A Novel Mechanism by Which Thiazolidinediones Facilitate the Proteasomal Degradation of Cyclin D1 in Cancer Cells*

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This study identifies a novel mechanism by which thiazolidinediones mediate cyclin D1 repression in prostate cancer cells. Based on the finding that the thiazolidinedione family of peroxisome proliferator-activated receptor γ (PPARγ) agonists mediated PPARγ-independent cyclin D1 degradation, we developed a novel PPARγ-inactive troglitazone derivative, STG28, with high potency in cyclin D1 ablation. STG28-mediated cyclin D1 degradation was preceded by Thr-286 phosphorylation and nuclear export, which however, were independent of glycogen synthase kinase 3β. Mutational analysis further confirmed the pivotal role of Thr-286 phosphorylation in STG28-induced nuclear export and proteolysis. Of several kinases examined, inhibition of IκB kinase α blocked STG28-mediated cytoplasmic sequestration and degradation of cyclin D1. Pulldown of ectopically expressed Cul1, the scaffold protein of the Skp-Cul-lin-F-box E3 ubiquitin ligase, in STG28-treated cells revealed an increased association of cyclin D1 with β-TrCP, whereas no specific binding was noted with other F-box proteins examined, including Skp2, Fbw7, Fbx4, and Fbxw8. This finding represents the first evidence that cyclin D1 is targeted by β-TrCP. Moreover, β-TrCP expression was up-regulated in response to STG28, and ectopic expression and small interfering RNA-mediated knockdown of β-TrCP enhanced and protected against STG28-facilitated cyclin D1 degradation, respectively. Because cyclin D1 lacks the DSG destruction motif, mutational and modeling analyses indicate that cyclin D1 was targeted by β-TrCP through an unconventional recognition site, EEVDLACpT286, reminiscent to that of Wee1. Moreover, we obtained evidence that this β-TrCP-dependent degradation takes part in controlling cyclin D1 turnover when cancer cells undergo glucose starvation, which endows physiological relevance to this novel mechanism.

Substantial evidence indicates that overexpression of the cell cycle control gene CCND1 represents a key mechanism underlying tumorigenesis, tumor progression, and metastasis in a variety of human cancers (1–6). Cyclin D1 serves as the regulatory subunit of cyclin-dependent kinases (CDKs) 4 and 6 and exhibits the ability to bind and sequester the CDK inhibitor p27 (5, 6). Together, these functions facilitate cyclin-dependent kinase-mediated phosphorylating inactivation of the retinoblastoma protein (pRb), resulting in G1/S progression. Moreover, cyclin D1 may regulate gene transcription through physical associations with a plethora of transcriptional factors, coactivators, and corepressors that govern histone acetylation and chromatin remodeling proteins (5). The concerted action of these cyclin-dependent kinase-dependent and -independent functions underscores the oncogenic potential of cyclin D1 in many forms of cancer (7). Transcriptional suppression of cyclin D1 expression has been shown to block tumorigenesis or to reverse the transformed phenotype of human esophageal (8), lung (9), colon (10), pancreatic (11), gastric (12), melanoma (13), and squamous cancer cells (14) in mice.

Considering its oncogenic role, targeting cyclin D1 expression represents a promising strategy for cancer therapy (15). Intracellular levels of cyclin D1 are regulated by a balance between mitogenic signal-activated gene expression and ubiquitin-dependent proteasomal degradation (16). Consequently, the mechanism that regulates cyclin D1 stability has been the focus of many recent investigations. Early studies indicate that during S phase, cyclin D1 is phosphorylated at Thr-286 by glycogen synthase kinase-3β (GSK3β), resulting in nuclear export and subsequent ubiquitin-dependent proteasomal degradation (17). More recently, at least three additional kinases have been shown to mediate the Thr-286 phosphorylation, including IκB kinase α (IκKα) (18), p38 (19), and extracellular signal-regulated kinase 1/2 (ERK1/2) (20). With regard to the identity of the E3 ligase that targets Thr-286-phosphorylated cyclin D1, multiple F-box proteins of the Skp-Cullin-F-box (SCF) E3 ubiquitin ligase, including Skp2 (21), Fbx4-αB crystalline (22), and Fbxw8 (20), have been shown to take part in cyclin D1 ubiquitination and degradation.

To date, a number of small-molecule agents have been shown to exhibit the ability to down-regulate cyclin D1 expression, 2

