Adenomatous Polyposis Coli Is Down-regulated by the Ubiquitin-Proteasome Pathway in a Process Facilitated by Axin*

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The adenomatous polyposis coli (APC)† gene encodes a large (~300-kDa) multidomain protein that has crucial roles in regulation of the Wnt signaling pathway. Germ line mutations of APC lead to an inherited form of colon cancer known as familial adenomatous polyposis coli, while inactivation of APC is an early event in the development of many sporadic tumors of the colon (Refs. 1 and 2 and references therein).

Developmental defects in mouse embryos carrying mutations in Axin and ectopic expression of Axin in Xenopus embryos have shown that Axin plays an essential role in vertebrate axis formation by modulating Wnt signaling (3, 4). Identification of mutations in Axin in diverse human cancer patients suggests that Axin is a tumor suppressor (5–7). Both the developmental defects and the tumorigenic effects are due to the lack of a central function of Axin, the down-regulation of β-catenin, which is a key mediator of canonical Wnt signaling (Refs. 8 and 9 and references therein). Axin forms a complex by directly binding to many proteins involved in the Wnt signaling pathway, such as APC, Dvl, low-density lipoprotein receptor-related proteins 5 and 6, GSK3β, and β-catenin (10–12). In the absence of a Wnt signal, cytoplasmic β-catenin is down-regulated by the Axin complex. Axin promotes phosphorylation of β-catenin by GSK3β, and the phosphorylated form of β-catenin is then recognized by β-TrCP, an E3 ubiquitin ligase, and destroyed by a ubiquitination-proteasome-dependent pathway. However, in the presence of a Wnt signal, the phosphorylation of β-catenin is blocked, allowing it to escape destruction and accumulate in the cytoplasm. Stabilized cytoplasmic β-catenin is translocated into nuclei and interacts with high mobility group box transcription factors of the Tcf/LEF family, leading to induction of specific target genes such as c-myc or cyclin D1. Uncontrolled expression of these cell cycle-related genes is generally considered an important mechanism for tumorigenesis due to over-activation of a Wnt signal (Refs. 9 and 13 and references therein).

Cancer cells carrying mutations in the APC gene have high levels of cytoplasmic β-catenin (14). Since APC can form a complex with β-catenin and exogenous expression of APC reduces the cytoplasmic level of β-catenin, APC is considered a negative regulator of the Wnt pathway (15). While the mechanism for the down-regulation of β-catenin by Axin is well understood as described above (10, 16), the biochemical mechanism for down-regulation of β-catenin by APC is unclear. However, recent findings suggesting a role of APC in shuttling β-catenin from the nucleus to the cytoplasm or vice versa may provide another explanation for the tumorigenic effects of mutations in APC (17, 18). Although it is still controversial whether APC contains real nuclear export sequences, it has been claimed that truncation of APC abolishes specific nuclear export sequences, which blocks the APC-dependent export of β-catenin from the nucleus, resulting in constitutive activation of downstream target genes, which leads to tumor formation (19).

The levels of key regulatory proteins that are involved in cell cycle regulation (cyclins, Sic1, p27, etc.), Wnt signaling (β-catenin), and NFκB signaling (IkBα) are controlled in part by ubiquitination-proteasome-mediated degradation (Refs. 20 and 21 and references therein). In a series of enzymatic reactions, free ubiquitin is first activated by linkage between activating enzyme E1 in an ATP-dependent manner, and the activated ubiquitin is then transferred to ubiquitin-conjugating enzyme (E2). Finally E2 transfers ubiquitin to a ubiquitin ligase (E3),...
which catalyzes linkage of ubiquitin to a lysine residue of the target protein. Repeated cycles of this process result in formation of a polyubiquitinated target protein, which is recognized by the 26 S proteasome and rapidly degraded into short peptides. It has been shown that the levels of several proteins in the regulation of Wnt signaling pathway, such as Axin, APC, and β-catenin, are controlled at a post-transcriptional level. However, it is not known whether the ubiquitination-proteasome-mediated pathway controls the level of APC or Axin.

In the studies reported here, we show that APC is downregulated by a direct ubiquitination-proteasome-mediated pathway and that Wnt signaling can inhibit the process. The APC, stabilized upon Wnt3a treatment, accumulates in nuclei. We identify the domain of APC responsible for this process. Our data also suggest an unexpected role for Axin, facilitation of the ubiquitination-proteasome-mediated destruction of APC through the oligomerization of Axin. These findings suggest a novel mechanism for the regulation of the protein level of APC upon Wnt3a treatment and a cross-regulation between APC and Axin.

