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Purification and characterization of polyclonal antibodies against tumour suppressor protein APC

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Abstract

An important part in the fight against cancer is finding high affinity reagents for proteomics research. One protein extensively studied is the tumour suppressor adenomatous polyposis coli or APC, involved in colon cancer. In this project polyclonal antibodies against APC from immunized rabbits were purified. The antibodies were found to be useful in western blot analysis, immunoprecipitation of native APC and immunocytochemistry.

Keywords

Antibody purification, APC, Biosensor analysis, proteomics

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Purification and characterization of polyclonal antibodies against tumour suppressor protein APC

Maria Kärrlander

Sammanfattning

En av de vanligaste formerna av cancer i västvärlden är tjocktarmscancer eller koloncancer. Man beräknar att nästan en miljon människor drabbas varje år och nära hälften dör till följd. Trots att förbättrad diagnostik och behandling har minskat antalet dödsfall är dödligheten ändå hög. Den främsta orsaken till detta är svårigheten att upptäcka sjukdomen i ett tidigt skede. En viktig del i cancerforskningen är att förstå de proteiner och gener som är inblandade i uppkomsten av tumörer. Sådana proteiner är ofta tumörsuppressorer, vilket innebär att när de är defekta ger upphov till cancer. Deras normala funktioner i celler undertrycker (suppress på engelska) cellulära aktiviteter som kan orsaka cancer. Vanliga sådana aktiviteter är celldelning och tillväxt, cellmigration d.v.s. att flytta på sig, och förmågan för celler att fästa vid varandra, s.k. celladhesion.

Ett exempel på en tumör suppressor är adenomatos polyposis coli eller APC. Genen för APC upptäcktes som orsaken till en ärftlig form av koloncancer och har sedan funnits inblandad i majoriteten av sporadiskt uppkomna tumörer i kolon. APC kontrollerar bland annat celldelning, cellmigration och celladhesion i det yttersta cellagret i tarmen. Mutationer i genen gör att APC proteinet inte kan fungera som det ska. APC:s funktion i cellen går då förlorad vilket gör att celldelningen ökar, cellerna kan inte migrera och håller stramare fast vid varandra. Detta leder alltså till att vi får en okontrollerad celldelning och cellerna stannar där de är som i en klump. När denna klump blir tillräckligt stor har en tumör bildats.

Antikroppar är ett mycket vanligt använt reagens inom forskning. De egenskaper man utnyttjar är att de är mycket specifika för vad de binder till och att de binder mycket starkt. Antikroppar bildas som en del av immunförsvarets reaktion då något främmande kommer in i kroppen. Det som antikropparna bildas mot vid en sådan immunrespons och som de sedan binder till kallas antigen. I detta projekt användes två bitar av APC proteinet som antigen för att ta fram antikroppar. De renades och testades för deras användning i olika vanligt använda experimentella metoder inom proteinforskning. Antikropparna jämfördes med en kommersiellt producerad antikropp. Båda visade sig fungera bra i jämförelse med den kommersiella antikroppen. Antikropparna kommer därmed att vara användbara vid framtida studier av APC.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet April 2005

Contents

1 Introduction

Colon cancer is one of the leading causes of death by cancer in western society[1]. About a million people around the world develop colorectal cancer each year and almost half of them die from it. Improvements in diagnostic techniques and treatments have decreased the mortality but it is still very high. The high mortality rate is mainly due to the difficulty in detecting the cancer early. The majority of colon tumors are adenocarcinomas. They start as intestinal polyps which eventually becomes cancerous. Most of the environmental risk factors, e.g. diet rich in meat and fat and lacking in fibre, folate and calcium, smoking, obesity and high alcohol intake, are strongly connected with the lifestyle of developed countries. A main focus in trying to understand tumor formation is the study of genes involved in their development. One important gene is the *adenomatous polyposis coli* (*APC*) gene encoding the protein with the same name (APC). The gene was discovered as the cause of familial adenomatous polyposis (FAP), a dominant inherited form of colon cancer with close to 100% penetrance. The disease is caused by a germline mutation in the *APC* gene. FAP is clinically characterized by adenomatous polyps in the colon and rectum which if untreated will develop into adenomas[1]. The risk of developing cancer is considered to be related to the number of polyps. Significantly, somatic mutations in *APC* have also been found in 80% of sporadic colon tumors[2].

1.1 The APC gene

The *APC* gene is localized at position 5q21 and consists of 21 exons covering 8535 base pairs. [3,4] The encoded protein has 2843 amino acids, most of which (75%) are encoded by exon 15.[5] In most germline mutations the protein product is truncated due to a nonsense or frameshift mutation. Tumor development follows the "two hit" mechanism with a somatic mutation or loss-of-heterozygosity (LOH) as the second hit.[6] Germline mutations are spread between codon 200-1600 with two hot spots at codon 1061 and 1309.[5] In contrast 60% of somatic mutations are concentrated within the mutation cluster region (MCR) resulting in a truncated protein. The MCR region is located between codon 1286-1513 with hot spots at codon 1309 and 1450.[5,7] The fact that both FAP and sporadic tumors have a hot spot at codon 1309 indicates some sort of selection for mutations in that position. In both FAP and sporadic tumors the second hit is often dependent on the first mutation. If the germline mutation lies between codon 1194-1392 there is a selection for allelic loss as a second mutation.[6] In the case of sporadic tumors LOH is connected with mutations in MCR.[8] If the first mutation is located outside these regions in their respective case the second hit is mostly a truncating mutation.

1.2 The APC protein

APC is a multifunctional tumor suppressor protein involved in signal transduction, cytoskeletal organization, chromosomal segregation and cell adhesion. It has different protein-protein interaction domains with several binding sites important for its functions (*Fig. 1*).

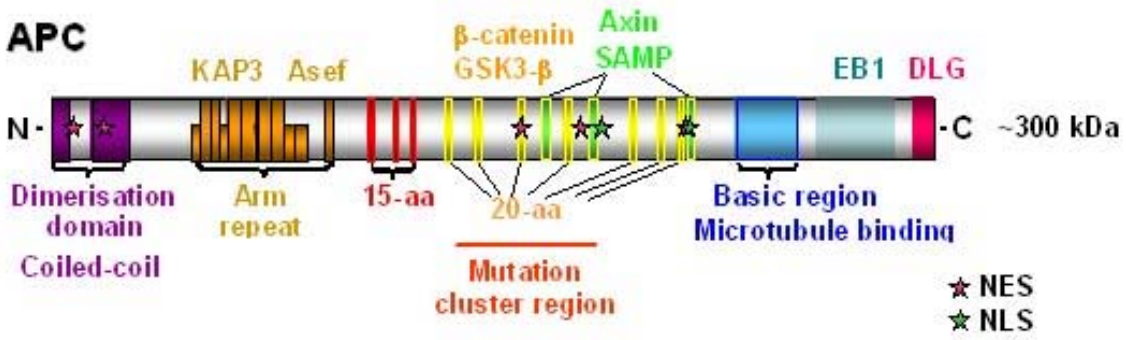

Figure 1: APC contains several domains with important protein and DNA binding sites. There are also five nuclear export signals (NES) and three nuclear localization signals (NLS).

1.2.1 Domains

The first 55 amino acids form a coiled-coil structure which can form dimers with wild-type APC as well as C-terminally truncated forms. There is a second coiled-coil between amino acids 129-250, but this region is not involved in dimerization of the protein.[9] The N-terminal region also contains two nuclear export signals (NES) required for shuttling APC between the cytoplasm and the nucleus.[10]

The next important part is the armadillo region containing several repeats. Armadillo is the Drosophila homolog for β-catenin. These proteins have a segment with very specific structural and sequential characteristics. The region between amino acids 450-760 in APC has a high homology to this segment and is therefore called an armadillo repeat region. The armadillo region binds to the B56 regulatory subunit of phosphatase 2a (PP2A).[11] PP2A is a heterotrimeric complex consisting of A, B and C subunits. Axin, a negative regulator of Wnt signaling, binds to the C subunit of PP2A.[12] In the armadillo region there is also a binding sites for Asef, the APC-stimulated guanine nucleotide exchange factor (GEF) for Rho proteins[13] and Kap3, a member of the kinesin superfamily of motor proteins.[14]

The 15 amino acid repeats contain binding sites for β-catenin that are unique for APC.[2] These binding sites however do not appear to be involved in β-catenin degradation.[15] The seven 20 amino acid (aa) repeats though are essential for degradation of β-catenin. Protein fragment studies have shown that only one repeat is needed for β-catenin binding but three are required for downregulation of the protein.[16] Within the 20 aa repeat region APC also binds to Axin via three SAMP (Ser-Ala-Met-Pro) repeats. Truncated mutant proteins lack all or most of these 20 aa repeats and the Axin binding sites.

The C-terminal region has a basic domain, which interacts with microtubules, and binding sites for EB1, a microtubule binding protein, and hDLG, human homolog to *Drosophila* lethal discs large.

