Downregulation of β-catenin by human Axin and its association with the APC tumor suppressor, β-catenin and GSK3β

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Background: Inactivation of the adenomatous polyposis coli (APC) tumor suppressor protein is responsible for both inherited and sporadic forms of colon cancer. Growth control by APC may relate to its ability to downregulate β-catenin post-translationally. In cancer, mutations in APC ablate its ability to regulate β-catenin, and mutations in β-catenin prevent its downregulation by wild-type APC. Moreover, signaling by the protein product of the *wnt*-1 proto-oncogene upregulates β-catenin and promotes tumorigenesis in mice. In a *Xenopus* developmental system, Wnt-1 signaling was inhibited by Axin, the product of the murine *fused* gene. This suggests a possible link between Axin, the Wnt-1 signaling components β-catenin and glycogen synthase kinase 3β (GSK3β), and APC.

Results: Human Axin (hAxin) binds directly to β-catenin, GSK3β, and APC *in vitro*, and the endogenous proteins are found in a complex in cells. Binding sites for Axin were mapped to a region of APC that is typically deleted due to cancer-associated mutations in the APC gene. Overexpression of hAxin strongly promoted the downregulation of wild-type β-catenin in colon cancer cells, whereas mutant oncogenic β-catenin was unaffected. The downregulation was increased by deletion of the APC-binding domain from Axin, suggesting that APC may function to derepress Axin activity. In addition, hAxin dramatically facilitated the phosphorylation of APC and β-catenin by GSK3β *in vitro*.

Conclusions: Axin acts as a scaffold upon which APC, β-catenin and GSK3β assemble to coordinate the regulation of β-catenin signaling.

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Background

The prevalence of mutations in the adenomatous polyposis coli (APC) gene in colon cancers suggests that the loss of APC function may be a prerequisite to tumor formation in the colon [1–8]. Precisely how inactivation of APC contributes to tumor progression is unknown, but probably relates to its ability to downregulate β-catenin. Mutant APC lacks this activity [9] and, more recently, mutant β-catenins that are refractory to downregulation by wild-type APC have been identified in cancers [10,11]. Deregulation of β-catenin appears relevant to actual human tumors as high levels of β-catenin have been detected by immunohistochemical staining of tumor tissue sections [12]. Moreover, the *wnt*-1 proto-oncogene promotes murine tumor progression and also upregulates β-catenin levels in cell culture [13–16]. Thus, the regulation of β-catenin, which directly impacts upon cell adhesion, morphology, motility, and gene transcription, is an important facet of epithelial cell growth control.

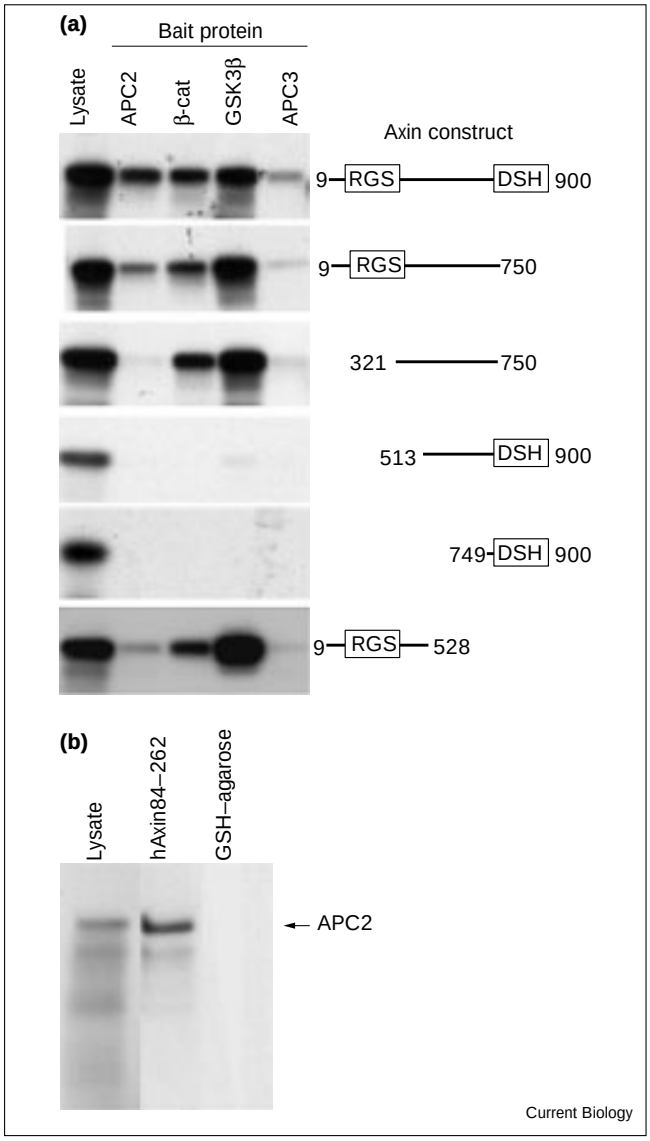
Both APC and glycogen synthase kinase 3β (GSK3β) have been implicated in the regulation of β-catenin in mammalian cells. APC is phosphorylated by GSK3β *in vitro* in a region of the protein that is essential for its ability to downregulate β-catenin levels [17]. This phosphorylation also greatly increases the binding affinity of β-catenin for APC. GSK3β has also been reported to phosphorylate β-catenin at serine and threonine residues that are critical for the rapid turnover of β-catenin in the cell [18]. The premise for examining GSK3β as a regulator of β-catenin was largely derived from developmental studies in *Xenopus* and *Drosophila* [18–23]. In these systems, the Wnt-1/Wingless signaling pathway suppresses GSK3β/Zeste-white 3 activity leading to a transcriptional output mediated by the effector protein β-catenin/Armadillo. A recent study in *Xenopus* identified Axin as a new potential component of the regulation of β-catenin signaling [24]. It was shown that murine Axin, the product of the mouse *fused* gene, inhibited the dorsal axis formation in *Xenopus* that is normally observed on expression of Wnt-1. Here, we have examined the role of human Axin (hAxin) in the regulation of β-catenin in cancer cells. We have found that hAxin contains independent binding sites for APC, β-catenin, and GSK3β, and that overexpression of hAxin in cancer cells post-translationally downregulates β-catenin.

