Fbw7 Isoform Interaction Contributes to Cyclin E Proteolysis

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Abstract

The ubiquitin proteasome system plays important roles in regulating cell growth and proliferation. Many proteins that function in ubiquitin-mediated destruction have been linked to tumorigenesis. The putative tumor-suppressor protein Fbw7 (hAgo/hCdc4) is a specificity factor for the Skp1-Cul1-F-box protein ubiquitin ligase complex and targets a number of proto-oncogene products for ubiquitin-mediated destruction, including the cell cycle regulator cyclin E. In mammals, there are three splice variants of Fbw7 that use distinct first exons, resulting in proteins that have unique NH$_2$ termini but are otherwise identical. Here, we show that the Fbw7 splice variants interact with each other through an NH$_2$-terminal region common to all of the Fbw7 isoforms. Other F-box proteins have been shown to regulate substrate binding or turnover by forming homodimeric or heterodimeric complexes, which are dependent on a sequence motif called the D domain. Fbw7 and its orthologues exhibit significant sequence similarity to such F-box proteins, including the D domain. Fbw7 mutants that lack the region encompassing the D domain fail to bind other Fbw7 isoforms, despite being properly localized and binding both cyclin E and Skp1. Finally, we show the functional significance of this region as mutants lacking the NH$_2$-terminal region involved in Fbw7 binding exhibit reduced rates of cyclin E protein turnover, indicating that Fbw7 isoform interaction is important for the efficiency of cyclin E turnover. Overall, this study contributes to the current understanding of the regulation of the Fbw7 tumor-suppressor protein. (Mol Cancer Res 2006;4(12):935–43)

Introduction

Ubiquitin-mediated proteolysis plays an important role in regulating cell cycle transitions; thus, proteins that function in these pathways are often found mutated in cancer cells. The WD40-repeat, containing F-box protein Fbw7 (hAgo/hCdc4), functions as a specificity factor for the modular Skp1-Cul1-F-box (SCF) protein ubiquitin ligase complex (1-3). Fbw7 is proposed to function as a tumor suppressor; mutations have been identified in the *Fbw7* locus in a number of breast, endometrial, ovarian, and pancreatic cell lines and primary tumors (2-10). In mice, $Fbw7^{+/-}$ heterozygotes exhibit increased incidence of tumor formation relative to wild-type animals (11).

The SCF^{Fbw7} complex is associated with the ubiquitination, and subsequent destruction via the proteasome, of at least four proto-oncogene protein products: cyclin E, c-Jun, c-Myc, and Notch (1-3, 12-17). The interaction of Fbw7 with several of its substrates has been linked to a phosphodegron motif first identified in cyclin E (18, 19). Conserved arginine residues in the WD40-repeat region are important for binding to the phospho-degron motif (1) and have been shown to contact the phosphate group in a crystal structure of Cdc4, the yeast orthologue of Fbw7, bound to an idealized consensus phosphopeptide (20). Many of the Fbw7 mutations found in tumors are in regions that disrupt binding to this phosphodegron motif, including changes at the conserved arginine residues (3, 8, 9, 21). Recent evidence suggests that proline isomerization may also be important in the recognition of cyclin E by Fbw7 (21, 22).

In humans, there are three splice variants of Fbw7—α, β, and γ—which arise from the use of independent first exons (9). Whereas expression of individual Fbw7 isoform mRNAs do show some tissue specificity, there are examples of tissues and cell lines in which all three are expressed (9). Interestingly, some of the Fbw7 tumor mutations are found in isoform-specific sequences, suggesting that individual isoforms may have distinct roles in preventing tumorigenesis (8, 9, 21). Differential localization of the Fbw7 isoforms may also regulate the function of each. Fbw7-α is largely nuclear; Fbw7-β is primarily cytoplasmic; and Fbw7-γ is reported to accumulate in the nucleolus (12, 23). The nucleolar-localized fraction of Fbw7-γ has been suggested to be responsible for the ubiquitination of c-Myc (12). However, the precise role and contribution of each isoform to the ubiquitination of most Fbw7 substrates remain to be established.

For other WD40-repeat containing F-box proteins, there is evidence that the formation of homodimeric complexes, or heterodimeric complexes with highly related F-box proteins, can regulate substrate recognition and ubiquitination. In *Schizosaccharomyces pombe*, the F-box proteins Pop1 and Pop2 form heterodimeric complexes that regulate the ubiquitination and subsequent destruction of the cyclin-dependent kinase inhibitor Rum1, the S-phase regulator Cdc18, and the S-phase cyclin Cig2 (24-27). In humans, βTrCP1 and βTrCP2

homodimers are reported to bind and be responsible for the ubiquitination of the $SCF^{\beta TrCP}$ substrate IκB, whereas hetero-dimers fail to bind IκB (28). Dimerization of the βTrCP proteins occurs through a region upstream of the F-box domain called the D domain. Fbw7 and its orthologues show significant sequence similarity to βTrCP homologues, including the D domain, but the existence of homo- or hetero-oligomeric complex formation among Fbw7 isoforms has not been previously reported.

In this study, we show that the Fbw7 splice variants can interact with each other in both mammalian and insect cells. We also identify an NH$_2$-terminal region common to all the Fbw7 isoforms that is required for the interaction. This finding is similar to studies of βTrCP1/2 as the region required for the interaction includes the D domain. Furthermore, this region has functional implications for the Fbw7 protein as cells expressing Fbw7 mutants that fail to interact exhibit a reduced rate of cyclin E protein turnover. Our results broaden the current understanding of Fbw7 protein regulation and may have implications for investigating the tumor-suppressor function of Fbw7.

