Previously, we have demonstrated that major histocompatibility class II trans-activator (CIITA) is crucial in mediating interferon-γ (IFN-γ)-induced repression of collagen type I gene transcription. Here we report that CIITA represses collagen transcription through a phosphorylation-dependent interaction between its proline/serine/threonine domain and co-repressor molecules such as histone deacetylase (HDAC2) and Sin3B. Mutation of a serine (S373A) in CIITA, within a glycogen synthase kinase 3 (GSK3) consensu site, decreases repression of collagen transcription by blocking interaction with Sin3B. *In vitro* phosphorylation of CIITA by GSK3 relies on a casein kinase I site three amino acids C-terminal to the GSK3 site in CIITA. Both GSK3 and casein kinase I inhibitors alleviate collagen repression and disrupt IFN-γ-mediated recruitment of Sin3B and HDAC2 to the collagen start site. Therefore, we have identified the region within CIITA responsible for mediating IFN-γ-induced inhibition of collagen synthesis.

Extracellular matrix plays a critical role in maintaining the homeostasis of the vertebrate organisms. Increases in collagen expression not only further the understanding of the molecular mechanisms underlying the aforementioned diseases but provide insights into potential therapeutics. Type I collagen, which consists of two α1 chains (α1(I)) and one α2 chain (α2(I)), is the most abundant member of the collagen family. Type I collagen is the major component of the extracellular matrix in bones, tendons, and ligaments. It is the major component of heterotypic collagen fibrils and includes ~84% of the collagen synthesized by fibroblasts and myofibroblasts.

Diverse collagen structure and distribution combined with complex interactions with other components of the extracellular matrix make the regulation of collagen expression an extremely complicated, yet critical process. Although regulation of collagen expression occurs at multiple levels, including transcription, processing of RNA, translation, and post-translation, collagen type I gene expression is regulated primarily at the transcriptional level. One of the common key features of fibrotic/atherosclerotic diseases is the infiltration of immune cells after injury, which release cytokines, such as IFN-γ, to challenge local cells that produce collagen. The research in our laboratory has focused on unveiling the molecular mechanisms whereby IFN-γ represses collagen synthesis. The investigations over the past few years in our laboratory as well as in others’ have led to the identification of class II trans-activator (CIITA) as a crucial factor in mediating IFN-γ-induced repression of collagen production (1–4).

CIITA was first cloned from patients with bare lymphocyte syndrome, a hereditary immunodeficiency with a complete absence of major histocompatibility complex class II (MHC II) expression because of dysfunctional proteins responsible for activating MHC II expression at the transcriptional level. The indispensability of CIITA to the expression of genes involved in antigen presentation, MHC II, invariant chain (II), human leucocyte antigen DM, was further confirmed by studies in CIITA knock-out (CIITA−/−) mice (5–7) and studies in cells where expression and function of CIITA are affected (8–12). Therefore, CIITA was established as the master regulator of MHC II activation (13–15). The expression of CIITA and hence MHC II is not only constitutive in some immune cell lines like B cells or dendritic cells, but also highly inducible by IFN-γ in multiple cell types such as fibroblasts (4, 16) and smooth muscle cells (1).

CIITA (also named MHC2TA/C2TA/NLRA) has been placed as the first member within a nucleotide-binding domain and leucine-rich receptor family, NLR (17–19), because it has a conserved GTP-biding domain as well as a leucine-rich domain, suggesting the possibility that its cytoplasmic function involves inhibition of proliferation and/or activation of apoptosis (20). CIITA also has several activation domains such as an acidic domain and a proline-, serine-, and threonine-rich region (PST) (21). The activation domain activates transcription (22, 23) through interactions with several proteins, including TFIIIB, TAFII55, CREB-binding protein (CBP), and p300 (22, 25–28). The PST region contains a bipartite nuclear localization signal that is regulated by histone acetyltransferase-medi-
CIITA Mediates Repression of Collagen

aded acetylation events at Lys-141 and Lys-144 (29). The PST domain is important for oligomerization of CIITA (30) and repression of collagen (4).

Although CIITA was initially described as a critical activator of MHC II transcription, it is now clear that it also suppresses transcription of several genes such as collagen, IL-4, Fas ligand, cathepsin E, IL-10, and thyroid-specific genes (2, 25, 31–35). The mechanism for repression is largely controversial and a matter for debate. One of the possible scenarios wherein CIITA represses transcription, as in the case of IL-4 gene, involves competition of CIITA with CBP/p300 for binding to NF-AT (25). A similar mechanism involving CBP interaction has been proposed for collagen transcription repression.

As a trans-activator, GIITA forms protein-protein interactions rather than bind to the DNA. It relies on other transcription factors such as regulatory factor for X-box (RFX) to be recruited to the individual response elements to exert its regulatory function (36). Our previous reports demonstrate that RFX5 brings CIITA to the type I collagen transcription start site to mediate IFN-γ repression (4), and that the RFX5-CIITA complex associates with co-repressors such as histone deacetylase 2 (HDAC2) and Sin3B during the IFN-γ response (37). Other investigators have also suggested that there is an interaction between CIITA and co-repressors (38). Here we report that at least part of the mechanism whereby IFN-γ represses collagen synthesis is through phosphorylation-dependent interaction between CIITA and co-repressor molecules.

EXPERIMENTAL PROCEDURES

Cell Culture Maintenance—Human lung fibroblasts, IMR-90 (IMR, NJ), and human embryonic kidney cells 293FT (Invitrogen) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin G-streptomycin (Sigma). Human aortic smooth muscle cells (SMC) (Cambrex, Charles City, IA) were maintained in the media supplied by Cambrex.

Plasmids, Transfections, and Luciferase Assays—The col1a2-luciferase construct (pH 20) (39) contains sequences from -221 to +54 bp of mouse col1a2 promoter fused to the luciferase reporter gene. A larger col1a2-luciferase construct (pGL3-Col-Luc) containing sequences from -357 to +55 bp of mouse col1a2 promoter fused to the luciferase reporter gene was a gift from Dr. Jenny Ting (2). The human MHC II promoter (pGL2-DRA300) was also a gift from Dr. Ting (40). The human COL1A1 (collagen α1 type I gene) promoter plasmids were all cloned into a pGL2-luciferase vector from the human col1a2 promoter fused to the luciferase reporter gene (pH 20). The luciferase construct (pH 20) (39) contains sequences from -221 to +54 bp of mouse col1a2 promoter fused to the luciferase reporter gene. A larger col1a2-luciferase construct (pGL3-Col-Luc) containing sequences from -357 to +55 bp of mouse col1a2 promoter fused to the luciferase reporter gene was a gift from Dr. Jenny Ting (2). The human MHC II promoter (pGL2-DRA300) was also a gift from Dr. Ting (40). The human COL1A1 (collagen α1 type I gene) promoter plasmids were all cloned into a pGL2-luciferase vector from the human col1a2 promoter fused to the luciferase reporter gene (pH 20). The luciferase construct (pH 20) (39) contains sequences from -221 to +54 bp of mouse col1a2 promoter fused to the luciferase reporter gene. A larger col1a2-luciferase construct (pGL3-Col-Luc) containing sequences from -357 to +55 bp of mouse col1a2 promoter fused to the luciferase reporter gene was a gift from Dr. Jenny Ting (2). The human MHC II promoter (pGL2-DRA300) was also a gift from Dr. Ting (40). The human COL1A1 (collagen α1 type I gene) promoter plasmids were all cloned into a pGL2-luciferase vector from the human col1a2 promoter fused to the luciferase reporter gene (pH 20).

