Alternative Splicing of the APC Gene and Its Association with Terminal Differentiation

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ABSTRACT

The human tumor suppressor gene, APC, is composed of at least 21 exons, 7 of which are alternatively expressed. Sixteen APC transcripts that differ in their 5'-most regions and arise by the alternative inclusion of 6 of these exons have been identified by reverse transcription-PCR analysis of RNA prepared from human, mouse, and rat cell lines and tissues. Tissue-specific differences were observed in the expression of APC transcripts without exon 1, a coding region for a heptad repeat that supports APC homodimerization. Transcripts without exon 1 were observed at high levels in postmitotic, differentiated tissues and in two cell lines following the induction of differentiation. Sequence analysis of these novel open reading frames predicts APC proteins with different amino-terminal domains and therefore potentially different abilities to associate with other proteins. Our findings suggest that the alternative splicing of APC leads to alternative forms of APC proteins with potentially unique functions in growth control and differentiation.

INTRODUCTION

APC is an autosomal dominant disorder characterized by an early onset of multiple adenomatous polyps of the colon and rectum and a predisposition to colorectal cancer (1). Inactivating mutations of the APC gene are found in the germline of patients with APC and in sporadic colorectal adenomas and carcinomas (2–4). Likewise, mice carrying germline mutations of APC are predisposed to intestinal tumors (5, 6). These observations, as well as the finding that the introduction of full-length APC into colon carcinoma cell lines suppresses tumorigenicity, shows that APC can function as a tumor suppressor gene (7).

The APC gene is expressed ubiquitously and contains an open reading frame, predicted to begin in exon 1, that encodes a 2843-amino acid polypeptide (2). It contains at least 21 exons (8–10); 17 of these are predicted to be coding exons because they are downstream of an initiating methionine codon in exon 1. Thiliveris et al. (8) identified three exons 5' of exon 1 (0.3, 0.1, and 0.2) in cDNAs isolated from fetal retina and fetal brain libraries. Horii et al. (9) also identified these exons as well as an additional brain-specific exon (BS) in RT-PCR analyses. These four exons are alternatively expressed (8, 9).

Although disruption of normal APC clearly plays an important role in colorectal tumorigenesis, the function of the protein remains unclear. Several domains of the APC protein have been identified by biochemical and peptide sequence analysis (2, 4, 11). The middle of the APC protein interacts directly with /3-catenin (12, 13), whereas the carboxyl-terminal end may associate with tubulin (14, 15). The amino-terminal domain contains 9 or 10 heptad repeat regions, suggesting that this protein can form homo- or heterodimers through the formation of α-helical rods (2, 4). The first heptad repeat, encoded primarily by exon 1, has been shown to support protein homodimerization (11, 16). Therefore, transcripts lacking exon 1 may produce protein isoforms with unique amino-terminal ends that are unable to homodimerize.

In this report, the tissue-specific expression patterns of APC that arise by alternative splicing were examined using RT-PCR amplification of cDNA prepared from several tissues and cell lines from human, mouse, and rat. A novel APC isoform that is ubiquitously expressed in humans was identified. APC transcripts with and without exon 1 were expressed at an equivalent level in brain, heart, and skeletal muscle from both mouse and human tissues. Other cell lines and tissues primarily contain transcripts with exon 1. Following the induction of terminal differentiation in two myoblast cell culture systems, a change in APC isoform expression was observed. Consequently, by the use of the open reading frames encoded by the 5' exons and by alternative splicing of exon 1, novel amino-terminal domains of the APC protein can be expressed. These proteins are predicted to have an altered ability to dimerize.

