Ubiquitination of Free Cyclin D1 Is Independent of Phosphorylation on Threonine 286*

Doris Germain‡§, Adrian Russel‖§, Anne Thompson‡, and Joy Hendley‡

From the ‡Peter MacCallum Cancer Institute, St. Andrew’s Place, East Melbourne, Melbourne, Victoria 3002 and §Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, Victoria 3002, Australia

Cyclin D1 binds and regulates the activity of cyclin-dependent kinases (CDKs) 4 and 6. Phosphorylation of the retinoblastoma protein by cyclin D1-CDK4/6 complexes during the G1 phase of the cell cycle promotes entry into S phase. Cyclin D1 protein is ubiquitinylated and degraded by the 26 S proteasome. Previous studies have demonstrated that cyclin D1 ubiquitination is dependent on its phosphorylation by glycogen synthase kinase 3β (GSK-3β) on threonine 286 and that this phosphorylation event is greatly enhanced by binding to CDK4 (Diehl, J. A., Cheng, M. G., Roussel, M. F., and Sherr, C. J. (1998) Genes Dev. 12, 3499–3511). We now report an additional pathway for the ubiquitination of free cyclin D1 (unbound to CDKs). We show that, when unbound to CDK4, a cyclin D1-T286A mutant is ubiquitinylated. Further, we show that a mutant of cyclin D1 that cannot bind to CDK4 (cyclin D1-KE) is also ubiquitinylated in vivo. Our results demonstrate that free cyclin D1 is ubiquitinylated independently of its phosphorylation on threonine 286 by GSK-3β, suggesting that, as has been shown for cyclin E, distinct pathways of ubiquitination lead to the degradation of free and CDK-bound cyclin D1. The pathway responsible for ubiquitination of free cyclin D1 may be important in limiting the effects of cyclin D1 overexpression in a variety of cancers.

Mammalian cell cycle progression is regulated by a family of cyclin-dependent kinases (CDKs). Among the cyclin subunits, D-type cyclins associate with CDK4 and -6 to phosphorylate the retinoblastoma (Rb) protein during the G1 phase of the cell cycle. Hyperphosphorylation of Rb promotes the release of the E2F family of transcription factors that then promote entry into S phase through activation of key target genes (1). Although this function of cyclin D1 is well established, several recent observations suggest that additional roles may exist for free cyclin D1 in the regulation of transcription. First, cyclin D1 has been shown to associate with the estrogen receptor and to promote its transcriptional activity (2–4). Second, cyclin D1 has been shown to associate with the transcription factor DMP1 and to antagonize its ability to induce cell cycle arrest (5). Third, cyclin D1 was shown to affect the transcriptional activity of Sp1 (6), the androgen receptor (7), and v-Myb (8). Although the impact of cyclin D1 interactions with these transcription factors remains to be demonstrated, the fact that the interactions are independent of binding to CDK4 suggests that cyclin D1 may affect cell cycle progression independently from its role in the regulation of CDKs and the phosphorylation of Rb.

D-type cyclins have been suggested to be rate-limiting for the progression from G1 into S phase of the cell cycle, based on the observation that their constitutive overexpression leads to a shorter G1 phase (9, 10). Further, constitutive overexpression of cyclin D1 in mouse mammary glands leads to carcinoma, indicating that cyclin D1 acts as an oncoprotein (11). In human breast cancer, abnormal accumulation of cyclin D1 is observed in as many as 35% of cases (12). However, despite overexpression of cyclin D1 gene, cyclin D1 protein accumulation remains modest because of the ubiquitination and rapid turnover of cyclin D1 by the 26 S proteasome (13). Linkage of ubiquitin to a protein requires the sequential action of the ubiquitin-SKP1 activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3). The SCF complex is an ubiquitin ligase composed of three proteins: CKP1, a Cullin, Rbx 1, and an F-box protein (for a review, see Refs. 14 and 15), where the F-box protein is thought to provide substrate specificity to the ligase complex (16, 17). In yeast, the SCFGrp1 complex is responsible for the ubiquitination of the G1 cyclins Cln1 and Cln2 (18–21). Although human cyclins D1 and D3 can both associate with the cullin Cul-1 (13) and expression of an antisense against the F-box protein SKP2 leads to the accumulation of cyclin D1 (22), the direct involvement of these proteins in the ubiquitination of cyclin D1 remains to be demonstrated.

