Expression of NAG-1, a Transforming Growth Factor-β Superfamily Member, by Troglitazone Requires the Early Growth Response Gene EGR-1*

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Troglitazone (TGZ) and 15-deoxy-Δ^{12,14}-prostaglandin J_2 (PGJ_2) are peroxisome proliferator-activated receptor-γ (PPARγ) ligands that have been shown to possess pro-apoptotic activity in human colon cancer. Although these compounds bind to PPARγ transcription factors as agonists, emerging evidence suggests that TGZ acts independently of PPARγ in many functions, including apoptosis. We previously reported that TGZ induces an early growth response transcription factor (EGR-1) by the ERK phosphorylation pathway rather than by the PPARγ pathway (Baek, S. J., Wilson, L. C., Hsi, L. C., and Eling, T. E. (2003) J. Biol. Chem. 278, 5845–5853). In this report, we show that the expression of the antitumorogenic and/or pro-apoptotic gene NAG-1 (nonsteroidal anti-inflammatory drug-activated gene-1) is induced by TGZ and correlates with EGR-1 induction. In cotransfection and gel shift assays, we show that EGR-1-binding sites are located within region −73 to −51 of the NAG-1 promoter and have an important role in the transactivation of TGZ-induced NAG-1 expression. In contrast, PGJ_2 induced NAG-1 protein expression, but PGJ_2 and TGZ may not affect the same region that TGZ does in the NAG-1 promoter. The effect of PGJ_2 is probably PPARγ-dependent because a PPARγ antagonist inhibited the PGJ_2-induced expression of NAG-1. TGZ-induced NAG-1 expression was not inhibited by the PPARγ antagonist. The fact that TGZ-induced NAG-1 expression was accompanied by the biosynthesis of EGR-1 also suggests that EGR-1 plays a pivotal role in TGZ-induced NAG-1 expression. Our results suggest that EGR-1 induction is a unique property of TGZ, but is independent of PPARγ activation. The up-regulation of NAG-1 may provide a novel explanation for the antitumorogenic property of TGZ.

The peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that can be activated by a specific ligand (1). Three isoforms (α, β/δ, and γ) have been identified and are encoded by separate genes. PPARs have been further characterized into three subtypes, γ1, γ2, and γ3 (2, 3). Each of the subtypes forms a heterodimeric complex with the retinoid X receptor and then binds to the PPAR response element (PPRE). This interaction can regulate cellular differentiation (4), apoptosis (5), inflammatory response (7, 8), and lipid metabolism (9).

Ligands of PPARγ include prostaglandins of the J series such as the natural prostaglandin 15-deoxy-Δ^{12,14}-prostaglandin J_2 (PGJ_2), the synthetic antidiabetic thiazolidinediones, and oxidative metabolites of polyunsaturated fatty acids. Previous studies have reported evidence for antitumorogenic activity of PPARγ ligands (10–14). Among the PPARγ ligands, the antitumorogenic activity of troglitazone (TGZ) and PGJ_2 has been well established (15, 16). For example, TGZ and PGJ_2 significantly inhibit tumor growth of human colorectal cancer cells (HCT-116), human breast cancer cells (MCF-7), and human prostate cancer cells (PC-3) in immunodeficient mice (15–18). Furthermore, TGZ and PGJ_2 affect several pathways in a PPARγ-independent manner. TGZ up-regulates nitric oxide synthesis (19), induces the p53 pathway (20), inhibits cholesterol biosynthesis (21), and has antioxidant function (22), whereas PGJ_2 induces apoptosis (23) and affects signaling pathways that utilize ERK kinase and NF-κB (24) independently of PPARγ. In addition, we have recently demonstrated that TGZ induces the early growth response gene EGR-1 independently of the PPARγ transcription factor (25). However, the molecular mechanism by which TGZ and PGJ_2 exhibit antitumorogenesis, other than by PPARγ activation, is not known.

The EGR-1 transcription factor (also known as NGFI-A, TIS8, krox-24, and zif268) is a member of the immediate-early gene family and encodes a nuclear phosphoprotein involved in the regulation of cell growth and differentiation in response to signals such as mitogens, growth factors, and stress stimuli. EGR-1 has been proposed as a tumor suppressor gene (26, 27). EGR-1 activates the PTEN (phosphatase and tensin homolog) tumor suppressor gene during UV irradiation (28), and re-expression of EGR-1 suppresses the growth of transformed cells both in soft agar and in athymic nude mice (29). EGR-1 induction is both p53-dependent and p53-independent (30–32). Moreover, EGR-1 is down-regulated in several types of neoplasia as well as in an array of tumor cell lines (33, 34). These results suggest that EGR-1 has a role in growth suppression.