Results

Fbw7 Splice Variants Interact with Each Other

As an initial experiment to determine whether the Fbw7 splice variants might interact with each other, we used the baculovirus expression system. Using glutathione S-transferase (GST) and Flag-tagged baculoviruses for each Fbw7 isoform, we examined their ability to coimmunoprecipitate with each other using insect cells infected with each pairwise combination of Fbw7 isoforms. We also tested for the ability of each variant to form homointeractions. In each case, when any Flag-tagged Fbw7 isoform is immunoprecipitated, the coexpressed GST-tagged isoform is also precipitated (Fig. 1A, *lanes 1-6*). None of the GST-tagged isoforms are able to interact with the anti-Flag agarose in the absence of expression of a Flag-tagged isoform, indicating that the interaction we observed is specific (Fig. 1A, *lanes 7-9*). Reciprocal coprecipitation experiments confirm that the Fbw7 isoforms interact with each other (data not shown).

To test whether this interaction also occurs in mammalian cells, we coexpressed myc-tagged and Flag-tagged Fbw7 variants in 293T cells. As shown in Fig. 1B, myc-tagged Fbw7-α coimmunoprecipitates with Flag-tagged Fbw7-α, Fbw7-β, and Fbw7-γ (*lanes 1-3*). Similar results are observed with myc-tagged Fbw7-γ (Fig. 1C, *lanes 1-3*). As a negative control, we cotransfected cells with empty Flag vector and either myc-tagged Fbw7-α or Fbw7-γ (Fig. 1B, *lane 4*, and C, *lane 4*). We did not observe any nonspecific interaction in these samples. Reciprocal coimmunoprecipitation experiments confirm that the Fbw7 isoforms interact with each other (data not shown). We conclude that the Fbw7 isoforms can immunoprecipitate with each other in mammalian cells in the following combinations: α/α, α/β, α/γ, β/γ, and γ/γ. We have been unable to generate a myc-tagged version of Fbw7-β that expresses at levels comparable with Fbw7-α and Fbw7-γ and thus have been unable to determine whether the Fbw7-β homo-oligomeric interaction occurs in mammalian cells. However, based on the results observed in insect cells, it seems likely that such an interaction occurs.
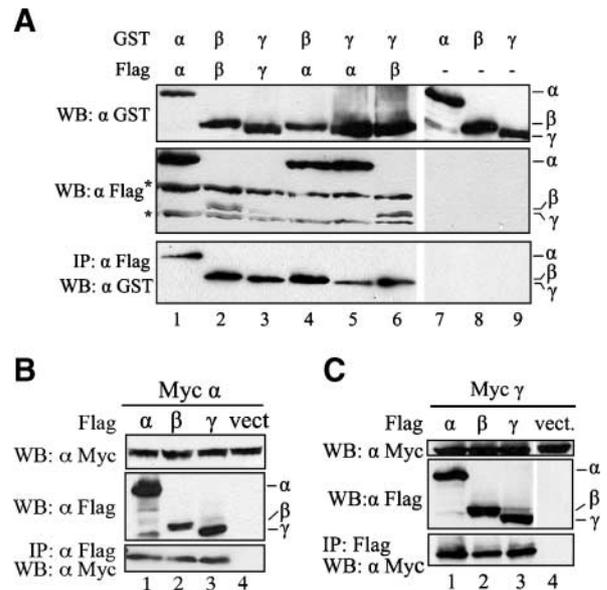
FIGURE 1. The splice variants of Fbw7 interact with each other *in vitro* and *in vivo*. **A.** The three isoforms of Fbw7 interact in insect cells. Individual isoforms of Fbw7 tagged with either GST or Flag were overexpressed in Hi5 insect cells. Top and middle, expression of GST-tagged and Flag-tagged isoforms tested by Western blot (*WB*); bottom, coimmunoprecipitation (*IP*) results of pairwise combinations of the isoforms. Hi5 insect cells were infected with baculovirus for 40 h and cells were harvested for lysis. Equal amounts of total protein extracted from each insect cell lysate were incubated with 20 μL of anti-Flag M2 affinity agarose beads at 4°C for 2 h followed by Western blot analysis with anti-GST antibodies. Lanes 7 to 9, cells that were only infected with GST-tagged baculovirus and were used to exclude the possibility that GST-tagged proteins nonspecifically bind to the anti-Flag agarose. *, nonspecific bands recognized by the anti-Flag antibodies. **B** and **C.** The Fbw7 isoforms interact in mammalian cells. Myc-tagged Fbw7-α (**B**) or Fbw7-γ (**C**) was cotransfected with the indicated Flag-tagged isoforms in 293T cells. Equal amounts of total protein extracted from cell lysates were incubated with 20 μL of anti-Flag M2 agarose for 2 h followed by Western blot analysis with anti-Myc antibodies. Lane 4, controls for nonspecific binding to the beads (**B** and **C**).

A Conserved Domain Is Necessary for Fbw7 Splice Variant Interaction

We used deletion analysis to map the region necessary for the interaction between Fbw7 isoforms (Fig. 2A). The interaction of other F-box proteins, most notably βTrCP1 and βTrCP2, has been shown to be dependent on the D domain, a motif located immediately upstream of the F-box domain in these proteins (28). We compared the amino acid sequence of Fbw7 with its homologues in yeast, worms, and flies. We also compared the amino acid sequence of human Fbw2, as well as βTrCP1, with its respective homologues. We find that there is significant homology among these proteins in the D domain, particularly at the COOH-terminal end of the domain (Fig. 2B). Interestingly, only a subset of F-box proteins seems to contain the D domain. For example, a search of several F-box proteins bearing leucine-rich repeats, including human Skp2 and budding yeast Dia2, shows no significant homology to the D domain (data not shown).

