Mullerian-Inhibiting Substance Induces Gro-β Expression in Breast Cancer Cells through a Nuclear Factor-κB–Dependent and Smad1-Dependent Mechanism

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Abstract

Mullerian-inhibiting substance (MIS), a transforming growth factor-β family member, activates the nuclear factor-κB (NF-κB) pathway and induces the expression of B-cell translocation gene 2 (BTG2), IFN regulatory factor-1 (IRF-1), and the chemokine Gro-β. Inhibiting NF-κB activation with a phosphorylation-deficient IκBα mutant abrogated MIS-mediated induction of all three genes. Expression of dominant-negative Smad1, in which serines at the COOH-terminal SSVS motif are converted to alanines, suppressed MIS-induced Smad1 phosphorylation and impaired MIS-stimulated Gro-β promoter-driven reporter expression and Gro-β mRNA. Suppressing Smad1 expression using small interfering RNA also mitigated MIS-induced Gro-β mRNA, suggesting that regulation of Gro-β expression by MIS was dependent on activation of NF-κB as well as Smad1. However, induction of IRF-1 and BTG2 mRNAs by MIS was independent of Smad1 activation. Characterization of κB-binding sequences within Gro-β, BTG2, and IRF-1 promoters showed that MIS stimulated binding of p50 and p65 subunits to all three sites, whereas phosphorylated Smad1 (phospho-Smad1) protein was detectable only in the NF-κB complex bound to the κB site of the Gro-β promoter. Consistent with these observations, chromatin immunoprecipitation assays showed recruitment of both phospho-Smad1 and p65 to the Gro-β promoter *in vivo*, whereas p65, but not phospho-Smad1, was recruited to the BTG2 promoter. These results show a novel interaction between MIS-stimulated Smad1 and NF-κB signaling in which enhancement of NF-κB DNA binding and gene expression by phospho-Smad1 is dependent on the sequence of the κB consensus site within the promoter. [Cancer Res 2007;67(6):2747–56]

Introduction

Mullerian-inhibiting substance (MIS) induces regression of the Mullerian duct in male embryos (1). The presence of MIS receptors in the mammary gland and prostate (2, 3) suggests that these tissues are likely targets for MIS action. MIS inhibits breast and prostate cancer cell growth through a nuclear factor-κB (NF-κB)-dependent mechanism (2, 3). The NF-κB family of transcriptional activators, p65, p50, p52, c-rel, and RelB, shares a rel homology domain. NF-κB in its inactive state exists in the cytosol bound to inhibitory IκB molecules. Activation of the pathway leads to phosphorylation and degradation of IκB with subsequent nuclear localization of NF-κB (4–6). The IκB kinase (IKK) complex consisting of IKKα and IKKβ, two protein kinases with high degree of sequence similarity, and the regulatory subunit IKKγ (NF-κB essential modulator), mediates site-specific phosphorylation of IκB proteins (7–9). On nuclear import, homodimeric or heterodimeric NF-κB transactivators bind to decameric DNA consensus motifs to activate transcription.

The κB sites within promoters of NF-κB–responsive genes do not display a strict consensus (10). However, the sequence of κB sites within a given gene is evolutionarily well conserved (11), suggesting that the κB sequence may play a critical role in defining gene activation by NF-κB. A strict sequence requirement for NF-κB inducibility is observed in the κB site within the IFN-β promoter (GGGAAATTCC), which when substituted by a closely related κB site from the Igκ light gene enhancer (GGGACTTTCC) becomes nonresponsive to NF-κB activation (12). Structural studies revealed that differences in κB sequences of IFN-β and the Igκ light chain gene enhancers contribute to structural changes in the NF-κB dimer/DNA complex, leading to altered gene expression (13, 14). Leung et al. (11) showed that the κB site sequence specifies which coactivators will result in productive interactions with bound NF-κB dimers. BRCA1 (15), IFN regulatory factor (IRF)-3, Bcl-3 (11), and HMG 1(Y) (16) are some of the cofactors reported to regulate NF-κB activity. Deciphering the cues provided by the κB sites to specify NF-κB subunit and cofactor requirement in stimulus-specific gene expression is an area of active investigation.

MIS, in addition to activating NF-κB, also stimulates Smad1/5/8 phosphorylation; phosphorylated (phospho) Smad1/5/8 complexes with Smad4 and localizes to the nucleus to activate transcription (17). Several lines of evidence indicate interactions between NF-κB and Smad pathways. Transforming growth factor-β (TGF-β) signaling is suppressed by tumor necrosis factor-α (TNF-α), which through NF-κB activation induces Smad7 to suppress TGF-β/Smad-mediated transcription (18). Bone morphogenetic protein (BMP)-2 and BMP-4 suppress TNF-α–induced caspase-8 activation and apoptosis through a Smad-dependent pathway (19). In addition to such antagonistic effects, functional cooperation between NF-κB and Smad3 can also regulate gene expression (20). Although these observations show a cross-talk between NF-κB and Smad pathways, a functional interaction between Smad1 phosphorylation and NF-κB activation has never been shown.

We report that MIS and BMPs induce Gro expression in mammary epithelial cells. The Gro genes encode 8- to 10-kDa secretory proteins and belong to a family of genes, such as platelet factor 4

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and neutrophil-activating peptide/interleukin (IL)-8 (21). The three related Gro genes, *Gro-α, Gro-β*, and *Gro-γ*, which encode ~1.1 kb mRNAs, are closely linked on chromosome 4. Gro-β and Gro-γ share 90% and 86% sequence homology with Gro-α (22). The CXCR2 receptor, a member of the serpentine receptor superfamily, binds multiple chemokines, including Gro-β, and signals through heterotrimeric G proteins (23).

Gro-β is constitutively expressed in normal mammary epithelial cells, but not in most breast cancer cell lines, suggesting that it may be a negative regulator of epithelial cell growth (24). Although IL-1, TNF-α, and fibronectin fragments induce Gro-β through activation of NF-κB (25–27), little is known about additional mechanisms that regulate Gro-β expression. Our results show that MIS induces Gro-β expression through a Smad1-dependent and NF-κB–dependent mechanism defined by the sequence of the κB site in the promoter, suggesting a novel functional interaction between Smad1 and NF-κB pathways in differential regulation of gene expression.

Materials and Methods

Reagents, cell lines, MIS production, and purification. Culture conditions for cell lines are described (2, 3, 28). Production of human recombinant MIS is described (29). Gro-β (human recombinant) was purchased from Sigma-Aldrich (St. Louis, MO); BMP-2, BMP-4, and BMP-6 were from R&D Systems (Minneapolis, MN). T47D and LNCaP cells stably expressing dominant-negative IκBα (IκBαDN) are described (3, 30).

The dominant-negative Smad1 (Smad1DN) transgene, in which the SSVS phosphorylation sequence was converted to AAVA, was a kind gift from Dr. Jeffrey Wrana (Mount Sinai Hospital, Toronto, Canada). Cells expressing Smad1DN were generated by transfecting 1 μg of hygromycin resistance plasmid and 5 μg Smad1DN. Cells were grown in medium containing 100 μg/mL hygromycin. Smad1DN-expressing clones were identified by Northern blot and by lack of Smad1 phosphorylation following MIS treatment. Characterization of Smad1DN-expressing LNCaP cells is described (31).

Western blot analysis. Anti–phospho-Smad1/5/8 and anti–E-cadherin antibodies were from Cell Signaling Technology (Danvers, MA) and Zymed (South San Francisco, CA), respectively. Western protocols are as described previously (32).

