Protein Kinase C-dependent Regulation of NAG-1/Placental Bone Morphogenic Protein/MIC-1 Expression in LNCaP Prostate Carcinoma Cells*

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NAG-1 (nonsteroidal anti-inflammatory drug-activated gene), a member of the transforming growth factor β superfamily, is involved in cellular processes such as inflammation, apoptosis/survival, and tumorigenesis and is regulated by p53, Sp1, and Egr-1. In the current study, the regulation of NAG-1 expression in LNCaP human prostate carcinoma cells by 12-O-tetradecanoylphorbol-13-acetate (TPA) was examined. TPA treatment increased NAG-1 protein and mRNA levels in a time- and concentration-dependent manner as well as NF-κB binding/transcriptional activity in LNCaP cells. Pretreatment with protein kinase C (PKC) inhibitor blocked the TPA-induced increase in NAG-1 protein levels and NF-κB binding/transcriptional activity, whereas an inhibition of p38, JNK, MEK activity had no effect on TPA-induced NAG-1 levels and NF-κB transcriptional activity. Expression of constitutively active PKCs induced an increase in NF-κB transcriptional activity and NAG-1 protein levels in LNCaP cells. The expression of NF-κB p65 induced NAG-1 promoter activity, and chromatin immunoprecipitation assay for p65 showed that NF-κB binds the NAG-1 promoter in LNCaP cells. Inhibition of TPA-induced NAG-1 expression by NAG-1 short interfering RNA blocked TPA-induced apoptosis in LNCaP cells, suggesting induction of NAG-1 negatively affects LNCaP cell survival. These results demonstrate that NAG-1 expression is up-regulated by TPA in LNCaP cells through a PKC-dependent pathway involving the activation of NF-κB.

Apoptosis, the programmed cell death, is critical for cellular homeostasis and prevention of tumor development. In the prostate carcinoma, cells are initially dependent upon androgens for growth and survival, whereas they become resistant to apoptotic stimuli such as androgen deprivation in the advanced stages of prostate carcinoma (1). LNCaP (2), an androgen-responsive human prostate carcinoma cell line, has been used as a model for the progression of prostate carcinoma from the androgen-sensitive to -insensitive stage. 12-O-Tetradecanoylphorbol-13-acetate (TPA),1 an agonist of pro-

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; NAG-1, nonsteroidal anti-inflammatory drug-activated gene; PKC, protein kinase C; TGF β, transforming growth factor β; siRNA, short interfering RNA; JNK, c-Jun NH2-terminal kinase; CHIP, chromatin immunoprecipitation; HA, hemagglutinin.

NF-κB is a transcription factor that mediates the immune responses, inflammation, proliferation, and apoptosis by regulating the expression of cytokines, growth factors, and adhesion molecules. NF-κB functions as homo- or heterodimers among Rel family proteins, all of which share Rel homology domains. There are five members of the Rel family identified as follows: p50 (p105), p52 (p100), Rel A (p65), c-Rel, and Rel B. The inducible form of NF-κB is a heterodimer between p50 and p65, and NF-κB can be activated by a variety of stimuli through a cascade of kinase signaling, which leads to release of NF-κB from IκB, an inhibitory protein, and subsequent translocation to the nucleus. Many studies have shown that the apoptotic signal activates NF-κB, and activation of NF-κB promotes cells from apoptosis. However, other data indicate NF-κB promotes apoptosis. For example, NF-κB activates Fas ligand (FasL), a member of tumor necrosis factor family, promoter during activation- or chemotherapeutic agent-induced apoptosis in T lymphocytes (14, 15). Inhibition of NF-κB in LNCaP cells renders cells resistant to apoptosis induced by death ligands in the presence or absence of irradiation (16). Recently, Fujioka et al. (17) have shown that NF-κB is essential in activating p53 to initiate proapoptotic signaling in response to overgeneration of superoxide.

