Identification of a gadd45β 3′ Enhancer That Mediates SMAD3- and SMAD4-dependent Transcriptional Induction by Transforming Growth Factor β§

Michael B. Major† and David A. Jones¶§†

From the Departments of †Oncological Sciences and ¶Medicinal Chemistry, Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah 84112

GADD45β regulates cell growth, differentiation, and cell death following cellular exposure to diverse stimuli, including DNA damage and transforming growth factor-β (TGFβ). We examined how cells transduce the TGFβ signal from the cell surface to the gadd45β genomic locus and describe how GADD45β contributes to TGFβ biology. Following an alignment of gadd45β genomic sequences from multiple organisms, we discovered a novel TGFβ-responsive enhancer encompassing the third intron of the gadd45β gene. Using three different experimental approaches, we found that SMAD3 and SMAD4, but not SMAD2, mediate transcription from this enhancer. Three lines of evidence support our conclusions. First, overexpression of SMAD3 and SMAD4 activated the transcriptional activity from this enhancer. Second, silencing of SMAD protein levels using short interfering RNAs revealed that TGFβ-induced activation of the endogenous gadd45β gene required SMAD3 and SMAD4 but not SMAD2. In contrast, we found that the regulation of plasminogen activator inhibitor type I depended upon all three SMAD proteins. Last, SMAD3 and SMAD4 reconstitution in SMAD-deficient cancer cells restored TGFβ induction of gadd45β. Finally, we assessed the function of GADD45β within the TGFβ response and found that GADD45β-deficient cells arrested in G2 following TGFβ treatment. These data support a role for SMAD3 and SMAD4 in activating gadd45β through its third intron to facilitate G2 progression following TGFβ treatment.

Normal epithelial cells are in constant communication with their surrounding environment, largely through the detection, interpretation, and response to extracellular signaling molecules. The TGFβ superfamily of growth factors comprises 42 such signaling molecules in humans, many of which play fundamental roles in development and adult tissue homeostasis. The epithelial response to members of this family is highly varied and includes such diverse cellular processes as proliferation, movement, differentiation, and apoptosis. Indeed, cells harboring mutations within the signal transduction proteins or the TGFβ target genes either fail to respond or respond inappropriately to the TGFβ signal, often leading to developmental problems, oncogenesis, fibrotic disease, metastasis, and autoimmune disorders. Greater understanding of how cells interpret the TGFβ signal will facilitate the prevention, detection, and treatment of various human diseases.

The central elements of TGFβ signal transduction are now known (1, 2). TGFβ activates the serine/threonine kinase activity of a multimeric receptor complex. Activation of this complex initiates a cascade of intracellular events that culminate in altered gene expression. The SMAD proteins form the foundation of this signaling network, since they are the only proteins directly phosphorylated by the receptor complex. However, these transcription factors are by no means sufficient to impart a TGFβ response. To specifically target a gene for transcriptional regulation, the SMADs require assistance by accessory factors. Consequently, the presence and activity of these accessory factors is important to the TGFβ transcriptional program as are the SMAD proteins. By designing the system in such a way, cell-specific responses to TGFβ can be achieved. Further, the logic of the TGFβ signaling network explains how the cell integrates multiple signals to generate highly specific phenotypic responses.

In an attempt to better understand how TGFβ regulates gene transcription and how those gene products contribute to TGFβ biology, we have partially defined the TGFβ transcriptional profile in normal human mammary epithelial cells (HMEC). cDNA microarray expression analysis of TGFβ-treated HMEC revealed a set of genes involved in cellular proliferation, differentiation, and apoptosis. One of these genes, gadd45β/hMyD118, is regulated by TGFβ in multiple cell types, thus suggesting that this gene is of central importance to the TGFβ response.

GADD45β and two similar small acidic nuclear proteins, GADD45α and GADD45γ, make up the GADD45 family (3). All three proteins regulate diverse cellular mechanisms including cell growth, DNA repair, differentiation, and apoptosis, four phenotypes that are also controlled by TGFβ signaling. Aside from sequence similarity, these genes share transcriptional regulation by DNA damage insult and growth factors. gadd45β is, however, the only member of this family regulated by TGFβ (4, 5). gadd45β was first discovered as a transcript rapidly induced by either TGFβ treatment or the onset of terminal differentiation in M1 murine myeloid cells (6, 7). Subsequent studies employing antisense-mediated silencing established
GADD45β as an important regulator of the G_{2}/M checkpoint following genotoxic stress (8) and apoptosis during M1 myeloid cell terminal differentiation (6, 9). Human GADD45β, which was first identified in a complex containing the p38-activating kinase MKK1 (MEKK4), is now a well-established regulator of p38 activity and consequently p38-regulated biology (5, 10, 11). GTFβ activates p38 kinase activity and induces apoptosis in normal murine hepatocytes, but not in hepatocytes derived from gadd45β knockout mice (11). An initial characterization of the molecular mechanism by which TGFβ induces gadd45β transcription has recently been reported. First, reconstitution of SMAD4 expression in SMAD4-null pancreatic cell lines restored gadd45β induction by TGFβ (5). The nature of the TGFβ-SMAD-gadd45β link appears to be direct; exogenously expressed SMAD2 and SMAD3 or SMAD3 and SMAD4 induce gadd45β proximal promoter activity 3–4-fold (11). However, the relative importance and function of each SMAD protein to the transcriptional activation of the endogenous gadd45β gene is not known.

Utilizing RNA interference and reconstitution of SMAD3 and SMAD4 protein expression in SMAD-deficient cell lines, we exclude SMAD2 and include SMAD3 and SMAD4 as transcription factors involved in the TGFβ induction of gadd45β. Additionally, through a genomics-based approach, we identified a SMAD-dependent TGFβ-responsive enhancer encompassing the third intron of gadd45β. The importance of this enhancer is indicated by a 3-fold greater transcriptional induction following TGFβ treatment than transcriptional effects mediated by 5′ promoter sequences. Finally, using a cell system that does not undergo TGFβ-induced apoptosis but does respond to TGFβ by gadd45β transcriptional induction, we establish an apoptosis-independent role for GADD45β as an important mediator of G_{2}/M progression following TGFβ treatment.

