The transcription factors Smad2 and Smad3 mediate a large set of gene responses induced by the cytokine transforming growth factor β (TGFβ), but the extent to which their function depends on chromatin remodeling remains to be defined. We observed interactions between these two Smads and BRG1, BAF250b, BAF170, and BAF155, which are core components of the SWI/SNF chromatin-remodeling complex. Smad2 and Smad3 have similar affinity for these components in vitro, and their interactions are primarily mediated by BRG1. In vivo, however, BRG1 predominantly interacts with Smad3, and this interaction is enhanced by TGFβ stimulation. Our results suggest that BRG1 is incorporated into transcriptional complexes that are formed by activated Smads in the nucleus, on target promoters. Using BRG1-deficient cell systems, we defined the BRG1 dependence of the TGFβ transcriptional program genomewide. Most TGFβ gene responses in human epithelial cells are dependent on BRG1 function. Remarkably, BRG1 is not required for the TGFβ-mediated induction of SMAD7 and SNON, which encode key mediators of negative feedback in this pathway. Our results provide a genome-wide scope of the participation of BRG1 in TGFβ action and suggest a widespread yet differential involvement of BRG1/SNF remodeler in the transcriptional response of many genes to this cytokine.

Genomic DNA is packed into chromatin units or nucleosomes whose accessibility determines the ability of particular genes to respond to regulatory inputs (1–3). Various classes of ATP-dependent chromatin remodeling factors (remodelers) have been identified that control the accessibility of DNA. Remodelers act on histone-DNA contacts to induce nucleosome sliding, DNA looping, or complete dissociation of histones from DNA (4–9). Although the role of remodelers in developmental stages and during the cell cycle has been characterized in some detail, little is known about their role in the genome-wide response to extracellular cytokine signals. Recent identification of interactions between Smad transcription factors and a SWI/SNF remodeler complex (10, 11) provides an opportunity to investigate this question.

SW1/SNF refers to a set of remodeler complexes consisting of up to 12 evolutionarily conserved protein subunits (12–15). Mammalian SWI/SNF complexes are nucleated by either BRG1 or BRM, which are mutually exclusive ATPase subunits that recognize acetylated lysines in histone tails. Other SWI/SNF core subunits include either BAF250 or BAF180 and the shared subunits BAF170, BAF155, BAF60, BAF57, BAF53, actin, and SNF5/INI1 (12, 16). SWI/SNF remodelers have been shown to function in association with developmental factors such as MyoD, NeuroD, and GATA-1; cell cycle checkpoint components such as p53, pRB and BRCA1; and nuclear receptors for androgen, estrogen, or glucocorticoid (17–20). Brm-null mice are viable and show only a subtle phenotype (21), whereas brg1-null mice die early during embryogenesis, and hemizygotes are tumor-prone (22). These phenotypes imply important roles for the BRG1/SNF remodeler complex in development and cancer.

Smad transcription factors are central mediators of many actions of transforming growth factor β (TGFβ)2 and other members of this morphogen and cytokine family (23). TGFβ transduces signals from the plasma membrane by interacting with two receptor subunits or TGFβ receptors type I and type II, which are serine/threonine kinases. On ligand-induced phosphorylation and activation by the type II receptor, the type I receptor phosphorylates Smad2 and Smad3 at C-terminal serine residues, inducing these proteins to accumulate in the nucleus to regulate gene expression (24–26). Activated Smad factors target different genes by forming complexes with other transcription factors. The DNA binding activity of each particular Smad cofactor combination directs such complex to a particular subset of target genes. Specific histone-modifying enzymes such as p300/CBP and others are recruited to these complexes for either transcriptional activation or repression (23, 24). Up to several hundred genes are directly regulated in this manner by TGFβ in any given cell type. The choice of target genes and the ultimate biological consequences of TGFβ stimulation depend on the repertoire of Smad transcriptional partners that are present in the cell, as a function of the context and
the cell type (27, 28). This signaling system controls many aspects of cell division and behavior and is essential for embryonic developmental and tissue homeostasis, and its malfunction underlies many inherited and somatic disorders from congenital malformations to fibrosis, inflammation, and cancer.

The Smad pathway is subject to an intricate set of functional and regulatory inputs through diverse protein-protein interactions. In a recent search for Smad-binding proteins, we noticed an interaction between Smad2 and several SWI/SNF components (10). In vitro this interaction was independent of the activation state of Smad2, sharing this property with the interaction of Smad2 with the histone acetyl-transferase CBP (10). An interaction between Smad2 and BRG1 in cell culture and a requirement for BRG1 in the activation of two TGFβ target genes (lefty and nodal) were recently reported by others (11). These observations suggested that Smad proteins may bring to DNA not only histone acetylating enzymes to relax the chromatin structure but also SWI/SNF complexes to provide chromatin remodeling activity.

The identification of protein interactions between Smad2 and SWI/SNF provided an opportunity to investigate the role of this chromatin remodeling complex in the cellular response to a cytokine. The present work is focused on this question at a genome-wide scale to define the requirement for BRG1 in TGFβ gene responses in human epithelial cells. The results provide a global view of the dependence of the TGFβ transcriptional program on BRG1 SWI/SNF function.