MATERIALS AND METHODS

Cell Culture. All cell lines, with the exception of C2C12, the mammary epithelial cells and primary fibroblasts, were obtained from the American Type Culture Collection and cultured according to American Type Culture Collection specifications at 37°C and 5% CO2, without antibiotics. The C2C12 cell line was obtained from Dave Wiezorek at the University of Cincinnati and was cultured in DMEM (Life Technologies, Inc.) supplemented with 20% FCS (HyClone). Differentiation medium that contained DMEM supplemented with 5% horse serum (Life Technologies, Inc.) was used to induce the differentiated phenotype. The fibroblast and mammary epithelial cultures were obtained from Leslie Jerominski and Ray White at the University of Utah. The fibroblasts were cultured in DMEM supplemented with 10% FCS (HyClone), and the mammary epithelial cultures were grown in mammary epithelial growth medium (Clonetics).

RNA Preparation and RT-PCR. RNA was prepared from cells in culture or human and mouse tissues obtained from autopsy by the guanidinium-acid-phenol method (17). cDNA was synthesized in a 20 μl reaction using 1 μg of total RNA. The RNA was heated for 5 min at 70°C with random hexamers (Life Technologies, Inc.) and cooled. RT was performed at 42°C for 1 h and 50°C for 20 min after the addition of 4 μl of 5X reverse transcriptase buffer (Stratagene), 1 μl of 10 mM dNTPs, 1 μl of 0.1 M DTT, and 30 units of Stratascript reverse transcriptase (Stratagene). Amplification of 20% of the cDNA sample was performed using custom primers (Table 1) that amplify APC, embryonic skeletal myosin heavy chain 3'-untranslated regions or glyceraldehyde-3-phosphate dehydrogenase. PCR was performed for 40 cycles as follows: initial denaturation at 94°C for 3 min, 94°C for 45 s, 60°C for 1 min, and 72°C for 1 min, unless otherwise stated in the figure legends. PCR products were separated by agarose gel electrophoresis in 3% NuSieve 3:1 agarose (FMC) in 1X Tris-acetate-EDTA buffer.

Sequencing. PCR products for sequencing were gel purified using GeneClean (Bio 101, Inc.) and sequenced using the M13 universal and reverse sequencing primers by the dyeodeoxy termination method on an Applied Biosystems 373A DNA Sequencer.

Southern Blot Analysis. Southern blot analysis was performed using a positively charged nylon membrane, Hybond-N+ (Amersham Corp.) using the protocol recommended by the manufacturer. PCR products were separated in
Table 1  Sequences of primers used for RT-PCR analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V58.UP</td>
<td>UP-GAGACAGAATGAGGTGCTGC</td>
<td>Exon 0.3</td>
</tr>
<tr>
<td>BS1.UP</td>
<td>UP-GCTTTACCCCATTTGAAAGGC</td>
<td>Exon BS</td>
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<tr>
<td>0.2.UP</td>
<td>UP-TCGGCTGGATCTGGTCCCA</td>
<td>Exon 0.1</td>
</tr>
<tr>
<td>V59.UP</td>
<td>UP-TGAGACACTGATTGCTCCCA</td>
<td>Exon 0.2</td>
</tr>
<tr>
<td>E.Skel.</td>
<td>GCATGGGAAAAGTGATACCTG</td>
<td>MHC</td>
</tr>
<tr>
<td>G3PDH5</td>
<td>TTGTGACAGGCGTACCAT</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Antisense primers</td>
<td></td>
<td></td>
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<td>V38.RP</td>
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</tr>
<tr>
<td>MUSEX3.RP</td>
<td>RP-TCCTCTCTTCTCACTTCTCTCCTA</td>
<td>Exon 3</td>
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<tr>
<td>G3PDH2</td>
<td>GAGCTGTGACGATATCGCC</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
</tbody>
</table>

* Originally published in Thiliveris et al. (8).
* Originally published in Sanchez et al. (37).
* Originally published in Spirio et al. (36).