MATERIALS AND METHODS

Cell Culture and Drug Treatments—The following cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2.0 μM l-glutamine, 1.0 μM sodium pyruvate, penicillin, and streptomycin and split every third day or at 80% confluence: MvILu (CCL64), HaCaT, HaLu, 293, and 10T1/2. HT29 adenocarcinoma colon cells were cultured in McCoy’s medium supplemented with 10% fetal bovine serum. The HepG2 and JAR cell lines were cultured in minimal essential medium and RPMI supplemented with 10% fetal bovine serum, respectively. We obtained all of the cell lines from ATCC except for the HaCaT immortalized keratinocyte cell line, which was a kind gift from D. Grossman (University of Utah, Salt Lake City, UT). The immortalized keratinocyte cell line, which was a kind gift from D. Ayer (University of Utah), and the JAR cells were a kind gift from E. Adashi (University of Utah). Human mammary epithelial cells (HMEC) were obtained from BioWhittaker (Walkersville, MD) and cultured in complete mammary epithelial growth medium. HMEC were seeded at passage 7 or 8 and harvested at no greater than 80% confluence for all experiments. For treatments with TGFβ (isofrom type 1; Peprotech, Rocky Hill, NJ), we found little to no difference with respect to gene transcription if the cells had been previously serum-starved. The vehicle control for TGFβ comprised 4 mM HCl, 1 mg/ml bovine serum albumin, cyclohexamide and actinomycin D (Calbiochem) were used at 10 and 5 μg/ml, respectively, and treated as described in Fig. 2.

RNA Interference—siRNAs were designed to specifically target either smad2, smad3, or smad4 in accordance with the guidelines developed by Tuschl et al. (12). Because the sequences of mink smad2 and smad3 cDNAs is unknown, siRNAs were designed against the human sequences. The human-designed smad2 and smad3 siRNAs efficiently and specifically silenced mink SMAD2 and SMAD3 protein expression, thus indicating that these sequences are conserved in mink. We designed the smad4 siRNA-A and siRNA-B against the mink sequence, and consequently they do not silence human smad4 (data not shown). The control siRNA (scrambled siRNA; siScr) specifically recognizes human smad4 and thus does not affect mink SMAD2, SMAD3, or SMAD4 expression. The sequences of the chemically synthesized and high pressure liquid chromatography-purified RNA oligomers are as follows (sense strand shown): Smad2 5′-UCUUUGUGGAGCAGCGC-GUAUGUUCUUGACUGAGT-3′; Smad3 5′-GGAGCA-AUAGUUGUCAGAGTtt; Smad4 5′-UGGUACUUUAUAAACAGGTT; Scir 5′-GGAUGAUAUGUGCAACTT. To silence gadd45β expression, three siRNAs were designed (sense strand shown): siGadd45β-A (5′-GUU GAU GAA UGU GGA ACC ACGG); siGadd45β-B (AUC CAC UUC ACG CUC AUC Ctt); and siGadd45β-C (CUU GGU UGG UCC UUG UCC UCU ttt). For the SMAD silenced (Invitrogen) for the ensembl-transport RNA or protein was isolated 40–48 h after transfection. In time course experiments, we found that maximal silencing occurred 36 h after transfection for all three SMAD proteins (data not shown). For Gadd45β silencing, 3 h after the start of Gadd45β siRNA transfection, cells were treated with vehicle or TGFβ for an additional 2 h prior to RNA isolation or 12 h prior to flow cytometry.

Plasmids and Genomic Alignments—We electronically cloned the human, mouse, and rat gadd45β genomic loci from publicly available sequence databases. Approximately 8 kb of the genomic loci, starting at 5000 kb upstream of the transcriptional start site, were aligned using the MAVID alignment algorithm (13, 14). The portion of this piece of genomic DNA showing conservation among all three species is shown in Fig. 7A. The G45-1 (1470 bp, +362 bp), G45-2 (1972 bp, +362 bp), G45-3 (1746 bp, +362 bp), G45-A (1353 bp, −1042 bp), G45-B (572 bp, −79 bp), and G45-C (1941 bp, +1428 bp) reporter constructs were created as follows. The indicated region of the human gadd45β genomic locus was PCR-amplified from HMEC genomic DNA and cloned into the pCMV2-TOPO vector (Invitrogen). These DNAs were then subcloned into pGL3basic, sequence-verified, and utilized in subsequent dual luciferase assays. J. Massague generously provided the STATluc reporter construct (Memorial Sloan-Kettering Cancer Center, New York). The murine Smad7 cDNA (generously provided by R. Derynck, University of California, San Francisco, CA) was subcloned into pG3A3.1. Similarly, the FLAG-tagged Smad3 expression vectors used in the reporter experiments were created by subcloning the cDNAs from constructs provided by D. Satterwhite into pCMV2-FLAG (University of Utah, Salt Lake City, UT). For luciferase assays, all reporters were co-transfected with an SV40-Renilla luciferase reporter plasmid that was used to normalize transcription efficiencies. For retroviral infections, we PCR-amplified the smad2 or smad4 open reading frames from HMEC cDNA and then cloned them into the pBabe retroviral vector. D. Ayer generously provided the GFP-pBabe vector (University of Utah, Salt Lake City, UT).

Luciferase Assays—Fugene 6 (Roche Applied Science) was used to transfect HaCaT cells as instructed by the manufacturer. We seeded 20,000 cells in 24-well plates and transduced them the next day. Transfections were performed using 0.56 μg of DNA (including either 0.1 μg of normalization vector and 0.5 μg of reporter vector or 0.1 μg of normalization vector, 0.2 μg of reporter vector, and 0.3 μg of expression vector) and harvested 20 h after the start of transfection. For TGFβ treatment, medium containing either TGFβ (200 pg) or an equal volume of vehicle was added to cells 8 h after the start of transfection. Luciferase activities were analyzed using a dual luciferase assay system (Promega). Dividing the firefly luciferase activity from each well by the Renilla luciferase activity from the same well normalized transcription efficiencies. Data in each experiment are presented as the mean ± S.D. of triplicates from a representative experiment. Each experiment was performed at least three times, producing qualitatively similar results.