We generated Flag-tagged versions of Fbw7-α, Fbw7-β, and Fbw7-γ in which the D domain and the NH$_2$-terminal common region are deleted in frame such that the unique NH$_2$ terminus for each isoform is immediately followed by the F-box domain
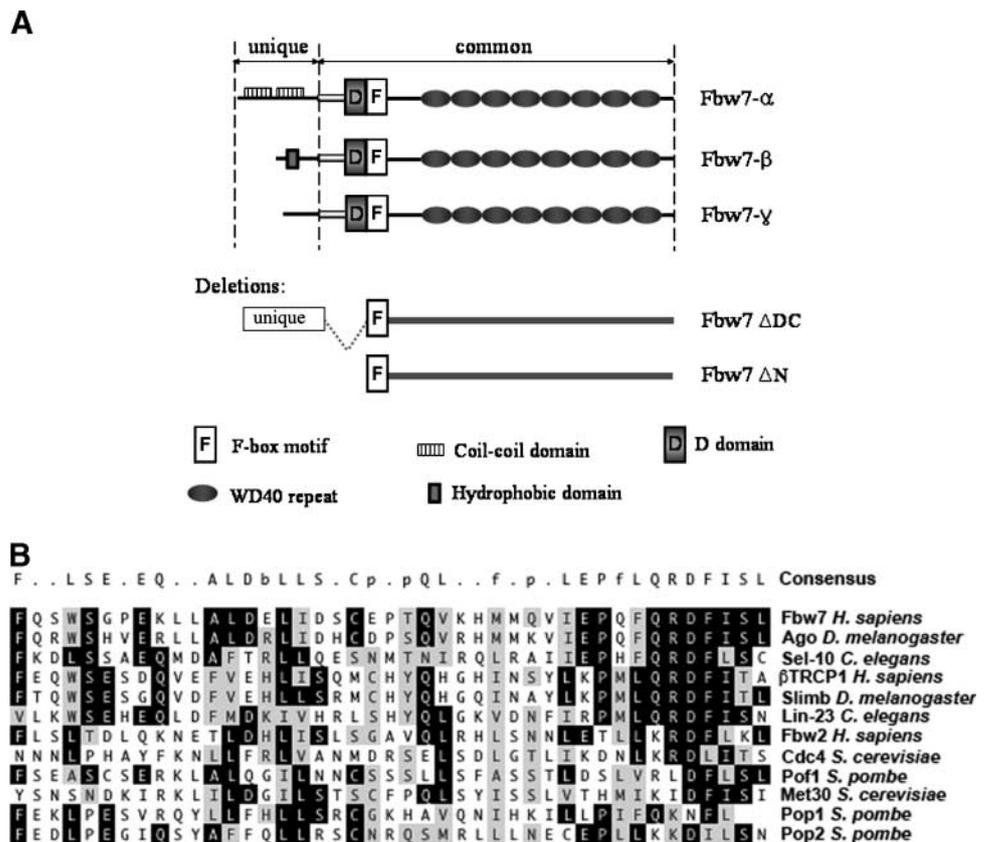
(ΔDC, D domain plus common NH$_2$-terminal region) as well as a form that deletes all residues upstream of the F-box domain (ΔN; Fig. 2A). The deletion mutants were then cotransfected into 293T cells with myc-tagged full-length Fbw7-α and used for coimmunoprecipitation experiments. Each of these mutants is expressed and shows comparable abundance to the full-length Fbw7-α protein (Fig. 3A and B, *top*). Whereas the Flag-tagged Fbw7-α coimmunoprecipitates myc-tagged Fbw7-α, none of the ΔDC or ΔN mutants are able to immunoprecipitate myc-tagged Fbw7-α (Fig. 3A and B).
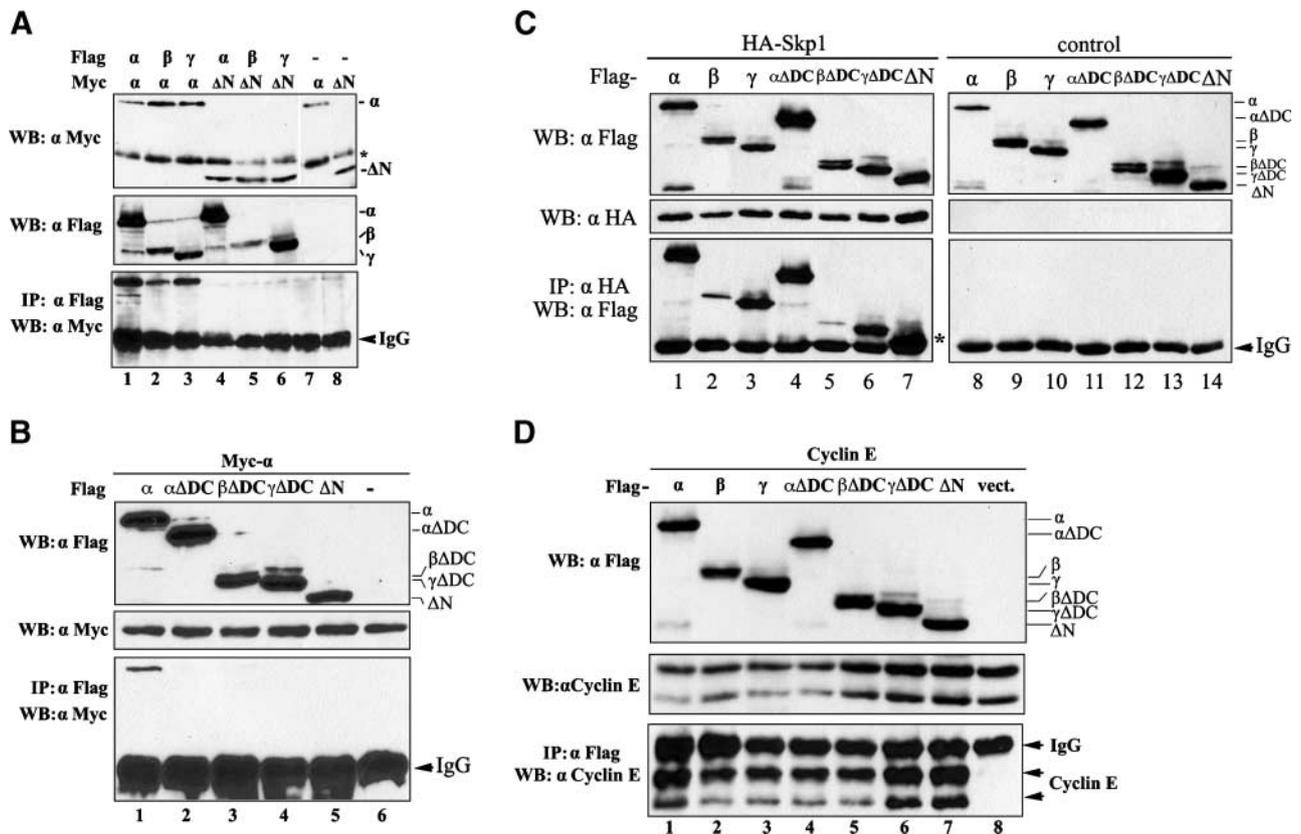
We anticipated that the region required for the Fbw7 isoform interaction would not interfere with recognized domains of the protein, such as the WD40-repeat region or the F-box motif. To test this, we examined the ability of the ΔDC and ΔN mutants to bind cyclin E and Skp1. The WD40-repeat region of Fbw7 has been implicated in binding cyclin E, whereas the F-box domain has been shown to be important for binding Skp1 (1, 29). Our deletion mutants were distinct from these domains; therefore, we predicted that binding to cyclin E and Skp1 would be retained. To test this, we cotransfected 293T cells with each mutant and either a hemagglutinin (HA)–tagged Skp1 expression vector or a cyclin E expression vector. As shown in Fig. 3C, the Fbw7-α, Fbw7-β, and Fbw7-γ ΔDC mutants as well as the ΔN mutant are each able to coimmunoprecipitate with HA-tagged Skp1 in a manner comparable with the respective wild-type isoforms. Likewise, in Fig. 3D, each mutant also coimmunoprecipitates with cyclin E. The interactions that we observed seem to be specific for Skp1 and cyclin E as we did not

