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Regulation and function of the interaction between the APC tumour suppressor protein and EB1

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The interaction between the adenomatous polyposis coli (APC) tumour suppressor and the microtubule-associated protein EB1 was examined. Immunoprecipitation suggested that APC and EB1 were not associated in cultures of HCT116 cells arrested in mitosis. The *C*-terminal 170 amino acids of APC, purified as a bacterial fusion protein, precipitated EB1 from cell extracts, significantly refining the location of the EB1 interaction domain in APC. *In vitro* phosphorylation of this fusion protein by either protein kinase A or p34^{cdc2} reduced its ability to bind to EB1. Expression of GFP fusions to *C*-terminal APC sequences lacking or including the APC basic domain but encompassing the EB1 binding region in SW480 cells revealed a microtubule tip association which co-localized with that of EB1. Expression of the basic domain alone revealed a non-specific microtubule localization. *In vitro* interaction studies confirmed that the APC basic domain did not contribute to EB1 binding. These findings strongly suggest that the interaction between APC and EB1 targets APC to microtubule tips, and that the interaction between the two proteins is down-regulated during mitosis by the previously described mitotic phosphorylation of APC. *Oncogene* (2000) **19,** 1950–1958.

Keywords: APC; EB1; cell cycle; mitosis; phosphorylation; microtubules

Introduction

The adenomatous polyposis coli (APC) protein is a tumour suppressor protein with a pivotal role in colorectal tumorigenesis. APC has been shown to associate with EB1 (Su *et al*., 1995), a 30 kDa protein which we (Morrison *et al*., 1998a) and others (Berrueta *et al*., 1998; Juwana *et al*., 1999) have recently shown to be associated with microtubule tips. APC is itself a microtubule tip-associated protein (Munemitsu *et al*., 1994; Smith *et al*., 1994; Näthke *et al*., 1996; Morrison *et al*., 1997), a feature ascribed to the presence of a microtubule-binding basic domain towards the *C*-terminus of the protein. Aberrant cell migration is an early feature of tumorigenesis initiated by APC mutation (Oshima *et al*., 1995), and the forced overexpression of APC also causes disordered cell migration in the intestinal epithelium (Wong *et al*., 1996). It has therefore been hypothesized that APC may play a role in directed microtubule assembly during cell migration via the stabilization of extending microtubule tips (Näthke *et al*., 1996; Morrison *et al*., 1997; Pollock *et al*., 1997). Interestingly, several yeast homologues of EB1 have been shown to play a role in mitotic spindle function, in the maintenance of chromosomal transmission fidelity and in a novel late mitotic checkpoint (Beinhauer *et al*., 1997; Schwartz *et al*., 1997; Muhua *et al*., 1998). We have suggested that the interaction between EB1 and APC may be downregulated during mitosis (Morrison *et al*., 1998a), a hypothesis based in part on the observation that no association of APC with the mitotic spindle has yet been reported despite the cytoplasmic localization of APC in interphase cells being dependent upon microtubules (Näthke *et al*., 1996; Morrison *et al*., 1997). We have further speculated that the phosphorylation of APC at mitosis, for example by the p34^{cdc2} mitotic kinase (Trzepacz *et al*., 1997), may negatively regulate the association with EB1. In this study we present data which suggests that the association between APC and EB1 is indeed modulated in cells undergoing mitosis. We also present data which suggests that the interaction with EB1 is responsible for directing APC to the tip of microtubules in interphase cells.

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Results

The *C*-terminus of APC has previously been shown to be phosphorylated by p34^{cdc2} in HCT116 colorectal carcinoma cells arrested at mitosis (Trzepacz *et al*., 1997). Immunofluorescence analysis demonstrated that EB1 in HCT116 cells was associated with the microtubule cytoskeleton throughout the cell cycle (results not shown), as previously shown by ourselves (Morrison *et al*., 1998a) in a variety of other cell types. Furthermore, Western blotting of PBS/TX100 solubilized HCT116 cell extracts confirmed that a single, completely soluble EB1 species was expressed in these cells (results not shown). HCT116 cells were therefore adopted as a suitable model in which to study the interaction between APC and EB1.

Immunoprecipitations were carried out on extracts from asynchronous (AS) cultures of HCT116 cells, cultures arrested at the G2-M checkpoint by nocodazole treatment and cultures arrested at the G1-S boundary by incubation with mimosine (Trzepacz *et al*., 1997). Immunoprecipitates prepared using APC or EB1-specific antibodies were subjected to SDS–PAGE and Western blotting with antibodies specific for APC, β-catenin and EB1. β-catenin was easily detected in APC immunoprecipitates from these cultures, demonstrating that it was associated with APC throughout

the cell cycle (Figure 1). However, no EB1 could be detected in immunoprecipitates made using a number of different APC-specific antibodies, followed by immunoblotting with two different EB1 antibodies (results not shown). In EB1 immunoprecipitates, EB1 itself was easily detected (Figure 1), confirming that it was present throughout the cell cycle. Unfortunately, no APC was detected in EB1 immunoprecipitates (results not shown). The difficulty of detecting endogenous cellular APC/EB1 complexes by immunoprecipitation using the same or similar antibody reagents has been noted and discussed previously by other workers (Su *et al*., 1995; Berrueta *et al*., 1999). However, β-catenin was detected in EB1 immunoprecipitates from AS and G1-S arrested cultures (Figure 1). Since both proteins can complex to APC simultaneously (Morin *et al*., 1996) it seemed likely that the detected β-catenin was co-immunoprecipitated as a consequence of its binding to APC and was not a non-specific association. In support of this, no β-catenin was detected in immunoprecipitates obtained using up to 20 μg of non-specific mouse IgG in place of 5 μg of EB1 monoclonal antisera (results not shown). Crucially, no β-catenin was detected in EB1 immunoprecipitates from SW480 cells, in which a truncated APC protein cannot bind EB1 (results not shown). This strongly suggests that the detection of β-catenin in EB1 immunoprecipitates represents confirmation of an APC/EB1 interaction in cells containing full-length APC.

