PKR and PKR-like Endoplasmic Reticulum Kinase Induce the Proteasome-dependent Degradation of Cyclin D1 via a Mechanism Requiring Eukaryotic Initiation Factor 2α Phosphorylation

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Cyclin D1 plays a critical role in controlling the G1/S transition via the regulation of cyclin-dependent kinase activity. Several studies have indicated that cyclin D1 translation is decreased upon activation of the eukaryotic initiation factor 2α (eIF2α) kinases. We examined the effect of activation of the eIF2α kinases PKR and PKR-like endoplasmic reticulum kinase (PERK) on cyclin D1 protein levels and translation and determined that cyclin D1 protein levels decrease upon the induction of PKR and PERK catalytic activity but that this decrease is not due to translation. Inhibition of the 26 S proteasome with MG132 rescued cyclin D1 protein levels, indicating that rather than inhibiting translation, PKR and PERK act to increase cyclin D1 degradation. Interestingly, this effect still requires eIF2α phosphorylation at serine 51, as cyclin D1 remains unaffected in cells containing a non-phosphorylatable form of the protein. This proteasome-dependent degradation of cyclin D1 requires an intact ubiquitination pathway, although the ubiquitination of cyclin D1 is not itself affected. Furthermore, this degradation is independent of phosphorylation of cyclin D1 at threonine 286, which is mediated by the glycogen synthase kinase 3β and mitogen-activated protein kinase pathways as described in previous studies. Our study reveals a novel functional cross-talk between eIF2α phosphorylation and the proteasomal degradation of cyclin D1 and that this degradation is dependent upon eIF2α phosphorylation during short, but not prolonged, periods of stress.

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(7) The abbreviations used are: eIF4E, eukaryotic initiation factor; 4E-BP1, eIF4E-binding protein; dsRNA, double-stranded RNA; PKR, protein kinase activated by dsRNA; ER, endoplasmic reticulum; PERK, PKR-like ER kinase; GCN2, general control non-derepressible 2; TG, thapsigargin; MEF, mouse embryonic fibroblast; eIF2α, A/A, MEFs containing a homozygous S51A mutation in the eIF2α protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; NQO1, NAD(P)H quinone oxidoreductase 1; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; WT, wild type; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ab, antibody; GSK-3β, glycogen synthase kinase 3β.

cyclin D1 protein levels and that this down-regulation requires eIF2α phosphorylation at serine 51. We further demonstrate using polysome profiles that it is not translation of the cyclin D1 message that is affected by activation of the kinases, as the cyclin D1 message is translated equally well regardless of the state of eIF2α kinase activity or the inhibition of global protein synthesis. We determine that PKR and PERK function to induce the proteasomal degradation of cyclin D1 independent of any previously described mechanism but still requiring cyclin D1 ubiquitination. Furthermore, our data also shows that eIF2α kinases themselves may activate different pathways to regulate the “early” and “late” degradation of cyclin D1. Our study reveals a novel ability of PKR and PERK to promote the proteasome-dependent degradation of cyclin D1 in a manner that depends on eIF2α phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—HT1080 cells expressing GyrB.PKR wild type (WT) were established as described (32). HT1080 parental and GyrB.PKR WT cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Invitrogen) and 100 units/ml of penicillin-streptomycin (Wisent). eIF2α S/S and A/A immortalized mouse embryonic fibroblasts (MEFs) were generated and maintained as described (33). SV-40 immortalized PERK+/+ and PERK−/− MEFs (34) as well as temperature-sensitive ts20 and control E36 cells (35) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and the latter was maintained at the permissive temperature of 31°C. GyrB.PKR WT cells were treated with 100 ng/ml coumermycin (Sigma) to activate the fusion protein. Thapsigargin (Sigma) treatment was performed at a concentration of 1 μM, and MG132 treatment was performed at a concentration of either 10 or 20 μM as indicated in the figure legends. Treatment with Sal003 (gift from Dr. J. Pelletier) occurred at a concentration of 75 μM, 1-azakenapaulone (Calbiochem) at a concentration of 5 μM and U0126 (BIOSOURCE) at 10 μM. Cells were treated with dicoumarol (Sigma) at a concentration of 200 μM. dsRNA transfection occurred at a concentration of 10 μg/ml for 4 h as previously described (36).

Vaccinia Virus Transfection—WT and T286A mutant cyclin D1 constructs in pcDNA3 were kindly provided by Dr. B. Law. 3 × 105 HT1080 GyrB.PKR cells were plated in 60-mm plates and infected with vaccinia virus/17 as described in Li and Koromilas (37). Cells were co-transfected with 1 μg of cyclin D1 WT or T286A vector DNA and 1 μg of HA-tagged ubiquitin. 24 h post-transfection cells were treated with coumermycin and MG132 as indicated.

