

Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 β phosphorylation

Jürg Zumbunn*, Kazuhisa Kinoshita[†], Anthony A. Hyman[†] and Inke S. Näthke*[‡]

Truncation mutations in the adenomatous polyposis coli protein (APC) are responsible for familial polyposis, a form of inherited colon cancer. In addition to its role in mediating β -catenin degradation in the Wnt signaling pathway, APC plays a role in regulating microtubules. This was suggested by its localization to the end of dynamic microtubules in actively migrating areas of cells and by the apparent correlation between the dissociation of APC from polymerizing microtubules and their subsequent depolymerization [1, 2]. The microtubule binding domain is deleted in the transforming mutations of APC [3, 4]; however, the direct effect of APC protein on microtubules has never been examined. Here we show that binding of APC to microtubules increases microtubule stability in vivo and in vitro. Deleting the previously identified microtubule binding site from the C-terminal domain of APC does not eliminate its binding to microtubules but decreases the ability of APC to stabilize them significantly. The interaction of APC with microtubules is decreased by phosphorylation of APC by GSK3 β . These data confirm the hypothesis that APC is involved in stabilizing microtubule ends. They also suggest that binding of APC to microtubules is mediated by at least two distinct sites and is regulated by phosphorylation.

Addresses: *School of Life Sciences, University of Dundee, Dundee, Scotland. [†]European Molecular Biology Laboratory, Heidelberg, Germany.

[‡]Correspondence: Inke S. Näthke
E-mail: i.s.nathke@dundee.ac.uk

Received: 24 July 2000
Revised: 14 November 2000
Accepted: 15 November 2000

Published: 9 January 2001

Current Biology 2001, 11:44–49

0960-9822/01/\$ – see front matter
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Results and discussion

APC protein stabilizes microtubules in vitro

To measure the effect of APC on microtubule stability in vitro, microtubules were polymerized in the absence or presence of APC4 protein fragment (residues 1034–2843,

