The SCF complex containing Skp1, Cul1, and the F-box protein FWD1 (the mouse homologue of Drosophila Slimb and Xenopus β-TrCP) functions as the ubiquitin ligase for IkBa. FWD1 associates with Skp1 through the F-box domain and also recognizes the conserved DSG\textsubscript{XXS} motif of IkBa. The structural requirements for the interactions of FWD1 with IkBa and with Skp1 have now been investigated further. The D31A mutation (but not the G33A mutation) in the DSG\textsubscript{XXS} motif of IkBa abolished the binding of IkBa to FWD1 and its subsequent ubiquitination without affecting the phosphorylation of IkBa. The IkBa mutant D31E still exhibited binding to FWD1 and underwent ubiquitination. These results suggest that, in addition to site-specific phosphorylation at Ser\textsubscript{32} and Ser\textsubscript{36}, an acidic amino acid at position 31 is required for FWD1-mediated ubiquitination of IkBa. Deletion analysis of Skp1 revealed that residues 61–143 of this protein are required for binding to FWD1. On the other hand, the highly conserved residues Pro\textsubscript{149}, Ile\textsubscript{160}, and Leu\textsubscript{164} in the F-box domain of FWD1 were dispensable for binding to Skp1. Together, these data delineate the structural requirements for the interactions among IkBa, FWD1, and Skp1 that underlie substrate recognition by the SCF ubiquitin ligase complex.

The ubiquitin-proteasome pathway is essential for several key biological processes, including cell cycle progression, gene transcription, and signal transduction (1–3). The formation of ubiquitin-protein conjugates requires three components that participate in a cascade of ubiquitin transfer reactions as follows: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3).\textsuperscript{1} The specificity of protein ubiquitination is often determined by E3 enzymes, and proteins polyubiquitinated by these enzymes are subjected to degradation by the 26 S proteasome. Recent genetic and biochemical studies of yeast have led to the identification of a new class of E3 ligases, termed SCF complexes, that are required for degradation of cyclins and their inhibitors (4, 5). However, in addition to cell cycle-related proteins, an increasing number of molecules important in other biological processes in yeast have been identified as substrates for the SCF complexes (6). These complexes consist of invariant components such as Skp1 and Cdc53 as well as variable components known as F-box proteins, which bind to Skp1 through the F-box domain (7–9). F-box proteins function as receptors for the target protein, which is usually phosphorylated (8, 9). Thus, the substrate specificity of the SCF complex is thought to be determined by F-box proteins. The physiological roles of the SCF complex in multicellular organisms remain to be elucidated.

Various short-lived regulatory proteins undergo ubiquitination in mammalian cells. One of the most extensively studied of these molecules is IkBa, an inhibitory protein that associates with the dimeric nuclear factor-κB (NF-κB) and thereby prevents it from entering the nucleus. NF-κB plays a central role in the regulation of genes that function in inflammation, cell proliferation, and apoptosis (10–14). It is located in the cytoplasm of resting cells but enters the nucleus in response to various stimuli, including viral infection, ultraviolet radiation, and inflammatory cytokines such as tumor necrosis factor-α and interleukin-1. In response to these external signals, IkBa is rapidly phosphorylated by a protein kinase complex known as IKK (IkBa kinase) (15–23). The IKK complex contains at least four kinases (IKKa, IKKβ, IKKγ (or NEMO), and NIK) as well as the scaffold protein IKAP (24). IKK specifically phosphorylates the two serine residues (Ser\textsubscript{32} and Ser\textsubscript{36}) of the DSG\textsubscript{XXS} motif present in the NH\textsubscript{2}-terminal region of IkBa (16–21). Phosphorylated IkBa is then ubiquitinated on Lys\textsubscript{33} and Lys\textsubscript{32}, which triggers the rapid degradation of the protein by the 26 S proteasome (25–29). The liberated NF-κB then translocates to the nucleus and activates the expression of target genes.