Phosphorylation of cyclin D1 on threonine 286 is required for its ubiquitination, nuclear export, and degradation in the cytoplasm (23, 24). This phosphorylation is mediated by the glycogen synthase kinase 3β (GSK-3β) and is greatly enhanced by the binding of cyclin D1 to CDK4 (23, 24). We report here that non-CDK4-bound cyclin D1 is ubiquitinylated independently of phosphorylation on threonine 286. These results indicate that, as has been shown for cyclin E (25), distinct pathways exist for the ubiquitination of free and bound cyclin D1. This mechanism may have an important role in limiting the level of free cyclin D1 and hence in negatively regulating G1/S cell cycle progression.

EXPERIMENTAL PROCEDURES

Tissue Culture Conditions, Plasmids, and Transfection—U2OS cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, glutamine, and antibiotics (Life Technologies, Inc.). The vectors expressing wild type cyclin D1, cyclin D1-KE mutant,
HA-tagged cyclin D1, and Myc-tagged ubiquitin under the cytomegalovirus promoter and the plasmid expressing the murine cyclin D1-T286A mutant have been described previously (13, 23). Human cyclin D1-T286A-T288A mutant was generated in the cyclin D1-pCDNA3 plasmid by site-directed mutagenesis as described previously (13). Transfections were performed using Fugene6 according to the manufacturer’s protocol (Roche Molecular Biochemicals).

In Vitro Ubiquitination Assay—Human wild type HA-tagged cyclin D1 and murine or human T286A mutant were labeled with [35S]methionine in an in vitro transcription/translation reaction using the manufacturer’s protocol (Promega). Exponentially growing U2OS cells were trypsinized, and U2OS cell pellets were obtained by centrifugation at 1,000 rpm for 5 min at room temperature. Cell pellets were washed twice with ice-cold phosphate-buffered saline and kept on ice. For cell lysis, pellets were resuspended in 100 μl of 10 mM Tris, pH 8.0, for 10 min and sonicated briefly. Cells debris was removed by centrifugation at 14,000 rpm for 20 min at 4 °C. The protein concentration of the resulting supernatant was determined by spectrophotometry (BCA protein assay, Pierce).

For the ubiquitination assay, 200 μg of total protein was mixed with 10 μl of [35S]-labeled cyclin D1, and the volume was adjusted to 125 μl using reaction buffer (10 mM Tris, pH 7.5, 5 mM CaCl₂, 5 mM MgCl₂). Following incubation at 30 °C for various periods of time, 20 μl of the ubiquitination mix was retrieved and the reaction stopped by adding 5 × SDS sample buffer. For the 26 S proteasome, LLaL was added to the ubiquitination mix to a final concentration of 100 μM. Samples were boiled for 3 min and separated on a 12% SDS-polyacrylamide gel. Following migration, the gel was fixed in 20% methanol, 10% acetic acid for 30 min, incubated with Amplify (Amersham Pharmacia Biotech) for 20 min, dried and exposed to Kodak XR film overnight at −70 °C.

Immunoprecipitation, Western, Pulse-Chase, and Kinase Assays—The protocol used for protein extraction and immunoprecipitation was described previously (13). For immunoprecipitation of cyclin D1, 2 μl of the rabbit polyclonal anti-Myc antibody M-20 (Santa Cruz) was used. For Western analysis, the mouse monoclonal anti-Myc antibody 9E10, the rat monoclonal anti-HA antibody 3F10 (Roche Molecular Biochemicals), the rabbit polyclonal anti-ubiquitin antibody (Sigma), and the rabbit polyclonal anti-Cdk4 antibody H-22 (Santa Cruz) were used at a dilution of 1:100. For Western analysis following the in vitro ubiquitination assay, proteins were transferred in 10 mM CAPS, pH 11, to nitrocellulose for 2 h at 60 V to ensure transfer of high molecular weight proteins. Western blots were developed by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech). [35S] Pulse-chase labeling and immunoprecipitation of cyclin D1 were performed as described previously (13).

