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Steroid Receptor Coactivator 1 Links the Steroid and Interferon γ Response Pathways

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We show here that steroid receptor coactivator 1 (SRC-1) is a coactivator of MHC class II genes that stimulates their interferon γ (IFNγ) and class II transactivator (CIITA)-mediated expression. SRC-1 interacts physically with the N-terminal activation domain of CIITA through two regions: one central [extending from amino acids (aa) 360–839] that contains the nuclear receptors binding region and one C-terminal (aa 1138–1441) that contains the activation domain 2. Using chromatin immunoprecipitation assays we show that SRC-1 recruitment on the class II promoter is enhanced upon IFNγ stimulation. Most importantly, SRC-1 relieves the inhibitory action of estrogens on the IFNγ-mediated induction of class II genes in transient transfection assays. We provide evidence that inhibition by estradiol is due to multiple events such as slightly reduced recruitment of CIITA and SRC-1 and severely inhibited assembly of the preinitiation complex. (*Molecular Endocrinology* 17: 2509–2518, 2003)

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) class II antigens are essential determinants in the regulation of the immune response. Their respective genes are expressed in a tightly regulated manner: constitutive in professional antigen-presenting cells and cytokine induced in most other cell types. Transcription of MHC class II genes requires the assembly of the enhanceosome that contains regulatory factor X (RFX), X2BP/cAMP response element binding protein, and nuclear factor Y multimeric factors on the S-X-X2-Y enhancer. Enhancers of the S-X-Y type are also found in MHC class I genes (1) and other genes that control assembly and transport of MHC class II proteins (2–5). Thus the class II enhanceosome appears to have a general role in the regulation of antigen presentation and immune response.

The analysis of bare lymphosite syndrome mutants (6, 7) has allowed the cloning of the three RFX subunits and most importantly class II transactivator (CIITA). CIITA is expressed constitutively in professional antigen-presenting cells and is induced by interferon γ (IFNγ) in most other cell types. CIITA has four different promoters [I–IV (8)] that drive the synthesis of at least three different mRNAs. pIV is mainly responsible for induction by IFNγ, which occurs at the transcriptional level and is preceded by the activation of the Janus family of tyrosine kinases (Jak)/signal transducer and activator of transcription (Stat) pathway. IFNγ-induced synthesis of CIITA type IV depends on STAT1, IFN-regulated factor 1, and upstream stimulatory factor (9–11).

In addition to being the major positive regulator of class II gene expression (12, 13), CIITA is the target of many factors that negatively regulate these genes such as TGFβ (14) prostaglandins (15, 16), and statins (17). CIITA does not bind to DNA but is recruited onto the class II promoter via multiple interactions with the enhanceosome (18, 19) and activates transcription through interactions with various components of the basal transcription machinery, *i.e*. TATA-binding protein (TBP) (20) and TBP-associated factor$_{II}$32 (21), as well as members of the acetyl-transferase (AT) type versatile coactivators cAMP response element binding protein (CREB)-binding protein (CBP)/p300 and pCAF (p300/CBP-associated factor) (22–24).

The nuclear receptor coactivators nuclear coactivator 1 (NcoA-1)/steroid receptor coactivator 1 (SRC-1) (25, 26), NcoA-2/SRC-2/transcriptional intermediary factor 2 (TIF-2)/glucocorticoid receptor interacting protein 1 (GRIP-1) (27), and NcoA-3/RAC-3 (receptor-associated coactivator 3)/activator of the thyroid and retinoic acid receptor (28, 29) constitute a separate class, which shows gene type specificity and ligand binding dependence of their ability for receptor binding. However, recent evidence that implicates NcoAs in nonnuclear receptor-mediated transcription *i.e.* nuclear factor-κB (NFκB) (30, 31), serum response factor

Abbreviations: aa, Amino acids; CBP, cAMP response element binding protein (CREB)-binding protein; CIITA, class II transactivator; E2, estradiol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GRIP, glucocorticoid receptor interacting protein; GST, glutathione-*S*-transferase; HLA, human leukocyte antigen; IFN, interferon; MHC, major histocompatibility complex; NcoA, nuclear coactivator; NFκB, nuclear factor-κB; NP-40, Nonidet P-40; pCAF, p300/CBP-associated factor; RAC, receptor-associated coactivator; RFX, regulatory factor X; SRC, steroid receptor coactivator; Stat, signal transducer and activator of transcription; TBP, TATA-binding protein; TIF, transcriptional intermediary factor.

(32), activator protein 1 (33), STAT6 (34), STAT3 (35), has lately expanded their role in gene regulation.

Because the NcoA family of nuclear receptor coactivators (29) interact with CBP/p300 and PCAF, which have been shown independently to interact and synergize with CIITA (22–24), we investigated the role of NcoA coactivators in IFNγ- and CIITA-mediated transactivation. We demonstrate that two noncontiguous regions that contain the nuclear receptor interaction and the carboxy-terminally located activation domain 2 (AD2) region of SRC1 bind independently to the activation domain of CIITA. SRC-1 and the other members of the p160 coactivator family synergize with IFNγ and CIITA to induce MHC class II transcription. SRC-1 is recruited on the class II promoter upon IFNγ induction. Interestingly, estradiol inhibits the IFNγ-induced transcription of MHC class II genes and this inhibition is relieved by overexpression of SRC-1. We show here that the observed competition between IFNγ and estradiol on the class II DRA gene transcription is due to defective preinitiation complex assembly and, to a lesser degree, reduced CIITA and SRC-1 recruitment. Thus, contrary to an expected SRC-1 squelching mechanism, multiple events contribute and lead to the estradiol-mediated inhibition of IFNγ induction of the DRA gene.

