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Recent evidence suggests that the adenomatous polyposis coli (APC) gene participates in breast tumorigenesis. The APC protein interacts with β-catenin and plakoglobin *in vivo*. β-catenin and plakoglobin are components of two specialized anchoring junctions, the adherens junction, a site of attachment for bundles of actin filaments, and the desmosome, a site of attachment for intermediate filaments (e.g. keratin). A direct correlation has been shown between loss of adherens junction components and the metastatic potential of breast cancer. I have used a combination of immunofluorescence microscopy and biochemical fractionation to determine that APC protein is located in the nucleus and the cytoplasm of all breast epithelial cells tested. APC protein concentrated at the edge of breast epithelial cells was eliminated by disruption of keratin filaments and microtubules, but not by actin disruption. APC protein appeared tightly associated with intermediate filaments of the normal breast epithelial cell following sequential extraction. APC was co-immunoprecipitated with keratin but not with actin protein. LiCl-treatment of breast cells led to a delay in both tight and adherens junction formation. These findings are consistent with APC protein interacting with intermediate filaments, but not with actin filaments, and APC protein playing a role in cell polarization.

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Kristi Neufeld

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INTRODUCTION

Recent evidence suggests that the adenomatous polyposis coli (APC) gene participates in breast tumorigenesis. *APC* gene loss is reported in as many as 28% of human breast tumors (1). Female mice carrying mutant *APC* genes show an increased rate of breast hyperplasia and neoplasia (2). In addition, APC has been linked to the murine proto-oncogene Wnt-1, which was originally identified as a frequent target for insertional activation by mouse mammary tumor virus in mammary carcinomas. In vertebrates, APC protein is proposed to be a component of the Wnt-1 signaling pathway. Together, these data suggest a key role for APC in the breast cancer pathway.

β-catenin and plakoglobin both bind to APC protein and are components of two specialized anchoring junctions. Anchoring junctions mechanically attach epithelial cells (and their cytoskeleton) to neighboring cells or to the extracellular matrix. β-catenin and plakoglobin are found in the adherens junction, a site of attachment for bundles of actin filaments. Plakoglobin is also a component of the desmosome, a site of attachment for intermediate filaments (e.g. keratin). Anchoring junctions also serve an important role in signal transduction, mediating changes in cyto-architecture and proliferation. A direct correlation has been shown between loss of adherens junction components and the metastatic potential of breast cancer. The purpose of this study is to determine whether APC interacts directly with adherens junctions or desmosomes through actin and keratin respectively. If APC protein can be demonstrated to be a component of this junction then this could explain the link between APC loss and breast cancer. In addition this proposal explores the possible role of APC protein in cell polarization.

BODY OF REPORT

Technical Objective 1: Identify breast cancer cell lines that do not express wild-type APC protein. Further characterize the polarization defect in these breast epithelial cells.

Task 1: Plate cells from breast cancer lines established in the lab and purchased from ATCC on glass chamber slides. Test cells for wild-type APC protein expression by immunofluorescence analysis. Characterize localization of APC protein in the cancer lines grown on a slide.

Task 10: Sequentially fractionate normal HMECs. Determine APC fractionation profile by Western immunoblot and immunofluorescence microscopy.

Unless otherwise stated, the data described for Tasks 1 and 10 appeared in my second annual report and is not shown again in this final report. Some of this data was also published (Neufeld and White, 1997). The distribution pattern for endogenous, full-length APC protein in various cell lines was determined using indirect immunofluorescence microscopy with antibodies that recognize the C-terminus of APC protein. The staining pattern in four breast cancer cell lines, BT549, MDA-MB468, MCF7 and T47D, was compared to that seen in cells from primary outgrowth of normal breast epithelial tissue (BE20, BE-21) and the "normal" breast cell line 184A1. Because of technical difficulties we have been unable to establish cell lines from any of the seven breast cancer tissue specimens we have received in the past year, therefore, we focused on lines available from ATCC. 184A1 is an immortalized human mammary epithelial cell line that displays keratin staining patterns and phenotypic characteristics reminiscent of normal human mammary epithelial cells (10). These cells expressed full-length APC protein as determined by Western blot analysis and were used as representative of normal epithelial cells.

Of seven monoclonal and three polyclonal antibodies tested in immunofluorescence assays with the 184A1 cell line, the three monoclonal

antibodies raised against the C-terminus of APC protein (monoclonal Ab-2, Ab-4 and Ab-6), and the polyclonal antibody APC64, also raised against the C-terminus, gave similar and reproducible staining patterns. These antibodies localized full-length APC protein to two distinct compartments of the epithelial cell. A punctate staining pattern was visible in the cytoplasm, with especially prominent staining at cell edges. In migrating cells, the APC staining concentrated at the leading edge. This cytoplasmic distribution of APC protein provided additional support for the very similar results reported recently in MDCK cells (11). In addition to the cytoplasmic staining, however, there was significant nuclear staining. Much of the total staining seemed to be concentrated in discrete nuclear regions. Normal primary epithelial cells that migrated from human breast tissue specimens (BE20 and BE21 data shown in second annual report and BE198 and BE212 experiments were performed this past year, not shown) had identical APC distribution patterns as the 184A1. As further proof of the specificity of the immunofluorescence signal using the APC antibodies, DLD-1 cells were stained using the C-terminal APC antibodies. As expected, since DLD-1 is a colon cancer cell line that expresses no full-length APC protein, these cells gave no APC signal.

Cells from the four breast cancer lines also showed a similar staining pattern to that seen in 184A1 cells. In each case, cells were stained using antibodies directed against the C-terminus of APC protein (monoclonal Ab-4 and polyclonal sera, APC64) and IgG1 as a negative control. T47D and MDA-MB468 cells displayed both punctate cytoplasmic stain, concentrated at the leading edge, and nuclear stain. A similar pattern was seen in BT549 cells, with additional filamentous staining visible in the cytoplasm. Non-confluent MCF7 cells also displayed the punctate cytoplasmic and nuclear

staining pattern. However, in areas of higher cell confluency, the staining pattern was strikingly different. While the nuclear staining was still clearly visible, a significant portion of the APC protein appeared to be located at cell-cell junctions.

Biochemical fractionation was employed as an independent test of the several cellular locations of APC protein in normal breast epithelial cells. 184A1 cells were subjected to lysis by detergent (>99% cell lysis as determined by trypan blue exclusion) followed by purification of the nuclei from the cytosolic fraction. The cytosolic fraction was further separated into a membrane/cytoskeletal fraction and a soluble, cytoplasmic fraction. Nuclear matrix/scaffold proteins were further purified from the nuclear fraction by DNAse treatment, stabilization with $CuSO_4$, precipitation in 0.2M ammonium sulfate, and washes. The fractions were verified by Western Immunoblot using antibodies directed against compartment-specific proteins: α-tubulin fractionated with the cytoplasm, α-adaptin with membranes, and lamins A, B, and C with the nuclear and nuclear matrix/scaffold fractions.

184A1 cells contained full-length APC protein in both the membrane/cytoskeletal and the nuclear fractions. The slowest band recognized by the APC antibody migrated at the molecular size predicted for full-length APC protein relative to the molecular weight markers and was recognized by antibodies raised to both N-terminal and C-terminal regions of APC protein. Band intensities were greatly diminished by preincubation of the APC antibody with an APC peptide.

Western Immunoblot analysis of APC protein in breast cancer cell lines.

Task 2: Test cells found to have no detectable level of APC protein in Task 1 for APC expression by Western immunoblot analysis. Determine percentage of breast cancer lines that have decreased APC protein compared to normal.

In the colon, >99% of the inactivating APC mutations result in production of a truncated APC protein product, thus implying that point mutations are not sufficient to inactivate the very large APC protein. In addition, surveys of colon polyps (colon cancer precursors) show that the vast majority of polyps have inactivated both copies of the APC gene (12). Assuming that the same is true for epithelial cells of the breast, it was possible to catalogue the percentage of breast cancer cell lines with fully-inactivated APC by testing these lines for the presence of full-length APC protein.

Because all four breast cancer cell lines showed significant immunofluorescence staining using antibodies directed against the C-terminus of APC, it was likely that they all expressed full-length APC protein. In order to confirm the presence of full-length APC protein by a second method, cells from two of the four breast cancer lines were tested for APC expression by Western blot analysis. Lysates from both BT549 and MDA-MB468 contained a 320 kDa protein that was recognized by an antibody raised against APC protein's N-terminus. Based on the combined immunofluorescence and Western immunoblot data, I concluded that four out of four breast cancer cell lines tested expressed full-length APC protein.

Task 3: Test cells found to have no detectable level of APC protein in Task 1 for ability to polarize. If no such cells were found, move on to Objective 2.

Since no breast cancer cell lines were found that expressed mutant APC protein, I moved on to Objective 2.

Technical Objective 2: Creation of "null" breast epithelial cells for use in polarization assay

Task 4: Clone APC antisense construct into retroviral vector. Infect normal HMECs with APC-antisense retroviral construct in order to prevent APC protein expression. Grow infected cells in selection media. Estimate infection efficiency by detection of GFP by immunofluorescence microscopy. Test APC expression level (Western immunoblot and immunofluorescence).

Since all four breast cancer cell lines tested appeared to have normal APC protein, it was necessary to create an APC "null" cell line in order to fully test potential APC functions. Several methods have been employed thus far, each based on the ability of an antisense oligonucleotide to block translation of the APC protein. The first method involved design and synthesis of DNA oligomers, 18 nucleotides long, that corresponded to sequences "antisense" to APC mRNA, either in the 5' untranslated region (APC-A5) or spanning the initiation codon (APC-AC) (as shown in second annual report). "Sense" oligos were synthesized in parallel for use as negative controls (APC-S5 and APC-SC). The oligos were purified by HPLC, dialyzed, then added to the growth media of 184A1 cells, both in the presence and absence of a lipofection reagent. Lipofection has been reported to improve permeability of DNA oligos into some types of cells (13). Although a range of oligo concentrations was tested, using two different antisense oligos, I was unable to detect changes in APC protein level as determined by immunofluorescence analysis.

For the second antisense approach, the entire APC gene was cloned in reverse orientation into the expression vector pCDNA3.1 (Invitrogen) (schematic in second annual report). Cells were co-transfected with the antisense APC construct and a green fluorescent protein- (GFP) expression construct so that APC protein level could be monitored specifically in those cells that were successfully transfected. Although GFP-expressing cells

could be identified after co-transfection, no reduction in APC protein level could be detected by immunofluorescence analysis.

