Neurite outgrowth involves adenomatous polyposis coli protein and β-catenin

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Summary
Neuronal morphogenesis involves the initial formation of neurites and then differentiation of neurites into axons and dendrites. The mechanisms underlying neurite formation are poorly understood. A candidate protein for controlling neurite extension is the adenomatous polyposis coli (APC) protein, which regulates membrane extensions, microtubules and β-catenin-mediated transcription downstream of Wnt signaling. APC is enriched at the tip of several neurites of unpolarized hippocampal neurons and the tip of only the long axon in polarized hippocampal neurons. Significantly, APC localized to the tip of only one neurite, marked by dephospho-tau as the future axon, before that neurite had grown considerably longer than other neurites. To determine whether neurite outgrowth was affected by β-catenin accumulation and signaling, a stabilized β-catenin mutant was expressed in PC12 cells, and neurite formation was measured. Stabilized β-catenin mutants accumulated in APC clusters and inhibited neurite formation and growth. Importantly, these effects were also observed was independently of the gene transcriptional activity of β-catenin. These results indicate that APC is involved in both early neurite outgrowth and increased growth of the future axon, and that β-catenin has a structural role in inhibiting APC function in neurite growth.

Keywords
Adenomatous polyposis coli; β-Catenin; Axon; Microtubule; Neurite; Neuronal polarity

Introduction
During morphogenesis, neurons undergo dramatic shape changes that are unmatched by any other cell type in the body. Typically, neurons extend long membrane processes, termed neurites, one of which will outgrow the others to form the axon. The growth cone at the end of the axon eventually forms synapses with target cells. The role of the cytoskeleton during these morphological changes has been studied intensively for many years. Axon formation is affected by several microtubule-associated proteins (e.g. dephospho-tau, MAP1B, CRMP-2) as well as microtubule drugs and actin-microtubule interactions (Baas, 2002; Dent and Gertler, 2003). Although axon specification requires increased neurite outgrowth and may use a similar mechanism to initial neurite outgrowth, little is known about initial neurite outgrowth, specifically how signaling pathways regulate cytoskeletal networks and how these in turn are involved in the necessary membrane changes.

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Adenomatous polyposis coli (APC) protein and its binding partners are good candidates to link the cytoskeletal dynamics with membrane extension. APC is specifically enriched at the tips of neurites in neurons (Shi et al., 2004; Morrison et al., 1997) and is required to form neurites in PC12 pheochromocytoma cells (Dobashi et al., 2000). APC localization to the axon tip is required for Par3 localization to the axon tip, where Par3 and its binding partner Par6 are required for neuronal polarity (Shi et al., 2004; Shi et al., 2003). APC is a large multi-domain protein that localizes with bundles of microtubules at the tips of membrane extensions in many cell types (Nathke et al., 1996; Etienne-Manneville and Hall, 2003; Zhou et al., 2004). APC binds microtubules directly and indirectly through EB1, a microtubule plus-end-binding protein. APC promotes microtubule assembly and bundling in vitro (Zumbrunn et al., 2001; Munemitsu et al., 1994), and APC association with microtubules in the growth cone is important during axon outgrowth induced by nerve growth factor (NGF) (Zhou et al., 2004). APC also regulates β-catenin levels (Logan and Nusse, 2004). APC facilitates phosphorylation of β-catenin by GSK3β and targeting of β-catenin for ubiquitin-mediated degradation. Wnt signals inhibit GSK3β, leading to accumulation of β-catenin that can then mediate transcription by the Tcf/Lef family of transcription factors. Significantly, stabilized β-catenin inhibits membrane extension in epithelial cells (Pollack et al., 1997), but it is unknown how APC and β-catenin are involved in more complex patterns of membrane extension during neuronal morphogenesis.

We examined the role of APC clusters in axon and neurite outgrowth in hippocampal neurons and PC12 cells, respectively. Detailed analysis of APC localization, particularly at an intermediate stage of neuronal polarization, signified its role in microtubule-dependent neurite outgrowth. We defined the localization of APC relative to β-catenin in hippocampal neurons and PC12 cells. Significantly, expression of a stabilized β-catenin mutant that separated structural from transcriptional roles of β-catenin inhibited neurite extension.

Materials and Methods

Cell culture and transfection

E18 embryonic rat hippocampi were trypsinized and plated onto poly-L-lysine-coated coverslips, which were suspended above a dish of glial feeder cells, as described (Goslin et al., 1998). Hippocampal neurons were imaged after 1.5 or 2 days in vitro. Animals were handled ethically, according to standard protocols. Rat PC12 pheochromocytoma cells were cultured on tissue culture plates in high-glucose Dulbecco’s modified Eagle’s medium plus 10% horse serum plus 5% calf serum. Cells were transfected with Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA).

Tcf/Lef-mediated transcription was measured in uninduced, transfected PC12 cells by the TOPFLASH assay as described (Barth et al., 1999). Transfection efficiency of pTOPFLASH or pFOPFLASH luciferase reporters (kind gift from M. van de Wetering and H. Clevers, Center for Biomedical Genetics, Utrecht, The Netherlands) (Korinek et al., 1997) and β-catenin constructs were normalized by activity of co-transfected β-galactosidase. Luciferase/β-galactosidase ratios were then normalized within each experiment to controls co-transfected with pTOPFLASH and GFP.