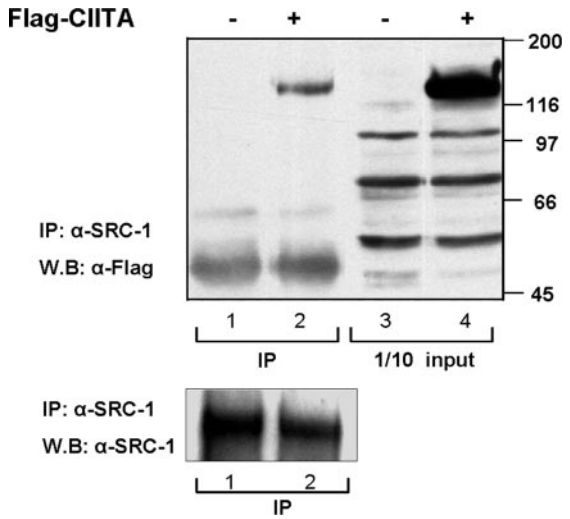

Fig. 1. *In Vivo* Interaction Between SRC-1 and CIITA

Whole-cell extracts from COS-7 cells transfected with a plasmid expressing Flag-CIITA (+) or empty vector (−) were immunoprecipitated with anti-SRC-1 antibody (M341, Santa Cruz Biotechnology, Inc.), followed by Western blot analysis using an anti-Flag (Eastman Kodak) monoclonal antibody and subsequently an anti-SRC-1 antibody (lanes 1 and 2) as control for immunoprecipitation efficiency. Inputs in lanes 3 and 4 represent 10% of the extract that was used for immunoprecipitation.

RESULTS

SRC-1 Interacts with CIITA

To examine the possibility of SRC-1 involvement in MHC class II transcription, we first asked whether it could interact with CIITA, which is the main transcriptional activator of these genes (12). To investigate whether CIITA and SRC-1 interact *in vivo* we used immunoprecipitation experiments using cell extracts from COS-7 transfected with a Flag-tagged CIITA expressing plasmid. Figure 1 shows that immunoprecipitation of endogenous SRC-1 with a specific antibody was able to coprecipitate transfected Flag-CIITA as revealed by Western blotting with anti-Flag antibody (lane 2).

To detect whether the observed interaction between CIITA and SRC-1 is direct, we used glutathione-*S*-transferase (GST) pull-down experiments employing the GST fusions of CIITA depicted in Fig. 2 and ^{35}S-labeled SRC-1. Figure 2 shows that a full-length CIITA interacts with SRC-1 (lane 1). The main interacting region is located to the amino terminal 1–408 aa (lane 4). Within the amino-terminal region, the first 114 aa of CIITA interacted strongly with SRC-1 (Fig. 2 lane 5), whereas the region extending from aa 114–550 (lanes 6 and 7) showed a weak interaction. The amino-terminal CIITA region that interacts with SRC-1 was further narrowed down to the first 80 aa (lane 13).

Using GST fusions of SRC-1 (Fig. 3), we show that the amino-terminal region of CIITA (aa 1–114 and 114–408) interacted with two SRC-1 regions: one containing the nuclear receptors interaction domain (fragment B) and one containing the carboxy-terminal AD2 (fragment D). The carboxy-terminal part of CIITA spanning aa 408-1130 (Δ408) interacted weakly with fragment D (as assessed and by data not shown). The specificity of these interactions was further verified by the ability of CBP to interact with regions C and C1 as expected (36).

Members of the p160 Family Potentiate the IFNγ-Induced Transcription of MHC Class II Genes

We studied the effect of overexpressed SRC-1 on the IFNγ-induced transcription of MHC class II genes (37, 38). HeLa cells were transfected with a luciferase reporter driven by an Eα class II promoter along with a construct expressing human SRC-1α. After transfection, cells were stimulated with IFNγ. Figure 4A shows that IFNγ induced the activity of Eα class II promoter, and SRC-1 was able to further stimulate it by 3-fold. Importantly, SRC-1 addition had no effect on the uninduced Eα expression levels.

Since IFNγ-induced transcription of MHC class II genes is mediated by the class II transactivator CIITA, we next checked whether CIITA was targeted by SRC-1. Figure 4B shows that expression of CIITA in HeLa cells led to activation of the class II promoter comparable to the IFNγ-induced levels. When increasing amounts of SRC-1 were also expressed, the CIITA-mediated transcription of MHC class II genes was further induced up to 8-fold (Fig. 4B), thus establishing

