The adenomatous polyposis coli protein (APC) exists in two distinct soluble complexes with different functions

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Accepted 14 July 2005

Journal of Cell Science 118, 4741-4750 Published by The Company of Biologists 2005

doi:10.1242/jcs.02589

Summary

Mutations resulting in the truncation of the adenomatous polyposis coli (APC) protein are common to most colonic tumours. The APC protein has emerged as a multifunctional protein that contributes to cytoskeletal organisation and is involved in the regulation of β-catenin. Both, changes in transcription due to increases in β-catenin, as well as defects in directed cell migration and cell division contribute to cancer when APC is mutated. Little is known about how separate functions of APC are coordinated. In this study, we identified two distinct soluble protein pools containing APC. We found that one of these pools represents the fully assembled β-catenin-targeting complex. The second pool contained at least two different forms of APC: APC that was bound to partially assembled β-catenin-targeting complexes and APC that could bind microtubules. Consistent with the previously proposed role for glycogen synthase kinase 3β (GSK3β) in modulating the assembly and activity of the β-catenin-targeting complex, formation of the fully assembled complex was reduced by inhibitors of GSK3β. Similarly, tumour cells with truncated APC only contained the partially assembly β-catenin-targeting complex. We also found that highly elevated levels of β-catenin in tumour cells containing wild-type APC correlated with a decrease in the ability of the endogenous APC protein to bind microtubules. Additionally, APC lacking the direct microtubule binding site was more effective at downregulating β-catenin. Together, our data suggest that the interaction of APC with microtubules and the β-catenin-targeting complex are mutually exclusive, and indicate that the distribution of endogenous APC between different pools is dynamic, which allows cells to distribute it as required.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/20/4741/DC1

Key words: APC, Protein complexes, Microtubules, β-Catenin

Introduction

Loss of the wild-type adenomatous polyposis coli (APC) protein is common to most colon cancers and truncation mutations in the APC gene occur early in the progression of this disease (Polakis, 1997; Polakis, 1999). Interactions between APC and a variety of cellular proteins implicate APC in the regulation of cytoskeletal elements, in cell migration and division, but also in signalling pathways that regulate gene transcription and cell differentiation (reviewed in Näthke, 2004). As a consequence, C-terminal truncations of APC that are commonly found in human colon cancer lead to a number of defects due to loss of function. For example, the loss of microtubule-binding sites located in the C-terminal part of APC correlates with changes in cell migration and defects in mitotic spindles (Dikovskaya et al., 2004; Green and Kaplan, 2003). In addition, truncation mutations in APC compromise the ability of APC to act as a scaffold protein in a protein complex that regulates the targeting of β-catenin for degradation in a Wnt-dependent manner. As a consequence, β-catenin levels are usually elevated in cells with mutated APC, which changes the transcription of genes involved in differentiation and proliferation (reviewed in Giles et al., 2003).

The protein complex that regulates β-catenin contains APC, Axin, glycogen synthase kinase 3β (GSK3β) and β-catenin and is regulated by Wnt signalling such that it is only active in the absence of Wnt (Hart et al., 1998; Hinoi et al., 2000; Kawahara et al., 2000; Kishida et al., 1998; Rubinfeld et al., 1996). In the active complex, β-catenin is phosphorylated by GSK3β (Hinoi et al., 2000), subsequently poly-ubiquitylated by βTrCP and then degraded by the 26S proteosome (Aberle et al., 1997; Kitagawa et al., 1999; Orford et al., 1997). GSK3β also phosphorylates APC and Axin to stabilise the β-catenin-targeting complex (Ikeda et al., 2000; Yamamoto et al., 1999). One of the genes that is upregulated in response to the accumulation of β-catenin is *Axin-2/conductin*, an Axin homologue. This provides a negative feedback loop for this pathway as Axin-2 can substitute for Axin in the β-catenin-targeting complex (Jho et al., 2002; Lustig and Behrens, 2003).

Little is known about how the activities of APC as a cytoskeletal protein and as a component of the Wnt-signalling pathway are temporally and spatially coordinated. To

investigate how different functions of APC are related, we aimed to identify and characterise protein complexes that contain APC and establish their relationship. To this end, we used a simple fractionation method. A similar approach has previously led to the identification of two different APC-containing protein complexes in cells (Reinacher-Schick and Gumbiner, 2001). Our fractionation method extends these previous results because it resolved one of the previously identified complexes into two distinct complexes. We identified 10S-APC and 23S-APC-containing protein complexes and were able to assign distinct functions to the APC in each of these pools. A proportion of APC in the 10S complex was competent to bind microtubules. However, some of the APC in this complex was part of a separate complex that appeared to be an intermediate of the β-catenin-targeting complex (Lee et al., 2003). Consistent with this idea, the 10S APC complex was still present in colon cancer cell lines that express only a truncated form of APC, which can no longer bind to microtubules but retains some binding sites for β-catenin. The 23S complex contained the fully functional β-catenin-targeting complex and APC in this pool did not bind to microtubules. This complex was greatly diminished in colon cancer cell lines only expressing mutant APC. Furthermore, inhibiting GSK3β resulted in disappearance of the 23S complex, as predicted by the requirement for GSK3β in stabilising the interactions that govern the assembly of the fully functional complex. In addition, we found that APC in cells with high levels of β-catenin did not bind microtubules efficiently, suggesting that the equilibrium in these cells is shifted towards β-catenin-associated APC, which has a reduced ability to interact with microtubules. Consistently, APC that lacks the direct microtubule binding site was more effective at downregulating β-catenin when introduced into tumour cells. Together, our data show that, cytoskeletal interactions of APC and its participation in the β-catenin-targeting complex may be mutually exclusive, and that the distribution of APC protein in cells between different complexes and thus functions can be dynamically regulated by phosphorylation.

Materials and Methods

Antibodies

The polyclonal antibody against APC used for immunoblots was directed against the N-terminal residues 3-347 (Midgley et al., 1997) and diluted 1:1000. The monoclonal antibody against the N-terminal domain of APC (ALi-12-28) used for immunoprecipitations, was obtained from Cancer Research UK (Efstathiou et al., 1998). The polyclonal antibody against the C-terminal peptide of β-catenin was diluted 1:1000 (Hinck et al., 1994). Antibodies against Axin-1 and Axin-2 were from Zymed (cat. nos 34-5900 and 34-7000, respectively) and diluted 1:100. GSK3β, GSK3β Y-216, PP2A catalytic and B56α were detected with antibodies from Transduction Laboratories; cat. nos 610202, 612313, 610556 and 610616, respectively, and diluted 1:100. The tubulin antibody was from Sigma (cat. no. T9026) and was diluted 1:500.

Cell lines and tissue culture

HeLa, HCT 116, SW480, DLD-1 and HT-29 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with non-essential amino acids, 10% FCS and penicillin-streptavidin. MDCK cells were grown in DMEM supplemented with 10% FCS, penicillin-streptavidin and 1 g/l $NaHCO_3$.

Drug treatments

To inhibit GSK3β, SB216763 and lithium chloride (LiCl) were added to growth media for 12 hours at 5 μM and 20 mM, respectively. Two-hour treatments were performed using SB216763 at 20 μM.

Preparation of cell lysates

Tissue culture dishes were placed on ice and washed twice with cold PBS. Media was drained and cells were lysed in MEBC (50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.5% NP-40, 40 mM β-glycerol phosphate) containing 10 mM sodium orthovanadate, 10 mM sodium fluoride and 100 μg/ml of leupeptin, pepstatin A and chymotrypsin. After 5 minutes cells were harvested by scraping. Lysates were spun at 20,800 ***g*** at 4°C for 20 minutes to pellet any insoluble material, the supernatant was transferred into a fresh tube and both, pellet and supernatant, were flash-frozen in liquid nitrogen and stored at –80°C until required.

