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Axin-independent phosphorylation of APC controls β-catenin signaling via cytoplasmic retention of β-catenin

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Abstract

It has been shown that accumulation of free β-catenin leads to mobility shift of adenomatous polyposis coli (APC) protein and that Axin facilitates this process. Here we show that the β-catenin-mediated mobility shift of APC is due to phosphorylation of two domains of APC by casein kinase 1ε/glycogen synthase kinase 3β and unknown kinase(s), respectively. Interestingly, our results suggest that this process does not require Axin. The phosphorylated APC showed higher affinity to β-catenin *in vivo*, and fragments of APC containing the phosphorylated domains can inhibit β-catenin/Tcf-mediated reporter activity regardless of their ability to reduce the level of β-catenin. From our data we propose a new role of APC: accumulation of excessive cytoplasmic β-catenin induces phosphorylation of APC and the phosphorylated APC retains β-catenin in cytoplasm to prevent excessive β-catenin signaling. The retained β-catenin in cytoplasm by APC may be down-regulated by Axin 2, which is induced by β-catenin/Tcf signaling.

Keywords: Wnt; β-Catenin; APC; Axin; Phosphorylation

Mis-regulation of canonical Wnt/β-catenin signaling leads to severe embryonic developmental defects and causes diverse human cancers [1]. The crucial point for the regulation of Wnt/β-catenin signaling is controlling the level of cytoplasmic and nuclear β-catenin. Accumulation of cytoplasmic and nuclear β-catenin due to the mutations in adenomatous polyposis coli (APC) protein and Axin in diverse human cancers suggests that both APC and Axin are tumor suppressors [2].

Axin serves as a scaffolding protein to bring β-catenin and GSK3β in close vicinity, which enhances phosphorylation of β-catenin by GSK3β. The phosphorylated β-catenin is recognized by an E3 ligase, β-TrCP, and down-regulated by the ubiquitin–proteasomal mediated process (Ref. [3]). Although the biochemical role of APC in the Axin complex for the down-regulation of β-catenin is not clear, recent structural analysis suggest that APC helps release of the phosphorylated β-catenin from the Axin complex for ubiquitin–proteasome mediated degradation and allows recycling of Axin for new Axin–β-catenin–GSK3β complexes [4].

Another major role of APC in the regulation of Wnt/β-catenin signaling is to shuttle β-catenin in and out of nuclei [5]. The nuclear β-catenin interacts with a transcription factor Tcf/LEF and enhances the expression of downstream target genes such as *c-myc* and *cyclin D1*. Continuing expression of these genes leads to cell cycle abnormalities that can be a main reason for initiation of tumors (Ref. [1]). While the exact mechanism to terminate expression of these target genes is barely understood, APC is considered one of the major players in this process. The nuclear APC interacts with β-catenin and facilitates the export of β-catenin out of nuclei [6]. The exported β-catenin can be down-regulated in the Axin/Axin 2 complex through the ubiquitin–proteasome mediated pathway, which may lead to terminating β-catenin signaling [7].

It has been shown that accumulation of the free form of β-catenin leads to stabilization of the β-catenin/APC

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complex via phosphorylation of APC by GSK3β, and that Axin facilitates the phosphorylation of APC, although the biological significance of these observations was not well characterized [8–10]. In this report, we re-examined the biochemical mechanism of APC phosphorylation in the presence of high level of β-catenin, and we suggest a biological explanation for this process.

Materials and methods

Plasmids. Myc epitope-tagged human APC constructs and several deletion constructs (hAPC2, hAPC21, and hAPC25) were provided by Dr. P. Polakis. hAPC 1–733, hAPC 1–1038, hAPC 331–1337, hAPC 733–1337, hAPC 960–1337, hAPC2 AxBsm 3, hAPC2 AxBsm 123, and RGS domain constructs (Axin 194–353) have been previously described [11]. hAPC 960–1260, EGFP-Axin, and EGFP-Axin 2 were constructed via routine molecular biological techniques. Wild-type and kinase dead for HA-human GSK3β were provided by Dr. J. Woodgett. Dr. A. Kikuchi provided both the wild-type and kinase-dead forms of pEGFP-hCKIε. HA-tagged mouse Axin, pSUPER-human Axin RNAi were provided by Dr. S.C. Lin [12].

