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An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of β-catenin

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β-catenin plays an essential role in the Wingless/Wnt signaling cascade and is a component of the cadherin cell adhesion complex. Deregulation of β-catenin accumulation as a result of mutations in adenomatous polyposis coli (APC) tumor suppressor protein is believed to initiate colorectal neoplasia. β-catenin levels are regulated by the ubiquitin-dependent proteolysis system and β-catenin ubiquitination is preceded by phosphorylation of its N-terminal region by the glycogen synthase kinase-3β (GSK-3β)/Axin kinase complex. Here we show that FWD1 (the mouse homologue of Slimb/βTrCP), an F-box/WD40-repeat protein, specifically formed a multi-molecular complex with β-catenin, Axin, GSK-3β and APC. Mutations at the signal-induced phosphorylation site of β-catenin inhibited its association with FWD1. FWD1 facilitated ubiquitination and promoted degradation of β-catenin, resulting in reduced cytoplasmic β-catenin levels. In contrast, a dominant-negative mutant form of FWD1 inhibited the ubiquitination process and stabilized β-catenin. These results suggest that the Skp1/Cullin/F-box protein FWD1 (SCF^{FWD1})–ubiquitin ligase complex is involved in β-catenin ubiquitination and that FWD1 serves as an intracellular receptor for phosphorylated β-catenin. FWD1 also links the phosphorylation machinery to the ubiquitin–proteasome pathway to ensure prompt and efficient proteolysis of β-catenin in response to external signals. SCF^{FWD1} may be critical for tumor development and suppression through regulation of β-catenin protein stability.

Keywords: β-catenin/F-box protein/FWD1/SCF complex/ubiquitin ligase

Introduction

The Wingless/Wnt pathway is critical for development and organogenesis (Huber *et al*., 1996b; Miller and Moon, 1996; Cadigan and Nusse, 1997). Cytoplasmic β-catenin plays an essential role in Wnt signaling and membrane-associated β-catenin serves as a lining protein for the cadherin cell adhesion complex (Aberle *et al*., 1996). In this pathway, which starts with Wnt, inactivation of glycogen synthase kinase-3β (GSK-3β) leads to stabilization and accumulation of hypo-phosphorylated β-catenin, which then interacts with and activates T cell factor/lymphocyte enhancer binding factor (TCF/LEF) transcription factors (Huber *et al*., 1996a; Clevers and Vandewetering, 1997; Bauer *et al*., 1998). In the absence of the Wnt signal, GSK-3β constitutively phosphorylates β-catenin, leading to low expression levels as a result of ubiquitin-mediated proteolysis of β-catenin. Regulation of cytoplasmic β-catenin levels is clinically important, because deregulation of this system may play a critical role in the pathogenesis of colorectal cancer. Mutations of the adenomatous polyposis coli (*APC*) tumor suppressor gene are the most common genetic events in colorectal cancers and one of the molecular functions of the APC protein is to bind β-catenin to GSK-3β, making it a target for destruction (Ilyas and Tomlinson, 1997; Korinek *et al*., 1997; Peifer, 1997). Colorectal tumors with intact *APC* genes, as well as melanoma cell lines, contain β-catenin mutations which functionally alter significant phosphorylation sites, and such alterations have been implicated in making β-catenin stable (Morin *et al*., 1997; Rubinfeld *et al*., 1997). GSK-3β, in concert with Axin, which associates with GSK-3β (Ikeda *et al*., 1998), appears to phosphorylate β-catenin at these sites. Axin also interacts directly with APC, suggesting that Axin functions as a scaffold protein for the assembly of the molecules that regulate β-catenin stability (Kishida *et al*., 1998). A recent study showed that β-catenin is a target for the ubiquitin–proteasome pathway and that phosphorylation of serine/threonine residues at positions 29, 33, 37, 41 and 45 by GSK-3β appears to be a prerequisite for ubiquitination (Aberle *et al*., 1997; Orford *et al*., 1997). These findings suggest that the abnormal accumulation of β-catenin resulting from deregulation of the proteolytic machinery via phosphorylation/ubiquitination is the most likely cause of tumorigenesis in colorectal cancers. The molecular mechanism by which β-catenin is specifically marked for degradation by ubiquitination in response to phosphorylation, however, is unclear.

The ubiquitin–proteasome pathway plays a key role in diverse biological processes, such as cell proliferation, differentiation and development, and protein levels which are determined in a substrate-specific manner by this system (Hershko and Ciechanover, 1992; Weissman, 1997). The formation of ubiquitin–protein conjugates involves three components which participate in a cascade of ubiquitin-transfer reactions: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a

ubiquitin ligase (E3). The specificity of protein ubiquitination often derives from the latter and proteins polyubiquitinated by these enzymes are subject to degradation by the 26S proteasome. Recent genetic and biochemical studies on yeast have led to the identification of a novel class of E3 ligases (termed the Skp1/Cullin/F-box proteins, SCF complex) required for degradation of cyclins and their inhibitors. As well as cell-cycle-related proteins, an increasing number of molecules in other biological systems of yeast have been identified as substrates for the SCF–E3 complex, which consists of invariable components, such as Skp1 and Cdc53, and variable components called F-box proteins, which bind to Skp1 through the F-box motif (Elledge and Harper, 1998; Krek, 1998). F-box proteins serve as receptors for the target proteins, which are usually phosphorylated (Feldman *et al*., 1997; Skowyra *et al*., 1997). Thus, the substrate specificity of the SCF complex is believed to depend on F-box proteins.

Although the physiological roles of the SCF complex in multicellular organisms have not been elucidated, a recent study showed that a mutation of the F-box/WD40-repeat protein Slimb in *Drosophila* led to accumulation of Armadillo, a β-catenin homologue (Jiang and Struhl, 1998). This genetic evidence prompted us to investigate whether the mammalian homologue of Slimb is involved in β-catenin degradation. In this study, we isolated FWD1, an F-box/WD40-repeat protein, as a mouse homologue of *Drosophila* Slimb, and demonstrated that FWD1 associated with β-catenin/Axin/GSK-3β/APC and promoted β-catenin ubiquitination and degradation. The biological roles of FWD1 and its involvement in cancer are discussed.

Results

Association of FWD1 with β-catenin and Axin

We searched a mouse expression sequence tag (EST) database for DNA sequences homologous with the genes for *Drosophila* Slimb and identified a clone showing significant homology with Slimb. Full-length DNA sequencing revealed that this clone encodes a protein with features of an F-box domain followed by seven WD40 repeats (Hatakeyama *et al*., 1999). This clone, designated FWD1 (F-box/WD40-repeat protein 1) is very similar to Slimb (*Drosophila melanogaster*), βTrCP (*Xenopus laevis*) and h-βTrCP (*Homo sapiens*) (Spevak *et al*., 1993; Jiang and Struhl, 1998; Margottin *et al*., 1998; Hatakeyama *et al*., 1999). Such prominent evolutionary conservation of FWD1 implies that it is biologically important. Recently, we demonstrated that FWD1 forms an SCF complex with Skp1 and Cul1 (referred to as SCF^{FWD1}), and that SCF^{FWD1} functions as a ubiquitin ligase for IκBα, which partially shares homology with β-catenin around the signal-induced phosphorylation site (Hatakeyama *et al*., 1999).