Results

A hAxin cDNA was isolated from a fetal brain cDNA library. This cDNA encoded a polypeptide initiating at the ninth amino acid with respect to a previously reported human Axin clone [24] and terminating at amino acid 900 and has therefore been designated hAxin9–900. To determine whether hAxin would bind directly to β-catenin, GSK3β, or APC, radiolabeled full-length hAxin and fragments of hAxin were produced by *in vitro* transcription and translation in wheat germ lysate and tested for binding to the purified bait proteins APC2, β-catenin, GSK3β and APC3. The central region of the APC protein, designated APC2 and representing amino acids 1034–2130, β-catenin, and GSK3β all associated with hAxin, whereas only low-level, non-specific binding was detected with the carboxy-terminal fragment of APC, termed APC3 and containing amino acids 2130–2843 (Figure 1a). The background signal observed with APC3 was comparable to that observed using purified Rap1 GTPase activating protein as an additional negative control (data not shown). Deletion of amino acids 9–320 of hAxin, which contains the regulator of G-protein signaling (RGS) domain [24], eliminated binding to APC2, but not to β-catenin or GSK3β. None of these bait proteins associated with the hAxin fragments 513–900 or 749–900, whereas all three associated with hAxin9–528. To demonstrate the direct binding of the hAxin RGS domain to APC, the APC2 fragment was produced in wheat germ lysate and tested for binding to a purified GST–hAxin84–262 fusion protein. Radiolabeled APC2 was recovered with GST–hAxin84–262 protein on glutathione agarose beads but no APC2 was recovered on beads alone (Figure 1b). GST–hAxin84–262 did not associate with either β-catenin or GSK3β in this assay (data not shown). These results indicate that β-catenin, GSK3β and APC bind directly to the amino-terminal half of hAxin.

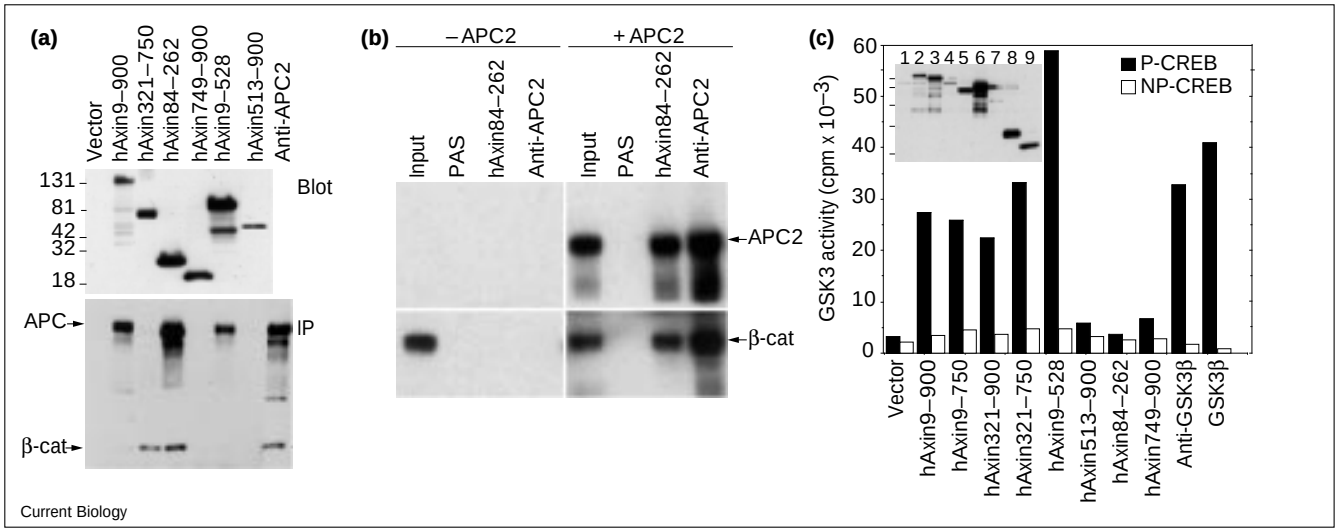
The binding interactions were also observed when Myc-tagged hAxin was expressed in 293 cells and anti-Myc immunoprecipitates were analyzed for β-catenin and APC (Figure 2a). Wild-type APC co-immunoprecipitated with hAxin fragments corresponding to amino acids 9–900, 84–262 and 9–528. Very little β-catenin was associated with hAxin9–900 and hAxin9–528 despite their association *in vitro*. This was also the case for wild-type APC (Figure 2a, anti-APC2), however, and may be due to the low levels of free β-catenin contained in these cells. By contrast, β-catenin, but not APC, co-immunoprecipitated with hAxin321–750, which lacks the RGS domain. No association of APC or β-catenin was seen with hAxin749–900 or hAxin513–900. Low levels of β-catenin were detected in the immunoprecipitates of hAxin84–262, but this may have resulted from its indirect association through APC. We therefore determined whether β-catenin could simultaneously bind to the Axin RGS domain and APC. Purified GST–hAxin84–262 was incubated with purified β-catenin in the presence or absence of purified APC2 and then recovered on glutathione–agarose beads. Analysis of the recovered GST–hAxin84–262 revealed that the association of β-catenin was dependent upon APC2 and that the amount of β-catenin relative to APC2 was

Figure 1



In vitro binding of hAxin to APC, β-catenin (β-cat) and GSK3β. **(a)** The indicated hAxin constructs were expressed as ^{35}S-labeled proteins by *in vitro* transcription and translation in wheat germ lysate and then incubated with the indicated Glu–Glu epitope-tagged bait proteins. Bait proteins were recovered by anti-Glu–Glu immunoprecipitation and analyzed by SDS–PAGE and autoradiography. The lysate sample represents 20% of that used in the binding analysis. DSH; Dishevelled homology domain. **(b)** Radiolabeled APC2 was produced by *in vitro* transcription and translation in wheat germ lysate and incubated with 2 μg purified GST–hAxin84–262 fusion protein: hAxin84–262 was recovered on glutathione–agarose beads and analyzed by SDS–PAGE and fluorography. Glutathione–agarose beads (GSH–agarose), in the absence of GST–hAxin84–262, were used a negative control.