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Nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) is a distant member of the TGF β superfamily (18) and is also known as macrophage inhibitory cytokine-1 (MIC-1) (19), placental TGF-β (20), prostate-derived factor (21), novel placental bone morphogenic protein (22), or growth differentiation factor-15 (GDF-15) (23). NAG-1 was identified from cyclooxygenase inhibitor (indomethacin)-treated HCT-116 colon cancer cell lines using subtractive hybridization (18). NAG-1 is highly expressed in human placenta and prostate, whereas it is expressed in kidney and pancreas at a lower level (21). NAG-1 protein is synthesized as a 308-amino acid propeptide with RXXR cleavage site and is secreted as a 30-kDa dimeric mature protein after cleavage at the RXXR site and formation of disulfide bond between cysteine residues. Pro-region of NAG-1 has been shown to be N-glycosylated during the processing of the NAG-1 through endoplasmic reticulum and Golgi (24). NAG-1 regulates a wide range of cellular functions in distinct cellular contexts. For example, NAG-1 is induced by TPA treatment in macrophages and blocks the late stage of macrophage activation (19). NAG-1 inhibits proliferation of breast carcinoma cells (25), mink lung epithelial cells, and prostate carcinoma cells (26). Treatment with NAG-1 induced a decrease in cell adhesion and an increase in apoptosis in the prostate cancer cell line (27), and forced expression of NAG-1 in HCT-116 colon cancer cells resulted in reduced soft agar growth and tumor growth in nude mice (18). In addition, NAG-1 has been identified as a p53 target gene (25, 26). In contrast to the role of NAG-1 as an anti-tumorigenic, pro-apoptotic molecule, several reports have shown that NAG-1 is positively associated with tumor development. Up-regulation of NAG-1 has been reported in serum from patients with metastatic breast, prostate, and colorectal carcinomas (28) and in transition from androgen-dependent to androgen-independent prostate carcinoma cells (29).

In this study, we examined the regulation of NAG-1 expression during TPA-induced apoptosis in LNCaP human prostate carcinoma cells. TPA induced NAG-1 expression as well as NF-κB activation in LNCaP cells by a PKC-dependent mechanism. We found that p65 directly binds/activates the NAG-1 promoter, and inhibition of NAG-1 expression diminished TPA-induced apoptosis in LNCaP cells. These data suggest that the expression of NAG-1 provides the novel mechanism for understanding the downstream effectors for TPA-induced apoptosis in LNCaP cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Akt antibody and phospho-Akt antibody were purchased from Cell Signaling Technology. Ly294002, GF109203X, PD98059, SB203580, and SP600125 were purchased from Calbiochem. TPA was purchased from Sigma.

**Cell Culture**—LNCaP human prostate carcinoma cells were purchased from American Type Culture Collection and cultivated in RPMI1640 media supplemented with 10% fetal bovine serum (Hyclone) and 10 μg/ml gentamycin (Invitrogen).

**Western Blot Analysis**—Cells were lysed in RIPA buffer, and protein concentration was measured by BCA reagent (Pierce). Equal amounts of protein were solubilized and heated at 65 °C in LDS sample buffer (Invitrogen) with sample reducing agent (Invitrogen) for 10 min, and then separated by SDS-PAGE. The separated proteins were transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer (TBS with 5% non-fat dry milk and 0.1% Tween 20) for 1 h at room temperature, the membranes were probed overnight at 4 °C with rabbit polyclonal anti-NAG-1 (1:2000), monoclonal anti-actin (Sigma, 1:5000), or anti-HA (Roche Applied Science, 1:2000) antibody. The membranes were washed and then probed with a horseradish peroxidase-linked secondary antibody (Amersham Biosciences, 1:2500) for 1 h at room temperature. Detection was made with an enhanced chemiluminescence reagent followed by exposure of membrane to film.

**Northern Blot Analysis**—Total RNA was isolated using SV total RNA isolation kit (Promega) or mRNA isolation kit (Qiagen). NAG-1 cDNA was labeled with (α-32P)dCTP by using Dcaphere II kit (Ambion) according to the manufacturer’s protocol. RNA was electrophoresed on agarose gel containing formaldehyde, transferred to Hybond XL membrane (Amersham Biosciences), and UV cross-linked. Blots were incubated at 65 °C in Rapid Hybridization buffer (Amersham Biosciences) and exposed to X-ray film after being washed with washing buffer 1 (0.5% SDS, 0.1% SSC) and washing buffer 2 (0.1% SDS, 0.1% SSC) at 65 °C. Films were exposed to membranes at −80 °C and developed.