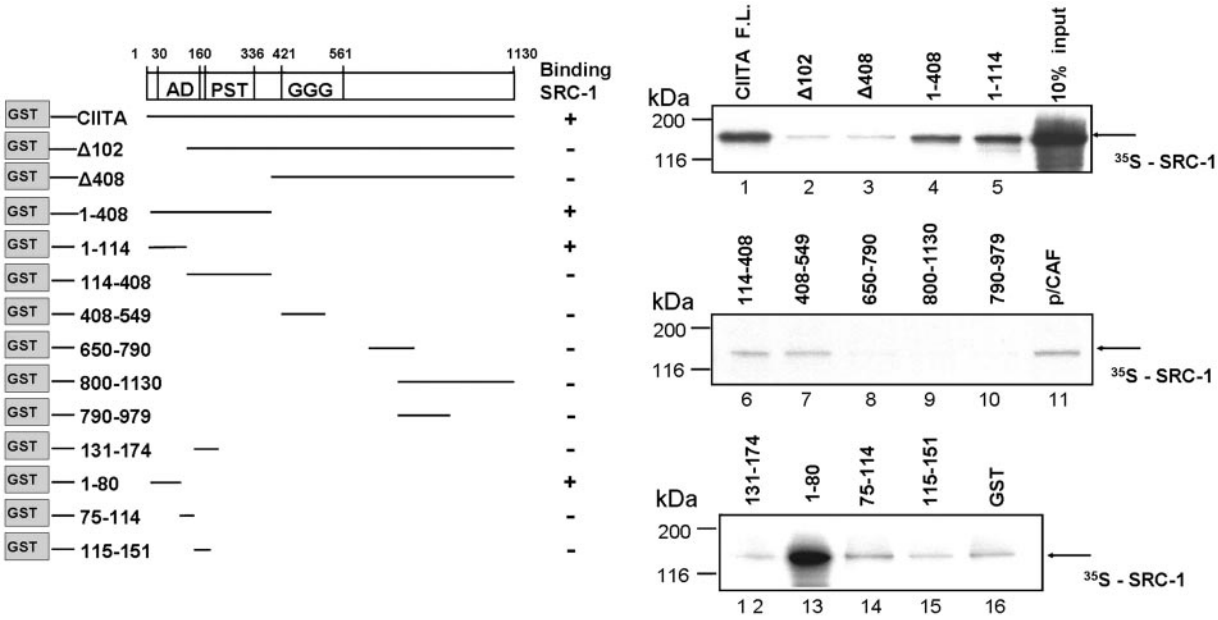

Fig. 2. The N-Terminal Domain of CIITA Binds SRC-1 *in Vitro*

GST fusions of different parts of CIITA (shown diagrammatically on the *left*) were tested for interaction with SRC-1. CIITA contains an acidic transcriptional activation domain (AD: aa 30–160), a region rich in proline, serine, and threonine (PST: aa 163–336) and three GTP-binding motifs (GGG: aa 421–561). *In vitro* translated ^{35}S-labeled hSRC-1α was used in GST pull-down assays with equal amounts (2 μg) of GST alone or fusions to the indicated CIITA fragments. Autoradiography was performed after SDS-PAGE analysis of the interacting pairs.

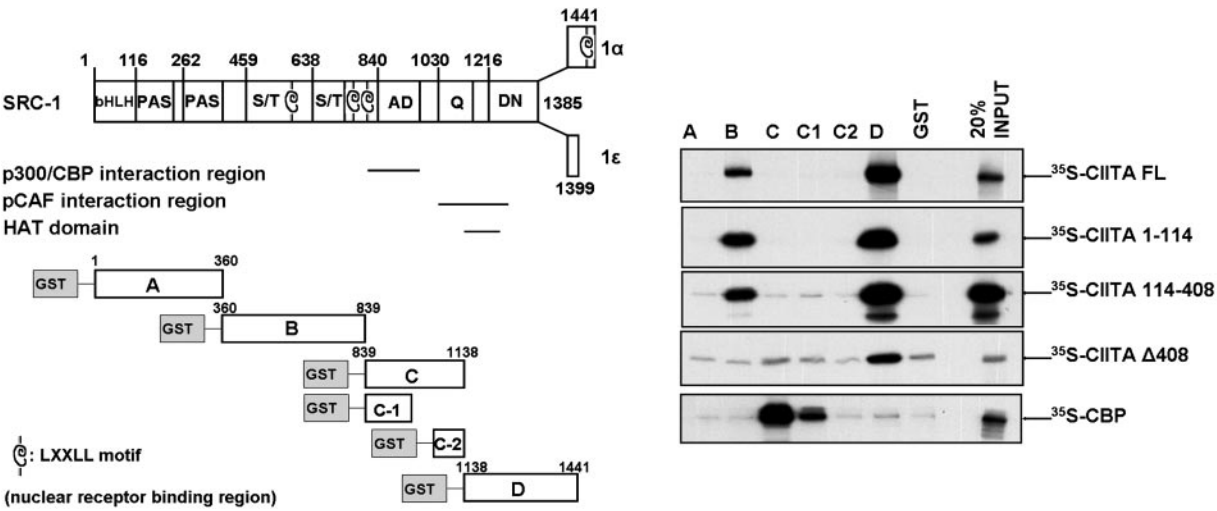

Fig. 3. Two Distinct Regions of SRC-1 Interact with CIITA *in Vitro*

A schematic drawing of SRC-1 characteristic regions along with the fragments that were fused to the GST are shown on the *left*. *In vitro* ^{35}S-labeled CIITA, full length or the indicated fragments, were used in a GST pull-down assay with equal amounts (2 μg) of GST alone or GST fused to the indicated regions of SRC-1. The retained proteins were separated by SDS-PAGE and subjected to autoradiography.

the role of SRC-1 on CIITA function. Previous studies have characterized the N-terminal activation domain of CIITA and its ability to interact with coactivators using fusions with the Gal4-DNA binding domain (22, 39). To investigate whether SRC-1 affects the transactivation function of CIITA, we used Gal4-fusions of diverse CIITA fragments directed to a reporter under the control of Gal4 sites. Figure 4C shows that full-length CIITA or its amino-terminal region (aa 1–408), which contains the activation domain, when fused to Gal4, led to high levels of promoter activity. In the presence of SRC-1, this activation was further augmented by 2.5-fold, indicating that SRC-1 can interact and cooperate with the activation domain of CIITA. A

