Phosphorylation of class II transactivator regulates its interaction ability and transactivation function

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Abstract

The MHC class II transactivator (CIITA) plays a central role in adaptive immune responses by controlling the expression of MHC class II genes. CIITA binds DNA-binding proteins and co-activator proteins to form an enhanceosome complex necessary for MHC class II gene expression. Here we demonstrate that CIITA interactions depend upon the phosphorylation status of CIITA. Hyper-phosphorylated CIITA interacts with co-activator p300, RFX5 and CIITA itself, which in turn results in induction of MHC class II promoter activity. Moreover, the C-terminal region of CIITA containing leucine-rich repeats (LRR) is a regulatory domain for CIITA self-association and LRR binding to CIITA is negatively regulated by phosphorylation. cAMP-dependent protein kinase (PKA) phosphorylates CIITA, and serine residues residing in a region between the proline/serine/threonine-rich domain and the GTP-binding domain are phosphorylated by PKA in vitro. The maximum transactivation potential of CIITA requires PKA phosphorylation as demonstrated by reduced transactivation activities of the mutant bearing substitutions of serine residues at the PKA site.

Introduction

Class II transactivator (CIITA) is a critical transcription factor necessary for both constitutive and inducible expression of MHC class II genes (1–3). CIITA does not bind to the class II promoter DNA directly, rather it has been shown to interact with the DNA-binding proteins that bind the cis-acting elements of the promoter (4,5). In addition, CIITA binds the co-activators p300/CBP (6–8), members of the basal transcriptional machinery (9,10) and itself (11,12).

CIITA contains at least four domains: acidic, proline/serine/threonine-rich (PST), GTP-binding domain (GBD) and leucine-rich repeat (LRR), all of which are required to activate the MHC class II promoter. The acidic transcriptional activation domain binds the co-activator p300/CBP, and this interaction has been reported to lead to synergistic activation of the MHC class II promoter and the repression of the IL-4 promoter (6–8). The acidic domain also interacts with members of the basal transcriptional machinery (9,13). The PST domain is important for CIITA function, but its exact role remains unknown (14). The binding of GTP to the GBD is required for efficient translocation of CIITA from the cytoplasm to the nucleus (15). The GBD also contains two LxxLL motifs which are crucial for the self-association and transactivation function of CIITA (12,16). CIITA interacts with the MHC class II promoter DNA-binding proteins including RFX5, RFXANK, NF-YB, NF-YC and CREB (17). Except for RFXANK and NF-YC, which bind the N-terminal region of CIITA, the GBD of CIITA serves as the likely domain for these interactions (17). The C-terminal domain of CIITA containing LRR is important for translocation to the nucleus (5) and transactivation function (14). The LRR has been shown to interact with a central portion of CIITA including the GBD (11,12).

Signaling via the second messenger cAMP initiates a wide array of physiologic responses including cellular proliferation and survival, neuronal signaling, and metabolic processes (18). These effects are brought about by the phosphorylation of specific nuclear factors, which in turn regulate the expression of a wide variety of genes. Elevation of intracellular levels of cAMP activates the cAMP-dependent protein kinase (PKA), which is required for cAMP to stimulate transcription (19–21). PKA phosphorylates several members of the MHC class II-
specific enhanceosome complex, including CREB (22,23) and all three subunits of the RFX complex (24). The effect of phosphorylation of these proteins in the context of the MHC class II promoter is not known, however.

There appear to be differences in the effect of cAMP/PKA on the expression of MHC class II genes, which is cell-type dependent. Studies have shown that stimulation of the cAMP pathway results in a reduction of MHC class II RNA in B cells (25) and IFN-γ-induced macrophages (26); yet another study concluded the cAMP/PKA pathway does not play a role in MHC class II expression in macrophages (27). Finally, PKA signaling induced MHC class II protein and mRNA expression in HeLa cells and a glioblastoma multiforme line respectively (28,29).

