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Oxidative stress-induced cyclin D1 depletion and its role in cell cycle processing

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ABSTRACT

Background: Cyclin D1 is immediately down-regulated in response to reactive oxygen species (ROS) and implicated in the induction of cell cycle arrest in G2 phase by an unknown mechanism. Either treatment with a protease inhibitor alone or expression of protease-resistant cyclin D1 T286A resulted in only a partial relief from the ROS-induced cell cycle arrest, indicating the presence of an additional control mechanism.
Methods: Cells were exposed to hydrogen peroxide (H_2O_2), and analyzed to assess the changes in cyclin D1 level and its effects on cell cycle processing by kinase assay, *de novo* synthesis, gene silencing, and polysomal analysis, etc.
Results: Exposure of cells to excessive H_2O_2 induced ubiquitin-dependent proteasomal degradation of cyclin D1, which was subsequently followed by translational repression. This dual control mechanism was found to contribute to the induction of cell cycle arrest in G2 phase under oxidative stress. Silencing of an eIF2α kinase PERK significantly retarded cyclin D1 depletion, and contributed largely to rescuing cells from G2 arrest. Also the cyclin D1 level was found to be correlated with Chk1 activity.
Conlclusions: In addition to an immediate removal of the pre-existing cyclin D1 under oxidative stress, the following translational repression appear to be required for ensuring full depletion of cyclin D1 and cell cycle arrest. Oxidative stress-induced cyclin D1 depletion is linked to the regulation of G2/M transit via the Chk1–Cdc2 DNA damage checkpoint pathway.
General significance: The control of cyclin D1 is a gate keeping program to protect cells from severe oxidative damages.

1. Introduction

The cell cycle is a precisely regulated global process, and ultimately determines the fate of organisms. The cell cycle is very sensitive and can be altered by factors present in the extracellular environment including growth factors, nutrients and other stress signals. Each step of the cell cycle has been clearly defined with 'check point' molecules such as cyclins and cyclin dependent kinases (CDK) [1–3]. These different cyclin/cdk complexes drive initiation of phase transition, DNA replication and mitosis at each stage. In actively proliferating cell, cyclin proteins are indispensable components to control the cell cycle. Therefore, expression, modification and elimination of cyclins are tightly regulated by environmental conditions [1,4].

Among the various cyclins, D-type cyclin has been shown to be a more hypersensitive protein than other cyclins because their post-transcriptional and post-translational modulations are very dynamic in response to a variety of extracellular stimuli [5], including oxidative stress [6,7]. D-type cyclin plays a role in the progression of the G0/G1 phase and this activity is associated with Cdk4 and 6 [8,9]. These molecules form complexes with cyclin A/E and Cdk2 and promote the G1/S phase transition through retinoblastoma phosphorylation and E2F-dependent gene induction [9,10]. Therefore, D-type cyclin, especially cyclin D1, is found typically in the G1 phase. However, there is increasing number of recent reports suggesting the necessity of cyclin D1 induction in G2 phase in many cases [11,12], including Ras-dependent mitosis progression [13]. Generally, the level of cyclin D1 is regulated by many factors and multiple steps including transcription, translation, RNA stability, and protein stability [13,14]. Particularly, post-translational modification of cyclin D1 is the most well-known mechanism required for the G1/S transition and occurs via phosphorylation of its Thr 286 by the activation pathway including glycogen synthase kinase 3β (GSK3) [15]. This phosphorylation triggers nuclear export and ubiquitin-mediated proteolysis of cyclin D1 during the S phase and this process can be controlled by additional kinases, such as ATM, MAPK and IKK [16–18]. A recent report indicated that eIF2α phosphorylation was required for the regulation of cyclin D1 activity [19]. Although it is still controversial, some reports have demonstrated that the accumulation of abnormal cyclin D1, such as mutant or splicing variant of cyclin D1, in the nucleus causes genomic instability [20], and may contribute to tumorigenesis [21,22]. In fact, a cyclin D1 splicing variant, cyclin D1b, was found in some cancer cell

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lines and tissues. This alternative product lacks the C-terminal region including the PEST domain and T286 residue, which are required for nuclear export and ubiquitin-dependent proteolysis. Therefore, change in cyclin D1 level or its structural alteration was suggested to be critical for cell transformation and tumor growth [23].

