Osmotic Stress Regulates the Stability of Cyclin D1 in a p38SAPK2-dependent Manner*

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We report here that different cell stresses regulate the stability of cyclin D1 protein. Exposition of Granta 519 cells to osmotic shock, oxidative stress, and arsenite induced the post-transcriptional down-regulation of cyclin D1. In the case of osmotic shock, this effect was completely reversed by the addition of p38SAPK2-specific inhibitors (SB203580 or SB220025), indicating that this effect is dependent on p38SAPK2 activity. Moreover, the use of proteasome inhibitors prevented this down-regulation. Thus, osmotic shock induces proteasomal degradation of cyclin D1 protein by a p38SAPK2-dependent pathway. The effect of p38SAPK2 on cyclin D1 stability might be mediated by direct phosphorylation at specific sites. We found that p38SAPK2 phosphorylates cyclin D1 in vitro at Thr286 and that this phosphorylation triggers the ubiquitination of cyclin D1. These results link for the first time a stress-induced MAP kinase pathway to cyclin D1 protein stability, and they will help to understand the molecular mechanisms by which stress transduction pathways regulate the cell cycle machinery and take control over cell proliferation.

Mammalian cell cycle progression depends on the sequential activation of different members of a family of serine-threonine kinases named cyclin-dependent kinases (CDKs).1 The activity of these kinases is positively regulated by cyclin binding, and phosphorylation by the CDK-activating kinase. The activity is also modulated negatively by phosphorylation at specific residues of the CDKs and by the binding of CDK inhibitors (1–3). Progression through G1 phase is controlled first by CDK4 and CDK6, which bind combinatorially to cyclins D1, D2, and D3; and later on by CDK2, which associates with cyclin E (4). During G1, these complexes are responsible for the phosphorylation of different members of the pocket proteins family, which includes the retinoblastoma protein, p107, and p130 (5–8). The hyperphosphorylation of the pocket proteins leads to the trans-activation of genes that are necessary for the onset and progression of DNA replication (9–11).

Quiescent cells contain low levels of d-type cyclins. After growth factor stimulation, their synthesis is induced, and then cyclin D1-CDK4/6 complexes can be formed during G1 (12, 13). Cyclin D1 expression, assembly of cyclin D-CDK complexes, and their nuclear translocation require the activation of Ras, Raf1, MAP kinase kinase 1/2, ERKs, and the transcription factor c-Ets-2 (14–17). The maintenance of active cyclin D1-CDK4/6 complexes requires persistent mitogenic signaling, and mitogen withdrawal cancels cyclin D1 synthesis and cyclin D1-CDK4 complexes rapidly dissipate (18). Cyclin D1 turnover is regulated by degradation, mediated by phosphorylation of a specific threonine residue (Thr286) located near the carboxyl terminus. This phosphorylation promotes its polyubiquitination and subsequent degradation by the 26 S proteasome (19). Recently, an alternative mechanism of cyclin D1 ubiquitination, independent of Thr286 phosphorylation, has been described (20). The Thr286 phosphorylation of cyclin D1 is catalyzed by glycogen synthase kinase 3β (GSK3β), which is active in quiescent cells, but its activity is strongly decreased during proliferation (21). Its inactivation is mediated by site specific phosphorylation by c-Akt (also named protein kinase B), which in turn is controlled by a Ras-activated pathway that includes phosphatidylinositol-3-OH kinase (22, 23). Thus, Ras-activated pathways increase cyclin D1 stability by inhibiting GSK3β activity in proliferating cells (21).

A variety of stresses induce growth arrest in bacteria and yeast but also in mammalians. Hyperosmolarity causes growth arrest in murine kidney cells, although the mechanisms involved in the proliferation blockade remain unknown (24, 25). Low level oxidative stress causes a mitotic arrest by selective activation of MAP kinase kinase 3/6 and p38 SAPK stress signaling pathway (26). Other reports indicate that lipopolysaccharide blocks CSF 1-induced cyclin D1 and CDK4 expression and proliferation in macrophages (27, 28).

