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1,1-Bis(3′-indolyl)-1-(*p*-substitutedphenyl)methanes Induce Peroxisome Proliferator-Activated Receptor γ-Mediated Growth Inhibition, Transactivation, and Differentiation Markers in Colon Cancer Cells

Sudhakar Chintharlapalli,[1] Roger Smith III,[2] Ismael Samudio,[3,4] Wei Zhang,[5] and Stephen Safe[3,4]

[1]Departments of Biochemistry and Biophysics, [2]Veterinary Pathobiology, and [3]Veterinary Physiology and Pharmacology, Texas A&M University, College Station, Texas; [4]Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, Texas; and [5]Division of Pathology and Laboratory Medicine, M. D. Anderson Cancer Center, Houston, Texas

ABSTRACT

1,1-Bis(3′indolyl)-1–(*p*-substitutedphenyl)methanes containing *p*-trifluoromethyl (DIM-C-pPhCF$_3$), *p*-*t*-butyl (DIM-C-pPhtBu), and *p*-phenyl (DIM-C-pPhC$_6$H$_5$) groups induce peroxisome proliferator-activated receptor γ (PPARγ)-mediated transactivation in HT-29, HCT-15, RKO, and SW480 colon cancer cell lines. Rosiglitazone also induces transactivation in these cell lines and inhibited growth of HT-29 cells, which express wild-type PPARγ but not HCT-15 cells, which express mutant (K422Q) PPARγ. In contrast, DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$ inhibited growth of both HT-29 and HCT-15 cells with IC$_{50}$ values ranging from 1 to 10 μmol/L. Rosiglitazone and diindolylmethane (DIM) analogues did not affect expression of cyclin D1, p21, or p27 protein levels or apoptosis in HCT-15 or HT-29 cells but induced keratin 18 in both cell lines. However, rosiglitazone induced caveolins 1 and 2 in HT-29 but not HCT-15 cells, whereas these differentiation markers were induced by DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ in both cell lines. Because overexpression of caveolin 1 is known to suppress colon cancer cell and tumor growth, the growth inhibitory effects of rosiglitazone and the DIM compounds are associated with PPARγ-dependent induction of caveolins.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and members of the nuclear receptor family (1–3). Ligand-bound PPARs form nuclear heterodimers with retinoid X receptors, which modulate transcription by interactions with cognate PPAR response elements in target gene promoters. PPARγ has been extensively investigated and ligands for this receptor typically induce adipocyte differentiation and mediate tissue-specific anti-inflammatory responses. Synthetic thiazolidinediones (TZDs) such as rosiglitazone and pioglitazone are PPARγ agonists that have been developed for treatment of insulin-resistant type 2 diabetes (1–4).

Ikezoe *et al.* (5) reported expression of wild-type PPARγ mRNA in tumors from multiple tissues, including lung, breast, colon, prostate, osteosarcomas, acute myelogenous leukemia, adult T-cell leukemia, glioblastomas, B-cell acute lymphoblastic leukemia, non-Hodgkin's lymphoma, and myelodisplastic syndrome. In addition, PPARγ mRNA was also expressed in 71 different cancer cell lines derived from hematopoietic and nonhematopoietic (lung, duodenal, prostate, breast, glioblastomas, and colon) tumors. Surprisingly, the reported PPARγ mutations in colon cancer (6) were not observed in the 10 colon cancer cell lines and 58 clinical samples examined in this study.

Studies in this laboratory have characterized a series of 1,1-bis(3′-indolyl)-1-(*p*-substitutedphenyl)methanes [methylene or C-substituted diindolylmethanes (DIMs)] that activate PPARγ-dependent transactivation and inhibit growth of MCF-7 breast cancer cells (7). Compounds containing *p*-CF$_3$ (DIM-C-pPhCF$_3$), *p*-*t*-butyl (DIM-C-pPhtBu), and *p*-phenyl (DIM-C-pPhC$_6$H$_5$) were the most potent activators of PPARγ and inhibitors of cell growth. Growth inhibition by C-substituted DIMs in MCF-7 cells was associated, in part, with proteasome-dependent down-regulation of cyclin D1.

A recent study reported that several colon cancer cells expressed either wild-type or mutant PPARγ containing a point mutation at codon 422 (K422Q; ref. 8). HCT-15 and other cells that express mutant PPARγ were resistant to the growth inhibitory and differentiation-inducing effects of rosiglitazone, whereas these responses were induced by rosiglitazone in cells (*e.g.*, HT-29) expressing wild-type PPARγ. Results of this study show that PPARγ-active C-substituted DIMs induce PPARγ-dependent transactivation and differentiation-induced genes/proteins in both rosiglitazone-responsive (HT-29) and -nonresponsive (HCT-15) colon cancer cell lines. Caveolin 1 and caveolin 2 proteins were induced by rosiglitazone only in HT-29 cells, whereas C-substituted DIMs induced these proteins in both HT-29 and HCT-15 cells. The cell context-dependent growth inhibitory responses of rosiglitazone and PPARγ-active C-substituted DIMs correlated with induction of caveolins, which exhibit tumor suppressor activity.

MATERIALS AND METHODS

Cell Lines and Reagents. Human colon carcinoma cell lines RKO, DLD1, and SW480 were provided by M. D. Anderson Cancer Center; HT-29 and HCT-15 were obtained from American Type Culture Collection (Manassas, VA). RKO, DLD1, SW480, and HT-29 cells were maintained in DMEM: Ham's F-12 (Sigma, St. Louis, MO) with phenol red supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, and 5% fetal bovine serum (FBS) and 10 mL/L of 100× antibiotic antimycotic solution (Sigma). HCT-15 cells were maintained in RPMI 1640 (Sigma) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 mL/L of 100× Antibiotic Antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO_2. Rosiglitazone was purchased from LKT Laboratories, Inc. (St. Paul, MN). Antibodies for PPARγ (sc-7273), Sp1 (sc-59), bcl-2, bax, poly(ADP-ribose) polymerase, cyclin D1 (sc-718), p21 (sc-756), p27 (sc-528), and caveolin 1 (sc-894) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Keratin-18 antibody was purchased from NeoMarkers (Fremont, CA). Caveolin 2 antibody was obtained from Transduction Laboratories (Lexington, KY). Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA). Lipofectamine reagent and Plus reagent were supplied by Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagent were from Perkin-Elmer Life Sciences (Boston, MA). The C-substituted DIMs were prepared in this laboratory by condensation of indole with *p*-substituted benzaldehydes, and compounds were >95% pure by gas chromatography-mass spectrometry.

Plasmids. The Gal4 reporter containing 5× Gal4 response elements (pGal4) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4DBD-PPARγ construct (gPPARγ) was a gift of

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Requests for reprints: Stephen Safe, Department of Veterinary Physiology and Pharmacology, Texas A&M University, 4466 TAMU, Veterinary Research Building 409, College Station, TX 77843-4466. Phone: (979) 845-5988; Fax: (979) 862-4929; E-mail: ssafe@cvm.tamu.edu.