**Transfection and Luciferase Assay**—pcDNA3.1-HA-PKCα ΔNPS and pcDNA3.1-HA-PKCa ΔNPS were the kind gifts from Dr. Jae-Won Soh (Inha University, Incheon, Korea). NF-κB p65 expression plasmid was the kind gift from Dr. Dean Ballard (Vanderbilt University School of Medicine, Nashville, TN). Construction of the NAG-1 reporter plasmid has been described previously (30). LNCaP cells were plated at 1.25 × 10^6 cells/well in a 12-well culture plate. Two days after plating, LNCaP cells were transfected in triplicate with the specified reporter plasmid as described in the text and pRL-null plasmid using Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocol. Forty-eight hours later, cells were harvested, and the luciferase activity was determined and normalized to Renilla luciferase activity with a dual luciferase assay kit (Promega).

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared as described previously by Schreiber et al. (31). Nuclear extracts were incubated at room temperature for 30 min with 10 μl of master binding mix with 32P-labeled NF-κB probe (Santa Cruz Biotechnology). Samples were loaded onto 6% native polyacrylamide gel (Invitrogen) and subjected to electrophoresis in 0.25 × TBE buffer at 100 V for 1 h. The gel was transferred to Whatman paper, dried in a 80 °C gel dryer for 1 h, and then exposed to film for an appropriate time to check the DNA binding pattern.

**Generation of PKC Isoform-expressing LNCaP Cells**—ΔNx cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and transfected with pcDNA3.1-HA-PKCα (Clontech) by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Virus-containing medium was collected from Δnx cell cultures three times, 6 h apart, filtered through a 0.45-μm filter, and overlaid onto LNCaP cells with 10 μg/ml Polybrene (Sigma). Cultures were maintained with viral containing medium overnight, and 24 h later cells were shifted to a selection medium containing 500 μg/ml G418. A pool of G418-resistant LNCaP cells (Tet-On LNCaPs) was infected with virus containing medium from ΔNx cells that were transfected with pRev-TRE-PKCα ΔNPS or pRev-TRE-PKCa ΔNPS. Cells were subjected to a selection with 500 μg/ml hygromycin and 250 μg/ml G418. Hygromycin/G418-resistant cells were pooled and used for Western blot analysis.

**Chromatin Immunoprecipitation (CHIP) Assay**—LNCaP cells were plated on 60-mm culture dishes at 1 × 10^6 cells/dish, and 24 h later were transfected with pRev-Tet-On (Promega) by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After phenol/chloroform extraction and ethanol precipitation, the pellet was resuspended in 50 μl of H_2O. For the PCR, 5 μl of immunoprecipitated DNA or total input DNA was amplified using primers for NAG-1 promoter sequence.

**Generation of NAG-1 siRNA Expressing LNCaP Cells**—The NAG-1 siRNA vector (pSuper-retro-puro-Si NAG1) was constructed using a pSuper-retro-puro and a synthetic oligonucleotide targeting 5’-ACAT-CCAGCGCGAGATGTTA-3’ corresponding to positions 780–789 on NAG-1 mRNA. ΔNx cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and transfected with pSuper-retro-puro-Si NAG1 by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Virus-containing medium was collected from Δnx cell cultures three times, 6 h apart, filtered through a 0.45-μm filter, and overlaid onto LNCaP cells with 10 μg/ml Polybrene (Sigma). Cultures were maintained with viral containing medium overnight, and 24 h later cells were shifted to a selection medium containing 2 μg/ml puromycin (Sigma). Puromycin-resistant LNCaP cells were pooled and used for Western blot analysis.

**RESULTS**

**TPA Increases NAG-1 mRNA and Protein Levels in LNCaP Cells**—To study the role of NAG-1 during TPA-induced apoptosis in LNCaP cells, we first determined the effect of TPA on NAG-1 levels in LNCaP cells. LNCaP cells were treated with various concentrations of TPA, and 24 h after TPA treatment, total cell lysates were prepared, and NAG-1 protein levels were determined by Western blot analysis. As shown in Fig. 1A, TPA induced an increase in NAG-1 protein...
levels in a concentration-dependent manner. TPA also induced an increase in NAG-1 protein in a time-dependent manner, reaching the maximum level at 12 h after TPA treatment (Fig. 1B). To examine whether this increase in NAG-1 protein levels resulted from increased NAG-1 mRNA levels, we performed Northern blot analysis for NAG-1. TPA treatment caused an increase in NAG-1 mRNA levels in a concentration- and time-dependent manner (Fig. 1, C and D), indicating that TPA regulates NAG-1 levels at the transcriptional level. To ascertain if TPA treatment activates the NAG-1 promoter, we transfected LNCaP cells with a luciferase reporter gene fused to NAG-1 promoter (Fig. 1E). These results demonstrate that TPA regulates NAG-1 protein levels at the transcriptional level.