Strikingly, no β-catenin was detected in EB1 immunoprecipitates from HCT116 cultures arrested at the G2-M spindle assembly checkpoint (Figure 1). We therefore conclude that under these conditions little or no EB1 is associated with APC, since the β-catenin/APC association in the same cell extract is unaffected by cell synchronization (Figure 1). We also note that the slight increase in immunoprecipitated β-catenin obtained from G1-S arrested cell extracts when compared to asynchronous cultures is consistent with this conclusion (Figure 1), since asynchronous cultures contain a proportion of cells in mitosis.

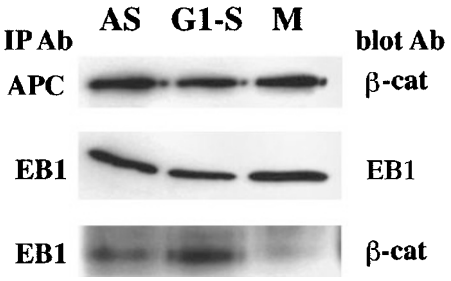

Figure 1 The EB1/APC association is downregulated in mitotic cells. Cells were synchronized at G1-S and mitosis (M), or left asynchronous (AS), before extraction. Immunoprecipitation with an APC-specific antibody was performed, and the precipitate subjected to Western blotting with a β-catenin-specific antibody. β-catenin was detected in precipitates from all three cell cultures. Immunoprecipitation from a portion of the same extract was also performed with an EB1-specific antibody, and the precipitate subjected to SDS–PAGE and Western blotting analysis with EB1 and β-catenin-specific antibodies. EB1 was detected in all three precipitates. β-catenin was detected in precipitates from AS or G1-S arrested cells, with slightly more seen in G1-S extracts, but little was detected in precipitates from G2-M arrested cells

It has previously been demonstrated that the *C*-terminal 284 amino acids of APC encompass the EB1 binding region (Su *et al*., 1995). We produced the fusion protein GST-APC-C1, comprising the final 170 amino acids of human APC fused to GST. This fusion protein was capable of specifically precipitating EB1 from extracts of SW480 cells, significantly refining the EB1 binding region in APC (Figure 2a). No EB1 was detected in precipitations performed using a higher concentration of GST coupled to glutathione-sepharose, or glutathione sepharose alone (Figure 2a).

Since both EB1 and APC are microtubule-associated proteins, it was possible that the observed interaction between the two proteins was an indirect one mediated via the independent association of both proteins with microtubules. The GST-APC-C1 fusion protein used here to precipitate EB1 does not include the basic region of APC previously shown to bind to microtubules *in vitro* (Deka *et al*., 1998), suggesting that the APC/EB1 interaction is indeed direct. Confirming this, the GST-APC-C1 fusion protein precipitated EB1 from

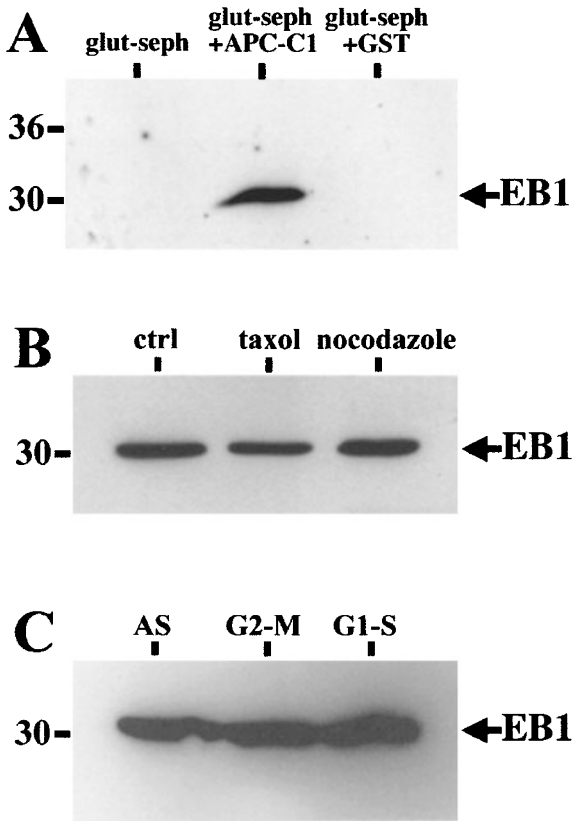

Figure 2 EB1 binds within the final 170 amino acids of APC independently of microtubules. (**a**) SW480 cell extracts were incubated with either glutathione sepharose beads (lane 1), GST-APC-C1 bound to glutathione sepharose beads (lane 2) or GST bound to glutathione sepharose beads (lane 3). Precipitates were analysed by Western blotting with an EB1-specific antibody. EB1 was detected only in the GST-APC-C1 precipitates. (**b**) HCT116 cells were incubated with nocodazole or taxol and extracts prepared in the presence of the drugs. A GST-APC-C1 precipitation was carried out and the precipitates analysed by Western blotting with an EB1-specific antibody. EB1 was precipitated equally well from all three extracts. (**c**) Extracts were prepared from HCT116 cells arrested at G2-M, G1-S or asynchronous cultures. A GST-APC-C1 precipitation was performed and the precipitates analysed by Western blotting with an EB1-specific antibody. EB1 was precipitated equally well from all three extracts

extracts of cells treated with either nocodazole or taxol (Figure 3b), either of which abrogate the association of both EB1 and APC with the microtubule cytoskeleton (Näthke *et al*., 1996; Morrison *et al*., 1998a). This experiment demonstrates that the interaction between APC and EB1 does not require both proteins to be associated with microtubules, or indeed the presence of an intact microtubule cytoskeleton.

Although EB1 contains few putative phosphorylation sites, it was possible that a cell-cycle dependent post-translational modification of EB1 contributed to the observed dissociation of EB1 and APC during mitosis. If such a modification did occur then the GST-APC-C1 protein would precipitate less EB1 from cells arrested in mitosis, but in cells arrested at G1-S the precipitation would be unaffected. As shown in Figure 2c, the APC fusion protein precipitated EB1 equally well from extracts of asynchronous cells and cells arrested at either G1-S or G2-M, supporting the hypothesis that modification of APC and not EB1 is responsible for the dissociation of these proteins during mitosis.