Immunoblotting

Protein samples were separated by PAGE using 4-12% Novex gels. Gradient samples were resolved on 5-15% Bio-Rad criterion gels. Proteins were transferred to 0.1 μm Protran® nitrocellulose (Schleicher and Schuell, cat. no. 10402096) using a Bio-Rad Mini trans-blot transfer cell. The transfer was performed in 384 mM glycine, 50 mM Tris, 7.5% methanol, 0.02% SDS for 16-18 hours in the cold at constant voltage (35 V). Criterion gradient gels were transferred using a Criterion plate blotter (Bio-Rad). The transfer was carried out in the same buffer at 4°C for 48 hours at constant current (600 mA) with continual mixing.

Immunoprecipitation

Antibody (5 μg) was bound to protein-G sepharose (Pharmacia) and incubated for 2 hours at 4°C with 500 μg total cell lysate while rotating. Precipitates were washed twice with MEBC, then twice with HSB-1M (0.1% SDS, 1% deoxycholate, 0.5% Triton X-100, 20 mM Tris-HCl pH 7.5, 1 M NaCl, 25 mM KCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM DTT) once with low salt buffer to remove salts. They were then boiled in 1× Novex® sample buffer.

Glycerol-gradient fractionation

Total cell lysate (2 mg) was layered on top of a 12 ml glycerol gradient (10% to 40%) prepared with MEBC. The gradients were spun in a Beckman SW41 rotor at 280,000 ***g*** for 12 hours at 4°C. Fractions of 200 μl were collected and proteins in every other fraction were precipitated by adding nine volumes (1.8 ml) –20°C methanol. Precipitations were left overnight at 4°C before collecting precipitated proteins by centrifugation for 1 hour at 20,000 ***g***. The remaining fractions were flash-frozen and stored at –80°C.

Microtubule binding of proteins

Binding of proteins to microtubules in gradient fractions or whole cell lysates was measured as previously described (Hyman et al., 1991). In brief, taxol-stabilised microtubules were incubated for 15 minutes at room temperature with 70 μl of gradient fractions corresponding to the 10S or 23S complexes, or with 100 μg of total protein lysate in 20 μl in which case the reaction was made up to 80 μl to bring the final tubulin concentration to 10 μM. The samples were incubated at room temperature for 20 minutes before sedimentation through a 200-μl cushion of 40% glycerol in 1× BRB80 (80 mM K-Pipes pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) at 285,000 ***g*** for 10 minutes in a Beckman TLA100 ultra centrifuge rotor. Supernatant (unbound material) was removed and precipitated with methanol as described above. Pelleted material (microtubule-associated) was re-suspended in gel sample buffer.

Control samples had identical buffer composition, but lacked microtubules.

Luciferase reporter assay

SW480 cells were seeded onto 6-well plates for overnight incubation and transfected at sub-confluence density the next day. Transfection was carried out using 9 μl of FuGene 6 reagent (Roche, IN) per sample. Two micrograms of pEGFP-C1 or 1 μg of the pEGFP-C/APC constructs were transfected, together with 0.2 μg of pGL3/TOP or pGL3/FOP plasmid, which contain the luciferase gene controlled by a TCF-responsive promoter or a scrambled promoter, respectively (kind gifts from Bert Vogelstein, Johns Hopkins Oncology Center, Baltimore, MA).

Fourty-eight hours after transfection cells were lysed and assayed for luciferase levels with luciferase assay system reagents (Promega). Luciferase activity was measured with a luminometer (Lumat LB9507, EG & G Berthold). Ratios of readings from TOP co-transfection versus FOP co-transfection were compared for β-catenin transcriptional activity.

Results

Fractionation of APC reveals two main complexes

To identify protein complexes containing APC and to examine the relationship between such complexes, we fractionated cell lysates by using glycerol gradients of 10-40% and determined how endogenous APC, β-catenin and GSK3β protein were distributed on these gradients. Our fractionation revealed two distinct complexes that contained APC with sedimentation values of 10S and 23S in cells with full length APC (HeLa and MDCK, Fig. 1). In tumour cells expressing only truncated APC (HT29, SW480, DLD-1) or a non-degradable form of β-catenin (HCT 116 cells), the relative amount of APC in the larger, 23S complex was reduced or completely absent (Fig. 1). In cells with full-length APC, the relative distribution of APC and these two complexes varied slightly in different experiments because the cell density affects this parameter. To compensate for this, we used cells grown to approximately 80% confluency. The truncated APC in SW480 cells was distributed more broadly across the gradient, possibly because it is present in large amounts. Alternatively, the elevated levels of β-catenin in these cells may permit the binding of β-catenin to its lower-affinity partners such that additional protein complexes of different sizes can form. In HT29 cells, the truncated APC was detected in a 10S a complex but also in a complex of approximately 19S. Those cells express the largest N-terminal fragment of APC of all the cells we examined. It is possible that this longer APC fragment retains some of the binding sites required for protein interactions involved in the protein complexes that are contained in the 23S complex.

The fractionation pattern of β-catenin was distinct from that of APC. The predominant peak for β-catenin was detected in a complex of approximately 4-7S. In cells with high levels of β-catenin, the fractionation of β-catenin was more extended, indicating that it was also present in larger complexes of up to 10 or 11S so that it co-sedimented partially with APC. Higher amounts of β-catenin were present in cells with truncated APC (HT-29, SW480, DLD-1), mutated β-catenin (HCT 116) or in cells with well-established cell-cell adhesions (MDCK) (see also supplementary material, Fig. S1). This suggests that these larger protein complexes, which contain β-catenin result from two possible interactions. They could represent β-catenin bound to APC in cells that contain only truncated APC and thus only have a partially assembled

