Retinoic Acid Inhibits β-Catenin through Suppression of Cox-2

A ROLE FOR TRUNCATED ADENOMATOUS POLYPOSIS COLI*

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Mutations in adenomatous polyposis coli (APC) underlie the earliest stages of colorectal carcinogenesis. Consequences of APC mutation include stabilization of β-catenin, dysregulation of cyclooxygenase-2 (COX-2) expression, and loss of retinoic acid production, events with poorly defined interactions. Here we showed that treatment of zebrafish expressing a truncated form of ApC with either retinoic acid or a selective COX-2 inhibitor decreased β-catenin protein levels and downstream signaling events. Interestingly, the destruction of β-catenin in apc mutant embryos following Cox-2 inhibition required the presence of truncated Apc. These findings support roles for retinoic acid and Cox-2 in regulating the stability of β-catenin following Apc loss. Furthermore, truncated Apc appears to retain the ability to target β-catenin for destruction, but only in the absence of Cox-2 activity. This novel function of truncated Apc may provide a molecular basis for the efficacy of COX-2 inhibitors in the treatment of colon cancer.

Retinoids are important regulators of differentiation and cell proliferation. Induction of differentiation by retinoic acid has been observed in endothelial, melanoma, neuroblastoma, and lung cancer cells (1). Retinoic acid has been shown to inhibit the growth of breast cancer cell lines and to reduce the average number and incidence of tumors in an animal model of breast cancer (2–4). The anti-tumor potential of retinoids has been observed in endothelial, melanoma, neuroblastoma, and lung cancer cells (1). Retinoic acid has been shown to inhibit the growth of breast cancer cell lines and to reduce the average number and incidence of tumors in an animal model of breast cancer (2–4). The anti-tumor potential of retinoids has been observed in endothelial, melanoma, neuroblastoma, and lung cancer cells (1).

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4 The abbreviations used are: RAR, retinoic acid receptor; APC, adenomatous polyposis coli; COX-2, cyclooxygenase-2; TCF, T-cell factor; PGE2, prostaglandin E2; hpf, hours post-fertilization; RT, reverse transcription; RXR, retinoid X receptor; GSK-3β, glycogen synthase kinase-3β; GFP, green fluorescent protein.

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**EXPERIMENTAL PROCEDURES**

*Embryo Culture and Zebrafish Stocks*—Wild-type, *apc* mutant, and *nls* mutant *Danio rerio* (zebrafish) were maintained on a 14:10-h light:dark cycle. Fertilized embryos were collected following natural spawns and allowed to develop at 28.5 °C. Control and experimental embryos were raised in 0.003% phenylthiourea to inhibit pigment formation.

*Whole Mount in Situ Hybridization*—Zebrafish embryos were fixed in sucrose-buffered 4% paraformaldehyde, rinsed in phosphate-buffered saline, dehydrated in methanol, and stored at −20 °C. The riboprobe for green fluorescent protein (GFP) was generated by linearization of pCRII (Invitrogen) containing GFP cDNA followed by *in vitro* transcription with SP6 or T7 RNA polymerase (Roche Diagnostics). The embryos were cleared in 70% glycerol/phosphate-buffered saline and photographed using an Olympus DP12 digital camera.

*Quantitative RT-PCR*—We isolated RNA from embryos and cell lines using an RNeasy kit (Qiagen) and then synthesized single-stranded cDNA from 1 μg of total RNA using Superscript III (Invitrogen). PCR was performed using a Light Cycler instrument and software (version 3.5, Roche Diagnostics). The sequences of PCR primers used were as follows: GFP forward, 5′-CCAGATCCGCAACATCG-3′; reverse, 5′-GTCCA-TGCGAGGTGATCC-3′. We performed duplicate PCRs using the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics). The amplification conditions for GFP included 10 s of denaturation at 95 °C, 5 s of annealing at 57 °C, and 10 s of extension at 72 °C, for a total of 35 cycles. A template-free negative control was included in each experiment. Quantitative RT-PCR primers and conditions for amplification of 28 S rRNA, *cox-2*, and *mmp9* were described previously (31, 32).