Alternative Splicing of the APC gene

Alternative Splicing of the APC Gene Is Tissue Specific. The tissue-specific expression patterns of APC were examined in RNA isolated from several human tissues and cultured cell lines. Exon-specific PCR primers were designed to amplify each of the 5' exons (0.3, BS, 0.1, and 0.2) and paired with reverse primers in exon 1 or 2 of APC. The following human cell lines were examined: melanocyte (RPMI 7951), normal dermal fibroblast (6390), colon carcinoma (HCT 116, DLD-1, SW480, SW48, HT29, and CACO 2), normal mammary epithelium (184Al), glioblastoma (T98G), medulloblastoma (DAOY), astrocytoma (SW1088) and retinoblastoma (WERI Rb-1 and Y79). Six isoforms, named by the exons they contain (0.3+1+2, 0.3+2, 0.1+0.2+1+2, 0.1+1+2, 0.1+0.2+2, and 0.1+2), were detected in cDNA from all these cell lines and are shown in Fig. 1. In addition, two other isoforms, BS+1+2 and BS+2, were detected in all cell lines examined (Fig. 1B). Isoform BS+2 has not been reported previously. These isoforms contain the BS exon previously identified by other investigators in normal human brain (9). This exon is ubiquitously expressed in all tissues examined (Fig. 1B). We were unable to observe the expression of exon 0.3+BS, as was reported by Hori et al. (9), in any tissue or cell line examined.

The composition of each isoform was confirmed by hybridization to an APC exon 2-specific probe that detected isoforms amplified with a different exon 2-specific primer (Fig. 1, bottom panels, Lanes 5–8)
and by sequencing of the PCR products (data not shown). The extra bands observed in Fig. 1 are heteroduplexes formed by the annealing of the complementary regions in the different isoforms; this was confirmed by the fact that only the expected sizes were observed when we analyzed RT-PCR products on denaturing gels. In every cell line examined, a difference between the level of each isoform containing exon 1 and the level of those without exon 1 was observed: the isoforms without exon 1 were expressed at much higher levels than those containing exon 1 in all cultured cell lines studied.

Several human tissues (brain, heart, lung, liver, kidney, lymph node, ovary, spleen, stomach, testis, uterus, mammary gland, and colon), were also examined for APC expression using RT-PCR. The same isoforms were observed in these tissues as in the cell lines previously described with the exception that the expression of each isoform was dramatically different in some tissues. The isoforms without exon 1 were expressed at similar or higher levels than isoforms containing exon 1 in brain RNA (Fig. 1). This was also observed in heart tissue (data not shown). All other tissues displayed a pattern similar to that observed in the cell lines (data not shown).

Each 5' APC exon is expressed in transcripts with and without exon 9. Exon 9 of APC has previously been identified as an alternatively spliced exon (2). APC-specific primers derived from sequence in exons 0.3, BS, 0.1, and 0.2 were paired with a reverse primer in exon 9A, and RT-PCR was used to study the expression of the 5' exons in association with exon 9. Each of the 5' exons exists in APC isoforms both with and without exon 9 in all cell lines and tissues examined (data not shown). The exonic composition of each of these transcripts was determined by the size of the RT-PCR products and by Southern hybridization of the amplification products to APC exon-specific probes. In brain and heart tissue, four transcripts were observed that contained exon 0.3 with and without exon 1 and with and without exon 9; four transcripts were identified that contained the 5' exon BS. Eight unique transcripts were identified using a primer specific to exon 0.1. These transcripts contained every combination of the alternatively expressed exons 0.2, 1, and 9. A total of 16 different human transcripts were detected in all tissues examined (Fig. 2). We observed that the postmitotic and terminally differentiated tissues, such as heart and brain, were characterized by higher levels of the eight isoforms lacking exon 1 when compared with the other cell types and cell lines studied by RT-PCR analysis.

APC primers were designed to test whether other exons were alternatively spliced. We could not detect the expression of any other alternative transcripts by RT-PCR analysis using one series of 40 PCR cycles, although these have been reported by others using different methodologies (10, 19).