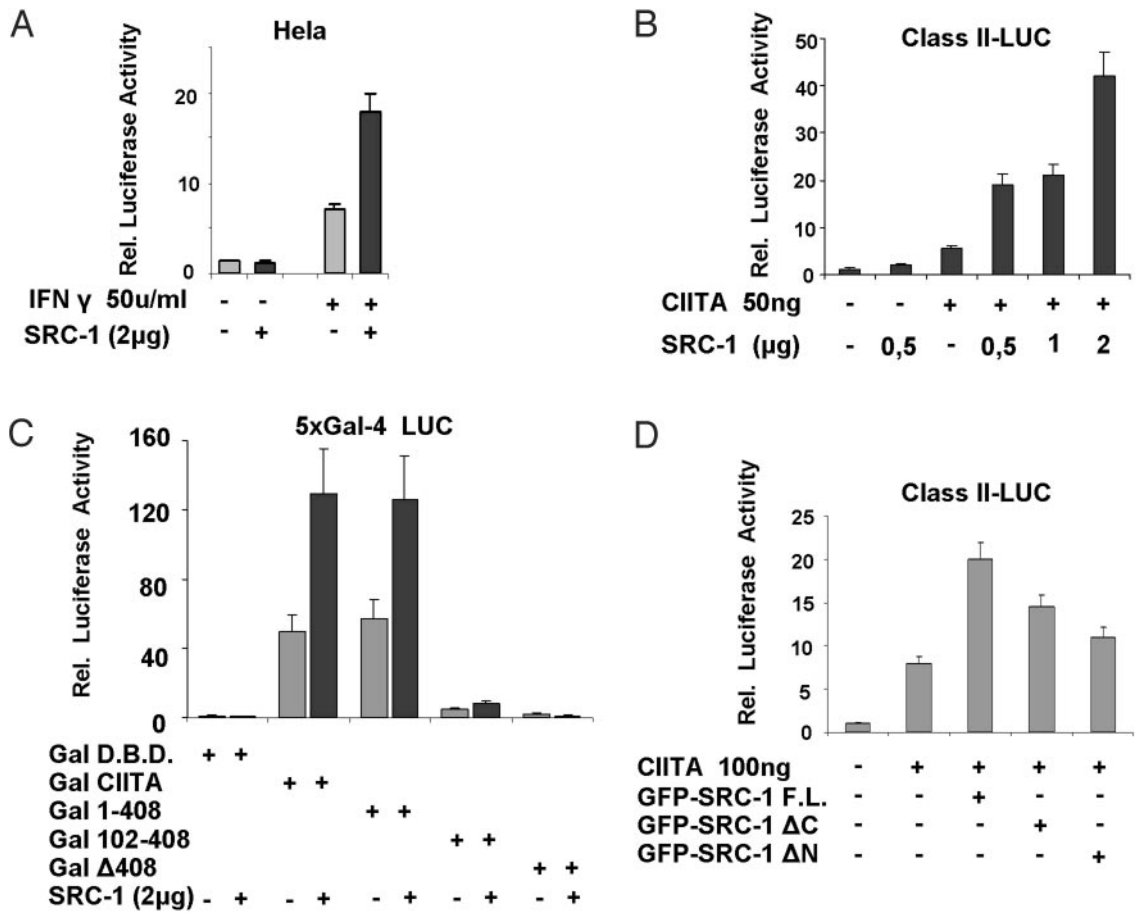

Fig. 4. SRC-1 Augments the IFNγ and CIITA-Induced Expression of MHC Class II Genes

A, HeLa cells were transiently transfected with 1 μg of a class II promoter-luciferase reporter as well as 1 μg pCMV-β-gal and 2 μg PCR3.1 hSRC-1α (+) or empty vector (−) as indicated. Cells were treated with 50 U/ml IFNγ (R&D Systems) for 20–24 h before harvesting, luciferase activity was measured 36 h posttransfection, and values were normalized relative to the untreated control vector cells, set to 1. B, MHC class II-luciferase reporter activity was assayed in the presence of 50 ng CIITA expressing plasmid and increasing amounts of SRC-1 expressing plasmid (HeLa cells). C, HeLa cells were transfected with 100 ng of the indicated Gal4BD-CIITA fusion constructs in the presence or absence of 2 μg SRC-1α expressing plasmid, along with 1 μg 5x Gal4-luciferase reporter and 1 μg pCMV-β-gal as internal control for value normalization. D, MHC class II-luciferase reporter activity was measured in the presence of 100 ng CIITA expressing plasmid and 2.5 μg of the indicated GFP-SRC-1 expressing plasmids (full length or deletion). The activity of reporter plasmids was assayed 36 h posttransfection, and luciferase activity was normalized for β-gal (HeLa cells).

Gal4 fusion of CIITA (aa 102–408) displayed a weak intrinsic activation ability that was also slightly augmented by SRC-1. Finally, the Gal4 fusion of the C-terminal (aa 408-1130) region of CIITA that is devoid of activation function was not affected by coexpression of SRC-1. To determine the relative importance of each one of the two CIITA interacting domains of SRC-1, we used two SRC deletions fused to green fluorescent protein (GFP): SRC-1-ΔC and SRC-1-ΔN lack the C- and N-terminal parts of the molecule, respectively. Inspection by direct microscopy and quantitation by flow cytometry and immunoblotting showed that the SRC truncations were expressed at similar levels in comparison to the full-length SRC-1 (not shown). These truncations were cotransfected with CIITA, and their coactivation potential was determined. Figure 4D shows that the coactivation ability of ΔC and ΔN was reduced to about 66% and 50% of that obtained by the full-length SRC-1. Thus, both CIITA interaction domains of SRC-1 contribute significantly to its coactivation function.

We next ask whether members of the p160 family other than SRC-1 have the same effect on MHC class II gene transcription. Figure 5A shows that SRC-2/TIF-2 and SRC-3/RAC-3 behave similarly to SRC-1 and potentiate the IFNγ-induced transcription of a class II gene. We also examined whether SRC-2/TIF-2 and SRC-3/RAC-3 could synergize with CIITA in the transcriptional activation of a class II gene. Figure 5B shows that all three categories of p160 family of coactivators are equally capable to potentiate CIITA's transactivation function.

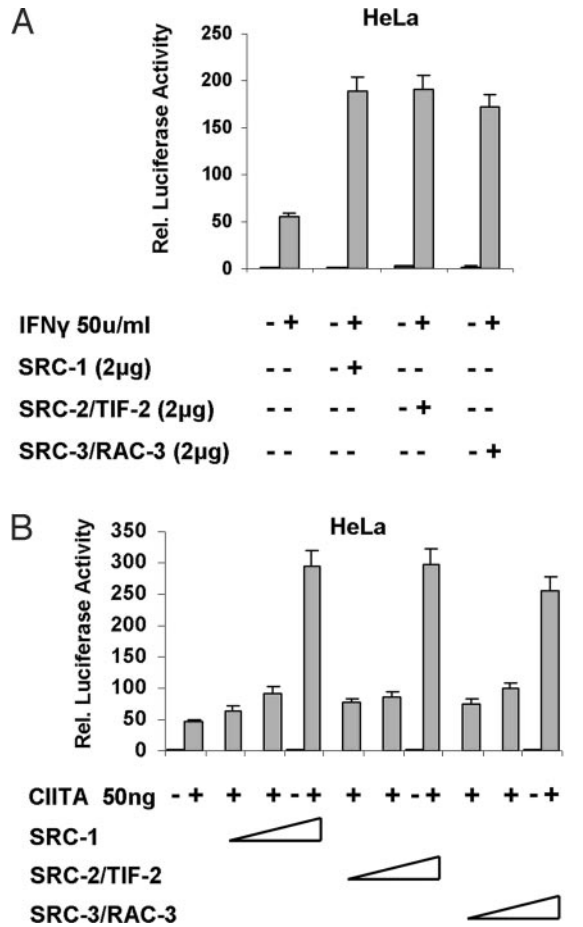

Fig. 5. p160 Family Members as Coactivators of the IFNγ- and CIITA-Induced Transcription of MHC Class II Genes

