Phosphorylation and Ubiquitination of Degron Proximal Residues Are Essential for Class II Transactivator (CIITA) Transactivation and Major Histocompatibility Class II Expression*

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Major histocompatibility (MHC) class II molecules are cell surface glycoproteins that present extracellular antigens to CD4+ T cells and are essential for initiation of the adaptive immune response. MHC class II expression requires recruitment of a master regulator, the class II transactivator (CIITA), to the MHC class II promoter. Post-translational modifications to CIITA play important roles in modulating CIITA mediated transcription of various genes in different cell types. We have previously linked regulation of CIITA to the Ubiquitin Proteasome System (UPS), and we and others have demonstrated that mono-ubiquitination of CIITA dramatically increases its trans-activity whereas poly-ubiquitination leads to CIITA degradation. Here we identify three degon proximal lysine residues, Lys-315, Lys-330, and Lys-333, and a phosphorylation site, Ser-280, located within the CIITA degon, that regulate CIITA ubiquitination, stability, and MHC class II expression. Together, these findings contribute to the developing post-translational modification code for CIITA.

Major histocompatibility (MHC) class II proteins present processed exogenous antigens on the cell surface to activate CD4+ T cells (1) and are thus critical contributors to both cell and antibody-mediated immune responses (2). In addition to eliciting pathogen-mediated immune responses, MHC class II molecules play important roles in anti-tumor immunity as tumor-specific CD4+ T cells recruit and activate CD8+ T cells at tumor sites (3). Because MHC class II is a critical regulator of both pathogen- and tumor-mediated adaptive immune responses, its expression is tightly regulated (4), and this regulation is primarily at the level of transcription. CIITA is a non-DNA binding cofactor required for initiation of MHC class II gene transcription (5). CIITA is expressed from three distinct promoters, (pl, pIII, and pIV), which direct the synthesis of CIITA isoforms I, III, and IV to ensure CIITA expression in different cell types under different conditions (6). CIITA isoform I (IFI) is expressed from pl in dendritic cells and macrophages, IF III is expressed from pIII in B cells and is up-regulated in response to IFN-γ (7), and IF IV is expressed from pIV in IFN-γ induced cells (6). CIITA is not only essential for MHC Class II transcription, but also plays crucial roles in transcriptional regulation of additional diverse immune response genes including IL-4 (8), IL-10 (9), E-cathepsin (10), MMP-9 (11), plexin (12), and Fas ligand (13). As expected for a critical regulator of diverse inflammatory genes, dysregulated expression and activity of CIITA is implicated in diseases including cancer, autoimmune disease, atherosclerosis, and many others (14–19). However, because of a complex domain structure, multiple roles in transcriptional regulation, and differential expression in various cell types, CIITA post-translational regulation remains enigmatic.

Post-translational regulation of CIITA is a crucial regulatory point as functional CIITA requires multiple post-translational modifications (20–25) including phosphorylation, ubiquitination, and acetylation (21–26). Among these, phosphorylation of CIITA is critical for CIITA transactivity, nuclear localization, oligomerization, and for interactions with transcription factors and cofactors (24, 26–28). CIITA ubiquitination is equally important as mono-ubiquitinated CIITA displays increased transactivity, association with the MHC class II enhanceosome complex, and drives enhanced MHC class II transcription (29). Poly-ubiquitinated CIITA has been shown to be degraded by the ubiquitin-proteasome pathway (30). Whereas ubiquitination is a known regulator of CIITA, sites of ubiquitination in CIITA remain to be identified, in part due to its large size and 42 lysine residues, each of which serves as a potential ubiquitination site. We identify here a regulatory phosphorylation site at serine 280 within the CIITA degon as a gatekeeper of CIITA mono-ubiquitination. We further identify three lysine residues, Lys-315, Lys-330, and Lys-333, which are proximal to the degon sequence in CIITA isoform III, as sites of mono-ubiquitination. Mutating these lysine residues to arginine reduces CIITA mono-ubiquitination, protein stability, transactivity, and MHC class II expression. These findings identify novel sites in CIITA isoform III that regulate CIITA transactivation and CIITA-mediated MHC class II expression.
**EXPERIMENTAL PROCEDURES**