There are numerous environmental factors that can influence the cell cycle control. Oxidative stress is a likely efficient contributor to the cell cycle. Reactive oxygen species (ROS) are regarded as the most suitable biomarker to evaluate the balance in the redox (reduction-oxidation) state of tissues. ROS produced by normal metabolism can be associated with cell proliferation or apoptosis via cell cycle factors, kinases and transcription factors [24–26], and are considered second messengers [27,28]. However, oxidative stress with an excess of ROS has been emerged in a variety of cellular events, including pathogenic viral infections [29], protein mistranslation [30], diabetic-related disease [31], cardiovascular disease [32] and phagocytic cells-mediated inflammation [33,34]. Also, overexpression of ROS can be induced by extracellular stimuli, such as UV irradiation [35], heavy metal [36] and chemotherapeutic drugs [37]. It has been reported that intracellular H_2O_2 concentration is approximately ten-fold lower than extracellular [38,39] and thus higher H_2O_2 concentration than theoretical concentration has been treated to derive oxidative stress condition in many previous reports [29,34,40–42]. Therefore, in culture system, it is likely that treatment of cells with H_2O_2 for reproducing acute oxidative stress requires a tricky control because pre-existing ROS scavenging enzymes may cause the difference between the concentration of extracellularly administrated H_2O_2 and that of intracellular H_2O_2 [43]. When cells are exposed to a relatively high level of ROS for a prolonged period, the cell cycle progression of most cells is dysregulated, which subsequently can lead to cell cycle arrest or cell death [44]. In response to various genotoxic stresses, checkpoints kinases (Chks) showed rigorous quality control of cell cycle to maintain genomic integrity. Although recent studies on the ATM (Ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) protein kinases suggested that Chks are involved in the redox regulation [42], relevance of Chks in modulation of oxidative stress-mediated cell cycle arrest remains largely unexplored.

A number of studies reported the effects of relatively low levels of ROS on cell cycle control including G1 arrest [45–48]. As mentioned above, however, cells can produce high levels of ROS in certain local areas in response to a variety of extracellular stimuli. In this study, we focused our attention on effects of exposure of ROS on the regulation of cyclin D1. We demonstrated that ROS induces proteasomal degradation of cyclin D1 followed by PERK activation. Also, we showed that the ROS-induced regulation of cyclin D1 is linked to the processing of G2/M cycle via the Chk1 pathway. Based on these experiments, we found that cyclin D1 is a pivotal effector in the Chk1-Cdc2 DNA damage checkpoint pathway and consequently controls the cell cycle under oxidative stress.

2. Materials and methods

2.1. Cell culture and reagents

HeLa (human cervical cancer cell) and HEK293 (human embryo kidney cell) were maintained in exponential growth at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 or DMEM (GIBCO) with 10% fetal bovine serum and antibiotics (100 μg/ml streptomycin, 100 IU/ml penicillin). *Perk*$^{+/+}$ MEF (murine embryonic fibroblast) and MEF *Perk* KO cells were kindly provided by Dr. Randal J. Kaufman. H_2O_2 and thymidine (Sigma) were diluted in PBS. ER stress inducer, thapsigargin (Sigma), and proteasome inhibitor MG132 (AG Scientific) were dissolved in DMSO as recommended by the manufacturers.