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‡ The abbreviations used are: GSK3β, glycogen synthase kinase-3β; SCF, Skp-Cullin-F-box; PPAR, peroxisome proliferator-activated receptor γ; NT, p-n-terminal; CT, p-c-terminal; IκKα, IκB kinase α; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; p-, phosphorylated; CMV, cyclo-megalovirus; PBS, phosphate-buffered saline; HA, hemagglutinin; GST, glutathione S-transferase; DMSO, dimethyl sulfoxide.
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including retinoic acid (23), curcumin (24), peroxisome proliferator-activated receptor γ (PPARγ) agonists (25–29), aspirin (30), and the histone deacetylase inhibitor trichostatin A (31), although the underlying mechanisms remain largely undefined. Data from this and other laboratories indicate that troglitazone, a thiazolidinedione PPARγ agonist, at high doses mediated the ubiquitin-dependent proteosomal degradation of cyclin D1 in MCF-7 breast cancer cells (25, 26, 28, 32). Moreover, we observed that troglitazone mediated cyclin D1 proteolysis independently of PPARγ activation (32). These findings provided a molecular basis for the pharmacological exploitation of troglitazone to develop a novel class of PPARγ-inactive, cyclin D1-ablative agents, among which STG28 represents a structurally optimized agent (33). Albeit devoid of PPARγ activity, STG28 retains the ability of troglitazone to repress cyclin D1 and a series of cell cycle regulatory proteins, including β-catenin (34) and androgen receptor (35). In light of the therapeutic potential of STG28 in cancer therapy, we embarked on investigating the mechanism underlying the effect of STG28 on facilitating the proteosomal degradation of target proteins. In this study we report a new pathway that involves SCFβ-TrCP in STG28-facilitated cyclin D1 ablation. It is noteworthy that cyclin D1 lacks the DSG destruction motif commonly found in other β-TrCP target proteins. Mutational and molecular modeling analyses indicate that the β-TrCP recognition of cyclin D1 was achieved through an unconventional motif, 279EEVDLA286.

EXPERIMENTAL PROCEDURES

Cell Culture—LNCaP prostate cancer cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in T-75 flasks with RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% CO2.

Reagents—MG132, leptomycin B, PD98059, Bay11-7082, lithium chloride, and SB216763 were purchased from Sigma-Aldrich. SB203580 was obtained from Rockland (Gilbertsville, PA). The PPARγ antagonist, SB203580 was obtained from Calbiochem. Glucose-lithium chloride, and SB216763 were purchased from Sigma-Aldrich. The IkBα kinase dominant negative mutant (IkBαΔN) was obtained from Rockland (Gilbertsville, PA). The IkBα kinase dominant negative mutant (pIkK2M) was obtained as described (36).

Plasmid Constructions, Site-directed Mutagenesis, and Semi-quantitative PCR Analysis—The cDNA encoding full-length human cyclin D1 was PCR-amplified from a human testicular cDNA library with primers 5'-GCGATCTATGGGAACAC-CAGCTCTCG-3' (forward) and 5'-CGGAATTTCACAT-GTCCAGCTCCGG-3' (reverse) that were flanked by BgIII and EcoRI restriction sites. The resulting fragments were subcloned into a cytomegalovirus (CMV)-driven GFP vector, pEGFP-C1 (Invitrogen), with a GFP protein fusion at its N terminus. This full-length cyclin D1 plasmid was named as pWT-cyclin D1-GFP and used as template to create a series of truncated or mutated cyclin D1 constructs. The pN-terminal (NT)-, pCT-terminal (CT)-, pPEST-cyclin D1-GFP plasmids were constructed by using a PCR approach and subsequently cloned into pEGFP-C1 (see Fig. 2A, lower panel). Plasmids encoding various cyclin D1 mutations were generated by site-directed mutagenesis from pWT cyclin D1-GFP by using the QuikChange site-directed mutagenesis kit from Stratagene (Cedar Creek, TX). The pMyc4-hSkp2-CMV14 plasmid encoding Myc-tagged full-length Skp2 (37) was subcloned into EcoRI/HindIII sites of pGEX vector for glutathione transferase (GST)-Skp2 fusion protein expression. All constructs were verified by DNA sequencing. Semiquantitative PCR analysis was performed by extracting total mRNA from STG28-treated cells followed by reverse transcriptase PCR according to a previously described procedure (32).

Cell Cycle Analysis—LNCaP cells were exposed to 10 μM STG28 in 10% FBS-supplemented RPMI 1640 medium for various time intervals, collected by trypsinization, and fixed in ice-cold 80% ethanol, PBS at 4 °C overnight. Cells were then centrifuged for 5 min at 1000 × g at room temperature, and after decanting the ethanol without disturbing the pellet, the cells were stained with propidium iodide (5 μg/ml) and Rhodamine A (50 units/ml) in PBS. Cell cycle phase distributions were determined on a FACSsort flow cytometer and analyzed by the ModFitLT V3.0 program.

Immunocytochemical Analysis—LNCaP cells growing on slides in 6-well plates (2.5 × 106 cells/well) in 10% FBS-supplemented RPMI 1640 medium were exposed to 10 μM STG28 for different intervals. Cells were then fixed with 3.7% formaldehyde at room temperature for 20 min, washed with PBS twice, permeabilized with PBS containing 0.1% Triton X-100 for 1 h, and blocked for 30 min in medium containing serum. After another wash, immunostaining was performed by incubating cells with mouse anti-cyclin D1 (1:100 dilution) or rabbit anti-β-TrCP (Santa Cruz; 1:100) primary antibody at room temperature for 12 h. Primary antibodies were diluted in PBS containing 0.1% Triton X-100, 0.2% bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. After washing with PBS, the bounded primary antibodies were detected using AlexaFluor 488 goat anti-mouse or AlexaFluor 555 goat anti-rabbit antibody (Molecular Probes; 1:100) at room temperature for 2 h. The nuclear counterstaining was performed using a 4,6-diamidino-2-phenylindole-containing mounting medium (Vector Laboratories, Burlingame, CA) before examination. Images of immunocytochemically labeled samples were observed using a Nikon microscope (Eclipse TE300).