We and others (30–42) have recently shown that FWD1, the mouse homologue of Drosophila Slimb and Xenopus β-TrCP, is a member of the F-box- and WD40 repeat-containing family of proteins and that it specifically recognizes IkBa and β-catenin as substrates only when they are phosphorylated at the serine residues in the conserved DSG\textsubscript{XXS} motif. FWD1 also interacts with the Skp1-Cul1 complex through its F-box domain, thereby forming the SCF complex SCFFWD1. Mutation of the serine residues in the DSG\textsubscript{XXS} motif stabilizes IkBa and β-catenin by inhibiting both their binding to FWD1 and their subsequent ubiquitination (30, 31). It is therefore likely that phosphorylation of the DSG\textsubscript{XXS} motif serves to recruit FWD1 and thereby to link IkBa to the ubiquitination machinery. However, it has remained unknown whether other conserved residues, including Asp\textsubscript{31} and Gly\textsubscript{33} of the DSG\textsubscript{XXS} motif, are also essential for ubiquitin-dependent degradation of IkBa.

We have now investigated the binding motifs that mediate the interactions of FWD1 with IkBa and Skp1. We molecularly dissected the pathway of IkBa ubiquitination into two steps as follows: phosphorylation of IkBa and subsequent binding of FWD1 to the phosphorylated DSG\textsubscript{XXS} motif. We identified the
region of Skp1 required for binding to FWD1 and performed a mutation analysis of the F-box domain. Our results reveal the structural requirements for complex formation among IκBα, FWD1, and Skp1 during the ubiquitination of IκBα. Furthermore, they may facilitate the identification of new substrates for SCFFWD1 by screening of sequence data bases.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293T cells were grown at 37 °C under an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.).

**Construction of Expression Plasmids and Mutagenesis**—Molecular cloning of Skp1 and FWD1 cDNAs was described previously (30, 31). Complementary DNAs encoding Skp1 and its deletion mutants, FWD1, and the mutant FWD1(ΔF), each protein-tagged at its NH₂ terminus with the Myc or FLAG epitope, were generated by the polymerase chain reaction with the high fidelity thermostable DNA polymerase KOD (Toyobo, Tokyo, Japan). Complementary DNAs encoding one- or two-site FWD1 mutants, each tagged at its NH₂ terminus with the FLAG epitope, were generated with the use of a Chameleon site-directed mutagenesis kit (Stratagene). IκBα and IKKβ cDNAs were kindly provided by H. Nakano. Complementary DNAs encoding IκBα mutants, each tagged at its NH₂ terminus with the Myc epitope, were generated with the use of a QuickChange site-directed mutagenesis kit (Stratagene). Complementary DNAs encoding all mutant proteins were sequenced and subcloned into pcDNA3 (Invitrogen).

**Transfection, Immunoprecipitation, and Immunoblot Analysis**—293T cells were transfected by the calcium phosphate method (43). After 48 h, the cells were lysed with a solution containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 0.5% (v/v) Triton X-100, aprotinin (10 μg/ml), 10 μg leupeptin (10 μg/ml), 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.4 mM Na₃VO₄, 0.4 mM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. The cell lysates were treated with 50 μl of each tagged at its NH₂ terminus with the Myc or FLAG epitope, were generated by the polymerase chain reaction with the high fidelity thermostable DNA polymerase KOD (Toyobo, Tokyo, Japan). Complementary DNAs encoding one- or two-site FWD1 mutants, each tagged at its NH₂ terminus with the FLAG epitope, were generated with the use of a Chameleon site-directed mutagenesis kit (Stratagene). IκBα and IKKβ cDNAs were kindly provided by H. Nakano. Complementary DNAs encoding IκBα mutants, each tagged at its NH₂ terminus with the Myc epitope, were generated with the use of a QuickChange site-directed mutagenesis kit (Stratagene). Complementary DNAs encoding all mutant proteins were sequenced and subcloned into pcDNA3 (Invitrogen).