Figure 2



In vivo binding of hAxin to APC, β-catenin and GSK3β. (a) The hAxin constructs were transiently transfected into 293 cells and the expressed proteins recovered by immunoprecipitation via an encoded Myc epitope tag. The immunoprecipitates were examined by SDS–PAGE and immunoblotting for the presence of APC and β-catenin. The upper panel shows the anti-Myc blot and the lower panel the anti-Myc immunoprecipitation. Molecular weights in kDa of standard proteins are indicated to the left of the upper panel. (b) Purified β-catenin (100 ng) was incubated with 2 μg purified GST–hAxin84–262 in the absence (– APC2) or presence (+ APC2) of 0.5 μg purified APC2. GST–hAxin84–262 was recovered on glutathione–agarose and APC2 was immunoprecipitated with anti-APC2 antibody. Protein-A–Sepharose (PAS) was used as a negative control and the lane marked input represents 10% of the total reaction mixture. Precipitates were analyzed by immunoblotting with antibody to APC (upper panel) and β-catenin (lower panel). (c) The hAxin constructs were expressed in SW480 cells and the proteins were recovered as in the legend to (a). Each immunoprecipitate was divided in half and assayed for GSK3β activity using the specific peptide substrate P-CREB or the non-specific control peptide NP-CREB; both peptide substrates are derived from the cAMP-response-element-binding protein (CREB). Anti-GSK3β indicates direct immunoprecipitation of GSK3β from control lysate, and 2 ng purified GSK3β was assayed as a positive control. The inset shows an anti-Myc immunoblot of the cell lysates where lanes 1–9 represent vector through hAxin749–900, in the same order as for the histogram.

comparable to that observed by direct immunoprecipitation of APC2 (Figure 2b). Fragments of hAxin were also expressed in the SW480 colorectal carcinoma cell line to investigate their association with GSK3β. Because detection of GSK3β in immunoprecipitates is obscured due to the co-migration of GSK3β with immunoglobulin G heavy chain, we assayed the immunoprecipitates for GSK3β activity (Figure 2c). Specific GSK3β activity was associated with the hAxin fragments 9–900, 9–750, 321–900, 321–750 and 9–528 but not with fragments 513–900, 84–262 and 749–900.

Analysis of the APC gene in colon cancer has delineated a mutation cluster region in which the majority of the polypeptide chain-terminating mutations are localized [25,26]. The 3′ border of this region resides approximately at codon 1500 and may demarcate the minimal deletion of corresponding amino acid sequence that is required to compromise the tumor suppressor activity of APC. To correlate this mutational border with Axin binding, we tested binding in a yeast two-hybrid assay with a series of APC polypeptides that progressively traverse the border of the mutation cluster region (Figure 3a). We first tested three large APC fragments that encompass the entire 2843 amino-acid polypeptide chain. The central APC fragment containing amino acids 957–2130 reacted with hAxin whereas the amino-terminal and carboxy-terminal fragments corresponding to amino acids 1–1210 (APC1) and 2130–2843 (APC3), respectively, did not. Subdivision of the central region of APC revealed that an Axin-binding site resided between APC amino acids 1554 and 1698, located near the carboxy-terminal border of the mutation cluster region. APC fragments that began after amino acid 1698 also interacted with hAxin, revealing the presence of more than one Axin-binding site in the central region of APC.

The results suggest that Axin binding is selected against by the polypeptide truncations resulting from mutations in the APC gene in cancer. The mutant APC in the SW480 colorectal cancer cell line is known to be truncated at codon 1337 [27] and thus should not associate with the APC-binding fragment of hAxin. Indeed, expression and recovery of the hAxin84–262 fragment in SW480 cells demonstrated that the mutant APC was not associated with this fragment of hAxin (Figure 3b). This is in contrast to the association of hAxin84–262 with wild-type APC from the 293 human embryonic kidney cell line