Experimental Procedures

Construction of Plasmids—Full-length Xenopus VSVG epitope-tagged APC and Myc-epitope tagged Axin and deletion constructs and PC2SMT-6TP have been described previously (10). Myc-epitope-tagged human APC constructs and several deletion constructs (human APC2, hAPC21, and hAPC25) were kindly provided by Dr. P. Polakis (22). hAPC (1–733), hAPC (1–1038), hAPC (331–1337), hAPC (733–1337), and hAPC (960–1337) were constructed by using routine molecular biology techniques. Myc epitope was tagged to the N terminus of all constructs, PCMV-HA Ub expression construct was a gift from Dr. D. Bohmann (23). HA epitope-tagged human GSK3β/kinase-dead human GSK3β and FLAG epitope-tagged mouse β-catenin were obtained from J. Woodgett (Ontario Cancer Institute, Toronto, Canada) and Dr. J. Kitajewski (Columbia University), respectively. Site-directed mutants were introduced by standard PCR techniques using Pfu DNA polymerase (Stratagene). The reading frame of all constructs and introduction of site-directed mutations were confirmed by sequencing and detection of expected sized bands in Western blot. Plasmids for transfection were isolated using the midigprep kit (Qiagen Inc., Valencia, CA).

Cell Culture and Transfection—Human kidney epithelial cell line 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc.) and gentamicin. For transfection experiments, each plasmid DNA was applied by the calcium phosphate precipitation method. The amount of DNA in each transfection was kept constant by each plasmid DNA was applied by the calcium phosphate precipitation method.

Pulse-chase Analysis—MyHC-tagged hAPC and HA-ubiquitin-expressing plasmids were transiently transfected into 293T cells in 60-mm dishes. At 16 h after transfection, cells were starved for 60 min in methionine- and cysteine-free medium and then labeled for 1 h with 0.1 mg/ml of [35S]methionine and [35S]cysteine (PerkinElmer Life Sciences). The labeling medium was replaced with cold medium, and cells were incubated for 30 min, 2, or 4 h. Cells were lysed by radioimmuno precipitation assay buffer supplemented with protease inhibitors at the indicated times. The lysates were subjected to anti-Myc immunoprecipitation and analyzed by SDS-PAGE and autoradiography.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from control and Wnt3a-CM treated 293T cells. After treatment for 24 h, medium was replaced with the serum-free DMEM. Cells were harvested 0, 2, 4, and 8 h and after changing the medium and processed using a modified protocol according to Dennler et al. (24). Briefly, cells were harvested at 4°C in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μM leupeptin, aprotime, and pepstatin) on ice for 10 min to swell. Subsequently, the cells were lysed by 30 strokes of a Dounce all glass homogenizer. Nuclei were pelleted by brief centrifugation and resuspended in 150 μl of cold buffer C (1% NP-40, 1% bovine serum albumin in phosphate-buffered saline), 420 mM NaCl, and 20% glycerol. The nuclear membrane was ruptured by 15 strokes of a Dounce all glass homogenizer, and the lysates were incubated on ice for 60 min with tapping. The nuclear lysates were aliquoted and frozen at −80 °C.

Immunofluorescence Staining—Cells (1.5 × 105) were plated in four chamber slides and allowed to achieve about 60% confluency. Cells were treated with Wnt3a-conditioned or control medium for 24 h, fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min, and subsequently permeabilized and blocked with buffer A at room temperature for 15 min. The subsequent incubation was carried out with buffer A, and all washes were carried out in phosphate-buffered saline at room temperature. Cells were incubated with anti-APC monoclonal antibody (1:200, Santa Cruz Biotechnology, Inc.) for 1 h at 37 °C and then incubated with rhodamine-conjugated horse anti-rabbit immunoglobulin (1:200, Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 1 h at 37 °C in the dark. After extensive washing, the slides were mounted in a drop of Aqua-Poly/Mount (Polyscience Inc., Warrington, PA) to reduce photobleaching. Signal was visualized by epifluorescence using a confocal laser scanning microscopy (MRC1024, Bio-Rad).

Results

The Level of APC Is Up-regulated by Wnt3a—A previous study demonstrated that the expression of Wnt-1 resulted in an increased steady-state level of APC protein as well as an increase in the amount of β-catenin and suggested that APC might be regulated by Wnt-1 at the post-translational level in a fashion similar to β-catenin (25). In the present study, we first investigated the effect of Wnt3a on the level of endogenous APC protein. The level of endogenous APC was increased by 2.13 ± 0.15-fold in 293T cells treated with Wnt3a-conditioned medium.
medium compared with cells treated with control medium. In 293T cells transfected transiently with Myc-tagged human APC plasmid and treated with Wnt3a-CM, the level of exogenous APC protein was increased by 1.86 ± 0.10-fold compared with control (Fig. 1A). In contrast to APC, the level of endogenous Axin was decreased by treatment with Wnt3a-CM (Fig. 1A, 1.72 ± 0.11-fold reduction) as shown previously (26, 27). Treatment with Wnt3a-CM or co-transfection of APC did not change the level of GSK3β, and the level of GSK3β was used as a control for even loading in subsequent experiments (Fig. 1A). Quantitative real time PCR analysis showed that the level of endogenous APC mRNA was unchanged, while Axin2, which is known to be a target gene by Wnt/β-catenin signaling (28–30), was enhanced by treatment with Wnt3a-CM (Fig. 1B, 0.995 ±

