GTP-dependent Recruitment of CIITA to the Class II Major Histocompatibility Complex Promoter*

Received for publication, December 22, 2006, and in revised form, June 12, 2007 Published, JBC Papers in Press, July 10, 2007 DOI 10.1074/jbc.M611747200

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We previously established that the class II transactivator CIITA binds GTP and disruption of the GTP binding ability of CIITA results in increased cytoplasmic CIITA, loss of nuclear CIITA, and thus diminished class II major histocompatibility complex transcription. Because of its role in facilitating nuclear localization, whether GTP binding is also required for CIITA-mediated transactivation of major histocompatibility class II genes remains unclear. We now show that recruitment of CIITA to the human leukocyte antigen (HLA)-DR promoter and activation of HLA-DR transcription is also GTP-dependent. After restoration of nuclear expression, CIITA mutants defective in GTP binding lack full transcriptional activation capacity. Although the availability of the activation domain of CIITA is unaltered, GTP mutants no longer cooperate with CREB-binding protein, p300, and pCAF and are defective in recruitment to the HLA-DR promoter.

Class II major histocompatibility complex (MHC II) molecules are essential for T cell- and antibody-mediated immune responses through presentation of exogenous antigen-derived peptides to CD4+ T cells. Although professional antigen-presenting cells (B cells, monocytes, macrophages, and dendritic cells) express MHC II constitutively, interferon-γ will induce MHC II on most negative tissues/cells. Expression of both constitutive and inducible MHC II is regulated by a master transcriptional co-activator, CIITA (1). CIITA also regulates expression of MHC II-related genes such as HLA-DM and invariant chain through the W, X, and Y elements common to this family of promoters (2, 3). Defects in CIITA (as in some MHC II deficiency patients or CIITA knock-out mice) nearly abolish all MHC II expression and are accompanied by a near complete lack of CD4+ T cells, causing a severe and lethal combined type immunodeficiency (4). Class II MHC expression levels, both constitutive and cytokine-induced, are in large part regulated via transcriptional control of CIITA through multiple promoters, each with particular tissue restriction and induction patterns (5–7). However, post-translational modification of CIITA function, either directly via signaling events (e.g. phosphorylation and ubiquitination) or indirectly through histone deacetylase association during infection also influences MHC II expression, suggesting that CIITA is highly regulated (8–13).

CIITA likely shuttles between the cytoplasm and the nucleus, a process regulated in part by at least three nuclear localization sequences, export sequences, GTP binding, and C-terminus leucine-rich repeats of CIITA (14–18). CIITA can self-associate and likely forms larger complexes mediated by homotypic and heterotypic interactions between the GTP-binding domain and the leucine-rich repeats (19, 20). These interactions may be important for transcription activation, but it remains unclear whether they are critical for nuclear import. Once in the nucleus, CIITA participates in chromatin remodeling and activates MHC II transcription. Although a non-DNA-binding protein, CIITA directs MHC II transcription by forming a scaffold or enhanceosome at the promoter through interactions with RFX, CREB, NF-Y, and components of the basal transcription machinery (21–24). The histone acetyltransferases CBP (CREB-binding protein), p300, and pCAF cooperatively activate CIITA-driven gene expression, and CIITA may have its own acetyltransferase activity (25). Moreover, CBP and pCAF acetylation of CIITA enhances nuclear import (16). Conversely, CIITA can be negatively regulated by phosphorylation at a number of different serine residues (8–10, 26).

