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Recognition of Phosphodegron Motifs in Human Cyclin E by the SCF^{Fbw7} Ubiquitin Ligase*

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Turnover of cyclin E is controlled by SCF^{Fbw7}. Three isoforms of Fbw7 are produced by alternative splicing. Whereas Fbw7α and -γ are nuclear and the β-isoform is cytoplasmic in 293T cells, all three isoforms induce cyclin E destruction in an *in vivo* degradation assay. Cyclin E is phosphorylated on Thr^{62}, Ser^{88}, Ser^{372}, Thr^{380}, and Ser^{384} *in vivo*. To examine the roles of phosphorylation in cyclin E turnover, a series of alanine point mutations in each of these sites were analyzed for Fbw7-driven degradation. As expected, mutation of the previously characterized residue Thr^{380} to alanine led to profound defects of cyclin E turnover, and largely abolished association with Fbw7. Mutation of Thr^{62} to alanine led to a dramatic reduction in the extent of Thr^{380} phosphorylation, suggesting an indirect effect of this mutation on cyclin E turnover. Nevertheless, phosphopeptides centered at Thr^{62} associated with Fbw7, and residual binding of cyclin E^{T380A} to Fbw7 was abolished upon mutation of Thr^{62}, suggesting a minor role for this residue in direct association with Fbw7. Mutation of Ser^{384} to alanine also rendered cyclin E resistant to degradation by Fbw7, with the largest effects being observed with Fbw7β. Cyclin E^{S384A} associated more weakly with Fbw7α and -β isoforms but was not defective in Thr^{380} phosphorylation. Analysis of the localization of cyclin E mutant proteins indicated selective accumulation of cyclin E^{S384A} in the nucleus, which may contribute to the inability of cytoplasmic Fbw7β to promote turnover of this cyclin E mutant protein.

Flux through signaling pathways is controlled, in large part, by regulated protein destruction and reversible protein phosphorylation. During the last few years, it has become clear that, in many cases, protein destruction is initiated by site-specific phosphorylation of the target protein, which then facilitates the interaction of the target protein with the destruction machinery. Much of the ubiquitination that occurs in response to protein phosphorylation occurs via the SCF ubiquitin ligase pathway. Phosphorylated proteins are ubiquitinated by an E1-E2[1] thiol-ester cascade, wherein the E2 is brought to the phosphorylated substrate via an SCF E3 (1–3). SCF complexes are composed of a core ubiquitin ligase containing the scaffold Cul1, a ring finger protein called Rbx1/Roc1, and an adaptor protein called Skp1 (4–8). Rbx1 associates with and activates E2s including Cdc34 (7, 9), whereas Skp1 interacts simultaneously with Cul1 and with a member of the F-box family of proteins (10). F-box proteins constitute a large family (more than 70 members in humans) of specificity factors that link diverse substrates with ubiquitination machinery (3, 11–13).[2] Many F-box proteins contain C-terminal protein-protein interaction domains including WD40 and leucine-rich repeats that allow for specific target recognition. Several F-box proteins, including Cdc4 and Grr1 in yeast and β-TRCP (β-transducin-repeat containing protein), Skp2, and Fbw7 in humans, have been demonstrated to interact with target proteins in a phosphorylation-dependent manner (5, 14–19, 20–23). Structural and biochemical analysis of the cyclin-dependent kinase inhibitor Sic1, a target of the SCF^{Cdc4} complex, has revealed a complex relationship between Sic1 phosphorylation events and recognition by WD40 repeats in Cdc4 (18, 24). In essence, a minimum of six phosphorylation events are required for recognition through a single phosphodegron binding site on Cdc4. Each of these phosphorylation events generates phosphodegrons that are, in isolation, suboptimal for tight binding with Cdc4 (18, 24). In contrast, structural analysis of the β-TRCP F-box protein responsible for destruction of IκBα and β-catenin indicates that two phosphorylation events are recognized as a unit by a specific network of basic residues (25). The extent to which other substrates of the Cdc4 class of F-box proteins require multiple phosphorylation events is unknown.

In this work, we have examined how phosphorylation is used to regulate the interaction of the human G_1 cyclin, cyclin E, with the WD40 containing F-box protein, Fbw7. Fbw7 is most closely related to Cdc4 in budding yeast (17, 21, 26) and has

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[1] The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; Cdk, cyclin-dependent kinase; MS, mass spectrometry.

[2] J. Jin and J. W. Harper, unpublished data.

been implicated in the turnover of not only cyclin E but Notch (27), c-Myc (28–30), and c-Jun (31) as well. RNAi against Fbw7 in humans or its *Drosophila* ortholog *Ago* leads to increased levels of cyclin E in tissue culture cells (17, 21), and mutations in *Ago* affect cyclin E levels in the *Drosophila* (26). In addition, mice deficient in Fbw7 die during embryogenesis (~10.5 days postcoitum) and both the embryo and the placenta display stabilization of cyclin E and Notch (32). Finally, Fbw7 can ubiquitinate cyclin E *in vitro* (17, 21). Mammalian cyclin E functions to activate cyclin-dependent kinase 2 (Cdk2) at the G_1/S transition. Previous work has demonstrated that phosphorylation of Thr^{380} in cyclin E, in part via an autophosphorylation mechanism, is required for its rapid turnover (33, 34). This residue can also be phosphorylated by GSK3β to promote turnover (35). Although mutation of Thr^{380} to alanine greatly stabilizes cyclin E, its turnover *in vivo* or ubiquitination *in vitro* is not completely eliminated (17, 21). Recent studies have also implicated phosphorylation of Thr^{62} and Ser^{384} in cyclin E turnover by Fbw7β (21, 35), but whether this reflects direct or indirect effects in Fbw7 recognition is not clear. Thr^{62} conforms to a Cdk2 consensus site but the identity of kinases involved in Thr^{62} phosphorylation are currently unknown (35). Available data indicates that phosphorylation of Thr^{62} is required for phosphorylation of Ser^{58} by GSK3β (35). Phosphorylation of Ser^{384} is dependent upon interaction of cyclin E with active Cdk2, and it has been proposed that Ser^{384} is directly phosphorylated by Cdk2 (35).

To understand in greater detail how Fbw7 recognizes cyclin E and promotes its turnover, we have used mass spectrometry to confirm and extend recent peptide mapping studies of cyclin E phosphorylation and have performed a series of biochemical and mutagenic experiments that examine the role of multiple phosphorylation events in recognition and turnover of cyclin E by three specific isoforms of Fbw7, α, β, and γ. The data indicate both direct and indirect roles for Thr^{62} in binding to Fbw7. Whereas synthetic peptides encompassing Thr^{62} bind Fbw7 in a phosphorylation-dependent manner, the major effect seen upon mutation of Thr^{62} to alanine is a dramatic reduction in the extent of Thr^{380} phosphorylation, suggesting an indirect effect of the T62A mutation on cyclin E degradation through the Thr^{380} degron. Nevertheless, residual association of cyclin E^{T380A} with Fbw7 is reduced upon mutation of Thr^{62} to alanine. Mutation of Ser^{384} to alanine reduced turnover of cyclin E by all three isoforms of Fbw7, although the effects seen with Fbw7β were much greater than that observed with α and γ. Cyclin E^{S384A} associated more weakly than wild-type cyclin E with both Fbw7α and Fbw7β. Unlike other cyclin E mutants examined, cyclin E^{S384A} appeared to be preferentially retained in the nucleus, partially explaining the reduced ability of cytoplasmic Fbw7β to promote cyclin E turnover.

MATERIALS AND METHODS

Plasmids, Transfections, and Immunofluorescence—The pCS2-Myc-cyclin E expression plasmid employed was from a previous study (33). Mutations were generated using the Gene Editor System (Promega). Vectors for expression of FLAG-Fbw7α, -β, and -γ were created in pCMV-FLAG (Sigma). To create an Fbw7γ expression plasmid, PCR was used to amplify Fbw7γ-specific sequences from a brain cDNA library. The oligonucleotides used were as follows: forward, GATCAAGCTTATGTCAAAACCGGGAAAACCTACTC; reverse, TGTCTGAGCTGCTTGTAGCAGGTC. The 570-bp PCR product was digested with HindIII and BspMI and ligated into the pCMV-FLAG-Fbw7 backbone previously digested with HindIII and BspMI, and the product was confirmed by DNA sequencing. Plasmids used for expression of untagged Fbw7 mutants were described previously (17). To generate an expression plasmid for dominant negative Cul1, sequences encoding amino acids 1–453 were cloned into pcDNA3 (12). 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The indicated quantities of DNA were used in either calcium phosphate- or Fugene 6 (Roche Applied Science)-mediated transfections. Thirty-six hours after transfection, cells were either lysed in 25 mM Tris-HCl, 100 mM NaCl, 0.1% Triton X-100, 10 mM β-glycerol phosphate, 10 mM NaF, and protease inhibitors (Roche Applied Science) or were processed for immunofluorescence.

