

Interplay among coactivator-associated arginine methyltransferase 1, CBP, and CIITA in IFN- γ -inducible MHC-II gene expression

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Class II major histocompatibility (MHC-II) genes are prototype targets of IFN- γ . IFN- γ activates the expression of the non-DNA-binding master regulator of MHC-II, class II transactivator (CIITA), which is crucial for enhanceosome formation and gene activation. This report shows the importance of the histone methyltransferase, coactivator-associated arginine methyltransferase (CARM1/PRMT4), during IFN- γ -induced MHC-II gene activation. It also demonstrates the coordinated regulation of CIITA, CARM1, and the acetyltransferase cyclic-AMP response element binding (CREB)-binding protein (CBP) during this process. CARM1 synergizes with CIITA in activating MHC-II transcription and synergy is abrogated when an arginine methyltransferase-defective CARM1 mutant is used. Protein-arginine methyltransferase 1 has much less effect on MHC-II transcription. Specific RNA interference reduced CARM1 expression as well as MHC-II expression. The recruitment of CARM1 to the promoter requires endogenous CIITA and results in methylation of histone H3-R17; hence, CIITA is an upstream regulator of histone methylation. Previous work has shown that CARM1 can methylate CBP at three arginine residues. Using wild-type CBP and a mutant of CBP lacking the CARM1-targeted arginine residues (R3A), we show that arginine methylation of CBP is required for IFN- γ induction of MHC-II. A kinetic analysis shows that CIITA, CARM1, and H3-R17 methylation all precede CBP loading on the MHC-II promoter during IFN- γ treatment. These results suggest functional and temporal relationships among CIITA, CARM1, and CBP for IFN- γ induction of MHC-II.

class II MHC | histone | interferon | methylation | transactivator

Class II major histocompatibility (MHC-II) proteins play a key role in the development of specific immune responses through the presentation of exogenously derived antigens to CD4⁺ T cells (1–5). Constitutive expression of MHC-II molecules is restricted to a subset of cells, but the induced expression of MHC-II by cytokines, particularly by IFN- γ , remains a key regulatory step. During an IFN- γ response, CIITA is transcriptionally activated by IRF-1 and STAT1 (1–3). Class II transactivator (CIITA) then leads to MHC-II promoter transcriptional complex assembly and histone acetylation, ultimately resulting in MHC-II activation (4).

CIITA is the master regulator of MHC-II and a member of the *CATERPILLER* family of genes (5, 6). Thus far, all CIITA-induced genes are in the MHC-II antigen presentation pathway except for *plexin-A1*, which nonetheless is important for the interaction of T cells and dendritic cells (7). CIITA is a scaffolding protein that bridges the MHC-II requisite transcription factors (RFX5, CREB, and NFY) (8) to various chromatin modifiers, including acetyltransferases [CREB-binding protein (CBP)/p300, P300/CBP-associated factor (PCAF), and steroid receptor coactivator-1 (SRC-1)] (9–13), ATP-dependent remodeling factors (BRG-1) (14, 15), and histone deacetylases (HDAC1) (16). These interactions are critical for the formation of a stable enhanceosome complex on the MHC-II promoter (17, 18).

Although roles for histone acetyltransferases and deacetylases in MHC-II regulation have been described, the possible implication of histone methylation has not been explored. Recent studies have demonstrated that arginine-specific methylation of histones H3 and H4 is an important modification modulating chromatin structure and gene transcription (19–21). In addition, methylation of non-histone substrates involved in transcription, such as CBP/p300, has also emerged as a critical feature for transcriptional regulation (22, 23). There are seven arginine methyltransferases in mammals: protein-arginine methyltransferase (PRMT) 1, PRMT3, CARM1/PRMT4, PRMT5/JBP1, PRMT6, PRMT7, and PRMT8 (24, 25). The coactivator-associated arginine methyltransferase 1 (CARM1) preferentially methylates histone H3 (19, 26, 27) and cooperates with hormone receptor coactivators such as GRIP1 and SRC-1 and with the acetyltransferases CBP/p300 and P300/PCAF to enhance the ability of nuclear receptors to activate transcription (28–31). Recently, CARM1 was also shown to be a promoter-specific regulator of NF- κ B-dependent gene expression (32). Mutations in the catalytic domain of CARM1 dramatically reduce both its methyltransferase and coactivator activities, suggesting that arginine methylation is the basis of transcriptional coactivation by CARM1 (26, 28). In addition to histones, CARM1 also methylates the transcriptional coactivator CBP/p300 (22, 23, 33, 34).

