

PPAR δ Is an APC-Regulated Target of Nonsteroidal Anti-Inflammatory Drugs

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Summary

PPAR δ was identified as a target of APC through the analysis of global gene expression profiles in human colorectal cancer (CRC) cells. PPAR δ expression was elevated in CRCs and repressed by APC in CRC cells. This repression was mediated by β -catenin/Tcf-4-responsive elements in the PPAR δ promoter. The ability of PPARs to bind eicosanoids suggested that PPAR δ might be a target of chemopreventive nonsteroidal anti-inflammatory drugs (NSAIDs). Reporters containing PPAR δ -responsive elements were repressed by the NSAID sulindac. Furthermore, sulindac was able to disrupt the ability of PPAR δ to bind its recognition sequences. These findings suggest that NSAIDs inhibit tumorigenesis through inhibition of PPAR δ , the gene for which is normally regulated by APC.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer deaths in the United States. Recent advances in the understanding of CRC have raised expectations that this growing knowledge might lead to improved cancer prevention. In this regard, the identification of genetic alterations that underlie the initiation of colorectal tumors and of drugs which can prevent colorectal tumors show particular promise. Here, we suggest a molecular basis for the convergence of these two heretofore separate lines of investigation.

A growing body of evidence has shown that nonsteroidal anti-inflammatory drugs (NSAIDs) can suppress colorectal tumorigenesis in both humans and rodents (reviewed in Smalley and DuBois, 1997; Thun, 1997). In the general population, epidemiological studies have documented a decreased risk of CRC deaths associated with use of the NSAID aspirin. In individuals with familial adenomatous polyposis (FAP), an inherited predisposition to multiple colorectal polyps, the NSAID sulindac can reduce both the size and number of colorectal tumors. Likewise, sulindac and other NSAIDs have proven to be effective in prevention of intestinal tumorigenesis in mouse models of FAP. The molecular basis for these striking chemopreventive effects has been attributed to inhibition of cyclooxygenases (COX) and the resulting decrease in prostaglandin production (reviewed in Prescott and White, 1996). Consistent with this, expression

of COX-2 is elevated in human colorectal tumors (Eberhart et al., 1994; Sano et al., 1995), and inactivation of the COX-2 gene in mice is associated with decreased intestinal tumorigenesis (Oshima et al., 1996).

However, other observations are difficult to reconcile with COX being the sole target of NSAIDs in the colon. For example, NSAID derivatives that lack the ability to inhibit COX have been shown to inhibit colon tumor growth in vivo and in vitro (Piazza et al., 1995, 1997; Mahmoud et al., 1998; Reddy et al., 1999). Conversely, colon cancer cells totally devoid of COX activity are growth inhibited as effectively as cells producing COX (Hanif et al., 1996; Elder et al., 1997). Likewise, COX-1 and COX-2 null mouse embryo fibroblast cells remain sensitive to the antiproliferative and antineoplastic effects of NSAIDs (Zhang et al., 1999). In those colon cancer cells producing COX, COX-produced prostaglandins cannot rescue cells from NSAID-associated growth arrest in vivo or in vitro (Narisawa et al., 1984; Hanif et al., 1996; Chan et al., 1998). The concentration of NSAIDs that inhibit growth is 10 to 100 times higher than that required to inhibit COX activity, suggesting the existence of additional cellular targets (Hanif et al., 1996; Ahnen, 1998; Charalambous et al., 1998; Simmons et al., 1999). Finally, many studies have demonstrated that the COX-2 protein is elevated in the neoplastic epithelial cells of human tumors (Eberhart et al., 1994; Sano et al., 1995), while COX-2 expression in mouse intestinal tumors is confined to nonneoplastic stromal cells (Oshima et al., 1996; Shattuck-Brandt et al., 1999). Thus, the mouse and human COX-2 proteins are located within disparate cellular populations of the colon, yet tumorigenesis in both species is prevented by the same NSAIDs. This suggests either that it is coincidental that the same agents can exert chemopreventive effects in mouse and human, or that other targets of NSAIDs, common to the neoplastic cells of both species, might exist and provide a link to the molecular pathogenesis described below.

Molecular genetic studies have identified a series of genetic alterations associated with the development of benign tumors (adenomas) and their progression to malignant disease (carcinomas) (reviewed in Kinzler and Vogelstein, 1996). In terms of prevention, the alterations that occur early in this process are of most interest. Inactivating mutations of the APC tumor suppressor pathway occur early and are found in most colorectal adenomas and carcinomas. Moreover, inherited mutations of APC cause FAP, characterized by the development of hundreds to thousands of colorectal adenomas. Several studies have suggested that APC's association with β -catenin (Rubinfeld et al., 1993; Su et al., 1993) might be critical to its tumor-suppressive effects. In the colon, β -catenin binds to the Tcf-4 transcription factor, providing a domain that activates genes containing Tcf-4-binding sites in their regulatory regions (Behrens et al., 1996; Molenaar et al., 1996). Wild-type APC can promote the degradation of β -catenin (Munemitsu et al., 1995) and inhibit β -catenin/Tcf-4 regulated transcription

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(CRT), while disease-associated APC mutants are deficient in this ability (Korinek et al., 1997; Morin et al., 1997). In tumors lacking APC mutations, oncogenic mutations of β -catenin can lead to increased CRT (Morin et al., 1997; Rubinfeld et al., 1997).

The targets of this increased CRT are therefore likely to provide insights into APC's suppressive effects. We have recently used SAGE technology to analyze changes in gene expression following inhibition of CRT by APC in human CRC cells and identified the *c-myc* oncogene as a direct target of CRT (He et al., 1998a). However, like many other critical regulators of cell growth, APC is likely to exert its effects through several effectors. Here, we report the identification of another target of the APC pathway, peroxisome proliferator-activated receptor δ (*PPAR δ* ; a.k.a. *PPAR β* , *NUC1*, and *FAAR*) (Schmidt et al., 1992; Amri et al., 1995; Jow and Mukherjee, 1995), which provides a link between NSAID-mediated chemoprevention and the genetic alterations identified in colorectal tumors.

PPAR δ belongs to the nuclear receptor superfamily, which includes the steroid hormone, thyroid hormone, retinoid, and PPAR subfamilies as well as a growing number of orphan receptors (Kastner et al., 1995; Mangelsdorf et al., 1995; Lemberger et al., 1996). The PPAR subfamily comprises at least three distinct subtypes found in vertebrate species: *PPAR α* , *PPAR δ* , and *PPAR γ* . The nuclear receptor family members function as ligand-dependent sequence-specific activators of transcription (Mangelsdorf et al., 1995; Lemberger et al., 1996). We found that the NSAIDs sulindac and indomethacin could mimic the effects of APC by downregulating the transcriptional activity of *PPAR δ* . This inhibition appeared to be due to disruption of the DNA binding ability of *PPAR δ* /RXR heterodimers. These observations demonstrate that APC and NSAIDs inhibit a mutual target, *PPAR δ* , thereby providing a link between the genetic alterations underlying tumor development and cancer chemoprevention.