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**In Vitro Kinase Assay**—Complementary DNAs encoding Myc-tagged IκBα and its mutants were subcloned into pGEX-6P1 (Amersham Pharmacia Biotech). The recombinant glutathione S-transferase fusion proteins containing the Myc-IκBα sequences were purified from bacteria with the use of glutathione beads and then treated with PreScission protease (Amersham Pharmacia Biotech) to remove the glutathione S-transferase sequence. Recombinant AU1-tagged IKKβ was generated as described previously (30). In *in vitro* kinase reactions were performed for 30 min at 30 °C with purified AU1-tagged IKKβ and bacterially expressed Myc-tagged IκBα proteins in a reaction mixture containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 4.5 mM 2-mercaptoethanol, 1 mM EGTA, 100 μM ATP, and 1 μCi of [γ-³²P]ATP. The reaction was terminated by adding SDS sample buffer and boiling. The samples were then subjected to SDS-PAGE on a 9% gel, which was then dried and exposed to x-ray film.

**Pulse-Chase Experiments**—Transfected 293T cells were metabolically labeled with Trans-³⁵S (ICN, Costa Mesa, CA) at a concentration of 100 μCi/ml for 1 h. After incubation for various times in the absence of isotope, the cells were lysed and subjected to immunoprecipitation with antibodies to Myc (9E10) and protein G-Sepharose. The immunoprecipitates were fractionated by SDS-PAGE on a 9% gel, which was then dried and exposed to x-ray film.

**RESULTS**

**Effect of Mutations in the DSGXXS Motif on IκBα Phosphorylation**—We and others (30–42, 44) have recently shown that FWD1 mediates the ubiquitination of IκBα, IκBβ, IκBε, β-catenin, and Vpu by functioning as an intracellular receptor that links these substrates to the core complex of the SCF E3 ubiquitin ligase. All five of these substrates share the DSGXXS motif (Fig. 1A), the two serine residues of which undergo signal-induced phosphorylation that is a prerequisite for protein ubiquitination and degradation. This shared property suggests that FWD1 may recognize the phosphorylated DSGXXS motif in each of these proteins. To investigate further the role of this motif in IκBα ubiquitination, we mutated conserved residues and thereby created the mutants D31A, D31E, G33A, S32A/S36A, D31A/S32A, and D31A/S36A (Fig. 1B). IKKβ specifically and equally phosphorylates the two serine residues (Ser⁴⁶ and Ser⁴⁸) of the DSGXXS motif in the NH₂-terminal region of IκBα (18, 19, 21). To examine the effects of the DSGXXS motif mutations on IκBα phosphorylation, we performed *in vitro* kinase assays (Fig. 2). Myc-tagged wild-type and mutant IκBα proteins were incubated with or without purified AU1-tagged IKKβ in the presence of [γ-³²P]ATP. The extent of phosphorylation of D31A, D31E, and G33A in the presence of AU1-tagged IKKβ was virtually identical to that of the wild-type protein (Fig. 2A). Although IKKβ has also been shown to phosphorylate the COOH-terminal region of IκBα (16, 18), such activity was not apparent under our experimental conditions, as indicated by the fact that phosphorylation of S32A/S36A by IKKβ was not detected. Taken together, these results suggest that the amino acid substitutions at Asp³¹ or Gly³⁵ did not affect IκBα phosphorylation at Ser⁴⁶ and Ser⁴⁸ by IKKβ.

**Requirement of an Acidic Residue at Position 31 for Association of IκBα with FWD1**—We next examined whether FWD1 binds to the IκBα mutants in *vivo*. Expression vectors encoding Myc-tagged IκBα or its mutants and FLAG-tagged FWD1 were introduced into 293T cells, with or without a vector encoding FLAG-tagged IKKβ, and immunoprecipitation assays were performed (Fig. 3). As shown previously, the interaction of wild-type IκBα with FWD1 required coexpression of IKKβ (30). The D31A mutant did not associate with FWD1 even in the presence of recombinant IKKβ. In contrast, the D31E mutant, in which Asp³¹ was replaced by another acidic amino acid (Glu), interacted with FWD1 in the presence of IKKβ, although the extent of the interaction was slightly reduced compared with that observed with the wild-type protein. The G33A mutant also interacted with FWD1. Given that mutations at positions 31 or 33 did not affect the phosphorylation of IκBα by...
Characterization of Interactions among IκBα, FWD1, and Skp1

