

AD _____

Award Number: DAMD17-99-1-9197

TITLE: A New APC-Like Gene Involved in Regulation of
B-catenin/LEF

PRINCIPAL INVESTIGATOR: Tagvor G. Nishanian
Todd Waldman, M.D., Ph.D.

CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20057

REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2003	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 1999 - 30 Jun 2003)	
4. TITLE AND SUBTITLE A New APC-Like Gene Involved in Regulation of B-catenin/LEF			5. FUNDING NUMBERS DAMD17-99-1-9197	
6. AUTHOR(S) Tagvor G. Nishanian Todd Waldman, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Medical Center Washington, DC 20057 E-Mail: nishanit@gunet.georgetown.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
20040421 065				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) <p>Here we describe two different approaches to better understanding the functions of Wnt and TGF-B signaling in cancer pathogenesis. First, we have identified a second adenomatous polyposis coli (APC)-like gene, APC2/APCL. Interphase fluorescence in situ hybridization analysis revealed an APC2 allelic imbalance in 19 of 20 ovarian cancers screened and indicates that APC2 could be a potential tumor suppressor gene in ovarian cancer. Antibodies against the NH2 terminus of human APC2 show that endogenous APC2 is diffusely distributed in the cytoplasm and colocalizes with both the Golgi apparatus and actin filaments. Second, we have identified a protein that interacts with the intracellular downmain of the TGF-B receptor BMPR1A. This protein, known as SAP49, is a developmentally-regulated splicing factor. The interaction was identified in a yeast two-hybrid analysis and confirmed via immunoprecipitation/Western Blot analysis in transfected human cells. The interaction has been localized to the GS-domain of BMPR1A and the the proline rich-doamin of SAP49 via mutational analysis. Further studies will address the biological relevance of the interaction in human cancer pathogenesis.</p>				
14. SUBJECT TERMS APC, Beta-Catenin, LEF, BMP, BMPR1A			15. NUMBER OF PAGES 9	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	3
Body.....	3-6
Key Research Accomplishments.....	6-7
Reportable Outcomes.....	7-8
Conclusions.....	8
References.....	N.A.
Appendices.....	N.A.

INTRODUCTION

APCL/APC2, an APC homologue located on chromosome 19p13.3, has also been shown to interact with β -catenin and can decrease β -catenin levels and signaling activity in SW480 colon cancer cells. hAPC2 is expressed in many different tissues and cell lines including brain, breast, colon, and ovary. APC family members have similar N-terminal dimerization domains, armadillo repeats, and β -catenin binding and regulatory domains but are less conserved at the C-terminus, which contains the basic domain, microtubule binding domain, and disc large binding site in hAPC. In addition, hAPC2 lacks the three 15 amino acid β -catenin binding sites. A phylogenetic tree of the highly conserved armadillo repeat domain suggests independent drosophila and vertebrate APC gene duplications. Drosophila APC (dAPC) is known to regulate wingless signaling in the eye but has not been associated with other tissues. Drosophila APC2 (dAPC2)/E-APC negatively regulates wingless signaling in the epidermis. These results suggest that APC and APC2 could be tissue specific and/or have different roles in β -catenin regulation.

BMPR1A is a membrane-bound receptor for BMP4, a TGF-B family member. Recently, inherited mutations in BMPR1A have been found in juvenile polyposis kindreds, demonstrating that BMPR1A is a tumor suppressor gene. BMPR1A is a serine/threonine kinase, phosphorylating second messengers known as SMADs that transduce BMP signals to the nucleus. Once in the nucleus, SMADS partner with various transcription factors to modulate changes in gene expression. Here we wondered whether, in addition to its kinase activity, whether BMPR1A might interact with any novel proteins.

RESULTS

Chromosomal Localization and Fine Mapping.