The third antisense approach utilized the retroviral construct pLXSN (Clontech) containing the complete cDNA for APC in reverse orientation. Transfection of a colon cancer cell line that contained endogenous full-length APC protein with this construct did not result in alteration of cellular morphology. I concluded from the inability to induce cellular morphology changes with repeated attempts and various methods of APC antisense DNA introduction, that the antisence approach would not be a viable one for APC protein knock-out purposes and therefore, I moved on to **task 5.**

Task 5: Introduce APC antibodies into normal HMECs by phospholipid-mediated antibody delivery. Determine antibody transfection efficiency by immunofluorescence microscopy using FITC-conjugated secondary antibody and no primary antibody. Test APC expression level (Western immunoblot and immunofluorescence).

As with task 4, we were unable to detect differences in cellular morphology between cells with introduced APC antibodies (monoclonal APC Ab-4, Oncogene Science, or polyclonal APC64, a gift from A. Levine) versus cells with introduced nonspecific isotype control antibodies or sera (IgG1 or normal rabbit sera). This was likely due to the fact that lysophosphatidyl choline (LPC)-treatment alone effects cell morphology. Alternatively, the APC antibodies might not recognize native APC protein within the cell or might not bind and block APC protein function. Both APC Ab-4 and APC64 antibodies have been used successfully for immunostaining and Western Immunoblot analysis. However, in this case, instead of recognizing native protein, the APC antibodies are binding to APC protein that was fixed or reduced. We have successfully used APC Ab-5 (N-terminal mouse monoclonal) and Ab-6 (C-terminal mouse

monoclonal) for immunoprecipitations from cell lysates. This likely represents the binding of antibody to native APC protein. It might therefore still be possible to optimize this technique using a mixture of these antibodies. I concluded that this method was not going to prove successful and therefore turned to a new approach.

It is thought that one of the major roles of APC protein in a normal epithelial cell is to down-regulate the cell-cell junction protein β-catenin (13, 14, 15). In addition to its role linking E-cadherin and actin at adherens junctions, β-catenin participates in the Wnt signaling pathway, which is important for axis formation in vertebrate embryos and can play an important role in neoplastic transformation in mammalian cells (16, 17). In the presence of Wnt signal, free β-catenin accumulates in the cytoplasm, migrates into the nucleus, and, in conjunction with T cell-factor/lymphoid-enhancer-factor (TCF/Lef-1), activates the transcription of genes such as cyclin D1, c-myc, c-jun, and fra-1. In the absence of Wnt, β-catenin binds to an APC-axin complex, where it is phosphorylated by glycogen synthase kinase 3β (GSK3β), and targeted for ubiquitin-mediated degradation in the cytoplasm (18, 19). I therefore reasoned that up-regulation of β-catenin signal in normal breast epithelial cells might mimic a knock-out of APC protein function and might be easier to facilitate.

One way to up-regulate β-catenin protein is to express a mutant form of β-catenin that is unable to be down-regulated by APC protein. To this end, Nori Matsunami in the lab created a retrovirus construct that expressed dominant β-catenin. Unfortunately, primary breast cells infected with this virus did not show altered mRNA expression profiles by microarray analysis, therefore we think that the dominant β-catenin might not be

behaving "typically" in these cells. Nori is exploring this possibility further and once he achieves successful dominant β-catenin expression, I will infect primary breast cells with this construct and test for polarization defects. In the meantime, I have used a different aproach to upregulate β-catenin and thus mimic a loss-of-function mutation in the APC gene. LiCl is a potent inhibitor of GSK3β activity. Phosphorylation of β-catenin and APC by GSK3β is required for β-catenin down-regulation. I therefore reasoned that LiCl-treatment might result in a similar phenotype as an APC loss-of-function.

Treatment of MDCK cells with LiCl for 7 days resulted in upregulation of β-catenin protein expression as observed by immunofluorescence microscopy. Cells were grown on transwell membranes in the presence or absence 100mM LiCl for 7days, and then fixed and stained for β-catenin protein. Pictures taken for the same exposure times revealed a slight increase in expression of cytoplasmic β-catenin in the LiCl-treated cells compared to untreated cells (data not shown). A similar result was obtained from two primary human breast tissue cell outgrowths (Fig. 4D, BE198). In Task 6 we used MDCK cells as well as the two primary breast cell populations for polarization assays.

Task 6: Perform polarization assay on breast cancer cells with no wild-type APC (identified in Objective 1) or in APC "null" cells (created in Tasks 4 or 5). Compare breast cancer cells with normal breast cells. Compare "null" cells to parental line.

Since primary breast cell outgrowth is a limited resource, I began these studies using the well-characterized Madin-Darby canine kidney cell line (MDCK). MDCK cells form polarized sheets when grown on filters, and have therefore been a favorite model system for epithelial cell

polarization studies. MDCK cells were seeded onto clear transwells in the presence or absence of LiCl (100 mM). Cells were fixed and analyzed for signs of polarization 24 h, 3 days and 7 days post-seeding. Antibodies used for immunofluoresence analyisis included α-ZO-1 and occludin which recognize tight junction proteins, α-E-cadherin and β-catenin which recognize cell-cell adherense junction proteins, and α-APC.

The results from two independent experiments with MDCK cells are as follows. As early as 24 hours post-plating, Occludin-positive and ZO-1-positive junctions, were visible (Fig. 1A and B respectively). The junctions had the typical appearance of gap junctions, as they were not detected throughout the lateral region but were instead limited to a narrow band of stain near the apical surface of the cells. Both occludin- and ZO-1-positive junctions were visible in untreated cells (Fig. 1A and B, left panel) and in cells treated with LiCl for 24 hours (right panel). E-cadherin protein appeared punctate and cytoplasmic in both Li-treated and untreated cells (Fig. 1C). APC protein was visible in both the cytoplasm and nucleus of untreated MDCK cells (Fig. 1D, left panel). LiCl-treatment appeared to result in a net decrease in the cytoplasmic APC staining (Fig. 1D, right panel).

By 3 days post-plating (Fig. 2), differences between untreated and Li-treated MDCK cells were more evident. Occludin and ZO-1 junctions were maintained in both cases, however, the cells treated with LiCl were much larger than the untreated MDCK cells (Fig. 2 A and B). By day three, the untreated MDCK cells displayed lateral E-cadherin staining, whereas in the Li-treated MDCK cells, most of the E-cadherin appeared as it had day 1, punctate cytoplasmic (Fig. 2C). Although there was slight evidence of lateral E-cadherin staining in Li-treated cells, the overall lack of lateral

staining leads me to conclude that the LiCl is having an affect on cell polarity. APC staining appeared similar in untreated and LiCl-treated MDCK cells after 3 days (Fig. 2D).

By seven days post plating (Fig. 3) antibodies to occludin and ZO-1 continued to stain tight junctions (A and B), and MDCK cells treated with LiCl were increasing in size compared to their untreated counterparts. E-cadherin was distributed laterally in the Li-treated MDCK cells (Fig. 3C), suggesting that LiCl does not prevent formation of adherense junctions but only delays it. β-catenin had a similar lateral staining pattern in both treated and untreated MDCK cells by day 7 (Fig. 3D). APC protein displayed no differences in staining (data not shown).

We repeated the polarization test in the presence or absence of LiCl using primary epithelial outgrowth from two different normal human breast samples, BE198 and BE212, grown in our lab. Since both cell types behaved similarly, I will only present data for BE198 and will refer to the cells as human mammary epithelial cells (HMECs). Occludin and ZO-1 stained the cytoplasm of HMECs plated for 1-day in the presence and absence of LiCl with no junctional staining visible (data not shown). APC protein appeared to be located in the nucleus and at the cell edge of untreated HMECs. LiCl-treatment resulted in a loss of edge staining (not shown). E-cadherin and β-catenin had similar distributions in untreated HMEC and cells treated with LiCl for 24 hours. By 3 days, there were some visible differences between treated and untreated HMECs. These differences persisted for 7 days. Occludin and ZO-1 junctions were forming in both cases, but there were far fewer in the LiCl-treated HMECs (Fig. 4A and 4B). E-cadherin and β-catenin were beginning to distribute to the lateral region of the HMECs, with more lateral staining visible in the

untreated versus the LiCl-treated cells (Fig. 4C and 4D). APC protein appeared to be located junctionally in the untreated HMECs at 7 days post-plating (Fig. 4E).

I conclude that, although the formation of junctions seemed delayed in HMECs compared to MDCK cells, both cell types displayed a similar phenomenon. LiCl treatment appeared to delay formation of both tight and adherense junctions.

Task 7: Express full-length APC protein in breast cancer cells with no wild-type APC (identified in Objective 1) or in colon cancer cell lines SW480 and DLD-1.
Task 8: Test cells created in Task 7 for expression of wild-type APC by Western immunoblot and immunofluorescence. Perform polarization assay on breast cancer cells created in Task 7.

We did not identify breast cancer cells lacking full-length APC protein in objective 1; therefore, we proceeded to perform task 7 using the colon cancer cell line, SW480. Full-length FLAG-tagged-APC protein was introduced into SW480 by transfecting cells with an APC expression construct. 24 hours after transfection, cells were fixed and stained for full-length APC protein, using a FLAG-antibody. In the majority of transfected SW480 cells, exogenous APC protein distributed to the cytoplasm, often displaying a filamentous pattern (data not shown). I used several different transfection protocols and was only able to achieve a transfection efficiency of about 1% in SW480 or DLD-1 colon cancer cell lines. With this low transfection efficiency, it appears that this system will not be amenable to the study of cell polarization. For the polarization studies that I have proposed, adjacent cells must be transfected with the APC expression construct. A possible alternative to transient transfections is to make a cell line that stably expresses full-length APC protein. As published by Groden

et al (1998) and experienced in our lab as well, no stable cell lines have been created that expressed full-length APC in colon cancer cell lines that previously lacked the wild-type protein. We attempted to overcome this obstacle with the use of an inducible expression system. Although we produced a cell line that inducibly expressed full-length APC protein, after several passages the APC expression was lost.

Retrovirus expression systems have proven efficient methods to introduce expression constructs into whole populations of cells. However, the APC coding region is too large to introduce into a retrovirus. We have therefore begun to construct an adenovirus vector that encodes full-length APC. When the adenovirus APC expression construct is completed, I will use it to express full-length APC protein in the colon cancer cell line SW480. Initially infected cells will be assayed by western immunoblot for APC protein production. Once APC protein has been identified, I will use adenovirus-infected cells to study affects of APC protein expression on cell morphology and polarization.

Technical Objective 3: Test directly for interactions between APC protein and the cytoskeletal components (actin and keratin) of the cell-cell anchoring junctions in normal breast epithelial cells

Task 9: Precipitate keratin and actin proteins from normal HMEC lysate. Test for co-precipitation of APC protein

In order to demonstrate interaction between APC protein and the filament proteins keratin and actin, I have tested for co-immunoprecipitation. These experiments have been performed by precipitation with APC antibodies followed by Western immunoblot with keratin and actin antibodies as well as the converse.