Microscopy and Image Analysis—For immunofluorescence, cells transfected with cyclin E or Fbw7α/β/γ plasmids were grown on ultrathin cover slides (Fisher) and fixed in methanol or 4% paraformaldehyde in phosphate-buffered saline followed by permeabilization with 0.2% Triton X-100 for 10 min. Cells were then blocked with 5% goat serum (Sigma) for 30 min, incubated with primary antibodies (anti-Myc or anti-FLAG at 1:100) for 1 h, washed five times with phosphate-buffered saline, incubated with fluorochrome-conjugated secondary antibody for 1 h, and generously washed. Nuclei were then counterstained with 4′,6′-diamidino-2-phenylindole, and cells were mounted in Slow-Fade Light Antifade reagent (Molecular Probes, Inc., Eugene, OR). To visualize subcellular localization of Fbw7 isoforms, images were taken on a Nikon/DeltaVision deconvolution microscope (Applied Precision) as a series of 0.2-μm-thick Z-sections and processed with a Softworx image work station. Each image represents single transcellular Z-section.

For fluorescence signal quantification, cells were fixed and immunostained as described above 48 h post-transfection with the indicated wild type and mutant cyclin E-expressing plasmid. Images were recorded with an empirically chosen, constant exposure time on an Olympus BX60 microscope fitted with a Hammamatsu CCD camera. For processing, an ImagePro Plus work station (Media Cybernetics Inc.) was employed. Fluorescence intensity profiles were measured and averaged along intranuclear or intracytoplasmic linear tracks, and obtained values were normalized against internal background controls for each visual field. Results were calculated as $y = \Delta(x(N_{norm}/C_{norm}); \mathrm{wt}(N_{norm}/C_{norm}))$, using averaged *N/C* ratios for 15 cells/construct. Statistical significance was confirmed by analysis of variance.

Immunoprecipitation and Protein Interactions—To examine cyclin E degradation, lysates from transfected cells were subjected to immunoblotting using anti-Myc (9E10) antibodies (Covance). To examine cyclin E turnover, cells were transfected with the indicated plasmids, and 36 h later, cells were cultured for 1 h in methionine-free medium prior to pulse labeling with [^{35}S]methionine (0.1 mCi/ml). Twenty minutes later, [^{35}S]methionine-containing medium was replaced with fresh medium containing 10 mM cold methionine. Extracts made at the indicated times were used for immunoprecipitation with anti-Myc antibodies. Immune complexes were subjected to SDS-PAGE, autoradiography, and quantitation using a PhosphorImager (Amersham Biosciences). To examine association of FLAG-Fbw7α with cyclin E post-transfection, extracts were subjected to immunoprecipitation with anti-FLAG antibodies, and immune complexes were immunoblotted with anti-Myc antibodies. To examine the extent of cyclin E phosphorylation at Thr^{380}, anti-cyclin E immune complexes were separated by SDS-PAGE and immunoblotted with anti-phospho-Thr^{380} antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the blot was subsequently stripped and reprobed with anti-Myc antibodies. The absence of reactivity of anti-phospho-Thr^{380} antibodies with cyclin E^{T380A} points to the specificity of the antibodies employed. For peptide binding experiments, the indicated synthetic peptides were coupled to Affi-Gel-10 beads (Bio-Rad) at a concentration of 1 mg/ml. Immobilized peptides (7 μl) were incubated with the indicated *in vitro* translation products (5 μl) in a total volume of 120 μl of NETN (25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, and 0.1% Nonidet P-40). Complexes were washed three times with 1 ml of NETN prior to SDS-PAGE and autoradiography. In some cases, Thr^{62}-containing peptides were employed as competitor. Antibodies against a synthetic peptide containing Thr^{62} (SLIPpTPDK) were made by Phospho-Solutions, Inc. (Aurora, CO).

Mass Spectrometry—Recombinant cyclin E was produced in insect cells as a glutathione *S*-transferase fusion protein as described (17). To produce mammalian cell-derived cyclin E for mass spectrometry, pCS2-Myc-cyclin E was transfected into four 150-mm dishes using Fugene 6. Subsequently, Myc-cyclin E was isolated using immobilized 9E10 antibodies and purified by SDS-PAGE for mass spectral analysis. Approximately 500 ng of cyclin E was recovered for mass spectral analysis, based on Coommassie Blue staining (data not shown). Mass spectral analysis of phosphopeptides in cyclin E was performed using matrix-assisted laser desorption ionization mass spectrometry with delayed extraction (Voyager-DE; Perseptive Biosystems, Framingham, MA). Unless otherwise noted, an electrospray ion trap mass spectrometer (LCQ; Finnigan, San Jose, CA) coupled on-line with a capillary high pressure liquid chromatograph (Magic 2002, Auburn, CA) was used for identification of phosphorylation sites. Cyclin E$^{81–98}$ was sequenced by

TABLE I
Analysis of cyclin E phosphorylation by mass spectrometry

Tryptic fragment	Calculated mass	Observed mass	
		293T	Insect cells
	Da	*Da*	
F14–15 (aa 81–98)[a]	1934.3	2014.2 (1P)	2014. 5[b] (1P)
F41–43 (aa 362–386)	2608.0	2848.2 (3P), 2768.5 (2P), 2688.1 (1P)	2848.3[b] (3P), 2768.2 (2P), 2688.2 (1P)
F42–43 (aa 363–386)	2479.8		2640.0[b] (2P), 2560.0 (1P)

[a] aa, amino acids.
[b] Phosphopeptides sequenced by liquid chromatography/MS/MS.

liquid chromatography/mass spectrometry/mass spectrometry (liquid chromatography/MS/MS) using a Q-TOF micro (Micromass).

RESULTS

Analysis of Cyclin E Phosphorylation in Vivo—Previous studies of cyclin E have demonstrated that mutation of Thr^{380} or Thr^{62} to nonphosphorylatable alanine leads to partial stabilization of cyclin E in transfected cells, whereas simultaneous mutation of both leads to further stabilization (21). Additionally, these mutations lead to reduced ubiquitination efficiency *in vitro* (17, 21). Precisely how these phosphorylation events facilitate recognition by Fbw7 is unclear. In addition, it is possible that additional phosphorylation events in cyclin E are important for recognition by Fbw7. Recent studies have identified five cyclin E phosphorylation sites, Ser^{58}, Ser^{75}, Ser^{372}, Thr^{380}, and Ser^{384}, by conventional peptide mapping (35). Using mass spectrometry, we confirmed and extended these results in cyclin E purified from mammalian cells after transfection with a pCMV-Myc-cyclin E expression plasmid and in cyclin E expressed in insect cells with Cdk2 (Table I). In brief, various forms of cyclin E$^{363–386}$ were found to contain either two or three phosphates, as determined by treatment with calf intestinal phosphatase. Peptide sequencing by liquid chromatography/MS/MS identified Ser^{372}, Thr^{380}, and Ser^{384} as sites of phosphorylation in the triply phosphorylated peptide (Table I). Peptide sequencing of the cyclin E$^{81–98}$ peptide by liquid chromatography/MS/MS demonstrated phosphorylation on Ser^{88} (Table I and data not shown). Ser^{88} in cyclin E has not been previously shown to be phosphorylated but does conform to a minimal Cdk2 consensus site. In these experiments, we did not observe phosphopeptides containing either Ser^{58}, which is known to be phosphorylated (35), or Thr^{62}, possibly due to the large size and cysteine-rich character of the predicted tryptic peptide. Therefore, antibodies directed at phospho-Thr^{62} were generated using a synthetic peptide as antigen. Immunoblotting of affinity-purified Myc-tagged wild-type or T62A cyclin E with these antibodies revealed specific interaction with wild-type but not T62A cyclin E (Fig. 1*C*). Moreover, treatment of Myc-cyclin E with λ-phosphatase resulted in loss of reactivity toward the phospho-Thr^{62} antibody, consistent with a specific phosphorylation-dependent interaction (Fig. 1*D*). These data indicate that cyclin E is phosphorylated on Thr^{62} in tissue culture cells. Overall, these data lead to the conclusion that cyclin E is phosphorylated on at least three sites in the C terminus and at least four sites near the N terminus (Fig. 1*B*).