**Figs. 2-3.** Effects of IκBα mutations at Asp31 or Gly33 on the phosphorylation of Ser32 and Ser36. *A. In vitro* phosphorylation of IκBα mutants by IKKβ. Myc-tagged wild-type IκBα (WT) and its mutants D31A, D31E, G33A, and S23A/S36A were incubated either alone or together with an equal amount of purified recombinant AU1-IKKβ protein, as indicated, in the presence of [γ-32P]ATP. The reaction mixtures were then subjected to SDS-PAGE and autoradiography (upper panel). The D31A mutant migrated faster than did the other Myc-tagged IκBα proteins, probably because of the removal of the negative charge of Asp31. The IκBα proteins were also subjected to Coomassie Blue (CB) staining to verify the equal abundance of substrates in the assay mixtures (lower panel). The positions of IκKβ, IκBα, and phosphorylated IκBα are indicated. B. Effect of the D31A mutation on phosphorylation of Ser32 and Ser36 by IKKβ. Myc-tagged IκBα proteins were incubated either alone or together with an equal amount of purified recombinant AU1-IKKβ protein, as indicated, in the presence of [γ-32P]ATP. The reaction mixtures were then analyzed as in A.

IKKβ (Fig. 2A), these results suggest that the phosphorylation of IκBα at Ser32 and Ser36 is not sufficient and that an acidic residue at position 31 is also required, for binding to FWD1. The possibility remained, however, that only a single serine residue, either Ser32 or Ser36, was phosphorylated in D31A. To exclude this possibility, we generated the double mutants D31A/S32A and D31A/S36A (Fig. 1B). The *in vitro* kinase assay revealed that the extent of phosphorylation of D31A/S32A and D31A/S36A was similar and approximately half that of D31A (Fig. 2B), suggesting that the replacement of Asp31 with Ala did not affect the phosphorylation of either Ser32 or Ser36.

**FWD1-induced Ubiquitination of IκBα Mutants**—We next examined whether the various IκBα mutants are susceptible to ubiquitination. Immunoblot analysis with antibodies to ubiquitin of immunoprecipitates prepared from transfected cells with antibodies to Myc revealed a correlation between the pattern of ubiquitination of IκBα proteins and the pattern of FWD1 binding (Fig. 3). Thus, interaction with FWD1 was associated with a marked increase in the extent of ubiquitination of wild-type IκBα, D31E, and G33A in the presence of IκKβ, with the extent of ubiquitination of D31E and G33A being slightly less than that of the wild-type protein. The D31A mutant neither associated with FWD1 nor underwent ubiquitination. These results indicate that FWD1-induced ubiquitination of IκBα requires not only site-specific phosphorylation but also an acidic residue at position 31. Substitution of Gly33 of IκBα prevented neither its binding to FWD1 nor its ubiquitination mediated by FWD1, although the extent of both reactions was slightly reduced, suggesting that Gly33 might be necessary for maximal ubiquitination. The intracellular abundance of wild-type, D31E, and G33A IκBα proteins was markedly reduced, compared with that of D31A and S23A/S36A, in the presence of FWD1 and IκKβ, likely reflecting an increased rate of turnover of these proteins in the transfected cells (see Fig. 4).

**FWD1-induced Degradation of IκBα Mutants**—To examine whether mutations in the DSGXXS motif of IκBα affect the turnover rate of the protein, we performed pulse-chase experiments (Fig. 4). When expressed in the presence of FWD1 alone, the wild-type and mutant IκBα proteins showed similar turnover rates. However, when coexpressed with both FWD1 and IκKβ, the rates of degradation of the wild-type protein and the D31E and G33A mutants were markedly increased, although the kinetics of degradation of D31E and G33A were slightly slower than those of wild-type IκBα. In contrast, the D31A mutant was relatively stable and, with the exception of the accumulation of a phosphorylated form, showed a pattern of degradation similar to that of the S23A/S36A mutant. This latter result is consistent with our observation that the D31A mutant has lost the ability to bind FWD1 and to undergo ubiquitination but is still phosphorylated on Ser32 and Ser36. Together, these data demonstrate that an acidic residue at position 31 in the DSGXXS motif of IκBα is important for binding to FWD1, for ubiquitination, and for protein degradation but not for phosphorylation.