EXPERIMENTAL PROCEDURES

Cell Culture, Antisera, and Plasmids—HaCaT keratinocytes were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. NCI-H522 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. H29 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.02 μg/ml doxycycline, 300 μg/ml G418, and 2 μg/ml puromycin. Cell culture media also contained 100 units/ml penicillin/streptomycin, 2 mM l-glutamine, and 1 μg/ml fungizone.

Antibodies from commercial source include rabbit polyclonal anti-phospho-Smad2 antibody and anti-BAF170 antibody (Cell Signaling), rabbit polyclonal anti-BAF170 antibody (Santa Cruz), mouse monoclonal anti-c-Myc-peroxidase (Roche Applied Sciences), rat monoclonal anti-HA-peroxidase, rabbit polyclonal anti-histone H3-ChIP grade antibody (Bethyl), mouse monoclonal anti-RNA polymerase II antibody (Clone 8WG16) (Upstate Biotechnology Inc.), mouse monoclonal anti-FLAG M2 antibody, agarose-conjugated mouse monoclonal anti-HA clone 7, and anti-FLAG M2 affinity gel were purchased from Sigma. Anti-Smad2/3 antibody has been described previously (29).

Plasmid pCMV5-BRG1-FLAG was generated by digestion of plasmid pFastBac-BRG1 (16) with Sall, and the fragment was inserted into a Sall-digested modified pCMV5 vector (30). pCMV5-BAF170 was created by digestion of plasmid pFastBac-BAF170 with EcoRI and HindIII, and the fragment was inserted into EcoRI/HindIII sites of modified pCMV5 plasmid. To create pCMV5-BAF170-FLAG, the FLAG-tagged C terminus of BAF170 was amplified from its cDNA templates by PCR with primers containing the FLAG peptide coding region, then subsequently digested with PstI/HindIII, and inserted into the PstI/HindIII sites of pCMV5-BAF170. Myc-tagged BRG1 constructs, Myc-BRG1, Myc-BRG1–1–344, Myc-BRG1–342–598, and Myc-BRG1–597–1648 were kindly provided by K. L. Kroll (31). To generate Myc-BRG1–597–1407 and Myc-BRG1–1400–1648, the indicated coding region were amplified from corresponding cDNA templates by PCR, digested, and inserted into the EcoRI/BamHI sites of modified pCMV5 vector containing two copies of HA epitope. For QCXIP-TbRII, the NotI/BamHI fragment of pMEP4-TbRII/HA (32) was cloned into NotI/BamHI sites of pQCXIP (BD Bioscience). QCXIH-BRG1-FLAG was generated by inserting blunted Sall fragment of pCMV5-BRG1-FLAG into the BamHI-blunted site of pQCXIH. All of the PCR-amplified fragments have been verified by sequencing.

Transfection, Immunoprecipitation, and Immunoblotting—Transient transfections for coimmunoprecipitation experiments were carried out using Lipofectamine (Invitrogen), according to the manufacturer’s instructions. The cells were harvested 36 h post-transfection and were lysed by sonication in HNG lysis buffer (0.2% Nonidet P-40, 25 mM HEPES, pH 7.9, 100 mM NaCl, 10% glycerol, 5 mM MgCl₂, 10 mM NaF, 1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 1 tablet of protease inhibitor (Roche Applied Sciences)/10 ml) at 4°C. For the detection of endogenous protein complexes, one 15-cm plate of cells was treated with or without 100 pM TGFβ for 2 h and then lysed by sonication in 1 ml of HNG lysis buffer. The lysates were precleared with normal mouse or rabbit IgG (Upstate Biotechnology Inc.). Cleared supernatants were incubated with the indicated antibodies and protein A beads (Zymed Laboratories Inc.) for 4 h at 4°C. Normal rabbit IgG or mouse IgG were used as negative controls. Immunoprecipitates were resolved by SDS-PAGE gels and were electroblotted onto Immunobilon-P membrane (Millipore). Immunoblot analysis was carried out as described before (30).

RNA Interference—siRNAs targeting human BRG1 were obtained from the Memorial Sloan-Kettering Cancer Center High Throughput Screening Core Facility. The sense strands are: 5′-GGGUACCCUCAGGACAACATT-3′ and 5′-CGACGUAGGAUCUAUUTT-3′. HaCaT cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. The shRNA-expressing H1 retroviral system was kindly provided by R. Agami (Netherlands Cancer Institute). The pRetrosuper-Puro plasmid containing short hairpin against human BRG1 was generated by ligating the annealed oligonucleotides with BglII/HindIII-digested pRetrosuper-Puro vector. The sequences of BRG1 shRNA oligonucleotide pairs are as follows, with 19 nucleotide target sequences underlined: shRNA1-sense, 5′-GATCCCCCGACGTCAGGTACATCATTTTTCAAGAGAATGATGTTACTCGTACGTCGTTT-TTGGAAA-3′; shRNA1-antisense, 5′-AGCTTTTCAAACTTCTTCGTTTACAAAGAGAATGATGTTACTCGTACGTCGTTT-TTGGAAA-3′.
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CGACGTACGATGATCATTCTTCTTGAATGATGTATAC-TGTACGTGGGG-3; shRNA2-sense, 5’-GATCCCCGGGT-ACCCCTGAGAACATTAATGAGGAGATTTGCTTCTGAG-GTGACCTTCTTGGGAAA-3; and shRNA2-antisense, 5’-AGCT-TTTCCAAAAAGGGTACCCTAGAACATCTCTTTGAA-TGGTTGTCTTGGATCCGGG-3’.

