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Translational Control of Protein Kinase Cη by Two Upstream Open Reading Frames▽

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Protein kinase C (PKC) represents a family of serine/threonine kinases that play a central role in the regulation of cell growth, differentiation, and transformation. Posttranslational control of the PKC isoforms and their activation have been extensively studied; however, not much is known about their translational regulation. Here we report that the expression of one of the PKC isoforms, PKCη, is regulated at the translational level both under normal growth conditions and during stress imposed by amino acid starvation, the latter causing a marked increase in its protein levels. The 5′ untranslated region (5′ UTR) of PKCη is unusually long and GC rich, characteristic of many oncogenes and growth regulatory genes. We have identified two conserved upstream open reading frames (uORFs) in its 5′ UTR and show their effect in suppressing the expression of PKCη in MCF-7 growing cells. While the two uORFs function as repressive elements that maintain low basal levels of PKCη in growing cells, they are required for its enhanced expression upon amino acid starvation. We show that the translational regulation during stress involves leaky scanning and is dependent on eIF-2α phosphorylation by *GCN2*. Our work further suggests that translational regulation could provide an additional level for controlling the expression of PKC family members, being more common than currently recognized.

Studies over the last several years have focused on the activation of protein kinase C (PKC) members and their regulation by phosphorylation, membrane recruitment, and down-regulation by proteolysis; however, not much is known about the regulatory mechanisms leading to PKC synthesis. Most cellular mRNAs have short 5′ untranslated regions (UTRs), sufficient for efficient initiation of translation by the cap-dependent ribosome scanning model. The 5′ UTRs of many mRNAs encoding oncogenes and cell cycle regulators are extraordinarily long and highly structured (32, 68). Cumbersome 5′ UTRs are often associated with complex translational control mechanisms mediated by upstream open reading frames (uORFs) or internal ribosome entry sites (IRESs). uORFs were shown to usually function as translation inhibitors of downstream ORFs (48). They are found in about 10% of eukaryotic mRNAs, but are common in the majority of oncogenes, with a rough estimation of about two-thirds (29). Among the PKC isoforms, the presence of a regulatory uORF was shown only for PKCε. This uORF suppressed the expression of PKCε in an in vitro system of rabbit reticulocyte lysates, but these effects could not be fully recapitulated in vivo in growing fibroblasts (50).

It is generally accepted that mRNAs containing repressive uORFs may have an advantage during translation under stress conditions (25, 27). Exposure of cells to various types of stresses, such as UV irradiation, nutrient limitations, oxidative stress, hypoxia, and exposure to various drugs or toxins, induces specific cascades, which are mediated by four distinct protein kinases (15, 31), each of which directly phosphorylates Ser51 of eIF-2α. Phosphorylation on eIF-2α inhibits eIF-2B activity and thus the exchange of GDP to GTP on eIF2, leading to decreased levels of the ternary complex (Met-tRNA^{Met}–eIF-2–GTP) and consequent inhibition of global protein synthesis (27). Under conditions whereby eIF-2α is phosphorylated and translation of most mRNAs is suppressed, the translation of a subclass of mRNAs is enhanced due to specific features including uORFs within the 5′ UTR. The first and best-studied example is the yeast transcriptional activator *GCN4* mRNA, which contains four uORFs in its 5′ UTR, playing a role in translational control under conditions of amino acid starvation (26, 51). In mammalian cells, two regulatory uORFs were found in mRNAs encoding proteins involved in the stress response, such as the transcription factors ATF4 and ATF5 and the eIF-2 phosphatase GADD34 (44, 65, 67).

PKC represents a family of phospholipid-dependent serine/threonine kinases that are key mediators in signal transduction pathways, involved in a wide variety of cellular processes, including cell proliferation, differentiation, and apoptosis (14, 64). Based on their primary structure, cofactors, and enzymatic properties, PKC members are divided into three subgroups: the conventional PKCs (α, βI, βII, and γ), the novel PKCs (δ, ε, η, and θ), and the atypical PKCs (ζ and λ) (47, 54). Understanding the cellular functions of PKCs is hampered by the fact that they represent a family of 10 members, which differ in their primary structures, biochemical properties, tissue distributions, and subcellular localizations (13, 52). Thus, elucidat-

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ing the biological roles of individual PKC enzymes and the molecular mechanisms controlling their expression is crucial for understanding their specific contribution to tumor development, progression, or apoptosis.

The novel PKCη isoform has a unique tissue distribution and is primarily expressed in epithelial tissues and cells undergoing high turnover (3). It is implicated in diverse cellular functions, including a role in terminal differentiation (16, 23, 43, 55, 56), proliferation (20, 28) and secretion (8, 19, 24). In some of these studies, the mechanism of action involved modulation of cell cycle components (20, 21, 30, 42, 43, 61). Recent studies suggest that PKCη has a special role in the response to stress and regulation of apoptosis (2, 46). It provides protection against apoptosis induced by the chemotherapeutic drugs camptothecin and doxorubicin in Hodgkin's lymphoma lines and breast adenocarcinoma MCF-7 cells (1, 59). In addition, PKCη expression was found to correlate with drug resistance and drug resistance-associated genes in patients with breast cancer (4), ovarian cancer (5), and acute myeloid leukemia blasts (6).

Here we show that the expression of PKCη is regulated at the translational level both under normal growth conditions and during stress imposed by amino acid starvation. The human 5′ UTR of PKCη is unusually long (659 nucleotides [nt]), is GC rich, and contains two conserved small uORFs. Using a reporter gene system, we demonstrate that each of these two uORFs suppresses expression of PKCη in growing cells, thus maintaining its low basal expression levels. However, this suppression is relieved during amino acid starvation by leaky scanning causing its translational upregulation. Using wild-type and knockout *GCN2* mouse embryonic fibroblasts (MEFs), we demonstrate that the *GCN2* kinase is required for the stress-induced upregulation. This is the first report demonstrating that the uORFs of one of the PKC family members has a regulatory role under stress.