The cellular MAP kinase modules that are responsible for stress signaling transduction are JNK and p38SAPK families, but their specific implication in the regulation of cell cycle machinery is still poorly understood. The stress-activated serine-threonine kinase p38SAPK2 promotes the transcriptional down-regulation of cyclin D1, thus having opposite effects to those triggered by the Ras-activated pathways (14). Cells overexpressing p38SAPK2 have reduced levels of cyclin D1, whereas blocking p38SAPK2 activity by the specific inhibitor SB203580 enhances cyclin D1 transcription and protein levels (14). Thus, it is clear that p38SAPK2 negatively regulates the expression of cyclin D1 at the transcriptional level, although it...
remains to be established whether p38SAPK2 modulates cyclin D1 post-transcriptionally.

We report here that different cellular stresses regulate the stability of cyclin D1 protein. Likewise, we demonstrate that, in the case of osmotic shock, this down-regulation is mediated by p38SAPK2 and is dependent on proteasome degradation. The effect of p38SAPK2 on cyclin D1 stability might be mediated by direct phosphorylation at specific sites. We found that p38SAPK2 phosphorylates cyclin D1 in vitro at Thr286, and this phosphorylation triggers its ubiquitination and targets it for degradation by the proteasome. These results link for the first time a stress-induced MAP kinase pathway to cyclin D1 protein stability, and they will help in understanding the molecular mechanisms by which stress transduction pathways regulate the cell cycle machinery and take control over cell proliferation.

EXPERIMENTAL PROCEDURES

Cell Cultures—Granta 519 cell line was obtained from the German Tissue Bank and was grown in Dulbecco’s modified Eagle’s medium (Biological Industries) supplemented with 10% fetal calf serum under a 10% CO2 atmosphere. Molt-4 lymphoblastoid cell line was grown in RPMI 1640 (Biological Industries) supplemented with 10% fetal calf serum. Osmotic shock was performed by the addition of 50 mM NaCl, 50 mM CaCl2, or 50 mM MgCl2 to the culture media. Oxidative stress and arsenite treatment were performed by the addition of 500 μM of H2O2 or sodium arsenite (NaAsO2) (Sigma), respectively, to the culture media. The specific p38SAPK2 inhibitors SB203580 and SB220025 (Calbiochem) were used at final concentrations of 10 μM.

Expression and Purification of Recombinant Proteins—All recombinant proteins were obtained as glutathione S-transferase (GST) fusion proteins. Cyclin D1 cDNA was obtained by digesting pET3d-cyclin D1 (29, 30) with HindIII digestion and the pGEX-KG vector (31) at the same sites. Cyclin D1 fragments were obtained by subcloning into pGEX-KG the NcoI digestion and the NcoI-HindIII digestion from pGEX-cyclin D1 for cyclin D1(1–200) and cyclin D1(201–295) fragments, respectively.

For the expression and purification of all these recombinant proteins, the resulting plasmids were transformed into Escherichia coli BL21 (DE3) strain carrying the pLysS plasmid. The expression and purification was performed as described in Ref. 32 with minor modifications. After purification, all recombinant proteins were resuspended in Kicine Buffer (25 mM HEPES, pH 7.4, and 10 mM MgCl2).

p38SAPK2 Activation in Molt-4 Cells—To activate p38SAPK2 in Molt-4 cells, 0.4 mM NaCl was added to the cell culture, and 10 min later cells were harvested and washed once with cold phosphate-buffered saline. The mixture was incubated for 1 h at 37 °C, and then the recombinant GST-fusion substrates were re-purified using glutathione-agarose beads (Amersham Pharmacia Biotech) as described by the manufacturer. The GST-fusion substrates were separated by SDS-polyacrylamide gel electrophoresis and the presence of ubiquitinated cyclin D1 forms was analyzed by Western blotting using an anti-GST antibody (Santa Cruz, SC-138) and an anti-ubiquitin antibody (Sigma U-5379).