To determine whether FWD1 associated with β-catenin, Flag-tagged FWD1 with or without Myc-tagged Axin were transfected into 293T cells, and co-immunoprecipitation assays were performed (Figure 1A). Very little β-catenin was detected in the FWD1 immunoprecipitate, but the association of FWD1 and β-catenin was significantly augmented by the co-introduction of Axin, which interacts with β-catenin, GSK-3β and APC (Ikeda *et al*., 1998; Kishida *et al*., 1998) (Figure 1A, lanes 3 and 4). The slight association between FWD1 and β-catenin without the introduction of Axin was probably due to the presence of endogenous Axin. FWD1 also associated with Axin (Figure 1A, lane 4). The negative control, $p57^{Kip2}$, did not interact with β-catenin or Axin. This association of FWD1 with β-catenin and Axin was confirmed by reciprocal co-immunoprecipitation analysis with anti-β-catenin and anti-Myc (Axin) antibodies. FWD1 was detected in the immunoprecipitate with the anti-β-catenin antibody only when Axin was co-introduced (Figure 1B, lane 4). Axin was also present in this complex. Therefore, β-catenin and FWD1 did not interact efficiently in the absence of Axin. FWD1 and β-catenin co-immunoprecipitated with Axin (Figure 1B, lane 8), whereas β-catenin did not bind efficiently to Axin in the absence of FWD1 (Figure 1B, lanes 2 and 6). Taken together, these results indicate that FWD1, β-catenin and Axin form a ternary complex and that the lack of any component, at least *in vivo*, significantly reduces the stability of this complex. We used FWD2, another F-box/seven WD40-repeat protein from the mouse EST database which was previously identified as an F-box protein of unknown function, called MD6 (Bai *et al*., 1996) (DDBJ/EMBL/GenBank accession No. X54352), as a negative control. FWD2 did not interact with β-catenin even when Axin was introduced (Figure 1B, lanes 2 and 6). Another F-box protein, Skp2, was also subjected to this assay, but it failed to interact with β-catenin or Axin (data not shown). These data suggest that the association between FWD1 and β-catenin is specific, but is not mediated by the F-box motif.

These *in vivo* results were confirmed by *in vitro* binding experiments. Consistent with the *in vivo* studies, β-catenin and FWD1 did not interact each other in the absence of Axin *in vitro* (Figure 1C, lane 2). The amount of FWD1 associated with β-catenin increased in proportion to the Axin level (lanes 3–5), suggesting that FWD1 requires Axin to interact with β-catenin. In contrast to the *in vivo* data, however, β-catenin associated with Axin *in vitro* in the absence of FWD1 (lane 6). This discrepancy between *in vivo* and *in vitro* experiments may possibly be explained by the extra physiological amount of proteins in the *in vitro* setting which may have resulted in non-specific binding, or that β-catenin and Axin might be spatially separated *in vivo*, and FWD1 may link β-catenin to Axin as a docking molecule. Moreover, a phosphorylated synthetic peptide corresponding to the N-terminal region of β-catenin competitively inhibited the association of FWD1 with β-catenin *in vitro* without affecting the binding between Axin and β-catenin (see Figure 4C). This result indicates that FWD1 binds directly to β-catenin with the aid of Axin. Axin probably serves as a scaffold protein linking β-catenin and GSK-3β to phosphorylate β-catenin. FWD1 barely interacted with Axin in the absence of β-catenin *in vitro* (data not shown). Mutations on the phosphorylation sites in β-catenin abrogated the binding between FWD1 and β-catenin (see below). We conclude from this data that FWD1 seems to be in immediate contact with phosphorylated β-catenin.

In order to determine the region required for β-catenin/Axin complexation, the abilities of FWD1 mutants to form complexes with β-catenin/Axin were tested (Figure 2A). A mutant FWD1 lacking the WD40-repeats (ΔWD) did not associate with β-catenin or Axin, whereas the mutant FWD1(ΔF) from which the F-box domain had

been removed complexed with β-catenin and Axin (Figure 2B). In contrast, the FWD1(ΔWD) mutant interacted with Skp1, whereas the FWD(ΔF) mutant did not (Hatakeyama *et al*., 1999). These results confirm that the F-box domain is essential for binding to Skp1, but not to β-catenin/Axin. A deletion mutant lacking the N-terminal region and F-box domain (ΔN/F) failed to associate with β-catenin/Axin (data not shown; the results are summarized in Figure 2A), suggesting that both the N-terminal region and WD40-repeat domain of FWD1 are necessary for complex formation with β-catenin/Axin.

Previous studies and our unpublished data suggest that ubiquitin-mediated degradation of β-catenin is largely dependent on the phosphorylation of serine/threonine residues at positions 29, 33, 37, 41 and 45 (Aberle *et al*., 1997; Orford *et al*., 1997). In order to establish whether phosphorylation is important for complex formation, the association between FWD1 and SA mutant β-catenin, in which the all five serine/threonine residues were replaced with alanine, was examined. The SA mutant β-catenin associated with neither FWD1 nor Axin (Figure 2C). Thus, formation of the trimolecular complex is dependent on phosphorylation of the serine/threonine residues at positions 29, 33, 37, 41 and 45 of β-catenin by the GSK-3β/Axin complex. Furthermore, the association of FWD1 with β-catenin was blocked by a phosphorylated synthetic peptide corresponding to the N-terminal region of β-catenin, whereas an unphosphorylated peptide was unable to inhibit it (see Figure 4C). These results indicate that FWD1 binds exclusively to the phosphorylated β-catenin.

Interaction of FWD1 with GSK-3β and APC

In previous studies, we demonstrated that Axin interacted directly with GSK-3β and APC (Ikeda *et al*., 1998; Kishida *et al*., 1998). Therefore, we examined whether FWD1 formed a complex with GSK-3β and APC through Axin. Hemagglutinin (HA)-tagged GSK-3β expressed in 293T cells with Myc-tagged Axin and Flag-tagged FWD1 or FWD2 was immunoprecipitated with anti-HA antibody (Figure 3A). Axin and GSK-3β were co-immunoprecipitated together regardless of the presence of FWD1. The interaction between FWD1 and GSK-3β was probably mediated through Axin. It is noteworthy that GSK-3β bound to β-catenin only when FWD1 was co-transfected (Figure 3A, lane 2). Similarly, the FWD1 immunoprecipitate contained APC and Axin (Figure 3B, lane 3) and the Axin immunoprecipitate contained APC and FWD1 (Figure 3B, lane 6). The association between FWD1 and APC required Axin, because APC was not detected in the FWD1 immunoprecipitate when Axin was not co-transfected (Figure 3B, lane 1). In contrast, Axin and APC