Sucrose Gradient Polysome Profiles—Polysome profiles were performed as previously described (38). Briefly, after the indicated treatments, 100 μg/ml cycloheximide was added directly to growth media and then immediately removed, and the plates kept on ice. Plates were washed 3 times with ice-cold 1× phosphate-buffered saline plus 100 μg/ml cycloheximide and lysed directly on the plate. After a 10-min incubation on ice, samples were cleared by centrifugation at 13,000 rpm for 10 min, and the lysate was loaded onto 10–55% sucrose gradients prepared on the Teledyne ISCO Density Gradient System. Gradients
were centrifuged in a Beckman L7–65 vacuum ultracentrifuge with a SW40.Ti rotor at 40,000 rpm for 2.5 h at 4 °C under vacuum. 18 fractions were collected per sample using the Foxy Jr. Fraction Collector.

**RNA Isolation and Reverse Transcriptase (RT)-PCR**—RNA was isolated from collected polysome fractions as follows. 600 μl of Trizol reagent (Invitrogen) and 120 μl of chloroform (Sigma) were added to each sucrose fraction and centrifuged, and the aqueous phase was removed to a separate tube. 600 μl of isopropanol was added to each fraction, and RNA was precipitated overnight at −20 °C. Samples were centrifuged at 13,000 rpm for 30 min, and the pellets were washed with ice-cold 75% ethanol and resuspended in 10 μl of Milli-Q H2O. 3 μl of RNA was used as a template for RT reactions with the AncT primer (5′-TTTTTTTTTTTTTTTTVN-3′). cDNA samples were diluted 10-fold, and 2 μl of template used to carry out PCR reactions for CCND1 (forward human primer, 5′-GCC GCC TCT GGT GTC CTA CTT-3′; reverse human primer, 5′-ACG CTC CCC GCT GCC ACC AT-3′; forward mouse primer, 5′-CTG GAG GTC TGT GAG GAG CA-3′; reverse mouse primer, 5′-GCC GTA GCA GGA GAG GAA GTT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer, 5′-CAT CAT CTC TGC CCC CTC TCG T-3′; reverse primer, 5′-CAG CGG TCG T ACC ACC TTC T-3′). It should be noted that the fractions collected from these polysome profiles were also used in a previous publication by our laboratory studying the effect of eIF2α kinases on p53 (38). In that case RT-PCR was performed for different mRNAs.

**[35S]Methionine Labeling**—HT1080 cells were either treated with MG132 for 1 h followed by coumermycin treatment for 2 h or treated with coumermycin for 3 h with MG132 added at the 2-h mark. Both treatments were performed in Dulbecco’s modified Eagle’s medium lacking methionine and supplemented with 10% dialyzed fetal bovine serum. Tran35S-label (ICN) was then added to the cells at a concentration of 100 μCi/106 cells, and the culture was continued for an additional 30 min in the presence of MG132 and coumermycin. Precipitation of radio-labeled proteins was carried out as follows; 250 μg of total protein was aliquot into microcentrifuge tubes in triplicate. Trichloroacetic acid was added to each sample to a final concentration of 10% w/v and incubated on ice for 3 h. Samples were centrifuged at 13,000 rpm for 15 min, washed with 300 μl of ice-cold acetone, and centrifuged again. Pellets were re-suspended in 50 μl of 1× phosphate-buffered saline plus 2% SDS and spotted on filter paper. CytoScint ESTM liquid scintillation fluid (MP Bio) was added to each sample, and the counts/min were determined.

**Immunoblotting**—Protein extraction and immunoblotting was performed as described (39). For immunoprecipitation and/or immunoblotting, the following antibodies were used: anti-cyclin D1 monoclonal Ab (BD Biosciences), anti-cyclin D1 Thr(P)-286 polyclonal Ab, anti-ERK 1/2 phospho-Thr-202/Tyr-204 polyclonal Ab and anti-ERK 1/2 total polyclonal Ab (Cell Signaling Technology), anti-eIF2α Ser(P)-51 polyclonal Ab (37), and anti-eIF2α (FL-315; Santa Cruz Biotechnology). All antibodies were used at a final concentration of 0.1–1 μg/ml. After incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (1:10,000 dilution; Amersham Biosciences), proteins were visualized with the enhanced chemiluminescence (ECL) detection system according to the manufacturer’s instructions (PerkinElmer Life Sciences). Quantification of the bands in the linear range of exposure was performed by densitometry using the NIH Image 1.54 software.