For counting neurite extensions, cells were split 1 day after transfection at 1:2 or 1:4 onto collagen-coated coverslips. To induce extensions, 20 ng/ml nerve growth factor (NGF 2.5S) (Upstate, Lake Placid, NY) was added to fresh complete medium for 3 days. To quantify cells with extensions, tubulin-stained, non-contacting, non-mitotic, mononucleate cells at 40× magnification were counted blind to which construct was transfected.
Myc-tagged β-cat-eng was a kind gift from P. McCrea at University of Texas, Houston, TX (Montross et al., 2000; Tepera et al., 2003). Stabilized β-catenin mutants, indicated by *, were generated by mutating four GSK3β phosphorylation sites (Ser33, Ser37, Thr41 and Ser35) to alanine and cloning into the ‘tet-off’ expression vector pUDH10-3, as described for ΔGSK-β-catenin (Barth et al., 1999). ΔGSKΔC-β-catenin, a vector for the expression of stabilized β-catenin without the C-terminal transcriptional transactivation domain (amino acids 696-781 of mouse β-catenin), was obtained by replacing the 3′-terminal BglII/XbaI in ΔGSK-β-catenin with the corresponding fragment from ΔNΔC-β-catenin as described (Barth et al., 1997). These KT3-tagged constructs were expressed in ‘tet-off’ PC12 cells in Fig. 4A,B (ΔGSK-β-catenin represented by *β-catenin and ΔGSKΔC-β-catenin, *β-catΔC-term). For all other experiments, stabilized *β-catenin mutant proteins were expressed as fusion proteins with green fluorescent protein (GFP) using expression vector pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA). For *β-catenin/pEGFP-C1 the SacII/BanHI fragment encoding ΔGSK-β-catenin was removed from pUDH10-3 and cloned into pEGFP-C1, and the endogenous β-catenin stop codon was restored. The C-terminal Spel/BanHI fragment in *β-catenin/pEGFP-C1 was replaced with the corresponding KT3-tagged fragment of ΔGSKΔC to obtain *β-catΔC-term.

Immunostaining

Cells were fixed with either 100% methanol at −20°C for 5 minutes (e.g. Fig. 3B) or, to preserve growth cone morphology or dephospho-tau epitopes or GFP fluorescence, 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS) at room temperature for 15-20 minutes (e.g. Fig. 1). Fixed cells were rinsed once in PBS with 0.1% Triton X-100, rinsed three times with PBS, blocked for at least 1 hour in PBS plus 2% bovine serum albumin plus 1% goat serum, then incubated with antibodies in same buffer. Antibodies used were affinity-purified rabbit anti-APC-2 (Nathke et al., 1996) (identical localization to the axon tip was observed using affinity-purified rabbit anti-APC-3) (V.V., unpublished observations), mouse anti-β-catenin (BD Transduction Laboratories, San Diego, CA), mouse anti-α-tubulin clone DM 1A (Sigma, St Louis, MO), rat anti-α-tubulin YL1/2 (Accurate Chemical and Scientific Corp., Westbury, NY) and mouse anti-myc clone 9E10 (Jou and Nelson, 1998), or mouse anti-tau-1 (Chemicon, Temecula, CA), which recognizes axon-specific dephospho-(Ser-199/202)-tau (Mandell and Banker, 1996).

Microscope images were recorded as described (Louie et al., 2004), using a Zeiss Axioplan fluorescence microscope, unless noted as deconvolved in figure legend. Resolution and brightness/contrast of all images were enhanced with Adobe Photoshop (Adobe, San Jose, CA). Micrographs of 150 dpi (dots per inch) or 72 dpi (for Deltavision deconvolved images) were changed to 600 dpi, free transformed, and checked for accurate representation of original image.

Fluorescence quantification

Line scans of neurons were performed on unenhanced TIFF files with ImageJ (http://rsb.info.nih.gov/ij/) by drawing a pixel-wide line along a neurite, starting from the edge of the cell body and passing through the areas of most intense APC immunofluorescence. We measured gray values of pixels along the line, then subtracted as background the gray values of the same line moved to an adjacent cell-free area. We calculated APC enrichment from line scans of 13 neurons fixed after 1.5, 2 or 3 days in vitro. The length of the line sampled was the same in the tips and shafts of both axon and next-longest neurite (which is the longest dendrite) of the same neuron. Total fluorescence along the line was measured in an unsectioned image, so differences in cytoplasmic volume would affect fluorescence quantification; however, because axonal growth cones are thinner
than axon shafts (Lee and Hollenbeck, 2003), this effect would only lead to underestimation of the APC tip enrichment observed qualitatively.

To quantify mutant β-catenin expression levels, PC12 cells transfected with CFP-β-catΔC-term or CFP-β-catenin were enriched for expressing cells by antibiotic selection and FACS sorting. After treating with NGF for 24 hours, cells were fixed, co-stained with APC and imaged through a GFP filter for the same exposure time (2 seconds). Each non-contacting cell was outlined, and mean fluorescence intensity and length of longest neurite were measured with ImageJ software.