Using PAC clone 22K8, we performed FISH analysis on the SKOV3 ovarian cancer cell line, which had reduced levels of APC2 protein, and a variety of other cancer cell lines including those derived from breast and colon. These pilot studies showed that most had two or more copies on chromosome 19. However, although SKOV3 cells had on average 3 signals, none of them were on chromosome 19. To continue this study, we screened 20 sporadic breast and ovarian tumors by interphase FISH analysis. Remarkably, 19 of 20 specimens from ovarian cancer patients exhibited marked allelic imbalance. In contrast no significant allelic imbalance was observed in normal ovarian or breast tissue. The number of signals of the 19p telomeric marker was also significantly less than 2 in the ovarian cancer specimens. Even taking into account the bias toward slightly underestimating hybridization signals using interphase FISH, these data are consistent with published studies showing a marked loss of chromosome 19ptel in ovarian cancers and indicate an even more marked loss of APC2. Although the overall ratio of APC2/19p when averaged over 20 breast cancers was not markedly reduced, 4 of 20 breast cancer specimens did actually exhibit allelic imbalance at this locus.

Exogenous expression of APC2 in SKOV3 cells.

In pilot studies we found that many cells expressed significant levels of APC2. However, the ovarian cancer cell line SKOV3 expressed low levels of APC2 as demonstrated by western blot and immunocytochemistry. We used SKOV3 cells to examine the distribution of exogenously expressed APC2. Upon APC2 transfection, the intensity of several bands was markedly

increased as detected by western blot using a chicken antibody to APC2. Immunocytochemistry showed that APC2 transfected cells could easily be detected against the background of non-transfected cells. Exogenously expressed APC2 localized around the nucleus and co-localized with PKC μ , a kinase known to associate with the Golgi apparatus. Co-localization of APC2 and PKC μ was observed for much of the Golgi stack indicating that APC2 is associated with certain regions of the Golgi where it co-localizes with PKC μ even though the Golgi apparatus is somewhat disrupted in the transfected cells. APC2 co-localized with actin filaments at the cell membrane. SKOV3 cells have very few actin filaments in the cytoplasm compared to the MDA-MB-157 cells shown in Figure 5. β -catenin co-localized with exogenous APC2 in the aggregates at the Golgi apparatus whereas in untransfected cells β -catenin is localized only at the membrane. This could be indicative of a transport function for APC2 as suggested in recent studies. Exogenously expressed APC2 also appears to co-localize with some perinuclear microtubules.

APC2 Expression.

APC2 expression was determined by RT-PCR, Northern analysis and Western analysis of both cell lines and tissue. For RT-PCR 1 ug of total RNA from each cell line or tissue was used for amplification, for Northern analysis 10 ug of total RNA and for Western analysis 60 ug of protein from each cell line. We confirmed that APC2 is expressed in a variety of tissues, including breast, colon, brain, and ovary, at both the RNA and protein level. In some cells APC2 levels were reduced but still detectable (+/-). APC2 expression, like APC, is greatest in the brain; however, there are differing levels in different brain regions with very little expression in the cerebellum and cerebral cortex. Lymphoid tissues and lymphoma cell lines had no detectable APC2 at the mRNA or protein level with the exception of K-562 leukemia cells, which express low levels of APC2.

Both rabbit and chicken hAPC2 antibodies were affinity purified on an antigen coupled CNBr column. Western blot analysis determined that both antibodies were specific to hAPC2 with no cross-reactivity to APC. This was confirmed using the SW480 colon cancer cell line that contains a C-terminally truncated form of APC. Neither rabbit nor chicken hAPC2 antibodies detect this truncated APC protein even though it contains the conserved N-terminus. The predicted molecular weight of full-length APC is ~310 K and that of APC2 is ~245 K. In addition, APC2 is present in T84 cells, which have a homozygous deletion of the APC gene. To further determine specificity, we blocked the antibody with recombinant antigen before Western blot analysis and found that all bands are specific to APC2. Similarly, when preimmune IgY was used to probe Western blots no staining was observed. Western blot analysis showed that APC2 is expressed in many cell lines including SKBR3, SW480, MDCK, MDA-MB-157 and 436. HL-60 lymphoma cells have no detectable APC2 protein, which correlates with the mRNA data. A characteristic pattern of immunoreactive species was observed. Three major bands larger than 200 kD and several smaller molecular weight species of ~121, 81, and 51 kD were present consistently. Other cell lines, for example MDA-MB-157 and SKOV3, have significantly less of all bands. The presence of multiple bands by western blotting with N-terminal APC2 antibodies is similar to that observed for APC using N-terminal APC antibodies. In the case of APC the multiple banding pattern has been ascribed to a combination of multiple splice variants and to degradation products. Because several lower molecular weight species are present in cells that are transfected with full-length APC2 it is likely that these bands represent degradation products.