A, HeLa cells were transfected with 1 μg of a class II-luciferase reporter as well as 2 μg of different SRC family members expressing plasmids as indicated. Cells were treated or not with 50 U/ml IFNγ (R&D Systems) for 20–24 h before harvesting, and luciferase activity was measured 36 h posttransfection. B, HeLa cells were transfected with 50 ng CIITA expressing vector and increasing amounts (0.5–2 μg) of the indicated SRC family members.

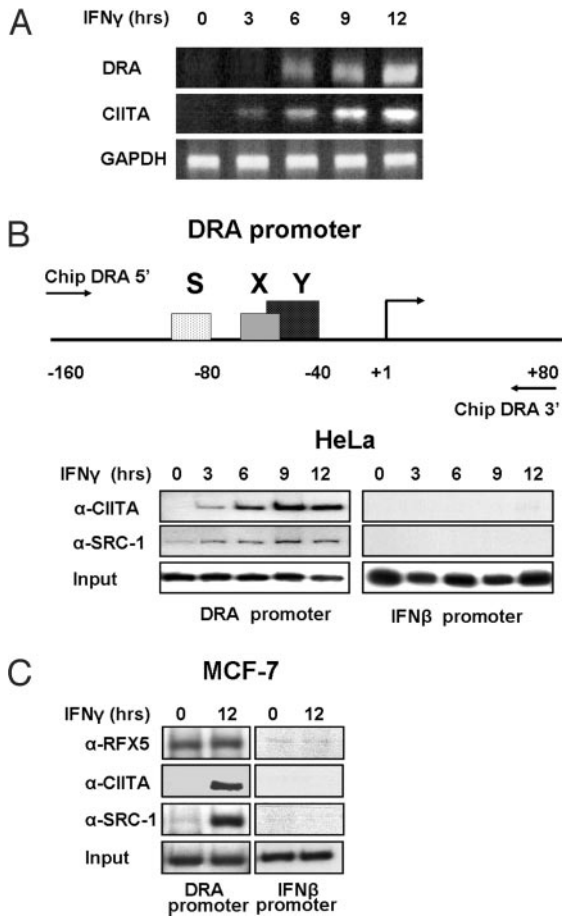

Fig. 6. SRC-1 Is Recruited to the MHC Class II Promoter by IFNγ Induction

A, Time course of IFNγ-induced mRNA for DRA class II gene and CIITA transactivator. RT-PCR analysis was performed on HeLa cells treated with 100 U/ml IFNγ for the indicated time periods. GAPDH mRNA was also checked as internal control. B and C, Chromatin immunoprecipitation assays employing HeLa (B) or MCF-7 (C) cells treated with 100 U/ml IFNγ for the indicated time periods. The occupancy of HLA-DRA and IFNβ promoters (negative control) by CIITA, SRC-1, and RFX-5 was evaluated by radioactive semiquantitative PCR analysis of the immunoprecipitated material using the primers described in *Materials and Methods*. Input represents 4% of the immunoprecipitated material.

IFNγ Enhances Occupancy of the DRA Class II Promoter by SRC-1

We next examined whether SRC-1 is bound to the class II promoter under physiological conditions. HeLa cells were induced with IFNγ and after cross-linking *in vivo*, chromatin immunoprecipitation experiments were performed using the indicated antibodies. Kinetic experiments performed in HeLa cells showed that DRA mRNA is first detected 6 h after IFNγ addition (Fig. 6A). CIITA mRNA is synthesized 3 h after IFNγ addition (Fig. 6A), and at the same time CIITA protein is bound on the DRA promoter (Fig. 6B) and its binding further increased at 6 and 9 h. Low levels of SRC-1 were detected on the promoter before induction and increased in a way that paralleled the occupancy by CIITA to reach a maximum at 9 h postinduction (Fig. 6B). Similar experiments were conducted in steroid-responsive MCF-7 breast carcinoma cells. In this cell line, a 12-h induction by IFNγ led to high amounts of SRC-1 and CIITA bound on the class II promoter whereas the X box binding factor RFX5 that was abundant before IFNγ treatment did not increase (Fig. 6C). Using an antibody against another member of the p160 coactivator family, SRC-2/GRIP-1, similar data were obtained (data not shown). The specificity of the chromatin immunoprecipitation was examined by PCR using primers for the IFNβ promoter, a virus-inducible promoter, which, in our experimental context, serves as negative control for the factors examined (Fig. 6, B and C, *right panels*).

SRC-1 Relieves the Repressive Effect of Estradiol on the IFNγ-Induced Expression of Class II Genes

We showed that the nuclear receptors interaction region of SRC-1 (fragment B) contacts CIITA. Because SRC-1 is a coactivator of steroid-responsive genes (36), and CIITA interacts with SRC-1 via its nuclear hormone receptor region, we asked whether estrogen stimulation could affect MHC class II gene transcription. To this aim we used MCF-7 cells that express estrogen receptors. Figure 7A shows that the presence of 10^{-6} M estradiol (E2) reduced by 50% the induction of class II genes by IFNγ in MCF-7 cells (compare lanes 5 and 7). Interestingly, overexpressed SRC-1 relieved the negative effect of E2 on the inducible class II activity (Fig. 7A, lane 8), while retaining its relative coactivation ability (compare lanes 6 and 7 *vs.* lanes 7 and 8). These results are consistent with E2-mediated squelching of SRC-1 required for MHC class II activation. To verify that the above conclusions reflect endogenous gene behavior, the induction of the DRA gene by IFNγ in the presence or absence of estradiol was examined by quantitative real time PCR. Figure 7B shows that, in consistence with transient transfection results, E2 reduced the amount of IFNγ-induced DRA message to 38% of the control. In comparison, we observe that the mRNA levels of CIITA are marginally reduced in the presence of E2. Assays in Fig. 7, A and B, were repeated for T47D cells and similar results were obtained.