MATERIALS AND METHODS

Cell culture and stress conditions. MCF-7 human breast adenocarcinoma and MEFs, established from wild-type (WT) and knockout *GCN2* animals, previously described (25) and kindly provided by D. Ron (Kimmel Center for Biology and Medicine of the Skirball Institute, New York University School of Medicine, New York, NY), were maintained in Dulbecco's modified Eagle's medium containing high glucose (D-glucose; Biological Industries, Beit Haemek, Israel), supplemented with 10% fetal bovine serum (Biological Industries), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and L-glutamine (2 mM) in a 5% CO_2 humidified atmosphere at 37°C. MCF-7 and MEF cells were subjected to amino acid starvation by the addition of Earl's balanced salt solution (Biological Industries) for the indicated time points with or without the addition of 20 μg/ml cycloheximide (Sigma).

Cell lysis and immunoblot analyses. Cell lysates were prepared using radioimmunoprecipitation assay lysis buffer containing 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EGTA, 10 mM EDTA, 0.1% sodium dodecyl sulfate, 1% NP-40, 45 mM β-mercaptoethanol, and 50 mM NaF. The protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and the phosphatase inhibitors (100 μM sodium orthovanadate, 50 mM β-glycerolphosphate, and 5 mM sodium pyrophosphate) were added just before the lysis. Lysates were placed on ice for 30 min and sheared several times through a 21-gauge needle. Lysates were centrifuged at 14,000 × *g* for 15 min at 4°C, and protein concentrations were determined by the Bio-Rad protein assay. Aliquots of 50 to 100 μg protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (Bio-Rad). The following antibodies were used for immunoblotting: anti-PKCη (Santa Cruz), anti-PKCα (Sigma), anti-PKCδ (Santa Cruz), anti-PKCε (Santa Cruz), anti-β-actin (ICN Biomedicals), anti-eIF-2α (provided by O. Elroy-Stein, Tel Aviv University, Israel), and anti-phospho-eIF-2α (Biosource). For the detection of primary antibodies, blots were incubated with horseradish peroxidase coupled to donkey anti-rabbit immunoglobulin or sheep anti-mouse immunoglobulin (Amersham Pharmacia), and the immobilized antibodies were detected by the ECL enhanced chemiluminescence system (Amersham Pharmacia). Quantitative densitometry analysis was achieved by the EZ-Quant software.

RNA isolation and Northern blot analysis. Total RNA was prepared using TRI-reagent (Invitrogen) according to the manufacturer's instructions. Equal amounts of total RNA were separated by electrophoresis on a 1% morpholinepropanesulfonic acid (MOPS)-formaldehyde-agarose gel and visualized by ethidium bromide staining and UV light. RNA was transferred to a HyBond membrane by capillary diffusion, fixed by UV irradiation, and hybridized to digoxigenin-labeled DNA probes for the PKCη probe as previously described (3). After posthybridization washes, the blot was submitted to immunological detection using antidigoxigenin antibody conjugated to alkaline phosphatase and CDP Star (Roche, Germany). The membrane was exposed to an X-ray film, and quantitative densitometry analysis was performed with EZ-Quant software.

Polysomal profile analysis and quantitative real-time PCR from sucrose gradient fractions. Polysomal profiles and RNA extraction were performed as described in reference 62. Equal volumes from fractions and from pools of fractions were used to perform cDNA synthesis followed by real-time PCR. The first DNA strand was reverse transcribed from 0.5 to 1 μg of total RNA using a Reverse-iT first strand synthesis kit and random hexamer primers (ABgene). Quantitative real-time PCR was performed with an ABI 7000 real-time PCR system (Applied Biosystems). PKCη mRNA levels were quantified by TaqMan PCR (Assay on Demand primer set hCG22361). PKCη expression was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) using TaqMan GAPDH control reagents (Applied Biosystems).

Cloning of the 5′ UTR and the 3′ UTR of PKCη. *Escherichia coli* (DH10B) carrying a 184-kbp bacterial artificial chromosome (BAC), that contains the genomic segment of human chromosome 14 in which the 5′ UTR of PKCη is encoded on a single exon (RZPD, Berlin, Germany), was grown on LB agar containing ampicillin (50 μg/ml) and chloramphenicol (12.5 μg/ml). DNA purification of the BAC was performed using the BACMAX DNA purification kit (Epicentre). The 5′ UTR of PKCη (659 nt) was amplified from the BAC using the following primers: 5′-GCAAGCTTCCAGACCCAGCGCTACAAGG-3′ (forward) and 5′-CAGACCATGGGCCGGCGCCGCTGCC-3′ (reverse). The resulting amplicon was then digested with HindIII and NcoI and cloned into the corresponding sites of the pGL3-Promoter vector (Promega).

The 3′ UTR of PKCη (1,122 nt, not including the polyadenylation site) was amplified from the λL-17 plasmid, which contains the entire 3′ UTR of PKCη, as previously described (3), using the following primers: 5′-GCAATCTAGACC TTATGGGGAGTG-3′ (forward) and 5′-CTTTTCTAGATGCCCTGTTTACA A-3′ (reverse). The resulting amplicon was digested with XbaI and cloned into the 5′ UTR pGL3-Promoter vector to generate the WT plasmid (see Fig. 3). All PCRs were accomplished using the *Pfu* DNA polymerase enzyme (Promega), and all cloned plasmids were confirmed by sequencing.