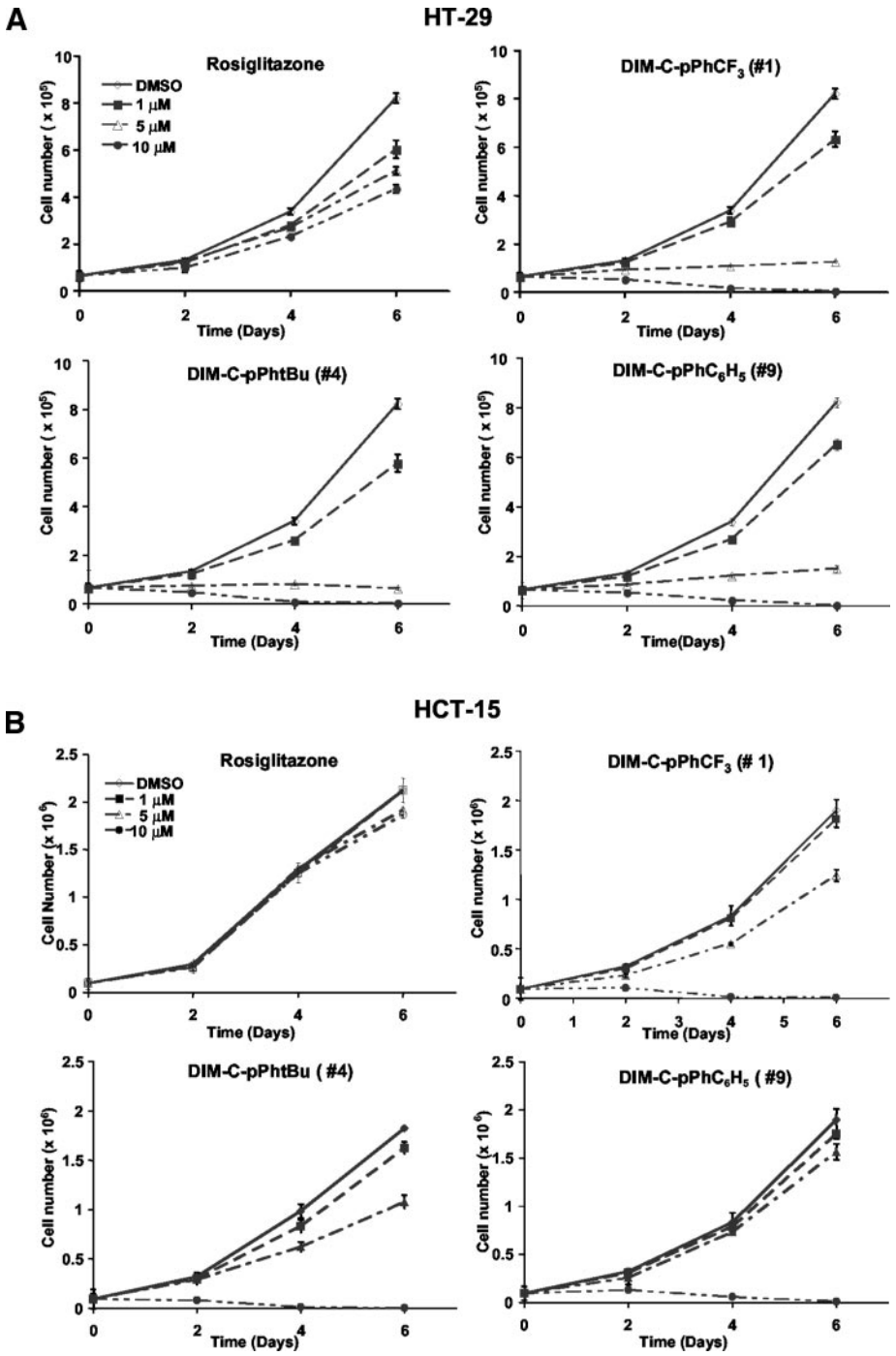

Fig. 1. Growth inhibition studies. HT-29 (*A*) and HCT-15 (*B*) colon cancer cells were treated with 1 to 10 μmol/L rosiglitazone, DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$ for 6 days, and cell numbers were determined using a Coulter Counter as described in the Materials and Methods. Results are expressed as means ± SE for three separate determinations at each time point.

Dr. Jennifer L. Oberfield (Glaxo Wellcome Research and Development, Research Triangle Park, NC). The PPARγ-VP16 fusion plasmid (VP-PPARγ) contained the DEF region of PPARγ (amino acids 183–505) fused to the pVP16 expression vector and the GAL4-coactivator fusion plasmids pM-SRC1, pMSRC2, pMSRC3, pM-DRIP205, and pM-CARM-1 were kindly provided by Dr. Shigeaki Kato (University of Tokyo, Tokyo, Japan).

Transfection and Luciferase Assay. Colon cancer cells were plated in 12-well plates at 1×10^5 cells/well in DMEM:Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After growth for 16 hours, various amounts of DNA, *i.e.*, Gal4Luc (0.4 μg), β-gal 0.04 μg), VP-PPARγ (0.04 μg), pM SRC1 (0.04 μg), pMSRC2 (0.04 μg), pMSRC3 (0.04 μg), pM-DRIP205 (0.04 μg), and pMCARM-1 (0.04 μg) were transfected by Lipofectamine or Lipofectamine Plus (Invitrogen) according to the manufacturer's protocol. After 5 hours of transfection, the transfection mix was replaced with complete media containing either vehicle (DMSO) or the indicated ligand for 20 to 22 hours. Cells were then lysed with 100 mL of 1× reporter lysis buffer, and 30 μL of cell extract were used for luciferase and β-gal assays. Lumicount was used to quantitate luciferase and β-gal activities, and the luciferase activities were normalized to β-gal activity.

Cell Proliferation Assay. Cells were plated at a density of 2×10^4/well in 12-well plates and replaced the next day with DMEM:Ham's F-12 media containing 2.5% charcoal-stripped FBS and either vehicle (DMSO) or the indicated ligand and concentration. Fresh media and compounds were added every 48 hours. Cells were counted at the indicated times using a Coulter Z1 cell counter. Each experiment was done in triplicate and results are expressed as means ± SE for each determination.

Fluorescence-Activated Cell Sorting Analysis. HT-29 and HCT-15 cells were synchronized in serum-free media for 3 days and then treated with either the vehicle (DMSO) or the indicated compounds for 24 hours. Cells were trypsinized, centrifuged, and resuspended in staining solution containing 50

μg/mL propidium iodide, 4 mmol/L sodium citrate, 30 units/mL RNase, and 0.1% Triton X-100. After incubation at 37°C for 10 minutes, sodium chloride was added to give a final concentration of 0.15 mol/L. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), using CellQuest (Becton Dickinson Immunocytometry Systems) acquisition software. Propidium iodide (PI) fluorescence was collected through a 585/42-nm bandpass filter, and list mode data were acquired on a minimum of 20,000 single cells defined by a dot plot of PI width *versus* PI area. Data analysis was performed in ModFit LT (Verity Software House, Topsham, ME) using PI width *versus* PI area to exclude cell aggregates.