HMECs are a rather limited resource; therefore, using two cell lines that express full-length APC protein (MCF-7 and HCT116) we have completed a study to characterize interaction of APC protein with actin and keratin proteins. Whole cell lysates were probed with several antibodies raised against APC protein. Using either a mixture of monoclonal antibodies Ab-5 and Ab-6 (Oncogene Science) or a rabbit sera raised by Covance Laboratories (Denver, PA) against an APC peptide (APC1-270) purified in our lab we were able to immunoprecipitate APC protein (Fig. 6B and 6D and data not shown). Control precipitations performed with mouse IgG1 or rabbit sera yielded no APC protein (Fig. 6B and 6D). APC protein recovery was less efficient when precipitations were performed using the APC270 sera, therefore co-immunoprecipitation experiments were carried out with the monoclonal antibodies Ab-5 and Ab-6.

An antibody raised against actin (clone AC-40, Sigma) identified a band of the correct molecular mass in the whole cell lysate from MCF-7 cells (Fig. 5). We were unable to identify actin protein among the proteins co-immunoprecipitated with APC (Fig. 5) and therefore conclude that APC and actin do not interact in MCF-7 cells.

A PAN-cytokeratin antibody (clone PCK-26, Sigma) identified several keratin species in whole cell lysates from MCF-7 cells (Fig. 6A). We were able to immunoprecipitate keratin with the antibody (Fig. 6A). Full-length APC protein co-immunoprecipitated with the keratin proteins (Fig. 6B). Conversely, proteins that immunoprecipitated with APC antibodies included at least one band that was recognized by PAN-keratin antibody (Fig. 6A). Based on the estimated molecular weight (~52 kDa) and the relative abundance of the keratin species in breast and colon cells, we hypothesized that the keratin co-immunoprecipitating with APC protein

was keratin 8. To test this hypothesis, we used an antibody specific for keratin 8 to probe immunoblots of APC-co-precipitated proteins. Keratin 8 antibody recognized a band of the correct size in the lane of APC-precipitated proteins but not in the control lane, precipitated with mouse IgG1 (Fig. 6C). We used the Keratin 8 antibody to immunoprecipitate proteins from HCT116 cell lysates and showed co-immunoprecipitation of full-length APC protein (Fig. 6D).

These results demonstrate that APC protein can bind to keratin protein in both breast and colon cells. We have identified at least one of the keratins that binds to APC protein as Keratin 8.

Task 10: Sequentially fractionate normal HMECs. Determine APC fractionation profile by Western immunoblot and immunofluorescence microscopy.

The following aim was completed and presented in the annual report for the first year of this award. As the first of two alternative method to test for possible interactions between APC protein and keratin or actin filaments I chose to disrupt the filaments in 184A1 cells with either cytochalasin B (for actin) or inactivating antibodies (for keratin) and test for redistribution of the APC protein by immunofluorescence microscopy. Cytochalasin B, which disrupts actin microfilaments, did not alter the distribution of APC protein. In contrast, when breast epithelial cells were subjected to keratin antibody treatment which induced collapse of the keratin filaments, APC protein at the leading edge was almost completely lost. This treatment had no effect on actin or tubulin organization, suggesting that the APC protein concentrated at the leading edge was dependent on intact keratin filaments. 184A1 cells treated with microtubule depolymerizing agent cochicine, as well as with nocodazole and vinblastine, two other microtubule depolymerizing agents also showed elimination of the APC staining at the

leading edge. γ-lumicolchicine, a derivative of colchicine that did not disrupt microtubules but had the same nonspecific effects as colchicine, had no affect on APC localization. Tubulin, actin and keratin were shown to be redistributed or eliminated by the specific drug or antibody treatments by antibody staining.

As a second alternative approach, immunofluorescence microscopy provided further support for the association of APC protein with the keratin intermediate filament network. Proteins not tightly bound to intermediate filaments were sequentially removed from 184A1 cells, using conventional extraction techniques (9). This extraction procedure removed components not associated with the intermediate filament or nuclear scaffold network, including microtubules, soluble proteins, actin, histone H1, and DNA-binding and RNA-binding proteins. Immunofluorescence microscopic analysis of cells either with no treatment or fully extracted revealed that APC protein remained cell-associated following complete extraction. Comparing the relative staining intensities and adjusting for differences in exposure rate, it appeared that the APC protein signal increased in intensity following extraction. This increase in staining intensity occurred after the first extraction step, which released soluble proteins and tubulin, leading to the suggestion that the increase was due to the release of proteins that mask the APC protein signal in untreated cells. Following extraction, APC protein remained associated, not only with the nuclear matrix, but also with the intermediate keratin filaments of the cytosol. In contrast, tubulin, actin, and DNA were quantitatively released.

REFERENCES

1. Frixen, U. H., et. al., *J. Cell. Biol.* **113**: 173-185 (1991).

2. Gabbert, H., Wagner, R., Moll, R. and Gerharz, C.-D, *Exp Metastasis* **3**: 257-279 (1985).

3. Gamallo, C., et al., *Am. J. Pathol.* **142**: 987-993 (1993).

4. Groden, J., G. Joslyn, W. Samowitz, D. Jones, N. Bhattacharyya, L. Spirio, A. Thliveris, and et. al., *Cancer Res.* **55**:1531-1539 (1995).

5. Groden, J., A. Thliveris, W. Samowitz, M. Carlson, L. Gelbert, H. Albertsen, G. Joslyn, J. Stevens, and et. al., *Cell.* **66**: 589-600 (1991).

6. Taylor-Papadimitriou, J., and M. R. Stampfer., *Culture of Human Mammary Epithelial Cells. In Culture of Epithelial Cells.* Vol. 1. R. I. Freshney, editor. A John Wiley and Sons, Inc., New York. 107-133 (1992).

7. Shea, T.B, and M L Beerman, *BioTechniques* **10**: 288-294, (1991).

8. Sprott, S., K. Hammond, and N. Savage, *Anal. Biochem.* **194**:407-412 (1991).

9. Nickerson, J., G. Krockmalnic, and S. Penman, *Cell Biology: A Laboratory Handbook. In Cell Biology A Laboratory Handbook.* Vol. 1. J. E. Celis, editor. Academic Press, San Diego. 622-627 (1994).

10. Walen, K. H. and M. R. Stampfer, *Cancer Genet Cytogenet.* **37**: 249-61 (1989).

11. Nathke, I. S., C. L. Adams, P. Polakis, J. H. Sellin, and J. Nelson, *J. Cell Biol.* **134**:165-179 (1996).

12. Powell, S. M., N. Zilz, B. Y. Beazer, T. M. Bryan, S. R. Hamilton, S. N. Thibodeau, B. Vogelstein and K. W. Kinzler, *Nature.* **359**: 235-7 (1992).