For in vitro phosphorylation of cyclin D1 by GSK-3β, cyclin D1-Cdk4 immune complex was diluted into 20 μl of kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 2 mM DTT), 100 μM ATP, and 1 μCi of [γ-32P]ATP and mixed with 1 unit of recombinant GSK-3β (Calbiochem). Reactions were incubated at 30°C for 10 min and stopped by boiling in SDS sample buffer.

RESULTS

Cyclin D1 Is Ubiquinated in Vitro—We have previously obtained evidence that the degradation of cyclin D1 may be defective in 15% of breast cancers (13). However, proof of this hypothesis required the ability to monitor directly the ubiquitination activity in these tumors. As a first step toward this end, we developed an in vitro ubiquitination assay for cyclin D1. Two cell lines were selected to establish this assay. First, U2OS cells were selected, as cyclin D1 is ubiquitinated (IP) and the presence of ubiquitinated cyclin D1 (cyclin D1-[Ub]) detected by immunoblotting (IB) using anti-Myc antibody. To increase the detection of ubiquitinated cyclin D1, cells were treated for 4 h with the 26 S proteasome inhibitor LLaL before protein extraction. For the ubiquitination assay, 200 μl of total protein extract from U2OS (lanes 1–4) and SKUT (lanes 5–8) cells for the indicated periods of time at 30 °C. Following electrophoresis, [35S]-cyclin D1HA was visualized by autoradiography. A ubiquitination assay of 35S-cyclin D1 was performed in the presence of excess GST-cyclin D1 (lane 9) or a control GST-fusion protein, GST-p70 (lane 10). C, following an in vitro assay, cyclin D1HA was immunoprecipitated from the U2OS extract, and Western blotting was developed with either anti-ubiquitin or anti-HA antibodies. As a control, immunoprecipitation was also performed using non-immune serum (NIS).

In Vitro Ubiquitination Assay—In Vitro ubiquitination of cyclin D1. A, U2OS and SKUT cells were transfected with plasmids expressing cyclin D1 and Myc-ubiquitin. 24 h after transfection, cyclin D1 was immunoprecipitated (IP) and the presence of ubiquitinated cyclin D1 (cyclin D1-[Ub]) detected by immunoblotting (IB) using anti-Myc antibody. To increase the detection of ubiquitinated cyclin D1, cells were treated for 4 h with the 26 S proteasome inhibitor LLaL before protein extraction. B, 10 μl of in vitro translated [35S]-cyclin D1HA was incubated in the presence of 200 μg of total protein extract from U2OS (lanes 1–4) and SKUT (lanes 5–8) cells for the indicated periods of time at 30 °C. Following electrophoresis, [35S]-cyclin D1HA was visualized by autoradiography. A ubiquitination assay of 35S-cyclin D1 was performed in the presence of excess GST-cyclin D1 (lane 9) or a control GST-fusion protein, GST-p70 (lane 10). C, following an in vitro assay, cyclin D1HA was immunoprecipitated from the U2OS extract, and Western blotting was developed with either anti-ubiquitin or anti-HA antibodies. As a control, immunoprecipitation was also performed using non-immune serum (NIS).
migrating species of cyclin D1 were detected by both anti-ubiquitin and anti-HA antibodies, confirming that this band represents ubiquitinated forms of cyclin D1HA. However, this band was not detected by either of the antibodies following immunoprecipitation using rabbit non-immune serum (NIS, Fig. 1C). These results indicate that cyclin D1 is ubiquitinated in the presence of U2OS extract in vitro.