observe any binding in mock immunoprecipitations (Fig. 3C, *lanes 8-14*, and D, *lane 8*). These results suggest that the deletion mutants we generated do not interfere with the F-box or WD40 domains and are not grossly misfolded. We therefore conclude that the NH$_2$-terminal region of Fbw7 that contains the D domain is independent of the F-box and WD40 domains. Furthermore, this region is required for the interaction that we observed between the Fbw7 isoforms.

The Fbw7 Interaction Domain Is Important for Cyclin E Proteolysis

We assessed the functional contribution of the region required for the interaction using a cyclin E stability assay. For these experiments, we focused on the Fbw7-α isoform as it exhibited the most robust response in the assay (data not shown). For the stability assay, we cotransfected 293T cells with equal amounts of the Flag-tagged Fbw7-α, Fbw7-α ΔDC, and Fbw7 ΔN, or the empty Flag vector with a cyclin E expression vector. Cycloheximide was added to the cells 36 h after transfection, and samples were collected at 30, 90, and 180 min to assess cyclin E protein abundance by immunoblotting. The same blots were probed with anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies as a loading control. A typical cyclin E stability assay for each Fbw7-α form is shown in Fig. 4A and the quantification of the assay is shown in Fig. 4B. We quantified the amount of cyclin E signal relative to the GAPDH signal for this experiment and used the ratio for each time point, where the ratio for the zero time point is

FIGURE 2. A. Schematic diagram of Fbw7 domains in each splice variant and the deletion mutants used in this study. **B.** Alignment of the D-domain sequences from Fbw7 and its homologues and a number of other WD40-repeat containing F-box proteins, including βTrCP1 and its homologues. Letters above the alignment, consensus sequence. Black, identical residues; gray, residues of similar functional groups. The alignment was generated using Clustal W and the Megalign program of the Lasergene Navigator Suite.

FIGURE 3. The domain required for Fbw7 isoform interaction encompasses the D domain. **A.** The NH$_2$ terminus is required for the Fbw7 isoform interaction. 293T cells were cotransfected with vectors expressing Flag-tagged full-length Fbw7 isoforms and either Myc-tagged Fbw7-α or Myc-tagged Fbw7 ΔN. Top and middle, Western blot analyzing the expression of the proteins; bottom, immunoprecipitation result. Equal amounts of total protein extracted from each lysate were incubated with 20 μL of anti-Flag agarose beads before SDS-PAGE and Western blot analysis using anti-Myc antibodies. Lanes 7 and 8, empty Flag vector transfected as a negative control. *, nonspecific band recognized by the anti-Myc antibodies. **B.** Fbw7 ΔDC no longer binds Fbw7 isoforms. 293T cells were transfected with full-length Flag-tagged Fbw7-α or the Fbw7 ΔDC mutant of each isoform together with Myc-tagged Fbw7-α. Top and middle, Western blot analyzing the expression of the proteins; bottom, immunoprecipitation result. Equal amounts of total protein extracted from each lysate were incubated with 20 μL of anti-Flag agarose beads before SDS-PAGE and Western blot analysis. **C.** The deletion mutants of Fbw7 coimmunoprecipitate with Skp1. 293T cells were transfected with the Fbw7 deletion mutants and HA-tagged Skp1. Top and middle, expression of Flag-tagged full-length Fbw7 isoforms and the ΔDC deletion mutants and HA-tagged Skp1; bottom, immunoprecipitation result. Equal amounts of total protein extracted from each cell lysate were incubated with anti-HA antibody for 2 h followed by incubation with 20 μL prewashed protein A/G agarose beads. Lanes 8 to 14, control experiments in which no HA-Skp1 was transfected. *, the Fbw7 ΔN band, which runs just above the IgG heavy chain. **D.** The Fbw7 deletion mutants coimmunoprecipitate cyclin E. 293T cells were transfected with Flag-tagged full-length Fbw7 isoforms, ΔDC deletion mutants, or Flag vector together with equal quantities of the cyclin E expression construct. Top and middle, the expression of the indicated forms of Fbw7 and cyclin E; bottom, immunoprecipitation result. Equal amounts of total protein from each cell lysate were incubated with 20 μL of prewashed anti-Flag M2 agarose beads at 4°C overnight.