Sequence analysis reveals open reading frames and initiation codons in transcripts lacking exon 1. Exon 1 contains an initiating codon for the known APC protein. Therefore, transcripts lacking exon 1 must use alternative initiating codons in order to be encoded into protein (20). Sequence analysis of the extreme 5' exons has identified in-frame initiation methionine codons in exons 0.3, BS, and 0.2 (Fig. 2). These initiation codons were analyzed for Kozak consensus sequences, GCCGCGA/GCCAUGG, in which a strong consensus is defined as having at least a purine in the −3 position and a guanine at +4 (20). Both of the codons in exons 0.3 and BS conform to this rule, whereas the codon in exon 0.2 does not. Because an initiation codon is not present in exon 0.1, we predict that exon 0.1 may not be translated. In addition, single-strand conformation polymorphism analysis of this exon in a sample set of 20 normal individuals has revealed a polymorphism that disrupts the open reading frame in exon 0.1. This also suggests that exon 0.1 may not be translated.

Sequence analysis had previously revealed a stop codon 6 base pairs upstream of the initiation codon in exon 1. Therefore, regardless of the exons included 5' to exon 1, this stop encoded by exon 1 will prevent translation of these open reading frames. Only transcripts lacking exon 1 have the ability to encode differentially expressed APC peptides with novel amino-terminal domains.

The 0.3-containing tissue-specific APC transcripts observed in humans are also observed in the mouse. The coding region for the human APC gene (exons 1−15) is highly conserved in the mouse APC gene (6). All the amino acid motifs, such as the heptad repeat regions previously predicted from human APC, are conserved in the mouse as well. The murine APC sequence for exon 0.3 was identified by sequencing RT-PCR products generated by amplifying exon 0.3 in mouse brain and heart using human-specific primers (Fig. 3). The murine and human APC exon 0.3 sequences were 90 and 82% identical at the nucleotide and amino acid levels, respectively. The initiation codon with a good Kozak consensus in human exon 0.3 also was conserved in the mouse (20). Exons BS and 0.1/0.2 have not yet been identified or sequenced from the mouse.

RT-PCR analysis with mouse-specific APC primers designed from exons 0.3 and 3 sequences revealed an alternatively spliced transcript lacking exon 1 in the following murine tissues and cell types: fibroblasts, spleen, skeletal muscle, heart, cerebrum, cerebellum, and intestine (Fig. 4). The exonic composition of these murine transcripts was identified by sequencing the RT-PCR products. The 0.3 isoform lacking exon 1 (Fig. 4, 0.3+2+3) is expressed at high levels in

4 I. M. Santoro, J. Groden, and K. Steigerwald, unpublished data.
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A

| HU Ex .3 | 1 TGGATGCGcGACCAGGCCGCTCCCATATCAGTAGTGCGGCCTGGGCTGtG|
| MUR Ex .3 | 1 TGGATGCGcGACCAGGCCGCTCCCATATCAGTAGTGCGGCCTGGGCTGtG|

| HU Ex .3 | 61 GcGCACGTGACCCGCATGCTGCTGTTAGCAGCAGGTCGTCCTGAGCTGGAGACAG|
| MUR Ex .3 | 61 GcGCACGTGACCCGCATGCTGCTGTTAGCAGCAGGTCGTCCTGAGCTGGAGACAG|

| HU Ex .3 | 122 AATGGAGGGCTGCCCCAGAATGGG|
| MUR Ex .3 | 122 AATGGAGGGCTGCCCCAGAATGGG|

Fig. 3. Alignment of the mouse and human exon 3 nucleotide and amino acid sequences. Identity is depicted by vertical bars and capital letters. Sequence information has been submitted to GenBank (accession no. U66412).

B

| HU Ex .3 | MWLYWCPSPGCWHRQNGAAG|
| MUR Ex .3 | MWLYWRLPGCHWRQNGAAG|

Fig. 4. Alternative isoforms of the murine APC gene. An agarose gel of the RT-PCR products amplified with 0.3 and 3 exon-specific primers is shown. These primers were used to identify the APC isoforms in RNA from the following mouse cell types and tissues: F, primary fibroblast cells; S, spleen; Sk, skeletal muscle; Ce, cerebrum; Cl, cerebellum; L, liver; K, kidney; I, intestine; and M, Life Technologies, Inc., 100-bp ladder.

