An Inducible Pathway for Degradation of FLIP Protein Sensitizes Tumor Cells to TRAIL-induced Apoptosis*

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TRAIL (Apo2 ligand) is a member of the tumor necrosis factor (TNF) family of cytokines that induces apoptosis. Because TRAIL preferentially kills tumor cells, sparing normal tissues, interest has emerged in applying this biological factor for cancer therapy in humans. However, not all tumors respond to TRAIL, raising questions about resistance mechanisms. We demonstrate here that a variety of natural and synthetic ligands of peroxisome proliferator-activated receptor-γ (PPARγ) sensitize tumor but not normal cells to apoptosis induction by TRAIL. PPARγ ligands selectively reduce levels of FLIP, an apoptosis-suppressing protein that blocks early events in TRAIL/TNF family death receptor signaling. Both PPARγ agonists and antagonists displayed these effects, regardless of the levels of PPARγ expression and even in the presence of a PPARγ dominant-negative mutant, indicating a PPARγ-independent mechanism. Reductions in FLIP and sensitization to TRAIL-induced apoptosis were also not correlated with NF-κB, further suggesting a novel mechanism. PPARγ modulators induced ubiquitination and proteasome-dependent degradation of FLIP, without concomitant reductions in FLIP mRNA. The findings suggest the existence of a pharmacologically regulated novel target of this class of drugs that controls FLIP protein turnover, and raise the possibility of combining PPARγ modulators with TRAIL for more efficacious elimination of tumor cells through apoptosis.

Considerable interest has emerged in the possibility of exploiting the apoptotic effects of TRAIL for the treatment of cancer. TRAIL is a member of the tumor necrosis factor (TNF) family of cytokines that is capable of inducing apoptosis (1).

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§ The abbreviations used are: TRAIL, TNF-related apoptosis inducing ligand; TNF, tumor necrosis factor; CDDO, 2-cyano-3,12-dioxygenolane-1,9-dien-28-oic acid; 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; FLIP, FLICE-inhibitory protein; IKK, IκB kinase; PPAR, peroxisome proliferator-activated receptor; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; GST, glutathione S-transferase; DAPI, 4,6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assays; GFP, green fluorescent protein; PARP, poly(ADP-ribose) polymerase; PPREs, PPAR-response elements; E3, ubiquitin-protein isopeptide ligase.

The apoptosis-inducing receptors for TRAIL include TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which are transmembrane type I receptors expressed on the surface of many types of cell. However, TRAIL also binds to non-apoptosis-inducing decoy receptors, which compete with death receptors for ligand and suppress apoptosis, including DcR1, DcR2, and osteoprotegerin (reviewed in Refs. 2 and 3). Empiric analysis of the effects of TRAIL on normal and malignant cells has provided compelling evidence that recombinant TRAIL protein preferentially induces apoptosis of cancer cells without harming most types of untransformed cells (reviewed in Ref. 2). When properly prepared and purified, recombinant trimeric TRAIL also lacks significant toxicity in primate species that possess receptors capable of binding human TRAIL (4, 5).

Preclinical studies of recombinant TRAIL (extracellular domain) in mice have demonstrated impressive anti-tumor activity and synergy with cytotoxic anticancer drugs (6). However, not all tumors respond to TRAIL. This lack of response may be attributed either to unfavorable ratios of death and decoy receptors or because of intracellular resistance mechanisms (3, 7–10). With respect to intracellular resistance mechanisms, the FLIP protein has been identified as a blocker of apoptosis induced by TNF family death receptors (reviewed in Ref. 11). FLIP binds to and neutralizes adapter proteins and procaspases normally recruited to the cytosolic domains of apoptosis-inducing TRAIL receptors upon ligand stimulation, thus interrupting early steps in TRAIL signaling. Furthermore, overexpression of FLIP protein has been documented in cancers (12).

PPARγ is a member of the steroid/retinoid superfamily of ligand-activated transcription factors. Agonistic ligands of PPARγ include modified fatty acids, cyclopentenone-containing prostaglandins, triterpenoids, and the thiazolidinediones, a class of insulin-sensitizing drugs used in the treatment of type II diabetes (reviewed in Ref. 13). Anti-tumor properties of PPARγ agonists have been reported. For example, thiazolidinediones have been shown to suppress the growth of human colon and breast cancer cell lines in vitro and in vivo in the mouse (14, 15), and a member of a new class of PPARγ agonists (tyrosine analogs) suppresses mammary carcinogenesis in a standard rat model (16). However, troglitazone increases incidence of colonic polyps in a mouse model in which one allele of adenomatous polyposis coli is inactive (17, 18), suggesting complex effects on neoplasia. Moreover, the concentrations of thiazolidinediones required for some apoptotic effects are beyond clinically attainable ranges (15).

Here we explored the effects of PPARγ ligands on TRAIL-induced apoptosis in epithelial cancers cell lines. Our findings demonstrate a new PPARγ-independent mechanism for these compounds, resulting in rapid decreases in FLIP protein without concomitant reductions in c-FLIP mRNA, and causing sen-
sitzation of tumor but not several types of normal cells to TRAIL-induced apoptosis. The mechanism invoked by these PPARγ modulatory compounds involves ubiquitination and proteasome-dependent degradation of FLIP, thus revealing the existence of an inducible pathway for reducing FLIP expression that might be exploited for promoting death receptor-induced apoptosis of neoplastic cells.