Site-directed mutations. Point mutations were introduced by a PCR-based mutagenesis method (9). The PCR products containing the mutated sequences were inserted in the NheI and BsrGI restriction sites. The uAUG initiation codons were altered to ACG codons (Fig. 3). The mut1 plasmid was generated by combining two amplicons produced by the following primers: (i) 5′-CCCTA ACTCCGCCCAGTTCC-3′ (forward) and 5′-AGCTGCCGCCGCCGCGTCCC CG-3′ (reverse) and (ii) 5′GGACTCTGGCACAAAATCGT-3′ (forward) and 5′-CCGGGGACGCGGCGGCGGCAGCTGC-3′ (reverse). The mut2 plasmid was generated by using the following primers: (i) 5′-CCCTAACTCCGCCCAG TTCC-3′ (forward) and 5′-CCTCGACTGGCCGTTCTGCCTCCTC-3′ (reverse) and (ii) 5′GGACTCTGG CACAAAATCGT-3′ (forward) and 5′-GAG GAGGCAGAACGGCCAGTCGAGG-3′ (reverse).

Transient transfection and dual-luciferase reporter assay. MCF-7 and MEF cells were seeded in 24-well plates (1×10^5 cells/well) in supplemented Dulbecco's modified Eagle's medium 24 h before transfection. Cells were transiently cotransfected with the cloned firefly plasmids (described above) and the *Renilla* plasmid as a control for transfection efficiencies (30:1 ratio), using the jetPEI kit (Poly transfection, France) according to the manufacturer's instructions. Twenty-four to 48 h posttransfection, the medium was changed to fresh complete medium or starved to amino acids. The cells were harvested using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

The firefly and *Renilla* luciferase activities were measured with a luminometer (Turner Designs model 20/20; Promega). Assays were repeated three times with triplicate samples. Statistical analysis of the differences between the groups was performed using Student's *t* test, with $P < 0.05$ considered statistically significant.

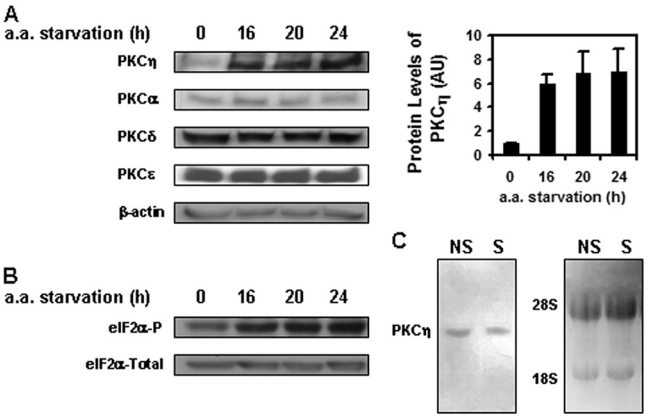

FIG. 1. PKCη is specifically induced upon amino acid starvation of MCF-7 cells. (A) MCF-7 cells were starved for amino acids (a.a.) for the indicated time points. The protein levels of PKCη, PKCα, PKCδ, and PKCε were determined by Western blot analysis as described in Materials and Methods. The results shown represent three independent experiments. Western blot images of three experiments were quantified using EZ-Quant software; the average is represented by a bar diagram of the induction (fold) of PKCη expression relative to the nontreated cells normalized to β-actin levels. AU, arbitrary units. (B) MCF-7 cells subjected to amino acid starvation showed increased phosphorylation of eIF-2α at Ser51, demonstrating that cells are under stress. (C) mRNA levels of PKCη were examined using Northern blot analysis with 28S and 18S as loading controls in nonstarved (NS) and amino-acid-starved (S) cells, as described in Materials and Methods. The results shown represent three independent experiments.

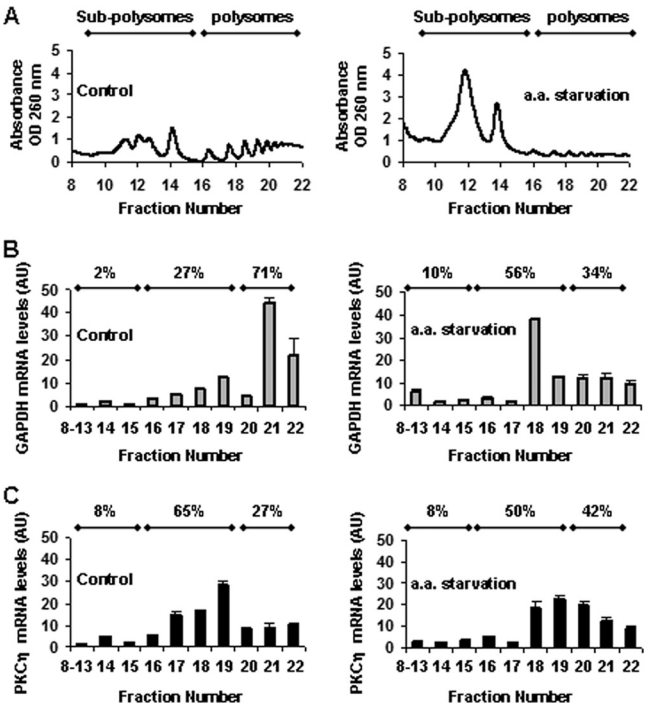

FIG. 2. PKCη mRNA is redistributed to heavier polysomes during amino acid starvation. MCF-7 cells were either not starved (control) or starved for amino acids for 24 h and analyzed for polysomal profile and mRNA distribution. (A) Polysomal profile of control (left) and amino-acid-starved (right) cells. Subpolysome and polysome fractions are indicated. (B and C) Distribution of GAPDH (B) and PKCη (C) mRNA levels along the polysomal profile of control cells (left) and amino-acid-starved cells (right). Graphs display the mean ± standard deviation of triplicate real-time PCR measurements of each mRNA in fraction or pools representing two independent experiments. The percentages of GAPDH and PKCη mRNA levels in fractions 8 to 15, fractions 16 to 19, and in most heavy polysomes (fractions 20 to 22) are indicated above the bars. AU, arbitrary units.