Cerebrum, cerebellum, skeletal muscle, and heart tissues but at very low levels in spleen, intestine, and fibroblasts. This isoform was also expressed at high levels in the olfactory bulb (data not shown). In liver and kidney, the transcript lacking exon 1 was not seen (Fig. 4). The middle product in Fig. 4 in Lanes Sk, H, Ce, Cl, and I is a heteroduplex of the two isoforms expressed.

Oshima et al. (21) have identified four alternatively spliced mouse APC transcripts in which exons 7 and/or 9 are alternatively spliced. These alternative forms of APC were identified in our analysis as well (data not shown). However, we have been unable to identify the alternate expression of exon 7 in human APC.

Differences in Two Cell Culture Systems Is Accompanied by a Change in APC Isoform Expression. The observation of tissue specificity in the expression of APC isoforms with and without exon 1 in postmitotic, terminally differentiated tissues suggested that changes in APC isoform expression could be induced by growth inhibition or differentiation of cells. Human primary fibroblasts were cultured in 0.1% serum to inhibit mitosis. These cells also were grown to confluency and contact inhibited. RNA was prepared, and RT-PCR analysis was used to identify the isoforms containing 0.3, BS, 0.1, and 0.2 in association with exon 1. No differences in isoform expression were observed when the two culture conditions were compared to growing primary fibroblasts (data not shown).

The mouse myoblast cell line, C2C12, which can be induced to differentiate into myotubes, was chosen as a skeletal muscle differentiation system. RNA was prepared from both myoblast cultures and myotubes differentiated in culture (Fig. 5, A and B). RT-PCR analysis was used to identify the expression of the APC isoforms containing exon 0.3 in myotube cultures after 8 days in differentiation medium and compared to that of the myoblast cultures. Differentiation was confirmed by successful RT-PCR of cDNA from the myosin heavy chain embryonic skeletal muscle gene from the differentiated myotube cell cultures only (data not shown). The glyceral 3-phosphate dehydrogenase gene also was amplified to control for the amount of RNA prepared from each culture (Fig. 5D). Only APC with exon 1 could be detected in the myoblast cultures using primers that amplified exon 0.3 to 3 (Fig. 5C, Lanes 1 and 2). Interestingly, in the differentiated myotube cultures, both products containing exon 1 and products lacking exon 1 were detected (Fig. 5C, Lanes 3 and 4). Another skeletal muscle differentiation system, rat cell line L6E9, was used to confirm that the 0.3-containing, exon 1-lacking isoform was induced following terminal differentiation. Using the same primers to amplify the L6E9 cDNA, only APC with exon 1 could be detected in cDNA from rat myoblast cultures. Amplification products containing exon 1 and products lacking exon 1 were detected in the differentiated L6E9 cells following RT-PCR analysis (data not shown).

DISCUSSION

Sixteen unique APC transcripts have been identified by RT-PCR analysis of RNA from a variety of human and mouse cell types. Differences in the expression levels of these transcripts in a number of cell types were observed. Transcripts that do not contain exon 1 were shown to vary in different samples. Brain and heart RNA from both human and mouse and skeletal muscle RNA from mouse exhibit changes in APC isoforms without exon 1. These three tissues expressed high levels of the transcripts that lack exon 1 when compared to other tissues and cell types examined. These tissue types also are characterized by their inability to reenter the cell cycle and hence are considered postmitotic or terminally differentiated. Therefore, expression of APC transcripts without exon 1 are associated with postmitotic, terminal differentiation. We also were able to observe an increase in the abundance of these transcripts in two cell culture systems following differentiation (Fig. 5). Exon 0.3 APC isoforms were observed in human and mouse tissues, suggesting functional importance by their conservation. The murine exon 0.3 genomic sequence shows a high degree of conservation to the human exon 0.3 sequence, suggesting that this exon, in both human and mouse, is protein-coding (Fig. 3). In total, these findings suggest that novel amino-terminal domains of the APC protein may be differentially expressed in cells.

