Oncogene (1998) 17, 3471–3477

http://www.stockton-press.co.uk/onc

EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle

EE Morrison, BN Wardleworth, JM Askham, AF Markham and DM Meredith

Molecular Medicine Unit, University of Leeds, Clinical Sciences Building, St. James's University Hospital, Leeds LS9 7TF, UK

The characteristics of the adenomatous polyposis coli (APC) associated protein EB1 were examined in mammalian cells. By immunocytochemistry EB1 was shown to be closely associated with the microtubule cytoskeleton throughout the cell cycle. In interphase cells EB1 was associated with microtubules along their full length but was often particularly concentrated at their tips. During early mitosis, EB1 was localized to separating centrosomes and associated microtubules, while at metaphase it was associated with the spindle poles and associated microtubules. During cytokinesis EB1 was strongly associated with the midbody microtubules. Treatment with nocodazole caused a diffuse redistribution of EB1 immunoreactivity, whereas treatment with cytochalasin D had no effect. Interestingly, treatment with taxol abolished the EB1 association with microtubules. In nocodazole washout experiments EB1 rapidly became associated with the centrosome and repolymerizing microtubules. In taxol wash-out experiments EB1 rapidly re-associated with the microtubule cytoskeleton, resembling untreated control cells within 10 min. Immunostaining of SW480 cells, which contain truncated APC incapable of interaction with EB1, showed that the association of EB1 with microtubules throughout the cell cycle was not dependent upon an interaction with APC. These results suggest a role for EB1 in the control of microtubule dynamics in mammalian cells.

Keywords: EB1; APC; microtubules; mitosis; cancer

Introduction

The adenomatous polyposis coli (APC) tumour suppressor protein has recently been shown to associate with EB1, a 30 kDa protein of unknown function (Su *et al*., 1995). The *APC* gene encodes a protein originally identified as a tumour suppressor because of the association between mutations in *APC* and familial adenomatous polyposis (FAP), a syndrome characterized by multiple colonic polyps and colorectal carcinoma (Levy *et al*., 1994; Oshima *et al*., 1995). APC has recently been shown to mediate the turnover of β-catenin in normal cells via binding to a central repeat region, thus downregulating a signalling pathway involving the interaction of β-catenin with the Tcf family of transcription factors (for a recent review see Gumbiner, 1997). The most common APC mutations found in colorectal cancer produce truncated proteins which lack this region, and this specific loss of function has been linked to carcinogenesis.

However, APC also associates with and stabilizes microtubules (Smith *et al*., 1994; Munemitsu *et al*., 1994), and may play a role in directed microtubule assembly during cell locomotion and neuronal growth cone exploration (Nathke *et al*., 1996; Pollock *et al*., 1997; Morrison *et al*., 1997a,b). Furthermore, both EB1 and human homologs of the *Drosophila* discs large tumour suppressor protein (hDLG) bind at the *C*-terminus of APC (Su *et al*., 1995; Matsumine *et al*., 1996). Critically, loss of only the final 200 amino acids of APC has been demonstrated to produce a phenotype similar to that arising with mutations which disrupt the β-catenin turnover function (Groden *et al*., 1993), although the expression of such a minimally truncated APC protein has been shown to successfully downregulate Tcf/β-catenin signalling (Morin *et al*., 1997). Therefore, loss of functions localized to the *C*-terminus of APC may also contribute to carcinogenesis. Deletion of this *C*-terminal region would disrupt hDLG binding and potentially the interactions with microtubules and EB1. An EB1 homolog has been identified in activated T-lymphocytes (Renner *et al*., 1997), while the interaction between EB1 and APC can occur as part of a complex with β-catenin (Morin *et al*., 1996). Several yeast homologs of EB1 have also been characterized (Beinhauer *et al*., 1997; Schwartz *et al*., 1997). These latter studies have created intense speculation about the role of EB1, but nothing has been reported concerning the function of this protein in mammalian cells to date. In this paper therefore, we present an initial characterization of the properties of EB1.

Results

Western blotting analysis of a range of cell and tissue samples revealed that EB1 appeared to be a ubiquitously expressed protein present in a range of human cell lines (Figure 1a and b). It should be noted that this blot was intended as a simple detection screen; relative expression levels of EB1 should not be inferred from this data. EB1 was also recognized in an extract of embryonic mouse brain, suggesting that the mouse homolog cross-reacted with the antibody used in this blot, clone EA3. In confirmation of this, EB1 was detected in Neuro2A mouse neuroblastoma cells by blotting with this antibody and also by immunofluorescence with the clone 5 EB1-specific antibody (results not shown). However, EB1 was not detected in baby hamster kidney cells by immunoblotting with clone EA3 (Figure 1a). In all cases but one, the EB1 species