*Cell Culture—HeLa cells (human epithelial) from ATCC (Manassas, VA) were maintained using high-glucose Dulbecco’s modified Eagle (DMEM) medium (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, 50 μg/ml of streptomycin and 2 mM...*
Flag-CIITA 1–335aa plasmids were kindly provided by Dr. J. CO2.

of domain structure of CIITA. CIITA is an 1130 amino acid protein with four functional domains: an N-terminal transcriptional acidic activation domain (AAD), a proline/serine/threonine rich (P/S/T) domain, a GTP-binding domain (GBD), and a C-terminal leucine-rich region (LRR). CIITA has three nuclear localization signals (NLS) dispersed through the length of the protein. The P/S/T rich region of CIITA contains a proteolytic signal site termed a degron (D) (top panel).

Site-directed mutagenesis was performed to mutate serine 280 to alanine (Flag-CIITA S280A, Flag-Mono-Ub-CIITA, or Flag-Mono-Ub-CIITA K3R (K315, 330, 333R) plasmids using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Eighteen hrs following transfection, cells were lysed with 100 µl of 1× cell culture lysis reagent (Promega, Madison, WI) supplemented with complete EDTA-free protease inhibitors (Roche). Following lysis, cells were scraped and the suspension was centrifuged for 2 min at 12,000 rpm (Thermo electron 851, Thermo INC, Needham Heights, MA) at 4 °C, and luciferase assays were performed using Lmax II 384 (Molecular Devices, Sunnyval, CA) according to the manufacturer’s instructions. Luciferase readings were normalized to protein content in the lysates by Bradford Assay. Western blots were performed to confirm equal expression of expressed plasmids.

RNA Expression—HeLa cells were plated at a cell density of 8 × 10^5 cells/plate. Following adhesion, cells were transfected with pcDNA, Flag-CIITA, Flag-CIITA K1R (K315R), Flag-CIITA K2R (K330, 333R), Flag-CIITA K3R (K315, 330, 333R), or Flag-CIITA S280A plasmids using Fugene 6. Cells were stimulated with interferon-γ and 18 h post-stimulation, cells were harvested, washed with cold PBS, centrifuged at 3,000 rpm at 4 °C for 5 min, and 9/10th of the cells were used to extract RNA.

Total RNA was prepared with 1 ml of TRizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA was reconstituted in 30 µl of DEPC water (MP Biomedicals, LLC, Aurora, OH) and stored at -80 °C. The Omniscript reverse transcription kit (Qiagen) was used to reverse transcribe 1 µg of RNA into cDNA. Gene-specific antisense primers (Sigma) were used for reverse transcription (RT). PCR was performed using a Mastercycler thermal cycler (Eppendorf, Hauppauge, NY). Real-time PCR reactions were carried out on an ABI prism 7900 (Applied Biosystems, Foster City, CA) using primers and probes for MHC-II (31–32) and GAPDH (33). GAPDH RNA was used to normalize mRNA values. Presented values from real-time PCR reactions were calculated on the basis of standard curves generated for each gene, were run in triplicate reactions, and were analyzed using the SDS 2.0 program. 1/10th of cells were lysed in 100 µl of 1% Nonidet P-40 buffer supplemented with complete EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged and Bradford assays were performed to determine total protein content in the lysates. Western blots confirmed equal expression of expressed plasmids.
Ubiquitination Assay—HeLa cells were plated at a cell density of $8 \times 10^5$ in 10-cm tissue culture plates. Following adhesion, cells were co-transfected as indicated with Flag-CIITA, 1–224 or 1–335aa truncation mutants of CIITA, Flag-CIITA 1–335aa K3R (K315, 330, 333R), Flag-CIITA K3R (K315, 330, 333R), or Flag-CIITA S280A, and HA-Mono-Ub or HA-WT-Ub. Cells were treated with the proteasome inhibitor MG132 for 4 h, harvested, and lysed in 1% Nonidet P-40 buffer supplemented with complete EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged, normalized for protein concentration, precleared with 50 μl of mouse IgG (Sigma-Aldrich), and immunoprecipitated with 30 μl of anti Flag-agarose beads (Sigma-Aldrich). Immune complexes were denatured with Laemmli buffer, boiled, and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-HA monoclonal antibodies (Sigma-Aldrich). HRP conjugates were detected with Immobilon Western Chemiluminescent substrate (Millipore) to determine ubiquitination patterns of various CIITA constructs. Bradford assays were used to normalize for protein and equal loading was determined in non-immunoprecipitated lysates.