Western Blot Analysis. HT-29 and HCT-15 cells were seeded in DMEM: Ham's F-12 media containing 2.5% charcoal-stripped FBS for 24 h and then treated with either the vehicle (DMSO) or the indicated compounds. For the apoptosis experiments, cells were treated for 72 h with various compounds. Western blot analysis of whole cell lysates was determined as described previously (7, 9). Band intensities were evaluated by scanning laser densitometry (Sharp Electronics Corporation, Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corporation, Billerica, MA).

Statistical Analysis. Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean ± SD for at least three separate determinations for each treatment.

RESULTS

Inhibition of Colon Cancer Cell Growth by Rosiglitazone and PPARγ-Active C-Substituted DIMs. The effects of rosiglitazone, DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$ on the growth of HT-29, HCT-15, SW480, and RKO colon cancer cells are summarized in Fig. 1 and Table 1. The results obtained in HT-29 and HCT-15 cells, which express wild-type and mutant (K422Q) PPARγ (8), are illustrated in Fig. 1, *A* and *B*. Rosiglitazone significantly inhibited growth of HT-29 cells at concentrations of 1, 5, and 10 μmol/L; however, the concentration required for IC$_{50}$ was >10 μmol/L. In contrast, 1 μmol/L concentrations of DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$ significantly ($P < 0.05$) inhibited growth of HT-29 cells, and IC$_{50}$ values were <5 μmol/L for all three compounds. Rosiglitazone did not inhibit proliferation of HCT-15 cells (8); the PPARγ-active C-substituted DIMs all significantly inhibited growth at 5 or 10 μmol/L concentrations, and IC$_{50}$ values were <10 μmol/L. The results in Table 1 show that rosiglitazone treatment (1 to 10 μmol/L) minimally affected growth of SW480 or RKO cells, whereas IC$_{50}$ values for the PPARγ-active C-substituted DIMs varied from 1 to 5 or 5 to 10 μmol/L with DIM-C-pPhCF$_3$ as the most potent compound in both cell lines.

Table 1 *Percentage inhibition of SW480 and RKO colon cancer cell growth (6 days) by rosiglitazone and PPARγ-active C-substituted DIMs*

Treatment (μmol/L)	SW480	RKO
Rosiglitazone		
1	7.85 ± 0.92	0.39 ± 1.73
5	8.13 ± 1.34	2.79 ± 2.89
10	7.75 ± 2.21	2 ± 1.55
DIM-C-pPhCF$_3$		
1	22.35 ± 3.87*	4.36 ± 0.18
5	104.83 ± 0.29*	101.65 ± 0.06*
10	104.93 ± 0.25*	101.71 ± 0.09*
DIM-C-pPhtBu		
1	1.43 ± 3.47	3.22 ± 1.70
5	65.52 ± 1.85*	41.04 ± 1.76*
10	102.03 ± 0.37*	103.82 ± 0.06*
DIM-C-pPhC$_6$H$_5$		
1	2.184 ± 2.44	6.34 ± 2.20
5	24.328 ± 1.67*	39.77 ± 2.33*
10	103.91 ± 0.08*	102.83 ± 0.052*

NOTE. Cells were treated with different concentrations of the test compounds, and cell numbers were determined as described in the Materials and Methods, and the percent inhibition of cell growth was compared with DMSO (set at 100%). Results are means ± SD for three replicate determinations for each treatment group, and significant ($P < 0.05$) inhibition (*) is indicated.

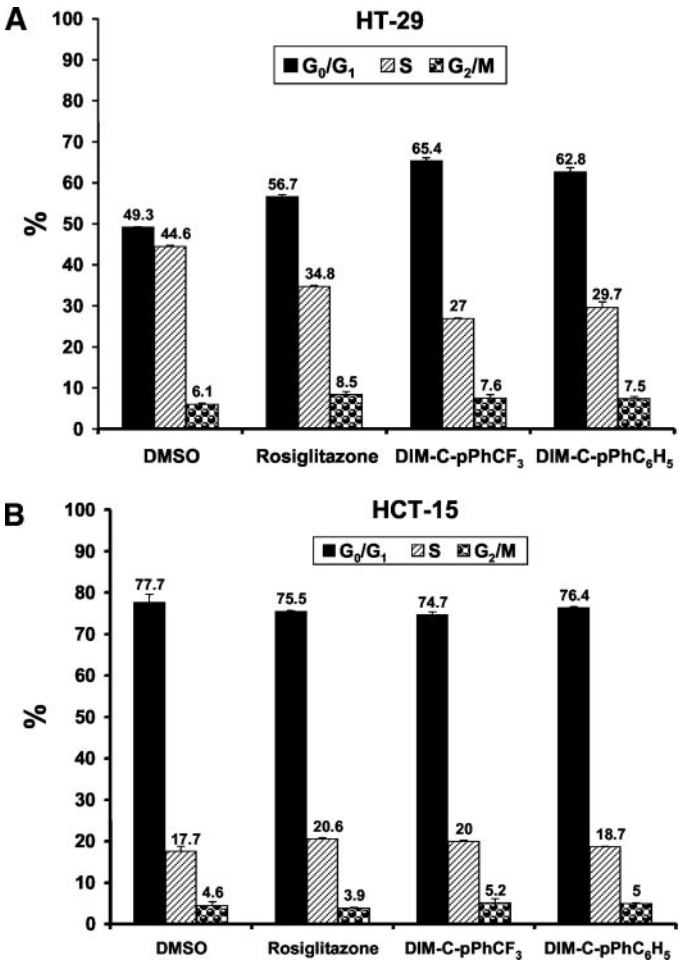

Fig. 2. Fluorescence-activated cell sorting analysis. HT-29 (*A*) and HCT-15 (*B*) cells were growth arrested for 3 days and then treated for 24 hours with 10 μmol/L rosiglitazone, DIM-C-pPhCF$_3$, or DIM-C-pPhC$_6$H$_5$ and analyzed by fluorescence-activated cell sorting analysis as described in the Materials and Methods. Results of a single experiment are presented and were also observed in two (HT-29) or three (HCT-15) separate studies.

HT-29 and HCT-15 cells were cultured in serum-free media for 3 days and then grown in 2.5% charcoal-stripped serum and treated with DMSO (solvent control), 10 μmol/L rosiglitazone, DIM-C-pPhCF$_3$, or DIM-C-pPhC$_6$H$_5$. Three days after treatment, the percentage distribution of cells in G$_0$-G$_1$, S, and G$_2$-M phases was determined by fluorescence-activated cell sorting analysis (Fig. 2). The PPARγ agonists decreased the percentage of HT-29 cells in S phase and increased the percentage in G$_0$-G$_1$. Rosiglitazone did not affect the percentage of HCT-15 cells in G$_0$-G$_1$, S, or G$_2$-M phase of the cell cycle, and this was consistent with the failure of this compound to inhibit proliferation of this cell line (Fig. 1*B*). Surprisingly, 10 μmol/L DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ also did not alter the percentage distribution of HCT-15 cells in G$_0$-G$_1$, S, or G$_2$-M phase, although both compounds significantly inhibited growth of these cells (Fig. 1). Previous studies with HCT-15 cells showed that the PPARγ agonist BRL-49653 did not inhibit growth of these cells or block G$_0$-G$_1$→S-phase progression (10).