Retroviral Transduction—Expression of the GFP, SMAD3, or SMAD4 retroviral constructs was verified by Western blot in a transient assay prior to virus production. To produce the retrovirus, Phoenix helper cells were seeded in 60-mm2 plates 24 h prior to transfection. The virus was transduced to MCFAMINE 2000/10-cm2 plate according to manufacturer's instructions. For the SMAD silenced (Invitrogen) for the ensembl-transport RNA or protein was isolated 40–48 h after transfection. In time course experiments, we found that maximal silencing occurred 36 h after transfection for all three SMAD proteins (data not shown). For Gadd45β silencing, 3 h after the start of Gadd45β siRNA transfection, cells were treated with vehicle or TGFβ for an additional 2 h prior to RNA isolation or 12 h prior to flow cytometry.

Quantitative Reverse Transcription-PCR—Trizol (Invitrogen) was used to isolate the total RNA from the HT29 and JAR retroviral poly-
clonal stables according to the manufacturer’s guidelines. cDNA was synthesized from 2 μg of total RNA using Superscript III (Invitrogen). Real time PCR was performed using the Roche Light Cycler instrument and software, version 3.5 (Roche Applied Science). Intron-spanning primers (Gadd45α, forward (5’-CGGTGAGGAGGTCCCTTGGT-G-3’) and reverse (5’-CACCAGCTGAGTTTGAGTG-3’); 18 S rRNA, forward (5’-GTTGAATCTTCTGACCGGC-3’) and reverse (5’-GACTTGGTTTCCCGGAAGC-3’)) were designed to amplify 200-bp products in order to minimize contamination from genomic DNA. PCR was performed in duplicate (or triplicate for 18 S rRNA) with a master mix consisting of cDNA template, buffer (500 mM Tris, pH 8.3, 2.5 mM MgCl2, 30 mM MgCl2), dNTPs (2 μM), TaqStart antibody (Clontech), Biolase DNA polymerase (Bioline), gene-specific forward and reverse primers (10 μM), and SYBR Green I (Molecular Probes, Inc., Eugene, OR). The PCR conditions are as follows: 5 cycles of amplification with 1 s denaturation at 95 °C and 5 s annealing at 57 °C for Gadd45α and 53 °C for 18 S rRNA. A template-free negative control was included in each experiment. We determined the copy number by comparing gene amplification with the amplification of standard samples that contained 103 to 106 copies of the gene or 103 to 106 for 18 S rRNA. The relative expression level of each gene was calculated by averaging the replicates and then dividing the average copy number of Gadd45α by the average copy number of 18 S rRNA. S.E. of the ratios was calculated using a confidence interval.

**RESULTS**

**gadd45α** is a Primary TGFβ-responsive Gene in Normal Human Mammary Epithelial Cells—TGFβ induces a G1 cell cycle arrest and epithelial to mesenchymal transition, but not apoptosis, in primary normal human mammary epithelial cells grown in culture (15) (data not shown). To understand the mechanisms behind these TGFβ-induced phenotypes, we partially defined the TGFβ transcriptome in normal human mammary epithelial cells (HMEC). Specifically, we used cDNA microarray expression analysis to determine the relative expression of 7000 genes at 2 and 12 h after TGFβ treatment in HMEC. Data analysis revealed 54 up-regulated and 10 down-regulated TGFβ-regulated genes. Genes included in this list had a fold change of greater than 1.3 or less 0.7 at both time points and a p value of less than 0.05 at both time points (Supplemental Table 1 and methods therein). Next, we identified genes within this data set that were in common to TGFβ-regulated genes identified through transcriptional profiling in other TGFβ-responsive cell systems. We surmised that because genes in this subgroup were regulated by TGFβ irrespective of cell origin or transformation status, they would be of central importance to the TGFβ cyostatic program. Plasminogen activator inhibitor-1 (PAI1) is a well established TGFβ-induced gene and was induced 8-fold 2 h after TGFβ treatment in HMEC (Supplemental Table 1). Consequently, PAI1 served as an important positive control in the microarray, in Northern blotting and serves as the loading control. B, randomly cycling HMEC were treated with either TGFβ (200 pM) or an equal volume of vehicle. At the indicated time, total RNA was isolated from the cells. Following mRNA purification, Northern blot analysis was performed to visualize the relative transcript abundance of the indicated genes. Both the 3.2- and 2.2-kb alternatively spliced forms of the mature PAI1 mRNA are shown. C, HMEC were treated with TGFβ (200 pM) or BMP-2 (4 nM) for the indicated times before RNA isolation and Northern blot analysis for the gadd45α transcript. GAPDH serves as a loading control. The gadd45α and GAPDH signals were quantitated using a PhosphorImager, and the resulting gadd45α/GAPDH ratio was plotted below the Northern blots.
eloid, breast, skin, breast, pancreas, and bone (4, 5, 11, 16). Because of its frequent presence in the TGFβ transcriptional response and because of its previously described role in growth arrest, differentiation, and apoptosis, we chose to characterize the upstream signal transduction pathway necessary for gadd45β transcriptional induction and, second, to examine the role of GADD45β in the TGFβ response.