RNA isolation, cDNA synthesis, and quantitative real-time PCR. Total RNA was isolated and purified using a Versagene RNA isolation kit (Gentra Systems) according to the manufacturer's protocol. Carryover DNA was eliminated by DNase (Turbo DNA-free; Ambion). The first-strand DNA was reverse transcribed from 0.5 to 1 μg of total RNA using Reverse-iT first-strand synthesis kit and random hexamer primers (ABgene). Quantitative real-time PCR was performed using an ABI 7000 real-time PCR system (Applied Biosystems). Firefly luciferase mRNA levels were quantified using ABsolute QPCR SYBR green reagents (ABgene), using specific primers for luciferase (5′-GGATTACCAGG GATTTCAGTC-3′ [forward] and 5′-CTCACGCAGGCAGTTCTAT-3′ [reverse]) normalized to GAPDH using specific primers (5′-CATCCCTGCCTCT ACTGG-3′ [forward] and 5′-CTGCTTCACCACCTTCTTG-3′ [reverse]). The PCR thermocycling parameters were 95°C for 15 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. All gene-specific primers used were examined for efficiency, displaying an amplification slope of -3.33 ± 0.3 ($r \geq 0.98$). Data analysis was performed using the cycle threshold ($\Delta\Delta C_T$) method. Statistical analysis of the differences between the groups was performed using Student's t test, with $P < 0.05$ considered statistically significant.

RESULTS

PKCη is specifically induced following amino acid starvation. A screen conducted to explore stress conditions that may alter the expression of PKCη in MCF-7 cells revealed that it is markedly induced upon prolonged amino acid starvation in a time-dependent manner. As shown in Fig. 1A, the protein levels of PKCη increased by six- to sevenfold following amino acid starvation for 16 to 24 h. Expression of other PKC isoforms expressed in these cells, PKCα, PKCδ, and PKCε, was not altered under the same conditions, indicating that the exhibited effect is specific to the PKCη isoform. Steady-state mRNA levels of PKCη, quantified in control and amino-acid-starved cells by Northern blot analysis, showed similar PKCη mRNA levels (Fig. 1C), indicating that the observed increase is obtained by posttranscriptional mechanisms. Amino acid starvation induces a stress response that is mediated by the phosphorylation of eIF-2α (27). The fact that MCF-7 cells were indeed subjected to amino acid starvation was confirmed by the increased phosphorylation of eIF-2α (Fig. 1B).

PKCη mRNA is preferentially translated in response to amino acid starvation. To determine whether the increase in PKCη expression is at the translational level, we tested the association of PKCη mRNA with polyribosomes under normal and amino acid starvation conditions by fractionations on sucrose gradients. To learn more about the translational status of the mRNA under both conditions, we monitored its distribution along the entire polysomal profile by testing its level in each fraction of the gradient. As expected, the polysomal profile of amino-acid-starved cells showed disassembly of heavy polysomes, which were not observed in cells under normal conditions (Fig. 2A). Translation repression under stress conditions was further demonstrated by the redistribution of GAPDH mRNA from heavier polysomes to lighter polysomes (Fig. 2B). In contrast, PKCη mRNA distribution shifted toward heavier polysomes, implying that the translation of PKCη was upregulated upon amino acid starvation, overriding the global translation repression (Fig. 2C).

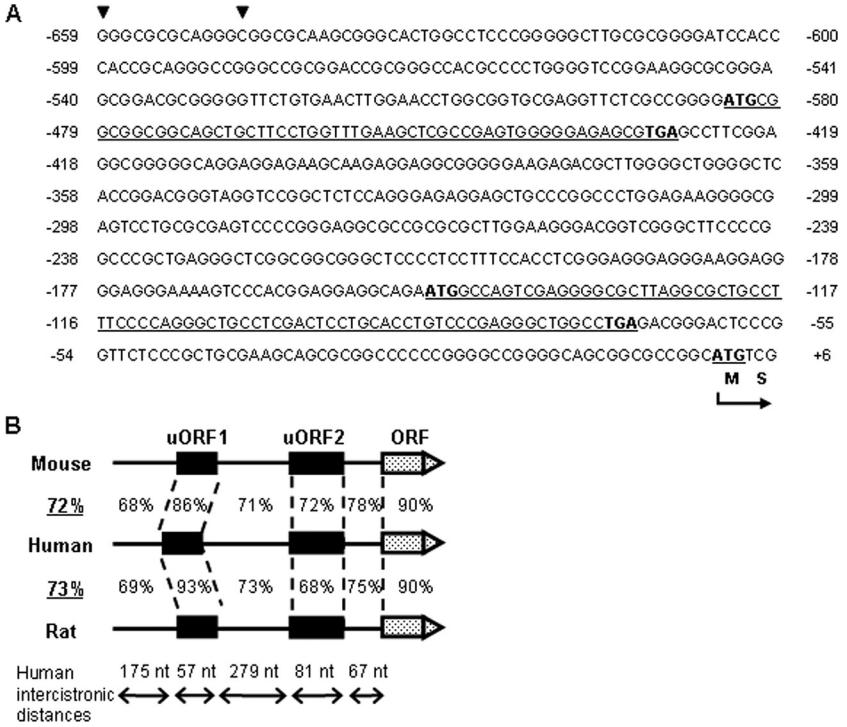

FIG. 3. The 5′ UTRs of the human, mouse, and rat PKCη orthologs are conserved and contain two uORFs. (A) The nucleotide sequence of the 5′ UTR of human PKCη is shown in relation to its main ORF. The two alternative start sites are indicated by solid triangles. The two uORFs (uORF1 and uORF2) are underlined. The ATG codons and stop codons are shown in boldface. (B) Comparison of the nucleotide sequences of mouse, human, and rat orthologs. Sequences were extracted from the NCBI database, and the nucleotide identities were determined using the Lalign software (shown as percentages). uORFs were found by the ORF-finder software (NCBI).

The 5′ UTRs of PKCη orthologs are highly conserved and possess two uORFs. It is widely accepted that elements in the 5′ UTR can mediate translational control (38). A previous study mapped two transcription start sites for human PKCη, at positions −647 and −659 relative to the AUG initiation codon (Fig. 3A). The presence of shorter transcripts starting downstream to these transcription start sites was also excluded (58). Our analysis of the 5′ UTR revealed that it is GC rich (74%), suggesting that it can adopt strong secondary structures (data not shown). Comparison of the 5′ UTR sequences of human, mouse, and rat orthologs showed high conservation (72 to 73% identity), indicating that this region could be involved in a conserved regulatory mechanism.