Although binding GTP, CIITA does not appear to be a GTPase in vitro (27). A point mutation that confers GTPase activity noticeably diminishes CIITA function. Mutants with defective GTP binding have been used to demonstrate the importance of the GTP-binding domain of CIITA in nuclear import and export, dimerization, and intrinsic acetyltransferase activity (18, 19, 25, 27). Because GTP-binding domain mutations render CIITA largely cytoplasmic, whether GTP binding is actually required for CIITA transcription activation potential remains unclear. In this report we explore the GTP dependence of CIITA-mediated MHC II transcription activation using CIITA constructs containing the strong SV40 nuclear localization signal sequence (NLS). Under conditions of enforced nuclear expression, activation of MHC II transcription by CIITA appears to be largely GTP-dependent. Further GTP binding appears important for recruitment to the MHC II

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*This work was supported by American Cancer Society Grant IRG32 (to J. A. H.) and National Institutes of Health Grant K01CA095582 (to J. A. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡‡The abbreviations used are: MHC II, class II major histocompatibility complex; CIITA, class II transactivator; HLA-DR, human leukocyte antigen-DR; NLS, nuclear localization signal sequence; ChIP, chromatin immunoprecipitation; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein.
promoter and may mediate CIITA interactions with promoter-bound transcription factors.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture Cells and Conditions**—COS-7 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (with 4.5 g/liter glucose) supplemented with 10% fetal calf serum, 5 mM L-glutamine, and 0.1% streptomycin-penicillin. All cells were grown at 37 °C with 5% CO₂.

**Plasmid Constructs**—pcDNA3 (Invitrogen) and FLAG-tagged versions of wild-type CIITA and the CIITA GTP binding mutants GTP1, GTP2, GTP3, C2RASG1, and C2RG1L465Q have been described previously (27, 28). To generate the chimera C2RAS, an Eco47III site at nucleotides 1279–1284 and a ClaI site at nucleotides 1707–1712 of wild-type CIITA were generated using QuikChange mutagenesis (Stratagene). Following digestion, an amplified Eco47III/ClaI-flanked fragment of the GTPase-inactive RasQ61L oncogene was inserted. All the NLS-tagged forms of these mutants were generated by transfecting the AocI/SacII fragment containing the entire nucleotide-binding domain into AocI/SacII-digested NLS-CIITA (14). The sequences of all DNA amplification-generated constructs were confirmed by fluorescent dye terminator sequencing.

**Luciferase Assays**—Transient cotransfection was performed using FuGENE 6 (Roche Applied Science). Briefly, 5 × 10⁴ cells/well were plated in 24-well plates, cultured overnight, and transfected with DRA300Luc (a luciferase reporter driven by the MHC class II HLA-DR promoter) and either pcDNA3 (vector control), FLAG-tagged NLS-CIITA, or NLS-CIITA mutant plasmids (14). The transfected cells were lysed 16–18 h post-transfection for lysis buffer (Promega), and luciferase assays were performed as previously described (29). Each transfaction was performed in triplicate, and luciferase data were normalized to total protein.

**Western Blotting**—Following transfection of cells with 1 μg of DNA, cell were lysed in 1× reporter lysis buffer (Promega) containing 1× Complete™ (Roche Applied Science) protease inhibitors. Protein concentrations were normalized, and 5 μg of protein was subjected to SDS-PAGE (8%) and transferred to nitrocellulose. Blotting for the FLAG epitope was performed as described (19).

**Immunofluorescence Microscopy**—Immunofluorescent staining of transiently transfected COS-7 cells was performed as previously described (14). Briefly, 5 to 8 × 10⁴ cells were plated on two-well chamber slides (Nunc), incubated overnight at 37 °C, and transfected with 1 to 1.5 μg of plasmid DNA using FuGENE 6. After a second overnight incubation, the cells were washed with 1× phosphate-buffered saline, blocked in 1× phosphate-buffered saline, 1% bovine serum albumin, 10% goat serum, and stained with anti-FLAG(M5) (Sigma) and goat anti-mouse IgG-fluorescein isothiocyanate (Southern Biotech). To ensure representative data in these experiments, prior to taking representative photomicrographs for each experiment the entire slide was examined under 400–500× magnification to observe the pattern of nuclear and cytoplasmic expression.