We first determined the scope of gadd45β transcriptional activation. Specifically, we monitored its induction by TGFβ in several cell lines and by other members of the TGFβ superfamily in HMEC. To determine whether other TGFβ-responsive cell lines responded similarly to HMEC with respect to gadd45β transcription, several cell lines were treated with TGFβ or vehicle for 1 h. The gadd45β and PAI1 transcripts were induced by TGFβ in the following cell lines: HMEC, HaCaT, Mv1Lu, PANC-1, primary breast organoid outgrowths, and to a lesser extent in HepG2 and HeLa cells (data not shown) (Fig. 1A). The Madin Darby canine kidney and 293 cell lines did not respond to TGFβ stimulation by inducing either gadd45β or PAI1. TGFβ treatment of 10T1/2 murine fibroblasts caused a moderate increase in PAI1 transcription but did not affect gadd45β mRNA levels. We also asked whether other members of the TGFβ superfamily of growth factors could regulate gadd45β transcription. Fig. 1C illustrates that both TGFβ and BMP2 induced gadd45β transcription. However, the kinetics of gadd45β induction as well as the strength of induction differed between the two ligands. Finally, of the three genes that comprise the gadd45 family, only gadd45β was found to be TGFβ-inducible in HMEC; gadd45α was not affected by TGFβ treatment, and Gadd45β was not detected (Fig. 1B). Gadd153/Chop10, a GADD family member by virtue of its induction by cellular stress, was transiently repressed by TGFβ.

To distinguish whether TGFβ treatment resulted in increased gadd45β transcription or increased gadd45β mRNA stability, we measured the gadd45β mRNA half-life before and after TGFβ treatment. HMEC were treated with TGFβ for 1 h before the addition of the transcription inhibitor actinomycin D for various periods of time. Quantitative analysis of the Northern blot revealed that TGFβ failed to stabilize the gadd45β mRNA (Fig. 2, A and B). The accumulation of gadd45β mRNA within 2 h of TGFβ treatment suggested that it is an immediate early TGFβ-induced target gene. To test this idea, we pretreated HMEC with the protein translation inhibitor cyclohexamide 15 min before a 3-h combined TGFβ/cyclohexamide treatment. We found that the levels of gadd45β increased in a TGFβ-dependent manner irrespective of cyclohexamide pretreatment, indicating that new protein synthesis is not required for TGFβ induction of gadd45β (Fig. 2C). These data indicate that gadd45β is a direct TGFβ transcriptional target.

*gadd45β Is Partly Dependent upon SMAD3 and Independent of SMAD2 in Its Regulation by TGFβ.*—We first sought to determine whether specific inhibition of SMAD2, SMAD3, and SMAD4 abrogated gadd45β responsiveness to TGFβ. To approach this, we employed siRNA-mediated silencing of the SMAD2, SMAD3, and SMAD4 proteins. Because we were unable to achieve silencing greater than 60% of wild-type levels in HMEC, we chose to use Mv1Lu cells for our siRNA studies. Transfection of Mv1Lu cells with siRNAs specific to SMAD2 or SMAD3 reduced the respective protein expression to nearly undetectable levels (Fig. 3A). Loss of SMAD2 caused a 70% decrease in the induction of PAI1 by TGFβ. In contrast, siRNA silencing of SMAD2 had no significant effect on gadd45β induction following TGFβ treatment (Fig. 3A). SMAD3-deficient cells, however, responded to TGFβ stimulation with reduced levels of induction for both gadd45β and PAI1. Although the decrease in PAI1 induction by TGFβ observed in the SMAD2 and the SMAD3 single-knockout cells was enhanced in the double-knockout cells, the SMAD2/SMAD3 double-knockout cells behaved similarly to SMAD3-deficient cells with respect to gadd45β induction (Fig. 3A). Dose-response curves with the Smad3 siRNA (IC50 = 1 nM) further demonstrated that TGFβ activates PAI1 and gadd45β through a mechanism that is partly dependent upon SMAD3 (Fig. 3B).

**SMAD4 Silencing Prevents gadd45β and PAI1 Induction by TGFβ.—**Of the many proteins involved in mediating the different facets of TGFβ signal transduction, SMAD4 is considered central to many of the responses. Two different siRNAs were designed against mink smad4, and the efficacy of their silencing was tested in Mv1Lu cells by Western blot (Fig. 4A). Consistent with the central role of SMAD4 in TGFβ signaling, siRNA silencing of SMAD4 resulted in a dramatic loss of gadd45β transcriptional induction following TGFβ treatment (Fig. 4B). As a confirmation of specificity, a human-specific SMAD4 siRNA, which contains mismatches at two positions relative to the mink sequence, did not affect SMAD4 protein expression or TGFβ-regulated transcription of gadd45β or PAI1. siSmad4-A and siSmad4-B both robustly silenced SMAD4 protein expression and did not interfere with SMAD3 protein expression (Fig. 4A). Examination of the gadd45β and PAI1 transcript levels in these SMAD4-deficient cells revealed a clear necessity for SMAD4 in targeting these genes for tran-
scription following TGFβ stimulation (Fig. 4B). The small difference between siSmad4-A and siSmad4-B in silencing SMAD4 protein expression directly reflected the levels of gadd45β and PAI1 induction by TGFβ. The siSmad4-A silences SMAD4 protein expression with an IC_{50} of less than 1 nM, which is consistent with the IC_{50} of silencing imparted by siSmad3 (compare Fig. 4C with Fig. 3B). The induction of gadd45β and PAI1 transcripts by TGFβ in these cells showed close correlation with each other and with the levels of SMAD4 protein (Fig. 4C).

Finally, we asked whether Mv1Lu cells lacking SMAD2, SMAD3, and SMAD4 responded differently to TGFβ with respect to gadd45β transcriptional induction than cells deficient in only one or two of the SMADs. Mv1Lu cells were transfected with siRNAs directed against each of the SMADs alone and in all combinations thereof (Fig. 5A). Northern blot analysis of
gadd45β again demonstrated a SMAD3 and SMAD4 dependence for TGFβ-induced transcription. Loss of SMAD2 in these SMAD3/SMAD4-deficient cells had no further effect on gadd45β induction. Interestingly, although PAI1 depends partly upon SMAD2 for TGFβ-induced transcription (Fig. 3A), loss of SMAD2 did not affect PAI1 induction in cells lacking SMAD3 and SMAD4 (Fig. 5B).