PPARγ-Dependent Transactivation and Coactivator Interactions by Rosiglitazone, DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$. PPARγ is expressed in SW480, RKO, HT-29, and HCT-15 cells (data not shown), and ligand-dependent activation of PPARγ in HCT-15 and HT-29 colon cancer was determined in cells transfected with chimeric PPARγ-GAL4 and a GAL4 response element plasmid (pGAL4) containing five tandem GAL4 response

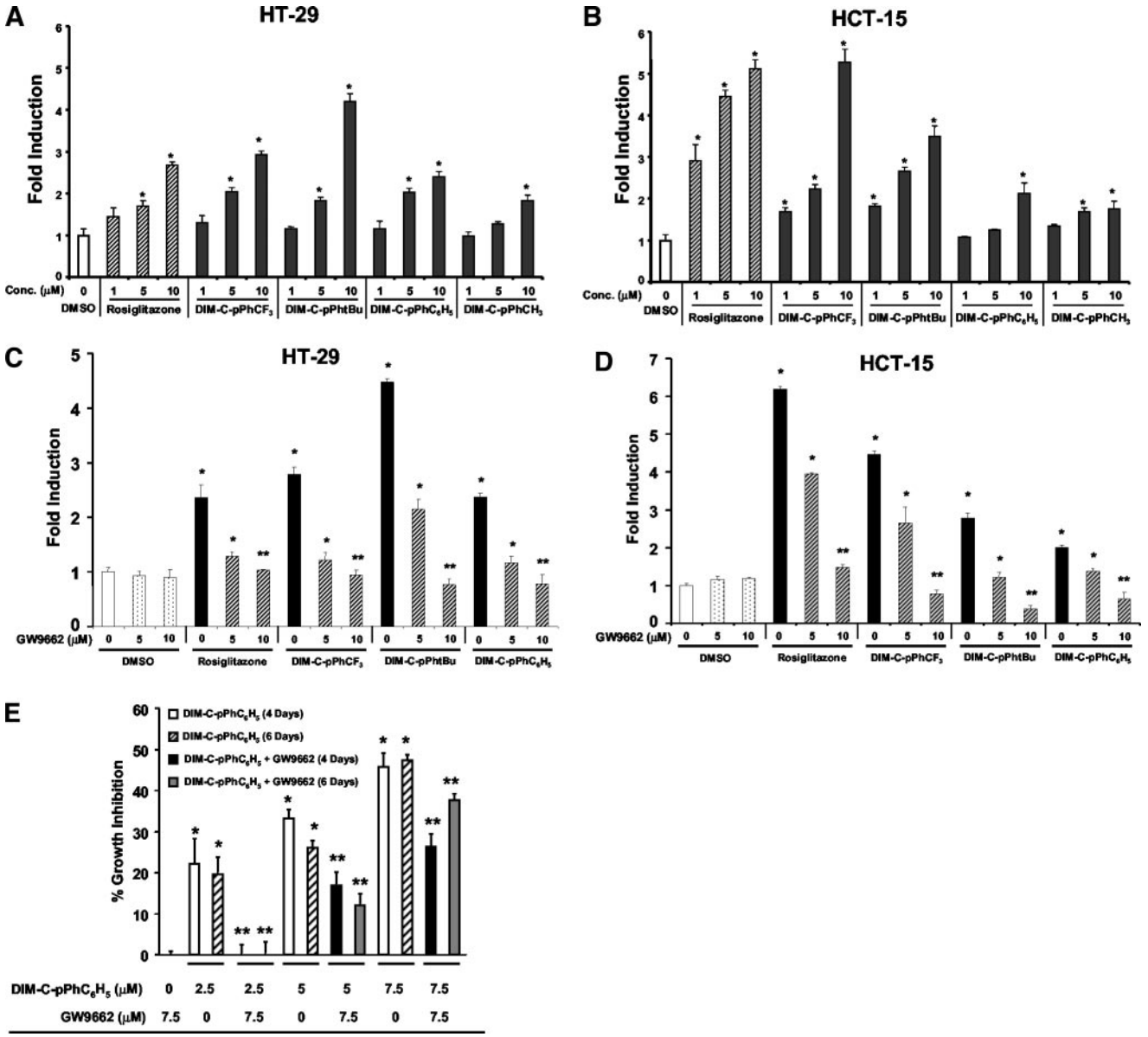

Fig. 3. Ligand-induced activation of PPARγ and effects of PPARγ antagonists. *A*, ligand-dependent activation of PPARγ-GAL4/pGAL4 in HT-29 cells. Cells were transfected with PPARγ-GAL4/pGAL4, treated with 1, 5, or 10 μmol/L of the PPARγ agonists, and luciferase activity was determined as described in Materials and Methods. Results of all transactivation studies in this figure are presented as means ± SE for at least three separate determinations for each treatment group, and significant ($P < 0.05$) induction compared with solvent (DMSO) control is indicated by an *asterisk*. *B*, ligand-dependent activation of PPARγ-GAL4/pGAL4 in HCT-15 cells. This experiment was carried out as described in *A*. Inhibition of transactivation in HT-29 (*C*) and HCT-15 (*D*) cells by GW9662. Cells were transfected with PPARγ-GAL4/pGAL4, treated with 10 μmol/L rosiglitazone or PPARγ-active C-substituted DIMs alone or in combination with 5 or 10 μmol/L GW9662, and luciferase activities were determined as described in *A*. Significant ($P < 0.05$) inhibition of induced transactivation by GW9662 is indicated (**). *E*, inhibition of HCT-15 cell growth by DIM-C-pPhC$_6$H$_5$: effects of GW9662. The effects of DIM-C-pPhC$_6$H$_5$ alone and in combination with GW9662 on proliferation of HCT-15 cells were determined as described in the Materials and Methods and outlined in the caption for Fig. 1. Significant ($P < 0.05$) inhibition of cell proliferation (*) and antagonism of this response by GW9662 (**) are indicated.

elements linked to a luciferase reporter gene (Fig. 3, *A* and *B*). Rosiglitazone (1 to 10 μmol/L) and 1 to 10 μmol/L DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$ induced reporter gene activity in HCT-15 and HT-29 cells, and similar results were observed in RKO and SW480 cells (data not shown). The magnitude of the induction responses were <6-fold in the HCT-15 and HT-29 cells, and the relative potencies of the individual compounds were variable and dependent on cell context. DIM-C-pPhCH$_3$ exhibited lower activity in the transactivation assays in the colon cancer cells as previously reported in breast cancer cell lines (7). Results in Fig. 3, *C* and *D*, show that induction of luciferase activity by rosiglitazone and PPARγ-active C-substituted DIMs in HT-29 and HCT-15 cells transfected with PPARγ-GAL4/pGAL4 was inhibited by GW9662, a PPARγ antagonist. Results summarized in Fig. 3*E* show that 7.5 μmol/L GW9662 also significantly blocks the growth inhibitory effects of 2.5, 5.0, or 7.5 μmol/L DIM-C-pPhC$_6$H$_5$ in HCT-15 cells. In contrast, GW9662 alone was growth inhibitory in HT-29 cells. These data confirm the role of PPARγ in mediating inhibition of HCT-15 cell growth by DIM-C-pPhC$_6$H$_5$.