Results

APC Represses *PPAR δ* Expression

The effects of APC on gene expression were explored using SAGE as previously reported (He et al., 1998a). In brief, gene expression was examined in a human CRC cell line with inducible APC (HT29-APC) and a control cell line with an inducible *lacZ* gene (HT29- β -gal) 9 hr after induction. SAGE analysis of 55,233 and 59,752 tags from APC-expressing and control cells, respectively, led to the identification of 14,346 different transcripts, the majority of which were not differentially expressed. Because biochemical studies have indicated that APC directly represses CRT, we focused on the repressed transcripts. One of the most highly repressed tags corresponded to *PPAR δ* (24 tags in HT29- β -gal versus 5 tags in HT29-APC).

To confirm the SAGE data, we performed Northern blot analysis of RNA from HT29-APC and HT29- β -gal cells using *PPAR* probes (Figure 1A). Repression of *PPAR δ* was evident as early as 3 hr after APC induction, whereas no change was detectable in HT29 β -gal cells even 9 hr after induction. In contrast, expression of

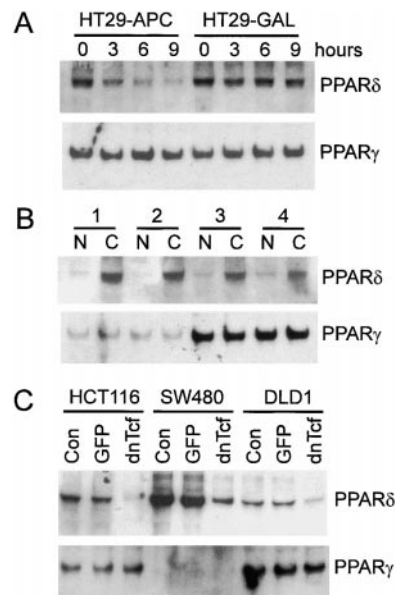


Figure 1. Expression of *PPAR δ* in Human CRC Cells

(A) Decreased expression of *PPAR δ* following induction of APC in CRC cells. Expression of APC (HT29-APC) or β -galactosidase (HT29-GAL) was induced with (110 μ M) ZnCl₂ for the indicated times in HT29 CRC cells containing the respective genes under the control of a modified metallothionein promoter. Total RNA (10 μ g) was isolated and analyzed by Northern blot analysis with probes specific for *PPAR δ* and *PPAR γ* .

(B) Increased expression of *PPAR δ* in primary CRCs. Northern blot analyses with probes specific to *PPAR δ* and *PPAR γ* were performed on total RNA (10 μ g) isolated from matched primary CRCs (C) and normal colon epithelium (N) removed from four different patients.

(C) Expression of *PPAR δ* in CRCs is dependent on Tcf-4-mediated transcription. CRC cells with increased β -catenin/Tcf-4-mediated transcription due to either APC (SW480, DLD1) or β -catenin (HCT116) mutations were either mock infected (Con) or infected with adenovirus expressing GFP (GFP) or a dominant-negative mutant of Tcf-4 (dnTcf). Total RNA (10 μ g) was isolated and analyzed by Northern blot analysis with probes specific for *PPAR δ* and *PPAR γ* .

PPAR γ was not affected by expression of APC, and the other known PPAR subfamily member, *PPAR α* , was not expressed at detectable levels (Figure 1A and data not shown). The ability of APC to repress *PPAR δ* expression suggested that expression of *PPAR δ* should be elevated in primary CRCs, where CRT is often increased by mutations in APC or β -catenin. Northern blot analysis of *PPAR* expression in CRCs and normal colorectal mucosa revealed a marked increase in *PPAR δ* expression in each of four cancers studied (Figure 1B). In contrast, there was no increase in *PPAR γ* expression in the cancers of these patients (Figure 1B).

APC Inhibits CRT of the *PPAR δ* Gene

To explore the basis for the repression of *PPAR δ* , we isolated and sequenced a 3.1 kb genomic region upstream of the *PPAR δ* transcription start site (GenBank accession #AF187850) and used it to analyze APC responsiveness (Figure 2). A luciferase reporter construct containing this fragment (BE) upstream of a minimal promoter was markedly repressed by APC expression (Figures 2A and 2B). Analysis of nested deletions and promoter fragments identified two APC-responsive frag-