MATERIALS AND METHODS

Cell Cultures—Human prostate cancer, PPC-1 and LNCaP, ovarian cancer, OVCAR-3, and SK-OV-3 cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin, and 0.1 U/ml streptomycin. Cells were maintained at 37 °C with 5% CO2.

For immunoprecipitation experiments, OVCAR-3 cells were transiently transfected with 5 μg of plasmid DNA encoding IKKα/β (Stratagene), and c-FLIP, CrmA, Bcl-2, PPARγ, and FLIP protein degradation by PPARγ-modulating Drugs 22321

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts were prepared from cells, and EMSAs were carried out as described previously (26). In brief, oligonucleotides containing a consensus NF-κB-binding site, 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Promega) were end-labeled with [γ-32P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Amersham Biosciences). When the assay was performed with MicroSpin G-25 columns (Amersham Biosciences), the labeled probe (15 fmol) was incubated with 3 μg of nuclear extract for 25 min at room temperature and separated by electrophoresis in nondenaturing 5% polyacrylamide gels in 0.25× TBE (22.5 mM Tris borate, 0.5 mM EDTA) at 4 °C. After drying, gels were exposed to x-ray film at −70 °C.

In Vitro Kinase Assays—Cells were pretreated with PPARγ modulators or treated with TNF (20 ng/ml) for 20 min. Cytosolic extracts (200 μg) prepared as above were incubated with 2 μg of anti-IκKs (BD Pharmingen) for 2 h, and 30 μl of protein A-Sepharose (Sigma) was added overnight at 4 °C. After centrifugation at 15,000 × g for 15 min, the lysates were mixed with 20 μl of anti-DR5 antibody (Alexis) and 50 μl of protein A-Sepharose at 4 °C for 6 h. The immune complexes (immunoprecipitations) were washed four times with the binding buffer, and the samples from immunoprecipitations and total lysates were subjected to SDS-PAGE/immunoblot analysis using antibodies specific for (α) TRADD (BD PharMingen) at 1:1000, (Bio-Rad) at 1:250; anti-FADD (BD PharMingen) 1:1000; anti-DcR1 (Bio-Rad) at 1:250; anti-FLIP (NF-6) 1:1000; anti-caspase 8 at 1:3000 (v/v) (21) or at 1:1000 from Alexis; anti-ARB (BD Pharmingen, La Jolla, CA) at 1:1000; anti-FLIP (BD Pharmingen) at 1:1000; anti-PPARγ (Santa Cruz Biotechnology Inc.) at 1:200; anti-TRADD (Santa Cruz Biotechnology Inc.) at 1:250; anti-FADD (BD Pharmingen) 1:1000; anti-DeR1 (ProSci Inc., Poway, CA) at 1:500; anti-DeR2 (Calbiochem) at 1:1000; anti-DAP3 (Transduction Laboratories, San Diego, CA) at 1:500; anti-ubiquitin (Santa Cruz Biotechnology Inc.) at 1:200, and anti-α-tubulin (Sigma) at 1:1000 with 25 μl of TRAIL-buffered blocking buffer containing 5% nonfat skim milk for 1–3 h at room temperature, followed by washing with T-TBS for 30 min. Goat anti-rabbit or mouse IgGs coupled with horseradish peroxidase (Bio-Rad) were used as secondary antibodies at 1:3000 (v/v). Immunospecific bands were detected by using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

For immunoprecipitation experiments, OVCAR-3 cells were treated with either CDDO (5 μM) or TRAIL (100 ng/ml) alone or both for 2 h. Cytosolic extracts were treated with iced-cold phosphate-buffered saline once and lysed in 1 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl [pH 7.5], 2 mM EDTA, 0.5 mM sodium orthovanadate, 10 mM β-glycerophosphate, and protease inhibitor mixture) at 4 °C for 30 min. In parallel, 10% of cell pellets were lysed in RIP buffer for immunoblot analysis. After centrifugation at 15,000×g for 15 min, the lysates were mixed with 20 μl of anti-DR5 antibody (Alexis) and 50 μl of protein A-Sepharose at 4 °C for 6 h. The immune complexes (immunoprecipitations) were washed four times with the binding buffer, and the samples from immunoprecipitations and total lysates were subjected to SDS-PAGE/immunoblot analysis using antibodies specific for (α) TRADD (BD PharMingen) at 1:1000, (Bio-Rad), and anti-TRAIL (FLIP) (NF-6) at 1:200 (v/v).

In Vitro Kinase Assays—Cells were pretreated with PPARγ modulators for 15 min and then treated with TNF (20 ng/ml) for 20 min. Cytosolic extracts (200 μg) prepared as above were incubated with 2 μg of anti-IκKs (BD Pharmingen) for 2 h, and 30 μl of protein A-Sepharose (Sigma) was added overnight at 4 °C. Immunoprecipitates were washed three times with 50 mM Tris-HCl (pH 7.4), 10% NaCl, 10 μg/ml trypsin inhibitor, 0.5 mM sodium orthovanadate, 5 mM NaF, and 50 mM HEPES (pH 7.4), containing protease inhibitor mixture (Roche Molecular Biochemicals) and washed once more with the kinase buffer (20 mM HEPES [pH 7.4], 2 mM MgCl2, 2 mM MnCl2, 1 mM dithiothreitol, 0.5 mM sodium orthovanadate, 2 mM NaF, 10 mM β-glycerophosphate, containing 10 mM ATP, 20 μl of [γ-32P]ATP, and 1 μg of GST-1xIκBα (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 30 min at 30 °C. The samples were subjected to 12% SDS-PAGE. Gels were dried and exposed to x-ray film.