arbitrarily set to 1, to plot the amount of cyclin E signal remaining on a logarithmic scale versus time (Fig. 4B). Although they are expressed at approximately equivalent levels as wild-type and retain their ability to bind cyclin E and Skp1, the Fbw7-α ΔDC and ΔN mutants show a reduced rate of cyclin E turnover compared with the full-length Fbw7-α. We did these experiments thrice; each time, the ΔDC and ΔN mutants exhibited reduced cyclin E turnover rates, indicating that the trends we observed are reproducible.

To determine whether this reduced rate of turnover is due to mislocalization of the mutant proteins or the loss of the ability to bind other Fbw7 molecules, we did indirect immunofluorescence of 293T cells transfected with each Flag-tagged expression vector (Fig. 4C). We find that the ΔDC mutant localizes to the nucleus like the wild-type Fbw7-α protein; thus, the effect of the ΔDC mutant on cyclin E stability cannot be due to localization differences. The ΔN mutant exhibits both nuclear

and cytoplasmic staining, consistent with previous work indicating that the Fbw7-α nuclear localization signal is in the NH$_2$ terminus (12). However, the ΔN mutant and the ΔDC mutant exhibited similar effects on cyclin E stability; thus, we did not observe a significant correlation between cytoplasmic localization and reduced cyclin E turnover in this assay.

If the Fbw7 ΔN and ΔDC mutants were unstable, that might explain the decreased rate of cyclin E turnover we observed. To test this possibility, we did stability assays with 293T cells expressing Flag-tagged Fbw7 mutants. Cycloheximide was added to cells 36 h after transfection with Fbw7-α, Fbw7α ΔDC, or Fbw7 ΔN. Then, samples were collected at 45, 120, and 210 min to assess Fbw7 abundance by immunoblotting with anti-Flag antibodies. The same blots were probed with anti-GAPDH antibodies as a loading control (Fig. 4D). We observed no change in the stability of the Fbw7 mutants compared with the full-length protein, indicating that the

reduced rate of cyclin E turnover in the Fbw7-α ΔDC and Fbw7 ΔN mutants is not due to changes in the stability of the Fbw7 protein itself. Instead, our results strongly suggest that it is the failure of the Fbw7-α mutants to interact with other Fbw7-α molecules that has a deleterious effect on the degradation of the cyclin E protein.

Discussion

The results of this study indicate that the Fbw7 splice variants can form both homotypic and heterotypic complexes with each other and suggest that at least the homotypic interactions are important for the efficient proteolysis of the SCF^{Fbw7} substrate, cyclin E. An NH$_2$-terminal domain common to all three Fbw7 splice variants is required for the isoforms to interact with each other; however, it does not interfere with protein abundance or binding to Skp1 and cyclin E. It is possible that the reduced rate of cyclin E protein turnover, caused by the inability of Fbw7 mutants to interact with each other, may have deleterious effects on cells that could contribute to tumor initiation, as misregulated cyclin E levels have been linked to chromosome instability and tumorigenesis (30). Interestingly, a recent study of primary ovarian tumors with elevated levels of cyclin E protein identified a mutation, S245T (Fbw7-α residue number), in the region of Fbw7 required for its interaction with other isoforms (8). This serine is conserved in Fbw7 homologues from multicellular organisms. Additional studies will be necessary to determine if there is a link between the defect in the S245T mutant and the interaction of Fbw7 with other isoforms.