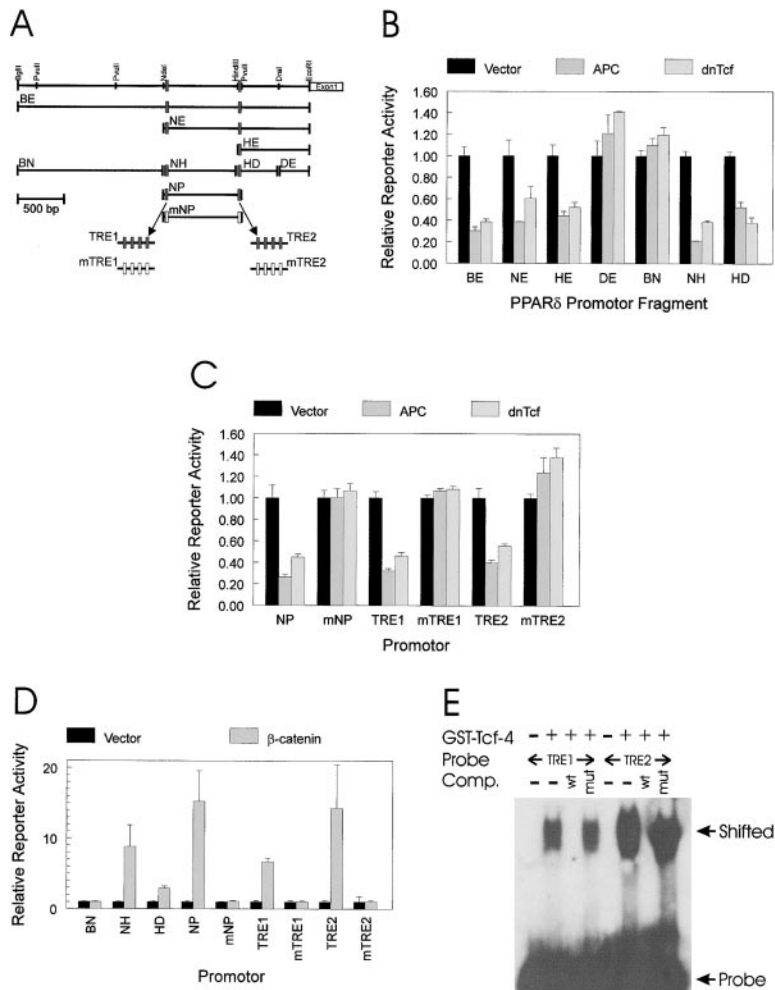


Figure 2. APC Regulates *PPAR* δ Expression through CRT

(A) *PPAR* δ promoter. A restriction map of the 3.1 kb region upstream of the first exon of *PPAR* δ is shown. Restriction fragments BE, NE, HE, DE, BN, NH, HD, and NP were used to construct reporters for measuring APC and β -catenin responsiveness. Filled boxes represent potential Tcf-4-binding sites, and open boxes represent the same sites engineered to contain mutations that abolish Tcf-4 binding. mNP represents fragment NP with both potential Tcf-4-binding sites mutated. TRE1 and TRE2 contain four repeats of the two Tcf-4-binding sites, respectively. mTRE1 and mTRE2 are mutant forms of TRE1 and TRE2. (B) The *PPAR* δ promoter is repressed by APC and dnTcf. SW480 CRC cells were transfected with the indicated *PPAR* δ promoter luciferase reporters (0.4 μ g), with a β -galactosidase expression vector (0.2 μ g pCMV β), and with 1.0 μ g of either a control (Vector), APC, or dnTcf expression vector. Luciferase activity is reported relative to the vector control after normalizing for transfection efficiency through β -galactosidase activity. Bars represent the means of three independent replicates with error bars representing the unbiased standard deviations.

(C) APC and dnTcf responsiveness is mediated by two putative Tcf-4-binding sites. *PPAR* δ promoter fragments with intact and mutated Tcf-4-binding sites were tested for APC and dnTcf-4 responsiveness as described in (B). Bars represent the means of three independent replicates, with error bars representing the unbiased standard deviations.

(D) β -catenin transactivation maps to the same promoter regions mediating APC and dnTcf responsiveness. 293 cells were transfected with the indicated *PPAR* δ promoter

luciferase reporters (0.4 μ g), with a β -galactosidase expression vector (0.2 μ g pCMV β), and with 1.0 μ g of either a no insert control (Vector) or an oncogenic β -catenin (β -catenin) expression vector. Luciferase activity was reported as described for (B). Bars represent the means of three independent replicates, with error bars representing the unbiased standard deviations.

(E) Putative Tcf-4-binding sites in the *PPAR* δ promoter bind Tcf-4. GEMSA was performed using 32 P-labeled probes containing either putative Tcf-4-binding sites TRE1 or TRE2. GEMSA was performed in the presence of a GST fusion protein containing the Tcf-4 DNA-binding domain as indicated. Wild-type (wt) or mutant (mut) competitors corresponding to the Tcf-4-binding sites were used as indicated.

ments (fragment NH and HD, Figures 2A and 2B). The sequence of these fragments revealed two putative Tcf-4-binding sites, one (TRE1) located 1543 bp upstream of the *PPAR* δ transcription start site in fragment NH and the other (TRE2) located 759 bp upstream in fragment HD. A fragment spanning these two sites conferred marked APC repression, which was completely abrogated by disruption of the putative Tcf-4-binding sites (NP versus mNP, Figure 2C). Moreover, either of the putative Tcf-4-binding sites in isolation could confer APC responsiveness in a sequence-specific manner (TRE1 versus mTRE1, and TRE2 versus mTRE2 in Figure 2C).

As noted above, an obvious basis for the APC responsiveness of *PPAR* δ would be inhibition of CRT. Consistent with this, there was a perfect concordance between the ability of APC to repress *PPAR* δ promoter fragments (Figures 2B and 2C) and the ability of oncogenic β -catenin to induce transcriptional activity (Figure 2D). Likewise, there was a perfect concordance between

APC responsiveness and the ability of a dominant-negative Tcf-4 (dnTcf) expression vector to inhibit transcriptional activity (Figures 2B and 2C). As with APC responsiveness, the β -catenin transactivation and the dnTcf repression were abrogated by mutation of the putative Tcf-4-binding sites (Figures 2C and 2D). The ability of Tcf-4 to directly bind to the *PPAR* δ TRE sites was demonstrated by gel electrophoresis mobility shift assays (GEMSA). Both putative binding sites demonstrated significant Tcf-4 binding, which was inhibited by their cognate wild-type binding sequences but not by their mutant counterparts (Figure 2E).

The above results suggested that APC repressed *PPAR* δ expression by interfering with CRT and that alterations in this pathway could lead to increased expression of *PPAR* δ in CRCs. To further evaluate the generality of this pathway, we examined the ability of dnTcf to interfere with *PPAR* δ expression in other human CRC cell lines. Like the HT29 cells in which *PPAR* δ expression was first identified (Figure 1A), SW480 and DLD1 cells

contain inactivating mutations of APC. HCT116 cells have an activating mutation of β -catenin. As expected from the study of primary tumors (Figure 1B), *PPAR δ* expression was detected in all the lines (Figure 1C). Moreover, *PPAR δ* expression was inhibited in each line by infection with an adenovirus containing a dnTcf expression cassette but not by a control adenovirus containing a GFP expression cassette (Figure 1C). In contrast, *PPAR γ* expression was barely detectable in SW480 cells, and dnTcf had no effect on *PPAR γ* expression in any of the lines tested.

Definition of *PPAR δ* -Responsive Elements

To explore the functional significance of *PPAR δ* repression, we developed reporters for *PPAR δ* function. Although downstream targets of *PPAR δ* are unknown, studies of other PPAR family members have defined a prototypic response element. Maximum DNA binding and activation are achieved through heterodimerization between a PPAR protein and RXR (Gearing et al., 1993). Accordingly, the prototypic PPAR response element ACO from the acyl-CoA oxidase gene promoter contains two copies of the core binding sequence AGGTCA separated by one base pair (Tugwood et al., 1992; Mangelsdorf et al., 1995; Lemberger et al., 1996; Juge-Aubry et al., 1997). *PPAR α* and *PPAR γ* bind this consensus efficiently, whereas *PPAR δ* does not (see below). To define a *PPAR δ* -responsive element, we performed *in vitro* binding site selection for both *PPAR δ* and *RXR α* . Analysis of 28 binding sites selected with a *RXR α* GST fusion protein identified (A/G)GGTCA as the core consensus for *RXR* (Figure 3A). Analysis of 20 sites selected with a *PPAR δ* GST fusion protein revealed a novel binding consensus (CGCTCAC), which was distinct from the previously defined *PPAR α/γ* consensus (Figure 3B).