A previous article (11) showed that 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2) but not TZDs induced transactivation in a mammalian two-hybrid assay in COS-1 cells transfected with VP-PPARγ and GAL4-coactivator chimeras (coactivators = SRC-1, SRC-2, SRC-3, and DRIP205/TBP/TRAP220). Therefore, we investigated ligand-dependent interactions of VP-PPARγ with chimeric GAL4-coactivator in colon cancer cells transfected with the pGAL4-luc reporter gene

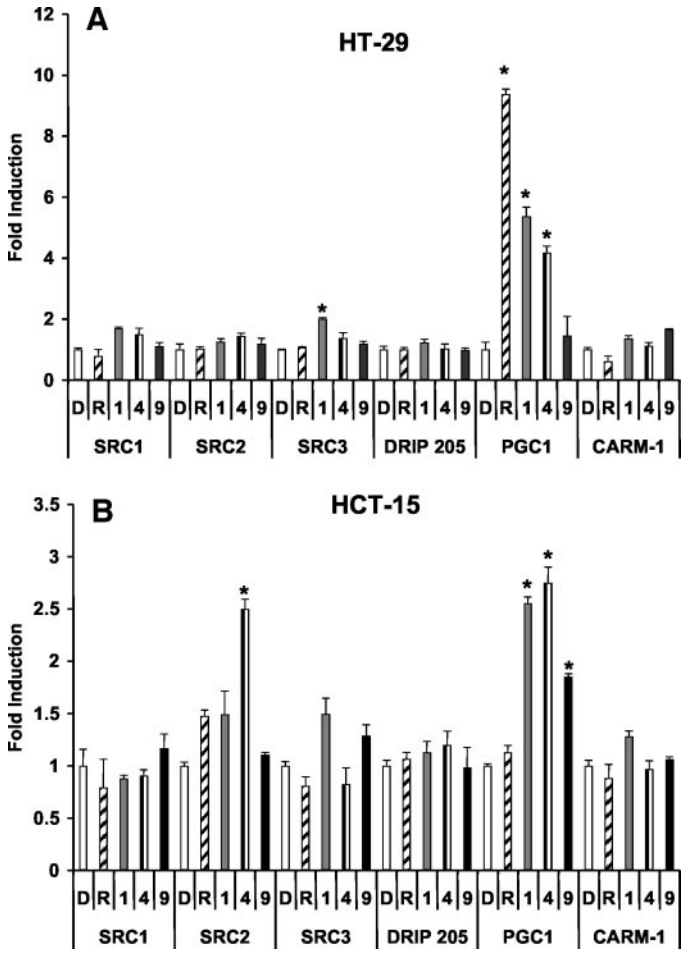

Fig. 4. Ligand-induced PPARγ-coactivator interactions. HT-29 (*A*) and HCT-15 (*B*) were transfected with VP-PPARγ, coactivator-GAL4/pGAL4, treated with 10 μmol/L rosiglitazone, DIM-C-pPhCF$_3$ (1), DIM-C-pPhtBu (4), or DIM-C-pPhC$_6$H$_5$ (9), and luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) induction is indicated by an *asterisk*.

construct (Fig. 4). PPARγ-active C-substituted DIMs and rosiglitazone induced transactivation in HT-29 cells transfected with VP-PPARγ and GAL4-PGC-1, and minimal interactions were observed using chimeric proteins containing SRC-1, SRC-2, SRC-3, DRIP205, or CARM-1. DIM-C-pPhCF$_3$ also induced transactivation (< 2-fold) in cells transfected with GAL4-SRC-3, and DIM-C-pPhC$_6$H$_5$ did not significantly induce activity in cells transfected with GAL4-PGC-1. PPARγ-active C-substituted DIMs induced transactivation in HCT-15 cells transfected with GAL4-PGC-1 and with one exception (DIM-C-pPhtBu/GAL4-SRC2), minimal interactions with other coactivators were observed. In SW480 and RKO colon cancer cells, a variable pattern of PPARγ-coactivator interactions were observed and the PPARγ-active C-substituted DIMs but not rosiglitazone-induced interactions with PGC-1 (data not shown).

Modulation of Cell Cycle Proteins, Differentiation Markers, and Apoptosis by PPARγ Agonists. The effects of rosiglitazone and PPARγ-active C-substituted DIMs on cyclin D1, p21, and p27 protein expression were investigated in HT-29 and HCT-15 after treatment with DMSO (control) and 5 or 7.5 μmol/L concentrations of these compounds for 24 hours. The results show that none of the treatments significantly affected levels of cyclin D1, p21, or p27 proteins (Fig. 5), and protein levels at other time points (6 and 12 hours) were also unchanged.

PPARγ agonists induce genes associated with differentiation in preadipocytes and cancer cell lines, and results in Fig. 6 show that keratin 18 is constitutively expressed in both HT-29 and HCT-15 cells. In the former cell line, 5 and 7.5 μmol/L rosiglitazone, DIM-C-pPhCF$_3$, and DIM-C-pPhC$_6$H$_5$ significantly induced keratin 18 protein, and a maximal 2-fold induction response was observed for 7.5 μmol/L DIM-C-pPhC$_6$H$_5$. Rosiglitazone and PPARγ-active C-substituted DIMs also induced a 2.5 to 3-fold increase in keratin 18 protein in HCT-15 cells, showing that induction of this differentiation marker did not correlate with expression of wild-type or mutant PPARγ. A recent study showed that caveolins 1 and 2 were induced by TZDs in HT-29 cells (12). Rosiglitazone, DIM-C-pPhCF$_3$, and DIM-C-pPhC$_6$H$_5$ also significantly induced caveolins 1 and 2 in this cell line, and protein levels were increased by 5 to 9-fold after treatment for 72 hours. Similar results were observed in HT-29 cells treated for only 48 hours with the PPARγ agonists (data not shown). In contrast, DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$, but not rosiglitazone, induced caveolins 1 and 2 in HCT-15 cells after treatment for 72 hours (Fig.