**Delineation of the Region of Skp1 Required for Binding to FWD1 in Vivo**—Although it has been established that F-box
proteins bind to Skp1 through the F-box domain (7), it remains unclear which region of Skp1 interacts with the F-box domain in vivo. To identify the region of Skp1 required for binding to FWD1 in vivo, we generated a series of Myc-tagged NH2- and COOH-terminal deletion mutants of Skp1 (Fig. 5A). Myc-tagged wild-type Skp1, its deletion mutants, or p21\(^{Cip1/Waf1}\) (negative control) was expressed in 293T cells together with FLAG-tagged FWD1, FWD1(ΔF), or p27\(^{Kip1}\) (negative control). Immunoprecipitation assays revealed that wild-type Skp1 as well as the ΔN40 and ΔC20 mutants were detected in FWD1 immunoprecipitates, whereas the ΔN80 and ΔC30 mutants were not (Fig. 5B). The ΔN60 mutant was also coprecipitated with FWD1, although to a slightly reduced extent compared with that of wild-type Skp1. These results were confirmed by a reciprocal immunoprecipitation analysis (Fig. 5B). Thus, FLAG-tagged FWD1 was present in the wild-type Skp1, ΔN40, and ΔC20 immunoprecipitates but not in those containing ΔN80 or ΔC30 Skp1 mutants. Again, the amount of FWD1 in the ΔN60 immunoprecipitate was slightly less than that in the wild-type Skp1 precipitate. None of the Skp1 proteins interacted with the FWD1(ΔF) mutant, which comprises residues 1–140 fused to residues 194–569 of FWD1 and therefore lacks the F-box domain. These observations suggest that residues 61–143 of Skp1 are required for binding to FWD1 in vivo.

**Mutational Analysis of the F-Box Domain**—Several amino acid residues are highly conserved among the F-box domains of many F-box proteins (Fig. 6A). To determine which residues in the F-box domain are necessary for binding to Skp1 in vivo, we replaced the highly conserved amino acids Pro\(^{149}\), Ile\(^{160}\), and Leu\(^{164}\) in the F-box domain of FWD1 with Ala (Fig. 6B). FLAG-tagged FWD1 derivatives or p27\(^{Kip1}\) (negative control) were expressed in 293T cells together with Myc-tagged Skp1 or p21\(^{Cip1/Waf1}\) (negative control). Immunoprecipitation assays revealed that similar amounts of wild-type FWD1 and its various point mutants were present in Skp1 immunoprecipitates (Fig. 6C). Again, the FWD1(ΔF) mutant, which lacks the entire F-box domain, did not interact with Skp1. These results were confirmed by reciprocal immunoprecipitation analysis (Fig. 6C). Thus, virtually identical amounts of Skp1 were detected in the immunoprecipitates containing FWD1 and its various point mutants, whereas Skp1 was not present in the FWD1(ΔF) immunoprecipitate.

The highly conserved residues in the F-box domain of FWD1 are dispensable for binding to Skp1 in vivo.

![Image](https://example.com/image.png)

**Fig. 4. Importance of an acidic residue at position 31 for IκBα degradation.** 293T cells were transfected with vectors encoding the indicated Myc-tagged IκBα proteins and FLAG-FWD1 in the absence or presence of a vector encoding FLAG-IKKβ. The cells were subsequently pulse-labeled with \(^{35}\)S-methionine and \(^{35}\)S-cysteine and then incubated in the absence of isotope for the indicated chase periods. Cell lysates were then subjected to immunoprecipitation with antibodies to Myc, and the resulting precipitates were subjected to SDS-PAGE and autoradiography. The positions of IκBα and phosphorylated IκBα proteins are indicated. WT, wild type.