Results

APC localizes to sites of neurite growth

To examine a role for APC in neurite growth, we first asked if APC was localized to distal tips of neurites, where microtubule dynamics are required for neurite growth (Tanaka et al., 1995). APC was enriched at the tip of the axonal growth cone of hippocampal neurons (Fig. 1A). Note that in this deconvolved image APC formed discrete clusters that localized along microtubules and at the distal tips of microtubules (Fig. 1A). These data confirm that APC is localized to the distal tip of axons where it might play a role with microtubules in regulating axon outgrowth (Shimomura et al., 2005; Zhou et al., 2004).

We next examined whether the enrichment of APC at the tip of neurites changed as neurons developed polarity, that is, differentiated one of several apparently equivalent neurites into a distinct long axon. We designated that a neuron was polarized if it met two criteria: (1) the axon marker protein dephospho-tau was enriched in only one neurite; and (2) the longest neurite, the axon, was at least twice as long as the next-longest neurite (Shi et al., 2003; Mandell and Banker, 1996). APC distribution was determined by immunostaining of primary hippocampal neurons 36 hours after dissociation and plating. Cultures contained both unpolarized early-stage neurons and polarized later-stage neurons. In unpolarized neurons, APC was localized at the cell body, along neurites emanating from the cell body, and enriched at the tips of several neurites. In contrast, APC was enriched specifically at the tip of the axon in polarized neurons; note that at this stage of development, dephospho-tau-negative neurites, which will become dendrites, contained relatively little or no APC staining at their distal tips (Fig. 1B) (Shi et al., 2004). The relative amount of APC along neurites was quantified by measuring the intensity of APC staining along a line through the neurite (Fig. 1C). APC enrichment was observed at the distal end of three neurites of the unpolarized neuron, N2, N3, N4, as well as the axon of the polarized neuron.

We tested whether APC enrichment was greater at the tips of faster-growing axons than at the tips of dendrites, as exemplified by the polarized neuron shown in Fig. 1C. For each line scan of APC staining intensity along an axon or next-longest neurite, we calculated APC enrichment as the ratio of maximum APC intensity in the tip to that in an equivalent length of the shaft. In dendrites, APC was only 1.5 times as intense as APC staining in the shaft, representing minimal APC enrichment at the growing tip (Fig. 1D). In contrast, in axons, APC staining was 3.4 times (range, 2-6 times) as intense as APC staining in the shaft. Given that axonal growth cones are usually flatter than the axon shaft (Lee and Hollenbeck, 2003), the actual APC enrichment per volume in the axonal growth cone is probably even greater. On average, axons showed 2.2-fold greater APC enrichment at the tip compared to the longest dendrites (Fig. 1D). Therefore, APC enrichment correlated with sites of increased neurite growth in polarized neurons.
APC enrichment in axonal growth cone precedes increased axonal elongation

We reasoned that if APC enrichment were required for the increased growth that differentiates the future axon from other neurites, it would be enriched early in axonal outgrowth and exclusively at the tip of the future axon. In a polarized neuron, the axon is defined as the neurite that meets both length and tau criteria, i.e. is exclusively enriched for dephospho-tau staining and is longer than twice the next-longest neurite. Of these two criteria for axon identification, dephospho-tau enrichment was more likely to first mark the future axon, because specification of a single future axon only by dephospho-tau enrichment was four times more prevalent than specification only by two-fold increased length (113 cells polarized only by tau criterion, compared to 31 cells polarized only by length criterion, from the experiments in Fig. 2). Therefore, we defined polarizing neurons as those with a ‘future axon’ specified by exclusive enrichment of dephospho-tau without twofold length increase over other neurites.

The distribution of APC among neurite tips was examined in polarizing neurons with a future axon marked only by dephospho-tau enrichment (Fig. 2B, middle bar). Polarizing neurons had APC enriched only or mostly in the tip of the longest neurite of the neuron (66±2.7% neurons); more than 90% of these neurites were dephospho-tau-enriched and therefore the future axons. Similarly, in the majority of fully polarized neurons, APC was preferentially enriched in the longest neurite (90±3.3%; Fig. 2B, right bar, black), which was the dephospho-tau-enriched axon. In contrast, most unpolarized neurons without a dephospho-tau-marked future axon had APC enriched at the tips of multiple neurites (Fig. 2B, left bar, gray), as in the example in Fig. 1C. Therefore, the accumulation of APC changes from several neurite tips to the tip of the future axon, which is marked by dephospho-tau, even before the increase in axonal length.

We conclude that early during neurite outgrowth APC was enriched at the tips of all growing neurites, whereas later during neuronal polarization APC localized preferentially to the future axon. Significantly, APC enrichment at the tip of a single dephospho-tau-marked neurite preceded much of the increased growth that distinguishes that neurite as an axon. Thus, growth of early neurites and growth of the future axon both involve APC tip enrichment. Enrichment of APC at tips of early neurites in unpolarized hippocampal neurons, together with previously published data that APC depletion inhibits initial neurite outgrowth in PC12 cells (Dobashi et al., 2000) indicate that APC is required at the tips of all growing neurites.