Some differences in the pattern of immunoreactive species were observed when endogenous APC2 was compared in different cell lines and when compared to exogenously expressed APC2. These variations are likely to represent variable degrees of proteolytic degradation in the different situations. However it is possible that like APC, APC2 may also have many splice variants.

The N-terminal region of APC2 contains the highly conserved dimerization domain. This region in APC has been shown to dimerize *in vitro*. To determine whether APC associates with APC2, APC2 was immunoprecipitated with the purified rabbit antibody and APC detected using APC Ab-1 in SW480 and HBL-100 cells. Full-length APC was detected in HBL-100 cells and the 150 kD truncated form of APC was detected in SW480 cells. This result demonstrates that APC and APC2 can either dimerize or associate in a complex in a detergent soluble lysate.

Sub-cellular Localization of APC2. To investigate the localization of endogenous APC2 in the cell, we performed immunocytochemistry on several cell lines including SKBR3 breast cancer cells, MDCK normal canine kidney cells, SW480 colon cancer cells, and A549 lung carcinoma cells. Although both rabbit and chicken antibodies exhibited a similar staining pattern by immunocytochemistry, the chicken antibody was exceptional and was used for these studies. Preimmune chicken IgY and antigen blocked antibody, as well as IgY prior to antigen affinity purification, were completely negative. Specific APC2 staining was similar in all cell lines and was observed diffusely in the cytoplasm as well as being associated with tubular structures adjacent to the nucleus that resembled the Golgi apparatus. Staining was also concentrated along filamentous structures and in what appeared to be lamellipodial membranes.

APC2 Association with Actin Filaments. As was observed with exogenously expressed APC2 in SKOV3 cells, endogenous APC2 co-localized with PKC μ at the Golgi apparatus. A relationship between APC2 and actin filaments was observed in cells stained with phalloidin. However, not all actin filaments stained and APC2 appeared to be concentrated near actin-associated membrane ruffles and lamellipodia as well as cell-cell contact sites. Treatment with cytochalasin D, an actin-disrupting agent, causes actin filaments to collapse and coalesce mostly at the cell periphery but also throughout the cell. Following treatment with cytochalasin D, APC2 remained associated with the actin filaments in MDA-MB-157 cells.

Discovery of Novel BMPR1A Interacting Proteins. A yeast two hybrid bait was prepared that included 232 amino acids of the BMPR1A intracellular domain fused in frame with Gal4 DNA binding domain. This bait was co-transformed into *S. cerevisiae* strain MAV203 together with a human fetal brain Gal4 activation domain library. 62 putatively interacting colonies were recovered. After further testing, 24 were purified and sequences. 23/24 corresponded to human SAP49, a splicosome associated protein. The remaining interactor was FKBP12.6, already known to interact with the intracellular domains of TGF-B type I receptors. To confirm the interaction in human cells, expression vectors for both BMPR1A and SAP49 were created, in which BMPR1A was tagged with HA and SAP49 was tagged with FLAG. After co-transfection of these plasmids into HEK293 cells, SAP49 was immunoprecipitated with FLAG. Western blot analysis with HA antibodies demonstrated that BMPR1A interacted strongly with the SAP49 protein. Finally, mutational analysis was performed to identify the regions of interaction. This

demonstrated that the proline-rich domain of SAP49 and the GS domain of BMPR1A were required for the interaction.

DISCUSSION

Subcellular localization of hAPC2

hAPC2 is diffusely distributed in the cytoplasm, is localized to the Golgi apparatus, and is associated with actin filaments. In some instances, such as lamellapodia or membrane ruffles, APC2 exhibits a punctate staining at the ends of actin filaments. Unlike APC, APC2 remains associated with the disrupted actin filaments following treatment with cytochalasin D. Recent studies show that E-APC/dAPC2 co-localizes with actin caps during *Drosophila* development and negatively regulates wingless signaling in the epidermis. These data suggest that even though sequence similarity is low, hAPC2 and dAPC2/E-APC may be functional homologues and that both may be involved in actin-associated events such as motility as well as in β -catenin signaling.