RESULTS

Different Cellular Stresses Regulate Cyclin D1 Stability—To study whether cellular stresses regulate the stability of cyclin D1 protein, the effect of osmotic shock, oxidative stress, and sodium arsenite on cyclin D1 protein levels was analyzed in proliferating Granta 519 cells. This cell line was derived from a case of high grade non-Hodgkin’s lymphoma, has a mature B cell immunophenotype, and harbors a translocation t(11; 14)(q13;q32) (33). This translocation is one of the most frequent alteration in mantle cell lymphomas and juxtaposes the bcl-1 (cyclin D1) locus to immunoglobulin gene sequences that lead to deregulation of cyclin D1 transcriptional expression (34). Thus, Granta 519 cells overexpress cyclin D1 in a constitutive manner, being a helpful model to analyze the post-transcriptional regulation of cyclin D1. Osmotic shock (50 mM NaCl, 50 mM CaCl2, or 50 mM MgCl2), oxidative stress (500 μM H2O2), or arsenite (100 μM NaAsO2) induced a down-regulation of cyclin D1 protein levels (Fig. 1). Interestingly, cyclin D1 protein decrease induced by oxidative stresses was higher than those observed by oxidative stress or arsenite. To study whether deregulation was implicated in these cyclin D1 protein decreases, three proteasome inhibitors (aLLnL, lactacystin, or MG132) were added to stressed Granta 519 cells. In all cases, the proteasome inhibitors prevented the down-regulation of cyclin D1 and even caused different levels of accumulation of this protein (Fig. 1). Thus, these cellular stresses down-regulate cyclin D1 by increasing its degradation by the proteasome.

To analyze the involvement of stress MAP kinase pathways in the regulation of cyclin D1 stability, we measured the effects of the p38SAPK2 inhibitors SB203580 and SB220025 (Calbiochem) on untransfected and on cyclin D1 transfected Granta 519 cells. The inhibition studies were performed in proliferating Granta 519 cells. In all cases, the proteasome inhibitors prevented the down-regulation of cyclin D1 and even caused different levels of accumulation of this protein (Fig. 1). Thus, these cellular stresses down-regulate cyclin D1 by increasing its degradation by the proteasome.

In Vitro Ubiquitination Assay—GST-cyclin D1 was phosphorylated with recombinant p38SAPK2 as described above but using cold ATP instead of [γ-32P]ATP. After phosphorylation reaction, 60 μl of reticul-ocyte cell lysate (Promega) were added and the sample incubated for 1 h at 37 °C. After stopping the reaction with SDS-sample buffer, the samples were electrophoresed and the proteins transferred to Immobilon membranes. The band corresponding to phosphorylated GST-cyclin D1 was excised and treated with 6% HCl at 110 °C for 1.5 h. The samples were then lyophilized, and the hydrolyzed amino acids were resuspended in the presence of 1 M HCl, 30% TCA, and 1 M phosphothreonine, and phosphoeryosine (Sigma), which were used as mobility standards. Finally, the samples were subjected to two-dimensional running (1000 V, 80 min, pH 1.9; and 1000 V, 80 min, pH 3.5).

Point Mutations—The Thr156→Ala mutation was obtained by two-stage PCR using megaprimers. The first PCR reaction was performed using a 21-mer middle forward oligonucleotide (5′-ggGGTGCAAGTCCGCCGCACTGGA-3′) carrying the mutation T156A (shown in bold) and a 30-mer reverse terminal oligonucleotide (5′-gACCTCGAGATTACAGTCGGCCATCTCCCG-3′). The second PCR product was used as a 30-mer initial forward oligonucleotide (5′-AACCCGgggATCCATggaACACAgCTCTg-3′) and the first PCR product (megaparam of 448 base pairs) as reverse mutated primer. The T156A substitution was obtained by single PCR amplification using a 30-mer initial forward oligonucleotide (5′-AACCCgCggATCCATggAAACAgCTCTg-3′) and a 50-mer terminal reverse oligonucleotide (5′-gGATCCgTggACgTgTAACgATgTggCCgTACCgCaGCgTgTgggTgCgAAgCCgC-3′) carrying the mutation T286A (shown in bold).

Phosphoamino Acid Analysis—First, phosphorylation reactions were performed using 200 ng of recombinant active kinase and 2 μg of recombinant GST-cyclin D1 as substrate in Kinase Buffer containing 2 mM dithiothreitol. Reactions were initiated by the addition of 2 μCi/μl [γ-32P]ATP and performed during 15 min at 37 °C. After stopping the reaction with SDS-sample buffer, the samples were electrophoresed and the proteins transferred to Immobilon membranes. The band corresponding to phosphorylated GST-cyclin D1 was excised and treated with 6% HCl at 110 °C for 1.5 h. The samples were then lyophilized, and the hydrolyzed amino acids were resuspended in the presence of 1 M HCl, 30% TCA, and 1 M phosphothreonine, and phosphoeryosine (Sigma), which were used as mobility standards. Finally, the samples were subjected to two-dimensional running (1000 V, 80 min, pH 1.9; and 1000 V, 80 min, pH 3.5).