![Image](https://example.com/image.png)

**Fig. 5. Delineation of the region of Skp1 responsible for binding to FWD1.** A. Schematic representation of Skp1 deletion mutants. ΔN and ΔC denote deletions from the NH2- and COOH-terminal regions, respectively, and the numbers correspond to amino acid positions of Skp1. Whether or not the various mutants interact with FWD1 is also indicated by the plus and minus signs. B. Interactions between Skp1 deletion mutants and FWD1. 293T cells were transfected with expression plasmids encoding Myc-tagged wild-type Skp1 (Skp1 WT), its deletion mutants, or p21 (negative control) together with a plasmid encoding FLAG-tagged wild-type FWD1, FWD1(ΔF), or p27 (negative control), as indicated. Cell lysates were subjected to immunoprecipitation (IP) with antibodies to the FLAG epitope or to Myc, and the resulting immunoprecipitates were subjected to immunoblot (IB) analysis with antibodies to Myc or to FLAG, respectively, as indicated. Portions of the cell lysates corresponding to 10% of the input for immunoprecipitation were also subjected to immunoblot analysis with antibodies to Myc or to FLAG in order to indicate the level of expression of the various recombinant proteins. The relative positions of ΔN40, ΔN60, ΔC20, and ΔC30 do not appear to correspond to their molecular sizes, probably because of the charge of these proteins.

**WFD1-mediated Association of Skp1 with IκBα**—We have...
and a yeast two-hybrid assay of the interaction between the various tagged wild-type FWD1 (293T cells were transfected with expression plasmids encoding FLAG-precipitation assays of the interaction of FWD1 mutants with Skp1. Amino acids that are identical in all proteins are boxed, and residues mutated in the present study are indicated by arrows. Residue numbers are shown on the left. B, schematic representation of F-box mutants generated in the present study. The black and gray boxes indicate the F-box domain and the WD40 repeats, respectively. Mutated amino acids are indicated by asterisks. The results of immunoprecipitation (IP) assays and a yeast two-hybrid assay of the interaction between the various mutants and Skp1 are summarized (n.d., not determined). C, immunoprecipitation assays of the interaction of FWD1 mutants with Skp1. 293T cells were transfected with expression plasmids encoding FLAG-tagged wild-type FWD1 (WT), FWD1 mutants, or p27 (negative control), together with plasmids encoding Myc-tagged wild-type Skp1 or p21 (negative control), as indicated. Cell lysates were subjected to immunoprecipitation with antibodies to Myc or to FLAG, and the resulting immunoprecipitates were subjected to immunoblot (IB) analysis with antibodies to Myc. The resulting immunoprecipitates were subjected to immunoblot (IB) analysis with antibodies to FLAG. Portions of the cell lysates corresponding to 10% of the input for immunoprecipitation were also subjected to immunoblot analysis with antibodies to FLAG or to Myc in order to indicate the expression levels of the various recombinant proteins.

Fig. 6. Effect of mutation of conserved amino acids in the F-box domain of FWD1 on its interaction with Skp1. A, alignment of amino acid sequences of the F-box domains in FWD1 (mouse), cyclin F (human), Cdc4, and Grr1 (S. cerevisiae), and Skp2 (mouse). Amino acids that are identical in all proteins are boxed, and residues mutated in the present study are indicated by arrows. Residue numbers are shown on the left. B, schematic representation of F-box mutants generated in the present study. The black and gray boxes indicate the F-box domain and the WD40 repeats, respectively. Mutated amino acids are indicated by asterisks. The results of immunoprecipitation (IP) assays and a yeast two-hybrid assay of the interaction between the various mutants and Skp1 are summarized (n.d., not determined). C, immunoprecipitation assays of the interaction of FWD1 mutants with Skp1. 293T cells were transfected with expression plasmids encoding FLAG-tagged wild-type FWD1 (WT), FWD1 mutants, or p27 (negative control), together with plasmids encoding Myc-tagged wild-type Skp1 or p21 (negative control), as indicated. Cell lysates were subjected to immunoprecipitation with antibodies to Myc or to FLAG, and the resulting immunoprecipitates were subjected to immunoblot (IB) analysis with antibodies to Myc. The resulting immunoprecipitates were subjected to immunoblot (IB) analysis with antibodies to FLAG. Portions of the cell lysates corresponding to 10% of the input for immunoprecipitation were also subjected to immunoblot analysis with antibodies to FLAG or to Myc in order to indicate the expression levels of the various recombinant proteins.