Cell Fractionation—Nuclear protein extraction was carried out by using the nuclear extract kit (Active Motif). In brief, STG28-treated LNCaP cells were harvested in ice-cold hypotonic buffer for 15 min followed by 14,000 × g centrifugation at 4 °C. Supernatant was collected as cytoplasmic fraction, and the pellet was further incubated with complete lysis buffer at 4 °C.
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for 30 min. The lysate was centrifuged at 14,000 × g for 10 min at 4 °C to obtain supernatant as nuclear fraction. Protein concentrations were quantitated, and immunoblotting was performed for cyclin D1 detection. Nucleolin and β-actin served as markers and internal controls for nuclear and cytoplasmic compartments, respectively.

Transfection and RNA Interference—Transfection was carried out by electroporation using an Amaza Nucleofector (Amaza Biosystems, Cologne, Germany) according to a reported procedure (34). In brief, LNCaP cells were nucleofected with various plasmids by using the Nucleofector kit R (Amaza), seeded in 6-well plates (5 × 10^5 cells/well), and incubated in 10% FBS-containing medium for 24 h before drug treatment. Transfection efficiency was >75% in LNCaP cells as determined by co-transfection with pmaxGFP plasmids followed by fluorescence microscopic visualization of the GFP-positive cells population. For small interfering RNA (siRNA) experiments, cells were electroporated with scrambled, IKKα (Upstate Biotechnology, Lake Placid, NY), β-TrCP (Santa Cruz), or Fbw8 (Origene, Rockville, MD) and seeded in 6-well plates (5 × 10^5 cells/well). After exposure to 10 µM STG28, cell lysates were collected and subjected to immunoblotting analysis.

Immunoprecipitation and Immunoblotting—LNCaP cells were nucleofected with 5 µg of plasmids encoding FLAG-tagged Cul1, Myc-tagged F-box proteins (β-TrCP, Skp2, or Fbw7) or various GFP-tagged cyclin D1 constructs and treated with 10 µM STG28 for different time intervals followed by 4 h of co-treatment with proteasomal inhibitor MG132 before harvest. After washing with PBS, drug-treated cells were cross-linked by incubating cells with dithiobis(succinimidyl-propionimod) (150 µg/ml in PBS) for 1 h in 4 °C. Dithiobis(succinimidyl-propionimod) was then quenched by adding 100 µl of 1 M glycine for 15 min at 4 °C, and cells were lysed by commercial lysis buffer (M-PER mammalian protein extraction reagent; Pierce) in the presence of a 1% protease inhibitor mixture (Calbiochem). After centrifugation at 13,000 × g for 10 min, the supernatant was collected, preincubated with protein A-agarose (Sigma) for 15 min, and centrifuged at 1000 rpm for 5 min. One-tenth of the supernatant was reserved as input, and the remainder was exposed to anti-cyclin D1 or anti-Myc antibodies in the presence of protein A-agarose at 4 °C for 12 h. After a brief centrifugation, immunoprecipitates were washed, combined with an equal volume of 2× SDS-polyacrylamide gel electrophoresis sample loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.1% bromphenol blue), and boiled for 10 min. Equal amounts of proteins were resolved in 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes using a semidy transfer cell. The transblotted membrane was washed twice with Tris-buffered saline containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 1 h, the membrane was incubated with the appropriate primary antibody (diluted 1:1000) in 1% TBST-nonfat milk at 4 °C overnight. After incubation with the primary antibody, the membrane was washed 3 times with TBST for a total of 30 min followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (diluted 1:2500) for 1 h at room temperature. After three thorough washes with TBST for a total of 30 min, the immunoblots were visualized by enhanced chemiluminescence.

In Vivo Ubiquitination Assay—LNCaP cells were nucleofected with 5 µg of HA-ubiquitin plasmids alone or in combination with β-TrCP-Myc plasmids, incubated in 6-well plates for 24 h, and treated with 10 µM STG28 for different time intervals followed by co-treatment with the proteasome inhibitor MG132 for 4 h. After harvest, cells were lysed by a 1% protease inhibitor mixture containing M-PER buffer. Cell lysates were centrifuged at 13,000 × g for 20 min, and the supernatant was collected, preincubated with protein A-agarose for 15 min, and centrifuged at 1000 × g for 5 min. One-tenth of the supernatant was stored at 4 °C as input, and the remainder was incubated with anti-HA (Roche Applied Science) or anti-FLAG (Sigma) affinity matrix at 4 °C overnight. After a brief centrifugation, immunoprecipitates were collected, washed, suspended in 2× SDS sample buffer, and subjected to Western blot analysis with antibodies against cyclin D1 and Myc.