**RESULTS**

**Cyclin D1 Protein Levels Are Decreased upon Endoplasmic Reticulum Stress**—Previous reports have indicated that translation of cyclin D1 is affected by the induction of ER stress and the unfolded protein response (28–30). To further investigate the role of eIF2α phosphorylation in regulating cyclin D1 in our system, we treated HT1080 cells with the pharmacological agent thapsigargin, which induces the UPR by promoting calcium release from the ER. Treatment of these cells with thapsigargin induced a decrease in cyclin D1 levels (Fig. 1A, panel a) coincident with increased eIF2α phosphorylation (Fig. 1A, panel b). To investigate the specific effect of eIF2α phosphorylation on cyclin D1 regulation, we used the compound Sal003, which inhibits eIF2α dephosphorylation (40). Cyclin D1 levels decreased significantly upon Sal003 treatment in HT1080 cells (Fig. 1B, panel a), once again accompanied by an increase in eIF2α phosphorylation (Fig. 1B, panel b), indicating a strong relationship between eIF2α phosphorylation and the level of cyclin D1. To determine whether the observed effect on cyclin D1 was due to decreased translation as previously proposed, we examined translation of cyclin D1 mRNA using polysome profiles on HT1080 cells treated with either thapsigargin or Sal003. This technique involves the separation of large cellular components using a sucrose gradient and the monitoring of the A254 across the gradient. In this manner the 40 S and 60 S ribosomal subunits as well as monosomes and polysomes can be isolated and studied. Although GAPDH mRNA underwent a shift from the translating polysomes to the stalled monosomes (Fig. 1C, panel a), cyclin D1 mRNA remained associated with the polysome fractions (Fig. 1C, panel a). The observable shift in GAPDH, but not in cyclin D1, confirms that a general translation is being inhibiting but suggests that a mechanism other than translation regulation contributes to the down-regulation of cyclin D1.

**Down-regulation of Cyclin D1 Requires eIF2α Phosphorylation**—Because phosphorylation of eIF2α is believed to be essential for the observed regulation of cyclin D1 (30), we sought to elucidate whether it alone can affect cyclin D1 translation. We treated eIF2α A/A MEFs in which Ser-51 was replaced with a non-phosphorylatable alanine (33) and their wild type eIF2α S/S counterparts with thapsigargin and immunoblotted for cyclin D1 (Fig. 2A, panel a). Cyclin D1 was quickly and significantly decreased by more than 50% in eIF2α S/S MEFs (Fig. 2A, lanes 1–5, and B) but remained steady in eIF2α A/A knock-in cells (Fig. 2A, lanes 6–10, and B). Once again we performed polysome profiles to examine the translation of cyclin D1 in these cells (Fig. 2C, panels i–iv). Treatment with thapsigargin induced inhibition of global translation initiation in eIF2α S/S, but not A/A cells (compare panels ii and iv), but cyclin D1 mRNA remained associated with the polysome fractions (Fig. 2C, panel a). It is interesting to note that although
inhibition of general translation does not result in a shift of cyclin D1 mRNA to earlier polysome fractions, the CCND1 transcript is localized to earlier fractions in eIF2α/S/S cells compared with their knock-in counterparts. This may be due to slight differences in the overall translational activity of these cell lines, as the profiles themselves also show slight variances. Together these data suggest that although the observed regulation of cyclin D1 protein levels is not a result of the ability of eIF2α to inhibit translation, phosphorylation of this factor is essential.

Activation of a Conditionally Active Form of PKR Decreases Cyclin D1 Protein Levels—The role of several eIF2α kinases in regulating cyclin D1 has been investigated in a number of previous studies. However, the treatments used to induce stress in these cases could potentially activate a large number of other cellular responses in addition to eIF2α kinase activity. Because eIF2α phosphorylation at Ser-51 is predominantly associated with inhibiting global translation initiation, we therefore investigated whether cyclin D1 was translationally regulated in a system containing a conditionally active form of PKR (41).

HT1080 cells stably expressing a chimeric protein in which the catalytic domain of PKR was fused to the first 220 amino acids of the Escherichia coli gyrase B protein were analyzed (32). Treatment of these cells with the antibiotic coumermycin caused dimerization of the GyrB domain and autophosphorylation and activation of PKR (32). Cells were treated with coumermycin, and the protein extract immunoblot was probed with an anti-cyclin D1 antibody (Fig. 3A, panel a). As early as 3 h after activation of GyrB.PKR, as shown by an induction of eIF2α phosphorylation at serine 51 (Fig. 3A, panel b), cyclin D1 protein levels decreased significantly. This down-regulation was maintained for at least 12 h after coumermycin treatment (data not shown). To determine whether this regulation occurred at the translational level, we performed polysome profiles as described above. HT1080 GyrB.PKR cells were left untreated (Fig. 3B, panel i) or treated with coumermycin for 4 h (Fig. 3B, panel ii). RT-PCR performed on cyclin D1 transcripts associated with monosomes and polysomes (panel a) revealed that upon coumermycin treatment cyclin D1 mRNA undergoes no shift and remains bound with the late polysomes. Translation of

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Cyclin D1 protein levels are decreased upon endoplasmic reticulum stress. HT1080 cells were treated with TG (1 μM) (A) or Sal003 (75 μM) (B) for the times indicated. Cell lysates (50 μg of protein) were resolved by SDS-PAGE and immunoblot for cyclin D1 (panel a), eIF2α Ser(P)-51 (panel b), and total eIF2α (panel c). C, HT1080 cells were left untreated (i) or treated with TG (1 μM, 2 h) (ii) or Sal003 (75 μM, 5 h) (iii). Lysates were subjected to sucrose gradient centrifugation, and 18 fractions collected per sample. RT-PCR for CCND1 (panel a) and GAPDH (panel b) was performed on RNA isolated from each fraction.
GAPDH was evaluated as a control (panel b) and undergoes a significant shift from fraction 15 in the polysomes to fraction 8 in the stalled monosomes upon activation of GyrB.PKR and subsequent eIF2\alpha/H9251 phosphorylation. These observations suggest that, contrary to the conclusions of previous studies (28–30), the decrease in cyclin D1 protein levels upon activation of eIF2\alpha/H9251 kinases is due to a mechanism other than translational inhibition.