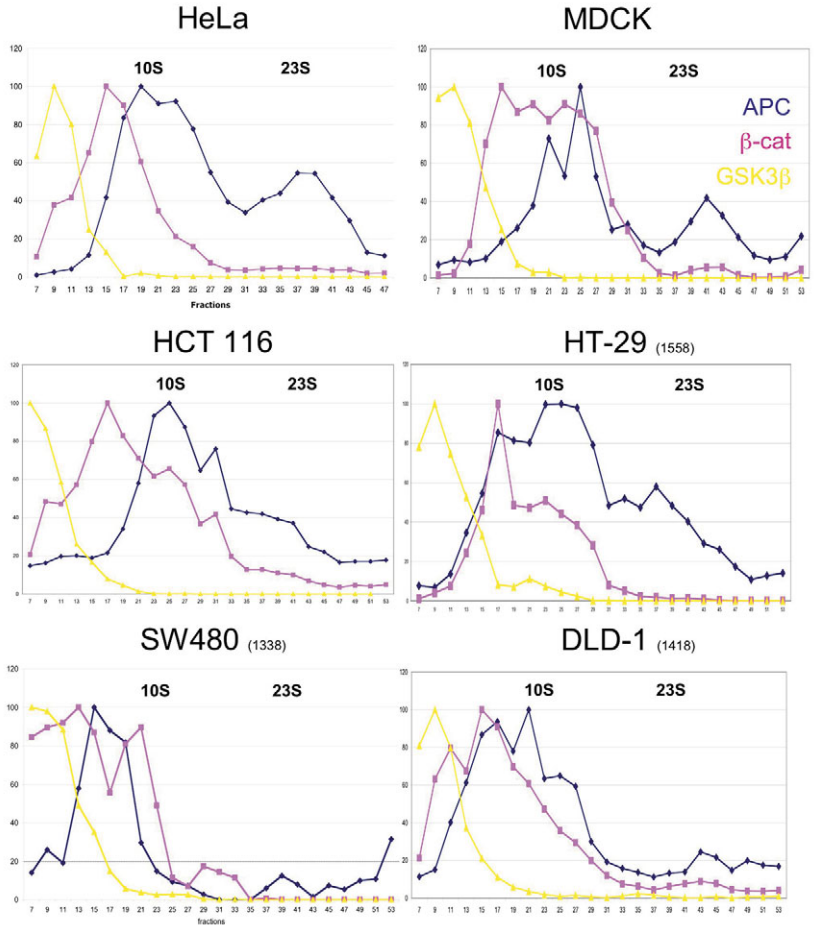

Fig. 1. Fractionation of APC, β-catenin and GSK3β from different cell lines on glycerol gradients. Cell lysates from the indicated cells were fractionated on 10-40% glycerol gradients. Every other fraction was subjected to PAGE, transferred to nitrocellulose probed with antibodies against APC, β-catenin and GSK3β. The relative intensity of each protein was determined using a charge-coupled-device (CCD)-based enhanced chemiluminescence (ECL) detection system and was plotted for each fraction. Sedimentation of proteins with known S-values was carried out in parallel for each experiment to allow calibration for each gradient. In HeLa and MDCK cells, which express the intact β-catenin-targeting complex, APC was present in two pools that sedimented at 10S and 23S. However, in cells that only express truncated APC or mutated β-catenin, which cannot be phosphorylated, the relative amount of APC in the 23S complex was reduced or absent (SW480, DLD-1, HCT 116) or was shifted to a smaller size complex (HT-29).

targeting complex, resulting in elevated β-catenin levels. In addition, complexes containing β-catenin and cadherins are known to fractionate with S-values of around 7-12S in sucrose-density gradients and may also contribute to the larger protein complex we observed in our gradients that contains β-catenin (Hinck et al., 1994). Consistent with this idea, the distribution of β-catenin in SW480 cells was restricted to the top of the gradient (Fig. 1), suggesting that it exists as free protein in these cells as described previously (Stewart and Nelson, 1997). SW480 cells have the most severely truncated form of APC (truncated at residue 1338) of all cells examined by us. APC proteins in HT-29 and DLD-1 cells [truncated at residues 1558 and 1408, respectively (Beroud and Soussie, 1996)] might bind better to β-catenin, compared with the APC in SW480 cells. Furthermore, SW480 cells do not efficiently form adherens junctions because they only express low levels of cadherin (Faux et al., 2004). Together, this could explain why the β-catenin in SW480 cells fractionates in a smaller complex possibly representing free protein.

The distribution of GSK3β was restricted to the top of the gradient in all cell types examined by us and fractionated with sedimentation values of approximately 3-4S, suggesting that

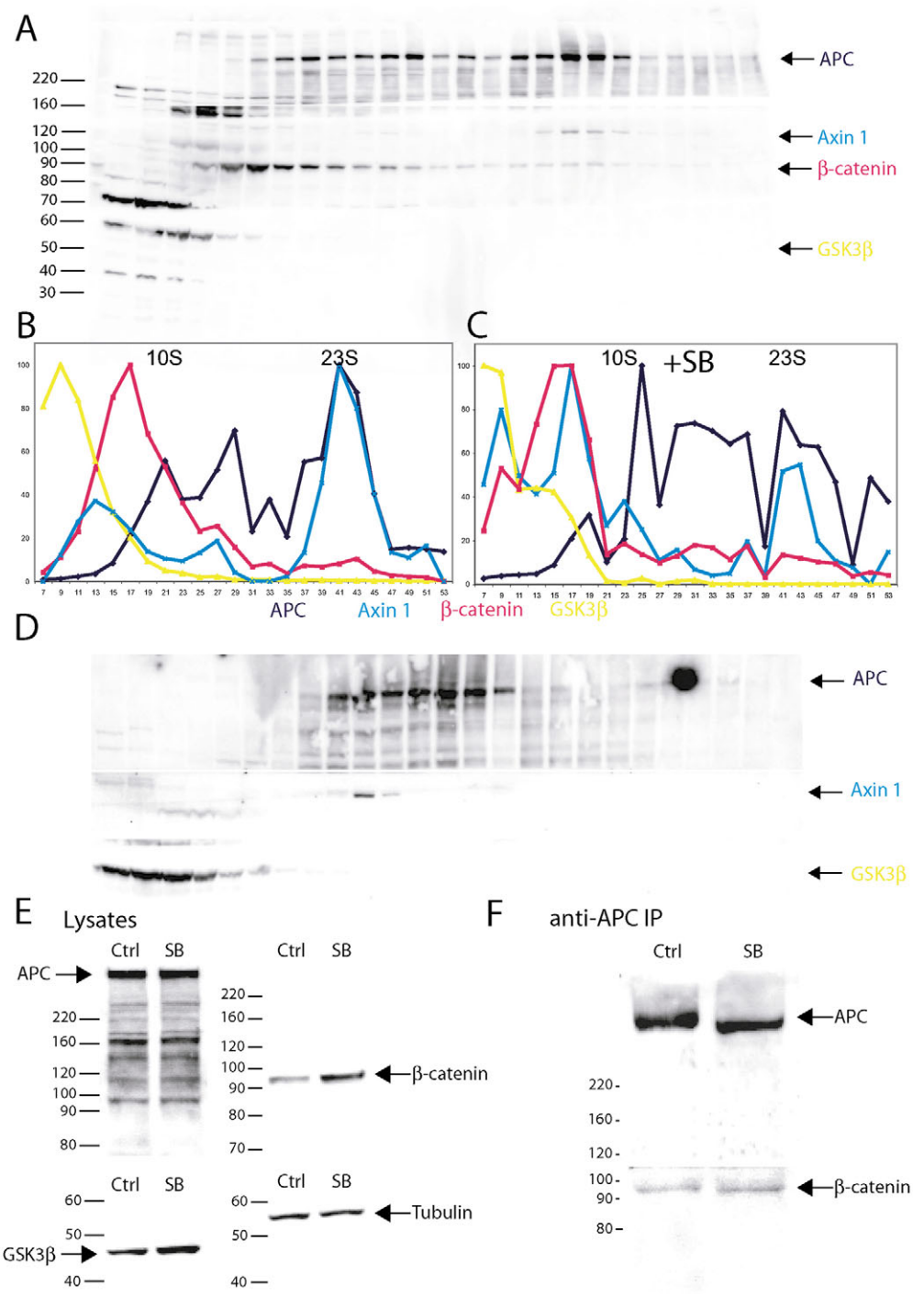

Fig. 2. Axin-1 co-sediments with the 23S APC-containing complex and is re-distributed to the 10S complex after cells were treated with GSK3β inhibitor. Cell lysates were fractionated on 10-40% glycerol gradients as in Fig. 1. (A) Every other fraction was subjected to PAGE and probed with antibodies against APC, β-catenin, GSK3β, and Axin-1. (B) Intensities of the bands were measured using a CCD-based ECL detection system and then plotted as indicated. The majority of cellular Axin-1 co-sedimented with the 23S APC-containing complex. (C) Parallel samples of cells were treated with the GSK3β inhibitor SB216763 (20 μM) for 2 hours and processed as described for A. Treatment of cells with SB216763 resulted in a relative increase in the proportion of APC and Axin-1 in smaller complexes. (D) Cell lysates from HeLa cells treated with SB for 12 hours were fractionated and probed for APC, Axin-1 and GSK3β. Axin-1 distributes almost exclusive to the 10S complex after 12 hours of GSK3β inhibition, similar to APC. (E) Cell lysates of cells treated with SB216763 for 2 hours were probed with antibodies against APC, β-catenin, GSK3β and tubulin. Only β-catenin was increased whereas the levels of the other proteins remained constant. (F) Cell lysates from control or SB216763-treated cells were immunoprecipitated with a monoclonal antibody against APC. Immunoprecipitated material was probed with antibodies against APC and β-catenin. Despite the elevated levels of β-catenin in SB216763-treated cells, there was no concomitant increase in the amount of β-catenin bound to APC.

the bulk of the protein was present as free protein and that only a small amount of the total GSK3β in cells is associated with APC-containing and/or β-catenin-containing complexes (Fig. 1B).