Half-life Assay—HeLa cells were plated at a cell density of $8 \times 10^5$ in 10 cm tissue culture plates. Following adhesion, cells were transfected as indicated with 5 μg of Flag-CIITA, Flag-CIITA K3R (K315, 330, 333R), or Flag-CIITA S280A. Transfected cells were treated with 100 μM cycloheximide (Sigma-Aldrich) for 0–20 h. Following cycloheximide treatment, cells were lysed in 1% Nonidet P-40 buffer supplemented with complete EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged and normalized for protein concentration. As controls, HeLa cells were transfected with indicated plasmids and treated with
100 μM cycloheximide and MG132 (EMD Biosciences, San Diego, CA) for 8 h. Proteins were denatured with Laemmli buffer, boiled, and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-Flag antibodies (Sigma-Aldrich). HRP conjugates were detected with Immobilon Western Chemiluminescent substrate (Millipore) to determine half-life of CIITA constructs. Proteins were normalized by Bradford assay, and equal loading was determined in lysates.

**Flow Cytometry**—HeLa cells were plated at a cell density of 8 × 10^5 in 10 cm tissue culture plates. Following adhesion, cells were transfected with the 1–224aa or 1–335aa truncation mutants of CIITA or WT-CIITA, and HA-mono-ubiquitin (HA-Mono-Ub) or HA-WT-Ubiquitin (HA-WT-Ub). Cells were treated as indicated with the proteasome inhibitor MG132 for 4 h prior to harvesting. Following lysis, cells were IP’d with anti-Flag antibody and IP’d samples were IB’d with anti-HA antibody (top panels). Lysates confirmed expression of Flag-CIITA (bottom panels). Comparisons were made between samples run on different percent gels simultaneously exposed to one film. Results shown are representative data of three experiments.

**RESULTS**

**Mutating Serine 280 in the CIITA Degron Reduces CIITA Ubiquitination and Enhances CIITA Half-life**—CIITA is large protein with four functional domains, an N-terminal acidic activation domain (AAD) required for CIITA transactivity, a proline/serine/threonine rich (P/S/T) domain required for binding factors and cofactors, a GTP-binding domain (GBD), and a C-terminal leucine rich region (LRR) required for localization and oligomerization (5, 22, 34) (Fig. 1A, top). The P/S/T domain of CIITA isoform 1 (IF1) contains a degron (D) (20), a sequence which typically targets proteins for ubiquitination at proximal lysine residues and degradation (35–36). CIITA IF1 serine 357 lies within the degron sequence and is phosphorylated by ERK1/2 prior to IF1 ubiquitination at as yet unknown sites (20). Our sequence analysis revealed the presence of a degron in CIITA isoform III (IF3), so we first determined if the conserved serine at position 280 is important for CIITA IF3 ubiquitination and stability. CIITA IF3 serine 280 was mutated to alanine using site-directed mutagenesis (Fig. 1A, bottom), and ubiquitination assays were performed. Expression of WT-CIITA or CIITA S280A with mono-ubiquitin (PE)-labeled anti-human HLA-DR (clone L243, Biologic, San Diego, CA) antibody or PE mouse IgG2a isotype control antibody (Biologic) was added to the cell suspension and rotated at 4 °C. Following antibody incubation, cells were washed twice with PBS, fixed with 2% paraformaldehyde, and stored at 4 °C. MHC class II cell surface expression was measured by FACS-Canto (Becton Dickinson, San Jose, CA) and analyzed using FlowJo. All samples were analyzed using 10,000 events per sample. 1/10th of the HeLa cells harvested for flow analysis were lysed in 1% Nonidet P-40 buffer supplemented with Complete EDTA-free protease inhibitors (Roche) on ice. Proteins were denatured with Laemmli buffer, boiled, and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-Flag antibodies (Sigma-Aldrich). HRP conjugates were detected with Immobilon Western Chemiluminescent substrate (Millipore) to determine expression of CIITA constructs. Proteins were normalized by Bradford assay and equal loading was determined in lysates.