These studies of cAMP/PKA-mediated MHC class II gene regulation were performed prior to the functional studies of CIITA. Therefore, we have investigated the role of cAMP/PKA in CIITA-mediated MHC class II gene expression. We show that CIITA is a phosphoprotein, and that phosphorylation of CIITA induced by PKA regulates CIITA’s transactivation potential by controlling CIITA self-association and interaction with other transcription factors.

**Methods**

**Transfections and functional assays**

293T was maintained as previously described (30). RAW264.7, murine macrophages, were purchased from ATCC and maintained in DMEM supplemented with 10% FBS and 2 mM L-glutamine. 293T cells were transfected using a standard calcium phosphate method with 1 x 10^5 cells and 33 ng of each DNA. Cells were analyzed for luciferase activity 2 days after transfection. For RAW264.7 cell transfection, 5 x 10^5 cells in a six-well plate were transfected with 1 µg of each DNA using FUGENE 6 (Roche). Luciferase assay was performed on the next day. The cytomegalovirus (CMV) promoter-driven β-galactosidase expression vector was used in all transfections and luciferase values were normalized to β-galactosidase activity. Fold induction was calculated using the luciferase activity of cells transfected with the reporter DNA alone as 1. Values in all transfections represent the average of at least three independent experiments performed in duplicate.

**Plasmid constructs**

Plasmids containing wild-type CIITA, Eα-luciferase, CMV-β-gal, ΔLRR/CIITA(1–872), ΔAcidic/CIITA(157–1130), PST, LRR and GBD/CIITA(407–856) (12), ΔPST and the C-terminal Δ71 (14,31), expression plasmid for the catalytic subunit of PKA/ PKAc, PKAc mutant, and PKI (32), Nod1 (33) and RFX5 (34) have all been described. The recombinant CREB and purified, recombinant PKAc have also been described (35). All constructs were made using the expression vector pCDNA3 containing an HA- or a FLAG-epitope at the N-terminus. PCR-mediated mutagenesis was used to generate internal deletion and amino acid substitution mutants. The primers used to generate mutants are shown below. The number indicates the starting position of the primer that corresponds to the published human CIITA cDNA sequence (1). 5’ and 3’ indicates the direction of priming. 5’-1230: GAGCGCCAG-CACAAGCCTGGA; 3’-1252 for S374A: TCCAGGGCTTGC-GTGGCCCTC; 5’-1082 with FLAG epitope and KpnI site: CGGGGTATCAGCTACTAAACAGCACTGATGACGACTTGA- ACCAGTTT; R1 was cloned by PCR with 5’-1082 and 3’-1519, and the PCR product digested with KpnI and NcoI, and cloned into pCDNA3 KpnI and NcoI sites. R1 S374A mutant was generated by overlapping PCR. The first PCR reactions used 5’-1230 and 3’-1519, and 5’-1077 and 3’-1252. These PCR reactions were mixed and followed by PCR with 5’-1082 and 3’-1519, followed by digestion with KpnI and NcoI, and cloned into pCDNA3. R1 S374/377 mutant was generated by overlapping PCR. The first PCR reactions used 5’-1227 and 3’-1519, and 5’-1077 and 3’-1252. These PCR reactions were mixed and followed by PCR with 5’-1082 and 3’-1519, followed by digestion with KpnI and NcoI, and cloned into pCDNA3. R2 S374A and KpnI and NotI sites. The acidic domain construct contained the sequence between the EcoRI and PvuII site (nucleotide position 596) of wild-type CIITA in pCDNA3. Mutants with deletions from the C-terminus were generated by introducing a stop codon at the nucleotide position 3466 (A13), 3164 (A113) and 2702 (ΔLRR). An XbaI site was added after the stop codon to facilitate the cloning. The integrity of the mutants was confirmed by sequencing.