In summary, these data show that APC protein is normally present in two distinct protein complexes with sedimentation values of 10S and 23S. Truncated APC in tumour cells does not efficiently partition into the 23S complex, tumour cells therefore lack one of the APC-protein complexes usually present in cells.

Previously, when a different fractionation protocol was employed, APC was described in a 60S complex (Reinacher-Schick and Gumbiner, 2001). In our gradients, this complex was expected to pellet. To confirm this assumption, and to determine how much of the soluble APC in HeLa cells distributed into this larger complex, we performed a set of gradient fractionations under our conditions while reducing the centrifugation time to 6 hours. Under these conditions, we detected approximately 30% of the soluble APC in complexes withS values above 40 (see supplemental material, Table S1). This was not altered by treating cells with GSK3β inhibitors for 12 hours (see below).

The 23S APC-containing complex contains Axin and is reduced after inhibiting GSK3β

The presence of two protein complexes containing APC raised the question of the function of these different APC complexes. The best-studied roles for APC are as a scaffolding protein in the Wnt-regulated β-catenin-targeting complex and as a microtubule binding protein. Previously an APC complex of approximately 20S was described to be the Wnt-regulated β-catenin-targeting complex (Reinacher-Schick and Gumbiner, 2001). To determine whether the 10S or the 23S complex represented the β-catenin-targeting complex, two methods were employed. First, the fractionation of Axin-1, another scaffolding protein central for the function of the targeting complex and the limiting factor in its assembly (Lee et al., 2003) was determined.

Fig. 2 shows a fractionation profile of HeLa cell lysates probed with antibodies against APC, Axin-1, β-catenin and GSK3β. The relative intensities of β-catenin and GSK3β were similar to other gradients described above and APC was detected in two complexes of 10S and 23S as before (Fig. 2). However, in this particular example, the relative amount of APC in the two complexes was more equally distributed. The majority of cellular Axin-1 cofractionated with APC in the 23S peak (Fig. 2), suggesting that the 23S complex represented the fully assembled β-catenin-targeting complex.

To obtain additional evidence for the idea that the 23S complex was the β-catenin-targeting complex, we measured the effect of inhibiting GSK3β on the fractionation of APC, β-catenin, GSK3β and Axin-1. To inactivate GSK3β, cells in a parallel culture were treated with 20 μM of the specific GSK3β inhibitor SB216763 for 2 hours prior to lysis and fractionation. The distribution of GSK3β did not change under these conditions (Fig. 2C). In contrast, treatment with SB216763 for two hours resulted in a shift of some of the Axin-1 to a smaller pool at 5-10S where it cofractionated with β-catenin and some APC (Fig. 2C). After 12 hours of exposure to SB216763 Axin-1 was exclusively present in the smaller pool (Fig. 2D). On the whole, APC was less abundant in the 23S complex in cells that were treated with SB216763 for two hours (Fig. 2C) and was completely shifted to smaller complexes after 12 hours of treatment with SB216763 (Fig. 2D).

To further examine the immediate effect of inhibiting GSK3β we also compared the relative amounts of β-catenin, APC and GSK3β in control and treated cells. Although the amount of β-catenin increased after cells were treated with SB216763 for 2 hours (Fig. 2E), there was little or no increase in the amount of β-catenin bound to APC under these conditions (Fig. 2F).

Together, these observations suggest that after treating cells with the GSK3β inhibitor SB216763 for two hours, some of the fully assembled β-catenin-targeting complexes are still present. One possibility is that GSK3β is not inhibited completely by this time. However, please notice the increase in the electrophoretic mobility of APC in the immunoblot for APC in Fig. 2F, suggesting a decrease in the phosphorylation of APC after the treatment with SB216763. These observations are consistent with the idea that APC is normally phosphorylated by GSK3β, and further suggest that the kinase is inactivated under these conditions (see also supplementary material Fig. S3).

Alternatively, the time required to competely abolish the fully formed targeting complexes in these cells may exceed the time frame of this experiment. This suggests that, in HeLa cells the level of β-catenin must either dramatically increase before the β-catenin-targeting complex is brought into action or the time of this increase has to be sufficiently long to induce the machinery that controls such excess levels. In addition, the nature of this response probably varies between cell types because of differences in the availability of cadherins or other β-catenin binding proteins that could 'buffer' any excess β-catenin.

Importantly, this finding demonstrates that the disassembly of the targeting complex might not be able to immediately respond to global activity changes of GSK3β, or that a sustained increase in β-catenin is necessary before the assembly of partially assembled targeting complexes is substantially increased.

Inhibiting GSK3β for extended times abolishes formation of the 23S complex

To establish that inhibiting GSK3β indeed results in the disassembly of the 23S complex, we measured the effect of longer treatment with GSK3β inhibitors and applied the general inhibitor lithium chloride (LiCl) or the specific inhibitor SB216763 for 12 hours. The most noticeable effect of inhibiting GSK3β with SB216763 on the fractionation profile of APC was the complete disappearance of APC in the 23S complex (Fig. 3A), consistent with the disappearance of Axin-1 in this pool (Fig. 2D). Treatment with LiCl also produced a reduction in the relative amount of APC in this pool (Fig. 3B) so that the distribution of APC in cells after inhibition of GSK3β was remarkably similar to that in colorectal-cancer-derived cell lines (compare Fig. 1 with Fig. 3A,B). The major proportion of β-catenin was distributed in a slightly broader peak than the one obtained in control conditions, so more β-catenin cofractionated with APC in the 10S complex, consistent with the idea that more β-catenin may bind to APC under these