Metal Affinity Capture—SK-OV-3 cells were co-transfected with plasmids encoding FLIP and His-tagged ubiquitin (pCW). After 18 h, cells were treated with 5 μg CDDO for 4 h in the absence or presence of 400 nm epoxomicin and lysed in 1 ml of GTN buffer (6 mM guanidine HCl, 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 20 mM imidazole, and 0.1% Triton X-100). His-tagged ubiquitin was recovered from lysates by incubation for 30 min at 25 °C with 80 μl of cobalt chelate resin (CLONTECH). Captured proteins were eluted in 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 200 mM imidazole, and 1.0% Triton X-100 after 3 washes of GTN buffer containing 10 mM imidazole, and analyzed by SDS-PAGE/immunoblotting using anti-FLIP antibody.

RNase Protection Assays—PPC-1 or OVCAR-3 cells were treated with the ligands for 1 and 6 h, and then total RNA was isolated with TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. The probe for c-FLIP was synthesized by using the human Apo3b Multi-Probe template set (BD Pharmingen) with [α-32P]UTP and-

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bridized with 10 μg of RNA at 56 °C overnight. After RNase treatment, the protected transcripts were separated by denaturing PAGE (5%). Gels were dried and exposed to x-ray film at −70 °C.

RESULTS

PPARγ Modulators Increase Sensitivity of Tumor Cell Lines to TRAIL—To explore preliminarily the effects of PPARγ modulators on tumor cell responses to TRAIL, we contrasted the effects of three different classes of PPARγ ligands as follows: (a) 15d-PGJ2, a natural cyclopentenone prostaglandin having PPARγ agonist activity; (b) ciglitazone (Cig) and troglitazone (Tro), representing synthetic thiazolidinediones PPARγ agonists; and (c) the triterpenoids CDDO and CDDO-Me, which function as a weak agonist and as an antagonist of PPARγ, respectively (28). When tested against a variety of solid tumor cell lines (PPC-1, PC3, OVCAR-3, SK-OV-3, HT29, COLO205, LNCaP, HeLa, HEY3, and HT1080) at concentrations of ≤1 μM, most of these PPARγ modulators had little effect on cell viability, as measured by MTT dye reduction assays (Fig. 1 and data not shown). For example, relative numbers of viable cells were within 80% of control following 1 day of treatment with 15d-PGJ2, Cig, Tro, CDDO, or CDDO-Me at ≤1 μM, although cytotoxic activity was observed at higher concentrations (5–25 μM). An exception was CDDO-Me, which exhibited anti-tumor activity at ≤1 μM in occasional tumor lines (data not shown). The sensitivity of these tumor cell lines to TRAIL was also tested. When treated with TRAIL, ≤80% of the tumor cells remained viable after 1 day. In fact, of 10 tumor lines treated with 100 ng/ml TRAIL, only one was significantly inhibited (data not shown). Thus, neither PPARγ modulators nor TRAIL by themselves was generally effective at killing tumor cells. However, combined treatment of epithelial cancer cell lines with TRAIL and PPARγ modulators resulted in synergistic reductions in relative numbers of viable cells. Fig. 1 shows examples for several PPARγ modulators and tumor cell lines. Synergy was observed both when holding the concentration of TRAIL fixed (100 ng/ml) and varying the concentration of PPARγ modulators and, conversely, when holding the concentration of PPARγ modulators fixed and varying TRAIL (Fig. 1). In contrast to tumor cell lines, the combination of TRAIL and PPARγ modulators was not cytotoxic to normal cells, including pri-

FIG. 1. Effect of PPARγ ligands on cell viability. Cell viability was measured by MTT assay and expressed as % relative to control cultures. Data represent mean ± S.D. of triplicate cultures and are representative of 2–5 independent experiments. A, human cancer cells OVCAR-3, COLO205, HT29, SK-OV-3, PPC-1, and LNCaP were plated in 96-well plates 48 h prior to treatment (5–6 × 104/well) and then treated with the indicated concentrations of CDDO for 24 h with (closed circles) or without TRAIL (open circles) at either 25 (COLO205 and PPC-1), 100 (OVCAR-3, HT29, and SK-OV-3), or 250 ng/ml (LNCaP). B, SK-OV-3 cells were treated with the indicated concentrations of ligands for 24 h with (closed circles) or without TRAIL (open circles) (100 ng/ml). C, SK-OV-3 cells, hepatocytes from cynomologus monkeys and human umbilical vascular endothelial cells (HUVEC) were treated with CDDO (0.5 μM) or troglitazone (20 μM) along with the increasing amounts of TRAIL.
mary cultures of hepatocytes, endothelial cells, peripheral blood leukocytes, and bone marrow (Fig. 1C and data not shown).

To explore whether these results were attributed to induction of apoptosis, tumor cell lines were treated with TRAIL in combination with PPARγ modulators, and apoptosis was measured by DAPI staining (counting percentages of cells with apoptotic nuclear morphology, as determined by chromatin condensation and nuclear fragmentation). Also, caspase activity was measured in cell lysates (based on cleavage of the fluorogenic caspase substrate, Ac-DEVD-AFC), and cleavage of the caspase substrate poly(ADP-ribose) polymerase (PARP) was monitored (by immunoblotting). Fig. 2 shows some representation data. Note that tumor lines such as prostate cancer PPC-1, ovarian cancer OVCAR-3, and cervical cancer HeLa are triggered by the combination of PPARγ modulators and TRAIL to undergo apoptosis (Fig. 2, A and B), activate caspases (Fig. 2C), and cleave the caspase substrate PARP (Fig. 2D). In contrast, culturing these cells with either TRAIL or PPARγ modulators alone had little effect. The broad spectrum caspase inhibitor, Z-VAD-fmk, potently suppressed TRAIL-induced cell death (not shown), further supporting an apoptotic mechanism. The synergistic effect of TRAIL and PPARγ modulators was not attributable to a mere shift in the kinetics of apoptosis induction, as demonstrated by time course analysis (Fig. 2E).