TPA Induces NAG-1 Protein by a PKC-dependent Mechanism in LNCaP Cells— Whereas TPA has been shown to activate PKCs in LNCaP cell (6, 32), TPA can affect various signaling pathways via the activation/inhibition of other kinases, such as JNK (7), p38 (8), and Akt (8) in LNCaP cells. To test if TPA induces NAG-1 expression by a PKC-dependent mechanism, LNCaP cells were pretreated with GF109230X, which is reported to be an inhibitor of PKC (33). LNCaP cells were then treated with TPA, and total lysates were subjected to Western blot analysis. Whereas GF109230X pretreatment completely blocked TPA-induced NAG-1 protein expression, pretreatment with the JNK inhibitor (SP600125), p38 kinase inhibitor (SB203580), or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor (PD98059) did not affect TPA-induced NAG-1 expression (Fig. 2A). Tanaka et al. (8) showed that TPA induces apoptosis in LNCaP cells by activation of p38 mitogen-activated protein kinase and inhibition of Akt. In addition, Yamaguchi et al. (34) recently showed that NAG-1 is a downstream target of the Akt/GSK3 pathway in HCT-116 human colorectal carcinoma cells. To examine whether the TPA-induced increase in NAG-1 protein levels resulted from an inhibition of Akt, we treated LNCaP cells with the phosphatidylinositol 3-kinase inhibitor Ly294002 (35), and
total cell lysates were subjected to Western blot analysis. Because phosphatidylinositol 3-kinase is an immediate upstream kinase of Akt, Ly294002 treatment blocks the phosphorylation of Akt. Whereas TPA or Ly294002 treatment blocked the phosphorylation of Akt, Ly294002 treatment did not affect NAG-1 protein levels, suggesting an inhibition of Akt is not involved in TPA-induced increase in NAG-1 protein levels (Fig. 2B) in LNCaP cells. These results indicate that the induction of NAG-1 by TPA is dependent on PKCs.

TPA Induces NF-κB Activity in LNCaP Cells—We have cloned the promoter region of NAG-1, and subsequent sequence analysis identified potential NF-κB binding sites in the NAG-1 promoter. TPA activates NF-κB (36, 37), and Rameshwar et al. (38) have shown that NF-κB positively regulates TGF-β1 expression from monocytes with idiopathic myelofibrosis. To identify whether NF-κB is associated with TPA-induced NAG-1 expression, LNCaP cells were treated with TPA, and nuclear extracts were isolated at various time points and subjected to electrophoretic mobility shift assay. As shown in Fig. 3A, TPA treatment significantly increased NF-κB binding activity. To ascertain if the TPA-induced increase in NF-κB binding activity was also accompanied by an increase in NF-κB transactivation activity, we transfected LNCaP cells with pxB-luciferase, a reporter plasmid, that has three consecutive NF-κB-binding sites upstream of luciferase, and we then treated these cells with TPA. As shown in Fig. 3B, TPA treatment resulted in a significant increase in NF-κB transcriptional activity. To determine whether PKCs are associated with TPA-induced increase in NF-κB transcriptional activity, we transfected LNCaP cells with pxB-luciferase reporter plasmid and treated with GF109230X in the presence or absence of TPA. Whereas GF109230X treatment blocked TPA-induced increase in NF-κB transcriptional activity, JNK inhibitor, p38 kinase inhibitor, or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor did not affect TPA-induced NF-κB activation (Fig. 3B). To provide further evidence that PKCs activate NF-κB transcriptional activity in LNCaP cells, we determined the effect of PKC expression on NF-κB transcriptional activity. Powell et al. (4) showed that four PKC isozymes (α, δ, ε, and γ) are expressed in LNCaP cells among classical and novel PKC isozymes. Because PKCs and δ have been shown to be involved in TPA-induced apoptosis in LNCaP cells, we co-transfected LNCaP cells with the NF-κB reporter plasmid and the constitutively active form of PKCα or δ (39). As shown in Fig. 3C, constitutively active PKCα and δ activated NF-κB reporter; whereas expression of the constitutively active PKCδ caused more significant increases in NF-κB transcriptional activity. These results demonstrate that TPA-induced activation of NF-κB is dependent on PKCs in LNCaP cells.