1.2.2 APC and Wnt signaling

One important part of APCs function as a tumor suppressor is its involvement in the Wnt signaling pathway (*Fig. 2*). Without a Wnt signal APC binds to β-catenin and axin in a complex with several other proteins. GSK-3β bound to axin phosphorylates APC which leads to a tighter binding to β-catenin. β-catenin is also phosphorylated by GSK-3β which

creates a recognition signal for ubiquitin ligase, triggering degradation of β-catenin by the proteasome. APC also actively transports β-catenin out of the nucleus. Wnt is a glycoprotein that acts on a transmembrane receptor. When the receptor is activated Dishevelled (Dsh) becomes phosphorylated and through Dsh´s association with Axin GSK-3β is inactivated. APC has been found to be dephosphorylated by the Axin/PP2A-complex at the sites phosphorylated by GSK-3β. This prevents degradation of β-catenin, which can accumulate in the cytoplasm and nucleus. Inactivation of APC also results in accumulation of β-catenin, probably due to mutation of Axin and β-catenin binding sites. In the nucleus β-catenin associates with TCF/LEF (T-cell factor/lymphoid enhancer factor) and activates the expression of several genes. Two genes activated by β-catenin/TCF/LEF are *c-myc* and *cyclin-D1*, which are involved in proliferation, apoptosis and cell cycle regulation. Other possible targets are caspases, known to be involved in apoptosis. Mutant APC have been shown to decrease caspase activity and β-catenin/TCF/LEF has been implicated in their regulation.

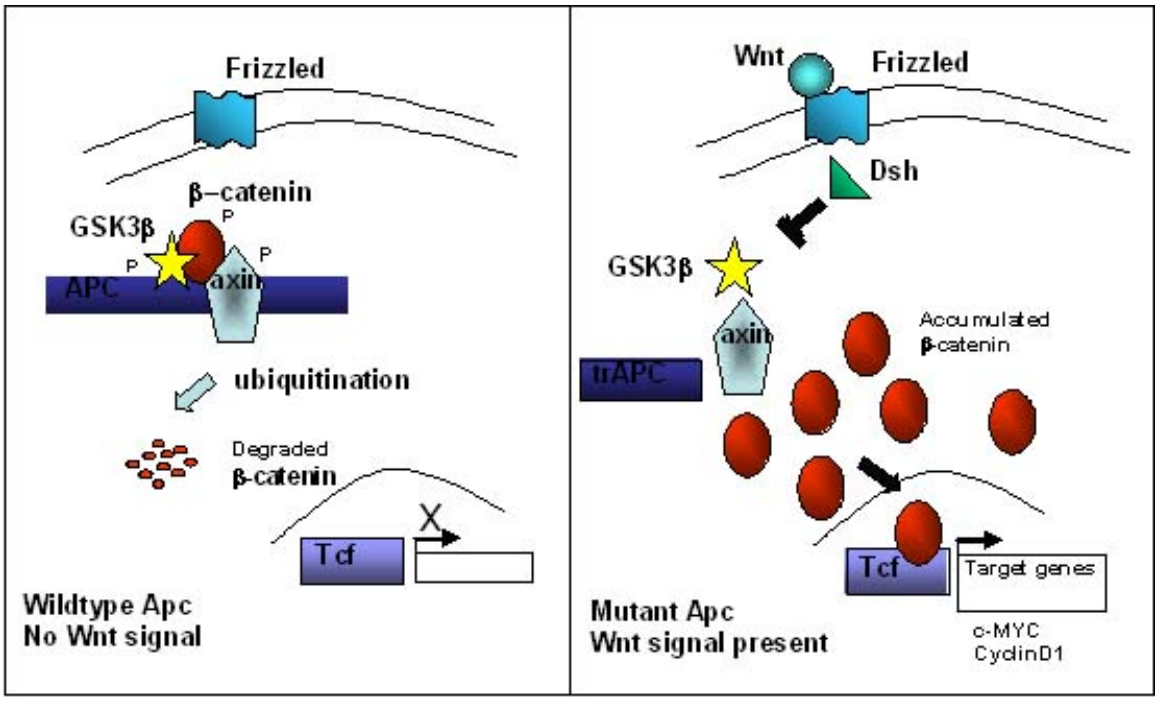

Figure 2: Without a Wnt signal wild type APC can form a complex with Axin, GSK3 and β-catenin. This will lead to the degradation of β-catenin. When there is a Wnt signal or if APC is mutated β-catenin is not marked for degradation. β-catenin will accumulate in the nucleus and activate transcription of target genes.

1.2.3 Cell adhesion

Cell-cell adhesion in epithelial cells is dependant on E-cadherin. At adherens junctions β-catenin interacts with E-cadherin to connect E-cadherin to α-catenin and provide a link to the actin cytoskeleton. The mechanism of APCs involvement in cell adhesion is not yet known. However, restoration of wild-type APC in colon cancer cells expressing a truncated form of APC has been shown to enhance cell adhesion.[17] APC and E-cadherin

also have separate but overlapping binding sites on β-catenin therefore competing for its binding.[18]

1.2.4 EB1 and microtubules

EB1, a small microtubule binding protein, was first identified through its binding to the C-terminal end of APC. Current research suggests that EB1 and APC form a complex at the centrosome and follow the growing ends of the microtubules in the mitotic spindle and assist in their attachment to the kinetochores. Experiments have shown that EB1 and APC co-localize at the centrosome[19] and growing ends (+-ends) of microtubules and stabilize them. A failure in formation of the EB1/APC complex can result in aberrations in the mitotic spindle. It is essential for successful chromosome segregation to have a functional mitotic spindle. A mutant APC can also result in microtubules that can not attach properly to the kinetochores[20, 21]. This will cause disruption in the chromosome segregation leading to chromosomal abnormalities and aneuploidy. APC also stabilizes microtubules in resting cells[22] and clusters at the +-ends of microtubules.[23] This indicates an involvement for APC in mitosis as well as cytoskeletal organization and cell migration.

1.2.5 Other functions

Another link with APC and cell migration is through its interaction with Asef. Asef has a Rac specific GEF activity which is activated by APC.[13] Rac is a Rho GTPase which regulates cell morphology by controlling the organization of the F-actin cytoskeleton.[24] Overexpressed Asef promotes cell migration. The majority of APC mutants still have the binding site for Asef. Even though it can still bind to Asef it is likely that the complex can not localize properly. This will prevent the function of Asef and thus interrupt cell migration.

APC also affects cell proliferation through its ability to block cell cycle progression from G_0/G_1 to S phase.[25] For this signal to occur APC needs to form a complex with hDLG.[26] Overexpression of either has been shown to suppress cell proliferation.[25, 26] Mutating the binding site for the complex on one of the proteins will eliminate this inhibition.[26]

1.3 APC and cancer

All epithelial cells in the gut apart from stem cells have a very short life span due to the toxic environment. Therefore it is important that the cells are constantly replaced. This makes the maintenance of the gut epithelium very dependant on differentiation, cell migration, adhesion and proliferation. These processes need to be balanced and maintained at all times. APCs involvement in Wnt-signaling provides an important role in the maintenance of the gut epithelium. Loss of APC increases proliferation and disrupts apoptosis signals, two key processes known to be involved in cancer development. In addition APC may play separate roles in cells migration and cell adhesion that may also be related to tumor formation. Increased cell-cell adhesion will anchor the cells and with the loss of migratory abilities this will lead to accumulation of proliferating cells. Chromosomal instability is a common occurrence in colorectal cancers

and again APC is involved. APC mutation has been described as the first event in a chain which will lead to colon cancer (*Fig. 3*).

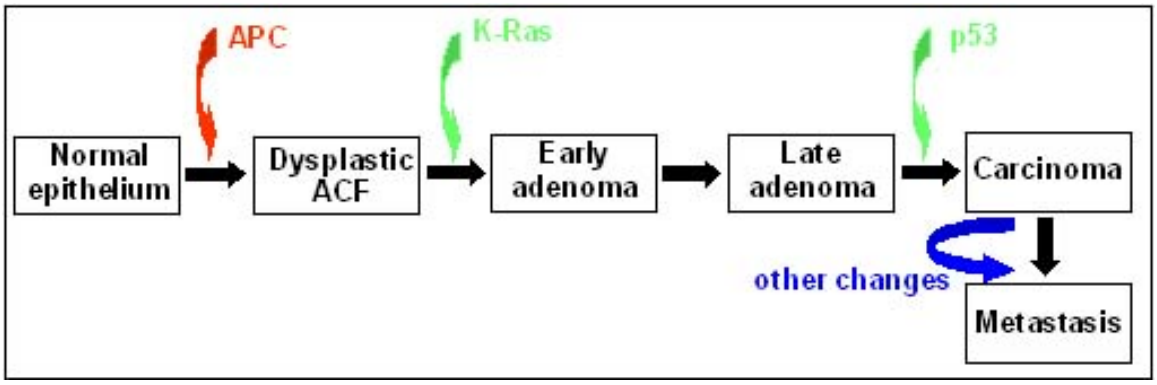

Figure 3: APC mutation is the first step in a chain of events leading to colon cancer. Adapted from Kinzler and Vogelstein Cell 1996, 87, 159-170

2 The project

The aim of this project was to purify and characterize antibodies against APC for their use in proteomics research. Antigens were produced in *E. Coli* bacteria from a GST-fusion expression vector, which includes an ampicillin resistance as a marker. Protein expression by the bacteria is induced by introducing IPTG (isopropyl-beta-D-thiogalactopyranoside). The proteins have a GST (glutathione-S-transferase)-tag which was utilized in the purification. When purifying the antigens the GST ligand glutathione was used in an affinity column. The proteins produced are segments of the APC protein. APC-1 is the N-terminal amino acids (aa) 1-219 and contains the coiled-coil domain (*Fig 4).* The APC-2 protein is aa 1011-1470 and has the 15 aa repeats and two 20 aa repeats (*Fig 4).*