Osmotic Stress Regulates Cyclin D1 Stability

To study whether cellular stresses regulate the stability of cyclin D1 protein, the effect of osmotic shock, oxidative stress, and sodium arsenite on cyclin D1 protein levels was analyzed in proliferating Granta 519 cells. This cell line was derived from a case of high grade non-Hodgkin’s lymphoma, has a mature B cell immunophenotype, and harbors a translocation t(11;14)(q13;q32) (33). This translocation is one of the most frequent alteration in mantle cell lymphomas and juxtaposes the bcl-1 (cyclin D1) locus to immunoglobulin gene sequences that lead to deregulation of cyclin D1 transcriptional expression (34). Thus, Granta 519 cells overexpress cyclin D1 in a constitutive manner, being a helpful model to analyze the post-transcriptional regulation of cyclin D1. Osmotic shock (50 mM NaCl, 50 mM CaCl2, or 50 mM MgCl2), oxidative stress (500 μM H2O2), or arsenite (500 μM NaAsO2) induced a down-regulation of cyclin D1 protein levels (Fig. 1). Interestingly, cyclin D1 protein decrease induced by oxidative stresses was higher than those observed by oxidative stress or arsenite. To study whether deregulation was implicated in these cyclin D1 protein decreases, three proteasome inhibitors (aLLnL, lactacystin, or MG132) were added to stressed Granta 519 cells. In all cases, the proteasome inhibitors prevented the down-regulation of cyclin D1 and even caused different levels of accumulation of this protein (Fig. 1). Thus, these cellular stresses down-regulate cyclin D1 by increasing its degradation by the proteasome.
Osmotic Stress Regulates Cyclin D1 Stability

in the down-regulation of cyclin D1, we first determined the activation of p38\textsuperscript{SAPK2} in Granta 519 cells subjected to all those stress treatments. Results revealed that all the treatments activated p38\textsuperscript{SAPK2} as determined by Western blotting using an anti-phospho-p38\textsuperscript{SAPK2} antibody that specifically recognizes the active (dually phosphorylated) form of p38\textsuperscript{SAPK2} (data not shown). Then, a time-course analysis of cyclin D1 protein levels in Granta 519 cells subjected to osmotic stress (50 mM NaCl) were performed in the presence or in the absence of the specific p38\textsuperscript{SAPK2} inhibitor SB203580. Results indicated that cyclin D1 decrease was very rapid and that, when cells were pre-incubated with SB203580, this effect was completely reversed (Fig. 2). To further demonstrate that this effect was specific for p38\textsuperscript{SAPK2}, another chemically different and more potent p38\textsuperscript{SAPK2} inhibitor SB220025 was used (35, 36). Results indicated that this inhibitor also reversed cyclin D1 decrease. The addition of the proteasome inhibitor aLLnL to the incubation medium prevented the osmotic shock-derived cyclin D1 decrease. These results indicate that osmotic shock induces a p38\textsuperscript{SAPK2}-dependent cyclin D1 decrease by triggering its proteasomal degradation.

Similar experiments performed with the same cells under oxidative stress (500 \mu M H\textsubscript{2}O\textsubscript{2}) or arsenite treatment (500 \mu M Na\textsubscript{AsO\textsubscript{2}}) revealed that SB203580 or SB220025 only produced a very low reversion of cyclin D1 degradation (data not shown). These results indicate that, in these cases, p38\textsuperscript{SAPK2} protein did not significantly mediate cyclin D1 degradation.