Endogenous β-catenin partially colocalizes with APC

Neurite growth mediated by nerve growth factor (NGF) involves local inhibition of GSK3β, which phosphorylates APC and modifies its binding to β-catenin and microtubules (Zhou et al., 2004; Rubinfeld et al., 1996; Zumbrunn et al., 2001). These data indicate that β-catenin could regulate APC during neurite growth.

To probe the role of APC/β-catenin during early neurite growth, we took advantage of the ability to induce neurite formation, and to express exogenous proteins prior to inducing neurite formation, in the well-studied PC12 pheochromocytoma cell line (Greene et al., 1998). PC12 cells induced with NGF are a good model for APC cluster function in hippocampal neurons because, as in neurons (Fig. 3A, Fig. 1A) (Shi et al., 2004), PC12 cells accumulated APC clusters at the tips of neurites (Fig. 3B).

Endogenous β-catenin and APC occasionally overlapped at the tips of neurites in hippocampal neurons (Fig. 3A) and in PC12 cells (Fig. 3B). Some neurite tips had no enrichment of β-catenin (Fig. 3A, arrow), whereas some had β-catenin enriched in the entire growth cone (Fig. 3A, solid arrowhead) or in a part of the growth cone just proximal to and
partly overlapping with enriched APC (Fig. 3A, hollow arrowheads). Partial overlap of APC and β-catenin was most common in both hippocampal neurons and PC12 cells (Fig. 3A,B).

**Stabilized β-catenin strongly co-clusters with APC**

We asked whether β-catenin that cannot be degraded upon binding to APC colocalized more strongly with APC than endogenous β-catenin and thereby would amplify any effect of endogenous β-catenin on APC function. We expressed ‘stabilized’ β-catenin, termed *β*-catenin, which lacks GSK3β phosphorylation sites (see Materials and Methods) and thus cannot be targeted for ubiquitin-proteasome degradation. Note that in PC12 cells, Wnt-induced β-catenin accumulation also activates β-catenin-mediated transcription (Chou et al., 2000) and prevents NGF-induced neurite outgrowth (Shackleford et al., 1993). Thus, to distinguish between transcriptional and other effects of β-catenin on neurite outgrowth, we expressed *β*-catΔC-term, a stabilized β-catenin mutant lacking the transcriptional transactivation domain, and a non-stabilized chimeric protein, termed β-cat-eng, in which the transcriptional transactivation domain was replaced with an active transcriptional repression domain from the Drosophila engrailed gene (Montross et al., 2000).

Each of these three β-catenin mutants often co-clustered with APC at the distal ends of microtubules at the neurite tip in PC12 cells (Fig. 4). In hippocampal neurons, *β*-catenin was enriched at the tips of neurite extensions (Yu and Malenka, 2003), and each β-catenin mutant co-clustered with APC (V.V., unpublished observations), similar to the localization in PC12 cells. This result indicates that transient overexpression of non-stabilized β-catenin (β-cat-eng) is sufficient to induce its accumulation in the APC complex.

We then confirmed the activity of β-catenin mutants in Tcf/Lef-mediated transcription. β-catenin stabilized by Wnt binds to and coactivates transcription with Tcf/Lef (van de Wetering et al., 1997). To test transcriptional activity of β-catenin mutants, we coexpressed a luciferase reporter with Tcf/Lef-binding sites. As expected, full-length stabilized β-catenin (*β*-catenin) activated Tcf/Lef-dependent transcription of the reporter, in a manner dependent on functional Tcf/Lef-responsive elements in the reporter (Fig. 5A). Neither *β*-catΔC-term nor β-cat-eng activated Tcf/Lef-dependent transcription (Fig. 5A).

**Stabilized β-catenin inhibits neurite growth**

We tested whether stabilized β-catenin, which accumulated with APC clusters at neurite tips independent of Tcf-mediated transcriptional activity, affected formation of neurite extensions. In PC12 cells, expression of stabilized β-catenin, compared with GFP as a control, significantly decreased the proportion of cells that formed neurites in response to NGF (*β*-catenin in Fig. 5B). To test the effect of stabilized β-catenin on neurite elongation, we quantified the proportion of cells with long neurites, defined as neurites at least twice the length of the cell body width (Fig. 5C). Expression of stabilized β-catenin also decreased the proportion of cells with long neurites (Fig. 5C). Thus, stabilized β-catenin inhibits neurite initiation as well as elongation (Table 1).

To determine whether inhibition of neurite extension required Tcf/Lef-mediated transcription, we compared the effects of different β-catenin mutants. Expression of transcriptionally inactive β-catenin (*β*-catΔC-term or β-cat-eng) significantly reduced the percentage of cells with neurites (Fig. 5B), and the percentage of cells with neurites longer than twice the cell body width (Fig. 5C). Together these data indicate that accumulation of β-catenin at APC clusters inhibits neurite extension.