Correspondence: EE Morrison
Received 5 May 1998; revised 24 June 1998; accepted 25 June 1998

detected by Western blotting appeared to be of a uniform size which comigrated with a 30 kDa SDS–PAGE molecular weight marker. On DATD cross-linked acrylamide gels, which give an excellent resolution of different protein isoforms, the form of EB1 recognized in the 293T human kidney-derived cell line migrated slightly slower than the form detected in the other cells and tissues (Figure 1a). The size differential between this band and the others on the blot was too small to be accurately determined. As shown in Figure 1b, EB1 is a relatively abundant protein in both COS-7 cells and several human colon cancer cell lines known to exhibit mutations in *APC*. EB1 is a very soluble protein in both the COS-7 and SW480 cell lines. In concentrations of Triton X-100 as low as 0.05% (v/v) in the presence of 100 mM NaCl, EB1 appeared to be completely soluble (Figure 1c). In view of data presented later in this paper, we wished to test the effects of cell division upon EB1 expression. Paju cells were derived from a human primitive neuroectodermal tumour (PNET) and can be induced to cease cell division and differentiate down a melanocytic pathway by treatment with retinoic acid or a neuronal pathway by incubation with phorbol ester (Zhang *et al*., 1996). As shown in Figure 1d, the expression levels of EB1 were essentially identical in actively dividing or differentiated Paju cells, suggesting that cell division is not essential for EB1 expression.

Immunofluorescence analysis of COS-7 cells revealed that EB1 was localized to the microtubule network throughout interphase cells and was particularly concentrated at the centrosome (Figure 2). The microtubule staining was not always uniform, and often seemed more intense at what appeared to be the microtubule tip (Figure 2a–d). This is of interest because endogenously expressed APC has previously been shown to associate with the tips of microtubules (Näthké *et al*., 1996; Morrison *et al*., 1997a). In some cells membraneous extensions were seen, which

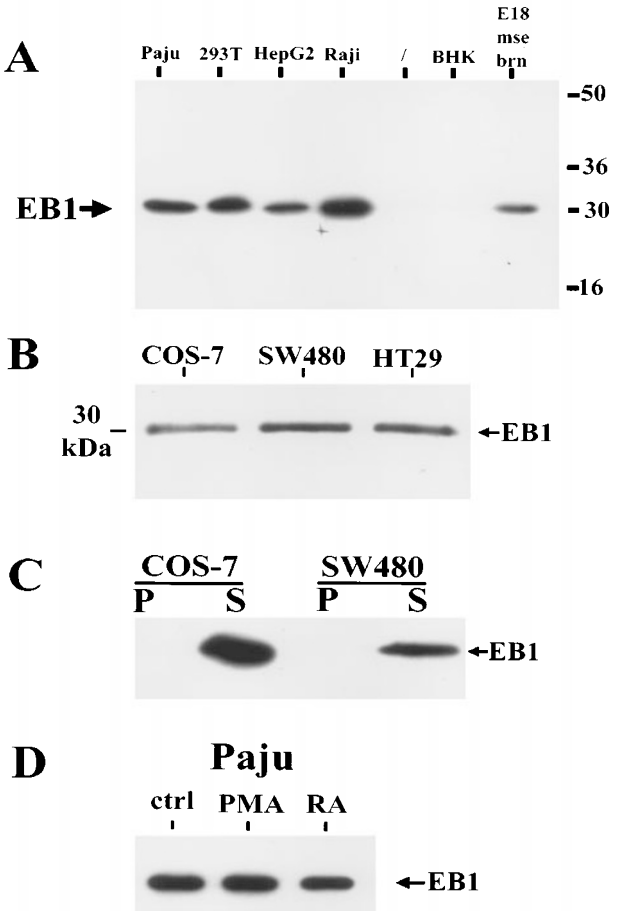

Figure 1 Western blotting analysis of EB1. (**a**) EB1 is expressed in Paju (PNET-derived), 293T (kidney-derived), HepG2, (liver-derived) and Raji (B-cell derived) cells. No EB1 was detected in BHK cells. EB1 was, however, detected in an extract of E18 embryonic mouse brain. (**b**) EB1 is visualized as a single 30 kDa band in COS-7, SW480 and HT29 cells. (**c**) EB1 is completely soluble in low concentrations of non-ionic detergent in COS-7 and SW480 cells. P = pelleted, insoluble cellular fraction, S = soluble cellular fraction. (**d**) EB1 expression is identical in dividing and differentiated cells. Similar amounts of EB1 were detected in dividing Paju cells (lane 1) and in cells differentiated by 72 h incubation with PMA (lane 2) or retinoic acid (lane 3)