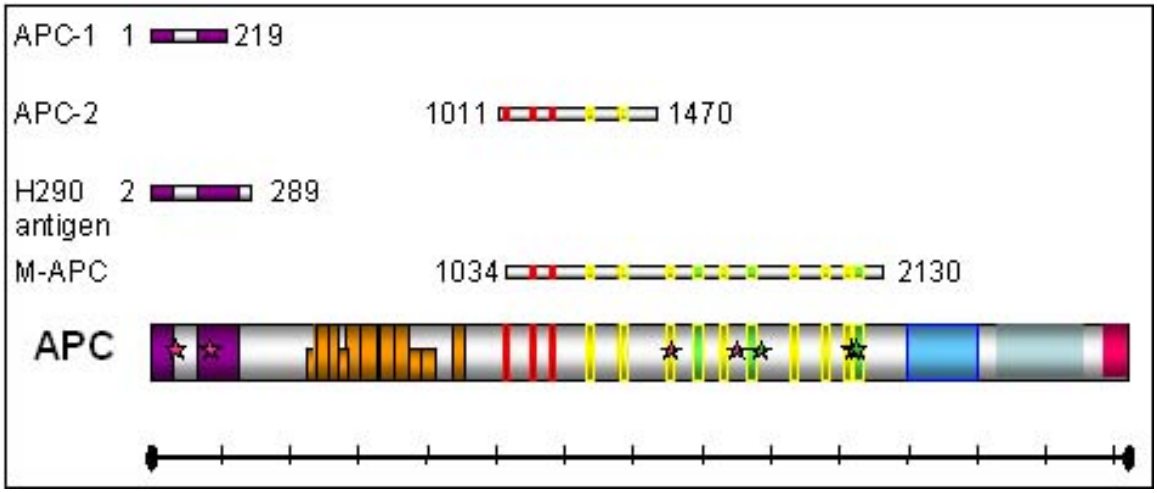

Figure 4: Antigens used in antibody production against APC with the full protein as shown in fig. 1. Numbers indicate amino acids included in the fragments. Every 200 amino acid is indicated in the bar at the bottom.

The antibodies were produced by immunizing rabbits with the antigens. Serum was collected from the rabbits and purified for the antibodies. In the first purification step the serum was passed through a protein A-sepharose column. Protein A caught the antibodies in the serum which after washing were eluted. To separate the antibodies of interest from all the rest that were caught by the protein A the sample was then put through an affinity column with the antigen used in the immunization coupled to the beads. The elution now contained the antibodies of interest but also antibodies against GST. So in the last step the antibodies were run through a GST affinity column. Now the desired antibodies were in the break through and the eluted antibodies are GST specific.

2.1 Biosensor

The purity of the antibodies can be tested on a BIAcore2000 biosensor. The BIAcore2000 is an optical biosensor that uses the principle of surface plasmon resonance for detection of interactions between molecules. The resulting signal can be used to study binding kinetics.

The instrument has two liquid microsyringe pumps, which supply a pulse-free flow. One pump keeps the buffer flow rate constant and the second is connected to the autosampler. A thermostatically controlled integrated fluidic cartridge (IFC) is brought against the sensor surface forming four parallel flow cells or channels 60nl in volume. The BIAcore2000 can be used to analyze up to all four flow cells at the same time. The sample racks have a capacity for up to 192 samples in two 96-well microtitre plates. (*Fig 5*)

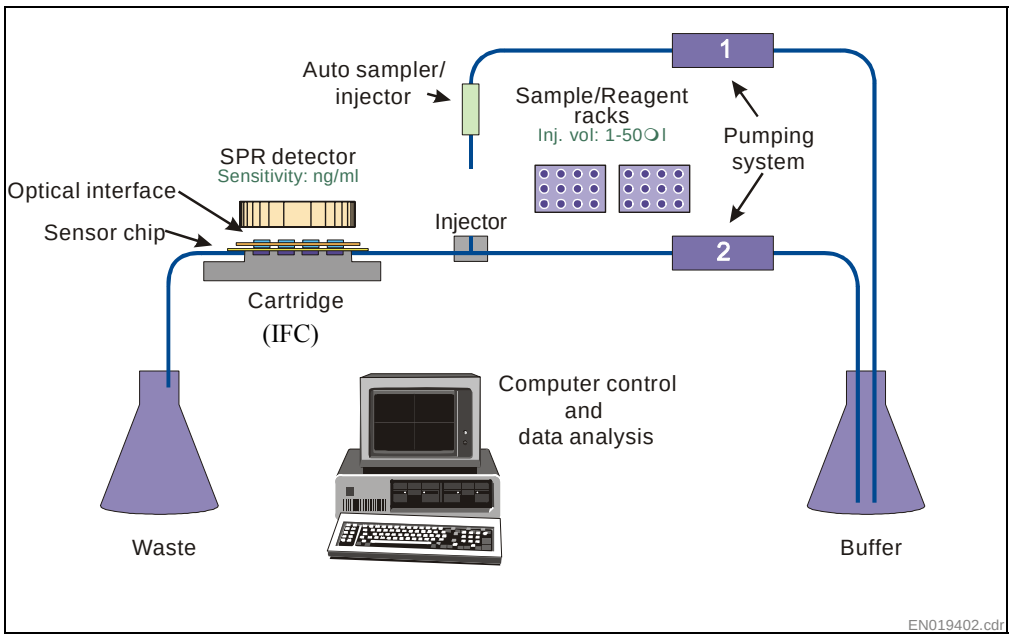

Figure 5: Schematic of a biosensor. (Illustration used with permission from E. Nice.)

The make up of the sensor chip is a 50 nm gold film on a glass slide. The gold surface has an alkanethiol linker layer, in this case carboxymethyl dextran (CM5), to which one of the binding partners can be attached. Before coupling with the protein the surface is activated by N-ethyl-N-dimethylaminopropyl-carbodiimide (EDC) and N-

hydroxysuccinimide (NHS). The protein is then linked to the surface via its primary amino groups.

Polarized light is directed on the chips gold surface opposite where the protein is linked and the reflected light is detected (*Fig 6*). Photons can interact with free oscillating electron (plasmons) in the gold. At a critical angle there is a resonance where energy from the light is transferred to the electrons resulting in a decrease in the reflected light. Changes in the refractive index near the surface toward the flow cell affect the critical angle. Binding to or dissociation from the immobilized protein cause changes in refractive index that are measured by the instrument. The results are displayed in a sensogram with resonance units (RU) against time. A signal of 1000 RU for a protein corresponds to 1 ng/mm^2 in surface concentration. As the sample is injected the RU signal increases if anything binds to the immobilized protein. When the injection is finished dissociation causes the signal to decrease. Kinetic constants can be calculated from the association and dissociation phases of the sensogram.

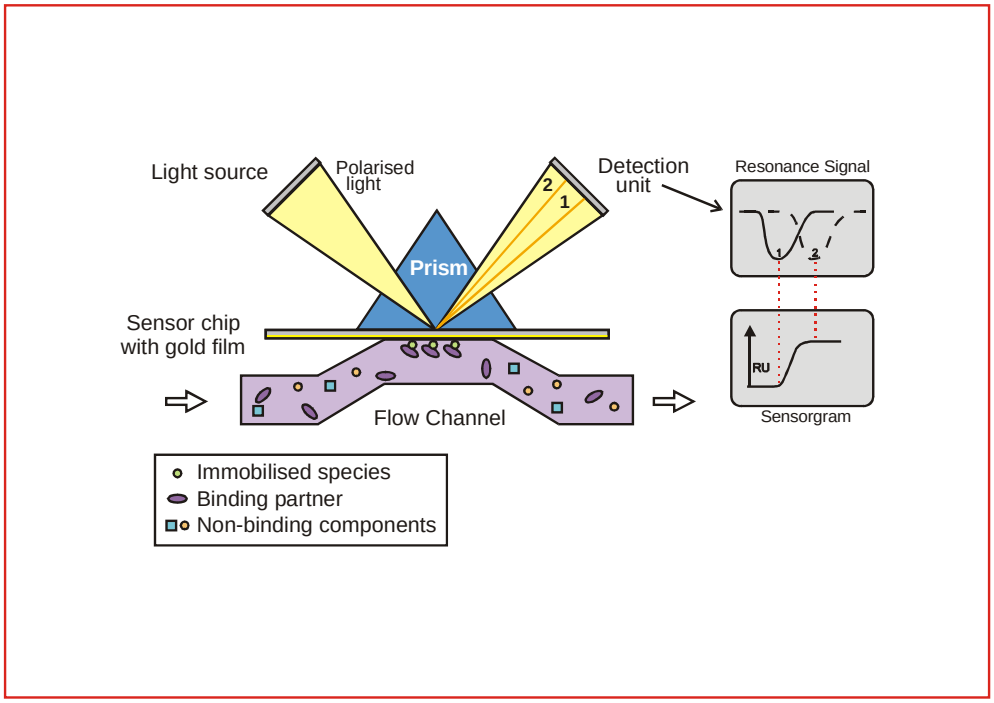

Figure 6: Surface plasmon resonance detection. Polarized light is directed on the chips gold surface opposite where the protein is linked and the reflected light is detected. Photons can interact with free oscillating electron in the gold. A resonance occurs at a critical angle where energy from the light is transferred to free oscillating electrons (plasmons) in the gold resulting in a decrease in the reflected light. This critical angle depends on the refractive index near the surface toward the flow cell. Binding to or dissociation from the immobilized protein cause changes in refractive index that are measured by the instrument. The results are displayed in a sensogram with resonance units (RU) against time. (Illustration used with permission from E. Nice.)

In the project sensor chips are used with the GST-tagged antigens APC-1 or APC-2 immobilized to the surface. After the first affinity purification the biosensor is used to make sure all or most of the α-APC-1 and α-APC-2 antibodies were caught by the column. When the GST antibodies have been removed the antibodies are tested on both

chips. For α-APC-1 samples the APC-2 surface will then work as a control for GST antibodies and for α-APC-2 on APC-1.