**CHIP Assay**—HeLa cells were plated (1 × 10⁶ cells/100-mm plate) 24 h prior to transfection. Cells were transfected with 3 μg of the indicated plasmids using FuGENE 6 (Roche) according to the manufacturer’s recommendations. Chromatin immunoprecipitations (ChIP) were performed in accordance with the ChIP assay kit (Upstate Biotechnology). Briefly, 18–24 h post-transfection DNA-protein cross-linking was accomplished at room temperature with 0.37% formaldehyde for 10 min and stopped by addition of 0.125 M glycine for 5 min. Cells were lysed on ice for 15 min with SDS cell lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% sodium dodecyl sulfate) supplemented with Complete EDTA-free protease inhibitors (Roche). After lysis, chromatin was sheared to an average size of 200–1200 bp by sonication. Lysates were diluted 1:2 with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL, pH 8.1, 167 mM NaCl) and precleared with Protein G Plus A-agarose beads (Santa Cruz). Immunoprecipitation was carried out with 10 μg of anti-FLAG M5 (Sigma). DNA-protein-antibody-bead complexes were then washed at room temperature using the following: TSE-500 wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL, pH 8.1), LiCl wash buffer (500 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 100 mM Tris-HCL, pH 8.1) and 1× TE buffer (10 mM Tris-HCL, pH 7.5, 1 mM EDTA). In addition, no-antibody control immunoprecipitations were performed. Elution of the DNA-protein complexes was accomplished with a 1% SDS and 50 mM NaHCO₃ buffer solution. Cross-links were reversed overnight at 65 °C. Samples were then incubated with 1.0 μg of Proteinase K for 1 h, and DNA was purified using phenol-chloroform extraction. Recovered HLA-DR promoter DNA was assayed by real-time PCR using 15% of the recovered DNA.

**Real-time PCR**—Real-time PCR was performed using a MyQ Thermocycler (Bio-Rad) with the following MHC II promoter primers: sense primer, 5’-GAT CTC TTG TGT CCT GGA CCC TTT GCA A-3’; antisense primer, 5’-CCC AAT TAC TCT TTG GCC AAT CAG AAA A-3’. Quantification of the HLA-DR promoter was conducted in a total volume of 25 μl with 2× Sybr Green (Bio-Rad). Thermal cycler parameters were 3 min at 95 °C and 40 cycles of denaturation at 95 °C for 15 s, annealing/extension at 55 °C for 1 min.

**RESULTS**

The GTP-dependent functions of CIITA are not fully understood. Previously we demonstrated that CIITA mutants with decreased abilities to bind GTP remain principally cytoplasmic and thus unable to effectively activate MHC II transcription (Table 1) (27). Interestingly, these observations may be due to increased nuclear export as other studies suggest enhanced binding of the same mutants to CREM1, the major protein responsible for nuclear export (18). Diminished self-association of certain CIITA-GTP mutants suggests that molecular interactions between CIITA molecules may be disrupted or non-productive if GTP is not bound (19, 20). Thus it remains unclear what role, if any, GTP binding actually plays in transcriptional activation. To directly test whether CIITA requires GTP binding to activate transcription of MHC II, we generated NLS-tagged versions of several GTP binding mutants and examined their ability to activate transcription from the HLA-DRA promoter (Fig. 1). All of the NLS-tagged constructs display nuclear localization comparable with that seen with wild-type NLS-CIITA (Fig. 1A). Consistent with our previous results where we
observed no apparent nuclear localization of the untagged mutants, the phosphate-binding domain (GTP1) and guanine ring-binding domain (GTP3) mutants essentially lack the ability to activate MHC II transcription (Fig. 1B). Surprisingly, however, the GTP2 mutation that eliminates the consensus magnesium ion coordination site (DAYG) retains substantial ability to activate transcription from the HLA-DRA promoter. We have previously observed that reducing the amount of transfected DNA can reveal the effects of nuclear import-related differences in activation potential relative to wild-type controls (17, 30). However, transfections of cells with a tenth of the DNA yielded essentially the same pattern, suggesting that NLS-GTP2 is functionally impaired, retaining only ~50% activity relative to wild-type, and that the reduced activity is not a consequence of import differences. These data strongly suggest that CIITA requires GTP for the full activation of HLA-DRA transcription.