GST Fusion Protein Preparation—The GST-β-TrCP and GST-Skp2 fusion proteins were expressed in Escherichia coli strain BL21 (DE3) by isopropyl-1-thio-β-D-galactopyranoside induction for 3 h at 37 °C. After centrifugation at 7000 rpm for 10 min, bacteria were pelleted, suspended with 10 ml STE buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), and lysed by sonication on ice for 10 s 5 times. The lysates were centrifuged for 20 min at 16,000 rpm and dissolved in 10 ml 1.5% N-laurylsarcosine (sarkosyl)-containing STE buffer at 4 °C for 1 h. After centrifugation at 16,000 rpm for 20 min, supernatant was transferred and neutralized by adding 2% Triton X-100. Recombinant GST fusion protein were purified by incubating 200 µl of glutathione-Sepharose beads with gentle rocking at 4 °C for 30 min followed by 10 times ice-cold PBS washing. The fusion proteins immobilized onto glutathione beads were used for the following GST pulldown assay.

GST Pulldown Assay—LNCaP cells were nucleofected with GFP, wild-type cyclin D1-GFP, or E279A/E280A/T286A cyclin D1-GFP plasmids and incubated in 6-well plates (5 × 10^5 cells/well). One-tenth of the supernatant was collected, preincubated with protein A-agarose (Sigma) for 15 min, and centrifuged at 1000 × g for 5 min. One-tenth of the supernatant was reserved as input, and the remainder was exposed to anti-cyclin D1 or anti-Myc antibodies in the presence of protein A-agarose at 4 °C for 12 h. After a brief centrifugation, immunoprecipitates were washed, combined with an equal volume of 2× SDS-polyacrylamide gel electrophoresis sample loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.1% bromphenol blue), and boiled for 10 min. Equal amounts of proteins were resolved in 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes using a semidy transfer cell. The transblotted membrane was washed twice with Tris-buffered saline containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 1 h, the membrane was incubated with the appropriate primary antibody (diluted 1:1000) in 1% TBST-nonfat milk at 4 °C overnight. After incubation with the primary antibody, the membrane was washed 3 times with TBST for a total of 30 min followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (diluted 1:2500) for 1 h at room temperature. After three thorough washes with TBST for a total of 30 min, the immunoblots were visualized by enhanced chemiluminescence.

Molecular Modeling—The interface structure between the β-TrCP1 WD40 domain and the doubly phosphorylated YLDSGHSGAT motif of β-catenin was retrieved from the published crystal structure (38) (PDB code of 1P22), whereas the interface between the WD40 domain and cyclin D1 was a representative model structure calculated by an energy minimization using the Modeler program (39) with the CHARMM force field (40). The β-catenin bound β-TrCP WD40 domain structure was first subjected to the addition of hydrogens and the assignment of atomic charges. The target 27^00E00EEV遂D-
LACT^280 motif in cyclin D1 was superimposed on the crystal structure of the YLDSGHSGAT motif of β-catenin by using their sequence alignments and under the control of steric and
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RESULTS

STG28-induced Cyclin D1 Degradation Is Preceded by Cyclin D1 Phosphorylation and Nuclear Export Independently of GSK3β—Despite a lack of PPARγ activity, STG28 exhibited multifold higher potency in suppressing cyclin D1 expression than its parent compound troglitazone in LNCaP cells (Fig. 1A), which is in line with that noted in MCF-7 cells (32). Previously, we demonstrated that STG28 treatment also led to the degradation of β-catenin in LNCaP cells (34), raising a possibility that STG28-induced cyclin D1 repression might be attributable to the effect of decreased β-catenin expression on inhibiting cyclin D1 gene transcription (41). This mechanistic link, however, was refuted by two lines of evidence. First, although 10 μM STG28 suppressed cyclin D1 protein expression in a time-dependent manner, no appreciable changes in the mRNA level were noted (Fig. 1B, right panel), suggesting that STG28-induced cyclin D1 repression was mediated at the post-translational level. Second, the proteasome inhibitor MG132 was effective in rescuing the drug-induced cyclin D1 ablation (Fig. 1B, left panel). The ability of STG28 to modulate the stability of cyclin D1 protein was confirmed by the significantly shortened half-life of cyclin D1 in drug-treated LNCaP cells relative to the untreated cells (≤20 min versus 60 min) (Fig. 1C).

As ubiquitin-dependent degradation of cyclin D1 is preceded by phosphorylation and nuclear export (16), we examined the effect of STG28 (10 μM) on the phosphorylation state and cellular distribution of cyclin D1. Western blot and immunocytochemical analyses indicate that STG28-facilitated cyclin D1 repression was accompanied by increases in Thr-286 phosphorylation and cytoplasmic sequestration of ectopically expressed GFP-cyclin D1 in response to STG28 treatment. DAPI, 4,6-diamidino-2-phenylindole.

electrostatic effects. Accordingly, molecular mechanical and dynamics simulations were carried out to delineate the interactions between cyclin D1 and the β-TrCP WD40 domain.