2.2 Proteomics

Western blotting, immunoprecipitation (IP) and immunocytochemistry are commonly used in proteomics and rely on efficient antibodies. Unspecific binding in these techniques can give false positives. Two cell lines were used in this project, to test the purified antibodies for their use in these techniques, MDCK and SW480. MDCK, Madin Darby Canine Kidney, is a dog cell line commonly used as a model for epithelial cells. These cells express full length APC. SW480 is a colon cancer cell line that has a truncated APC protein of approximately 140 kDa spanning aa 1-1339(*Fig. 7*). The α-APC-1 and α-APC-2 antibodies were compared with commercial antibody H290 (Santa Cruz). H290 is a rabbit polyclonal against a peptide containing aa 2-289 of APC (*Fig. 4*).

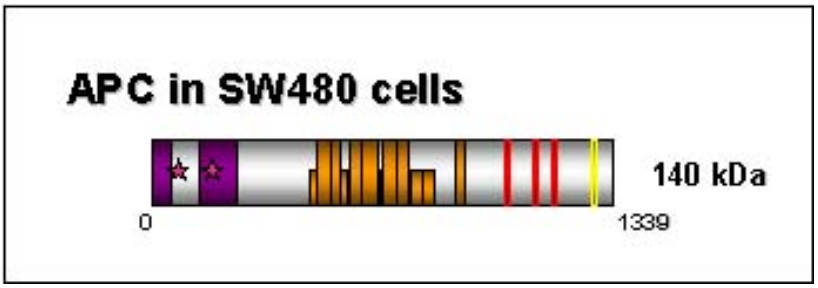

Figure 7: SW480 cells have a mutation at codon 1339 in the APC gene. This mutation results in a truncated APC protein of approximately140 kDa.

In western blotting cell lysates were size separated with SDS-PAGE. The proteins were then transferred to a membrane which was blotted (or probed) with the purified antibodies. An ideal antibody should only give one strong distinct band of the correct size on the blot.

IP uses the antibodies to catch protein in cell lysates. The antibodies were added to the lysates and allowed to bind to proteins. The formed complexes are then extracted from the lysates and the bound proteins analyzed. Here the only protein desired is off course APC.

In immunocytochemistry the antibodies were used to fluorescently stain cells. The fluorescent dyes used here were ALEXA 488-conjugated goat-anti-mouse and ALEXA 546-conjugated goat anti-rabbit. The numbers indicate the approximate excitation maximum with 488 being a green dye and 546 a red. Previous staining using H290 (unpublished results) show staining at the tips of cell extensions and at the cell membrane between adhered cells. Previously produced antibodies against a peptide called M-APC, aa 1034-2130 (*Fig. 4)* have shown similar results.

3 Materials and methods

3.1 Affinity column

3.1.1 Bacteria culture

LB media with ampicillin was inoculated with glycerol stock of antigen producing bacteria at a 1:1000 dilution. The cultures were grown overnight at 37°C on a shaker. The culture was diluted 1:20 and grown at 37°C until OD_{595nm} was 0.5-0.6. Protein expression was induced with 0.2 mM IPTG and grown for 2.5-4 h at 37°C. Cultures were spun at 3000 rpm for 10 min, the supernatant was discarded, and the pellets stored at -20°C.

3.1.2 Antigen purification

GST-fused antigens (APC1-GST and APC2-GST and GST proteins) were purified from antigen producing bacteria. Bacteria pellets were resuspended in CelLytic B bacterial cell lysis/extraction reagent (Sigma) with protease inhibitors (inhibitor pellet 1/50ml (Roche), trasylol 1:500, pefa block 1:1000) 20ml/pellet and transferred to 50ml tubes. Tubes were rotated at 4°C for 15 minutes and then centrifuged (13000 rpm, 10 min) and the supernatant collected. The glutathione sepharose column was first washed with water and then TBS-T (tris buffered saline with Tween 20). The beads were mixed with the supernatant in 50ml tubes and incubated over night at 4°C. The beads were spun down, supernatant removed, washed with TBS-T, spun down again, supernatant removed and beads put back in column. The column was washed with TBS-T until no reaction with Bradford reagent in the wash. The Bradford reagent has a brown color and turns blue in reaction to proteins. The antigens were eluted with 20mM glutathione in TBS (tris buffered saline) and fractions tested for Bradford reaction. Positive fractions were collected and concentrated using vacuum centrifuge at 56°C. The column was cleaned with 0.1 M Tris in 0.5 M NaCl, pH 8.5, and 0.1 M sodium acetate in 0.5 M NaCl, pH 4.5, and finally washed with water and stored in 20% ethanol. To further purify the antigen sample after concentration they were run through a size exclusion column, Superdex 75, on the ÄKTA machine (Amersham Biosciences). The fractions were run on a gel for coomassie staining (see Gel and western blot analysis).

3.1.3 Preparation of APC1, APC2 and GST -affinity columns

NHS-activated Sepharose™ 4 Fast Flow beads (Amersham Biosciences), stored in isopropanol, were washed twice with 1mM HCl and twice with HBS (Hepes buffered saline). The beads were then mixed with equal volume antigen (APC1-GST, APC2-GST or GST) and incubated over night at 4°C. Next day the beads were spun and the supernatant tested for Bradford reaction. No Bradford reaction indicated that the proteins were conjugated to NHS-Sepharose beads. The beads were then blocked with Tris pH 8 and incubated for 2h at 4°C. The beads were then spun down and washed with PBS (phosphate buffered saline) and stored in PBS with sodium azide.

3.2 Antibody purification

3.2.1 Protein A column

Antibodies were purified from serum from immunized rabbits. The serum was spun to pellet any debris. PBS pH 8.2 was added to the supernatant and the pH adjusted to approximately 8.2 with 1M Tris pH 8. The protein-A column (stored in 20% ethanol) was initially washed with water and equilibrated with PBS pH 8.2. The sample was injected and the column washed with PBS pH 8.2 until no reaction with Bradford reagent. The antibodies were eluted using 50mM glycine pH 3.5 and fractions tested for Bradford

reaction. Positive fractions were pH adjusted using 1M Tris pH 7.5 (50µl/ml) and pooled. The column was washed with 10mM HCl and water and stored in 20% ethanol.

3.2.2 Affinity purification

Antibodies were mixed with either APC1-GST or APC2-GST affinity beads and incubated over night at 4°C. Beads were put back in an empty column and break through collected by gravity flow. The column was washed with PBS until no Bradford reaction. The antibodies were eluted from the column with 10mM HCl into 1 ml fractions which were tested with Bradford reagent. Positive fractions were pH adjusted using Tris pH 7.5 (50µl/ml) and pooled. Column was washed with PBS and stored in PBS with sodium azide. Starting material, break through and eluate were tested on Biosensor.

3.2.3 GST-affinity purification

Antibodies immunopurified with APC1-GST and APC2-GST Sepharose were then incubated overnight at 4°C with GST-Sepharose beads in order to specifically remove anti-GST IgG. After incubation, beads were put back in empty column and the break through containing the antibodies of interest (APC1 or APC2 IgG) was collected by gravity flow. The column was washed with PBS until no Bradford reaction was seen in the wash. The GST antibodies were eluted from the column with 10mM HCl into 1 ml fractions which were tested with Bradford reagent. Positive fractions were pH adjusted using Tris pH 7.5 (50 µl/ml) and pooled. Column was washed with PBS and stored in PBS with sodium azide. Break through and eluate were tested on Biosensor.

3.2.4 Biosensor analysis

BIAcore 2000 Biosensor (BIAcore AB) was used for all biosensor analyses. Antigens were immobilized on CM5 Chip (BIAcore AB) via amine coupling. First 60 µl EDC/NHS was injected on the surface. Then the purified antigen diluted 1:2 in sodium acetate pH 3.5 was injected until a sufficient increase in activity was observed. Non-linked proteins were washed away with 30µl glycine pH 2.1 and finally the surface was blocked with 60µl 1M ethanolamine-HCl (BIAcore AB).

Each sample was diluted 1:2 in HBS and 30µl (60µl diluted) were injected into the biosensor at a flow rate of 10µl/min. The surface was regenerated by injecting 30µl of glycine pH 2.1.

3.3 Gel and western blot analysis

Nu-PAGE® 4-12% Bis-Tris 1.0 mm gels (Invitrogen) and Nu-PAGE® MOPS SDS running buffer (Invitrogen) were used all experiments except protein extraction and immunoprecipitation. The 4x Nu-PAGE® LDS sample buffer (Invitrogen) was added to the samples which were then boiled for 5 minutes before loading on the gel. The SeeBlue Plus2® prestained protein marker (Invitrogen) was used on the gel. The gel was loaded with 20µl of each sample and protein marker. The gel was run at 150-200 V until the front of the samples reached the end of the gel. The gels were either used for coomassie staining or western blot. Proteins were transferred onto an immobilon P membrane at 110 V for 1-2.5 h. Following transfer, the membrane was blocked in TBS-T with 3% BSA (bovine serum albumin) for 30 min at room temperature. Incubation with the primary

antibody in TBS-T with 1% BSA was performed overnight at 4°C. Next day the membrane was washed 3x 20min in TBS-T with 1% BSA. It was incubated with secondary antibody (e.g. anti-rabbit HRP-conjugated) in TBS-T with 1% BSA for at least 1 hour at room temperature. The membrane was then washed in TBS-T 5-6x 5min. The ECL™ Western Blotting Detection Reagent (Amersham Biosciences) was used to develop onto medical x-ray film (Fuji).