CIITA has been shown to synergize with CBP/p300, P300/PCAF, and most recently the steroid receptor coactivator SRC-1, thereby enhancing expression of MHC-II (12, 13, 35). CARM1 also cooperates with these same coactivators to enhance transcription by nuclear receptors (28, 30). These observations prompted us to examine the possible involvement of CARM1 and protein methylation in transcription of MHC-II genes by CIITA.

Materials and Methods

Tissue Culture, Cells, and Conditions. COS7, 293T, HeLa, Raji, and RJ2.2.5 cells were maintained as described in refs. 7 and 16.

Reagents. CARM1 and E267Q expression vectors were obtained from M. Stallcup (University of Southern California, Los Angeles). PRMT1 expression vector was obtained from Y. Zhang (University of North Carolina). GST-CIITA was obtained from J. Papam-

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Abbreviations: CIITA, class II transactivator; CARM1, coactivator-associated arginine methyltransferase 1; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; CREB, acetyltransferase cyclic-AMP response element binding; HA, hemagglutinin epitope; HDAC, histone deacetylase; Me-R17, dimethyl-H3-R17; MHC-II, major histocompatibility class II; PRMT, protein arginine methyltransferase; P/CAAF, P300/CBP-associated factor; siRNA, small interfering RNA.

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atheakis (University of Crete). FLAG-CIITA, MYC-CIITA, MHC-II-Luc, HA-CBP, HA-CBP- Δ 685–774, and HA-CBPR3A have been described in refs. 8, 23, 36, and 37. Anti-hemagglutinin epitope (HA) was obtained from Roche. Anti-FLAG M5 was obtained from Sigma. Anti-MYC 9E10, anti-CARM1 and anti-methyl-H3-R17 (Me-R17) were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-CBP was obtained from Santa Cruz Biotechnology; anti-CIITA was obtained from Rockland Immunochemicals (38); and IFN- γ was obtained from PeproTech (Rocky Hill, NJ).

Transient Transfections and Luciferase Assays. COS7 cells (5×10^4) were plated in six-well plates and then transfected 18–24 h later by using FuGene 6 transfection reagent (Roche). Cells were lysed and luciferase assays were performed 18–24 h after transfection as described in ref. 39.

Chromatin Immunoprecipitation (ChIP) Assays. Chromatin from 1×10^7 Raji, 5×10^6 HeLa, or 1×10^6 293 T cells was prepared as described in ref. 38, and ChIPs were performed by using the ChIP assay kit (Upstate Biotechnology) as described in ref. 16. The following antibodies were used: 5 μ g of anti-HA, 10 μ l of anti-CARM1, 10 μ l of anti-Me-R17, 10 μ g of anti-CBP, and 20 μ g of anti-CIITA. Analysis of the immunoprecipitated products was done by real-time PCR.

Real-Time PCR. cDNA synthesis and real-time PCR were performed as described in ref. 40. Values were calculated based on standard curves generated for each gene. Samples were normalized by dividing copies of MHC-II RNA (HLA-DRA) or CIITA by copies of 18S rRNA. Analysis of chromatin immunoprecipitated products was performed by using MHC-II (HLA-DRA) promoter-specific primers and probe, and values were determined by subtracting values obtained from beads-only immunoprecipitations and normalizing to the total amount of MHC-II promoter DNA (input). All primer and probe sequences have been described in ref. 16.