We then wished to examine the effect of simultaneous action of IFNγ and E2 on DRA gene in terms of *in vivo* factor recruitment using chromatin immunoprecipitation assays (Fig. 7C). When E2 was added along with IFNγ, we observed a slight reduction in SRC-1 and CIITA recruitment in comparison to the IFNγ control. In contrast, no difference was observed for the DNA binding factor RFX5, which is part of the class II enhanceosome. To further study the dynamic state of the promoter, we examined the recruitment of TBP and RNA polymerase II. Both these factors are inducibly recruited and this recruitment is severely inhibited by E2 (Fig. 7C). Therefore, multiple E2-mediated events cause reduced DRA gene transcription.

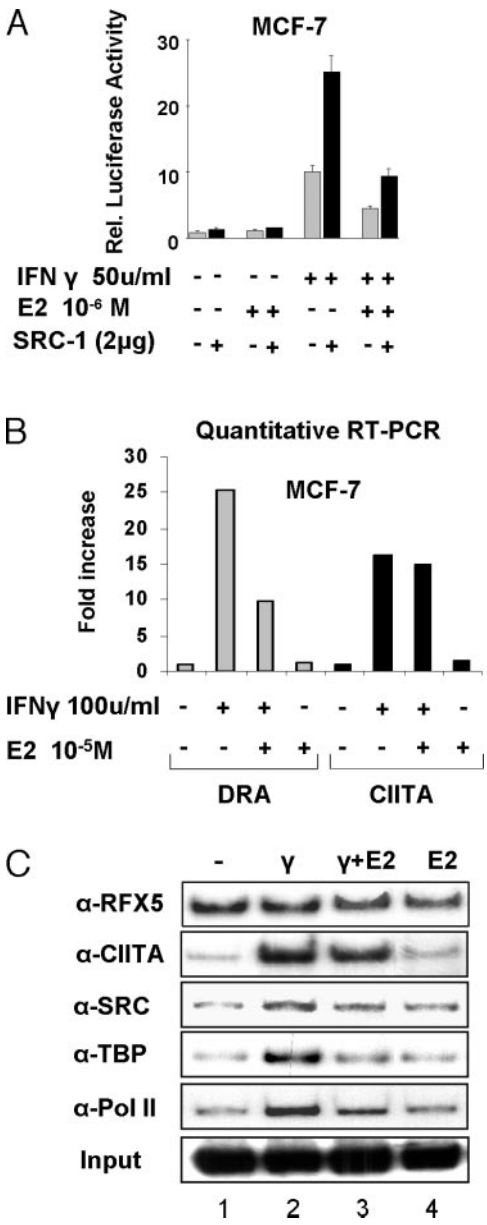

Fig. 7. SRC-1 Relieves the Inhibitory Effect of E2 on IFNγ-Induced MHC II Transcription

A, MCF-7 cells were transfected with 1 μg MHC class II-luciferase reporter in the presence or absence of 2 μg SRC-1-expressing plasmid and treated with 50 U/IFNγ and/or 10^{-6} M E2 for 20 h before harvesting. Luciferase activity was measured 36 h posttransfection, and values were normalized relative to the untreated control vector cells, set to 1. B, MCF-7 cells, cultured in 5% dextran-charcoal-stripped serum-supplemented medium, were treated with 100 U/ml IFNγ and/or 10^{-5} M E2 for 20 h before harvesting. Total RNA was extracted and after reverse transcription real-time PCR reactions were performed for DRA and GAPDH transcripts. Relative quantitation values for DRA (normalized to GAPDH) are shown diagrammatically. C, MCF-7 cells were treated with 100 U/ml IFNγ for 18 h and/or 10^{-5} M E2. Chromatin immunoprecipitation assays using the antibodies indicated on the *left* were performed to evaluate changes triggered by the combined action of IFNγ/E2 in the presence/recruitment of the respective proteins on the DRA promoter.

DISCUSSION

We show here that CIITA, the master regulator of MHC class II genes (12), synergizes with the p160/SRC/NcoA family of coactivators in the transcriptional regulation of MHC class II genes. The N-terminal activation domain of CIITA contacts two discrete regions of SRC-1, a central one that contains the nuclear receptor binding domain and the C-terminal region that harbors the AD2. Both CIITA-interacting SRC-1 regions are required for coactivation of class II genes (Fig. 4D and data not shown). However, the centrally

located one is most important because a deletion that removes it, SRC-1 ΔN, retains marginal coactivation (Fig. 4D and data not shown) although it is still able to bind CBP (Fig. 3, *scheme*).

SRC-1 binds to a CIITA region (aa 1–408) that is broader than the region previously shown to be required for CBP and PCAF interaction aa 1–114). It is therefore possible that SRC-1 might be involved in the activatory function of CIITA region 102–408 (22, 40). These interaction properties imply that CIITA may contact both CBP and SRC-1 at the same time through the N-terminal 1–102 aa and the neighboring 102–408 aa regions, respectively. In addition, CIITA and CBP bind to nonoverlapping parts of SRC-1 (36), and the same situation holds for the binding of SRC-1 and CIITA to CBP (22, 23, 26). This may allow the formation of a very stable trimeric complex between CIITA, CBP, and SRC-1 where each one contacts the other two proteins at the same time.