Contribution of Phosphorylation to Fbw7-mediated Cyclin E Turnover in Vivo—Currently, three distinct isoforms of Fbw7 (α, β, and γ) have been described (17, 21, 36). These isoforms employ distinct 5′ exons encoding unique N termini fused with 10 common exons. We first asked where these proteins are localized in the cell. However, because anti-Fbw7 antibodies suitable for immunofluorescence are not available, we used transient transfection of Fbw7 expression vectors in which the N terminus was tagged with a FLAG epitope. In 293T cells, we found that both Fbw7α *and Fbw7*γ are localized primarily in the nucleus (Fig. 1, *A* and *C*). In contrast, Fbw7β is almost exclusively found in the cytoplasm (Fig. 1*B*). We previously reported that Fbw7β has an apparent transmembrane domain near the N terminus (17), and this may be involved in localizing Fbw7β to endoplasmic reticulum membranes. An in depth analysis of cis-acting signals important for proper localization will be reported elsewhere.[3]

To examine the contribution of multisite phosphorylation to cyclin E turnover, we employed an *in vivo* degradation assay wherein Myc-cyclin E and Cdk2 are transiently expressed in 293T cells in the presence or absence of Fbw7 isoforms (35). Expression of Myc-cyclin E and Cdk2 alone led to readily detectable Myc-cyclin E, as determined by immunoblotting of crude cell extracts (Fig. 2, *A–C*). Co-expression of increasing levels of FLAG-Fbw7α led to a dramatic decrease in the steady-state abundance of Myc-cyclin E. All three Fbw7 isoforms, when expressed at comparable levels, were capable of reducing the abundance of cyclin E but had no effect on Cdk2 abundance. These results extend previous results from multiple laboratories indicating that Fbw7β overexpression can drive cyclin E degradation when overexpressed (21, 35). As expected, cyclin E^{T380A} and cyclin E$^{T62A/T380A}$ levels were largely unaffected by expression of all three isoforms of Fbw7. This is consistent with a major role for Thr^{380} phosphorylation in controlling cyclin E turnover, as determined by pulse-chase (Fig. 2*D*). We then examined the susceptibility of T62A, S88A, S372A, and S384A mutations to elimination by Fbw7 isoforms. We found that T62A was substantially defective in elimination by Fbw7β and -γ, but this defect was much less obvious with the Fbw7α isoform under these conditions (Fig. 2, *A–C*). However, using lower Fbw7α expression plasmids revealed clear defects in cyclin E^{T62A} turnover (Fig. 2*F*). We also found that Fbw7β was profoundly defective in eliminating cyclin E^{S384A} (Fig. 2*B*), whereas turnover of cyclin E^{S384A} by Fbw7α and -γ was less affected (Fig. 2, *A* and *C*). At higher levels of cyclin E^{S384A} expression plasmid used for transfection, cyclin E^{S384A} was substantially more resistant to turnover by Fbw7α than was wild-type cyclin E (Fig. 2*G*). Cyclin E^{S372A} and cyclin E^{S88A} were efficiently degraded by all three Fbw7 isoforms (Fig. 2, *A–C*, and data not shown for cyclin E^{S372A}). Control experiments demonstrated comparable levels of all three Fbw7 isoforms in transient transfections (Fig. 2*E*).

Phosphorylation of Thr^{380} in the C-terminal Phosphodegron of Cyclin E Is Sufficient for Interaction with Fbw7—Given the multisite phosphorylation of cyclin E in residues flanking Thr^{380}, we next examined the contribution of individual phosphorylation events to recognition by Fbw7 in the context of short peptide phosphodegrons. Previous studies have demonstrated that short phosphopeptides are specifically recognized by WD40-containing F-box proteins (17, 18, 22). Synthetic peptides spanning amino acids 361–388 of cyclin E (cyclin E$^{361–388}$) were synthesized in various phosphorylated and unphosphorylated forms that mimic phosphorylation *in vivo* (Fig. 3*A*), coupled to agarose beads, and then tested for binding to *in vitro*

[3] B. E. Clurman and M. Welcker, unpublished data.

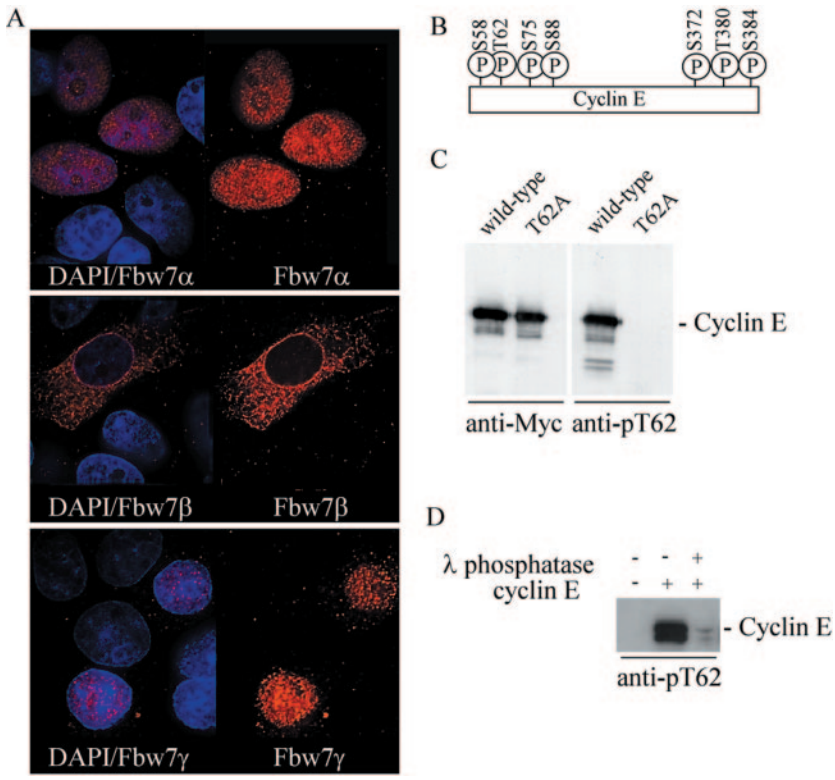

FIG. 1. *A*, differential localization of Fbw7 isoforms. Expression plasmids encoding the indicated N-terminally FLAG-tagged Fbw7 isoforms were transiently transfected into 293T cells using Fugene 6. After 36 h, cells were subjected to indirect immunofluorescence. Anti-FLAG immunofluorescence is shown in *red*. Nuclei, stained with 4′,6′-diamidino-2-phenylindole, are shown in *blue*. *B*, schematic representation of phosphorylation sites found in cyclin E (see Table I). *C* and *D*, antibodies directed at phospho-Thr^{62} of cyclin E selectively interact with wild-type cyclin E purified from 293T cells after transient transfection but do not react with cyclin E^{T62A} or with cyclin E treated previously with λ-phosphatase. *C*, 293T cells were transfected with pCMV-Myc-cyclin E DNA, and after 48 h, cell lysates were immunoprecipitated with anti-Myc antibodies prior to immunoblotting with either anti-Myc antibodies or anti-phospho-Thr^{62} antibodies. In *D*, Myc-cyclin E immune complexes were incubated in the presence or absence of λ-phosphatase prior to immunoblotting with anti-phospho-Thr^{62} antibodies.

translated Fbw7 (Fig. 3*B*). As expected, unphosphorylated cyclin E$^{361–388}$ did not associate with Fbw7 (Fig. 3*B*, *lane 2*). In addition, we found that phosphorylation of either Ser^{372} of Ser^{384} alone did not support Fbw7 binding (Fig. 3*B*, *lanes 4* and *6*). In contrast, cyclin E$^{361–388}$ peptides containing phosphorylation at Thr^{380} alone (*lane 4*) or in combination with Ser^{372} and Ser^{384} (*lanes 5* and *7*) interacted efficiently with Fbw7. In the context of Thr^{380} phosphorylation, phosphorylation at either Ser^{372} or Ser^{384} did not enhance association with Fbw7 (Fig. 3*B*, *lanes 5* and *7*). Thus, within the context of immobilized peptides *in vitro*, phosphorylation of Thr^{380} appears to be the major determinant in Fbw7 binding to the C-terminal phosphodegron.

Interaction of Fbw7 with a Phosphodegron Centered at Thr^{62}—Mutation of Thr^{62} to alanine leads to reduced cyclin E turnover upon expression of Fbw7 (Fig. 2) (21). A major question that emerges is whether cyclin E employs phosphorylated Thr^{62} as a phosphodegron or whether the effects on cyclin E abundance are indirect. The budding yeast Fbw7 isolog Cdc4 is known to preferentially interact with sequences having the consensus (I/L)(P/I/L)pTPP (where pT represents phosphotyrosine) (18). Whereas positions −2 and −1 in the potential Thr^{62} degron are compatible with this consensus, peptide array data would indicate that the potential Thr^{62} phosphodegron would be blocked from binding, due to the presence of lysine at +3 (18). In model peptides, those containing lysine or arginine at +3 were found to not interact with Cdc4. To directly examine whether the Thr^{62} region can act as a phosphodegron, we synthesized peptides encompassing Thr^{62} in phosphorylated and unphosphorylated forms and tested them for binding to Fbw7 as described above (Fig. 3, *A* and *B*). In addition, we also synthesized peptides that contained phosphoserine at position 58 (35). Fbw7 did not interact with unphosphorylated cyclin E$^{50–69}$ or with cyclin E$^{50–69}$ phosphorylated at Ser^{58} (Fig. 3*A*, *lanes 8* and *10*). However, Fbw7 did interact with Thr^{62}-phosphorylated cyclin E$^{50–69}$ (Fig. 3*B*, *lane 9*). The extent of interaction was similar to that seen with Thr^{380} phosphorylated cyclin E$^{361–388}$ (Fig. 3*B*, *lane 3*). The interaction of Thr^{62}-phosphorylated cyclin E$^{50–69}$ was not enhanced by phosphorylation at Ser^{58} in the context of this synthetic phosphodegron (Fig. 3*B*, *lane 11*). Thus, phosphorylation of Thr^{62} could, in principle, serve to directly target cyclin E to Fbw7.