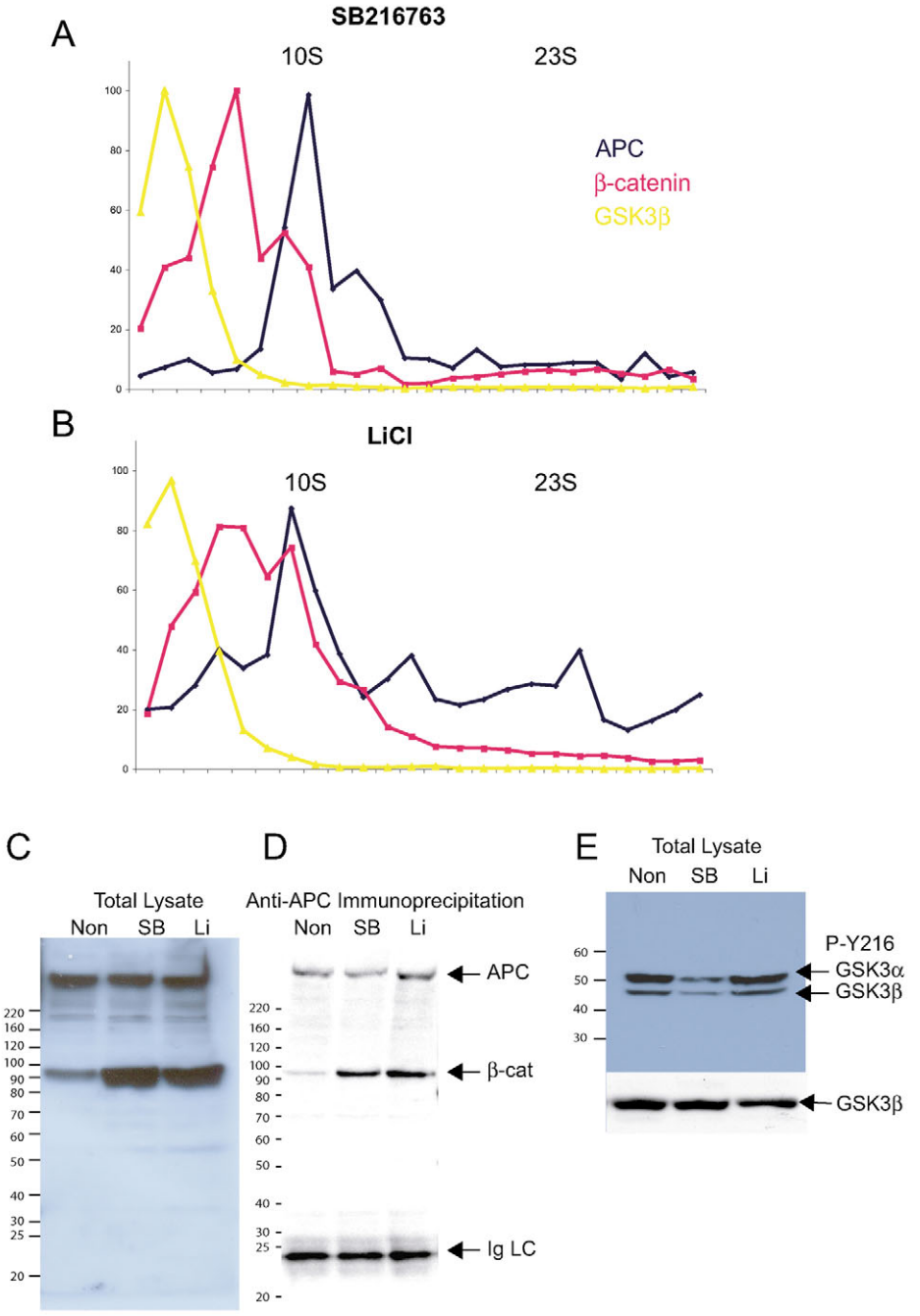

Fig. 3. Extended inhibition of GSK3β causes a robust reduction of APC in the 23S complex. (A,B) HeLa cells were treated with the GSK3β inhibitors, SB216763 (5 μM) (A) or LiCl (20 mM) (B) for 12 hours. Cell lysates were fractionated and the distribution of APC and β-catenin determined as above. Inhibiting GSK3β for 12 hours greatly reduced the amount of APC in the 23S complex and also resulted in a higher proportion of β-catenin cofractionating with APC in the 10S complex. This effect was produced more effectively by SB216763, the more specific of the two inhibitors. These plots represent the distribution profiles of two independent experiments. (C) Immunoblots of equal amounts of total cell lysates were probed with antibodies against APC and β-catenin. This confirmed that both GSK3β inhibitors produced an increase in the cellular β-catenin content, whereas the amount of APC remained constant. (D) APC was immunoprecipitated from cell lysates of SB216763 and LiCl treated HeLa cells as indicated. Probing the immunoprecipitated material with antibodies against β-catenin revealed an increased amount of β-catenin bound to APC when GSK3β was inhibited. (E) Total cell lysates were also probed with an antibody against the phospho-epitope Y-216 of GSK3 or a GSK3β-specific antibody (lower panel). Only SB216763 produced a decrease in the activating phosphorylation of GSK3α and GSK3β at Y-216, consistent with the fact that LiCl and SB216763 inhibit GSK3 by different mechanisms.

conditions. The distribution of GSK3β across the gradient was not altered by treatment with the inhibitors for 12 hours.

To directly measure the effect of inhibiting GSK3β on the amount of β-catenin bound to APC, we immunoprecipitated APC from cell lysates after inhibiting GSK3β for 12 hours and determined the amount of β-catenin that coimmunoprecipitated (Fig. 3D). These experiments confirmed that the increased levels of β-catenin in these cells correlated with an increase in the amount of β-catenin associated with APC (compare Fig. 3C,D). Importantly, GSK3β inhibition did not lead to changes in the total amount of APC or Axin-1 and did not alter the distribution of these protein between different cellular compartments (Fig. 3C, and see supplementary material, Figs S2 and S4).

These experiments also revealed that only treatment with SB216763 but not LiCl led to a detectable decrease in the amount of GSK3β phosphorylated at tyrosine-216, an activating phosphorylation site within GSK3β (Fig. 3E). This is consistent with the fact that SB216763 and LiCl inhibit GSK3β by different mechanisms (Meijer et al., 2004).

The increased association of β-catenin with APC in cells with inactivated GSK3β seemed contradictory to our conclusion that the 23S complex represented the fully assembled targeting complex. Furthermore, after GSK3β inhibition, the relative amount of APC in the 10S complex increased, whereas the 23S complex disappeared. We therefore propose that the 10S complex represents a partially assembled targeting complex. This conclusion is also based on the fact

that GSK3β activity is required for the assembly of the fully functional complex and our finding that Axin-1 cofractionated most efficiently with the 23S complex, not the 10S complex. Consistent with this, the amount of β-catenin available to bind APC increased when β-catenin levels were significantly increased due to prolonged inactivation of GSK3β (Fig. 3D).

Only APC in the 10S complex is capable of binding microtubules

The data presented so far are consistent with the idea that the 10S and 23S complexes represent partially and fully assembled β-catenin-targeting complexes, respectively. However, APC also associates with microtubules, and exogenously expressed GFP-APC predominantly localises to microtubules (Mimori-Kiyosue et al., 2000; Zumbrunn et al., 2001). To test whether APC in the 10S or 23S complex is capable of binding microtubules, fractions from each complex were selected and the ability of the APC in each of these fractions to bind to microtubules was measured (Fig. 4A). These experiments revealed that only APC in the 10S complex, but not APC in the 23S complex, co-sedimented with polymerised microtubules (Fig. 4A). β-Catenin was not bound to polymerised microtubules (Fig. 4A) although it was detectable in the 10S pool.

These data indicate that the 10S complex contains at least two distinct forms of APC: one that is part of an intermediate Wnt-regulated β-catenin-targeting complex, and one that has the ability to bind to microtubules. These data also suggest that these two functions of APC are separate, so that APC bound to β-catenin does not bind to microtubules and vice versa.

To further investigate the relationship between APC binding to microtubules and β-catenin, we compared the amount of APC in total cell lysates that can co-sediment with microtubules (Fig. 4B). In HeLa cells ⩾50% of the soluble APC sedimented with microtubules. This did not change after treatment with the GSK3β inhibitor (Fig. 4B), although an increase in the ability of APC to bind microtubule was predicted by the previous finding that phosphorylation of APC by GSK3β decreases its interaction with microtubules (Zumbrunn et al., 2001). Involvement of GSK3β is probably only one of many mechanisms that regulate APC-microtubule interactions and to interfere with this one mechanism is not sufficient to globally affect cellular APC. Alternatively, the increased levels of β-catenin that had accumulated after 12 hours of SB treatment and the concomitant increase in the amount of APC bound by β-catenin (Fig. 3D) might have compromised the ability of APC to bind microtubules; this would counteract any effect the inactivation of GSK3β might have produced on this function of APC.