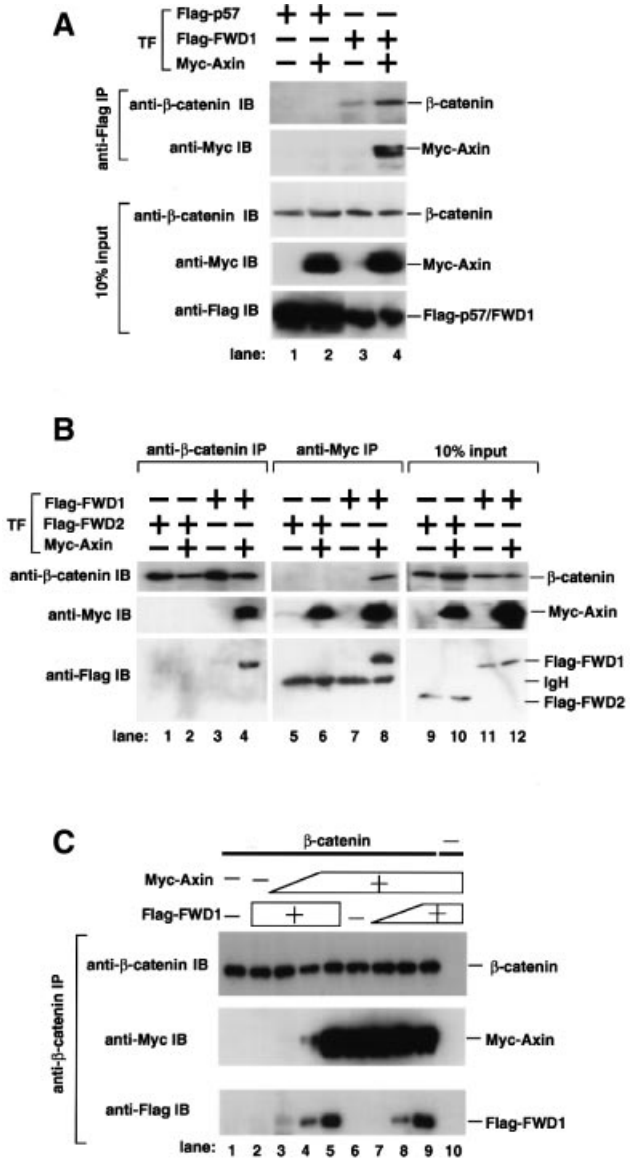

Fig. 1. Association of FWD1 with β-catenin and Axin *in vivo*. (**A**) The FWD1 immunoprecipitate contained β-catenin and Axin. Transfection of 293T cells was carried out with expression plasmids encoding Flag-p57 or Flag-FWD1 in combination with vector alone or Myc-Axin (indicated by the plus and minus signs at the top of each lane), the cell lysates were immunoprecipitated via the Flag-tag on p57 or FWD1, immunoblotted and probed with anti-β-catenin or anti-Myc antibodies to detect Axin. The bands represent β-catenin or Axin associated with p57 or FWD1. Ten percent of each input lysate was immunoblotted and probed with anti-β-catenin, anti-Myc or anti-Flag antibodies to show the expression levels of endogenous β-catenin, Myc-Axin and Flag-p57 or Flag-FWD1, respectively. The positions of β-catenin, Myc-Axin, Flag-p57 and Flag-FWD1 are indicated. The reasons why the amount of β-catenin as shown by the 10% input is not reduced when Axin and FWD1 are co-transfected are that transfected cells may be a small part of the total cells, and that only cytosolic β-catenin is degraded by FWD1/Axin while a substantial amount of membrane-associated β-catenin remains unaffected. (**B**) The β-catenin and Myc-Axin immunoprecipitates contained FWD1. Transfection of 293T cells were performed with expression plasmids encoding Flag-FWD1 or Flag-FWD2 in combination with vector alone or Myc-Axin (indicated by the plus and minus signs at the top of each lane), the cell lysates were immunoprecipitated with the β-catenin antibody (lanes 1–4) or via the Myc tag on Axin (lanes 5–8), immunoblotted and probed with anti-β-catenin antibody (top panels), anti-Myc antibody to detect Axin (middle panels) or anti-Flag antibody to detect FWD2 and FWD1 (bottom panels). Ten percent of each input lysate was immunoblotted and probed with anti-β-catenin, anti-Myc or anti-Flag antibodies to show the expression levels of endogenous β-catenin, Myc-Axin and Flag-FWD1 or Flag-FWD2, respectively (lanes 9–12). The positions of β-catenin, Myc-Axin, Flag-FWD1, and Flag-FWD2 are indicated. (**C**) *In vitro* binding assays. Recombinant β-catenin, Myc-Axin, and FWD1 produced in the baculoviral expression system were mixed in the combination as indicated. The reaction mixture was immunoprecipitated with anti-β-catenin antibody, immunoblotted and probed with anti-β-catenin (top), anti-Myc to detect Axin (middle) or anti-Flag antibody to detect FWD1 (bottom). TF, transfection; IP, immunoprecipitation; IB, immunoblotting.