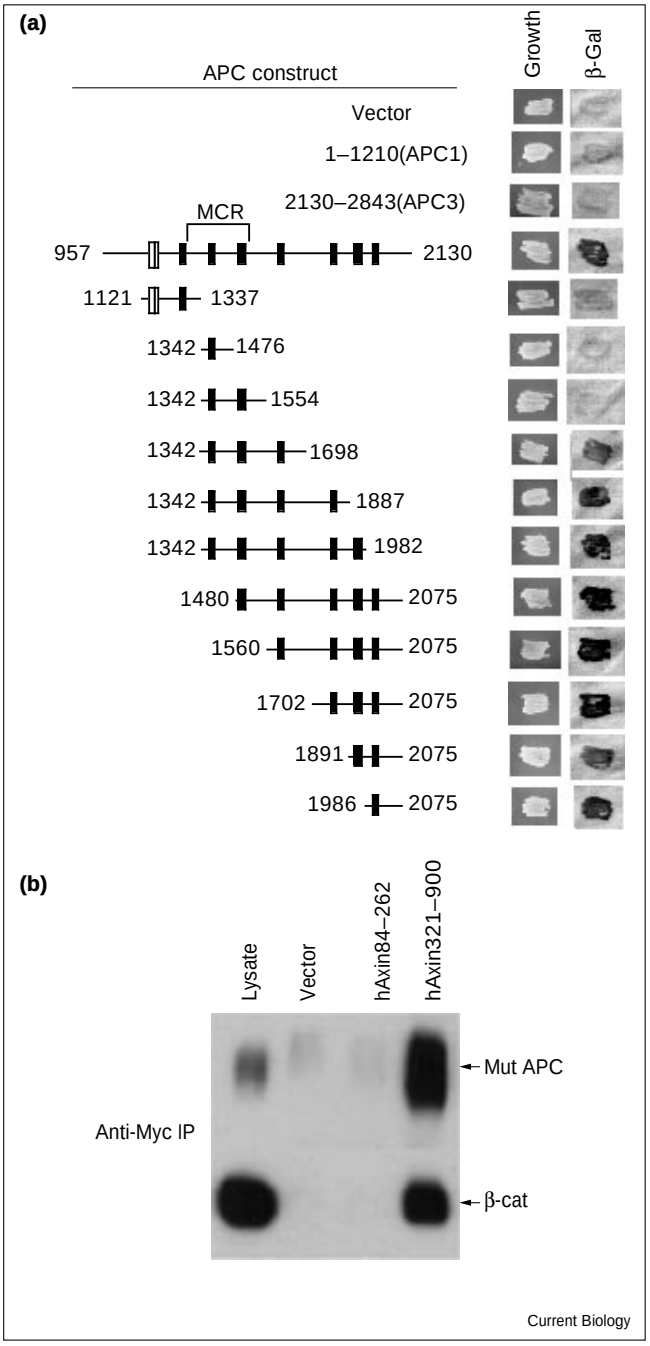


Figure 3

Localization of Axin-binding sites on APC. (a) Yeast were cotransformed with one plasmid encoding the indicated APC fragment fused to the GAL4 DNA-binding domain and another encoding hAxin84–726 fused to the GAL4 transactivation domain. The presence of two plasmids permits growth in selective media (Growth) and a positive APC–Axin interaction produces β-galactosidase detected by color development (β-Gal). The central region of APC is depicted schematically where open and closed rectangles represent the 15 and 20 amino acid repeat sequences, respectively, and MCR denotes the mutation cluster region. (b) Empty vector, hAxin84–262, or hAxin321–900 were transfected into SW480 cells and the proteins recovered by immunoprecipitation with Myc antibody (anti-Myc IP). Immunoprecipitates were analyzed by immunoblotting with antibodies to APC (upper panel) or β-catenin (lower panel). Mut APC indicates the endogenous truncated APC protein present in SW480 cells.

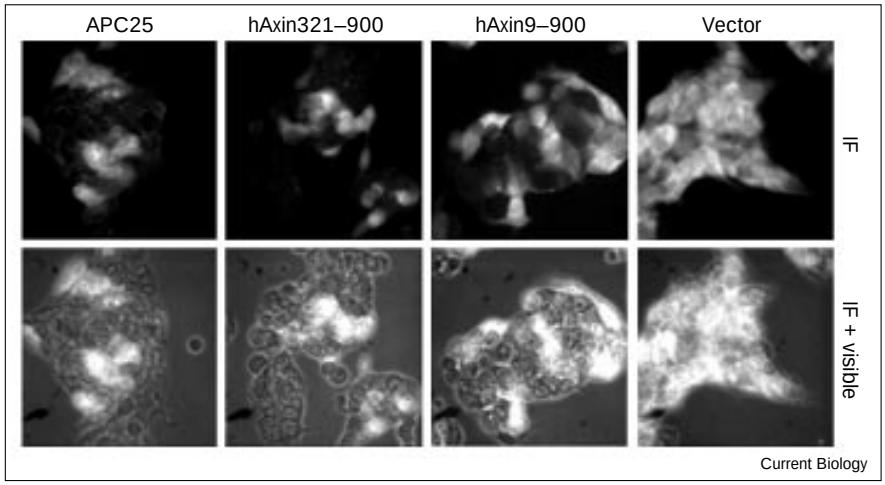
(Figure 2a). Interestingly, the mutant APC from SW480 cells was co-immunoprecipitated with the hAxin321–900 fragment. As SW480 cells contain an ample supply of free β-catenin to which the mutant APC can bind, it is likely that the mutant APC was indirectly associated with hAxin321–900 through the simultaneous association of these proteins with β-catenin.

The interaction of hAxin with both APC and β-catenin, together with the previous finding that Axin inhibited Wnt-1 signaling in *Xenopus* [24], prompted us to examine the effect of Axin expression on the intracellular levels of β-catenin. Immunofluorescence staining of endogenous β-catenin was analyzed in SW480 cells following overexpression of hAxin9–900 or, as a positive control, the APC25 fragment comprising amino acids 1342-2075 of the APC protein (Figure 4). The transient transfection efficiency in these cells is greater than 50% [9], and a high percentage of cells exhibited a marked diminution of β-catenin immunofluorescence staining following transfection with either hAxin9–900 or APC25. Surprisingly, hAxin321–900, which lacks the APC-binding domain, also strongly downregulated intracellular β-catenin. Additional experiments involving quantitative immunoblotting indicated that hAxin321–900 was a more potent downregulator of β-catenin than hAxin9–900 (data not shown). This was also apparent from pulse-chase experiments in which hAxin was co-expressed with Myc-tagged β-catenin in SW480 cells and the rate of β-catenin turnover was examined (Figure 5). The half-life of the Myc–β-catenin was approximately 90 minutes when co-expressed with hAxin9–900 compared with a half-life of several hours when co-expressed with vector control. The half-life was reduced further to approximately 45 minutes upon co-expression of hAxin321–900. The half-life of the β-catenin mutant S37A, which contains a serine to alanine mutation at amino acid 37 and has previously been shown to be refractory to APC-dependent turnover [11], was unaffected by hAxin9–900. The results demonstrate that hAxin downregulates β-catenin in a post-translational manner and that deletion of the APC-binding domain of hAxin does not ablate this activity but instead might even enhance it.

To demonstrate that endogenous Axin was associated with APC, β-catenin and GSK3β, antibodies specific to Axin were raised in rabbits. Immunoprecipitates obtained with affinity-purified antibodies to Axin, APC, GSK3β and β-catenin were examined by immunoblotting for the presence of Axin (Figure 6a). A strongly reactive band co-migrating with purified recombinant hAxin9–900 was detected in the Axin immunoprecipitates from HCT116

Figure 4

Downregulation of β-catenin. SW480 cells were transiently transfected with APC25, hAxin9–900, hAxin321–900, or vector control and endogenous β-catenin was visualized by immunofluorescence staining (IF). The same image was then recorded in the presence of both fluorescent and visible light (IF + visible).



colorectal carcinoma cells. A reactive polypeptide of equivalent mobility was also detected in the APC, β-catenin and GSK3β immunoprecipitates, but not in the anti-Rap1 and anti-BRCA1 control immunoprecipitates. To assess whether active GSK3β was associated with endogenous Axin, GSK3β assays were performed on Axin immunoprecipitates (Figure 6b). Anti-Axin immunoprecipitates from the HCT116 and SW480 cells phosphorylated the specific, but not the non-specific, GSK3β peptide substrate. These results indicate that endogenous Axin exists in a complex with APC, β-catenin and active GSK3β.