In DLD-1 cells, APC co-sedimented with microtubules with variable efficiency. Importantly, in each experiment significantly less APC was recovered in the microtubule-associated pool when compared with HeLa cells (Fig. 4B). This observation is consistent with the fact that the APC in these cells lacks the direct microtubule-binding site but can still be recruited to microtubules to some degree by interacting with KAP3. Surprisingly, the APC in HCT 116 cells did not efficiently sediment with microtubules (Fig. 4B). Based on the ideas outlined above, the most likely explanation for this observation is that the high amount of β-catenin in these cells drives the formation of a partially assembled complex containing β-catenin and APC that prevents APC from binding to microtubules. The mutation of β-catenin in HCT 116 renders it insensitive to phosphorylation by GSK3β and, as a consequence, the processing required to generate the fully assembled complex cannot occur and the system is 'trapped'.

To further investigate the relationship between APC bound to microtubules and bound to the targeting complex, we compared in the TOP/FOP luciferase assay the ability of full-length APC and APC lacking the N-terminal third of the protein (which retains full ability to participate in Wnt signaling) with or without the direct microtubule-binding site to downregulate β-catenin (Fig. 4C). We found that the ability of APC to downregulate β-catenin was increased when APC lacked the direct microtubule-binding site. This supports our suggestion that microtubule binding by APC and its participation in the β-catenin-targeting complex are mutually exclusive events.

Discussion

We show that endogenous APC protein can be separated into two distinct complexes with sedimentation values of 10S and 23S (Fig. 1). Truncated APC found in colon cancer cells did not partition into the 23S complex efficiently and Axin-1 cofractionated with APC in the 23S complex. Inhibiting GSK3β for 12 hours resulted in a lack of APC and Axin-1 in the 23S complex in cells with wild-type APC. Shorter inhibition of GSK3β only resulted in a partial reduction of APC and Axin-1 in the 23S complex. However, coimmunoprecipitation of APC from each of the peak fractions showed that APC in the 10S complex associated with β-catenin and that only a negligible amount of β-catenin could be detected in the 23S pool (data not shown and Fig. 4A, respectively). These data led us to propose that the 10S complex represents a partially assembled and the 23S complex a fully assembled β-catenin-targeting complex. Our data also showed that only APC in the 10S complex was able to bind to microtubules. Although β-catenin could be coimmunoprecipitated with APC and was abundant in this pool, it did not bind to microtubules suggesting that at least two different APC-containing protein complexes were present in the 10S complex.

It is important to notice that a previous study also detected two biochemically distinct, high molecular weight APC complexes of approximately 20S and 60S in HCT 116 and MCF-7 cells (Reinacher-Schick and Gumbiner, 2001). We used a different fractionation method, which provided greater resolution of complexes between 5S and 30S but allowed the previously described 60S complex to be pelleted (supplementary material, Table S1). Our observations are entirely consistent with the results presented in this earlier study. Similar to our observations, the previous experiments showed that GSK3β fractionated in an apparently uncomplexed form at 4-5S (Reinacher-Schick and Gumbiner, 2001) and that the complex of approximately 20S contained components of the β-catenin-targeting complex. Importantly, we were able to extend this latter observation because our fractionation protocol resolved the complex of approximately 20S into two distinct complexes of 10S and 23S. We therefore propose that they represent the β-catenin-targeting complex at

different stages of assembly but also differentiate APC that can bind microtubules from that involved in the regulation of β-catenin.

The existence of intermediates that contain APC bound to β-catenin has been proposed as part of a recently established model. This model is based on the rate constants for the