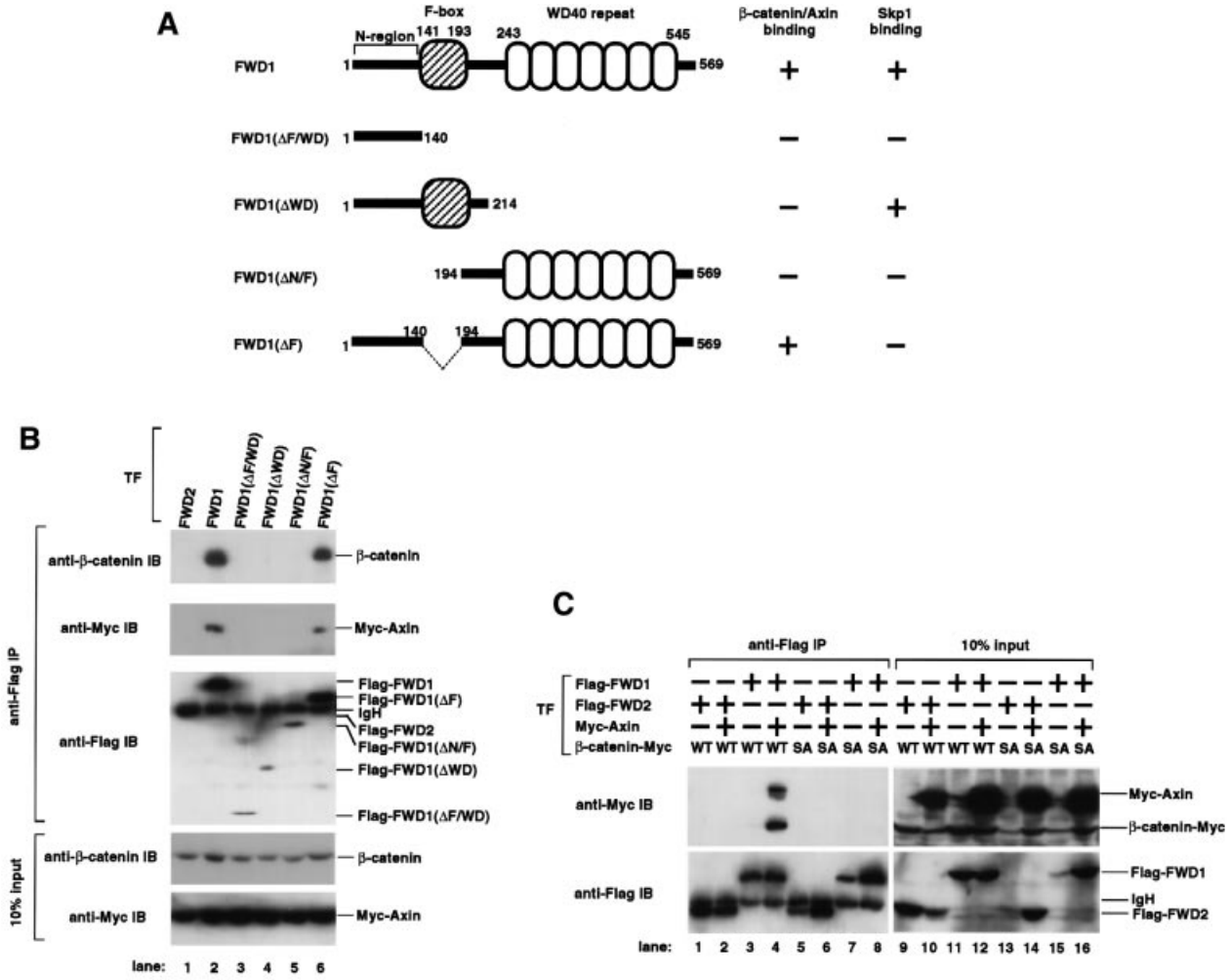

Fig. 2. Determination of the interacting regions of FWD1 and β-catenin. (**A**) Schematic representation of FWD1 deletion mutants. The hatched and white boxes indicate the F-box domain and WD40-repeats, respectively, and the interactions with β-catenin/Axin and Skp1 are summarized (indicated by plus and minus signs). (**B**) FWD1 deletion mutant expression and interactions with β-catenin and Axin. Wild-type FWD1/2 and all the deletion mutants were tagged with the Flag epitope at their N-termini and lysates of 293T cells expressing FWD2 (lane 1), wild-type FWD1 (lane 2), FWD1(ΔF/WD) (lane 3), FWD1(ΔWD) (lane 4), FWD1(ΔN/F) (lane 5), FWD1(ΔF) (lane 6), and Myc-Axin (all lanes) were immunoprecipitated with the anti-Flag antibody, immunoblotted and probed with anti-β-catenin, anti-Myc, or anti-Flag antibodies. Ten percent of each input lysate was immunoblotted and probed with the anti-β-catenin or anti-Myc antibody to show the expression levels of endogenous β-catenin and Myc-Axin, respectively. The positions of β-catenin, Myc-Axin and the Flag-FWD1 mutants are indicated. (**C**) FWD1 did not associate with SA mutant β-catenin. Transfection of 293T cells were carried out with expression plasmids encoding Myc-Axin, Flag-FWD2 and Flag-FWD1 (indicated by the plus and minus signs at the top of each lane) in combination with wild-type β-catenin-Myc (indicated as WT) or SA mutant β-catenin-Myc (indicated as SA). Cell lysates were immunoprecipitated via the Flag-tag on FWD2 or FWD1 (lanes 1–8), immunoblotted and probed with the anti-Myc antibody to detect β-catenin and Axin (upper) or with the anti-Flag antibody to detect FWD2 and FWD1 (lower). Ten percent of each input lysate was immunoblotted and probed with anti-Myc and anti-Flag antibodies to show the expression levels of β-catenin-Myc/Myc-Axin and Flag-FWD1/Flag-FWD2, respectively (lanes 9–16). The positions of β-catenin-Myc, Myc-Axin, Flag-FWD1 and Flag-FWD2 are indicated. TF, transfection; IP, immunoprecipitation; IB, immunoblotting.

formed a complex in the absence of FWD1 (Figure 3B, lane 5). Again, FWD2 interacted with neither GSK-3β nor APC, indicating that FWD1 associated specifically with GSK-3β and APC. Collectively, these data suggest that the Axin/GSK-3β/APC is complexed constitutively, whereas the interaction of β-catenin with Axin/GSK-3β/APC is dependent on FWD1.

Promotion of β-catenin ubiquitination by FWD1

If FWD1 is involved in β-catenin ubiquitination, overexpression of wild-type or mutant FWD1 may alter β-catenin ubiquitination. Treatment of cells with the proteasome inhibitors *N*-acetyl-Leu-Leu-norleucinal (LLnL) and lactacystin was reported to lead to the accumulation of ubiquitinated β-catenin (Aberle *et al*., 1997; Orford *et al*., 1997). We found that the introduction of FWD1 significantly enhanced the formation of multi-ubiquitinated β-catenin, whereas very little ubiquitinated β-catenin was detected in mock- (data not shown) and FWD2-transfectants when 293T cells were treated with LLnL or lactacystin (Figure 4A and B). In contrast to wild-type FWD1, the FWD1(ΔF) mutant suppressed β-catenin ubiquitination relative to the levels in the mock- (data not shown) and FWD2-transfectants, suggesting that FWD1(ΔF) functions as a dominant-negative mutant (Figure 4B). These data suggest that an increase in FWD1-induced ubiquitination is dependent on the association of FWD1 with the Skp1/Cullin/E2 complex through the

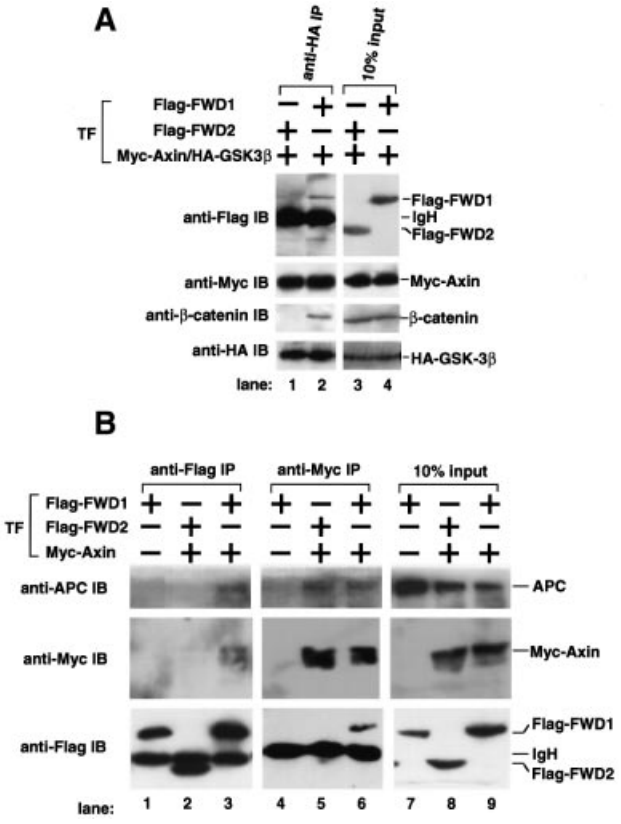

Fig. 3. Association of FWD1 with GSK-3β and APC *in vivo*. (**A**) The GSK-3β immunoprecipitate contained FWD1, β-catenin and Axin. Transfection of 293T cells were carried out with expression plasmids encoding Flag-FWD1 (lanes 2 and 4) or Flag-FWD2 (lanes 1 and 3) in combination with Myc-Axin and HA-GSK-3β (every lane), the cell lysates were immunoprecipitated via the HA-tag on GSK-3β (lanes 1 and 2), immunoblotted and probed with the anti-Flag antibody to detect FWD1 and FWD2, anti-Myc antidody to detect Axin or the anti-β-catenin or anti-HA antibody to detect GSK-3β. The bands represent FWD1, β-catenin or Axin associated with GSK-3β. Ten percent of each input lysate was immunoblotted and probed to show the expression levels of Flag-FWD1, Flag-FWD2, Myc-Axin, endogenous β-catenin and HA-GSK-3β (lanes 3 and 4). The positions of Flag-FWD1 and Flag-FWD2, Myc-Axin, β-catenin and HA-GSK-3β are indicated. (**B**) The FWD1 and Axin immunoprecipitates contained APC. Transfection of 293T cells were performed with expression plasmids encoding Flag-FWD1 or Flag-FWD2 in combination with vector alone or Myc-Axin (indicated by the plus and minus signs at the top of each lane), the cell lysates were immunoprecipitated with the anti-Flag antibody on FWD1 or FWD2 (lanes 1–3) or the anti-Myc tag on Axin (lanes 4–6), immunoblotted and probed with anti-APC antibody (top panels), anti-Myc antibody to detect Axin (middle panels) or anti-Flag antibody to detect FWD1 and FWD2 (bottom panels). Ten percent of each input lysate was immunoblotted to show the expression levels of endogenous β-catenin, Myc-Axin, Flag-FWD1 and Flag-FWD2 (lanes 7–9). The positions of β-catenin, Myc-Axin, Flag-FWD1 and Flag-FWD2 are indicated.
TF, transfection; IP, immunoprecipitation; IB, immunoblotting.