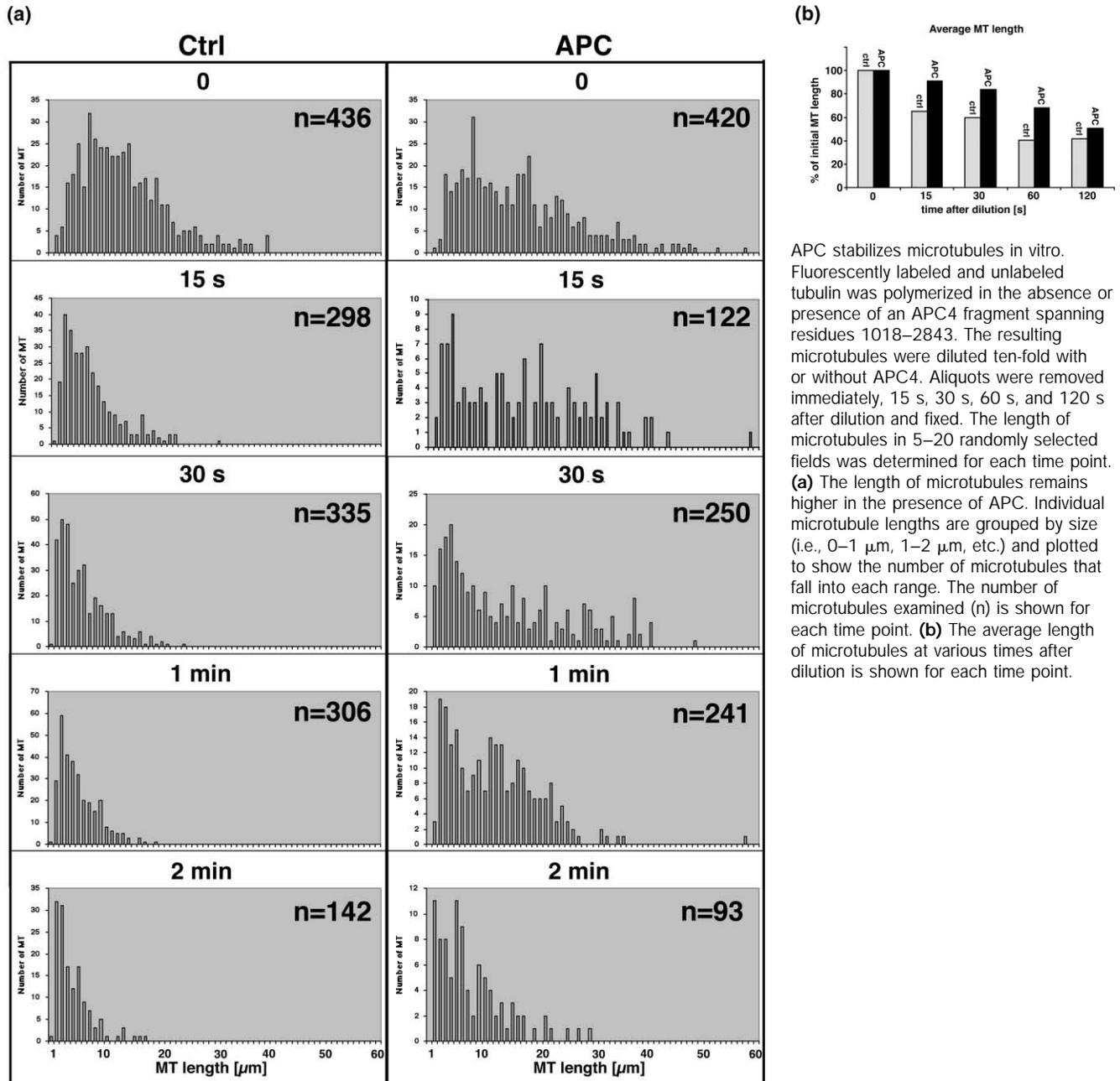
Figure 2) [5] at concentrations of APC4 that do not induce microtubule bundling. These microtubules were then diluted into buffer alone or buffer containing APC4 protein corresponding to the presence of APC4 in the assembly reaction. The length of microtubules in 5–20 randomly selected fields was recorded. Figure 1a shows the distribution of microtubule lengths immediately after polymerized microtubules were diluted or 15, 30, 60, and 120 s later. The data show that in the presence of APC4, microtubules remained longer and thus depolymerized more slowly. Consistent with this, the average length of the remaining microtubules after various times was greater in the presence of APC4 protein than in control samples (Figure 1b), confirming that APC stabilizes microtubules. When no APC4 was included in the dilution buffer, the rate of microtubule disappearance was independent of whether APC4 had been included in the assembly step (data not shown). The average microtubule length immediately after samples were diluted was larger in samples containing APC4 by about 20% (data not shown). The microtubule length is expressed as a percentage of the value obtained at time 0 to correct for this difference, which most likely reflects the increased depolymerization in the absence of APC4 that occurs during the short time (approximately 5–8 s) required for handling before fixation.

APC protein stabilizes microtubules in vivo

To measure the effect of APC on microtubules in vivo, the distribution of a variety of GFP-tagged APC constructs (summarized in Figure 2 and Table 1) was compared to that of tubulin in the presence and absence of nocodazole (Figure 3 and summarized in Table 1). GFP-APC protein localized to clusters near the cell membrane and also decorated the microtubule lattice, similar to endogenous APC protein (Figure 3a,b). The C-terminal third of APC, C-APC, only decorated microtubules and did not accumulate in clusters (Figure 3i,k), consistent with previous findings in other cell types [1, 5, 6]. Microtubules in cells expressing APC or C-APC were protected against depolymerization by nocodazole treatment confirming that APC stabilizes microtubules (Figure 3e,f and n,o).

The microtubule binding site of APC has been mapped to a highly positively charged region in the C-terminal third of APC [1, 5, 6] and an APC fragment containing residues 2219–2580 binds microtubules in vitro [7]. Deleting this domain from APC altered but did not abolish the

Figure 1



APC stabilizes microtubules in vitro. Fluorescently labeled and unlabeled tubulin was polymerized in the absence or presence of an APC4 fragment spanning residues 1018–2843. The resulting microtubules were diluted ten-fold with or without APC4. Aliquots were removed immediately, 15 s, 30 s, 60 s, and 120 s after dilution and fixed. The length of microtubules in 5–20 randomly selected fields was determined for each time point. **(a)** The length of microtubules remains higher in the presence of APC. Individual microtubule lengths are grouped by size (i.e., 0–1 μm , 1–2 μm , etc.) and plotted to show the number of microtubules that fall into each range. The number of microtubules examined (n) is shown for each time point. **(b)** The average length of microtubules at various times after dilution is shown for each time point.

