Novel Double-negative Feedback Loop between Adenomatous Polyposis Coli and Musashi1 in Colon Epithelia

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Loss of tumor suppressor adenomatous polyposis coli (APC) is thought to initiate the majority of all colorectal cancers. The predominant theory of colorectal carcinogenesis implicates stem cells as the initiating cells. However, relatively little is known about the function of APC in governing the homeostasis of normal intestinal stem cells. Here, we identify a novel double-negative feedback loop between APC and a translation inhibitor protein, Musashi1 (MSI1), in cultured human colonocytes. We show APC as a key factor in MSI1 regulation through Wnt signaling and identify APC mRNA as a novel target of translational inhibition by MSI1. We propose that APC/MSI1 interactions maintain homeostatic balance in the intestinal epithelium.

Two subpopulations of stem cells have recently been identified in the intestinal epithelium (1–3). It has been shown that loss of APC in one of these stem cell subpopulations results in intestinal adenomas (4). Loss of APC throughout the small intestine leads to expansion of undifferentiated, stem cell-like epithelium into the normally well differentiated villus compartment and a >12-fold increase in Msi1 mRNA (5).

Musashi proteins function to inhibit translation initiation by binding to the 3′-untranslated region (3′-UTR) of target mRNAs and competing with poly(A)-binding protein for eukaryotic initiation factor 4G binding (6). The two best characterized targets of Musashi proteins in the canonical Wnt signaling pathway (12). A complex containing APC, Axin, and glycogen synthase kinase 3β binds to and phosphorylates β-catenin, leading to ubiquitination and degradation of β-catenin by the proteasome. Wnt binding to its cognate receptor or APC loss each lead to β-catenin accumulation in the cytoplasm, translocation into the nucleus, and interaction with co-activator TCF/LEF-1, resulting in transcription of Wnt target genes. In addition, there is evidence that nuclear APC also brings the transcriptional repressor C-terminal binding protein to the TCF-β-catenin complex (13), sequesters β-catenin from TCF/LEF-1 (14) and facilitates nuclear export of β-catenin (15–17). Approximately 80% of all colorectal cancers are associated with mutation of both APC alleles, resulting in APC truncation and loss of this tumor suppressor function (18).

In the current study, we used cultured human colonocytes to examine the functional relationship between APC and MSI1, first observed in mouse intestines (5). Msi1 was recently shown to be a Wnt target gene in mouse intestinal epithelium (19), and our studies in human colon cells support this finding. Our study further reveals that MSI1 regulates APC levels, providing the first evidence for a double-negative feedback loop between APC and MSI1. We have confirmed that MSI1 regulates APC translation. We hypothesize that this double-negative feedback system is central to the maintenance of homeostasis, the critical balance of proliferation and differentiation, in the intestinal epithelium.

EXPERIMENTAL PROCEDURES

Plasmids and Cloning—SureSilencing short-hairpin RNA (shRNA) plasmids (SuperArray, Frederick, MD) were used to reduce levels of APC and MSI1. For Msi1 overexpression, pcDNA3-Flag-Msi1 (Flag-Msi1) and pcDNA3-Flag-Msi1mutR1 (Flag-mutR1) were generously provided by Hideyuki Okano (Tokyo, Japan). The mutR1 Msi1 mutant contains three phenylalanine-to-leucine substitutions in the first RNA binding domain (8). These phenylalanines are required for RNA binding and subsequent translational inhibition by Msi1, and these substitutions have been shown to ablate binding of the mutant to Numb mRNA (8, 20). Mouse Msi1 cDNA was employed for these studies. Mouse and human cDNAs are 93% identical, and the proteins differ by two amino acids (Q127H and T251S) that do not affect the first RNA recognition motifs, required for RNA binding (20). The Numb 3′-UTR-luciferase reporter, pGVP2-Numb, was provided by H. Okano. The APC 3′-UTR luciferase reporter was made by replacing the Numb 3′-UTR in pGVP2-Numb3′-UTR with the APC 3′-UTR amplified from HCT116 genomic DNA using the following primers: APC 3′-UTR (forward), 5′-AAGAGAGGAAGAATGAAACTAAG-3′ and APC 3′-UTR (reverse), 5′-GCATGTATCCTCATTGTTATG-3′.

The pGVP2-APC 3′-UTR 5′ deletion mutant was made by cutting the APC 3′-UTR reporter DNA with the XbaI restriction enzyme, gel-purifying the fragments, and religating the truncated APC 3′-UTR into the pGVP2 backbone.