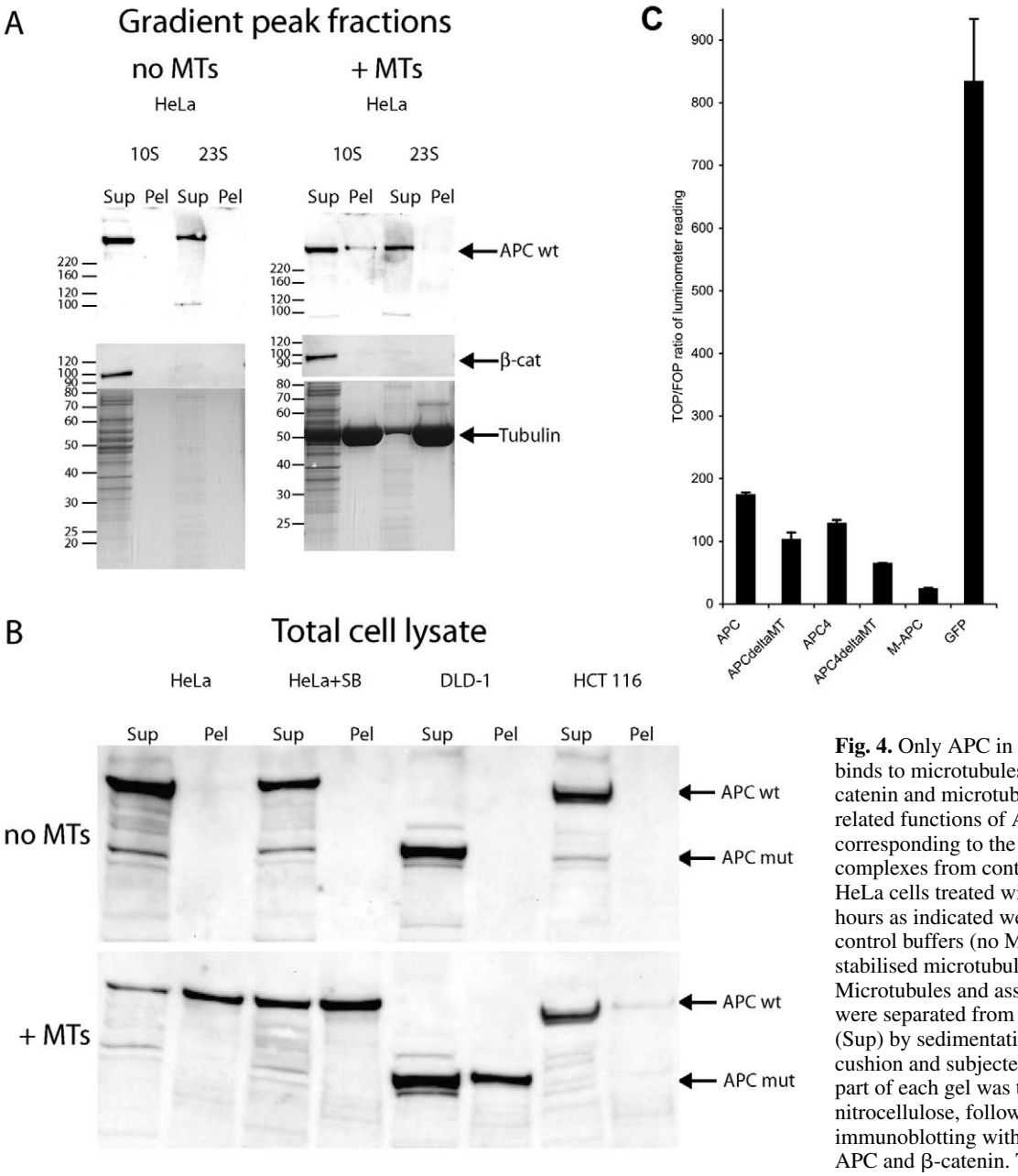

Fig. 4. Only APC in the 10S complex binds to microtubules, binding to β-catenin and microtubules are inversely related functions of APC. (A) Fractions corresponding to the 10S and 23S complexes from control HeLa cells and HeLa cells treated with SB216763 for 12 hours as indicated were incubated with control buffers (no MTs) or taxol stabilised microtubules (+ MTs). Microtubules and associated proteins (Pel) were separated from unbound material (Sup) by sedimentation through a glycerol cushion and subjected to PAGE. The top part of each gel was transferred to nitrocellulose, followed by immunoblotting with antibodies against APC and β-catenin. The bottom part of each gel was stained with Coomassie blue to show the distribution of tubulin. Upon addition of polymerised tubulin only APC in the 10S complex was detected bound to microtubules. β-Catenin was not recovered bound to microtubules. (B) Total cell lysates from HeLa control cells or SB-treated HeLa cells, or DLD-1 and HCT 116 cells were incubated with control buffer (no MTs) or taxol stablised microtubules (+ MTs). Microtubules and associated proteins were recovered by centrifugation through a glycerol cushion and probed with antibodies against APC. APC co-sedimented specifically with microtubules in HeLa cells, only slightly in DLD-1 cells and extremely poorly in HCT 116 cells. (C) SW480 cells were co-transfected with GFP or GFP-tagged APC constructs and a TOP reporter plasmid carrying a TCF-specific promoter, or the FOP plasmid, a control plasmid carrying a mutated promoter, for 48 hours. The APC constructs encoded the following proteins: full length APC, APC lacking the direct microtubule binding site (residues 2168-2451) (APCdeltaMT), APC4, a fragment lacking the N-terminal 1000 amino acids, or APC4 lacking the direct microtubule binding site (APC4deltaMT), M-APC (residues 1014-2038) that have been described in detail (Zumbrunn et al., 2001), or GFP. Luciferase activity was measured in FOP- and TOP-transfected cells and the TOP:FOP ratio was calculated to control for transfection efficiency and background. Luciferase activity was significantly reduced by all APC proteins compared with GFP alone. However, APC that lacks the direct microtubule binding site was more efficient in producing this effect, regardless of whether the N-terminal domain was present or not. Similar results were observed after 24 hours of transfection. Data represent triplicate readings.

interactions required for the formation of the β-catenin-targeting complex and suggests that the complex is assembled in a series of steps (Lee et al., 2003). Further evidence for the existence of such intermediates comes from a recently published crystal structure of the components of the β-catenin-targeting complex. The structure supports a model with Axin-1 linking GSK3β and β-catenin via APC to facilitate phosphorylation of these proteins. In this model, the resulting phosphorylation of the 20 amino acid repeats of APC by GSK3β initially promotes binding of β-catenin to APC but also induces the subsequent dissociation of phosphorylated β-catenin so that the process can be repeated (Xing et al., 2003). This model is further supported by previous crystallography data that confirmed the existence of two different modes of interaction between the 20-amino acid repeats of APC and β-catenin which may represent these two different states (Eklof Spink et al., 2001). The two complexes we identified in our experiments may represent these two different states.

The formation of the fully assembled β-catenin-targeting complex requires GSK3β to phosphorylate APC, Axin-1 and β-catenin. The phosphorylation of Axin-1 increases its stability and thus promotes its inclusion in the complex and the phosphorylation of APC increases its affinity for β-catenin, although unphosphorylated APC stills binds β-catenin (Yamamoto et al., 1999; Rubinfeld et al., 1996). As expected, inhibiting GSK3β resulted in increased levels of total of β-catenin and this correlated with a spread of the distribution of β-catenin over the gradient in cells treated with GSK3β inhibitors. This was similar to the situation in cells expressing truncated APC unable to form a fully functional targeting complex (Fig. 1). Both, long (12 hours) and short (2 hours) treatment with GSK3β inhibitors led to an increase in the amount of soluble β-catenin, although longer treatment was more effective (Figs 2 and 3). However, only the longer treatment resulted in a detectable increase in the amount of β-catenin that was bound to APC (Fig. 3). These data suggest that the time required to assemble additional complexes to respond to an increase in cellular β-catenin in HeLa cells is longer than two hours. Alternatively, the levels of β-catenin that are tolerated before such a response is initiated might vary between cells types. One factor that probably determines the threshold for cellular β-catenin that activates its degradation is the amount of β-catenin that can be recruited by cadherins. In addition, our data confirm that high levels of β-catenin can drive its binding to APC even in the absence of GSK3β activity.

The relative lack of detectable β-catenin in the 23S complex seems contradictory to our idea that the 23S complex represented the β-catenin-targeting complex because β-catenin should be detectable in the 23S complex. However, β-catenin dissociates and is degraded rapidly as soon as it becomes part of the fully functional targeting complex and this is probably the reason for the lack of detectable β-catenin in the 23S complex.

The fractionation of GSK3β was similar in all cell types we examined and did not change after treatment with inhibitors. It fractionated with S-values of 3-4 suggesting that the bulk of cellular GSK3β was free. We could not detect GSK3β protein in the 23S complex most probably because the small fraction of the total cellular GSK3β that is present in the fully assembled β-catenin-targeting complex was below our detection limit.

Our data reveal GSK3β as a key regulator of APC that can provide the temporal and spatial regulation of APC by directing it to different functions. Indeed, phosphorylating APC with GSK3β in vitro renders it less able to interact with microtubules (Zumbrunn et al., 2001), and inhibiting GSK3β by enhancing its phosphorylation on serine-9 by PKC leads to the association of APC with the plus-ends of microtubules (Etienne-Manneville and Hall, 2003). Interestingly, β-catenin can also be redistributed to sites where GSK3β is inactivated and APC is bound to microtubule plus ends suggesting that APC and β-catenin can together associate with microtubules under some circumstances. However, our biochemical data showed that APC that is bound to β-catenin in the partially assembled 10S complex did not efficiently bind to microtubules. One explanation for this discrepancy is that, the relative proportion of β-catenin that is recruited to microtubule ends when GSK3β is inactivated locally is too small to be detected in our experiments. Alternatively, global inhibition of GSK3β might have a different effect on cells than activation of PKC.

Importantly, our finding that APC in HCT 116 cells was less efficient in binding microtubules (Fig. 4B) is consistent with our conclusion that the majority of endogenous APC is unable to bind to the β-catenin-targeting complex and microtubules at the same time. Elevated levels of β-catenin in these cells might shift the equlibrium to favour partially assembled APC-β-catenin complexes as indicated by the increase in APC in the 10S complex in these cells (Fig. 1). Because the β-catenin in these cells cannot be phosphorylated by GSK3β, maturation to the fully assembled complex cannot proceed; therefore APC is trapped by β-catenin and unavailable to bind to microtubules. Consistent with this idea, most of the Axin-1 in HCT116 cells sediments in a complex that is less than 21S (supplementary material Table S2). Similarly, when we examined the ability of APC to downregulate β-catenin – as measured by the ability of β-catenin to drive expression of luciferase in a TCF-dependent reporter assay – we found that APC that lacked the direct microtubule binding site was more effective at reducing β-catenin available for transcriptional regulation. These data further confirm our hypothesis that the ability of APC to bind microtubules and its ability to participate in the β-catenin-targeting complex are opposing events.