Activation of PKR and PERK Leads to Cyclin D1 Degradation—Because our studies, thus, far strongly indicated that a mechanism other than translational inhibition was responsible for the decrease in cyclin D1 protein levels, we investigated whether protein degradation may contribute to the observed effect. Transfection of HT1080 cells with dsRNA caused a significant decrease in cyclin D1 levels (Fig. 4A, panel a, lane 3), but this effect was absent when cells were pretreated with the proteasome inhibitor MG132 (Fig. 4B, panel a, lane 6). A similar effect was observed in HT1080 GyrB.PKR WT cells pretreated with MG132 before coumermycin treatment, as no decrease in cyclin D1 protein was observed (Fig. 4B, panel a, lanes 6–10) compared with coumermycin treatment alone, which caused a dramatic decrease in cyclin D1 levels. Cyclin D1 levels remained stable for the entire 8-h time course of GyrB.PKR activation (Fig. 4C). eIF2\alpha phosphorylation was equally induced by coumermycin in the presence or absence of MG132 treatment (Fig. 4B, panel b), indicating that the lack of cyclin D1 down-regulation is not due to a lack of translation inhibition. Activation of PERK upon ER stress also appears to induce the degradation of cyclin D1 in an eIF2\alpha/H9251 phosphorylation-dependent manner, as pretreatment with MG132 prevented a cyclin D1 decrease upon thapsigargin treatment of eIF2\alpha/S/S cells (Fig. 4D, panel a, compare lanes 2 and 4). Cyclin D1 levels remained unchanged in eIF2\alpha/A/A cells regardless of treatment (Fig. 4, D, panel a, lanes 5–8, and E). Several pathways have been implicated in regulating cyclin D1 degradation by inducing phosphorylation of cyclin D1 on threonine 286, including the glycogen synthase kinase 3\beta (GSK-3\beta) pathway (42), PTEN (43), activated Ras (44), and the mitogen-activated protein kinase (MAPK) pathway (45). Using 1-azakenpaullone, a specific inhibitor of GSK-3\beta (46), we determined that cyclin D1 degradation upon GyrB.PKR activation does not proceed through GSK-3\beta (supplemental Fig. 1A, panel a), as cyclin D1 levels were not rescued upon inhibition of GSK-3\beta activity. Although previous studies have demonstrated that GSK-3\beta can phosphorylate the e-subunit of eIF2B (47), the guanine nucleotide exchange factor that targets eIF2, in our experiments eIF2\alpha phosphorylation remained unaffected by 1-azakenpaullone treatment (supplemental Fig. 1A, panel b). The same lack of effect was observed when GSK-3\beta was inhibited by treatment with LiCl (data not shown) or when GSK-3\beta\textsuperscript{−/−} and GSK-
3β−/− MEFs were used (data not shown). We also examined signaling through the MAPK pathway and observed that inhibition of MEK activity by the inhibitor U0126 did not rescue cyclin D1 levels after coumermycin treatment (supplemental Fig. 1B, panel a). Similar to our results obtained with 1-azakavllone, eIF2α phosphorylation was not affected by the MEK inhibitor (supplemental Fig. 1B, panel b). Combined, these data indicate that cyclin D1 degradation via the proteasome pathway is not mediated by one of the traditional pathways. 