Cell culture and transfection. Human kidney epithelial 293T cells and hepatoma SNU475 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Cambrex Bio Science Walkersville, Inc.), respectively. The 293T cells were transfected via calcium phosphate precipitation. In order to induce human β-catenin-HA (S37A) using a doxycyclin-inducible system [13], the 293T cells were treated with 10–50 ng of doxycyclin (Sigma). The transfections were conducted in SNU475 cells using WelFect-EX™ PLUS transfection reagent (WelGENE).

Immunoprecipitation and Western. Immunoprecipitation and Western blot analysis were conducted as previously described [11]. The antibodies used were as follows: anti-c-myc antibody (9E10, Oncogene Research Products), anti-HA, anti-APC (APC(C-20)) and anti-α-tubulin antibodies (Santa Cruz Biotechnology), anti-GSK3β antibody (Transduction Laboratories), anti-lamin A antibody (Cell Signaling Technology Inc.), anti-Axin polyclonal antibody (provided by Dr. S.C. Lin, [12]).

λ-Phosphatase treatment. Fifty microgram of total lysates were mixed in λ-Ppase Buffer (50 mM Tris–HCl (pH7.5), 0.1 mM Na_2EDTA, 5 mM dithiothreitol, 0.01% Brij 35, 2 mM $MnCl_2$) with 400 U of λ-Ppase (New England Biolabs, Inc.), and then the mixture was incubated for 30 min at 30 °C.

Luciferase assay. In order to determine the effects of the expression of hAPC deletion constructs on β-catenin/Tcf signaling, luciferase assays were conducted as described previously [7].

Immunofluorescent analysis. The 293T cells transfected with myc epitope-tagged APC deletion constructs were fixed and stained with anti-myc monoclonal antibody as previously described [13]. The nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) were incubated with secondary antibody and examined via fluorescence microscopy (LEICA).

Results

β-Catenin mediated phosphorylation of APC

To verify the interaction between APC and other proteins, which were identified by yeast two-hybrid screening using APC fragment as bait (Kim et al., manuscript in preparation), co-immunoprecipitation assay was performed and the interaction of β-catenin and APC was used as a positive control. Interestingly, a clear mobility shifted band of APC was observed when β-catenin was co-expressed (Supplementary Fig. 1A). It has been known that accumulation of free β-catenin promoted the association of GSK3β with APC and it led to phosphorylation of the 20 amino acid repeat region of APC [14]. More recently, it has been shown that Axin could facilitate the GSK3β- or CK1ε-dependent phosphorylation of APC [9,10].

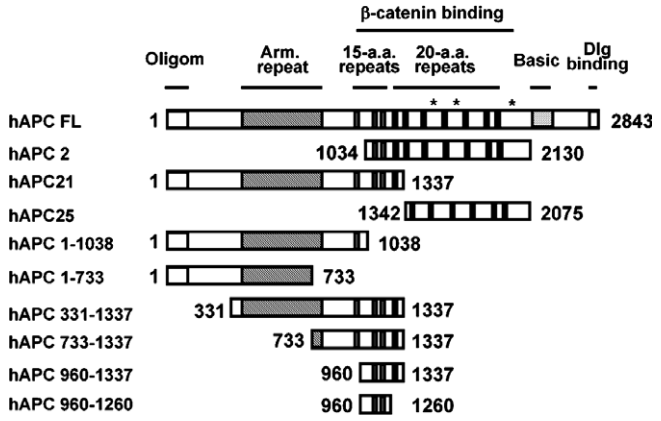

Fig. 1. A diagram of myc-tagged full-length human APC (hAPC FL) and other deleted versions of human APC, which were employed in this study.