Cyclin D1 Is Degraded by the 26 S Proteasome in Vitro—As ubiquitinated proteins are targeted for degradation by the 26 S proteasome, we tested whether inhibition of the proteasome affects the accumulation of ubiquitinated cyclin D1HA in the in vitro assay. The presence of the proteasome inhibitor LLnL led to an enhanced accumulation of ubiquitinated [35S]-cyclin D1HA (Fig. 2A). In addition, although the disappearance of the [35S]-cyclin D1HA band correlated with the accumulation of ubiquitinated [35S]-cyclin D1HA, this effect was not observed in the presence of LLnL (Fig. 2A). This result suggested that ubiquitinated cyclin D1HA is targeted for degradation in vitro. This observation was confirmed by the disappearance of [35S]-cyclin D1HA upon incubation with the U2OS extract for an extended period of time (Fig. 2B). However, cyclin D1 remained stable during this period in the presence of the SKUT extract (Fig. 2B), indicating that the degradation correlates with the ability of cellular extracts to promote the formation of ubiquitinated intermediates. These results confirmed that cyclin D1HA is ubiquitinated and degraded by the 26 S proteasome in this assay.

Human Cyclin D1 Threonine 286 and 288 Residues Are Important for Its Ubiquitination—Previous studies have shown that GSK-3β phosphorylates murine cyclin D1 on threonine 286 and that, when co-expressed with CDK4, the ubiquitination of murine cyclin D1-T286A mutant is abolished (23, 24). Human cyclin D1 has one threonine at this position and another at position 288; to test whether human cyclin D1 behaves in a similar manner as described for murine cyclin D1, we mutated these residues to alanine. The half-lives of human wild type cyclin D1 and cyclin D1-T286A-T288A were determined by pulse-chase analysis (Fig. 3A). The half-life of cyclin D1-T286A-T288A was increased to ~180 min compared with 60 min for wild type cyclin D1. These data show that, as also shown for murine cyclin D1, these residues are important for the half-life of human cyclin D1. However, although greatly reduced, the turnover of cyclin D1-T286A-T288A was not entirely abolished, indicating the presence of some residual proteolytic activity. As this residual degradation was not observed when murine cyclin D1-T286A mutant was co-expressed with CDK4, we next tested the effect of CDK4 co-expression on the turnover of cyclin D1-T286A-T288A mutant. As observed for murine cyclin D1 in the presence of CDK4, cyclin D1-T286A-T288A was completely stabilized over the time course tested, indicating that the residual degradation observed in the absence of CDK4 expression may be because of the turnover of non-CDK4 bound cyclin D1. To address whether the residual activity was associated with the formation of ubiquitinated intermediates, plasmids expressing cyclin D1-T286A-T288A and Myc-ubiquitin were co-transfected in U2OS cells, and the ubiquitination of the cyclin D1-T286A-T288A mutant was assessed by immunoprecipitation and Western blotting. Under these conditions, cyclin D1-T286A-T288A was ubiquitinated (Fig. 3B) although to a lesser extent than observed for wild type cyclin D1 (Fig. 1A). The weak ubiquitination of cyclin D1-T286A-T288A mutant is consistent with the reduced but residual degradation activity observed toward this mutant (Fig. 3A). This result indicates that, analogous to what was described previously for murine cyclin D1, mutations affecting threonine 286 and 288 in human cyclin D1 greatly increase the half-life of cyclin D1. However, our results also show the presence of a residual ubiquitination activity that is independent of phosphorylation at these residues.

Phosphorylation on Thr286 and Thr288 Is Not Required for Ubiquitination of Free Human Cyclin D1 in Vitro—GSK-3β has been shown to phosphorylate preferentially cyclin D1 when bound to CDK4 (24). This result would seem to predict that the turnover of CDK4-bound cyclin D1 is faster than the turnover of free cyclin D1. However, in Rb-negative cells, where cyclin D1 is free as a result of its failure to assemble with CDK4 in the presence of an elevated level of p16, the half-life of free cyclin

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**Fig. 2.** In vitro degradation of cyclin D1. A, assays were performed as described in Fig. 1B in the presence or absence of 5 μm proteasome inhibitor LLnL. B, degradation of [35S]-cyclin D1HA was monitored during a period of 6 h in the presence of U2OS and SKUT extracts.