*Morpholino and RNA Microinjections*—We obtained morpholino oligonucleotides from Gene Tools LLC. Sequences for the *apc* and *cox-2* morpholinos were described previously (27, 33). The *raldh2* morpholino (5′-GTTCACTCTGGAG-GTCATC-3′) and the control morpholino (5′-CTCTTACCTCGTACATATATA-3′) were dissolved in 1× Danieau buffer and adjusted to a final concentration of 1.0 mM. For microinjections, 1.0 nl of 1.0 mM morpholino was injected into wild-type embryos at the one- to four-cell stage. Messenger RNA for Cox-2 and Apc-(1–1318) were synthesized using message mMachine (Ambion) according to the manufacturer’s instructions. Cox-2 mRNA and Apc-(1–1318) mRNA were injected into embryos at the one-cell stage.

*Drug Treatments*—To investigate the role of retinoic acid in β-catenin expression, we incubated embryos with 900 nM all-trans-retinoic acid (Sigma) in Me2SO at 50% epiboly for 1 h. After washing with embryo water, we treated the embryos with 20 nM all-trans-retinoic acid at 30 and 54 hpf for 1 h and then repeated the washing step. Inhibition of Cox-2 was accomplished by treating embryos at 50% epiboly, 30 and 54 hpf with the selective COX-2 inhibitor NS-398 (Cayman Chemical). Embryos were incubated with 5 μM PGE2 (Cayman Chemical) in Me2SO for the time periods described in the figure legends. To antagonize the EP2 receptor, embryos were treated with 3 μM AH-6809 (Cayman Chemical) in Me2SO at 50% epiboly, 30 and 54 hpf. Inhibition of the proteasome was accomplished by incubating 54-hpf embryos with 20 μM MG-132 for 18 h before harvesting.

*Antibodies and Western Blotting*—We obtained extracts from zebrafish embryos in 1× Reporter Lysis Buffer (Promega) with the aid of a Dounce homogenizer. Lysates were frozen at −80 °C and then centrifuged at 12,000 × g for 2 min to remove the insoluble components. We assessed protein concentration in the supernatants (using the BCA protein assay; Pierce) and then subjected 20–60 μg of protein to electrophoresis on 7.5% denaturing polyacrylamide gels. After transfer to polyvinylidene difluoride membranes (PolarScreen), we stained the proteins using Ponceau S solution for 2 min and then blocked the remaining protein-binding sites with 5% nonfat dry milk in TBST. The primary antibodies used throughout this study were obtained from Cayman Chemical (anti-COX-2), Cell Signaling (anti-β-catenin), and ICN Biomedicals (anti-actin). We then probed the membranes using horseradish peroxidase-labeled secondary antibody (BIOSOURCE) and a chemiluminescence detection system (Western Lightning) to reveal the presence of immunoreactive proteins.

**RESULTS**

*Retinoic Acid Negatively Regulates Expression of β-Catenin*—We reported previously that APC is also an essential regulator of retinoic acid biosynthesis (26–28) and that retinoic acid suppressed the expression COX-2 (31). Interestingly, parallel work has shown that PGE2, a COX-2 product, promotes the stabilization of β-catenin in colon cancer cell lines by displacing GSK-3β from the β-catenin destruction complex (29, 30). Based on these findings, we hypothesized that APC control of retinoic acid and COX-2 may function to regulate the stability of β-catenin and consequent downstream signaling. To examine this possibility, we supplemented developing *apc* mutant zebrafish embryos with retinoic acid and found that this reduced levels of β-catenin protein (Fig. 1A). The functional consequences of reduced β-catenin were reflected by remarkably reduced transcript levels of two β-catenin target genes, *mmp9* (Fig. 1B) and *mmp13* (not shown) (34, 35).