The precise mechanism by which an Fbw7 homo-oligomeric interaction promotes cyclin E degradation remains to be determined. Because Fbw7 is the specificity factor for an SCF complex that ubiquitinates cyclin E, presumably the Fbw7-Fbw7 interaction promotes the rate of ubiquitination of cyclin E. One possibility is that the processivity of the reaction is enhanced, perhaps by reducing lag time between ubiquitination of individual cyclin E molecules. For example, it is possible that an Fbw7-Fbw7 dimer, bound through the interaction domain upstream of the F-box domain, could potentially dock with the SCF complex via the binding of one F-box domain. In this way, two cyclin E molecules would be available for ubiquitination by the same complex.
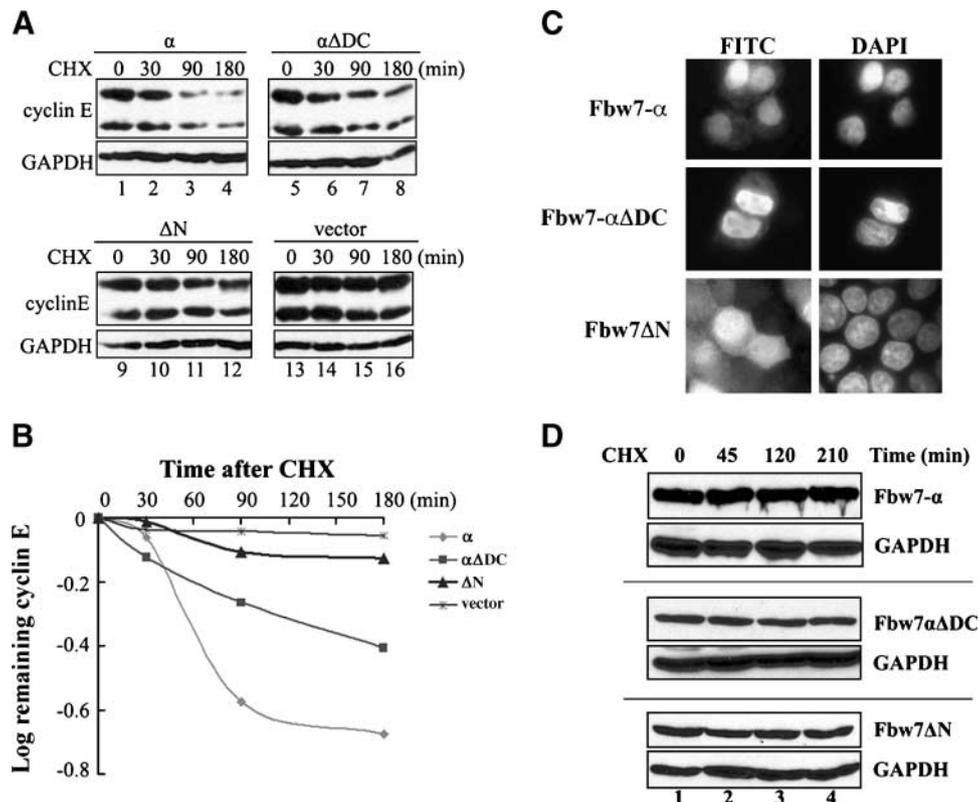
FIGURE 4. The homo-oligomeric interaction of Fbw7 isoforms is important for cyclin E proteolysis. **A.** Examples of cyclin E stability assays. 293T cells were cotransfected with the same amount of cyclin E expression construct together with Flag-tagged Fbw7-α, Fbw7-α ΔDC, Fbw7 ΔN, or empty vector. Thirty-six hours after transfection, cycloheximide (*CHX*; final concentration, 30 μg/mL) was added to stop protein synthesis (time point 0). Cells were collected at the indicated time points and cyclin E protein abundance was analyzed by Western blot. Anti-GAPDH antibodies were used as a loading control. **B.** Quantitation of cyclin E stability. The results of the cyclin E stability experiments in **A** were analyzed by Image J software. The log of the ratio of cyclin E (both bands) to the loading control is plotted versus time. **C.** The Fbw7-α ΔDC mutant shows similar localization to the full-length protein. 293T cells were grown on coverslips and transfected with Fbw7-α, Fbw7-α ΔDC, and Fbw7 ΔN. Thirty-six hours after transfection, cells were fixed in 3% paraformaldehyde and immunostained with anti-Flag antibodies. DAPI, 4′,6-diamidino-2-phenylindole. **D.** 293T cells were transfected with Flag-tagged Fbw7-α, Fbw7-α ΔDC, or Fbw7 ΔN. Thirty-six hours after transfection, cycloheximide (final concentration, 30 μg/mL) was added to stop protein synthesis (time point 0). Cells were collected at the indicated time points and Fbw7 protein abundance was analyzed by Western blot using anti-Flag antibodies. Anti-GAPDH antibodies were used as a loading control.

Alternatively, dimerization or oligomerization of F-box proteins might influence the neddylation-deneddylation cycle proposed for Cul1 (31). In this cycle, deneddylation of Cul1 by the Cop9 signalosome releases Cul1 from the SCF complex, upon which it is bound by the CAND1 protein and held inactive. Neddylation of Cul1 leads to its release from CAND1 and subsequent binding by a new F-box protein (32, 33). There is evidence that cyclin E turnover is regulated by the Cop9 signalosome in both *Drosophila* and mice (34, 35). Intriguingly, recent work on the SCF^{Skp2} complex indicates that increasing the amount of the Skp1-Skp2 subcomplex enhances Cul1 dissociation from CAND1 and that increased Skp2-substrate subcomplex inhibits Cul1 deneddylation (36). Likewise, perhaps Fbw7 dimers are more efficient than monomers at dissociation of Cul1 from CAND1 or an Fbw7-Fbw7 dimer bound to cyclin E might prevent Cul1 from being deneddylated by the Cop9 signalosome for a longer period of time. In either case, the result would be an increase in the active pool of Cul1 and a decrease in the inactive pool of Cul1.

Recent results indicate that blocking the activity of the Cop9 signalosome leads to enhanced turnover of a subset of F-box proteins, including Fbw7, presumably via increased auto-ubiquitination as the F-box protein remains bound to the catalytic core of the SCF complex because it cannot be dissociated by deneddylation of Cul1 (37). If the Fbw7 ΔDC mutant were to prevent the activity of the Cop9 signalosome on neddylated Cul1, perhaps by altering the conformation of the catalytic complex, that might explain the decreased rate of cyclin E turnover that we observed with this mutant. This explanation predicts that the Fbw7 ΔDC mutant should be less stable than wild-type Fbw7. However, as we find that this mutant shows no appreciable difference in protein stability than wild-type Fbw7, we think that this possibility is unlikely.

Finally, it is possible that the ΔDC mutant has a reduced binding affinity for cyclin E that our assay is not sufficiently sensitive to detect, although how the NH$_2$ terminus might affect the substrate-binding domain in the WD40 region is unclear. Crystal structures of SCF complexes indicate that several α-helices of the F-box domain and Skp1 are interdigitated; however, unfortunately, no structural information for the NH$_2$ terminus of any Fbw7 homologue that includes the D domain is available (38, 39).

