C-terminal Sequences Direct Cyclin D1-CRM1 Binding*

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GSK-3β-dependent phosphorylation of cyclin D1 at a conserved C-terminal residue, Thr-286, promotes CRM1-dependent cyclin D1 nuclear export. Herein, we have identified a short stretch of residues adjacent to Thr-286 that mediates CRM1 association and thus cyclin D1 nuclear export. We found that disruption of this hydrophobic patch, stretching from amino acids 290 to 295 within cyclin D1, results in constitutively nuclear cyclin D1-CDK4 complexes with an increased propensity to potentiate transformation of murine fibroblasts. Our data support a model wherein deregulation of cyclin D1 nuclear export might contribute to human neoplastic growth.

Progression through G1 phase is initiated by mitogenic stimulation, which in turn initiates the expression and assembly of the D-type cyclins (D1, D2, D3) with their catalytic partner CDK4. The cyclin D/CDK4 kinase has two functions necessary for cell cycle progression: phosphorylation-dependent inactivation of the retinoblastoma protein family members such as pRb, p107, and p130 (1–5) and the stoichiometric association with Cip/Kip family proteins (6–9). This association facilitates Kip access to cyclin E-CDK2 complexes, which would result in the inhibition of the cyclin E/CDK2 kinase (9–14).

Although cyclin D1 accumulates in the nucleus during the G1 interval, it relocates to the cytoplasm during S phase. The essential functions of cyclin D1 require its nuclear localization, and thus the redistribution of cyclin D1 complexes to the cytoplasm following G1 implies that regulation of cyclin D1 nucleocytoplasmic distribution is necessary for maintaining cellular homeostasis. These alterations in the subcellular distribution of cyclin D1 during the cell cycle are subject to mitogenic regulation (15, 16). It is now clear that phosphorylation of cyclin D1 at Thr-286 by GSK-3β promotes CRM1 binding, which then shuttles cyclin D1 to the cytoplasm (17) for subsequent degradation via the 26S proteasome (15). Although these data imply that phosphorylation of Thr-286 enables CRM1 binding, the residues within cyclin D1 that direct CRM1 binding have not been elucidated.

Although cyclin D1 is overexpressed in a number of malignancies, it is clear that overexpression of the wild type protein is not by itself sufficient to induce a transformed cellular phenotype (17, 18). In contrast, we found previously that neither the cyclin D1 mutant, cyclin D1-T286A, nor an alternatively spliced cyclin D1 variant lacking the fifth exon can be phosphorylated by GSK-3β, and thus both are refractory to phosphorylation-dependent nuclear export and are capable of driving transformation of murine fibroblasts in the absence of a collaborating oncogene (17, 20). This suggests that deregulation of cyclin D1 nuclear export results in increased cyclin D1 oncogenic capacity.

Herein we describe the identification of residues within cyclin D1 that direct CRM1 association. Our results demonstrate that hydrophobic residues adjacent to Thr-286 and within the C-terminal nine amino acids of cyclin D1 mediate CRM1 binding.

**EXPERIMENTAL PROCEDURES**

Cell Culture Conditions and Transfections—NIH-3T3 cells were maintained in Dulbecco’s modified Eagle’s medium containing glucose supplemented with antibiotics (Cellgro) and 10% FCS (Gemi). Procedures for manipulation of baculoviruses were described previously (21). Site-directed mutagenesis of D1-V290/295A mutant was achieved by using QuikChange (Stratagene), substituting valines 290 and 293 and isoleucine 295 to alanines with the following primers: forward 5′-TCGACCCCTACGAGTCGCTGATGTTCACTG TGGAGCCGCA-CGG-3′ and reverse 5′-CGCGTGGGCCCTGAGGTCGATCGATCGT GC-CGGCGTTGTCGGT-3′. The derivation of NIH-3T3 cells over-expressing FLAG-tagged cyclin D1 and D1 mutants was as described previously (17). Cell lines were maintained on a passage protocol wherein 9 × 106 cells were passaged per 60-mm dish every third day (22). Transient expression of HA-tagged GSK-3β or HA-tagged CRM1 was performed as described previously (15).