**Degron Proximal CIITA Ubiquitination Regulates MHC II Expression**

**FIGURE 3.** CIITA is highly ubiquitinated downstream of the N-terminal degron. A, schematic representation of CIITA truncation mutants. Full-length Flag-CIITA was used to generate truncation constructs Flag-CIITA 1–224aa and Flag-CIITA 1–335aa. B, CIITA is ubiquitinated between residues 225 and 335. Ubiquitination assay, HeLa cells were transfected with the 1–224aa or 1–335aa truncation mutants of CIITA or WT-CIITA, and HA-mono-ubiquitin (HA-Mono-Ub) or HA-WT-Ubiquitin (HA-WT-Ub). Cells were treated as indicated with the proteasome inhibitor MG132 for 4 h prior to harvesting. Following lysis, cells were IP’d with anti-Flag antibody and IP’d samples were IB’d with anti-HA antibody (top panels). Lysates confirm expression of Flag-CIITA (bottom panels). Comparisons were made between samples run on different percent gels simultaneously exposed to one film. Results shown are representative data of three experiments. C, densitometric quantitation. Western blots from ubiquitination assays were quantified using Multi-Gauge V3.1 and graphed. Comparisons were made between samples run on different percentage gels simultaneously exposed on one film. Results shown are representative data of three experiments.
uitin in HeLa cells demonstrated that CIITA S280A is less mono-ubiquitinated than WT-CIITA (Fig. 1B, compare lanes 2 and 5). Densitometric quantitation revealed a 65% decrease in CIITA S280A mono-ubiquitination (Fig. 1C) and a 25% decrease in CIITA S280A wt-ubiquitination (Fig. 1B, compare lanes 3 and 6 and Fig. 1D) in comparison to that of WT-CIITA.
Half-life assays indicated CIITA S280A is more stable than WT-CIITA (Fig. 1E, top) and controls using a combined 8 h of treatment of cycloheximide and proteasome inhibition via MG132 equally reconstituted expression of WT-CIITA and CIITA S280A (Fig. 1E, bottom) demonstrating equal transfection and a dependence of WT-CIITA and CIITA S280A on proteasome-mediated degradation.

**Despite Increased Stability, CIITA S280A Is Less Active than WT CIITA**—We have recently identified a novel N-terminal 19 S proteasomal ATPase binding domain in CIITA that lies within the P/S/T region of CIITA, encompasses a majority of the degron, and includes the serine at amino acid 280. Absence of this domain increases CIITA half-life, but dramatically decreases MHC class II surface expression indicating roles for the 19 S ATPase binding domain in regulating CIITA transactivation (37). Serine 280 lies within this newly identified ATPase binding domain of CIITA. Observations in Fig. 1 indicate that mutating serine 280 to alanine reduces CIITA mono-ubiquitination and enhances CIITA half-life. As these observations link to our previous findings indicating roles for the ATPase binding domain in MHC class II expression, we next determined if mutating serine 280 to alanine affects CIITA-mediated MHC class II transcription. MHC class II mRNA levels were measured in HeLa cells transfected with WT-CIITA or with CIITA S280A and either untreated or stimulated with IFN-γ (Fig. 2A). In unstimulated cells, expression of WT-CIITA resulted in increased MHC class II expression as compared with pcDNA or CIITA S280A-transfected cells. Upon IFN-γ stimulation, endogenous MHC class II transcript levels were increased, and expression of WT-CIITA further enhanced transcript levels beyond cells transfected with pCDNA. However, expression of CIITA S280A did not significantly increase endogenous MHC class II transcript levels beyond that of IFN-γ stimulated cells transfected with pCDNA (Fig. 2A, left) despite equal expression of WT-CIITA and CIITA S280A (Fig. 2A, right). Flow cytometric analysis demonstrated MHC class II cell surface expression to be similarly negatively impacted by expression of the CIITA serine 280 to alanine mutant when compared with expression of WT-CIITA (Fig. 2B). Together, these data indicate that despite its increased stability, the CIITA S280A mutant is less active than WT-CIITA.