Induction of eIF2α Phosphorylation Does Not Significantly Affect Global Proteasome-dependent Protein Degradation—The effect we observed regarding the ability of eIF2α kinases to mediate the proteasome-mediated degradation of cyclin D1 led us to investigate whether there were other proteins regulated in a similar manner. [35S]Methionine labeling revealed that a significant amount of labeled proteins were up-regulated in cells pretreated with MG132 (Fig. 5A, panel a, lanes 3 and 4) compared with MG132-untreated cells (lanes 1 and 2) either before (lanes 1 and 3) or after activation of GyrB.PKR with coumermycin (lanes 2 and 4). However, GyrB.PKR activation was still capable of decreasing the amount of 35S-labeled protein even when MG132 was present, as assessed by autoradiography and scintillation counting (Fig. 5A, panels a and c). We obtained a similar result when the effects of MG132 were examined after GyrB.PKR activation by coumermycin (Fig. 5A, panel a, lanes 5 and 6). These data indicated that the eIF2α kinases exert their inhibitory effects on protein synthesis mainly at the level of translation. It is important to note, however, that a few radio-
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FIGURE 5. Induction of eIF2α phosphorylation does not alter global proteasome-dependent protein degradation. A, HT1080 GyrB.PKR WT cells were left untreated (lanes 1 and 2), pretreated with MG132 (20 μM; 1 h), and subsequently treated with coumermycin (coum; 100 ng/ml; 3 h) or treated with coumermycin (100 ng/ml; 3 h) with MG132 (20 μM) added 2 h after the addition of coumermycin for an additional hour. The [35S]methionine labeling of the cells was performed as described under "Experimental Procedures." Whole cell extracts (50 μg of protein) were separated by SDS-PAGE, and novel protein synthesis was evaluated by autoradiography (panel a). Coomassie Blue staining of the protein extracts are shown in panel b. The level of labeled protein in each lane was quantified by trichloroacetic acid precipitation and scintillation counting and is shown in panel c. The values in panel c represent the average of two experiments performed in triplicate. HT1080 GyrB.PKR WT cells were treated with coumermycin (100 ng/ml) (B) or cycloheximide (CHX; 20 μg/ml) (C) for the times indicated followed by MG132 (20 μM) treatment 2 h after initial coumermycin or cycloheximide treatment for the total times indicated. D, HT1080 GyrB.PKR WT cells were left untreated (lanes 5–8) or were pretreated with coumermycin (100 ng/ml) for 2 h (lanes 1–4 and 9–12). Cycloheximide (20 μg/ml) was added (lanes 5–12) for the times indicated. Extracts were subjected to SDS-PAGE and immunoblot for cyclin D1 (panel a), eIF2α Ser(P)-51 (panel b), or total eIF2α (panel c).

active bands retained their original intensity upon GyrB.PKR activation in the presence of MG132 (panel a), suggesting that in some cases eIF2α kinases can also regulate the degradation of specific proteins. The resistance of specific labeled proteins to degradation in the presence of MG132 as well as the ability of MG132 pretreatment to prevent cyclin D1 degradation upon eIF2α phosphorylation prompted us to examine whether MG132 post-treatment could similarly rescue cyclin D1 protein levels. HT1080 GyrB.PKR cells were treated with coumermycin followed by MG132 treatment 2 h after induction of GyrB.PKR activity. Inhibition of proteasome-dependent degradation caused a substantial rescue of cyclin D1 protein levels in cells treated with coumermycin (Fig. 5B, panel a) but not in cells treated with cycloheximide (Fig. 5C, panel a), a compound that inhibits the elongation step of protein synthesis and is, therefore, independent of eIF2α. To further confirm that induction of eIF2α kinase activity targeted the degradation and not translation of cyclin D1, we studied the two events separately and in conjunction. GyrB.PKR cells were treated either with coumermycin alone for 2 h, cycloheximide alone for short time points as controls, or both reagents together. These treatments were performed at shorter than normal intervals due to the fact that prolonged treatment with either reagent rapidly degrades cyclin D1 signal. Coumermycin treatment alone caused a gradual decrease in cyclin D1 protein levels as previously observed (Fig. 5D, panel a, lanes 1–4), whereas cycloheximide caused a more rapid decline in cyclin D1 (lanes 5–8). When cells were treated with cycloheximide after coumermycin treatment, the decrease in cyclin D1 protein levels was immediate and dramatic, falling below detectable levels after only 30 min of treatment (lanes 9–12). Because cycloheximide inhibits translation by a mechanism independent of eIF2α phosphorylation, if PKR targeted the translation of cyclin D1 we would not expect to see the additive effect of the two reagents observed in this experiment. This indicates that the proteasomal degradation of cyclin D1 is dependent upon eIF2α phosphorylation but not on the inhibition of overall protein translation. It should also be noted that in both experiments treatment of GyrB.PKR cells with MG132 alone caused an increase in the basal levels of cyclin D1, confirming that cyclin D1 is a very labile protein subject to a high rate of turnover.