**Immunoblotting**—For Western analysis, cells were lysed in EBC buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 units/ml aprotinin, 5 μg/ml leupeptin, 0.4 mM NaVO₃, 0.4 mM NaF). Total cellular proteins (200 μg) were resolved on denaturing polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (MSI, Westborough, MA), and blotted with the cyclin D1 monoclonal antibody (D1-17-13G). Detection of GSK-3β was performed as described previously (15).

**Expression and Purification of Proteins**—FLAG-D1 mutants were cloned into the pVDL-1395 baculoviral expression vector (Pharmingen), and virus was isolated according to established procedures as described previously (15). After infection of insect S9 cells at high multiplicity, cells were lysed in EBC buffer and clarified by sedimentation in a microcentrifuge for 10 min. Recombinant proteins were then subjected to precipitation with epitope-specific antibodies and detected by immunoblot.

**Immunofluorescence**—NIH-3T3 cells seeded on glass coverslips were transfected with expression vectors encoding the indicated cDNAs. Cells were fixed at 48 h after transfection using methanol-acetone (1:1). For visualization of cyclin D1, coverslips were stained with a mouse-specific cyclin D1 monoclonal antibody (D1-17-13G) in phosphate-buffered saline containing 10% FCS. Secondary antibody staining was performed for 30 min using fluorescein isothiocyanate-conjugated anti-mouse (Amersham Biosciences). DNA was visualized using Hoechst 33258 dye at a 1:500 dilution. Coverslips were mounted on glass slides with Vectashield (Vector Laboratories).

**Protein Turnover Analysis**—Equivalent numbers of NIH-3T3 cells overexpressing either FLAG-tagged wild type or mutant cyclin D1 were seeded in 10-cm dishes. The following day, cells were treated with the

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‡ The abbreviations used are: CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; GSK-3β, glycogen synthase kinase 3β; HA, hemagglutinin; FCS, fetal calf serum.
protein synthesis inhibitor cycloheximide (100 μg/ml, Sigma), incubated for the indicated times, and harvested in SDS-sample buffer. Cyclin D1 was detected by Western blot analysis.

**RESULTS**

**Cyclin D1 Residues 290–295 Mediate CRM1 Binding**—CRM1 directs nuclear export of target proteins via direct binding to leucine/hydrophobic stretches of amino acids referred to as a nuclear export signal (23). Because phosphorylation of Thr-286 is required for CRM1 association with cyclin D1, we reasoned that phosphorylation of this residue might result in a conformational change that allows CRM1 access to a region of cyclin D1 adjacent to this site of phosphorylation. A stretch of 10 highly conserved amino acids exists within the C terminus of cyclins D1 and D2 (286TPDVRDVFL in cyclin D1) (24) with the hydrophobic residues (in boldface) being conserved in cyclin D3 as well (286TPDVRDVFL in cyclin D3). Anchorage-independent growth of NIH-3T3, D1–3T3, D1-T286A–3T3, and D1-V290/295A was performed as described previously (17).