Examination of the amino acid sequence of the final 170 amino acids of APC revealed a number of consensus phosphorylation sites for protein kinases (Figure 3a). Of particular note were the presence of two motifs containing overlapping consensus phosphorylation sites for PKA and $p34^{cdc2}$, between amino acids 2789–2794 and 2830–2835 respectively, with the conserved sequence S-P-R/K-R/K-(X)-S (Figure 3a). These are the only two $p34^{cdk2}$ sites in GST-APC-C1 and the motifs include two of the three PKA sites present in this fragment of the protein. Both of these motifs are conserved in the mouse Apc sequence. A third motif located between amino acids 2813–2818, with the amino acid sequence T-K-K-R-D-S, lacks only a proline residue adjacent to the initial threonine

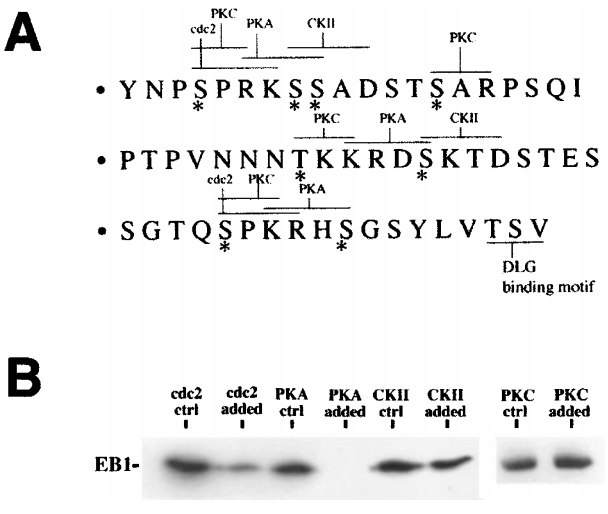

Figure 3 Regulation of APC/EB1 binding *in vitro*. (**a**) Consensus protein kinase phosphorylation sites in APC-C1. Note the repeated motif consisting of overlapping consensus phosphorylation sites for $p34^{cdc2}$ and PKA. Putative sites for PKC and CKII phosphorylation are also indicated. (**b**) Aliquots of GST-APC-C1 on glutathione sepharose beads were incubated with $p34^{cdc2}$, PKA, CKII or PKC and used to precipitate EB1 from SW480 cell extracts. Precipitates were analysed by Western blotting with an EB1-specific antibody. Preincubation with both $p34^{cdc2}$ and PKA significantly inhibited the ability of GST-APC-C1 to precipitate EB1, whereas PKC and CKII had no effect

to create a third conserved repeat. This third motif contains the last PKA site in GST-APC-C1. To test the hypothesis that phosphorylation at these sites might regulate the association between APC and EB1, equal aliquots of GST-APC-C1 were incubated with a number of different recombinant kinases. After extensive washing, the fusion proteins were used to precipitate EB1 from SW480 cell extracts. Pre-incubation with both PKA and $p34^{cdc2}$ greatly reduced the ability of GST-APC-C1 to precipitate EB1, whereas pre-incubation with CKII or PKC had no effect (Figure 3b).

From previous studies (Näthke *et al*., 1996; Morrison *et al*., 1997, 1998a) it seemed plausible that the interaction between APC and EB1 served to target APC to microtubule tips. To test this hypothesis a number of expression constructs consisting of defined regions from the *C*-terminus of APC fused to eGFP were produced. Two fragments encompassing the final 307 or 717 amino acids of APC (APC-C2 and APC-C3 respectively) were fused to eGFP (Figure 4). APC-C2-GFP does not contain the basic domain of APC previously hypothesized to solely mediate the microtubule association of the protein (Munemitsu *et al*., 1994; Smith *et al*., 1994), but encompasses the EB1 binding region. APC-C3-GFP includes both of these domains of the protein. Finally a construct comprising amino acids 2127–2420 fused to eGFP was created (APC-basic-GFP). This construct contains only the basic region of APC, and lacks the EB1 binding region (Figure 4).

To investigate whether these GFP fusion proteins could interact with EB1, the APC-GFP fusion proteins were *in vitro* transcribed and translated and assessed for their ability to bind GST-EB1. SDS–PAGE and autoradiography confirmed the production of radiolabelled proteins of the correct molecular weight (Figure 5a, arrows). Precipitations of the radiolabelled GFP fusion proteins were then performed using either GST alone (Figure 5b) or a GST-EB1 fusion protein (Figure 5c). GST did not specifically precipitate any of the APC-GFP fusion proteins. GST-EB1 precipitated APC-C2-GFP and APC-C3-GFP (Figure 5c, arrows), but not APC-basic-GFP. This confirms that the EB1 binding region in APC lies *C*-terminal to the basic domain in the protein and demonstrates that the basic domain alone cannot specifically interact with EB1. Furthermore, it confirms that the addition of a GFP tag to the APC-C2-GFP and APC-C3-GFP proteins does not interfere with the EB1 interaction.

Using the same plasmid expression vectors, the APC-GFP fusion proteins were transiently expressed in SW480 cells, which lack full-length APC. This allowed the interaction between endogenous EB1 and the APC-GFP fusion proteins to be investigated without interference from endogenous APC. To allow clear visualization of the distribution of the fusion proteins transfected cells were fixed in methanol at −20°C, conditions previously demonstrated to preserve the association of EB1 with microtubules (Morrison *et al*., 1998a). Fixed cells were co-immunostained with antibodies specific for eGFP and EB1. The cells were then analysed by confocal microscopy. In cells transfected with eGFP alone, the immunoreactivity was diffusely distributed throughout the cell and no specific microtubule association was seen (results not shown). However, the staining patterns observed for