Retroviral Infection—H29 packaging cells or amphotropic Phoenix packaging cells were transfected with pRetroSuper or pQCXIP or pQCXIH (BD Bioscience) retroviral vectors using Phoenix packaging cells were transfected with pRetrosuper or pQCXIP or pQCXIH (BD Bioscience) retroviral vectors using Phoenix packaging cells were transfected with pRetrosuper or pQCXIP or pQCXIH (BD Bioscience) retroviral vectors using Phoenix packaging cells were transfected with pRetrosuper or pQCXIP or pQCXIH (BD Bioscience) retroviral vectors using Phoenix packaging cells were transfected with pRetrosuper or pQCXIP or pQCXIH (BD Bioscience) retroviral vectors using Phoenix. Retroviral supernatants were collected 48 h post-transfection, passed through a 0.45-mm filter, concentrated at 18000 rpm for 2 h, and used for overnight infection of cells in the presence of 5 μg/ml of polybrene. The cells were allowed to recover for 24 h in fresh medium and selected with 5 μg/ml puromycin for 48 h or 300 μg/ml hygromycin B for 72 h.

Microarray Analysis—RNA was collected in duplicate and labeled as described previously (27). Each sample was hybridized to an Affymetrix human genome U133A plus 2.0 microarray at the Memorial Sloan-Kettering Cancer Center Genomics Core Laboratory. The hybridization images were preprocessed by the Robust multiarray analysis (33), provided by the Affy package of R programming software. Differentially expressed genes were identified using a Bayes model implemented by the limma package of R (34).

Chromatin Immunoprecipitation—HaCaT cells were grown to 70% confluence, incubated with or without TGFβ (100 pg/ml) for 2 h, and then cross-linked with 1% formaldehyde at 37 °C for 15 min. ChIP was performed by using a ChIP assay kit (Upstate Biotechnology Inc.) as described by manufacturer’s protocol. The antibodies used were anti-smad2/3, anti-BRG1, anti-histone H3, and anti-RNA polymerase II. The primer pairs used to amplify the open reading frame or promoter regions of indicated genes are: for CTGF distal site, 5’-GCTCTCTTCTTTTTTGAG-GAAATGC-3’ (forward) and 5’-CTGGTTTGGTGGCGAAGAGA-TGG-3’ (reverse); for the CTGF SBE region, 5’-GGCGACTGGGAGTGTGCGACGC-3’ (forward) and 5’-GAGGGCTTTTAT-ACGCTCCGGG-3’ (reverse); for the CTGF open reading frame, 5’-CTTACCGATGGAGACAGC-3’ (forward) and 5’-CTTCATCAAGCCAGTACGTTCC-3’ (reverse); for the SMAD7 SBE region, 5’-CCCGTCTCCTCCTCCCTGGTTTCC-3’ (forward) and 5’-CCCTCTGCTGTCCTCGGCTTCC-3’ (reverse); for the SNOV SBE region, 5’-CTCGAGGAGGAGGGAAGGG-3’ (forward) and 5’-TACACACACCTCCTCAGG-3’ (reverse); for the β-ACTIN promoter, 5’-AAACATCTTCCCCTCTTCCTCC-3’ (forward) and 5’-GCCATAAA-GGCAACCTTCGGAAC-3’ (reverse); and for PAI1 SBE region, 5’-CTCTCAGCCAGGAGACAGC-3’ (forward) and 5’-CCACGCGCAACACGCAGG-3’ (reverse).

Quantitative Reverse Transcription-PCR Analysis—qRT-PCR was carried out as described previously (27). Primer sequences are available upon request.

RESULTS

Smad2 and Smad3 Bind a BRG1 SWI/SNF Remodeler Complex—We used affinity purification followed by mass spectrometry to identify proteins that bind to Smad2 and Smad3. Recombinant glutathione S-transferase fusion proteins containing the transcriptional regulatory regions (linker region and MH2 domain, L+MH2) of Smad2 or Smad3 were bound to glutathione S-transferase beads and incubated with human HeLa cell extracts. Coomassie staining of electrophoresis gels containing the eluates from these beads reproducibly revealed a similar pattern of proteins using the Smad2 and Smad3 baits (Fig. 1A). Mass spectrometry analysis of the excised bands identified these proteins as the SWI/SNF complex subunits BRG1, BAF155, BAF170, and BAF250b (also known as OSA2) in both eluates (Fig. 1A). In addition, both eluates contained the histone acetyl transferase CBP, the ErbB2-interacting protein Erbin, and the transcription factors NCoA3 and TIF1γ/TRIM33. All of these proteins were previously observed in affinity purification of Smad2-binding proteins (10). The interactions of Smad2/3 with CBP, Erbin, and TIF1γ have been characterized (10, 23, 35). The Smad3 eluate additionally contained NCoR1 and NCoR2/SMRT, which were previously identified as nuclear receptor corepressors (36, 37) (Fig. 1A). Of these proteins, only TIF1γ was increased in abundance when the experiments were repeated using activated (C-terminal acidic mutant) versions of Smad as baits (data not shown and Ref. 10). These results demonstrate a similar, intrinsic affinity of Smad2 and Smad3 for these SWI/SNF components and additionally identify NCoA3 and NCoR as potentially important partners of Smad transcriptional complexes.