Nu-PAGE® 3-8% Tris-Acetate gels (Invitrogen) and Nu-Page® Tris-Acetate running buffer (Invitrogen) were used during protein extraction and immunoprecipitation experiments. A 2x sample buffer was added to the samples which were then boiled for 5 minutes before loading on the gel. Samples (40µg or maximum 20µl total volume) were loaded on the gel. The SeeBlue Plus2® prestained protein marker (12µl) (Invitrogen) protein marker was used on the gel. The gel was run at 150 V until the front of the samples reached the end of the gel. The blot was performed with wet transfer onto an immobilon P membrane at 110 V for 4 h. The membrane was blocked in TBS-T with 2% skim milk overnight at 4°C. It was blotted with the primary antibody in TBS-T with 2% skim milk at 4°C over night. Next day the membrane was washed 3x 20min in TBS-T. It was incubated with secondary antibody in TBS-T for at least 1 h at room temperature. The membrane was then washed in TBS-T 5-6x 5min. The ECL solution was used to develop onto x-ray film.

3.4 APC pulldown

3.4.1 Antibody columns

Columns were made by coupling α-APC-1 and α-APC-2 antibodies separately to sepharose beads. NHS-activated Sepharose™ 4 Fast Flow beads (Amersham Biosciences), stored in isopropanol, were washed twice with 1mM HCl and twice with HBS. Pure antibodies were concentrated from 5 ml to approximately 1.5 ml and buffer exchanged with PD-10 column to HBS. The antibodies were mixed with approximately 1ml pre-washed beads each and incubated over night at 4°C. Next day the beads were spun and the supernatant tested for protein content with Bradford reaction. The supernatant was discarded if no reaction was observed. The beads were blocked with Tris pH 8 and incubated for 2h at 4°C. The beads were then spun down and washed with PBS and stored in PBS with sodium azide.

3.4.2 Pulldown

Cells from 10x 15cm dishes of SW480 cells were lysed in 12ml lysis buffer (10mM Tris, 2% CHAPS (Sigma), 10mM MgCl2, 10mM EGTA) with protease inhibitors. They were rotated at 4°C for 30min and then centrifuged at 13000 rpm for 30min. The supernatant was retrieved and 6ml were mixed with the beads of each antibody column and incubated at 4°C overnight. Remaining supernatant was saved as starting material. The beads were put back in the columns and washed with TBS-T (20x column volume) and PBS (30x column volume). Proteins were eluted with 10mM HCl in 1ml fractions and elution tested using Bradford reagent. Protein containing fractions were concentrated to approximately 100µl and loaded under reducing conditions using Bond-Breaker™ TCBP (Pierce) onto SDS-PAGE gel. Ten microlitres of samples and starting material were loaded on gel. Western blot analysis was performed using anti H290 APC IgG

(1/1000 dilution) and HRP-conjugated goat-anti-rabbit (1:3000 dilution) as secondary antibody. Sample aliquots were also loaded on a gel for coomassie staining (see Gel and western blot analysis).

3.5 Protein extraction

Two methods were employed to harvest cells from 10cm plates for lysis. Either cells were scraped directly into ice cold lysis buffer (30mM Tris pH 7.4, 250mM NaCl, 5mM EDTA, 50mM NaF, 0.1% Triton X-100) with protease inhibitors or into ice cold PBS. When scraped into PBS, the cells were pelleted by centrifugation at 1500rpm for 15min and lysis buffer added to the cell pellet. Appropriate lysis buffer volume was added to obtain maximum protein yield in a minimum volume. The lysed cells were passed through a 26G needle 10 times and then incubated for 15 min on ice. Cell lysates were then spun for 30 min at 13000 rpm and 4°C and the supernatant was collected. A protein concentration assay was performed on the lysates. Three blots were done with different primary antibodies. Antibodies used were α-APC1, α-APC2 and H290 all at 1:1000 dilutions. A goat anti-rabbit at dilution 1:3000 was used as secondary antibody. Full-length APC with a Gln-epitope tag was expressed in insect cells using the baculuvirus expression system. APC was immunoprecipitated with anti-Gln-Gln antibodies and used as a positive control in this experiment.

For one extraction two lysis buffers were used buffer 1 (2% CHAPS (Sigma), 10mM Tris, 2% CHAPS, 10mM $MgCl_2$, 10mM EGTA) and buffer 2 (same as above). Lysate aliquots were taken for protein assay and Western blot analysis and remaining lysates were used for IP.

3.6 Immunoprecipitation

MDCK and SW480 lysates were pre-cleared by adding 100µl protein-A sepharose (10% beads v/v in PBS) and incubated rotating at 4°C for 30 min. The beads were centrifuged at 5500 rpm for 1 min at 4°C and the supernatants retained. The pre-cleared lysates were aliquoted and mixed with 1-2µg of each antibody separately, α-APC1, α-APC2, H290 and negative control with no antibody. The IP samples were incubated rotating at 4°C overnight. One hundred microlitres of protein A sepharose (10 % beads v/v in PBS) was then added to the samples and incubated rotating at 4°C for 30-60 min. The beads were spun down at 5500rpm for 1 min at 4°C and the supernatant aspirated through a 26G needle. Beads were washed with 0.5 ml cold PBS, centrifuged at 5500 rpm for 1 min at 4°C and the supernatant aspirated through a 26G needle. The wash was repeated three times. After the final wash 30 µl 2x sample buffer was added to the beads to elute and boiled for 5 min before western blot analysis. The full-length APC (see protein extraction) was used as a positive control on the blots. H290 was used as primary antibody (1:1000 dilution) and HRP-conjugated goat anti-rabbit antibody (1:3000 dilution) was utilized as secondary reagent.

3.7 Immunocytochemistry

Cells were plated on cover slips in a 6-well plate at a suitable density. Two days later the cells (on the cover slips) were washed twice in PBS and fixed with 3.7% formaldehyde in PBS for 5-10 min. The cells were then washed again twice in PBS

before being permeabilized with 0.2% TritonX-100 in PBS for 5 min. The cover slips were washed a third time twice in PBS and then blocked with 0.2% BSA in PBS for 30 min. The cells were incubated with primary antibody at different dilutions in 0.2% BSA in PBS for 1 hour and then washed three times in 0.2% BSA in PBS. The cells were then incubated with secondary antibody at 1:500 dilution in 0.2% BSA in PBS for 1 hour and washed three times in 0.2% BSA in PBS. The coverslips were then washed by dipping 10 times in three vials with 100% ethanol followed by 10 washes in two vials with xylene. A drop of D.P.X neutral mounting medium (Aldrich) was added and the coverslips were mounted on microscope slides. The slides were analyzed with a confocal microscope.

In the first immunocytochemical experiment MDCK cells were plated on coverslips at a density of $5.6*10^4$ cells/well and staining performed two days later. The primary antibodies used were α-APC-1 at dilutions 1:50 and 1:200, α-APC-2 at dilutions 1:100 and 1:200 and H290 at a 1:200 dilution. As a negative control cells were incubated without primary antibody. As secondary antibody a goat-anti-rabbit Alexa 488 with green fluorescence was used at a 1:500 dilution.

The second immunocytochemical experiment was performed on MDCK cells and SW480 cells. The MDCKs were plated on coverslips at a density of $2.5*10^5$ cells/well and the SW480 at a density of $5*10^5$ cells/well. Fluorescent staining was performed the following day. As primary antibodies α-APC-1, α-APC-2 and H290 were used at dilutions 1:50, 1:200 and 1:200 respectively. Cells were also co-stained for β-tubulin at a 1:500 dilution. α-APC-1 was also tested for different dilutions 1:50, 1:100 and 1:200. The secondary antibody used was goat-anti-rabbit Alexa 488 and for the co-stained cells also goat-anti-mouse Alexa 546 with red fluorescence both at a 1:500 dilution.

4 Results

4.1 Antibody purification

Antibodies were purified from rabbit serum first through a protein-A column and then affinity-purified using APC-1-GST and APC-2-GST fusions proteins coupled to Sepharose beads. The eluted antibodies were further purified through a GST-affinity column in order to remove anti-GST antibodies. Following immunopurification, break through and eluate were tested using biosensor technology. APC-1-GST, APC-2-GST and GST were immobilized onto the sensor surfaces. After affinity purification using APC1-GST and APC-2-GST beads, the eluate gave a strong signal on the biosensor as compared to the weak signal obtained with the break through (*Fig. 8A, Fig. 9A*). Anti-APC-1-GST and APC2-GST IgG have therefore been purified with an insignificant amount left in the break through.

It is worth noting that cross reactivity is observed between these antibody fractions due to the anti-GST IgG. Anti-GST IgG recognized the GST domain of both immobilized APC-1-GST and APC-2-GST proteins. These antibodies were then injected onto GST-Sepharose beads. The break through from the GST-affinity purification resulted in the separation of α-GST IgG (bound fraction) from α-APC-1 and α-APC-2 IgG (break through).

The immunopurified IgG recognized specifically their corresponding immobilized antigen using biosensor analysis: Immunopurified α-APC-1 IgG bound to immobilized APC-1-GST antigen but did not recognize immobilized APC-2-GST-antigen (*Fig. 8B*). Immunopurified α-APC-2 IgG recognized specifically APC2-GST antigen and did not react with immobilized APC1-GST (*Fig. 9B*). Eluted α-GST IgG recognized both immobilized APC-1-GST and APC-2-GST (*Fig. 8C, Fig. 9C*).

This shows that the antibodies were immunopurified into a homogeneous pool of α-APC IgG, that specifically recognized their respective antigens.