The nonsteroidal anti-inflammatory drug-activated gene kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase.
Troglitazone-induced NAG-1 Expression Is Mediated by EGR-1

NAG-1 was identified from an indomethacin-induced gene library (35). NAG-1 (also known as MIC-1, GDF-15, placental transforming growth factor-β (TGF-β), and PLAB) represents a divergent member of the TGF-β superfamily. NAG-1 has anti-tumorigenic and pro-apoptotic activities as assessed by in vivo and in vitro assays (35–38). The expression of NAG-1 in human colon tissue was seen only in the tips of the villi, where apoptosis occurs (39). Although the expression of NAG-1 is regulated by several nonsteroidal anti-inflammatory drugs independent of cyclooxygenase (40), it is also regulated by several immunomodulatory agents, including resveratrol (38), retinol (41), and the retinoid 6-(3,1-adamantyl)-4-hydroxyphenyl)-2-phenanthrene carboxylic acid (42). We have previously reported the cloning and characterization of the 3.5-kb NAG-1 promoter (43). Although Sp1 and chicken ovalbumin upstream promoter transcription factor 1 are essential factors in the regulation of the basal level of NAG-1 expression, compound-induced NAG-1 expression at the transcriptional level has not been fully characterized.

In this study, we examine the relationship between PPARγ ligands and NAG-1 expression. PPARγ ligands, including TGZ and PGJ2, induce NAG-1 expression in human colorectal cancer cells. We found that TGZ induces EGR-1 expression, followed by induction of NAG-1 at the transcriptional level, whereas PGJ2 does not induce EGR-1. Rather, NAG-1 seems to be induced by PGJ2 through the PPARγ transcription factor because a PPARγ antagonist inhibited NAG-1 expression. EGR-1 induction by TGZ appears to be independent of PPARγ because other PPARγ ligands did not induce EGR-1, and PPARγ-binding sites are not located in the TGZ response element in the NAG-1 promoter. These data suggest that the expression of NAG-1 provides a novel mechanism for understanding how TGZ exerts its antitumorigenic activity.

MATERIALS AND METHODS

Cell Lines and Reagents—Human colorectal carcinoma cells (HCT-116) were purchased from American Type Culture Collection (Manassas, VA) and maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and gentamycin (10 µg/ml). Rosiglitazone (Invitrogen), PGJ2, Wy-14643, 13-hydroxyoctadecadienoic acid, and the PPARγ antagonist GW9662 were purchased from Cayman Chemical Co., Inc. (Ann Arbor, MI). All-trans-retinoic acid (RA), 9-cis-RA, and retinol were purchased from Sigma. Recombinant human TGF-β1 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). TGZ was obtained from WY Laboratories (Beverly, MA). NAG-1(antisense) was generated using primers 5′-TCCAGATCTCTGGATTTTGGTG-3′ (sense) and 5′-TGAGGACCCATTCCGTCTGAATGC-3′ (antisense). After PCR, the fragment was cloned into the TA vector (Invitrogen), sequenced, and further cloned into the pcDNA3 basic vector digested with XhoI and HindIII restriction enzymes. The NGFI-A binding protein NAB1 cDNA in the expression vector was cloned by PCR from the IMAGE:843249 clone (Invitrogen) using primers 5′-CTCCAGATCTCTGGATTTTGGTG-3′ (sense) and 5′-ATCACGAGCTTTGAATCGTTTTC-3′ (antisense). The amplified products were cloned into the pCR2.1 TOPO vector (Invitrogen), followed by cloning into the pcDNA3.1neo expression vector.

Transfection and Luciferase Assay—HCT-116 cells were plated in 6-well plates at 2 × 10⁵ cells/well in McCoy's 5A medium supplemented with 10% fetal bovine serum. After growth for 24 h, the cells were transfected with LipofectAMINE (Invitrogen) according to the manufacturer's protocol. For the cotransfection experiment, plasmid mixtures containing 0.5 µg of NAG-1 promoter linked to luciferase and 0.1 µg of pHRE-luc were transfected with LipofectAMINE (Invitrogen) according to the manufacturer's protocol. A 3×10⁴ cells/well were transfected with LipofectAMINE (Invitrogen) according to the manufacturer's protocol. The transfection efficiency was determined and normalized to the pRL-null luciferase activity with a dual luciferase assay kit (Promega). For PPARγ ligand treatments, the cells were treated with the ligand in the absence of serum for 24 h and then assayed for luciferase activity.