We have identified two uORFs in the human 5′ UTR, designated uORF1 and uORF2, which are also conserved at the nucleotide level in the rat and mouse orthologs (Fig. 3A and B), as indicated by nonsynonymous/synonymous substitution ratio (K_a/K_s) studies (63; data not shown). The human, rat, and mouse uORF1s start at positions −584, −567, and −579, respectively, upstream of the AUG start codon of the main PKCη ORF and code for small putative peptides of 18 (human and rat) and 22 (mouse) amino acids. uORF2 is highly conserved among all three orthologs, with regard to both their length and distance from the main ORF. It encodes a small putative peptide of 26 amino acids, starting at position −148 relative to the first AUG of the main ORF. The nucleic acid contexts adjacent to the initiation codons of these two uORFs revealed that position −3 of uORF1 matches the Kozak consensus sequence for efficient translation initiation, whereas uORF2 matches the consensus at both the −3 and +4 positions (37, 39), suggesting that both uORFs might be translated. Notably, all three orthologs of uORF2 have an optimal match to the Kozak consensus sequence (A/GNNAUGG), even stronger than the main ORF (Table 1). Northern blot analysis of PKCη (Fig. 1C) indicated that the sizes, as well as the steady-state levels, of the native PKCη transcript were not altered by amino acid starvation, which also ruled out the presence of shorter transcripts that begin 3′ of one or both uORFs.

Expression of PKCη is repressed via its uORFs under normal growth conditions. In order to determine whether the uORFs in the 5′ UTR of PKCη affect its expression, the 5′ UTR and 3′ UTR were cloned upstream and downstream of a

TABLE 1. Nucleic acid contexts of the initiation codons of the uORFs and the main ORF of PKCη compared to the Kozak consensus sequence

Species	Important sequence position(s) in[a]:		
	uAUG1	uAUG2	Main AUG
Human	**G**GGAUG*C*	**A**GAAUG**G**	**G**GCAUG*T*
Rat	**G**GGAUG*C*	**G**GAAUG**G**	**A**GCAUG*T*
Mouse	**G**GGAUG*C*	**G**GAAUG**G**	**G**GCAUG*T*

[a] The optimal Kozak consensus is **A/G**NNAUG**G**. Differences from the optimal sequence are highlighted in italic. The translation initiation codon is underlined.

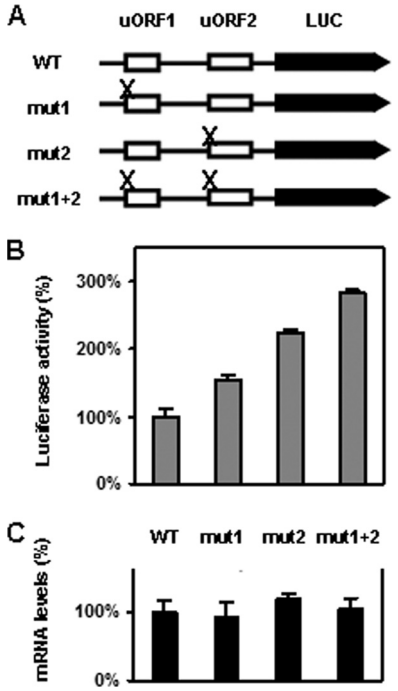

FIG. 4. The two uORFs in the 5′ UTR of PKCη repress its expression in MCF-7 growing cells. (A) Schematic representation of the generated constructs of PKCη. The 5′ UTR and the 3′ UTR of PKCη were fused upstream and downstream to a firefly reporter gene (WT). Point mutations of uAUG initiation codons were introduced into uORF1 (mut1), uORF2 (mut2), or both (mut1 + 2). (B) Mutations of uORF1 or uORF2 alone or both derepress the luciferase activities of the transfected constructs. Cells were transiently cotransfected with each of the cloned firefly reporter plasmids and with the *Renilla* plasmid as a control for transfection efficiencies. The firefly luciferase activities were assessed and normalized to *Renilla* values. WT luciferase activities were defined as 100%. The data shown are means of three separate experiments in triplicates. (C) mRNA levels of transfected constructs are similar. Cells were transiently transfected with each of the cloned firefly reporter plasmids. The luciferase mRNA levels were examined using real-time PCR with GAPDH as an endogenous control. The results represent the average of three independent experiments.