**Disruption of Cyclin D1 Nuclear Export**

Overexpression of CRM1 can drive wild type cyclin D1 into the cytoplasm, whereas the phosphorylation-deficient D1-T286A mutant is refractory to the enforced nuclear export driven by CRM1 overexpression (17). It stands to reason that cyclin D1 mutants deficient in CRM1 binding, such as D1-V290/295A, should be refractory to CRM1-mediated nuclear export and thus constitutively nuclear. To address this possibility, we examined the subcellular localization of D1-V290/295A in asynchronously proliferating cells. The localization of cyclin D1 varied, with ~50% of the cells exhibiting primarily nuclear localization, whereas in the remaining cells it localized either to the cytoplasm or to both the nucleus and the cytoplasm (Fig. 1, C and D). This distribution of nuclear cyclin D1 approximates the percentage of cells in G1 phase in an asynchronous population (15). In contrast, D1-V290/295A was nuclear in more than 90% of the cells (Fig. 1, C and D). To assess the capacity of CRM1 to regulate localization, we determined whether ectopic CRM1 could drive wild type cyclin D1 or D1-V290/295A into the cytoplasm. Whereas expression of CRM1 shuttled wild type cyclin D1 out of the nucleus (Fig. 1, C and D (quantitation)), D1-V290/295A remained nuclear in the presence or absence of ectopic CRM1 (1, C and D (quantitation)).
Although these results suggest that this region of cyclin D1 mediates CRM1 binding, its close proximity to Thr-286 could disrupt GSK-3β-mediated phosphorylation and thereby indirectly disrupt CRM1 association. To examine the phosphorylation state of D1-V290/295A, we performed immunoblot analysis using an antibody specific for phosphorylated Thr-286. We have previously demonstrated that recognition of cyclin D1 by this antibody is strictly dependent upon phosphorylation of Thr-286 (17). FLAG-D1, FLAG-D1-T286A, or FLAG-D1-V290/295A was precipitated from cells with the M2 monoclonal antibody. Although the D1-T286A mutant was not phosphorylated, both wild type cyclin D1 and D1-V290/295A were phosphorylated on Thr-286 (Fig. 2A). In fact D1-V290/295A phosphorylation was increased relative to wild type cyclin D1, suggesting decreased turnover of this mutant cyclin D1 protein (see Fig. 3). These results are consistent with the notion that hydrophobic residues between amino acids 290 and 295 direct CRM1 association with cyclin D1. Furthermore, these results demonstrate that phosphorylation of cyclin D1 at Thr-286 alone is insufficient to direct CRM1-mediated cyclin D1 nuclear export in the absence of these hydrophobic residues.

We next determined whether these alanine substitutions specifically interfere with CRM1 binding or whether they have a general effect on cyclin D1 folding that would be reflected in its capacity to associate with other known cyclin D1 interacting proteins or, alternatively, in its capacity to support CDK4 catalytic activity. We first assessed the association of D1-V290/295A with GSK-3β. We have previously demonstrated that kinase-defective GSK-3β will form stable complexes with cyclin D1 in insect Sf9 cells (15). Insect Sf9 cells were infected with baculoviruses encoding kinase-defective GSK-3β along with cyclin D1, D1-T286A, or cyclin D1-V290/295A. GSK-3β-cyclin D1 complexes were isolated from lysates prepared from these cells by precipitation with a GSK-3β-specific monoclonal antibody. Immunoblot analysis confirmed the presence of D1-V290/295A within GSK-3β precipitates (Fig. 2B).

Overexpression of GSK-3β will drive wild type cyclin D1 into the cytoplasm (15, 17), whereas the phosphorylation-deficient D1-T286A mutant is refractory to this enforced nuclear export. Because D1-V290/295A retained both binding to and phosphorylation by GSK-3β, it was important to determine whether D1-V290/295A was refractory to GSK-3β-mediated nuclear export. We examined the subcellular localization of cyclin D1 and D1-V290/295A in asynchronously proliferating cells transfected with GSK-3β. As shown in Fig. 2C, cyclin D1 was efficiently shuttled out of the nucleus and cytoplasmically localized in the presence of GSK-3β. In contrast, D1-V290/295A remained nuclear and is thus refractory to GSK-3β-mediated nuclear export. These results are consistent with the notion that the hydrophobic residues between amino acids 290 and 295 are important for CRM1 association with cyclin D1.