This particular finding has important implications for the role of APC in cancer. It suggests that the accumulation of β-catenin resulting from changes in Wnt-signalling might indirectly produce a defect in the ability of APC to regulate microtubules. This, in turn, makes it difficult to separate the functions of APC in Wnt signaling and as a cytoskeletal regulator, but might explain why restoring E-cadherin in APC-mutant cells confers a partial rescue, not only on the transcriptional activity of β-catenin but also on the cytoskeletal effect of APC. E-cadherin has a higher affinity for β-catenin than APC and increasing its expression would release APC from β-catenin allowing APC to bind to microtubules.

In summary, our data suggest that of APC interacts either with components of the Wnt-signalling pathway or with microtubules. However, it can be recruited from one complex to the other when and where required.

We would like to thank all the members of the Näthke laboratory for technical and moral support. In particular Dina Dikovskaya for constructive comments on the manuscript. We would also like to thank all the colleagues who provided reagents as mentioned in the text. G.P. was funded by a project grant from Cancer Research UK and I.S.N is a Cancer Research UK Senior Research Fellow.

References

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). Beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16**, 3797-3804.

Beroud, C. and Soussie, T. (1996). APC gene: database of germline and somatic mutations in human tumors and cell lines. *Nucleic Acids Res.* **24**, 121-124.

Dikovskaya, D., Newton, I. P. and Nathke, I. S. (2004). The Adenomatous Polyposis Coli protein is required for the formation of robust spindles formed in CSF Xenopus extracts. *Mol. Biol. Cell* **15**, 2978-2991.

Efstathiou, J. A., Noda, M., Rowan, A., Dixon, C., Chinery, R., Jawhari, A., Hattori, T., Wright, N. A., Bodmer, W. F. and Pignatelli, M. (1998). Intestinal trefoil factor controls the expression of the adenomatous polyposis coli-catenin and the E-cadherin-catenin complexes in human colon carcinoma cells. *Proc. Natl. Acad. Sci. USA* **95**, 3122-3127.

Eklof Spink, K., Fridman, S. G. and Weis, W. I. (2001). Molecular mechanisms of beta-catenin recognition by adenomatous polyposis coli revealed by the structure of an APC-beta-catenin complex. *EMBO J.* **20**, 6203-6212.

Etienne-Manneville, S. and Hall, A. (2003). Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. *Nature* **421**, 753-756.

Faux, M. C., Ross, J. L., Meeker, C., Johns, T., Ji, H., Simpson, R. J., Layton, M. J. and Burgess, A. W. (2004). Restoration of full-length adenomatous polyposis coli (APC) protein in a colon cancer cell line enhances cell adhesion. *J. Cell Sci.* **117**, 427-439.

Giles, R. H., van Es, J. H. and Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim. Biophys. Acta* **1653**, 1-24.

Green, R. A. and Kaplan, K. B. (2003). Chromosome instability in colorectal tumor cells is associated with defects in microtubule plus-end attachments caused by a dominant mutation in APC. *J. Cell Biol.* **163**, 949-691.

Hart, M. J., de los Santos, R., Albert, I., Rubinfeld, B. and Polakis, P. (1998). Downregulation of β-catenin by human axin and its association with the APC tumor suppressor, b-catenin and GSK3β. *Curr. Biol.* **8**, 573-581.

Hinck, L., Näthke, I. S., Papkoff, J. and Nelson, W. J. (1994). Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J. Cell Biol.* **125**, 1327-1340.

Hinoi, T., Yamamoto, H., Kishida, M., Takada, S., Kishida, S. and Kikuchi, A. (2000). Complex formation of adenomatous polyposis coli gene product and axin facilitates glycogen synthase kinase-3 beta-dependent phosphorylation of beta-catenin and down-regulates beta-catenin. *J. Biol. Chem.* **275**, 34399-34406.

Hyman, A. A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Pfeffen, P., Wordeman, L. and Mitchison, T. J. (1991). Preparations of modified tubulins. *Methods Enzymol.* **196**, 478-485.

Ikeda, S., Kishida, M., Matsuura, Y., Usui, H. and Kikuchi, A. (2000). GSK-3beta-dependent phosphorylation of adenomatous polyposis coli gene product can be modulated by beta-catenin and protein phosphatase 2A complexed with Axin. *Oncogene* **19**, 537-545.

Jho, E. H., Zhang, T., Domon, C., Joo, C. K., Freund, J. N. and Costantini, F. (2002). Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol. Cell. Biol.* **22**, 1172-1183.

Kawahara, K., Morishita, T., Nakamura, T., Hamada, F., Toyoshima, K. and Akiyama, T. (2000). Down-regulation of beta-catenin by the colorectal tumor suppressor APC requires association with Axin and beta-catenin. *J. Biol. Chem.* **275**, 8369-8374.

Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S. and Kikuchi, A. (1998). Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. *J. Biol. Chem.* **273**, 10823-10826.

Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A. and Nakayama, K. (1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. *EMBO J.* **18**, 2401-2410.

Lee, E., Salic, A., Kruger, R., Heinrich, R. and Kirschner, M. W. (2003). The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. *PLoS Biol.* **1**, E10.

Lustig, B. and Behrens, J. (2003). The Wnt signaling pathway and its role in tumor development. *J. Cancer Res. Clin.* **129**, 199-221.

Meijer, L., Flajolet, M. and Greengard, P. (2004). Pharmacological inhibitors of Glycogen Synthase Kinase 3. *Trends Pharmacol. Sci.* **25**, 471-480.

Midgley, C. A., White, S., Howitt, R., Save, V., Dunlop, M. G., Hall, P. A., Lane, D. P., Wyllie, A. H. and Bubb, V. J. (1997). APC expression in normal human tissue. *J. Pathol.* **181**, 426-433.

Mimori-Kiyosue, Y., Shiina, N. and Tsukita, S. (2000). Adenomatous Polyposis Coli (APC) protein moves along microtubules and concentrates at their growing ends in epithelial cells. *J. Cell Biol.* **148**, 505-517.

Näthke, I. S. (2004). The adenomatous polyposis coli protein: the Achillis heel of the gut epithelium. *Annu. Rev. Cell Dev. Biol.* **20**, 337-366.

Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M. and Byers, S. W. (1997). Serine phosphorylation-regulated ubiquitination and degradation of beta- catenin. *J. Biol. Chem.* **272**, 24735-24738.

Polakis, P. (1997). The adenomatous polyposis coli (APC) tumor suppressor. *Biochim. Biophys. Acta* **1332**, F127-F147.

Polakis, P. (1999). APC protein. In *Cytoskeletal and Motor Proteins*, (ed. T. E. Kreis and R. D. Vale), 197pp. Oxford: Oxford University Press.

Reinacher-Schick, A. and Gumbiner, B. M. (2001). Apical membrane localization of the adenomatous polyposis coli tumor suppressor protein and subcellular distribution of the beta-catenin destruction complex in polarized epithelial cells. *J. Cell Biol.* **152**, 491-502.

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P. (1996). Binding of GSK3β to the APC-beta-catenin complex and regulation of complex assembly. *Science* **272**, 1023-1026.

Stewart, D. B. and Nelson, W. J. (1997). Identification of four distinct pools of catenins in mammalian cells and transformation-dependent changes in catenin distributions among these pools. *J. Biol. Chem.* **272**, 29652-29662.

Xing, Y., Clements, W. K., Kimelman, D. and Xu, W. (2003). Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex. *Genes Dev.* **17**, 2753-2764.

Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S. and Kikuchi, A. (1999). Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3b regulates its stability. *J. Biol. Chem.* **274**, 10681-10684.

Zumbrunn, J., Inoshita, K., Hyman, A. A. and Näthke, I. S. (2001). Binding of the Adenomatous Polyposis Coli protein to microtubules increases microtubule stability and is regulated by GSK3β phosphorylation. *Curr. Biol.* **11**, 44-49.