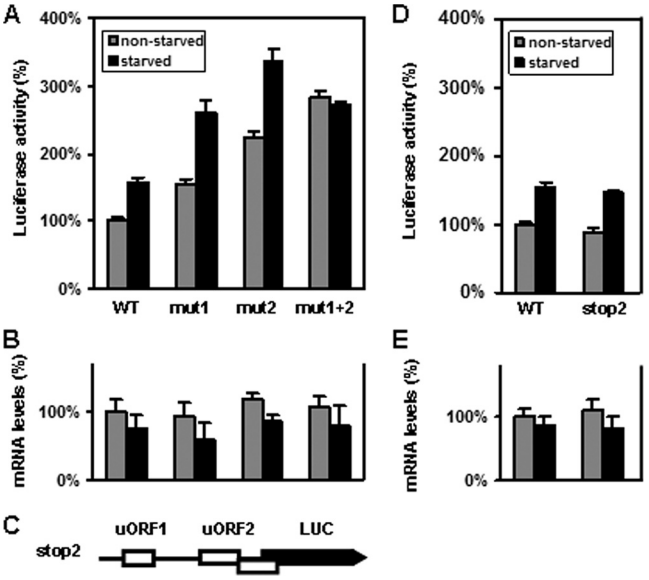

FIG. 5. The uORFs of PKCη are required for induction of its expression upon amino acid starvation in MCF-7 cells. (A) MCF-7 cells were transfected with each of the constructs described in the legend to Fig. 4A. Transfection efficiencies were monitored by cotransfection with a *Renilla* luciferase reporter plasmid. Twenty-four to 48 h posttransfection, the cells were starved for amino acids as described in Materials and Methods. The firefly luciferase activities were assessed and normalized to *Renilla* values. The induction (fold) due to starvation was determined for each construct relative to the nonstarved WT construct. The data shown are means of three separate experiments in triplicates. (B and E) mRNA levels of transfected constructs are similar. Cells were transiently transfected with each of the cloned firefly reporter plasmids. Twenty-four hours posttransfection, the cells were starved for amino acids as described in Materials and Methods. The luciferase mRNA levels were examined using real-time PCR with GAPDH as an endogenous control. The results represent the average of three independent experiments. (C) Schematic representation of the stop2 construct. LUC, luciferase. (D) The firefly luciferase activities of the WT and stop2 constructs are similar under nonstarved and starved conditions.

firefly luciferase reporter. Next, each of the two uAUG initiation codons, or both, was altered to an ACG triplet to eliminate their translational potential (Fig. 4A). The resulting constructs were transfected into MCF-7 cells, and their expression was assessed. Transfection efficiencies were normalized by cotransfection with a *Renilla* luciferase reporter. Each of the mutations in the AUG initiation codons of the uORFs caused an increase in the luciferase activity as compared to that of the WT plasmid (Fig. 4B). uAUG mutations in the first and second uORFs caused 1.5- and 2.2-fold increases, respectively, while a double mutation of both uAUGs caused an increase of almost 3-fold. This suggests that each of the uORFs can independently suppress expression of PKCη, although at different efficiencies since uORF2 has a stronger repressive effect on translation than uORF1. Overall, our results indicate that under normal growth conditions expression of PKCη is repressed by both uORFs. This could explain, at least in part, the low protein basal expression of PKCη in MCF-7 cells (Fig. 1A, control lane). Real-time PCR of the RNAs transcribed from these constructs showed no quantitative differences (Fig. 4C), suggesting that the variable expression of the reporter protein was not due to differences in mRNA levels (Fig. 4B).

Induction of PKCη following amino acid starvation requires the presence of its two uORFs. Since expression of PKCη is induced upon amino acid starvation (Fig. 1 and 2), we tested the possibility that the uORFs are involved in this induction. The constructs described above (Fig. 4A), containing mutations within the uAUG1 and uAUG2 initiation codons, were transfected into MCF-7, and their luciferase activity was tested under normal conditions and following amino acid starvation. As shown in Fig. 5A, the luciferase activity of the plasmid harboring the authentic PKCη 5′ UTR increased in response to amino acid starvation, as compared to that under the control nonstressed conditions. Mutations of either uAUG1 or uAUG2 exhibited similar levels of induction during amino acid starvation. Thus, abolishing translation of only one of the two uORFs did not abrogate the stress-induced increase in luciferase activity. However, eliminating the translation of both uORFs resulted in constitutive high activity levels under amino acid starvation, similar to those exhibited under nonstress con-

ditions. Hence, suppression of the basal luciferease activity in nonstressed growing cells was maximally relieved by the double mutations (Fig. 4) and the luciferase activity was not further increased in response to amino acid depletion. Taken together, our results indicate that each of the uORFs plays a role in the translational control mechanism that increases expression of PKCη following stress of amino acid starvation. Real-time PCR of the RNAs obtained from these constructs under amino acid starvation conditions showed no quantitative differences, demonstrating that the increase in luciferase activity depicted in Fig. 5A were not the result of differences in mRNA levels (Fig. 5B). The magnitude of the increase in protein levels (Fig. 1) was not fully recapitulated by the luciferase reporter assays. Additional control mechanisms could be involved in enhancing expression of PKCη under stress, such as protein stabilization by phosphorylation (53).

Our data show that translation of PKCη is regulated during stress of amino acid starvation by a mechanism that is dependent on two repressive uORFs, unlike the case of *GCN4*/ATF4 wherein uORF1 is a positive element that allows reinitiating ribosomes to bypass the downstream inhibitory uORFs when eIF-2 is phosphorylated. Next, we aimed to better understand the translational mechanism responsible for PKCη regulation and to assess the involvement of leaky scanning during stress. Since uORF2 is a stronger repressor element, we chose to mutate its stop codon. A frameshift mutation was introduced (TGA was replaced with TCGA) in order to generate an extended uORF that is not in frame with the luciferase gene. This extended uORF terminates at the next stop codon located 83 nt downstream of the luciferase initiation codon (stop2 construct) (Fig. 5C). This mutation should prevent the translation of the luciferase ORF by reinitiation after translation of uORF2; thus, translation of the luciferase ORF would occur only by leaky scanning of uORF2 of these specific constructs. As depicted in Fig. 5D, the introduction of a stop codon to uORF2 did not alter the luciferase activity under normal growth conditions compared to the WT plasmid, indicating that reinitiation at the main ORF does not normally occur following translation of uORF2. Furthermore, following amino acid starvation (black bars) the luciferase induction was not hampered by this mutation, providing evidence that under these conditions the ribosomes bypass uORF2.

***GCN2* is required for the translational induction of PKCη under stress of amino acid starvation.** Exposure of cells to amino acid starvation induces the phosphorylation of eIF-2α by *GCN2* kinase, causing inhibition of global mRNA translation (27) and enhanced translation of selected uORF-containing mRNAs which code for proteins that function in the adaptation to stress (26, 44, 51, 65, 67). To test if *GCN2* functions to induce the translation of PKCη mRNA, wild-type MEF cells ($GCN2^{+/+}$) and *GCN2* knockout cells ($GCN2^{-/-}$) were transfected with the luciferase plasmids containing the authentic or the double mutant 5′ UTR of PKCη (Fig. 4A). As shown in Fig. 6A, amino acid starvation elicited translational induction of PKCη in $GCN2^{+/+}$ cells, similar to the results observed in MCF-7 cells. This induction was completely abrogated in $GCN2^{-/-}$ cells, demonstrating that *GCN2* is required for translational upregulation of PKCη. When both uORFs were mutated, luciferase expression was constitutively derepressed, regardless of the stress conditions, in either $GCN2^{+/+}$ or $GCN2^{-/-}$ cells.