To test the hypothesis that the higher levels of β-catenin seen in *apc* mutant embryos resulted from a lack of retinoic acid production, we next examined the converse situation. We measured the levels of β-catenin in zebrafish *neckless* (*nls*) mutants in which disruption of the retinal dehydrogenase 2 (*raldh2*) gene results in profound retinoic acid deficiency (36). We found that *nls* embryos expressed higher levels of β-catenin compared with wild-type embryos, a result that is consistent with our observations in *apc* mutants. These findings indicated that retinoic acid deficiency alone increased β-catenin levels (Fig. 1C). This conclusion was further substantiated when we utilized TopdGFP zebrafish, a genetically engineered reporter line used as a measure of the state of activation of β-catenin (37). We found that antisense morpholino knockdown of Raldh2 in TopdGFP embryos robustly increased dGFP expression.
PGE$_2$ Regulates $\beta$-Catenin Signaling

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** The expression of $\beta$-catenin is suppressed by retinoic acid. A, immunoblot analysis for $\beta$-catenin in wild-type (WT) embryos and apc mutant embryos treated with vehicle (Veh) (Me$_2$SO) or retinoic acid (RA). $\beta$-Actin protein levels were determined for normalization purposes. Data shown are representative of at least three independent experiments. B, mmp9 mRNA levels were determined by quantitative RT-PCR using specific primers and RNA isolated from wild-type embryos, apc mutant embryos treated with vehicle (Me$_2$SO), and apc mutant embryos treated with retinoic acid (RA). Each transcript measurement was first normalized to the levels of 28 S rRNA. Relative changes in expression were determined by comparing the normalized number of mmp9 transcripts in apc mutants treated with Me$_2$SO or retinoic acid to the number of normalized mmp9 transcripts in wild-type embryos. The data are expressed as the mean ± S.D., n = 3.

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** Down-regulation of Cox-2 is required for Ap-mediated suppression of $\beta$-catenin. A, immunoblot analysis for $\beta$-catenin in wild-type (WT) embryos, and in apc mutant embryos injected with control (Con) or Cox-2-specific morpholinos (MO). $\beta$-Actin protein levels were determined for normalization purposes. Data shown are representative of at least three independent experiments. B, mmp9 mRNA levels were determined by quantitative RT-PCR using RNA isolated from wild-type embryos and apc mutant embryos injected with control (CD) or Cox-2-specific morpholinos. Each transcript measurement was first normalized to the levels of 28 S rRNA. Relative changes in expression were determined by comparing the number of normalized mmp9 transcripts in apc mutants injected with control morpholino or Cox-2-specific morpholino to the number of normalized mmp9 transcripts in wild-type embryos. The data are expressed as the mean ± S.D., n = 3.

indicating $\beta$-catenin activation (Fig. 1D). Taken together, our data suggested that Apc controls $\beta$-catenin levels by a mechanism that involves retinoic acid.

**Down-regulation of Cox-2 Is Required for Ap-mediated Inhibition of $\beta$-Catenin Expression**—Because retinoic acid suppressed Cox-2 expression in apc mutant zebrafish and given that two groups recently demonstrated that PGE$_2$ stabilized $\beta$-catenin in human colon cancer cell lines (29, 30), we next tested whether Cox-2 and PGE$_2$ served as intermediates between retinoic acid and $\beta$-catenin. Consistent with this idea, we first found that antisense morpholino-mediated knockdown of Cox-2 in apc mutant embryos significantly reduced $\beta$-catenin protein levels (Fig. 2A) (33). Similarly, inhibition of Cox-2 activity with the selective antagonist NS-398 (33) strongly suppressed $\beta$-catenin expression compared with Me$_2$SO-treated embryos (Fig. 2C). Decreased activation of the $\beta$-catenin target genes mmp9 (Fig. 2, B and D) and mmp13 (not shown) paralleled the reduced levels of $\beta$-catenin. These results demonstrated that the catalytic activity of Cox-2 was necessary for up-regulation and activation of $\beta$-catenin when the function of Apc was impaired.