Mutation of the phosphate-binding domain of CIITA to that of Ras (C2RasG1) does not diminish activation of HLA-DRA transcription; however, the addition of the L465Q point mutation to either C2RASG1 (C2RG1L465Q) or to wild-type CIITA imparted intrinsic GTPase activity to CIITA (27). We previously demonstrated that CIITA GTPase activity correlated with diminished transcriptional activation ability and an apparent loss of nuclear translocation. Presumably, limited nuclear import of C2RG1L465Q is sufficient to partially activate transcription but insufficient for successful immunofluorescent staining. If the diminished capacity of this mutant is due principally to the import defect, restoration of nuclear localization would be expected to completely restore function. The NLS-tagged forms of both C2RASG1 and C2RASG1L465Q display nuclear localization comparable with wild-type NLS-CIITA (Fig. 2A), but the GTPase active form of CIITA has ~50% of the activity of the parent construct (Fig. 2B). Thus, even with detectable and apparently normal nuclear localization, GTPase-active CIITA is impaired in its ability to activate transcription. This observation further supports the hypothesis that the GTP-bound state of CIITA influences its transcriptional activity.

### Table 1

Summary of transactivation potential and nuclear localization for CIITA GTP-binding domain mutants

| Construct | G1       | G3   | G4       | GTPase activity | Relative activation | Nuclear localization |
|-----------|----------|------|----------|-----------------|---------------------|---------------------|
| CIITA     | G1AGQGKS | DAYG | L SKAD   | ND              | 100                 | C/N                 |
| GTP1      | AGQGKS   | DAYG | L SKAD   | ND              | 38                  | C                   |
| GTP2      | G1AGQGKS | ---  | L SKAD   | ND              | 10                  | C                   |
| GTP3      | G1AGQGKS | DAYG | L SKAD   | ND              | 21                  | C                   |
| C2RASG1   | GAGVGGKS | DAYG | L SKAD   | ND              | 95                  | C/N                 |
| C2RASG1L465Q | GAGVGGKS | DAYG | Q SKAD   | ND              | 30                  | C                   |

*FIGURE 1.* NLS-tagged CIITA and GTP domain mutants are imported to the nucleus and are defective in their ability to activate HLA-DRA transcription. A, following transfection with wild-type FLAG-tagged NLS-CIITA or the indicated mutants, CIITA was detected using anti-FLAG as indicated under “Experimental Procedures.” Nuclear targeting of CIITA reveals GTP-dependent HLA-DRA transcription. Results are representative of two independent experiments. B, COS-7 cells were co-transfected with either 1 or 100 ng of the indicated constructs and the HLA-DR luciferase reporter. Transactivation ability was normalized to that of wild-type NLS-CIITA (100%; relative activation). Data shown represent the mean ± S.E. for three independent experiments.

*FIGURE 2.* The GTPase-active CIITA mutant C2RG1L465Q decreases CIITA-mediated transactivation. COS-7 cells were co-transfected with either 1 µg (A) or 100 ng (B) of the indicated constructs and the HLA-DR luciferase reporter. Transactivation ability was normalized to that of NLS-C2RASG1 (100%; relative activation), the functional, non-GTPase parent construct. Data shown represent the mean ± S.E. for three independent experiments.
How GTP binding imparts transactivation capacity to CIITA is unclear. One possibility is that GTP binding confers a transactivation-permissive conformation. To test this, wild-type CIITA and GTP mutants were fused to the DNA-binding domain of the yeast transcription factor GAL4 and tested for their ability to activate a GAL4-luciferase promoter/reporter construct (Fig. 3). From previous studies, it is known that phosphorylation of CIITA by cAMP-dependent protein kinase inhibits the ability of GAL4-CIITA to activate a GAL4-luciferase promoter (8). However, deletion of critical GTP binding elements in GAL4-CIITA had no effect on activation of GAL4-luciferase, suggesting that GTP binding defects do not impair the availability of the activation domain. Interestingly, GAL4-C2RASG1 has an increased ability to activate transcription whereas that of GAL4-C2RG1L465Q is decreased relative to its parent construct (C2RASG1). Although some correlation with GTP binding may account for the increased activity of C2RasG1, the lack of differences between wild type and deletion or GTPase-active mutants strongly suggests that GTP binding differences do not alter transactivation domain availability.