Fig. 7. Association of Skp1 with IxBo mediated through FWD1. 293T cells were transfected with expression plasmids encoding FLAG-tagged wild-type IxBo or its S32A/S36A mutant together with the indicated combinations of vectors encoding FLAG-FWD1, FLAG-IKKβ, and either Myc-Skp1 or Myc-p21. Cell lysates were subsequently prepared and subjected to immunoprecipitation (IP) with antibodies to Myc. The resulting immunoprecipitates were subjected to immunoblot (IB) analysis with antibodies to FLAG. Portions of the cell lysates corresponding to 10% of the input for immunoprecipitation were also subjected to immunoblot analysis with antibodies to FLAG or to Myc in order to indicate the expression levels of the various recombinant proteins.

analyzed in detail the interactions of FWD1 with IxBo and with Skp1. Finally, we investigated whether IxBo, FWD1, and Skp1 form a trimolecular complex in vivo. FLAG-tagged IxBo and Myc-tagged Skp1 were coexpressed in 293T cells in the absence or presence of exogenous FWD1 and IKKβ. Immunoprecipitation assays revealed that IxBo was present in Skp1 immunoprecipitates only when both proteins were expressed together with FWD1 and IKKβ (Fig. 7). The fact that the IxBo-Skp1 association was not observed in the absence of FWD1 confirms that it is mediated through FWD1. This association was also not detected with the S32A/S36A mutant of IxBo or in the absence of coexpression of IKKβ, indicating the importance of phosphorylation of the two serine residues by IKKβ for its manifestation. The negative control protein p21 (Cip1/Waf1) did not interact with IxBo even in the presence of both FWD1 and IKKβ, indicating that the association between IxBo and Skp1 is specific. These data demonstrate that FWD1 binds simultaneously to Skp1 through its F-box domain and to the serine-phosphorylated DSGXXS motif of IxBo.

DISCUSSION

Protein ubiquitination mediated by the SCF complex requires the same core complex, containing a ubiquitin-conjugating enzyme, Cul1 (or Cdc53), and Skp1, together with different F-box proteins that function as substrate-tethering molecules (4–6). Skp1 is thus able to participate in multiple ubiquitination pathways by assembling with different F-box proteins that recruit specific substrates for ubiquitination. In addition to the F-box domain, which mediates interaction with Skp1, all F-box proteins are thought to contain a motif that is necessary for substrate recognition. The F-box proteins Cdc4 and Met30 (Saccharomyces cerevisiae), Pop1 and Pop2 (Schizosaccharomyces pombe), SEL-10 (Caenorhabditis elegans), Slimb (Drosophila melanogaster), β-TrCP (Xenopus laevis), and FWD1 and FWD2 (Mus musculus) all contain WD40 repeats (7, 30, 31, 49–54); we designate this group of proteins the FLR family. In addition, we have identified many F-box proteins that do not possess known
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structural features that mediate protein-protein interactions (data not shown).

In the present study, we investigated the structural features required for the interaction between IκBα and FWD1 as a model system for interaction of F-box proteins with their corresponding substrates. Our results indicate that an acidic residue is required immediately upstream of the phosphorylated serines of the DSGXXS motif of IκBα for interaction with FWD1. Furthermore, our data suggest that the interaction between the F-box domain of FWD1 and Skp1 is mediated by a broad range of contacts between the two proteins.

IκBα, a substrate of SCFPKD1, is one of the most studied of proteins that are regulated by the ubiquitin-proteasome pathway. Previous studies have shown that site-specific phosphorylation of IκBα on Ser32 and Ser36 is a prerequisite for ubiquitination and subsequent degradation of the protein (25–27, 29). Consistent with this observation, FWD1 recognizes IκBα only when both Ser32 and Ser36 in the DSGXXS motif are phosphorylated (30). It has therefore been thought that site-specific phosphorylation of IκBα is the key signal for ubiquitination of IκBα. However, our results now show that, in addition to site-specific phosphorylation, an acidic residue at position 31 in the DSGXXS motif of IκBα is required for binding to FWD1 and subsequent ubiquitination.