**TRAIL Sensitization by PPARγ Modulators Is Not Mediated by Effects on NF-κB**—In addition to their effects on PPARγ activity, several PPARγ modulators have been reported to directly inhibit the IkB kinases, IKKa and IKKβ, thereby suppressing NF-κB induction (29). Because NF-κB plays important roles in suppressing apoptosis induced by TNF, affecting expression of antiapoptotic and proapoptotic genes (30, 31), we explored whether inhibition of IKK activity or suppression of NF-κB induction correlated with the ability of various PPARγ modulators to sensitize tumor cells to TRAIL.

For IKK activity assays, tumor cell lines such as PPC-1 were treated for 15 min with four different PPARγ modulators which we had demonstrated sensitize tumor cells to TRAIL, including 15d-PGJ2, ciglitazone, CDDO, and CDDO-Me. Because TRAIL did not induce significant IKK activation (not shown), TNF was added to cultures for 20 min to stimulate IKKs. Cells were then lysed, and IKKa was recovered by immunoprecipitation, measuring its activity by *in vitro* kinase assay using GST-IκBα as an *in vitro* substrate. Loading of equal amounts of IKKa protein was confirmed by immunoblotting. Although 15d-PGJ2 was a potent inhibitor of IKKα activity, consistent with previous reports (29, 32), and CDDO reduced IKKα activity by about half, the other PPARγ modulators ciglitazone and CDDO-Me had no effect on IKKα activity (Fig. 3A). Thus, suppression of IKKα activity cannot explain the ability of ciglitazone and CDDO-Me to sensitize tumor cells to TRAIL.

Similar conclusions were reached by assessing NF-κB induction using EMSAs, where binding of NF-κB to 32P-labeled oligonucleotide probes containing NF-κB-binding sites was measured (Fig. 3B). For example, when TNF was used as a stimulus for inducing NF-κB DNA binding activity, ciglitazone and CDDO-Me had little or no effect. In contrast, 15d-PGJ2 completely inhibited NF-κB induction and CDDO reduced levels of NF-κB DNA binding activity by about half, consistent with the kinase data (Fig. 3A). Thus, some PPARγ modulators that sensitize tumor cells to TRAIL reduce IKKα activity and inhibit NF-κB induction (15d-PGJ2, CDDO), but others do not (ciglitazone; CDDO-Me).

To probe further the relationship of NF-κB to effects of PPARγ modulators on TRAIL sensitivity, levels of NF-κB were also measured in PPC-1 cells following stimulation with TRAIL (instead of TNF) and correlated with induction of apoptosis, using caspase-mediated cleavage of PARP as a surrogate marker for apoptosis (Fig. 3B). Similar to when TNF was employed, 15d-PGJ2 caused striking reductions in NF-κB levels in TRAIL-stimulated cells, and this correlated with sensitization to TRAIL-induced apoptosis, as evidenced by PARP cleavage. In contrast, levels of NF-κB in TRAIL-stimulated cells were not reduced by ciglitazone, CDDO, and CDDO-Me (and may have even been slightly increased), yet all of these PPARγ modulators sensitized PPC-1 cells to TRAIL-induced apoptosis.

We also tested additional prostaglandins, some of which inhibit NF-κB and others which do not, correlating their effects on NF-κB with TRAIL-induced PARP cleavage (Fig. 3B).
Among the seven prostaglandins tested (PGA1, PGD2, PGE2, PGF2α, PGJ2, 12d-PGJ2, and 15d-PGJ2), three of them (PGA1, PGJ2, and 15d-PGJ2) completely and one (12d-PGJ2) partially inhibited NF-κB. Thus, reductions in NF-κB-induced apoptosis (Fig. 3) do not correlate with sensitization to TRAIL. Regardless, PPARγ modulators reduced the levels of both FLIPL and FLIP (not shown).

**PPARγ Modulators Enhance Assembly of TRAIL Receptor Signaling Complexes**—The FLIP protein inhibits recruitment and activation of pro-caspase 8 at ligand-activated TNF-family death receptor complexes (33, 34). Thus, if PPARγ modulators sensitize cells to TRAIL by reducing FLIP levels, then we would expect to observe enhanced recruitment of pro-caspase 8 to TRAIL receptors and increased caspase 8 activation. To explore this hypothesis, OVCAR-3 cells were cultured with or without TRAIL and PPARγ modulator CDDO individually and in combination, and then the TRAIL receptor DR5 was immunoprecipitated and associated caspase 8, FLIP, and FADD (a pro-caspase 8-binding adapter protein) were examined by SDS-PAGE/immunoblotting (Fig. 5). Control experiments confirmed successful immunoprecipitation of DR5 by the anti-DR5 but not by control antibodies (not shown).