GCTGCTG(G/T)CCTTCCAGCTTGGC-3′. The presence of the intended mutations was verified by sequencing. Cells were plated at the density of 3 × 10⁵ cells/well (for IMR-90 cells) or 2 × 10⁶ cells/well (for SMC) in 6-well tissue culture dishes, and 5 × 10⁶ cells per 100 tissue culture dish (for 293FT cells). Transfections were performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were harvested 48 h post-transfections, and luciferase assays were performed with a luciferase reporter assay system (Promega).

Immunoprecipitations—Whole cell lysates (IMR-90 or 293FT with transfected constructs as indicated where applicable) were obtained by resuspending cell pellets in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor (Roche Applied Science) and phenylmethylsulfonyl fluoride (100 μg/ml RIPA). Anti-CIITA (7-1H, Santa Cruz Biotechnology) antibodies were added to and incubated with IMR-90 cell lysate overnight before being absorbed by protein A/G plus-agarose beads (Santa Cruz Biotechnology). Precipitated immune complex was released by boiling with 1× SDS electrophoresis sample buffer. Alternatively, FLAG-conjugated beads (M2, Sigma) were added to and incubated with 293FT cell lysate overnight. Precipitated immune complex was eluted with 3× FLAG peptide (Sigma).

Westerns—Proteins were quantified with the BCA reagent (Pierce) according to manufacturer’s protocol and separated by 10% PAGE with pre-stained markers (Bio-Rad) for estimating molecular weight and efficiency of transfer to blots. Proteins were transferred to nitrocellulose membranes (Bio-Rad) in a Mini-Trans-Blot cell (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline buffer (TBST) (0.05% Tween 20, 150 mM NaCl, 100 mM Tris-HCl, pH 7.4) at 4°C for several hours and incubated overnight with monoclonal anti-FLAG (1:5000) (Sigma), monoclonal anti-CIITA (7-1H, 1:200) (Santa Cruz Biotechnology), monocular anti-Myc (1:1000) (Santa Cruz Biotechnology), polyclonal anti-HDAC2 (1:1000) (Santa Cruz Biotechnology), or polyclonal anti-Sin3B antibody (1:1000) (Santa Cruz Biotechnology). After three washes with TBST, the membranes were incubated with appropriate secondary antibodies, either anti-goat IgG (Sigma), anti-mouse IgG, or anti-rabbit IgG (Amersham Biosciences) conjugated to horseradish peroxidase for another hour at room temperature. Then protein blots were visualized using the Supersignal ECL reagent (Pierce) on a Kodak image station (PerkinElmer Life Sciences).

Chromatin Immunoprecipitation—Chromatin in control and treated cells were cross-linked with 1% formaldehyde for 8 min at room temperature, sequentially washed with phosphate-buffered saline, solution I (10 mM HEPES, pH 7.5, 10 mM EDTA, 0.5 mM EGTA, 0.75% Triton X-100), and solution II (10 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris, pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor tablet (Roche Applied Science) and phenylmethylsulfonyl fluoride. DNA was fragmented into ~500 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-CIITA (7-1H, Santa Cruz Biotechnology), anti-Sin3B (AK-12, Santa Cruz Biotechnol-
The PST domain of CIITA interacts with co-repressors in a phosphorylation-dependent manner. A, PST region of CIITA is required for interaction with HDAC2. FLAG-tagged WT and mutated CIITA constructs (shown in diagrams) were transfected into 293FT cells. Protein lysates were harvested 24 h post-transfection and treated with (+) or without (−) phosphatase before being subject to immunoprecipitation. One of two independent experiments is shown. B, the PST domain of CIITA interacts with HDAC2 and Sin3B. Two FLAG-tagged CIITA constructs (shown in diagrams) were transfected into 293FT cells. Protein lysates were harvested 24 h post-transfection and co-immunoprecipitated (IP). Westerns were immunoblotted (IB) with HDAC2 antibodies as described under “Experimental Procedures.” One of two independent experiments was shown. C, CIITA interaction with co-repressors requires phosphorylated PST domain. The FLAG-tagged CIITA PST construct was transfected into 293FT cells. Protein lysates were harvested 24 h post-transfection and treated with (+) or without (−) phosphatase before being subject to immunoprecipitation. One of two independent experiments is shown.

RESULTS

The PST Domain of CIITA Interacts with Co-repressors in a Phosphorylation-dependent Manner—Previously, our results demonstrated that CIITA is an important mediator of the tran-
CITTA Mediates Repression of Collagen

CIITA represses collagen transcription primarily through its N-terminal PST domain. More recently, our laboratory discovered that an HDAC2/Sin3B containing co-repressor complex is necessary for complex formation. CIITA represses collagen transcription primarily through its PST domain. Abrogations of COL1A2 repression but not MHC II activation when the PST domain (1–410) was deleted (Fig. 1), and the S373A mutant lost much of its repression activity when compared with the WT mutant (collagen α2 type I gene) promoter activity (−220-bp COL1A2 promoter–luciferase) similarly to the wild type (WT), a Ser to Ala mutant (S373A) completely lost its capability of repressing collagen transcription, whereas a Ser to Asp mutant at the same site repressed the collagen promoter slightly better than the WT. Similar patterns were also observed with a larger COL1A2 promoter containing 357 bases of promoter (pGL3-COL-LUC, −357) as well as a COL1A2 promoter (COL1A1-LUC, −311) in fibroblasts and smooth muscle cells (data not shown). Interestingly, all the CIITA constructs tested here activated the MHC II promoter (Fig. 2B, pGL2-DRA300), suggesting that association with co-repressors is necessary for repression but not for activation.