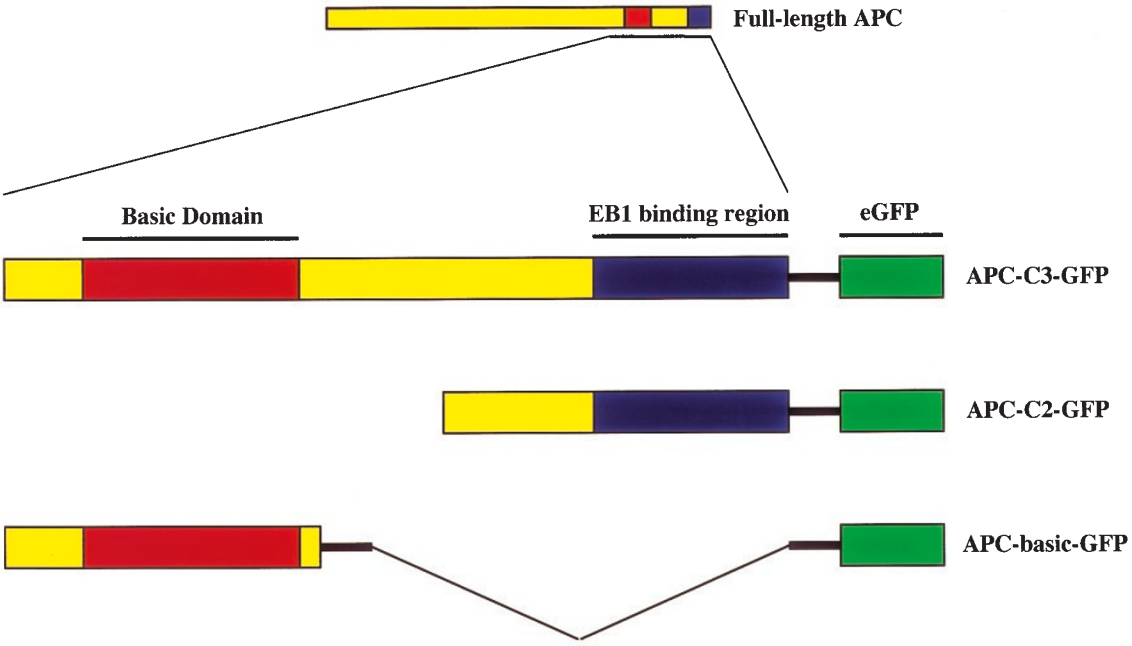

Figure 4 Schematic representation of the APC-GFP fusion constructs used in this study

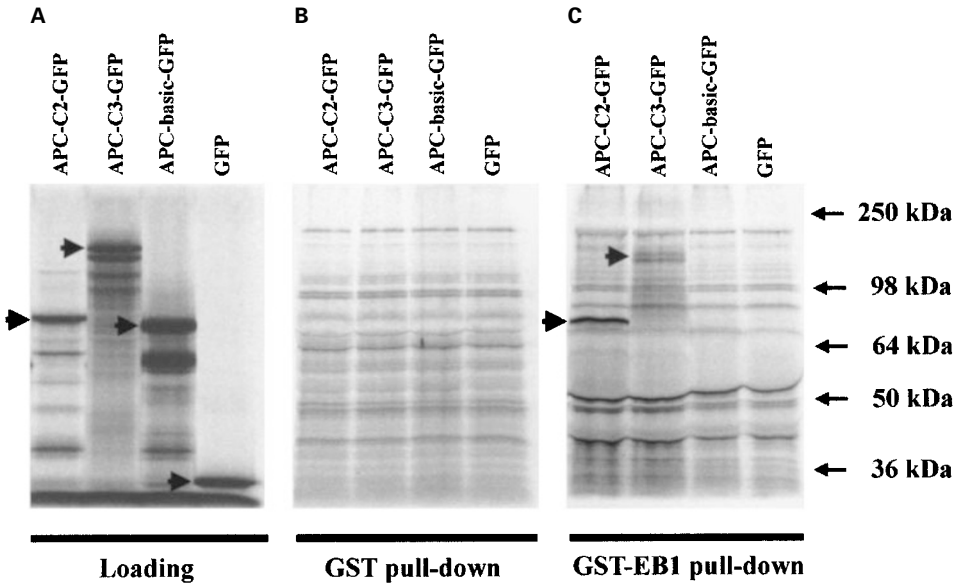

Figure 5 Analysis of APC-GFP fusion protein interactions with EB1 *in vitro*. APC-GFP fusion proteins were *in vitro* transcribed and translated in the presence of radiolabelled cysteine/methionine. A portion of the reaction mix was subjected to SDS–PAGE, and the gel dried and visualized using autoradiography. The APC-GFP-specific products are indicated in (**a**, arrows). The remainder of the reaction mix was subjected to precipitation using either GST or a GST-EB1 fusion protein. The precipitates were subjected to SDS–PAGE and visualized using autoradiography. GST alone did not specifically precipitate any radiolabelled products (**b**). The GST-EB1 fusion protein specifically precipitated the APC-C2-GFP and APC-C3-GFP fusion proteins (**c**, arrows), but not the APC-basic-GFP fusion protein

both APC-C2-GFP (Figure 6a–c) and APC-C3-GFP (Figure 6d–f) were different to this, as the eGFP immunoreactivity localized to short filamentous structures within the cell. Crucially, the immunostaining obtained for both of these fusion proteins co-localized with that obtained for EB1 (Figure 6a–c,d–f, arrows). In contrast, the APC-basic-GFP construct was seen to localize to microtubules in a non-specific manner, with no accumulation at microtubule tips and no specific co-localization with EB1 (Figure 6g–i, arrows).