RNA analysis. Ten cases of human breast carcinoma and adjacent matched normal tissues were obtained from the tissue bank of Massachusetts General Hospital (Charlestown, MA) according to protocols approved by the Human Research Committee at Massachusetts General Hospital. The expressed sequence tag clones to detect human Gro-β, IRF-1, B-cell translocation gene 2 (BTG2), and Smad1 were purchased from Incyte Genomics, Inc. (Palo Alto, CA). Northern blot was done as described (33).

To block Smad1 expression using small interfering RNA (siRNA), cells were transfected with Oligofectamine (Invitrogen, Carlsbad, CA); 8 μL siRNA (20 μmol/L) and 6 μL Oligofectamine were diluted with 132 and 34 μL of Opti-MEM, respectively. After 5 min, they were combined and incubated for 15 min. Reaction mixtures were overlaid on cells for 24 h. MIS was added for 2 h and RNA was extracted. The siRNA against human Smad1, CAGG-ACUUUGUGUACAGUUAA, and control siRNA, AAUUCUCCGAACGUGU-CACGU, was purchased from Qiagen (Valencia, CA). The following primers were used to amplify cDNAs: Smad1, 5′-ACTTGACCTCTGTGACCAACTG-3′ (sense) and 5′-GCTTGGCCATCTCTCCTTTA-3′ (antisense; generated a 191-bp fragment); Gro-β, 5′-CCCCGAATTCACGGAGCTCCTTGCCAGCT-CT-3′ (sense) and 5′-CCCCCTCGAGCCTTCAGGAACAGCCACCAATAAG-3′ (antisense; generated a 422-bp fragment); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCAGTCAGGGAGGAAATAAAGCC-3′ (sense) and 5′-GATGCGTCCAAATCCATTGATGCC-3′ (antisense).

RNase protection assay. The primers used to generate Gro-α were CCCC(GAATTC)CCAGTAGGACAAACAGCAACAGG (forward) and CCCC(CTCGAG)TTCAGGAACAGCCACCAGTGAG (reverse). The primers used to generate Gro-β were CCCC(GAATTC)TCAAACCCAAGTTAGTT-CAATCCTG (forward) and CCCC(CTCGAG)ATAGACACACATACATTT-CCCTGCC (reverse). The sequences within parentheses in forward and reverse primers represent *Eco*R1 and *Xho*1 restriction sites, respectively.

cDNA generated from MIS-treated T47D cells was used for PCR amplification. The Gro-α and Gro-β primers gave rise to 330- and 192-bp fragments, respectively, which were cloned into *Eco*R1 and *Xho*1 sites of pCDNA3.1(−). Constructs were sequenced to confirm insert boundaries and linearized with *Hin*DIII. Antisense transcripts were obtained using T7 polymerase (MAXIscript In Vitro Transcription kit, Ambion, Austin, TX). RNase protection assays were done as described (28).

NF-κB electrophoretic mobility shift assays. Protocols for gel shifts are described (2). Supershifts were done by adding 1 to 2 μg of rabbit anti-p65 or p50 antibodies to binding reactions. To detect phospho-Smad1 in complexes bound to the NF-κB site, 4 μL of phospho-Smad1 antibody were added to reactions. The phospho-Smad1 antibody supplied by Cell Signaling Technology was at a concentration of 0.055 μg/μL. Therefore, the amount of phospho-Smad1 used in gel shift was ~18-fold lower compared with the concentrated p50 and p65 antibodies (1–2 μg/μL; TransCruz, Santa Cruz Biotechnology, Santa Cruz, CA) specifically designed for use in gel shifts.

The sense and antisense oligonucleotides containing κB sites in Gro-β, IRF-1, and BTG2 promoters were annealed, radiolabeled, and used as probes for gel shifts. The κB sequence within each oligonucleotide is italicized: Gro-β, 5′-GAGCTCC*GGGAATTTCC*CTGG-3′ (sense); mutant Gro-β, 5′-GAGCTCC*GGGCACTTCC*CTGG-3′ (sense); IRF-1, 5′-CAGGGC-TG*GGGAATCCCG*CTA-3′ (sense); BTG2, 5′-GCTGCCCG*GGGAAAGTCC*G-GG-3′ (sense); and Igκ light chain gene enhancer (Promega, Madison, WI), 5′-AGTTGAG*GGGACTTTCC*CAGGC-3′ (sense).

The cytoplasmic and nuclear proteins of untreated and MIS-treated cells were analyzed by Western blot to assess nuclear localization of p65.

Luciferase assays. Cells grown in 24-well plates were transfected with 0.18 μg of BMP response element (BRE)-driven luciferase; *Renilla* luciferase (0.006 μg) was used to control for transfection efficiency. After 48 h, cells were treated with 1 ng/mL BMP-2 for 6 h and assayed for luciferase activity (n = 4).

Gro-β promoter-luciferase constructs were derived from Gro-β promoter-chloramphenicol acetyl transferase (CAT) constructs described in ref. 26. Briefly, the 500-bp fragment containing the promoter region of Gro-β between nucleotides −428 and +76 (designated SS1), the 150-bp fragment containing nucleotides −80 to +76 (designated SS2), and a deletion fragment containing a mutation within the NF-κB site located within −76 and −67 (designated SS4) were released from Gro-β-CAT constructs with *Xba*1 and *Hin*DIII and cloned between *Nhe*1 and *Hin*DIII sites of the pGL3-basic luciferase construct (Promega). To determine the effect of Smad1 on Gro-β transactivation, Gro-β-luciferase constructs (0.18 μg) were transfected into cells; 48 h after transfection, cells were treated with 35 nmol/L MIS for 6 h and luciferase activity was measured (n = 6). *Renilla* luciferase (0.006 μg) was used to control for transfection efficiency.

To ensure that loss of MIS-inducible Gro-β promoter activity in Smad1DN-expressing cells did not result from clonal variation, transient transfections were done. Cells were transfected with 0.18 μg of Gro-β promoter-luciferase and either (0.18 μg) vector or (0.18 μg) Smad1DN construct and analyzed as described above (n = 6).

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were done as described in ref. 34 with some modifications. Briefly, cells were cross-linked with 1% formaldehyde for 15 min. Cross-linker was quenched using 0.125 mol/L glycine. Cells were lysed in radioimmunoprecipitation assay buffer and sonicated. Chromatin was immunoprecipitated with anti-p65 (Santa Cruz Biotechnology) or anti–phospho-Smad1/5/8 antibodies overnight at 4°C. Anti–green fluorescent protein (GFP; Santa Cruz Biotechnology) was used as control. Antibody complexes were incubated with protein A-Sepharose for 1 h at 4°C, washed five times, and eluted with 0.1% SDS/0.1 mol/L $NaHCO_3$. NaCl was added to a final concentration of 200 mmol/L. Eluates were reverse cross-linked at 65°C for 6 h. DNA was precipitated with ethanol overnight. Following RNase A and proteinase K treatment, DNA was reprecipitated and analyzed by PCR. "Input" templates were purified from 10% of the original lysates. Primers used to amplify 150 and 252 bp of Gro-β and BTG2 promoter

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regions containing κB-binding sites are the following: Gro-β, 5′-CTCCG-GGAATTTCCCT-3′ (forward) and 5′-TCAGCAGGCGGTTCGAGCGGCTG-3′ (reverse); BTG2, 5′-GTTCTTAGCACTGACGACAG-3′ (forward) and 5′ AAAA-CAGCGTTACCCTGGCC-3′ (reverse). Primers used to amplify a region of Gro-β promoter outside of the κB site are 5′-CGGTATCTCTGAGAGCAG-3′ (forward) and 5′-AGTTCCAGATCGATCCCGAG-3′ (reverse).