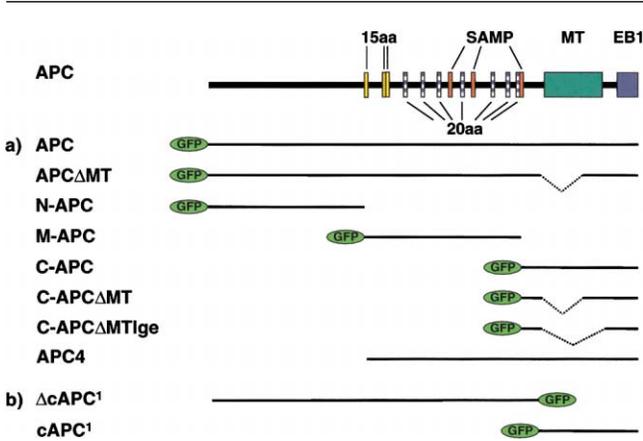
association of APC with microtubules. APC- Δ MT was restricted to clusters at the end of microtubules near the cell periphery (Figure 3c,d) and C-APC Δ MT still decorated the microtubule lattice, similar to C-APC (Figure 3l,m). Treatment with nocodazole caused the dispersal of APC Δ MT into small clusters that appeared to line the small number of remaining microtubules (Figure 3g,h). In general, deleting residues 2168–2451 resulted in a significant decrease in the ability of APC to stabilize microtubules toward nocodazole as judged by reduced number and length of microtubules in cells expressing APC Δ MT or

C-APC Δ MT compared to cells expressing APC or C-APC after nocodazole treatment (Figure 3 and Table 1).

Together, these data suggest that the basic region of APC contained within residues 2168–2451 is important for microtubule stabilization but that other regions in the APC molecule contribute to its ability to bind microtubules and to cluster at the dynamics end of microtubules.

It is important to note that the distribution of a C-terminal APC fragment missing residues 2168–2590, C- Δ MTlge, is

Figure 2

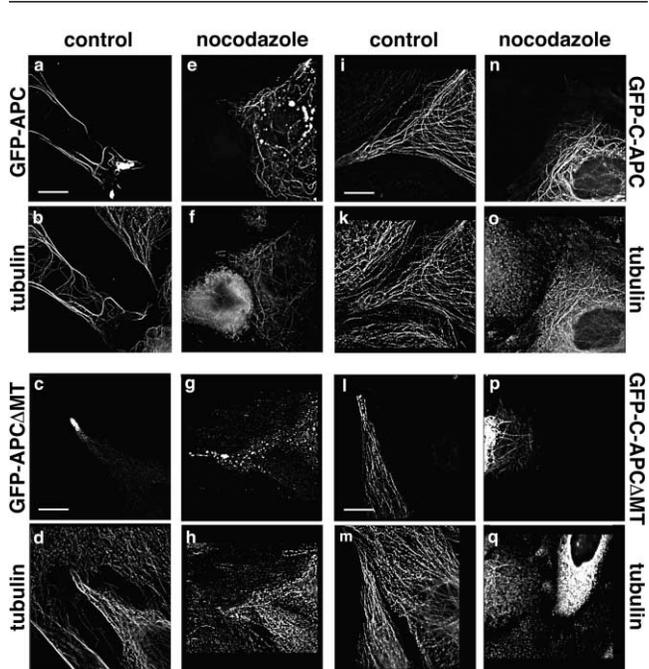


Schematic of GFP-APC constructs. A schematic of the APC sequence indicating the positions of the classical microtubule binding site (MT), the binding site for EB1 (EB1), the 15 and 20 amino acid repeats (15 aa and 20 aa) involved in binding to β -catenin, as well as SAMP repeats, binding sites for axin. **(a)** APC constructs used or **(b)** described in this manuscript are represented schematically. Deletions in the different constructs are marked by dashed lines. (Table 1 shows exact residue numbers.)

identical to that of C- Δ MT (see Supplementary material), confirming that deleting residues 2168–2451 is sufficient to inactivate the classical microtubule binding site.