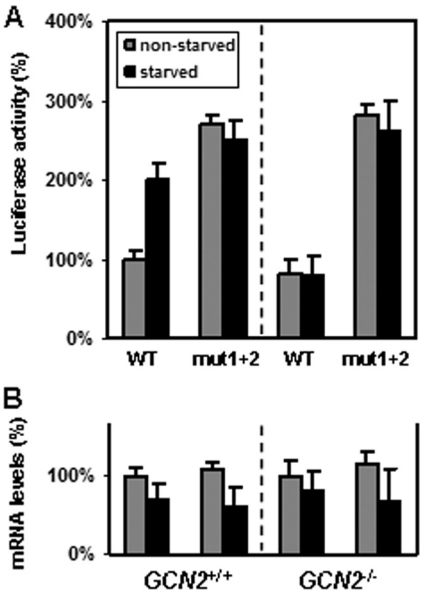

FIG. 6. *GCN2* is required for the translational induction of PKCη. (A) WT ($GCN2^{+/+}$) and *GCN2* knockout ($GCN2^{-/-}$) MEF cells were transfected with the WT and mut1 + 2 constructs described in the legend to Fig. 4A. Transfection efficiencies were monitored by cotransfection with a *Renilla* luciferase reporter plasmid. Twenty-four to 48 h posttransfection, the cells were starved for amino acids as described in Materials and Methods. The firefly luciferase activities were assessed and normalized to *Renilla* values. The induction (fold) due to starvation was determined for each construct relative to the nonstarved WT construct. The data shown are means of two separate experiments in triplicates. (B) mRNA levels of transfected constructs are similar. WT ($GCN2^{+/+}$) and *GCN2* knockout ($GCN2^{-/-}$) MEF cells were transiently transfected with each of the cloned firefly reporter plasmids. Twenty-four hours posttransfection, the cells were starved for amino acids as described in Materials and Methods. The luciferase mRNA levels were examined using real-time PCR with GAPDH as an endogenous control. The results represent the average of three independent experiments.

DISCUSSION

The posttranslational control of the PKC family has been extensively studied and was shown to play a key role in its regulation (reviewed in reference 52). However, there is hardly any information on the translational regulation of PKCs. Here we report that two uORFs in the 5′ UTR of PKCη mRNA function as regulators of its expression under both normal growth conditions and upon stress induced by amino acid starvation. Under normal growth conditions, both uORFs act as *cis*-repressor elements that suppress translation of PKCη and maintain its low basal levels. However, upon amino acid starvation, the translation of PKCη is upregulated in the presence of active *GCN2* and uORFs. PKCη appears to be among the very limited number of mammalian proteins described in the literature the expression of which is upregulated by the presence of uORFs under stress conditions. This is the first report demonstrating that the uORFs of one of the PKC family members have a functional regulatory role under stress.

Leader sequences of mRNAs that possess uORFs were pre-

viously documented in key regulatory proteins, such as growth factors, proto-oncogenes, and transcription factors. According to the scanning model for 5′-cap-dependent translational initiation in eukaryotes (38), uORFs offer physical blockage for scanning ribosomes, leading to reduced translation efficiency (48). This could serve as a mechanism that limits the expression of proteins that are harmful if overproduced and are therefore maintained at low levels (36). Our results demonstrate that PKCη, a signaling molecule, has two functional conserved uORFs that constitutively suppress its expression under normal growth conditions. Mutagenesis of each or both of the two PKCη uAUG initiation codons increased the expression of a luciferase reporter plasmid (Fig. 4), relieving the repression imposed by these two uORFs. This may explain, at least in part, the low basal levels of PKCη expression in MCF-7 cells grown under basal conditions (Fig. 1A, control lane). The fact that both uORFs function as repressive elements suggests a safety mechanism to ensure low expression levels of PKCη under nonstress conditions.

uORF2 has a stronger repression potential than uORF1 in growing cells (Fig. 4), which is also consistent with its optimal Kozak consensus sequence (Table 1). After translation of uORF2, the ribosomes can either terminate translation, leading to dissociation from the mRNA, or resume scanning, resulting in translation reinitiation of the main ORF. In order to determine the reinitiation efficiency, the stop codon of uORF2 was altered to form an out-of-frame uORF that extends beyond the main ORF, thereby precluding reinitiation (Fig. 5C). This mutation did not exhibit a significant effect on the luciferase activity (Fig. 5D), suggesting that after translation of uORF2, a major portion of the scanning ribosomes do not reinitiate translation at the main ORF. Two major factors control the extent of reinitiation: the length of the uORFs and the intercistronic distance between the ORFs, whereby expanded intercistronic distances increase translation efficiency (35). Efficient reinitiation is thought to occur following translation of only very short uORFs, presumably due to proximal availability of initiation factors (33, 45). However, when longer uORFs are translated, these factors fall off the mRNA, thus, preventing reinitiation. The lengths of human uORF1 and uORF2 of PKCη are 18 and 26 amino acids, respectively, lowering the probability of reinitiation. Following translation of a uORF, the probability of reinitiation at the downstream ORF depends on the length of the intercistronic distance to allow the scanning ribosomes to reacquire the ternary complex. The intercistronic region between the two uORFs of PKCη (279 nt) is probably long enough for reinitiation under normal conditions. The distance between the termination codon of uORF2 and the main ORF is shorter (67 nt), which may not favor efficient reinitiation (34). Moreover, as the 5′ UTR of PKCη confronts the scanning ribosomes with three alternative start sites, it is plausible that the 5′ UTR is inhibitory to translation initiation, with ribosome flow decreasing following encounters with each uORF, as previously shown for the tie2 receptor (57).