Our data generated with siRNA-mediated silencing have revealed no differences between gadd45β and PAI1 with respect to their regulation by SMAD3 and SMAD4 (Figs. 3 and 4). To examine the role of SMAD3 and SMAD4 more closely, we asked whether loss of SMAD3 in a SMAD4-reduced background would further inhibit gadd45β and PAI1 induction by TGFβ. Mv1Lu cells were transfected with a constant amount of siSmad4-A (15 nM) in the presence of an increasing concentration of siSmad3 (at 0.01, 0.1, 1.0, 10.0, and 15.0 nM) for 40 h prior to TGFβ treatment for 1 h. As before, the RNA and protein were harvested in parallel for Northern and Western blot analysis, respectively. D, the PAI1 and gadd45β Northern blots seen in Fig. 5C were quantitated by PhosphorImager analysis and normalized to the -fold induction observed in Mv1Lu cells transfected with a scrambled siRNA (-fold induction in lane 1 versus lane 2).

**gadd45β Enhancer Mediates TGFβ Induction**

**FIG. 5.** SMAD3 silencing in SMAD4-deficient cells represses gadd45β transcriptional induction by TGFβ. A, Mv1Lu cells were transfected with the indicated siRNA at 15 nM for 40 h. The siSmad4-A siRNA was used to silence Smad4 expression. Total cellular protein was harvested and analyzed by Western blot for the indicated proteins. B, RNA was harvested from either vehicle or TGFβ-treated Mv1Lu cells transfected with the indicated siRNAs (15 nM) in parallel to the protein seen in Fig. 5A. The gadd45β and PAI1 transcripts were detected by Northern blot. A graphical representation of the quantitated gadd45β and PAI1 Northern blots is shown below. For both bar graphs, all values were normalized to the transcript expression level in the siScr- and vehicle-treated sample. The 18S ribosomal band serves as a loading control. C, Mv1Lu cells were transfected with either the scrambled siRNA (15 nM) alone or the Smad4-A siRNA (15 nM) in the presence of increasing concentrations of Smad3 siRNA (at 0.01, 0.1, 1.0, 10.0, and 15.0 nM) for 40 h prior to TGFβ treatment for 1 h. As before, the RNA and protein were harvested in parallel for Northern and Western blot analysis, respectively. D, the PAI1 and gadd45β Northern blots seen in Fig. 5C were quantitated by PhosphorImager analysis and normalized to the -fold induction observed in Mv1Lu cells transfected with a scrambled siRNA (-fold induction in lane 1 versus lane 2).

SMAD3 and SMAD4 Expression in SMAD3- and SMAD4-null Cancer Cells Reconstitutes TGFβ-mediated Induction of gadd45β—The second approach we utilized to study the transcriptional regulation of gadd45β by TGFβ relied upon the preponderance of inactivating mutations within the SMAD proteins in human cancer cell lines. HT29 colon adenocarcinoma cells do not express SMAD4 protein because of a nonsense mutation that renders the transcript unstable (17). JAR cells, on the other hand, do not express SMAD3 (18). TGFβ treatment of these cell lines results in the phosphorylation of SMAD2, indicating that both cell lines express functional TGFβ receptor complexes and that SMAD2 phosphorylation is not dependent upon SMAD3 or SMAD4 (Fig. 6A). Retroviral transduction followed by polyclonal selection of these cells with either a GFP-encoding retrovirus or a SMAD3- or SMAD4-encoding virus provided an experimental approach to further examine the role of the SMADs in gadd45β transcription. Two weeks after the transduced cells were placed under selection, expression of the transduced genes was verified by fluorescence microscopy (for GFP expression; data not shown) and Western blot (Fig. 6B). RNA harvested in parallel to the protein samples analyzed in Fig. 6B was reverse transcribed and used in real time quantitative PCR to measure the gadd45β transcript levels. TGFβ treatment of JAR-SMAD3 cells revealed a small but statistically significant increase in gadd45β message levels (Fig. 6C). The SMAD4-HT29 cells responded to TGFβ through
a robust induction of gadd45β (Fig. 6C). Northern blot analysis of these RNAs confirmed the quantitative PCR results (data not shown).

**gadd45β Contains a TGFβ-responsive Enhancer That Encompasses the Third Intron**—Next, we analyzed the gadd45β genomic locus for transcriptional responsiveness to TGFβ. First, 1500 bp of the proximal promoter of gadd45β was cloned upstream of firefly luciferase for use in reporter assays (G45β-1) (Fig. 7A). TGFβ stimulation of HaCaT or Mv1Lu cells increased the transcriptional activity of G45β-1, G45β-2, and G45β-3–2-fold (Fig. 7B) (Mv1Lu data not shown). In contrast, the endogenous gadd45β transcript levels increased 8–15-fold in responsive cell lines following TGFβ treatment (Fig. 1A). We were unable to see increased reporter activity when other portions of this 5′-flanking region were analyzed or in numerous other cell lines or when the cells were treated for different lengths of time with TGFβ (Fig. 7A) (data not shown). We reasoned that because the gadd45β coding sequence is highly conserved between human, mouse, rat, and zebrafish, the region of the genomic locus mediating TGFβ responsiveness might also be conserved. To address this possibility, we aligned the human, mouse, and rat gadd45β genomic sequences and plotted the degree of conservation utilizing the MAVID algorithm (13, 14). In addition to the coding regions, three domains of the gadd45β genomic locus demonstrate high conservation between species. Each of these regions was cloned upstream of Firefly luciferase and used in reporter experiments (Fig. 7A). Remarkably, TGFβ robustly activated transcription from the G45β-C enhancer, which contains part of the third exon, the complete third intron, and part of the fourth exon, but not from G45β-A or G45β-B (Fig. 7B, Supplemental Fig. 1) (Mv1Lu data not shown). We took two approaches to test if SMAD proteins were mediating TGFβ-dependent transcriptional induction off of G45β-C. First, the inhibitory SMAD7 protein was overexpressed to block SMAD activation. SMAD7 overexpression inhibited TGFβ-induced activation of the 3TPLux reporter, which contains the PAI1 promoter, and the G45β-C reporter, but did not affect an SV40-driven luciferase construct (Fig. 7C). Second, overexpression of SMAD3 and SMAD4 greatly enhanced G45β-C reporter activity in HaCaT cells (Fig. 7C) and in HeLa cells (data not shown). In contrast, SMAD2 expression did not affect the transcriptional activity. Interestingly, the increase in reporter activity was dependent upon both SMAD3 and SMAD4, because neither one alone significantly affected the G45β-C transcriptional activity. These data support a role for SMAD3 and SMAD4 in regulating Gadd45β transcription through a 3′ enhancer that contains the third intron. Indeed, sequence analysis of G45β-C revealed four conserved putative SMAD binding elements (SBEs) (Supplementary Fig. 1).