S373A Mutant Does Not Interact with Sin3B—Because the phosphorylation of the CIITA PST domain governs its interaction with HDAC2 and Sin3B (Fig. 1), and the S373A mutant lost much of its repression activity when compared with the WT (Fig. 2), the next experiment was designed to examine whether the mutation affects the interactions between CIITA and the co-repressors. In addition, other investigators demonstrated that the PST domain interacts with CIITA-forming oligomers (30). To test whether this serine is important both for oligomerization and co-repressor complex interaction, FLAG-tagged CIITA constructs (WT, S373A, and S373D) were co-transfected with a Myc-tagged CIITA construct, which contains the complete PST domain (253–410 amino acids) was present. The partial presence of PST domain had interactions with both protein concentration and GFP fluorescence. This representative experiment was repeated at least three times. The region in CIITA where the mutations are located are on top of the figure. Serines that were mutated are circled. The important area identified is underlined.

A Serine Mutation in CIITA (S373A) in the PST Domain Abrogates COL1A2 Repression but Not MHC II Activation—Because CIITA needs to be phosphorylated in the PST domain to interact with co-repressors, it was hypothesized that, if certain serine sites within the PST domain were mutated, the interactions with co-repressors would be diminished and the repression of collagen transcription by CIITA would be ameliorated. Therefore, a series of serine to alanine and/or serine to aspartic acid mutations were introduced into various sites within or near the PST domain (circled in Fig. 2). The mutants were co-transfected with different promoter constructs into human lung fibroblasts to test their transcriptional activities. As depicted in Fig. 2A, while most mutants repressed COL1A2 (collagen α2 type I gene) promoter activity (−220-bp COL1A2 promoter–luciferase) similarly to the wild type (WT), a Ser to Ala mutant (S373A) completely lost its capability of repressing collagen transcription, whereas a Ser to Asp mutant at the same site repressed the collagen promoter slightly better than the WT. Similar patterns were also observed with a larger COL1A2 promoter containing 357 bases of promoter (pGL3-COL-LUC, −357) as well as a COL1A2 promoter (COL1A1-LUC, −311) in fibroblasts and smooth muscle cells (data not shown). Interestingly, all the CIITA constructs tested here activated the MHC II promoter (Fig. 2B, pGL2-DRA300), suggesting that association with co-repressors is necessary for repression but not for activation.

S373A Mutant Does Not Interact with Sin3B—Because the phosphorylation of the CIITA PST domain governs its interaction with HDAC2 and Sin3B (Fig. 1), and the S373A mutant lost much of its repression activity when compared with the WT (Fig. 2), the next experiment was designed to examine whether the mutation affects the interactions between CIITA and the co-repressors. In addition, other investigators demonstrated that the PST domain interacts with CIITA-forming oligomers (30). To test whether this serine is important both for oligomerization and co-repressor complex interaction, FLAG-tagged CIITA constructs (WT, S373A, and S373D) were co-transfected with a Myc-tagged CIITA construct, which contains the PST domain (1–410) into 293FT cells. Protein-protein interactions were examined by co-immunoprecipitation followed by Westerns (Fig. 1A). Indeed, HDAC2 co-precipitated with CIITA if the complete PST domain (253–410) came down with all three CIITA proteins, indicating that association with co-repressors is necessary for repression but not for activation.

S373A Mutant Does Not Interact with Sin3B—Because the phosphorylation of the CIITA PST domain governs its interaction with HDAC2 and Sin3B (Fig. 1), and the S373A mutant lost much of its repression activity when compared with the WT (Fig. 2), the next experiment was designed to examine whether the mutation affects the interactions between CIITA and the co-repressors. In addition, other investigators demonstrated that the PST domain interacts with CIITA-forming oligomers (30). To test whether this serine is important both for oligomerization and co-repressor complex interaction, FLAG-tagged CIITA constructs (WT, S373A, and S373D) were co-transfected with a Myc-tagged CIITA construct, which contains the PST domain (1–410) into 293FT cells. Protein-protein interactions were examined by co-immunoprecipitation followed by Westerns. As shown in Fig. 3A, an equal amount of Myc CIITA (1–410) came down with all three CIITA proteins, indicating that CIITA self-association was not impaired (30). On the other hand, interaction between Sin3B and S373A was completely disrupted compared with WT and S373D, whereas HDAC2 association was greatly diminished.

Our previous results demonstrated that during IFN-γ response when CIITA is stimulated and recruited to the collagen transcription start site, it also brings Sin3B and HDAC2 to
CIITA Mediates Repression of Collagen

**GSK3 Inhibitors Alleviate Repression of Collagen Transcription by IFN-γ**—Our previous results demonstrate that CIITA mediates the collagen transcriptional repression by IFN-γ (4). Because GSK3 is important for collagen repression by CIITA, we hypothesized that GSK3 was also involved in the IFN-γ repression of collagen synthesis. Human aortic SMCs were transfected with different collagen promoter constructs and treated with IFN-γ for 24 h. IFN-γ potently down-regulated all three collagen promoter activities, which were significantly recovered by the GSK3 inhibitor LiCl (Fig. 5A). Endogenous expression of both collagen and MHC II genes was also measured. IFN-γ down-regulated both COL1A1 and COL1A2 mRNAs to ~40% of the control level (Fig. 5B and data not shown). Two GSK3 inhibitors, LiCl and SB216763, partially

FIGURE 3. S373A mutant does not interact with Sin3B. A, serine to alanine mutation at amino acid 373 retains the self-association ability of CIITA but disrupts its interaction with co-repressors. Myc-CIITA (1-410) and FLAG-tagged CIITA constructs were transfected into 293FT cells as indicated. Protein lysates were harvested 24 h post-transfection and co-immunoprecipitated. Westerns were immunoblotted (IB) with FLAG, HDAC2, mSin3B, and Myc antibodies as described under “Experimental Procedures.” One of two independent experiments is shown. B, CIITA S373A mutant has reduced ability to recruit co-repressors to the collagen transcription start site. FLAG-tagged CIITA constructs or an empty vector (pcDNA3) was transfected into 293FT cells. Protein lysates were harvested 24 h post-transfection, and chromatin immunoprecipitation assays were performed as described under “Experimental Procedures.” Precipitated genomic DNA was amplified by primers surrounding the collagen transcription start site (44). Inset, 10% of the eluted IP samples was separated by SDS-polyacrylamide gel and immunoblotted (IB) for different proteins as indicated to verify that the immunoprecipitation efficiencies were similar across the board.

the same site (4, 37). Therefore, chromatin immunoprecipitation was performed to assess whether the mutation of CIITA serine 373 would reduce recruitment of co-repressors to the collagen promoter. Indeed, although all three CIITA forms (WT, S373A, and S373D) bound to the collagen transcription start site with equal affinity, the S373A mutant failed to recruit as much co-repressor as either the WT or the S373D mutant (Fig. 3B). Interestingly, the S373D mutant with a negative charge used to mimic phosphorylation recruited more co-repressor molecules. This suggests that phosphorylation of CIITA serine 373 is partially responsible for recruiting co-repressors to the collagen start site to repress transcription.