FIG. 1. The level of APC protein, but not mRNA, is enhanced by Wnt3a. A, endogenous APC and transfected Myc-tagged human APC levels in 293T cells grown in control L-CM or Wnt3a-CM were analyzed by Western blot. In A and B, the treatment of L-CM and Wnt3a-CM is indicated by - and +, respectively. Wnt3a treatment enhanced the levels of both endogenous (top panel, lanes 1 and 2) and transfected APC (top panel, lanes 3 and 4) for Myc-tagged full-length human APC (Myc-hAPC) and lanes 5 and 6 for VSV-tagged full-length Xenopus APC (VSV-xAPC). Treatment with Wnt3a-CM reduced the level of endogenous Axin protein (middle panel). Endogenous GSK3β was used as a loading control (bottom panel). B, total RNAs from 293T cells either treated with control L-CM or Wnt3a-CM for 24 h were purified, and real time PCR was performed for the analysis of APC, Axin2, or β-actin (left panel). The normalized levels of Axin2 and APC mRNAs based on the level of β-actin mRNA show that the level of APC is unchanged, while Axin2 is induced with the treatment of Wnt3a-CM (right panel). C, endogenous APC levels in 293T cells transfected with empty vector, human GSK3β, kinase-dead human GSK3β, and Dvl or treated with either 20 mM NaCl or LiCl for 12 h were analyzed by Western blot. Induction of cytoplasmic β-catenin showed that LiCl worked as expected, and endogenous α-tubulin was used as a loading control (lanes 5 and 6, middle and bottom panels). The endogenous level of APC was not changed by any of the treatments. WT, wild type; KD, kinase-dead; ENDO, endogenous.
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0.10 for APC and 1.83 ± 0.22 for Axin 2). This suggests that Wnt3a may modulate the level of APC at a post-transcriptional level. Ectopic expression of Dvl1, GSK3β, or kinase-dead GSK3β or treatment with the specific GSK3β inhibitor LiCl did not cause any changes at the level of APC in 293T cells (Fig. 1C). These results suggest that the regulation of APC by Wnt may use mechanisms other than the well studied Wnt-Dvl-GSK3β pathway.

Down-regulation of APC Is Mediated by the Ubiquitin-Proteasome Pathway—The increased level of APC upon Wnt3a treatment led us to suspect that APC, like β-catenin, may be down-regulated by the ubiquitination-proteasome pathway in the absence of Wnt signaling. To test this possibility, HA-tagged ubiquitin was transiently expressed in 293T cells along with either Myc-tagged full-length hAPC or VSV-tagged full-length Xenopus APC (xAPC). Interestingly the protein level of hAPC (Fig. 2A) and xAPC (data not shown) was dramatically decreased (about 4-fold) by co-transfection with ubiquitin, whereas the level of transfected GFP protein was unchanged. The peptides ALLN (calpain inhibitor I) and MG132 (benzoyloxycarbonyl-Leu-Leu-aldehyde) have been widely used to inhibit the 26 S proteasome (31, 32). ALLM (calpain inhibitor II) is structurally related to ALLN but does not inhibit the proteasome (33). The reduction of hAPC level by co-transfection of ubiquitin was partially blocked by the treatment with ALLN or MG132 (Fig. 2B). However, treatment with ALLM or the protease inhibitor leupeptin did not block ubiquitin-mediated down-regulation of APC (Fig. 2B, lanes 2 and 5). Increasing the amount of the transfected ubiquitin further reduced the protein levels of hAPC and xAPC in the presence of ALLM, while this reduction was blocked by treatment with ALLN (Fig. 2C and data not shown for xAPC). However, the protein level of transfected GFP was unaffected by the amount of transfected ubiquitin (data not shown). The down-regulation of hAPC was blocked by as little as 1 h of pretreatment with ALLN, and the extent of blocking was enhanced by 2 h of ALLN pretreatment (Fig. 2D). 35S]Methionine metabolic labeling and pulse-chase experiments, which showed that the addition of ALLN stabilized APC protein considerably compared with the cells treated with ALLM, further confirmed that the proteasome is involved in the down-regulation of hAPC (Fig. 2E). From these data, we conclude that the ubiquitination-proteasome-mediated pathway can regulate the protein level of APC.

Because the APC level is down-regulated by co-transfection of ubiquitin but enhanced by Wnt3a-CM treatment, we wondered whether the effect of Wnt3a was due to an inhibition of the ubiquitin-mediated down-regulation of APC. To test this idea, 293T cells, co-transfected with hAPC and ubiquitin, were treated with Wnt3a-CM immediately after transfection. As shown in Fig. 2F, this treatment significantly blocked ubiquitin-mediated down-regulation of APC (1.2-fold reduction instead of 4-fold), whereas control conditioned medium had no effect.