Our data suggested that GTP does not alter availability of the distal N-terminal transactivation domain; however, GTP-dependent conformational changes affecting residues near the motifs mediating GTP binding might be important. To explore this idea, the GTP-binding domain of Ras61L (GTPase-deficient) was cloned into CIITA to generate C2RAS (Fig. 4A). C2RAS retains the phosphate binding loop (G1) of CIITA but contains the nucleotide-binding domain of the oncogenic, GTPase-defective Ras61L mutant from residue 18 through 127 and displays GTP binding comparable with Ras in an in vitro exchange assay (not shown). C2RAS fails to activate HLA-DRA transcription (not shown) and has defective nuclear import (Fig. 4B). To rule out failed nuclear import as a reason for lack of transactivation ability, C2RAS was tagged with the SV40 NLS. NLS-C2RAS enters the nucleus but still fails to activate transcription (Fig. 4, B and C). These observations suggest that residues within the domain, but not directly involved in nucleotide binding, are required for activation, even in the presence of an intact GTP-binding domain.

Various studies support the contention that CIITA drives both opening of the promoter and activation of transcription (25, 31–34). This led us to consider whether the defect was in recruitment of the GTP-binding domain mutants to the promoter or in the ability of the mutants to transactivate. CIITA interacts cooperatively with the histone acetyltransferases CBP, pCAF, and p300 leading to increased transactivation and requires the N terminus of CIITA (16, 17, 35–37). We reasoned that if CIITA mutants were recruited to the promoter but

**FIGURE 3.** GTP domain mutations do not adversely affect CIITA activation domain function. COS-7 cells were transfected with DNA coding for the GAL4 DNA-binding domain (pSG424), GAL4 fused to wild-type CIITA (GAL4-CIITA), or the indicated GTP-binding domain mutants and a Gal4 luciferase reporter with five copies of the Gal4 DNA binding motif. Luciferase activity was normalized to the parent construct, GAL4-CIITA (100%; relative activation). Luciferase activity in triplicate transfections is shown as the mean ± S.E. and is representative of results from one of three similar experiments.

**FIGURE 4.** CIITA nuclear localization and HLA-DRA transactivation require the CIITA GTP-binding domain. A, schematic of the C2RAS construct that contains the GTP-binding domain of the Ras Q61L, non-GTPase-active oncogene. The wild-type phosphate-binding site (G1) from CIITA is maintained and the G2-G5 motifs derive from RasQ61L. B, anti-FLAG immunofluorescence staining is shown for COS-7 cells expressing transfected wild-type CIITA and C2RAS, without and with the addition of an N-terminal NLS sequence. C, HLA-DR luciferase reporter activation by NLS-CIITA versus NLS-C2RAS. Data are the mean ± S.E. of three independent experiments. GTP binding by C2RAS in in vitro exchange assays was similar to that of the GTPase-impaired RasV12 (not shown).
Unable to activate transcription, their intact N-terminal domain should still interact with these coactivators (in excess), resulting in increased transcriptional activity. Expression of the NLS-tagged CIITA constructs is comparable with wild type in transfected cells (Fig. 5A), with the exception of NLS-GTP2. NLS-GTP2, like the parent GTP2 construct, consistently displays reduced expression relative to other constructs (27, 28). Although no cooperation was observed between wild-type NLS-CIITA and CBP, pCAF and p300 both substantially enhanced the ability of NLS-CIITA to activate transcription (Fig. 5B). Of the mutants, only NLS-GTP2 displayed cooperation with both pCAF and p300, despite its lower expression. This confirms the initial observation that NLS-GTP2 is partially active and supports our conclusion that NLS-GTP2 is recruited to the promoter. Neither GTP1 nor GTP3 displayed any cooperativity with pCAF or p300, suggesting that they are unlikely to be recruited to the promoter. Curiously, C2RAS, although unable to appreciably activate HLA-DRA transcription, displayed some cooperation with p300. Although small, cooperation between C2RAS and p300 was consistent in multiple assays, suggesting that C2RAS may have some interaction with p300, the HLA-DRA promoter, or both. Given that the histone acetyltransferase coactivators tested all interact with regions of CIITA distinct from the GTP-binding domain, we conclude from these experiments that GTP binding is most likely required for recruitment to the HLA-DRA promoter.