**Overexpression of Cox-2 Increases Expression of $\beta$-Catenin in Wild-type Zebrafish**—To investigate if Cox-2 regulated the expression of $\beta$-catenin when the function of Apc was intact, we overexpressed Cox-2 in wild-type zebrafish embryos by injection of cRNA and then assessed the expression of $\beta$-cate-
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FIGURE 3. Overexpression of Cox-2 increases β-catenin levels in wild-type zebrafish. A, immunoblot analysis for Cox-2 and β-catenin in wild-type embryos injected with water or with cox-2 cRNA. β-Actin protein levels were determined for normalization purposes. Data shown are representative of at least three independent experiments. Con, control. B, mmp9 mRNA levels were determined by quantitative RT-PCR using specific primers and RNA isolated from wild-type embryos injected with water or with cox-2 cRNA. Each transcript measurement was first normalized to the levels of 28S rRNA. Relative changes in expression were determined by comparing the normalized number of mmp9 transcripts in wild-type embryos injected with cox-2 cRNA to the normalized number of transcripts in wild-type embryos. The data are expressed as the mean ± S.D., n = 3. C, whole mount in situ hybridization with a GFP-specific probe following injection of TOPdGFP embryos with COX-2 cRNA. Embryos shown are typical of ~50 examined of each type.

FIGURE 4. PGE₂ induction of β-catenin. A, PGE₂ levels in 72-hpf wild-type (WT) and apc mutant zebrafish embryos were analyzed by a PGE₂ enzyme immunoassay. The data are expressed as the mean ± S.D., n = 3. B, immunoblot analysis for β-catenin in wild-type and in apc mutant embryos treated with either vehicle (Veh) (Me₂SO) or an EP2 receptor antagonist (AH-6809). β-Actin protein levels were determined for normalization purposes. Data shown are representative of at least three independent experiments. C, treatment of wild-type embryos with 5 μM PGE₂, at 50% epiboly and at 24 hpf for 0.5–2 h increased β-catenin protein levels compared with treatment with vehicle (Me₂SO). β-Actin protein levels were determined for normalization purposes. D, mmp9 mRNA levels were determined by quantitative RT-PCR using specific primers and RNA isolated from wild-type embryos treated with vehicle (Me₂SO) or with 5 μM PGE₂ for 6 h. Each transcript measurement was first normalized to the levels of 28S rRNA. Relative changes in expression were determined by comparing the number of normalized mmp9 transcripts in wild-type embryos treated with PGE₂ to the number of normalized transcripts in wild-type embryos treated with Me₂SO. The data are expressed as the mean ± S.D., n = 3. E, whole mount in situ hybridization with a GFP-specific probe following treatment of TOPdGFP embryos with vehicle (Me₂SO) or with 5 μM PGE₂ for 6 h. F, apc mutant zebrafish embryos were injected with a wild-type Apc construct (Apc-(955–2075)). The levels of β-catenin were decreased as judged by immunoblot analyses. Treatment of Apc-(955–2075)-injected embryos with 5 μM PGE₂, for 6 h inhibited the ability of wild-type Apc to suppress β-catenin, resulting in increased expression of the protein. β-Actin protein levels were determined for normalization purposes. Data shown are representative of at least three independent experiments.