To determine whether Wnt3a signaling blocks the ubiquitination process on hAPC, the following experiments were performed. 293T cells were co-transfected with Myc-tagged full-length hAPC and HA-tagged ubiquitin, and lysates from the transfected cells were subjected to anti-Myc immunoprecipitation followed by immunoblotting using anti-HA antibody. Smeared high molecular weight forms of APC protein and enhancement of the intensity of the signal when the cells were treated with proteasome inhibitor ALLN were detected (Fig. 2G, lanes 1 and 2; further study for direct ubiquitination on APC is described below (Fig. 4)). These results imply that hAPC is directly ubiquitinated, and the enhanced signal upon ALLN treatment might be due to blocking of proteasomal degradation, which causes an increased level of ubiquitinated APC. The signal below the intact transfected APC might originate from the incomplete blocking of APC degradation by ALLN (Fig. 2G, **). Most importantly, Wnt3a treatment caused a reduction of the signal for the smeared high molecular weight forms of APC protein (Fig. 2G, *). This suggests that Wnt signaling inhibits the process of direct ubiquitination on APC (Fig. 2G, compare lanes 2 and 3). Overall our data suggest that the level of APC protein is regulated by a ubiquitin-proteasome-mediated pathway and that Wnt signaling causes inhibition of APC ubiquitination, leading to an increase in the APC level.

Identification of the Domain Responsible for the Down-regulation of APC—APC is a large (~300-kDa) protein with diverse domains and motifs responsible for interactions with other proteins (Fig. 3A). The N-terminal region of APC is known to be involved in the homo-oligomerization of APC (34). APC contains seven repeated armadillo motifs, which show homology to the Drosophila β-catenin homolog armadillo. These armadillo motifs and a basic motif interact with the kinesin superfamily (KIF) proteins and microtubules, respectively, suggesting a role of APC in the regulation of cytoskeleton (Ref. 9 and references therein). Three 15-amino acids repeats and seven 20-amino acids repeats are domains for interaction with β-catenin (35, 36). Most APC mutations associated with tumorigenesis cause truncation of five of seven 20-amino acid repeats, suggesting that the 20-amino acid repeats confer the ability to down-regulate β-catenin (Ref. 37 and references therein). The C-terminal end of APC includes a domain that interacts with the homolog of Drosophila discs large protein, which is a known tumor suppressor (38).

To identify the domain of APC responsible for its down-regulation, several hAPC mutant constructs were co-transfected in 293T cells together with HA-tagged ubiquitin. Initially the available three mutant forms of hAPC were used (22). Full-length hAPC and the mutant forms hAPC2 and hAPC21 were clearly down-regulated by co-transfection with ubiquitin, while mutant APC25 was not (Fig. 3A). Comparison of all four constructs suggested that amino acids 1034–1337 of APC might be responsible for its down-regulation by ubiquitin. Consistent with this idea, ubiquitination-mediated down-regulation of APC was completely blocked in mutants missing this region (Fig. 3B). In contrast, two mutants lacking the known APC oligomerization domain (34) remained sensitive to down-regulation by ubiquitin, suggesting that the oligomerization domain of APC is not involved in this process (Fig. 3B). hAPC (733–1337), which is missing most of the armadillo repeat domain (2), and hAPC (960–1337) were still down-regulated (Fig. 3B). There was no effect of ubiquitin on the protein level of GFP co-transfected with each of the APC constructs, showing that the reduction in the levels of certain APC constructs is not due to differential efficiency of transfection (Fig. 3B). Overall the experimental data with deletion constructs suggest that the region of APC including amino acids 1034–1337 is necessary for the ubiquitination-proteasome-mediated down-regulation of APC.

