Redefining the subcellular location and transport of APC: new insights using a panel of antibodies

Mariana Brocardo¹, Inke S. Näthke² & Beric R. Henderson¹⁺

¹Westmead Institute for Cancer Research, Westmead Millennium Institute at Westmead Hospital, University of Sydney, Westmead, New South Wales, Australia, and ²Department of Cell & Developmental Biology, University of Dundee, Dundee, UK

Adenomatous polyposis coli (APC) is a tumour suppressor involved in colon cancer progression. We and others previously described nuclear–cytoplasmic shuttling of APC. However, there are conflicting reports concerning the localization of endogenous wild-type and tumour-associated, truncated APC. To resolve this issue, we compared APC localization using immunofluorescence (IF) microscopy and cell fractionation with nine different APC antibodies. We found that three commonly used APC antibodies showed nonspecific nuclear staining by IF and validated this conclusion in cells where APC was inactivated using small interfering RNA or Cre/Flox. Fractionation showed that wild-type and truncated APC from colon cancer cells were primarily cytoplasmic, but increased in the nucleus after leptomycin B treatment, consistent with CRM1-dependent nuclear export. In contrast to recent reports, our biochemical data indicate that APC nuclear localization is not regulated by changes in cell density, and that APC nuclear export is not prevented by truncating mutations in cancer. These results verify that the bulk of APC resides in the cytoplasm and indicate the need for caution when evaluating the nuclear accumulation of APC.

Keywords: APC; antibodies; subcellular localization; colon cancer; mutations

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INTRODUCTION

Mutations in the adenomatous polyposis coli (APC) gene are associated with the development of colorectal cancer (Polakis, 2000; Lustig & Behrens, 2003). APC interacts with proteins involved in the Wnt signalling pathway and in cytoskeletal organization (Dikovskaya *et al*, 2001). Activation of the Wnt pathway results in the β -catenin/lymphoid-enhancing factor 1

(LEF1)-dependent activation of transformation-inducing genes and cancer initiation (Lustig & Behrens, 2003). Tumour-associated mutations in APC affect several functions, including its role in stabilizing β -catenin (Polakis, 2000). APC can shuttle between the nucleus and cytoplasm (Henderson, 2000; Neufeld *et al*, 2000; Rosin-Arbesfeld *et al*, 2000), and its nuclear export may affect β -catenin localization and turnover (Henderson & Fagotto, 2002). In addition, APC is a cytoskeletal regulator and accumulates at the ends of microtubule bundles near the plasma membrane where it may contribute to cell migration (Näthke *et al*, 1996). The diversity of function and localization of APC raise the question of how these different pools of APC are inter-related and coordinated. A crucial factor in addressing this issue is the ability to determine unambiguously the localization of endogenous APC.

Endogenous APC has been described at several microtubule-associated locations, including membrane protrusions (Näthke *et al*, 1996; Mimori-Kiyosue *et al*, 2000) and kinetochores (Fodde *et al*, 2001; Kaplan *et al*, 2001), using different antibodies and immunofluorescence (IF) microscopy. APC has also been observed at the apical membrane (Reinacher-Schick & Gumbiner, 2001). However, the antibody used in the latter study was subsequently found to crossreact with the DNA-binding protein Ku (Mogensen *et al*, 2002; Roberts *et al*, 2003), potentially questioning the specificity of other antibodies used for detection of APC at specific sites. This issue may be a factor in some of the discrepancies described in the accumulation of APC in the nucleus. For example, although APC has been detected predominantly in the nucleus of certain cell types by cell staining (Neufeld & White, 1997; Rosin-Arbesfeld *et al*, 2003), biochemical fractionation methods showed a predominantly cytoplasmic distribution of APC (Smith *et al*, 1993; Galea *et al*, 2001). In addition, a shift of endogenous APC from the nucleus to the cytoplasm in response to increased cell density (Brocardo *et al*, 2001; Zhang *et al*, 2001; Fagman *et al*, 2003; Davies *et al*, 2004) or proliferation (Fagman *et al*, 2003; Olmeda *et al*, 2003) has been observed using antibodies, such as M-APC antisera by cell staining, but has not yet been confirmed by biochemical methods. Our aim was to compare the localization of APC using a panel of the most commonly used APC antibodies, using biochemical fractionation as well as fluorescence microscopy. We found that endogenous forms of APC are predominantly cytoplasmic, and