Small Interfering RNA (siRNA) Transfections and Luciferase Assays. The siRNA sequence targeting CARM1 corresponded to the coding region 129–149 relative to the first nucleotide of the start codon. CARM1-specific siRNA or the control siRNA (200 nM) (Dharmacon RNA Technologies, Lafayette, CO) was transfected into U2OS by using Oligofectamine reagent (Invitrogen). Cells were trypsinized and seeded at a density of 5×10^4 cells in 12-well plates 24 h after transfection. Twelve hours later, U2OS cell lines were transfected by the calcium phosphate coprecipitation procedure with 1 μ g of MHC-II-luc reporter vector and 50 ng of CIITA expression vector. Empty vector was included to keep the total amount of DNA per well constant. pCMV-lacZ was included in each experiment as a control for transfection efficiency. Cells were harvested 30 h after transfection. Luciferase and β -galactosidase activities were measured with Promega and Tropix (Bedford, MA) kits, respectively.

Results

CARM1 Enhances CIITA-Mediated MHC-II Activation in a Methylation-Dependent Manner. To investigate the possible role of histone methyltransferase in *MHC-II* transcription, we tested the effect of CARM1 on the function of CIITA in a transient transfection experiment in COS7 cells. Cotransfection of increasing amounts of CARM1 with CIITA resulted in the synergistic activation of an MHC-II promoter-driven reporter (Fig. 1A). A CARM1 mutant that lacks methyltransferase activity, E267Q (28), was also tested. The synergy between CIITA and CARM1 was abolished when the mutant E267Q was used (Fig. 1B). In contrast to CARM1, the arginine-specific methyltransferase PRMT1 exerted a modest effect on CIITA-mediated transcription, indicating an important role of CARM1 in *MHC-II* gene regulation (Fig. 1B). To further assess the

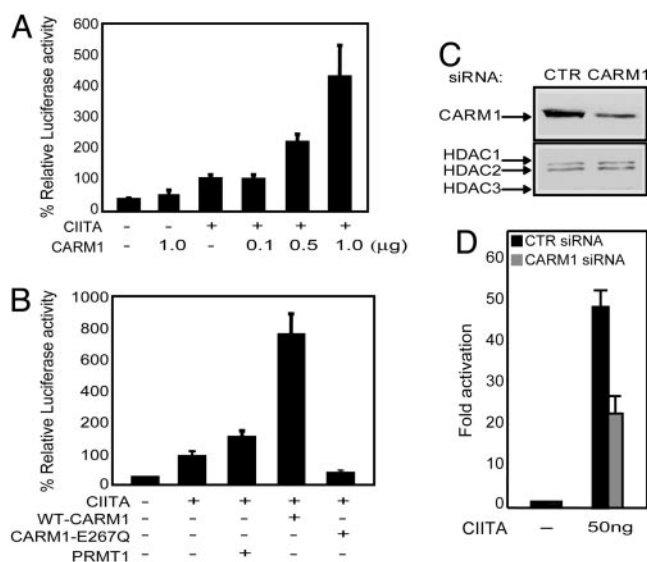


Fig. 1. CARM1 enhances CIITA transactivation in a methylation-dependent manner. (A) COS7 cells were cotransfected with 10 ng of CIITA, increasing amounts of CARM1, and 500 ng of the MHC-II-luc reporter construct. Cells were harvested 24 h later. Luciferase activity is reported as percent activation relative to that by CIITA alone. Values are shown as mean percent relative luciferase activity \pm SEM for three experiments, each of which was repeated in triplicate. (B) COS7 cells were cotransfected with 10 ng of CIITA, 1 μ g of CARM1, 1 μ g of PRMT1 or the methylation-defective mutant E267Q, and 500 ng of the MHC-II-luc reporter construct. Luciferase activity was assayed and reported as in A. (C) Endogenous CARM1 expression in U2OS cells transfected with control or CARM1 siRNA was detected by immunoblotting with anti-CARM1 (Upper). CARM1 siRNA successfully reduced the endogenous gene in U2OS, hence this cell line was used in these experiments. Endogenous HDACs (1–3) served as controls (Lower). (D) U2OS cells described in C were transfected with 50 ng of CIITA and 1 μ g of the MHC-II-luc reporter construct. Luciferase activity is reported as fold activation relative to that of empty vector and was normalized to β -galactosidase.