**CIITA Is Significantly Ubiquitinated Downstream of Ser-280 and the Degron Sequence**—Gene transcription involves highly coordinated and sequential post-translational modifications of activators with activator phosphorylation frequently preceding mono-ubiquitination. Serine phosphorylation within the degron of CIITA isoform I has been shown to precede CIITA mono-ubiquitination and subsequent poly-ubiquitination, however, sites of ubiquitination within CIITA remain unknown (20). Amino acid sequence analysis reveals the presence of 42 lysine residues in full-length CIITA. Of these, 7 lysine residues lie upstream of the degron while the remaining 35 lysine residues are located downstream of the degron. Of these 35 lysine residues, there are 3 proximal lysine residues immediately downstream of the degron. These three lysines are potential sites for ubiquitination as degron proximal lysine residues are frequently targets of ubiquitination (35–36). To identify if these lysine residues are targeted for ubiquitination within CIITA, we generated two CIITA truncation mutants: CIITA 1–224aa that lacks both the degron and degron proximal downstream lysines at residues 315, 330, and 333, and CIITA 1–335aa that contains both the degron and the degron proximal lysine residues (Fig. 3A) and compared ubiquitination of these truncation mutants to ubiquitination of WT-CIITA (Fig. 3B). Ubiquitination assays indicate low level mono-ubiquitination of CIITA 1–224aa compared with robust mono-ubiquitination of CIITA1–335aa (Fig. 3B, compare lanes 2 and 5) and WT-CIITA (Fig. 3B, compare lanes 2 and 8). Importantly, CIITA 1–335aa contains only 3 lysine residues more than CIITA 1–224aa. By comparison, WT-CIITA contains an additional 35 lysine residues more than CIITA 1–224aa and 32 more lysine residues than CIITA truncation 1–335aa. As the difference of 3 lysine residues between the two CIITA truncations results in a substantial difference in their levels of mono-ubiquitination (Fig. 3C), these 3 lysine residues likely serve as sites for prominent CIITA mono-ubiquitination.

**Mutating Degron Proximal Lysines 315, 330, and 333 to Arginine Reduces CIITA Mono-ubiquitination and Half-life**—As lysine residues proximal to degrons are preferentially targeted for ubiquitination by E3 ligases (35), we sought to further define the ubiquitination status of lysine residues 315, 330, and 333. Ubiquitination assays comparing WT full-length CIITA to full-length CIITA with mutated degron proximal lysines (Flag-CIITA K315,330,333R) (Fig. 4A), demonstrated decreased mono-ubiquitination (supplemental Fig. S1A, compare lanes 2 and 5, and supplemental Fig. S1B). The relatively small differences in ubiquitination between WT and mutant full-length CIITA can be attributed to the fact that WT full-length CIITA has a additional 39 lysine residues, which may also serve as sites of ubiquitination (29). Further evidence that this may be the case comes from ubiquitination assays comparing CIITA1–335 to the triple lysine mutant CIITA 1–335 K315, 330, 333R (Fig. 4B).
Degron Proximal CIITA Ubiquitination Regulates MHC II Expression

4A), which demonstrate dramatic decreases in mono-ubiquitination when degron proximal lysines are mutated to arginine (Fig. 4B, compare lanes 2 and 5, and Fig. 4C). Importantly, mutation of degron proximal lysines to arginine did not decrease poly-ubiquitination of CIITA 1–335 K315, 330, 333R in comparison to that of WT-CIITA 1–335 (Fig. 4B, compare lanes 3 and 6, and Fig. 4D). Together, these data indicate the three degron proximal lysine residues are targets for mono-ubiquitination, and highlight the possibility that other lysine residues may serve as sites for mono-ubiquitination if lysines 315, 330, and 333 are unavailable for ubiquitination.

Mono-ubiquitination has been shown to protect target proteins from the “stripping” activity of 19 S proteasomal ATPases, thus preventing transcription factor degradation and increasing transactivation potential (38–39). If degron proximal lysine residues Lys-315, Lys-330, and Lys-333 serve as sites for pro-
pective mono-ubiquitination, then mutating the three lysine residues to arginine may render them susceptible to rapid degradation and increased vulnerability to the "stripping" activity of 19 S proteasomal ATPases. Indeed, cycloheximide chase assays indicate the CIITA K3R mutant has a shorter half-life than WT-CIITA (Fig. 4E, top panels). Controls using a combined 8 h of treatment of cycloheximide and the proteasome inhibitor MG132 equally reconstituted expression WT-CIITA and CIITA K3R (Fig. 4E, bottom panel), demonstrating equal transfection and a dependence of each protein on proteasome-mediated degradation.