Results

Gro-β expression is induced by MIS and BMPs. *Gro-β* was identified as a target gene induced by MIS in human breast cancer cells using a HG-U95Av2 oligonucleotide array. To validate this observation further, estrogen receptor–positive human breast cancer cell line, T47D, nontumorigenic human mammary epithelial cell line, MCF10A, primary human prostate epithelial cells, PrEC, and prostate cancer cell line, DU145, were treated with MIS. MIS induced Gro mRNA in all cell lines following 1 to 2 h of treatment; expression remained elevated above basal levels at later time points (Fig. 1*A*).

Because Gro-β is highly homologous to Gro-α, RNase protection assays were done to determine whether MIS was inducing Gro-β and/or Gro-α transcripts. The Gro-β probe protected by the transcript was 83 bp shorter than the full-length probe due to unrelated sequences at the 5′ and 3′ ends. The Gro-α fragment has a *Hin*DIII site around position 226 of its sequence. Thus, the protected Gro-α fragment was 42 bp shorter than the full-length probe due to unrelated sequences at the 5′ end. The 192-bp Gro-β probe protected by Gro-β mRNA was induced by MIS, whereas the 226-bp Gro-α fragment protected by Gro-α mRNA was barely detectable in T47D cells before or after MIS treatment (Fig. 1*B*), suggesting that MIS was predominantly inducing Gro-β in breast cancer cells. We examined Gro-γ expression using PCR analysis and observed that it was neither detectable nor induced in the cell types analyzed.

In addition to MIS, BMP-2, BMP-4, and BMP-6 also up-regulated Gro mRNA expression in MCF10A cells although with different kinetics. BMP-2 and BMP-6 strongly induced Gro as early as 1 h and enhanced levels persisted for up to 24 h (Fig. 1*C, top*). BMP-4 suppressed Gro mRNA at early time points, but expression was induced above basal level at 24 h (Fig. 1*C, bottom*). To identify whether BMPs were inducing Gro-α or Gro-β, RNase protection assays were done. Unlike MIS, BMP-2 induced both Gro-α and

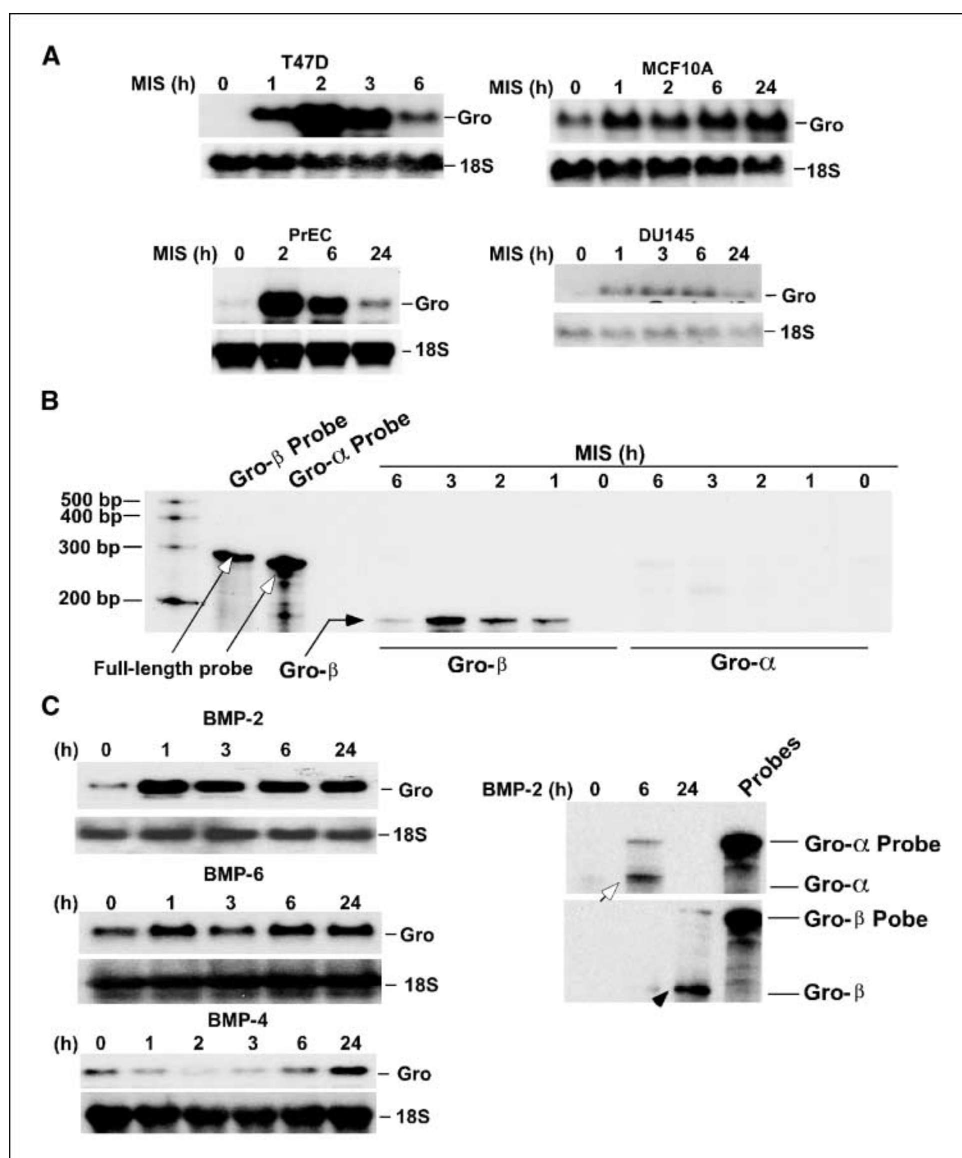

Figure 1. MIS induces Gro-β expression. *A,* total RNA from MIS-treated cells was analyzed by Northern blot for Gro-β expression. Hybridization to 18S is shown as loading control. *B,* RNA isolated from MIS-treated T47D cells was analyzed by RNase protection assay using probes specific for Gro-β and Gro-α. Positions of full-length probes (*open arrows*) and protected Gro-β fragments (*closed arrow*). *C,* MCF10A cells were treated with 1 nmol/L of BMP-2, BMP-4, and BMP-6, and total RNA was analyzed by Northern blot. Hybridization to 18S is shown as loading control. *D,* RNA isolated from T47D cells treated with BMP-2 was analyzed by RNase protection assay using probes specific for Gro-β and Gro-α. Positions of Gro-α (*open arrow*) and Gro-β (*closed arrow*) transcripts.

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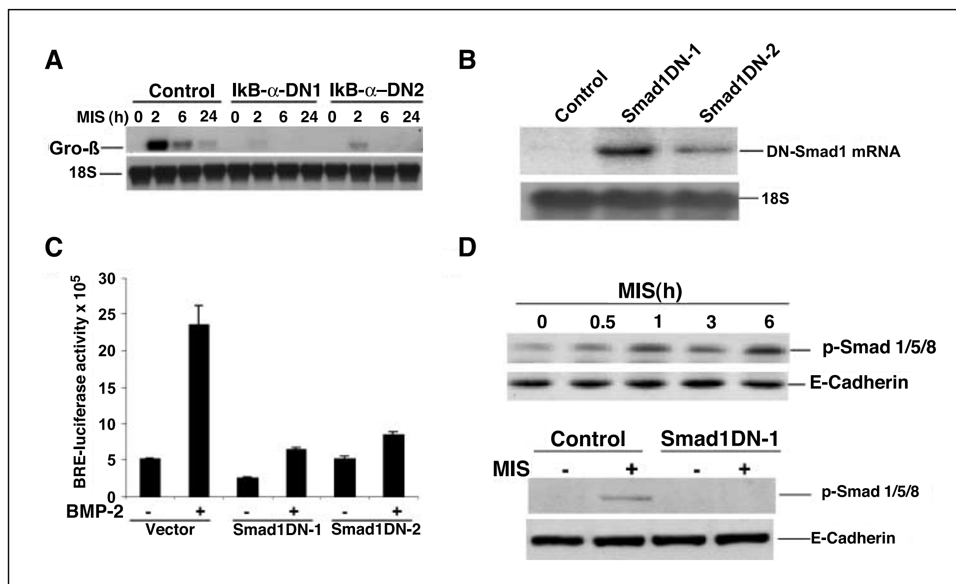