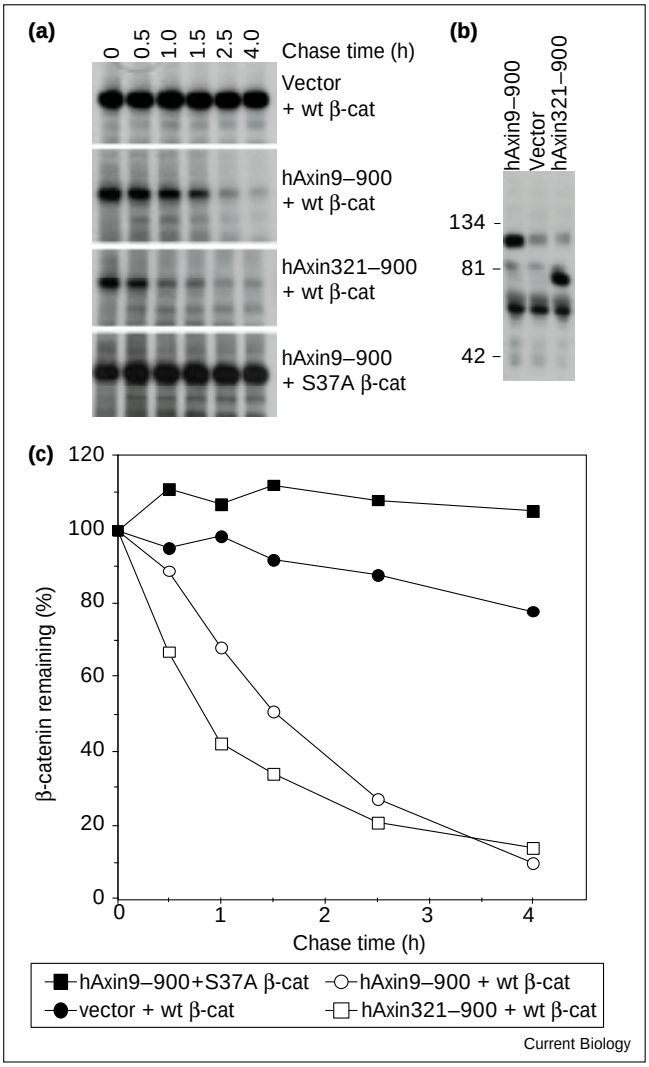
The finding that the phosphorylation of APC by GSK3β greatly enhanced its ability to bind β-catenin led to the proposal that GSK3β played a role in the regulation of β-catenin by APC [17]. In these experiments, GSK3β was unable to phosphorylate APC efficiently unless prephosphorylation of APC was first performed using protein kinase A. The binding of both GSK3β and APC to Axin suggests that Axin may serve to facilitate the phosphorylation of APC by GSK3β. To test this proposal, we performed phosphorylation of APC by GSK3β in the presence or absence of purified hAxin9–900. The purified APC fragment APC25 was used in this assay and was dephosphorylated and repurified as previously described [17]. In the presence of GSK3β alone, a low level of APC25 phosphorylation was detected; however, APC25 phosphorylation was dramatically increased on inclusion of hAxin9–900 (Figure 7). In the absence of GSK3β, low levels of APC25 phosphorylation were still observed on inclusion of hAxin9–900, suggesting the presence of a kinase in the hAxin9–900 preparation. However, the level of APC25 phosphorylation detected in the presence of both GSK3β and hAxin9–900 was far greater than the sum of the phosphorylations observed by GSK3β and hAxin9–900 alone. Moreover, only when both hAxin9–900 and GSK3β were included did APC25 undergo a bandshift indicative of quantitative phosphorylation (Figure 7). These results suggest that Axin greatly enhances the phosphorylation of APC by GSK3β *in vitro*.

While this paper was under review a report by Ikeda *et al.* [28] showed that Axin facilitated the phosphorylation of β-catenin by GSK3β. Indeed, the inclusion of hAxin9–900 also appears to enhance the phosphorylation of β-catenin by GSK3β, confirming the findings of Ikeda *et al.* [28].