2.2. Plasmids and transfection

To construct a Flag tagged expression vector, pcDNA6-Flag-Cyclin D1, Flag fragment and cyclin D1 cDNA were inserted into *Pst*I-*Eco*RV and *Eco*RV sites of pcDNA6 (Invitrogen), respectively. Site directed mutagenesis of cyclin D1 with change Thr 286 to Ala 286 (T286A) was performed by PCR to generate pcDNA6-Flag-Cyclin D1 T286A. To construct pGEX4T3-GST-eIF2α, cDNA encoding eIF2α was amplified by PCR, and inserted to *Bam*HI-*Xho*I sites of pGEX-4T3 (GE Healthcare). All vectors were verified by automated DNA sequencing. pMT2-eIF2α mutant (S51A) was described elsewhere [16]. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) and ExGen 500 (Fermentas) as recommended by the manufacturers.

2.3. Antibodies and Western blotting

Normalized cell lysates were separated by SDS-PAGE and immunoblotted with antibody against PKR, PERK, actin, cyclin D1, cyclin A1 or cyclin E1 (Santacruz). Antibody against phospho-eIF2α, phospho-Chk1, phospho-Chk2, phospho-GSK3β and phospho-Cdc2 were purchased from Cell Signaling. Antibody against cyclin B1, Cdc25, GADD153 and eIF2α were purchased from GeneTex.

2.4. shRNAs and siRNAs

For stable knockdown of PKR and PERK, Mission shRNA™ vectors were purchased from Sigma. Target cells were transfected with these vectors and subcultured under puromycine. For transient knockdown, GFP, PKR and PERK, and Chk1 siRNA mixture were chemically synthesized (Genolution) as the following sequences.: GFP sense (5′-GCAGCACGACUUCUUCAAGUU-3′), GFP antisense (5′-CUUGAAGAAGUCGUGCUGCUU-3′), PKR sense (5′-GGUGAAGGUAGAUCAAAGAUU-3′), PKR antisense (5′-UCUUUGAUCUACCUUCACCUU-3′), PERK sense (5′-GUGGCAAGAAAAGAUGGAUUU-3′), PERK antisense (5′-AUCCAUCUUUUCUUGCCACUU-3′), GCN2 sense (5′-GGGAAAUGUAUUGGCAGUGUU-3′), GCN2 antisense (5′-CACUGCCAAUACAUUUCCCUU-3′), Chk1 sense (5′-GGGCUAUCAAUGGAAGAAAUU-3′), Chk1 antisense (5′-UUUCUUCCAUUGAUAGCCCUU-3′). Cellular uptake of siRNAs was performed using Lipofectamine 2000 (Invitrogen) or INTERFERin (Polyplus) following the manufacturer's instructions.

2.5. In vitro kinase assay

Cells were solubilized in 1% NP-40 lysis buffer containing protease and phosphatase inhibitors. Whole lysates were pre-clearing with rabbit IgG and protein agarose A/G (Santacruz) for 30 min at 4°C. PERK kinases were collected by immunoprecipitation with anti-PERK (Santacruz) from total lysates. The kinase assay was performed using recombinant GST-eIF2α with 10 μCi of [γ-^{32}P]ATP for 15 min at 30°C. Reactions were resolved on SDS-PAGE and visualized using autoradiography.

2.6. Cell synchronization and cell cycle analysis

When necessary, cells were synchronized at G2/M or G1/S with treatment of thymidine-nocodazole or double-thymidine as previously described [49]. In most experiments, however, synchronization was omitted to exclude the possibility of cyclin D1 decrease due to the synchronization itself.

For cell cycle analysis, cells were harvested and washed with cold PBS and then fixed with 70% ethanol for 24 hr at −20°C. After collecting the fixed cells by centrifugation, cells were resuspended with PBS containing RNase A (500 ng/ml) and then incubated in 37°C for 1 hr. Finally, cells were stained with propidium iodide (400 ng/ml) and then DNA content was immediately analyzed using flow cytometry. Analysis was performed on FACSCalibur (BD Bioscience). The ratio of cell cycle phase was calculated by Cell Quest™ and FlowJo™ software.