13. Munemitsu, S., I. Albert, et al. Proc Natl Acad Sci USA **92**(7): 3046-50 (1995).

14. Rubinfeld, B., I. Albert, et al. Science **272**: 1023-1026 (1996).

15. Rubinfeld, B., I. Albert, et al. Cancer Res **57**(20): 4624-30 (1997).

16. Dale, T. C. Biochem J **329**(Pt 2): 209-23 (1998).

17. Dierick, H. and A. Bejsovec Curr Top Dev Biol **43**: 153-90 (1999).

18. Aberle, H., A. Bauer, et al.EMBO Journal **16**: 3797-3804 (1997).

19. Orford, K., C. Crockett, et al. J Biol Chem **272**(40): 24735-8 (1997).

APPENDICES

Figure 1

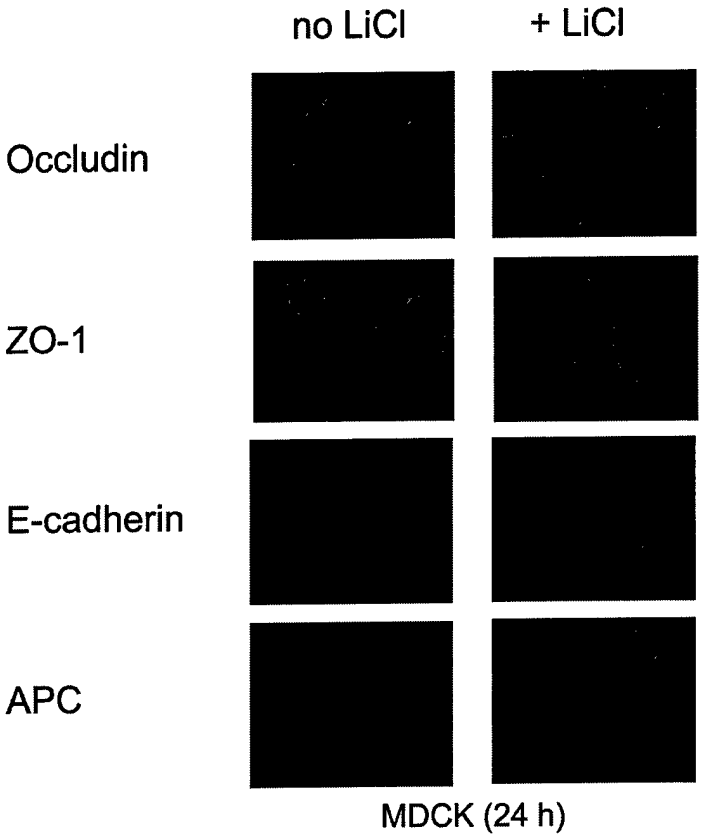


Figure 2

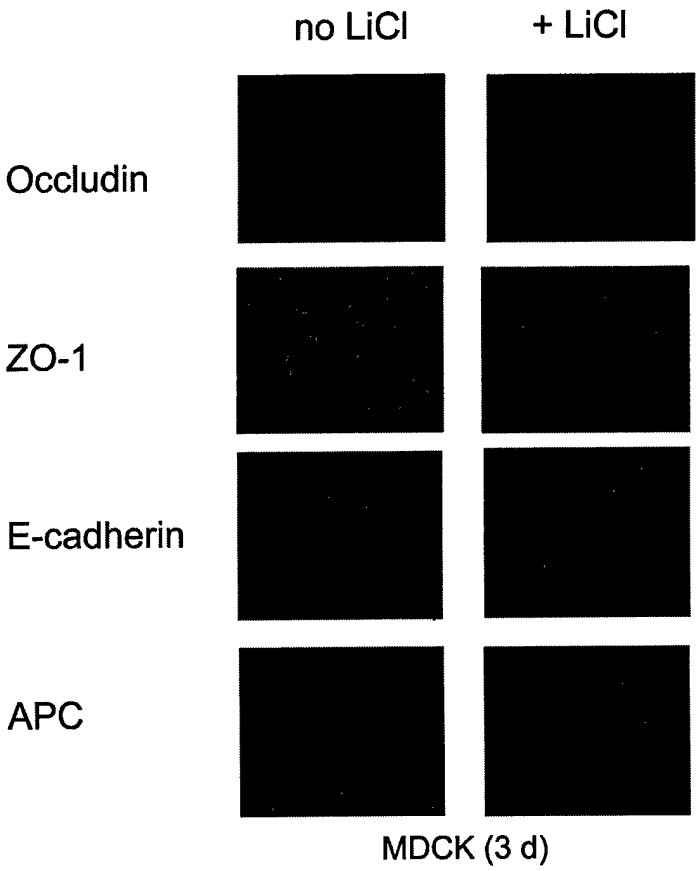
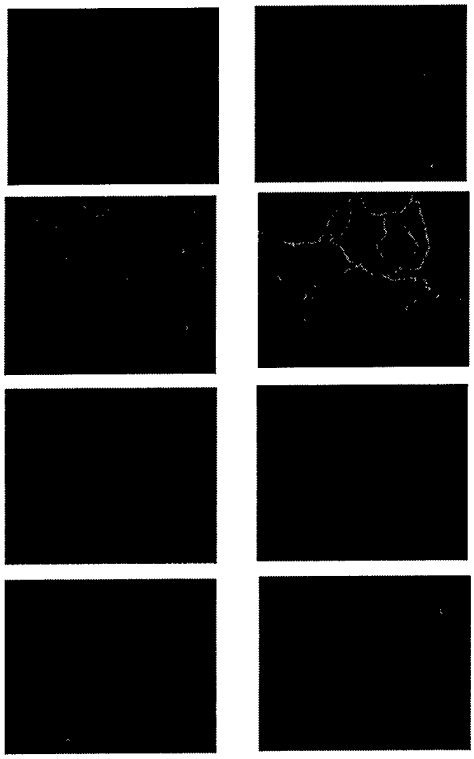
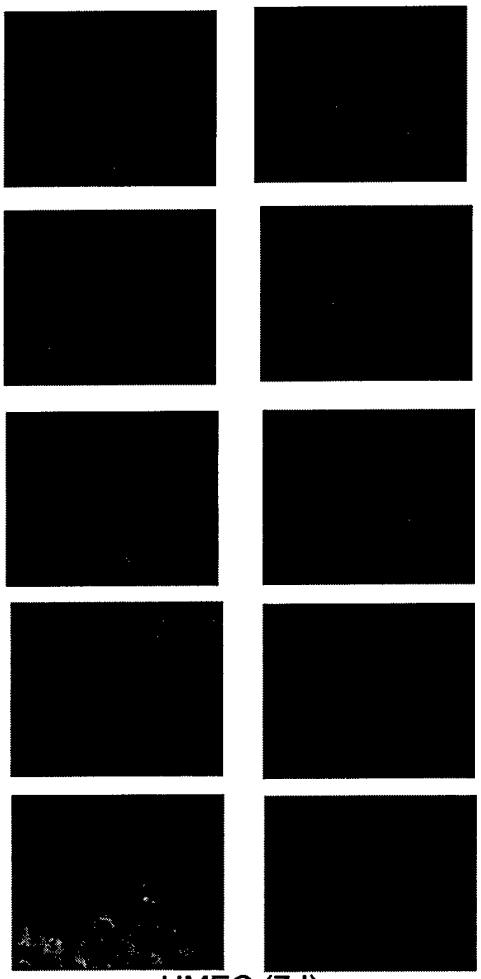


Figure 3



MDCK (7d)

Figure 4



HMEC (7d)

Figure 5

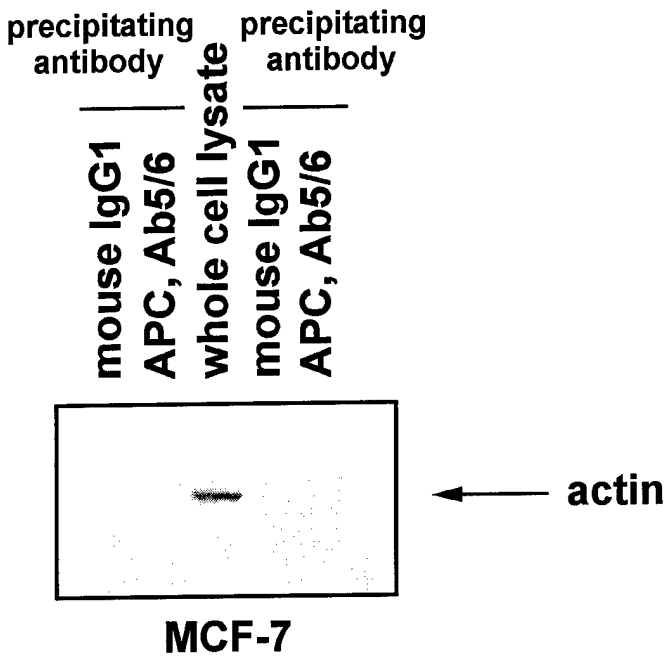
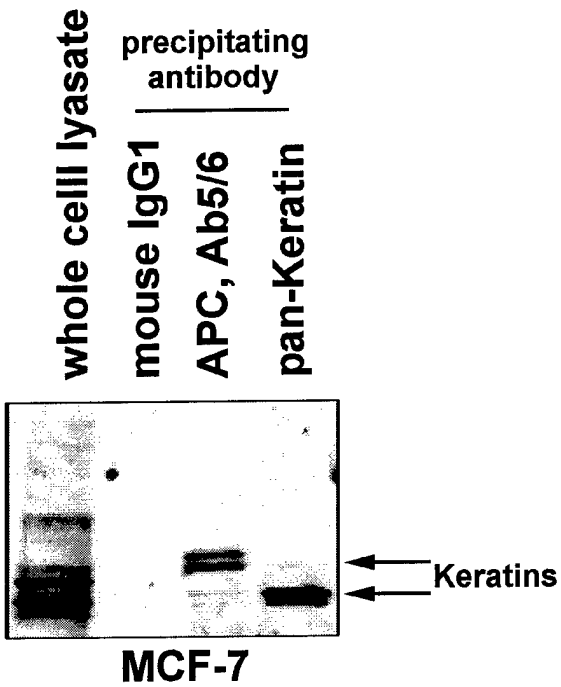
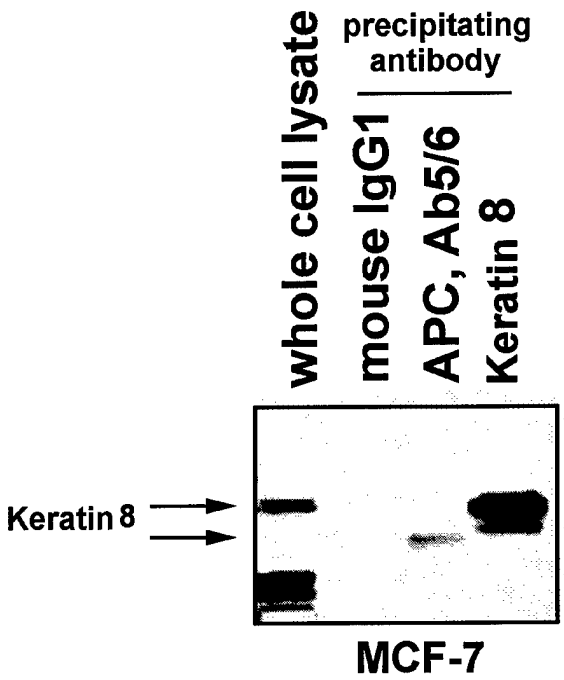

precipitating antibody
precipitating antibody
mouse IgG1
APC, Ab5/6
whole cell lysate
mouse IgG1
APC, Ab5/6
actin
MCF-7

Figure 6

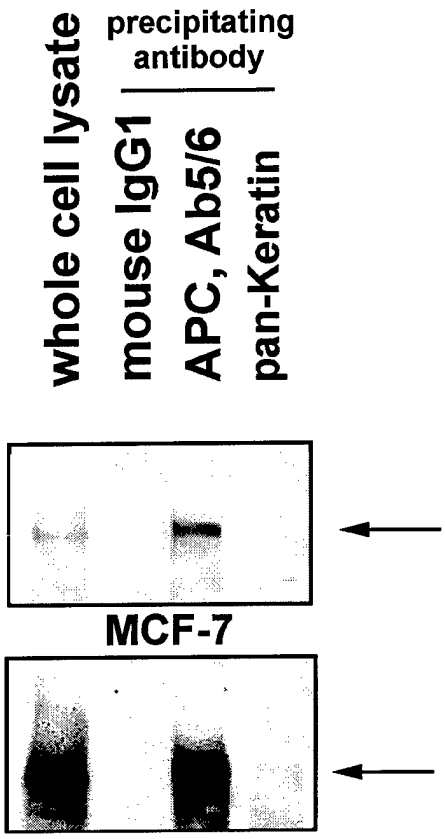
A.



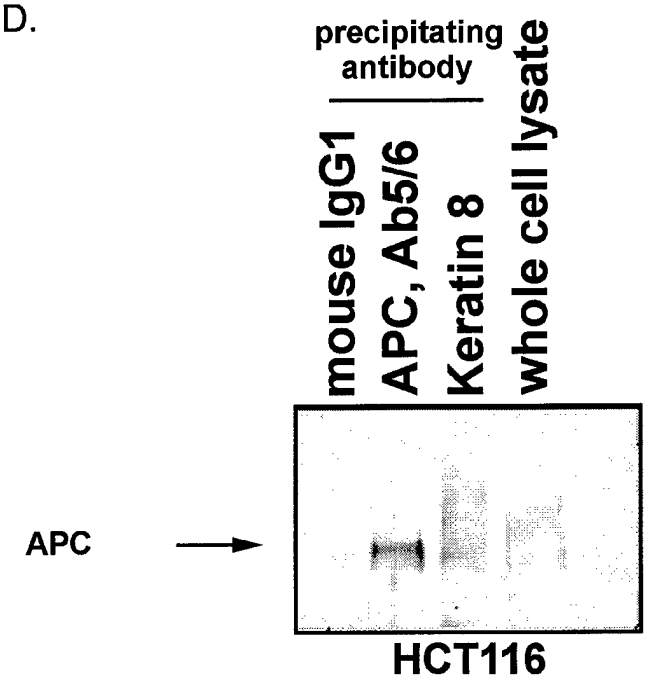
C.