Distinct Phosphodegrons in Cyclin E Interact with an Overlapping Binding Site on Fbw7—The finding that Fbw7 can interact with two distinct phosphodegrons in cyclin E in the context of synthetic peptides raises the question of whether these distinct motifs interact with Fbw7 through the same recognition site. To examine this issue, two experiments were performed. One approach took advantage of a collection of point mutations in the WD40 propeller of Fbw7, which are known to reduce binding to phosphorylated cyclin E. In particular, three arginine residues (Arg^{385}, Arg^{425}, and Arg^{463}, using the Fbw7β protein as reference) (Fig. 3*E*) have been shown to be important for interaction with human cyclin E (17), and the equivalent residues in Cdc4 have been shown to be important for binding to Sic1 and phosphodegrons derived from Sic1 (18) (Fig. 3*F*). We reasoned that if Thr^{62}- and Thr^{380}-derived peptides interact in a similar way with Fbw7, then mutations in these arginine residues would decrease binding to both phosphopeptides. To examine this, these three Fbw7 mutants were produced by *in vitro* translation and tested for interaction with phospho-Thr^{62}- and phospho-Thr^{380}-containing peptides. We found that the R385A mutant was strongly defective in association with both the Thr^{62}- and the Thr^{380}-based peptides, with the interaction being undetectable (Fig. 3*C*, *lanes 6* and *10*). Mutations in Arg^{425} and Arg^{463} displayed reduced interactions compared with wild-type controls, but interaction with both the Thr^{62}- and Thr^{380}-derived peptides were reduced to similar extents. These data are consistent with the idea that overlapping binding sites are used to bind both peptides.

If similar binding sites are used by both peptides, one would expect that the presence of one peptide in solution would compete with Fbw7 for binding to the second peptide immobilized to agarose. To establish this competition assay, Fbw7 was added to mixtures of immobilized phospho-Thr^{380} cyclin E$^{361–388}$ peptide and increasing amounts of either phos-

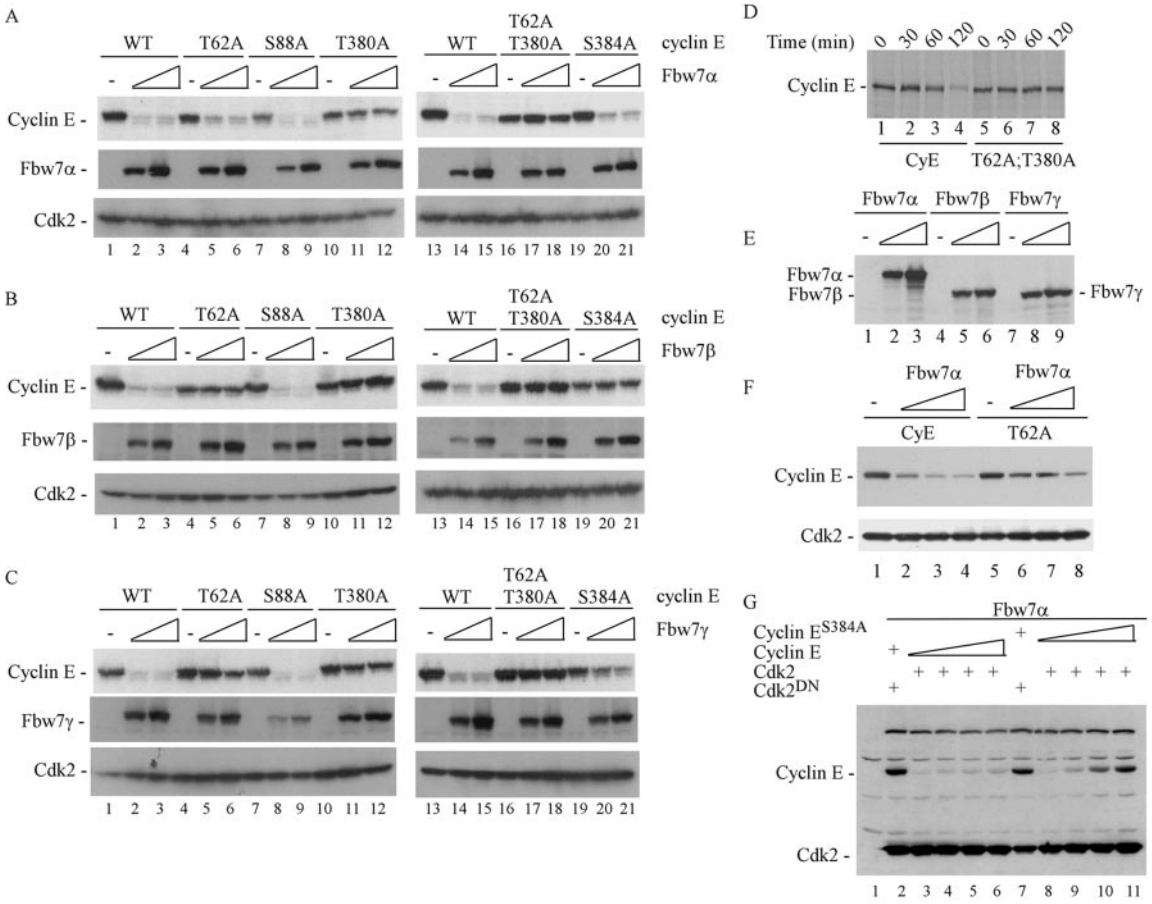

FIG. 2. ***In vivo*** **degradation of cyclin E phosphorylation site mutants by Fbw7 isoforms.** *A–C*, plasmids expressing the indicated FLAG-tagged Fbw7 isoforms were transfected into 293T cells (6-cm dish) together with vectors expressing Myc-tagged cyclin E mutants and Cdk2. After 36 h, cells were lysed, and immunoblots were probed with either anti-FLAG, anti-Cdk2, or anti-Myc antibodies. The quantities of plasmids employed were 1.5 and 3.0 μg for pCMV-FLAG-Fbw7, 0.3 μg for pCS2-Myc-cyclin E, and 1.5 μg of pCMV-Cdk2. *D*, pulse-chase analysis of Fbw7α-driven cyclin E turnover in 293T cells. Pulse-chase experiments were performed as described under "Materials and Methods." *E*, similar levels of Fbw7 proteins are expressed in cyclin E turnover assays. Extracts from co-transfection studies performed in Fig. 3, *A–C* (*lanes 1–3*), were subjected to immunoblotting using anti-FLAG antibodies to demonstrate comparable levels of Fbw7 proteins. *F*, analysis of cyclin E^{T62A} turnover by Fbw7α at lower Fbw7α/cyclin E ratios. Transfections were performed in 10-cm dishes using 2 μg of pCS2-Myc-cyclin E, 4 μg of pCMV-Cdk2, and 0, 2, 4, and 8 μg of pCMV-Fbw7α expression plasmids. *G*, constant amounts of pCMV-Fbw7α (0.25 μg) and pCMV-Cdk2 (3 μg) expression plasmids were co-transfected with increasing amounts of pCMV-Myc-cyclin E or pCMV-Myc-cyclin E^{S384A} plasmids (0.3, 0.6, 0.9, or 1.2 μg). After 48 h, extracts were subjected to immunoblotting with anti-cyclin E and anti-Cdk2 antibodies.

phorylated or unphosphorylated cyclin E$^{50-69}$ peptide in solution. After mixing, the extent of association of Fbw7 with immobilized cyclin E$^{361-388}$ was determined by SDS-PAGE (Fig. 3*D*). Whereas unphosphorylated Thr^{62} cyclin E$^{50-69}$ had no effect on the association of Fbw7 with cyclin E$^{361-388}$, phospho-Thr^{62} cyclin E$^{50-69}$ dramatically decreased the efficiency of Fbw7 association with cyclin E$^{50-69}$ (Fig. 3*D*). Taken together, these data indicate that Fbw7 is capable of interacting with phospho-Thr^{62} and phospho-Thr^{380} peptides through a single interaction site composed of a cluster of arginine residues.