To clarify the contribution of structural and transcriptional functions of β-catenin to neurite inhibition, we calculated inhibition of neurite formation as the proportion of cells with neurites compared to GFP-expressing cells. Expression of *β*-catenin and β-cat-eng proteins

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inhibits neurite formation by 59% and 25%, respectively (from Fig. 5B). Therefore, Tcf-mediated transcription, which is activated by *β-catenin but not β-cat-eng, accounts for about half of the inhibition of neurite formation by β-catenin, while nontranscriptional effects account for the other half. Expression of *β-catenin and β-cat-eng proteins inhibit neurite elongation (Fig. 5C) by 89% and 70%, respectively, indicating a much greater role for structural (non-transcriptional) effects of β-catenin in neurite elongation compared to neurite initiation. Furthermore, both transcriptionally active and transcriptionally inactive β-catenin mutants inhibited neurite growth in proportion to protein expression level (Fig. 5D,E). Cells expressing high levels of *β-catenin or *β-catΔC-term (i.e. an arbitrary fluorescence value greater than 50) formed significantly shorter neurites than cells expressing low amounts of stabilized β-catenin (Table 2). Note the similarity in distribution of expression levels and effects on neurite growth inhibition in cells expressing *β-catenin or *β-catΔC-term (Fig. 5E). We conclude that stabilized β-catenin has structural effects, in addition to its transcriptional effects, that inhibit neurite growth.

Discussion

We propose that endogenous β-catenin is rapidly turned over at APC clusters in neurite tips and that this regulates APC function in neurite growth (see Fig. 6). APC clusters in neuronal extensions are very similar to the previously described APC clusters at microtubule ends in epithelial extensions (Nathke et al., 1996). In epithelial extensions, proteasome inhibition causes accumulation of endogenous β-catenin and axin, another component of the APC destruction complex (Hart et al., 1998; Ikeda et al., 1998) at APC clusters (A.B., unpublished results). This further indicates that APC clusters represent part of the destruction complex engaged in β-catenin turnover. Overexpression or stabilization of β-catenin causes accumulation of β-catenin in the APC clusters and may disrupt the normal function of APC clusters in promoting neurite outgrowth.

APC associated with microtubules marks the future axon

Axon growth is an example of neurite growth, and axon specification involves increased relative growth of one neurite. APC within axonal growth cones is at distal microtubule ends (Fig. 1A), consistent with recent work showing that microtubule-associated APC at axon tips functions in axon growth (Zhou et al., 2004). Overexpression of APC dominant-negative mutants disrupts axon specification and axon growth (Shi et al., 2004; Zhou et al., 2004), but it is unclear whether enrichment of endogenous APC predicts increased neurite growth. Here we show that APC is enriched at the tip of a neurite marked to be the axon by dephospho-tau staining, even before that neurite has grown significantly longer than neurites destined to become dendrites. This result indicates that APC function is important early in axonal outgrowth.

What initially signals one of the morphologically equivalent neurites to become the axon? Par3 and Par6 are important for axon formation (Shi et al., 2003). Like APC, Par3 localizes to the tips of several neurites in unpolarized hippocampal neurons and the tip of the axon in polarized hippocampal neurons (Shi et al., 2003). Par3 localization to the axon tip depends on APC localization (Shi et al., 2004), so APC is an earlier signal for axon determination. Although NGF signaling results in both APC localization to the axon tip and increased microtubule density at the axon tip (Zhou et al., 2004), it is unclear if APC and Par3 and Par6 form a complex at the axon tip before microtubule recruitment.

Several other microtubule-associated proteins are also important for axon determination (Baas, 2002). Tau, for example, binds and bundles microtubules, unless phosphorylated by GSK3β (Wagner et al., 1996). However, GSK3β inhibition promoted changes in APC localization and microtubule organization such that APC is a more likely substrate for
GSK3β during axon growth (Zhou et al., 2004). Our data leave open the possibility that APC localizes preferentially to the future axon even before dephospho-tau enrichment. APC enrichment at neurite tips definitely does not require dephospho-tau, or dephospho-tau-mediated microtubule changes, because unpolarized neurons have little dephospho-tau staining and still localize APC to neurite tips (Fig. 1B,C).

Importantly, this APC localization to neurite tips of unpolarized neurons indicates that APC may be involved in initial outgrowth of neurites as well as increased growth characteristic of the axon. To separate neurite formation from axon differentiation, we studied PC12 cells, which require APC to form neurites (Dobashi et al., 2000), but do not differentiate a morphologically distinct axon.

β-catenin associates with APC

In the absence of Wnt signaling, APC forms a multi-protein ‘β-catenin degradation complex’ that includes GSK3β, axin and PP2A (Ikeda et al., 2000). In this complex, GSK3β phosphorylates β-catenin and APC. Of the components in this complex that could mediate APC cluster function in neurites, spatially regulated GSK3β activity is required for axon growth, morphology and differentiation (Jiang et al., 2005; Goold and Gordon-Weeks, 2004) (see also Fig. 6). However, little is known about the effect of β-catenin on initial neurite outgrowth. Both β-catenin and APC localize generally to neurite tips in primary mouse neuronal cultures (Morrison et al., 1997). Here we examined localization of β-catenin in APC clusters using deconvolution microscopy. We show varying degrees of colocalization of endogenous APC and β-catenin in hippocampal neurons and PC12 cells (Fig. 3, see also supplementary material Fig. S1). This variability may indicate transient interaction of APC and β-catenin as endogenous β-catenin is degraded or binds with lower affinity to unphosphorylated APC when NGF inhibits GSK3β (see Fig. 6). The model that β-catenin degradation and turnover in the APC complex regulate β-catenin/APC colocalization is supported by the enrichment of undegradable (stabilized) or overexpressed β-catenin mutants in APC clusters at the tips of neurites (Fig. 4). In epithelial cells, stabilized β-catenin similarly accumulates at APC clusters at extension tips (Barth et al., 1997; Nathke et al., 1996), and stabilized β-catenin that associates with APC has delayed turnover compared to normal β-catenin (Barth et al., 1999). APC co-immunoprecipitated a stabilized β-catenin mutant identical to *β-catenin (with the exception of the GFP tag) from stably-expressing MDCK epithelial cells, even 18 hours after expression of stabilized β-catenin was repressed by doxycycline (Barth et al., 1999), indicating a very stable association of this mutant β-catenin with APC.