However, the mobility shift of hAPC21, which does not have most of the 20 amino acid repeat region or the Axin-binding sites (Fig. 1 and Supplementary Fig. 2A), was somewhat unexpected [9,10]. Therefore, we re-examined the mechanism for the β-catenin mediated phosphorylation of APC. To show the mobility shift was due to phosphorylation, the lysate was incubated with lambda phosphatase, which clearly eliminated the mobility shift of APC while incubation of the lysate without lambda phosphatase did not (Supplementary Fig. 1B). These results imply that overexpression of β-catenin causes phosphorylation of APC.

The kinases responsible for the β-catenin-mediated phosphorylation of APC

To identify the domain of APC phosphorylated due to the accumulation of β-catenin, various APC deletion constructs were co-transfected with β-catenin (Fig. 1). As shown in Supplementary Fig. 2A, the mobility of all constructs containing the 20 amino acid repeat region was shifted, consistent with previous findings by others [9,10]. The hAPC21 (1–1337), which includes the 15 amino acid repeat region but only one 20 amino acid repeat at the C-terminal end also showed a mobility shift. While the constructs lacking both the 15 and 20 amino acid repeat regions did not show a mobility shift (hAPC 1–1038 and hAPC 1–733), a construct that has only this region (960–1337) showed the mobility shift (Supplementary Fig. 2B). The lack of induction of mobility shift in hAPC 960–1260 suggests that the region (1261–1337) contains the phosphorylation site (Supplementary Fig. 2B).

While it was reported that stabilization of β-catenin and Axin-dependent phosphorylation of APC can be mediated

by either GSK3β or casein kinase 1ε, there were inconsistencies between the results of different groups [9,10]. Therefore, we re-examined which kinases are responsible for the β-catenin mediated phosphorylation of APC. Despite high expression of GSK3β, the mobility shift of hAPC2 was not detected (Supplementary Fig. 3A). Co-transfection of casein kinase 1ε (CK1ε WT) causes a slight mobility shift (although far less pronounced than that induced by β-catenin), while a kinase-dead mutant of casein kinase 1ε (CK1ε KD) did not. Since phosphorylation of GSK3β often requires priming phosphorylation by other kinases, the possibility for priming phosphorylation by CK1ε was tested. When hAPC2 was co-transfected with GSK3β and CK1ε the mobility shift was obvious, although it was not exactly the same as that induced by β-catenin (Fig. 2A). Co-transfection of kinase-inactive mutants of GSK3β and CK1ε significantly reduced β-catenin mediated phosphorylation of hAPC2 (Fig. 2A, lanes 2 and 3). These results suggest that both GSK3β and CK1ε are involved in the β-catenin-mediated phosphorylation of APC *in vivo*. Since we identified two separated domains that can be phosphorylated, we tested whether both domains are phosphorylated by GSK3β and CK1ε. GSK3β and CK1ε caused mobility shift of hAPC25 and co-expression of kinase-dead mutants of GSK3β and CK1ε completely blocked the β-catenin-mediated phosphorylation of APC (Fig. 2B lanes 7 and 8). These results suggest that the region containing 20 amino acid repeats is phosphorylated by GSK3β and CK1ε (Supplementary Fig. 3B). However, the mobility shift of hAPC 960–1337 was not induced by GSK3β and CK1ε, and co-expression of kinase-inactive mutants of GSK3β and CK1ε could not block the β-catenin-mediated phosphorylation of APC (Fig. 2B lanes 3 and 4). These results imply that the region of hAPC 960–1337 is phosphorylated by kinase(s) other than GSK3β and CK1ε (Supplementary Fig. 3B).