Western Blot Analysis—The level of protein expression was evaluated by Western blot analysis with anti-EGR-1 and anti-NAG-1 antibodies. Cells were grown to 60–80% confluency in 10-cm plates, followed by 16 h of additional growing in the absence of serum. After treatment with the indicated compounds, total cell lysates were isolated using precipitation assay buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). After sonication for 30 s, aliquots were separated by SDS-PAGE and transferred for 1 h onto nitrocellulose membrane (Schleicher & Schuell). The blots were blocked for 1 h with 5% skim milk in Tris-buffered saline and Tween 0.05% and probed with each antibody for 2 h at room temperature. After washing with Tris-buffered saline and Tween 0.05%, the blots were treated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed several times. Proteins were detected by the enhanced chemiluminescence system (Amersham Biosciences).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as described previously (43). For the gel shift assay, double-stranded oligonucleotides (Invitrogen) were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (New England Biolabs Inc., Beverly, MA). Assays were performed by incubating 10 µg of nuclear extracts in binding buffer (Geneka Biotechnology) containing 2 × 10⁻¹⁰ cm of labeled probe for 20 min at room temperature. To assure the specific binding of transcription factors, the probe was chased with 1-, 10-, and 50-fold molar excesses of unlabeled wild-type oligonucleotide. For the supershift experiments, anti-EGR-1 antibody (Geneka Biotechnology) was incubated with nuclear extracts on ice for 30 min before addition to the binding reaction. Samples were then electrophoresed on 5% non-denaturing polyacrylamide gels with 0.5× Tris borate/EDTA, and gels were dried and subjected to autoradiography.

RNA Isolation and Northern Blot Analysis—After reaching 60–80% confluency in 10-cm plates, the cells were treated at the indicated concentrations with PPARγ ligands in the absence of serum. For the cycloheximide experiment, the cells were treated with 5 µg/ml compound for 30 min prior to TGZ treatment. Total RNAs were isolated with TRizol reagent (Invitrogen) according to the manufacturer's protocol. Ten µg of total RNA was denatured at 55 °C for 15 min, separated on a 1.2% agarose gel containing 2.2 M formaldehyde, and then transferred to Hybond-N membrane (Amersham Biosciences). After fixing the membrane by UV, blots were prehybridized in hybridization solution (Rapid-Hyb buffer, Amersham Biosciences) for 1 h at 65 °C, followed by hybridization with cDNA labeled with [α-32P]dCTP by random primer extension (DECAGON III kit, Ambion Inc., Austin, TX). The digoxigenin-labeled probe was obtained from PGJ2 (sense 110) and anti-act (sc-1615) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-NAG-1 antibody was described previously (43).

Construction of Plasmids—The full-length EGR-1 cDNA in the pcDNAs expression vector was described previously (25). The luciferase constructs containing the NAG-1 promoter and Sp1 in the pcDNAs expression vector were prepared as described previously (43). The pNAG133′/+70 constructs were previously generated (38). The pNAG14′/+70 construct was generated using primers 5′-AGTTCGGGACTATAAGCCGGG-TCCGGC-3′ (sense) and 5′-TGGAGGCCATTCGCTCGAGTCT-3′ (antisense). After PCR, the fragment was cloned into the TA vector (Invitrogen), sequenced, and further cloned into the pGLBasic3 vector digested with Xhol and HindIII restriction enzymes. The NGFI-A binding protein NAB1 cDNA in the expression vector was cloned by PCR from the IMAGE:843249 clone (Invitrogen) using primers 5′-TCAGA-GTAATGCGGATTCCGGC-3′ (sense) and 5′-ATCACGAGCTTTGAATCGTTTTC-3′ (antisense). The amplified products were cloned into the pCR2.1 TOPO vector (Invitrogen), followed by cloning into the pcDNA3.1 neo expression vector.

RESULTS

PPARγ Ligands PGJ2 and TGZ Induce NAG-1 Expression by Different Pathways—PPARγ ligands have an antitumorigenic activity that is either dependent on binding to the ligand to PPRE or independent of PPARγ transcriptional binding (25, 44–46). One mechanism by which PPARγ ligands exert antitumorigenesis may involve the transcriptional up-regulation of antitumorigenic proteins. We measured Pten and p53 tumor suppressor gene expression. The PTEN protein is only marginally induced, whereas the level of p53 is not altered by TGZ in HCT-116 cells (25). Interestingly, NAG-1, which has antitumorigenic activity, was significantly induced by the PPARγ ligands. As shown in Fig. 1A, PGJ2 and TGZ, which are both PPARγ ligands, induced NAG-1 mRNA in a concentration-dependent manner (3-fold at 1 and 5 µM, respectively). HCT-116 cells were also treated with 1 µM PGJ2 or 5 µM TGZ for different times. Both PGJ2 and TGZ induced NAG-1 protein expression.
as early as 6 h (Fig. 1B), and a marked increase in NAG-1 was observed at 24 and 48 h, indicating that PGJ2 and TGZ induce NAG-1 expression in a dose- and time-dependent manner. In addition, the PPARγ ligand WY-14643 did not induce NAG-1 expression at concentrations up to 100 μM (data not shown), indicating that induction of NAG-1 is specific for this PPARγ ligand. We then examined whether NAG-1 induction by PPARγ ligands is dependent on the PPARγ transcription factor in HCT-116 cells expressing intact PPARγ (25). HCT-116 cells were treated with a combination of PPARγ ligands and/or GW9662, a selective PPARγ inhibitor. Western analyses suggest that the PPARγ antagonist suppressed the PGJ2-induced NAG-1 expression, but did not suppress TGZ-induced NAG-1 expression. These findings suggest that TGZ-induced NAG-1 expression may be PPARγ-independent, whereas PGJ2 increased NAG-1 expression through activation of PPARγ (Fig. 2).