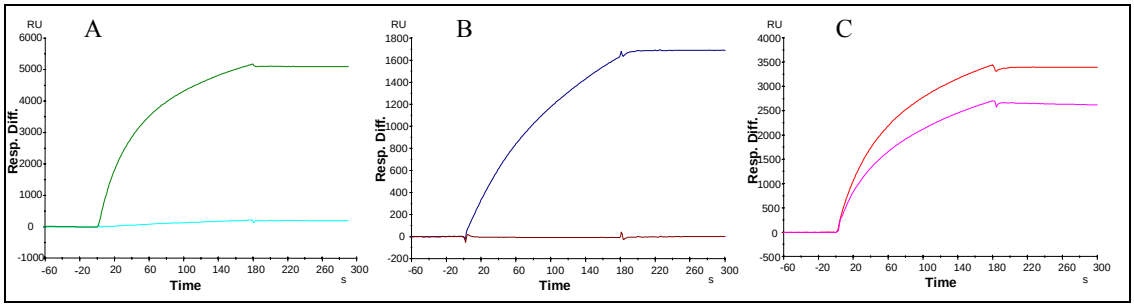

Figure 8: Samples from affinity purification of α-APC-1 IgG were analyzed on biosensor. (A) Break through (turquoise) and eluate (green) after affinity purification using APC-1-GST proteins coupled to Sepharose beads was injected into the biosensor with APC-1-GST linked to the chips surface. (B) Break through from GST-affinity purification was injected on surfaces with APC-1-GST (blue) or APC-2-GST (dark red) immobilized. (C) Eluate from GST-affinity purification was injected on surfaces with APC-1-GST (red) or APC-2-GST (purple) immobilized.

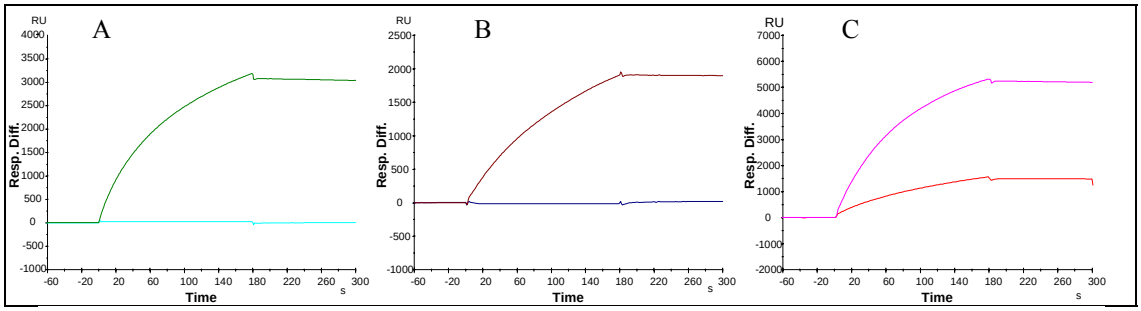

Figure 9: Samples from affinity purification of α-APC-2 IgG were analyzed on biosensor. (A) Break through (turquoise) and eluate (green) after affinity purification using APC-2-GST proteins coupled to Sepharose beads was injected into the biosensor with APC-2-GST linked to the chips surface. (B) Break through from GST-affinity purification was injected on surfaces with APC-1-GST (blue) or APC-2-GST (dark red) immobilized. (C) Eluate from GST-affinity purification was injected on surfaces with APC-1-GST (red) or APC-2-GST (purple) immobilized.

4.2 APC pulldown

Affinity columns were made with the purified antibodies coupled to beads. The columns were used on SW480 cell lysate. The resulting samples were analyzed by western blot and coomassie stained gel and compared with the lysate. The protein yield as seen on the western blot (results not shown) was too low for both antibodies compared with the lysate. This makes the antibodies not useful in this method to capture APC and associate proteins.

4.3 Protein extraction

The purified antibodies were tested for their ability to detect APC, wild type and a truncated mutant, on western blots. Wild type APC was detected in MDCK cell lysates migrating at 300 kDa and the truncated form in SW480 cell lysates, migrating at 140 kDa. Full length APC was used as a positive control. The antibodies were compared with the commercial antibody H290 (Santa Cruz).

The first protein extraction was performed on MDCK by scraping them into the lysis buffer. This made the lysates too diluted. Overall only very faint bands were seen and none for the wild type APC in MDCK cells.

In the second extraction MDCK cells and SW480 cells were scraped onto PBS and less lysis buffer was used which gave much better results (*Fig. 10*). All blots show a faint band at the level of wtAPC at approximately 310 kDa for the MDCK lysate (lane 1, *Fig. 10*). The SW480 lysates contain a band migrating just above 111 kDa molecular weight marker which corresponds with the truncated form of APC in these cells (approximately 140 kDa in size). The α-APC-2 antibody detected a strong band migrating just above the 210 kDa marker in both MDCK and SW480 cells. In the MDCK cells this may be an alternative form of APC, i.e. proteolytic breakdown product. This is not the case for SW480 cells, however, as these cells contain only the truncated APC of 140 kDa that is also detected by a-APC-2 but more weakly. This band at approximately 210 kDa is therefore likely to represent an unrelated, non-specific protein. This indicates an unspecific binding of α-APC-2 to some protein other than APC.

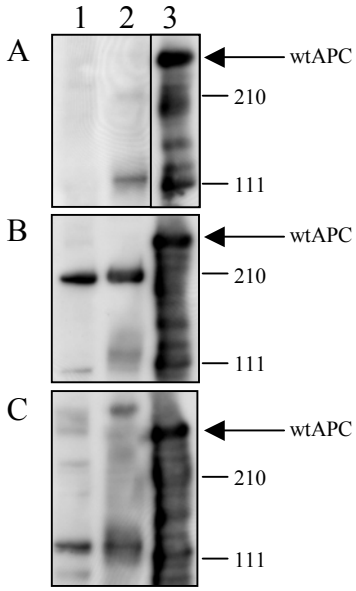

Figure 10: Protein was extracted from MDCK (lane 1) and SW480 (lane 2) cells. Western blots were performed on the lysates with full length APC as a positive control (lane 3). The blots were incubated with antibodies α-APC-1 (A), α-APC-2 (B) and H290 (C). The MDCK lysate has a faint band in the level of full length APC on all blots. The truncated form in SW480 cells can also be seen on all blots. On the α-APC-2 blot the SW480 lysate show a strong band at size 210 kDa which indicates an unspecific binding. Lane 3 in A is inserted from an ECL development with less exposure time.

The final extraction (*Fig. 12*) was done to compare two lysis buffers, one based on CHAPS (buffer 1) and one on Triton X-100 (buffer 2). Again they were scraped in PBS to give a more concentrated lysate. MDCK lysates have a band corresponding to wtAPC on all the blots, as indicated by the positive control (lane 6, *Fig. 12*). The SW480 lysates show a band at the predicted size for the truncated form, migrating just above the 111 kDa marker. Again there is a prominent band migrating above the 210 kDa marker

detected by α-APC-2 (blot B, *Fig. 12*). In this experiment, α-APC-1 also detected a band at approximately 210 kDa in SW480 cell lysates (lanes 4&5) as well as the major species corresponding to the truncated APC protein. In addition, in MDCK lysates immunoblotted with α-APC-1 there is a major band migrating above the 210 kDa marker that may represent a proteolytic breakdown product of wtAPC. The bands for APC are slightly stronger with lysis buffer 2 than buffer 1.

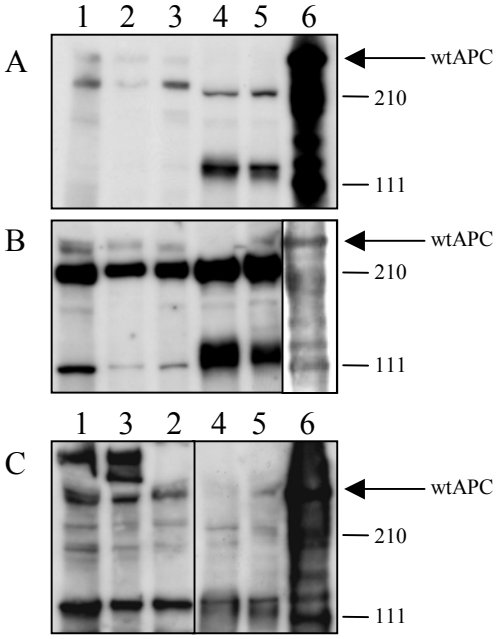

Figure 11: Two lysis buffers were used, buffer 1 (lanes 3 and 5) and buffer 2 (lanes 1, 2 and 4), for protein extraction from MDCK (lanes 1-3) an SW480 (lanes 4-5) cells. Western blots were performed on the lysates with full length APC as a positive control (lane 6). The blots were incubated with antibodies α-APC-1 (A), α-APC-2 (B) and H290 (C). Lane 6 in B is inserted from an ECL development with less exposure time.

4.4 Immunoprecipitation

In order to determine the ability of the purified APC antibodies to recognize APC in solution, immunoprecipitation was performed on lysates from MDCK and SW480 cells with α-APC-1, α-APC-2, H290 (Santa Cruz) and a negative control without primary antibody. Western blots on immunoprecipitates were probed with the H290 anti-APC antibody (Santa Cruz) (*Fig. 12*). Full length APC was used as a positive control (lane5, *Fig. 12*). All APC Antibodies immunoprecipitated full length and truncated APC from MDCK and SW480 cell extracts, respectively. The immunoprecipitations for MDCKs showed a clear and distinct band corresponding to the predicted size of wtAPC (lane 5) and the immunoprecipitations for SW480s contain strong bands for truncated APC. The immunoprecipitations performed with lysis buffer 2 gave stronger bands than when buffer 1 was used. With buffer 2 the antibodies show little difference between them in their ability to immunoprecipitate APC. Furthermore the immunoprecipitations performed with α-APC-1 and α-APC-2 appeared to give cleaner results as compared to those performed with the commercial H290 antibody. These experiments shows that we could use these anti-APC antibody to immunopurify native APC from various colonic cell lines.