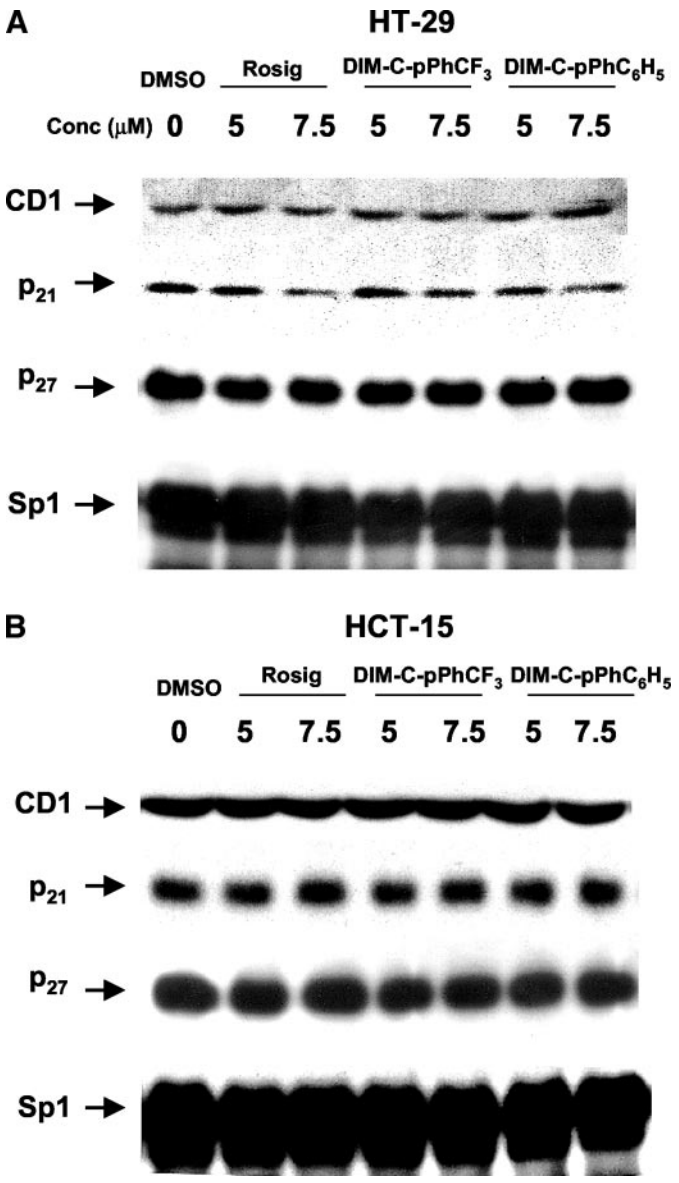

Fig. 5. Modulation of cell cycle proteins. HT-29 (*A*) or HCT-15 (*B*) cells were treated with 5.0 or 7.5 μmol/L rosiglitazone, DIM-C-pPhCF$_3$, or DIM-C-pPhC$_6$H$_5$ for 24 hours, and whole cell lysates were analyzed by Western blot analysis as described in Materials and Methods. Cyclin D1, p21, and p27 protein levels were similar in all treatment groups in duplicate experiments after treatment for 24 or 12 hours (data not shown).

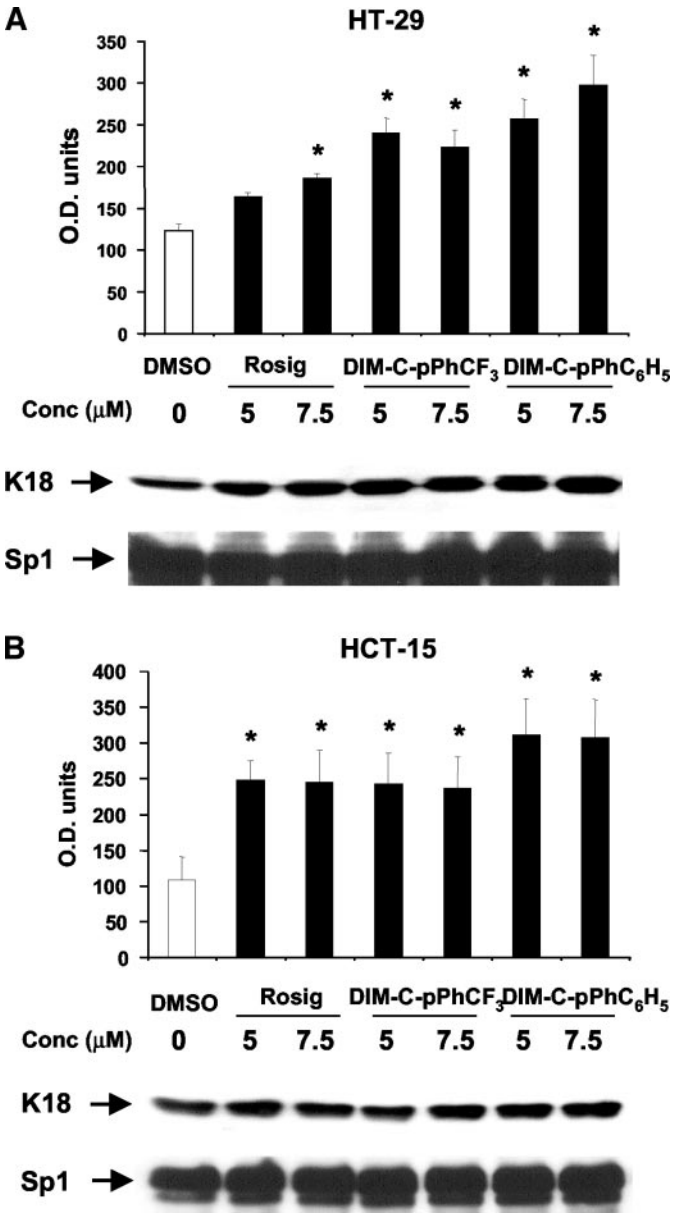

Fig. 6. Expression of keratin 18. HT-29 (*A*) and HCT-15 (*B*) cells were treated with 5.0 or 7.5 μmol/L rosiglitazone, DIM-C-pPhCF$_3$, or DIM-C-pPhC$_6$H$_5$ for 3 days, and whole cell lysates were analyzed by Western blot analysis as described in Materials and Methods. Keratin 18 levels in each treatment group were quantitated relative to an Sp1 protein loading control, and results are expressed as means ± SE for three replicate determinations for each treatment group. Significant ($P < 0.05$) induction is indicated by an *asterisk*.

7, *C* and *E*) and 48 hours (data not shown). Induction of caveolins 1 and 2 by DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ in HT-29 and HCT-15 cells was inhibited by cotreatment with the PPARγ agonist GW9662; similar inhibition was observed for rosiglitazone in HT-29 cells (Fig. 7, *E* and *F*). Because caveolin 1 exhibits tumor suppressor and growth inhibitory activities, the results suggest that induction of caveolins may be important for the antiproliferative effects of PPARγ-active C-substituted DIMs and rosiglitazone in colon cancer cells (Fig. 1).

PPARγ agonists also induce apoptosis in some cancer cell lines (13–15), and therefore, this response was also investigated in HT-29 and HCT-15 cells. PPARγ-active compounds (10 μmol/L) did not induce poly(ADP-ribose) polymerase cleavage (a marker of apoptosis) or alter levels of bax or bcl-2 protein expression in HCT-15 or HT-29 cells (Fig. 8). In contrast, the proteasome inhibitor MG132 induced poly(ADP-ribose) polymerase cleavage in both cell lines but did not affect levels of bcl-2 or bax proteins. Thus, differences in the observed antiproliferative activities of rosiglitazone and PPARγ C-substituted DIMs in HT-29 and HCT-15 cells (Fig. 1) was not due to apoptosis or modulation of cell cycle proteins but correlated with their cell context-dependent differential induction of caveolins 1 and 2 but not keratin 18.