Cyclin D1 Phosphorylated at Thr-286 Is Not Subject to Degradation upon eIF2α Kinase Activation—Although known upstream pathways are not affected in our system, we examined phosphorylation of cyclin D1 at a key threonine residue (Thr-286) in the carboxyl terminus of the protein. This phosphorylation causes a relocalization of cyclin D1 from the nucleus to the cytoplasm where it can be processed by the proteasome. Therefore, since we have demonstrated that the regulation of cyclin D1 in our system is at the level of degradation and not translation, we examined the effect of eIF2α kinase activity on cyclin D1 phosphorylation at this site. Using a phosphospecific antibody, we observed no increase in phosphorylation at Thr-286 (Fig. 6A, panels a and b). In fact, the level of phosphorylated cyclin D1 remained stable, whereas that of total cyclin D1 decreased (compare panels a and b with panel c). Specificity of this antibody was verified in quality control experiments probing extracts of cells overexpressing either wild type or T286A mutant forms of cyclin D1 (supplemental Fig. 2). Because cyclin D1 is rapidly degraded upon treatment with coumermycin, stimulation was also performed in the presence of MG132 to prevent this degradation (Fig. 6A, lanes 5–8). No discernable induction of Thr-286 phosphorylation is observed up to 4 h of coumermycin in cells pretreated with MG132, indicating that
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PKR and PERK mediate cyclin D1 degradation. Although degradation of cyclin D1 was not affected by the lack of phosphorylation at Thr-286, it does not necessarily follow that the proteasomal degradation of cyclin D1 is not dependent on protein ubiquitination.
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FIGURE 7. eIF2α kinases promote the ubiquitin-dependent degradation of cyclin D1 but do not directly affect its ubiquitination. (A) and E36 cells (B) were incubated at 31 °C (lanes 1–5) or 39 °C (lanes 6–10) and treated with thapsigargin (1 μM) for the times indicated. Extracts were separated by SDS-PAGE and immunoblotted for cyclin D1 (panel a), eIF2α Ser(P)-51 (panel b), eIF2α total (panel c), and tubulin (panel d). C, HT1080 GyrB.PKR WT cells were transfected with HA-tagged ubiquitin (Ub) and either cyclin D1 WT (lanes 4–6) or cyclin D1 T286A (lanes 7–9) by vaccinia virus infection. Cells were treated with coumermycin (100 ng/ml) for the times indicated, and extracts were separated by SDS-PAGE. Membranes were probed with anti-HA (panel a), anti-cyclin D1 (panel b), or anti-actin (panel c) antibodies.

FIGURE 8. Additional pathways contribute to the degradation of cyclin D1. A, HT1080 GyrB.PKR WT cells were treated with coumermycin (100 ng/ml; lanes 1–5) or dicoumarol (200 μM; lanes 6–10) for the times indicated. eIF2α S/S and A/A (B) and PERK+/+ and PERK−/− (C) cells were treated with TG (1 μM) or dicoumarol (200 μM) as indicated. Extracts were separated by SDS-PAGE and probed for cyclin D1 (panel a), eIF2α Ser(P)-51 (panel b), and eIF2α total (panel c).

Discussion

In this study we observed a similar negative regulation of cyclin D1 protein levels after eIF2α kinase activation as noted previously (28–30, 51) and further revealed a novel property of eIF2α kinases in promoting the proteasome-dependent degradation of cyclin D1. Our data provide evidence that the decrease in cyclin D1 levels observed upon activation of PKR and PERK is not due to their ability to inhibit translation initiation but via their ability to induce degradation of the protein. Results obtained from both a conditionally active PKR system and human fibrosarcoma cells demonstrate that whereas cyclin D1 protein levels decrease upon activation of the eIF2α kinases (Figs. 1, A and B, and 3A), translation of the CCND1 transcript does not differ once overall

ined the regulation of cyclin D1 in cells treated with dicoumarol, a competitive inhibitor of NQO1. We determined that in GyrB.PKR WT cells dicoumarol could indeed induce cyclin D1 degradation but with kinetics much slower than that of coumermycin (Fig. 8A, panel a, compare lanes 1–5 with lanes 6–10). Coumermycin caused a decrease in cyclin D1 levels as early as 2 h post-treatment, whereas it took 8 h for dicoumarol to take effect. This suggests that eIF2α kinase activation and inhibition of NQO1 binding do not function through related pathways and that the two mechanisms may be responsible for early and late cyclin D1, respectively. To confirm this and to investigate whether eIF2α phosphorylation affects the response to dicoumarol, we treated eIF2α S/S and A/A cells with either thapsigargin or dicoumarol for prolonged periods (Fig. 8B). As previously observed, both treatments induced the decrease of cyclin D1 protein levels but at different rates (Fig. 8B, panel a, compare lanes 2–4 with lanes 5–7 and lanes 9–10 with 12–14). Furthermore, the efficacy of dicoumarol treatment was independent of eIF2α phosphorylation, and dicoumarol itself did not induce eIF2α phosphorylation (Fig. 8B, panel b). Interestingly, we also observed that prolonged thapsigargin treatment could induce cyclin D1 degradation in eIF2α A/A as well as S/S cells (Fig. 8B, compare lanes 1–4 with 8–11). This is in contrast to short thapsigargin treatments in the same cell lines, in which no decrease in cyclin D1 protein levels is detected (Fig. 2A, panel a). To determine whether this late regulation is dependent upon eIF2α kinase activity, we performed thapsigargin and dicoumarol treatments in PERK+/+ and PERK−/− cells for 24 h (Fig. 8C). In PERK wild type cells, as in eIF2α S/S cells, both thapsigargin and dicoumarol induced the down-regulation of cyclin D1 (Fig. 8C, panel a, lanes 2 and 3). Prolonged treatment with both reagents also induced the degradation of cyclin D1 in PERK−/− cells (Fig. 8C, panel a, lanes 5 and 6). Together this data indicates that the degradation of cyclin D1 upon prolonged thapsigargin treatment occurs independently of both eIF2α phosphorylation and kinase activity.