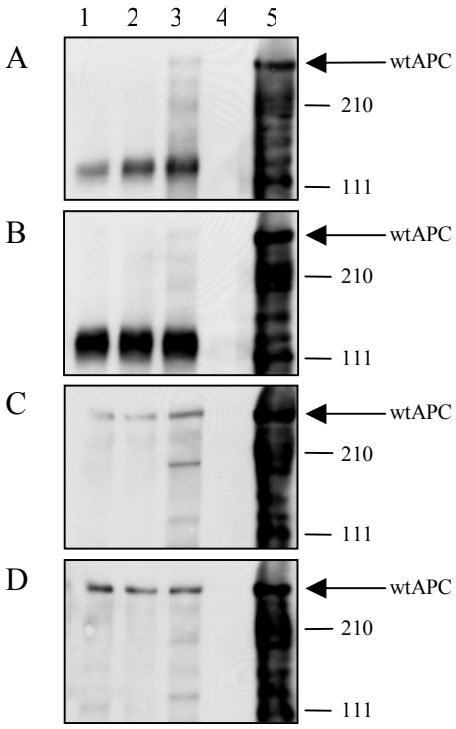

Figure 12: Cells were lysed using two different lysis buffers, buffer 1 (A and C) and buffer 2 (B and D). APC was immunoprecipitated from the SW480 (A and B) and MDCK (C and D) lysates using α-APC-1 (lane 1), α-APC-2 (lane 2), H290 (lane 3) and no antibody as a negative control (lane 4). Full length APC was used as a positive control (lane 5) on the blots.

4.5 Immunocytochemistry

MDCK cells were stained using α-APC-1, α-APC-2, H290 and no primary antibody as a control (*Fig 13*). The negative control shows no staining of the cells. All three antibodies gave distinct staining of the tips of cellular extensions indicating accumulation of APC in punctuate clusters. H290 also shows staining at the plasma membrane which is not observed in cells stained by α-APC-1 or α-APC-2. There is a clear nuclear staining by α-APC-1 and some by H290.

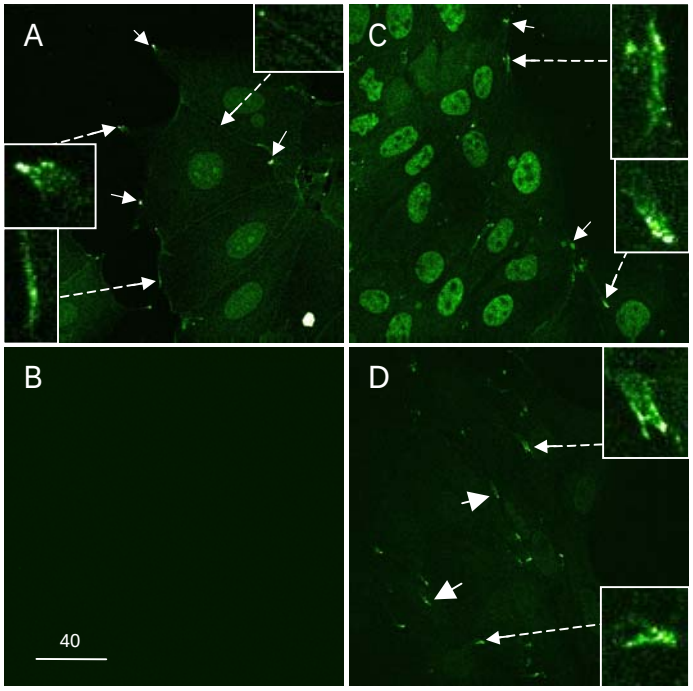

Figure 13: MDCK cell were immunostained using H290 (A), no primary antibody (B), α-APC-1 (C) and α-APC-2 (D). Inserted boxes are 5x magnification of arrow indicated staining. Bar equals 40 μm.

In order to see more clearly the localization of APC within the cells, MDCK cells were then stained using α-APC-1, α-APC-2, H290 and co-stained for β-tubulin (*Fig. 14).* Again there is apparent staining of the tips of the cells by all three antibodies. Together with the β-tubulin staining, this suggests an accumulation of APC at the ends of the microtubules. The peripheral staining observed with the H290 antibody may be consistent with adherens junctions. Both H290 and α-APC-1, but not a-APC-2, demonstrate nuclear staining, the latter more distinct. This nuclear staining may however be non-specific.

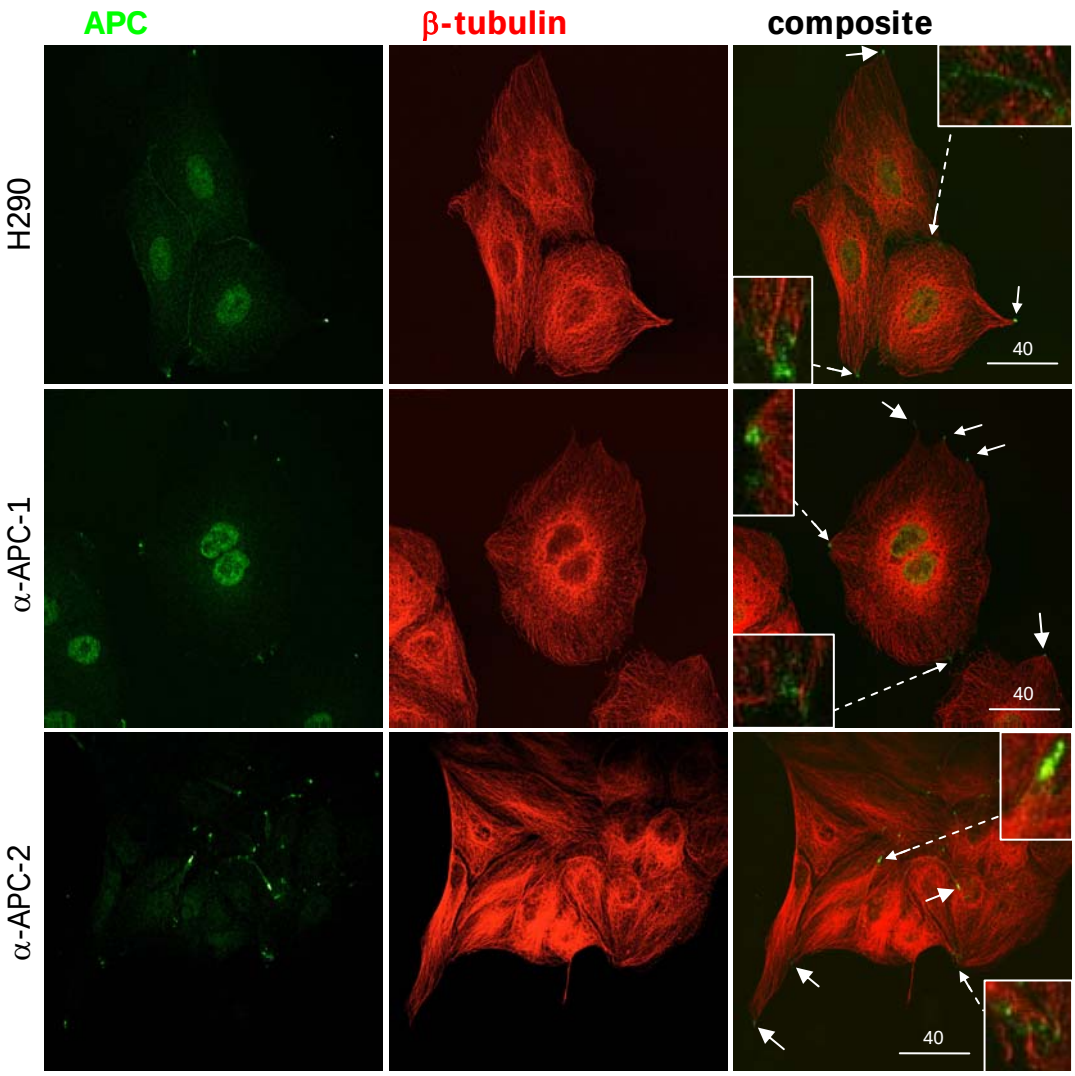

Figure 14: MDCK cells were immunostained for APC (green) using H290, α-APC-1 and α-APC-2 and co-stained for β-tubulin (red). Inserted boxes are 5x magnification of arrow indicated staining. Bars equal 40 μm.

As a comparison SW480 cells were stained using α-APC-1, α-APC-2 and H290 antibodies (*Fig. 15*). There was no specific staining of the SW480 cells by α-APC-2. The tip staining present in the MDCK cells was not observed in SW480. The staining by α-APC-1 and H290 was more spread within the cell with some accumulation in the nucleus.