¹Westmead Institute for Cancer Research, Westmead Millennium Institute at Westmead Hospital, University of Sydney, Darcy Road (PO Box 412), Westmead, New South Wales 2145, Australia

²Department of Cell & Developmental Biology, University of Dundee, Dundee DD1 5EH, UK

⁺Corresponding author. Tel: +61 2 9845 9057; Fax: +61 2 9845 9102; E-mail: beric_henderson@wmi.usyd.edu.au

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Fig 1 | Detection of ectopic and endogenous APC with different antibodies by IF microscopy. (A) Diagram of APC protein showing the different epitopes recognized by antibodies tested in this study. (B) Full-length pAPC–YFP was transfected into SW480 colon cancer cells. After 48 h, ectopic YFP localization of APC–YFP was compared using different antibodies against APC protein by immunostaining. Positive colocalization of each antibody with APC–YFP expression is indicated (+). (C) Cellular staining patterns observed with different APC antibodies. SW480, HCT116 or MDCK cells were fixed on glass coverslips in formalin and analysed by IF microscopy. The staining shows variability between different antibodies, although all except for Ab4 detected APC at membrane protrusions in MDCK cells.

Fig 2 | M-APC nuclear staining is not specific for APC. (A) SW480 cells, untransfected or transfected with an anti-APC siRNA for 48 h, were fixed and stained with affinity-purified M-APC antibody. APC siRNA did not affect the cell staining profile of M-APC antibody. (B) In parallel, equivalent amounts of cytoplasmic or nuclear extracts from cells treated $\pm$ siRNA (APC, or control BRCA1 siRNA) were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and analysed by immunoblot. Truncated APC (150 kDa) was detected with antibody M-APC. The same membrane was reprobbed for topoisomerase II α (170 kDa) as a fractionation control. APC siRNA effectively reduced cytoplasmic and nuclear APC by 80% relative to untransfected cells. Similar results were observed in three independent experiments. (C) Comparison of APC nuclear expression as measured by immunoblots quantified by densitometry, and for cell staining with quantification of nuclear fluorescence using ScionImage software. The degree of nuclear APC inhibition was expressed in arbitrary units and shows that the nuclear staining signal obtained with M-APC antibody was not specific for APC. (D) We confirmed the lack of specificity of M-APC antibody in floxed mouse fibroblasts in which APC expression was inducibly silenced by Cre recombinase (Sansom *et al*, 2004). In such cells, the loss of full-length APC (as shown by immunoblot with N-APC antibody, see Midgley *et al* (1997); the lower band is nonspecific and confirms equal loading of proteins in the samples) was accompanied by the predicted increase in cellular β -catenin levels, but there was no loss of nuclear fluorescence with M-APC antibody.

that APC nuclear export activity is not abolished by truncating cancer mutations. Most of the antibodies tested, including M-APC, were found to be unsuitable for the specific detection of APC in the nucleus, and could not be used to determine the relative distribution of APC between the nucleus and cytoplasm by IF microscopy.

RESULTS AND DISCUSSION

Detection of APC by IF microscopy

We examined several commonly used antibodies targeted to epitopes in the amino terminus, middle or carboxyl terminus of APC (Fig 1A) for their ability to detect overexpressed full-length APC–YFP (yellow fluorescent protein) in SW480 colon tumour

Fig 3 | Nuclear export of endogenous APC in different cell lines. (A) SW480 cells were treated $\pm$ 5 h LMB, fixed with formalin and stained with antibodies Ali 12–28 (1:50) and Ab7 (1:50) followed by Texas red secondary conjugate. LMB induced a visible shift to the nucleus as shown by IF. The nucleus is stained with Hoechst chromatin dye. (B) Nuclear and cytoplasmic cell extracts were prepared from SW480 (APC^{mut/mut}), HT29 (APC^{mut/mut}) and HCT116 (APC^{wt/wt}) colon cancer cell lines. The endogenous truncated or full-length forms of APC were separated by SDS–PAGE (or agarose gels for HCT116 extracts, see Methods) and detected by western blot using M-APC antibody. The APC bands were quantified by densitometry and the cytoplasmic/nuclear ratios and LMB-dependent nuclear shift are shown. Similar results were observed in two independent experiments.