DISCUSSION

In this study we observed a similar negative regulation of cyclin D1 protein levels after eIF2α kinase activation as noted previously (28–30, 51) and further revealed a novel property of eIF2α kinases in promoting the proteasome-dependent degradation of cyclin D1. Our data provide evidence that the decrease in cyclin D1 levels observed upon activation of PKR and PERK is not due to their ability to inhibit translation initiation but via their ability to induce degradation of the protein. Results obtained from both a conditionally active PKR system and human fibrosarcoma cells demonstrate that whereas cyclin D1 protein levels decrease upon activation of the eIF2α kinases (Figs. 1, A and B, and 3A), translation of the CCND1 transcript does not differ once overall...
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translation is inhibited (Figs. 1C and 3B). We further demonstrated that although cyclin D1 is not inhibited at the translational level after PKR or PERK activation, eIF2α phosphorylation is still required for the down-regulation of cyclin D1 protein (Fig. 2A). How cyclin D1 mRNA bypasses the global inhibitory effects of eIF2α phosphorylation on translation is not immediately clear. Previous work demonstrated an important role of eIF4E in stimulating cyclin D1 mRNA translation (52). We recently reported that activation of eIF2α kinases can also lead to the phosphorylation of 4E-BP1 (53). Inasmuch as 4E-BP1 phosphorylation results in the dissociation of eIF4E/4E-BP1 complex (54), the possibility remains that free eIF4E selectively maintains cyclin D1 mRNA translation under conditions of increased eIF2α phosphorylation. The presence of the proteasome inhibitor MG132 ablated the down-regulation of cyclin D1 upon induction of PKR and PERK activity, strongly supporting the hypothesis that eIF2α kinases induce degradation of the protein rather than inhibiting its synthesis (Fig. 4, A, B, and E, and 5B). Cyclin D1 levels also decreased upon treatment of cells with the translation inhibitor cycloheximide (Fig. 5C, panel a, lanes 1–3), but this down-regulation could not be rescued by MG132 (Fig. 5C, panel a, lanes 4 and 5). Furthermore, when the same cells were treated with both courmymycin and cycloheximide in conjunction, cyclin D1 levels decreased more rapidly than when either agent used alone (Fig. 5D). This suggested that activated GyrB.PKR promotes the proteasomal degradation of cyclin D1 that remains in the cells after the blockade of mRNA translation by cycloheximide. Our study also indicated that this function of eIF2α kinases is limited to a small number of specific proteins. That is, activation of GyrB.PKR did not exhibit a global effect on proteasome-dependent degradation of cellular proteins (Fig. 5A), strongly suggesting that the effect of eIF2α kinases in mediating the degradation of cyclin D1 is specific rather than general.

A number of different cellular pathways have been identified that contribute to the ubiquitination and subsequent degradation of cyclin D1. As described above, phosphorylation at Thr-286 by GSK-3β is required for the ubiquitination of cyclin D1 (42). The SCFβTrCP/αB-crystallin complex has been identified as an E3 ubiquitin ligase-targeting cyclin D1 (55). Our group recently published a study in which we demonstrated that GSK-3β is downstream of the eIF2α kinases (38). However, recent studies have called into question the significance of the contribution of GSK-3β to cyclin D1 regulation (56). Furthermore, data obtained in our study indicate that eIF2α kinases do not induce the degradation of cyclin D1 via this particular pathway (supplemental Fig. 1A).

Further studies have also implicated another well characterized signaling pathway, the MAPK pathway in regulating the degradation of cyclin D1. In cancer cells increased signaling through the Ras/Raf/MEK/MAPK pathway leads to increased phosphorylation at Thr-286 and the subsequent destabilization of cyclin D1 (44). A separate study also implicated another SCF E3 ubiquitin ligase, the S-phase kinase-associated protein (SKP1), and the scaffolding protein Cullin 7 (CUL7) in this regulation (57). SKP1 and CUL7 associate via the F-box protein FBXW8, and the co-operative action of these pathways regulates cyclin D1. Inhibition of the MAPK pathway using a specific inhibitor of MEK indicates that the action of eIF2α kinases is not exerted via MAPK activation (supplemental Fig. 1B). In combination, these data indicate that the regulation of cyclin D1 stability is not mediated by either of these previously described mechanisms. This hypothesis is further supported by the fact that although cyclin D1 is rapidly degraded upon induction of eIF2α phosphorylation, inhibition of 26 S proteasome activity after activation of PKR is sufficient to cause a nearly complete rescue of cyclin D1 levels (Fig. 5B). This indicates that the upstream pathways that contribute to cyclin D1 ubiquitination and degradation remain functional. Indeed, our study revealed that upon eIF2α kinase activation, ubiquitination of cyclin D1 occurs and remains unchanged. Based on this evidence, however, it cannot be concluded that PKR and PERK act in an ubiquitin-independent manner. On the contrary, PERK activation was unable to induce the degradation of cyclin D1 in temperature-sensitive cells defective in E1 ubiquitin ligase activity (Fig. 7A).