To directly assess the presence of wild-type or mutant CIITA at the HLA-DRA promoter, ChIP was performed (Fig. 6). As anticipated, NLS-CIITA contact with the HLA-DRA promoter was readily detected. Consistent with the promoter/reporter and coactivator experiments, NLS-GTP1, NLS-GTP3, and NLS-C2RAS mutants were essentially unable to bind the MHC II promoter, whereas NLS-GTP2 promoter interaction was greatly diminished relative to NLS-CIITA. Collectively, these data are highly consistent with a mechanism of CIITA recruitment to the HLA-DRA promoter that is GTP-dependent.
DISCUSSION

Regulated binding of GTP is a common mechanism influencing a broad spectrum of cellular processes. We and others have previously demonstrated the importance of GTP binding by the unique transcriptional coactivator CIITA in nuclear translocation (27), acetyltransferase activity (25), oligomerization (19), and nuclear export (18). In addition, the GTP-binding domain interacts with the transcription factors RXF5, NF-YB, and BRG1 (21, 38, 39). Our initial study strongly suggested defective nuclear import of GTP-binding-deficient CIITA (27). Subsequently, Raval et al. suggested that GTP mutants enter the nucleus but are rapidly exported by CREM1 (18). Thus, nuclear CIITA should be GTP-bound, but whether GTP binding contributes to CIITA-activated MHC II transcription remains unclear. Here we demonstrate a requirement for the GTP binding function of CIITA in HLA-DR transcriptional activation.

NLS tagging of previously characterized CIITA GTP binding mutants conferred nuclear expression, revealing that nuclear GTP1 and GTP3 were essentially unable to activate HLA-DRA transcription. Unexpectedly, NLS-GTP2 was partially active, although its untagged form was the least active and the least able to bind GTP in vitro (27, 28). The higher activity of NLS-GTP2 once in the nucleus might reflect increased nuclear availability of GTP or differences in the amount of GTP binding required for nuclear import versus transactivation. Alternatively, because CIITA-GTP exchange assays appear less sensitive than those for Ras (27) and may not accurately detect subtle affinity differences, GTP binding by GTP2 may be better in vivo. Further, the presence of intact phosphate- and guanine-binding domains and an adjacent polar residue (Asp-457) near the deletion might generate a low-affinity GTP-binding site, supporting the latter possibility.

Because both NLS-tagged and -untagged C2RASG1 are functionally comparable with their wild-type counterparts, our interpretation is that GTP binding is essential and not an interaction dependent upon non-consensus residues in the G1 motif. Despite binding GTP, CIITA does not possess intrinsic GTPase activity, but GTPase activity is conferred by the introduction of a single point mutation, L465Q, alone or in combination with the RASG1 replacement (27). Even when nuclear localization is enforced, GTPase active CIITA has diminished activity, further supporting a conclusion of GTP-dependent transactivation.

Phosphorylation by cAMP-dependent protein kinase impairs CIITA transactivation by influencing the availability or function of the activation domain (8). In contrast, GTP binding defects do not affect GAL4-CIITA activation of the GAL4 promoter. Surprisingly, the Ras replacement mutant GAL4-C2RASG1 is more active in this assay, but GTPase activity abolishes the increase. The involvement of the Rh P-loop in interactions with nucleotide exchange factors suggests that the Ras P-loop might contribute a different, positive interaction (40). However, a larger portion of the Ras GTP-binding domain was unable to substitute for that of CIITA, displaying defects in both nuclear localization and transactivation regardless of the NLS addition. Primary sequence differences between Ras and CIITA, motif spacing, and/or potential steric hindrance effects of Ras-binding proteins (e.g., GEFs, GAPs, Raf) disrupting necessary CIITA interactions are possibilities raised by this observation. Nevertheless, some aspect of this region is required for normal function, and GTP binding alone is likely insufficient. Collectively, these data suggest that CIITA requires GTP binding to activate transcription, that the intrinsic activity of the activation domain of CIITA is not adversely affected by diminished or absent GTP binding, and that specific features of the GTP binding region are involved.