Fig. 2. Cyclin D1-V290/295A is subject to GSK-3β-mediated phosphorylation. A, lysates prepared from cells expressing cyclin D1, D1-T286A, or D1-V290/295A were subjected to precipitation with the M2 antibody. Thr-286 phosphorylation was assessed by immunoblot using a phospho-specific antibody (pT286, upper panel), and total cyclin D1 was assessed using a monoclonal antibody that recognizes both phosphorylated and unphosphorylated protein (lower panel). IP, immunoprecipitate. B, GSK-3β co-precipitates with wild type and mutant cyclin D1 isoforms. Sf9 lysates co-expressing kinase-defective GSK-3β along with FLAG-D1, D1-T286A, or D1-V290/295A were subjected to precipitation with the M2 antibody. Cyclin D1 was assessed by immunoblot using a nitrocellulose membrane. The nitrocellulose membrane was processed for Western blot analysis for detection of cyclin D1 (upper panel) and cyclin D1 tagged protein. Following precipitation with the M2 antibody, proteins were separated on a denaturing polyacrylamide gel and transferred onto the nitrocellulose membrane. Corresponding Hoechst DNA staining is also shown. C, constitutive nuclear localization of phosphorylated D1-V290/295A. The effect of GSK-3β overexpression in NIH-3T3 cells stably expressing either wild type D1 (a and b) or D1-V290/295A (c and d) was determined by immunofluorescence. Following transient transfection of GSK-3β cDNA, the respective cell lines were fixed, and cyclin D1 localization in the presence of GSK-3β was detected using monoclonal cyclin D1 antibody and fluorescein isothiocyanate-conjugated secondary antibody. Corresponding Hoechst DNA staining is also shown. D, CRM-1 binding-deficient mutant retains association with p21Cip1. Cyclin D1, D1-T286A, and D1-V290/295A were immunoprecipitated from NIH-3T3 cells stably overexpressing each respective FLAG-tagged protein. Following precipitation with the M2 antibody, proteins were separated on a denaturing polyacrylamide gel and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was processed for Western blot analysis for detection of cyclin D1 (upper panel) and co-precipitating p21Cip1 (lower panel).
presence or absence of MG132, and ubiquitinated cyclin D1 was de-
clin D1 or D1-V290/295A was immunoprecipitated from cells in the
phospho-T286 antibody.
phorylated cyclin D1 proteins was assessed by immunoblot with the
from cells treated with cycloheximide as indicated, and decay of phos-
D1 proteins were treated with cycloheximide (CHX) for the indicated

tervals. Lysates prepared from the respective cells were subjected to
mutants.
A
B
C.

FIG. 3. Increased stability of constitutively nuclear cyclin D1
mutants. A, NIH-3T3 cells stably overexpressing the indicated
D1 proteins were treated with cycloheximide (CHX) for the indicated
intervals. Lysates prepared from the respective cells were subjected to
Western analysis using the cyclin D1 monoclonal antibody. Results
shown are representative of multiple independent experiments. B, wild
type cyclin D1 or D1-V290/295A was precipitated from lysates prepared
from cells treated with cycloheximide as indicated, and decay of phos-
phorylated cyclin D1 proteins was assessed by immunoblot with the
phospho-T286 antibody. C, HA-ubiquitin co-transfected with either
cyclin D1 or D1-V290/295A was immunoprecipitated from cells in the
presence or absence of MG132, and ubiquitinated cyclin D1 was de-
tected by Western blot. poly-Ub, poly-ubiquitin laddering.
nuclear export (Fig. 2C, panels c and d). GSK-3β expression
was confirmed by indirect immunofluorescence in parallel
(data not shown).

We subsequently assessed the capacity of D1-V290/295A to
associate with and activate CDK4 as well as to retain binding
to p21Cip1. Similar to wild type cyclin D1 and D1-T286A, cyclin
D1-V290/295A assembled with CDK4 and supported CDK4-de-
pendent phosphorylation of Rb (data not shown). Moreover,
D1-V290/295A also associated with p21Cip1 (Fig. 2D), indicat-
ing that the alanine substitutions have not significantly per-
turbed the structural or functional integrity of cyclin D1-V290/
295A. These data support the hypothesis that residues 290–
295 of cyclin D1 mediate CRM1 association and together with
phosphorylated Thr-286 constitute the cyclin D1 nuclear export
signal.