Mutating CIITA Lysines 315, 330, and 333 to Arginine Reduces CIITA Transactivity, MHC Class II mRNA Levels, and MHC Class II Surface Expression—Mono-ubiquitination of CIITA has previously been shown to enhance CIITA transactivity and MHC class II expression (29). As mutating lysine 315, 330, and 333 to arginine reduces CIITA mono-ubiquitination and half-life, we next performed luciferase reporter assays to determine if these mutations also affect CIITA mediated MHC class II transactivation. Results from reporter assays indicate expression of WT-CIITA dramatically increases transactivation from a MHC class II HLA-DR promoter construct. However, expression of any of the following combinations of CIITA lysine mutants: CIITA K315R, CIITA K330,333R, or CIITA K315,330,333R, results in dramatic decreases in CIITA transactivation (Fig. 5A, left). Western blots of transfection controls demonstrate expression of each of the indicated Flag-tagged constructs (Fig. 5A, right).

We next determined if endogenous MHC class II transcript levels were similarly negatively affected by the degron proximal lysine mutations. MHC class II mRNA levels were measured in HeLa cells transfected with WT-CIITA, CIITA K315R, CIITA K330,333R, or CIITA K315,330,333R and either left untreated or stimulated with IFN-γ. In unstimulated HeLa cells, expression of WT-CIITA resulted in an increase in MHC class II transcript levels as compared with cells transfected with any of the CIITA degron proximal lysine mutants (Fig. 5B, 0 h). When HeLa cells were stimulated with IFN-γ, endogenous MHC class II transcript levels increased and were further enhanced by expression of WT-CIITA (Fig. 5B, 18 h WT-CIITA). Expression of the degron proximal lysine mutants failed to significantly enhance MHC class II transcript levels beyond levels seen in cells stimulated with IFN-γ and transfected with the pcDNA control (Fig. 5B, 18 h). Western blots of transfection controls demonstrate expression of each of the indicated Flag-tagged constructs (Fig. 5B, bottom panels). Finally, MHC class II cell surface expression was similarly reduced by each of the CIITA lysine to arginine mutations in unstimulated HeLa cells (Fig. 5C, i-iv). Western blots of transfection controls demonstrate expression of each of the indicated Flag-tagged constructs (Fig. 5C, bottom panel).

As mutating the degron proximal lysine residues to arginine reduces CIITA activity and transactivation, we next genetically fused mono-ubiquitin to the N terminus of CIITA and CIITA K3R constructs in hopes of overcoming the lack of mono-ubiquitination at the degron proximal lysine residues. However, we observed substantial decreases in CIITA transactivation when either WT CIITA (Mono-Ub-CIITA) (Fig. 6A, left panel) or CIITA K315,330,333R (Mono-Ub-K315,330,333R) (Fig. 6A, right panel) was fused to mono-ubiquitin in luciferase reporter assays using these fusion constructs. Western blot analysis of lysates isolated from cells transfected with these fusion-constructs further demonstrated CIITA was degraded in these fusions as indicated by the formation of a smear as compared with a single band for WT-CIITA (Fig. 6B).