F-box domain and that FWD1 serves as an intracellular receptor in part of the SCF complex to attract phosphorylated β-catenin to the ubiquitination machinery.

This promotion of β-catenin ubiquitination by FWD1 *in vivo* was also confirmed in an *in vitro* ubiquitination assay (Figure 4C). The synthetic peptides corresponding to the N-terminal region of β-catenin (amino acids 24–42) were tested to inhibit the binding between β-catenin and FWD1, as well as β-catenin ubiquitination. We prepared the unphosphorylated peptide and the peptide phosphorylated on serine residues at positions 33 and 37. Addition of the phosphorylated peptide to the reaction inhibited the binding between FWD1 and β-catenin, as well as ubiquitination of β-catenin (Figure 4C, lanes 2 and 5). In contrast, the unphosphorylated peptide affected neither the binding nor ubiquitination (lanes 3 and 6), suggesting that FWD1 recognizes and ubiquitinates the phosphorylated β-catenin.

Facilitation of β-catenin degradation by FWD1

A pulse–chase experiment was performed to determine whether FWD1 affects the turnover rate of β-catenin (Figure 5). Introduction of Axin alone significantly accelerated the turnover rate of β-catenin, and the combination of FWD1 with Axin slightly enhanced the degradation. It was probable that endogenous FWD1 might be sufficient for the degradation when Axin was overexpressed, because introduction of the dominant-negative mutant FWD1(ΔF) into the cells significantly inhibited the degradation of β-catenin evoked by Axin. A similar result was obtained from the *in vitro* degradation assay in which cell lysates prepared from the human colon cancer cell line SW480, which lacks functional APC, resulting in abnormal accumulation of β-catenin, were incubated in degradation mixtures (data not shown). Acceleration of β-catenin degradation in response to Axin and FWD1 was demonstrated by carrying out an immunofluorescence assay (Figure 6). Introduction of the vector only (data not shown) or FWD2 into SW480 cells did not affect the β-catenin levels. In contrast, cytoplasmic β-catenin disappeared when Axin or FWD1 was introduced into SW480 cells, whereas the β-catenin levels of non-transfected control cells remained constant. Collectively, these data suggest that FWD1 controls ubiquitination-mediated β-catenin stability.

Discussion

The ubiquitin–proteasome system plays a critical role in the regulation of the levels of many regulatory proteins (Hershko and Ciechanover, 1992; Weissman, 1997; Elledge and Harper, 1998; Krek, 1998). A key issue in this field is how the target specificity is determined. Although ubiquitin ligases (E3s) are thought to play critical roles in the determination of substrate specificity, few E3s correlating with specific targets have been identified in higher eukaryotes. E6-AP and Mdm2 function as ubiquitin ligases which target p53 and result in proteolysis of the ubiquitinated product; however, they are not SCF-type ubiquitin ligases (Scheffner *et al*., 1993, 1995; Hatakeyama *et al*., 1997; Honda *et al*., 1997). The involvement of the SCF–E3 complex in substrate-specific ubiquitination in multicellular organisms has been largely unclear. Skp2, an F-box protein identified in mammals, was isolated as a cyclin A/Skp1-binding protein and it may link cyclin A to the Skp1/Cullin complex to execute ubiquitination and degradation of cyclin A (Zhang *et al*., 1995; Bai *et al*., 1996). Although recent reports implicated Skp2 in the ubiquitination and/or degradation of cyclin A (Lisztwan *et al*., 1998), no direct evidence demonstrating this has been presented.

In *Drosophila*, genetic evidence suggested that Slimb, a *Drosophila* homologue of FWD1, is functionally