Association of Cyclin E Phosphorylation Site Mutants with Fbw7 in Vivo—The data described above suggested the possibility that Thr^{62}, in addition to Thr^{380}, could be employed for Fbw7 association with cyclin E *in vivo*. To examine whether dual modes of interaction occur with intact cyclin E, binding experiments were performed with a series of cyclin E mutants and Fbw7α after transfection in 293T cells. The ability to accurately assess binding interactions requires that comparable levels of cyclin E mutants be expressed. However, mutation of Thr^{380}, and to a lesser extent Thr^{62}, leads to increased steady-state levels of cyclin E in the presence of FLAG-Fbw7 expression (data not shown). Therefore, to achieve approximately equal levels of cyclin E expression, we also co-transfected vectors expressing a dominant negative form of Cul1 (Cul1^{DN}), which contains the Skp1 binding site but lacks the Rbx1 binding site. This form of Cul1 sequesters Skp1·F-box complexes and leads to stabilization of SCF targets (16, 37). As expected, expression of Cul1^{DN} leads to equal accumulation of all cyclin E mutants examined, despite the presence of FLAG-Fbw7α (Fig. 4*A*, *lanes 1–5*). Extracts from cells expressing mutant cyclin E proteins and FLAG-Fbw7α were then subjected to immunoprecipitation using anti-FLAG antibodies, and the levels of associated cyclin E were examined by immunoblotting. Cyclin E efficiently associated with Fbw7α (Fig. 4*A*, *lane 1*). Interestingly, cyclin E^{T380A} was found to associate weakly with Fbw7α (*lane 3*), but mutation of Thr^{62} to alanine in the context of the T380A mutant further reduced the interaction with Fbw7α (Fig. 4*A*, *lane 4*). Mutation of Thr^{62} in cyclin E to alanine also led to a reduction in the extent of Fbw7α binding (Fig. 4*B*, *lane 2*).

The reduced association between Fbw7α and cyclin E^{T62A} could reflect either a significant utilization of phosphorylated Thr^{62} in binding to Fbw7α or could potentially reflect alterations in the phosphorylation of Thr^{380}. To examine this issue, we tested the extent of phosphorylation of Thr^{380} in the context of the T62A mutation under the same conditions employed in Fig. 4*A*. Lysates from transfected cells were immunoprecipitated with anti-Myc antibodies, and the levels of total cyclin E-

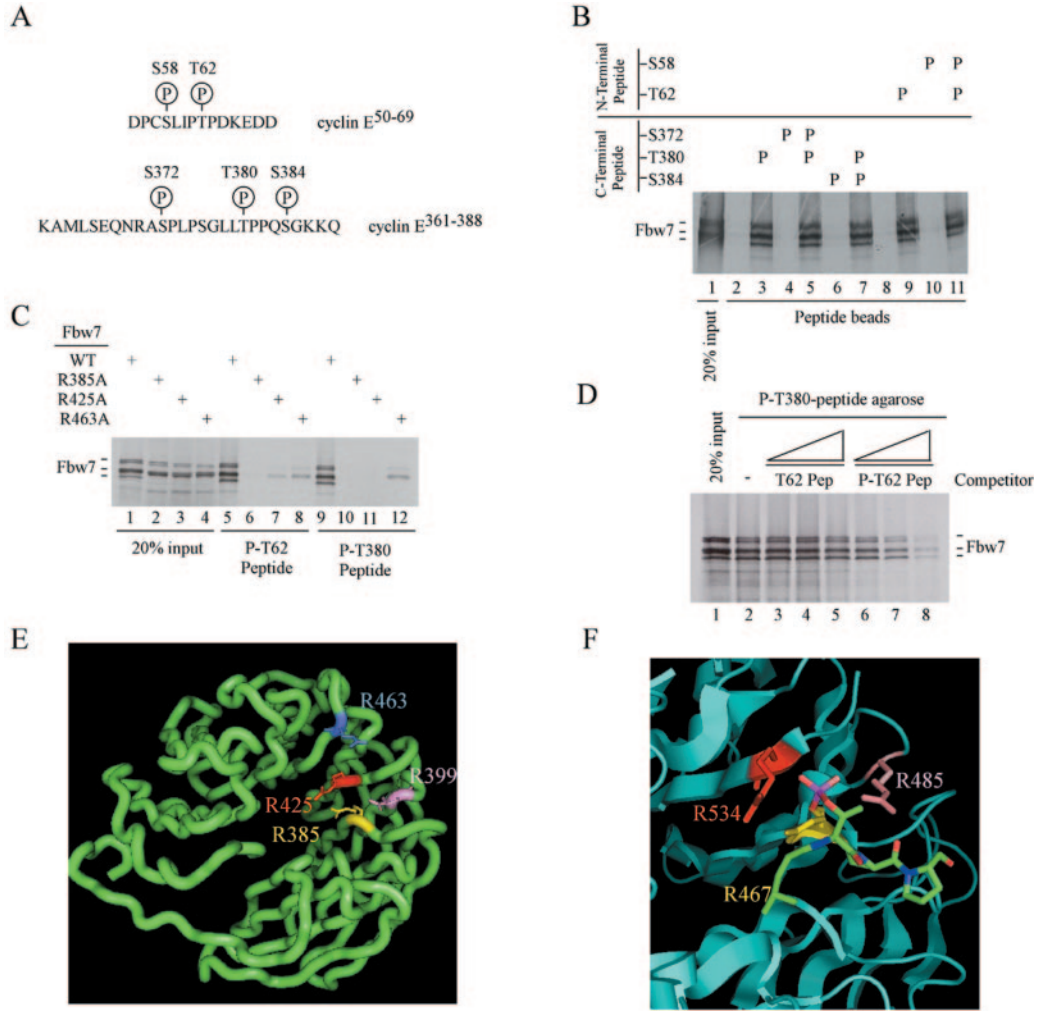

FIG. 3. **Recognition of Fbw7 by cyclin E phosphodegrons centered at Thr^{62} and Thr^{380}.** *A*, amino acid sequences of phosphopeptide degrons used in these studies. *B*, phosphorylation dependence for degron association. Cyclin E$^{361-388}$ and cyclin E$^{50-69}$, phosphorylated at the indicated positions, were immobilized on agarose beads and used in *in vitro* binding reactions with Fbw7β. With cyclin E$^{361-388}$, phosphorylation at Thr^{380} was sufficient for interaction (*lane 3*). With cyclin E$^{50-69}$, phosphorylation of Thr^{62} was sufficient for interaction (*lane 9*). *C*, arginine residues in the WD40-propeller of Fbw7β are required for association of the cyclin E$^{50-69}$ phosphodegron. Binding assays using the indicated immobilized peptides were performed using *in vitro* translated Fbw7 mutants. *D*, phospho-Thr^{62} cyclin E$^{50-69}$ competes with phospho-Thr^{380} cyclin E$^{361-388}$ for association with Fbw7. Fbw7 binding with immobilized phospho-Thr^{380} cyclin E$^{361-388}$ was performed in the presence of phosphorylated or unphosphorylated cyclin E$^{50-59}$. The quantities of soluble peptide used were 0, 25, 50, and 100 μg. *E*, model of the human Fbw7 WD40 propeller generated using Swissmodel with yeast Cdc4 as the template. Arg^{385}, Arg^{425}, and Arg^{463} implicated in association with cyclin E-derived phosphodegrons are shown, as is Arg^{399}, which is implicated on the basis of the Cdc4/phosphodegron structure. *F*, structure of Cdc4 bound to a phosphodegron containing the sequence LpTPP (24). The phosphodegron is shown in *green*, with the phosphate in *purple*. Arg^{467}, Arg^{485}, and Arg^{534} in Cdc4 correspond to Arg^{385}, Arg^{399}, and Arg^{425} in human Fbw7. Graphics were generated using Pymol.

and Thr^{380}-phosphorylated cyclin E determined by immunoblotting (Fig. 4*B*). We found that replacement of T62 with alanine significantly reduced the extent of Thr^{380} phosphorylation. The extent of reduction was comparable with the reduction seen in the association of Fbw7α with cyclin E^{T62A}. Transfection of cyclin E mutants in the absence of Fbw7 and Cul1^{DN} demonstrated that the effect on Thr^{380} phosphorylation was independent of these two components (data not shown). Taken together, these data indicate that mutation of Thr^{62} affects Thr^{380} phosphorylation, and the majority of its contribution to Fbw7-mediated turnover appears to be indirect.

Involvement of Ser^{384} in Recognition of Cyclin E by Fbw7—Results described above indicate that cyclin E^{S384A} is partially defective in degradation by Fbw7 with turnover by Fbw7β being affected to the greatest extent. To examine whether this reflects recognition by Fbw7, we compared the ability of Fbw7α and -β isoforms to immunoprecipitate cyclin E^{S384A} in transfected 293T cells (Fig. 5). Interestingly, cyclin E^{S384A} bound more weakly to both Fbw7 isoforms (*lanes 3* and *6*) than did wild-type cyclin E, suggesting a significant decrease in affinity in the context of full-length proteins *in vivo*. Association of cyclin E with Fbw7β was substantially lower than with the α isoform, possibly reflecting the fact that Fbw7β is largely cytoplasmic, whereas cyclin E is largely nuclear. Importantly, cyclin E^{S384A} maintained wild-type levels of Thr^{380} phosphorylation, as determined by immunoblots of cyclin E immune complexes using anti-phospho-T380 antibodies (Fig. 4*B*).