**GADD45β Regulates G2 Progression following TGFβ Stimulation**—To examine the contribution of GADD45β to the TGFβ phenotype, a siRNA was designed to silence gadd45β expression. Dose-response analysis revealed potent (IC50 ~ 1 nM) and specific knockout of TGFβ-induced gadd45β expression (Fig. 8A). TGFβ rapidly induces a G1 cell cycle arrest, but not apoptosis, in Mv1Lu cells. We asked whether Mv1Lu cells deficient in GADD45β would undergo a G1 cell cycle arrest. Introduction of a scrambled siRNA had no detectable effect on TGFβ-induced cell cycle arrest (Fig. 8B). However, cells containing reduced levels of gadd45β demonstrated a slight reduction in G1 accumulation and failed to progress through G2 following TGFβ treatment (Fig. 8B). Loss of the gadd45β transcript did not affect cell cycle progression in the absence of TGFβ treatment. Dose-response analysis further verified this finding: 0.01 and 0.1 nM siRNA did not significantly affect gadd45β transcript levels or cell cycle progression following TGFβ stimulation. These findings indicate that GADD45β is an important regulator of cell cycle progression following TGFβ treatment.

**DISCUSSION**

The intracellular domain of a ligand-bound TGFβ receptor complex ignites an intertwined cascade of signaling events that induces one of many possible phenotypic responses (1, 2). Consequently, the mechanism by which a cell decides how to respond to TGFβ is fundamental to many aspects of eukaryotic life. One approach to decipher the cellular interpretation of the TGFβ signal and how that interpretation might be altered in a diseased tissue is to define and utilize the TGFβ target genes as a starting point in a retrograde molecular characterization of the upstream transcriptional program. Concurrent studies would assess the gene function as it contributes to the phenotypic response. We have employed this approach to the gadd45β gene. We found that gadd45β transcriptional induction by TGFβ was dependent upon SMAD4 and to a lesser extent on SMAD3 but independent of SMAD2. Further, SMAD3 and SMAD4 mediated the transcriptional induction of gadd45β through an enhancer that encompasses the third intron of the gadd45β gene. Finally, TGFβ stimulation of...
**A.**

**gadd45β Enhancer Mediates TGFβ Induction**

**FIG. 7.** *gadd45β* is activated by TGFβ through a 3′ enhancer. A, schematic representation of the *gadd45β* genomic locus. The relative position of the exons and introns are indicated above the graph. The MAVID algorithm was used to determine the relative degree of sequence conservation between human *gadd45β* (x axis) and rat *gadd45β* (bottom half of plot; y axis) and murine *gadd45β* (top half of plot; y axis). The degree of genomic conservation is indicated by the height of the black curve. Below the graph is a schematic diagram of six pieces of the *gadd45β* genomic locus that were cloned upstream of firefly luciferase for use in subsequent reporter assays. Of note, G45β-C contains 93 bp of exon 3, the complete third intron (237 bp), and 98 bp of exon 4 of *gadd45β* (Supplementary Fig. 1). B, HaCaT cells were transfected with the indicated reporter construct for 24 h in the presence or absence of TGFβ (200 pM) before firefly luciferase values were quantitated, normalized, and plotted as -fold induction by TGFβ. Qualitatively similar results were obtained with Mv1Lu cells (data not shown). C, reporter assay in HaCaT cells that were transiently co-transfected with an empty vector (Vector) or a SMAD7 expression vector and the indicated luciferase reporter construct. TGFβ or vehicle control was added 4 h after the transfection began. Luciferase values were read 24 h after the TGFβ was administered. D, HaCaT cells co-transfected with G45β-C and the indicated SMAD expression construct in the presence (black bars) or absence (open bars) of TGFβ. All firefly luciferase values were normalized to *Renilla* luciferase before data analysis. The error bars represent the S.D. values from three independent experiments. All luciferase reporter experiments were repeated a minimum of five times (each time in triplicate) and produced qualitatively identical results.
**Fig. 8. gadd45g regulates cell cycle progression following TGFβ treatment.** A, Mv1Lu cells were transfected with a siScr or a gadd45g-specific siRNA (siGadd45g) at the indicated concentration. Three hours after the start of transfection, the cells received an equal volume of vehicle or TGFβ (200 pm). TGFβ treatment lasted 2 h, at which point RNA was extracted and analyzed for the gadd45g or GAPDH transcript by Northern blot. Quantitative representation of the GAPDH-normalized gadd45g Northern blot is plotted below. B, Mv1Lu cells were transfected with gadd45g siRNA at the indicated concentration for 3 h prior to vehicle or TGFβ (200 pm) treatment for an additional 12 h. Samples were then harvested for flow cytometry analysis. The error bars represent the S.D. across three independent experiments.

gadd45g-deficient cells, but not of gadd45g-expressing cells, resulted in the activation of a G2/M checkpoint.