role of CARM1 in CIITA-dependent transcription of *MHC-II*, we designed a siRNA duplex to specifically knock down endogenous CARM1 expression. As a control, we used a siRNA duplex, which does not anneal to any known mRNA. siRNA-mediated reduction of endogenous CARM1 by \approx 50% relative to the control was verified by Western blot analysis (Fig. 1C Upper). The expression of several control proteins (HDACs 1–3) was not reduced by the CARM1 siRNA (Fig. 1C Lower). In control cells that express normal levels of CARM1, CIITA efficiently activated a MHC-II driven luciferase reporter, whereas in cells transfected with CARM1-specific siRNA, activation was reduced by half (Fig. 1D). This level of reduction correlates with the extent of CARM1 reduction seen in Fig. 1C.

CARM1 Enhances IFN- γ -Inducible and CIITA-Dependent Expression of Endogenous MHC-II. The experiments above used an artificial promoter-reporter system. To address the role of CARM1 in endogenous *MHC-II* gene expression, real-time PCR was used to measure endogenous *MHC-II* mRNA in HeLa cells. HeLa cells were used in all experiments measuring endogenous MHC-II expression because MHC-II is IFN- γ -inducible in these cells.

CARM1 significantly enhanced the expression of endogenous *MHC-II* but only in the presence of CIITA. The level of induction of *MHC-II* by CIITA is within the range of induction observed with physiologic inducers such as IFN- γ (41, 42). Furthermore, the coactivating effect of CARM1 is similar to that observed in another system (32). In contrast, the methyltransferase-deficient mutant, E267Q, did not enhance *MHC-II* expression (Fig. 2A). CIITA expression was not altered by CARM1 or the E267Q mutant as

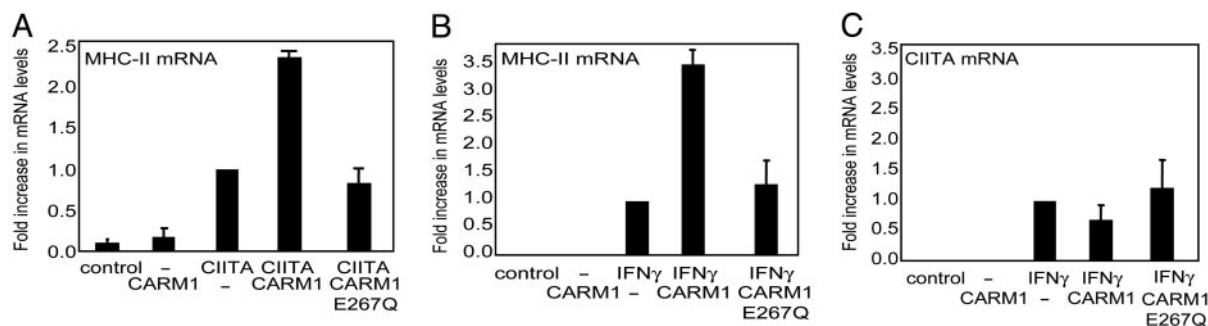


Fig. 2. Enhancement of CIITA-mediated and IFN- γ inducible expression of endogenous MHC-II depends on CARM1 methyltransferase activity. (A) HeLa cells were cotransfected with 0.5 μ g of CIITA vector and 3 μ g of CARM1 or CARM1-E267Q vector. MHC-II mRNA was measured by real-time PCR. Values are reported as fold increase in mRNA expression over a sample transfected with CIITA only. Samples were normalized to the number of 18S rRNA copies. (B) HeLa cells were induced with IFN- γ (25 ng/ml) for 24 h. Real-time PCR analysis was performed to measure endogenous mRNA levels of MHC-II in the presence of 3 μ g of CARM1 or CARM1-E267Q vector. The value of the sample treated only with IFN- γ is set as 1. (C) CIITA mRNA levels from B were measured by real-time PCR. Values are reported as in B. All data shown are averages of three experiments.

determined by immunoblot analysis (data not shown). To investigate the role of CARM1 with a physiologic stimulus of *MHC-II* gene, real-time PCR was used to measure endogenous MHC-II mRNA in HeLa cells after treatment with IFN- γ . CARM1 significantly enhanced endogenous MHC-II induced by IFN- γ (Fig. 2B). This enhancement was eliminated with the methyltransferase-deficient E267Q mutant. The level of endogenous CIITA transcript remained unaffected by CARM1 or the E267Q mutant (Fig. 2C).