**Immunoprecipitations, Western blotting and binding assays**

Immunoprecipitation and Western blotting were performed as previously described (12). For binding assays of proteins made in different plates of cells, 8 µg of each plasmid DNA was transfected into separate plates of cells. Two days later, cells were harvested, placed on ice and whole-cell lysates were made. These lysates were then normalized to each other based on the amount of protein and placed at 37°C for 1.5 h with or without 100 U of alkaline phosphatase (Roche) per 150 µg of lysate. An equal amount of each lysate was saved for analysis of input protein, while the remainder of the lysates were equilibrated to 4°C and then mixed with the appropriate partner lysate and brought up to a total volume of 600 µl with ice-cold binding buffer (150 mM KCl, 20 mM HEPES pH 7.4, 5 mM MgCl2, and 0.1% NP-40 plus protease inhibitors). These lysates were incubated on ice for 2 h, followed by immunoprecipitation reactions and Western blotting.
**Phosphorylation of CIITA**

In vitro kinase assays

Plasmids containing FLAG-tagged CIITA domains were used in in vitro transcription and translation reactions. Typically, 40 μl reactions were performed according to the manufacturer’s recommendations (T7 TNT kit; Promega, Madison, WI) using non-labeled methionine. Upon completion of the reactions, tubes were placed on ice and 300 μl of ice-cold binding buffer was added. Then 2 μg of anti-FLAG (M2; Sigma) was added and immunoprecipitation reactions carried out as described above. Immunoprecipitation pellets were then resuspended in kinase assay buffer (50 mM MOPS, pH 6.8, 50 mM NaCl, 2 mM MgCl2, and 1 mM DTT). One-third was analyzed for protein synthesis using Western blotting (anti-FLAG, M2; Sigma) and one-third for negative control of kinase assay ([γ-32P]ATP added to final concentration of 1 mM, no recombinant PKAc added) and one-third for the kinase assay (1 mM [γ-32P]ATP plus 100 ng of rPKAc). Kinase assay and control reactions were incubated for 30 min at 30°C. Samples were run on SDS-PAGE gels, and exposed to film or transferred to PVDF membrane (Millipore) and Western blotted for FLAG-tagged proteins.

**Results**

**Phosphorylated CIITA self-associates**

We have noted that when CIITA plasmid DNA is expressed in cells, the CIITA protein detectable by Western blotting appears as a doublet. One possibility is that the CIITA doublet is due to migration differences between CIITA proteins which have been post-translationally modified. To test if phosphorylation was responsible for the migration difference, FLAG-CIITA plasmid was transfected into 293T cells and the lysates were treated with or without alkaline phosphatase. The samples were then separated by SDS-PAGE followed by Western blotting using anti-FLAG antibodies. Without alkaline phosphatase treatment, CIITA appeared as a doublet (Fig. 1A, lane 1). However, alkaline phosphatase treatment caused the disappearance of the slower migrating upper band (Fig. 1A, lane 2), suggesting that the upper band is a phosphorylated form of CIITA, and that CIITA protein normally exists in both hyper- and hypo-phosphorylated forms.

We and others have recently shown that CIITA forms complexes with itself (11,12). To determine if the phosphorylation of CIITA played a role in self-association, we examined the interaction between HA- and FLAG-tagged CIITA with or without alkaline phosphatase treatment. While both HA- and FLAG-CIITA expressed comparable amounts in 293T cells (Fig. 1B, bottom two panels), the interaction between the two was not detectable from the sample that was treated with alkaline phosphatase (Fig. 1B, upper panel). These data indicate that self-association of CIITA is phosphorylation dependent.

**Phosphorylated CIITA interacts with p300**

CIITA has been proposed to comprise an integral part of an enhanceosome complex on the MHC class II promoter.
Therefore, we asked whether phosphorylation of CIITA would affect its association with components of the enhanceosome, such as p300. FLAG-CIITA and HA-p300 were co-transfected to 293T cells, and cell lysates were prepared followed by immunoblot. As shown in Fig. 1(C), lysates contained both the hyper- and hypo-phosphorylated forms of CIITA (Fig. 1C, lane 2, top two panels), but only the slower migrating, hyper-phosphorylated form of CIITA co-immunoprecipitated with p300 (Fig. 1C, lane 1, top two panels). When the cell lysates were treated with alkaline phosphatase, the interaction of CIITA to p300 was greatly reduced (Fig. 1D, upper panel). Again, alkaline phosphatase-treated CIITA showed one band that corresponds to the hypo-phosphorylated form (Fig. 1D, middle panel). p300 did not show a difference in its migration pattern upon phosphatase treatment (data not shown). It is possible, however, that different forms of p300 could not be resolved due to its high mol. wt.