NF-κB Activates NAG-1 Promoter—Because TPA induced an increase in NAG-1 levels as well as NF-κB transcriptional activity in LNCaP cells, we examined the direct effect of NF-κB on the NAG-1 promoter. LNCaP cells were co-transfected with p65 expression plasmid and the NAG-1 promoter reporter construct. Expression of p65 induced a 4-fold increase in NAG-1 promoter activity, whereas Egr-1, which has been shown to mediate tretinoin-induced increase in NAG-1 levels (40), increased NAG-1 promoter activity 2-fold (Fig. 4A). To localize NF-κB-binding site(s) in the NAG-1 promoter, we co-transfected LNCaP cells with different lengths of NAG-1 promoter reporter plasmid and p65 expression plasmid. NAG-1 promoter activation by p65 was most significant in the reporter plasmid that has −474/40 region of NAG-1 promoter, indicating that this region of the NAG-1 promoter (∼133 to −474) has NF-κB-binding site(s) (Fig. 4B). NF-κB binding to the NAG-1 promoter in vivo (in LNCaP cells) was determined by CHIP assay. LNCaP cells were collected after formaldehyde treatment, and isolated chromatin was subjected to sonication followed by an immunoprecipitation with antibodies against NF-κB p65 subunit. After immunoprecipitation, the NAG-1 promoter region from −309 to +63 was amplified (Fig. 4C), indicating that NF-κB binds to the NAG-1 promoter in LNCaP cells. These results demonstrate that NF-κB binds and activates the NAG-1 promoter.

PKC Induces NAG-1 in LNCaP Cells—To examine the direct effect of PKCs on NAG-1 levels, we measured NAG-1 levels in LNCaP cells that express the constitutively active PKCα or δ isoform. To make the pools of LNCaP cells that express constitutively active PKCα or constitutively active PKCδ plasmid, NF-κB reporter activity was normalized to Renilla luciferase activity. CTL, control. C, LNCaP cells were co-transfected with NF-κB reporter plasmid, pRL-null, and empty vector, constitutively active PKCα, or constitutively active PKCδ plasmid. NF-κB reporter activity was normalized to Renilla luciferase activity.
PKC, NAG-1, and Apoptosis

PKC without the addition of tetracycline, and this may be due to the use of pooled cells rather than a single clone. As shown in Fig. 5, forced expression of constitutively active PKCa or -b resulted in induction of NAG-1, whereas constitutively active PKC8 was more potent in inducing NAG-1 protein levels, indicating PKC is the upstream kinase for NAG-1 expression in LNCaP cells.