One possible mechanism by which CARM1 enhances *MHC-II* transcription is by interaction with CIITA. To explore this interaction, coimmunoprecipitations of overexpressed CIITA and CARM1 were performed. CIITA efficiently associated with both wild-type CARM1 and E267Q (see Fig. 6A and B, lanes 2 and 3, which is published as supporting information on the PNAS web site) but not to another nuclear MHC-II enhanceosome-associated protein, NF-YA (Fig. 6B, lane 4). This finding indicates that CARM1 interacts specifically with CIITA *in vivo* and that the loss of *MHC-II* transcriptional enhancement by E267Q is due not to the lack of its association with CIITA but to the lack of methyltransferase activity. However, thus far we have been unable to show the association of endogenous CIITA and CARM1, which is likely due to the low level of endogenous proteins and to the lack of appropriate antibodies for endogenous CIITA. Nevertheless, *in vitro* evidence for a physical interaction between CIITA and CARM1 was obtained by GST pull-down experiments. *In vitro*-translated 35 S-labeled CARM1 and E267Q mutant efficiently interacted with full-length CIITA immobilized on glutathione-Sepharose beads (Fig. 6C, lanes 3 and 6).

Recruitment of CARM1 to the Endogenous *MHC-II* Promoter Is Induced by IFN- γ . To test whether CARM1 is recruited to an endogenous MHC-II promoter, HLA-DRA, ChIP assay was used. HeLa cells were treated with IFN- γ (25 ng/ml) and endogenous CARM1 was immunoprecipitated. *MHC-II* promoter DNA sequences were detected by real-time PCR. IFN- γ significantly enhanced CARM1 association with the promoter (Fig. 3A) and the recruitment of CARM1 correlated with the methylation of histone H3-R17 (Me-R17), a known target of CARM1 (Fig. 3B) (19, 27), indicating that arginine methylation of histone H3 at the MHC-II promoter occurs during IFN- γ -induced MHC-II expression. To exclude the possibility that the results of Fig. 3A were due to altered expression of CARM1 after IFN- γ induction, extracts from cells untreated or treated with IFN- γ (25 ng/ml) for 24 h were immunoblotted with an anti-CARM1 antibody. No dramatic change in CARM1 expression was observed in these conditions (see Fig. 7, which is published as supporting information on the PNAS web site).

CIITA Mediates CARM1 Promoter Association in a B Cell Line. The Raji and RJ2.2.5 cell lines respectively represent a CIITA-expressing, MHC-II⁺ B cell line and its CIITA-defective and MHC-II-negative variant (43). These cell lines provide another biologic system to analyze the effect of endogenous CIITA on CARM1. To investigate the mode of CARM1 recruitment to the MHC-II promoter in B cells, ChIP assays were performed. Both CIITA and CARM1 were associated with MHC-II promoter in Raji cells (Fig. 3C, left bar, and Fig. 3D, left bar). In the CIITA-deficient cell line RJ2.2.5, CIITA (Fig. 3C, right bar) and CARM1 (Fig. 3D, right bar) were not detected by ChIP on the promoter, indicating that a functional CIITA is required for optimal association of CARM1 with the *MHC-II* promoter. Me-R17 was also found at the *MHC-II* promoter in Raji cells but not in RJ2.2.5 cells (Fig. 3E). As a control for specificity, ChIP analysis of the β -actin promoter showed that neither CARM1 nor CIITA interacted with the β -actin promoter, and methylation that produces Me-R17 was not observed (data not shown). These data demonstrate that CARM1 is specifically recruited to the promoter in a CIITA-dependent fashion, resulting in histone methylation at the *HLA-DRA* promoter.