To study the role of SRC-1 in class II gene activation, under physiological conditions, we used chromatin immunoprecipitation using IFNγ-induced HeLa cells. There is weak class II promoter occupancy by SRC-1 before IFNγ induction. Upon IFNγ treatment, enhanced SRC-1 recruitment is observed, with temporal kinetics similar to CIITA. These results point to the importance of direct interaction with CIITA for the stable integration of SRC-1 into the class II enhanceosome. Class II promoter occupancy by CBP and GCN5 upon IFNγ induction follows similar kinetics with SRC-1 [Spilianakis *et al.* (40a)]. Therefore, all three coactivators function at the same step during IFN-induced transcription, as opposed to the estrogen response where p160 coactivators precede CBP and pCAF on target promoters (41).

One issue that requires elucidation is whether all these coactivators are simultaneously required during class II genes activation and/or whether they occupy a single promoter at the same time. Differences in the histone specificities of the various histone acetyltransferase activities, the need for the assembly of a scaffold to support efficient recruitment of RNA polymerase II, and also distinct protein modification functions may explain the advantage of multiple CIITA-coactivator interactions. SRC-1 bears a weak histone acetyltransferase activity (42) and was shown to potentiate transcription mainly through bridging activators with CBP (43, 44). Interestingly, a novel function has been recently assigned by showing that p160 coactivators can link activators to the coactivator-associated arginine methyltransferase (45). In addition both CIITA and SRC-1/NcoA interact with positive transcription elongation factor-b (P-TEFb) (46, 47), which is a critical complex for transcriptional elongation.

All three categories of the p160 SRC coactivator family (48) were shown to be equally strong as coactivators of MHC class II transcription in transient transfection assays. In addition both SRC-1 (Fig. 6) and SRC-2/GRIP-1 (data not shown) were found to be tethered on the class II promoter upon IFNγ treatment. However, the specific contribution of each one in the class II gene expression *in vivo* remains in question. Gene inactivation techniques employing neutralizing antibodies, siRNA, or cells from knockout mice are required to definitively establish whether different p160 coactivators are interchangeable or not in the IFNγ-induced transcription of class II genes. These approaches, when employed in other gene systems, have already provided valuable information about redundant or specific functions of these coactivators (49–53).

Because CIITA binds to the nuclear receptor-binding region of SRC-1, the question that arises is whether activation of a nuclear receptor pathway could affect IFNγ- or CIITA-mediated transcription. Diverse members of the nuclear receptors family, such as glucocorticoids (54, 55) and steroids (56, 57), behave as negative regulators of the IFNγ-inducible expression of MHC class II genes. In a previous report, dexamethasone was shown to inhibit the action of CIITA through competition for binding to CBP (23). Because dexamethasone was reported as a negative regulator of SRC-1 (58), we chose to use another MHC class II inhibitory steroid, E2, that also employs SRC-1, among other coactivators, to drive transcription (36). E2 treatment inhibits the induction of class II DRA transcription by IFNγ in MCF-7/T47D cells. At the same time E2 inhibits the IFNγ-inducible activity of a class II promoter. Importantly, overexpression of SRC-1 can compensate this effect as it would be expected if activation of the steroid pathway limited the availability for SRC-1 coactivator.

To determine the molecular mechanism underlying the competition between the signaling pathways of IFNγ and estradiol, we employed chromatin immunoprecipitation assays. Addition of E2 along with IFNγ produced a small reduction in the amount of SRC-1 and CIITA that is bound to the class II promoter. Therefore, the E2 inhibitory action cannot be attributed merely to squelching SRC-1 away from the promoter. Other mechanisms, such as posttranslational modifications and/or involvement of additional factors, might be involved. Furthermore, we found that the negative action of E2 on the IFNγ-induced transcriptions is accompanied by defective preinitiation complex assembly: both TBP and RNA Polymerase II show reduced occupation of the DRA promoter, which quantitatively correlates with transcription inhibition as measured by mRNA levels. Therefore cross-competition between estrogens and IFNγ pathways is caused by multiple pathways.

MATERIALS AND METHODS

Plasmids

The –2 kb to +14 region of the murine class II Ea gene was fused to the luciferase vector (Promega Corp., Madison, WI). Full-length CIITA or its derivatives were expressed from

PBXG1 (in frame with Gal4 binding domain), eukaryotic expression vector pCDNA3 (Invitrogen, San Diego, CA), or prokaryotic pGEX (in frame with GST) vector (Pharmacia Biotech, Piscataway, NJ). The 5x Gal4-luciferase reporter was a gift from D. Thanos, and the PCR3.1-hSRC-1α (full length) expressing construct was kindly provided by I. Talianidis. The hSRC-1α expression plasmid was used as a template for the purification of conveniently digested fragments cloned in frame with glutathione-*S*-transferase (GST) into pGEX vectors (Pharmacia Biotech). GFP-SRC-1 was constructed in the GFP-C1 vector (CLONTECH). GFP-SRC-1 ΔC (aa 1–839) was generated by *Bam*HI digestion of GFP-SRC-1 and religation. GFP-SRC-1 ΔN was created by cloning the *Bam*HI fragment (aa 840-1441) in frame with the GFP. SRC-2/GRIP-1, SRC-3/RAC-3 expression constructs, and αGRIP-1 (M343, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were kindly provided by I. Talianidis.

Cell Culture and Transfections

HeLa, MCF-7, and Cos-7 cell lines were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum. In experiments where E2 was added (1 μM, Sigma Chemical Co., St. Louis, MO), cells were cultured with the addition of 5% dextran-charcoal-stripped serum for 24–30 h before transfection.

Cells were transfected using various amounts of expression vectors, as indicated, along with 1 μg luciferase-reporter plasmid and 1 μg pCMV-β-gal, by the calcium-phosphate-DNA precipitation method. Where indicated, cells were treated with 50 U/ml IFNγ (R&D Systems, Minneapolis, MN) and/or 10^{-6} M E2 for 20–24 h before harvesting. Thirty-six hours after transfection the cells were harvested, and luciferase and β-galactosidase activities were assayed as recommended by the manufacturer (Promega Corp.).