PGE₂ Regulates β-Catenin Signaling

Truncated Apc Is Required for β-Catenin Degradation Following Inhibition of COX-2—Mutations in APC are thought to impair formation of a functional β-catenin degradation complex. Surprisingly, we found that retinoic acid treatment, or inhibition of Cox-2 activity, each caused loss of β-catenin protein expression in apc mutant zebrafish despite the presence of a typical, truncated form of Apc. This suggested that truncated Apc may retain some ability to form a functional β-catenin degradation complex. To address this issue we first asked whether β-catenin degradation following Cox-2 inhibition required the action of the proteasome. To do so, we decreased PGE₂ accumulation in apc mutants using NS-398 but also tested the effects of MG-132, an inhibitor of the proteasome. We found that MG-132 treatment attenuated the ability of NS-398 to decrease β-catenin levels, indicating that apc mutant embryos degraded β-catenin through a proteasome-dependent process (Fig. 5A).
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To investigate whether some form of Apc was essential for degradation of β-catenin following Cox-2 inhibition, we suppressed the expression of full-length Apc in wild-type embryos using an Apc-specific splice-blocking antisense morpholino. As expected, Apc morphants expressed higher total levels of β-catenin, reflecting functional down-regulation of Apc (Fig. 5B) and higher levels of Cox-2 (data not shown and see Ref. 31). However, in contrast to our observations in apc mutant embryos, blockade of PGE₂ synthesis in Apc morphants failed to reduce levels of β-catenin (Fig. 5B). We could rescue this defect by co-injection of a cRNA encoding amino acids 1–1318 of Apc, the region precisely representing the truncated form expressed in apc mutant zebrafish. Injection of Apc morphants with Apc-(1–1318) cRNA alone had no effect on β-catenin protein levels. In contrast, injection of Apc morphants with Apc-(1–1318) cRNA along with treatment by NS-398 robustly decreased β-catenin protein levels (Fig. 5B) and expression of mmp9 (Fig. 5C). These observations indicated that Apc-(1–1318) is required for the degradation of β-catenin seen in the presence of Cox-2 inhibition. This conclusion is consistent with results shown in Fig. 2, C and D, demonstrating that inhibition of PGE₂ synthesis with a Cox-2 inhibitor restored β-catenin to nearly basal levels in the presence of truncated Apc.

DISCUSSION

We previously reported that the tumor suppressor APC is an essential regulator of retinoic acid biosynthesis (27). Additional studies indicated that APC control of retinoic acid biosynthesis occurs through the transcriptional regulator CtBP-1 and that this control is independent of β-catenin (39, 40). Although the regulation of retinoic acid biosynthesis appears independent of the actions of β-catenin, the converse may not hold true. Indeed, a number of studies suggest that retinoic acid may antagonize the actions of β-catenin. First, studies in SW480 cells indicated that activated RARs sequestered β-catenin from the TCF transcription factor, thus preventing transcription of β-catenin/TCF target genes (11). Similarly, RXR agonists appear to accelerate the destruction of β-catenin through a proteasome-dependent process (12). The mechanism leading to the destruction of β-catenin following RXR activation is currently unclear.

Recent evidence implicates retinoic acid in suppressing the expression of Cox-2 downstream of Apc, a process that presumably leads to decreased PGE₂ accumulation (31). Given the recent demonstrations that the COX-2 product, PGE₂, stabilized β-catenin (29, 30), we reasoned that retinoic acid may antagonize β-catenin by a mechanism that required attenuation of Cox-2 expression and decreased accumulation of PGE₂. We tested this possibility using three independent approaches as follows: down-regulation of Cox-2 expression, inhibition of Cox-2 enzymatic activity, and Cox-2 overexpression. The results of our studies consistently supported a role for Cox-2 as a positive regulator of β-catenin signaling. These findings extend previous studies and support a model wherein retinoic acid suppresses β-catenin signaling by inhibiting the expression of Cox-2.

Castellone et al. (29) recently elucidated the mechanism by which PGE₂ stabilizes β-catenin in colorectal carcinoma cells. They showed that PGE₂ promoted the dissociation of GSK-3β from axin within the β-catenin destruction complex. This dissociation prevented GSK-3β-mediated phosphorylation and degradation of β-catenin. Details concerning the interplay between other complex members were not addressed. Our findings showed that the enzymatic activity of Cox-2 was necessary for stabilization of β-catenin in vivo. We found that treatment of wild-type zebrafish embryos with PGE₂ increased β-catenin protein levels and activated β-catenin target genes in vivo. Conversely, reducing PGE₂ levels in apc mutant zebrafish by inhibiting Cox-2 resulted in degradation of β-cate-
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