When treated with TRAIL or CDDO individually, little pro-caspase 8 was associated with anti-DR5 immune complexes. However, DR5 immune complexes recovered from TRAIL-treated cells did contain cleaved p43-FLIP protein, as expected from prior studies of FLIPL-expressing cells (33, 34), showing that FLIPL can become cleaved by caspase 8 when recruited to the CD95 death-inducing signaling complex (34, 35). The full-length, uncleaved p55 FLIP protein was not visible in these experiments because of its co-migration with the immunoglobulin heavy chain band (see Fig. 5 for details). In contrast, treating OVCAR-3 cells with the combination of CDDO and TRAIL resulted in disappearance of FLIP and increased association of pro-caspase 8 with DR5 complexes. Moreover, a partially processed ~41–43-kDa form of caspase 8 was found at the receptor complex (Fig. 5). Analysis of lysates from the same cells by immunoblotting demonstrated detectable levels of fully processed ~18-kDa caspase 8 (catalytic large subunit) and partially processed 41–43-kDa caspase 8 only in cells treated with the combination of CDDO and TRAIL but not in cells treated with either agent alone. Levels of FADD were unchanged in cell lysates, thus providing an internal control for equal loading. Interestingly, recruitment of FADD to DR5 also was apparently enhanced by CDDO treatment, based on these co-immunoprecipitation experiments (Fig. 5). Thus, CDDO enhances proper assembly of the TRAIL-mediated death-inducing signaling complex and activation of caspase 8.

**Genetic Manipulation of FLIP Levels Correlates with Sensitivity to TRAIL**—To explore the functional significance of the observed reductions of FLIP protein in tumor cell lines treated with PPARγ modulators, we performed gene transfer experiments, asking what effect overexpression of FLIP has on the ability of PPARγ modulators to sensitize cells to TRAIL. Tran-
was added to cultures instead of CDDO.

Human ovarian cancer OVCAR-3 cells were treated with 15d-PGJ2 (10 μM) or CDDO (5 μM) for 6 h. Cell lysates were subjected to immunoblot analysis using c-FLIP, RIP, and α-tubulin antibodies. Control, treatment with vehicle; −, lysates from time 0. B, human ovarian cancer OVCAR-3 cells were treated with 15d-PGJ2 (10 μM) or CDDO (5 μM) for 6 h. Immunoblot analysis was performed using antibodies for c-FLIP, DR4, DR5, and α-tubulin. C, control and untreated cells. C, PPC-1 cells were treated with 15d-PGJ2 (10 μM), ciglitazone (20 μM), CDDO (5 μM), CDDO-Me (0.5 μM), or troglitazone (10 μM) for 6 h. Cell lysates were normalized for total protein content and analyzed by immunoblotting using antibodies specific for FLIP, RIP, TRADD, caspase 8, FADD, DAP3, DcR1, and DcR2. D, OVCAR-3 cells were cultured for 24 h with various concentrations of CDDO with or without (control) TRAIL (top). The percentage of apoptotic cells was determined by DAPI staining (mean ± S.D.; n = 3). Lysates were also prepared from cells treated with CDDO alone for 6 h, normalizing for total protein content (35 μg), and analyzed by SDS-PAGE/immunoblotting using antibodies specific for FLIP or α-tubulin (bottom). E, OVCAR-3 cells were cultured and analyzed as in D except that troglitazone was added to cultures instead of CDDO.

FIG. 4. Down-regulation of c-FLIP protein by PPARγ ligands. A, PPC-1 cells were treated with 50 μM arachidonic acid, PGA₂, PGD₂, PGE₂, and PGG₂, PGJ₂, or 10 μM 12d-PGJ₂, and 15d-PGJ₂ for 6 h. Cell lysates were subjected to immunoblot analysis using c-FLIP, RIP, and α-tubulin antibodies. Control, treatment with vehicle; −, lysates from time 0. B, human ovarian cancer OVCAR-3 cells were treated with 15d-PGJ2 (10 μM) or CDDO (5 μM) for 6 h. Immunoblot analysis was performed using antibodies for c-FLIP, DR4, DR5, and α-tubulin. C, control and untreated cells. C, PPC-1 cells were treated with 15d-PGJ2 (10 μM), ciglitazone (20 μM), CDDO (5 μM), CDDO-Me (0.5 μM), or troglitazone (10 μM) for 6 h. Cell lysates were normalized for total protein content and analyzed by immunoblotting using antibodies specific for FLIP, RIP, TRADD, caspase 8, FADD, DAP3, DcR1, and DcR2. D, OVCAR-3 cells were cultured for 24 h with various concentrations of CDDO with or without (control) TRAIL (top). The percentage of apoptotic cells was determined by DAPI staining (mean ± S.D.; n = 3). Lysates were also prepared from cells treated with CDDO alone for 6 h, normalizing for total protein content (35 μg), and analyzed by SDS-PAGE/immunoblotting using antibodies specific for FLIP or α-tubulin (bottom). E, OVCAR-3 cells were cultured and analyzed as in D except that troglitazone was added to cultures instead of CDDO.