The combination of the *PPAR δ* and *RXR α* consensus sequences should form a *PPAR δ* -binding element *in vitro* and a *PPAR δ* -responsive element *in vivo*. We generated a putative *PPAR δ* -responsive element (DRE, 5'-CGCTCACAGGTCA-3') by joining the *PPAR δ* - and *RXR α* -binding sites. GEMSA analysis of DRE revealed binding to *PPAR δ* GST fusion protein but not to *PPAR α* or *PPAR γ* GST fusion proteins (Figure 3C). In contrast, the prototypic PPAR-responsive element ACO (5'-AGG ACAAGGTCA-3') bound *PPAR α* and *PPAR γ* but not *PPAR δ* GST fusion proteins (Figure 3C). *RXR α* GST fusion protein demonstrated weaker binding to both responsive elements. To further test the specificity of these elements, we performed GEMSA analysis with *in vitro* translated *PPAR δ* /*RAR α* and *PPAR γ* /*RAR α* heterodimers in the presence or absence of ligand stimulation. Under the conditions used, binding of *PPAR δ* /*RXR α* and *PPAR γ* /*RXR α* heterodimers to their cognate elements could not be detected in the absence of ligand. However, *PPAR δ* /*RXR α* binding to DRE was markedly induced by the *PPAR δ* ligand cPGI, and *PPAR γ* /*RXR α* binding to ACO was induced by the *PPAR γ* ligand BRL49653 (Figure 3D). In contrast, *PPAR γ* /*RXR α* heterodimers did not bind DRE in the presence of BRL49653 nor did *PPAR δ* /*RXR α* bind ACO in the presence of cPGI. To test the specificity of these response elements in cells, we constructed luciferase reporters containing either the DRE or ACO elements. Transfection of 293 cells with *PPAR δ* resulted in activation of the DRE reporter that was enhanced by cPGI (Figure 3E) but did not activate the ACO

reporter even in the presence of cPGI (Figure 3F). In contrast, expression of *PPAR γ* did not activate the DRE reporter (Figure 3E) but did activate the ACO reporter that was enhanced by BRL 49653 (Figure 3F).

PPAR δ Function Is Regulated by the APC/ β -Catenin/Tcf-4 Pathway

The above findings suggested that *PPAR δ* activity was regulated by APC/ β -catenin/Tcf-4 pathway at the transcriptional level. To address the functional consequences of this transcriptional regulation in CRC cells, we used the *PPAR δ* -specific reporters described above. Transfection of *APC* into a human CRC cell line resulted in downregulation of the *PPAR δ* reporter DRE but had no effect on the *PPAR α/γ* -responsive reporter ACO (Figure 4A). The lack of any effect on *PPAR α/γ* demonstrated the specificity of this inhibition and made it unlikely that the effects were due to nonspecific toxicity of a tumor suppressor. Transfection of a *dnTcf-4* expression vector also specifically repressed the *PPAR δ* reporter but not the *PPAR α/γ* reporter. To further eliminate the possibility of nonspecific toxic effects, we tested β -catenin's ability to positively regulate *PPAR δ* activity. Expression of oncogenic β -catenin mutants activated the *PPAR δ* reporter but did not activate the *PPAR α/γ* reporter (Figure 4B).

NSAIDs Suppress *PPAR δ* Activity

Suppression of colorectal tumorigenesis by NSAIDs suggested that these compounds may be linked to the genetic alterations that drive tumorigenesis. The identification of *PPAR δ* as a target of the APC tumor suppressor pathway suggested a specific link. Both precursors and products involved in eicosanoid metabolism have been shown to be ligands for PPARs (Keller et al., 1993; Yu et al., 1995; Forman et al., 1997; Kliewer et al., 1997). The ability of NSAIDs to perturb eicosanoid metabolism suggested that PPARs may be an ultimate target of NSAIDs in suppressing tumorigenesis (Prescott and White, 1996), and the above findings suggest that *PPAR δ* could be a specific target. To explore this possibility, we tested the effects of the NSAID sulindac on *PPAR δ* function. As noted in the Introduction, sulindac has been shown to suppress intestinal tumorigenesis in humans and mice. In culture, sulindac sulfide, the active metabolite of sulindac, has been shown to induce apoptosis in CRC cells (Piazza et al., 1995; Shiff et al., 1995; Hanif et al., 1996; Chan et al., 1998). Sulindac sulfide treatment resulted in a dose-dependent repression of *PPAR δ* activity in CRC cells, as assessed with the DRE reporter (Figure 4C). A greater than 2-fold repression was observed at low concentrations of sulindac sulfide, and a greater than 10-fold reduction was noted at levels of sulindac sulfide that induced substantial degrees of apoptosis in these cells (Figures 4C and 5D). In contrast, sulindac sulfide had only a modest effect (less than 25% repression at the highest concentration tested) on *PPAR α/γ* activity, assessed with the ACO reporter (Figure 4C). A similar dose-dependent suppression of *PPAR δ* was observed with indomethacin, another NSAID (Figure 4C).