**GSK3 Inhibitors Alleviate Repression of Collagen Transcription by CIITA**—Analysis of the CIITA PST domain by PROSITE revealed that serine 373 was a putative glycogen synthase kinase 3 (GSK3) site (Fig. 4A). To investigate whether GSK3 was indeed involved in collagen repression by CIITA, lithium chloride (LiCl), a commonly used GSK3 inhibitor, was utilized to treat cells co-transfected with a collagen promoter and a CIITA expression construct. Neither LiCl nor NaCl, a negative control, altered the collagen promoter by itself (Fig. 4B). However, LiCl, but not NaCl, alleviated the repression of the collagen promoter activity by WT CIITA but not the mutants. This strongly suggests that serine 373 is indeed a GSK3 site, and its phosphorylation is at least partially necessary for CIITA repression of collagen transcription. The lithium effect was slightly stronger in human smooth muscle cells than in human lung fibroblasts (data not shown). Similar results were also obtained with a larger COL1A2 promoter (pGL3-COL-LUC, −357) as well as a COL1A1 promoter (COL1A1-LUC, −311) and with another GSK3 inhibitor SB216763 (data not shown).

To determine whether decreased collagen repression by CIITA in the presence of GSK3 inhibitors was because of disruption of interactions between CIITA and co-repressors, co-immunoprecipitations were conducted. Both Sin3B and HDAC2 co-immunoprecipitated with ectopically expressed CIITA as expected in 293FT cells (Fig. 4C). When the cells were treated with LiCl, however, interactions between CIITA and co-repressors were greatly diminished.

Next, to determine whether CIITA and Sin3B also function together, they were co-transfected with a collagen promoter construct (COL1A2, −220) into human lung fibroblast cells. Sin3B did not significantly repress collagen promoter activity by itself even at the highest concentration tested. However, Sin3B enhanced the repression by CIITA significantly in a dose-response manner (Fig. 4D), suggesting that part of the mechanism whereby CIITA represses collagen transcription is probably through cooperative interaction with Sin3B. Sin3B, however, had minor repression activity by itself on a COL1A1 promoter (COL1A1-LUC, −311) and in human smooth muscle cells. Nonetheless, Sin3B consistently increased the repression by CIITA (data not shown).

Finally, a similar experiment was performed to determine whether inhibition of GSK3 activity would relieve the synergistic repression of collagen transcription by CIITA and Sin3B. As shown in Fig. 4E, the presence of GSK3 inhibitor LiCl blocked the repression of the COL1A2 promoter activity (−220) by CIITA, strongly suggesting that full repression of collagen transcription depends on CIITA-Sin3B interaction, which in turn relies on the phosphorylation of CIITA by GSK3.
abrogated the collagen repression bringing the collagen message up to 75% of the control level. On the other hand, stimulation of HLA-DR/H9251 molecules by IFN-γ/H9253 was not altered by either GSK3 inhibitor, suggesting that phosphorylation of CIITA by GSK3 is most likely unnecessary for MHC II activation.

Next, co-immunoprecipitations were performed to assess whether the interactions between endogenous IFN-γ-induced CIITA and co-repressors would be disrupted if GSK3 activity was inhibited. CIITA was precipitated when cells were treated with IFN-γ and both Sin3B and HDAC2 co-precipitated with CIITA (Fig. 5C). When LiCl was added to the cells, very little association was observed between CIITA and co-repressors, indicating that GSK3 phosphorylation is necessary for CIITA to interact with co-repressors.

When CIITA is stimulated by IFN-γ, it translocates to the nucleus and binds to the collagen transcription start site (4). Meanwhile, occupancy of HDAC2 and Sin3B is enhanced at the same site (37), which partly accounts for the mechanism whereby CIITA represses collagen synthesis. Because our results demonstrate that GSK3 inhibitors prevented CIITA from interacting with co-repressors and hence dampening its ability to mediate IFN-γ-stimulated down-regulation of collagen transcription, it was hypothesized that recruitment of

FIGURE 4. GSK3 inhibitors alleviate repression of collagen transcription by CIITA. A, sequences surrounding kinase site. B, LiCl alleviates the repression of collagen promoter activity by CIITA. A col1a2 promoter construct (pH20, 0.5 μg) was co-transfected with various CIITA constructs as indicated, along with GFP (0.1 μg) for normalization, into IMR-90 cells in triplicate wells as described under “Experimental Procedures.” 24 h later, cells were treated with LiCl or NaCl (5 mM) for an additional 24 h before harvesting. Average luciferase activities were normalized by both protein concentration and GFP fluorescence. This representative experiment was repeated at least three times and the same for luciferase assays in D and E. C, LiCl disrupts interaction between CIITA and co-repressors. FLAG-tagged WT CIITA construct was transfected into 293FT cells. 24 h later, cells were treated with LiCl or NaCl (5 mM) as indicated for an additional 24 h before harvesting. Whole cell lysates were prepared and immunoprecipitated (IP), and Westerns were immunoblotted (IB) as described under “Experimental Procedures.” One of two independent experiments is shown. D, Sin3B enhances CIITA repression of the collagen promoter activity in a dose-response manner. A col1a2 promoter construct (pH20, 0.5 μg) was co-transfected with WT CIITA construct (0.25 μg) as well as with increasing doses of Sin3B construct, along with GFP (0.1 μg) for normalization, into IMR-90 cells in triplicate wells as described under “Experimental Procedures.” Average luciferase activities were normalized by both protein concentration and GFP fluorescence. Inset, protein lysates (20 μg) from this transfection experiment were separated by SDS-polyacrylamide gel and blot for Myc-Sin3B and β-actin to examine the expression of Sin3B. E, LiCl abrogates the cooperation between CIITA and Sin3B in down-regulating collagen promoter activity. A col1a2 promoter construct (pH 20, 0.5 μg) was co-transfected with WT CIITA construct (0.25 μg) as well as with Sin3B construct (0.25 μg), along with GFP (0.1 μg) for normalization, into IMR-90 cells in triplicate wells as described under “Experimental Procedures.” 24 h later, cells were treated with LiCl or NaCl (5 mM) for an additional 24 h before harvesting. Average luciferase activities were normalized by both protein concentration and GFP fluorescence. LiCl significantly decreases the repression of collagen promoter activity by CIITA with or without Sin3B (**, p < 0.01).
Ciita Mediates Repression of Collagen

repressors to the collagen transcription start site would be impaired if GSK3 could not phosphorylate Ciita. Therefore, chromatin immunoprecipitation experiments were performed in human SMCs. As expected, Ciita occupancy on the collagen transcription start site was greatly stimulated 24 h after IFN-γ treatment (>10-fold), which was not changed by the addition of GSK3 inhibitor LiCl (Fig. 5D). Both Sin3B and HDAC2 were recruited to the collagen site along with Ciita, and the increased binding was diminished in the presence of LiCl, suggesting that the disrupted interaction between Ciita and co-repressors also extended to the promoter of a specific gene where Ciita acts as repressor. On the other hand, although Ciita occupancy on the HLA-DRα promoter was increased to a similar extent as compared with the collagen transcription start site (>10-fold), very little co-repressor was present (Fig. 5D), indicating the activation of genes and repression of genes are two separate functionalities of Ciita.