Axin-independent phosphorylation of APC

As mentioned above, others reported that the β-catenin forms a complex with Axin and that Axin facilitates the phosphorylation of APC [9,10]. However, recent findings suggest that Axin may not be involved in the β-catenin-stimulated phosphorylation of APC (see Discussion). Therefore, we tested the requirement of Axin in the β-catenin-mediated phosphorylation of APC.

Consistent with previous finding [9], overexpression of Axin caused the mobility shift of APC (Supplementary Fig. 4A, arrow head). However, despite a reduction of endogenous Axin by siRNA specific for Axin [12], β-catenin-mediated mobility shift of APC was not affected (Supplementary Fig. 4B). Axin is known to interact with APC through SAMP sequences in the middle region of APC and introduction of mutations in these sites blocks the interaction between Axin and APC [10,11]. Mutants of hAPC2, which contain either mutations in individual or all three known Axin-binding sites, also showed clear mobility shifts when they are co-transfected with β-catenin (Supplementary Fig. 4C). It was reported that hepatic carcinoma cell line SNU475 does not express Axin due to mutation and our RT-PCR analysis data is consistent with that finding ([15], Supplementary Fig. 4D). Co-transfection of hAPC2 and β-catenin into SNU 475 cells caused clear mobility shift of hAPC2 and the mobility shifted APC band could be eliminated by treatment with lambda phosphatase (Supplementary Fig. 4E and F). These findings strongly suggest that Axin is not necessary in the β-catenin-mediated phosphorylation of

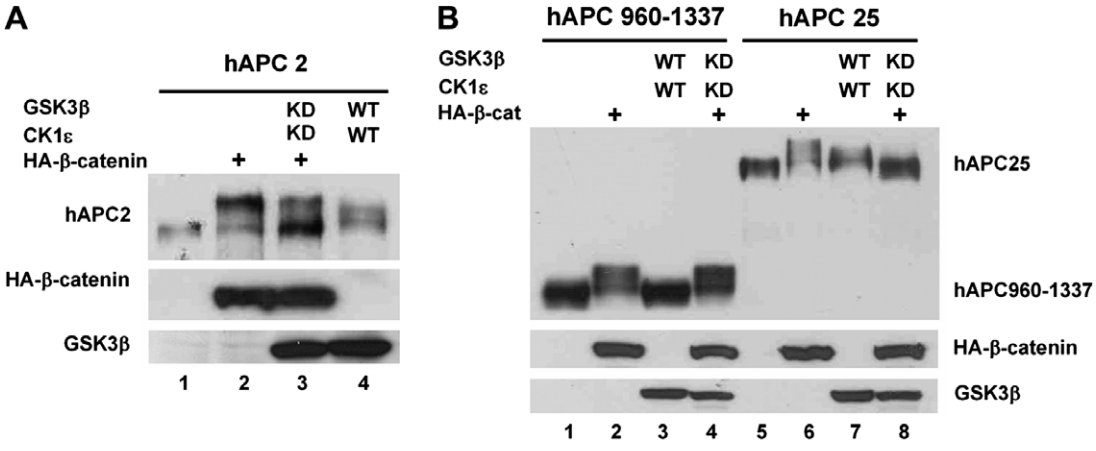

Fig. 2. Kinases responsible for the mobility shift of hAPC due to β-catenin accumulation. (A) The co-transfection of hAPC2 with β-catenin induced a mobility shift of hAPC2 (compare lanes 1 and 2). The intensity of the mobility-shifted band of hAPC2 was reduced by the co-transfection of the kinase-dead form of GSK3β and pEGFP-hCKIε (lane 3), and a similar mobility shift of hAPC2 was induced by the co-transfection of wild type GSK3β and pEGFP-hCKIε (lane 4) without β-catenin overexpression. (B) hAPC-(960–1337) or hAPC25 were co-transfected with plasmids, as shown in the figure, into 293T cells. While a similar mobility shift of hAPC25 was induced by the expression of wild-type GSK3β and pEGFP-hCKIε as the overexpression of β-catenin, no such mobility shift of hAPC-(960–1337) was induced (compare lanes 3 and 7). Whereas the ectopic expression of the kinase-dead variant of GSK3β and pEGFP-hCKIε had no effect on the β-catenin-mediated mobility shift of hAPC-(960–1337), the mobility shift of hAPC25 was clearly blocked (compare lanes 4 and 8). *WT*, wild-type; *KD*, kinase-dead.