Our results are consistent with those of DiDonato et al. (56), showing that the D31A mutant of IκBα is more stable than is the wild-type protein, although the reason for the increased stability of the mutant was not evident at the time of this previous study. We molecularly dissected the pathway of IκBα ubiquitination into two steps as follows: phosphorylation of Ser32 and Ser36 by the IKK complex and subsequent binding of FWD1 to the phosphorylated DSGXXS motif. An acidic residue at position 31 of IκBα appears to be required for the second step but not for the first step. The other conserved residue of the DSGXXS motif, Gly35, is dispensable for the binding of IκBα to FWD1 as well as for its ubiquitination and degradation. Therefore, we propose that the (D/E)/S*/XXXS* sequence (S*, phosphorylated serine) represents the primary destruction motif for the binding of IκBα to FWD1 and its subsequent ubiquitination. These results may facilitate the identification of other substrates that are regulated by SCFPKD1 from sequence data bases. In the case of β-catenin, another substrate of SCFPKD1, mutation of Asp32 (the residue corresponding to Asp31 in IκBα) has been frequently detected in human neoplasms, including synovial sarcomas (D32Y) (57), hepatocellular carcinomas (D32Y and D32G) (60), and prostate cancer (D32Y) (57), hepatoblastomas (D32T) (59), pilomatrixomas (D32Y and D32G) (60), and prostate cancer (D32Y) (60). Although it has not been determined whether all of these β-catenin mutants have lost the ability to bind FWD1, these observations suggest that the (D/E)/S*/XXXS* motif functions as the primary determinant of destruction in β-catenin. Thus, it is likely that these natural mutations of Asp32 in β-catenin prevent both binding to FWD1 and subsequent ubiquitination and thereby promote tumorigenesis. None of the known substrates for FWD1 contains Glu instead of Asp in the (D/E)/S*/XXXS* motif, suggesting that the slight reduction in binding to FWD1 and in ubiquitination observed with the D31E mutant of IκBα might not be negligible under physiological conditions.

Ng et al. (62) recently delineated the region of Skp1 required for binding to the F-box protein Skp2 in vitro. They showed that deletion of 35 amino acids from the NH2 terminus or 49–98 amino acids from the COOH terminus of Skp1 significantly reduced the extent of binding to Skp2. Although these in vitro results are roughly in agreement with our in vivo delineation of the region of Skp1 required for binding to FWD1, it remains unclear whether other F-box proteins also bind to Skp1 through this region. We showed that about half of the Skp1 protein is required for binding to FWD1. Another component of the SCF complex, Cul1, has been shown to bind to the NH2-terminal half of Skp1 (63). Furthermore, a kinetic component has also been shown to associate with Skp1 (64). Given that Skp1 is a small protein (~19 kDa), it is unlikely that it interacts with many molecules simultaneously; the various binding regions might overlap with each other, or some of the described interactions of Skp1 might be indirect.

Unlike the situation for Skp1, the region of F-box proteins responsible for binding to Skp1 has been well established as the F-box domain (7). A previous mutational study of the yeast F-box protein Cdc4 showed that replacement of Pro279 Ile286, or Leu290 (corresponding to Pro149 Ile166, and Leu144 of FWD1, respectively) with Ala significantly reduced or abolished binding to yeast Skp1 in vitro (7). However, we have now shown that the corresponding mutations did not affect the interaction of FWD1 with Skp1 in vivo. This apparent discrepancy might be attributable to promotion of the FWD1-Skp1 interaction by other SCF constituents, such as Cul1 or Rbx1, in vivo (35, 45–48). The possibility remains, however, that all of the F-box domain is not required for binding to Skp1. Our observations that the interaction between Skp1 and the F-box domain of FWD1 requires a large portion of the Skp1 molecule and is not affected by single or double point mutations in the F-box domain suggest that the two proteins interact through a broad range of intermolecular contacts.

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