Discussion

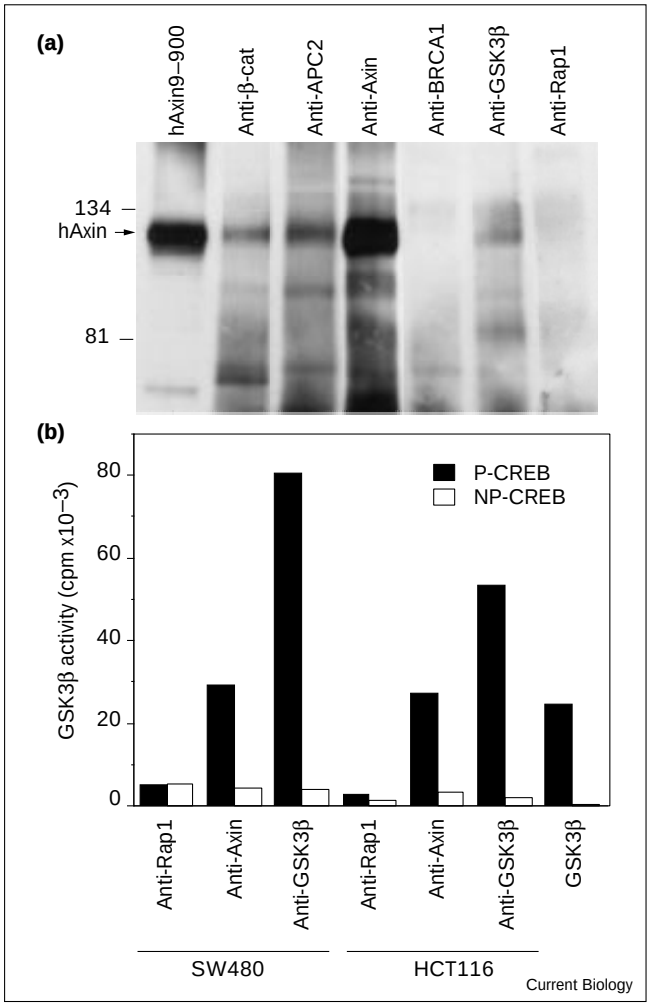
Our results indicate that hAxin is intimately involved in the process of β-catenin regulation. It binds to the Wnt-1 signaling components β-catenin and GSK3β and strongly downregulates β-catenin when overexpressed in cancer cells. The binding of hAxin to both APC and GSK3β is consistent with a previous report describing a multimolecular complex containing APC, β-catenin and GSK3β [17]; in that report, APC was shown to be phosphorylated by GSK3β in a region of the protein essential for its ability to downregulate β-catenin. Here, we found that the phosphorylation of APC *in vitro* by GSK3β is enhanced by the inclusion of hAxin protein. In a separate study it was shown that GSK3β also phosphorylated β-catenin at serine and threonine residues known to be critical for its turnover [18]. Ikeda *et al.* [28] have recently reported that Axin facilitates this phosphorylation and our data are also in agreement with their finding. Thus, the binding of APC and β-catenin to Axin may serve to establish a local proximity and orientation that facilitates their phosphorylation by GSK3β *in vivo* (Figure 8). Moreover, the association of both GSK3β and APC with Axin provides an explanation for a previously described multimolecular complex containing APC and GSK3β [17]. Although this complex could be demonstrated using cell lysates, we were unable to observe direct binding

Figure 5



Pulse-chase analysis of ectopically expressed wild-type (wt) β-catenin and the S37A β-catenin mutant. SW480 cells were transiently cotransfected with the indicated plasmids, pulse-labeled with ^{35}S-methionine, and then chased with cold media. At the indicated times, cells were lysed and the expressed β-catenin was recovered by immunoprecipitation via a Myc epitope tag fused to the β-catenin protein. (a) Analysis of immunoprecipitates by SDS–PAGE and fluorography. (b) Expression levels of the Glu–Glu-tagged hAxin fragments detected by anti-Glu–Glu immunoblotting of a normalized lysate aliquot. (c) Plot of the pulse-chase analysis quantitated by β-scanning.

Figure 6

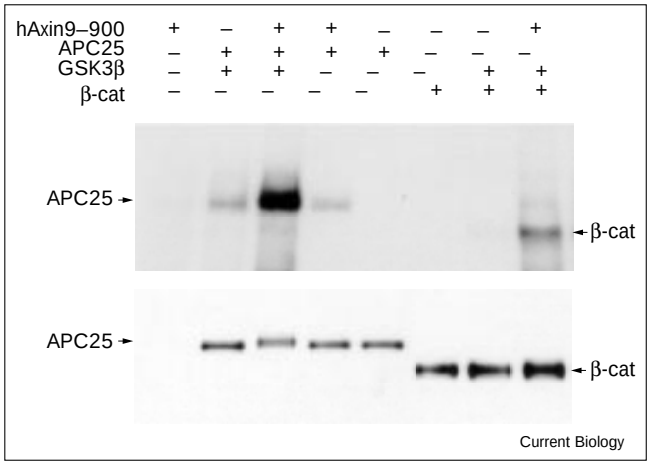


Association of endogenous hAxin with APC, β-catenin and GSK3β. (a) Whole cell lysates from HCT116 and SW480 cells were subjected to immunoprecipitation with antibodies specific to Axin, APC, β-catenin, GSK3β or, as controls, Rap1 and BRCA1. The immunoprecipitates were analyzed by SDS–PAGE and immunoblotting with anti-Axin antibody. Approximately 20 ng purified recombinant hAxin9–900 was loaded as a positive control. Molecular weights in kDa of standard proteins are indicated to the left. (b) Immunoprecipitates of endogenous Axin, GSK3β or Rap1 from whole cell lysates from HCT116 and SW480 cells were assayed for GSK3β activity. Purified GSK3β (2 ng) was used as a positive control.

of GSK3β to either β-catenin or APC using purified proteins or using the yeast two-hybrid interaction assay (our unpublished observations). Thus, Axin provides the missing link for the connection of APC to GSK3β.

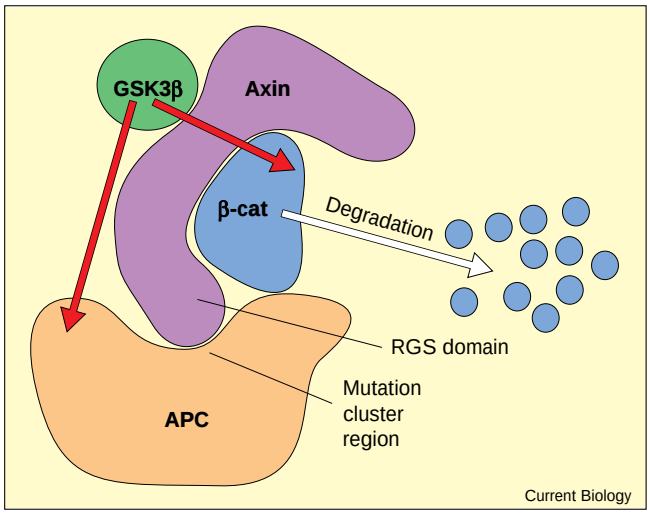
The phenotype observed on expression of the full-length hAxin9–900 in mammalian cells agrees with that observed for ectopic expression of murine Axin in *Xenopus* development [24]. We found that hAxin downregulated β-catenin and Zeng *et al.* [24] reported that Axin inhibited β-catenin cell signaling in *Xenopus*. It is thought that GSK3β negatively regulates β-catenin in this signaling pathway, as expression of a dominant-negative form of GSK3β resulted in a positive signal [22,29]. The expression of β-catenin, but not dominant-negative GSK3β, overcame the inhibition by Axin, leading Zeng *et al.* [24] to conclude that Axin acted upstream of β-catenin and downstream of GSK3β in the Wnt-1 pathway. However, our results showing direct binding of GSK3β to hAxin suggest that

Figure 7



Axin facilitates phosphorylation of APC and β-catenin *in vitro*. Purified proteins produced using the baculovirus/Sf9 cell expression system were combined as indicated and incubated in the presence of [γ-^{32}P]ATP. Autoradiography (upper panel) was performed following SDS–PAGE and electroblotting. Immunoblotting for APC and β-catenin was performed on 5% of each reaction mixture (lower panel).