Increased Stability of Constitutively Nuclear Cyclin D1 Mu-
tants—Our previous work has suggested that cyclin D1 prote-
oysis is a cytoplasmic event. This conclusion was based on the
increased stability of constitutively nuclear cyclin D1-T286A.
However, it was not possible to determine the role of Thr-286
phosphorylation versus nuclear export in mediating cyclin D1-proteolysis. In characterizing D1-V290/295A, we noted that it
accumulated as a Thr-286-phosphorylated protein (Fig. 2A, compare
lanes 1 and 3), suggesting that phosphorylation is not
sufficient to trigger proteolysis of this nuclear protein. Using
cycloheximide to inhibit nascent protein synthesis (30), we
investigated the rate of D1-V290/295A degradation versus that
of either wild type cyclin D1 or the D1-T286A mutant (Fig. 3A).
Our results revealed that both D1-V290/295A and D1-T286A
have increased protein stability in comparison with wild type
cyclin D1. To confirm reduced Thr-286 proteolysis of phospho-
yrylated D1-V290/295A, we measured decay of phosphorylated
isoforms of wild type cyclin D1 and D1-V290/295A. Although
the half-life of Thr-286 phosphorylated cyclin D1 was less than
15 min, the half-life of phospho-D1-V290/295A was greatly
extended (Fig. 3B).

Moreover, to corroborate the increased stability of D1-V290/
295A relative to wild type cyclin D1, we assessed ubiquitina-
ton of cyclin D1 versus D1-V290/295A. Either wild type or
D1-V290/295A was co-transfected with HA-ubiquitin into NIH-
3T3 cells. Transfected cells were treated with either vehicle or
MG132 to inhibit the 26 S proteasome. Cells were then har-
ested, and lysates were subjected to immunoblotting with the
cyclin D1 monoclonal antibody (Fig. 3C). Wild type cyclin D1
alone exhibited the characteristic poly-ubiquitin laddering
(poly-Ub) recognized for ubiquitinated proteins in the presence
of MG132 (Fig. 3C, lane 2). No higher molecular weight iso-
forms of D1-V290/295A were detected either in the presence or
absence of MG132. These data demonstrate that disruption of
CRM1 association promotes the accumulation of Thr-286 phos-
phorylated cyclin D1 that is inaccessible to and consequently
not degraded via cytoplasmic 26 S proteasomes.

Loss of CRM1 Binding Increases the Transforming Potential
of Cyclin D1—Cells engineered to overexpress cyclin D1 dis-
play a contracted G1 interval (18). These cells, however, do not
form foci, grow in soft agar, or promote tumor formation in
immunocompromised mice (18). As shown previously in our
laboratory, a cyclin D1 mutant that cannot be phosphorylated
at Thr-286 and is thus constitutively nuclear promotes cellular
transformation (17). This result suggests that disruption of
cyclin D1 nuclear export is an oncogenic event. We therefore
considered the possibility that the D1-V290/295A mutant,
which unlike D1-T286A retains phosphorylation at Thr-286,
might exhibit an increased potential to drive cell transformation
relative to wild type cyclin D1. For these experiments
NIH-3T3 cells were co-transfected with vectors encoding either
wild type FLAG-tagged cyclin D1 or the specified cyclin D1
mutant and a vector encoding puromycin as a selectable
marker. Transfectants selected in puromycin were then pooled
to eliminate potential clonal variation when assessing trans-
formation. NIH-3T3 cells (data not shown) or NIH-3T3 deriv-
atives engineered to overexpress either wild type FLAG-tagged
or mutant cyclin D1 isoforms were assayed for characteristics
of cell transformation: foci formation (refractory to contact in-
hibition) and growth in soft agar (capable of anchorage-inde-
pendent growth). Over the course of five independent experi-
ments, all NIH-3T3 cells overexpressing the C-terminal cyclin
D1 mutants D1-T286A and D1-V290/295A reproducibly formed
numerous foci (Fig. 4A; data not shown). Consistent with these
results, unlike wild type NIH-3T3 (data not shown) and D1–
3T3 cells, which were incapable of significant growth in soft
agar, NIH-3T3 cell lines overexpressing constitutively nuclear
C-terminal cyclin D1 mutants D1-T286A and D1-V290/295A reproducibly grew in soft agar, forming
numerous and robust colonies (Fig. 4, B and C (quantitation)).
Our data support the model wherein mutations in the CRM1
binding site and/or loss of Thr-286 phosphorylation in wild type
cyclin D1 leads to a constitutively nuclear cyclin D1 with in-
creased capacity to drive neoplastic transformation and, as a
result, a loss in G1/S homeostasis (Fig. 5).
Cyclin D1 localization is driven by the competing processes of nuclear import and export. Although the mechanisms of cyclin D1 nuclear import remain poorly characterized, it is now clear that CRM1-dependent nuclear export drives cyclin D1 complexes into the cytoplasm during S phase. CRM1 directs nuclear export of target proteins via direct binding to leucine/hydrophobic stretches of amino acids. However, the exact spacing of the leucine/hydrophobic patch is variable making it difficult to identify CRM1 binding sites by scanning the primary sequence of a given putative substrate. Although two stretches of residues closely conformed to previously documented nuclear export signal motifs, residues 87–94 (RFLSLEPL) and residues 290–295, the 290–295 sequence was an attractive target given its proximity to the site of GSK-3β-mediated phosphorylation, Thr-286, and its highly conserved nature among all three D-type cyclins. Mutation of valines and isoleucines to alanines in this region abrogates CRM1 binding and promotes nuclear retention without perturbing phosphorylation of Thr-286. This mutant, D1-V290/295A, accumulates in the nucleus as a Thr-286-phosphorylated protein. In contrast, the phosphorylated form of wild type cyclin D1 is highly labile and is detectable in the cytoplasm (9). Taken together, these data identify residues 290–295 as the CRM1 binding site in cyclin D1.