Stabilized β-catenin inhibits neurite outgrowth

We show that expression of stabilized β-catenin decreased neurite initiation and elongation in NGF-treated PC12 cells (Fig. 5). Several mechanisms could explain how stabilized β-catenin inhibits neurite outgrowth in PC12 cells. When β-catenin is stabilized by Wnt signals it can promote cadherin-mediated cell-cell adhesion (Hinck et al., 1994) in addition to Tcf/Lef-mediated transcription. Experiments expressing stabilized β-catenin in whole animals or in neuronal cultures directly contacting glial cells may mask the role of β-catenin in the APC complex with its role in adhesion (Yu and Malenka, 2004; Loureiro et al., 2001; Elul et al., 2003). Previous work on the role of β-catenin in branching of axons and dendrites uses neurons in direct cell-cell contact with a glial feeder layer, and β-catenin is thought to require N-cadherin for this effect (Yu and Malenka, 2003; Yu and Malenka, 2004). PC12 cells do not form distinct axons and dendrites (Greene et al., 1998) and, if treated with NGF for only 3-4 days as in our experiments, do not form significantly branched neurites (see Fig. 3B and Fig. 4). Furthermore, we minimized the effect of stabilized β-catenin on cell-cell adhesion by counting only PC12 cells that had not contacted other cells. Finally, neurite...
extension from PC12 cells was also inhibited by expressing stabilized β-catenin with or without the α-catenin-binding domain, which is necessary for strengthening cell-cell adhesions (A.B., unpublished observations). Thus, our experiments investigate a different structural role of β-catenin separate from its role in cell-cell adhesion.

However, we do not exclude the possibility that cadherin-mediated adhesion may regulate the amount of β-catenin in the APC complex when neurite/axon tips contact other cells. Binding of β-catenin to APC is mutually exclusive with its binding to cadherin because cadherin and APC compete for the same binding site in β-catenin (Ha et al., 2004). Therefore, recruitment of β-catenin to cell adhesion sites could regulate the amount of β-catenin associated with APC at neurite/axon tips and, thereby promote neurite extension on glial cells.

To test whether β-catenin accumulation inhibited neurite growth through Tcf/Lef-mediated gene transcription, we used two β-catenin mutants that could not induce transcription. Transcriptionally inactive β-catenins showed significant inhibition of neurite formation and elongation. Thus, inhibition by stabilized β-catenin did not require Tcf/Lef-mediated transcription. In support of β-catenin affecting the cytoskeleton instead, and specifically through microtubule-associated APC, transcriptionally active and inactive β-catenins colocalized with APC clusters at distal microtubule ends.

APC regulates microtubule growth, bundling and stability (Munemitsu et al., 1994; Zumbrunn et al., 2001). Neurite growth requires cycles of microtubule bundling and unbinding, and balance between microtubule stabilization and destabilization (Goold and Gordon-Weeks, 2004; Tanaka and Kirschner, 1991; Tanaka et al., 1995). The direct role of APC in these processes could be inhibited by constitutive rather than cyclical binding to β-catenin (Ha et al., 2004). The β-catenin-binding region of APC overlaps with the GSK3β-phosphorylated region; phosphorylation of APC decreases its ability to bundle microtubules in vitro (Zumbrunn et al., 2001). Similar to phosphorylation by GSK3β, binding of stabilized β-catenin to this region of APC may decrease interaction of APC with microtubules (see also Fig. 6).

Note that in our experiments, exogenous stabilized β-catenin prevents NGF-stimulated neurite growth, whereas β-catenin stabilization that is assumed to result from local inactivation of GSK3β after NGF stimulation allows neurite growth. Before NGF stimulation, active GSK3β phosphorylates APC, increasing its affinity for β-catenin (Rubinfeld et al., 1996) and inhibiting APC function in microtubule bundling (Zumbrunn et al., 2001). Turnover of β-catenin from this high-affinity APC/β-catenin complex is probably mediated by protein degradation (Fig. 6A). Conversely, after NGF stimulation, inactive GSK3β cannot phosphorylate APC or β-catenin, and therefore the endogenous β-catenin that accumulates does not bind as strongly to unphosphorylated APC (Ha et al., 2004), allowing APC to dissociate and bundle microtubules (Fig. 6B). Accordingly, NGF stimulation increases the localization of APC to neurite tips but does not significantly increase the level of endogenous β-catenin in APC clusters (see supplementary material Fig. S1).