**CIITA intra-molecular interactions are governed by phosphorylation**

Interactions between the C-terminal LRR and a central region of CIITA including the GBD have recently been characterized (11,12). Upon analysis of the self-association of CIITA, we consistently observed more self-association from the mutant lacking the LRR than the wild-type protein (Fig. 2B, top panel). We reasoned that the LRR binds to the central domain of CIITA on the same molecule, preventing the central region from associating with another CIITA molecule. To test this, we compared CIITA mutants with varying length of deletions at the C-terminus for their ability to interact with the LRR domain. The interaction of HA-LRR with the full-length CIITA was not detectable (Fig. 2C, lanes 1 and 2). However, mutants with C-terminal truncations showed interaction with the co-transfected LRR (Fig. 2C, lanes 3–10). The degree of interaction was inversely correlated with the length of LRR and the best interaction occurred with the mutant lacking the entire LRR (Fig. 2A and C). This supported a model in which the LRR acts as a regulatory domain for self-association.

We next set out to determine if the intra-molecular interaction between the LRR and the central region of CIITA might be regulated by phosphorylation. We co-transfected FLAG-ΔLRR and HA-LRR into the same population of cells, and then immunoprecipitated with anti-HA antibodies. Although two forms of the ΔLRR protein were present, the LRR co-immunoprecipitated only the faster-migrating, presumably hypo-phosphorylated form of the protein (Fig. 2D, top panel). These data suggest that, unlike the self-associating form of CIITA (Fig. 1B), the LRR seems to bind the hypo-phosphorylated form of CIITA.
PKA modulates inter- and intra-molecular interactions of CIITA

It has been shown that cAMP signaling has the ability to either inhibit or induce MHC class II gene expression in a cell-type-dependent manner (25–29), which was attributed to modulation of CIITA transactivation activity by PKA (38). Therefore, we asked whether PKA-mediated phosphorylation regulates CIITA interaction that could be responsible for differential activities of CIITA. To test this, CIITA was co-transfected with the vector plasmid or the expression vector encoding PKAc into 293T cells. As expected, CIITA protein by itself appeared as a doublet indicating the presence of both hyper- and hypo-phosphorylated forms (Fig. 3A, lane 1). When PKAc was co-transfected, there was a noticeable shift in the migration pattern, suggesting that PKA induces the phosphorylation of CIITA (Fig. 3A, lane 2). Again, alkaline phosphatase treatment resulted in CIITA that migrated predominantly as the faster form (Fig. 3A, lane 3).

To test whether PKA-mediated phosphorylation is responsible for CIITA self-association observed in Fig. 1(B), we co-transfected HA- and FLAG-tagged CIITA with or without PKAc. Co-transfection of PKAc enhanced the interaction of CIITA with itself (Fig. 3B, cf. lanes 5 and 6). As a negative control, FLAG-tagged CIITA was not co-immunoprecipitated when HA-CIITA was not present. These data suggest that the degree of CIITA self-association depends on PKA-mediated phosphorylation.

Next, we examined the role of phosphorylation in CIITA intra-molecular interactions. FLAG-ΔLRR and HA-LRR DNA were transfected separately into different plates of 293T cells. Whole-cell lysates were treated with alkaline phosphatase prior to mixing of lysates on ice. Post-incubation, immunoprecipitation reactions and Western blotting was performed. (D) Hyper-phosphorylated CIITA interacts with RFX5. RFX5 and FLAG-CIITA DNA without (lane 1) or with (lane 2) PKAc DNA were transfected separately into different plates of 293T cells. Anti-RFX5 immunoprecipitation reactions were followed by Western blotting as depicted.
Since co-transfecting PKAc with CIITA increased CIITA phosphorylation in vivo, we asked whether CIITA was a substrate for PKA in vitro. We used in vitro transcribed and translated FLAG-tagged wild-type and mutant forms of CIITA as a source of CIITA protein for the kinase assays (Fig. 4A, see Methods). Wild-type CIITA was phosphorylated in the presence of PKAc (Fig. 4B, lane 1). To determine the domain that is phosphorylated by PKA in vitro, we tested deletion mutants of CIITA shown in Fig. 4(A). All mutants were phosphorylated, indicating that the loss of one domain does not abolish PKA phosphorylation (Fig. 4B, lanes 3–8). PKA-mediated phosphorylation of CIITA seems to be specific since Nod1, a protein with a similar domain architecture to CIITA (30, 33), was not phosphorylated under similar conditions (Fig. 4B, lane 9). The amounts of CIITA proteins generated in vitro were similar (Fig. 4B, bottom panel).