FIGURE 1. STG28 facilitates cyclin D1 phosphorylation, nuclear export, and proteasomal degradation. A, chemical structure of troglitazone (TG) and STG28 and Western blot analyses of the dose-dependent effect of these two thiazolidinediones on suppressing cyclin D1 expression in LNCaP cells in 10% FBS-supplemented RPMI 1640 medium for 72 h. B, protective effect of proteasomal inhibitor, MG132, on STG28-mediated cyclin D1 degradation (left panel). LNCaP cells were exposed to 10 μM STG28 alone or in combination with 10 μM MG132 in 10% FBS-supplemented medium for the indicated time intervals followed by immunoblotting with anti-cyclin D1 antibodies. Right panel, STG28 does not affect cyclin D1 mRNA levels. RT, reverse transcription. C, STG28 shortens cyclin D1 protein half-life. Cells were pretreated with either DMSO or 10 μM STG28 for 6 h followed by exposure to 100 μg/ml cycloheximide for the indicated time intervals followed by immunoblotting with anti-cyclin D1 antibodies. D, time-dependent effect of 10 μM STG28 on cyclin D1 phosphorylation at the Thr-286 residue with normalized cyclin D1 levels. E, immunocytochemical evidence of STG28-induced nuclear export of cyclin D1. Left panel, intracellular distribution of endogenous cyclin D1 after treating LNCaP cells with 10 μM STG28 for the indicated time intervals. Right panel, time-dependent cytoplasmic sequestration of ectopically expressed GFP-cyclin D1 in response to STG28 treatment. DAPI, 4,6-diamidino-2-phenylindole.
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A progressive manner (Fig. 2A). Moreover, blockade of cyclin D1 nuclear export with leptomycin B, an inhibitor of CRM1-dependent nuclear export, provided partial protection against STG28-mediated cyclin D1 degradation in LNCaP cells (Fig. 2B). Together, these data suggest that the ability of STG28 to facilitate phosphorylation-dependent nuclear transport of cyclin D1 played an integral role in the subsequent proteasomal degradation.

It has been reported that nuclear export of cyclin D1 and the subsequent ubiquitin-dependent proteolysis requires GSK3β-mediated Thr-286 phosphorylation (17). However, in this study, the involvement of GSK3β in STG28-mediated cyclin D1 proteolysis was refuted by the lack of appreciable effect of two GSK3β inhibitors, LiCl and SB216763, on protecting cyclin D1 from degradation (Fig. 2C). This finding prompted an investigation of the mechanistic relationship between Thr-286 phosphorylation and STG28-induced cyclin D1 proteolysis.

In light of the dynamic role of cyclin D1 turnover in cyclin cycle regulation (1, 5), one might attribute the effect of STG28 on cyclin D1 degradation to dysregulated cell cycle function in drug-treated cells. Consequently, we assessed the time course of STG28-induced G1 arrest relative to that of cyclin D1 degradation. Flow cytometric analysis indicates that the drug-induced G1 arrest occurred at 24 h of drug treatment (Fig. 2D), whereas that of cyclin D1 repression and phosphorylation took place as early as 6 h post-treatment (Fig. 1D). This finding argued against the possibility that the effect of STG28 on cyclin D1 repression was cell cycle-dependent.

**Mutational Analysis of the Role of Thr-286 in STG28-mediated Cyclin D1 Nuclear Export and Proteolysis**—Thr-286 resides within the PEST domain, which is required for ubiquitin-dependent proteolysis of cyclin D1 (42) (Fig. 3A). To shed light on the role of Thr-286 in the pharmacological action of STG28, we assessed the susceptibility of a series of GFP-tagged cyclin D1 mutants *vis à vis* the wild-type counterpart to STG28-facilitated nuclear export and degradation. These mutations included those with a deleted C terminus (GFP-NT), deleted N terminus (GFP-CT), deleted PEST domain (GFP-ΔPEST), and substitution of Thr-286 with an alanine (T286A) or glutamic acid (T286E) (Fig. 3A). Ectopically expressed GFP-cyclin D1 was degraded at a rate comparable with that of the endogenous protein (Fig. 3C). Analysis of the subcellular distribution and metabolic fates of these GFP-tagged mutants revealed that Thr-286 was required for STG28-facilitated cyclin D1 nuclear export and degradation (Fig. 3, B and C). Of the GFP-cyclin D1 mutants, GFP-CT remained exclusively cytoplasmic irrespective of STG28 treatment but was readily subject to the drug-induced proteolysis. In contrast, GFP-NT, GFP-ΔPEST, and GFP-T286A, all of which lacked a progressive manner (Fig. 2A). Moreover, blockade of cyclin D1 nuclear export with leptomycin B, an inhibitor of CRM1-dependent nuclear export, provided partial protection against STG28-mediated cyclin D1 degradation in LNCaP cells (Fig. 2B). Together, these data suggest that the ability of STG28 to facilitate phosphorylation-dependent nuclear transport of cyclin D1 played an integral role in the subsequent proteasomal degradation.

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![Diagram of cyclin D1 structure and degradation](Image)

**FIGURE 3.** Evidence that the Thr-286 residue is integral to STG28-facilitated nuclear export and proteolysis of cyclin D1. **A**, upper panel, schematic representation of the structure of the GFP-cyclin D1 fusion protein. Lower panel, immunoblotting (IB) of GFP-cyclin D1 and various GFP-cyclin D1 mutants ectopically expressed in LNCaP cells. **B**, left panel, immunocytochemical analysis of the subcellular distribution of various GFP-cyclin D1 mutants in DMSO- and STG28-treated LNCaP cells. GFP-cyclin D1 mutants were ectopically expressed in LNCaP cells, and their subcellular localization was visualized by fluorescence microscopy after 36 h of treatment with DMSO or 10 μM STG28. Right panel, percentage nuclear distribution of GFP-tagged wild-type (WT) versus mutant cyclin D1 in STG28-treated LNCaP cells relative to that of DMSO-treated cells. Three random fields were inspected, and 10–20 transfected cells were counted in each field. Columns, mean (n = 3); bar, S.D. C, dose-dependent effect of STG28 on the degradation of the various GFP-cyclin D1 mutants in LNCaP cells. Cells ectopically expressing GFP-cyclin D1 mutants were treated with STG28 at the indicated concentrations for 48 h and immunoblotted for GFP and cyclin D1. Endogenous cyclin D1 degradation was used as an internal control for STG28 activity. DAPI, 4,6-diamidino-2-phenylindole.