APC. One possibility is that Axin 2 may substitute for the role of Axin in the absence of Axin [7]. To eliminate that possibility, the RGS fragment of Axin, which was known to block the interaction of both Axin and Axin 2 with APC [10], was co-expressed with β-catenin. As expected, overexpression of RGS clearly blocked the interaction between hAPC2 and Axin/Axin 2 (Fig. 3A). Interestingly, the Axin-mediated phosphorylation of APC was clearly eliminated by the overexpression of RGS (Fig. 3A, compare lanes 1 and 2 in lysates). However, we observed no effect on the β-catenin-mediated phosphorylation of APC despite high expression of RGS, suggesting that Axin and Axin 2 are not necessary in the β-catenin-mediated phosphorylation of APC (Fig. 3B). In addition, these data imply that Axin-mediated mobility shift (Supplementary Fig. 4A and Fig. 3A) and β-catenin-mediated phosphorylation of APC are executed as different ways. In summary, our data provide evidence that Axin is not necessary for the β-catenin-mediated phosphorylation of APC.

Retention of β-catenin in cytoplasm by phosphorylated APC leads to inhibition of β-catenin signaling

Phosphorylated APC is known to interact with β-catenin with higher affinity than the un-phosphorylated form *in vitro* [16]. Enrichment of the mobility-shifted band of APC in the immunoprecipitated complex with anti-HA antibody for ectopically expressed β-catenin suggests that phosphorylated form of APC interacts with β-catenin better *in vivo*, too (Supplementary Fig. 5, marked with arrowhead in lane 2).

To draw a clue as to the biological meaning of β-catenin-mediated phosphorylation of APC, the effects of several deleted forms of hAPC on Tcf/β-catenin signaling were tested. In the first experiment, either hAPC25 or hAPC 960–1337 was co-transfected with wild type β-catenin and reporter constructs, for the following reasons. First, hAPC25 is known to down-regulate β-catenin while hAPC 960–1337 does not [17]. Second, hAPC 960–1337 does not contain any known nuclear localization or export signals [18]. The main mechanisms for the APC mediated down-regulation of β-catenin/Tcf signaling are either facilitation of proteasomal degradation of β-catenin or blocking of Tcf and β-catenin interaction by APC. If co-transfection of hAPC 960–1337, which lacks both activities described above, blocked β-catenin/Tcf signaling, we might envision another role for APC. Interestingly, hAPC 960–1337 blocked β-catenin/Tcf mediated reporter activity even better than hAPC25 did (Fig. 4A).

To eliminate the possibility of β-catenin degradation by hAPC 960–1337, mutated β-catenin (β-catenin (S37A)), which is resistant to proteasomal degradation, was tested with APC deletion constructs. Even with the β-catenin (S37A), hAPCs could inhibit β-catenin/Tcf mediated reporter activity (Fig. 4B). All hAPC constructs that showed β-catenin-mediated mobility shift blocked reporter activity, while hAPC 1–1038 and 1–733, which did not show mobility shift, did not block (Fig. 4B). Indirect immunofluorescence experiments for transiently transfected hAPCs also showed that most of hAPCs are present in the cytoplasm (Supplementary Fig. 6).