p38\textsuperscript{SAPK2} Phosphorylates Cyclin D1 in Vitro—Since cyclin D1 stability is regulated by direct phosphorylation at threonine 286 (19, 21), we analyzed whether p38\textsuperscript{SAPK2} was able to directly phosphorylate cyclin D1. Thus, we performed an in vitro kinase assay using recombinant GST-cyclin D1 as a substrate. Active p38\textsuperscript{SAPK2} was immunoprecipitated from osmotically shocked Molt-4 lysates by using an antibody that specifically recognizes the N-terminal sequence of p38\textsuperscript{SAPK2} as described under "Experimental Procedures." The immunoprecipitated p38\textsuperscript{SAPK2} was active as checked by Western blotting using an anti-phospho-p38\textsuperscript{SAPK2} antibody that specifically recognizes the active (dually phosphorylated) form of p38\textsuperscript{SAPK2} (data not shown). In vitro kinase reactions demonstrated that p38\textsuperscript{SAPK2} efficiently phosphorylated purified GST-cyclin D1 but not purified GST-CDK4, GST-CDK2, GST-cyclin A, or GST (Fig. 3A). Myelin basic protein that was used as a control substrate was also efficiently phosphorylated by p38\textsuperscript{SAPK2}. Similar results were obtained when the immunoprecipitation was performed using another anti-p38\textsuperscript{SAPK2} antibody raised against a C-terminal sequence (data not shown). To confirm that no other kinase than p38\textsuperscript{SAPK2} was involved in this phosphorylation, kinase reactions were performed using purified active GST-p38\textsuperscript{SAPK2} instead of that obtained by immunoprecipitation. Purified active GST-p38\textsuperscript{SAPK2} also phosphorylated GST-cyclin D1 but not GST-CDK4, GST-CDK2, GST-cyclin A, or GST. Under these experimental conditions, cyclin D1 phosphorylation was abolished by the presence of SB203580 (Fig. 3B). The affinity constant of p38\textsuperscript{SAPK2} versus cyclin D1 was measured by kinetic analysis. Results showed that the \(K_m\) was 288 nM (Fig. 4). These results indicate that cyclin D1 is a specific substrate for p38\textsuperscript{SAPK2}, and its high affinity, compared with those of p38s to other substrates, suggests that cyclin D1 phosphorylation by this kinase may have physiological relevance (see "Discussion").

Identification of the Cyclin D1 Amino Acid Residues Phosphorylated by p38\textsuperscript{SAPK2}—Since p38\textsuperscript{SAPK2} is a proline-directed serine/threonine kinase, TP or SP motifs were searched in the primary sequence of D-type cyclins. Three putative phosphorylation sites for proline-directed kinases were found in the sequence of cyclin D1. Two of them are threonines (Thr\textsuperscript{286} and Thr\textsuperscript{296}), and the other one is a serine (Ser\textsuperscript{219}). Only one of these three sites (Thr\textsuperscript{296}) is conserved in all \(\beta\)-type cyclins. The Thr\textsuperscript{296} site is conserved in both cyclin D1 and cyclin D2, whereas the Ser\textsuperscript{219} site is only present in cyclin D1.

To analyze whether these sites may be phosphorylated by p38\textsuperscript{SAPK2} kinase assays were performed using two cyclin D1-
noprecipitated p38SAPK2 from Molt-4 lysates and the purified active GST-p38SAPK2 recovered and subjected to two-dimensional electrophoresis. The amino acids were then hydrolysed with HCl as described under "Experimental Procedures," and the phosphoamino acids separated by two-dimensional electrophoresis. The phosphoamino acids were visualized by autoradiography, and their relative position was determined by ninhydrin staining.

These kinase reactions revealed that both the immunoprecipitated p38SAPK2 from Molt-4 lysates and the purified active GST-p38SAPK2a efficiently phosphorylated both cyclin D1 fragments (Fig. 7). These results clearly confirm that the sites of p38SAPK2 phosphorylation were both Thr156 and Thr286.

To determine whether both sites where phosphorylated by p38SAPK2 in the full-length cyclin D1, we expressed two full-length cyclin D1 forms, one containing an Ala for Thr286 substitution and another one containing these substitutions plus an additional Ala for Thr156 substitution. Kinase reactions were performed using these two mutated forms of the full-length cyclin D1. Interestingly, results revealed that both the immunoprecipitated p38SAPK2a from Molt-4 lysates and the purified active GST-p38SAPK2a did not phosphorylate neither of the two mutated forms (cyclin D1-T286A and cyclin D1-T156A-T286A) (Fig. 8). Thus, the substitution of Thr286 by Ala completely abolished the phosphorylation by p38SAPK2a, indicating...