Inhibition of NAG-1 Expression Blocks TPA-induced Apoptosis in LNCaP Cells—To examine the biological significance of NAG-1 induction by TPA in LNCaP cells, we generated LNCaP cells that express NAG-1 siRNA using the retroviral system. To determine whether the expression of NAG-1 siRNA can block TPA-induced NAG-1 expression, vector or NAG-1 siRNA-infected LNCaP cells were pooled after the selection with puromycin and treated with different concentrations of TPA. Twenty-four hours after TPA treatment, total lysates were made and subjected to Western blot analysis for NAG-1. As shown in Fig. 6A, the stable expression of NAG-1 siRNA partially blocked TPA-induced NAG-1 expression in the pool of LNCaP cells infected with NAG-1 siRNA-containing retrovirus. To determine the effects of NAG-1 induction by TPA on LNCaP cell survival, vector or NAG-1 siRNA-containing retrovirus-infected LNCaP cells were plated and treated with various concentrations of TPA for 24 h. In vector-infected LNCaP cells, 2.5 ng/ml TPA treatment resulted in ∼50% reduction in cell number compared with control cells, whereas stable expression of NAG-1 siRNA resulted in 20% reduction in cell number after 2.5 ng/ml TPA treatment (Fig. 6B), suggesting the inhibition of NAG-1 expression renders LNCaP cells resistant to TPA-induced apoptosis. In addition, the stable expression of NAG-1 siRNA increased the rate of basal proliferation that is consistent with the role of NAG-1 as an inducer of growth arrest/apoptosis. To provide additional evidence for the role of NAG-1 in TPA-induced apoptosis in LNCaP cells, the vector or NAG-1 siRNA-infected pool of LNCaP cells was treated with various concentrations of TPA for 24 h, and DNA content was measured by fluorescence-activated cell sorter analysis. In all concentrations of the TPA tested, inhibition of TPA-induced NAG-1 expression by NAG-1 siRNA resulted in less amounts (30–40%) of the sub-G1 phase compared with vector-infected cells (data not shown). Together, these results suggest that the induction of NAG-1 may be one of the mechanisms by which TPA induces apoptosis in LNCaP cells.

FIG. 4. NF-κB p65 binds and activates NAG-1 promoter. A, LNCaP cells were transfected with NAG-1 promoter reporter plasmid (−966/70), pRL-null plasmid, and expression plasmid for each transcription factor. NAG-1 promoter reporter activity was normalized to Renilla luciferase activity. B, LNCaP cells were transfected with p65 expression plasmid, pRL-null, and the indicated NAG-1 promoter reporter plasmid. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured. NAG-1 promoter reporter activity was normalized to Renilla luciferase activity. C, LNCaP cells were treated with 1% formaldehyde in phosphate-buffered saline for 10 min. Cells were lysed in lysis buffer, and NF-κB p65-associated DNA was immunoprecipitated using NF-κB p65 antibody (Ab). NAG-1 promoter region was amplified using PCR with immunoprecipitated DNA or total input DNA as templates.

FIG. 5. PKC induces NAG-1 expression in LNCaP cells. LNCaP cells were infected with pRev-Tet-On retrovirus and selected with G418. G418-selected pools of LNCaP cells (Tet-On LNCaP) were subsequently infected with pHev-TRE retrovirus encoding each HA-tagged constitutively active PKC isoform and selected with hygromycin/G418. Total cell lysates were subjected to Western blot analysis for NAG-1. Membrane was stripped and reprobed for HA and actin.

DISCUSSION

NAG-1 is a divergent member of the TGF-β superfamily and has been shown to be induced by nonsteroidal anti-inflammatory drugs in our laboratory. The major role of NAG-1 is not clear; however, several studies suggest NAG-1 as an inducer of growth arrest/apoptosis in addition to other cellular functions. In this report, we showed that TPA induces NAG-1 protein by a PKC-dependent mechanism involving the activation of NF-κB, and this TPA-induced NAG-1 expression in LNCaP cells negatively affects cell survival.

TPA induced the expression of NAG-1 protein and mRNA in a concentration- and time-dependent manner. TPA-induced NAG-1 expression was significantly blocked by pretreatment with the PKC inhibitor, indicating that TPA induces NAG-1 expression by a PKC-dependent mechanism. In addition, forced expression of constitutively active PKCa or PKCδ in LNCaP cells induced NAG-1 expression, demonstrating PKC is an upstream signaling kinase in TPA-induced NAG-1 expression. Because PKCa and PKCδ are involved in TPA-induced apoptosis in LNCaP cells, the fact that the expression of constitutively active PKCa or PKCδ induces NAG-1 suggests that NAG-1 may be the downstream target in TPA-induced apoptosis in LNCaP cells. The expression of constitutively active...
Cells were harvested by trypsinization, and cell number was counted. Cells were treated with the indicated concentrations of TPA for 24 h. Total lysates were subjected to Western blot analysis for NAG-1. The location of unprocessed (38 kDa) and cleaved NAG-1 (16 kDa) is shown by arrows. B, empty vector or NAG-1 siRNA-expressing LNCaP cells were treated with the indicated concentrations of TPA for 24 h. Cells were harvested by trypsinization, and cell number was counted.