Direct Ubiquitination on APC—To determine whether APC is directly ubiquitinated, the same approach described above was used (Fig. 2G). Immunoprecipitation with anti-Myc antibody and subsequent immunoblotting with anti-HA antibody using lysates from 293T cells that were co-transfected with Myc-tagged full-length hAPC and HA-tagged ubiquitin showed smeared high molecular weight bands, whose intensity was enhanced by proteasome-specific inhibitor (Fig. 4, lanes 3 and 4 of hAPC- and hAPC2-transfected samples). These results suggest that APC is directly ubiquitinated (Figs. 2G and 4). APC
FIG. 2. Ubiquitin-proteasome-mediated down-regulation of APC is regulated by Wnt3a. A, Myc-tagged hAPC- or enhanced GFP-expressing plasmids were co-transfected with HA-tagged ubiquitin (Ub) into 293T cells, and lysates were obtained 36 h later. Western blot was performed using anti-Myc antibody. GSK3β was used as a loading control. B, proteasome-specific inhibitors block down-regulation of APC. 293T cells transfected with hAPC- and ubiquitin-encoding plasmids were treated with either 50 μM ALLM or ALLN, 30 μM MG132, or 10 μM leupeptin for 4 h, and Western analysis was performed. AM, ALLM; AN, ALLN; MG, MG-132; Leu, leupeptin. C, increased amount of HA-ubiquitin further down-regulated hAPC, and that process was blocked by ALLN (50 μM, 4 h). hAPC (2 μg) was co-transfected with either 0, 1, 2, or 4 μg of ubiquitin into 293T cells. These cells were treated with either 50 μM ALLM or ALLN for 4 h before harvest of the lysates. D, the blocking of APC down-regulation was steadily enhanced upon increased time of ALLN treatment before harvest of the cells. hAPC (2 μg) and ubiquitin (4 μg) were co-transfected into 293T cells, which were treated with 50 μM ALLN for 0, 1, and 2 h before harvest. The levels of transfected hAPC were detected using anti-Myc antibody. E, pulse-chase analysis of hAPC. 293T cells, transfected with Myc-tagged hAPC and ubiquitin, were labeled with [35S]Met in the presence of either 50 μM ALLM or ALLN followed by a chase of 2 or 4 h. The autoradiographic signal was quantified, and the values relative to the value at time 0 were plotted as a function of chase time. F, 293T cells co-transfected with hAPC and ubiquitin were treated with either control L-CM or Wnt3a-CM immediately after transfection. The treatment of Wnt3a-CM significantly blocked ubiquitin-mediated down-regulation of APC, whereas L-CM had no effect. In F and G, the treatment of L-CM and Wnt3a-CM is indicated by − and +, respectively. G, HA-tagged ubiquitin and
mutants hAPC2 and hAPC25, which lack the region responsible for the ubiquitination-proteasome-mediated down-regulation of the hAPC, were compared to show the specificity of smeared high molecular forms of APC (Fig. 4). The mutant hAPC2 showed the accumulation of similar smeared high molecular mass species, while the ubiquitin-conjugated protein was absent in the mutant hAPC25 (Fig. 4). To further confirm that direct ubiquitination occurs on amino acids 1034–1337 of Myc-tagged hAPC were co-transfected into 293T cells, and these cells were treated with ALLM, ALLN, or ALLN/Wnt3a-CM as indicated. Cell lysates were immunoprecipitated (IP) with anti-Myc antibody, and the resulting immunoprecipitates were subjected to immunoblot (IB) analysis with antibodies to HA (top panel) or to Myc (bottom panel) as indicated. The arrowhead shows the locations of ubiquitinated protein from transfected APC construct. The detection of smeared high molecular weight forms of APC (marked with * on the right side) was further enhanced by co-incubation with ALLN (lanes 1 and 2), and the enhanced signal was blocked by Wnt3a CM treatment (lanes 2 and 3). The signal below the intact APC might originate from the incomplete blocking of APC degradation by ALLN (marked with ** on the right side). TFed, transfected.

**FIG. 3.** The region of APC including amino acids 1034–1337 is responsible for the down-regulation of APC. A, Myc-tagged full-length hAPC (hAPC FL) or its deletion mutants, hAPC2, -21, and -25, were transiently co-transfected with empty vector or HA-ubiquitin (Ub). The Western blots were subjected to densitometric analysis. APC proteins from all constructs except hAPC25, which does not have the amino acid region 1034–1337, were severely down-regulated by co-transfection of ubiquitin. Top, schematic representation of hAPC and its deletion mutants, hAPC2, -21, and -25; the box marked with * indicates the potential domain responsible for the down-regulation of APC. B, hAPC21 and its deletion mutants were transiently transfected with HA-ubiquitin in 293T cells, and lysates were analyzed by Western blotting. All constructs besides hAPC-(1–1038) and hAPC-(1–733) showed clear reduction of protein levels by co-transfection of ubiquitin. Left, schematic diagram for hAPC21 or its deletion mutants, hAPC-(1–1038), hAPC-(1–733), hAPC-(331–1337), hAPC-(733–1337), and hAPC-(960–1337). The box marked with * indicates the domain responsible for the down-regulation of APC. a.a., amino acid; Oligom, oligomer; Arm., armadillo; Dlg, disc large protein; wt, wild type.
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Fig. 4. Direct ubiquitination on APC. HA-tagged ubiquitin and the indicated constructs (hAPC, hAPC2, hAPC25, or hAPC(960–1337)) were transfected into 293T cells, and these cells were treated with ALLM or ALLN as indicated. The box marked with an asterisk indicates the domain responsible for the down-regulation of APC. Cell lysates were immunoprecipitated (IP) with anti-Myc antibody, and the resulting immunoprecipitates were subjected to immunoblot (IB) analysis with antibodies to HA (top panel) or to Myc (bottom panel) as indicated. Arrowheads show the locations of ubiquitinated protein from transfected APC constructs. The detection of smeared high molecular weight forms of APC was further enhanced by co-incubation with ALLN (lanes 3 and 4 in all top panels). Ub, ubiquitin.