Phosphorylation of Ciita by Gsk3 in Vitro Requires Cki Activity—Because there was a strong implication that repression of collagen transcription by IFN-γ/Ciita required Gsk3 activity, possibly through Ciita serine 373, an in vitro kinase assay was performed to verify whether Ciita was a de facto Gsk3 substrate. Flag-tagged Ciita constructs (WT, S373A, and S373D), as well as a negative control (pcDNA3), were used as substrates for in vitro transcription-translation followed by purification using the Flag antibody. Gsk3 phosphorylation

FIGURE 5. GSK3 inhibitors alleviate repression of collagen transcription by IFN-γ. A, LiCl alleviates the repression of collagen promoter activity by IFN-γ. Three different collagen type I promoters (0.5 μg) were transfected, along with GFP (0.1 μg) for normalization, into human SMCs in triplicate wells as described under “Experimental Procedures.” 24 h later, cells were treated with LiCl (Li) or NaCl (Na) (5 mM) in the absence or presence of IFN-γ (100 units/ml) for an additional 24 h before harvesting. Average luciferase activities were normalized by both protein concentration and GFP fluorescence. This representative experiment was repeated at least three times. LiCl significantly decreases the repression of all three collagen promoter activities by IFN-γ (**, p < 0.01). B, LiCl and SB216763 (Sβ) alleviate the repression of endogenous collagen expression by IFN-γ. IMR-90 cells were treated with IFN-γ (100 units/ml) in the presence of either LiCl (5 mM) or SB216763 (5 μM), or NaCl (5 mM) or MeSO (D) as negative controls, as indicated for 24 h. Total RNA was extracted and transcribed to cDNA, and real time PCRs were performed with the cDNA samples using primers to detect COL1A2 mRNA (left) or HLA-DRA mRNA (right). Each experiment was repeated at least three times in triplicate wells. Data are expressed as relative RNA levels compared with control levels, normalized to 18 S RNA and presented as average ± S.D. Both LiCl and SB216763 significantly alleviate the repression of collagen mRNA (**, p < 0.01) by IFN-γ without altering MHC II expression. C, LiCl disrupts interaction between endogenous Ciita and co-repressors in the presence of IFN-γ. IMR-90 cells were treated with IFN-γ (100 units/ml) in the presence of either LiCl or NaCl (5 mM) as indicated for 24 h. Whole cell lysates were prepared and immunoprecipitated (IP) with anti-Ciita antibody, and Westerns were immunoblotted (IB) with Ciita, Sin3B, and HDAC2 antibodies (α) as described under “Experimental Procedures.” Immune complex was separated by SDS gel and blot for different proteins as indicated. D, LiCl decreases recruitment of co-repressors to the collagen transcription start site induced by IFN-γ. IMR-90 cells were treated with IFN-γ (100 units/ml) in the presence of either LiCl or NaCl (5 mM) as indicated for 24 h. Chromatin immunoprecipitation assays were performed using anti-Ciita, anti-Sin3B, anti-HDAC2, or pre-immune IgGs as indicated. Precipitated genomic DNA was amplified by primers surrounding the collagen transcription start site (left) or the MHC II X-box (right). These experiments were repeated three times in triplicate wells and plotted as average ± S.D. Data were expressed as picogram of DNA precipitated by indicated antibody per nanogram of total genomic DNA.
CIITA Mediates Repression of Collagen

A

B

C

FIGURE 6. Phosphorylation of CIITA by GSK3 in vitro requires CKI activity. A, GSK3 phosphorylation of CIITA requires CKI. In vitro translated and purified CIITA proteins were subject to in vitro kinase assay described under “Experimental Procedures.” WT CIITA is phosphorylated by GSK3 in the presence of CKI. One of two independent experiments is shown. B, CIITA phosphorylates CIITA directly in vitro. In vitro translated and purified CIITA proteins were subject to in vitro kinase assay with or without indicated CKI inhibitor (D4476) or GSK3 inhibitor (GSK3). One of two independent experiments is shown. C, CIITA and GSK3 inhibitors blocks CIITA phosphorylation in vitro. In vitro translated and purified CIITA proteins were subject to in vitro kinase assay with or without indicated CKI inhibitor (D4476) or GSK3 inhibitor (LiCl) as described under “Experimental Procedures.” One of two independent experiments is shown.

is often primed by kinases at a serine 3 amino acids downstream of the GSK3 site (51–53). A Prosite scan of the CIITA sequence revealed that there was indeed a serine within a potential casein kinase I (CKI) site whose phosphorylation presumably could be a prerequisite for GSK3 to act on serine 373. As shown in Fig. 6A, GSK3 kinase failed to phosphorylate all the substrates without CK1. However, when these substrates were preincubated with a recombinant CK1 before being subject to GSK3 treatment, the WT CIITA, but not the mutants, displayed a strong signal of phosphorylation.

Next, to verify whether CIITA was also a direct CKI substrate in vitro, a similar kinase assay was performed. As expected, all three CIITA substrates were phosphorylated in the presence of CKI and [γ-32P]ATP but not the negative control (Fig. 6B, lanes 1, 3, 5, and 7). Moreover, when a CKI-specific inhibitor, D4476, was added to the reaction system, the signals were completely gone (Fig. 6B, lanes 4, 6, and 8), suggesting that CIITA phosphorylation by CKI is specific.

Finally, to confirm that the specificity of both CKI and GSK3 phosphorylation, wild type CIITA (CIITA WT) was incubated with enzymes with and without specific inhibitors. As demonstrated in Fig. 6C, GSK3 could only phosphorylate CIITA WT when the substrate was preincubated with CKI (lane 5). When CKI and GSK3 inhibitors were included in the reaction system, phosphorylation was almost completely lost.