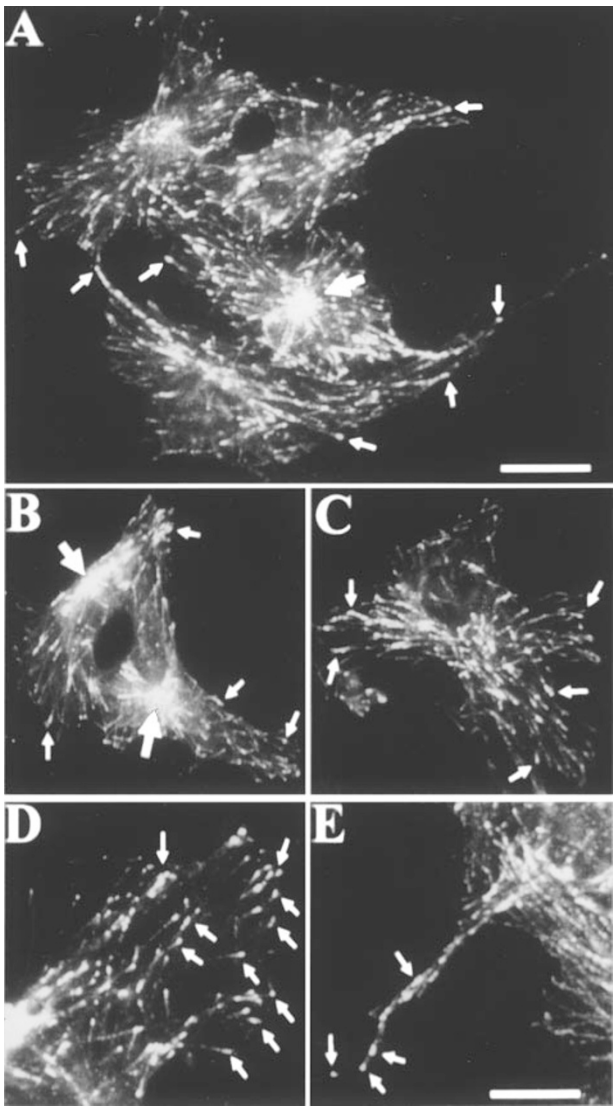

Figure 2 EB1 associates with the microtubule network in COS-7 cells. COS-7 cells were immunostained with monoclonal antibodies to EB1. (**a**–**c**) EB1 localizes to the microtubule network, and in particular to the tips of microtubules (arrows). Bar = 25 μm. (**d**) Magnified view of part of a COS-7 cell immunostained for EB1. Note again the increased localization of EB1 to microtubule tips (arrows). (**e**) Magnified view of a COS-7 membrane extension immunostained for EB1. Note the increased concentration of EB1 associated with the microtubule bundles (arrows). Bar for **d** and **e** = 10 μm

appeared to contain a higher proportion of thick, bundled microtubules. EB1 immunostaining was more intense in these structures when compared to the surrounding microtubule network (Figure 2e).

Strikingly, EB1 remained associated with the microtubule network even during the extensive remodelling which occurs during mitosis (Figure 3). During prophase EB1 intensely immunostained centrosomes (Figure 3a). In prometaphase EB1 stained the spindle poles and the astral and polar microtubules associated with them (Figure 3b), an association that was maintained throughout metaphase and anaphase (Figure 3c–e). After mitosis, EB1 intensely stained the midbody during cytokinesis (Figure 3f), before re-associating with the cytoplasmic microtubule network.

Nocodazole is a compound which inhibits microtubule assembly and incubation of cells with this drug leads to the collapse of the microtubule cytoskeleton. As might be expected, this resulted in the disappearance of the EB1 microtubule-associated staining, although EB1 was still associated with any remaining microtubules in the cell (Figure 4b). EB1 immunostaining of centrosomes also disappeared, possibly due to centrosome fragmentation (Jordan *et al*., 1992). Concurrent with this was the appearance of a speckled EB1 staining pattern associated with the nucleus.

Taxol is a clinically useful drug which inhibits microtubule dynamics, leading to the stabilization of the cellular microtubule network. Treatment of COS-7 cells with taxol dramatically decreased the localization of EB1 to microtubules, again leading to a speckled nuclear-associated staining pattern (Figure 4c). In contrast, treatment of COS-7 cells with cytochalasin D, a compound which destabilises the actin microfilament network, had no effect upon the cytoskeletal association of EB1 (Figure 4d).

When cells treated with nocodazole were rinsed clear of this drug and incubated in nocodazole-free medium, EB1 was seen to become associated with the regenerating microtubule network. Simply washing

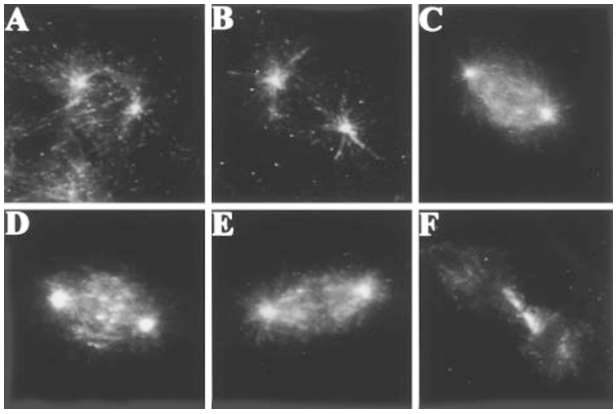

Figure 3 EB1 is associated with the mitotic spindle apparatus in COS-7 cells. COS-7 cells were immunostained for EB1 and images of the different stages of mitosis, as assessed by cellular morphology, were captured. (**a**) Prophase: EB1 is associated with the separating centrosomes and associated microtubules. (**b**) Prometaphase: EB1 is associated with the spindle poles and the associated astral microtubules. (**c** and **d**) Metaphase. EB1 is associated with the spindle poles, spindle microtubules (polar and kinetochore) and astral microtubules. (**e**) Anaphase: EB1 association remains as described for **c** and **d**. **e**; cytokinesis: EB1 is strongly associated with the midbody microtubules