**DISCUSSION**

We sought here to identify essential ubiquitination sites in CIITA and to link CIITA ubiquitination to a regulatory phosphorylation event. Protein ubiquitination is the result of a complex enzymatic cascade which covalently attaches an ubiquitin moiety to an internal lysine residue. Although E3 ligases can target different lysine residues for ubiquitination, lysine residues proximal to degron sites are preferentially targeted (35–36). Mutation of threonine 286 in the degron of cyclin D1 leads to a loss of phosphorylation dependent ubiquitination resulting in accumulation of cyclin D1 (40). Similarly, Chk1 ubiquitination on degron proximal lysines by the Fbx6-containing SCF ubiquitin ligase is critical for S-phase regulation (36). Here we have identified a conserved serine residue within the degron of CIITA isoform III that regulates CIITA ubiquitination, protein stability, and MHC class II expression. Mutating serine 280 to alanine reduces CIITA ubiquitination and stabilizes CIITA. Recent studies have identified serine residues in the CIITA degron and P/S/T domain as targets of phosphorylation by ERK1/2 (20, 26, 41). ERK1/2 mediated phosphorylation and subsequent ubiquitination of proteins, including cyclin D and Bim, has been shown to be essential in regulating cell cycle progression. In these experiments, we fused a Flag-ubiquitin fusion to the C terminus of CIITA and CIITA K3R constructs in hopes of overcoming the lack of mono-ubiquitination at the degron proximal lysine residues. However, we observed substantial decreases in CIITA transactivation when either WT CIITA (Mono-Ub-CIITA) (Fig. 6A, left panel) or CIITA K315,330,333R (Mono-Ub-K315,330,333R) (Fig. 6A, right panel) was fused to mono-ubiquitin in luciferase reporter assays using these fusion constructs. Western blot analysis of lysates isolated from cells transfected with these fusion-constructs further demonstrated CIITA was degraded in these fusions as indicated by the formation of a smear as compared with a single band for WT-CIITA (Fig. 6B).

**FIGURE 5.** Mutating lysines 315, 330, and 333 to arginine reduces CIITA transactivity, MHC class II mRNA levels, and MHC class II surface expression. A, mutating CIITA lysine residues Lys-315, Lys-330, and Lys-333 to arginine reduces CIITA transactivity. Luciferase reporter assays, HeLa cells were transfected with Flag-CIITA, Flag-CIITA K1R, Flag-CIITA K2R, Flag-CIITA K3R, or pcDNA and HLA-DR promoter reporter. Luciferase readings were normalized to total protein concentration. Results shown are an average of three experiments (top panel). Expression of Flag-CIITA, Flag-CIITA K1R, Flag-CIITA K2R and Flag-CIITA K3R. Western blot, Lysates were IB’ed with anti-Flag antibodies to confirm expression of Flag-tagged constructs. Results shown are representative of three experiments (bottom panels). B, mutating CIITA lysine residues Lys-315, Lys-330, and Lys-333 to arginine reduces MHC class II mRNA levels. Reporter assays, HeLa cells were transfected with Flag-CIITA, Flag-CIITA K1R, Flag-CIITA K2R, Flag-CIITA K3R or pcDNA. Following transfection, cells were left untreated or were stimulated with IFN-γ for 18 h. Cells were harvested, RNA extracted, and cDNA was prepared and quantified by real-time PCR. Results shown are an average of three experiments (top panels). Expression of Flag-CIITA, Flag-CIITA K1R, Flag-CIITA K2R, Flag-CIITA K3R, or pcDNA. Following transfection, cells were left untreated or were stimulated with IFN-γ for 18 h. Cells were harvested, RNA extracted, and cDNA was prepared and quantified by real-time PCR. Results shown are an average of three experiments (bottom panels). C, mutating CIITA lysine residues Lys-315, Lys-330, and Lys-333 to arginine reduces MHC class II surface expression. Flow cytometry, HeLa cells were transfected with Flag-CIITA, Flag-CIITA K1R, Flag-CIITA K2R, Flag-CIITA K3R, or pcDNA. Cells were fixed and stained with PE-labeled anti-human HLA-DR antibody. Following antibody incubation, cells were fixed and PE cell surface staining was measured by FACS. (i) HeLa cells transfected with Flag-CIITA (light gray line) and HeLa cells transfected with pcDNA (black line). (ii) HeLa cells transfected with Flag-CIITA K1R (light gray line) and HeLa cells transfected with pcDNA (black line). (iii) HeLa cells transfected with Flag-CIITA K2R (light gray line) and HeLa cells transfected with pcDNA (black line). (iv) HeLa cells transfected with Flag-CIITA K3R (light gray line) and HeLa cells transfected with pcDNA (black line). (v) HeLa cells transfected with Flag-CIITA K1R (light gray line) and HeLa cells transfected with pcDNA (black line). (vi) HeLa cells transfected with Flag-CIITA K2R (light gray line) and HeLa cells transfected with pcDNA (black line). (vii) HeLa cells transfected with Flag-CIITA K3R (light gray line) and HeLa cells transfected with pcDNA (black line). (viii) HeLa cells transfected with Flag-CIITA K1R (light gray line) and HeLa cells transfected with pcDNA (black line). (ix) HeLa cells transfected with Flag-CIITA K2R (light gray line) and HeLa cells transfected with pcDNA (black line). (x) HeLa cells transfected with Flag-CIITA K3R (light gray line) and HeLa cells transfected with pcDNA (black line). Flow cytometry was used to confirm expression of Flag-CIITA, Flag-CIITA K1R, Flag-CIITA K2R, and Flag-CIITA K3R (bottom panels). Results shown are representative of three experiments.
ubiquitination by E3 ligases (35). We have identified three lysine residues in CIITA isoform III, Lys-315, Lys-330, and Lys-333, proximal to the degron sequence, as sites for mono-ubiquitination. Mutating these lysine residues to arginine reduces CIITA mono-ubiquitination and half-life, indicating that mono-ubiquitination at these residues stabilizes CIITA. These lysine mutants also show reduced CIITA transactivity, leading to reductions in MHC class II transcript levels and in MHC class II surface expression. Thus, mono-ubiquitination at these lysine residues is crucial for CIITA transactivity. The mono-ubiquitination events at these three lysine residues are interchangeable; CIITA K315R; CIITA K330, 333R; and CIITA K315, 330, 333R all have similar effects on CIITA transactivity, indicating stearic hindrance may allow these sites to serve as alternate sites for ubiquitination.