B.



D.



Key Research Accomplishments / Demonstrations

1. APC protein was located to the nucleus and the cytoplasm of normal breast epithelial cells.

2. Breast cancer cells lines tested (4) showed expression of full-length APC protein with a similar distribution pattern as that seen in normal breast epithelial cells.

3. APC protein concentrated at the edge of breast epithelial cells was eliminated by disruption of keratin filaments and microtubules, but not by actin disruption.

4. APC protein appeared tightly associated with intermediate filaments of the normal breast epithelial cell following sequential extraction.

5. APC protein from breast cell lines co-immunoprecipitated with keratin 8 but not with actin.

6. MDCK cells or HMECs were treated with LiCl to mimic a defect in APC function. Treated cells displayed retardation in their ability to form tight and adherense junctions.

Reportable Outcomes:

Manuscripts, abstracts, and presentations

Neufeld, K. L. and White, R. (1997) Cytoplasmic and nuclear localizations of adenomatous polyposis coli protein. Proc. Natl. Acad. Sci. USA. 94: 3034 - 3039.

Endogenous APC Protein has a Tripartite Distribution Pattern in Normal Epithelial Cells
Cancer Genetics and Tumor Suppressor Genes
Cold Spring Harbor, NY. August 1996

Interaction of Adenomatous Polyposis Coli Protein with Structural Components of Breast Epithelial Cells
DOD Breast Cancer Research Program Meeting
Washington, DC. October 1997

The Adenomatous Polyposis Coli Tumor Suppressor is Associated with the Nuclear

Matrix
Keystone Symposia- The Nuclear Matrix: Involvement in Genomic Organization, Function and Cellular Regulation
Copper Mountain, CO. April 1998

Nucleocytoplasmic shuttling of the Adenomatous Polyposis Coli Protein
Guest Alumni Lectures, Bethel College
North Newton, KS. September 1998

The Tumor Suppressor Protein APC Contains Functional Nuclear Export Signals
Keystone Symposia-Molecular Mechanisms for Gastrointestinal Cancer
Keystone, CO. April 1999

Employment and Research Opportunities applied for and Received based on training supported by this award.

In July of 1998 I was promoted from post doctoral fellow to the position of research track assistant professor.

Copy of the manuscript

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 3034–3039, April 1997
Cell Biology

Nuclear and cytoplasmic localizations of the adenomatous polyposis coli protein

(tumor suppressor/immunofluorescence microscopy/cellular fractionation/colon cancer)

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Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112

Contributed by Raymond L. White, December 31, 1996

ABSTRACT **Mutation of the adenomatous polyposis coli (*APC*) gene is an early step in the initiation of colon cancer. Because the distribution pattern of a protein within the cell can provide important clues as to function, we have used a combination of immunofluorescence microscopy and biochemical fractionation to determine the location of APC protein in epithelial cells. Immunofluorescence microscopy placed full-length APC protein in both the nucleus and the cytoplasm. The nuclear APC protein was concentrated in discrete subnuclear regions, including nucleoli, whereas the cytoplasmic APC protein concentrated at the leading edge of migrating cells. Colocalization of APC protein with rRNA confirmed a nucleolar localization. These immunocytochemical findings have been supported by cell fractionation, which demonstrated that full-length APC protein was located in both the membrane/cytoskeletal and the nuclear fractions.**

Inherited tumor predispositions provide an opportunity to define critical early genetic events in the development of tumors. An inherited colon cancer predisposition, familial adenomatous polyposis, is caused by mutant alleles of the adenomatous polyposis coli (*APC*) gene (1–4). The early appearance of hundreds or thousands of colon polyps in this inherited disorder indicates that mutations in the *APC* gene can be rate-limiting in polyp development. The majority of sporadic colon polyps and carcinomas also carry mutated *APC* genes, indicating that somatic mutations in the *APC* gene are an early event in the development of most colon polyps and carcinomas.

The distribution pattern of a protein within the cell can provide important clues as to its function. Previous studies of the intracellular distribution of endogenous APC have focused on APC protein in the cytoplasm (5–7). Importantly, endogenous APC protein has recently been shown to localize near the ends of microtubules that extend into actively migrating regions of MDCK or IEC-6 cell membranes (10). A recent examination of APC protein overexpressed in mouse colonocytes, however, also suggested that APC may be present in the nucleus (11).

To provide a more detailed characterization of the intracellular distribution of APC protein in epithelial cells, in particular to determine whether APC protein might, in fact, be present in the nucleus, we have examined the subcellular distribution of endogenous APC protein in epithelial cells in culture. We have used three commercially available mAbs and one polyclonal antiserum for cell staining experiments. Immunofluorescence microscopy revealed APC protein in both the cytoplasmic and the nuclear regions of the epithelial cells. As seen by others in different cells (10), we have seen a punctate cytoplasmic pattern and a more intense particulate labeling of the leading edges of migrating cells. In addition to the cytoplasmic pattern, we have also observed a distinct pattern of APC protein within the nucleus, with focal staining of the nucleoli. These immunocytochemical findings were supported by cell fractionation, demonstrating that full-length APC protein is found in the nucleus as well as the membrane/cytoskeletal fraction.

Most disease-causing mutations in the *APC* gene result in a truncated protein product (12). In contrast to wild-type APC protein, we found no truncated APC protein in the nuclear fraction of colon cancer cells containing only mutant *APC*. This result indicates that such truncations abrogate nuclear localization of APC protein, suggesting that elimination of the nuclear location and function of this protein may be important in colon cancer progression.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. Cell lines used in these experiments were maintained at 37°C in CO_2 (5%) incubators. 184A1 cells are an immortalized human mammary epithelial cell line (13) (a gift from Martha Stampfer, Lawrence Berkeley Laboratory, Berkeley, CA). They were grown in MCDB 170 media (Clonetics, San Diego) supplemented as described (14). Primary epithelial or fibroblast outgrowths from normal breast tissue were grown in MCDB 170 media or MEM, 10% fetal bovine serum (FBS), respectively. Other cells were obtained from the American Type Culture Collection and were maintained in the following growth media: HCT116 (McCoy's 5a medium/10% FBS), LS174T (Eagle's MEM/10% FBS/1% nonessential amino acids), DLD-1 (RPMI medium 1640/10% FBS), T47D (RPMI medium 1640/10% FBS), and MDCK (MEM/10% FBS). HCT116 and LS174T are colon cancer cell lines that both express only full-length APC protein. DLD-1 is a colon cancer cell line that expresses no full-length APC protein but, rather, APC protein truncated at amino acid 1427. T47D is a breast cancer cell line that expresses full-length APC protein.

Immunofluorescence Microscopy. Cells were seeded onto tissue culture chamber slides (25–50% confluency) and allowed to grow for 36–48 hr before manipulation. Cells were rinsed in PBS (10 mM phosphate, pH 7.5/100 mM NaCl) then fixed with 2% paraformaldehyde in PBS for 30 min at 4°C. Following two PBS rinses, cells were permeabilized with 0.2% Triton X-100 in TBS (10 mM Tris, pH 7.5/100 mM NaCl/5 mM KCl) for 5 min at room temperature. Following two TBS washes, cells were incubated with 0.5% Na_2BH_3 in water for 10 min at room temperature. Cells were rinsed with TBS and then incubated with primary antibody diluted in antibody buffer

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Abbreviations: APC, adenomatous polyposis coli; DAPI, 4, 6-diamidino-2-phenylindole; DIC, differential interference contrast; FBS, fetal bovine serum.

*To whom reprint requests should be addressed.

(1% BSA/3% normal goat sera/0.2% Triton X-100 in TBS) for 90 min at room temperature. Cells were rinsed three times with TBS prior to incubation with secondary antibody conjugated to fluorescein isothiocyanate or Texas Red for 30 min at room temperature. Cells were rinsed three times with TBS and mounted with Pro Long antifade (Molecular Probes) for immunofluorescence microscopy. Antibodies and dilutions used for the experiments are as follows: APC (mouse IgG1, Ab-2, Ab-4, or Ab-6) 1:150 (Oncogene Science) or APC64 (rabbit sera, a gift from the Arnold J. Levine laboratory, Princeton University, Princeton) 1:1000, α-tubulin (mouse IgG1, DM-1A) 1:200 (ICN), goat anti-mouse IgG1-fluorescein isothiocyanate and goat anti-mouse IgG1-Texas Red 1:200 (Southern Biotechnology Associates), goat anti-rabbit-Texas Red 1:200 (Accurate Chemical and Scientific, Westbury, NY), and goat anti-rabbit-fluorescein isothiocyanate 1:200 (Boehringer Mannheim). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) following secondary antibody incubation. APC polyclonal serum was preadsorbed to DLD-1 cells that had been fixed and permeabilized as described for immunofluorescence staining. For the APC antibody blocking experiment, APC antibody was incubated with a peptide corresponding to APC protein amino acids 2717–2844 at 10-fold molar excess, in PBS for 12 hr at 4°C. Any precipitant protein was pelleted by centrifugation for 15 min prior to dilution of the peptide/antibody mixture in antibody buffer. Stained cells were examined using an Axioplan microscope (Zeiss) with a ×63 objective. Images of APC-stained cells and APC controls were captured using 400 ASA slide film (Eastman Kodak) and 2- to 4-min exposure times.

Cell Fractionation. Cell fractionation was performed essentially as described (15). For cell fractionation procedures, protease inhibitors (Boehringer Mannheim) were added to the buffers in the following concentrations: pefablock, 0.2 mg/ml; aprotinin, 0.01 mg/ml; pepstatin, 0.01 mg/ml; and leupeptin, 0.01 mg/ml. Cells grown in 150-cm^2 flasks were harvested by scraping into ice cold PBS. Cells were rinsed two times with cold PBS prior to lysis with detergent. Cells were resuspended in L-buffer (PBS/0.1% Triton X-100/0.1% Nonidet P-40) and incubated on ice for 10 min, or until they were determined to be >99% lysed using trypan blue exclusion. Nuclei were pelleted by centrifugation at 1000 × *g* for 10 min at 4°C. Supernatant was further fractionated by centrifugation at 100,000 × *g* for 60 min at 4°C. The supernatant fraction was collected and classified as cytoplasm. The pellet was resuspended in L-buffer and was classified as the membrane/cytoskeletal fraction. The nuclear pellet was purified from membrane contaminants by two rinses in L-buffer, passage through a 0.22-gauge needle three times, and passage through a 0.85 M sucrose cushion (15,000 rpm, microfuge, 15 min). Nuclei in the pellet were lysed by sonication (30 sec) in PBS prior to DNase (100 units/200 μl) treatment (45 min, 4°C). Nuclei were further sonicated two times, for 30 sec at 4°C to make a nuclear lysate. For nuclear scaffold/matrix isolation, nuclear pellets purified through the sucrose cushion were washed once, then resuspended in nuclei buffer (10 mM Tris, pH 7.4/20 mM KCl/0.125 mM spermidine/0.05 mM spermine/1% thiodiglycol). DNase I (100 units) and $MgCl_2$ (5 mM final concentration) were added prior to incubation on ice for 30 min. $CuSO_4$ (1 mM final concentration) was added, and the nuclei were incubated for 10 min at 37°C. Nuclear scaffold proteins were precipitated on ice by addition of an equal volume of 0.4 M $(NH_4)_2SO_4$ in 10 mM Tris·HCl/0.2 mM $MgCl_2$. Precipitate was raised in 15 ml TM-0.2 buffer [10 mM Tris·HCl, pH 7.4/0.2 mM $MgCl_2$/0.2 M $(NH_4)_2SO_4$] and pelleted at 1500 rpm for 15 min at 4°C. The pellet was washed three times with nuclei buffer and 70 mM NaCl prior to resuspension in L-buffer. Protein concentration was determined in all cell fractions using a Bio-Rad protein assay reagent as per manufacturer's instructions.