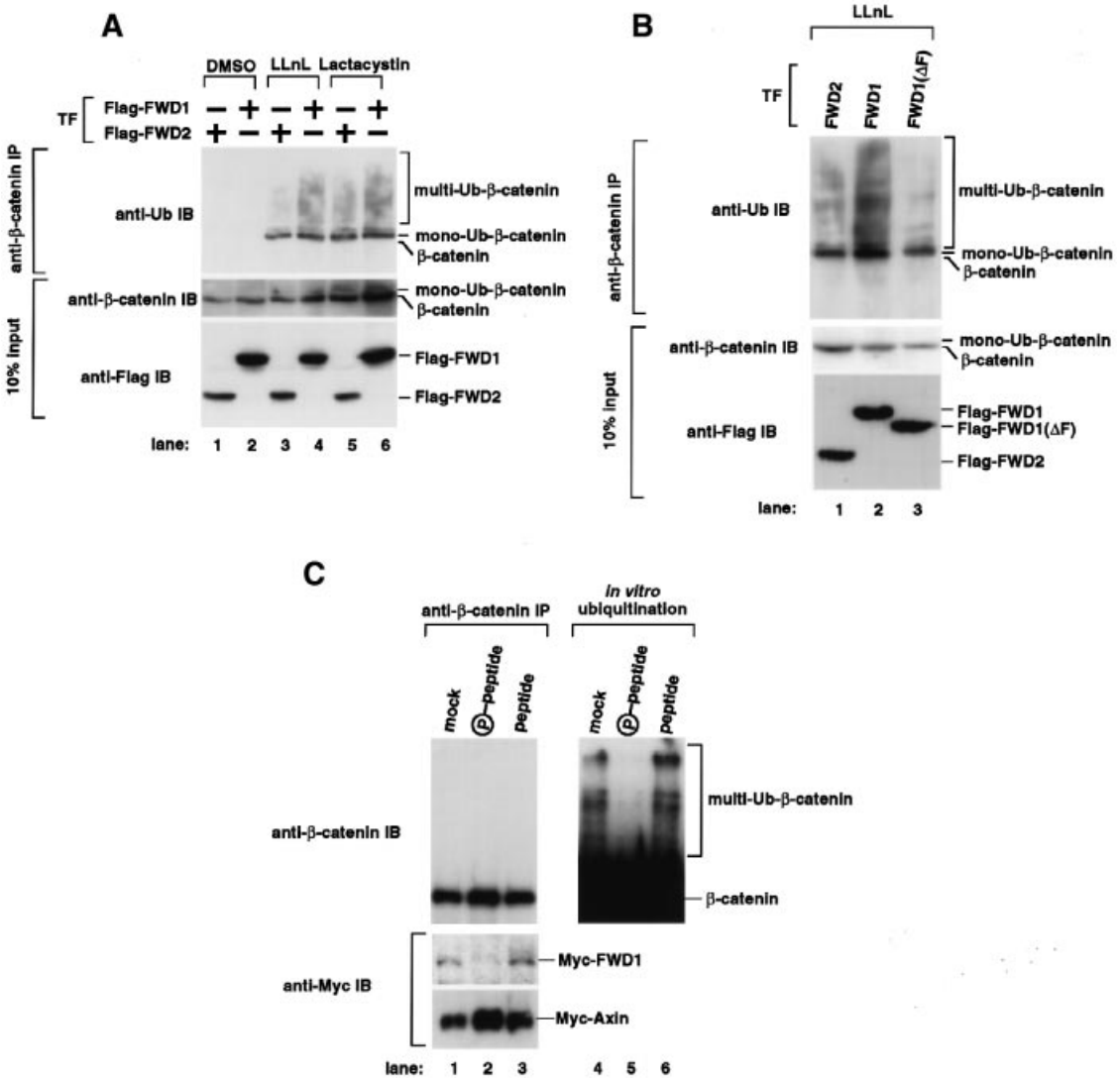

Fig. 4. Facilitation of β-catenin ubiquitination *in vivo*. (**A**) Promotion of β-catenin ubiquitination by FWD1. Transfection of 293T cells were performed with expression plasmids encoding Flag-FWD1 (lanes 2, 4 and 6) or Flag-FWD2 (lanes 1, 3 and 5), treated with control vehicle (dimethyl sulfoxide, DMSO; lanes 1 and 2), LLnL (lanes 3 and 4) or lactacystin (lanes 5 and 6), the cell lysates were immunoprecipitated with the anti-β-catenin antibody, immunoblotted and probed with the anti-ubiquitin antibody (top panel). Ten percent of each input lysate was immunoblotted and probed with the anti-β-catenin or anti-Flag antibody to show the expression levels of endogenous β-catenin, Flag-FWD1 and Flag-FWD2 (middle and bottom panels). (**B**) The F-box domain of FWD1 was required for β-catenin ubiquitination. Transfection of 293T cells were carried out with an expression plasmid encoding Flag-FWD2 (lane 1), Flag-FWD1 (lane 2) or Flag-FWD1(ΔF) (lane 3), the cell lysates were immunoprecipitated with the anti-β-catenin antibody, immunoblotted and probed with anti-ubiquitin antibody (top panel). Ten percent of each input lysate was immunoblotted and probed with anti-β-catenin or anti-Flag antibodies to show the expression levels of endogenous β-catenin, Flag-FWD1, Flag-FWD1(ΔF) and Flag-FWD2 (middle and bottom panels). (**C**) Phosphopeptide corresponding to the N-terminal region of β-catenin inhibit β-catenin ubiquitination *in vitro*. Recombinant β-catenin, Myc-Axin, and FWD1 produced in the baculoviral expression system were added in the ubiquitination mixture. No peptide (lanes 1 and 4), the phosphorylated peptide on Ser33 and Ser37 (lanes 2 and 5) or the unphosphorylated peptide (lanes 3 and 6) were added into the mixtures. The reaction mixture was immunoprecipitated with anti-β-catenin antibody, immunoblotted and probed with anti-β-catenin (left, top panel), anti-Myc antibody to detect FWD1 (left, middle panel) and Axin (left, bottom panel). On the other hand, the mixture was subjected to immunoblotting with the β-catenin antibody as a probe to show the multiple ubiquitin chain attached to β-catenin (right panel). TF, transfection; IP, immunoprecipitation; IB, immunoblotting.

involved in the degradation of Armadillo, a *Drosophila* homologue of β-catenin (Jiang and Struhl, 1998). However, this study did not show how Slimb controlled the expression level of Armadillo. Our study has provided a biochemical and molecular basis for the β-catenin degradation in mammals, and indicated that this system is highly evolutionarily conserved. Recently, we and others discovered that SCF^{FWD1} associates with and ubiquitinates IκBα (Yaron *et al*., 1998; Hatakeyama *et al*., 1999). Our data demonstrated that an F-box protein, FWD1, bound to β-catenin and IκBα in a phosphorylation-dependent manner and facilitated their degradation via the ubiquitin–proteasome pathway. Furthermore, a dominant-negative mutant form of FWD1 significantly inhibited the ubiquitination of β-catenin and IκBα. Taken together, our findings suggest that SCF^{FWD1} is a bona fide β-catenin/IκBα ubiquitin ligase. The possibility remains, however, that other E3s are also involved in β-catenin and IκBα ubiquitination. One approach to address this issue is to create *FWD1* gene-ablated mice, a project currently underway in our laboratory.

Previous studies on yeast suggested that F-box proteins

probably recognize multiple substrates. In *Saccharomyces cerevisiae*, the WD40-repeat containing the F-box protein Cdc4 was shown to interact with Sic1 (Feldman *et al*., 1997; Skowyra *et al*., 1997), Far1 (Henchoz *et al*., 1997) and Cdc6 (Piatti *et al*., 1996; Drury *et al*., 1997). Similarly, Pop1^{+} in *Schizosaccharomyces pombe* is required for ubiquitination of both Rum1 and Cdc18 (Kominami and Toda, 1997). Therefore, it is not surprising that SCF^{FWD1} also serves as a ubiquitin ligase for β-catenin and IκBα. This is supported by the structural evidence that β-catenin and IκBα share a similar amino acid sequence, the DSGXXS motif, at their phosphorylation sites for ubiquitination and degradation (Aberle *et al*., 1997; Orford *et al*., 1997). We propose that the DSGXXS motif is a consensus sequence for binding to the intracellular receptor FWD1 when the serine residues in this motif are phosphorylated. Mutations in this motif significantly affect the interactions of FWD1 with β-catenin and IκBα. Furthermore, h-βTrCP, a human homologue of FWD1, interacts with the DSGXXS motif in the human immunodeficiency virus (HIV)-1 protein Vpu (Margottin *et al*., 1998). The apparently multiple targets of FWD1 raise the question: what distinguishes these substrates? Possible candidates conferring specificity on the targets are the kinases that phosphorylate each substrate. β-catenin, IκBα and Vpu are phosphorylated by the GSK-3β/Axin (Ikeda *et al*., 1998), IκB kinases (reviewed by Ghosh *et al*., 1998) and casein kinase II (Schubert and Strebel, 1994; Paul and Jabbar, 1997), respectively. Phosphorylated DSGXXS motifs on these substrates may be the signals for the common ubiquitination pathway through FWD1. Target specificity during protein ubiquitination may also be determined by combinatorial interactions among components of ubiqui-

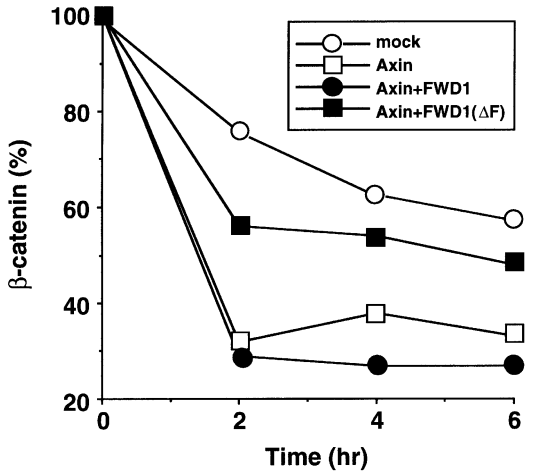

Fig. 5. Involvement of FWD1 in the β-catenin degradation. Pulse–chase analysis of the turnover rate of β-catenin radiolabeled with [^{35}S]methionine/cysteine in 293T cells which were transfected with expression plasmids alone (mock; denoted by open circles), that encoding Axin (open squares), Axin and FWD1 (closed circles) or Axin and FWD1(ΔF) (closed squares), in combination with the Myc-tagged β-catenin. Cell lysates were immunoprecipitated via a Myc tag on β-catenin, then subjected to SDS–PAGE and autoradiographed. Quantification of the autoradiogram was performed by BAS-2000, and the amount of β-catenin at 0 h was defined as 100%.