APC, which is the region responsible for down-regulation, a new deletion construct, hAPC-(960–1337), was tested. hAPC-(960–1337) showed the same pattern as full-length hAPC and hAPC2. Collectively these results confirm that the region including amino acids 1034–1337 is responsible for down-regulation through a process mediated by direct ubiquitination of APC on amino acids 960–1337.

Axin Facilitates the Ubiquitination-mediated Down-regulation of APC—Upon treatment of cells with Wnt3a-conditioned medium, the protein level of Axin is reduced, while the level of hAPC is increased (Fig. 1A and Refs. 25 and 27). Since Axin and APC are components of the same protein complex, we hypothesized that Axin may enhance the down-regulation of APC in the absence of Wnt signaling, whereas the reduction of Axin protein upon Wnt signaling could cause an increase in the level of APC. Axin appears not to be essential for the ubiquitin-proteasome-mediated down-regulation of APC for the following two reasons. First, hAPC21, which lacks the known Axin binding sites, was down-regulated by co-transfection of ubiquitin (Fig. 3) (39–41), and second, dhAPC2 was down-regulated by co-transfection of ubiquitin in a hepatocellular carcinoma cell line, SNU475 (ATCC CRL2236), which does not express Axin (Ref. 5 and data not shown). Nevertheless, Axin could still contribute to the down-regulation of APC. To address this question, we performed a series of co-transfection experiments (Fig. 5). The hAPC2, a deleted form of hAPC that contains both the domains responsible for ubiquitination and Axin binding, was co-transfected with HA-tagged ubiquitin and Myc-tagged Axin in 293T cells. Consistent with data presented above, the hAPC2 level was severely reduced upon co-transfection with ubiquitin (Fig. 5A, lanes 1 and 3). Co-transfection of hAPC2 with Axin alone had no effect on the level of hAPC2 (Fig. 5A, lanes 1 and 2). However, co-transfection of hAPC2, ubiquitin, and Axin further down-regulated the level of hAPC2 (Figs. 5A and 5D, lanes 3 and 4; 3.3 ± 0.84-fold reduction). On the other hand, the mutant hAPC21, which includes the domain responsible for ubiquitination but not the Axin-binding sites, was not further down-regulated by the co-transfection of Axin (Fig. 5B, lanes 3 and 4). These results suggest that Axin may facilitate the ubiquitin-mediated degradation of APC through direct interaction.

To further test the above idea, point mutations were introduced into the Axin binding sites of hAPC2 (hAPC2-AxBSm3 and hAPC2-AxBSm123; -SAMP- sequence was changed to -AALP-; Fig. 6). These mutated proteins, which cannot bind Axin (Refs. 39–41 and data not shown), were down-regulated by co-transfection of ubiquitin, consistent with the conclusion that Axin is not absolutely necessary for the ubiquitin-proteasome-mediated down-regulation of the APC (Figs. 6, B and C, lanes 1 and 3 in each construct). However, mutation of the Axin binding sites completely abolished the ability of Axin to enhance the down-regulation of hAPC2 by Axin (Fig. 6, B and C, lanes 3 and 4 in each construct). Overall these data suggest that Axin can facilitate the ubiquitin-mediated down-regulation of APC.

Facilitation of the Ubiquitin-Proteasome-mediated Down-regulation of APC by Axin Requires the DIX Domain—To further confirm that Axin can facilitate the ubiquitin-mediated down-regulation of APC, we next tested whether removal of the APC binding site on Axin (42, 43) resulted in loss of its ability to facilitate the down-regulation of APC. A mutant form of hAPC, hAPC2, was co-transfected with AxΔRGS, which lacks the APC binding domain (10). In contrast to our expectation, AxΔRGS enhanced the ubiquitin-mediated down-regulation of hAPC2 (Fig. 7B, lanes 3 and 4). It is known that Axin can dimerize through the C-terminal DIX domain (44–46). Since a high level of endogenous Axin is present in 293T cells in the absence of Wnt signaling (Fig. 1A), we thought that dimerization of AxΔRGS with endogenous Axin was one way to facilitate the ubiquitin-mediated down-regulation of hAPC2. To test that possibility, we used the deletion constructs Ax810ΔRGS and Ax672ΔRGS, which lack both the APC binding and DIX domains (Fig. 7A). Consistent with our expectation, co-transfection of these constructs failed to enhance the ubiquitin-mediated down-regulation of APC (Fig. 7, C and D, lanes 3 and 4). These results suggest that the oligomerization of Axin through the C-terminal DIX domain may be involved in facilitating the down-regulation of APC, although we still could not eliminate the possibilities for the interaction of other proteins with DIX domain.