Figure 8



Proposed interaction of Axin with APC, β-catenin and GSK3β. Axin interacts simultaneously with GSK3β, APC, and β-catenin, thereby promoting the phosphorylation of the latter two proteins by GSK3β (represented by red arrows). The interaction leads to the rapid degradation of β-catenin in the cell.

dominant-negative GSK3β may operate simply by displacing wild-type GSK3β from Axin. This may not have been observed in *Xenopus* if the level of ectopically expressed dominant-negative GSK3β did not substantially exceed that of the ectopically expressed Axin.

A more curious departure from the *Xenopus* study is apparent when comparing the effects of a deletion mutant of Axin that lacks an RGS domain. In mammalian cells, this mutant resulted in enhanced downregulation of β-catenin, whereas a similar protein promoted, rather than inhibited, β-catenin signaling in *Xenopus* [24]. The effect of Axin on the levels of β-catenin in the *Xenopus* system were not determined, however, and β-catenin levels in *Xenopus* may not be affected by Axin in the same way as is observed in mammalian cells. Manipulation of the Wnt-1 pathway in *Xenopus* has also led to paradoxical conclusions regarding the role of APC. Expression of wild-type APC in mammalian cells promotes the downregulation of β-catenin [9], whereas forced expression of wild-type APC in *Xenopus* results in positive β-catenin signaling [30]. The contrasts in mammalian and *Xenopus* signaling may indicate differences in the utilization of the Wnt-1 signaling components between these organisms. Indeed, Wnt-1 signaling components identified in *Caenorhabditis elegans* do not appear to operate in a similar manner as that described for these components in either *Xenopus* or mammalian cells [31]. Alternatively, differences in the experimental paradigms used to study the signaling pathways could account for the different outcomes.

In a previous study, we reported that the polypeptide chain-terminating mutations that are characteristic of the forms of APC associated with cancer progression correlate well with the loss of the downregulatory activity of APC on β-catenin [32]. It was concluded that a defined number of tandemly repeated amino acid sequences in the central region of APC were required for its ability to downregulate β-catenin. The present study, however, shows that the loss of this activity may instead relate to the inability of mutant APC to associate directly with cellular Axin. If association with Axin is required for APC tumor suppressor activity, it might be expected that Axin itself would be deleted or mutated in some cancers. This may not be the case for the SW480 or HCT116 colon cancer cell lines, as indicated by immunoblotting, as these cells already contain mutant APC and mutant β-catenin, respectively [10]. Interestingly, overexpression of hAxin promoted the downregulation of β-catenin in SW480 cells, despite the lack of wild-type APC expression in these cells. This suggests that endogenous levels of Axin are not sufficient to regulate β-catenin in the absence of wild-type APC and that Axin overexpression may compensate for this by mass action. Indeed, hAxin may not require APC as removal of its APC-binding domain did not impair its ability to regulate β-catenin, but instead increased it. Thus, APC may serve to derepress Axin by binding a regulatory domain that normally inhibits the ability of Axin to downregulate β-catenin.

Conclusions

Human Axin associates with the APC tumor suppressor protein at binding sites in the APC protein that are deleted due to cancer-causing mutations. Axin probably

plays a critical role in the downregulation of β-catenin by APC. The binding of β-catenin and GSK3β to Axin suggests that Axin assembles the protein components that are required for β-catenin degradation into a closely associated complex. The inability of APC to participate in this complex may lead to deregulated β-catenin signaling that then contributes to tumor progression.

Materials and methods

Plasmids and cell lines

A hAxin cDNA (Genbank accession number AF009674) was cloned from a human fetal brain cDNA library (Stratagene). A 1.9 kb PCR product was generated using the following primers: GGGAAAGGTGTTGGCATT and GTGGGGTCTTGGATGAAGA. Two radiolabelled cDNA probes were generated using this PCR product as a template and the following primer sets: GGGAAAGGTGTTGGCATT and TCGTTCGAGTCACAGGGC and AACGCCAGTGATGGCCTC and GTGGGGTCTTGGATGAAGA. A cDNA encoding hAxin initiating at amino acid 9, with respect to the published sequence [24], and terminating at amino acid 900 was isolated. This cDNA does not include the 36 amino acid insert previously shown to be present in human form 2 [24]. In addition, we noted three differences in our cDNA sequence which affect the amino acid sequence. One of these occurred at codon 397 (GCC to GTG) changing alanine to valine, one occurred at codon 520 (ACT to CCT) changing threonine to proline, and finally three nucleotides contained between codons 488 and 493 were absent from our sequence resulting in an in-frame deletion of alanine 493 and the following change in amino acid sequence; 488LCWTW492 to 488CVDMG492. The new amino sequence is now identical to the mouse sequence, indicating that the original human sequence [24] was probably an error. These cDNAs were subcloned into the pCDNA3 (Invitrogen) expression vector containing a Myc epitope tag: hAxin9–900 was subcloned as an *Eco*RI–*Xba*I fragment; hAxin9–750 was subcloned as an *Eco*RI–(*Asc*I, blunt) fragment into the *Eco*RI–*Eco*RV sites; hAxin321–750 was subcloned as an *Acc*I fragment into the *Eco*RV site; hAxin513–900 was generated by subcloning a PCR product into the *Eco*RI–*Xba*I sites; hAxin749–900 was subcloned as an (*Asc*I, blunt)–*Xba*I fragment into the *Eco*RV–*Xba*I sites; hAxin9–528 was made by subcloning a PCR product into the *Eco*RI–*Xba*I sites; hAxin321–900 was subcloned as a *Acc*I fragment into the *Eco*RV site; hAxin84–729 was a PCR product generated from a human fetal brain cDNA library. The PCR product was cloned into the TA cloning vector and then subcloned as an *Eco*RI fragment into pCDNA3; hAxin84–262 was obtained by digestion of hAxin84–729 with *Bst*XI and *Xba*I, followed by treatment with T4 polymerase and religation. For expression of hAxin in Sf9 cells, hAxin9–900 was subcloned into pFastBac as an *Eco*RI–*Xba*I fragment.