FIG. 3. In vitro phosphorylation of cyclin D1 by p38SAPK2a. Kinase assays were performed using p38SAPK2a obtained by IP from Molt-4 cell lysates (A) or purified active GST-p38SAPK2a (B). A, IPs were performed using a polyclonal anti-p38SAPK2 antibody (α-p38) or a normal rabbit serum (NRS) as a control. GST-cyclin D1, GST-CDK4, GST-CDK2, GST-cyclin A, and GST were used as substrates as indicated. Arrows mark phosphorylated substrates, whereas lines mark those not phosphorylated. B, kinase assays using purified active GST-p38SAPK2a as kinase and GST-cyclin D1, GST-CDK4, GST-CDK2, GST-cyclin A, and GST as substrates were performed in the presence or absence of the p38SAPK2 inhibitor SB203580 (20 μM) as indicated. Assays using myelin basic protein (MBP) as a substrate were performed as a control. Note that GST-p38SAPK2a became autophosphorylated.

FIG. 4. Kinetic analysis of cyclin D1 phosphorylation. Kinase assays were performed using p38SAPK2a obtained by IP from Molt-4 cell lysates, in the presence of growing concentrations of GST-cyclin D1 as a substrate. Results are shown as a Lineweaver-Burk representation where activity is represented as arbitrary units excluding fragments. One of the fragments, cyclin D1-(1–200), contained one of the three putative phosphorylation sites (Thr156) whereas the other fragment, cyclin D1-(201–295), contained the other two putative sites (Ser219 and Thr286). Kinase reactions performed in vitro demonstrated that both the immunoprecipitated p38SAPK2a from Molt-4 lysates and the purified active GST-p38SAPK2a efficiently phosphorylated both cyclin D1 fragments (Fig. 5). These results indicate that both cyclin D1 fragments contain at least one phosphorylation site for p38SAPK2a.

Cyclin D1 amino acid residues phosphorylated by p38SAPK2a were determined by phosphoamino acid analysis. GST-cyclin D1 was phosphorylated by the purified active GST-p38SAPK2a and subjected to acid hydrolysis. The amino acids were then recovered and subjected to two-dimensional electrophoresis. Results revealed that threonine was highly phosphorylated, whereas only slight phosphorylation of serine was observed (Fig. 6). These results suggested that Thr156 and Thr286 are good phosphorylation site candidates. To confirm this possibility, these two threonines were mutated to alanines. Then, we expressed the two fragments of cyclin D1 containing the mentioned mutations (cyclin D1-(1–200)-T156A and cyclin D1-(201–295)-T286A) and in vitro kinase assays were performed.

FIG. 5. In vitro phosphorylation of cyclin D1 fragments by p38SAPK2a. Kinase assays were performed using p38SAPK2a obtained by IP of Molt-4 cell lysates (A) or purified active GST-p38SAPK2a (B). A, IPs were performed using a polyclonal anti-p38SAPK2 antibody (α-p38) or a normal rabbit serum (NRS) as a control. GST-cyclin D1, GST-cyclin D1-(1–200), or GST-cyclin D1-(201–295) were used as substrates as indicated. B, kinase assays using purified GST-p38SAPK2a as kinase and GST-cyclin D1, GST-cyclin D1-(1–200), or GST-cyclin D1-(201–295) as substrates were performed. Experiments in the presence of the p38SAPK2 inhibitor SB203580 (20 μM) were performed as a control. Note that GST-p38SAPK2a became autophosphorylated.

FIG. 6. Phosphoamino acid analysis of in vitro phosphorylated GST-cyclin D1. Purified GST-p38SAPK2a was used to extensively phosphorylate purified GST-cyclin D1 in an in vitro phosphorylation assay. Phosphorylated GST-cyclin D1 was subjected to hydrolysis with HCl as described under "Experimental Procedures," and the phosphoamino acids were visualized by autoradiography, and their relative position was determined by ninhydrin staining.

These kinase reactions revealed that both the immunoprecipitated p38SAPK2a from Molt-4 lysates and the purified active GST-p38SAPK2a efficiently phosphorylated the wild type cyclin D1 fragments but not the mutated fragments (Fig. 7). These results clearly confirm that the sites of p38SAPK2a phosphorylation were both Thr156 and Thr286...
that, in the full-length cyclin D1, this is the major site of phosphorylation. Thr^{286} is not phosphorylated in the full-length cyclin D1, although it is a good phosphorylation site when cyclin D1 is fragmented.