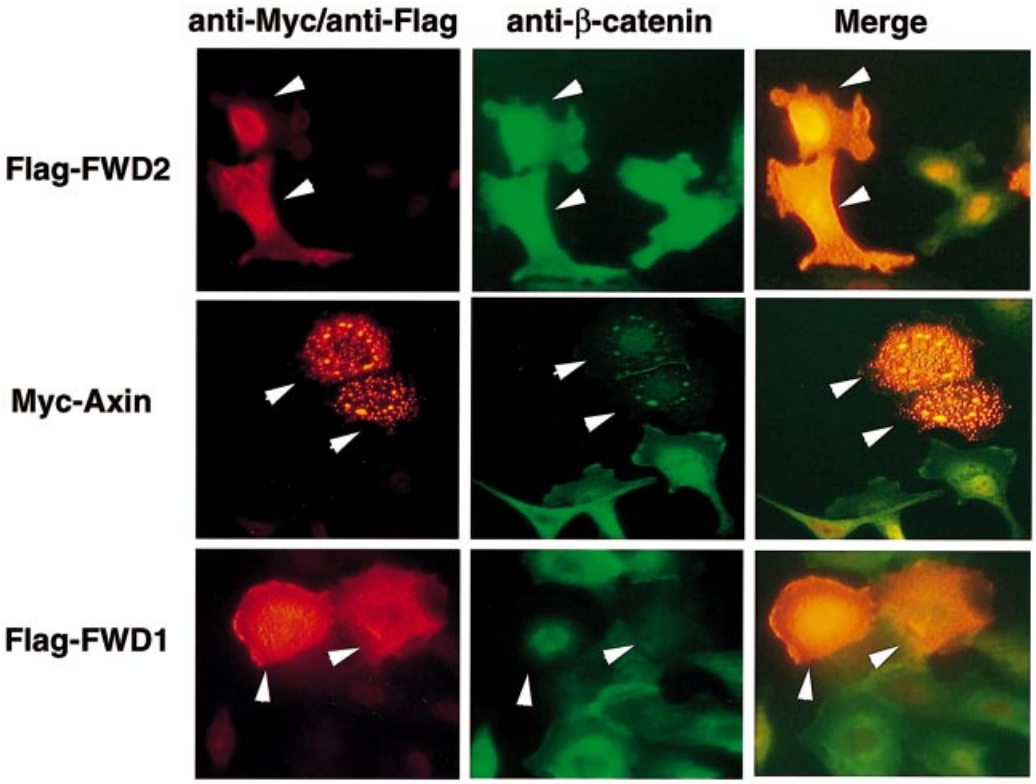

Fig. 6. Facilitation of cytoplasmic β-catenin degradation by FWD1 *in vivo*. SW480 cells were transfected with expression plasmids encoding Flag-FWD2 (top panels), Myc-Axin (middle panels) or Flag-FWD1 (bottom panels) and 48 h later, the cells were fixed and stained with anti-Myc or anti-Flag antibodies to identify the transfected cells (left panels) and with anti-β-catenin antibody (middle panels). Overlaid photographs are shown (right panels). The arrowheads indicate transfected cells. Note that cells expressing Axin or FWD1 show low β-catenin expression levels, whereas β-catenin was abundant in the cytoplasm of the cells expressing FWD2 and the non-transfectants (data not shown).

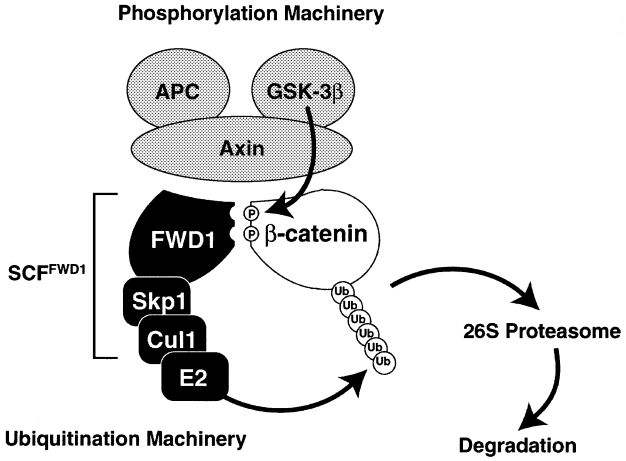

Fig. 7. Model of regulation of β-catenin degradation. Axin/GSK-3β/APC appears to be constitutively complexed to form a kinase complex for β-catenin (shown in gray). Axin, FWD1 and β-catenin form a trimolecular complex and all three components seem to be necessary for the association. FWD1 recruits the Skp1/Cul1/E2 ubiquitination apparatus (shown in black) to the complex, leading to multi-ubiquitination of phosphorylated β-catenin, the multi-ubiquitinated β-catenin is recognized by 26S proteasome and degraded.

tin-conjugating enzymes (E2s) and ubiquitin ligases (E3s). Fifteen E2-related genes have been found in *S.cerevisiae* and five cullins have been identified in mammals (Cul1–5) (Kipreos *et al*., 1996; Elledge and Harper, 1998). Moreover, several dozen F-box-containing proteins have been identified in several species, and many of them associate with Skp1 (Bai *et al*., 1996). Combinatorial interactions among these components may generate a large number of SCFs. It is possible that the SCF^{FWD1} for IκBα and β-catenin use different E2s and/or cullins; this remains to be examined.

Another important finding is that FWD1 also associated with the Axin/GSK-3β/APC complex, suggesting that FWD1 directly links the phosphorylation machinery to the ubiquitination apparatus (Figure 7). These results led us to the general hypothesis that phosphorylation and ubiquitination are efficiently coupled to each other through F-box proteins, ensuring prompt and efficient proteolysis in response to external signals. As FWD1 is involved in β-catenin instability, it is highly likely that loss of FWD1 function results in cancer. Therefore, FWD1 may be an oncosuppressor molecule, alterations of which may be found in cancer patients. Another potential medical application of FWD1 is gene therapy involving the introduction of FWD1 into colorectal cancers using an adenoviral vector, as described previously (Shibata *et al*., 1997).

Materials and methods

Cell culture

SW480 and 293T cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Rockville, MD) supplemented with 10% v/v fetal calf serum (Gibco-BRL) at 37°C in an atmosphere of air containing 5% CO_2.

Construction of expression plasmids and mutagenesis

A search of a mouse EST database yielded a mouse clone (DDBJ/EMBL/GenBank accession No. W61438) that showed significant homology with *Drosophila* Slimb. Sequencing revealed that this clone includes the full-length of mouse Slimb/FWD1 cDNA. The nucleotide sequences encoding mouse FWD1, mouse Skp1, mouse Cul1 and mouse Skp2 have been deposited in DDBJ/EMBL/GenBank under accession Nos AF081887, AF083214, AF083216 and AF083215, respectively.