Figure 2. MIS induces Gro-β expression through a NF-κB–dependent mechanism. *A,* vector and IκBαDN-expressing T47D cells were treated with MIS and RNA was analyzed for Gro-β expression. Position of Gro-β transcript. Expression of Smad1DN mitigates MIS-induced Smad1 phosphorylation. *B,* T47D clones expressing Smad1DN were identified by Northern blot. *C,* Smad1DN expression abolishes induction of BRE-luciferase by BMP-2. Vector and Smad1DN-expressing T47D cells were transfected with BRE-luciferase reporter. Cells were treated with 1 ng/mL BMP-2 for 6 h and assayed for luciferase activity ($n = 4$). *Columns,* mean; *bars,* SD. *D, top,* T47D cells were treated with 35 nmol/L MIS and protein was analyzed by Western blot using a phospho-Smad1 antibody. Hybridization to E-cadherin. *Bottom,* Smad1DN-expressing T47D cells were treated with MIS and proteins were analyzed by Western blot. Vector-transfected cells were used as control. Hybridization to E-cadherin showed equal loading of protein.

Gro-β in MCF10A cells (Fig. 1*D*). Thus, Gro-β expression in mammary epithelial cells may be under the regulation of multiple TGF-β family members.

MIS-induced Gro-β expression is dependent on activation of NF-κB and Smad pathways. Gro-β expression in cells is reported to be dependent on NF-κB activation (26, 27), and MIS strongly induces NF-κB activity in breast and prostate cancer cells (2, 3, 28). Therefore, we tested whether stimulation of Gro-β by MIS was mediated through NF-κB activation. Characterization of T47D cells expressing IκBαDN has been described (30). Stimulation of Gro-β by MIS was mitigated in T47D cells expressing IκBαDN, suggesting that NF-κB activation was required for this process (Fig. 2*A*).

Because MIS signaling is also transmitted through phosphorylation of Smad1 (17), we tested whether Smad1 phosphorylation plays a role in Gro-β induction. Cells were transfected with Smad1DN, in which three conserved serine residues at the COOH-terminal SSVS motif are converted to alanines. Two T47D clones stably expressing Smad1DN were identified by Northern blot (Fig. 2*B*). To ensure that Smad1DN transgene was functioning as a dominant negative, we transfected vector and Smad1DN-expressing T47D cells with BRE-luciferase. BMP-2 induced BRE-luciferase in vector-transfected cells, an effect suppressed by Smad1DN (Fig. 2*C*), suggesting that the Smad1DN transgene was indeed functioning as a dominant negative. Western blot analysis showed that MIS induced Smad1/5/8 phosphorylation in T47D cells by 0.5 to 1 h of treatment, an effect readily visible following 6 h of exposure (Fig. 2*D, top*); Smad1DN suppressed MIS-mediated phosphorylation of Smad1 (Fig. 2*D, bottom*).

Induction of Gro-β by MIS was decreased in T47D cells expressing Smad1DN compared with vector-transfected controls (Fig. 3*A*). To ensure that lack of Gro-β induction in Smad1DN-expressing cells was not due to Smad1 overexpression interfering with other functions, we used RNA interference to block Smad1. siRNA specific for Smad1 resulted in significant reduction in Smad1 mRNA expression compared with cells transfected with control siRNA (Fig. 3*B, left*). Induction of Gro-β by MIS was not affected in cells transfected with control siRNA, whereas loss of Smad1 led to ablation of MIS-mediated Gro-β induction (Fig. 3*B, right*).

We then determined whether Smad1 exerts its effects through the NF-κB site in the Gro-β promoter. Characterization of Gro-β promoter used in this assay is described (26). Vector and Smad1DN-expressing cells were transfected with Gro-β promoter-luciferase constructs, SS1, SS2, and SS4, described in Materials and Methods. MIS stimulated luciferase activity from SS1- and SS2-driven reporters but not from the reporter driven by SS4 in which the NF-κB site was mutated. However, none of the Gro-β promoters were responsive to MIS in Smad1DN-expressing clones. Therefore, decrease in MIS-stimulated Smad1 phosphorylation in breast cancer cells was associated with lack of MIS-induced Gro-β promoter activation, which occurs through the κB site in the promoter (Fig. 3*C*). To rule out the possibility of clonal variation, responsiveness of the Gro-β promoter constructs to MIS was analyzed following transient transfections. The results obtained were consistent with the results observed in T47D cells that stably expressed Smad1DN (Fig. 3*D*).

To ensure that these observations were not limited to a single cell line, we tested MIS-induced Gro-β expression in the human prostate cancer cell line LNCaP stably expressing IκBαDN and Smad1DN. Characterization of these cells is described (3, 31). As with T47D cells, IκBαDN and Smad1DN expression in LNCaP cells suppressed Gro-β induction by MIS (Fig. 3*E*). Therefore, Gro-β induction by MIS requires activation of both NF-κB and Smad1.

Activation of NF-κB by phospho-Smad1 is dependent on sequence of the κB site. MIS induces IRF-1 and BTG2 expression in breast cancer cells through a NF-κB–dependent mechanism (30, 33). To determine whether Smad1 activation is required for MIS-mediated stimulation of IRF-1 and BTG2, vector and Smad1DN-expressing cells were treated with MIS and analyzed for IRF-1 and BTG2 expression. Both IRF-1 and BTG2 mRNA were induced to similar levels in both vector and Smad1DN-expressing cells (Fig. 4*A*), indicating that Smad1 activation was not required for this process.

To further characterize this observation, sequence of the κB sites within IRF-1, BTG2, and Gro-β promoters was surveyed (Fig. 4*B*). Because sequences of NF-κB–binding sites within the three promoters were not identical, we analyzed whether blocking MIS-induced Smad1 phosphorylation would affect binding of NF-κB

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proteins to these sites. Both vector and Smad1DN-expressing cells were treated with MIS for 1 h, and nuclear proteins were analyzed by gel shift. MIS induced NF-κB binding to all three NF-κB sites (Fig. 4*C–E*). Expression of Smad1DN abrogated MIS-stimulated NF-κB binding to the κB site derived from Gro-β promoter (Fig. 4*C*) but did not affect binding to sequences derived from IRF-1 and BTG2 promoters (Fig. 4*D* and *E*).

We next investigated whether phospho-Smad1 was present within the p50, p65 complex that binds to κB sequences. Preincubation of nuclear lysates with phospho-Smad1 antibody led to a 80% reduction in DNA binding to the κB sequence derived from Gro-β promoter, suggesting that phospho-Smad1 was a component of the complex bound to this sequence (Fig. 4*C*). Conversion of two nucleotides within NF-κB sequence of the Gro-β promoter abrogated binding, confirming that protein-DNA complex detected in gel shift assays was indeed occurring through the κB site (Fig. 4*C*). However, phospho-Smad1 was not a component of the NF-κB complex bound to κB sites derived from IRF-1 and BTG2 promoters (Fig. 4*D* and *E*).