These observations were confirmed by performing measurements of fluorescence intensity along a line beginning in front of a microtubule tip and running back down the microtubule. Representative measurements taken from individual microtubules from the cells shown in Figure 6 are shown in Figure 7a. The red line indicates EB1 fluorescence along the microtubule, while the green line represents the GFP fusion protein-derived fluorescence. EB1 fluorescence in untransfected cells peaked rapidly at the microtubule tip, with a tail of decreasing intensity and variable length evident (not shown). This reflects the typical 'comet trail' appearance of EB1 microtubule tip labelling. In cells expressing APC-C2-GFP or APC-C3-GFP the EB1 fluorescence profile seen was similar to that in control cells (Figure 7a). In addition, the APC-C2-GFP

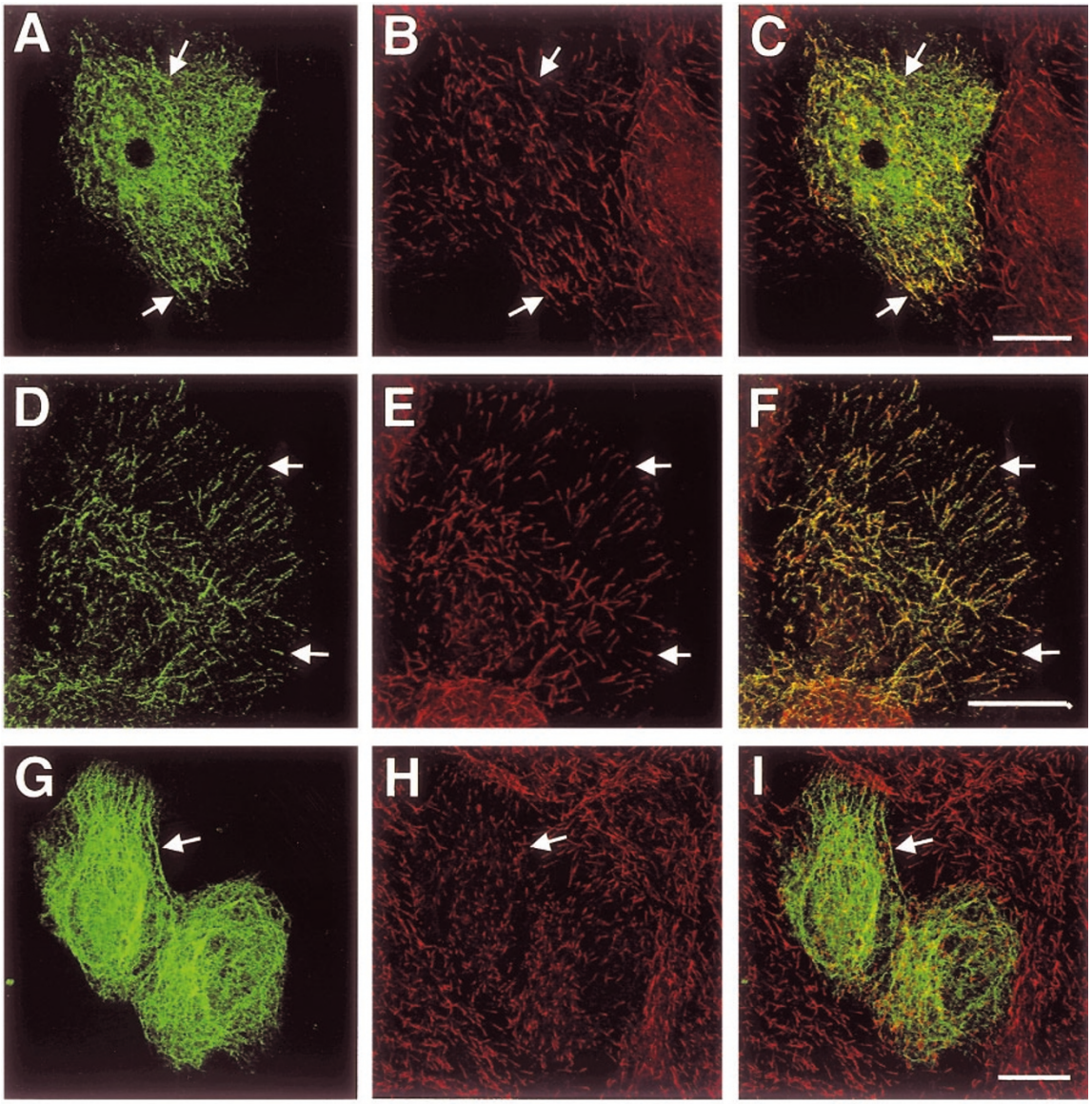

Figure 6 Analysis of APC-GFP fusion protein expression in SW480 cells. SW480 cells were transfected with APC-C2-GFP (**a–c**), APC-C3-GFP (**d–f**) and APC-basic-GFP (**g–i**) expression plasmids. The cells were co-immunostained with antibodies to eGFP (**a, d, g**) and EB1 (**b, e** and **h**), and analysed by confocal microscopy. **c, f** and **i** are overlays of the immunostaining patterns obtained for each antibody. APC-C2-GFP and APC-C3-GFP co-localized with EB1 at microtubule tips (**a–c** and **d–f**, arrows). APC-basic-GFP displayed a non-specific microtubule association, and no specific co-localization with EB1 was seen (**g–i**, arrows). Scale bars = 10 μm

and APC-C3-GFP fluorescence mirrored that seen for EB1 along the same microtubule, confirming the co-localization of both fusion proteins with EB1 at the microtubule tip. In contrast, no specific co-localization between EB1 and the APC-basic-GFP fusion protein was observed (Figure 7a).

Unexpectedly, in cells expressing the APC-basic-GFP protein the EB1 immunostaining observed at microtubule tips appeared shorter than that observed in untransfected cells, with the tail of the EB1 'comet trail' appearing absent (Figure 6g–i; see also the fluorescence plots in Figure 7a). Confirmation of this was obtained by measuring the length of EB1 microtubule tip labelling on at least 20 randomly chosen microtubules from cells expressing the APC-GFP fusion proteins. In cells expressing APC-basic-GFP the average length of EB1 microtubule tip labelling was reduced by approximately 1 μm in comparison to control cells (Figure 7b).

Importantly, these experiments demonstrate that although APC has a genuine capacity for associating with microtubules in cells via its basic domain, the interaction with EB1 is required for the specific targeting of APC to microtubule tips. The results obtained with the APC fusion proteins described in this study therefore support the hypothesis that the signals for microtubule targeting in APC are located at the *C*-terminus, as suggested by previous studies on truncated APC proteins (Munemitsu *et al*., 1994; Smith *et al*., 1994).