FIG. 5. CDDO enhances TRAIL-induced caspase 8 activation. OVCAR-3 cells were cultured with 100 ng/ml TRAIL, 5 μM CDDO, both, or neither of these agents, and then cells were lysed 2 h later, and DR5 was immunoprecipitated. Immune complexes and lysates were analyzed by SDS-PAGE using antibodies specific for caspase 8 (top), FLIP (middle), and FADD (bottom). The positions of unprocessed and fully processed caspase 8 are indicated. The p43 FLIP band arises from caspase 8-mediated cleavage (34, 35). The asterisk indicates contaminating immunoglobulin heavy chain which co-migrates with pro-caspase 8 and uncleaved p55 FLIP (33, 34) in co-immunoprecipitations (1st 4 lanes), thus obscuring these proteins from view.

sufficient transfection of PPC-1 cells with an expression plasmid encoding FLIP abrogated the ability of 15d-PGJ₂ (Fig. 6A) and CDDO (Fig. 6C) to sensitize tumor cells to TRAIL, as measured by reductions in apoptosis of the transfected cells. Immunoblotting confirmed that FLIP protein levels were restored in the transfected cells which received the FLIP expression plasmid (not shown). These data provide correlative evidence that the sensitization of tumor cells to TRAIL by PPARγ modulators could be due to decreases in FLIP levels. Overexpression of FLIP also inhibited TNF-mediated apoptosis (Fig. 6A), consistent with previous reports (36–38).

A gene transfer approach was also used to explore other aspects of the apoptotic mechanism induced by the combination of TRAIL and PPARγ modulators. To address further the issue of NF-κB, PPC-1 cells were transiently transfected with a plasmid producing IKKβ, which caused marked increases in NF-κB activity (not shown). When apoptosis was induced by the combination of TRAIL and 15d-PGJ₂, IKKβ overexpression failed to provide protection (Fig. 6B). In contrast, when apoptosis was induced by the combination of TNF and 15d-PGJ₂, then IKKβ overexpression potently suppressed apoptosis. These data are consistent with prior reports demonstrating an important role for NF-κB in regulating apoptosis induction by TNF (31) but indicate that NF-κB is not protective against TRAIL-induced apoptosis (39). Consistent with the documented role for caspases in TRAIL induced apoptosis, overexpression of the CrmA (a viral inhibitor of caspase 8 (40, 41) suppressed apoptosis induced by the combination of TRAIL and PPARγ modulators such as CDDO (Fig. 6C). In contrast, overexpression of Bcl-2 did not suppress apoptosis (Fig. 6C), consistent with evidence that TRAIL and other death receptors can trigger apoptosis through Bcl-2-independent mechanisms in many types of cells.

Finally, antisense oligonucleotides targeting FLIP were used to explore whether down-regulation of FLIP protein levels is sufficient to sensitize tumor cells to TRAIL. Treatment of PPC-1 cells with FLIP antisense oligonucleotides resulted in a
pronounced reduction in the levels of both the long and short isoforms of FLIP protein, compared with control oligonucleotide-treated cells (Fig. 6D). This antisense-mediated reduction in FLIP protein levels was correlated with enhanced sensitivity of PPC-1 cells to TRAIL-induced apoptosis (Fig. 6D). Therefore, we conclude that reducing FLIP expression is indeed sufficient to sensitize at least some tumor cell lines to TRAIL-induced apoptosis.

**PPARγ Levels and Activity Do Not Correlate with TRAIL Responses** — Although the agents used here are known to modulate PPARγ, we suspected a PPARγ-independent mechanism accounted for their ability to sensitize tumor cells to TRAIL, given that PPARγ agonists (15d-PGJ2, ciglitazone, and troglitazone), weak agonists (CDDO), and antagonists (CDDO-Me) were effective. We therefore explored the effects of overexpressing PPARγ on TRAIL-induced apoptosis, reasoning that if PPARγ modulators were functioning through a PPARγ-dependent process then they should be more potent when PPARγ levels are higher. HeLa cells were employed for these studies because of their relatively low endogenous levels of PPARγ protein (32). Overexpressing PPARγ had no effect on apoptosis induction by the combination of TRAIL and CDDO, despite successful transfection of >80% of cells as determined by a marker plasmid encoding green fluorescent protein (GFP). To confirm that these transfections resulted in higher levels of functional, bioactive PPARγ protein, reporter gene assays were performed in parallel, confirming that transfection of HeLa cells with PPARγ-encoding plasmids resulted in dose-dependent increases in ligand-activated expression of a luciferase reporter gene plasmid containing PPAR-response elements (PPREs) (Fig. 7B). We conclude therefore that although 15d-PGJ2 and CDDO are capable of activating PPARγ, their ability to sensitize tumor cells to TRAIL does not correlate with effects on PPARγ.

As an alternative approach, we employed a mutant of PPARγ that functions as a dominant-negative inhibitor of the endogenous protein (22). Overexpression of dominant-negative PPARγ failed to abrogate CDDO-mediated sensitization of PPC-1 cells to TRAIL (Fig. 7C), despite its ability to block completely 15d-PGJ2-mediated induction of PPARγ transcriptional activity (as determined by reporter gene assays using a PPARγ-inducible luciferase reporter gene) (Fig. 7D). We conclude therefore that the TRAIL-sensitizing effects of CDDO can be dissociated from its effects on PPARγ.

**PPARγ Modulators Reduce FLIP Protein Levels through a Non-transcriptional Mechanism Involving Protein Ubiquitination** — RNase protection assays were performed to measure relative levels of c-FLIP mRNA in PPC-1 and OVCAR-3 cells treated with PPARγ modulators. Although causing a profound decrease in FLIP protein levels (Fig. 4), corresponding decreases were not observed in c-FLIP mRNA levels or in levels of several other mRNAs that serve as comparison controls (Fig. 8A and not shown). Thus, PPARγ modulators appear to cause FLIP protein reductions through a transcription-independent process, lending further support for a PPARγ-independent mechanism.

Analysis of the kinetics of FLIP protein reductions in cells treated with CDDO (Fig. 8B) and other PPARγ modulators (not shown) revealed a rapid process, with decreases evident within 20 min and complete clearance of FLIP protein from cells by 2 h. Pulse-chase experiments using L-[35S]methionine-labeled cells confirmed that CDDO induced decreases in the rate of FLIP protein degradation (not shown).