PKA phosphorylates the region between the PST and GBD of CIITA

To further identify regions(s) within CIITA that are phosphorylated by PKA, we constructed individual domains and regions between the domains of CIITA and tested them in kinase assays as before (Fig. 5A). As a positive control, recombinant CREB protein was used since it is phosphorylated by PKA (22) (Fig. 5B, lane 1). PKAc phosphorylated R1 (325–408), a region between the PST and the GBD (Fig. 5B, lane 4). Other domains (PST, LRR and GBD) were not phosphorylated although the amounts of input protein were greater than that of R1 (Fig. 5B, bottom panels). The region between the GBD and the LRR, which we termed R2, also has a potential PKA site (Fig. 5C). However, R1, but not R2, was phosphorylated by PKAc in vitro (Fig. 5C, cf. lanes 1 and 3). This was not due to the lack of R2 protein (Fig. 5C, bottom panels). Taken together, the R1 region is a target of PKA in vitro.

The R1 sequences include a putative PKA site, RXS or RXXS (X: any amino acid, S: phosphorylated serine residue) (39). To test if serine residues were phosphorylated by PKA, we first substituted the serine at position 374 to alanine, resulting in the S1 mutant (Fig. 5D). This substitution did not affect phosphorylation by PKAc (Fig. 5E, cf. lanes 2 and 4). The S2 mutant with substitutions of two serine residues, S374/377A, displayed a significantly reduced phosphorylation (Fig. 5E, lane 2 versus 6). Substitution of all four serines, S4, residing near this PKA consensus site, S373/374/375/377A, resulted in the complete loss of phosphorylation (Fig. 5E, lane 8). This result indicates that serine residues residing in the R1 region are a target of PKA phosphorylation in vitro.

Maximum transactivation potential of CIITA requires PKA-mediated phosphorylation

To ascertain whether PKA-mediated phosphorylation of R1 had a functional significance, the S4 mutation was engineered into the context of full-length CIITA resulting in FL-S4. We then compared the transactivation potential of the mutant with the wild-type in 293T cells by co-transfecting with the MHC class II promoter-driven luciferase reporter plasmid. When we tested the wild-type CIITA for its transactivation potential in the presence of PKAc, CIITA or PKAc alone led to a moderate induction of the promoter (Fig. 6A, lanes 2 and 3). When CIITA and PKAc were co-transfected, we observed synergistic augmentation of luciferase activity (Fig. 6A, lane 4). The kinase activity inherent to PKAc was essential for this activity since co-transfection with a catalytic subunit mutated in the kinase domain did not augment CIITA mediated activity of the promoter (Fig. 6A, lanes 5 and 6). This enhanced activity correlated with an increase in CIITA phosphorylation, as indicated by slower molecular mobility in Western blot (Fig. 6B).
Fig. 5. PKA phosphorylates a region between the PST domain and the GBD of CIITA. (A) The FLAG-tagged CIITA domains and regions used are shown. (B) In vitro kinase assays were performed as in Fig. 4. Recombinant CREB protein (lane 1) was used as a positive control for the in vitro kinase reactions. Arrowheads point to phosphorylated proteins. The input proteins were marked with asterisks shown at the bottom panels. HC and LC denote heavy and light chain Ig respectively. (C) PKA phosphorylates the RSSS motif in R1, but not in R2. Comparison of a portion of the amino acid sequences of R1 and R2 is shown. Arrowheads point to phosphorylated proteins (top panels), and to in vitro transcribed and translated input control protein (bottom panels). HC and LC depict the antibody heavy chain and light chain bands detected during Western blotting. (D) Serine to alanine mutations were made in the R1 region as indicated. (E) Phosphorylation of R1 depends upon serine residues located in this region. The following mutants were used: S1, S374A, S2, S374/377A and S4, S373/374/375/377A. Kinase assays and Western blotting were performed as before.
The mutant FL-S4 CIITA showed a slightly reduced ability to transactivate the MHC class II promoter compared to that of the wild-type in 293T cells (Fig. 6C, lanes 1 and 2). PKAc co-transfection resulted in further induction of the MHC class II promoter by the mutant (Fig. 6C, lanes 2 and 4), although the level of induction was less than the wild-type (Fig. 6C, cf. lanes 3 and 4). To test whether PKA-mediated phosphorylation of CIITA has a functional significance in antigen-presenting cells, we used RAW264.7, a macrophage cell line, for the transfection experiment. Consistent with the data from 293T cells, wild-type CIITA activity was enhanced by PKA transfection (Fig. 6D, lane 3 and 4). In contrast, the degree of PKA responsiveness of the mutant CIITA was reduced in the macrophage cell line (Fig. 6D, lane 5 and 6).