Discussion

The work presented in this study suggests that the previously described phosphorylation of APC during mitosis (Trzepacz *et al*., 1997) regulates the association between APC and EB1, and that this interaction

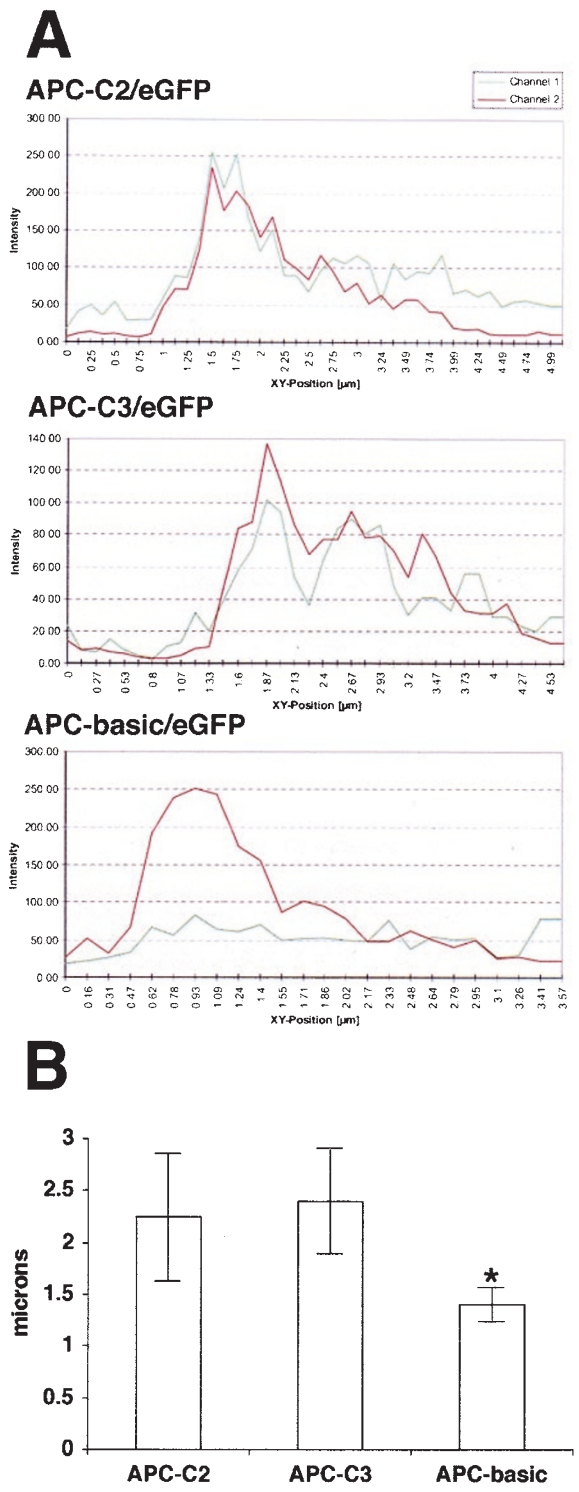

Figure 7 Analysis of APC-GFP fusion protein distribution in SW480 cells. (**a**) Measurements of fluorescence intensity were collected from the transfected cells in Figure 6 along a line beginning in front of a microtubule and running down its length. Representative plots are shown here, and the expressed fusion protein is indicated for each plot. Fluorescence intensity is indicated on the y-axis, and distance along the line on the x-axis. Red lines indicate EB1 fluorescence, green GFP fusion protein fluorescence. A clear correlation between APC-C2-GFP and APC-C3-GFP fluorescence and that of EB1 was seen. No correlation between APC-basic-GFP and EB1 fluorescence was seen. (**b**) The length of EB1 microtubule tip labelling on at least 20 randomly chosen microtubules from the transfected cells in Figure 6 was measured. This labelling was significantly shorter in cells expressing APC-basic-GFP when compared to cells expressing the other fusion proteins

mediates the targeting of APC to microtubule tips in interphase cells. The involvement of $p34^{cdc2}$ in regulating the function of a spindle microtubule-associated protein such as EB1 is not unusual, as it has long been noted that $p34^{cdc2}$ activity is closely linked to mitotic microtubule function (i.e. Alfa *et al*., 1990; Andreassen *et al*., 1994; see Cassimeris, 1999 for a recent review). Furthermore, the association of tau protein with microtubules is also modified during mitosis by phosphorylation at Ser-Pro and PKA consensus motifs (Illenberger *et al*., 1998). Interestingly, the *in vitro* biochemical and tubulin-binding properties of the basic domain of APC have recently been shown to be similar to that of tau (Deka *et al*., 1998). We note that a number of other $p34^{cdc2}$ consensus phosphorylation sites are present towards the *C*-terminus of APC, including a cluster of four sites within the basic domain of the protein (Trzepacz *et al*., 1997). By analogy with the tau model, phosphorylation at these sites may directly influence the ability of the APC basic domain to bind to microtubules. In this way both mechanisms of the APC interaction with microtubules, directly via its basic domain and indirectly via EB1, could be down-regulated during mitosis.

Our results suggest that APC microtubule tip-targeting is mediated via an interaction with EB1. Our work also, however, confirms a role for the basic domain in the microtubule association of APC. In the context of full-length APC, our observations could mean that EB1 targets APC to the extending microtubule tip (Näthke *et al*., 1996; Morrison *et al*., 1997). Once there, the APC basic domain could exert a stabilizing effect upon microtubule structure and/or assembly (Deka *et al*., 1998), perhaps by decreasing the catastrophe rate of the extending microtubule. The putative microtubule stabilizing effect of the APC basic domain may also explain the observed shortening of EB1 microtubule tip labelling in cells expressing APC-basic-GFP. One hypothesis to explain the localization of EB1 to microtubule tips is that the protein recognizes and preferentially binds to an unknown structural determinant found on newly-assembled microtubules. Ectopic expression of the APC basic domain could non-specifically stabilize microtubules soon after assembly, accelerating the loss of this determinant from the microtubule and therefore accelerating the loss of EB1 from regions immediately distal to the microtubule tip.