**Fig. 3.** Residual ubiquitination and degradation of cyclin D1-T286A-T288A mutant in vitro. A, U2OS cells were transfected with plasmids expressing the human wild type (WT) or T286A-T288A-cyclin D1 mutant alone (286) or with CDK4 (286 + K4). 24 h after transfection, cells were metabolically labeled with [35S]methionine for 30 min. Washed cells refed with complete medium containing unlabeled methionine were collected at the indicated times, and proteins were subjected to immunoprecipitation using anti-cyclin D1 antibody. B, plasmids expressing cyclin D1-T286A-T288A and Myc-ubiquitin were co-transfected in U2OS cells. Cyclin D1 was immunoprecipitated (IP) and ubiquitinated cyclin D1 was detected by immunoblotting (IB) using anti-Myc antibody. To enhance the detection of ubiquitinated forms of cyclin D1, the proteasome inhibitor LLnL was added 4 h before protein extraction.
D1 was found to be shorter compared with that of CDK4-bound cyclin D1 found in Rb-positive cells (27). This discrepancy indicates that a difference may exist in the ubiquitination of free and CDK4-bound cyclin D1. Although, to a lesser extent, GSK-3β can phosphorylate free cyclin D1 (24). Whether this phosphorylation is required for the ubiquitination of free cyclin D1 has not been determined. To address this question, we determined whether cyclin D1 was free or bound to CDK4 in our in vitro assay. 35S-cyclin D1 was mixed with extracts from U2OS cells, and CDK4 was immunoprecipitated from this mixture. The depletion of CDK4 from the extract was confirmed by Western analysis (Fig. 4A, top panel). To test whether the depletion of CDK4 resulted in the co-depletion of 35S-cyclin D1 from the extract, the presence of 35S-cyclin D1 in the CDK4-depleted extract was determined. The levels of 35S-labeled cyclin D1-T286A-T288A in the supernatant after CDK4 or after mock depletion were identical (Fig. 4A, bottom panel). This result indicates that exogenously added 35S-labeled cyclin D1-T286A-T288A does not assemble with the CDK4 present in the U2OS extract. We conclude that in this assay 35S-cyclin D1 is free from CDK4.

To determine whether phosphorylation of residues Thr286-Thr288 is required for the ubiquitination of free cyclin D1, we tested the ability of the 35S-labeled cyclin D1-T286A-T288A mutant to be ubiquitinated in vitro. As shown in Fig. 4B, this cyclin D1 mutant was efficiently ubiquitinated. The same result was obtained with murine cyclin D1-T286A mutant (data not shown). This result indicates that although GSK-3β is able to phosphorylate free cyclin D1 on threonine 286 at a low level (24), this phosphorylation event is not required for its ubiquitination. Further, this result suggests that the residual ubiquitination of cyclin D1-T286A-T288A mutant observed in vitro (Fig. 3B) is due to the presence of a low level of free cyclin D1-T286A-T288A in transfected cells.

Free Human Cyclin D1 Is Ubiquitinated in Vivo—Our finding that cyclin D1 is not bound to CDK4 in vitro indicates that free cyclin D1 is the predominant substrate for ubiquitination in this assay. To test whether free cyclin D1 is a substrate for ubiquitination in vivo, we co-transfected U2OS cells with plasmids expressing Myc-tagged ubiquitin and either wild type or a mutant form of cyclin D1 that cannot bind to CDKs (cyclin D1-KE) and then monitored cyclin D1-KE mutant ubiquitination. Despite its inability to bind CDK (Fig. 5A, bottom panel), cyclin D1-KE was ubiquitinated to an extent similar to wild type cyclin D1 (Fig. 5A, top panel). This result indicates that free cyclin D1 is a substrate for ubiquitination. In agreement with this result, the half-lives of cyclin D1 and cyclin D1-KE mutant were similar (Fig. 5B).

The observations that GSK-3β preferentially phosphorylates CDK4-bound cyclin D1 (24), and that phosphorylation at threonine 286 is not required for efficient ubiquitination of free cyclin D1 in vitro, raise the possibility that GSK-3β phosphorylation enhances the ubiquitination of cyclin D1 indirectly by promoting its dissociation from CDK4. In this model, CDK4-bound cyclin D1 and free cyclin D1 are ubiquitinated by the same pathway. Binding to CDK4 protects cyclin D1 from being degraded, and GSK-3β phosphorylation leads to cyclin D1 degradation by promoting its dissociation from CDK4. To test this hypothesis, U2OS cells were transfected with a plasmid expressing human cyclin D1. Cells were metabolically labeled with [35S]methionine for 30 min. Washed cells refed with complete medium containing unlabeled methionine were collected at the indicated times, and proteins were subjected to immunoprecipitation using anti-cyclin D1 antibody.