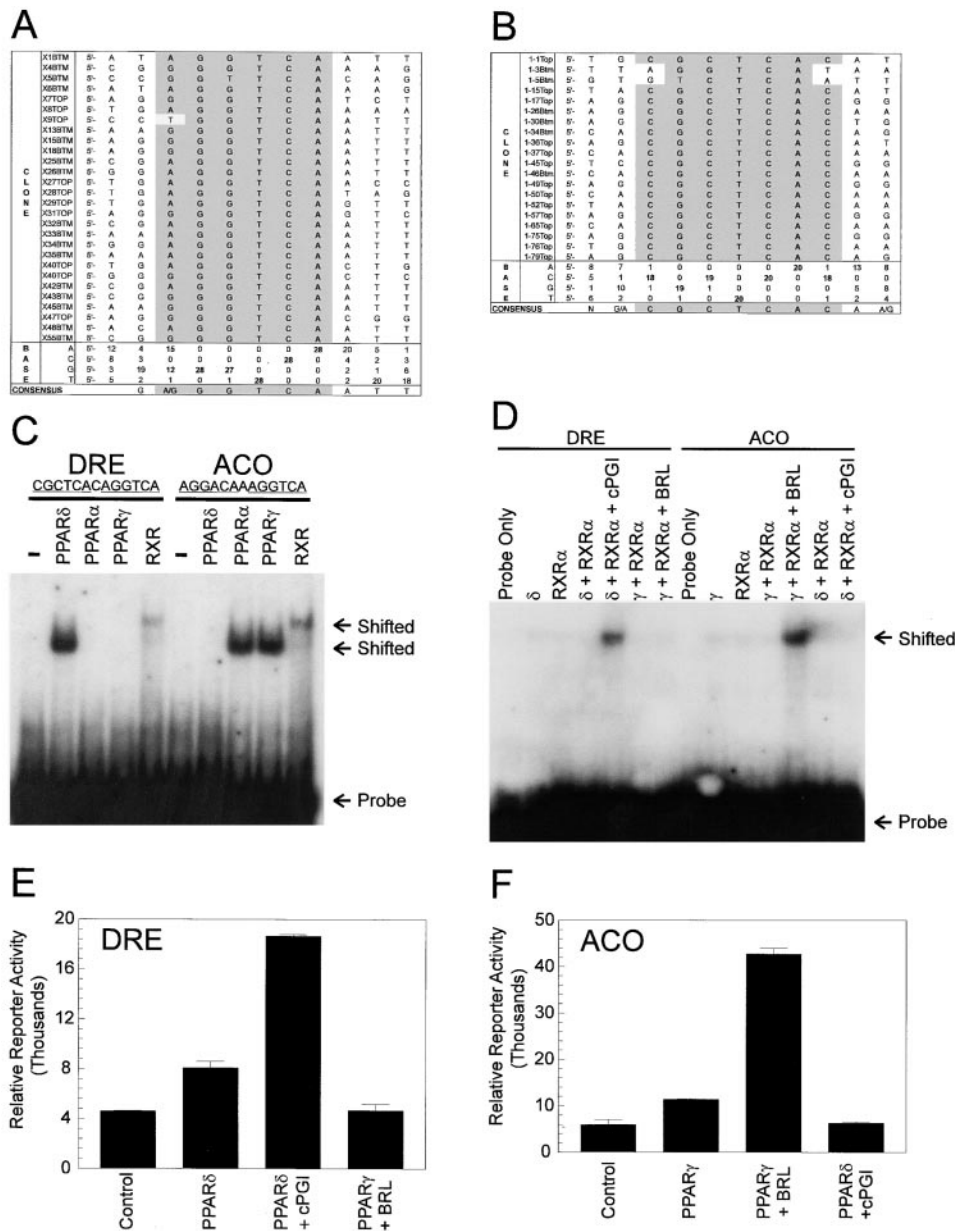


Figure 3. Development of a PPAR δ -Specific Reporter

(A) RXR α consensus binding site. PCR products of a randomized oligonucleotide template that bound a GST fusion protein containing the DNA-binding domain of RXR α were selected, cloned, and sequenced. The sequences of 28 clones are shown, manually aligned to derive the consensus binding sequence indicated at the bottom.

(B) PPAR δ consensus binding site. PCR products of a randomized oligonucleotide template that bound a GST fusion protein containing the DNA-binding domain of PPAR δ were selected, cloned, and sequenced. The sequences of 20 clones are shown, manually aligned to derive the consensus binding sequence indicated at the bottom.

(C) Binding specificity of PPAR α , PPAR δ , and PPAR γ . Oligonucleotides containing the indicated binding elements (DRE or ACO) were ³²P-labeled and incubated with GST fusion proteins containing either the PPAR α , PPAR δ , PPAR γ , RXR α , or no DNA binding domain (-). DNA binding was assessed by GEMSA, where "Probe" indicates the unbound probe and "Shifted" indicates bound probe.

(D) Binding specificity of PPAR δ /RXR α and PPAR γ /RXR α heterodimers. Oligonucleotides containing the indicated binding elements (DRE or ACO) were ³²P-labeled and incubated with in vitro translated PPAR δ , PPAR γ , and RXR α as indicated. Bindings were supplemented with PPAR δ ligand cPGI (10 μ M) or PPAR γ ligand BRL 49653 (10 μ M) as indicated. DNA binding was assessed by GEMSA, where "Probe" indicates the unbound probe and "Shifted" indicates bound probe.

(E) DRE confers PPAR δ but not PPAR γ responsiveness. 293 cells were transfected with DRE luciferase reporter (0.3 μ g), a β -galactosidase expression vector (0.2 μ g pCMV β), and with 1.0 μ g of either empty vector (Control), PPAR δ , or PPAR γ expression vectors. Where indicated, cells were treated with the PPAR δ ligand cPGI (20 μ M) or the PPAR γ ligand BRL 49653 (20 μ M). Luciferase activity was reported as relative luciferase activity after correction for transfection efficiency using β -galactosidase activity. Bars represent the means of three independent replicates with the error bars representing the unbiased standard deviations.

(F) ACO confers PPAR γ but not PPAR δ responsiveness. The same as in (E) except an ACO luciferase reporter was used.

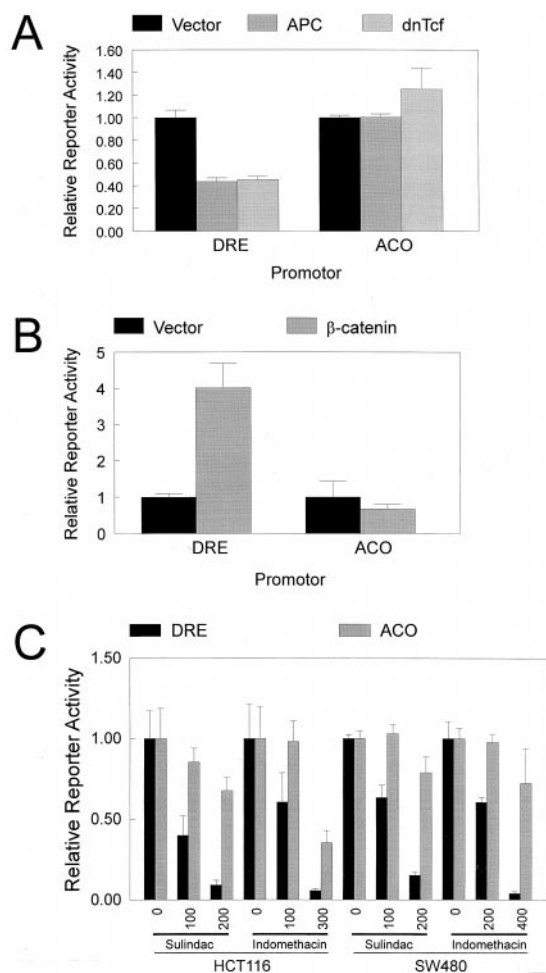


Figure 4. PPAR δ Activity Is Regulated by APC, β -Catenin, and Sulindac

(A) APC and dnTcf specifically repress PPAR δ activity. PPAR δ and PPAR γ activity was assessed with the DRE and ACO luciferase reporters, respectively. SW480 CRC cells were transfected with the indicated luciferase reporters (0.4 μ g of DRE or ACO), with a β -galactosidase expression vector (0.2 μ g pCMV β), and with 1.0 μ g of either a control (Vector), APC, or dnTcf expression vector. Luciferase activity was calculated as described in Figure 2. Bars represent the means of three independent replicates with the error bars being the unbiased standard deviations.

(B) β -catenin expression increases PPAR δ activity. The 293 human cell line was transfected with the indicated luciferase reporters (0.4 μ g of DRE or ACO), with a β -galactosidase expression vector (0.2 μ g pCMV β), and with 0.8 μ g of either a no insert control (Vector) or an oncogenic β -catenin expression vector.

(C) Sulindac and indomethacin specifically repress PPAR δ activity. PPAR δ and PPAR γ activity was assessed as transcriptional activity of the DRE and ACO luciferase reporters, respectively. HCT116 and SW480 CRC cells were transfected with the indicated luciferase reporters (1.0 μ g of DRE or ACO) and with a β -galactosidase expression vector (0.2 μ g of pCMV β). Cells were allowed to recover for 20 hr after transfection and then treated for 10 hr with the indicated concentrations (μ M) of sulindac sulfide or indomethacin. Luciferase activity was reported relative to the control (0) after normalizing for transfection efficiency.