If stabilization of β-catenin at APC clusters interferes with APC function and inhibits extension formation, and transient interaction of endogenous β-catenin with APC clusters properly regulates extension formation, the absence of β-catenin at APC clusters should inappropriately increase microtubule growth and extension formation. Although transfection of β-catenin siRNA was not successful in PC12 cells, siRNA-induced β-catenin knockdown in HeLa cells did result, as expected, in increased cell extension. That is, the average cell elongation (measured as the major axis of the ellipse best fitted to the cell) of HeLa cells
treated with a pool of siRNAs targeting β-catenin was 115±21.6 μm, whereas that of cells treated with a control siRNA against GFP was 66±5.1 μm (see Fig. S2 in supplementary material). Therefore, stabilized β-catenin or reduced APC (Dobashi et al., 2000) decreases cell extension, and reduced β-catenin increases cell extension. In HeLa cells depleted of β-catenin, APC clusters still form at extension tips (arrowheads in Fig. S2, supplementary material) and presumably are free to bundle microtubules.

APC is highly expressed in developing rat brain during neuronal maturation (Bhat et al., 1994). Brain development involves physiological signals that modulate the effects of GSK3-β and APC. For example, both Wnt and NGF signaling inactivate GSK3β (Logan and Nusse, 2004; Zhou et al., 2004), and local inactivation of GSK3β is required for APC localization to microtubule ends in extension tips in astrocytes and neurons (Etienne-Manneville and Hall, 2003; Zhou et al., 2004). Axons turn toward Wnt in a time frame suggestive of structural rather than transcriptional effects (Lyuksyutova et al., 2003). Other physiological signals inhibiting axonal growth, such as semaphorin, activate GSK3β (Eickholt et al., 2002). Furthermore, cadherins are involved in promoting axonal outgrowth on non-neuronal cells (Matsunaga et al., 1988). Future studies will determine how binding of β-catenin to APC clusters in growth cones responding to these diverse signals regulates microtubule changes necessary for neurite and axon outgrowth and potentially neuronal targeting and synaptogenesis.