SW480 and HCT116 cell lines were derived from human colorectal cancers (ATCC references CCL228 and CCL247, respectively), 293 is an immortalized embryonic kidney cell line (ATCC reference CRL1573).

Antibodies

Polyclonal antibodies to APC2, β-catenin, GSK3β, and monoclonal antibodies to the Myc and Glu–Glu epitopes have been described elsewhere [11]. Antibodies to Axin were raised in rabbits against the purified murine Axin fragment 811–992 fused to GST and produced in *Escherichia coli*. The murine cDNA (accession number 464191) was purchased from Genome Systems and subcloned as a *Eco*RI–*Xba*I fragment into a pGEX vector for expression in *E. coli*. Antibodies were affinity-purified against the antigen as described previously [33].

In vitro *binding*

The bait proteins APC2, β-catenin, GSK3β and APC3 all contained amino-terminal Glu–Glu epitope tags and were produced using baculovirus/Sf9 cell expression and then immunoaffinity-purified using immobilized Glu–Glu antibody. Affinity precipitation of *in vitro* translated proteins was performed by first transcribing and translating the indicated hAxin cDNAs using the wheat germ lysate system (Promega) in the presence of ^{35}S-methionine according to the manufacturers' instructions. A 5 μl aliquot of the lysate was diluted to 200 μl in 20 mM Tris (pH 7.5), 100 mM NaCl, and 1 mM dithiothreitol and incubated with 2 μg each of the purified bait proteins for 2 h on ice. Bait proteins were recovered by anti-Glu–Glu immunoprecipitation on protein-G–Sepharose; after three washes with buffer B (25 mM Tris (pH 7.5), 0.15 M NaCl and 0.1% NP-40), proteins were eluted from the Sepharose with SDS–PAGE sample buffer and analyzed by SDS–PAGE and fluorography. The *in vitro* binding of purified GST–hAxin84–262 to APC2 produced by *in vitro* translation was also performed as described as above for binding of bait proteins to *in vitro* translated hAxin fragments. The *in vitro* binding of purified proteins to each other was carried out in a final volume of 150 μl 25 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin and 0.1% NP-40. All binding reactions contained 100 ng of β-catenin and 2 μg GST–hAxin84–262 and incubations were performed for 30 min at 30°C in the presence or absence of 500 ng APC2. Protein-A–Sepharose, glutathione–agarose or protein-A–Sepharose and 2 μg anti-APC2 antibody were added to the reactions, as indicated, followed by a 1 h incubation with rocking at 4°C. The beads were recovered and washed as described above and the eluted proteins analyzed by SDS–PAGE and immunoblotting. The β-catenin and APC2 proteins were produced using the baculovirus/Sf9 cell expression system.

In vivo *binding*

For expression of hAxin in mammalian cells, 4 μg plasmid was transfected into a 3.5 cm dish of subconfluent SW480 or 293 cells as described previously [9,34]. Cells were lysed on the dish in 400 μl lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.4% Triton X-100, 25 mM NaF, 1 mM sodium vanadate, 30 μg/ml leupeptin and aprotinin, and 1 μM pefabloc) 48 h after transfection and, following centrifugation, the supernatants were incubated with 2 μg antibody specific to the Myc epitope. Immunocomplexes were recovered on protein-G–Sepharose, washed three times with buffer B, and analyzed by SDS–PAGE and immunoblotting as described previously [33]. Immunoprecipitation of endogenous APC, β-catenin and GSK3β was carried out as described in [17]. Affinity-purified Axin antibodies were used at a final concentration of 2 μg/ml for immunoprecipitation or 0.2 μg/ml for immunoblotting. Western blots were developed using the ECL system (Amersham). Yeast two-hybrid binding analysis was carried out as described previously [32]. GSK3β assays were performed as previously described [17] using a peptide substrate (CREB) which requires prephosphorylation by protein kinase A (P-CREB) for subsequent phosphorylation by GSK3.

Downregulation of β-catenin

Immunofluorescence detection of β-catenin in fixed whole cells was carried out as described previously [9]. The transfected SW480 cells were grown on coverslips, fixed in methanol and stained using mouse monoclonal antibodies to β-catenin (Transduction Laboratories). Pulse-chase analysis of β-catenin protein turnover was carried out as described previously [11]. Each time point was derived from a single 3.5 cm well of SW480 cells pulse-labeled for 30 min with 0.1 mCi each of ^{35}S-methionine. Cells were lysed on the dish with lysis buffer and following clarification by centrifugation, the β-catenin was recovered by immunoprecipitation via the Myc epitope tag.

In vitro *phosphorylation*

Purified proteins were incubated in a final volume of 20 μl 25 mM Tris (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 50 mM NaCl, 10% glycerol and 50 μM [γ-^{32}P]ATP (10,000 cpm/pmol). Proteins were produced using the baculovirus/Sf9 cell expression system and the following amounts were included in the reaction: APC25, 300 ng; β-catenin, 300 ng; GSK3β, 200 ng; hAxin9–900, 200 ng. Following a 20 min incubation at 30°C, the reactions were terminated by addition

of SDS–PAGE sample buffer. Autoradiograms were generated from PVDF filter membranes to which the proteins were electrophoretically transferred following SDS–PAGE.

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