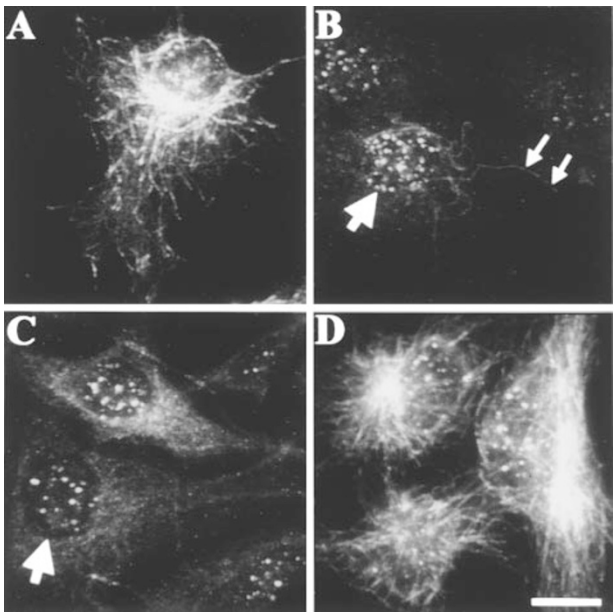

Figure 4 Disrupting microtubule dynamics causes EB1 dissociation from the microtubule cytoskeleton. (**a**) Control COS-7 cell immunostained for EB1. (**b**) COS-7 cell treated with nocodazole to disrupt the microtubule cytoskeleton. Note that the EB1 immunoreactivity redistributes to foci associated with the nucleus (large arrow). EB1 remains associated with intact microtubules (small arrows). (**c**) COS-7 cell treated with taxol to inhibit microtubule dissociation. The microtubule association of EB1 is abolished and a speckled nuclear-associated stain is again seen (large arrow). (**d**) COS-7 cells treated with cytochalasin D to disrupt the actin cytoskeleton. No effect is seen on EB1 distribution. Bar = 20 μm

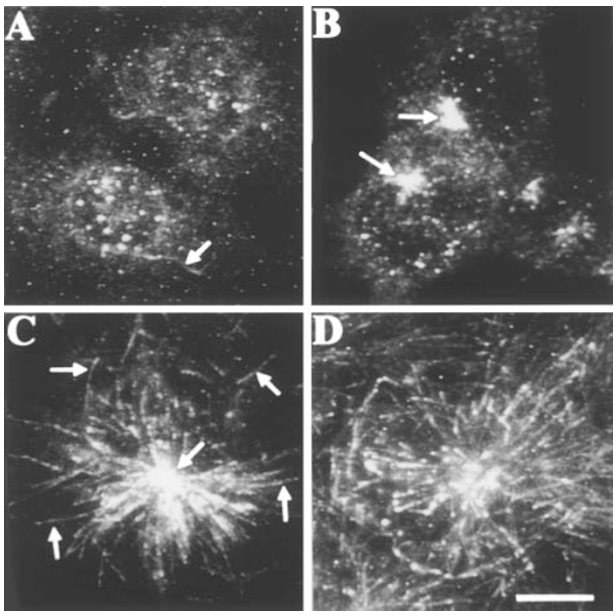

Figure 5 EB1 associates with assembling microtubules. COS-7 cells were incubated in the presence of nocodazole, which was then washed out and the cells incubated for up to 10 min in the absence of the drug. (**a**) Nocodazole treated control. The microtubule network has been abolished, except for occasional individual microtubules which immunostain for EB1 (arrow). (**b**) Nocodazole treated cells washed twice in warmed PBS. The speckled nucleus-associated staining has largely disappeared and EB1 is already associated with the centrosomes of the cell (arrows). (**c**) Following a further 1 min incubation in warmed media in the absence of nocodazole EB1 is associated with the rapidly re-polymerizing microtubule network (arrows). (**d**) After a further 10 min incubation EB1 immunostaining of the microtubule network resembles untreated controls. Bar = 25 μm

the cells in warmed PBS for approximately 30 s before processing for immunofluorescence resulted in the loss of the speckled nuclear staining and the intense immunostaining of the centrosomes within the cells (Figure 5b). This association was never seen in cells continuously incubated in nocodazole (Figure 5a). After a further 1 min incubation in warmed medium, EB1 was seen to be associated with microtubules growing from the centrosomes. This association continued as the network grew during further incubation (Figure 5c and d).

When cells were treated with taxol followed by a washing protocol similar to that described above, EB1 was seen to rapidly re-associate with the microtubule network (Figure 6). By 10 min incubation after washing the EB1 microtubule association appeared identical to that seen in untreated cells (Figure 6c).

APC has previously been shown to have an association with microtubules (Smith *et al*., 1994; Munemitsu *et al*., 1994). Although no reports have been presented showing binding of APC to the mitotic spindle, and since the intracellular staining patterns of APC and EB1 in interphase cells appear to overlap to some extent (although endogenously expressed APC is only reported to localize to microtubule tips, Näthke *et al*. (1996); Morrison *et al*. (1997a)), it was formally possible that the EB1 microtubule association was mediated through the interaction of EB1 with APC.