Differential Interaction of BRG1 with Smad2 and Smad3 in Vivo—Focusing on SWI/SNF, we verified its interaction with Smad2/3 in vivo by means of coimmunoprecipitation of the endogenous proteins from HaCaT human keratinocytes (Fig. 1B). We chose this cell line because it is a well established model for the analysis of TGFβ action. The interaction between endogenous Smad2/3 and BRG1 in the cells was increased by TGFβ stimulation. In response to TGFβ, Smad proteins accumulate in the nucleus (25), which is where SWI/SNF components reside (4, 6, 8, 9, 12).

Similar results were obtained by immunoprecipitation from cells overexpressing FLAG epitope-tagged BRG1 and HA-tagged Smad proteins (Fig. 1C). Despite the similar affinity of SWI/SNF for Smad2 and Smad3 in vitro (Fig. 1A), in vivo BRG1 interacted more robustly with Smad3 than with Smad2 (Fig. 1C). In contrast to Smad3, the main isoform of Smad2 lacks DNA binding activity (38). The stronger interaction of BRG1 with Smad3 may therefore reflect the recruitment of BRG1 to Smad transcriptional complexes that are competent to bind to DNA. HA-tagged Smad4 showed a very weak interaction with BRG1, which could be detected only on long exposures of immunoblots (Fig. 1C and data not shown). Smad4 coexpression did not increase the interaction of BRG1 with Smad 2 or 3 (data not shown).

BRG1 and BRM are mutually exclusive ATPases in SWI/SNF complexes, whereas BAF250, BAF170, and BAF155 are core subunits that bind to both ATPases. Because BRG1 but not BRM was identified among the Smad2/3-binding proteins (Fig. 1A), we postulated that BRG1 might be the main Smad-binding component in SWI/SNF complexes. Consistent with this possibility, BRG1 interacted with Smad3 more strongly than did BAF170 (Fig. 1D). BRG1 has four conserved domains, including a proline-rich domain (domain I), a conserved domain II, a DNA-
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FIGURE 1. Interaction of Smad2 and Smad3 with a BRG1 SWI/SNF complex. A, HaLa cell extracts were subjected to affinity purification with the indicated baits. Eluted proteins were resolved on SDS-PAGE and stained with Coomassie Blue. Bands were excised and identified by mass spectrometry analysis. Red letters indicate SWI/SNF components, B, the interaction between BRG1 and Smad2/3 was verified by immunoprecipitation (IP) of TGFβ-treated or untreated HaCaT cells with anti-Smad2/3 antibody or rabbit IgG, followed by Western immunoblotting (IB) with anti-BRG1 or anti-smad2/3 antibodies. C and D, HEK293 cells were transfected with vectors encoding FLAG-tagged BRG1 or FLAG-tagged BAF170, and HA-tagged Smad proteins, in the presence of either constitutive active (C) TGFβ type I receptor (HA-TβRII(AAD) construct) or kinase-dead (D) receptor (HA-TβRII(KR) construct), as shown. Whole cell lysates were immunoprecipitated and subsequently immunoblotted with antibodies as shown. Protein expression was monitored by immunoblot analysis of total cell extracts (input). E, scheme of the Smad3 and BRG1 domain structures; brackets indicate the interacting domains.

Differential BRG1 Sensitivity of TGFβ Gene Responses—In various cell types that have been studied, the acute stimulation with TGFβ typically leads to the rapid induction of over 100 genes and the repression of a smaller number of genes (23, 24). To determine the role of BRG1 in the genome-wide transcriptional response to TGFβ, we first determined the sensitivity of individual TGFβ gene responses to a partial depletion of BRG1. HaCaT cells were transfected with a combination of two siRNA oligonucleotides that decreased the level of BRG1 to ~20% of the level in cells transfected with siRNA control (Fig. 2A, inset). The knockdown of BRG1 did not affect the levels of Smad2/3 (Fig. 2A, inset) or the C-terminal phosphorylation of these proteins by TGFβ (data not shown). These cells were incubated with TGFβ for 2 h and subjected to transcriptomic analysis using Affymetrix HG-U133 plus 2.0 microarrays. Several comparisons were conducted to identify the effect of partial BRG1 depletion on the transcriptomic response to TGFβ. We first identified 128 genes (represented by 174 probe sets on the microarrays) whose expression in control cells was increased or decreased at least 2-fold (p < 0.05) upon TGFβ addition (data not shown). Among these genes, 106 were up-regulated and 22 were down-regulated. Genes whose basal expression in the absence of TGFβ was significantly affected by the BRG1 siRNA treatment were excluded from further analysis, because they would confound our interpretation of the results. This step left 84 up-regulated and 13 down-regulated genes (Fig. 2A). The response of these 97 genes to TGFβ was diminished to varying degrees, from 60% to no effect, in the BRG1-depleted cells compared with control cells. Fig. 2B shows a rank order of these genes according to the extent of this reduction. BRG1 depletion blunted many but not all the gene induction and repression responses to TGFβ. The affected responses included genes that are strongly responsive to TGFβ as well as genes that are minimally responsive (Fig. 2A). Thus, the BRG1 sensitivity of these gene responses was independent of the sign or magnitude of TGFβ response.