From the results described above we propose a new role of APC. Accumulation of high levels of β-catenin by Wnt

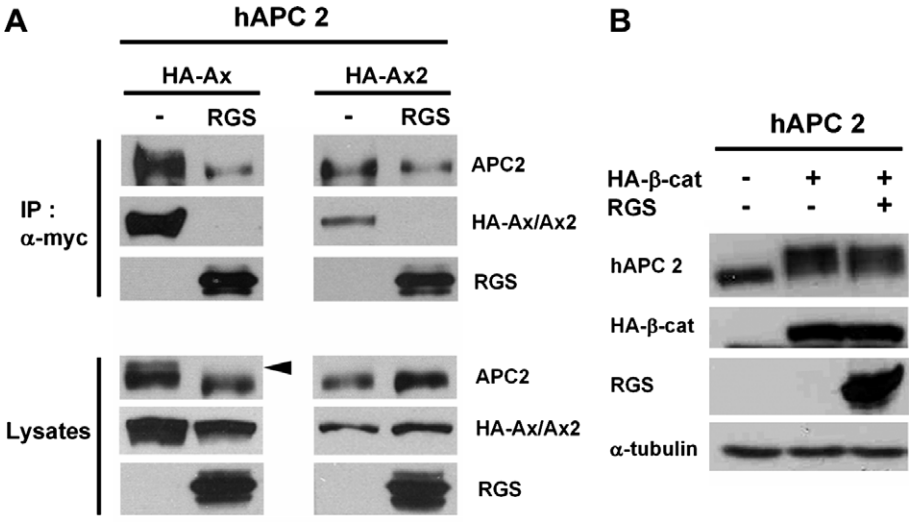

Fig. 3. Axin/Axin 2 independent phosphorylation of the hAPC *in vivo*. (A) hAPC2 and HA-tagged mouse Axin/Axin 2 plasmids were transfected into 293T cells with or without the plasmid expressing the RGS fragment of Axin, as shown in the figure. (Top panel) The cell lysates were immunoprecipitated using anti-myc monoclonal antibody, and Western analysis was conducted with the antibodies marked on the right side of the figure. Co-expression of the RGS domain of Axin blocks the interaction between APC and Axin/Axin 2. (Bottom panel) The cell lysates were probed with the antibodies marked on the right side of the figure. The co-expression of RGS clearly eliminated the Axin-mediated mobility-shifted band (arrowhead). (B) 293T cells were transfected with the plasmid expressing the RGS fragment of Axin, and others, as depicted in the figure. hAPC2 and RGS were probed with anti-myc monoclonal antibody. β-Catenin-mediated phosphorylation of hAPC2 was not blocked by the overexpression of RGS.