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2 The abbreviations used are: MSI1, Musashi1; APC, adenomatous polyposis coli; MCS, MSI1 consensus binding sequence; TCF, T cell factor; LEF, lymphoid enhancer-binding factor.
Cell Culture and Transfection—HCT116βw cells, a generous gift from Bert Vogelstein, were cultured as described (21). NIH3T3 cells (ATCC) were grown in DMEM:10% Cosmic Calf® serum. GeneExpress (Lab Supply Mall, Gaithersburg, MD) was used to transfect cells at 30–40% confluency, grown in 6-well plates. For RNA interference, 2.5 μg of shRNA plasmid was used; for overexpression, 0.5 μg and 1 μg of Flag-Msi1 or Flag-mutR1 were used with DNA content equalized by the addition of pcDNA3.1.

Protein Analysis—Cells were washed twice with cold PBS and then collected in reporter lysis buffer (Promega, Madison, WI) with protease inhibitors 48 h after transfection. Lysates were sonicated (10 s, Output 1) using a Heat Systems-Ultrasonics, Inc. cell disruptor (Plainview, NY). An aliquot was immediately removed, added to 4× protein sample buffer, and heated to 95 °C for SDS-PAGE. Another aliquot was added to 1 ml of TRIzol reagent (Invitrogen), incubated at room temperature for 5 min, and stored at −80 °C for RNA isolation. Proteins were separated using SDS-PAGE and 7% polyacrylamide gels and blotted onto nitrocellulose. Antibodies used for protein detection were as follows: APC (M2-APC (22)), rabbit anti-MSI1 (locally produced), and α-tubulin (12G10, Developmental Studies Hybridoma Bank (DSHB), University of Iowa. Images acquired with a Kodak image station 4000R (Molecular Imaging Systems, Rochester, NY) were analyzed using the ImageJ 1.4.1o software (rsb.info.nih.gov/ij).

RNA Analysis—Quantitative real-time reverse transcriptase polymerase chain reaction was used to assess mRNA content. RNA was isolated from TRIzol solution following the manufacturer’s instructions. First strand cDNA was prepared from 0.5 μg of RNA using 200 units of M-MuLV reverse transcriptase and Random 6 primer (New England Biolabs, Ipswich, MA). Quantitative PCR for APC, MSI1, and hypoxanthine-guanine phosphoribosyl-transferase (HGPRT; internal control) was performed using the DyNAmo HS SYBR Green quantitative PCR kit (New England Biolabs) and analyzed on a DNA Engine Opticon 2 thermal cycler for continuous fluorescence detection (Bio-Rad). Target mRNA estimates were made by comparing -fold change to levels in control transfections using the ΔΔC(T) calculation method.

Luciferase Reporter Assay—Numb and APC 3′-UTR-Luciferase reporters were co-transfected with Flag-Msi1 or Flag-mutR1 and Renilla luciferase (transfection control) expression constructs with total DNA adjusted to 2 μg using pcDNA3.1 vector. 48 h after transfection, cell lysates were prepared as described for protein analysis, and luciferase activity was assessed using a Dual-Luciferase reporter assay system (Promega) and a LMAXII™ microplate reader (Molecular Devices, Sunnyvale, CA). Firefly luciferase data were normalized to Renilla luciferase and expressed as a percentage of the negative control (vector only) luminescence. MSI1 shRNA experiments were performed similarly with 3 μg of total DNA.

RESULTS

Loss of APC Leads to Increased MSI1 Protein and mRNA in HCT116βw Cells—In mice, inducible, intestine-specific loss of Apc led to a greater than 12-fold increase in Msi1 mRNA in intestinal tissue. To further evaluate the interaction between APC and MSI1 in human colonocytes, we used HCT116βw cells, which have a stable karyotype and express wild-type APC. HCT116βw cells were also manipulated to only express wild-type β-catenin (21). The intact Wnt signaling pathway in HCT116βw cells is critical for our studies because both APC and MSI1 can impact Wnt signaling (19, 23). APC mRNA and protein levels were both reduced in HCT116βw cells transiently transfected with plasmids expressing shRNA corresponding to the APC mRNA sequence (Fig. 1). This knockdown of APC resulted in a reproducible increase in MSI1 protein (Fig. 1B) and a 2–4-fold increase in MSI1 mRNA (Fig. 1C).

Loss of MSI1 Leads to Increased APC Protein in HCT116βw Cells—The canonical Wnt signaling pathway is functional and stimulates target gene expression in HCT116βw cells (supplemental text and supplemental Fig. S1, A–C). Moreover, MSI1 expression is up-regulated in HCT116βw cells with overexpressed β-catenin (supplemental Fig. S1, D and E). MSI1 has recently been shown to activate the Wnt signaling pathway (19). To determine whether this regulation involves APC, we reduced MSI1 protein and mRNA in HCT116βw cells by transient transfection with plasmids expressing shRNA corresponding to the MSI1 mRNA sequence (Fig. 2). This knockdown of MSI1 resulted in a reproducible increase in APC protein (Fig. 2B) but no significant change in APC mRNA level (Fig. 2C), consistent with MSI1 functioning as a translational inhibitor of APC.