Regulation at the translational level allows for a rapid response to physiological changes that require alterations in protein levels, such as stress or apoptosis. Global translation is reduced in response to most types of cellular stresses, saving energy that is consumed during translation and also reducing the synthesis of proteins that could interfere with the cellular response to stress (27). This inhibition of global translation is often accompanied by a switch to the selective translation of a limited number of proteins that are required for the response of cells to stress. Selective stress-induced translational control involving uORFs was first demonstrated for *GCN4* (26, 51) and later for ATF4, ATF5, and GADD34 (25). In addition, IRES-based mechanisms were shown to be responsible for the induction of other genes, such as XIAP (41), Apaf-1 (11), CAT-1 (17, 18), HIAP2 (66), and SNAT2 (22).

Here we report that the PKCη gene is among the limited number of genes upregulated during stress via its two uORFs. Mutation of both uAUGs completely abrogated the translational induction during stress (Fig. 5). Furthermore, it appears that each uORF is independently sufficient for this increase since a single mutation of each uAUG was able to relieve repression and further enhance translation to a similar extent (Fig. 5A). Mutation of the stop codon of uORF2 did not interfere with luciferase induction following amino acid starvation, providing evidence that under these conditions ribosomes bypass uORF2 by leaky scanning (Fig. 5D). Our results with regard to uORF2 are in agreement with ATF4 and ATF5 (44, 70); when eIF-2α becomes phosphorylated and the ternary complex levels are limiting, the recruitment of the ternary complex by the ribosome is markedly reduced, and as a result the ribosomes have a higher probability of bypassing uORF2 by leaky scanning and reinitiating at the main ORF. However, in contrast to ATF4/ATF5, mutation of the initiation codon of uORF2 of PKCη resulted also in increased translation during amino acid starvation (Fig. 5A). This could be the result of leaky scanning on uORF1 alone or on both uORF1 and uORF2. During stress, we propose that when uORF1 is mutated, the scanning ribosomes bypass uORF2 by leaky scanning and reach the main ORF. When the initiation codon of uORF2 is mutated, leaky scanning of uORF1 results also in the enhanced translation of the main ORF to a similar extent. Mutation of both uORFs abolished induction since both uORFs are already fully derepressed. The two uORFs may have evolved as a fine-tuning mechanism, ensuring that PKCη will be optimally induced during stress. Since *GCN2* is probably involved (Fig. 6), it is possible that leaky scanning occurs under ternary complex-limiting levels. Enhanced leaky scanning of uORFs during eIF-2 phosphorylation was previously documented for C/EBP and GADD34 (7, 40). Leaky scanning of a single uORF of the transcriptional factors, C/EBPα and C/EBPβ, was shown in response to eIF-2α phosphorylation, responsible for the generation of truncated cellular C/EBP isoforms. In the case of GADD34, two uORFs were also identified; unlike our data, uORF1 was shown to be dispensable for the translational regulation and the enhanced leaky scanning of uORF2 was important for translational induction during stressed conditions. The molecular mechanism responsible for increased leaky scanning of uORFs during stress is still poorly understood (7, 40).

Our data indicate that the two uORFs of human PKCη, highly conserved in its mouse and rat orthologs, control translation through a mechanism that is distinct from the well-studied *GCN4*/ATF4 model. First, a crucial feature in the ATF4 model is the differential effect of the two uORFs: uORF1 serves as a positive element, and uORF2 is a strong

repressor. In contrast, our results suggest that both uORF1 and uORF2 of PKCη serve as repressor elements under normal growth conditions. Second, while according to the ATF4 model, both uORFs are required for translational induction during stress, a single uORF is sufficient for maximal upregulation of PKCη. Third, the translational induction of ATF4 is mediated by reinitiation, whereby translation of the positive element uORF1 is followed by leaky scanning of only uORF2 under induced eIF-2 phosphorylation. However, we propose that enhanced leaky scanning of either uORF2 or both uORF1 and uORF2 enables ribosomes to reach the main ORF, resulting in its increased translation under stressed conditions.

What could be the role of the increased expression of PKCη during stress? Recent studies reveal a linkage between PKC and induction and regulation of autophagy. Autophagy, an evolutionally conserved process for the bulk degradation of cytoplasmic proteins and organelles, and apoptosis are induced in response to cellular insults, such as endoplasmic reticulum stress, amino acid starvation, hypoxia, and oxidative stress. Activation of PKCθ was shown to be required for endoplasmic reticulum stress-induced autophagy (60). PKCδ also activates autophagy by promoting Jun N-terminal kinase 1 (JNK1)-mediated Bcl-2 phosphorylation and dissociation of the Bcl-2/Beclin 1 complex (10). The pharmacological agents safingol (12) and oridonin (69) were shown to trigger autophagy via PKC. The specific role of PKCη during stress and autophagy is currently being investigated.

Different PKC enzymes, including PKCη, were previously implicated in the cellular response to stress and cell death (see the introduction). Our data suggest that regulation at the translational level could provide another level for PKC regulation, when an increase in their protein levels is needed. The 5′ UTR of PKCδ was shown to direct translation by an IRES element that was most active when 5′-cap-dependent translation is reduced under high-density growing cells or tumor necrosis factor-induced apoptosis, but not during serum starvation (49). As mentioned above, the 5′ UTR of PKCε was shown to repress translation in rabbit reticulocyte lysate, and mutation of a uAUG motif in this region partially relieved repression (50), but a physiological role in stress or apoptosis was not examined. Noteworthy, our preliminary analysis indicate that in addition to PKCη reported here and PKCε (50), additional PKC isozymes also possess uORFs and out-of-frame uAUGs in their 5′ UTRs (data not shown). Thus, translational control may provide another level for regulating expression of PKC family members in mammalian cells, being more common than currently recognized.

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