DISCUSSION

PPARγ is overexpressed in multiple tumor types (5) and studies in several laboratories have demonstrated that PGJ2 and TZDs inhibit growth of human cancer cell lines *in vitro* and also in xenograft models *in vivo* (7–16). Two reports (17, 18) showed that treatment of Min mice, which express a mutated inactive form of the adenomatous polyposis coli (APC) tumor suppressor gene, with troglitazone or BRL-49653 induced colon polyp formation. These results contrast with xenograft studies where PPARγ agonists inhibit colon tumor growth (16). Moreover, a recent study reported that 100 or 200 ppm pioglitazone and bezafibrate in the diet of *Apc*-deficient mice reduced intestinal polyps and hyperlipidemia (19). Another study used PPARγ$^{+/-}$/Min mice and their crosses to show that inhibition of colon carcinogenesis in these mice by PPARγ agonists is Apc dependent (20).

Recent studies in this laboratory showed the C-substituted DIMs inhibited growth and G_0-$G_1 \rightarrow S$ progression of MCF-7 breast cancer cells and activated PPARγ-dependent transactivation (7). The growth inhibitory properties of rosiglitazone and three PPARγ-active C-substituted DIMs (DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$) were also determined in HT-29, HCT-15, SW480, and RKO colon cancer cell lines. At concentrations of 10 μmol/L, rosiglitazone significantly inhibited growth of HT-29 cells ($IC_{50} > 10$ μmol/L) but not the other cell lines, whereas the PPARγ-active C-substituted DIMs inhibited growth of all four colon cancer cell lines with IC_{50} values between 1 and 10 μmol/L. The growth inhibitory effects of DIM-C-pPhC$_6$H$_5$ were inhibited by GW9662 in HCT-15 cells (Fig. 3*E*). A recent study showed that troglitazone inhibited growth and induced differentiation genes in HCT-15 cells (21). However, these responses were observed using 50 μmol/L troglitazone, suggesting that activation of some mutant (K422Q) PPARγ-dependent responses in HCT-15 cells may be observed with higher concentrations of TZDs.

Rosiglitazone and the PPARγ-active C-substituted DIM activated PPARγ-GAL4/pGAL4 in all four cell lines (Fig. 3) with variable potencies, and transactivation was inhibited after cotreatment with the PPARγ antagonist GW9662. The pattern of coactivator-PPARγ interactions in HT-29 and HCT-15 cells paralleled, in part, their responsiveness to the growth inhibitory effects of rosiglitazone and the PPARγ-active C-substituted DIMs (Fig. 4). Both structural classes of PPARγ agonists induced interactions between VP-PPARγ and GAL4-PGC-1 in HT-29 cells, whereas only the C-substituted DIMs induced the same interaction in HCT-15 cells (Fig. 4). In contrast, the pattern of coactivator-PPARγ interactions in SW480 and RKO cells was more complex, and minimal induction was observed for rosiglitazone in both cell lines (data not shown). The differences in rosiglitazone-induced transactivation in the mammalian two-hybrid assay in HT-29 and HCT-15 were observed using VP-PPARγ (wild-type) in both cell lines. This suggests that differences in PGC-1-PPARγ interactions in HT-29 and HCT-15 cells are not attributable to expression of the PPARγ mutant (K422Q) in the latter cell line but related to differential expression in the two cell lines of other nuclear cofactors required for this response. Although PPARγ-active C-substituted DIMs preferentially induce PPARγ-PGC-1 interactions in HT-28 and HCT-15 cells, this was not uniformly observed for all ligands in these cells

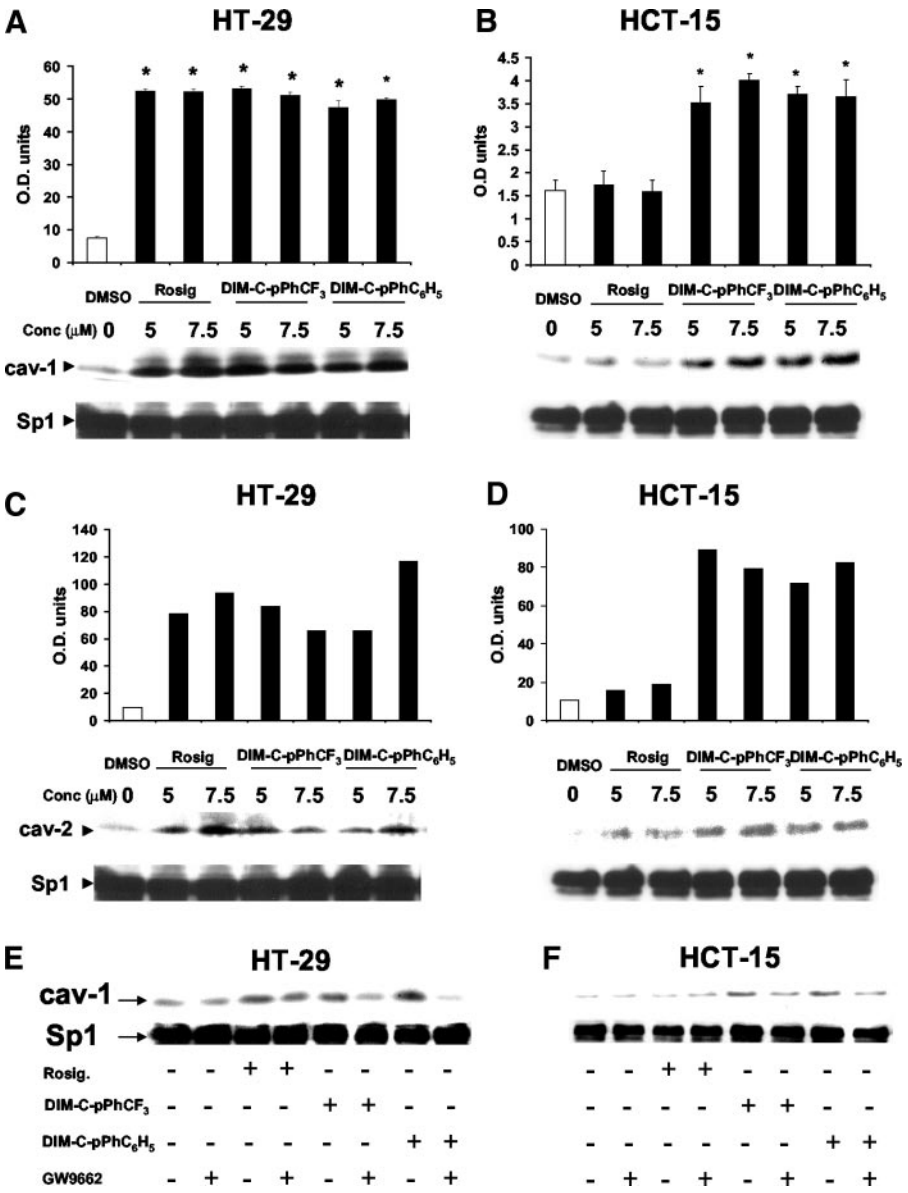

Fig. 7. Expression of caveolin 1 and caveolin 2. HT-29 (*A*) and HCT-15 (*B*) cells were treated with 5.0 or 7.5 μmol/L rosiglitazone, DIM-C-pPhCF$_3$, or DIM-C-pPhC$_6$H$_5$ for 3 days, and whole cell lysates were analyzed by Western blot analysis as described in Materials and Methods. Using a similar approach, caveolin 2 protein levels were determined in HT-29 (*C*) and HCT-15 (*D*) cells. Results are expressed as means ± SE for three replicate determinations (*A* and *B*) or averages of two determinations (*C* and *D*), and significant ($P < 0.05$) induction (*) (*A* and *B*) was determined as outlined in Fig. 6. Inhibition of caveolin 1 induction in HT-29 (*E*) and HCT-15 (*F*) cells by GW9662. The experiment was carried out as described in *A* and *B*; however, cells were also treated with the PPARγ agonists (7.5 μmol/L) in combination with GW9662. Comparable results were observed in duplicate experiments.