Western Immunoblot Analysis. Proteins (70 μg/lane; 35 μg/lane for scaffold fractions) were separated electrophoretically using either 6% or 4–12% gradient acrylamide Tris tricine gels (NOVEX, San Diego) and Laemmli buffer. Gels were run for 2 hr at 125 V with cold circulating water. Proteins were transferred to nitrocellulose (Schleicher & Schuell) for 16 hr at 30 V in transfer buffer (192 mM glycine/20% methanol/25 mM Tris base/0.1% SDS) with circulating cold water. Rainbow molecular weight markers (Amersham) were loaded in one lane of each gel for size standardization. Nitrocellulose membranes containing transferred proteins were blocked with 5% BSA in TBST (TBS/0.1% Tween 20) then incubated with primary antibody diluted in 1% BSA in TBST for 1 hr at 20°C. Following three 10-min rinses with TBST, blots were incubated with an appropriate secondary antibody conjugated to horseradish peroxidase in 1% BSA/TBST. Blots were rinsed three times with TBST and then probed using an enhanced chemiluminescence detection system (Amersham) as per manufacturer's instructions. Antibodies used for Western immunoblot analysis were as follows: APC (mouse IgG1, Ab-1, or Ab-2) 1:200 (Oncogene Science), α-tubulin 1:200 (ICN), lamin A/C (mouse IgG1, X-67), or lamin B (mouse IgG1, X233) 1:10 (American Research Products, Beltsville, MD), horseradish peroxidase–rabbit anti-mouse IgG1 1:20,000 (Zymed), β1,β2-adaptin (mouse IgG1) 1:2000 (Sigma), horseradish peroxidase–goat anti-rabbit IgG, and horseradish peroxidase–sheep anti-mouse 1:40,000 (Sigma).

RESULTS

Location of Full-Length APC Protein in Epithelial Cells. As a first step toward determining the distribution pattern for endogenous, full-length APC protein, we screened commercially available APC antibodies for their ability to stain epithelial cells using indirect immunofluorescence microscopy. Fig. 1 shows a schematic of the APC protein modified from ref. 12 showing the various epitopes recognized by the APC antibodies used in the present study. Ab-4 is an mAb made against the C-terminal 300 aa of APC protein. The precise location of its epitope has not been mapped. Additional features of the APC protein include a mutation cluster region, where 94% of all somatic and ≈62% of all inherited, disease-causing APC mutations occur (16), a β-catenin-binding region (17, 18), and an oligomerization region (19).

A panel of antibodies raised against various APC epitopes was used to stain 184A1 cells. 184A1 is an immortalized human mammary epithelial cell line that displays keratin staining

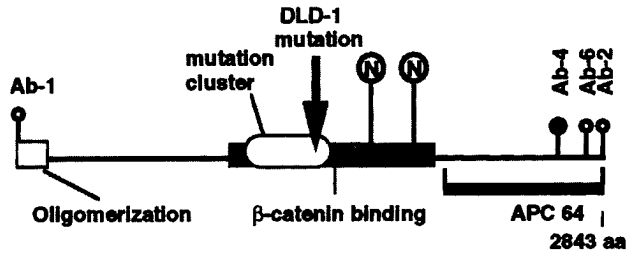

FIG. 1. Antibodies recognize different epitopes of the APC protein. Schematic representation of the APC protein as adapted from (12). The mutation cluster region is indicated by the oval. β-catenin-binding region is indicated by a shaded rectangle. □, Oligomerization region; ○, mAbs that recognize distinct APC epitopes used in the present study. Antibody Ab-4, whose epitope has not been mapped precisely, was made against a 300-amino acid peptide beginning at ● and proceeding to the end of the APC protein. The solid line represents the APC region that was used to produce the polyclonal rabbit sera APC64. The arrow marks the point of APC protein truncation in the colon cancer cell line DLD-1. The circled Ns indicate two potential nuclear localization sequences beginning at amino acids 1773 and 2054.

patterns and phenotypic characteristics very similar to normal human mammary epithelial cells (13). Of seven mAbs and three polyclonal antibodies tested in immunofluorescence assays with the 184A1 cell line, the three mAbs raised against the C terminus of APC protein (Ab-2, Ab-4, and Ab-6), and the polyclonal antibody APC64, also raised against the C terminus, gave similar and reproducible staining patterns, as seen in Fig. 2 *b*, *c*, and *g–i*. These antibodies localized full-length APC protein to two distinct compartments of the epithelial cell. A punctate staining pattern is visible in the cytoplasm, with especially prominent staining at cell edges (Fig. 2 *b* and *g–i*, solid arrowheads). In migrating cells, the APC staining concentrated at the leading edge (data not shown). This cytoplasmic distribution of APC protein provides additional support for the very similar results reported recently in MDCK cells (10). In addition to the cytoplasmic staining, however, there is significant nuclear staining, shown most clearly in Fig. 2*c* (open arrowhead), where the same cells viewed in Fig. 2 *a* and *b* are photographed at a slightly different focal distance. Much of the total staining seems to be concentrated in discrete nuclear regions (open arrowheads). In Fig. 2*a*, the cells shown in Fig. 2 *b* and *c* were stained with DAPI and viewed using differential interference contrast (DIC) optics to distinguish cell nuclei, cell edges, and general morphology. In addition, a similar APC protein location was seen in several other cells tested, including T47D (Fig. 2*j*), normal human fibroblasts, MDCK, mink lung epithelial cells, primary outgrowth from normal breast tissue, and Cos7 (data not shown). The remaining four mAbs (Ab-1, Ab-3, Ab-5, and Ab-7; Oncogene Science) and one of the polyclonal antibodies (N-15; Santa Cruz Biotechnology) showed essentially no staining. Interestingly, each of these antibodies had been raised against the N terminus of the APC protein. The other polyclonal serum raised against the C terminus of APC protein (C-20; Santa Cruz Biotechnology) also showed only minimal staining.

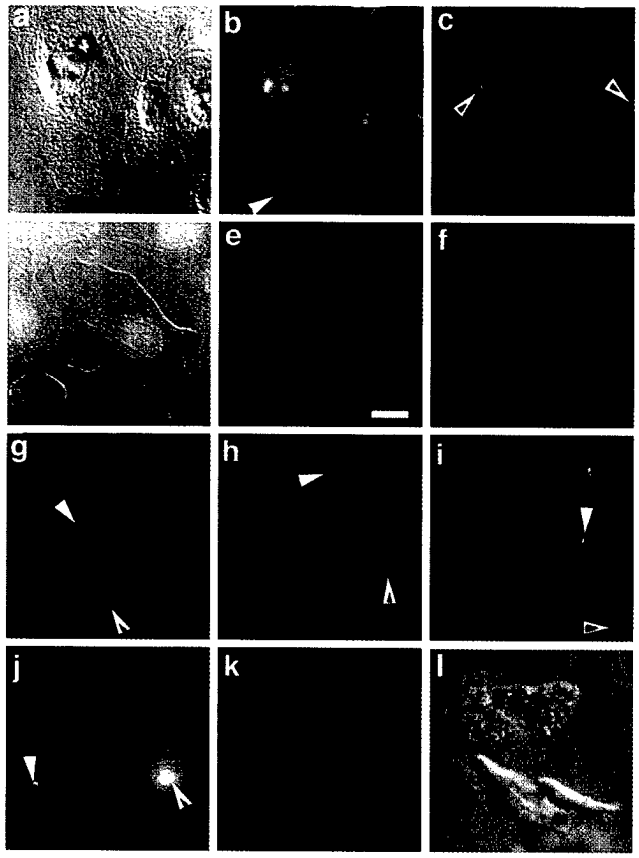

FIG. 2. Localization of APC protein in 184A1 cells using immunofluorescence microscopy. 184A1 cells were grown on glass slides prior to fixation and immunofluorescence microscopy using Ab-4, an antibody specific for APC protein (*b* and *c*) or using Ab-4 preincubated with an APC peptide (*e*). *b* and *c* are photographs of the same group of cells taken at two focal distances to more clearly capture cell edge staining (*b*, solid arrowhead) and nuclear staining (*c*, open arrowhead). APC protein appears in a punctate pattern throughout the cytoplasm with areas of protein concentration at one edge (solid arrowhead). In addition, APC protein appears throughout the nuclei with a few areas of concentration (*c*). *a* and *d* are DIC and DAPI views of the fluorescence views shown in *b*, *c*, and *e*, respectively. Controls include: *f*, 184A1 cells stained with nonspecific antibody IgG_1; *g–i*, 184A1 cells stained for APC using antibodies Ab-2 (*g*), Ab-6 (*h*) or APC64 (*i*); *j*, APC staining of T47D cells. For each antibody, both edge staining (solid arrowhead) and nuclear staining (open arrowhead) are apparent. In *k*, DLD-1 cells that express only truncated APC protein were stained using the C terminal antibody Ab-4 to demonstrate staining specificity. *l* is the corresponding DIC and DAPI view of the cells shown in *k*. (Bar = 10 μm.)

In addition to the observations that four independent antibodies give the same staining pattern, this result was further examined by several criteria. APC staining was eliminated by preincubation of the primary antibody with an APC peptide (Fig. 2*e*), while the same APC peptide did not affect immunofluorescence with an α-tubulin antibody (data not shown). In the absence of primary antibody (data not shown), or in the presence of a nonimmune mouse mAb of the same isotype, IgG1 (Fig. 2*f*), only a diffuse, very faint staining was seen, without the characteristic pattern obtained with APC antibody.