Based on the above and on previously unpublished data, we propose the following model for the regulation of the APC/EB1 association. During interphase EB1 is complexed to APC, and this association targets APC to microtubule tips and plays a role in promoting microtubule assembly or stability during cell migration. At the onset of mitosis the interaction between APC and EB1 is downregulated when APC is phosphorylated at its *C*-terminus by $p34^{cdc2}$ and possibly also PKA. The dissociation of the APC/EB1 complex prevents the targeting of APC to the tips of microtubules forming the mitotic spindle. If the APC/EB1 complex promotes microtubule plus-end stability, then this could be antagonistic to both the remodelling of the microtubule cytoskeleton involved in spindle assembly and to the highly dynamic behaviour of spindle microtubules. A similar hypothesis has recently been presented to explain modulations in the activity of other microtubule

stabilizing proteins (Cassimeris, 1999). Alternatively, EB1 may have a specific role to play during mitosis which would be inhibited by binding to APC. For example, EB1 has recently been shown to interact with components of the dynein/dynactin microtubule motor complex (Berrueta *et al*., 1999). When the $p34^{cdc2}$ kinase activity is inactivated late in mitosis, APC and EB1 re-associate.

An interesting question is whether mammalian EB1 proteins fulfil the same late mitotic checkpoint function demonstrated by their budding yeast homologue Bim1p (Muhua *et al*., 1998). It is tempting to speculate that the loss of the interaction between APC and EB1 which occurs upon APC truncation could compromise the putative mitotic checkpoint function of EB1. This could contribute to the chromosomal instability observed in some colorectal cancer cell lines (Lengauer *et al*., 1997) by a failure to arrest abnormal mitoses. This intriguing hypothesis deserves further investigation.

Materials and methods

Plasmid constructs

The *APC* cDNA was a kind gift from Professor B Vogelstein, John Hopkins University, Baltimore, USA. The *EB1* cDNA was a kind gift from Dr Ursula Fleig, Institute für Mikrobiologie und Molekularbiologie, Geissen, Germany. The GST fusion protein vector pJMAGST6 was constructed by polymerase chain reaction (PCR) amplification of *APC* bases 7606 to 8529 using the primer pair; 5′-dATCGGATCCCCTTCTAGACTTCCAATC (forward), and 5′-dAACCGCTCGAGTCAACAGATGTCACAAGGTA (reverse). The reverse primer introduces a *Xho*I restriction site. The 945 bp product was gel purified and digested with *Bgl*II and *Xho*I which cleave at an endogenous *Bgl*II site and at the site engineered into the reverse primer. The resulting 510 bp fragment was gel purified and cloned into the *Bam*HI and *Xho*I sites in plasmid pGEX-4T-3 (Pharmacia). The resulting plasmid, pJMAGST6, encodes the fusion protein GST-APC-C1 which comprises the extreme *C*-terminal 170 amino acids of APC *N*-terminally fused to GST.

An EB1-GST bacterial expression vector was also created. The bacterial plasmid expression vector pGEX-4T-3 (Pharmacia) was digested with the restriction enzymes *BamH*I and *Xho*I. A synthetic adapter molecule, A10, was directionally ligated into these sites. The adapter incorporates *BamH*I and *Xho*I cohesive ends (such that both sites are re-created upon ligation), a *Kpn*I site for cloning of PCR amplified *EB1*, and a unique *Eco*RV site to detect successful cloning of the adapter. The resulting vector was named pGEX-4T-A10. *EB1* cDNA bases 1–813 were amplified by PCR using the primer pair 5′-dAGAGGTACCATGGCAGTGAACGTATACTCA (forward) and 5′-dAACCGCTCGAGCCATACTCTTCTTGCTCCTC (reverse). The forward and reverse primers incorporate *Kpn*I and *Xho*I restriction sites respectively. After digestion of the 835 bp product with *Kpn*I and *Xho*I, the *EB1* cDNA was ligated into the same sites in the vector pGEX-4T-A10 creating the plasmid pGST-EB1 which encodes the fusion protein GST-EB1, which comprises full-length EB1 *N*-terminally fused to GST.

An eGFP eukaryotic expression vector was created by digesting the eukaryotic expression vector pcDNA3 (Invitrogen) with the restriction enzymes *Hind*III and *Xho*I. A synthetic adapter molecule, A2, was directionally ligated into these sites. The adapter molecule incorporates a *Hind*III cohesive end (such that a *Hind*III site is not re-created upon ligation), a Kozak consensus translation initiation sequence, restriction sites for sub-cloning, a unique *Sac*II restriction site to detect successful cloning of the adapter, and a *Xho*I cohesive end (such that the *Xho*I site is re-created upon ligation). The resulting vector was named pcDNA3A2. *eGFP* bases 4 to 719 were amplified by PCR using the primer pair 5′-dT TATC TCG AGA GTGA GCAAGGG CGAG GAG CT (forward), and 5′-dCTGAATGGGCCCATTACTTGTACAGCTCGTC (reverse) using the plasmid pEGFP-1 (Clontech) as a template. The forward and reverse primers incorporate *Xho*I and *Apa*I restriction sites, respectively. The 729 base pair product was digested with *Xho*I and *Apa*I and ligated into the same sites in the plasmid pcDNA3A2 to create the vector pJMA2eGFP.

Plasmid pAPC-C2-eGFP was made by PCR amplification of *APC* bases 7606 to 8531 using the primer pair; 5′-dATCGGATCCCCTTCTAGACTTCCAATC (forward), and 5′-dTCGGAATTCTAAACAG ATGTCACAAGGTAAGAC (reverse). The forward and reverse primers incorporate *Bam*HI and *Eco*RI restriction sites respectively. The 947 bp product was digested with *Bam*HI and *Eco*RI and cloned into plasmid pJMA2eGFP. The resulting plasmid encodes the fusion protein APC-C2-GFP and comprises the extreme *C*-terminal 307 amino acids of APC *C*-terminally fused to eGFP (Figure 4).