(Fig. 4), SW480 or RKO cells (data not shown). For example, DIM-C-pPhC$_6$H$_5$ inhibits HT-29 and HCT-15 cell growth (Fig. 1), induces caveolins 1/2 (Fig. 7), and both responses are inhibited by the PPARγ agonist GW9662 (Figs. 3*E*, 7*E*, and 7*F*). In contrast, 10 μmol/L DIM-C-pPhC$_6$H$_5$ does not induce transactivation in the mammalian two-hybrid assay in HT-29 cells using GAL4-PGC-1 and VP-PPARγ (Fig. 4). Current studies are investigating expression of PGC-1 and other coactivators and their role in PPARγ-dependent transactivation and growth inhibition in HT-29 and HCT-15 cells.

We further investigated the role of wild-type *versus* mutant (K422Q) PPARγ expression in mediating ligand-dependent responses in HT-29 and HCT-15 cells by examining their effects on proteins critical for cell cycle progression and differentiation. In HT-29 cells, treatment with rosiglitazone or PPARγ-active C-substituted DIMs inhibited G_0-G_1→S-phase progression (Fig. 2*A*). These effects were not observed in HCT-15 cells (Fig. 2*B*) and may be due to the high percentage of cells in G_0-G_1 with or without treatment (>74%). Despite differences in fluorescence-activated cell sorting analysis, neither rosiglitazone or the PPARγ-active C-substituted DIMs significantly changed levels of the cell cycle regulatory proteins p27, p21, or cyclin D1 in HT-29 or HCT-15 cells (Fig. 5). Moreover, we did not observe PPARγ agonist-dependent induction of apoptosis in either cell line (*e.g.*, Fig. 8), suggesting that the antiproliferative effects (Fig. 1) are not dependent on modulation of cell cycle proteins or induction of apoptosis in HT-29 or HCT-15 cells.

PPARγ agonists also induce genes/proteins associated with differentiation in colon cancer cells (8, 12, 19, 22, 23). For example, troglitazone induced villin and intestinal alkaline phosphatase mRNA levels in several colon cancer cell lines (23), and TZDs and PGJ2 induced caveolin 1 and caveolin 2 protein expression in HT-29 cells (12). Keratin 18 and 20 proteins and CEACAM6 mRNA levels were minimally expressed in HCT-15 cells but were not affected by treatment with rosiglitazone; however, after transfection of HCT-15 cells with wild-type PPARγ, rosiglitazone induced these responses (8). A direct comparison of PPARγ agonist-induced expression of differentiation markers in HCT-15 *versus* HT-29 cells has not been reported previously. The results in Fig. 6 show that rosiglitazone, DIM-C-pPhCF$_3$, and DIM-C-pPhC$_6$H$_5$ significantly induced keratin 18 in both HT-29 and HCT-15 cells, indicating that keratin 18 protein induction was independent of PPARγ agonist structure and expression of wild-type or mutant (K422Q) PPARγ. In contrast, induction of caveolin 1 and caveolin 2 in HT-29 and HCT-15 was dependent on both ligand structure and cell context (Fig. 7). Rosiglitazone induced

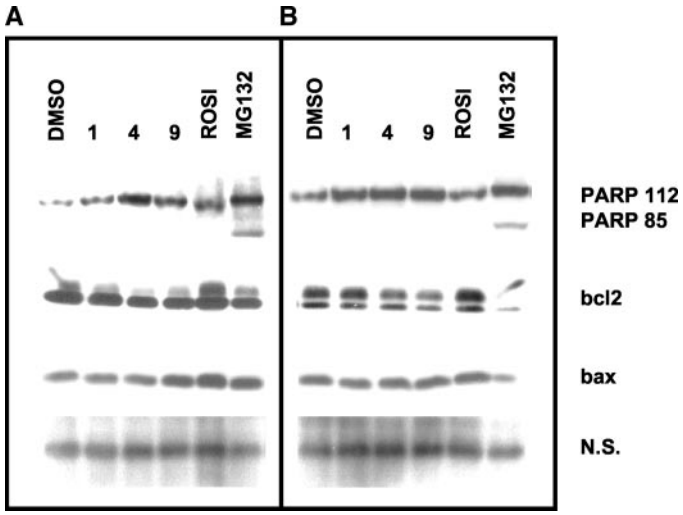

Fig. 8. Effects of PPARγ agonists on apoptosis. HCT-15 (*A*) or HT-29 (*B*) cells were treated with DMSO, 10 μmol/L DIM-C-pPhCF$_3$ (1), DIM-C-pPhtBu (4), DIM-C-pPhC$_6$H$_5$ (9), or rosiglitazone for 72 hours or 10 μmol/L MG132 for 20 hours, and whole cell lysates were analyzed by Western blot analysis for PARP112/85, bcl-2, bax, or a nonspecific (NS) (loading control) protein. Similar results were observed in duplicate analyses.

caveolins 1 and 2 in HT-29 but not in HCT-15 cells, whereas PPARγ-active C-substituted DIMs induced caveolins 1 and 2 in both HT-29 and HCT-15 cells, and these responses were inhibited after cotreatment with GW9662. This pattern of induced caveolin expression parallels the growth inhibitory effects of rosiglitazone and PPARγ-active C-substituted DIMs in HT-29 and HCT-15 cells (Fig. 1), suggesting that induced caveolin 1 and caveolin 2 expression may contribute to growth inhibition of colon cancer cells. This observation is consistent with a study by Bender *et al.* (24), showing that overexpression of caveolin 1 in HT-29 or DLD (HCT-15) cells significantly decreased their tumorigenicity in athymic nude mouse xenograft models.

In summary, this study demonstrates the PPARγ-active C-substituted DIMs induce PPARγ-dependent transactivation in colon cancer cells expressing wild-type (HT-29) or mutant (K422Q) (HCT-15) PPARγ. Moreover, these compounds also inhibit growth of HT-29 and HCT-15 cells, whereas equivalent concentrations of rosiglitazone are active only in the former cell line. The enhanced growth inhibitory activity of PPARγ-active C-substituted DIMs *versus* rosiglitazone in HCT-15 cells correlated with the induction of caveolins 1 and 2 by the former compounds and not by rosiglitazone (Fig. 7). Current studies are focused on the mechanisms of the growth inhibitory effects of this new class of PPARγ agonists in other colon cancer cell lines and the role of caveolins and other genes/proteins in mediating PPARγ-dependent antitumorigenic activities.

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