**NAG-1 Promoter Activity and PPARγ Ligands**—To evaluate the importance of cis-acting elements in conferring PPARγ-inducible NAG-1 expression, the 3.5-kb NAG-1 promoter and other deletion constructs were transfected into HCT-116 cells and then treated with either PGJ2 or TGZ. As an internal control, the plasmid pRL-null was used to determine the transfection efficiency. As shown in Fig. 3A, a large increase in luciferase activity was observed after TGZ treatment for all NAG-1 promoter constructs. However, in contrast, an increase in luciferase activity was not observed with PGJ2 treatment. In fact, the response of the different constructs to PGJ2 appeared

![Graph showing NAG-1 expression](image)

**Fig. 1.** PPARγ ligands induce NAG-1 expression in HCT-116 cells. A, PPARγ ligands regulate NAG-1 expression in a dose-dependent manner. HCT-116 cells were treated with vehicle (V; 0.2% Me2SO) or with PGJ2 or TGZ at different concentrations for 6 h in the absence of serum. Total RNAs were isolated, and Northern blotting was performed. B, time-dependent expression of the NAG-1 protein in HCT-116 cells. Cells were grown and treated with either 1 μM PGJ2 or 5 μM TGZ for varying times. At the same time, 0.2% Me2SO (DMSO) was added to HCT-116 cells, and cell lysates were isolated at the indicated time points. It has been reported that NAG-1 is marginally induced by Me2SO treatment in HCT-116 cells (38). Total protein (30 μg) was subjected to Western analysis as described under “Materials and Methods.”

![Graph showing NAG-1 expression](image)

**Fig. 2.** Effect of a PPARγ antagonist on PGJ2- and TGZ-induced NAG-1 expression. The quiescent cells were pretreated with or without the PPARγ antagonist GW9662 (1 μM) for 30 min prior to the addition of either TGZ (5 μM) or PGJ2 (1 μM). After 24 h, total proteins were isolated for Western blot analysis. Equal loading was confirmed by determining actin immunoreactivity. The relative NAG-1 levels normalized by actin are shown at the bottom.
to be the same as that to the vehicle. The pGLBasic3 promoterless vector was transfected into HCT-116 cells as a negative control, and no significant luciferase activity was observed with either PGJ2 or TGZ treatment. These data suggest the presence of a positive TGZ response element in the 3.5-kb NAG-1 promoter, but the absence of a PGJ2 response element. These results also indicate that TGZ and PGJ2 induce NAG-1 expression by different mechanisms, which is consistent with the finding from the Western analysis experiment in which we used a PPARγ antagonist (Fig. 2). To investigate whether this promoter region is responsive to other ligands, pNAG133/LUC-transfected cells were treated with several PPARγ ligands and other compounds for 24 h in the absence of serum, and luciferase activity was measured. The internal control vector (pRL-null) was used to normalize for transfection efficiency. The data represent the means ± S.D. of three different experiments. The concentrations of compounds used were as follows: vehicle (MeSO), 0.2%; TGZ, 5 μM; [(-)-5-[(4-[2-methyl-2(pyridylamino)ethoxy]methyl[2,4-thiazolidinedione)] (BRL), 5 μM; 13-hydroxyoctadecadienoic acid (HODE), 30 μM; PGJ2, 1 μM; 13-hydroxyoctadecadienoic acid (HODE), 30 μM; PGJ2, 1 μM; WY-14643 (Wy), 50 μM; all-trans-RA, 10 μM; 9-cis-RA, 10 μM; retinol, 10 μM; and TGF-β1, 5 ng/ml.