To explore the potential role of proteases in the reduction of FLIP protein induced by PPARγ modulators, tumor cells were cultured with inhibitors of caspases (Z-VAD-fmk), the 26 S proteasome (MG132, lactacystin, and MG115), and lysosomal proteases (TLCK and calpeptin). MG132, lactacystin, and MG115 partially prevented FLIP down-regulation by CDDO, suggesting a potential role for the proteasome in this mechanism. In contrast, inhibitors of caspases and lysosomal proteases had no effect (Fig. 8C).

Because the 26 S proteasome degrades ubiquitinated proteins, we investigated whether PPARγ modulators induce ubiquitination of the FLIP protein. Long exposures of immunoblots probed with anti-FLIP antibody demonstrated the presence of higher molecular weight conjugates of FLIP after treatment with CDDO (Fig. 8D, top). The appearance of these higher molecular weight forms of FLIP was evident within 1 h after CDDO treatment, coinciding with the approximate time when

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**FIG. 6. Genetic modulation of FLIP expression correlates with sensitivity to TRAIL-induced apoptosis.** PPC-1 cells were transiently transfected with pEGFP (1 μg) and 4 μg of plasmids encoding c-FLIP (A) or IKKβ (B). After 20 h, cells were treated with TRAIL (100 ng/ml) or TNF (20 ng/ml) for 6 h in the presence of 15d-PGJ2 (10 μM). C, cells were transiently transfected with 1 μg of pEGFP and 4 μg of plasmids encoding c-FLIP, IKKβ, CrmA, and Bcl-2 proteins. The next day, cells were treated with CDDO (5 μM) in the presence of TRAIL (100 ng/ml) for 6 h. The percentage of apoptotic GFP-positive cells was determined by DAPI staining (mean ± S.D.; n = 3). D, cells were transfected with FLIP or control antisense oligonucleotides for 14 h and then treated with TRAIL (25 ng/ml) for 7 h. Apoptotic cells were counted by DAPI staining. Inset show the levels of long and short forms of FLIP proteins in cells transfected with control (C) and FLIP antisense (AS) oligonucleotides, respectively. Levels of α-tubulin are shown as a control.
FLIP<sub>1</sub> and FLIP<sub>2</sub> protein levels began to decline. This higher molecular weight material was confirmed to represent polyubiquitinated FLIP, based on experiments where lysates from CDDO-treated cells were immunoprecipitated using anti-FLIP antibody, and the resulting immune complexes were analyzed by SDS-PAGE/immunoblotting using anti-ubiquitin antibodies (Fig. 8D, bottom). Treating cells with the proteasome inhibitors, such as epoxomicin, also induced a slight increase in ubiquitinated FLIP, indicative of a basal low rate of FLIP ubiquitation, but CDDO massively increased the abundance of
ubiquitin conjugates of FLIP (Fig. 8D). In contrast to FLIP, the extent of ubiquitination and steady-state levels of p53 and β-catenin (proteins known to be regulated by ubiquitin-proteasome pathways (42, 43)) were not altered by CDDO treatment of cells (not shown).

To provide further evidence of CDDO-inducible ubiquitination of FLIP, cells were transiently co-transfected with plasmids encoding FLIP and histidine-tagged (His6) ubiquitin. After culturing cells for 4 h with CDDO, epoxomicin, or the combination of these reagents, lysates were subjected to co-balt-chelation chromatography to recover His6-tagged proteins, followed by SDS-PAGE/immunoblotting using anti-FLIP antibody (Fig. 8E). As shown, His6-ubiquitin-conjugated FLIP products accumulated in cells treated with either CDDO or epoxomicin but not in untreated cells (Fig. 8E). Furthermore, the combination of CDDO and epoxomicin led to even higher increases in His6-ubiquitin-conjugated FLIP products, consistent with the hypothesis that CDDO induces increases in FLIP ubiquitination, whereas epoxomicin prevents degradation of the ubiquitinated FLIP proteins. Taken together, these results suggest that CDDO enhances ubiquitination of FLIP, thus accelerating the degradation of FLIP protein by the proteasome.

**DISCUSSION**

In this report, we reveal the existence of an inducible pathway that triggers ubiquitination and degradation of the FLIP protein and is capable of sensitizing at least some types of the transformed cells to death receptor-mediated apoptosis in vitro. Interestingly, T-cell receptor stimulation of T-lymphocytes has also been reported to diminish levels of FLIP protein without concomitant changes in mRNA levels (44). In addition, reductions in FLIP protein levels induced by p53 are reported negated by proteasome inhibitors (45). Thus, precedent exists for post-transcriptional regulation of FLIP expression. For many proteins whose levels are conditionally regulated by ubiquitination and proteasome-dependent degradation, ubiquitination is induced upon binding of E3 ubiquitin ligases to the target protein (reviewed in Ref. 46). In this regard, FLIP has been shown to interact with TRAF2, which contains a RING finger domain known to possess E3 ligase activity (33). It is conceivable therefore that PPARγ modulatory drugs influence the expression of TRAF2 or other types of FLIP-binding E3 ubiquitin ligases. Several other mechanisms could also be envisioned.