Wnt3a Treatment Leads to Accumulation of APC in Nuclei—Since it is well known that the level of β-catenin is increased upon Wnt signaling, the induced level of APC protein upon Wnt signaling does not seem consistent with its role in ubiquitin-proteasome-mediated down-regulation of β-catenin. To further investigate the significance of APC induction upon Wnt3a treatment, we examined the subcellular distribution of the induced APC. After culturing 293T cells in Wnt3a-conditioned
or control medium for 24 h, localization of the endogenous APC proteins was measured by indirect immunofluorescence analysis with anti-APC antibody. The induced APC was highly localized in nuclei (Fig. 8A). Immunoblot analysis clearly showed that treatment with Wnt3a-CM caused induction of APC and $\beta$-catenin in nuclear lysates, while the level of the nuclear protein lamin A was not changed (Fig. 8B). The accumulated nuclear APC increases until 4 h after removal of Wnt3a-CM and then decreases at 8 h similar to $\beta$-catenin (Fig. 8C, lanes 2, 4, and 8). Although the role of APC is controversial, it has been proposed that nucleocytoplasmic shuttling of APC can regulate $\beta$-catenin signaling (18, 19, 47). Co-immunoprecipitation analysis with nuclear extract showed that the accumulated APC and $\beta$-catenin can form a complex in the nucleus (Fig. 8D). Our findings showing a similar pattern of localization of APC and $\beta$-catenin upon Wnt signaling or after removal of Wnt and interaction between APC and $\beta$-catenin in the nuclear extract support the idea that APC has a role in shuttling $\beta$-catenin in and out of the nucleus.

**DISCUSSION**

Studies of the regulation of canonical Wnt signaling have been focused on identifying mechanisms that regulate the level of $\beta$-catenin. A tremendous amount of work by several groups has focused on the mechanisms for the down-regulation of $\beta$-catenin in the Axin complex, which includes APC, GSK3$\beta$, $\beta$-catenin, etc. (Refs. 8 and 9 and references therein). However, the mechanisms that regulate the levels of other components in the complex, such as Axin and APC, upon Wnt signaling have been little examined. Here we present evidence that APC is down-regulated by the ubiquitination-proteasome-mediated pathway and that Wnt signaling blocks that process. We found that the stabilized APC upon Wnt signaling is highly localized in nuclei and decreased upon removal of the Wnt signaling. The domain of APC that is responsible for its down-regulation and direct ubiquitination was identified. In addition to the well known role of Axin in down-regulation of $\beta$-catenin, we provide preliminary data showing that Axin can facilitate the ubiquitin-mediated down-regulation of APC.
Since induction of the protein level of APC by Wnt was shown in C57mg cells (25), the increased level of APC in Wnt-treated 293T cells was not a cell line-specific phenomenon. The observation that Wnt signaling increases the level of both \( \beta \)-catenin and APC, which is a negative regulator of \( \beta \)-catenin, appears to be contradictory. However, when we consider APC as a vector that shuttles \( \beta \)-catenin in and out of the nucleus (47–49), we can envisage the following model. Upon transient Wnt signaling, the accumulated \( \beta \)-catenin is translocated into nuclei and transiently induces downstream target genes. Subsequently the stabilized APC enters into nuclei and exports \( \beta \)-catenin, which forms a negative feedback loop for the precise control of the Wnt signaling. Transcriptional control of \( Tcf \) (50), \( \beta \)-TrCP (51), naked cuticle (52), and Axin2 (28–30) are known to serve as negative feedback mechanisms for Wnt signaling. Therefore, the regulation of APC may represent yet another mechanism for negative feedback in the Wnt signaling pathway; however, this is the first suggested negative feedback mechanism operating at the post-transcriptional level.

The localization and level of cytoplasmic \( \beta \)-catenin and APC depend on the cell cycle and proliferate state (53, 54). Olmeda et al. (53) showed that both APC and \( \beta \)-catenin accumulate in nuclei during S and G2/M phases, and interestingly they found that alteration of APC and \( \beta \)-catenin interaction occurs during the cell cycle. They suggested that the unproductive APC-\( \beta \)-catenin complexes could compete with other APC interactions required for further progression of the cell cycle (53). Their speculation might explain the significance of the accumulation of both APC and \( \beta \)-catenin in nuclei upon Wnt3a treatment, which is known to enhance proliferation.