Cyclin D1 Phosphorylation by p38SAPK2 Triggers Its Ubiquitination in Vitro—Phosphorylation of cyclin D1 in Thr^{286} by GSK3β triggers its ubiquitination and degradation by the 26 S proteasome (21, 37). Thus, we analyzed whether phosphorylation of this site by p38SAPK2 also triggers the ubiquitination of cyclin D1. In vitro kinase reactions were performed using the wild type and T286A-mutant forms of cyclin D1 as substrates and the purified active GST-p38SAPK2 as a source of kinase. Then, an *in vitro* ubiquitination assay was performed using the total volume of the kinase assay as a source of phosphorylated and unphosphorylated cyclin D1 and a reticulocyte cell lysate.

**FIG. 7.** *In vitro* phosphorylation of mutated fragments of cyclin D1 by p38SAPK2. Kinase assays were performed using p38SAPK2 obtained by IP of Molt-4 cell lysates (A) or purified active GST-p38SAPK2 (B). A, IPs were performed using a polyclonal anti-p38SAPK2 antibody (α-p38) or a normal rabbit serum (NRS) as a control. GST-cyclin D1, GST-cyclin D1-(1–200), GST-cyclin D1-(1–200/T156A), GST-cyclin D1-(201–295), or GST-cyclin D1-(201–295/T286A) were used as substrates as indicated. B, kinase assays using purified GST-p38SAPK2 as kinase and GST-cyclin D1, GST-cyclin D1-(1–200), GST-cyclin D1-(1–200/T156A), GST-cyclin D1-(201–295), or GST-cyclin D1-(201–295/T286A) were used as substrates as indicated. Experiments in the presence of the p38SAPK2 inhibitor SB203580 (20 μM) were performed as a control. Note that GST-p38SAPK2 became autophosphorylated.

**DISCUSSION**

The regulation of the levels of d-type cyclins is a critical step for G1 progression. Quiescent cells contain low levels of d-type cyclins, and mitogenic stimuli induce their increase by transcriptional and post-transcriptional mechanisms. Studies on the post-transcriptional regulation of cyclin D1 indicate that its phosphorylation has special relevance on the turnover of the protein. During G1, cyclin D1 turnover is rapid (t_{1/2} ~ 20–30 min), whereas that of its catalytic subunit CDK4 is relatively slow (t_{1/2} ~ 4 h) (19, 21, 37–39). Moreover, cyclin D1-CDK4 complexes exist in a dynamic equilibrium between bound and free cyclin D1 that permits continuous replacement of the cyclin subunit of the complex (38). For those reasons, slight changes in cyclin D1 protein levels can rapidly modulate cyclin D1-CDK4/6 activity; thus, the regulation of cyclin D1 protein stability is a crucial step for G1 progression.

Cyclin D1 degradation is regulated by GSK3β, which catalyzes the phosphorylation of a specific threonine residue (Thr^{286}), and this triggers its polyubiquitination and subsequent degradation by the 26 S proteasome (21). The low levels of cyclin D1 in quiescent cells are due to the low rate of synthesis and to the high instability of the protein. This instability is probably due to the high activity of GSK3β in cells deprived of growth factors (40). Mitogenic stimuli induce Ras-dependent signaling pathways that increase cyclin D1 transcription and also down-regulate GSK3β preventing cyclin D1 phosphorylation and degradation. These two mechanisms give a precise and fast control of the total amount of cyclin D1 protein that correlates with cyclin D1-CDK4/6 kinase activity in the cell.

Results reported here revealed that different cellular stresses regulate cyclin D1 stability *in vitro*. For these studies proliferating Granta 519 cells were used because they have an abnormal constitutive expression of cyclin D1. This characteristic makes this cell line very useful to analyze the post-transcriptional regulation of cyclin D1. Osmotic shock and, to a
lesser extent, oxidative stress or arsenite down-regulated cyclin D1 total protein levels in these cells. This effect was due to the increase of cyclin D1 degradation by proteasome, because the addition of different proteasome-specific inhibitors blocked cyclin D1 decrease.