Further work will be required to determine the stoichiometry of the Fbw7 complexes and to determine the extent of interaction among endogenously expressed Fbw7 isoforms. Other F-box proteins have been shown to form dimers; indeed, the D domain derives its name from its role in βTrCP dimerization (28). Fbw7 and its homologues in model organisms exhibit significant homology to the D domain and it is interesting that this domain appears in only a subset of F-box proteins. The region required for the interaction encompasses the D domain; however, we still observed substantial interaction among the Fbw7 isoforms when just the D domain is deleted in frame (Supplementary Fig. 1). Therefore, at least for the Fbw7 isoforms, residues in addition to the D domain are important for the formation of oligomeric complexes.

A functional role for the heterotypic interaction among the Fbw7 isoforms remains to be established. For βTrCP1 and

βTrCP2, it has been reported that the heterodimers are unable to bind substrate protein; in this way, dimerization might regulate substrate ubiquitination (28). The role of the heterotypic interaction with Fbw7 variants is complicated by the differential localization of each (12). For example, Fbw7-α is localized to the nucleus, whereas Fbw7-β is cytoplasmic. Our coimmunoprecipitation results suggest that the hetero-oligomeric interactions can occur with the mammalian proteins; however, if they do so in intact cells, it must either mean that only a small fraction of each population interacts or that Fbw7-α or Fbw7-β can shuttle between the nucleus and the cytoplasm.

In conclusion, we have shown that alternative splice variants of the F-box protein Fbw7 interact with each other and we have identified the region of the protein necessary for this interaction. This observation broadens the current understanding of F-box protein regulation, and it will be important to determine in the future if Fbw7 isoform interaction represents a common regulatory mechanism for other SCF^{Fbw7} substrates. In the case of the Fbw7α/α complex, our results may link F-box protein complex formation with tumorigenesis, as the cyclin E turnover rate is reduced in Fbw7-α mutants that fail to bind each other.

Materials and Methods

Cell Culture and Reagents

HEK293T cells were maintained in DMEM (Mediatech, Inc., Herndon, VA) with 10% newborn bovine serum. Sf9 insect cells were grown in Insect Xpress with L-Glutamine (BioWhittaker, Walkersville, MD) medium supplemented with 10% heat-inactivated newborn bovine serum. Hi5 insect cells were grown in ExCell 405 (JRH Biosciences, Lenexa, KS) without serum.

Cell Transfection and Infection

HEK293T cells were transfected with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the standard procedure provided by the manufacturer. Forty hours after transfection, cells were collected and washed with PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na$_2$HPO$_4$, 2 mmol/L KH$_2$PO$_4$). Baculovirus expressing GST- or Flag-tagged Fbw7 isoforms were generated in Sf9 cells using BD BaculoGold Linearized Baculovirus DNA (BD Biosciences, San Jose, CA). For expression of recombinant proteins, Hi5 insect cells were infected with recombinant baculovirus and incubated for 40 h before collection.

Generation of Expression Constructs

To generate either GST- or Flag-tagged Fbw7-expressing baculovirus, we used the Cre recombinase–based univector plasmid fusion system (40). Generation of Fbw7-β baculovirus has been described (1). Fbw7-α was amplified in two overlapping fragments using oligonucleotides DK396, DK398, DK399, and DK376. The NH$_2$ terminus of the Fbw7-γ isoform was cloned using three overlapping primers—DK373, DK373, and DK374—that were annealed, and a full-length product generated with primers DK373 and DK376. To generate Myc-tagged Fbw7-α and ΔN plasmids, these fragments were

Table 1. Plasmids Used in This Study

Name	Description	Source
p1212-Fbw7-α	GST-Fbw7-α for baculovirus production	This study
p1212-Fbw7-β	GST-Fbw7-β for baculovirus production	Ref. 1
p1212-Fbw7-γ	GST-Fbw7-γ for baculovirus production	This study
p1214-Fbw7-α	Flag-Fbw7-α for baculovirus production	This study
p1214-Fbw7-β	Flag-Fbw7-β for baculovirus production	Ref. 1
p1214-Fbw7-γ	Flag-Fbw7-γ for baculovirus production	This study
pcDNA3.1 Fbw7-α	CMV promoter, myc, his-tagged Fbw7-α	This study
p3XFlag-Fbw7-α	CMV promoter, Flag-tagged Fbw7-α	Ref. 12
p3XFlag-Fbw7-β	CMV promoter, Flag-tagged Fbw7-β	Ref. 12
p3XFlag-Fbw7-γ	CMV promoter, Flag-tagged Fbw7-γ	Ref. 12
Myc-Fbw7-γ	CMV promoter, Myc-tagged Fbw7-γ	This study
pcDNA3.1 Fbw7 ΔN	CMV promoter, myc, his-tagged Fbw7ΔN	This study
p3XFlag-Fbw7-α ΔDC	CMV promoter, Flag-tagged Fbw7-α ΔDC	This study
p3XFlag-Fbw7-β ΔDC	CMV promoter, Flag-tagged Fbw7-β ΔDC	This study
p3XFlag-Fbw7-γ ΔDC	CMV promoter, Flag-tagged Fbw7-γ ΔDC	This study
pRc-CycE	CMV promoter, cyclin E	J.W. Harper

Abbreviation: CMV, cytomegalovirus.

amplified using primer pairs DK397/DK400 and DK355/DK400, respectively. PCR products were digested with *Eco*RI and *Xba*I and cloned into pcDNA3.1-MycHis vector (Invitrogen). We also made 6× Myc-tagged Fbw7-γ plasmids (in pCS2+MT) by amplifying Fbw7-γ using primers DK221 and DK375 and cloning the PCR product into the *Eco*RI and *Xba*I sites.