Mutation of Ser^{384} Affects the Nuclear/Cytoplasmic Ratio of Cyclin E—As stated above, the Fbw7β isoform was profoundly defective in degradation of cyclin E^{S384A} in transfection experiments, whereas the α and γ isoforms were less defective. Because our previous experiment revealed that Fbw7α and Fbw7γ are predominantly nuclear, whereas Fbw7β is localized to the cytoplasm, we examined whether the inability of Fbw7β to degrade cyclin E^{S384A} could result from aberrant intracellular shuttling of this mutant. Previous studies have demonstrated that cyclin E shuttles from the nucleus to the cytoplasm (38). To determine whether cyclin E^{S384A} accumulates in the

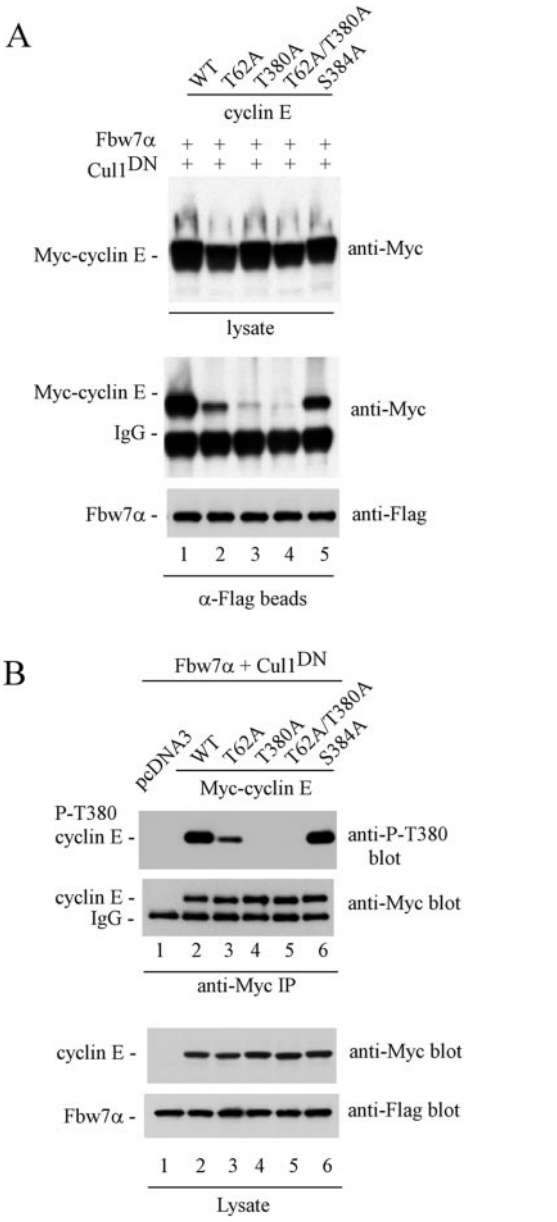

FIG. 4. **Role of Thr^{62} and Ser^{384} phosphorylation in assembly with Fbw7α *in vivo*.** Plasmids expressing the indicated Myc-tagged cyclin E proteins were co-transfected with plasmids expressing FLAG-Fbw7α in the presence of pCMV-Cul1^{DN}. After 36 h, lysates were either directly immunoblotted for cyclin E or subjected to immunoprecipitation using anti-FLAG antibodies prior to immunoblotting. *B*, anti-cyclin E immune complexes were subjected to immunoblotting with anti-phospho-Thr^{380} antibodies, and the blot was stripped and reprobed with anti-cyclin E antibodies. Corresponding cell extracts were probed with anti-Myc antibodies to detect cyclin E and anti-FLAG antibodies to detect Fbw7α.

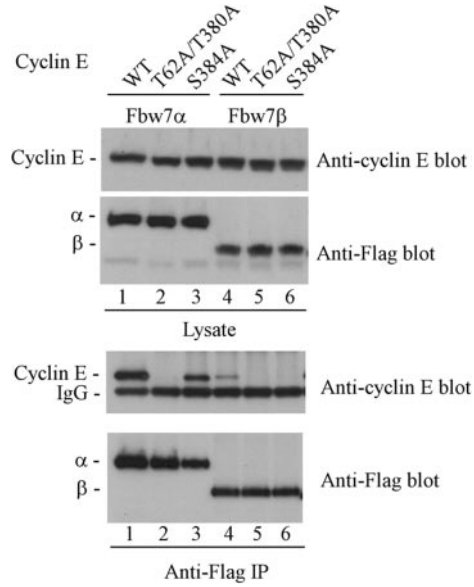

FIG. 5. **Mutation of Ser^{384} in cyclin E reduces association with Fbw7α and β isoforms.** Vectors expressing wild-type cyclin E and the indicated cyclin E mutants were cotransfected with vectors expressing Cul1^{DN} and either FLAG-Fbw7α or FLAG-Fbw7β. After 36 h, lysates were either directly immunoblotted for cyclin E or subjected to immunoprecipitation using anti-FLAG antibodies prior to immunoblotting.

nucleus more efficiently than wild-type cyclin E or other cyclin E mutants that are prone to Fbw7β-mediated degradation, we measured the nuclear/cytoplasmic ratios for cyclin E and the mutants used in this study (Fig. 6). The indicated plasmids were expressed in 293T cells and the levels of cytoplasmic and nuclear cyclin E were determined by immunofluorescence and quantitative image analysis (Fig. 6*B*). Wild-type cyclin E as well as the T62A, S88A, and T380A mutants displayed comparable nuclear/cytoplasmic ratios. In contrast, the nuclear/cytoplasmic ratio of cyclin E^{S384A} was 2-fold larger, indicating defects in nuclear export for this mutant protein (Fig. 6*A*). Thus, defects in turnover of cyclin E^{S384A} by Fbw7β may reflect both decreased affinity and decreased accessibility due to nuclear accumulation of this cyclin E mutant.

DISCUSSION

WD40 repeats in F-box proteins serve as receptors for recognition of phosphodegrons (5, 24, 25). Two distinct WD40/phosphodegron complexes have been examined structurally. The *Saccharomyces cerevisiae* Cdc4 WD40 propeller uses multiple arginine residues to interact with a single phosphothreonine in phosphodegrons derived from Sic1 or cyclin E (24), whereas human β-TRCP employs a large basic surface to interact with dual phosphoserines in a β-catenin-derived phosphodegron (25). Interestingly, the ability of Cdc4 to interact with its sole essential G_1 target, the Cdk inhibitor Sic1, depends upon the number of G_1 Cdk-generated phosphodegrons present in Sic1 (18). In general, each of these phosphodegrons binds weakly to Cdc4 in isolation, but occupancy of six sites allows for facile Sic1 ubiquitination and turnover. The use of a large number of relatively weak interactions allows for ultrasensitivity in the response of Sic1 turnover to G_1 Cdk activity (reviewed in Refs. 39 and 40). The extent to which other SCF^{Cdc4} substrates use multiple phosphorylation events to control turnover is unclear.

In this report, we examined how phosphorylation regulates the interaction of cyclin E with its cognate E3, SCF^{Fbw7}. Fbw7, the closest homolog of Cdc4 in the human genome, exists as three distinct isoforms, α, β, and γ, which display tissue-specific expression patterns (17, 21, 26, 36). Differences in Fbw7 isoforms are concentrated at the N terminus and are not expected *per se* to affect association with substrates via the C-terminal WD40 repeats. The localization properties of endogenous Fbw7 isoforms are unknown due to the absence of antibodies suitable for immunofluorescence. Therefore, we employed transient transfection of epitope-tagged versions of Fbw7α, -β, and -γ to examine the localization properties of these proteins. Fbw7α and -γ are found in the nucleus in 293T cells, whereas Fbw7β appears to be exclusively cytoplasmic. The localization of Fbw7β in the cytoplasm probably reflects the presence of a transmembrane domain not present in the α and γ isoforms (17, 36). Co-expression of Fbw7β with a vector expressing an endoplasmic reticulum marker (GFP-ER) dem-