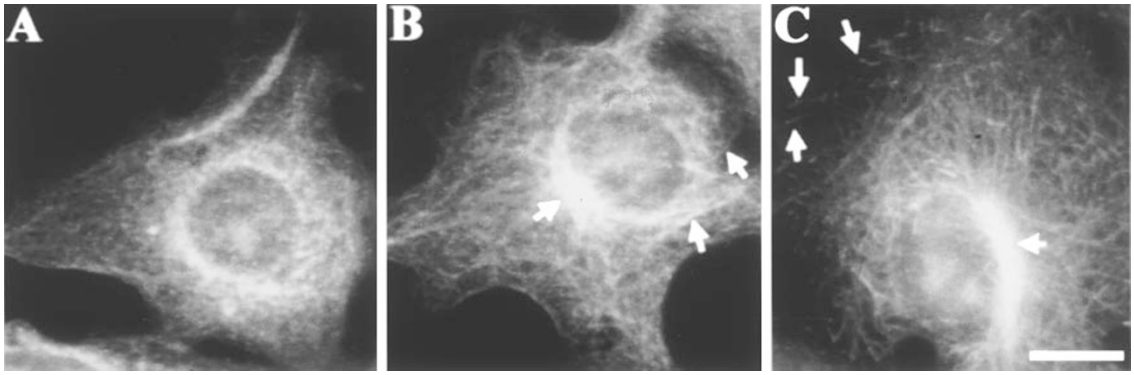

Figure 6 EB1 re-associates with microtubules following taxol withdrawal. (**a**) Taxol treated cell washed twice in warmed PBS. The EB1 microtubule association remains largely abolished, although the speckled nucleus-associated staining has disappeared. (**b**) After a further 1 min incubation EB1 has begun to re-associate with microtubules, but does not appear to be concentrated at microtubule tips. (**c**) After 10 min incubation in the absence of taxol, EB1 has re-associated with the entire microtubule network and is once again concentrated at microtubule tips (arrows). Bar = 25 μm

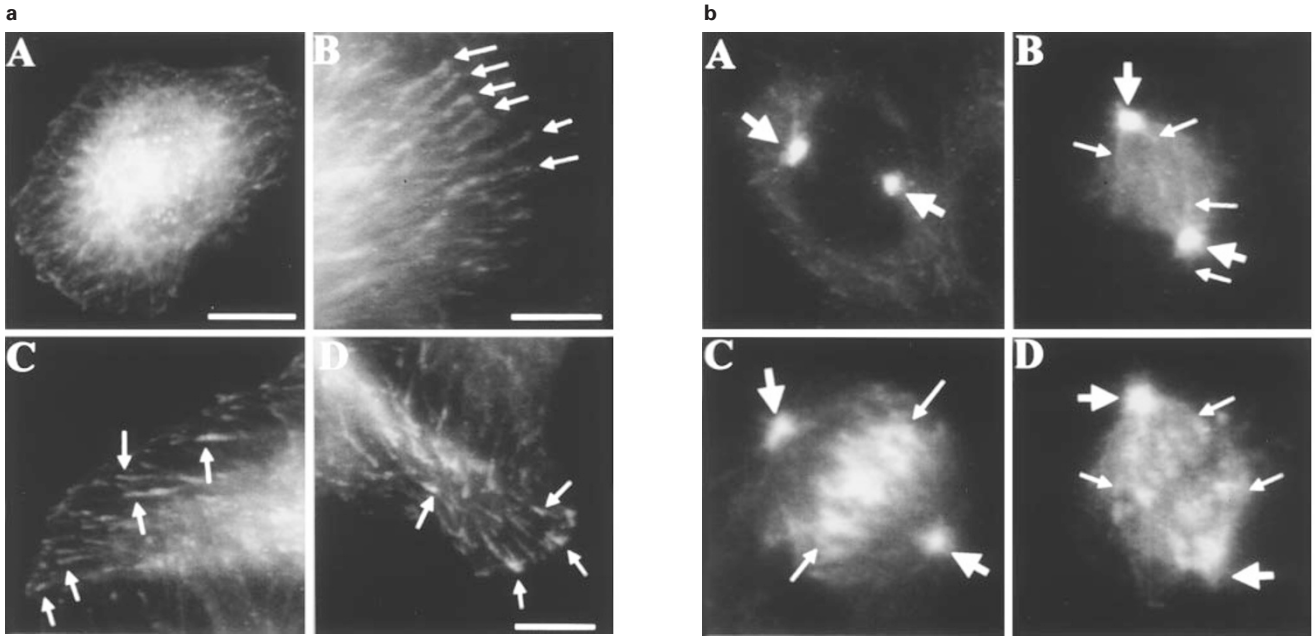

Figure 7 The EB1 microtubule association is not dependent upon an interaction with APC. (**a**) (Panel A) SW480 cells, which contain a truncated APC protein incapable of EB1 binding, immunostained for EB1. Note the extensive localization of EB1 to the microtubule network. Bar = 20 μm. (Panels b–d) higher magnification view of the EB1 microtubule association in SW480 cells. EB1 is again concentrated at the tips of microtubules in the absence of APC binding (arrows). Bar for Panels B–D = 10 μm. (**b**) EB1 is associated with spindle microtubules during mitosis in the absence of APC binding in SW480 cells. Panel A; prophase Panels B and C; metaphase. Panel D anaphase

SW480 cells have been incontrovertibly demonstrated to contain no full-length APC. Only a single form of APC, truncated at amino acid 1338, is expressed in these cells. This *N*-terminal fragment lacks both the region of APC implicated in EB1 binding (Su *et al*., 1995) and a basic region hypothesized to mediate the interaction of APC with microtubules (Smith *et al*., 1994; Munemitsu *et al*., 1994).