Comparing the localization of the panel of APC fragments described in this and other manuscripts (see Table 1) and assuming that binding to microtubules is mediated by a linear sequence led to the prediction that a second microtubule binding site in APC resides between residues 2038–2158. Examining this region of APC revealed the presence of four consecutive lysine residues (amino acids 2049–2052 in the human sequence). Such lysine-rich regions are important for binding of motor proteins to microtubules and may also contribute to the microtubule binding of APC [8]. It is noteworthy that four positively charged residues are found in the sequences of human, mouse, rat, and *Xenopus* APC at this site, which falls into

Figure 3



APC stabilizes microtubules in vivo. PTK2 cells transiently expressing **(a,b and e,f)** GFP-tagged APC, **(c,d and g,h)** APC Δ MT lacking residues 2168–2451, the **(i, k and n, o)** C-APC, or **(l,m and p,q)** C-APC Δ MT lacking residues 2168–2451 were left untreated (**a–d** and **i–m**) or maintained at 4°C for 30 min and subsequently incubated in the presence of 10 μ g/ml nocodazole to depolymerize microtubules (**e–h** and **n–q**). Cells were fixed and processed for immunofluorescence using antibodies against tubulin (**b,f,k,o,d,h,m,q**). The localization of the GFP-tagged APC proteins was visualized directly (**a,e,i,n,c,g,l,p**); tubulin was detected with secondary antibodies conjugated to Texas red. Images were obtained using a Delta-Vision microscope. Projections of 5–8 0.2 μ m thick optical sections are shown. The scale bar represents 10 μ m.

the third SAMP repeat. SAMP repeats in APC provide binding sites for axin [9, 10]. Binding of β -catenin to APC is greatly enhanced by the presence of axin [11, 12], and our observations raise the possibility that APC bound to axin and β -catenin does not bind to microtubules and

Table 1

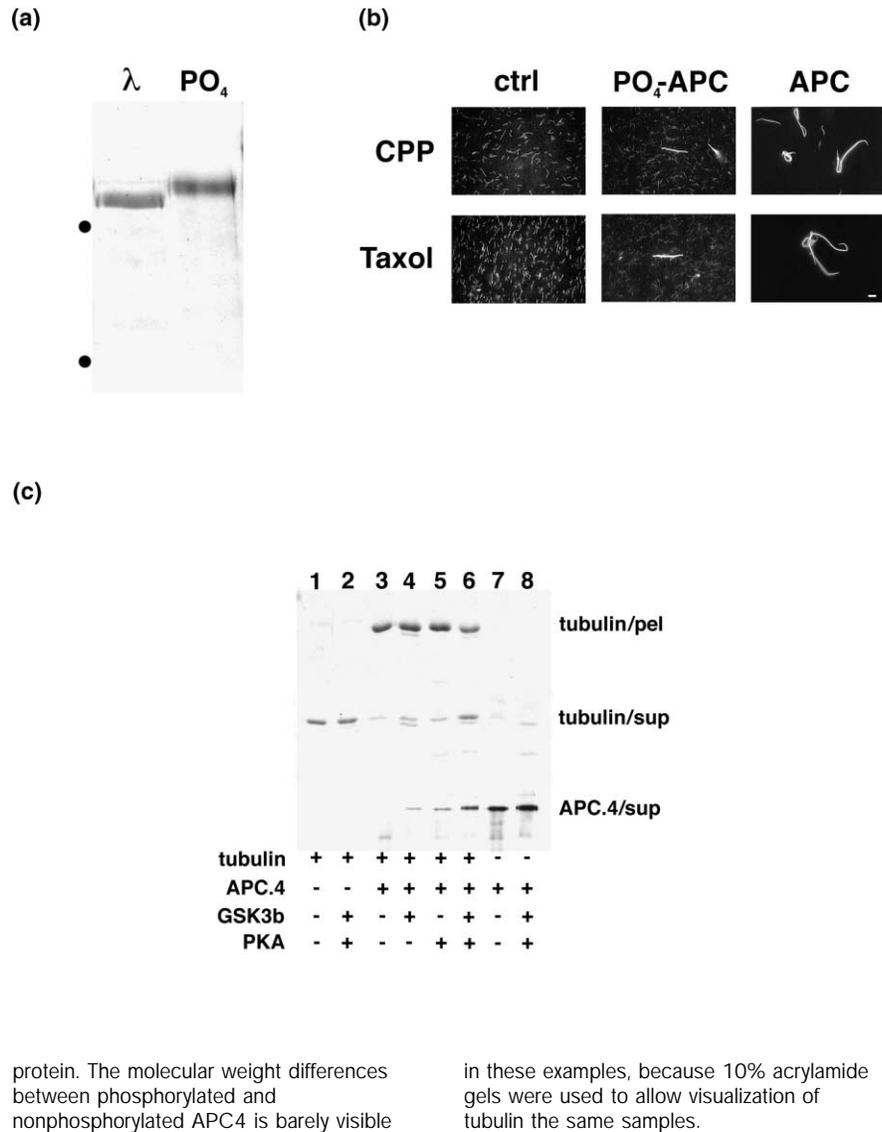
APC fragment; residues (name)	Colocalization w/MTs	Clusters	Increased MT stability
1-1018 (N-APC)*	–	–	–
1014-2038 (M-APC)*	–	–	–
2038-2843 (C-APC)	+	–	+
2-2843 (APC)	+	+	+
2-2167, 2452-2843 (APC Δ MT)	+	+	–
2038-2167, 2452-2843 (C-APC Δ MT)	+	–	–
2038-2167, 2591-2843 (C-APC Δ MTIge)	+	–	ND
1034-2843 (APC4) [†]	+	ND	ND
1-2158 (Δ cAPC) [†]	+	(+)	ND
2159-2843 (cAPC) [†]	+	(–)	ND