Supplementary Material

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Fig. 1. APC in neurite tips is along microtubules and changes during neuronal polarization. (A) An axonal growth cone of a hippocampal neuron immunostained for tubulin and APC. Projection of deconvolved planes is shown. (Note that deconvolution resolves APC enrichment into puncta.) (B) Immunostaining for the axon marker dephospho-tau (Tau) and APC show APC enrichment at several neurite tips in an unpolarized neuron and only at the tip of the axon (arrowhead), which is marked by dephospho-tau staining (arrow), in a polarized neuron. Magnified images below also show phase-contrast images of neurite tips N3 and N4 from the unpolarized neuron and the axon tip from the polarized neuron. A, axon; D, dendrite; N, neurite. (C) Line scans quantifying fluorescence intensity of APC (red) and dephospho-tau (Tau, green) along corresponding neurites from B. (D) From line scans of polarized neurons (including that in C), we calculated the ratio of maximum APC intensity at the neurite tip to that of equivalent length of the neurite shaft. Shown is the mean ±s.e.m. of the axon and the longest dendrite of 13 neurons. ***P<0.001 compared to APC enrichment in axons by Student’s t-test. Bar, 2 μm (A); 20 μm (whole-cell images in B); 2.5 μm (magnified images in B).
Fig. 2.
APC is enriched at the axon tip prior to significant length increase. (A) Hippocampal neurons immunostained for APC and dephospho-tau were classified by two neuronal polarization criteria: length + indicates that the length of the longest neurite is greater than or equal to twice the length of the next-longest neurite; tau + indicates increased dephospho-tau staining (represented by green) in one of the neurites. Polarizing neurons were defined as dephospho-tau +, length − neurons (see Results). (B) Unpolarized, polarizing and polarized neurons (left, middle and right bars, respectively) were further classified by which neurite tips had enriched APC. Black, longest extension only or most intensely; gray, two or more neurites evenly; white, no neurites or only one neurite other than the longest. Shown is the mean±s.e.m. of three independent experiments; n, total number of cells in each category.
Fig. 3.
Localization of endogenous β-catenin with APC at neurite tips. APC and β-catenin immunostaining in (A) hippocampal neurons (2 days in vitro) and (B) PC12 cells (treated for 4 days with NGF) as deconvolved planes. Arrow, no β-catenin enrichment at neurite tip; solid arrowhead, complete overlap of β-catenin enrichment with APC (see also magnified images i in A and i, ii and iii in B); open arrowhead, partial or no overlap (see also magnified images ii in A and i in B). Bar, 10 μm.
Fig. 4.
Stabilized β-catenin accumulates with APC clusters independently of the transcriptional activation domain. Transfected PC12 cells were treated with NGF for 3-4 days to induce extensions. (A,B) Deconvolved images of immunostaining for APC (first column) and KT3 epitope tag of *β-catenin or *β-catΔC-term (middle column). (C) Immunostaining for tubulin (first column) and myc tag of β-cat-eng (middle column). *β-catenin, full-length stabilized β-catenin; *β-catΔC-term, stabilized β-catenin lacking the C-terminal transcriptional transactivation domain; β-cat-eng, β-catenin lacking the transactivation domain and fused to a transcriptional repression domain from Engrailed. Arrows indicate neurite tips magnified in inset and arrowheads indicate all other neurite tips. Compared to endogenous β-catenin (Fig. 3B), stabilized β-catenin is enriched in APC clusters at neurite tips (A,B). Bar, 10 μm.
Fig. 5.
Transcriptionally active and inactive stabilized β-catenin mutants inhibit neurite outgrowth. PC12 cells were transfected with GFP alone or one of three stabilized β-catenin constructs shown on the left. Transcriptional activation (A) and NGF-induced neurite formation (B) and neurite growth (C) were measured. (A) Co-transfection of pTOPFLASH Tcf/Lef-driven luciferase reporter (gray bars, TOPFLASH) indicates that only full-length stabilized β-catenin (*β-catenin) increased Tcf/Lef-mediated transcription. pFOPFLASH (black bars, FOPFLASH) is a negative control for the reporter assay, with mutated Tcf/Lef-binding sites. Luciferase activity was normalized for transfection efficiency (see Materials and Methods). Shown is the mean±s.e.m. of three independent experiments. (B,C) One day after transfection, PC12 cells were passaged and treated with NGF for 3.5 days. Cells were stained for tubulin and >250 cells were counted for the presence of neurites (see Table 1). Expression of each stabilized β-catenin mutant reduced the percentage of cells with neurites of any length (B) and cells with neurites longer than twice cell body width (C). For the GFP control, *β-catenin and β-catenin-eng bars show the mean±s.e.m. of three independent experiments. For *β-cateninΔC-term, the bar represents the average of two independent experiments. Each mutant was significantly different from the GFP control by binomial test (*P<0.05). (D) Representative images of PC12 cells expressing CFP-tagged *β-cateninΔC-term and induced with NGF for 24 hours. High-expressing cells (arrowheads) usually had short or no neurites in contrast to untransfected cells. (E) Quantification of neurite length in cells, including those in D, which express varying levels of *β-cateninΔC-term and *β-catenin. For each cell, higher mean fluorescence intensity correlated with decreased length of the longest neurite. Bar, 10 μm.
Fig. 6.
A model for APC/β-catenin interaction during neurite outgrowth. (A) In uninduced cells, active GSK3β may phosphorylate APC and β-catenin. Phosphorylated APC (APC\(^P\)) has a higher affinity for β-catenin (Rubinfeld et al., 1996) and a lower affinity for microtubules (striped bar) (Zumbrunn et al., 2001). Phosphorylated β-catenin (β\(^P\)) is degraded. (B) In NGF-induced cells, NGF locally inactivates GSK3β (Zhou et al., 2004), so GSK3β may be inactive at the cell periphery and eventually the neurite tips, leading to local β-catenin stabilization. Unphosphorylated APC has lower affinity for β-catenin, so β-catenin in the APC complex turns over rapidly. Unphosphorylated APC also binds and bundles microtubules, and this function at neurite tips may be regulated by its binding to β-catenin. The ratio of free versus β-catenin-bound APC could thereby determine microtubule bundling and neurite growth rate. The equilibrium would further shift toward neurite growth if β-catenin levels are decreased, for example by being sequestered at adhesion sites, or if APC levels are increased, for example in response to NGF (Dobashi et al., 1996).
Table 1
Transcriptionally active and inactive stabilized β-catenin mutants inhibit neurite outgrowth

|                | Number of cells | Cells with neurites (%) | Cells with long neurites (%) |
|----------------|-----------------|-------------------------|-----------------------------|
| GFP            | 468             | 73.3                    | 17.7                        |
| *β-cat         | 491             | 34.8                    | 1.8                         |
| *β-catΔC-term  | 310             | 58.1                    | 7.7                         |
| β-cat-eng      | 266             | 54.9                    | 4.9                         |

Shown is the total number of transfected PC12 cells counted in two to three independent experiments (see Fig. 5B,C), the percentage with neurites of any length (Cells with neurites) and the percentage with neurites longer than twice cell body width (Cells with long neurites).
### Table 2
**Dose-dependent effect of stabilized β-catenin on neurite length**

| Stabilized β-catenin | Expression level | Longest neurite (μm) |
|----------------------|------------------|----------------------|
| *β-catΔC-term        | Low              | 16.6±2.0             |
| *β-catΔC-term        | High             | 4.3±2.0              |
| *β-cat               | Low              | 14.7±1.9             |
| *β-cat               | High             | 2.3±2.3              |

Data points from Fig. 5E were grouped by expression level of fluorescently tagged stabilized β-catenin. Low, <50 fluorescence intensity units; High, >50 fluorescence intensity units. Mean neurite length of the longest neurites of high-expressing cells was significantly shorter than that of low-expressing cells (t-test, P<0.01). This dose-dependence was significant for both transcriptionally inactive (*β-catΔC-term, n=12 cells total) and transcriptionally active (*β-cat, n=11 cells total) stabilized β-catenin mutants. Data are the mean lengths of the longest neurites of the cell ±s.e.m.