cells. The YFP fluorescence pattern of APC–YFP was compared with the staining pattern obtained with different APC antibodies. As shown for the formalin-fixed cells in Fig 1B, autofluorescent APC–YFP was localized predominantly in the cytoplasm where it associated with microtubules, as previously described (Mimori-Kiyosue *et al*, 2000; Henderson *et al*, 2002). A similar, overlapping staining pattern was observed with all antibodies except for the N-terminal targeted antibodies Ab1 (Calbiochem–Novabiochem Corporation, San Diego, CA, USA) and N-15 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), which detected ectopic APC poorly. The same results were observed using methanol fixation (supplementary information and Fig S1 online). We concluded that these two antibodies were not suitable for the detection of APC by IF microscopy, and such experiments (including our own, for example, figure 1 in Henderson, 2000) are unlikely to be accurate.

Next, we focused on detection of endogenous APC in the colon cancer cell lines SW480 (APC^{mut/mut}) and HCT116 (APC^{wt/wt}), and in canine MDCK (Madine–Derby canine kidney) epithelial cells. The different antibodies gave diverse staining patterns (Fig 1C). The C-terminal-targeted antibodies Ab4, C9.9 and C20 were used as negative controls in SW480 cells, because this cell line expresses a truncated form of APC (1–1338) that lacks the C terminus. Interestingly, Ab4 and C20 stained SW480 cells strongly, especially in the nucleus (Fig 1C), despite an inability to detect APC isoforms in these cells by western blot (supplementary Fig S3 online). The nonspecific C20 staining pattern included nuclear dots, similar to the profile described previously (Erdman *et al*, 2000). The N-terminal antibodies Ab7 and Ali 12–28 produced a weaker staining pattern that was mostly cytoplasmic in all cell lines, except for MDCK in which Ali 12–28 displayed a nuclear–cytoplasmic pattern. The antibody most commonly used for IF detection of APC, named M-APC, generated predominantly nuclear staining as recently described (Fagman *et al*, 2003; Rosin-Arbesfeld *et al*, 2003; Davies *et al*, 2004). In the cytoplasm, all antibodies, except for Ab4, detected accumulation of APC at microtubule-associated membrane protrusions (see the MDCK cells in Fig 1C).

Nuclear staining with M-APC antibody is not specific

On the basis of the now frequent use of the M-APC polyclonal antisera, we tested the specificity of the nuclear staining pattern observed with this antibody, a concern raised previously (Näthke *et al*, 1996). We used RNA interference, transfecting SW480 cells with small interfering RNAs (siRNAs) that target endogenous APC, and prepared nuclear and cytoplasmic cell extracts for analysis by immunoblotting with M-APC antibody. As shown in the immunoblot in Fig 2B,C, the amount of truncated APC protein in the cytoplasmic and nuclear fractions ($\sim$ 150 kDa) was decreased by $\sim$ 80% after transfection with APC-specific siRNA. Probing with other antibodies such as Ali 12–28 or Ab1 gave a similar result (data not shown). A different siRNA effective in silencing the breast cancer susceptibility gene (BRCA1) had little effect on APC levels (see control lanes in Fig 2B). In contrast to the immunoblot results, the nuclear fluorescence intensity of APC siRNA-treated cells did not diminish when examined using different dilutions of M-APC (Fig 2A,C), independent of the fixation method used (supplementary Fig S6 online). Thus, the nuclear signal detected with M-APC is not specific for APC in cell staining experiments. To confirm this result, we performed similar experiments in a different cell model, using mouse fibroblasts in which endogenous full-length APC was inducibly silenced by activation of the Cre recombinase (Sansom *et al*, 2004). In this system, loss of APC was verified by immunoblotting (lower panel of Fig 2D), and correlated with an increase in β -catenin expression as expected (Polakis, 2000). The nuclear staining observed with M-APC antisera did not diminish (Fig 2D), although cytoplasmic APC was reduced and peripheral clusters of APC disappeared (Fig 2D; K. Kroboth & I. Näthke, unpublished data).

Collectively, these results demonstrate that the M-APC antibody produces a nonspecific nuclear staining pattern by IF microscopy.