To validate the microarray results, we selected a functionally diverse set of TGFβ target genes and determine their mRNA level by quantitative real time qRT-PCR. The chosen genes include several that scored as BRG1-sensitive in the microarray assays and others that scored as BRG1-insensitive. These genes included the secretory factors PAII (plasminogen-activator-inhibitor 1) and CTGF (connective tissue growth factor), which are implicated in pericellular proteolysis control, extracellular matrix regulation, and fibrogenesis (41, 42); the BMP and activin membrane-bound inhibitor BAMBI, the inhibitory Smad SMAD7, and the Ski-like transcriptional regulator SNON, all of which participate in negative feedback control of TGFβ signaling (43–47); the transcriptional activator of cell growth and proliferation MYC (48, 49), which is repressed by TGFβ action; the MYC antagonist MXD1 (50); the phosphatidic acid phosphatase PPAP2B (7); the homeobox transcription factor DLX2, which is critical in bone formation (51); the protooncogene MN1 (meningioma 1), whose fusion with TEL causes myeloid leukemia (52); and the Jumonji domain-containing nuclear factor JMJ3, which is a H3K27 histone demethylase and has been linked to polycomb-group-protein-mediated suppression of Hox genes and animal body patterning. X-chromosome inactivation, and possibly maintenance of embryonic stem cell identity (53–56).

qRT-PCR analysis of the mRNA levels of all these genes confirmed that BRG1 depletion by either of two siRNA probes strongly inhibited the induction of BAMBI, CTGF, MXD1, and PPAP2B (Fig. 3A) and partially blunted the repression of MYC (Fig. 3B) and the induction of DLX2, JMJ3, MN1, and SMAD7 (Fig. 3A). In contrast, this level of
BRG1 depletion did not significantly alter the induction of PAI1 or SNON (Fig. 3, A and C).

Probing the Physical and Functional Interactions of BRG1 and Smad2/3 in Stable Knockdown Cells—To further probe the interaction of BRG1 with Smad2/3 and to avert possible confounding effects of acute, transient expression of siRNAs in functional assays cells, we repeated these experiments using HaCaT cells that stably expressed two different shRNA vectors targeting BRG1. Each shRNA achieved a reduction of 80% in BRG1 protein level (Fig. 4A). Neither shRNA altered the level of Smad2 or Smad3 or their phosphorylation in response to TGFβ, as confirmed by Western immunoblotting of cell lysates with anti-BRG1 or anti-(phospho) Smad antibodies (Fig. 4A). The level of BRG1 remained constant in both the control and the knockdown cells for at least 7 h after TGFβ addition (Fig. 4B).

The knockdown of BRG1 strongly diminished the level of endogenous Smad2/3-BRG1 complexes that were formed in TGFβ-treated cells (Fig. 4C). The level of BAF170 protein in the BRG1 knockdown cells was not decreased compared with the level in control cells. However, the interaction of Smad2/3 and BAF170 was decreased (Fig. 4C). This result is in agreement with the possibility that BRG1 is a direct and principal Smad-binding component in BRG1-dependent SWI/SNF complexes (refer to Fig. 1). This result also suggests that BRM-dependent SWI/SNF complexes, which also share BAF170 (12, 16), do not take over when BRG1 levels become limiting in HaCaT cells.

Under these stable knockdown conditions we confirmed the results obtained with the siRNA-transfected HaCaT cells, with the exception of the effect on PPAP2B, which was less pronounced in the shRNA-transfected cells. The effect of BRG1 depletion was sustained for most of the duration of the TGFβ response over a 12-h period (Fig. 4D).