Our data demonstrate that a hydrophobic patch within the C terminus of cyclin D1 mediates CRM1 binding. Mutations within this region do not perturb GSK-3β-mediated phosphorylation of cyclin D1 at Thr-286, a modification that increases the propensity for CRM1 recognition and binding. As our results indicate, however, phosphorylation in itself is insufficient for cyclin D1 nuclear export or degradation, which is consistent with cytoplasmic proteolysis of cyclin D1. Thus, in the absence of an intact CRM1 binding site, CRM1 fails to associate with cyclin D1, and the resulting protein is more stable and constitutively nuclear.

These data suggest that the C terminus of cyclin D1 is a critical modulator of cyclin D1 nuclear export and might therefore represent a potential “hot spot” for the activation of mutations in cyclin D1 that ultimately contribute to cancer genesis. In support of this notion, we have recently identified a splice variant of cyclin D1, referred to as cyclin D1b, which lacks the C-terminal residues that direct GSK-3β phosphorylation and CRM1-dependent nuclear export (20). As with D1-V290/295A described herein, cyclin D1b is constitutively nuclear and can drive cell transformation (20, 31). Furthermore, we have found that this protein is expressed in a significant fraction of primary cancers, highlighting the significance of maintaining temporal control of cyclin D1 subcellular localization for normal cell growth and proliferation (20).

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FIG. 4. Constitutively nuclear cyclin D1 mutants transform murine fibroblasts. Cells were plated in 6-well dishes in complete medium containing 5% FCS. Cells were grown for 21 days and stained with Giemsa to visualize foci. B, NIH-3T3 cells engineered to stably express the indicated cyclin D1 proteins were plated in semisolid medium and allowed to proliferate for 21 days. Colonies were visualized by 0.01% neutral red stain. C, quantitation of colonies scored in A.

FIG. 5. Cell transformation results from inhibition of cyclin D1 nuclear export. Wild type cyclin D1 is phosphorylated at Thr-286 (circled “P”) in late G1 phase, allowing for CRM1 recognition and binding that triggers the rapid nuclear export of cyclin D1-CDK4 complexes in S phase (top). Mutation of phosphorylatable Thr-286 or mutations directly within the CRM1 binding site of cyclin D1 results in abrogated CRM1 binding and inhibition of cyclin D1 nuclear export. Expression of constitutively nuclear C-terminal cyclin D1 mutants results in cellular transformation.
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