286 phosphorylation. In addition to GSK3β, a number of kinases have also been reported to phosphorylate cyclin D1 at Thr-286, including IKKα (18), ERK1/2 (20), and p38 (19). Western blot analysis indicates that STG28 (10 μM) increased the phosphorylation levels of ERK1/2, p38, and IKKα as well as GSK3β in a time-dependent manner (Fig. 4A, left panel). To discern the involvement of ERKs, p38, and IKKα, we assessed the effect of the respective pharmacological inhibitors PD98059, SB203580, and Bay11-7082 on rescuing STG28-mediated cyclin D1 degradation in LNCaP cells. Our data indicate that inhibition of IKKα, but not that of ERK1/2 or p38, provided a dose-dependent protection against STG28-facilitated cyclin D1 repression (Fig. 4A, right panel), suggesting that IKKα might play a key role in the STG28 effect on cyclin D1 degradation. To validate this finding we transfected LNCaP cells with IKKKα-specific siRNA or plasmids encoding the IKK dominant negative mutant (pIKK2M) to suppress the expression or activity of IKKα. As shown, IKKα inhibition via either approach conferred resistance to STG28-induced cyclin D1 proteolysis (Fig. 4B and C). Immunocytochemical analysis in pIKK2M-transfected LNCaP cells indicates that this protection was attributable to the inability of cyclin D1 to undergo cytoplasmic translocation in response to STG28 (Fig. 4D).

**SCFβ-TrCP** Facilitates STG28-mediated Cyclin D1 Degradation—Earlier studies have implicated a number of F-box proteins of the SCF E3 ubiquitin ligase in the nuclear export of the Thr-286 mutant. Likely, an additional mechanism might be required to account for the ability of STG28 to facilitate cytoplasmic sequestration of T286E. Alternatively, as cyclin D1 cellular distribution results from an equilibrium between nuclear import and nuclear export (17), it is also possible this drug-induced nuclear exclusion might in part be attributed to the effect of STG28 on interfering with nuclear import of the T286E mutant, of which the investigation is in progress.

**IKKα Regulates STG28-induced Cyclin D1 Nuclear Export and Degradation**—The findings described above suggest the dependence of STG28-induced cyclin D1 proteolysis on Thr-286 phosphorylation. In addition to GSK3β, a number of kinases have also been reported to phosphorylate cyclin D1 at Thr-286, including IKKα (18), ERK1/2 (20), and p38 (19). Western blot analysis indicates that STG28 (10 μM) increased the phosphorylation levels of ERK1/2, p38, and IKKα as well as GSK3β in a time-dependent manner (Fig. 4A, left panel). To discern the involvement of ERKs, p38, and IKKα, we assessed the effect of the respective pharmacological inhibitors PD98059, SB203580, and Bay11-7082 on rescuing STG28-mediated cyclin D1 degradation in LNCaP cells. Our data indicate that inhibition of IKKα, but not that of ERK1/2 or p38, provided a dose-dependent protection against STG28-facilitated cyclin D1 repression (Fig. 4A, right panel), suggesting that IKKα might play a key role in the STG28 effect on cyclin D1 degradation. To validate this finding we transfected LNCaP cells with IKKKα-specific siRNA or plasmids encoding the IKK dominant negative mutant (pIKK2M) to suppress the expression or activity of IKKα. As shown, IKKα inhibition via either approach conferred resistance to STG28-induced cyclin D1 proteolysis (Fig. 4B and C). Immunocytochemical analysis in pIKK2M-transfected LNCaP cells indicates that this protection was attributable to the inability of cyclin D1 to undergo cytoplasmic translocation in response to STG28 (Fig. 4D).

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FIGURE 4. Evidence that IKKα plays a pivotal role in STG28-mediated cyclin D1 nuclear export and degradation. A, left panel, time-dependent effect of 10 μM STG28 on the phosphorylation status of GSK3β, IKKα, ERKs, and p38 in LNCaP cells in 10% FBS-containing medium for 36 h. Right panel, effects of specific kinase inhibitors (PD98059, ERKs; SB203580, p38; Bay11-7082, IKKα) on STG28-mediated cyclin D1 degradation. LNCaP cells were exposed to STG28 and/or individual kinase inhibitors at the indicated concentrations for 36 h. B, siRNA-mediated knockdown of IKKα rescues STG28-induced cyclin D1 repression. C, IKKα inhibition by the ectopic expression of a dominant negative IKKα mutant (ICK2M) protected cyclin D1 from STG28-induced degradation. Percentages represent the relative expression levels of cyclin D1 compared with that at the 0-h time point and normalized to β-actin. D, immunocytochemical evidence that ectopic expression of IKK2M hindered STG28-mediated GFP-cyclin D1 nuclear export. DAPI, 4,6-diamidino-2-phenylindole.
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(2-4) in many of its target proteins, such as IκB, β-catenin, Cdc25A, and Emi1, after phosphorylation of both serine residues by different kinases (for review, see Ref. 43). As cyclin D1 lacks this binding motif, we rationalized that cyclin D1 contained an alternative sequence required for β-TrCP recognition. Based on our finding that Thr-286 was essential to the effect of STG28 on facilitating cyclin D1 nuclear export and degradation (Fig. 2), we hypothesized that the sequence preceding the phospho-Thr-286 (279EEVDLACpT286) mimicked the DSG destruction motif with Glu-280 serving as a phosphomimetic in place of the upstream phosphoserine.