BRG1 Recruitment to TGFβ/Smad Target Gene Promoters—Collectively our results with BRG1 knockdown cells suggest a widespread sensitivity of TGFβ gene responses to BRG1 depletion and a general dependence of these responses on BRG1 function. However, this sensitivity was absent in certain TGFβ gene responses, either because these responses can be supported by the residual levels of BRG1 that remain in knockdown cells or because the responses are truly independent from BRG1 function. To distinguish between these two possibilities, we investigated the recruitment of Smad proteins and BRG1 to TGFβ target promoters. We focused on CTFG as an example of a TGFβ-responsive gene that is highly sensitive to BRG1 depletion, SMAD7 as a partially sensitive gene, and PAI1 and SNON as BRG1 depletion-insensitive genes.
TGFβ/Smad-responsive regions have been defined in the promoters of PAI1 (57), SNON (58), and CTGF and SMAD7.3 ChIP assays using primers encompassing these regions demonstrated a specific, TGFβ-dependent recruitment of BRG1 to all four genes (Fig. 5A). Given that the interaction between BRG1 and Smad2/3 is stimulated by TGFβ treatment (refer to Fig. 1), these results suggest that TGFβ-activated Smad transcriptional complexes drive the recruitment of BRG1 to these promoter regions. The knockdown of BRG1 had little or no effect on the TGFβ-dependent recruitment of Smad2/3 to these regions, as determined by Smad2/3 ChIP (Fig. 5B), suggesting that Smad2/3 transcriptional complexes can gain access to these target promoters under limiting BRG1 conditions. The TGFβ-dependent recruitment of BRG1 to the CTGF and SMAD7 promoters was inhibited in the knockdown cells (Fig. 5B). This result is in keeping with the partial sensitivity of these TGFβ responses to BRG1 depletion. The recruitment of BRG1 to the SNON promoter was also strongly inhibited. The fact that the SNON response to TGFβ in these cells remained intact suggests that this gene response is independent of BRG1.

Surprisingly, the recruitment of BRG1 to the PAI1 promoter was not decreased in BRG1 knockdown cells. This result may explain the unaltered response of PAI1 to TGFβ under these conditions. This also indicates that the TGFβ-dependent Smad complex targeting PAI1 recruits BRG1 more avidly than do the Smad complexes targeting CTGF, SMAD7, or SNON. In agreement with the extent of the TGFβ response in each of these genes, the knockdown of BRG1 decreased the recruitment of RNA polymerase II almost completely in the case of CTGF, partially in the case of SMAD7, and not at all in the cases of PAI1 and SNON (Fig. 5B). Collectively these results show four distinct classes of TGFβ target genes that are distinguished by the avidity of the corresponding Smad complexes for BRG1 or the degree of their dependence on BRG1.

Restoration of TGFβ Responses in BRG1-null Lung Cancer Cells—As an independent approach to determine the dependence of the TGFβ transcriptional program on BRG1, we investigated the TGFβ response in BRG1-null cells. Unlike normal lung epithelial cells, which are sensitive to growth inhibition by TGFβ, a majority of human lung cancer cell lines examined lack this response (59, 60). The lung adenocarcinoma cell line NCI-H522 has been reported to lack expression of BRG1 (61, 62). Additionally, our analysis of this cell line revealed that it also lacks expression of the TGFβ type II receptor (TβR-II) (data not shown). To use this cell line for the analysis of the BRG1 dependence of various TGFβ gene responses, we employed a retroviral vector encoding the human TGFBRII cDNA. NCI-H522 cells that were transduced with this vector were able to respond to TGFβ with Smad phosphorylation, whereas control cells transduced with empty vector lacked this response (Fig. 6A). TGFβ addition did not alter the expression of the exogenous BRG1 in these transfected cells (Fig. 6B). Expression of BRG1 decreased the proliferation rate of NCI-H522 cells (Fig. 6C), suggesting that the loss of endogenous BRG1 expression conferred a selective advantage in this tumor cell line. TGFβ addition fully inhibited the proliferation of NCI-H522 cells expressing TβR-II and BRG1 in combination (Fig. 6C).

NCI-H522 cells that were transduced with BRG1, TβR-II, or both vectors combined were incubated with TGFβ and subjected to transcriptomic analysis. Genes whose basal expression was significantly affected by expression of BRG1 alone were excluded from the analysis. We identified 77 genes whose expression was increased or decreased by at least 2-fold upon TGFβ addition in cells expressing both TβR-II and BRG1 (Fig. 6D). Of these genes, 55 were up-regulated, and 22 were down-regulated. To determine the degree of dependence of these gene responses on BRG1, we ran this analysis in NCI-H522 cells expressing TβR-II but not BRG1. A comparison between the two data sets revealed that 43 of these 77 TGFβ gene responses in NCI-H522 are fully dependent on BRG1 function, because they were completely absent in cells expressing TβR-II but lacking BRG1 (Fig. 6D). The remaining 34 gene responses were at least partly independent of BRG1 function.

3 Q. Wang, C. Alarcón, and J. Massagué, unpublished observations.
because they still occurred in cells transduced with TβR-II in the absence of BRG1. These results further support the findings made with BRG1 knockdown HaCaT cells that a majority but not all of the TGFβ gene responses require BRG1 function.