We used RNA interference as a tool to probe the upstream signal transduction components necessary for gadd45g and PAI1 transcriptional induction following TGFβ stimulation. We chose Mv1Lu cells as a cell system for these studies rather than a human cell line such as HMEC or HaCaTs, because we found that in these cells our siRNAs were more efficacious as compared with a panel of TGFβ-responsive human cells. In fact, silencing SMAD protein expression in HaCaTs cells to 30% of wild-type levels resulted in no detectable effect on PAI1 or gadd45g transcription following TGFβ treatment. These results and our findings presented in Fig. 4 argue that with respect to the transcriptional activation of gadd45g and PAI1, the SMAD proteins are expressed in excess. Coupled to the immediate early transcriptional induction of gadd45g and PAI1 by TGFβ, these data suggest that the gadd45g and PAI1 promoters share a relatively high affinity for the SMAD proteins. Further, this provides a possible molecular mechanism explaining how gadd45g and PAI1 are regulated by TGFβ irrespective of tissue type. Analogous findings have recently been discovered in Caenorhabditis elegans where the FoxA protein, PHA4, achieves transcriptional discrimination among target genes through a differential affinity to gene promoter sequences (19). Consequently, high affinity PHA4 promoters are responsive to relatively low levels of PHA4 protein expression. Further studies are in progress to classify TGFβ transcriptional targets by their sensitivity to changes in SMAD protein expression.

With the exception of a few genes, such as p15 (20) and MMP2 (21), most well characterized immediate early TGFβ-regulated genes appear to depend upon SMAD3 and SMAD4, but not SMAD2, for TGFβ transcriptional regulation. Our work places gadd45g within this SMAD2-independent, SMAD3/SMAD4-dependent class of TGFβ-responsive genes. Our conclusion that gadd45g is a SMAD4-dependent TGFβ target gene agrees with the findings of Yoo et al. (11) and Takekewa et al. (5), who have also reported SMAD4 dependence in gadd45g regulation, although through different experimental approaches. Conversely, our findings that TGFβ regulated gadd45g independently of SMAD2 contradict previous findings. Yoo et al. (11) recently reported that overexpression of SMAD2 and SMAD4 together, but not separately, induced a gadd45g reporter construct in a TGFβ-dependent fashion. Although additional work is necessary to reconcile these outcomes, they could result from cell type-specific responses to TGFβ (hepatocytes versus keratinocytes and fibroblasts). It is important to note, however, that SMAD2-deficient fibroblasts show gadd45g transcriptional induction following TGFβ with kinetics and efficacy similar to that of wild-type cells, an observation that is consistent with a SMAD2-independent model of gadd45g regulation (22).

In contrast to gadd45g, we found that SMAD2, SMAD3, and SMAD4 all contributed to TGFβ regulation of PAI1, although to varying degrees (Figs. 3–5). Extensive research on PAI1 has not implicated SMAD2 (23–25) in its regulation with the notable exception that fibroblasts derived from smad2 knockout mice failed to induce PAI1 following TGFβ treatment (21). The ability of smad2 siRNAs to phenocopy the Smad2 knockout fibroblasts in this respect strongly supports the use of siRNA-mediated gene silencing in future TGFβ transcriptional studies. Clearly, genome-wide analysis of TGFβ responsiveness in SMAD-silenced or SMAD knockout cells will be of great importance.

Yoo et al. (11) have recently shown that 220 bp of the gadd45g proximal promoter is activated by TGFβ and that this activation is enhanced by overexpression of SMAD2, SMAD3, and SMAD4, but not dominant negative forms of SMAD2 or SMAD3. Our data support their results in that we have also found the 5′ promoter sequence to be TGFβ-responsive (Fig. 7B). However, through a genomics-based alignment strategy, we identified a second TGFβ-responsive domain encompassing the highly conserved third intron of the gadd45g gene. In contrast to the 2-fold activation we observed with 5′ promoter sequences, the 3′ enhancer is activated 5−7-fold following TGFβ treatment. It will be important to determine which of the conserved transcription factor binding sites within this enhancer account for induction by TGFβ. Notably, we identified four conserved SMAD binding elements, three of which are located in exonic sequence (Supplementary Fig. 1). The endogenous gadd45g gene may likely respond to TGFβ through a concerted action of the 3′ enhancer and 5′ promoter sequences. A similar transcriptional model has been reported for the gadd45a gene where highly conserved sequences within the third intron or fourth exon facilitate transcriptional induction following genotoxic stress (26, 27) and vitamin D3 (28), respectively. Thus, in addition to primary sequence and genomic organization, gadd45a, gadd45b, and gadd45γ might also

---

2 M. B. Major and D. A. Jones, unpublished results.
share an intronic/exonic enhancer as an important transcriptional regulatory element.

Last, utilizing the power of siRNA mediated gene silencing, we discovered that Mv1Lu cells made deficient for gadd45β arrested at the G2/M checkpoint following TGFβ treatment. Previous research has established GADD45β as a negative regulator of cell cycle progression, and several molecular mechanisms behind this inhibition have been put forth (3). Following genotoxic stress, GADD45β acts to inhibit Cdc2/cyclin B1 kinase to induce a G2/M cell cycle checkpoint in RKO lung carcinoma cells (8). In contrast, normal fibroblasts microinjected with a GADD45β expression vector fail to undergo a G2/M arrest, although GADD45β was found to associate with Cdc2 in these cells (29). Our findings support these previous data in that we also see a GADD45β/H9252-dependent cellular response to GADD45β expression vector fail to undergo a G2/M checkpoint following TGFβ treatment.

Perhaps the most well understood function of the GADD45 family of proteins is their ability to regulate apoptosis through the activation of MTK1 (MEKK4) (32) and subsequently p38 kinase (5, 10). Although gadd45β is rapidly induced by TGFβ in Mv1Lu and HMEC, we have not detected an apoptotic response following TGFβ treatment in these cells. An apoptosis-independent cellular response to GADD45β induction was recently shown, where tumor necrosis factor-α signaling through NF-κB induced gadd45β transcription to prevent c-Jun N-terminal kinase activation and cell death (33, 34). Further, several research laboratories have been successful in generating gadd45β overexpression systems and have not observed cell death (35). Future studies utilizing siRNA silencing of gadd45β following transcriptional agonists other than TGFβ will be invaluable in determining the functional consequences of GADD45β expression.