**Discussion**

Our current study demonstrated for the first time that phosphorylation of CIITA regulates CIITA’s ability to self-associate and to interact with other proteins, such as p300 and RFX5, found in the MHC class II enhanceosome complex. It was primarily the hyper-phosphorylated form of CIITA which associated with self and components of the enhanceosome, providing support for a positive role of CIITA phosphorylation in the activation of the MHC class II promoter. The fact that there was no detectable interaction of the hypo-phosphorylated form of CIITA with any of these proteins suggests that the phosphorylation may induce conformational changes in CIITA which allow protein–protein interactions and, likewise, that the
hypo-phosphorylated form of the protein displays a conformation rendering it incapable of these interactions.

Interaction between the LRR and the central portion of CIITA was studied in the context of the phosphorylation status of CIITA. Previously, we and others have documented that the LRR binds a small portion of the central region which includes the GBD (11,12). The LRR bound preferentially to the under-phosphorylated form of CIITA (Fig. 2D and 3C). Hence, the LRR binding to the under-phosphorylated form could keep this form of the protein in a conformation unable to interact with other proteins, acting as a negative regulatory domain. Upon CIITA phosphorylation, a conformational change may occur, preventing LRR intra-molecular interaction, and allowing CIITA to interact with other proteins and act upon the MHC class II promoter. Alternatively, LRR interaction with an unidentified protein may induce conformational changes that allow CIITA to be phosphorylated and be accessible to components of the enhanceosome.

A similar regulation of inter- and intra-molecular interactions by phosphorylation has been reported (40). The unphosphorylated NF-κB subunit p65 does not interact with p300/ CBP because the binding sites are masked by the intra-molecular interaction of p65. Phosphorylation weakens intra-molecular interaction of p65, analogous to that of the interaction between the LRR and the GBD for CIITA, creating the conformation accessible for CBP/p300 binding. Therefore, the transcriptional activity of both NF-κB and CIITA is regulated by a similar manner that involves the conformational change in intra-molecular interactions induced by phosphorylation. For NF-κB, PKA-mediated phosphorylation was responsible for regulation of conformational changes and transcriptional activity (40).