BRG1-dependent and -independent Gene Responses in the TGFβ Program—To validate these findings with TGFβ gene responses of interest, we focused on gene responses that were shared between NCI-H522 and at least two of four other human epithelial cell lines. These lines include HPL1 lung epithelial cells, HaCaT keratinocytes, MCF10A breast epithelial cells, and MDA-MB-231 breast carcinoma cells, all of which were previously subjected to transcriptomic analysis on the same microarray platform (63, 64). A set of 14 TGFβ gene responses fulfilled these criteria (Fig. 7 A). Within this set, six representative genes that scored with different degrees on BRG1 dependence in the microarray analysis of the TGFβ response were chosen for qRT-PCR analysis. These genes included PAI1, SMAD7, SNON, DLX2, the stress response factor GADD45B, and the Notch target gene HEY1 (hairy/enhancer of split 1) (Fig. 7A). None of these genes was induced by TGFβ in the absence of TβR-II, whereas TGFβ increased their expression severalfold in NCI-H522 cells with restored receptor and BRG1. In cells containing TβR-II but not BRG1, the response of these genes to TGFβ ranged from essentially none in the cases of PAI1, DLX2, and HEY1 to a partial response in the cases of GADD45B and SMAD7, and an essen-

FIGURE 4. BRG1-dependent gene responses in stable BRG1 knockdown cells. Stable BRG1 knockdown HaCaT cells were constructed using two different shRNAs. Knockdown and control cells were treated with TGFβ for different lengths of time. A, BRG1, phospho-Smad2, Smad2, and Smad3 protein levels in control and BRG1 knockdown HaCaT cells were detected by immunoblotting (IB) with the indicated antibodies. B, BRG1 levels after 7 h of treatment with TGFβ as determined by Western immunoblotting. Smad2/3 was used as loading control. C, the interaction between BRG1 or BAF170 and Smad2/3 was tested by immunoprecipitation (IP) of TGFβ-treated HaCaT control or BRG1 knockdown cells with anti-Smad2/3 antibodies or rabbit IgG, followed by Western immunoblotting with anti-BRG1, anti-BAF170, or anti-Smad2/3 antibodies. D, mRNA levels of selected genes at different time points after TGFβ treatment were measured by qRT-PCR. The fold change in mRNA is calculated and plotted against the indicated time course. The data are the mean values ± S.D. of triplicate determinations. The experiments were repeated at least twice, with similar results each time.

FIGURE 5. Differential recruitment of BRG1 to TGFβ/Smad target gene promoters. A, HaCaT cells were left untreated (−) or treated (+) with TGFβ for 2 h, and ChIP assays were performed with the indicated antibodies and PCR primers specific for the promoter regions of the indicated target genes, and β-actin as a control. B, HaCaT control (ctr) and stable BRG1 knockdown (KD) cells were subjected to assays as described under A. Acetyl H4, acetylated histone 4; H3, histone 3; POLII, RNA polymerase II.
tially full response in the case of SNON (Fig. 7B). PAI1, whose response was unaffected by an 80% depletion of BRG1 in HaCaT cells, showed a complete dependence on BRG1 in the BRG1-null NCI-H522. Thus, the TGFβ transcriptional program includes a majority of responses that are highly dependent on BRG1 function and the rest that are partially or fully independent. Interestingly, two of the most prominent mediators of negative feedback in the Smad pathway, SMAD7 and SNON, are in this latter group.

DISCUSSION

Prompted by our previous finding that BRG1 and other core components of the SWI/SNF complex bind to recombinant Smad2 (10) and findings by others that BRG1 is required for the activation of two genes by TGFβ in a mouse cell line (11), we have undertaken to define the requirement of BRG1 function for the genome-wide response of human cells to TGFβ. This is, to our knowledge, the first analysis on this scale of the role of a SWI/SNF core component in the transcriptional response to a cytokine-activated pathway.

In vitro Smad2 and Smad3 show a similar ability to bind a BRG1 SWI/SNF complex minimally consisting of BRG1, BAF155, BAF170, and BAF250b/OSA2. In vivo, the Smad-BRG1 interaction is enhanced upon TGFβ addition. TGFβ receptors act directly on Smads, enabling their accumulation in the nucleus and their association with other DNA-binding factors to specifically recognize target gene promoters. Smad2 is inferior to Smad3 at binding BRG1 in cell, which mirrors the restricted ability of the main Smad2 splice form to bind DNA (38). Thus, the properties of the BRG1-Smad interaction in vivo suggest that this interaction is consolidated upon the accumulation of Smad in the nucleus and the assembly of Smad transcriptional complexes on target gene promoters. Under conditions of RNA interference-mediated BRG1 depletion, Smad2/3 complexes still bind to target promoters in response to TGFβ, even though their ability to regulate transcription is inhibited. Therefore, BRG1 is not required for Smad binding to target promoters in vivo. Our results support the model that BRG1 is a direct and principal mediator of Smad-SWI/SNF interactions. The SWI/SNF complex component BAF170, which is also shared in BRM SWI/SNF complexes (12, 16), fails to be recruited to Smad2/3 complexes in BRG1 knockdown cells. This suggests that BRG1 plays a nonredundant role as a mediator of Smad-SWI/SNF interactions.