REFERENCES
1. Massague, J. (2000) Nat. Rev. Mol. Cell. Biol. 1, 169–178
2. Shi, Y., and Massague, J. (2000) Cell 113, 685–700
3. Liebermann, D. A., and Hoffman, B. (2002) Leukemia 16, 597–541
4. Zhang, W., Bae, I., Krishnaraju, K., Azam, N., Fan, W., Smith, K., Hoffman, B., and Liebermann, D. A. (1999) Oncogene 18, 4899–4907
5. Takekawa, M., Tatebayashi, K., Itoh, F., Adachi, M., Imai, K., and Saito, H. (2002) EMBO J. 21, 6473–6482
6. Selvakumaran, M., Lin, H. K., Sjin, R. T., Reed, J. C., Liebermann, D. A., and Hoffman, B. (1994) Mol. Cell. Biol. 14, 2352–2360
7. Abdelali, A., Lord, K. A., Hoffman-Liebermann, B., and Liebermann, D. A. (1991) Oncogene 6, 165–167
8. Vajrabapu, M., Balliet, A., Hoffman, B., and Liebermann, D. A. (2002) J. Cell. Biol. 192, 327–338
9. Zhan, Q., Lord, K. A., Alamo, I., Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A., and Fornace, A. J., Jr. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2361–2367
10. Takekawa, M., and Saito, H. (1998) Cell 95, 521–530
11. Yoo, J., Ghiassi, M., Jirmanova, L., Balliet, A. G., Hoffman, B., Fornace, A. J., Jr., Liebermann, D. A., Bottinger, E. P., and Roberts, A. B. (2003) J. Biol. Chem. 278, 43001–43007
12. Elbashir, S. M., Harborth, J., Weber, K., and Tuschl, T. (2002) Methods 26, 199–213
13. Bray, N., and Pachter, L. (2003) Nucleic Acids Res. 31, 3525–3526
14. Bray, N., Dubchak, I., and Pachter, L. (2003) Genome Res. 13, 97–102
15. Hosobuchi, M., and Stamps, M. H. (1989) In Vitro Cell Dev. Biol. 25, 751–713
16. Zawel, L., Yu, J., Torrance, C. J., Markowitz, S., Kinzler, K. W., Vogelstein, B., and Zhou, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2848–2853
17. Woodford-Richens, K. L., Rowan, A. J., Gorman, P., Halford, S., Bicknell, D. C., Wason, H. S., Roylande, B. R., Bedmer, W. F., and Tomlinson, I. P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9719–9723
18. Xu, G., Chakraborty, C., and Lala, P. K. (2002) Biochem. Biophys. Res. Commun. 294, 1079–1086
19. Gaudet, J., and Mange, S. E. (2002) Science 295, 821–825
20. Feng, X. H., Lin, X., and Derynck, R. (2000) EMBO J. 19, 5178–5193
21. Piek, E., Ju, W. J., Heyer, J., Escalante-Alcalde, D., Stewart, C. L., Weinstein, M. A., Deng, C., Kucherlapati, R., Bottinger, E. P., and Roberts, A. B. (2001) J. Biol. Chem. 276, 19945–19953
22. Yang, Y. C., Piek, E., Zavadil, J., Liang, D., Xie, D., Heyer, J., Pavlidis, P., Kucherlapati, R., Roberts, A. B., and Bottinger, E. P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10269–10274
23. Denuiller, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (2000) EMBO J. 19, 3599–3609
24. Song, C. Z., Siok, T. E., and Gelehrter, T. D. (1998) J. Biol. Chem. 273, 29287–29290
25. Strickstein, S. L., Wang, W., and Luo, K. (1999) J. Biol. Chem. 274, 9431–9441
26. Huang, M. C., Alamo, I., Jr., Liebermann, D. A., Bottinger, E. P., Wang, M. G., McBride, O. W., and Fornace, A. J., Jr. (1993) J. Biol. Chem. 268, 24385–24393
27. Chin, P. L., Momand, J., and Pfeifer, G. P. (1997) Oncogene 15, 87–99
28. Jiang, P., Li, P., Fornace, A. J., Jr., Nicotera, S. V., and Bai, W. (2000) J. Biol. Chem. 275, 48030–48040
29. Yang, Q., Manicone, A., Courson, D. J., Linke, S. P., Nagashima, M., Forgues, M., and Wang, X. W. (2000) J. Biol. Chem. 275, 36892–36898
30. Kovalsky, O., Lang, F. D., Roller, P. P., and Fornace, A. J., Jr. (2001) J. Biol. Chem. 276, 39330–39339
31. Vairapandi, M., Azam, N., Balliet, A. G., Hoffman, B., and Liebermann, D. A. (2000) J. Biol. Chem. 275, 16810–16813
32. Mita, H., Tsutsui, J., Takekawa, M., Witten, E. A., and Saito, H. (2002) Mol. Cell. Biol. 22, 4544–4555
33. Jin, R., De Smaele, E., Zazzaroni, F., Nguyen, D. U., Papa, S., Jones, J., Cox, C., Gelinas, C., and Frangos, G. (2002) DNA Cell Biol. 21, 491–503
34. De Smaele, E., Zazzaroni, F., Papa, S., Nguyen, D. U., Jin, R., Jones, J., Cong, R., and Frangos, G. (2001) Nature 414, 308–313
35. Yang, J., Zhu, H., Murphy, T. L., Ouyang, W., and Murphy, K. M. (2001) Nat. Immunol. 2, 157–164
36. Karpf, A. R., Peterson, P. W., Rawlins, J. T., Dalley, B. K., Yang, Q., Albertsen, H., and Jones, D. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14007–14012
37. Miller, G. S., and Fuchs, R. (1997) Comput. Appl. Biochem. 13, 81–87