To characterize the sequence requirement of the Gro-β κB site that renders Smad1 responsiveness, we analyzed NF-κB binding to the κ light chain enhancer κB site, which differs from the Gro-β NF-κB site by a single nucleotide. MIS-induced NF-κB complex that bound to this site contained both p65 and p50 subunits. The phospho-Smad1 antibody decreased the band intensity compared with control. Moreover, NF-κB binding to this site was decreased in Smad1DN-expressing clones compared with that observed in control cells (Fig. 4*F*). Overall κB site sequence comparison of Gro-β, BTG2, IRF-1, and Igκ light chain enhancer genes suggests that two T residues (italicized) within the Gro-β κB site (GGGAAT*TT*CC) are important to confer Smad1 responsiveness to the Gro-β promoter (Fig. 4*B*).

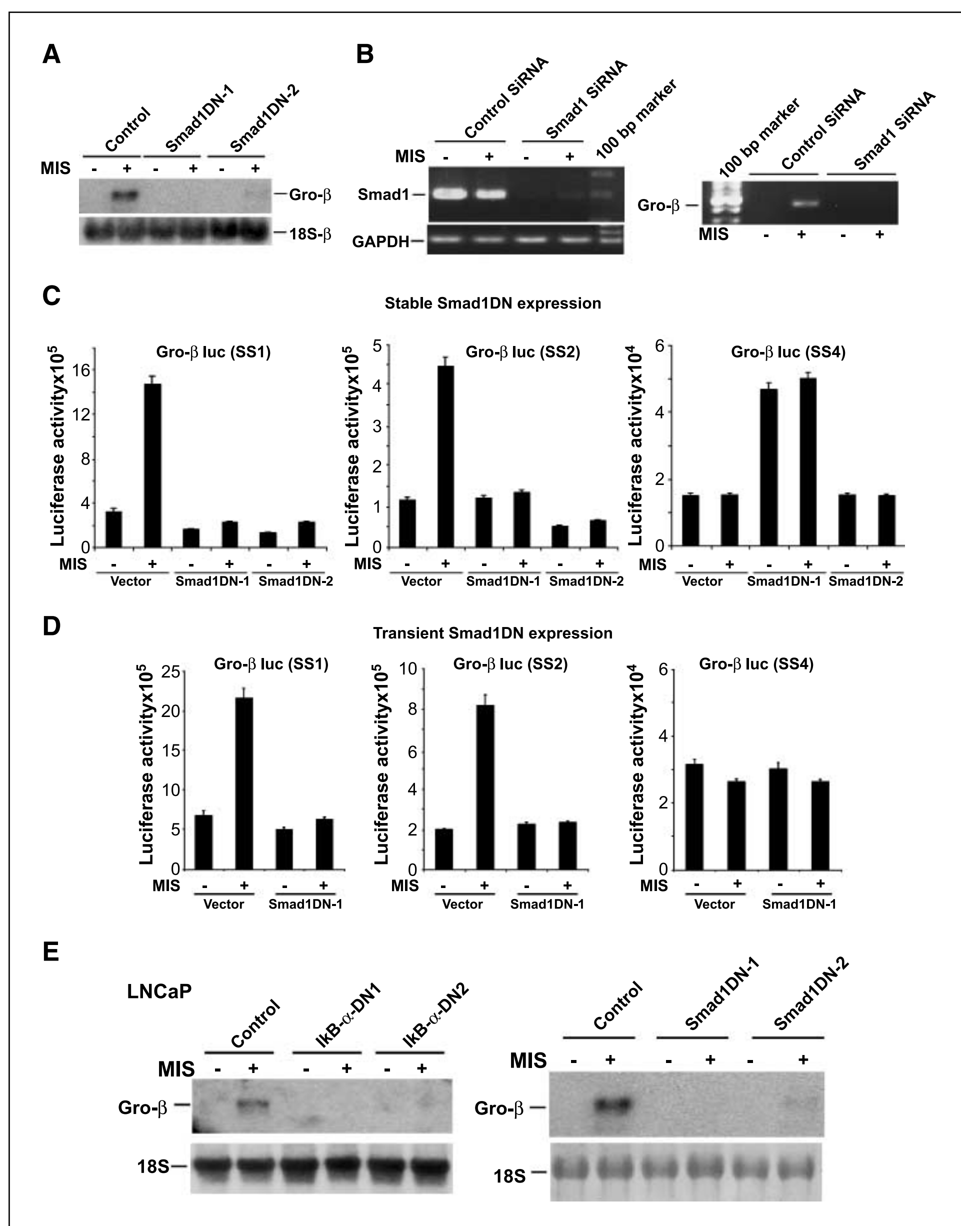

Figure 3. MIS-mediated induction of Gro-β requires Smad1 activation. *A,* vector-transfected and Smad1DN-expressing T47D cells were treated with MIS for 2 h and RNA was analyzed for Gro-β mRNA expression. Hybridization to 18S. *B,* T47D cells were transfected with control and Smad1 siRNA 24 h before addition of MIS. After 2 h of MIS treatment, RNA was isolated and analyzed by reverse transcription-PCR using primers specific for Smad1, Gro-β, and GAPDH. *C,* vector and Smad1DN-expressing T47D cells were transfected with Gro-β promoter-driven luciferase reporters. Cells were treated with 35 nmol/L MIS for 6 h and assayed for luciferase activity (*n* = 6). *Columns,* mean; *bars,* SD. *D,* T47D cells were transiently transfected with Gro-β promoter-luciferase reporters and either vector or Smad1DN construct. Cells were treated with 35 nmol/L MIS for 24 h and assayed for luciferase activity (*n* = 6). *Columns,* mean; *bars,* SD. *E,* vector, IκBαDN-expressing (*left*), and Smad1DN-expressing (*right*) LNCaP cells were treated with 35 nmol/L MIS for 2 h and total RNA was analyzed for Gro-β expression.

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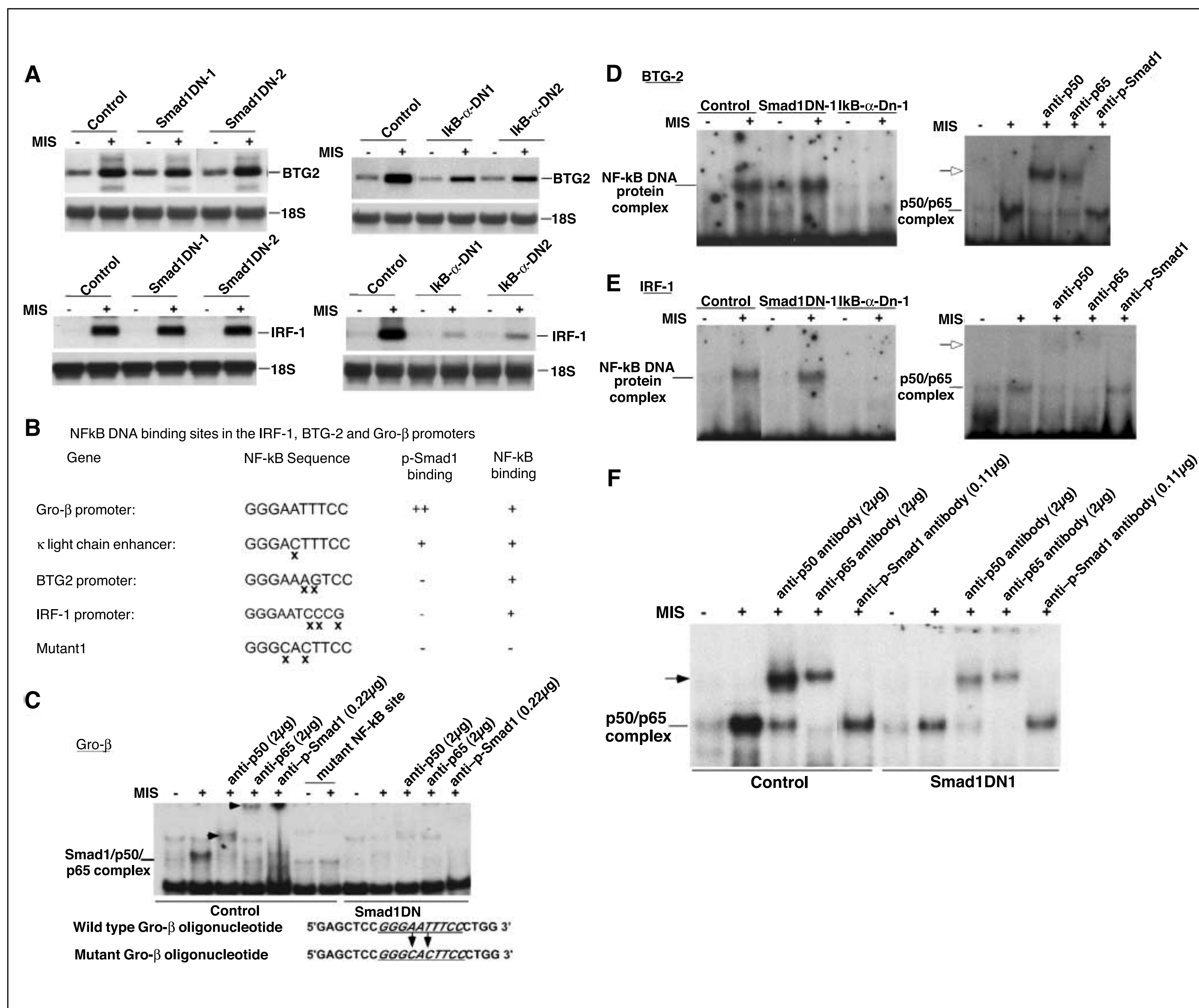