ND = not determined

*Näthke et al. unpublished data. [†][1]. [‡][5].

Figure 4

Phosphorylation by GSK3 β decreases APC-induced MT bundling. **(a)** Phosphorylation of APC with GSK3 β induces a significant mobility shift in APC. The APC4 fragment containing residues 1014–2843 was dephosphorylated with λ -phosphatase (λ) or rephosphorylated with a combination of PKA and GSK3 β (PO_4) prior to elution from affinity resin. (Prephosphorylation of APC with PKA is required for efficient phosphorylation by GSK3 β [15].) Ten microliters of each peak fraction was analyzed on a 6% acrylamide gel stained with Coomassie blue. The migration of molecular weight standards (200 and 120 kDa) is shown on the left. **(b)** Phosphorylated APC induces fewer and smaller bundles of microtubules. Taxol- or GMP-CPP (CPP)-stabilized microtubules were incubated in the presence of equal amounts of phosphorylated (PO_4 -APC) or dephosphorylated (APC) APC4 fragment. Samples were fixed and visualized using a Zeiss Axioplan microscope. Buffer controls (ctrl) contain single microtubules. The scale bar represents 10 μ m. **(c)** Phosphorylation by GSK3 β decreases the ability of APC to bundle microtubules. Dephosphorylated APC4 (lane 3) was rephosphorylated with GSK3 β (lane 4), PKA (lane 5), or a combination (lane 6) and added to GMP-CPP-stabilized microtubules. Microtubule bundles were separated from single microtubules by centrifugation. Tubulin in the supernatants and pellets was analyzed by PAGE and stained with Coomassie blue. APC4 in the supernatant fractions was detected by immunoblotting with APC-specific antibodies. To confirm that the presence of kinases did not affect microtubule bundling or APC4 solubility, control samples containing tubulin plus or minus the kinases but no APC4 (lanes 1 and 2) or APC4 plus or minus kinases but no tubulin (lanes 7 and 8) were included. The band visible just below tubulin in lanes 2, 4, 6, and 8 is GSK3 β



vice versa. This is consistent with our finding that β -catenin was never recruited to microtubules bound to exogenously expressed GFP-APC no matter how much exogenous APC was expressed (data not shown).

Clustering of APC at the growing (plus) end of microtubules may involve the association of APC with a kinesin-like motor protein, as suggested by the ATP-dependence of APC-GFP movement in permeabilized epithelial cells [1]. Our finding that APC Δ MT does not localize to the microtubule lattice but is restricted to clusters near the growing ends of microtubules suggests that it still binds to such a putative motor protein. The exclusive localization of APC Δ MT to the ends of growing microtubules could be explained if transport by such a motor protein was accelerated, because deleting the primary microtu-

bule binding site weakened the direct interaction of APC with the microtubule lattice.