**Fig. 3. NAG-1 promoter activity is induced by TGZ, but not by PGJ2.** A, the indicated promoter regions were fused to the luciferase reporter gene (LUC). Each construct (1 μg) was cotransfected with 0.1 μg of pRL-null vector into HCT-116 cells using LipofectAMINE, and the cells were treated with vehicle (white bars), 1 μM PGJ2 (hatched bars), or 5 μM TGZ (black bars) in the absence of serum. After 24 h of treatment, the promoter activities were measured by luciferase activity. Transfection efficiency for luciferase activity was normalized to Renilla luciferase (pRL-null vector) activity. The x axis shows relative luciferase units (RLU; luciferase activity/Renilla units). The results are the means ± S.D. of three independent transfections. B, shown is the promoter activity of pNAG133/LUC in the presence of TGZ and other antitumorigenic compounds. HCT-116 cells were transfected with pNAG133/LUC and treated with several PPARγ ligands and other compounds for 24 h in the absence of serum, and luciferase activity was measured. The internal control vector (pRL-null) was used to normalize for transfection efficiency. The data represent the means ± S.D. of three independent transfections. The concentrations of compounds used were as follows: vehicle (MeSO), 0.2%; TGZ, 5 μM; [(-)-5-[(4-[2-methyl-2(pyridylamino)ethoxy]methyl[2,4-thiazolidinedione)] (BRL), 5 μM; 13-hydroxyoctadecadienoic acid (HODE), 30 μM; PGJ2, 1 μM; 13-hydroxyoctadecadienoic acid (HODE), 30 μM; PGJ2, 1 μM; WY-14643 (Wy), 50 μM; all-trans-RA, 10 μM; 9-cis-RA, 10 μM; retinol, 10 μM; and TGF-β1, 5 ng/ml.

**Fig. 4.** A, the indicated promoter regions were fused to the luciferase reporter gene (LUC). Each construct (1 μg) was cotransfected with 0.1 μg of pRL-null vector into HCT-116 cells using LipofectAMINE, and the cells were treated with vehicle (white bars), 1 μM PGJ2 (hatched bars), or 5 μM TGZ (black bars) in the absence of serum. After 24 h of treatment, the promoter activities were measured by luciferase activity. Transfection efficiency for luciferase activity was normalized to Renilla luciferase (pRL-null vector) activity. The x axis shows relative luciferase units (RLU; luciferase activity/Renilla units). The results are the means ± S.D. of three independent transfections. B, shown is the promoter activity of pNAG133/LUC in the presence of TGZ and other antitumorigenic compounds. HCT-116 cells were transfected with pNAG133/LUC and treated with several PPARγ ligands and other compounds for 24 h in the absence of serum, and luciferase activity was measured. The internal control vector (pRL-null) was used to normalize for transfection efficiency. The data represent the means ± S.D. of three independent transfections. The concentrations of compounds used were as follows: vehicle (MeSO), 0.2%; TGZ, 5 μM; [(-)-5-[(4-[2-methyl-2(pyridylamino)ethoxy]methyl[2,4-thiazolidinedione)] (BRL), 5 μM; 13-hydroxyoctadecadienoic acid (HODE), 30 μM; PGJ2, 1 μM; 13-hydroxyoctadecadienoic acid (HODE), 30 μM; PGJ2, 1 μM; WY-14643 (Wy), 50 μM; all-trans-RA, 10 μM; 9-cis-RA, 10 μM; retinol, 10 μM; and TGF-β1, 5 ng/ml.
induced NAG-1 expression, we generated point/deletion mutation clones in the Sp1 and EGR-1-induced NAG-1 expression, we generated point/deletion mutation clones in the Sp1 and EGR-1 sites (Fig. 4B). The analysis of all mutant constructs revealed a dramatic reduction in luciferase activity compared with the wild-type construct, indicating that the TGZ response element may be located in region −73 to −51 of the NAG-1 promoter. Furthermore, both Sp1 and EGR-1 may be involved in TGZ-induced NAG-1 expression.