Our results reveal a requirement for BRG1 in the majority of TGFβ gene responses. This suggests that Smad binding to target promoters directs BRG1 SWI/SNF complexes to remodel nearby nucleosomal structures to enable transcriptional regu-
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FIGURE 7. BRG1-dependent and -independent gene responses. A, the heat map plot shows 14 TGFβ gene responses in NCI-H522 (BRG1/TβRII) cells that are shared in other human epithelial cell lines; cell lines responsive to TGFβ responses in cells shared with HaCaT, HPL1, MDA-MB-231, and MCF10A cells. B, NCI-H522 cells transduced with the indicated vectors were incubated with or without TGFβ for 2 h, and the expression level of six genes of interest was analyzed by qRT-PCR. The data are the averages ± S.D. of triplicate determinations.

These results are consistent with the recently reported ability of Smads to mediate transcription from chromatin templates in vitro (11). However, our results also show that the requirement for BRG1 is not universal among Smad target genes. Different TGFβ gene responses show a differential sensitivity to depletion of BRG1. This is exemplified by our comparative analysis of the response of two important TGFβ target genes, CTGF and PAI1. CTGF is implicated in extracellular matrix regulation, wound healing, fibrogenesis, and bone metastasis (42, 65), whereas PAI1 is a regulator of pericellular proteolysis (41). The induction of CTGF expression by TGFβ is highly dependent on BRG1, as shown by the high sensitivity of this response to BRG1 depletion in HaCaT cells. The PAI1 response is strictly dependent on BRG1, as evidenced by the absence of PAI1 response in BRG1-null cell line. However, this response was essentially unaffected under RNA interference conditions that cause an extensive (80%) depletion of BRG1 in HaCaT. Our ChIP results reveal that, under these conditions, the specific TGFβ-activated Smad complex that targets the PAI1 promoter still managed to recruit to this promoter enough BRG1 from the residual pool remaining in the knockdown cells. The more efficient recruitment of BRG1 to the PAI1 promoter compared with other promoters (e.g. the CTGF promoter) argues that Smad cofactors in different transcriptional complexes influence the strength of the Smad-BRG1-promoter interaction.

Although BRG1 is required for many TGFβ gene responses, our results show that this requirement is not universal. We identified several genes whose response to TGFβ is at least partly independent of BRG1. As determined by restoration of BRG1 function in the BRG1-null NCI-H522 lung adenocarcinoma cells, approximately one-quarter of the TGFβ responses fall in this category (refer to Fig. 6D). This suggests that the nucleosomal packing of these genes under basal conditions is favorable for the RNA polymerase II complex to proceed with transcription in the absence of nucleosomal remodeling. Of interest, this class of genes includes two of the most prominent mediators of negative feedback in the TGFβ pathway, namely SMAD7 and SNON. Smad7 is an inhibitory Smad that negatively regulates the TGFβ pathway by binding and targeting TβRII for degradation (46, 47, 66) and by interfering with Smad-DNA complex formation (67). Snon is a Ski-like transcription regulator that interacts with Smad2 and Smad4 and represses their ability to activate transcription (45, 68–70). The increased expression of these genes in response to TGFβ in NCI-H522 and HaCaT cells is partially independent of BRG1 in the case of SMAD7 and almost fully independent in the case of SNON. The recruitment of Pol II to the SNON promoter and the transcriptional activation of this gene by TGFβ were essentially undiminished in BRG1-depleted cells, despite the absence of BRG1 binding to this promoter under such conditions. Of note, in contrast to the context-dependent nature of many TGFβ responses, SMAD7 and SNON are induced by TGFβ in diverse cell types and conditions. The low requirement for BRG1 suggests a basal readiness of these genes to exert their crucial feedback function in this pathway.

TGFβ-activated Smad-BRG1 complexes bind to target promoters regardless of whether BRG1 is required for the transcriptional response of a particular gene. This is suggested by the case of SNON, whose promoter binds BRG1 along with Smads in response to TGFβ even though BRG1 is not required for the transcriptional response of SNON. Recruitment of Smad-BRG1 complexes to target promoters may be a general event in Smad pathways. Smads as mediators of the cellular response to the TGFβ cytokine therefore join other classes of transcriptional regulators, including developmental regulators, cell cycle checkpoint components, and nuclear hormone receptors, as factors that engage SWI/SNF remodeler complexes to effect transcriptional responses (9, 12, 71). Our results provide a genome-wide scope of the participation of BRG1 in TGFβ action, suggesting a widespread yet differential involvement of SWI/SNF components in the immediate transcriptional response of many genes to this cytokine.

Acknowledgments—We thank R. E. Kinston, K. L. Kroll, and R. Agami for reagents; members of this laboratory, C. Alarcón, and Q. Wang, for sharing unpublished information; and G. Sapkota, D. Nguyen, S. Tavazoie; and D. Marenstein for helpful discussion. We acknowledge the support of E. Kim and the High Throughput Screening Core Facility and A. Viale and the Genomic Core Facility of the Memorial Sloan-Kettering Cancer Center.

REFERENCES
1. Kornberg, R. D., and Thomas, J. O. (1974) Science 184, 865–868
2. Kayne, P. S., Kim, U. J., Han, M., Mullen, J. R., Yoshizaki, F., and Grunstein, M. (1988) Cell 55, 27–39