The involvement of this putative motif in regulating cyclin D1 degradation through β-TrCP binding was corroborated by a series of mutational analyses. The T286A mutation abrogated the interaction between ectopically expressed Myc-tagged β-TrCP and the GFP-tagged cyclin D1 mutant, whereas T286E, a phosphomimetic mutation, did not hinder the binding (Fig. 7A). Furthermore, when glutamates 279 and 280 were replaced with alanines in cyclin D1 (E279A/E280A) or in the T286A mutant (E279A/E280A/T286A), the resulting cyclin D1 was resistant to STG28-mediated degradation (Fig. 7B) and was unable to bind β-TrCP (Fig. 7C). Nevertheless, the E279A/E280A mutant retained a dose-dependent susceptibility to STG28-facilitated phosphorylation. Consequently, in contrast to cyclin D1T286A, which failed to exit the nucleus, the E279A/E280A mutant was sequestered into the cytoplasm in response to STG28 (Fig. 7D). Together, these findings underscore the obligatory role of Thr-286 phosphorylation in mediating the translocation of cyclin D1 into the cytoplasm in STG28-treated cells, where cyclin D1 could interact with β-TrCP to undergo ubiquitin-dependent degradation.

Pursuant to this finding, we performed an in vitro pulldown analysis to confirm the role of this recognition site in β-TrCP binding. GST, GST-β-TrCP, and GST-Skp2 were expressed in E. coli cells and purified by glutathione beads. Meanwhile, LNCaP cells were nucleofected with plasmids encoding GFP, GFP-cyclin D1, or GFP-E279A/E280A/T286A mutant, and the resulting cell lysates were exposed to immobilized GST, GST-β-TrCP, or GST-Skp2. The resulting complexes were washed and subjected to immunoblotting with GFP antibodies. Relative to wild-type cyclin D1, the cyclin D1 mutant exhibited substan-
tially lower binding affinity with GST-β-TrCP (Fig. 7E). In contrast, neither wild-type cyclin D1 nor the mutant displayed appreciable binding to either GST or GST-Skp2.

To envisage the mode of interaction of this recognition sequence with β-TrCP, we carried out molecular modeling analysis by docking the phosphorylated binding motif (S277EEEVDLASpT286) of cyclin D1 into the top face of the β-TrCP1 WD40 domain in a manner similar to that described for the doubly phosphorylated β-catenin destruction motif (S38YLDpSGHlPSGAT40) (38). As seen in Fig. 8, these two peptides exhibited a similar mode of binding into the channel of the WD40 domain. The charged side chains of Asp-32–phospho-Ser-33 (β-catenin) and Glu-279–Glu-280 (cyclin D1) form hydrogen bonding and electrostatic interactions with the guanidinium groups of Arg-285 and Arg-474. Despite differences in the length of the recognition motifs, the phosphate groups of phospho-Ser37 (β-catenin) and phospho-Thr-286 (cyclin D1) make contact with the guanidinium groups of Arg-431 and Arg-410 on the opposite end of the channel.

**Role of β-TrCP in Cyclin D1 Degradation in Glucose-starved LNCaP Cells**—Cyclin D1 accumulates in response to nutrients and mitogenic growth factors to sufficient levels through transcriptional activation, enhanced mRNA expression, and reduced proteasomal degradation (for review, see Refs. 3 and 44). We hypothesized that nutritional deprivation such as glucose starvation might accelerate cyclin D1 degradation at least in part through the β-TrCP-mediated pathway. To corroborate this premise, we examined the temporal relationship between glucose starvation and the expression levels of cyclin D1, β-TrCP, and known β-TrCP target proteins, including, β-catenin, IκBα, and Wee1 in LNCaP cells. Western blot analysis shows that glucose withdrawal from medium caused a time-dependent increase in β-TrCP expression accompanied by de-
creased levels of cyclin D1 and various β-TrCP target proteins, parallel the dose-dependent effects of STG28 on modulating the expression levels of these proteins (Fig. 9A). Moreover, glucose deprivation altered the expression of various F-box proteins in a manner similar to that of STG28 (Fig. 9B, left panel). Equally important, co-immunoprecipitation analysis shows that glucose starvation-induced cyclin D1 repression was associated by increased association of cyclin D1 with β-TrCP, confirming the proposed mechanistic link. Although a weak association was noted between cyclin D1 and Fbxw8, prolonged exposure did not increase the extent of complex formation. Moreover, cyclin D1 displayed no appreciable binding with Skp2, Fbw7, or Fbx4, refuting their involvement in glucose deprivation-facilitated cyclin D1 degradation.