Msi1 Overexpression Decreases APC Protein in HCT116βw Cells—As confirmation of the loss-of-function data, overexpression of wild-type but not mutant Msi1 in HCT116βw cells led to a decrease in APC protein (Fig. 3, A and B). Unexpectedly, APC mRNA did not decrease but rather showed an increase at the highest level of exogenous Msi1 (Fig. 3C). These data are consistent with the known function of MSI1 as
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FIGURE 2. Loss of MSI1 leads to an increase in APC protein, but not mRNA. A, a representative Western blot reveals decreased MSI1 protein and increased APC protein in cells expressing either of two MSI shRNAs as compared with cells with scrambled shRNA (NC). B, MSI1 and APC band intensities from 3 independent experiments were normalized to α-tubulin. Results are expressed as percentage of increase in APC or MSI1 in cells transfected with MSI1 shRNA as compared with cells transfected with scrambled shRNA ± S.E. C, quantitative real-time RT-PCR analysis was performed on cells transiently expressing MSI1 or scrambled shRNA. Results from 4–5 independent experiments are expressed as average -fold change of APC- or MSI1-specific mRNA in MSI1-shRNA-transfected cells as compared with scrambled shRNA-transfected cells (dashed line) ± S.E.

FIGURE 3. Overexpression of Msi1 results in decreased APC protein. A, representative Western blots of HCT116βw cells transiently transfected with Flag-Msi1 or mutant Msi1 (Flag-mutR1) expression constructs. B, APC band intensities for the lowest Msi1 cDNA concentrations, from 5 independent experiments, were normalized to α-tubulin and expressed as average (ave.) band intensity ± S.E. Asterisks indicate a statistically significant decrease in APC band intensity from vector alone (p < 0.05) and Flag-mutR1 controls. Flag-mutR1 overexpression did not alter APC band intensity when compared with vector alone control (p > 0.4). C, quantitative real-time RT-PCR analysis was used to measure APC mRNA in HCT116βw cells transfected with increasing concentration of Flag-Msi1 expression construct or empty vector. Results from 5 independent experiments are expressed as average -fold change of APC mRNA in Flag-Msi1-transfected cells as compared with empty vector-transfected cells (dashed line) ± S.E.

DISCUSSION

Here, we report a novel double-negative feedback loop between APC and MSI1 that has potential relevance to the homeostasis of intestinal epithelia (Fig. 5). Our data indicate that APC, acting through β-catenin in the canonical Wnt signaling pathway, is responsible for regulation of MSI1 in cultured human colonocytes. These results confirm the recent report that murine Msi1 is a direct target of β-catenin transcriptional activation (19) and extend these findings to human colonocytes. Furthermore, we show that the APC 3′-UTR is a novel target of translational inhibition by MSI1. This reciprocal regulation of APC by MSI1 is likely responsible for the dramatic effect on differentiation observed upon Apc mutation in the adult mouse intestine (5).