**Sp1 and TGZ Induce NAG-1 Expression**—Because region −73 to −51 of the promoter contains two Sp1 sites and two EGR-1 sites, the corresponding transcription factors might bind and transactivate the TGZ-induced NAG-1 expression. To evaluate the importance of these sites, Sp1 and EGR-1 expression vectors were generated and cotransfected along with the pNAG133/LUC reporter vector into HCT-116 cells. As shown in Fig. 5A, TGZ treatment of the Sp1-transfected cells did not enhance the induction of luciferase activity compared with the vector-transfected cells. Actually, the TGZ-induced increase in luciferase activity was less in the Sp1-transfected cells than in the vector-transfected cell (3.6-fold versus 2.3-fold, respectively). These data suggest that Sp1 may negatively interfere with TGZ-induced NAG-1 expression in HCT-116 cells. Because Sp1 proteins do not appear to be involved in TGZ-induced NAG-1 expression, we examined the effect of EGR-1 on TGZ-induced NAG-1 expression. EGR-1 contains a zinc finger motif that shares a DNA-binding site with Sp1. Indeed, the promoters of many genes contain a GC box, which may interact with Sp1 and EGR-1. To determine whether EGR-1 plays a pivotal role in TGZ-induced NAG-1 expression, we cotransfected an expression vector containing EGR-1. Interestingly, EGR-1 expression with the NAG-1 promoter resulted in an increase in luciferase activity after TGZ treatment (3.6- versus 6.7-fold, respectively) (Fig. 5A). Taken together, these data suggest that Sp1 and EGR-1 compete with each other in the NAG-1 promoter region and that the expression of EGR-1 is critical for TGZ-induced NAG-1 expression. To explore the functional influence of EGR-1 on the NAG-1 promoter and its interplay with Sp1, HCT-116 cells were transiently transfected with expression vectors encoding Sp1 and EGR-1 cDNAs, along with the reporter construct pNAG133/LUC. When increasing amounts of the EGR-1 expression vector were introduced into the HCT-116 cells, a dose-dependent increase was observed in the expression of NAG-1 promoter activity (Fig. 5B). Although both Sp1 and EGR-1 induced NAG-1 promoter activity, the overexpression of EGR-1 resulted in more luciferase activity than observed with Sp1. This result prompted us to clone NAB1 cDNA, a known EGR-1 repressor, and to construct an expression vector. As shown in Fig. 5C, EGR-1-mediated NAG-1 expression was inhibited by NAB1 expression. Indeed, NAB1 expression markedly reduced EGR-1-induced NAG-1 promoter activity, whereas Sp1-induced NAG-1 expression was not affected by NAB1 expression (Fig. 5C).

**NAG-1 Induction by TGZ Is Mediated by EGR-1**—Because EGR-1 has an important role in TGZ-induced NAG-1 expression in HCT-116 cells at the transcriptional level, we sought to determine whether EGR-1 expression is altered during TGZ treatment in HCT-116 cells. HCT-116 cells were treated with 5 μM TGZ at the indicated times, and cell extracts were analyzed for EGR-1 by Western analysis. EGR-1 expression was increased after 2 h of treatment with TGZ and then decreased after 12 h of treatment (Fig. 6A), whereas Sp1 was not changed during the TGZ treatment (data not shown). NAG-1 expression

**FIG. 4.** GC box located in the NAG-1 promoter has an important role in TGZ-induced NAG-1 expression. A, the constructs pNAG133+/−70 and pNAG41+/−70 were transfected into the pRL-null control vector. Both constructs contain the p53-binding site, but the pNAG41+/−70 construct does not contain the two Sp1-binding sites (Sp1-B and Sp1-C). The constructs pNAG133+/−70 and pNAG41+/−70 (1 μg each) were cotransfected into HCT-116 cells and treated with either vehicle or 5 μM TGZ. Transfection efficiency for luciferase activity was normalized to Renilla luciferase (pRL-null vector) activity. B, mutations in the Sp1/EGR-1-binding sites affect the activity of the NAG-1 promoter in the presence of TGZ. The construction of NAG-1 promoter vectors with point or deletion mutations has been described previously (43). Wild-type (pNAG133/ LUC) or Sp1/EGR-1 site mutant reporters (1 μg each) and pRL-null (0.1 μg) were cotransfected into HCT-116 cells. After 24 h of treatment with either vehicle or TGZ, the cell lysates were isolated, and luciferase (LUC) activity was measured. The x axis shows relative luciferase units (RLU; firefly luciferase/Renilla luciferase). The results are the means ± S.D. of four independent transfections. The point mutations are underlined.
was also increased by TGZ treatment, with the increase in EGR-1 expression by TGZ always preceding that in NAG-1 expression. EGR-1 can be phosphorylated by ERK kinases, but the importance of the phosphorylation in the biological function of EGR-1 is not understood (49). TGZ induces ERK1/2 phosphorylation and activity in these cells, which appear to regulate the expression of EGR-1 by post-transcriptional and post-translational mechanisms (25). Therefore, the effect of ERK1/2 inhibition on TGZ-induced NAG-1 expression was examined. As shown in Fig. 6B, TGZ did not increase the expression of NAG-1 in the presence of the MAPK inhibitor PD98059. The inhibition of NAG-1 expression was dependent on the concentration of the MAPK inhibitor (Fig. 6B). This result is consistent with a previous report (25) and further indicates that MAPK has a pivotal role in TGZ-stimulated induction of NAG-1. In addition, the pNAG133/LUC construct was cotransfected with the EGR-1 expression vector, and the cells were incubated with TGZ, PD98059, or both. Treatment with the MAPK inhibitor PD98059 resulted in the reduction of NAG-1 promoter activity in the presence of EGR-1 expression (Fig. 6C). These findings suggest that the ERK1/2 kinase may play an important role in the transactivation of EGR-1-induced NAG-1 expression. Finally, to determine whether TGZ-induced NAG-1 expression requires de novo synthesis, HCT-116 cells were pretreated with or without cycloheximide for 30 min, followed by treatment with 5 μM TGZ. NAG-1 mRNA was induced by TGZ treatment (Fig. 6D). However, in the presence of cycloheximide, TGZ did not increase the level of NAG-1 mRNA, suggesting that TGZ-induced NAG-1 expression requires de novo protein synthesis. These data are compatible with the notion that the increase in NAG-1 biosynthesis dependent on TGZ requires the de novo synthesis of EGR-1.