**DISCUSSION**

As cyclin D1 overexpression in human cancers can arise from increased protein stability, targeting cyclin D1 degradation by small-molecule agents represents a therapeutically relevant strategy for the treatment of cyclin D1-overexpressing tumors (16). Previously, based on our mechanistic finding that the effect of troglitazone and ciglitzone on promoting the ubiquitin-dependent proteolysis of cyclin D1 was dissociated from PPARγ activation (32), we have developed a novel class of cyclin D1-ablative agents (33). From a translational perspective, understanding how these agents mediate cyclin D1 degradation represents an integral step for their therapeutic development.

In this study we report a novel mechanism in which β-TrCP plays a pivotal role in cyclin D1 degradation in STG28-treated LNCaP cells. Mutational analysis indicates that phosphorylation at Thr-286 represented an obligatory step for STG28-facilitated nuclear export and proteasomal degradation of cyclin D1. STG28 exhibited a dichotomous effect on the activation of the triad Thr-286, Glu-279, and Glu-280 in the β-TrCP recognition and STG28-mediated degradation of cyclin D1.
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FIGURE 8. Molecular modeling analysis of β-TrCP WD40 domain and β-TrCP binding motif of cyclin D1, 279EEVDLaCT286. Interface structures of the doubly phosphorylated β-catenin peptide (left panel) and the singly phosphorylated cyclin D1 peptide (right panel) bound to the β-TrCP WD40 domain. The β-TrCP1 WD40 domain is shown as solid ribbons, with its involved side chains in stick form, and the peptides of β-catenin and cyclin D1 are represented as green and orange tubes, respectively, with their identified side chains shown in light green and yellow stick forms, respectively.

FIGURE 9. STG28 mimics the effect of glucose starvation on cyclin D1 degradation. A, dose-dependent effects of STG28 and time-dependent effect of glucose starvation on modulating the expression of cyclin D1, β-TrCP, and its known substrates, including β-catenin, IκBα, and Wst1. LNCaP cells were exposed to different concentrations of STG28 for 72 h or incubated in glucose-deprived, 10% FBS-supplemental RPMI 1640 medium for the indicated time intervals, and immunoblotting was performed as described under “Experimental Procedure.” B, glucose starvation promoted the association of cyclin D1 with β-TrCP. Cells were incubated in glucose-deprived medium for different time intervals followed by immunoprecipitation (IP) with anti-cyclin D1-agarose, and immunoblotting for cyclin D1, β-TrCP, Skp2, Fbw7, Fbw4, and Fbw8x were performed.

indicates that cyclin D1 interacted with β-TrCP in a Thr-286-dependent manner. Equally important, overexpression and siRNA-mediated knockdown of β-TrCP resulted in the enhancement and rescue of STG28-mediated cyclin D1 degradation. As cyclin D1 does not contain a DSG destruction motif common to many β-TrCP substrates, we obtained evidence that the sequence preceding Thr-286 (279EEVDlACT286) served as a β-TrCP recognition motif (DSG_X_S) with Glu-280 acting as a phosphomimetic of the upstream phospho-Ser. Consequently, phosphorylation at Thr-286 represents the sole molecular switch regulating the nuclear export and β-TrCP recognition of cyclin D1 without the requirement of second phosphorylation. This EEV motif is reminiscent of the unconventional β-TrCP recognition site (116EEFGpS121) reported in the Cdc2 inhibitory kinase Wee1 (45, 46).

Data from this and other laboratories indicate that there exists a multitude of mechanisms involving different kinases and F-box proteins of the SCF E3 ligase to govern cyclin D1 degradation, each of which might play a different functional role in cell-cycle regulation. Substantial evidence indicates that cyclin D1 serves as an active switch in the regulation of continued cell cycle progression (3). In addition to regulating cell cycle, cyclin D1 levels are elevated in response to proliferative signals such as nutrients and mitogenic growth factors, indicating its role as an important regulator of cell growth (for review, see Refs. 3 and 44). Contrarily, when these proliferative signals are withdrawn from the environment, cyclin D1 levels have to be suppressed to arrest cell proliferation. We provide the first evidence that that this β-TrCP-dependent degradation takes part in controlling cyclin D1 turnover when cancer cells undergo glucose starvation, which endows physiological relevance to this novel mechanism.

In conclusion, using STG28 as a probe, we have identified a novel IKKα-dependent, SCFβ-TrCP-mediated pathway for cyclin D1 degradation. This mechanism also accounts for the unique ability of troglitazone and STG28 to suppress the expression of β-catenin, NF-κB, and a series of cell-cycle regulatory proteins (34). Equally important, normal prostate epithelial cells are resistant to the effect of troglitazone and STG28 on up-regulating β-TrCP expression and are thereby less sensitive to the apoptosis-inducing activity of these agents (34). From a therapeutic perspective, the tumor cell-specific, pleiotropic effect of STG28 on multiple signaling pathways might underlie its translational potential in cancer therapy/prevention, which represents the current focus of this investigation.

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