Double-negative feedback is thought to be important in cells undergoing significant change, such as cells that are differentiating or undergoing asymmetric cell division, because the effect of double-negative feedback is similar to that of positive feedback (24). APC is involved in regulating cell division in the intestinal epithelium through inhibition of Wnt signaling (25). Loss of APC is an initiating event in a majority of colorectal cancers, further underscoring the importance of APC in the maintenance of the intestinal epithelium. MSI1 contains four putative MCSs, two near the 5′ end and two closer to the 3′ end (Fig. 4A). Of the four potential MCSs, only the first is conserved in sequence and position in human and mouse (Fig. 4B). This first MCS is predicted to locate in a hairpin structure, which is optimal for MSI1 binding (Fig. 4C). To test whether APC is a novel target of MSI1 translational inhibition, we generated a luciferase reporter that incorporated the entire APC 3′-UTR or a deletion mutant lacking the first two potential MCSs. NIH3T3 cells were used for this study because they lack endogenous MSI1 (8). Cells transfected with the APC 3′-UTR luciferase reporter showed a marked reduction in luciferase activity when co-transfected with the Flag-Msi1 expression construct (Fig. 4D). This reduction was similar to that seen using the Numb 3′-UTR reporter and did not occur in NIH3T3 cells expressing a mutant form of Msi1 (Fig. 4D). The APC 3′-UTR reporter lacking MCSI and −2 did not show a decline in activity significantly different from the complete APC 3′-UTR reporter in the presence of mutant Msi1 (Fig. 4D). HCT116βw cells transfected with the APC 3′-UTR luciferase reporter showed no significant decline in luciferase activity when cells co-expressed either Msi1 or mutant Msi1, nor was a decline apparent for the Numb 3′-UTR luciferase reporter, which served as a positive control (supplemental text and supplemental Fig. S2). To examine whether endogenous MSI1 in HCT116βw cells was suppressing translation of the APC 3′-UTR reporter, endogenous MSI1 levels were reduced using MSI1 shRNA (Fig. 4E). Reduced MSI1 levels were accompanied by an increase in APC 3′-UTR reporter activity. We conclude that the dampened response of the reporters to MSI1 overexpression seen in HCT116βw cells likely resulted from endogenous MSI1. Combined, these data indicate that MSI1 regulates APC levels in HCT116βw cells through its activity as an RNA sequence-specific inhibitor of translation.
inhibition of p21 translation has been shown to increase cell proliferation (7, 8). By activating Notch signaling, MSI1 has also been implicated in the maintenance of a dedifferentiated state in mammalian cells (9). In the intestinal epithelium, MSI1 is present in the stem cell compartment and is predicted to have a role in symmetric stem cell division (26). It is noteworthy that colorectal cancers show increased MSI1 levels, and decreasing MSI1 in colorectal cancer cell lines decreases tumorigenicity in mouse xenografts (27). It is likely that loss of differentiation observed in vivo following induced Apc mutation results from the double-negative feedback between APC and MSI1 that we have now identified (5). Imbalance in this double-negative feedback loop leads to loss of homeostasis in the intestinal epithelium and may ultimately result in cancer.

Colorectal cancer is unique because APC loss is associated with nearly 80% of all disease (18). Replacement of APC in colorectal cancers through gene therapy remains a complicated ther-

**FIGURE 4. MSI1 binds to APC 3′-UTR and inhibits translation.** A, schematic representation of mouse and human APC 3′-UTRs showing position of MCSs. A mutant with the first 360 bp deleted (human 3′-UTR del.1) was incorporated into a luciferase reporter. B, alignment of the first 360 bp of mouse and human APC 3′-UTR (mApc 3′-UTR and hAPC 3′-UTR, respectively). The asterisk indicates MCS conservation in both position and sequence. Secondary RNA structure surrounding MCS1 and −2 (shaded) as predicted by the RNAfold WebServer. Structural images were produced by the mfold Web Server. D, left panel, luciferase activity in NIH3T3 cells transfected with a luciferase reporter containing APC 3′-UTR (APC), APC 3′-UTR 5′ deletion (APC 5′ del.), and Flag-Msi1 cDNA (Msi) or a Flag mutant Msi1 cDNA (mutR1). Right panel, luciferase activity in NIH3T3 cells transfected with luciferase reporter containing the positive control Numb 3′-UTR (Numb) and Msi or mutR1. Data are expressed as the average percentage of luciferase reporter activity in Msi or mutR1 co-transfected cells versus Vector alone (% Control ± S.E.). Co-transfection of the wild-type APC 3′-UTR construct with Flag-Msi1 significantly decreased the luciferase activity when compared with mutR1 co-transfectants (p < 0.05). Co-transfection of APC 3′-UTR 5′ deletion with Msi1 did not significantly decrease luciferase activity from mutR1 (p > 0.05) and resulted in a significant loss of Msi1 repression (p < 0.05). E, luciferase activity in HCT116sw cells co-transfected with either scrambled (NC) or MSI shRNA and luciferase reporters containing either APC or Numb 3′-UTR. Data are expressed as average relative luminescence ± S.E. after normalizing for transfection efficiency with Renilla luciferase. In HCT116sw cells, reporters containing either the APC or the Numb 3′-UTR each produced more luciferase when co-transfected with MSI1 shRNA than when co-transfected with scrambled shRNA (p < 0.05).
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Under normal cellular conditions, APC represses Wnt target gene expression by targeting β-catenin for destruction (red line) and sequestering nuclear β-catenin from transcription co-factor TCF/LEF-1 (not shown). In the presence of Wnt or following loss of APC function, β-catenin drives expression of Wnt target genes including MSI1 (green lines and arrows). Current studies expand this model by demonstrating that MSI1 protein inhibits translation of APC mRNA, completing a double-negative feedback loop between APC and MSI1.

A therapeutic strategy. Our data indicate that APC loss results in the up-regulation of MSI1 and the disruption of a delicate balance between these two effectors. Thus, we propose that MSI1 represents a potential target for future colorectal cancer therapies.

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