Phosphorylation of APC by GSK3 enhances its affinity for \( \beta \)-catenin and regulates \( \beta \)-catenin signaling (55, 56). However, overexpression of GSK3 or kinase-dead GSK3 or treatment with lithium did not change the level of endogenous APC protein (Fig. 1C). In addition, the region of APC phosphorylated by GSK3 and casein kinase I was not included in the domain that was identified as the ubiquitination domain in our study (Refs. 40 and 56 and Fig. 3B). Therefore, although the significance of phosphorylation on APC by other unknown kinases for its stabilization or nuclear localization has not been tested, our data suggest that Dvl and GSK3 may not be involved in the induction of the protein level of APC upon Wnt signaling. Currently we are trying to identify the kinase that may be involved in regulating the level of APC.

Since the level of endogenous APC protein is very low in 293T cells and good antibodies for immunoprecipitation of APC protein and ubiquitin were not available, ectopic transfection of epitope-tagged APC and ubiquitin was used to show the ubiquitin-proteasome-mediated down-regulation of APC (Fig. 1C). In addition, the region of APC phosphorylated by GSK3 and casein kinase I was not included in the domain that was identified as the ubiquitination domain in our study (Refs. 40 and 56 and Fig. 3B). Therefore, although the significance of phosphorylation on APC by other unknown kinases for its stabilization or nuclear localization has not been tested, our data suggest that Dvl and GSK3 may not be involved in the induction of the protein level of APC upon Wnt signaling. Currently we are trying to identify the kinase that may be involved in regulating the level of APC.

Since the level of endogenous APC protein is very low in 293T cells and good antibodies for immunoprecipitation of APC protein and ubiquitin were not available, ectopic transfection of epitope-tagged APC and ubiquitin was used to show the ubiquitin-proteasome-mediated down-regulation and direct ubiquitination on APC in our study. For example, we used ectopically transfected APC instead of analyzing endogenous APC to show the blocking of degradation by proteasome-specific inhibi-
itors (Fig. 2B). When we measured endogenous APC, it was difficult to reproducibly show clear induction by proteasome inhibitors (data not shown). This might be because the effect of proteasome-specific inhibitors ALLN and MG132 was incomplete at the dose that we used in our experiments. Higher doses showed severe toxicity, which resulted in cell death (data not shown). While the in vivo role of this mode of APC regulation remains to be established, the significance of our finding is that we have provided the first evidence for the ubiquitin-proteasome-mediated regulation of APC protein upon Wnt signaling.

The analysis of germ line APC mutations in human tumors has identified two hot spots, one at position 1061 and the other at 1309, which showed the highest frequency of mutation in colon cancers (37, 57). It has been proposed that the tumorigenic effects of these mutations are due to either deletion of β-catenin binding sites or a dominant effect of the N terminus of APC (37, 58). Interestingly the domain that we identified for the down-regulation of APC contains both hot spots (a region from 960 to 1337, Figs. 3 and 4). Furthermore position 1061 encodes Lys and 1309 is in between two lysine residues (59), which can be targets for ubiquitin conjugation. Since there are many lysine residues between amino acids 960 and 1337, identification of the ubiquitination site(s) was beyond the scope of our study. Instead of examining all potential ubiquitination sites, we tested whether mutation of lysine residues on hot spots can block ubiquitin-proteasome-mediated down-regulation of APC. However, we found that the modified hAPC2 protein (amino acids 1034–2130, Fig. 3A), which has mutations in two hot spots (Lys at 1061 replaced by Asn and Lys-Glu-Lys at 1307–1309 replaced by Asn-Glu-Asn) was still down-regulated by the co-transfection of ubiquitin, suggesting that at least these hot spots are not sites for ubiquitination (data not shown).

It was recently reported that several colon cancer cell lines with truncation mutations in the mutation cluster region have high levels of truncated APC in nuclei, which was attributed to lack of a nuclear export signal (19). However, an additional mechanism, suggested by our findings, is that truncation of
Regulation of APC by Ubiquitination and Axin

APC blocks down-regulation of APC and leads to accumulation of APC in nuclei. Therefore, the high β-catenin signaling in colon cancer cells might be due to retention of β-catenin in nuclei caused by both an increased level of truncated APC and the lack of a nuclear export signal.

The opposite responses of APC and Axin, which are in the same complex, to the same Wnt signal led us to test whether Axin may have a role in the regulation of APC. The facilitation of APC down-regulation by Axin may explain the simultaneous induction of APC and down-regulation of Axin. As shown in the experiments presented in Figures 5–7, Axin could facilitate ubiquitin-mediated down-regulation of APC. However, the level of Axin may have a role in the regulation of APC. The facilitation of APC down-regulation by Axin may change the focus of APC tumor biology from identification of molecules that control the level of APC and Axin to the regulation of APC itself. Further understanding of the mechanism remains unknown, these observations suggest that APC and Axin may cross-regulate each other's protein level. One possibility is that the transfected APC fragment might titrate out unknown molecules that inhibit the degradation of Axin.
Adenomatous Polyposis Coli Is Down-regulated by the Ubiquitin-Proteasome Pathway in a Process Facilitated by Axin

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