Although the function of APC is unknown, recent studies on the
structure of APC, its localization in the cell, and the identification of interacting proteins establish a basis for understanding its function in cells. APC is a cytoplasmic protein that interacts directly with β-catenin and indirectly with α-catenin, cytoplasmic proteins essential for the cell adhesion activity of cadherins (12, 13, 22). APC also contains seven copies of the Armadillo motif, a protein motif believed to mediate protein-protein interactions that is also found in β- and γ-catenins. This suggests that APC and catenins may compete for common binding sites (23, 24). In addition, transient overexpression of wild-type, but not mutant, forms of APC have shown that normal APC protein can associate with the microtubule cytoskeleton via its carboxyl-terminal basic domain (14, 15). It is unknown whether homodimerization of APC is required for appropriate interaction with these proteins, although it has been shown using the yeast two-hybrid system that short APC protein domains will bind β-catenin (25). The expression of different forms of APC with novel amino-terminal domains without the dimerization domain may or may not affect the interaction of APC with catenins and tubulin.

Analysis of the putative amino acid sequences of the four novel human APC transcript amino-terminal domains by GCG, Intelligenetics, and a program that predicts coiled coil potential suggests that these domains are not α-helical and that they most likely do not contribute to the coiled coil structure originally proposed to occur at the amino-terminus of APC (Refs. 2, 26, and 27 and Fig. 6). This is not surprising given that the putative amino acid sequence of the novel APC amino-terminal ends includes numerous proline residues. In the absence of exon 1-encoded amino acids, the amino-terminal coiled coil domain encoded by exons 1 and 2 in the APC protein is eliminated (Fig. 6). The removal of exon 1 has previously been shown to disrupt the ability of APC protein to form a homodimer by the λ cl dimerization assay and by APC immunoprecipitation studies (11, 16). Because coiled coil structures are known to mediate homo- and heterooligomerization (28) and the first heptad repeat has been shown to support protein homodimerization, disruption of the first heptad repeat by alternative splicing of APC may alter the ability of APC to homodimerize and/or heterodimerize with as yet unknown proteins. This in turn would predict alterations of APC function when APC is alternatively spliced.

Many eukaryotic genes have been identified that express multiple gene products with different functions (29). For instance, multiple protein isoforms can be identified from many of the extracellular matrix genes, such as fibronectin, tropoelastin, and collagen (30), and in cytoskeletal components (31, 32). Alternative splicing leading to multiple proteins has also been observed with other tumor suppressors such as p53 and WT1 (33, 34). Alternative RNA processing can also result in the expression of proteins that differ in their amino-terminal ends and that elicit different functions such as the Mxi1 protein (35). APC may be similar with its extensive alternative splicing.

Because tissue specificity of APC expression is observed, the likelihood of alternate functions of the APC protein variants seems reasonable. The question remains, however, of whether the variant APC isoforms encode protein. The fact that transcripts without exon 1 include 5′ exons with initiation codons and open reading frames provides some evidence that these exons encode protein. Furthermore, the high degree of amino acid conservation between human and mouse APC provides an argument for the protein-coding ability of at least the exon 0.3-containing transcripts. The expression of these transcripts in postmitotic, terminally differentiated cells and their induction following differentiation in two cell culture systems also supports the argument that these transcripts encode novel APC proteins with unique functions. Finally, in preliminary experiments with anti-BS antiserum, we can identify APC-specific protein of the pre-
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We thank Chris Trzepacz, Olga Shamraz, and Laura Pajak for generously providing RNA samples; Greg Wernke and Rachel Fernandez for their computer assistance; and Dave Wizerek for providing the C2C12 cells and L6E9 RNA. We also thank Leslie Jerominski and Ray White for providing cell lines

and the DNA Core Laboratory at the University of Cincinnati for primer synthesis and sequencing.

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*Cancer Res* 1997;57:488-494.

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