A recent report (47) demonstrated that troglitazone can sensitize two human tumor cell lines to TRAIL; however, the responsible mechanism was not addressed here. We extended this observation to multiple solid tumor cell lines and to several classes of PPARγ modulators. Our data indicate that compounds previously recognized for their ability to bind and modulate the function of PPARγ have an additional PPARγ-independent mechanism, allowing them to reduce FLIP protein levels and sensitize cancer cells to apoptosis induction by TRAIL. The evidence arguing against a PPARγ-dependent mechanism for these compounds includes the following: (a) efficacy of both PPARγ agonists (15d-PGJ2, ciglitazone, troglitazone, and CDDO) and antagonists (CDDO-Me) (28); (b) failure of PPARγ overexpression and dominant-negative PPARγ to alter effects of compounds on TRAIL-induced apoptosis; and (c) decreases in FLIP protein levels without concomitant reductions in mRNA, suggesting a non-transcriptional mechanism uncharacteristic of PPARγ. Prior studies of effects of thiazolidinediones and 15d-PGJ2 on cells from PPARγ knock-out mice have demonstrated PPARγ-independent inhibition of cytokine production by activated macrophages (48), suggesting an alternative target of these agents. Moreover, it has been reported recently that the growth-suppressive effects of thiazolidinediones on cells in vitro and in vivo are independent of this receptor, based on experiments using PPARγ−/− cells (49). In this regard, the IκB kinases that control NF-κB activity have been identified as direct targets of 15d-PGJ2 and some other types of PPARγ modulators. However, IKK and NF-κB also are not the relevant targets of the TRAIL-sensitizing compounds studied here, because overexpression of IKK failed to restore TRAIL resistance and because some TRAIL-sensitizing compounds (CDDO-Me, ciglitazone, troglitazone) did not inhibit IKK or reduce NF-κB DNA binding activity. We speculate that natural PPARγ agonists (PGJ2, 12d-PGJ2, and 15d-PGJ2) and synthetic PPARγ modulators, including thiazolidinediones (troglitazone and ciglitazone) and triterpenoids (CDDO and CDDO-Me), interact with an unidentified cellular target, resulting in post-transcriptional reductions in FLIP protein levels, inducing FLIP protein degradation through a ubiquitin-proteasome pathway. If PPARγ modulators interact with a novel target protein, then medicinal chemistry efforts potentially could be used for identifying analogues of these compounds that retain the ability to reduce FLIP levels without affecting PPARγ or IKK activity. Specific analogues of this type might prove useful for identifying the relevant molecular target that controls FLIP ubiquitination. Given that triterpenoids such as CDDO are roughly 1 log more potent than the thiazolidinediones examined here at inducing FLIP degradation and sensitizing tumor cells to TRAIL, this class of chemical compounds should be given particular attention in the efforts to design selective agonists of the FLIP degradation pathway that lack effects on PPARγ. Interestingly, when used at higher concentrations (~5 μM), CDDO has recently been reported to trigger apoptosis of established leukemia and osteosarcoma cell lines through a pathway involving activation of caspase 8 but not caspase 9 and through a mechanism that is suppressible by CrmA but not by Bcl-XL (50, 51). These observations are consistent with activation of the “extrinsic” apoptosis pathway, which is commonly invoked by TNF family death receptors, as opposed to the “intrinsic” apoptosis pathway where mitochondria play a critical role (reviewed in Ref. 52). Thus, CDDO and related triterpenoids may possess the ability to activate the extrinsic pathway as single agents, without accompanying application of TRAIL or other death ligands, provided sufficiently high concentrations are employed. In such cases, it remains to be determined whether these compounds induce autocrine expression of TNF family death ligands or receptors, thus accounting for these observations. Regardless, such findings hint that CDDO and related molecules possess additional activities, besides induction of FLIP degradation, which promote activation of the extrinsic pathway. Moreover, because FLIP has been reported to regulate NF-κB and extracellular signal-regulated kinase signal transduction pathways (33) (in addition to suppressing caspase 8 activation), it is also possible that simply ablating FLIP expression can affect gene expression, thereby modulating apoptosis pathways in a cell context-dependent manner.

It has been reported that some preparations of recombinant TRAIL induce apoptosis of normal human hepatocytes, thus raising concerns about potential hepatotoxicity (reviewed in Ref. 53). Subsequent studies, however, have demonstrated that toxicity to hepatocytes is associated with aggregated TRAIL and is not seen with soluble trimeric TRAIL (5). Although we were unable to obtain suitable preparations of human hepatocytes to assess the effects of combined treatment with TRAIL and PPARγ modulators, we did perform testing using (as an alternative) hepatocytes from cynomolgus monkeys, but we failed to see induction of apoptosis. In this regard, the TRAIL recep-
tors of cynomologous monkey are 84–99% identical to their human counterparts (5). Moreover, it has been demonstrated that hepatocytes from these primates bind human TRAIL with high affinity (5). More importantly, these monkey and human hepatocytes are equivalent in their apoptotic responses to “good” (trimeric) and “bad” (aggregated) preparations of TRAIL, where trimeric TRAIL fails to induce apoptosis of both human and monkey cells, whereas aggregated TRAIL kills hepatocytes from both species (5). Thus, hepatocytes from cynomologous monkeys are a valid surrogate for human hepatocytes where TRAIL-induced apoptosis is concerned. The differential effects on normal versus malignant cells of combination treatment with TRAIL plus PPARγ modulators in vitro suggest that it could be possible to exploit these agents for the treatment of cancer, particularly because thiazolidinedione class PPARγ modulators are already in clinical use as insulin sensitizers for treatment of type II diabetes. Thus, although such sensitizers of cancer cells to TRAIL and other activa-

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