Immunostaining revealed that EB1 was still associated with the microtubule network within SW480 cells (Figure 7a). APC, therefore, cannot entirely mediate the association of EB1 with microtubules. The opposite possibility, however, that EB1 mediates the association of APC with microtubules, remains to be investigated. EB1 appeared to associate with the mitotic spindle throughout mitosis in SW480 cells in a similar manner to that seen in COS-7 cells (Figure 7b). No gross, obvious distortion of the spindle was observed. Essentially identical results were also obtained in the HCT116 human colorectal cancer cell line, which contains full length APC (Results not shown).

Discussion

At least three different members of the EB1 protein family have been identified in humans to date (Su *et al*., 1995; Renner *et al*., 1997; Su and Qi, 1998). It is therefore possible that the antibodies used in this study cross-react with more than one member of the family, although in all of the cells tested the forms would have to display an absolutely identical MW, which is unlikely. The human EB1 proteins have a similar *N*-terminal sequence but are more divergent elsewhere in the protein. Of the antibodies used in this study, one was raised against the *C*-terminal half of human EB1 (clone 5) while the epitope of the other (clone EA3) is undetermined. A recent study has demonstrated that three monoclonal antibodies, including the EA3 clone, do not cross-react with either of the two existing human EB1 homologs (Su and Qi, 1998), a finding which may indicate that monoclonal antibodies to individual family members will tend to be monospecific. On the basis of our Western blotting data, this is an assertion with which we would concur. The origin of the slightly larger EB1 protein in 293T cells is unclear. Since the difference in MW is slight and was only obvious on DATD cross-linked gels, we suspect that it represents either a polymorphism in the EB1 sequence or, more likely, a post-translational modification of the protein in this cell line. However, until definitive proof of antibody specificity is shown by the screening of all of the human EB1 family members, the results presented in this paper cannot be considered completely specific for human EB1.

The data presented here demonstrates that EB1 is a microtubule-associated protein (Figures 2–6). Drug-induced perturbation of the microtubule cytoskeleton suggested that EB1 is involved in microtubule assembly or the maintenance of microtubule stability, whilst actin disruption had no effect upon the distribution of EB1 (Figures 4–6). An association of EB1 with the mitotic spindle and with the midbody during cytokinesis has also been demonstrated (Figures 3 and 7b). All of this data agrees with recent studies characterizing two yeast homologs of EB1 (Beinhauer *et al*., 1997; Schwartz *et al*., 1997). Interestingly, one of these homologs, Mal3, was originally identified in a screen designed to isolate genes involved in chromosome segregation. Although null mutants of *mal3* were viable and displayed normal mitotic spindle morphology, *mal3* overexpression increased the number of cells in mitosis but caused defects in chromosome segregation to the poles. In some cells it also induced spindle disintegration, while cytokinesis was retarded and septa were malformed and misplaced. EB1 overexpression in yeast cells produced a similar phenotype. Deletion of the budding yeast EB1 homolog *BIM1* resulted in aberrant spindle formation, while overexpression was lethal, probably as a result of spindle disintegration.

All of these features are characteristic of a family of proteins whose function involves the promotion of microtubule stability. The data presented here indicates that EB1 is likely to fulfil a very similar role to its yeast homologs during mitosis in mammalian cells. Interestingly, as Beinhauer *et al*. (1997) point out, a feature of colorectal cancer is genetic instability within the tumour cells and this has been linked to defects in chromosome segregation (Lengauer *et al*., 1997). The observation that EB1 associates with the mitotic spindle, as well as interacting with a tumour suppressor commonly truncated in colon cancer, provides a potential link between mutant APC-induced tumorigenesis and this genetic instability. As noted in the Introduction to this paper, the commonest mutations in APC which have been linked to tumorigenesis would also ablate the APC/EB1 interaction.

EB1 decorated microtubules throughout their length during interphase and during growth after recovery from nocodazole-induced depolymerization. This is also very similar to the situation observed in fission yeast with Mal3. Null mutants of *mal3* were shown to display defects in cell shape caused by poor microtubule structure (Beinhauer *et al*., 1997). We have shown here that EB1 staining was more intense on rigid microtubule structures within membrane projections. These appeared to be composed of thick microtubule bundles (Figure 2e), again suggesting that EB1 contributes to microtubule stability. We would speculate therefore that EB1 or a closely related homolog may contribute to microtubule stability in specialized structures such as neuronal axons, and that aside from a putative role in regulating microtubule dynamics during mitosis EB1-like proteins may be involved in promoting microtubule stability and/or extension during neuronal neuritogenesis and cellular locomotion. Similar roles have also recently been postulated for APC (Näthke *et al*., 1996; Pollock *et al*., 1997; Morrison *et al*., 1997a,b). The fact that EB1 expression levels were essentially identical in both dividing and differentiated Paju cells supports the contention that EB1 plays a general role in the microtubule cytoskeleton, and that it is not a protein specifically expressed for mitotis. The observation that taxol, a drug which suppresses microtubule dynamics, decreases the amount of EB1 associated with the microtubule cytoskeleton may appear paradoxical. However, a similar effect of taxol upon the distribution of APC was noted in a previous study (Näthke *et al*., 1996), and the effect appears reversible (Figure 6).