Casein Kinase I Inhibitors Partially Abrogate the Repression of Collagen Transcription by CIITA—According to our in vitro kinase assay, CKI not only phosphorylated CIITA directly but also promoted CIITA phosphorylation by GSK3, implying that CKI might share a similar function with GSK3 as a regulator of CIITA activities. A series of experiments was performed to verify this hypothesis. First, two CKI-specific inhibitors, D4476 and CKI-7, were tested in co-transfection assays. CIITA repressed all three type I collagen promoters by more than 80%, all of which were ameliorated by both CKI inhibitors (Fig. 7A and data not shown). Next, ectopically expressed CIITA in 293FT cells co-immunoprecipitated with both Sin3B and HDAC2 (Fig. 7B, 4th lane). Both D4476 and CKI-7 interfered with these interactions (Fig. 7B, 5th and 6th lanes), suggesting that CKI phosphorylation is a prerequisite of CIITA-co-repressor interactions in vivo.

Finally, the effect of CKI inhibitors on IFN-γ-mediated collagen transcription was examined. IFN-γ down-regulates collagen transcription by activating CIITA expression, as evidenced both by transient transfections (Fig. 7D) and mRNA studies (Fig. 7E). D4476 partially blocked this effect on all three collagen promoters as well as collagen messages without affecting MHC II expression, possibly by keeping CIITA from interacting with co-repressors, here providing proof to our hypothesis that CKI enables CIITA to mediate IFN-γ repression of collagen transcription through its permissive phosphorylation by GSK3.

GSK3 and CK1 Inhibitors Additively Decrease Interactions between CIITA and Co-repressors—Because both GSK3 and CKI phosphorylation of CIITA are important for its function as a collagen repressor, the combinatorial effect of GSK3 and CKI on CIITA interaction with co-repressors was examined as well as its regulation of IFN-γ-mediated repression of collagen synthesis. When FLAG-tagged CIITA was immunoprecipitated from 293FT cell lysates, both Sin3B and HDAC2 co-precipitated with CIITA (Fig. 8A, 1st to 3rd lanes). GSK3 inhibitor LiCl, as well as CKI inhibitor D4476, strongly inhibited the interactions (Fig. 8A, 4th and 5th lanes). When these two inhibitors were added together into the cells, an even stronger disruption of interactions was observed (Fig. 8A, 6th lane), more so for Sin3B than HDAC2, which is consistent with our earlier findings (Figs. 3A, 4E, and 7B).

Next, recruitment of CIITA and co-repressors to the collagen transcription start site was examined (Fig. 8B). As expected, there was little CIITA occupancy on the collagen site in the absence of IFN-γ. In the presence of IFN-γ, however, occupancy of CIITA increased dramatically and was not altered by either GSK3 or CKI inhibitors. Both Sin3B and HDAC2 bound to the collagen promoter despite the absence of CIITA, and binding of both increased when CIITA was induced. GSK3 inhibitor LiCl reduced the recruitment of co-repressors by more than 50%, as did the CKI inhibitor D4476. When these...
two inhibitors were added together, there was a further reduction of co-repressor recruitment, suggesting that CIITA interaction with co-repressors on the collagen chromatin also depends on phosphorylation.

Finally, the effect of GSK3 and CKI inhibitors on collagen expression was evaluated (Fig. 8C). Neither LiCl nor D4476 affected COL1A2 mRNA on their own. IFN-γ down-regulated the collagen message by ~60%, which was partially blocked by both LiCl and D4476. In the presence of both inhibitors, repression of collagen synthesis by IFN-γ was further alleviated. Therefore, our results strongly implicate that sequential phosphorylation of CIITA by CKI and GSK3 is important for IFN-γ-mediated transcriptional repression of type I collagen genes.

DISCUSSION

Previously, our laboratory reported that the group of transcription factors, RFX, bind to the transcription start site of collagen type I genes and repress transcription (54). More specifically, one family member, RFX5, recruits CIITA to the collagen transcription start site during the IFN-γ response (4) along with co-repressor molecules such as HDAC2 and Sin3B (37). This is accompanied by the deacetylation of core histones.

FIGURE 7. Casein kinase I inhibitors partially abrogate the repression of collagen transcription by CIITA. A, D4476 alleviates the repression of collagen promoter activity by CIITA. Three different collagen type I promoters (0.5 μg) were co-transfected with the WT CIITA construct (0.5 μg), along with GFP (0.1 μg) for normalization, into IMR-90 cells in triplicate wells as described under "Experimental Procedures." Cells were treated for 24 h with or without D4476 (5 μM) for additional 24 h before harvesting. Average luciferase activities were normalized by both protein concentration and GFP fluorescence. This representative experiment was repeated at least three times. D4476 significantly decreases the repression of all three collagen promoter activities by CIITA (**, p < 0.01). B, CKI inhibitors disrupt interaction between CIITA and co-repressors. FLAG-tagged WT CIITA construct was transfected into 293FT cells. 24 h later, cells were treated with D4476 or CKI-7 (5 μM) as indicated for additional 24 h before harvesting. Whole cell lysates were prepared and immunoprecipitated (IP) with FLAG antibody (M2). Westerns were immunoblotted (IB) with FLAG, Sin3B, and HDAC2 antibodies as described under "Experimental Procedures." One of two independent experiments is shown. C, D4476 abrogates the cooperation between CIITA and Sin3B in down-regulating collagen promoter activity. A COL1A2 promoter construct (pH 20, 0.5 μg) was co-transfected with WT CIITA construct (0.25 μg) as well as with Sin3B construct (0.25 μg), along with GFP (0.1 μg) for normalization, into IMR-90 cells in triplicate wells as described under "Experimental Procedures." 24 h later, cells were treated with or without D4476 (5 μM) for additional 24 h before harvesting. Average luciferase activities were normalized by both protein concentration and GFP fluorescence. This representative experiment was repeated at least three times. D4476 significantly decreases the repression of collagen promoter activity by CIITA with or without Sin3B (**, p < 0.01). D, D4476 alleviates the repression of collagen promoter activity by IFN-γ. Three different collagen type I promoters (0.5 μg) were transfected, along with GFP (0.1 μg) for normalization, into IMR-90 cells in triplicate wells as described under "Experimental Procedures." 24 h later, cells were treated with or without D4476 (5 μM) in the absence or presence of IFN-γ (100 units/ml) for an additional 24 h before harvesting. Average luciferase activities were normalized by both protein concentration and GFP fluorescence. This representative experiment was repeated at least three times. D4476 significantly decreases the repression of all three collagen promoter activities by IFN-γ (**, p < 0.01). E, D4476 and CKI-7 alleviate the repression of endogenous collagen expression by IFN-γ. IMR-90 cells were treated with IFN-γ (100 units/ml) in the presence of either D4476 or CKI-7 (5 μM) as indicated for 24 h. Total RNA was extracted and transcribed to cDNA, and real-time PCRs were performed with the cDNA samples using primers to detect COL1A2 mRNA (left) or HLA-DRα mRNA (right). Each experiment was repeated at least three times in triplicate wells. Data are expressed as relative RNA levels compared with control levels, normalized to 18S RNA and presented as average ± S.D. Both D4476 and CKI-7 significantly alleviate the repression of collagen mRNA (**, p < 0.01) by IFN-γ without altering MHC II expression.
H3 and H4 around the same site (44), raising the possibility that CIITA may directly interact with co-repressors as a means of down-regulating the expression of target genes such as collagen.