**Fig. 4. Human cyclin D1-T286A-T288A mutant is ubiquitinated in vitro.** A, 10 µl of in vitro translated [35S]-cyclin D1 was mixed with 400 µg of total protein extract from U2OS and the volume adjusted to 500 µl with reaction buffer as described under “Experimental Procedures.” To immunodeplete CDK4, 4 µl of anti-CDK4 antibody was added and incubated on ice for 2 h. CDK4-containing complexes were depleted from supernatant by adding 50 µl of protein A-Sepharose slurry overnight by centrifugation. As a control, non-immune serum was used. The level of CDK4 remaining in the supernatant was tested by immunoblot analysis, and the level of cyclin D1 was detected directly by autoradiography. B, cyclin D1-T286A-T288A was in vitro translated and assayed for ubiquitination as described in the legend for Fig. 1B.

**Fig. 5. Ubiquitination of cyclin D1 in the absence of CDK4 binding.** A, plasmids expressing wild type or a mutant of cyclin D1 unable to bind to CDK4 (cyclin D1-KE) and Myc-tagged ubiquitin were co-transfected in U2OS. After 24 h, proteins were extracted, cyclin D1 was immunoprecipitated (IP), and immunoblotting (IB) analysis was performed using anti-Myc antibody. 50 µg of crude extracts were also analyzed by Western blotting against cyclin D1 to monitor the transfection efficiency. To test the binding of CDK4 to wild type and cyclin D1-KE mutant, the Western blot of cyclin D1 immunoprecipitation was stripped and reprobed with antibody against CDK4. B, U2OS cells were transfected with human wild type cyclin D1 or cyclin D1-KE mutant. 24 h after transfection, cells were metabolically labeled with [35S]methionine for 30 min. Washed cells refed with complete medium containing unlabeled methionine were collected at the indicated times, and proteins were subjected to immunoprecipitation using anti-cyclin D1 antibody.
CDK4 beads resuspended in buffer. The amount of cyclin D1 present in association with CDK4 beads and in the supernatant was determined by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 6A, cyclin D1 was found in the CDK4 beads fraction independently of GSK-3β phosphorylation. Therefore, GSK-3β phosphorylation did not result in the release of cyclin D1 from CDK4 beads. The ability of recombinant GSK-3β to phosphorylate cyclin D1 in vitro was confirmed (Fig. 6B). This result indicates that the ability of GSK-3β phosphorylation to affect cyclin D1 ubiquitination is not related to the dissociation of cyclin D1 from CDK4. Therefore, the pathways leading to the ubiquitination of free and CDK4-bound cyclin D1 are distinct.

**DISCUSSION**

This study describes an *in vitro* ubiquitination assay for cyclin D1 and provides evidence that free cyclin D1 is ubiquitinated independently of phosphorylation on threonine 286. The *in vitro* assay requires protein extract from cells that are proficient in cyclin D1 proteolysis *in vivo* and is therefore representative of the ubiquitination capacity of the extract being tested. Further, as observed *in vivo* (23), the *in vitro* ubiquitination leads predominantly to the formation of very high molecular weight forms of cyclin D1. The efficiency of the ubiquitination is reflected by the accumulation of ubiquitinated species in the stacking gel, a feature that was also observed in other *in vitro* ubiquitination assays (28). These observations suggest that long polyubiquitin chains are attached to cyclin D1. The length of the polyubiquitin chains could not be reduced by the presence of excess methylated ubiquitin, suggesting that preformed polyubiquitin chains are transferred onto cyclin D1. The presence of these preformed polyubiquitin chains has been documented (29).