The association of APC with other microtubule binding proteins may also contribute to the observed differences. One protein that has been suggested to interact with APC at microtubule ends is EB1 [13]. It is formally possible that the different APC fragments differ in their ability to bind to EB1. However, APC, APC Δ MT, C-APC, and C-APC Δ MT all contain the entire region required for binding to EB1 (the last 170 amino acids of APC [13]), making it unlikely that EB1 is responsible for the differences observed in the behavior of these fragments. In addition, it was recently demonstrated that the interaction between EB1 and APC at the ends of microtubules is

only permitted under restricted conditions that are not understood [14].

Binding of APC to microtubules is decreased by phosphorylation

Binding of APC to β -catenin is enhanced by GSK3 β phosphorylation of APC [15]. To determine whether phosphorylation of APC by GSK3 β also affects APC binding to microtubules, we compared the ability of completely dephosphorylated APC4 protein or APC4 protein phosphorylated by GSK3 β to bundle microtubules (Figure 4). A dephosphorylated APC4 fragment (residues 1034–2843, Figure 2) was generated by adding a combination of PKA (to prephosphorylate APC4) and GSK3 β to phosphatase-treated APC4. Prephosphorylation of APC with PKA is required for efficient phosphorylation by GSK3 β [15]. This treatment resulted in a large mobility shift between the two forms of the protein as shown in Figure 4a, confirming that the APC fragment was phosphorylated successfully. Both the dephosphorylated and phosphorylated form of APC4 resulted in the formation of microtubule bundles when added to microtubules at equal concentrations (Figure 4b), independent of whether taxol or GMP-CPP stabilized microtubules were used. However, dephosphorylated APC4 was more efficient at bundling microtubules and led to the formation of larger bundles with few or no single microtubules remaining (Figure 4b). To quantitate this effect, single microtubules were separated from microtubule bundles by centrifugation. Figure 4c shows the amount of tubulin recovered in the pellet and supernatant when APC4 phosphorylated with GSK3 β or PKA individually or both kinases together was used. Only phosphorylation with both PKA and GSK3 β caused a decrease in the amount of microtubule bundles detectable in the pellet, with a corresponding increase in the amount of single microtubules left in the supernatant (Figure 4c, lane 6). Phosphorylation with either kinase alone produced only a slight increase in the amount of nonbundled tubulin (Figure 4c, lanes 4 and 5). Similarly, the amount of APC4 that remained in the supernatant was significantly increased only when both kinases were used to phosphorylate APC4 (Figure 4c, lane 6). Comparing the relative amount of tubulin recovered in bundles in the presence of a range of concentrations of phospho- and dephospho-APC4 protein from five independent experiments revealed that two to three times as much phosphorylated APC4 was necessary to induce the same amount of microtubule bundling compared to dephosphorylated APC4.

These data indicate that phosphorylation of APC, especially by GSK3 β , decreases its association with microtubules. This effect is opposite to the effect on APC binding to β -catenin, suggesting that binding of APC to microtubules and β -catenin may be mutually exclusive events.

In summary, the experiments presented in this manuscript provide the first demonstration that APC stabilizes microtubules. The pool of APC protein that performs this function may be separate from the pool that is involved in regulating β -catenin, raising the possibility that GSK3 β may be a molecular switch that helps to shift the balance between these different APC pools. The ability of APC to bind to microtubules directly and indirectly is mediated by a number of different binding sites, and this could explain the unique dynamics of APC/microtubule interactions.

Supplementary material

Additional details describing the experimental methods are available at <http://current-biology.com/supmat/supatin.htm>.

Acknowledgments

I.S.N. is a Cancer Research Campaign Senior Fellow and is also supported by a Career Development award from the Burroughs Wellcome Fund and receives funding from Tenovus Scotland and the Royal Society. K.K. is supported by an HFSP long-term fellowship. We would like to acknowledge that these studies were initiated in the laboratory of Dr. W.J. Nelson at Stanford University and some of the experiments shown were performed in Dr. Timothy J. Mitchison's laboratory at Harvard University Medical School. We thank Kathy Siemers and Dr. Arshad Desai for their generous technical help and helpful discussions, Paul Polakis and Bonnee Rubinfeld for their generous gift of SF9 cells containing APC4, and Carol Midgely (University of Dundee) for the GFP-APC construct.

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