Here we report that CIITA interacts with the co-repressor Sin3B in a phosphorylation-dependent manner controlled by GSK3 and CK1 to mediate IFN-γ-induced transcriptional repression of type I collagen genes. CIITA directly interacts with co-repressors Sin3B and HDAC2 (Fig. 1A), which depend on the phosphorylation of the PST region (Fig. 1, B and C) and, more specifically, serine 373 (Fig. 3A). CIITA recruits Sin3B/HDAC2 to the collagen transcription start site during the IFN-γ response (Fig. 5D). The interaction between CIITA and this co-repressor complex defines the outcome of a specific transcription event because inhibition of CKI/GSK3 activity disrupts the interaction and attenuates CIITA-mediated repression of collagen synthesis (Figs. 4B and 7A). Low levels of Sin3B/HDAC2 were recruited to the MHC II promoter (Fig. 5D), which does not disrupt expression of MHC II (Figs. 5B and 7E). Accordingly, mutation of the critical serine 373 responsible for mediating the CIITA-Sin3B interaction did not significantly alter the activation of MHC II transcription as it did the collagen gene (Fig. 2, A and B). A model summarizes our current hypothesis of the mechanism for collagen repression and MHC II activation (Fig. 9). Our finding not only reveals a novel complex in which CIITA is involved, but it also provides specific functional relevance for this complex.

CIITA was initially established as the master regulator of MHC II transcription (55). One primary characteristic of CIITA is its ability to act as a scaffold protein and participate in multicomponent complexes. It is well documented that CIITA interacts with Sin3A and HDAC1/HDAC2 to down-regulate MHC II (38).
CIITA Mediates Repression of Collagen

CIITA is able to modulate the transcription of a variety of genes, including cytokines (25, 32), extracellular matrix components (2, 60), cell survival signaling molecule (35), antigen-processing proteaseinase (31), and a T cell activation gene (40). Whereas the mechanism by which CIITA activates MHC II transcription has been extensively studied, the mechanisms by which CIITA controls the transcription of other target genes remains controversial (61). It was postulated that CIITA might compete with transcription factors interacting with the universal co-activator CBP/p300 as a mechanism for its repression of the IL-4 transcription (25) and collagen type I transcription (2). The interaction between CIITA and co-repressor molecules demonstrated for collagen transcriptional repression may also be critical for repression of other genes.

CIITA is the target of multiple post-translational modifications, including phosphorylation (30), acetylation (29), and ubiquitination (62). In particular, the proline-serine/threonine (PST) domain is essential for the repression of collagen type I genes by CIITA (4, 41). This domain, which spans from amino acids 253 to 410, contains more than 20 putative phosphorylation sites, which could generate a large number of potential combinations of phosphorylation that would in turn dictate the specific outcomes of different transcriptional events involving CIITA. In this paper, in vitro kinase assays establish that CIITA is a substrate for GSK3 phosphorylation (Fig. 6A), adding CIITA to a list of transcriptional regulators that are under the influence of GSK3. In a number of cases, phosphorylation by GSK3 leads to proteasome-dependent degradation of its targets. The most canonical event occurs in the Wnt signaling pathway where β-catenin is targeted for breakdown following GSK3 phosphorylation. Other GSK3 substrates in the Wnt signaling pathway (63–65) or outside (66) are targets that get degraded. However, our experiments did not find CIITA expression levels to be altered by GSK3 activity (Fig. 5C and data not shown).

Alternatively, GSK3 phosphorylation changes the localization of target proteins inside the cells (67). Although this has not directly been examined here, it is highly unlikely that CIITA localization would be affected by GSK3 activity because MHC II expression, which depends on the recruitment of CIITA to its promoter in the nucleus, was not significantly changed whether

FIGURE 9. Model of collagen repression and MHC II during IFN-γ treatment by CIITA. This model is a schematic representation diagramming the current understanding for how CIITA can repress collagen and activate MHC II transcription. CIITA belongs to the NLR family containing a nucleotide-binding domain (NBD) and a leucine-rich repeat (LRR). IFN-γ-dramatically increases CIITA expression and activates RFX5 in mesenchymal cells. CK1 phosphorylates Ser-377 in the CIITA PST domain that is a priming site for GSK3 to phosphorylate Ser-373. Sin3B within an HDAC2 containing co-repressor interacts with CK1/GSK3-phosphorylated CIITA. RFX5 recruits CIITA-co-repressor complex to the collagen start site repressing collagen transcription and decreasing histone acetylation (AC). On the other hand, CIITA can interact with histone acetyltransferases (HAT) (22, 29), which is recruited through RFX5 to the MHC II enhanceosome. The two promoters have binding sites for three proteins (RFX5, TFIID, and NFY) in different relationships to each other that interact with CIITA.
CIITA Mediates Repression of Collagen

GSK3 was active or not (Fig. 5B). Moreover, the in vivo recruitment of CIITA to its target genes in the nucleus (COL1A2 and HLA-DRα) also remained unaffected regardless of the GSK3 status (Fig. 5D), suggesting that there was no altered trans-localization of CIITA. Instead, phosphorylation by GSK3 not only allows CIITA to interact with Sin3B (Figs. 3A, 4C, and 5C) but also necessitates the recruitment of co-repressors to the collagen transcription start site by CIITA during the IFN-γ response in vivo (Figs. 3B and 5D). More importantly, GSK3 activity is required to maintain the repression of collagen type I gene transcription by CIITA (Fig. 4B). Therefore, this investigation provides a novel mechanism by which GSK3 regulates the activity of its substrate.