Although all these different stresses induced the activation of p38SAPK2, only in the case of osmotic stress was the down-regulation of cyclin D1 clearly mediated by this kinase. This differential response might be due to the fact that the different stresses can activate distinct additional signaling pathways, which can differentially participate in the modulation of cyclin D1 degradation. Thus, it has been reported that oxidative stress also induced the protein kinase B, ERK, and JNK pathways (41–43). It has also been shown that arsenite activates ERK and JNK pathways in addition to p38SAPK2 (44). A possible involvement of these different pathways might explain why cyclin D1 degradation was not reversed by p38SAPK2 inhibitors in these cases. Alternatively, the participation of SB203580-insensitive isoforms of p38 (p38α or and p38γ) might also explain the lack of reversion by these inhibitors.

p38SAPK2 is a member of the MAP kinase family that is activated by environmental stresses such as osmotic shock and oxidative stress, but also by different agents as arsenite, pro-inflamatory cytokines or UV light (45). p38SAPK2 phosphorylates different protein substrates as GADD153 (46), transcription factors such as ATF2 (47), and protein kinases as MAP kinase-activated protein kinases 2 and 3 (48–50).

Taking together previous reports and results reported here, it appears that p38SAPK2 may be a dual regulator of cyclin D1 levels, because it inhibits cyclin D1 transcription (14), and it triggers cyclin D1 degradation in cells under osmotic stress. Thus, p38SAPK2 regulates cyclin D1 transcription and degradation in the opposite way to Ras during proliferation. These results suggest that stress activated pathways may counteract the effects of mitogen-induced pathways as has been described in Ref. 51. In fact it is known that a variety of stresses induce growth arrest in bacteria, yeast, and mammalian cells. For instance, osmotic stress blocks proliferation in murine kidney cells (24, 25) and lipopolysaccharide blocks CSF 1-stimulated macrophage proliferation (27, 28). Consistent with this anti-mitogenic action, lipopolysaccharide inhibits CSF 1-induced cyclin D1 and CDK4 expression and pRB phosphorylation (52, 53). Osmotic stress and lipopolysaccharide are potent activators of p38 family members. Thus, it is possible that cell cycle arrest in both cases could be mediated by cyclin D1 degradation induced by p38SAPK2.

We have demonstrated that p38SAPK2 phosphorylates cyclin D1 in vitro by using enzymes obtained from two different sources. On one hand p38SAPK2 was obtained by immunoprecipitation of Mol4 cell extracts using specific anti-p38SAPK2 antibodies. On the other hand, a recombinant purified kinase fragment containing the last 95 amino acid residues. Thus, p38SAPK2 phosphorylation of cyclin D1 triggers its ubiquitination and proteasomal degradation (21). This phosphorylation is catalyzed by GSK3β, which triggers in vivo cyclin D1 polyubiquitination and degradation by the proteasome (21). Taken together, these data and results reported here suggest that p38SAPK2 may regulate the stability of cyclin D1 in vivo by phosphorylation at Thr286 and subsequent proteasomal degradation.

Results obtained from the in vitro kinase assays, using the full-length cyclin D1, indicate that the only phosphorylated site was Thr286. These results are similar to those described in Ref. 21, using GSK3β. However, another amino acid residue (Thr156) was also phosphorylated when the protein was truncated. These results suggest that the Thr156 position is masked in the free full-length form of cyclin D1 but it is accessible when protein is truncated by elimination of the carboxyl-terminal fragment containing the last 95 amino acid residues. Thus, Thr156 seems not to be accessible for p38SAPK2 in the free form of cyclin D1. However, it remains to be explored whether in other physiological conditions, for instance in cyclin D1-CDK4 complexes, this residue can be unmasked and consequently physiologically phosphorylated by p38SAPK2.

In summary, results reported here indicate that different types of stresses down-regulate cyclin D1 levels by proteasomal degradation; in the case of osmotic stress, this degradation is mediated by p38SAPK2. The in vitro experimental analysis of cyclin D1 phosphorylation and ubiquitination suggests that this degradation might be induced by direct phosphorylation at Thr286 of cyclin D1 by p38SAPK2. The analysis of the physiological relevance of the phosphorylation of cyclin D1 by p38SAPK2 is currently under way in our laboratory.

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