We have recently identified a novel N-terminal ATPase binding domain in CIITA that encompasses a majority of the degron sequence (37). Absence of the ATPase binding domain increases CIITA half-life, but dramatically decreases MHC class II surface expression, indicating roles for the ATPase binding domain in regulating CIITA transactivity (37). Binding of 19 S ATPases to the ATPase binding domain of CIITA may have dual roles in regulating CIITA activity and stability: mono-ubiquitinated CIITA is protected from ATPase stripping and demonstrates enhanced transactivity, while poly-ubiquitinated CIITA is no longer protected and is targeted for degradation.

Despite increased accumulation of CIITA, mutating CIITA serine 280 to alanine decreases CIITA-mediated MHC class II expression and predominantly effects CIITA mono-ubiquitination. Previous studies have linked activator phosphorylation and downstream, degron proximal, mono-ubiquitination (20, 35). E3 ubiquitin ligases have specificity to particular lysine residues in substrate proteins (35, 44–45), and lysine residues proximal to degron sequences are preferentially targeted for phosphorylation and apoptosis (42–43). Future studies will determine if CIITA Ser-280 is an additional target of ERK1/2-mediated phosphorylation.

Degron Proximal CIITA Ubiquitination Regulates MHC II Expression

FIGURE 6. Genetically fusing mono-ubiquitin to WT or the triple lysine mutant of CIITA reduces transactivity because of rapid degradation of fusion products. A, Flag-mono-ubiquitin-CIITA and Flag-mono-ubiquitin-CIITA K3R show lower transactivity as compared with Flag-CIITA or Flag-CIITA K3R. Luciferase reporter assays, HeLa cells were co-transfected with Flag-CIITA/Flag-mono-ubiquitin-CIITA (left panel), Flag-CIITA K3R/Flag-mono-ubiquitin-CIITA K3R (right panel), or pcDNA (empty vector) and HLA-DRA luciferase reporter constructs. Cells were harvested, lysed in cell lysis buffer, and luciferase assays were performed. Luciferase readings were normalized to total protein concentration. Results shown are an average of three experiments. B, Flag-mono-ubiquitin-CIITA and Flag-mono-ubiquitin-CIITA K3R are rapidly degraded. Western blot, HeLa cells were transfected with the Flag-CIITA/Flag-mono-ubiquitin-CIITA (left panel) or the Flag-CIITA/Flag-mono-ubiquitin-CIITA K3R (right panel). Cells were harvested, lysed in Nonidet P-40, and Western blots were performed.
Degron Proximal CIITA Ubiquitination Regulates MHC II Expression

proteasomal ATPases, protects CIITA from degradation, increases CIITA transactivation, and up-regulates MHC class II transcription.

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