Plasmid pAPC-C3-eGFP was made by digesting the *APC* cDNA with *Hind*III and *Dra*I and ligating the 2.1 kb fragment comprising APC bases 6381–8530 into the *Hind*III and *Eco*RV sites in the plasmid pJMA2eGFP. The resulting plasmid encodes the fusion protein APC-c3-GFP and comprises the extreme *C*-terminal 717 amino acids of APC *C*-terminally fused to eGFP (Figure 4).

The plasmid pAPC-basic-eGFP was made by PCR amplification of *APC* bases 6361–7529 using the primers 5′-dGCTGCATGTTTATCTAGAC (forward) and 5′-dTGATCATGTCGAGGACATTCTAGAAAGTTC (reverse), the reverse primer incorporating a *Sal*I restriction site. The 911 bp product was digested with *Hind*III and *Sal*I, cutting at an endogenous *Hind*III and the incorporated *Sal*I site to produce a fragment of 883 bp which was cloned into the *Hind*III and *Xho*I sites of pJMA2eGFP. The resulting vector directs the expression of the fusion protein APC-basic-GFP, which comprises APC amino acids 2127–2420 *C*-terminally fused to eGFP (Figure 4). All constructs were fully sequenced before use.

Cell culture

HCT116 and SW480 cells were obtained from the ECACC and cultured according to the supplier's instructions. HCT116 cells were synchronized at G1-S or G2-M by incubation in mimosine or nocodazole respectively, precisely as described previously (Trzepacz *et al*., 1997). SW480 cells were transiently transfected using DOTAP (Boehringer Mannheim) according to the manufacturer's instructions. Cells were processed for immunocytochemistry 24 h post-transfection.

Antibodies

The anti-EB1 monoclonal antibodies used in this study were obtained from Oncogene Research Products (clone EA3) and Transduction Laboratories (clone 5). The anti-APC reagents used in this study were a monoclonal antibody obtained from Oncogene Research Products (clone CF11) and a polyclonal antibody obtained from Santa-Cruz Biotechnology (APC N-15). A monoclonal antibody specific for β-catenin was obtained from Sigma (clone 6F9). Anti-GFP polyclonal antibodies were obtained from Clontech.

Immunocytochemistry

Cells were processed for immunocytochemistry as described previously (Morrison *et al*., 1998a). Confocal images were

obtained using a Leica TCS SP confocal imaging system, and figures were assembled using Adobe Photoshop 4. All analyses of imaged cells were performed using the Physiology module of the Leica confocal microscopy software.

SDS–PAGE and Western blotting

SDS–PAGE and Western blotting were performed as described previously (Morrison *et al*., 1998a).

Immunoprecipitation

Cell cultures in 75 cm^2 flasks were scraped into PBS, pelleted by centrifugation and the pellet lysed in 2 ml of ice-cold PBS containing 1% Triton X-100, a cocktail of protease inhibitors, 50 mM sodium fluoride and 100 μM sodium orthovanadate (PBS/TX100). After 10 min incubation on ice insoluble material was pelleted by centrifugation at 10 000 *g* and discarded. The lysate was pre-cleared by incubation with 10 μg/ml of normal mouse IgG or an equivalent volume of normal rabbit serum for 2 h at 4°C followed by incubation with protein A-sepharose for 1 h. The protein A-sepharose was pelleted by centrifugation and the cleared lysate removed to a fresh tube. One ml of this lysate was used in each immunoprecipitation. Five μg of monoclonal primary antibody or an equivalent volume of rabbit polyclonal primary antibody was added to each tube, followed by overnight incubation at 4°C. Immune complexes were precipitated by the addition of protein A-sepharose for the final 2 h of incubation, followed by centrifugation. The precipitated complexes were washed five times for 15 min in 20 volumes of cold PBS/1% TX100. Finally, the complexes were resuspended in SDS–PAGE loading buffer containing 5 mM DTT and boiled for 5 min before SDS–PAGE.

In vitro *transcription/translation*

One μg of plasmid DNA was transcribed and translated *in vitro* using the Promega TNT coupled transcription/translation system according to the manufacturer's instructions.

GST fusion protein precipitations

The GST fusion proteins GST-APC-C1 and GST-EB1 were purified from PBS/TX100 extracts of sonicated bacterial cultures using glutathione-sepharose according to the manufacturer's instructions (Pharmacia). Cell extracts were prepared as described for immunoprecipitation, and pre-cleared using glutathione-sepharose beads. Five μg of purified fusion protein, *in vitro* phosphorylated fusion protein (see below) or 20 μg of GST, all on glutathione-sepharose beads, were added to 1 ml of lysate or an *in vitro* transcription/translation reaction mix. Precipitations were performed overnight at 4°C. Captured complexes were collected by centrifugation and washed five times for 15 min in 20 volumes of cold PBS/TX100. Finally, the complexes were resuspended in SDS–PAGE loading buffer containing 5 mM DTT and boiled for 5 min before SDS–PAGE.

In-vitro *phosphorylation*

The *in vitro* phosphorylation conditions used in this study have been described previously for protein kinase A (PKA), protein kinase C (PKC) and casein kinase II (CKII) (Morrison *et al*., 1998b). $p34^{cdc2}$ was obtained from New England Biolabs along with its 10X concentrated reaction buffer. $p34^{cdc2}$ incubations were performed for 2 h at 30°C in the presence of 10 U enzyme. Five μg of the purified fusion protein GST-APC-C1 or GST, on 20 μl of a 50% slurry of glutathione-sepharose beads in PBS/TX100, were typically used in each reaction. Following the incubations the fusion proteins on beads were washed three times for 15 min in 20 volumes of PBX/TX100 before use in precipitation experiments.

Abbreviations

APC, adenomatous polyposis coli; GST, glutathione-S-transferase; DTT, dithiothreitol; PBS, phosphate buffered saline; PCR, polymerase chain reaction; TX100, Triton X-100; AS, asynchronous; M, mitosis; PKA, protein kinase A; PKC, protein kinase C; CKII, casein kinase II; eGFP, enhanced green fluorescent protein.

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