Figure 4. NF-κB activation by phospho-Smad1 is dependent on sequence of the κB-binding site. *A,* induction of IRF-1 and BTG2 by MIS is NF-κB dependent but is not dependent on Smad1 activation. Vector and IκBαDN- or Smad1DN-expressing T47D cells were treated with MIS for 2 h and RNA was analyzed for IRF-1 and BTG2 expression. *B,* sequences of the κB-binding sites within Gro-β, Igκ light chain enhancer, IRF-1, and BTG2. *x,* sequence variation(s) from the Gro-β κB site. *C,* control and a pool of Smad1DN-expressing cells were treated with 35 nmol/L MIS for 1 h. Nuclear proteins were analyzed by gel shift assay. *Arrowheads,* positions of DNA-NF-κB protein complexes and supershifted complexes. To ensure that the protein complex of interest was binding to the κB site on the probe, a ^{32}P-labeled oligonucleotide probe in which the κB site is mutated was used in the binding assay. Sequence of wild-type and mutant oligonucleotide. *D,* nuclear proteins from MIS-treated vector and IκBαDN- and Smad1DN-expressing T47D cells were mixed with ^{32}P-labeled NF-κB DNA-binding oligonucleotide probe derived from the BTG2 promoter and separated on nondenaturing gels. *E,* nuclear proteins from MIS-treated vector and IκBαDN- and Smad1DN-expressing T47D cells were mixed with ^{32}P-labeled NF-κB DNA-binding oligonucleotide probe derived from the IRF-1 promoter and separated on nondenaturing gels. In both experiments (*D* and *E*), T47D cells were treated with 35 nmol/L MIS for 1 h. Nuclear proteins were incubated with TransCruz p50 and p65 or phospho-Smad1 antibodies before addition of radiolabeled probe. *Open arrows,* positions of DNA-NF-κB protein complexes and supershifted complexes. *F,* control and Smad1DN-expressing T47D cells were treated with 35 nmol/L MIS for 1 h. Nuclear proteins were preincubated with indicated amounts of antibody overnight at 4°C, mixed with ^{32}P-labeled NF-κB DNA-binding oligonucleotide derived from the Igκ light chain enhancer (Promega), and separated on a nondenaturing gel. *Arrow,* positions of DNA-NF-κB protein complexes and the supershifted complexes. The phospho-Smad1 antibody inhibited binding by 60% compared with that observed in the MIS-treated sample. Band intensities were quantified using Scion Image software.

Retention of p65 in the nucleus is impaired in Smad1DN-expressing T47D cells. To analyze the role of Smad1 phosphorylation in NF-κB activation, vector and Smad1DN-expressing T47D cells were treated with MIS for 30 and 60 min and nuclear localization of p65 was analyzed by Western blot. MIS enhanced the nuclear localization of p65 in control and Smad1DN-expressing cells. In Smad1DN-expressing cells, p65 localized to the nucleus after 30 min of exposure to MIS; levels returned to the basal state after 60 min. However, MIS-induced p65 nuclear localization occurred at 60 min in control cells (Fig. 5*A*). To determine whether p65 continues to be retained in the nuclei of control cells, we treated the cells with MIS for 2 h. MIS induced nuclear localization of p65 in control cells, but no difference in nuclear p65 expression was observed in Smad1DN-expressing cells. The blots were probed for tubulin and c-myc to ensure that the cytoplasmic fraction did not contaminate the nuclear fraction (Fig. 5*B*). These results indicate that loss of Smad1 activation impairs nuclear retention of p65 following MIS treatment.

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Phospho-Smad1 and p65 are bound to Gro-β promoter *in vivo*. Because gel shifts suggest that phospho-Smad1 is a component of the complex bound to the κB site derived from Gro-β promoter, we used ChIP assays to characterize the association of p65 and phospho-Smad1 with BTG2 and Gro-β promoters. Untreated and MIS-treated T47D cells were cross-linked and chromatin was immunoprecipitated with anti-p65, anti–phospho-Smad1, and anti-GFP antibodies. DNA eluted from immune complexes was analyzed by PCR using gene-specific primers flanking the κB sites in Gro-β and BTG2 promoters. PCR analysis using primers specific for BTG2 promoter amplified the BTG2 promoter from DNA immunoprecipitated by anti-p65 but not anti–phospho-Smad1 antibody, suggesting that phospho-Smad1 was not a component of the NF-κB complex bound to this region (Fig. 6*A*). MIS induced the binding of both p65 and phospho-Smad1 to the Gro-β promoter fragment containing the κB site (Fig. 6*B*). The PCR fragments were purified and sequenced to confirm that it matched the sequence of Gro-β and BTG2 promoters (data not shown). To determine whether the lack of Gro-β induction in Smad1DN and IκBαDN clones correlated with the lack of p65 and phospho-Smad1 binding to the Gro-β promoter, ChIP was carried out with untreated and MIS-treated Smad1DN- and IκBαDN-expressing T47D cells. Expression of Smad1DN abrogated MIS-induced recruitment of phospho-Smad1 to the Gro-β promoter. Moreover, compared with MIS-treated control cells, binding of p65 to the Gro-β promoter was mitigated in Smad1DN-expressing T47D cells. In IκBαDN-expressing cells, neither p65 nor phospho-Smad1 binding to the Gro-β promoter was observed in response to MIS (Fig. 6*B*). Together, these results suggest that p65 is likely to be recruited before phospho-Smad1 to the Gro-β promoter. The two entities may then functionally interact to form an active transcription complex.