EGR-1 Protein Binds to the NAG-1 Promoter

Because TGZ dramatically induces EGR-1 expression prior to NAG-1 induction, the EGR-1 proteins should bind to the NAG-1 promoter. To confirm that the EGR-1 protein binds to such promoter sites, a gel shift assay was performed with [γ-32P]ATP-radiolabeled oligonucleotide probes corresponding to region −73 to −44 of the human NAG-1 promoter (Fig. 7), and nuclear extracts were prepared from either vehicle- or TGZ-treated HCT-116 cells. In addition, unlabeled wild-type oligonucleotides were incubated at 10 and 50 times the concentration to compete...
with the labeled probe to confirm the specificity of protein binding to this region of the promoter (Fig. 7, lanes 3–5 and 9–11). The shifted complexes observed resulted from specific binding (Fig. 7, lane 2), which is similar to results shown previously (43). When nuclear extracts from vehicle-treated cells were incubated with the unlabeled oligonucleotide before the addition of the radiolabeled oligonucleotide probe, binding of the radiolabeled probe was reduced with increasing concentrations of the unlabeled wild-type oligonucleotides. Similarly, when nuclear extracts from TGZ-treated cells were incubated with the unlabeled oligonucleotide, shifted bands were competed out with unlabeled oligonucleotides (Fig. 7, lanes 9–11). These data indicate that the shifted bands represent a specific protein binding to the NAG-1 promoter sequence. In addition, we also performed a gel shift assay in the presence of anti-EGR-1 antibody to demonstrate supershifting. Shifted bands from nuclear extracts from vehicle-treated cells represent only Sp1 family proteins (and not EGR-1 proteins) because anti-EGR-1 antibody did not supershift these bands (Fig. 7, lane 6). However, when nuclear extracts from TGZ-treated cells were mixed with the labeled oligonucleotide, the addition of anti-EGR-1 antibody caused a supershift (SS). Thus, we observed EGR-1 binding to the NAG-1 promoter region only with nuclear extracts from TGZ-treated cells.

**DISCUSSION**

In this study, we report for the first time that EGR-1 induction by TGZ results in the increased expression of the nonsteroidal anti-inflammatory drug-activated gene NAG-1 (also known as MIC-1, ODF-15, PLAB, and placental TGF-β). NAG-1 is associated with pro-apoptosis (36–38, 50), anti-inflammatory activity (51), and antitumorigenesis (35) in several model systems. Although other PPARγ ligands induce NAG-1 expression in HCT-116 cells via PPARγ activation, TGZ uses a unique mechanism that requires EGR-1 to mediate NAG-1 expression.

