expression of RLUC from this RNA was similar to that seen for the wild-type construct, indicating that the mRNAs were expressed at similar levels and that cap-dependent translation was not affected. In contrast, IGR IRES-dependent expression of FLUC was barely detectable in the mutant, consistent with previous results (24). Moreover, ER stress caused by thapsigargin did not cause a measurable increase in IRES-mediated translation. Amino acid deprivation also did not stimulate translation mediated by the mutant IGR IRES (not shown).

We have shown that the stress-induced stimulation of translation mediated by the cat-1 IRES requires phosphorylation of eIF2α and that different stresses stimulate different kinases. Two experiments were carried out to determine whether this is also true for the stimulated translation from the IGR IRES. To prove the importance of eIF2α phosphorylation, we studied the effects of expressing a mutant eIF2α in which Ser^{51}, which is the substrate for eIF2α kinases, is mutated to Ala. This mutant, eIF2α S-A, functions as a dominant negative because it cannot be phosphorylated (31). Overexpression of eIF2α S-A prevented most of the stimulation of translation from the IGR IRES during amino acid starvation (Fig. 4*C*). These results demonstrate the importance of eIF2α phosphorylation in the regulation of translation mediated by the IGR IRES during cellular stress.

Three eIF2α kinases are involved in the regulation of translation mediated by the cat-1 IRES. To determine whether this was also true for the stimulation of translation from the IGR IRES, we tested the effects of expressing kinases with dominant-negative mutations. Overexpression of mutant GCN2 prevented increased IRES activity during amino acid starvation but did not interfere with the thapsigargin-induced stimulation (Fig. 4*C*). Conversely, overexpression of mutant PERK decreased the stimulation of IRES activity by thapsigargin but did not interfere with the stimulation of IRES activity by amino acid starvation. These results support the idea that enhanced translation of the IGR IRES is mediated by eIF2α phosphorylation. Moreover, activities of both the IGR and cat-1 IRESs are regulated by several kinases that phosphorylate eIF2α in response to distinct cellular stresses.