Localization predominantly to the cytoplasm

As several recently proposed mechanisms for regulating APC nuclear localization were based on cell-staining experiments

Fig 4 | The localization of endogenous APC is not influenced by cell density in SW480, HCT116 or MDCK cells. (A) SW480 and MDCK cells were seeded at different densities and harvested at 40% confluence (subconfluent) or confluence. Cells were fixed with formalin and stained with M-APC antibody (1:1,000 dilution). The M-APC staining pattern shifted from nuclear–cytoplasmic to cytoplasmic with increased cell density, as described by others (Fagman *et al*, 2003; Davies *et al*, 2004). (B) SW480, HCT116 and MDCK cells were grown under the same conditions as above, and processed for immunoblotting. Equivalent amounts of cytoplasmic (60 µg) or nuclear (20 µg) extracts were separated by SDS–PAGE or agarose gel (for HCT116 and MDCK) and analysed by western blot. Truncated and full-length APC were detected with M-APC antibody (1:4,000). Neither form of APC redistributed to the nucleus after changes in density. The same membrane was reprobed for topoisomerase II (TOP2II) as a fractionation control. The same results were obtained in two independent experiments.

with M-APC antibody, we felt it was important to use a panel of antibodies to determine the localization of APC in tumour cells. This was also warranted due to conflicting reports about the nuclear–cytoplasmic distribution of APC. Wild-type and truncated forms of endogenous APC have been observed predominantly in the cytoplasm by immunoblotting (Smith *et al*, 1993; Galea *et al*, 2001), whereas cell-staining experiments with M-APC antibody detected tumour-associated truncated APC in the nucleus, leading to the conclusion that mutant APC is not exported (Rosin-Arbesfeld *et al*, 2003). To resolve these discrepancies, we reappraised the localization of endogenous APC before and after treatment with the nuclear export inhibitor leptomycin B (LMB).

We stained SW480 cells with the antibodies M-APC, Ali 12–28 or Ab7, after formalin (Fig 3A) or methanol fixation (supplementary Fig S2 online). As described above, M-APC staining was strongly nuclear regardless of the test condition, although a modest increase in nuclear staining was observed after 5 h treatment with LMB in methanol-fixed cells (supplementary Fig S2 online). A more pronounced effect of LMB was observed with antibodies Ab7 and Ali 12–28 in formalin-fixed cells, with the staining pattern changing from mostly cytoplasmic to nuclear–cytoplasmic (Fig 3A). The LMB response was less obvious with methanol fixation, indicating that the fixation method influences the staining patterns obtained with different antibodies. To obtain a more direct measure of the cellular APC distribution, we used

immunoblotting to analyse fractionated cell lysates prepared from colon cancer cell lines that express full-length (HCT116) or truncated APC protein (SW480, HT29). We observed a significant shift of APC from cytoplasm to nucleus in all cell lines after LMB treatment. Intriguingly, the impact of LMB was strongest on the truncated APC in cancer cells (see C/N ratio in Fig 3B). One possible explanation is that truncated forms of APC lacking the C-terminal domains are not sequestered by microtubules (which bind to this region) as efficiently as full-length APC, and thus enter the nucleus more efficiently. Alternatively, full-length APC, but not truncated APC, may use additional LMB-insensitive nuclear export pathways. Similar immunoblot data were obtained with Ab1 and Ali 12–28 antibodies (data not shown). These results confirm that mutant and full-length APC are predominantly cytoplasmic, due in part to chromosome region maintenance 1 protein (CRM1)-dependent nuclear export. The dynamic nuclear export of endogenous APC(1–1338) in SW480 cells is consistent with the action of an N-terminal nuclear export signal (NES; Henderson, 2000; Neufeld *et al*, 2000), and suggests that APC nuclear export activity is not lost in colon cancer cells.

Nuclear localization of APC is not affected by cell density

Several reports have described a correlation between cell density and the localization of APC on the basis of cell staining (Brocardo *et al*, 2001; Zhang *et al*, 2001; Fagman *et al*, 2003; Davies *et al*, 2004); thus, in highly packed confluent cells, APC redistributes