The ΔDC deletion mutants of Fbw7-α, Fbw7-β, and Fbw7-γ were cloned into p3X FLAG-CMV 7.1 expression vector (Sigma, St. Louis, MO) by a two-step PCR. To delete the ΔDC region of each isoform, we first amplified the unique region of each isoform (α primers: WZ1, WZ2; β primers: WZ4, WZ5; γ primers: WZ7, WZ8) and the fragments starting from the F-box motif to the 3′ end by PCR (α primers WZ3/DK376; β primers: WZ6/DK376; γ primers: WZ9/DK376). The sequence at the 3′ of the each unique fragment and at the 5′ of the COOH-terminal fragment was complementary to each other. The two fragments of each isoform were annealed and the annealed fragment was used as the template for the next PCR. The final PCR products were digested by *Eco*RI and *Sal*I, and ligated into the p3X FLAG-CMV7.1 vector. A complete list of the constructs and primers used in this study are shown Tables 1 and 2, respectively.

Western Blot Analysis and Reagents

Cell lysates were prepared in NETN buffer [20 mmol/L Tris (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40] containing 1 mmol/L NaF, 2.5 mmol/L β-glycerophosphate, and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Cell lysates were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked in PBST (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4, 0.1% Tween 20) containing 5% milk for at least 40 min. Blots were probed with primary antibodies followed by labeling with horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA). Following antibody

incubation, blots were developed on film using an enhanced chemiluminescence kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Densitometry of immunoblot bands was measured by using NIH ImageJ. The primary antibodies used included anti-Flag M2 antibody (Sigma), anti-GST antibody (Z5, Santa Cruz Biotechnology, Santa Cruz, CA), anti-HA (HA.11, Covance Research, Princeton, NJ), anti-Myc (9E10, Covance Research), anti-cyclin E (HE-12, Santa Cruz Biotechnology), and anti-GAPDH (Abcam, Cambridge, MA).

Table 2. Oligonucleotides Used in This Study

Name	Sequence (5′ to 3′)
α-Specific	
WZ1	CGGAATTCCACCATGAATCAGGAACAGCTCTC
WZ2	CTCTTTAGGGAGTTTTGTTTTTGTATAGAATGG
WZ3	AAAACAAAACTCCCTAAAGAGTTGGCACTC
DK396	CGGAATTCCCACCATGAATCAGGAACTGCTCTC
DK397	CGGAATTCCACCATGAATCAGGAACTGCTCTC
DK398	GGTCCAACTTTCTTTTCATTTTTGTTGTTTTTGT ATAGAATGGGGAGG
DK399	CCTCCCCATTCTATACAAAAACAACAAAAATGA AAAGAAAGTTGGACC
β-Specific	
WZ4	TAATTGAATTCCATGTGTGTCCCGAGAAGC
WZ5	CTCTTTAGGGAGTTTGTAAAAAATCATTTTTAATG
WZ6	ATTTTTTTACAAACTCCCTAAAGAGTTGGCACTC
γ-Specific	
WZ7	CGGAATTCCACCATGTCAAAACCGGGAAAACCT
WZ8	CTCTTTAGGGAGCCGTCTTCGACAAAAAGGGAGG
WZ9	TGTCGAAGACGGCTCCCTAAAGAGTTGGCACTC
DK372	CGGAATTCCCACCATGTCAAAACCGGGAAAACCTA CTCTAAACCATGGCTTGGTTCCTGTTGATCTTAAAAG
DK373	AAGATATTTAGCATTAGCATCATTGCCCAAGGCCTCCCT TTTTGTCGAAGACGGATGAAAAGAAAGTTGGAC
DK374	GCTAATGCTAAATATCTTCATCACGGTTTGATGTGGTAG AGGCTCTTTTGCACTTTTAAGATCAACAGGAA
DK375	CGGAATTCCACCATGTCAAAACCGGGAAAACCT
Common region	
DK221	TCTAGACACTTCATGTCCACATC
DK376	ACGGGGTCGACTCACTTCATGTCCACATCAAG
DK400	ACGCCGTCGACCACTTCATGTCCACATC
ΔN	
DK355	CCGAATTCCGCCCACCATGCAAGTGATAGAACCCCA

Coimmunoprecipitation Assays

Two hundred to 500 μL of cell lysate were incubated with NETN buffer containing antibody at 4°C for 4 h; then, 20 μL of protein A/G beads (Santa Cruz Biotechnology) were added for another 2 h or overnight. The beads were washed thrice with NETN lysis buffer. Anti-Flag M2 (Sigma) agarose affinity gel was used to purify Flag-tagged proteins.

Protein Stability Assays

The cyclin E construct together with Fbw7 isoform or deletion mutant constructs were transfected into HEK293T cells. Thirty-six to 40 h after transfection, cycloheximide (Sigma) was added to a final concentration of 30 μg/mL to stop the protein synthesis (time 0). Cell extracts from each time point were analyzed by Western blotting.

Immunofluorescence Microscopy

HEK293T cells were transfected with Flag-tagged Fbw7, or deletion mutants were grown on coverslips for 40 h. Cells were fixed with 3% paraformaldehyde and 2% sucrose solution for 10 min at room temperature. Cells were permeabilized in ice-cold 0.5% Triton X-100 solution [0.5% Triton X-100, 20 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, 300 mmol/L sucrose] on ice for 5 min and blocked with 1% bovine serum albumin in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4) at 37°C for 30 min. Cells were incubated with anti-Flag antibody (1:2,000) at 37°C for 30 min followed by incubation with anti-mouse FITC (1:5,000) for 20 min at 37°C. Images were collected on a Zeiss Axioskop 2 microscope equipped with a Zeiss Axiocam R2 digital camera using Zeiss Axiovision software release 3.1 (Carl Zeiss, Thornwood, NY).

Acknowledgments

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