In a previous study, we identified a proximal promoter region spanning positions −133 to +41 that functions in the basal expression of NAG-1 in HCT-116 cells (43). Binding of the transcription factors Sp1, Sp2, Sp3, and chicken ovalbumin upstream promoter transcription factor-1 to this region is crucial for the regulation of basal level expression (43). To extend our initial studies, we performed, in the present investigation, a detailed functional analysis of the NAG-1 promoter region in the presence of PPARγ ligands. A TGZ response element is located between positions −73 and −51 in the NAG-1 promoter region. In addition to Sp family proteins, the EGR-1 transcription factor bound to the same region and transactivated the TGZ-induced NAG-1 expression (Figs. 5A and 7). Furthermore,
interplay between EGR-1 and Sp1 was required for TGZ responsiveness of NAG-1 promoter activity. As shown in Fig. 7, Sp1 controlled the basal level of NAG-1 expression, whereas EGR-1 fully controlled TGZ-induced NAG-1 expression. Because Sp1 and EGR-1 sites are located in the same region of the NAG-1 promoter, expression of Sp1 might compete with EGR-1 and result in the reduction of TGZ-induced NAG-1 expression. As shown in Fig. 5A, cotransfection of EGR-1 increased NAG-1 promoter activity after TGZ treatment, whereas Sp1 expression resulted in the reduction of NAG-1 promoter activity compared with empty vector transfection. Functional interplay between Sp1 and EGR-1 has been described for a number of human gene promoters (49, 52–54). The cis-acting element required for Sp1/EGR-1 binding is usually represented by a GC-rich sequence (GC box). In general, EGR-1 does not bind to Sp1 consensus sites, and conversely, Sp1 does not compete with EGR-1 at its recognition motifs; but the presence of overlapping Sp1/EGR-1 sites allows binding and functional interplay of both factors (55, 56). For example, Sp1 and EGR-1 interplay has been reported for the human platelet-derived growth factor α-chain gene (55). Upon stimulation by phorbol 12-myristate 13-acetate, EGR-1 displaces constitutively bound Sp1 in the promoter and stimulates the transcriptional activity of the platelet-derived growth factor α-chain gene. Fig. 8 illustrates a model for EGR-1 regulation of the NAG-1 promoter after TGZ treatment. The increase in ERK1/2 activity that occurs following treatment with TGZ increases the promoter activity and mRNA stability of EGR-1 (25). The increase in EGR-1 protein results in the specific association of this protein with cognate sites in the NAG-1 promoter, which then increases NAG-1 expression. The Sp1 transcription factor competes with EGR-1 at the same site in the promoter. In addition, the NAB1 repressor can also inhibit EGR-1-induced NAG-1 expression by binding to the EGR-1 protein. Overexpression of NAB1 blocks transcription mediated by EGR-1; and, furthermore, NAB1 does not act by blocking DNA binding or nuclear localization of EGR-1 (57). Thus, TGZ-induced NAG-1 expression is regulated in HCT-116 cells by at least three proteins, EGR-1, Sp1, and NAB1 (Fig. 8). Sp1 protein expression does not change during TGZ treatment in HCT-116 cells, and there is no obvious PPRE with a direct repeat within the −133 NAG-1 promoter, supporting the conclusion that NAG-1 induction by TGZ is not dependent on PPARγ activation or an Sp1 transcription factor. In contrast, PGJ2 may induce NAG-1 expression by the PPARγ transcription factor. The PPARγ antagonist GW9662 inhibited PGJ 2-induced NAG-1 expression, and cycloheximide treatment of HCT-116 cells did not suppress PGJ2-induced NAG-1 expression (data not shown). Taken together, these data suggest that PGJ2 elicits its effect by a PPARγ transcription factor, but a functional PPRE site in the NAG-1 promoter region has not been confirmed. The EGR-1 transcription factor (also known as NGFI-A, TIS8, krox-24, and zif(268)) is a member of a transcription factor family that contains three zinc fingers and preferentially binds to the GC-rich DNA core sequence 5′-GCGGGGGCG-3′; each finger contacts 3 bases within this sequence. However, the sequence 5′-TGCGGT/GAGCGGCT-3′ has been determined as a high affinity consensus site for EGR-1 (58), indicating that there is a variation in the core sequence. Indeed, the EGR-1 sites in the NAG-1 promoter contain 3 mismatch base pairs compared with the EGR-1 core sequence. These are apparently functional sites because they can bind the EGR-1 protein (Fig. 7). Furthermore, two EGR-1-binding sites may work together to transactivate NAG-1 expression because mutation of one EGR-1 site showed dramatic reduction of NAG-1 promoter activity (Fig. 4B). The exact mechanism of how two EGR-1 sites play a role in TGZ-induced NAG-1 expression is currently under investigation. TGZ-induced ERK1/2 activity appears to be critical in regulating EGR-1 expression (25). In addition, EGR-1 is a nuclear phosphoprotein (59), but the biological significance of this modification is unknown. Some reports suggest that the phosphorylated forms of EGR-1 are bound to DNA more efficiently than the non-phosphorylated forms (60). Our data suggest that the EGR-1 protein is not only induced after TGZ treatment, but also subsequently highly phosphorylated (25) by the ERK1/2...
pathway. The MAPK inhibitors, including PD98059 (MEK inhibitor), attenuated TGZ-induced NAG-1 protein expression and promoter activity (Fig. 6, B and C). These data indicate that MAPK is responsible for the expression and phosphorylation of EGR-1. On the other hand, TGZ-induced ERK1/2 phosphorylation may result in the phosphorylation of PPARγ and has been well documented that the phosphorylation of PPARγ results in the inactivation of PPARγ activity (61). Other PPARγ ligands such as PGJ2 up-regulate NAG-1 expression by a mechanism dependent on the PPRE. However, this additional activity of the PPARγ-ligand TGZ to stimulate ERK activity inhibits any increase in NAG-1 expression dependent on PPARγ. Thus, TGZ-induced expression of NAG-1 is essentially regulated by the increased expression of EGR-1 under these experimental conditions.

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Troglitazone-induced NAG-1 Expression Is Mediated by EGR-1
Expression of NAG-1, a Transforming Growth Factor-β Superfamily Member, by Troglitazone Requires the Early Growth Response Gene EGR-1
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