To further demonstrate the dependence of the staining pattern on the presence of full-length APC protein, cells from the colon cancer cell line DLD-1, which lack full-length APC protein, were used for immunofluorescence microscopy with each of the four antibodies directed against the C terminus of APC. DLD-1 cells express no full-length APC protein since they carry a mutation that truncates APC at amino acid 1427. This truncation deletes the C-terminal portion of the APC protein, thereby eliminating all APC epitopes recognized by antibodies directed toward APC protein's C terminus. The distinctive staining pattern previously observed was not seen in the DLD-1 cells stained with any of the C-terminal antibodies [Ab-4 (Fig. 2*k* with corresponding DAPI/DIC in Fig. 2*l*), Ab-2, Ab-6, and APC64; data not shown]. This finding supports the interpretation that the characteristic staining pattern we observe in cells carrying normal APC protein is due to epitopes within the C terminus of full-length APC protein.

APC Protein Within the Nucleus Localizes to the Nucleoli. Nuclei were identified with DAPI stain as shown in Fig. 2*a*, with the blue DAPI signal overlying the DIC image. The size and shape of the concentrated staining suggested that APC protein might be in nucleoli. This suggestion was confirmed using a fluorescein-conjugated DNA probe that recognizes rRNA, a major component of nucleoli. As shown in Fig. 3, most regions with concentrated nuclear APC protein, identified using a Texas Red-conjugated secondary antibody (Fig. 3*a*), overlapped with the nucleoli, visualized with a green, fluorescein-conjugated, antisense oligonucleotide rRNA probe (Fig. 3*b*). The micrographs shown in this figure were taken with the nuclear region of the cell in focus, eliminating most of the cytoplasmic and edge staining. The overlap of APC protein and rRNA is shown in Fig. 3*c*, where a double exposure shows the coincidence of red APC signal and green rRNA signal as yellow. In Fig. 3, solid arrows designate nuclear staining in a single cell, and open arrows indicate additional regions of staining overlap. Some regions that stained positive for APC protein did not stain positive for rRNA. The DIC view with DAPI signal overlay is shown in Fig. 3*d*. A fluorescein-conjugated oligonucleotide probe corresponding to the rRNA sense strand sequence did not show the nucleolar staining pattern (data not shown).

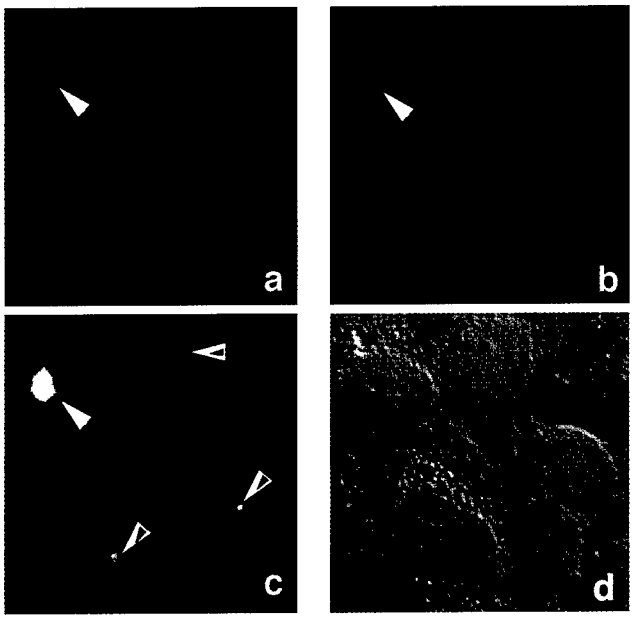

FIG. 3. Nuclear APC protein co-localizes with rRNA. 184A1 cells were stained simultaneously for APC protein (*a*) and rRNA (*b*) using antibodies specific for the APC protein and a fluorescein isothiocyanate-conjugated DNA oligo complementary to rRNA. The yellow staining in *c* reflects the colocalization of rRNA and APC protein. Solid arrowheads designate nuclear staining in a single cell (*a–c*). Open arrowheads designate additional regions of staining overlap. *d* is a DIC and DAPI view of the fluorescence views shown in *a–c*. (Bar = 10 μm.)

Biochemical Fractionation Locates Full-Length APC Protein to Both Membrane/Cytoskeletal and Nuclear Cell Fractions. Biochemical fractionation was employed as an independent test of the two cellular locations of APC protein. Because the colon cancer cell lines HCT116 and LS174T both carry normal APC alleles, and express 5–10 times more full-length APC protein than 184A1 cells, they were used to optimize the biochemical fractionation procedure. Both cell lines were subjected to lysis by detergent (>99% cell lysis as determined by trypan blue exclusion) followed by purification of the nuclei from the cytoplasmic fraction. The cytoplasmic fraction was further separated into a membrane/cytoskeletal fraction and a soluble fraction. Nuclear matrix/scaffold proteins were purified from the nuclear fraction by DNAse treatment, stabilization with $CuSO_4$, precipitation in 0.2 M ammonium sulfate, and washes. Because similar results were obtained with both LS174T and HCT116 cell lines, only data from fractionation of LS174T is shown. The fractions were characterized by Western immunoblot analysis using antibodies directed against compartment-specific proteins: α-tubulin fractionated with the cytoplasm and cytoskeleton, β-adaptins with cytoplasm and membranes, and lamins A, B, and C with the nucleus and nuclear matrix/scaffold.

As seen in Fig. 4*a*, LS174T cells contain full-length APC protein in both the membrane/cytoskeletal (lane M) and the nuclear (lane N) fractions with ≈one-sixth of the total APC protein residing in the nucleus. The majority of the nuclear APC protein further fractionated with the nuclear matrix (lane Sc). The slowest band recognized by the APC antibody migrated at the molecular size predicted for full-length APC protein relative to the molecular weight markers (Fig. 4*a*, lanes T, M, N, and Sc) and was recognized by antibodies raised to both N-terminal (Ab-1) and C-terminal (Ab-2) regions of APC protein. Band intensities were greatly diminished by preincubation of the APC antibody with an APC peptide (data not shown). The various cell fractions were characterized by stripping and re-probing the blot for the marker proteins

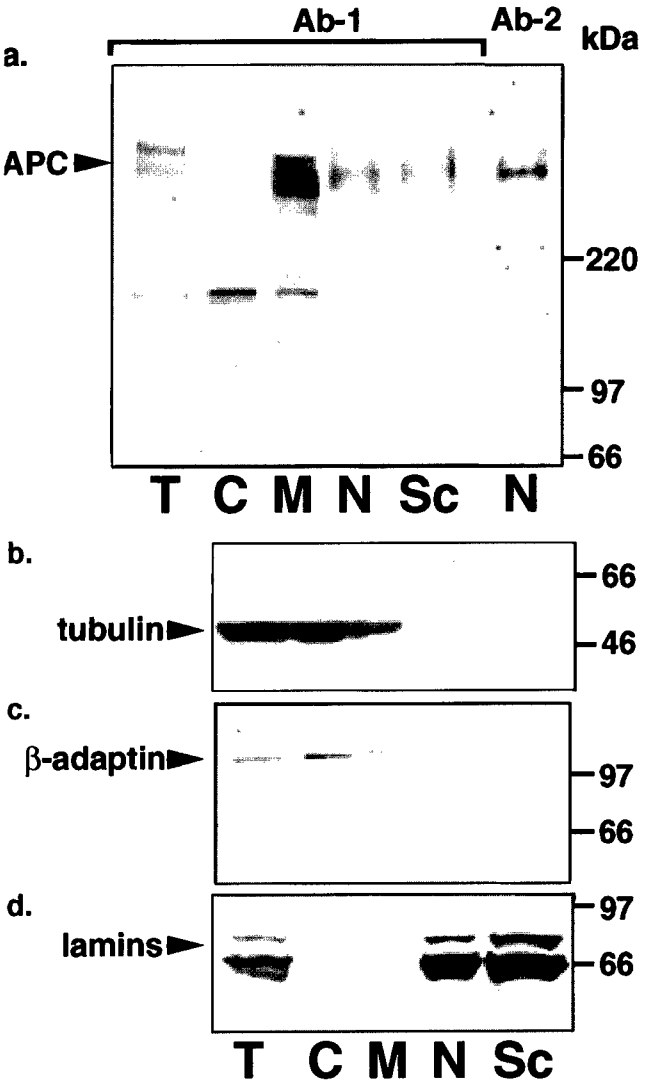

FIG. 4. Full-length APC protein locates to both membrane/cytoskeletal and nuclear cell fractions in LS174T cells. Proteins within the various fractions of LS174T cells were analyzed by SDS/PAGE and immunoblotting. Fractions are labeled at the figure bottom as follows: T, total; C, cytoplasm; M, membrane/cytoskeleton; N, nucleus; Sc, nuclear scaffold. The antibodies used for the Western immunoblots are as follows: *a*, APC;, *b*, tubulin as a cytoskeletal marker; *c*, β-adaptin as a membrane marker; and *d*, lamins as nuclear and nuclear matrix scaffold markers.

α-tubulin (Fig. 4*b*, lanes C and M), β-adaptin (Fig. 4*c*, lanes C and M), and lamins (Fig. 4*d*, lanes N and Sc).

Once fractionation conditions were optimized, they were applied to 184A1 cells, used previously for the immunofluorescence microscopy analysis. Although these cells express much lower levels of APC protein, we were able to show full-length APC protein in the membrane/cytoskeletal, the nuclear, and the nuclear matrix fractions (data not shown). The 184A1 cells showed a more even distribution of APC protein between the nuclear and the membrane/cytoskeletal fractions, consistent with the ratio seen using immunofluorescence microscopy (Fig. 2). The various cell fractions were again characterized by stripping and reprobing the blot for the marker proteins α-tubulin, α-adaptin, and lamins (data not shown).