We show that threonine 286 and 288 of human cyclin D1 are important residues that affect the rate of proteolysis. However, as mutation of these residues did not entirely abolish the ubiquitination and degradation of cyclin D1, our results revealed a residual activity, which is independent of phosphorylation at these residues. This observation was confirmed using the *in vitro* ubiquitination assay, in which cyclin D1-T286A-T288A mutant was found to be a substrate for ubiquitination. Further, we showed that *in vivo* cyclin D1 is not bound to CDK4 and that *in vivo* a mutant of cyclin D1 that is unable to bind CDK4 (cyclin D1-KE) is ubiquitinated at a level similar to wild type cyclin D1. Our observations indicate that although GSK-3β can phosphorylate free cyclin D1 to some extent (24), this phosphorylation is not required for the ubiquitination of free cyclin D1. Further, we show that phosphorylation of cyclin D1 by GSK-3β does not promote its dissociation from CDK4. Therefore, our results suggest that two distinct pathways exist for the ubiquitination of cyclin D1. One pathway, which is GSK-3β-independent, targets free cyclin D1 for ubiquitination. The second is a GSK-3β-dependent pathway targeting CDK4-bound cyclin D1 for degradation. Previous observations indicated that in UMSCC2 cells, which overexpress cyclin D1 because of the amplification of the cyclin D1 gene, the small fraction of cyclin D1 that co-immunoprecipitates with CDK6 was more stable than the fraction immunoprecipitated with cyclin D1 antisera (27). Further, in Rb-negative cells, where cyclin D1 is free from CDK4, cyclin D1 turnover was found to be more rapid than in Rb-positive cells where cyclin D1 is bound to CDK4. These results indicate that unbound cyclin D1 is degraded more rapidly than bound cyclin D1 (27). Although these results are consistent with our finding that free cyclin D1 is a substrate for ubiquitination, we did not observe a more rapid degradation of free cyclin D1-KE compared with wild type cyclin D1. This discrepancy may indicate that the responsible for the degradation of free cyclin D1 is more predominant in some cellular types than others.

What is the advantage of having distinct ubiquitination pathways for free and CDK4-bound cyclin D1? Interestingly, because the cyclin D1-T286A mutant is stabilized when bound to CDK4 (23), it appears that binding to CDK4 protects cyclin D1 from degradation by the GSK-3β-independent ubiquitination pathway. This protection mechanism may be important in regulating the cyclin D1 function, as it would ensure sufficient stability for the cyclin D1-CDK4 complex to perform its function during G1 phase. However, upon translocation of GSK-3β into the nucleus in S phase (24), the ubiquitination of CDK4-bound cyclin D1 is initiated, because the cyclin D1-CDK4 complex function is no longer required. Therefore, the GSK-3β-dependent ubiquitination pathway ensures a regulated destruction of CDK4-bound cyclin D1 at the G1/S boundary.

The GSK-3β-independent ubiquitination pathway may have a distinct role in the regulation of cyclin D1. Considering the growing evidence for the functions of non-CDK bound cyclin D1 in the activation of a variety of transcription factors (2–4), the GSK-3β-independent ubiquitination pathway may be important for the constitutive destruction of free cyclin D1, thereby preventing its accumulation in the nucleus during G1. This model is consistent with the observation that, in most cases, little free cyclin D1 is found in cells. The constitutive degradation of free cyclin D1 may be particularly important in limiting the effect of cyclin D1 overexpression observed in 35% of breast cancers (12). The situation in these tumors is similar to that of UMSSC2 cells, which overexpress cyclin D1 because of the amplification of the cyclin D1 gene, but in which most of the cyclin D1 is found unbound to CDKs (27) and therefore potentially able to modulate various transcriptional activities.

Our results also show similarities to the recent finding that cyclin E is degraded by two mechanisms. Free cyclin E is degraded independently of phosphorylation on threonine 380 by CDK2 and involves Cul-3 (25), whereas ubiquitination of CDK2 bound cyclin E requires phosphorylation at that residue and involves Cul-I (30, 31). Together, the findings that free and bound cyclin E and cyclin D1 ubiquitination pathways differ indicates that cellular mechanisms have evolved to tightly control the level of G1 cyclins. We have previously obtained...
evidence that ubiquitination of cyclin D1 may be defective in
15% of breast cancer (13), and so the challenge ahead is to
determine which ubiquitination pathway is defective in these
tumors.

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