Expression of PPAR δ Partially Rescues Sulindac Sulfide-Induced Apoptosis

If suppression of PPAR δ activity were contributing to sulindac-induced apoptosis, overexpression of PPAR δ might be expected to protect against sulindac sulfide-induced apoptosis. We constructed an adenovirus (AdPPAR δ) expressing PPAR δ and a green fluorescent protein (GFP) marker using AdEasy technology (He et al., 1998b). The ability of AdPPAR δ to suppress sulindac sulfide-induced apoptosis was compared to that of AdGFP, which contained only the GFP marker gene. AdPPAR δ produced nearly a 5-fold decrease in apoptosis in HCT116 cells treated with 100 or 125 μ M sulindac sulfide (Figures 5A–5D). Similar results were obtained with the SW480 cell line (Figure 5D). However, the suppression of apoptosis could be overridden at higher concentrations of sulindac sulfide (150 μ M, Figure 5D). The results were further confirmed and extended by the ability of AdPPAR δ to rescue inhibition of clonal cell growth by sulindac sulfide. Treatment of cells with sulindac sulfide resulted in a greater than 4-fold decrease in the number of colonies (Figure 5E). At 100 or 125 μ M sulindac sulfide but not higher doses, this decrease could be completely rescued by infection with AdPPAR δ , which actually resulted in a slightly increased number (~15%) of colonies. In contrast, the APC target and prototypic oncogene *c-myc* could not rescue the sulindac-mediated inhibition of clonal growth.

Sulindac Sulfide Disrupts the DNA Binding Ability of PPAR δ /RXR α Heterodimers

To determine whether sulindac could inhibit the transcription of PPAR δ like APC, we examined expression of PPAR δ following sulindac sulfide treatment. Concentrations of sulindac sulfide that resulted in suppression of PPAR δ activity and apoptosis had no effect on the level of PPAR δ transcripts, excluding this possibility (Figure 6A). Because PPARs can bind eicosanoids (Keller et al., 1993; Yu et al., 1995; Forman et al., 1997; Kliewer et al., 1997), the effects of NSAIDs on PPAR δ could be due to their ability to perturb eicosanoid metabolism. However, several studies (see Discussion) have suggested that the chemopreventive effects of NSAIDs are not simply related to their ability to suppress prostaglandin synthesis. We therefore considered the possibility that NSAIDs directly inhibit PPAR δ activity. This notion was supported by the ability of some NSAIDs to activate PPAR α/γ (Lehmann et al., 1997) and of indomethacin to alter the ligand responsiveness of TetR/PPAR δ fusion proteins (Yu et al., 1995). We tested the ability of sulindac to inhibit cPGI-stimulated PPAR δ /RXR α heterodimer DNA binding activity in vitro. Sulindac sulfide was able to inhibit binding of PPAR δ /RXR α heterodimers to the DRE element (Figure 6B). Binding to DRE was also inhibited by the NSAID indomethacin and, at higher concentrations, the sulindac sulfide-related compound sulindac sulfone (Figure 6B). The relative concentrations of sulindac sulfide, indomethacin, and sulindac sulfone required to inhibit binding to DRE were roughly concordant with the concentrations required to induce apoptosis in CRC cells, with sulindac sulfide being the most potent and sulindac sulfone the least (Figures 5D

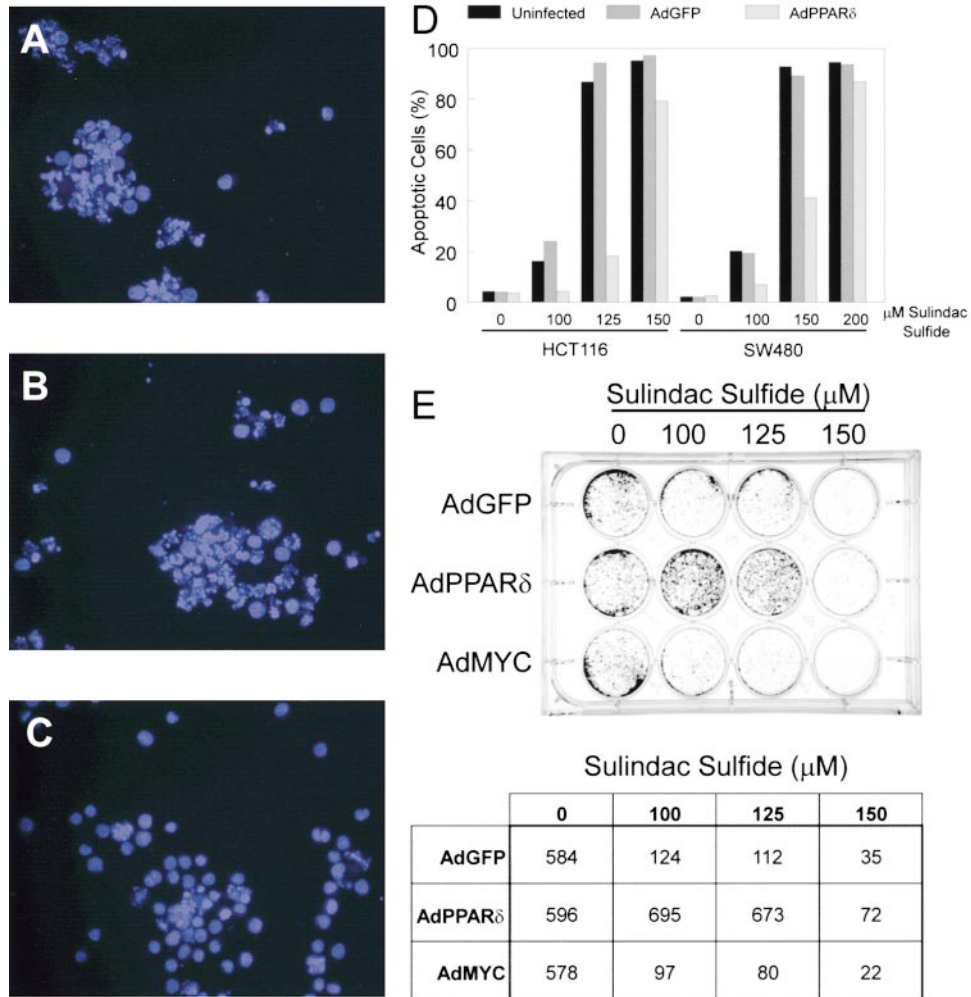


Figure 5. PPAR δ Can Protect Colon Cancer Cells from Sulindac-Induced Apoptosis

(A–D) PPAR δ inhibits sulindac sulfide–induced apoptosis. HCT116 and SW480 cells were either mock infected (Uninfected) or infected with adenovirus expressing GFP (AdGFP) or PPAR δ (AdPPAR δ). Twenty hours after infection, cells were treated for 42 hr with sulindac sulfide. Apoptosis was assessed by the presence of apoptotic nuclei (condensation and fragmentation) after Hoechst 33258 staining. (A–C) Fluorescence microscopy of uninfected (A), AdGFP (B), or AdPPAR δ (C) infected HCT116 cells treated with 125 μ M of sulindac sulfide. (D) Bars represent the fraction of apoptotic nuclei after treatment with the indicated adenoviruses and concentration of sulindac sulfide (μ M).