Truncated APC Protein from a Colon Cancer Cell Line Does Not Locate to the Nuclei. Cells from the DLD-1 colon carcinoma cell line, which carries only a mutant allele of the *APC* gene, were fractionated by the above procedure. As seen

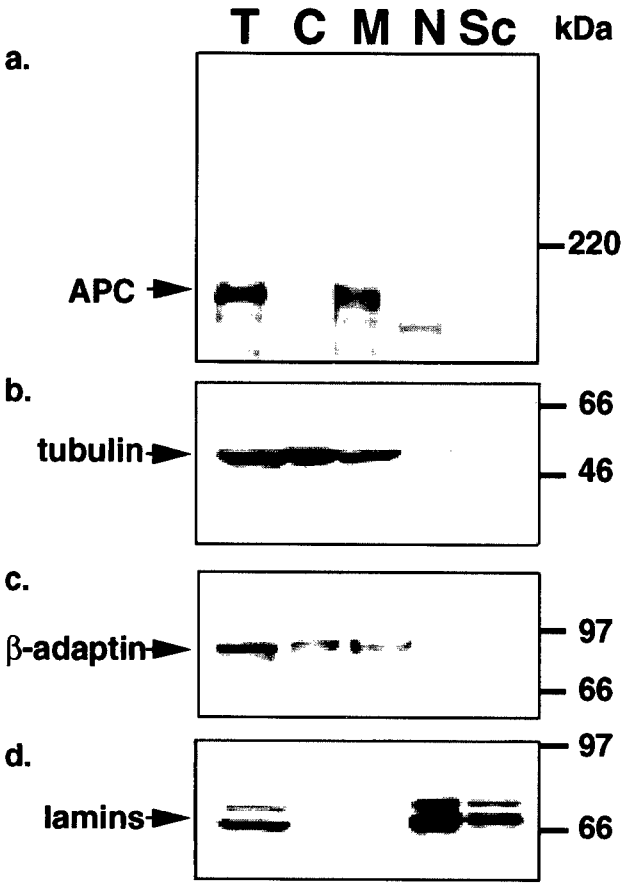

FIG. 5. Truncated APC protein does not fractionate with the nucleus of DLD-1 cells. Proteins within the various fractions of DLD-1 cells were analyzed by SDS/PAGE and immunoblotting. Fractions are labeled as follows: T, total; C, cytoplasm; M, membrane/cytoskeleton; N, nucleus; Sc, nuclear scaffold. The antibodies used for the Western immunoblots include: *a*, APC; *b*, tubulin as a cytoskeletal marker; *c*, β-adaptin as a membrane marker; and *d*, lamins as nuclear and nuclear matrix scaffold markers. Truncated APC protein is present in the membrane/cytoskeletal fraction, but not the nuclear or nuclear scaffold fractions.

in Fig. 5*a*, the truncated APC peptide is found in the membrane/cytoskeletal fraction. Longer exposure times reveal truncated APC protein in the cytoplasmic fraction but not the nuclear or nuclear scaffold fractions. The band appearing slightly below that of truncated APC protein represents cross-reactivity of the secondary antibody with a protein in the DLD-1 cell line as it is present in blots probed with an antibody to APC protein's C terminus and also with secondary antibody alone (data not shown). Compartment-specific proteins α-tubulin (Fig. 5*b*), β-adaptin (Fig. 5*c*), and lamins A, B, and C (Fig. 5*d*), had the expected distribution patterns.

DISCUSSION

Endogenous APC protein has been found in both the nucleus and the cytoplasm of cultured human epithelial cells. Immunocytochemistry revealed a particulate distribution of APC protein in distinct nuclear regions with foci in the nucleoli (Figs. 2*c* and 3*a*), and throughout the cytoplasm (Fig. 2*b*), with concentrations at the leading edge of migrating cells. Each of three mAbs and one polyclonal antibody directed against APC protein's C terminus consistently showed the same staining pattern. In contrast, five antibodies directed against the APC protein's N terminus failed to show significant staining, suggesting that the N-terminal epitopes might be masked *in vivo*, or that these antibodies might recognize denatured but not native protein.

Cell fractionation experiments provided further support for a nuclear as well as cytoskeletal/membrane localization for the APC protein. The relative cellular distribution of APC protein between nucleus and cytoskeletal/membrane fractions varied among cell types; 184A1 cells showed a high proportion of APC protein in the nucleus (up to half of the total), whereas the colon cancer cell line LS174T showed only ≈one-sixth of the total APC protein in the nucleus. Approximately 70% of the nuclear APC protein fractionated together with the nuclear matrix.

The observation that a significant fraction of APC protein localized to the nucleus of epithelial cells prompted a search for putative nuclear localization sequences within the APC amino acid sequence. As indicated in Fig. 1, APC protein has two stretches of basic amino acid residues, bordered by glycine or proline, typical of nuclear localization sequences. Interestingly, almost all mutations mapping to the APC gene in both familial adenomatous polyposis and sporadic colon cancer patients result in elimination of both putative nuclear localization sequences from the resulting APC protein.

Our findings extend previous studies that revealed primarily cytoplasmic components of the intracellular distribution of APC. Consistent with our results, a recent report using MDCK and rat intestinal epithelial cells has shown punctate cytoplasmic and leading edge localization of APC protein, although nuclear staining was not reported as significant (10). Previous studies differ from those reported here in fixation, staining, and in the cells examined, focusing on immunohistochemical analyses of frozen colon tissue sections (6, 20), overexpressed exogenous protein (8, 9), APC protein expressed in neural cells (21), canine and rat epithelial cells (10), or murine enterocytes (11). Our studies, focusing on human epithelial cells, indicate that the ability to clearly see details of the nuclear component of APC is highly dependent on protocol details: e.g., the use of paraformaldehyde vs. formaldehyde in cell fixation and DNAse treatment of nuclear lysates prior to immunoblot analysis.

Identification of β-catenin (17, 22), and plakoglobin (23, 24) as APC protein partners suggests that APC may modulate the WNT1 signaling pathway, perhaps by increasing the level of β-catenin turnover (18, 25). The threonine-serine kinase GSK3β was also recently found to bind and phosphorylate APC *in vitro* and this phosphorylation was necessary for β-catenin binding (25). Recent observations demonstrating the presence of β-catenin in the nucleus (26), in association with the transcription complex component, LEF-1 or XTcf-3 (27, 28), together with our observations reported here of APC protein in the cell nucleus, raise the possibility that APC protein interacts with β-catenin in both the cytoplasm and the nucleus.

It is intriguing to relate these observations to possible functional roles for APC in colonocyte development. The developing colonocyte must coordinate a number of cellular events as it migrates up the walls of the crypt. At early stages there are several cell divisions but at later stages, cell division ceases, with cessation of ribosomal synthesis, and specific differentiated functions such as mucin production are initiated, while cell migration continues up the crypt. Upon reaching the top of the crypt, the colonocyte initiates a program of apoptosis and is shed into the lumen of the colon. Several pieces of evidence point to a cell regulatory function for APC in this process. Removal of APC can initiate the deregulated growth of adenomatous colon polyps, characterized by continued cell division and a failure to complete the normal differentiation program. Localization of APC protein in the nucleus with foci in the nucleoli may suggest a regulatory role for APC in both mitosis and ribosomal RNA synthesis. Taken together, these results suggest that APC may be playing

a role as a global regulator of colonocyte development, orchestrating the activities of multiple systems.

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1. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., *et al.* (1991) *Cell* **66,** 589–600.
2. Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., *et al.* (1991) *Cell* **66,** 601–613.
3. Kinzler, K. W., Nilbert, M. C., Su, L.-K., Vogelstein, B., Bryan, T. M., *et al.* (1991) *Science* **253,** 661–665.
4. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., *et al.* (1991) *Science* **253,** 665–668.
5. Smith, S. A., Easton, D. F., Ford, D., Peto, J., Anderson, K., Averill, D., Stratton, M., Ponder, M., Pye, C. & Ponder, B. A. J. (1993) *Genetics* **52,** 767–776.
6. Miyashiro, I., Senda, T., Matsumine, A., Baeg, G., Kuroda, T., Shimano, T., Miura, S., Noda, T., Kobayashi, S. & Monden, M., *et al.* (1995) *Oncogene* **11,** 89–96.
7. Boman, B., Lovas, S., Abraham, C., Adrian, T., Murphy, R., Marbello, R. & Bhattacharya, G. (1995) *Biochem. Biophys. Res. Commun.* **206,** 909–915.
8. Munemitsu, S., Souza, B., Muller, O., Albert, I., Rubinfeld, B. & Polakis, P. (1994) *Cancer Res.* **54,** 3676–3681.
9. Smith, K., Levy, D., Maupin, P., Pollard, T., Vogelstein, B. & Kinzler, K. (1994) *Cancer Res.* **54,** 3672–3675.
10. Nathke, I. S., Adams, C. L., Polakis, P., Sellin, J. H. & Nelson, J. (1996) *J. Cell Biol.* **134,** 165–179.
11. Wong, M. H., Hermiston, M. L., Syder, A. J. & Gordon, J. I. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9588–9593.
12. Polakis, P. (1995) *Curr. Opin. Genet. Dev.* **5,** 66–71.
13. Walen, K. H. & Stampfer, M. R. (1989) *Cancer Genet. Cytogenet.* **37,** 249–261.
14. Taylor-Papadimitriou, J. & Stampfer, M. R. (1992) in *Culture of Epithelial Cells*, ed. Freshney, R. I. (Wiley, New York), Vol. 1, pp. 107–133.
15. Sprott, S., Hammond, K. & Savage, N. (1991) *Anal. Biochem.* **194,** 407–412.
16. Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., K, Muraoka, M., Takahashi, H., Amada, Y. & Fukayama, M. (1994) *Cancer Res.* **54,** 3011–3020.
17. Su, L. K., Vogelstein, B. & Kinzler, K. W. (1993) *Science* **262,** 1734–1737.
18. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B. & Polakis, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3046–3050.
19. Joslyn, G., Richardson, D., White, R. & Alber, T. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11109–11113.
20. Smith, K. J., Johnson, K. A., Bryan, T. M., Hill, D. E., Markowitz, S., Willson, J. K., Paraskeva, C., Petersen, G. M., Hamilton, S. R., Vogelstein, B. & Kinzler, K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2846–2850.
21. Matsumine, A., Ogai, A., Senda, T., Okumura, N., Satoh, K., Baeg, G.-H., Kawahara, T., Kobayashi, S., Okada, M., Toyoshima, K. & Akiyama, T. (1996) *Science* **272,** 1020–1023.
22. Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S. & Polakis, P. (1993) *Science* **262,** 1731–1734.
23. Rubinfeld, B., Souza, B., Albert, I., Munemitsu, S. & Polakis, P. (1995) *J. Biol. Chem.* **270,** 5549–5555.
24. Shibata, T., Gotoh, M., Ochiai, A. & Hirohashi, S. (1994) *Biochem. Biophys. Res. Commun.* **203,** 519–522.
25. Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. & Polakis, P. (1996) *Science* **272,** 1023–1026.
26. Funayama, N., Fagotto, F., McCrea, P. & Gumbiner, B. M. (1995) *J. Cell Biol.* **128,** 959–968.
27. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. & Birchmeier, W. (1996) *Nature (London)* **382,** 638–642.
28. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. & Clevers, H. (1996) *Cell* **86,** 391–399.