Methylation of CBP by CARM1 Is Required for CIITA-Dependent Transcription. The above data strongly suggest that CARM1 enhances the transcription of *MHC-II* genes in a methyltransferase-dependent manner. One possibility is that CARM1 regulates *MHC-II* transcription by methylating CIITA. To test this hypothesis, *in vitro* methylation assays were performed in which GST-CIITA was incubated with recombinant CARM1 in the presence of radiolabeled S-adenosyl-L-[methyl- 3 H]methionine (SAM). In contrast to the predicted result, CIITA was not methylated by CARM1 *in vitro* (see Fig. 8A, which is published as supporting information on the PNAS web site). In contrast, residues 685–774 of the histone acetyltransferase CBP were methylated by CARM1. The full-length CBP was also methylated by CARM1, and this methylation was not affected by including CIITA (Fig. 8B). This observation prompted us to examine whether CARM1-mediated methylation enhances MHC-II transcription by targeting CBP. CBP is known to enhance CIITA-dependent transcription and has been shown to interact and cooperate with CARM1 in nuclear receptor-dependent transcription (23, 28). However, the importance of CARM1-mediated methylation of CBP has not been explored in IFN- γ -regulated transcription.

To address the potential role of CARM1-mediated methylation of CBP in MHC-II transcription, we took advantage of previous studies that showed that CARM1 methylates CBP in a region that spans residues 685–774 and that specifically targets arginines 714, 742, and 768 (23). Luciferase reporter assays using a mutant of CBP

intact methyltransferase activity. CARM1 may have two functions, the methylation of H3 at arginine-17 and the methylation of CBP. The latter is supported by the requirement for intact methylation sites within CBP for its association with the endogenous MHC-II promoter and for its activation of *MHC-II* genes. Whereas CARM1 has been shown to be an important component of nuclear receptor-dependent transcription, this report specifically indicates its role in IFN- γ -induced transcription.

This study shows a dynamic series of events involving CIITA, CARM1, and CBP. CARM1 is not observed at the *MHC-II* promoter in the CIITA-negative RJ2.2.5 cells, indicating a role of CIITA in recruiting CARM1 to the endogenous MHC-II promoter. In turn, the molecular mechanism by which CARM1 enhances the expression of *MHC-II* genes depends on an intact methyltransferase domain. Me-R17 is highly enriched at the MHC-II promoter shortly after IFN- γ induction, correlating with the presence of CARM1 at the promoter. Although the functional consequence of Me-R17 on the localized chromatin structure is not yet fully understood, a prevailing view is that this modification could facilitate chromatin alterations necessary for transcription (45, 46). In addition to histone methylation, CARM1 also methylates CBP within a region containing arginines R714, R742, and R768. These residues are required for the association of CBP with the *MHC-II* promoter, suggesting that the methylation of CBP by CARM1 is likely important for promoter association. In addition, the kinetics study shows that whereas a maximal level of CARM1 was found at the *MHC-II* promoter 4 h after IFN- γ treatment, CBP-promoter association trailed that of CARM1.

Notably, methylation of CBP at arginines 714, 742, and 768 is not known to affect transcription by CREB, an important transcription

factor for MHC-II expression (23, 47). CBP methylation within the kinase-inducible (KIX) domain has been previously demonstrated to inhibit the interaction between CBP and CREB, and thus decrease CREB-mediated transcription (22). However, the methylation sites within the KIX domain are entirely different from the ones investigated here and do not affect CREB transactivation (23).

A recent paper by Gomez *et al.* (48) examined IFN- γ -induced chromatin changes, albeit at a later time point (16, 24, and 48 h). A host of modifications involving histone acetylation were documented. Whether these later modifications are predicated on CARM1-mediated methylation of H3-R17 to produce Me-R17 or whether they influence the function and release of CARM1 will be of future interest.

In summary, our results are consistent with the following model. Shortly after IFN- γ stimulation, CIITA expression is induced and is recruited to the MHC-II promoter. CIITA then enhances the recruitment of CARM1, which mediates methylation that produces Me-R17. CARM1 also methylates CBP, thereby facilitating its association with the promoter. Finally, CARM1 dissociates from the promoter, whereas promoter association with CIITA and CBP is more sustained. These results indicate a coordinated sequence of events involving the interplay of CIITA, CARM1, and CBP during IFN- γ -induced gene activation.

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