(E) PPAR δ rescues sulindac sulfide inhibition of clonal growth. Cells were infected with the indicated adenovirus, treated with the indicated concentrations of sulindac sulfide, and plated. Clonal growth was scored as colony formation after 6 days. Colonies were visualized by staining with Crystal Violet (upper panel) and enumerated (lower panel).

and 6B; data not shown). None of these drugs had any effect on BRL49653-stimulated binding of PPAR γ /RXR α heterodimers in an analogous assay performed with the ACO element (Figure 6C).

Discussion

The above results demonstrate that PPAR δ is a target of both APC and NSAIDs and suggest a model of how APC and NSAIDs operate to suppress intestinal tumorigenesis (Figure 7). In most CRCs, inactivating mutations of APC lead to elevated levels of CRT (Korinek et al., 1997; Morin et al., 1997). In rare CRCs without APC mutations, β -catenin mutations that render it resistant to APC-mediated degradation result in elevated β -catenin/Tcf-4-mediated transcription (Morin et al., 1997). In either case, this increased β -catenin/Tcf-4 activity leads

to increased transcription of growth-promoting genes. Accordingly, restoration of APC function to CRC cells with defective APC function results in growth suppression and apoptosis (Morin et al., 1996). The genes that have been postulated to mediate the growth-promoting effects of β -catenin/Tcf-4 activity include those encoded by the *c-myc* oncogene (He et al., 1998a), the *cyclin D1* gene (Tetsu and McCormick, 1999), and others (*WISP*, *c-jun*, and *fra-1*) (Pennica et al., 1998; Mann et al., 1999). The present findings suggest that PPAR δ represents a β -catenin/Tcf-4 target with particular importance for chemoprevention. Whereas APC or β -catenin mutations can result in increased PPAR δ activity, NSAIDs can compensate for this defect by suppressing PPAR δ activity and promoting apoptosis. This suppression of PPAR δ is mediated in part by the ability of some NSAIDs to directly inhibit the DNA binding activity of PPAR δ . In

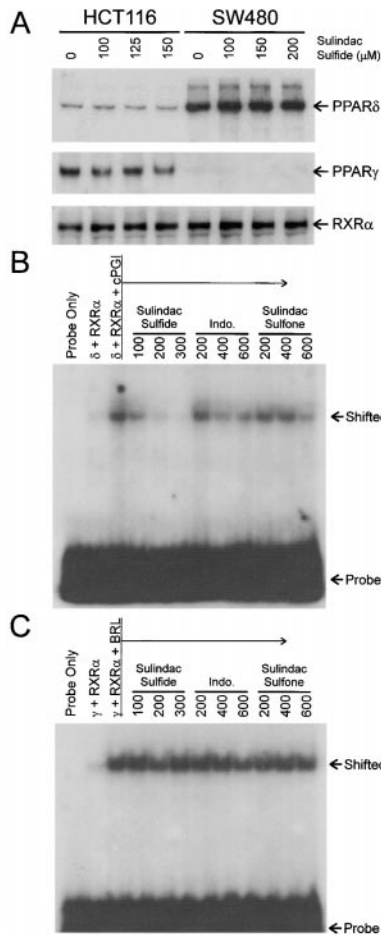


Figure 6. Mechanism of Suppression of PPAR δ by NSAIDs
(A) NSAIDs do not affect PPAR δ , PPAR γ , or RXR α expression. HCT116 and SW480 cells were treated with the indicated concentration (μ M) of sulindac sulfide for 36 hr, and RNA was isolated. Northern blot analysis was performed on 10 μ g of total RNA with a probe specific for PPAR δ , PPAR γ , or RXR α .
(B) NSAIDs suppress PPAR δ DNA binding. The DRE-binding element was 32 P-labeled and incubated with no lysate (Probe only) or in vitro translated PPAR δ (δ), RXR α (RXR α), or both (δ + RXR α). PPAR δ + RXR α + cPGI (10 μ M) was included in all lysates treated with the indicated NSAIDs. DNA binding was assessed by GEMSA, where "Probe" indicates the unbound probe and "Shifted" indicates bound probe.
(C) NSAIDs do not suppress PPAR γ DNA binding. The ACO-binding element was 32 P-labeled and incubated with no lysate (Probe only) or in vitro translated PPAR γ (γ), RXR α (RXR α), or both (γ + RXR α). PPAR γ + RXR α + BRL 49653 (10 μ M) was included in all lysates treated with the indicated NSAIDs. DNA binding was assessed by GEMSA, where "Probe" indicates the unbound probe and "Shifted" indicates bound probe.

addition, because fatty acids and eicosanoids can act as ligands and modifiers of PPAR activity (Keller et al., 1993; Yu et al., 1995; Prescott and White, 1996; Forman et al., 1997; Kliewer et al., 1997), PPAR δ activity might be repressed by the NSAID-mediated changes in eicosanoid metabolism.

The above model can help explain several features of NSAID-mediated chemoprevention. First, the effectiveness of some NSAIDs in the prevention of colorectal adenomas can now be linked to genetic defects that

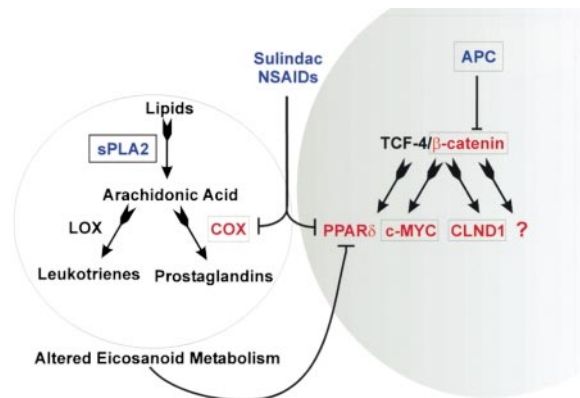


Figure 7. Unified Model for APC- and NSAID-Mediated Suppression of CRC

Elements indicated in blue have been shown to have a tumor-suppressive effect, whereas elements in red have been shown to promote tumor formation. The effects of items in boxes have been demonstrated by genetic alterations. LOX, 5-lipoxygenase; sPLA2, secretory phospholipase 2; and COX, cyclooxygenase.