It therefore seems that inhibiting microtubule dynamics disrupts the association of both APC and EB1 with the cytoskeleton. It is worth noting that taxol acts by stabilizing microtubules at the plus end of the tubulin polymer, where most of the growth of microtubules occurs (see Jordan and Wilson, 1998 for a recent review) and where we see a greater concentration of EB1 (Figure 2a–d). This further supports the hypothesis that EB1 may promote microtubule polymerization.

Previous studies have indicated that truncated APC cannot associate with microtubules *in vivo* (Munemitsu *et al*., 1994; Smith *et al*., 1994). We have shown here that EB1 still associates with microtubules and still appears to concentrate at microtubule tips in the absence of an interaction with APC in SW480 colorectal carcinoma cells (Figure 7a, b–d). Given the similar effect of taxol on the microtubule associations of both APC and EB1, it is possible that the association of APC with the microtubule cytoskeleton is mediated via its *C*-terminal binding to EB1, and not via a direct association of APC with the cytoskeleton using a putative *C*-terminal basic region as originally proposed (Munemitsu *et al*., 1994; Smith *et al*., 1994). Supporting this former contention is the observation that overexpression of APC or *C*-terminal fragments of this protein results in the decoration of the entire microtubule cytoskeleton in a pattern similar to that observed here for EB1 (Munemitsu *et al*., 1994; Smith *et al*., 1994), whereas endogenous APC appears to localize only to microtubule tips (Näthke *et al*., 1996; Pollock *et al*., 1997; Morrison *et al*., 1997a). Alternatively, both APC and EB1 may associate with microtubules and the interaction between the two proteins could serve to enhance microtubule stability and bundling through either the cross-linking of adjacent microtubules or the stabilization of individual microtubules. EB1 and APC could also possibly form part of a regulatory complex at the tip of a microtubule, which would have the potential to promote or inhibit microtubule extension in response to environmental cues.

We note that the *C*-terminal region of APC which includes the site for interaction with EB1 has recently been shown to be phosphorylated during mitosis by $p34^{cdc2}$ kinase (Trzepacz *et al*., 1997). It would therefore be interesting to investigate whether this phosphorylation regulates the association of APC with EB1. A model can be postulated in which APC phosphorylation regulates the association of EB1 and APC, modulating EB1 function during mitosis. Loss of the EB1 binding function of APC could lead to a potential misregulation of mitosis and contribute to genetic instability and thus the initiation of carcinogenesis. Implicit in this model is that the forced overexpression of APC would be antagonistic to cell division through interference with mitosis, and evidence exists for this (Morin *et al*., 1996). Regulation of the interaction between EB1 and APC may also play a role during the directed microtubule assembly seen in cellular locomotion and neuritogenesis. It has been demonstrated that upregulating APC expression in the colonic epithelium causes disordered cell migration (Wong *et al*., 1996) and that abnormal cell migration is an early event in polyp formation in a mouse model of FAP where the Apc/EB1 interaction is presumably ablated (Oshima *et al*., 1995). In summary, the EB1/APC complex may play an important role in the regulation of microtubule stability, and loss of this interaction could make an important contribution to the tumour development seen in familial and sporadic forms of colorectal cancer.

Materials and methods

Cells

The majority of the cell lines used in this study were obtained from the ECACC. The Paju PNET cell line was a kind gift from Professor LC Andersson, Department of Pathlogy, University of Helsinki, Finland, (Zhang *et al*., 1996). COS-7 cells, an SV40-transformed African Green monkey kidney cell line, were maintained in DMEM containing glutamax (Sigma) and 10% (v/v) FCS. SW480 human colon cancer cells and Paju cells were grown in RPMI 1640 medium containing glutamax (Sigma) and 10% (v/v) FCS. Other cell lines were maintained according to ECACC guidelines.

Antibodies

The anti-EB1 monoclonal antibodies used in this study were obtained from Oncogene Research Products (clone EA3; Su *et al*., 1995) and Transduction Laboratories (clone 5). Both antibodies gave identical results in immunofluorescence and Western blotting studies, although clone 5 was generally used for immunocytochemistry and clone EA3 was normally used in blotting experiments.

Immunocytochemistry

Cells were washed in PBS before fixation in ice cold methanol for 5 min. Following fixation the cells were washed in PBS before incubation in 1% non-fat dried skimmed milk in PBS for 30 min at room temperature to block non-specific binding. The cells were then incubated with primary antibodies in the same solution for 2 h at room temperature. Following this the cells were washed in PBS before incubation with secondary antibodies (goat anti-rabbit IgG, Vector Laboratories and goat anti-mouse immunoglobulins, Dako) in 1% skimmed milk. Coverslips were mounted using Mowiol (Sigma). Slides were viewed using a Zeiss Axiovert 135TV inverted fluorescence microscope. Images were captured using a Hamammatsu cooled CCD camera and the Fluo Vision fluorescence image analysis package (Impro Vision) running on an Apple Macintosh computer.

SDS–PAGE and Western blotting

SDS–PAGE gels contained 12% acrylamide cross-linked with N,N″-diallyltartardiamide (DATD). Samples were solubilized by boiling under reducing conditions in the presence of SDS and 5 mM dithiothreitol. For Western blotting, proteins were electrophoretically transferred to nitrocellulose membranes. Western blots were developed using HRP-conjugated secondary antisera (DAKO) and the Pierce SuperSignal ECL system used according to the manufacturer's instructions. Non-specific binding was minimized by the addition of 1% non-fat dried skimmed milk to primary and secondary antibody incubations and 0.1% Tween 20 to wash buffers.