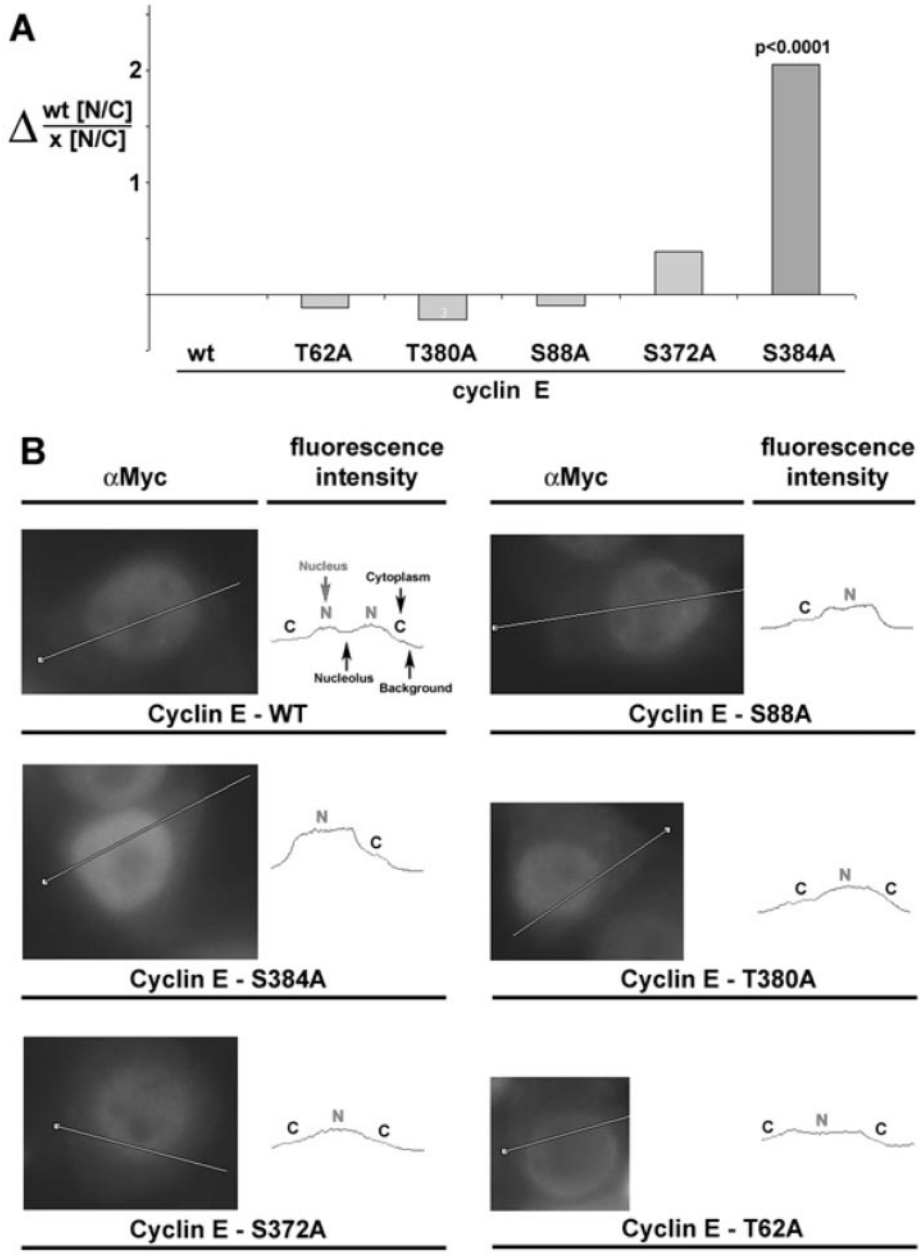

FIG. 6. **Mutation of Ser^{384} to alanine affects subcellular shuttling of cyclin E.** *A*, quantitative analysis of nuclear/cytoplasmic fluorescence intensity ratio in 293T cells subjected to indirect immunofluorescence 48 h post-transfection with five phosphorylation-deficient cyclin E point mutants (T62A, T380A, S88A, and S372A) and wild-type cyclin E reveals significantly enhanced nuclear accumulation of cyclin E^{S384A} protein as compared with wild-type cyclin E and four other cyclin E mutants. The quantities of all plasmids utilized and the exposure times were constant (1.0 μg and 1 s, respectively). Results represent averaged and normalized ($N_x/C_x - N_{wt}/C_{wt}$) values for 15 cells for each construct. *B*, representative cells expressing wild-type cyclin E as well as cyclin E phosphorylation-deficient mutants and respective immunofluorescence profiles are shown. *N*, nucleus; *C*, cytoplasm.

onstrated significant overlap of the fluorescence patterns, suggesting that Fbw7β is at least partially associated with endoplasmic reticulum membranes (data not shown).

Cyclin E has long been known to be phosphorylated on Thr^{380} through an autocatalytic function involving Cdk2 (33, 34), but recent experiments (35) also suggest a role for GSK3β in this process, functioning in an apparently redundant manner with Cdk2. Mutation of Thr^{380} to alanine leads to significant stabilization of cyclin E, implicating this phosphorylation event in cyclin E turnover. A role for this residue is also consistent with the finding that phosphopeptides encompassing Thr^{380} are capable of binding to Fbw7 (17). However, evidence of the involvement of Thr^{62} and Ser^{384} has also been presented (21, 35). Mutation of Thr^{62} partially stabilizes cyclin E to Fbw7β overexpression (35) and enhances the stabilization of the cyclin E^{T380A} mutant (21). However, Thr^{62} has not been demonstrated to be phosphorylated *in vivo*, and it is not clear whether it plays a direct or indirect role in cyclin E turnover. Likewise, mutation of Ser^{384} to alanine also leads to partial stabilization of cyclin E in an Fbw7β-driven turnover assay (35), again through an unknown mechanism. Although the finding that cytoplasmic Fbw7β overexpression can promote degradation of cyclin E (which is primarily nuclear) seems counterintuitive, we note that cyclin E is initially synthesized in the cytoplasm, where it assembles with Cdk2. In this context, newly synthesized cyclin E was generated by ectopic expression in the assays performed here and may be readily destroyed via an Fbw7β-mediated mechanism. Moreover, cyclin E is known to shuttle from the nucleus to the cytoplasm in a Crm1-independent pathway (38). Thus, elevated levels of Fbw7β, through the destruction of cytoplasmic cyclin E, could force the equilibrium in favor of nuclear export, thereby leading to depletion of the nuclear pool of cyclin E. Further studies are required to determine what Fbw7 isoforms are directly involved in cyclin E turnover *in vivo*.

In this paper, we systematically examined the role of various phosphorylation events in the interaction between cyclin E and Fbw7 as well as the ability of individual Fbw7 isoforms to promote turnover of various phosphorylation site mutants in cyclin E. We initially used mass spectrometry to establish

phosphorylation sites in cyclin E. Consistent with recent data using conventional peptide mapping (35), we found that cyclin E is phosphorylated on three sites near the C terminus (Ser^{372}, Thr^{380}, and Ser^{384}). Whereas Thr^{380} is phosphorylated directly by Cdk2, Ser^{384} does not conform to a Cdk2 site, yet its phosphorylation is Cdk2-dependent (35). We also detected phosphorylation of Ser^{88}, a candidate Cdk2 site that was not identified previously. As with other studies (35), we were unable to unequivocally identify Thr^{62}-phosphorylated peptides by mass spectrometry, possibly due to the large size of this tryptic peptide. However, phosphospecific antibodies against Thr^{62} unequivocally demonstrated that Thr^{62} in cyclin E is phosphorylated in 293T cells. However, the stoichiometry of phosphorylation of this residue and how this process is regulated is currently unknown.

Two parallel series of experiments were performed to examine the consequences of phosphorylation at these sites. First, we examined the ability of Fbw7 isoforms to promote cyclin E degradation *in vivo*. Wild-type cyclin E was efficiently degraded by all three Fbw7 isoforms, as were cyclin E^{S88A} and cyclin E^{S372A}. As expected, we found that mutation of Thr^{380}, and to a lesser extent Thr^{62}, led to defects in cyclin E turnover. This defect was independent of Fbw7 isoform. Cyclin E^{S384A} also displayed defects in turnover, but degradation by Fbw7β was affected to the greatest extent. We note that high levels of cyclin E^{S384A} are also substantially resistant to degradation induced by Fbw7α. The stoichiometric relationship between cyclin E and Fbw7 isoforms *in vivo* is not known, and further studies need to be performed to understand what Fbw7 isoforms are most important for cyclin E turnover *in vivo*.

We then went on to examine how various phosphorylation events in cyclin E promote the interaction with Fbw7. Initially, we used phosphopeptides centered at Thr^{380} and Thr^{62} and tested these for binding to Fbw7 *in vitro*. Phosphorylation of Thr^{380} is sufficient to allow for interaction of the C-terminal phosphodegron with Fbw7. Peptides phosphorylated at Ser^{372} or Ser^{384} alone were not capable of interacting with Fbw7, and phosphorylation at these sites did not enhance interaction of phospho-Thr^{380} peptides with Fbw7 in the context of the *in vitro* binding assay. Consistent with this, cyclin E^{T380A} demonstrated a greatly reduced ability to interact with Fbw7α in transfected cells. These data are consistent with previous studies indicating that a single phosphorylation event in a related cyclin E-derived peptide is sufficient for interaction with budding yeast Cdc4 *in vitro* (18). Interestingly, we found that peptides containing phospho-Thr^{62} were also capable of interacting efficiently with Fbw7, whereas phosphorylation of Ser^{58} did not support interaction with cyclin E. The interaction of phospho-Thr^{62}-containing peptides with Fbw7 required the same Fbw7 binding site as used by Thr^{380} peptides. Thus, in principle, this Thr^{62} phosphorylation could be involved directly in cyclin E recognition by Fbw7 independent of Thr^{380} phosphorylation. To examine this issue further, we determined the effects of replacement of Thr^{62} by alanine on the interaction of cyclin E with Fbw7 *in vivo*. The major effect found with cyclin E^{T62A} is a dramatic reduction in the extent of Thr^{380} phosphorylation, as measured using a phosphospecific antibody against Thr^{380}, indicating at least a partially indirect effect of this mutation on interaction with and turnover by Fbw7α. However, residual association of cyclin E^{T380A} with Fbw7α was reduced further upon mutation of Thr^{62}, indicating a direct, albeit small, contribution of Thr^{62} to Fbw7α recognition. The mechanism by which mutation of Thr^{62} to alanine affects phosphorylation of Thr^{380} is unknown. Although the direct contribution of Thr^{62} to cyclin E turnover appears to be small under the conditions examined here, we cannot exclude the possibility that Thr^{62} phosphorylation may be used in particular circumstances and be a major determinant of cyclin E degradation. For example, the stoichiometry of phosphorylation under the conditions examined here may be small relative to that of Thr^{380} phosphorylation, but this need not always be the case *in vivo*. Identification of the Thr^{62} kinase is required to address this issue in greater depth.