CIITA cooperates with the histone acetyltransferases CBP, pCAF, and p300 (16, 17, 25, 35, 42). This is believed to happen at the HLA-DR promoter via histone acetylation and chromatin remodeling, although CBP and pCAF histone acetyltransferase activities are not essential and CIITA itself is reported to have acetyltransferase activity (17, 25). Cooperation between histone acetyltransferases and NLS-CIITA GTP binding mutants would argue against promoter recruitment defects. NLS-CIITA did not cooperate with CBP, consistent with the role of CBP and pCAF in promoting CIITA nuclear import through acetylation of lysine 144 (16). However, pCAF cooperates well with NLS-CIITA, implying that the role of pCAF in CIITA import is secondary to that of histone acetylation/transcriptional activation and/or that CBP is the predominant histone acetyltransferase influencing the nuclear import of CIITA. Likewise, p300 cooperates. Although NLS-GTP1 and NLS-GTP3 failed to cooperate with pCAF or p300, NLS-GTP2 cooperated with both, correlating very well with promoter/reporter experiments and supporting our interpretation that NLS-GTP2 is simply less active. Further, they indicate that NLS-GTP2 is present at the HLA-DR promoter. NLS-C2RAS, however, consistently cooperated with p300, but not pCAF or CBP, yet failed to substantially activate the HLA-DR promoter. It may be possible that C2RAS together with p300 interacts in a limited fashion with the promoter, has increased histone acetyltransferase activity, or weakly sequesters histone deacetylases known to interact with CIITA (11, 43–45). The latter seems likely as the histone deacetylase inhibitor trichostatin A, which induces CIITA-independent HLA-DR transcription, leads to histone deacetylase displacement (45).

Chromatin immunoprecipitation experiments confirm that NLS-CIITA is recruited to the HLA-DR promoter but the GTP mutants and C2RAS are either defective or largely impaired. Of the mutants, NLS-GTP2 appears to retain limited ability to interact with the HLA-DR promoter, consistent with its ability to partially activate transcription and cooperate with coactivators. Considering the reasons suggested above for the partial activity of NLS-GTP, it is reasonable to conclude that NLS-GTP2 likely associates weakly with the promoter or its interaction is only stable transiently. Such transient association might suggest a GTP-dependent event required for stabilization of protein-protein interactions required for promoter activation. Collectively, these experiments largely substantiate that CIITA requires GTP binding to associate with the HLA-DR promoter. Because GTP binding induces a conformational change in Ras promoting Raf binding, it is likely that a similar change occurs in CIITA, although the GTP-dependent interaction partner is unknown.

The transcription factors RXF5, NF-YB, CREB, and the chro-
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matin-remodeling protein BRG1 interact with residues 335 to 886 flanking the GTP-binding site (21, 46). Although loss of a single interaction could result in failed promoter recruitment, other possibilities exist, including differences in phosphoryla-
tion or acetylation and failure to initiate promoter opening/ chromatin remodeling. The latter is supported by chromatin remodeling experiments where residues 323–548 of CIITA interact with BRG1 (46). Although an attractive hypothesis, a role for CIITA in promoter opening, as opposed to (or in con-
junction with) transcription activation, is controversial. We are currently exploring the possibility that one or more CIITA-protein interactions are GTP-dependent, but at present, the identity of the interacting protein(s) and whether these require a GTP-dependent conformation or specific sequences in the nucleotide-binding domain remain to be determined.

Our findings indicate the importance of GTP binding for transcriptional activation of MHC II genes by CIITA, further supporting the hypothesis that CIITA can be regulated through transcriptional activation of MHC II genes by CIITA, further

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