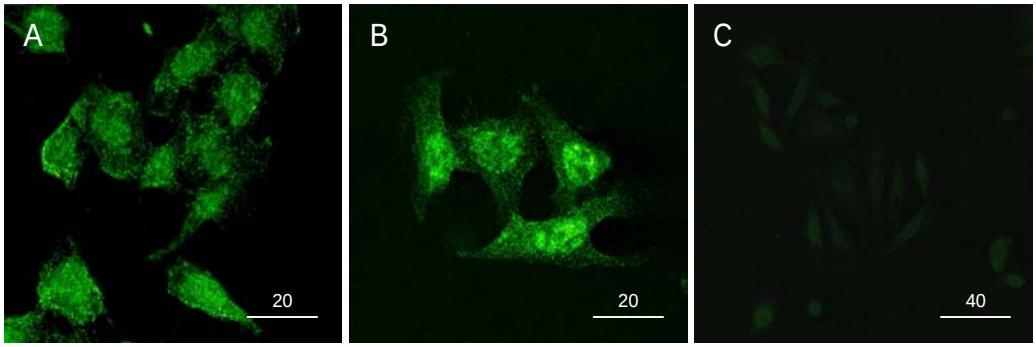

Figure 15: SW480 cells were stained using H290 (A), α-APC-1 (B) and α-APC-2 (C). Bars for A and B equal 20 μm and for C 40 μm.

SW480 cells were co-stained for β-tubulin as well (*Fig. 16*). Again α-APC-2 gave no staining. The loss of punctate staining confirms the previous results.

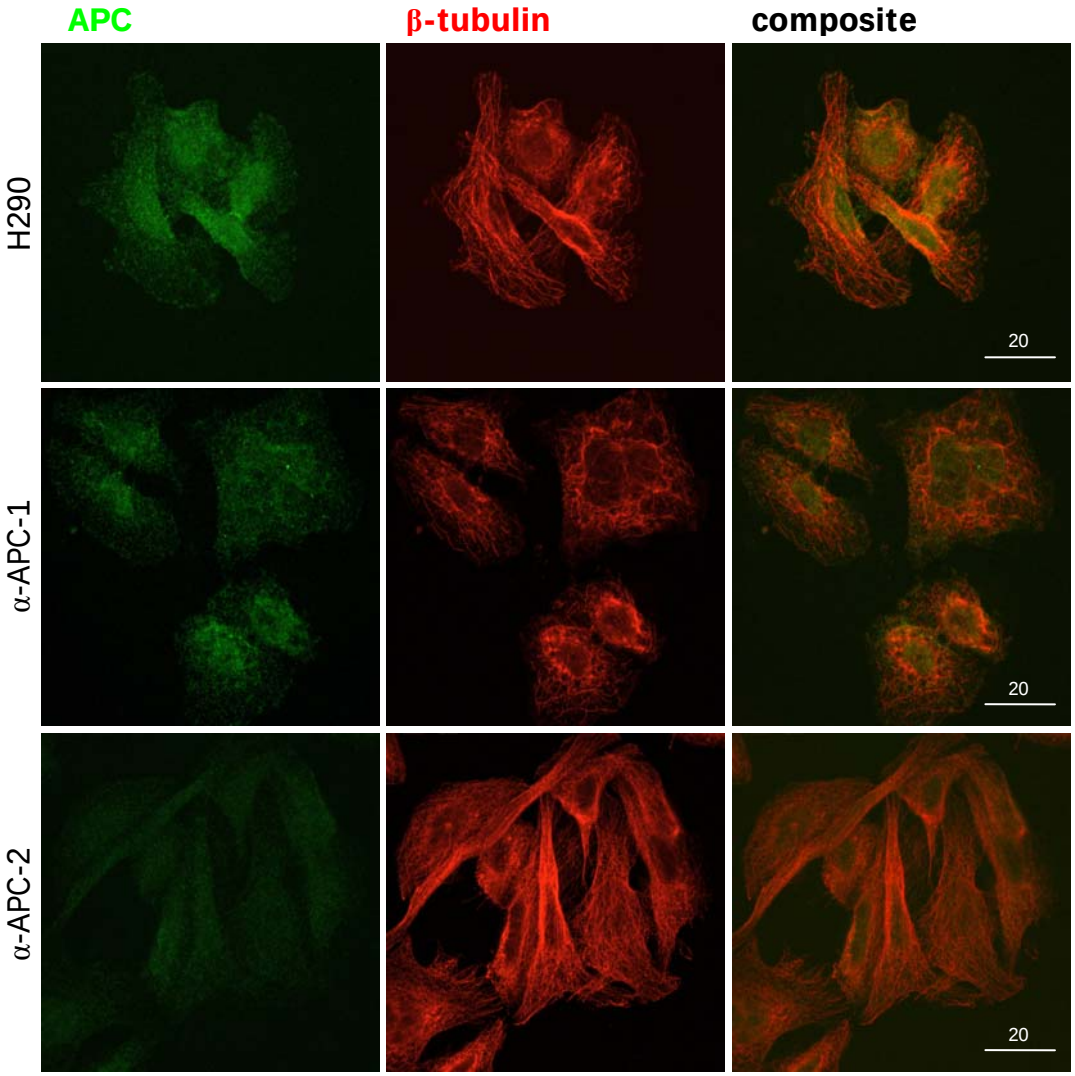

Figure 16: SW480 cells were immunostained for APC (green) using H290, α-APC-1 and α-APC-2 and co-stained for β-tubulin (red). Bars equal 20μm.

5 Discussion

In this work, polyclonal anti-APC antibodies (APC-1 and APC-2) were immunopurified to homogeneity using a succession of affinity columns. The different purification steps were followed using biosensor analysis with recombinant antigen immobilized onto the sensor surfaces. The immunopurified antibodies were characterized using biosensor, Western blot, immunoprecipitation and immunocytochemistry analyses of epithelial and colon cancer cell lines as well as recombinant APC protein. The α-APC-1 and α-APC-2 antibodies recognized full-length and truncated APC protein by immunoblot analysis that compared favourably with commercial antibodies. Furthermore, both APC antibodies immunoprecipitated endogenous APC protein in epithelial cells containing full-length APC and colon cancer cells with truncated APC. In addition the antibodies also enabled us to define proper lysis conditions to extract APC from different cell lines. Finally, immunoflourescent analysis demonstrated APC staining in epithelial cells corresponding to results.

The biosensor results show that the final antibodies are very pure and any residual contaminating antibodies can be neglected. They also show good affinity for the recombinant proteins.

The immunopurified APC antibodies clearly recognize endogenous APC and the truncated form of APC expressed in SW480 cells. Although neither antibody gave a clean signal by immunoblot analysis, immunoprecipitation of both full-length and truncated APC was extremely specific. α-APC-1 and α-APC-2 gave less background than he commercial H290. The fact that α-APC-2 recognized a very strong band in SW480 lysates that migrated at a higher molecular weight than the truncated form that the cells express was a concern. This suggests an unspecific binding to something other than APC. A BLAST search with the APC-2 sequence (results not shown) revealed no high enough similarity to anything other than APC. The comparison is not entirely relevant, though, since the antibodies are derived against folded APC-2. Since the proteins are denatured on the blots different epitopes are present than in the actual lysate. The folding of APC-2 might give epitopes that appear in other proteins when they are unfolded. Identifying the unknown band through bioinformatics may prove too difficult. This is because a) the folding of APC-2 is not known, b) there is no way as yet to compare denatured structures with folded. An experimental approach would be to identify the protein through masspectrometry. The trouble in this case however would be to get enough protein to get a sequence. Since this band did not show up on the immunoprecipitation blots it would not be possible to use this method to retrieve the protein. However, the blot was probed with the H290 antibody which would only be expected to recognize APC from immunoprecipitations with the other antibodies. This is also worth noting with regard to the purity of the immunoprecipitations. The immunoprecipitated samples for α-APC-1 and α-APC-2 appeared cleaner than for H290 but to be certain that is true, the blot should be probed with each antibody. There is, nonetheless, a strong indication that the antibodies are specific for APC in solution which is supported by the immunoflourescence results. It is possible that the bands detected in the protein extractions are denatured proteins which are not recognized endogenously.

Previous results have illustrated localization of full length APC to the tips of cells extensions and microtubule +-ends. All the antibodies displayed this punctate staining in the MDCK cells, entirely consistent with preceding findings. α-APC-1 also demonstrated

a distinct nuclear staining while α-APC-2 showed not other staining than the tips. Since they are derived against different parts of APC it is not surprising that they give different staining. Other antibodies against the N-terminal of APC have show nuclear staining but some have been proven to be non-specific. Nuclear staining should be regarded with some skepticism. Cross reactivity can give false positive results. A commercial antibody against APC has been shown to react with a DNA-binding protein more strongly than with APC. [27] Other antibodies have also been shown to give false staining of APC. Among these was the antibody produced against M-APC.

Immunostaining of SW480 to elucidate intracellular localization of APC has previously proven difficult. The results here though do show a distinct difference in staining between SW480 and MDCK cells. In the SW480 cells there is no staining of the tips of cell extensions which is so clear in the MDCK cells. The truncated APC in SW480 cells has lost the microtubule binding site and as previous research has shown the ability to localize at the +-ends. SW480 cells would therefore not show any staining of APC at the tips. The APC staining seen in SW480 cells is very dispersed and difficult to distinguish from unspecific staining. Staining of polyps and carcinomas, however, has demonstrated an increased cytoplasmic staining compared to normal cells which correlates to the staining seen in the SW480.

In conclusion, these antibodies can help us to design a chromatographic protocol for the purification of native APC. They will be used to monitor the different chromatographic steps involved in APC purification. These antibodies are valuable reagent as they can be used to immunopurify APC and APC associated protein complexes in human colonic carcinoma cell lines.

The antibodies can also be used in immunocytochemistry to study APC localization in various human colonic cell lines and to study the effect of the different APC mutation in the subcellular localization.

6 Acknowledgments

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