underlie the initiation of these tumors and to the ability of NSAIDs to counterbalance the consequences of these genetic defects. Second, although NSAID functions have been linked to their inhibition of COX activity and the resulting inhibition of prostaglandin synthesis, several studies have suggested that the chemopreventive and apoptosis-inducing activities of NSAIDs are not entirely related to the inhibition of COX or to the decreased levels of prostaglandins (see Introduction). These results may be explained by the ability of NSAIDs to directly inhibit PPAR δ . Indeed, the sulindac derivative sulindac sulfone, which is devoid of COX inhibitory activity, has apoptotic activity in vitro and chemopreventive activity in vivo and has been proposed as a chemopreventive agent that lacks the toxicity associated with traditional NSAIDs (Piazza et al., 1995, 1997; Mahmoud et al., 1998). Sulindac sulfone inhibited PPAR δ activity, albeit at higher concentrations than that required for sulindac sulfide, consistent with its reduced chemopreventive and apoptosis-promoting activity. Third, recent studies have demonstrated that PPAR γ agonists promote intestinal tumorigenesis in the Min mouse (Lefebvre et al., 1998; Saez et al., 1998), while the same agonists inhibit the growth of human CRC cells (Brockman et al., 1998; Sarraf et al., 1998). Although the conclusions of these studies were contradictory, they clearly demonstrated the ability of PPAR ligands to modify intestinal tumor growth. A role for PPARs in intestinal tumorigenesis is further suggested by the recent identification of loss-of-function mutations in one allele of PPAR γ in 4 of 55 sporadic CRCs (Sarraf et al., 1999). However, unlike PPAR δ , neither PPAR γ nor PPAR α is a target of the APC/ β -catenin pathway. Whereas PPAR δ is increased in expression in cancers, downregulated by APC, and upregulated by β -catenin, PPAR γ and PPAR α do not display these properties. Fourth, the ability of COX2 expression to modulate apoptosis (Tsuji and Dubois, 1995) and intestinal tumorigenesis (Oshima et al., 1996) may be partially related to its ability to alter the spectrum of ligands for PPAR δ and other PPARs. In this regard,

it is interesting to note that the PPAR δ ligand cPGI can partially rescue infertility resulting from COX-2 deficiency (Lim et al., 1999). Finally, the ability of dietary fatty acids and secreted phospholipases to modify the spectrum of PPAR δ ligands and thus alter PPAR δ activity could account for their ability to affect CRC risk (Willett et al., 1990; Dietrich et al., 1993; MacPhee et al., 1995; Vanden Heuvel, 1999).

In addition to explaining several features of NSAID-mediated chemoprevention, our observations may have important ramifications for the development of chemopreventive agents. In particular, it is conceivable that the development of drugs that specifically target PPAR δ might lead to more efficacious and less toxic means for CRC chemoprevention.

Experimental Procedures

Cell Lines and Chemicals

Human CRC cells (HT29, HCT116, SW480, and DLD1) and embryonic kidney cells (293) were obtained from ATCC (Manassas, VA). BRL49653 and cPGI were purchased from American Radiolabeled Chemicals and Cayman Chemical Company, respectively. Sulindac derivatives and indomethacin were purchased from BIOMOL. Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO).

In Vitro DNA-Binding Site Selection for PPAR δ and RXR α

GST fusion proteins containing the N-terminal DNA-binding domains of human PPAR δ and RXR α were constructed by PCR amplifying codons 1–249 of PPAR δ and 1–224 of RXR α and cloning them into pGEX-2TK vector. As controls, GST fusion proteins containing the DNA-binding domains of human PPAR α (aa 1–249) and PPAR γ (aa 1–248) were also constructed. To identify the potential consensus DNA sequence motifs recognized by PPAR δ and RXR α , a previously described in vitro site selection procedure was utilized (Zawel et al., 1998). Selected PCR products were cloned in to pZero2.1, tested for binding, and sequenced as described.

Gel Electrophoretic Mobility Shift Assays

DNA-binding assays supplemented with Poly dIdC (6 μ g/ml) were performed essentially as described (Zawel et al., 1998). For binding to PCR products derived from in vitro site selections, 1.0–1.5 μ g of protein and 50 ng of DNA were used. For competitions, a 100-fold excess of unlabeled probe was used. For EMSA with GST fusion proteins, 0.3–0.5 μ g of fusion protein and 0.5 ng of ³²P kinase labeled (~10⁶ dpm) DNA were used. The probes for Tcf-4 binding were as previously reported (Korinek et al., 1997). For EMSA with in vitro translated proteins, 0.1 to 0.2 μ l of programmed lysate and ³²P-labeled probe (~10⁶ dpm) was used. The DRE probe was formed by annealing 5'-GCGTGAGCGCTCACAGGTCAATTCCG-3' and 5'-CCGAATTGACCTGTGAGCGCTCACG-3'. The ACO probe was formed by annealing 5'-GCGGACCAGGACAAAGGTGACGTTCC-3' and 5'-CGAACGTGACCTTTGCTCCTGGTCCG-3'.

Construction of a PPAR δ -Responsive Reporter

The following oligonucleotides containing PPAR δ and RXR recognition motifs that were identified from in vitro site selection approach were synthesized: 5'-CTAGCGTGAGCGCTCACAGGTCAATTCCG TGAGCGCTCACAGGTCAATTCCG-3' and 5'-CTAGCGAATTGACCTGTGAGCGCTCACCGAATTGACCTGTGAGCGCTCACG-3'. As a control, the following oligonucleotides containing a PPAR α and PPAR γ -responsive element from the acyl-CoA oxidase promoter were also synthesized: 5'-CTAGCGGACCAGGACAAAGGTGACGTTCCGACCAGGACAAAGGTGACGTTCCG-3' and 5'-CTAGCGAACGTGACCTTTGCTCCTGGTCCGACGTTGCTCCTGGTCCG-3'. The oligonucleotide cassettes were dimerized and cloned into pBV-Luc, a luciferase reporter plasmid with very low basal activity (He et al., 1998a). All constructs were verified by DNA sequencing.

Constructions of PPAR δ Promotor Reporters

Three independent BAC clones containing the PPAR δ promoter sequence were obtained by screening a BAC library (Research Genetics). For the construction of PPAR δ promoter reporters, corresponding restriction fragments (illustrated in Figure 2A) were subcloned into pBV-Luc. The mNP reporter was constructed by cloning a PCR product into pBV-Luc, whereas p4XTRE1-Luc, p4XmTRE1-Luc, p4XTRE2-Luc, and p4XmTRE2-Luc were constructed from oligonucleotides. Details of construction and oligonucleotide sequences are available upon request or at www.coloncancer.org/ppar.htm.

Reporter Assays

Reporter plasmid, effector plasmid, and β -gal control plasmid were transfected into cells using LipofectAmine (Life Technologies). Twenty-four hours after transfection, cells were lysed and collected for assays of luciferase activity using Promega's Luciferase Assay System.

In Vitro Transcription and Translation Assays

The full-length proteins of PPAR δ , PPAR γ , and RXR α were generated by in vitro transcription-coupled translation of PCR products using the TNT T7 Quick-Coupled Transcription/Translation System (Promega). PCR primer sequences are available upon request or at www.coloncancer.org/ppar.htm.

Generation of Recombinant Adenovirus Expressing PPAR δ or c-Myc

A PCR product of human PPAR δ was cloned into pCMV-HAHA and verified by sequencing. This expression cassette was used to generate recombinant adenovirus using the AdEasy system as previously described (He et al., 1998b). Additional details of the construction are available upon request or at www.coloncancer.org/ppar.htm. AdMyc was generated in a similar fashion and was a gift of H. Hermeking.

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