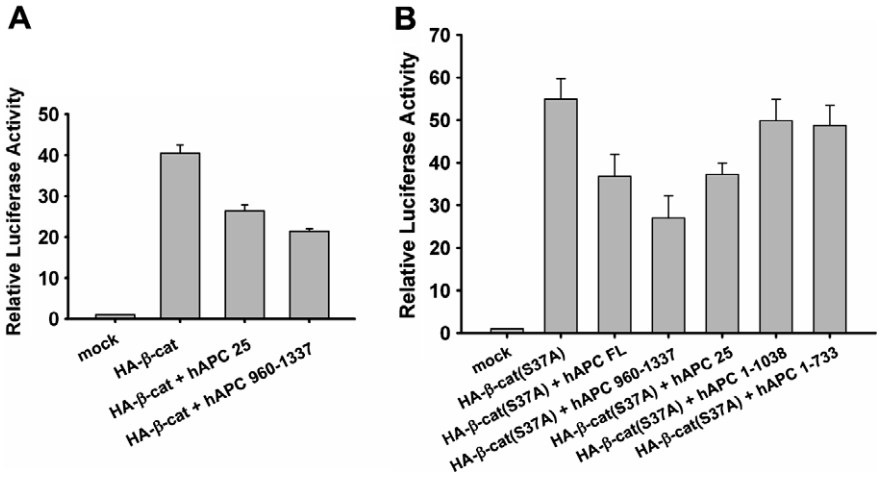

Fig. 4. hAPC proteins which showed the β-catenin-mediated mobility shift down-regulated β-catenin-induced Tcf reporter activity. (A) HA-β-catenin was co-transfected with hAPC FL or hAPC deletion constructs in order to evaluate their ability to down-regulate Tcf/Lef signaling, via a luciferase assay. This graph shows data from one representative of four independent experiments. The error bars indicate the standard deviations on the basis of triplicate luciferase assays. (B) In order to avoid the potential degradation of β-catenin by hAPC (960–1337), the same Tcf/Lef luciferase assay was conducted after the co-transfection of HA-β-catenin (S37A) with a variety of hAPC deletion constructs, as shown in the figure. Only hAPC (1–1038) and hAPC (1–733), which did not indicate a mobility shift as the result of ectopic β-catenin expression, showed incapable of significant blocking β-catenin-induced Tcf/Lef luciferase activity.

signal or in cancer cells may cause Axin-independent phosphorylation of APC. The phosphorylated APC, which has an increased affinity for β-catenin, could then retain β-catenin in the cytoplasm, blocking excessive β-catenin/Tcf signaling.

Discussion

Two widely accepted explanations for tumorigenesis in familial and somatic cases of colon cancer caused by APC mutations are: (1) the inability of mutant, truncated forms of APC to form a complex with Axin, which is believed to lead to an increase in the level of cytoplasmic β-catenin; and (2) the attenuated ability of the mutant APC proteins to export β-catenin out of nuclei, which is thought to extend the duration of β-catenin/Tcf signaling [2,5]. While these two mechanisms are widely accepted, they cannot explain two of our major findings described in this report. Instead, our results appear consistent with recent data suggesting that Wnt/β-catenin signaling can be regulated by the interaction of β-catenin with proteins that retain it in specific subcellular compartments, rather than by active transport into or out of the nucleus [19].

First, we found that accumulation of β-catenin causes phosphorylation of APC in an Axin-independent manner. Analysis of ectopically expressed EGFP-Axin in 293T cells with confocal microscopy shows that it is expressed in a punctuated pattern mainly in cytoplasm and some in nuclei (Supplementary Fig. 7). However, most of the ectopically expressed β-catenin and hAPCs are diffusely distributed in the cytoplasm, and are thus not co-localized with Axin. When the localization pattern and the low level of endogenous Axin are considered, the involvement of Axin in the β-catenin-mediated phosphorylation of APC may be implausible *in vivo*.

Second, the hAPC mutants, which showed mobility shift by the co-transfection of β-catenin in our experiments, inhibit reporter activity induced by the mutant form of β-catenin that is resistant to ubiquitination/proteasomal degradation (Fig. 4B). Interestingly, hAPC 960–1337, which is unable to down-regulate the level of β-catenin ([17] and data not shown) and is localized in cytoplasm, strongly inhibits reporter activity (Fig. 4B and Supplementary Fig. 6). Therefore the ability of hAPC 960–1337 to inhibit β-catenin signaling is neither due to down-regulation of β-catenin nor exportation of nuclear-localized β-catenin by APC. This suggests that there must be other mechanisms for the regulation of β-catenin signaling by APC.

We propose the following mechanism to explain our results: accumulation of free β-catenin in response to a Wnt signal or in cancer cells causes phosphorylation of APC, and the phosphorylated APC in the cytoplasm may retain the β-catenin to regulate excessive β-catenin/Tcf signaling. The level of Axin 2 is high in many cancer cell lines and it is considered as a negative feedback regulation of β-catenin/Tcf mediated signaling, since Axin 2 is a target gene of that signal [7]. The subcellular localization of EGFP-Axin 2 in 293T cells is quite different from that of Axin. Ectopically expressed EGFP-Axin 2 is diffused in the cytoplasm (Supplementary Fig. 7), which is similar as the subcellular localization of ectopically expressed APC and β-catenin (Supplementary Fig. 6). It may be possible that the role of APC is to retain β-catenin in the cytoplasm until Axin 2 joins to this complex for the down-regulation of β-catenin to the normal level.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.117.

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