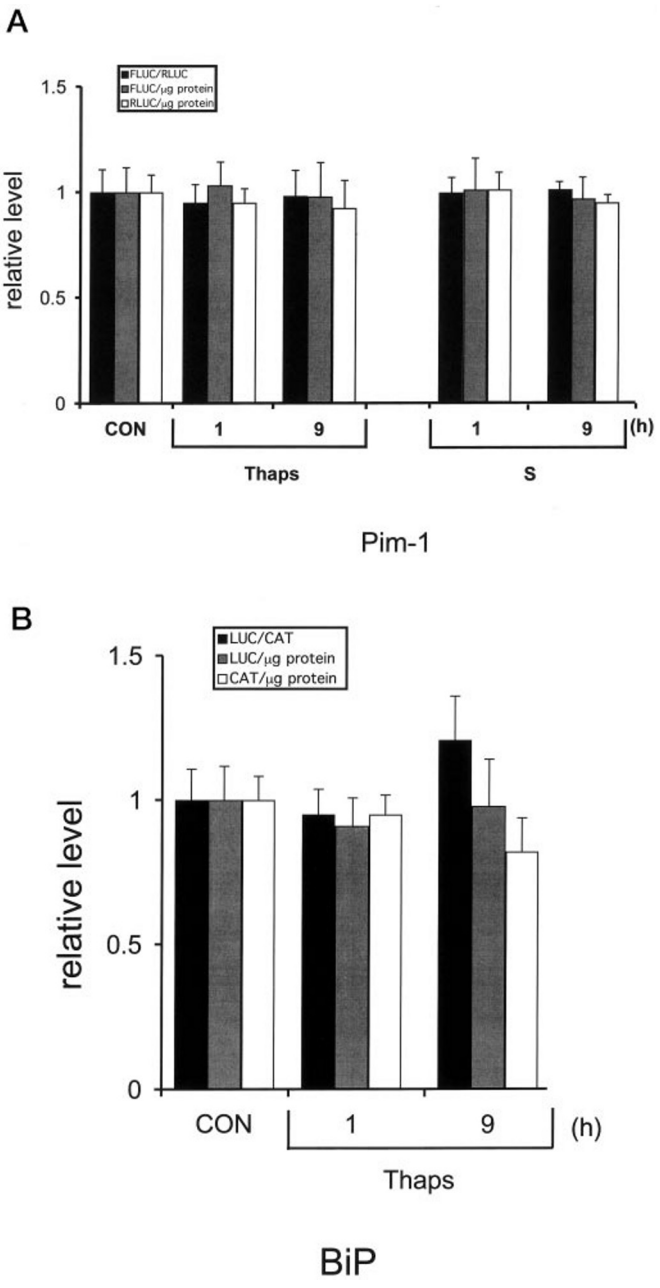

FIG. 5. **Translation mediated by the Pim-1 and BiP IRESs is not induced by amino acid starvation or ER stress.** C6 cells were transfected with bicistronic mRNA expression vectors containing either (*A*) the Pim-1 IRES or (*B*) the BiP IRES in the intercistronic regions. Cells were cultured in DMEM/F12 alone (*CON*), thapsigargin-containing media (*Thaps*), or amino acid-depleted (*S*) media for the times indicated. Cell extracts were prepared, and either FLUC and RLUC (*A*) or LUC and CAT (*B*) activities were measured. Results were analyzed as described in the legend for Fig 1. The *bars* represent the average ± S.E. of three independent experiments.

Translation Mediated by the Pim-1 and BiP IRESs Is Not Stimulated by Cellular Stress—To determine whether the stimulation of translation from IRESs is a general phenomenon, we tested the regulation of IRESs from two other cellular mRNAs, BiP and Pim-1. BiP is an ER chaperone whose levels are increased by ER stress (19). Pim-1 is a Ser/Thr protein kinase whose mRNA contains an IRES; this mRNA is translated when eIF4F activity is reduced (6). To test whether trans-

lation from these IRESs is regulated by the stress conditions that induce the cat-1 and IGR IRES, bicistronic vectors with the 5′-UTR of either BiP or Pim-1 in the intercistronic region were studied. C6 cells transfected with these vectors were treated with either amino acid-free medium or thapsigargin. Cell lysates were then assayed for CAT and LUC activities (BiP vector) or RLUC and FLUC (Pim-1 vector) to determine IRES activity. It was shown previously that amino acid starvation did not increase BiP IRES-mediated translation (12). As seen in Fig. 5, neither amino acid starvation nor treatment with thapsigargin for up to 9 h affected the activity of either IRES. Treatment with poly(IC) also had no effect (not shown). These results demonstrate that cellular stress has at least three different effects on IRESs. Some IRESs, such as BiP and Pim-1, are not regulated by these stresses. Some IRESs, such as the IGR, show immediate stimulation with a time course similar to the phosphorylation of eIF2α. The cat-1 IRES shows a third type of regulation since increased translation occurs with slow kinetics and persists after the phosphorylation of eIF2α returns to base-line levels.

DISCUSSION

In this report, we show that several cellular stresses stimulate translation mediated by the cat-1 IRES. These include ER stress, dsRNA, which mimics viral infection, and amino acid starvation (32). Moreover, the stress-induced increase in phosphorylation of the translation initiation factor, eIF2α is required for the stimulation of IRES activity. We show that at least three different eIF2α kinases are involved in this regulation. Our previous work demonstrated that the effects of amino acid starvation are mediated by GCN2 kinase (14). In this work, we show that PERK kinase mediates the effects of ER stress and that PKR mediates the effects of dsRNA.

An important finding of this study is that cellular mRNAs that contain IRESs within their 5′-UTRs have diverse regulatory patterns. Translation from the cat-1 IRES is stimulated by amino acid starvation, ER stress, and dsRNA. In contrast, we found that translation mediated by the IRESs from the BiP and Pim-1 mRNAs is not affected by these stresses. Other types of regulation have been reported for other IRES-containing cellular mRNAs. Apoptotic stress induces IRES-mediated translation of the IAP proteins (33), which are potent inhibitors of apoptosis (10, 11). However, we found that apoptosis was not induced by amino acid starvation or ER stress (data not shown). In addition, cell cycle-dependent regulation has been observed for some IRES-containing mRNAs, including c-Myc (34), ornithine decarboxylase (8), and the protein kinase PITSLRE (35). Tissue-specific regulation has also been shown for the IRESs in the c-Myc and fibroblast growth factor 2 (FGF2) mRNAs. These IRESs have higher activity in embryonic than in adult tissues (9). Recently, Johannes *et al.* (6) showed that 200 out of 7,000 cellular mRNAs examined remained associated with polysomes in poliovirus-infected cells. Among the gene products of these mRNAs were transcription factors, kinases, phosphatases, and protooncogenes. These are candidates for IRES-containing mRNAs because they are translated under conditions of reduced eIF4F activity. It will be interesting to see how many types of IRESs are contained in these mRNAs and how the initiation of translation from these IRESs is regulated.