N-terminal Flag-tagged FWD1 and deletion mutants of FWD1 were generated by PCR using high-fidelity thermostable DNA polymerase KOD (Toyobo, Tokyo, Japan), sequenced and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). pcDNA3-Flag-FWD1, -FWD1(ΔF/WD), -FWD1(ΔWD) and -FWD1(ΔNF) contain the cDNA encoding residues 1–569, 1–140, 1–214 and 194–569, respectively, and pcDNA3-Flag-FWD1(ΔF) contains the cDNA encoding residues 1–140 fused to 194–569.

FWD2 (DDBJ/EMBL/GenBank accession No. X54352) was isolated from the EST database and Flag-tagged FWD2 was generated by the PCR using KOD polymerase (Toyobo), sequenced and subcloned into pCIneo (Promega, Madison, WI).

The pBSSK/β-catenin containing full-length β-catenin cDNA in the pBlueScript II SK^+ vector (Stratagene, La Jolla, CA) and pUC19/EF1-α/β-catenin (SA) containing full-length β-catenin cDNA mutated by replacing Ser29, Ser33, Ser37, Thr41 and Ser45 residues with Ala (gifts from Dr A.Nagafuchi, with modification) were digested with *Kpn*I and *Eco*RI and subcloned into the *Kpn*I and *Eco*RI sites of pcDNA3.1/Myc-His A. The pEF-Bos-Myc/rAxin and pCGN/HA-GSK-3β were generated as described previously (Mizushima and Nagata, 1990; Ikeda *et al*., 1998) and pBSSK/β-catenin, pEF-Bos-Myc/rAxin and pcDNA3-Flag-FWD1 were digested with both *Kpn*I and *Xho*I, *Eco*RI and both *Eco*RI and *Xho*I, respectively, and subcloned into the *Kpn*I and *Xho*I, *Eco*RI and *Eco*RI and *Xho*I sites, respectively, of pBacPAK9 (Clontech, Palo Alto, CA).

Transfection, immunoprecipitation and immunoblot analysis

Using the calcium phosphate method (Wigler *et al*., 1977) or by lipofection with the LipofectAMINE reagent (Gibco-BRL), 293T cells were transfected according to the manufacturer's instructions. The cells were lysed with a buffer comprising 50 mM Tris–HCl (pH 7.6), 300 mM NaCl, 0.5% Triton-X 100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM iodoacetamide, 1 mM phenylmethyl sulfonylfluoride, 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF and 10 mM sodium pyrophosphate after incubation for 48 h. In order to detect multi-ubiquitinated β-catenin, the cells were treated with a proteasome inhibitor, 10 μM LLnL (Roche, Basel, Switzerland) or 30 μM lactacystin (Kyowa Medics, Tokyo, Japan), for a further 8 h after incubation for 48 h without the inhibitors. The cell lysates were pre-cleared with 50 μl protein G–Sepharose beads (Pharmacia, Uppsala, Sweden) for 1 h at 4°C and then incubated with 5 μg required antibody and protein G–Sepharose beads for 4 h at 4°C. The resulting immunoprecipitates were washed thoroughly four times with cold lysis buffer, separated by SDS–PAGE, immunoblotted with the following primary antibodies (1 μg/ml): anti-Myc (9E10; Roche Diagnostics K.K., Tokyo, Japan), anti-Flag (M5; Sigma, St Louis, MO), anti-HA (HA.11/16B12; Babco, Richmond, CA), anti-β-catenin (SC-1496; Santa Cruz, Santa Cruz, CA), anti-APC (Ab-1; Calbiochem, Cambridge, MA) or anti-ubiquitin (1B3; MBL, Nagoya, Japan), followed by a horseradish peroxidase-conjugated anti-mouse (Promega) or anti-goat IgG polyclonal antibody (Southern Biotechnology Associates, Birmingham, AL) and visualized using an enhanced chemiluminescence system (ECL; Amersham, Buckinghamshire, UK).

Baculovirus expression system

cDNAs for β-catenin-His and Myc-Axin-His were introduced in pBacPAK9 (Clontech) and transfected into Sf9 cells to obtain recombinant baculoviruses. Recombinant proteins with His-tag were prepared using Probond kit (Promega). cDNAs for Myc-FWD1 was introduced in pBacPAK9 as glutathione *S*-transferase (GST)–fusion forms. Recombinant GST–Myc-FWD1 was purified using glutathione beads and then treated with PreScission protease (Pharmacia) to obtain Myc-FWD1 protein.

In vitro ubiquitination and binding assays

Phosphorylated and unphosphorylated peptides encoding the N-terminal phosphorylation site of β-catenin, HWQQQSYLDS(PO_4H)GIHS(PO_4H)-GATTT and HWQQQSYLDSGIHSGATTT, were chemically synthesized to use in the following ubiquitination and binding assays as competitors. Purified β-catenin-His, Myc-Axin-His, Myc-FWD1, GSK-3β (New England Biolabs Inc., Beverly, MA) and S100 fraction of NIH 3T3 cell extracts were incubated with or without the peptides in ubiquitination mixture (50 mM Tris–HCl pH 8.3, 2 mM dithiothreitol, 5 mM $MgCl_2$, 2.5 mM ATP, 1 mM phosphocreatine, 500 U/ml phosphocreatine kinase, 4 mM LLnL, 25 μg/ml ubiquitin aldehyde, 1.9 mg/ml ubiquitin and 10 μg/ml protease inhibitors, 2 μM okadaic acid) at 37°C for 45 min.

Ten percent of the reaction mixtures were subjected to SDS–8% polyacrylamide gel electrophoresis then immunoblotted and probed with monoclonal anti-β-catenin antibody (Transduction Laboratories, Lexington, KY) to detect ubiquitinated β-catenin. In order to detect binding of β-catenin to Myc-FWD1 and Myc-Axin, the residual reaction mixtures were immunoprecipitated with polyclonal anti-β-catenin (Santa Cruz). The immunoprecipitates were subjected to SDS–8% polyacrylamide gel electrophoresis then immunoblotted and probed with monoclonal anti-β-catenin Ab or anti-Myc 9E10.

Pulse–chase experiments

Transfected 293T cells were metabolically labeled with Trans^{35}S (ICN Pharmaceuticals Inc., Costa Mesa, CA) at a concentration of 100 μCi/ml for 1 h and chased. Cell lysates were immunoprecipitated with anti-Myc antibody (9E10) followed by purification with protein G–Sepharose (Pharmacia), separated on SDS–PAGE, exposed and quantified by BAS-2000 (Fuji Film, Kanagawa, Japan).

Immunofluorescence staining

SW480 cells were grown on glass cover slips in growth medium, transfected using the calcium phosphate method (Wigler *et al*., 1977) and prepared as described elsewhere (Hatakeyama *et al*., 1997). Briefly, the cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and incubated with anti-Myc, anti-Flag or anti-β-catenin antibodies (1 μg/ml in PBS containing 0.1% bovine serum albumin and 0.1% saponin) for 1 h at room temperature and then with Cy3-conjugated anti-mouse or Cy2-conjugated anti-goat IgG (both Amersham), diluted 1:500, for 1 h at room temperature. Finally, the cells were covered with a drop of GEL/MOUNT™ (Biomeda Corp., Foster City, CA), viewed using a Nikon Eclipse E800M microscope and photographed with a color-chilled 3CCD camera C5810 (Hamamatsu Photoniques, Hamamatsu, Japan).

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