In Vitro Protein-Protein Interaction Experiments

Full-length and fragments of CIITA, as well as fragments of SRC-1 subcloned into pGEX vectors (Pharmacia Biotech) in frame with GST were expressed in *Escherichia coli* DH5a. For binding assays, approximately 2 μg fusion proteins were immobilized to glutathione sepharose beads (Amersham Pharmacia Biotech, Arlington Heights, IL) and incubated with *in vitro* translated (35), S-labeled (TNT, Promega Corp.) SRC-1 or CIITA protein in 400 μl buffer containing 150 mM KCl, 20 mM HEPES (pH 7.9), 0.1% Nonidet P-40 (NP-40), 5 mM $MgCl_2$, and 0.2% BSA and supplemented with protease inhibitors. Reactions were carried out at 4 C for 6 h and washed three times in the same buffer without BSA. Bound proteins were subjected to SDS-PAGE and detected by autoradiography.

Immunoprecipitation and Western Blot

Whole-cell extracts were prepared from transiently transfected Cos-7 cells (expressing Flag-CIITA or control vector), in lysis buffer containing 50 mM Tris HCl (pH 8), 170 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM dithiothreitol, and protease inhibitors. Cleared cell lysates, equivalent to about 5×10^6 cells, were incubated for 16 h at 4 C with 2.5 μg rabbit polyclonal anti-SRC-1 antibody (M-341, Santa Cruz Biotechnology, Inc.). Immunoprecipitates were collected with 20 μl protein A-Sepharose per sample (Amersham Pharmacia Biotech), and samples were washed three times with lysis buffer and subjected to SDS-PAGE. Western blotting analysis was performed using monoclonal anti-Flag (Eastman Kodak, Rochester, NY) or anti-SRC-1 (Santa Cruz Biotechnology, Inc.) antibodies. Chemiluminescence detection of coimmunoprecipitated proteins was performed using Super Signal (Pierce Chemical Co., Rockford, IL).

Chromatin Immunoprecipitation

HeLa or MCF-7 cells, having been treated with 100 U/ml IFNγ (R&D Systems) for the indicated time, were incubated with 1% formaldehyde for 10–15 min at room temperature, and subsequently cross-linking was stopped by the addition of 0.125 M glycine for 5 min. Cells were rinsed with cold PBS twice, collected, and lysed in a buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% NP-40, and protease inhibitors. Nuclei were pelleted and lysed in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.5% sarcosyl. Samples were centrifuged, and the cross-linked chromatin was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, and sonicated on ice to an average length of 400–1,000 bp, followed by two cycles of centrifugation at 15,000 $\times$ *g* for 15 min. The resulting chromatin supernatant was supplemented with 0.1% sarcosyl and stored at -80 C. Before Immunoprecipitation, chromatin supernatant was precleared by incubation with protein-A Sepharose beads. Immunoprecipitation was performed by adding 6 μg SRC-1 (Santa Cruz Biotechnology, Inc.) 3 μg RFX-5 (Rockland Immunochemicals), or 3 μg CIITA (Institute of Molecular Biology and Biotechnology) antibody to approximately 20 μg precleared chromatin supernatant diluted in immunoprecipitation buffer [20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride] and containing 100 μg/ml salmon sperm DNA and 2 mg/ml BSA. Samples were washed seven times in 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.5 M NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonylfluoride. The immunocomplexes were eluted in 100 μl Tris-EDTA containing 0.5% sodium dodecyl sulfate, and samples were treated with 200 μg/ml proteinase K at 55 C for 4 h followed by overnight incubation at 65 C to reverse the cross-links. After extraction with phenol/chloroform, DNA was precipitated with ethanol in the presence of 20 μg glycogen as a carrier and resuspended in Tris-EDTA. One tenth of immunoprecipitated DNA and 1/100 input DNA were analyzed by radioactive semiquantitative PCR using promoter-specific primer sets. The following primers were used: human leukocyte antigen (HLA)-DRA sense, 5′-GTTGTCCTGTTTGTTTAAGAAC-3′; HLA-DRA antisense, 5′-GCTCTTTTGGGAGTCAG-3′; IFNβ sense, 5′-GCTTTCCTTTGCTT TCTCCCA AGTC-3′; IFNβ antisense, 5′-CCTTTCTCCATGGGTATGGCC-3′

Real Time RT-PCR

RNA was prepared with the Trizol Reagent (Life Technologies, Inc., Gaithersburg, MD). Reverse transcription reactions were set up for 2 μg RNA per sample, using Omniscript RT kit (QIAGEN, Chatsworth, CA). One twentieth of a reverse transcriptase reaction was used as template for real-time PCR. The sequences of PCR primers used are the following: DRA.exon V sense, 5′-GAAAGCAGTCATCTTCAGCGTT-3′; DRA.exon V antisense, 5′-AGAGGCATTGCATGGTGATAAT-3′; CIITA sense, 5′-CTGAAGGATGTGGAAGACCTGGGA-AAGC-3′; CIITA antisense, 5′-ACCCTCGTCCCCGATCTT-GTTCTCACTC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5′-CCTTCCGTGTCCCCACTGC-CAAC-3′; GAPDH antisense, 5′-GTGTCGCTGTTGAAGTCA-GAGGAG-3′.

Real-time PCRs were performed using the Opticon (MJ Research Inc., Watertown, MA). Quantitation was accomplished by measuring the incorporation of the fluorescent dye SYBR-green-I into the PCR product. All PCRs were performed in triplicate and averaged.

DNA samples were heated to 94 C for 5 min, followed by 35 cycles of 30 sec at 94 C, 30 sec at 60 C, 30 sec at 72 C, and a final step of 5 min at 72 C. To compare samples, the results for DRA-RT-PCR assays were normalized to results

obtained for the corresponding GAPDH-RT-PCR assays, providing a relative quantitation value.

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