Interestingly, GSK3 phosphorylation often depends on priming by another kinase. For instance, phosphorylation of p53 by GSK3 requires prior phosphorylation at serine 37 by DNA-dependent protein kinase, which enhances its transcriptional activity (68). On the other hand, GSK3 has been demonstrated to act synergistically with casein kinase II (CKII) to provide feedback regulation for a tumor suppressor (69). There are also many examples in which GSK3 phosphorylation has been found to be preceded by CKI, including the canonical Wnt signaling pathway (53), and tumor suppressor von Hippel-Lindau (52). This investigation demonstrates that CKI phosphorylates CIITA directly in vitro (Fig. 6B). This event permits GSK3 phosphorylation to take place (Fig. 6C). Similar to GSK3, CKI phosphorylation results in diverse consequences, including target stability (70) and trans-localization (71). Our findings here demonstrate that CKI facilitated the interaction between CIITA and Sin3B (Fig. 7B) and that CKI activity was required to maintain CIITA-mediated collagen transcriptional repression (Fig. 7A), thus expanding the list of protein targets and functions of CKI.

Our results suggest that CIITA interacts through the phosphorylated GSK3 site most strongly with Sin3B. Sin3 was first isolated from budding yeast Saccharomyces cerevisiae as a regulator of the HO gene, encoding for an endonuclease involved in mating type switch (72). Two mammalian homologs, Sin3A and Sin3B, have been identified with Sin3B having a shorter N-terminal region (73). The functional difference between these two isoforms remains unknown. Sin3 protein usually associates with other factors to form a so-called “core complex,” which then establishes additional contacts with regulators to achieve specific effects (74–76). The exact composition of the core complex is ill defined in most organisms. Although the generally accepted viewpoint is that any given core complex should include Sin3A/B and HDAC1/2, there is evidence that one or more components could be missing. For instance, it has been shown that HDAC1 is absent from a Sin3A-containing Brg1 chromatin remodeling complex (77), suggesting that there is a certain level of flexibility in the composition of the complex, which may depend on the specific promoter it is binding to and the specific biological event it regulates.

Previously, others have reported that CIITA might interact with the HDAC1-Sin3A complex to terminate MHC II activation (38). On the other hand, HDAC2, but not HDAC1, is recruited to the collagen transcription start site along with CIITA during the IFN-γ response (37). Moreover, Sin3B occupancy on the collagen promoter increases significantly, whereas Sin3A binding decreases accordingly on the same site in response to IFN-γ (37), indicating that HDAC2-Sin3B, rather than HDAC1-Sin3A, is involved in repression of collagen transcription mediated by CIITA. Indeed, a distinctive Sin3-HDAC complex, different from previously described, is associated with CIITA on the collagen transcription start site and represses collagen transcription. The exact biological relevance of these phenomena still needs further elucidation.

Although Sin3 protein has been implicated in a wide range of biological events, it has no known enzymatic activity and is thought to play a structural role in stabilizing complexes. The specificity of the complex relies on the different factors with which Sin3 interacts. For instance, our earlier results indicate that Sin3A, but not Sin3B, is capable of associating with a DNA methyltransferase-containing complex and is involved in DNA methylation of collagen genes in a number of cancerous tissues (37, 78). Other investigators connected Sin3 to chromatin remodeling (77), transcriptional elongation (79), histone methylation (80), DNA damage repair (81), and replication control (82). Here we found Sin3B in a complex together with HDAC2 and CIITA (Figs. 1 and 3). In agreement with previous findings that Sin3 proteins mainly contribute to maintaining the integrity of the complex, Sin3B alone did not change collagen promoter activity. It did, however, significantly down-regulate collagen promoter activity in the presence of CIITA (Fig. 4D).

There has been accumulating evidence that phosphorylation of protein factors affects their association with Sin3. An Spr-1-like repressor (TIEG2) interacts with Sin3A, and epidermal growth factor-induced phosphorylation of TIEG2 disrupts its association with Sin3A relieving transcription repression (83). Similarly, phosphorylation of p53 also abrogates its association with Sin3A and targets it for degradation (24). Here phosphorylation of CIITA increases the stability of its association with Sin3 proteins. More importantly, the particular transcriptional event it regulates depends on the phosphorylation of CIITA by GSK3/CKI.

Previously, we have determined that CIITA mediates the repression of type I collagen transcription by IFN-γ (4). New data presented here show that two kinases, GSK3 and CKI, sustain IFN-γ-mediated repression of collagen synthesis (Fig. 5, A and B, Fig. 7, D and E, and Fig. 8C), possibly through phosphorylation-dependent interaction between CIITA and Sin3B. This suggests possible cross-talk between IFN-γ and GSK3/CKI. There have been very few reports where GSK3 is implicated in an IFN-γ-mediated event, whereas CKI has not been found to be involved with the IFN-γ signaling. GSK3 is usually active in resting cells but can be inactivated by several important signaling pathways (including PI3K/Akt, mTOR/S6K, and MAPK/MAPKAP-K1 and Wnt signaling) (48). In murine macrophages, IFN-γ stimulates GSK3 activity by decreasing phosphorylation of AKT downstream of GSK3 phosphorylation (46). In turn, IL-10 production is down-regulated in part through inhibiting GSK3-dependent DNA binding capability of an essential transcriptional factor, AP-1. Therefore, our data that IFN-γ down-regulates collagen synthesis through GSK3/CKI-dependent phosphorylation of CIITA provide a novel example of cross-talk between IFN-γ and kinases. Curiously, the specific GSK3 site serine 373, which was studied here by employing human constructs and human cell lines, is not con-
CIITA Mediates Repression of Collagen

served in mouse. This might help explain why IFN-γ does not repress collagen transcription in mouse cell culture quite as efficiently it does in human cells (3).

Despite that fact GSK3 and CK1 inhibitors reduced the interaction between CIITA and co-repressors (Figs. 4C, 5C, 7B, and 8A), they did not completely abolish the repression of collagen transcription by CIITA (Figs. 4E and 7A) and IFN-γ (Fig. 8C). There are several explanations to this apparent discrepancy. First, CIITA has more than 30 putative phosphorylation sites both activates and abrogates IFN-γ-mediated repression. It should be pointed out that these mechanisms can exist simultaneously to determine the end result of collagen transcription, it is a cumulative effect that determines the ultimate outcome of collagen synthesis. Finally, our focus of research has been directed to the PST region of CIITA, which we found to be sufficient to repress the basal collagen type I promoter (3, 4) and possibly through phosphorylation-dependent recruitment of co-repressors (45). At the same time, co-activators CBP/p300 as well as transcription factors STAT6/NF-AT1 are prevented from binding to the IL-4 promoter, possibly through CIITA activation domain-mediated interaction with these factors.

In summary, we provide new evidence here that transcription repression of type I collagen genes is mediated at least in part through GSK3/CK1-dependent phosphorylation of CIITA and its interaction with co-repressor molecules. Our data not only identify a novel Sin3B-HDAC2 complex associated with CIITA and link this complex to a particular transcription event, but they also shed light into possible cross-talk between the IFN-γ and kinase signaling.

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CIITA Mediates Repression of Collagen

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