Although the ubiquitin-dependent degradation of cyclin D1 has been extensively examined, a number of other, ubiquitin-independent pathways leading to cyclin D1 degradation have been discovered. A 2004 study also identified an alternative mechanism of cyclin D1 degradation (58) in which the regulatory protein antizyme that predominantly functions to regulate the degradation of another enzyme, ornithine decarboxylase (59), binds to cyclin D1 and directs it to the 26 S proteasome. Because it does not depend on the presence of ubiquitin, this regulation is also independent of phosphorylation of cyclin D1 at Thr-286. The T286A mutant form of the protein is degraded in an identical manner as wild type cyclin D1 upon induction of antizyme expression (58). We postulated that due to the unique mechanism in which antizyme is translated (60), the eIF2α kinases may induce antizyme expression, but induction of PERK and GyrB.PKR activity caused no significant change in antizyme expression (data not shown). The ubiquitin-independent degradation of ornithine decarboxylase can be regulated by a separate mechanism involving NQO1 (48). In contrast to the action of antizyme, NQO1 binds to ornithine decarboxylase and consequently stabilizes the protein. NQO1 has also been demonstrated to regulate the tumor suppressor protein p53 via the same mechanism (49, 50). We determined that in addition to ornithine decarboxylase and p53, NQO1 can also negatively regulate the degradation of cyclin D1 (Fig. 8, A–C). Treatment of cells with dicoumarol caused a decrease in cyclin D1 protein levels, albeit at a later time than either courmymycin or thapsigargin treatment. This suggests that the two pathways are capable of independently regulating cyclin D1. The existence of this additional mechanism of regulation is supported by our observation that prolonged treatment with reagents inducing eIF2α kinase activity (in this case, thapsigargin) can induce cyclin D1 degradation independent of eIF2α phosphorylation and PERK activity (Fig. 8, B and C). It is possible, therefore, that early degradation of cyclin D1 depends on eIF2α phosphorylation, whereas late degradation proceeds through a mechanism independent of eIF2α altogether.
This is the second instance we have documented of eIF2α kinases inducing the degradation of a protein (38). That is, we recently demonstrated that the eIF2α kinases can promote the degradation of tumor suppressor p53 (38). However, unlike cyclin D1, the proteasomal degradation of p53 is independent of eIF2α phosphorylation and involves the activation of GSK-3β. In regard to cyclin D1 and eIF2α phosphorylation, there is a precedent for a link between translational inhibition and protein degradation. For example, Rad23 and Rpn10 are two proteins that play a role in the recognition of ubiquitinated proteins by the proteasome by interacting with both the degradation targets and the proteasomal machinery. The yeast rad23Δrpn10Δ strain is hyper-sensitive to translational inhibitors; however, this sensitivity can be suppressed by the overexpression of the translation elongation factor 1A (eEF1A) (61). In addition to its traditional role of promoting the binding and release of aminoacyl tRNAs from the ribosome, eEF1A also binds to nascent and unfolded proteins and peptides (62, 63) and can escort them to the proteasome. This suggests that Rad23 and Rpn10 perform a type of translation quality control, promoting the degradation of damaged immature translation products in conjunction with eEF1A. Unlike eEF1A, the function of phosphorylated eIF2α in proteasomal degradation appears to be rather specific (Fig. 5A).

The implications of our study may be significant, as our data expand on the current knowledge of the function of eIF2α kinases. It suggests that in addition to regulating cellular protein synthesis, eIF2α also promotes the proteasome degradation of specific target proteins. Because it promotes progression through the cell cycle, expression of cyclin D1 is frequently increased in various forms of cancer (for review, see Ref. 2). Therefore, pharmacological activation of eIF2α phosphorylation, as is now possible with salubrinal (64) and its derivative Sal003 (40), may prove to be useful in the treatment of cancers overexpressing cyclin D1. Moreover, the ability of PKR to contain virus replication may not be linked to translation inhibition only but also to proteasomal degradation. For example, our previous studies showed the ability of PKR to down-regulate the human papilloma virus E6 protein (32) in a manner that is dependent on the proteasomal degradation of the viral oncoprotein.3 There may be other implications as well, as PERK also contributes to normal pancreatic function, and the loss of this kinase has been implicated in the development of diabetes (66). eIF2α phosphorylation itself has also been implicated in the induction of gluconeogenesis in the liver, possibly via GCN2 activity (67). Induction of proteasome-mediated degradation in response to eIF2α phosphorylation might play a role in maintaining the balance of proteins in the cell and contributing to glucose homeostasis given the emerging view about a link between proteasomal degradation and insulin resistance (65). Thus, the cross-talk between pathways regulating protein synthesis by eIF2α phosphorylation and protein degradation may have a significant impact in diseases such as viral infection and diabetes as well as cancer.

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