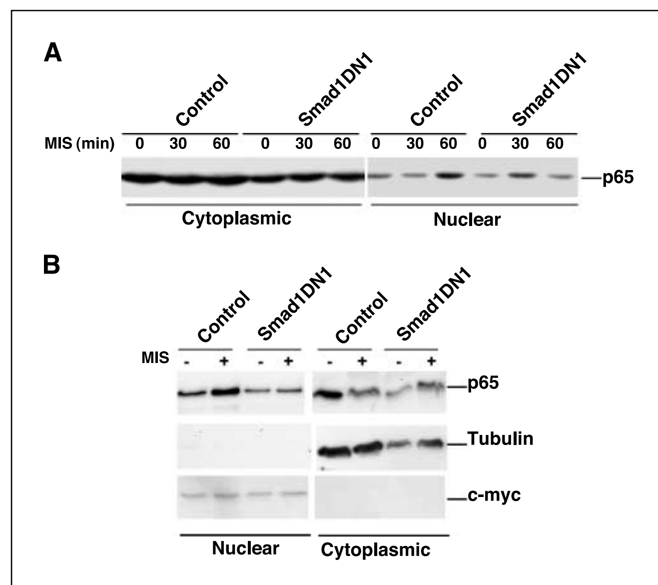

Figure 5. Nuclear retention of p65 is impaired in Smad1DN-expressing cells. *A,* control and Smad1DN-expressing T47D cells were treated with 35 nmol/L MIS for 30 and 60 min. Nuclear and cytoplasmic fractions were analyzed by Western blot for p65 expression. *B,* control and Smad1DN-expressing T47D cells were treated with 35 nmol/L MIS for 2 h. Nuclear and cytoplasmic proteins were analyzed by Western blot for p65 expression. The blots were stripped and reprobed for tubulin and c-myc to determine the purity of protein fractions.

Gro-β inhibits breast cancer cell growth. Consistent with previous reports (24, 35), Gro-β expression was readily detectable in immortalized nontumorigenic human mammary epithelial cells, MCF10A and 184A1, but almost undetectable in all breast cancer cell lines (Fig. 6*C*). Analysis of RNA from human breast carcinoma and matched normal tissue from 10 patients showed decreased Gro-β expression in tumors of 7 patients (Fig. 6*C*). Because MIS inhibits breast cancer cell growth (2), we tested whether Gro-β may be responsible for this effect. Equal numbers of T47D cells were treated with 0.1, 1, and 10 ng/mL of Gro-β for a week, and the plates were stained with crystal violet; Gro-β suppressed T47D growth in a dose-dependent manner.

Discussion

Gro-α and Gro-β are expressed in normal mammary epithelium and suppressed in breast tumors and cancer cell lines (24, 35), suggesting that they may be potential regulators of growth, differentiation, and morphogenesis in the mammary gland. However, little is known about factors that regulate their expression in mammary epithelial cells or breast cancer cells. Our results show that, in addition to IL-1, IL-17, and TNF-α (26, 27, 36) that regulate Gro-α/Gro-β in a variety of cell types, MIS and BMPs, members of the TGF-β family, also regulate Gro expression in mammary epithelial cells. Unlike MIS, which predominantly induced Gro-β, BMP-2 stimulated both Gro-α and Gro-β.

The Gro-β promoter, in addition to containing κB sites, also has putative binding sites for AP3, SP1, and CRE (26, 37) and a potential Z-DNA region (37). Although the SP1 and CRE sites in the Gro-β promoter may contribute to higher basal activity (26), TNF-α and IL-1 inducibility of Gro-β required NF-κB activation with p50, p65 subunits binding to its κB site (26, 27, 37); the AP3 site was not sufficient for induction, whether it cooperates with the NF-κB site is not known (37).

NF-κB–mediated induction of Gro-β by IL-1 was sensitive to genistein, an inhibitor of tyrosine phosphorylation (26). Gro-β induction by IL-17 in fibroblast-like synoviocytes is mediated by mitogen-activated protein kinase p38, and inhibitors of tyrosine kinases and protein kinase C significantly suppressed IL-17–induced Gro-β expression (36). Although our results suggest that NF-κB activation by MIS is necessary for Gro-β induction, characterization of MIS-mediated induction of Gro-β, BTG2, and IRF-1 revealed that κB sequence within the Gro-β promoter may subject it to further regulation by phospho-Smad1.

Smad proteins have relatively low DNA binding affinities and specificities but can regulate transcription either by functional cooperation with other transcription factors bound to adjacent sites or through association with DNA-bound transcription factors. Smad3 and Smad4 interact with jun family to activate activator protein-1 promoter sequences synergistically (38), and formation of a complex between signal transducers and activators of transcription 3 and Smad1, bridged by p300, is involved in cooperative signaling of leukemia inhibitory factor and BMP-2 and subsequent induction of astrocytes from neural progenitors (39). Although Smad and NF-κB signaling have been shown to interact with each other, this is the first report to show that the interaction between activation of Smad1 and NF-κB is functional. The presence of phospho-Smad1 within the MIS-induced complexes bound to κB site of Gro-β promoter and recruitment of p65 and phospho-Smad1 to Gro-β promoter *in vivo* suggest that NF-κB proteins and phospho-Smad1 may function as coactivators of MIS-induced Gro-β.

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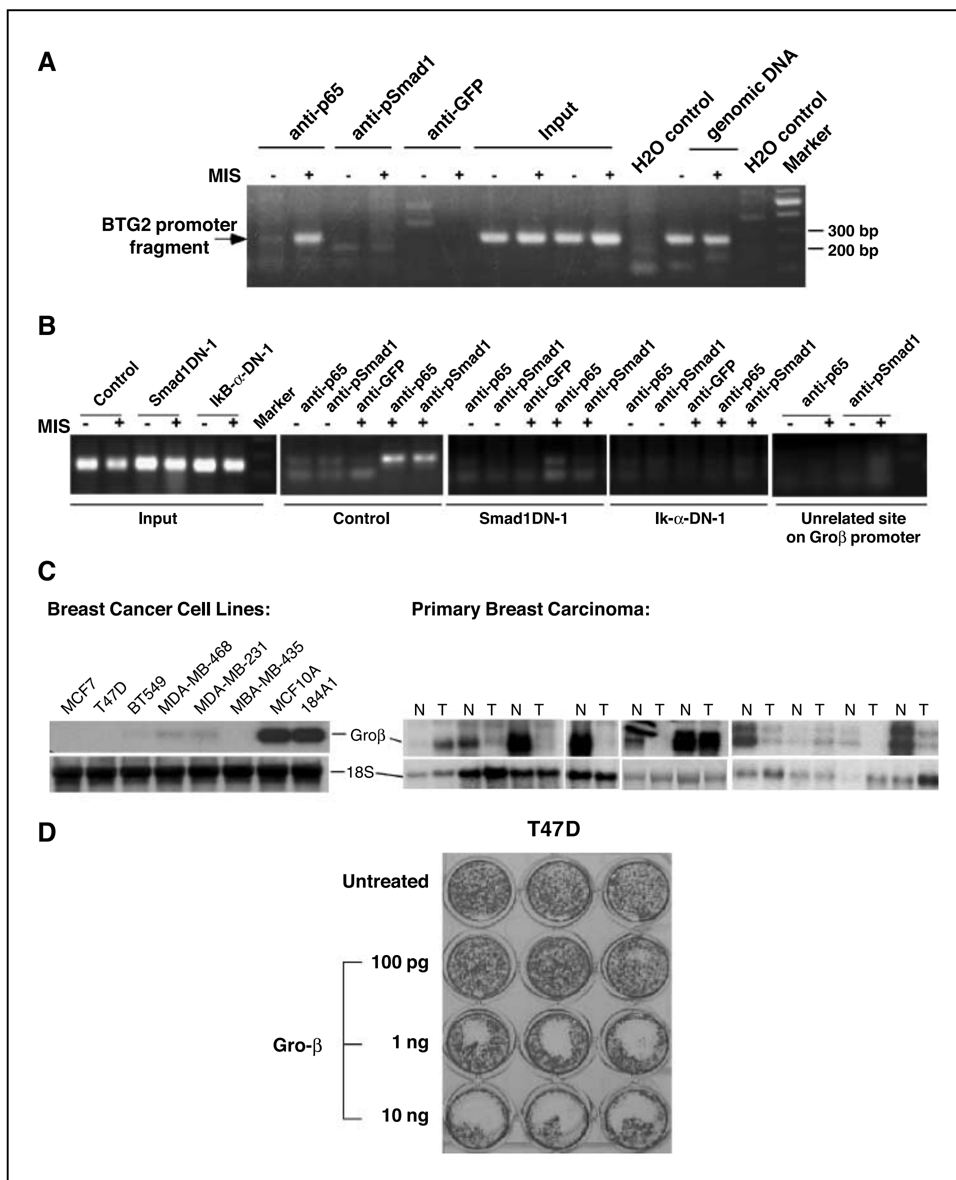