It is believed that IRES-containing cellular mRNAs are inefficiently translated from the 5′-cap under normal conditions due to the secondary structure of their IRESs (1). It is therefore assumed that translation of these mRNAs under normal conditions is partly cap-dependent and partly IRES-mediated. The mode of translation changes under stress conditions when cap-dependent initiation decreases and IRES-mediated initiation prevails. Significantly, we found that the activity of the BiP and Pim-1 IRESs did not increase during the stress conditions used in this report. It has been shown that the BiP and Pim-1 mRNAs are translated under conditions of increased eIF2α phosphorylation and decreased cap-dependent translation, suggesting that their IRESs function under these conditions (5, 6). However, our data suggest that the BiP and Pim-1 IRESs are not stimulated by amino acid limitation (15) or ER stress (13). Consequently, translation of these mRNAs during cellular stress may represent a switch from cap-dependent to cap-independent translation. In contrast, the cat-1 IRES shows a strong increase in IRES-mediated translation.

The studies in this report and our previous work (12–14) suggest that the regulation of IRES activity by amino acid starvation, ER stress, and dsRNA is complex. Despite the fact that eIF2α phosphorylation is required for increased cat-1 IRES activity, phosphorylation and IRES activity change with different kinetics. Phosphorylation increases and returns to the control level before large increases in translation from the cat-1 IRES are seen. The changes in eIF4E phosphorylation also do not correlate with the increased activity of the cat-1 IRES. It is concluded that induction of the cat-1 IRES activity depends on eIF2α phosphorylation but that maximum activation can occur at a time when eIF2α is dephosphorylated. One indirect mechanism that explains these results is that stress-induced phosphorylation of eIF2α causes the synthesis or accumulation of a protein that stimulates cat-1 IRES activity (14). This protein would reach effective levels after eIF2α phosphorylation levels have decreased.

Our data with dsRNA also support the idea that increased translation mediated by the cat-1 IRES can occur when the levels of phosphorylated eIF2α are high. Treatment of cells with dsRNA led to the sustained phosphorylation of eIF2α by PKR kinase. Because phosphorylation inhibits the activity of eIF2α, it is likely that the cat-1 IRES can function efficiently when the level of the eIF2·GTP·Met-tRNA^{Met} ternary complexes is low. In contrast, cap-dependent translation is inhibited when the level of ternary complexes decreases. This suggests that initiation from the 5′-cap and from the cat-1 IRES uses different requirements for certain translation initiation factors. This has been observed for the IRES from hepatitis C virus, which can function by binding eIF3, 40 S, and the ternary complex (36) in contrast to cap-dependent translation, where the ternary complex binds the 40 S ribosomal subunit and eIF3 before recruitment on the mRNA (36).

Because there are no other examples of IRES-containing cellular mRNAs that are regulated by eIF2α phosphorylation, we compared the regulation of the cat-1 IRES and the cricket paralysis virus IGR IRES under stress conditions. It is shown here that translation from the IGR IRES increases transiently during stress with kinetics that followed the phosphorylation of eIF2α, which is in agreement with previous findings (23). The IGR IRES can form an RNA structure that can recruit 40 S and 60 S ribosomal subunits directly and initiate translation at the Ala site of the ribosome in the absence of the eIF2·GTP·Met-tRNA^{Met} ternary complex (24). It has been suggested that the global decrease of protein synthesis caused by eIF2α phosphorylation should increase IGR IRES-mediated translation due to increased availability of 40 S ribosomal subunits (6, 37). Our results support this idea by showing that cellular stress causes a transient increase in translation mediated by the IGR IRES with kinetics that follow the transient increase in eIF2α phosphorylation. In contrast, cellular stress stimulated translation mediated by the cat-1 IRES with kinetics that did not follow eIF2α phosphorylation, making it likely that regulation of the two IRESs occurs by different mechanisms.

We conclude that IRES-mediated translation is important for regulation of gene expression and becomes crucial in the adaptive response of cells to nutritional and other stress conditions. It is shown here that the catabolic response of cells to stress by a global decrease of protein synthesis is a prerequisite for an anabolic response of increased IRES-mediated translation initiation of protein synthesis.

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