from the nucleus to the cytoplasm. The report by Zhang *et al* (2001) used cell staining with the antibodies Ab4, Ab1, C20 and N-15, none of which seems to specifically detect APC in the nucleus (Fig 1; supplementary Figs S3, S4 online). Moreover, our own previous findings (Brocardo *et al*, 2001) could not be reproduced (data not shown). Two other studies used M-APC antibody (Fagman *et al*, 2003; Davies *et al*, 2004), and using this antibody we also observed a redistribution of APC from nucleus to cytoplasm with increasing density of SW480 cells and MDCK cells (Fig 4A; supplementary Fig S5 online). However, when subconfluent or confluent SW480, MDCK or HCT116 cells were fractionated and the nuclear–cytoplasmic distribution of APC was measured by immunoblotting, a density-dependent redistribution of APC was not detected (Fig 4B), although nuclear accumulation was easily detectable after LMB treatment (Fig 3B). These results were highly reproducible and indicate that nuclear APC is not regulated by cell density.

CONCLUSIONS

We compared a panel of APC antibodies and conclude that most lack specificity for APC in cell-staining experiments. The least reliable antibodies for IF detection of nuclear APC are M-APC, Ab4, C20, N-15 and Ab1. Fixation method also affected the IF staining patterns. Several antibodies, including M-APC and Ab1, were effective in detecting APC specifically by immunoblotting, and detected APC at microtubules and membrane protrusions in IF experiments. Where is APC localized in the cell? Our results contradict several papers, including some of our own published data (such as figure 1 in Henderson, 2000). However, we conclude that full-length and mutant APC are localized most frequently in the cytoplasm. Our experiments refute the hypothesis that mutant APC lacking a C-terminal NES is trapped in the nucleus (Rosin-Arbesfeld *et al*, 2000, 2003), and instead show that such APC cancer-associated mutants shuttle in and out of the nucleus, even more efficiently than the full-length APC (Fig 3). We are at present in the process of a more extensive investigation into the role of nuclear–cytoplasmic transport of APC. In terms of the regulation of APC localization, our western blot data argue against several recent papers that proposed fluctuations in APC nuclear localization on the basis of changes in cell density (Brocardo *et al*, 2001; Zhang *et al*, 2001; Fagman *et al*, 2003; Davies *et al*, 2004). Again, the use of valid controls for the first time has an impact on the conclusions of one of our own papers as well (Brocardo *et al*, 2001). In conclusion, we believe that we finally have a clearer understanding of the location of APC in those cultured cell lines most commonly studied, and we urge extreme caution in the interpretation of any data obtained with antibodies against APC protein when using IF microscopy.

METHODS

A full version is available as supplementary information online.

Cell culture and transfection. Human SW480 (APC truncated at amino acid (aa) 1338), HT-29 (APC truncated at aa 853 and 1555), HCT116 (full-length APC) colon carcinoma cells and the MDCK cell line were cultured in DMEM with 10% fetal bovine serum. Cells were seeded onto coverslips and transfected with 1 µg of DNA per 2 ml of medium using Lipofectamine reagent as instructed by the supplier (Invitrogen Corporation, Carlsbad, CA, USA), and then processed 48 h later. The plasmid pAPC–YFP has

been described previously (Henderson *et al*, 2002). LMB cell treatments were for 5 h at 6 ng/ml drug. For cell density experiments, cells were grown to 40% confluence (subconfluent cells) or to confluence for 3–4 days.

Cell fractionation and immunoblotting. HCT116 and SW480 cells were separated into nuclear and cytoplasmic fractions using the NE-PER kit (Pierce Biotechnology Inc., Rockford, IL, USA), separated on agarose or polyacrylamide gels and probed with antibodies to detect endogenous APC by western blot as described in supplementary information online.

RNA interference. Double-stranded 21-mer RNA oligonucleotides homologous to sequences in human APC were purchased as purified duplexes (Qiagen Inc., Valencia, CA, USA). The DNA target sequence was 5867-AGGGGCAGCAACTGATGAAAA. Cells at medium density were transfected with 6 µg RNA duplexes in 2 ml of DMEM medium using Lipofectamine for 6 h and harvested 48 h post-transfection for analysis.

Antibodies and immunofluorescence microscopy. Cells were grown on coverslips at medium density and fixed in 3.7% formalin (tissue culture grade from Sigma)/PBS for 20 min or chilled 100% methanol for 10 min, followed by permeabilization with 0.2% Triton X-100/PBS for 10 min. Cells were blocked in 3% BSA/PBS for 30 min and incubated for 1 h at 20–25 °C with APC antibodies as described in supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboports.org>).

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