Figure 6. Phospho-Smad1 and p65 are bound to Gro-β promoter. *A,* T47D cells were treated with 35 nmol/L MIS for 2 h and analyzed by ChIP with anti-p65 and anti–phospho-Smad1 antibodies. Samples immunoprecipitated with anti-GFP antibody were used as negative control. Lysates used for immunoprecipitation were used as input control. Precipitated DNA was subjected to PCR analysis using primers specific for BTG2 promoter region containing the κB-binding site. Position of BTG2 promoter fragment. *B,* control and Smad1DN- and IκBαDN-expressing T47D cells were treated with MIS for 2 h and analyzed by ChIP as described above. Precipitated DNA was subjected to PCR analysis using primers specific for Gro-β promoter region containing the κB-binding site. Position of Gro-β promoter fragment. An unrelated site on the Gro-β promoter was amplified to control for specificity. The data shown represent an experiment in which control and IκBαDN- and Smad1DN-expressing T47D cells were concurrently processed for ChIP and simultaneously analyzed by PCR. The PCR products were run on lanes above and below on the same gel due to a single row having insufficient number of lanes for the entire analyses. *C,* expression of Gro-β in breast cancer cell lines. Total RNA from (7.5 μg) MCF10A, 184A1, BT549, T47D, MCF7, MDA-MB-231, MDA-MB-435, and MDA-MB-468 was analyzed by Northern blot for expression of Gro-β. Expression of Gro-β is suppressed in breast carcinoma. Total RNA isolated from 10 breast carcinomas and matched normal tissue was analyzed for Gro-β mRNA expression. Hybridization to 18S is shown to control for loading. *D,* equal number of T47D cells were cultured in 24-well plates and treated with 100 pg/mL, 1 ng/mL, and 10 ng/mL of Gro-β. Plates were stained with crystal violet after 7 d. Three representative wells for each sample.

Although NF-κB is stimulated by a myriad of reagents, differential regulation of NF-κB–responsive genes, which varies depending on cell type and stimulus, is not completely understood. Activation of different NF-κB/Rel subunits, phosphorylation of p65, and degree of difference in the κB sequence within promoters (11, 40–43) are likely factors in differential regulation of gene expression in response to NF-κB activation. Characterization of binding sites using gel shift assays, promoter transactivation assays, and ChIP analysis suggests that phospho-Smad1–dependent Gro-β expression is dictated by sequence of the κB site within the promoter. It is likely that this sequence plays a role in selective recruitment of the cofactor Smad1 and NF-κB dimers, leading to transactivation of Gro-β. Gro-β promoter ChIP of IκBαDN- and Smad1DN-expressing T47D cells suggests that p65 may be recruited to the Gro-β promoter before phospho-Smad1. Moreover, the weak NF-κB activation in response to MIS in Smad1DN-expressing cells suggests that phospho-Smad1 may be enhancing the affinity of NF-κB complexes to this site.

The fleeting nuclear localization of p65 in Smad1DN-expressing cells may be sufficient for NF-κB–mediated transactivation of BTG2 and IRF-1 promoters, which may rely on a different subset of MIS-inducible cofactors, whereas the lack of Smad1 activation in these cells may impair the formation of a productive transcription complex on the κB site of the Gro-β promoter.

It is likely that, in the gel shift assay shown in Fig. 4*D* and *E*, these MIS-inducible, unidentified cofactors lead to efficient recruitment of the residual NF-κB proteins to the BTG2 and IRF-1 κB sites.

Leung et al. showed that sequence of the κB site does play an important role in determining κB family member specificity. However, the sequence of the site, rather than determining the ability of a particular dimer to bind effectively, defined the coactivators that would form productive interactions with the DNA-bound NF-κB dimer. For example, the κB sites of the IP-10 promoter recruited IRF-3 to the promoter; lipopolysaccharide, by inducing IRF-3, functionally activated the p65 homodimers bound to the

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IP-10 κB site. The coactivator requirement was imposed by a single nucleotide in the sixth position of the κB site. It is noteworthy that the sequence of the upstream κB site (GGGAAATTCC) of the *IP-10* gene differs from the κB site of Gro-β (GGGAATTTCC) by single base at the sixth position and that the COOH-terminal region of IRF-3 is structurally and topologically similar to the Mad homology (MH2) domain of Smad proteins (44, 45). Whether the molecular mechanism by which the cofactors IRF-3 and phospho-Smad1 stimulate NF-κB–mediated transactivation are similar remains to be investigated.

Experimental evidence indicates that Gro-β may mediate varied functions. Fibronectin fragments generated from damaged extracellular matrix induce several cytokines, including Gro-β, and augment inflammatory responses, leading to progressive matrix destruction and cartilage degradation (25). Gro-β can also regulate angiogenesis. It inhibits fibroblast growth factor (FGF)-induced DNA synthesis and proliferation of bovine capillary endothelial cells and prevents vascularization in the chicken chorioallantoic membrane assay and FGF-induced corneal neovascularization in mice. Consistent with this observation, Gro-β suppressed the growth of Lewis lung tumors in immune-competent and immune-deficient mice by suppressing tumor vascularization, although it was ineffective in regulating the growth of Lewis lung carcinoma cells *in vitro* (46). However, expression of Gro-β (and Gro-α) in immortalized murine melanocytes enhanced colony formation in soft agar and tumorigenicity in nude mice with an associated induction of angiogenesis (47). Interestingly, Gro-α–induced transformation of these cells required Ras activation, whereas Gro-β only weakly induced Ras expression and activity (48). Thus, biological responses to Gro-β vary depending on the cellular context. Loss or decline in Gro-β mRNA expression in breast tumors compared with matched uninvolved tissue suggests that impaired autocrine/paracrine chemokine signaling may be a contributing factor in tumorigenesis of the breast.

Although constitutive activation of NF-κB has been reported in breast cancer cell lines and breast carcinoma (49, 50), the expression of *Gro-β*, a NF-κB–responsive gene (26, 27), is low in breast cancer cell lines and is suppressed in a large percentage of breast tumors compared with matched uninvolved tissue, suggesting that additional mechanisms besides NF-κB activation may be involved in regulating Gro-β expression in breast cancer. Although NF-κB activation is predominantly associated with survival signals, there is also evidence to the contrary. RelA null mouse embryonic fibroblasts are resistant to p53-induced apoptosis (51). Immortalized embryonic fibroblasts lacking RelA have a weakly transformed phenotype and form tumors in severe combined immunodeficient mice, suggesting that RelA under certain conditions can function as a tumor suppressor (52). MIS-mediated inhibition of both breast and prostate cancer cell growth was dependent on activation of NF-κB (2, 28). A likely explanation for this phenomenon could be selective induction of NF-κB–regulated growth-inhibitory genes stimulated by MIS and interaction between two distinct signaling pathways representing an additional level of specificity in gene regulation, which may not be achieved with either one alone.

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