Significance of the chromosomal location of APC2

The chromosomal localization of APC2 to chromosome 19p13.3 within 12 cM of the telomere is significant because this region is associated with Peutz-Jeghers Syndrome (PJS) and exhibits significant loss of heterozygosity (LOH) in several sporadic cancers. Patients with PJS are more susceptible to breast, testis, gastrointestinal, and ovarian cancers. Loss of 19p13.3 occurs in many sporadic cancers including those of the breast and is remarkably common in sporadic ovarian carcinomas (~50%). Ovarian cancers are also characterized by a high rate (~16%) of stabilizing β -catenin mutations. However, mutations in the PJS gene, LKB1, are not present in most of these sporadic cancers suggesting the existence of other tumor suppressor loci in this region of chromosome 19. Our fine-mapping analysis shows that APC2 is located in the region of markers D19S883 and WI-19632 between the LKB1 gene and the site of 100% LOH found in adenoma malignum of the uterine cervix. Importantly, we found significant allelic imbalance of APC2 in sporadic ovarian cancers. Therefore, like APC, APC2 could be a tumor suppressor gene important in several cancers particularly ovarian cancer.

Significance of the putative interaction of BMPR1A with SAP49

The fact that BMPR1A interacts with this developmentally regulated splicing factor demonstrates that BMP signaling may play a role in tissue-specific splicing. Further characterization of the functional relevance of the interaction is underway.

Key Research Accomplishments:

- Identification of a new APC-like gene, APC2 (~245 K), which is overall 35% identical to APC (~310K).

- APC2 is located on chromosome 19p13.3 in a region containing markers D19S883 and WI-19632, a region of LOH predisposing to several different cancers including breast and ovarian.
- APC2 is expressed in most cells and tissues, including breast, colon, and ovary with greatest expression in the brain, which is similar to APC.
- APC2 is localized diffusely in the cytoplasm, is localized to the golgi apparatus, and is associated with actin filaments.
- Upon retinoic acid (RA) treatment, APC2 co-localizes with β -catenin and actin filaments at the membrane in SKBR3 breast cancer cells.
- APC2 regulates β -catenin signaling activity similar to APC.
- APC2 and APC associate in a detergent soluble lysate.
- Upon overexpression, APC2 localizes at the Golgi apparatus, in aggregates containing β -catenin, and associates with both actin and microtubule filaments.
- There is a significant APC2 allelic imbalance in ovarian cancer.
- An APC2 allelic imbalance was found in 4 of 20 breast tumors, however these 4 were the most aggressive of the 20 specimens examined.

Reportable Outcomes

Manuscripts:

Albanese C, Wu K, D'Amico M, Jarrett C, Joyce D, Hughes J, Hult J, Sakamaki T, Fu M, Ben-Ze'ev A, Bromberg JF, Lamberti C, Verma U, Gaynor RB, Byers SW, Pestell RG. IKK α regulates mitogenic signaling through transcriptional induction of cyclin D1 via Tcf. *Mol Biol Cell.* 14:585, 2003.

Kim JS, Crooks H, Dracheva T, Nishanian TG, Singh B, Jen J, Waldman T. Oncogenic β -catenin is required for bone morphogenetic protein 4 expression in human cancer cells. *Cancer Res.* 62:2744, 2002.

Jarrett CR, Blancato J, Cao T, Bressette DS, Cepeda M, Young PE, King CR, Byers SW. Human APC2 localization and allelic imbalance. *Cancer Res.* 61, 7978, 2001.

Abstracts: None.

Presentations: None.

Patents and licenses applied for and/or issued: None.

Degrees obtained that are supported by this award:

Christy Jarrett – Ph.D., 2002.

Development of cell lines, tissue or serum repositories: None.

Infomatics such as databases and animal models, etc.: None.

Funding applied for based on work supported by this award: None.

Employment or research opportunities applied for and/or received based on experience/training supported by this award:

Christy Jarrett – Post-Doctoral Fellow with Dr. Arthur Murcurio, Harvard University School of Medicine.

Grants and other Funding:

APC2 in Breast Cancer, NIH 5 R21-CA87749. NIH grant awarded to Stephen Byers, 04/01/02 – 03/31/05.

CONCLUSIONS

We have made significant advances in the mechanisms of Wnt and BMP signaling in human cancer.