Our data as well as that of Welcker *et al.* (35) indicate that mutation of Ser^{384} to alanine blocks effective turnover of cyclin E by Fbw7. As assessed by immunoprecipitation in transfected cells, mutation of Ser^{384} to alanine substantially reduces the association of cyclin E with Fbw7, suggesting that Ser^{384} phosphorylation contributes to binding to Fbw7. Using quantitative imaging, we found that, with the exception of cyclin E^{S384A}, all of the other cyclin E proteins tested displayed indistinguishable nuclear/cytoplasmic ratios when transiently expressed in 293T cells. However, cyclin E^{S384A} displayed a dramatic (more than 2-fold) increase in the nuclear/cytoplasmic ratio. Thus, the defects seen in degradation of cyclin E^{S384A} by Fbw7β may reflect, in part, the inaccessibility of the cyclin E^{S384A} mutant with the Fbw7β isoform. Interestingly, c-Myc has recently been demonstrated to be ubiquitinated by SCF^{Fbw7} in a phosphorylation-dependent manner (28, 29). In this case, Thr^{58} is phosphorylated by GSK3β, and Ser^{62} is phosphorylated by a mitogen-activated protein kinase. In this case, Thr^{58} phosphorylation depends absolutely on prior phosphorylation of Ser^{62}. The phosphodegron in c-Myc (LPpTPPLpSP) is quite similar to that found in cyclin E (LLpTPPQpSG). Because of the dependence of Ser^{62} phosphorylation on Thr^{58} phosphorylation, it has not been possible to examine whether Ser^{62} phosphorylation contributes to association of c-Myc with Fbw7, but in the case of synthetic peptides, the Thr^{58} phosphopeptide binds to Fbw7 independently of Ser^{62} phosphorylation (28). Taken together, our studies reveal a complex interplay between phosphorylation of cyclin E and control of its degradation by Fbw7 and reveal that mutational analysis of cyclin E can in some cases lead to apparent indirect effects in its turnover by altering phosphorylation and/or localization.

REFERENCES

1. Deshaies, R. J. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 435–467
2. Koepp, D. M., Harper, J. W., and Elledge, S. J. (1999) *Cell* **97,** 431–434
3. Patton, E.E., Willems, A. R., and Tyers, M. (1998) *Trends Genet.* **14,** 236–243
4. Feldman, R. M., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. (1997) *Cell* **91,** 221–230
5. Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997) *Cell* **91,** 209–219
6. Patton, E. E., Willems, A. R., Sa, D., Kuras, L., Thomas, D., Craig, K. L., and Tyers, M. (1998) *Genes Dev.* **12,** 692–705
7. Seol, J. H., Feldman, R. M., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Nasmyth, K., Shevchenko, A., and Deshaies, R. J. (1999) *Genes Dev.* **13,** 1614–1626
8. Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M., Conaway, R. C., Conaway, J. W., Harper, J. W., and Pavletich, N. P. (2002) *Nature* **416,** 703–709
9. Skowyra, D., Koepp, D. M., Kamura, T., Conrad, M. N., Conaway, R. C., Conaway, J. W., Elledge, S. J., and Harper, J. W. (1999) *Science* **284,** 662–665
10. Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1999) *Cell* **86,** 263–274
11. Cenciarelli, C., Chiaur, D. S., Guardavaccaro, D., Parks, W., Vidal, M., and Pagano, M. (1999) *Curr. Biol.* **9,** 1177–1179
12. Kipreos, E. T., and Pagano, M. (2000) *Genome Biol.* **1,** 3002.1–3002.7
13. Winston, J. T., Koepp, D. M., Zhu, C., Elledge, S. J., and Harper, J. W. (1999) *Curr. Biol.* **9,** 1180–1182
14. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) *Nat. Cell Biol.* **1,** 193–199
15. Latres, E., Chiaur, D. S., and Pagano, M. (1999) *Oncogene* **18,** 849–854
16. Jin, J., Shirogane, T., Xu, L., Nalepa, G., Qin, J., Elledge, S. J., and Harper, J. W. (2003) *Genes Dev.* **17,** 3062–3074
17. Koepp, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., and Elledge, S. J. (2001) *Science* **294,** 173–177
18. Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F. B., Mendenhall, M. D., Sicheri, F., Pawson, T., and Tyers, M. (2001) *Nature* **414,** 514–521
19. Shirane, M., Hatakeyama, S., Hattori, K., Nakayama, K., and Nakayama, K. (1999) *J. Biol. Chem.* **274,** 28169–28174
20. Spencer, E., Jiang, J., and Chen, Z. J. (1999) *Genes Dev.* **13,** 284–294
21. Strohmaier, H., Spruck, C. H., Kaiser, P., Won, K.-A., Sangfelt, O., and Reed,

S. I. (2001) *Nature* **413,** 316–322
22. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) *Genes Dev.* **13,** 270–283
23. Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998) *Nature* **396,** 590–594
24. Orlicky, S., Tang, X., Willems, A. R., Tyers, M., and Sicheri, F. (2003) *Cell* **112,** 243–256
25. Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., and Pavletich, N. P. (2003) *Mol. Cell* **11,** 1445–1456
26. Moberg, K. H., Bell, D. W., Wahrer, D. C. R., Haber, D. A., and Hariharan, I. K. (2001) *Nature* **413,** 311–316
27. Hubbard, E. J., Wu, G., Kitajewski, J., and Greenwald, I. (1997) *Genes Dev.* **11,** 3182–3193
28. Welcker, M., Orian, A., Jin, J., Grim, J. A., Harper, J. W., Eisenman, R. N., and Clurman, B. E. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 9085–9090
29. Yada, M., Hatakeyama, S., Kamura, T., Nishiyama, M., Tsunematsu, R., Imaki, H., Ishida, N., Okumura, F., Nakayama, K., and Nakayama, K. I. (2004) *EMBO J.* **23,** 2116–2125
30. Moberg, K. H., Mukherjee, A., Veraksa, A., Artavanis-Tsakonas, S., and Hariharan, I. K. (2004) *Curr. Biol.* **14,** 965–974
31. Nateri, A. S., Riera-Sans, L., Da Costa, C., and Behrens, A. (2004) *Science* **303,** 1374–1378
32. Tetzlaff, M. T., Yu, W., Li, M., Zhang, P., Finegold, M., Mahon, K., Harper, J. W., Schwartz, R. J., and Elledge, S. J. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 3338–3345
33. Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M., and Roberts, J. M. (1996) *Genes Dev.* **10,** 1979–1990
34. Won, K. A., and Reed, S. I. (1996) *EMBO J.* **15,** 4182–4193
35. Welcker, M., Singer, J., Loeb, K. R., Grim, J., Bloecher, A., Gurien-West, M., Clurman, B. E., and Roberts, J. M. (2003) *Mol. Cell* **12,** 381–392
36. Spruck, C. H., Strohmaier, H., Sangfelt, O., Muller, H. M., Hubalek, M., Muller-Holzner, E., Marth, C., Widschwendter, M., and Reed, S. I. (2002) *Cancer Res.* **62,** 4535–4539
37. Donzelli, M., Squatrito, M., Ganoth, D., Hershko, A., Pagano, M., and Draetta, G. F. (2002) *EMBO J.* **21,** 4875–4884
38. Jackman, M., Kubota, Y., den Elzen, N., Hagting, A., and Pines, J. (2002) *Mol. Biol. Cell.* **13,** 1030–1045
39. Deshaies, R. J., and Ferrell, J. E., Jr. (2001) *Cell* **107,** 819–822
40. Harper, J. W. (2002) *Trends Cell Biol.* **12,** 104–107

Recognition of Phosphodegron Motifs in Human Cyclin E by the SCF^{Fbw7} Ubiquitin Ligase

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