PKCε or PKCδ in LNCaP cells induced growth inhibition as well as NAG-1 expression compared with the parental cell line (pTet-On LNCaP), suggesting NAG-1 expression may be involved in growth inhibition in LNCaP cells that express constitutively active PKCs or PKCδ (data not shown).

TPA treatment activated NF-kB binding and transcription activity by a PKC-dependent mechanism, and the transient expression of constitutively active PKCs activated NF-kB activity in LNCaP cells. We have also shown that p65 binds and activates the NAG-1 promoter by using CHIP assay and NAG-1 promoter reporter assay. TPA treatment induced an increase in binding activity of other transcription factors that have putative binding site(s) on the NAG-1 promoter such as AP-1, Egr-1, and C/EBP (data not shown). Although other transcription factors can be activated by TPA, co-transfection with NAG-1 promoter reporter showed that p65 was most efficient at activating NAG-1 promoter (Fig. 4A and data not shown). However, co-transfection of LNCaP cells with IκBα and NAG-1 promoter reporter resulted in a partial inhibition of TPA-induced increase in NAG-1 promoter activity (data not shown), suggesting other transcription factors are also involved in TPA-induced NAG-1 expression. NAG-1 promoter has two p53-binding sites, and NAG-1 is induced by signals that activate p53 (25, 26). Whereas LNCaP cell has wild type p53, PC-3, an androgen-insensitive prostate carcinoma cell, has truncated p53 (p53-null). To determine whether TPA-induced NAG-1 expression is associated with the activation of p53, we treated PC-3 cells with the same concentrations of TPA as in LNCaP and performed Western blot analysis for NAG-1. TPA treatment also induced NAG-1 protein in PC-3 cells (data not shown), indicating TPA-induced NAG-1 expression is p53-independent.

When we performed Western blot for NAG-1 in LNCaP cells, we found that unprocessed NAG-1 protein (38 kDa) migrates as a doublet on SDS-PAGE (Fig. 1A and B). We also observed the existence of NAG-1 protein doublet in lysates from other cell lines, such as HCT-116 colon cancer cell line. NAG-1 protein is N-glycosylated at Asn-41 and has the potential phosphorylation sites. Therefore, it is possible that this difference in electrophoretic mobility of unprocessed NAG-1 protein may result from the differential post-translational modifications. However, the treatment of LNCaP lysates with glycosidases or phosphatases did not affect the migration of unprocessed NAG-1 protein as a doublet (data not shown) on SDS-PAGE, suggesting post-translational modifications are not involved in differential electrophoretic mobility of NAG-1 protein. It is also possible that translation of NAG-1 protein may start at several sites, leading the generation of polypeptide with different lengths because NAG-1 mRNA has several in-frame ATG codons thus providing alternative translational start sites. Further study will determine the biological significance of the NAG-1 doublet and the mechanism by which the NAG-1 doublet is generated.

NAG-1 has been shown to negatively affect cell growth or survival in epithelial or other cancer cell lines and is believed to mediate the effect of several chemopreventive compounds (18, 27, 30, 40). We have shown that the pool of LNCaP cells that stably express NAG-1 siRNA was more resistant to the apoptotic effect of TPA, indicating that NAG-1 is associated with TPA-induced apoptosis in LNCaP cells. Along with TPA, a thapsigargin treatment has been known to induce apoptosis in LNCaP cells (7). When LNCaP cells were incubated with thapsigargin for 48 h, massive apoptosis was observed, but no NAG-1 expression was induced (data not shown), suggesting the increased expression of NAG-1 is not a consequence of apoptosis. In contrast to inducing apoptosis in LNCaP cells, TPA treatment induced an increase in cell growth as well as a decrease in NAG-1 levels in HCT-116 colon cancer cell lines (data not shown), supporting the notion that NAG-1 negatively affects cell growth/survival. In addition, PC-3 clones that stably transfected with NAG-1 displayed impaired growth and increased apoptosis.2

In conclusion our results demonstrate that TPA induces NAG-1 in LNCaP cells via a PKC-dependent pathway involving the activation of NF-kB. In addition, our finding identifies NAG-1 as a downstream molecule in TPA-induced apoptosis and contributes to the understanding of apoptotic pathways that can provide the approaches to prostate cancer therapeutics.

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