We chose to examine PKA phosphorylation of CIITA in detail because studies have shown that cAMP/PKA signaling affects MHC class II expression in various cell types (25–29). Our data suggest that this effect is at least partly due to changes in CIITA interactions and its transactivation potential that are modulated by PKA-mediated phosphorylation. While these experiments were underway, two other groups published work documenting the phosphorylation of CIITA (38,41). The data by Li et al. showed that transactivation function of CIITA is modulated by PKA-mediated phosphorylation in monocytes/macrophages although underlying mechanisms for this regulation were not investigated. Work by Li et al. also demonstrated that CIITA is phosphorylated by PKA in several regions of the protein, including the region that we have identified in this study. However, we did not see phosphorylation of any region of CIITA besides R1 using as much as 1 μg of recombinant PKAc in the in vitro kinase assay (data not shown). PKA may depend upon a conformation only exhibited by full-length, wild-type CIITA in order for phosphorylation at other residues to take place. Alternatively, PKA-mediated phosphorylation observed in the study of Li et al. may be indirect, through kinases activated by PKA. Both studies did not use recombinant CIITA and therefore further experiments using purified, recombinant CIITA are needed to clarify which serine residues within CIITA are directly phosphorylated by PKA.

The study of Li et al. also showed that CIITA activity was decreased when PKAc was co-transfected, whereas we observed slightly enhanced activity of CIITA in the presence of exogenous PKAc in the monocyte/macrophage cell line. This discrepancy between the two studies is not entirely clear. Possibilities for the differences observed include use of different cell lines, use of different class II promoter reporter constructs with differing amounts of upstream sequence or possible differences in the PKAc expression vector. CIITA interaction studies from macrophages could clarify this issue, although it is technically difficult to investigate CIITA interactions in macrophages as we have done in 293T cells since the transfection efficiency in macrophages is poor. In this regard, further investigation is also necessary to confirm whether endogenous CIITA is phosphorylated by PKA, and, moreover, whether phosphorylation is required for self-association and transactivation function of CIITA in antigen-presenting cells including B cells. Lack of a means to distinguish between two endogenous CIITA proteins challenges this task.

In contrast, the data of Tosi et al. (41) agrees with our model for a role of phosphorylation in CIITA self-association and functional activity. Their experiments found that phosphatase treatment reduced CIITA self-association, while treatment with the phosphatase inhibitor okadaic acid enhanced both self-association and functional activity. By using [32P]orthophosphate labeling of COS cells transfected with various deletion mutants, Tosi et al. map the region of constitutive phosphorylation to residues 253–321. This discrepancy with our in vitro mapping results (Figs 4 and 5) is most likely due to different kinases responsible for phosphorylation of CIITA at different positions.

We found that the mutant CIITA was phosphorylated in cells and activated the MHC class II promoter, suggesting that direct phosphorylation of CIITA by PKA is not responsible for all of PKA's effect on MHC class II gene expression. It is likely that the remaining PKA enhancement of MHC class II expression was due to PKA-mediated phosphorylation of other components of the enhanceosome. Alternatively, activation of other kinases could phosphorylate CIITA. CIITA over-expressed in 293T cells was constitutively phosphorylated without additional stimulation (Fig. 1A). This data further suggests that PKA may not be the primary kinase regulating CIITA interactions or the basal level of PKA activity in cells is sufficient for CIITA phosphorylation. Consistent with this notion, the PST domain, which contains many serine and threonine residues, was not phosphorylated by PKA in vitro. Nonetheless, the PST domain is most likely phosphorylated in cells in a PKA-independent manner, as the migration pattern of a mutant lacking this domain did not exhibit the distinguishable hyper- and hypo-phosphorylated forms seen with the wild-type (unpublished data). Additional studies are required to determine which kinases are responsible for generating the hyper-phosphorylated form that is capable of self-association.

In summary, this study demonstrates that the overall phosphorylation status of CIITA is important for CIITA interaction with self and other transcription factors. Our data showed that PKA is at least partly responsible for phosphorylation of CIITA to achieve the maximum level to transactivate the MHC class II promoter. Underlying mechanisms explaining the cell-type-specific effect of PKA in the context of CIITA interaction remain to be elucidated.
Phosphorylation of CIITA

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Abbreviations
CIITA class II transactivator
cAMP cyclic adenosine monophosphate
CMV cytomegalovirus
GDB GTP-binding domain
LRR leucine-rich repeat
PKA cAMP-dependent protein kinase
PKAc catalytic subunit of PKA
PST proline/serine/threonine-rich

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