Cell treatments

To stabilize the microtubule cytoskeleton, taxol (Sigma) was added to a final concentration of 10 μM and the cells

incubated for 45 min at 37°C. To disrupt microtubules, nocodazole (Sigma) was added to cells at a concentration of 2.5 μg/ml followed by incubation at 37°C for 45 min The actin cytoskeleton was disrupted by incubation with cytochalasin D (Sigma) at a final concentration of 2 μg/ml for 45 min at 37°C. In nocodazole and taxol wash-out experiments cells were incubated as described above before washing three times in warmed PBS and a further incubation for various times in medium without drug. Cells were then fixed and processed for immunocytochemistry as described above.

Cell extractions

Cells were scraped into PBS and pelleted by low-speed centrifugation. Cell pellets were resuspended in 50 mM Tris HCl pH 7.4, 100 mM NaCl containing 0.05–1% (v/v) Triton X-100 and a cocktail of protease inhibitors. After 5 min incubation at room temperature with occasional mixing the cell extracts were centrifuged at 12 000 *g* in a bench-top centrifuge for 5 min. The supernatants were kept as the detergent soluble cell extracts and the pellets were boiled in SDS–PAGE sample buffer under reducing conditions (5 mM DTT) and kept as the detergent-insoluble cell extract.

Acknowledgements

The authors wish to acknowledge the financial support of the Medical Research Council (UK) for this work. Research in the author's laboratories is also supported by Yorkshire Cancer Research and the West Riding Medical Research Trust.

References

Beinhauer JD, Hagan IM, Hegemann JH and Fleig U. (1997). *J. Cell. Biol.,* **139,** 717–728.

Gumbiner BM. (1997). *Curr. Biol.,* **7,** R443–R446.

Groden J, Gelbert L, Thliveris A, Nelson L, Robertson M, Joslyn G, Samowitz W, Spirio L, Carlson M, Burt R, Leppert M and White R. (1993). *Am. J. Hum. Gen.,* **52,** 263–272.

Jordan MA, Thrower D and Wilson L. (1992). *J. Cell. Sci.,* **102,** 401–416.

Jordan MA and Wilson L. (1998). *Curr. Opin. Cell. Biol.,* **10,** 123–130.

Lengauer C, Kinzler KW and Vogelstein B. (1997). *Nature,* **386,** 623–627.

Levy DB, Smith KJ, Beazer-Barclay Y, Hamilton SR, Vogelstein B and Kinzler KW. (1994). *Cancer Res.,* **54,** 5953–5958.

Matsumine A, Ogai A, Senda T, Okumura N, Satoh K, Baeg G-H, Kawahara T, Kobayashi S, Okada M, Toyoshima K and Akiyama T. (1996). *Science,* **272,** 1020–1023.

Morin PJ, Vogelstein B and Kinzler KW. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 7950–7954.

Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B and Kinzler KW. (1997). *Science,* **275,** 1787–1790.

Morrison EE, Askham J, Clissold P, Markham AF and Meredith DM. (1997a). *Neuroscience,* **81,** 553–567.

Morrison EE, Askham J, Clissold P, Markham AF and Meredith DM. (1997b). *Neuroscience Lett.,* **235,** 129–132.

Munemitsu S, Souza B, Muller O, Albert I, Rubinfeld B and Polakis P. (1994). *Cancer Res.,* **54,** 3676–3681.

Näthke IS, Adams CL, Polakis P, Sellin JH and Nelson JW. (1996). *J. Cell. Biol.,* **134,** 165–179.

Oshima M, Oshima H, Kitagawa A, Kobayashi M, Itakura C and Taketo M. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 4482–4486.

Pollock AL, Barth AIM, Altschuler Y, Nelson WJ and Mostov KE. (1997). *J. Cell. Biol.,* **137,** 1651–1662.

Renner C, Pfitzenmeier J-P, Gerlach K, Held G, Ohnesorge S, Sahin U, Bauer S and Pfreundschuh M. (1997). *J. Immunol.,* **159,** 1276–1283.

Schwartz K, Richards K and Botstein D. (1997). *Mol. Biol. Cell.,* **8,** 2677–2691.

Smith KJ, Levy DB, Maupin P, Pollard TD, Vogelstein B and Kinzler KW. (1994). *Cancer Res.,* **54,** 3672–3675.

Su L-K, Burrell M, Hill DE, Gyuris J, Brent R, Wiltshire R, Trent J, Vogelstein B and Kinzler KW. (1995). *Cancer Res.,* **55,** 2972–2977.

Su L-K and Qi Y. (1998). *Proc. Am. Assoc. Cancer Res.,* **39,** abstract no. 4193.

Trzepacz C, Lowy AM, Kordich JJ and Groden J. (1997). *J. Biol. Chem.,* **272,** 21681–21684.

Wong MH, Hermiston ML, Syder AJ and Gordon JI. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 9588–9593.

Zhang K-Z, Westberg JA, Hölltä E and Andersson LC. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 4504–4508.