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Leucine-Rich Repeats of the Class II Transactivator Control Its Rate of Nuclear Accumulation

Jonathan A. Harton, William O'Connor, Jr., Brian J. Conti, Michael W. Linhoff, and Jenny P.-Y. Ting

ABSTRACT: Activation of class II major histocompatibility complex (MHC) gene expression is regulated by a master regulator, class II transcriptional activator (CIITA). Transactivation by CIITA requires its nuclear import. This study will address a mechanistic role for the leucine-rich repeats (LRR) of CIITA in regulating nuclear translocation by mutating 12 individual consensus-motif "leucine" residues in both its α-motifs and β-motifs. While some leucine mutations in the LRR motif of CIITA cause congruent loss of transactivation function and nuclear import, other alanine substitutions in both the α-helices and the β-sheets have normal transactivation function but a loss of nuclear accumulation (*i.e.*, functional mutants). This seeming paradox is resolved by the observations that nuclear accumulation of these functional mutants does occur but is significantly less than wild-type. This difference is revealed only in the presence of leptomycin B and actinomycin D, which permit examination of nuclear accumulation unencumbered by nuclear export and new CIITA synthesis. Further analysis of these mutants reveals that at limiting concentrations of CIITA, a dramatic difference in transactivation function between mutants and wild-type CIITA is easily detected, in agreement with their lowered nuclear accumulation. These experiments reveal an interesting aspect of LRR in controlling the amount of nuclear accumulation. *Human Immunology 63, 588–601 (2002).* © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: CIITA; class II MHC; leucine-rich repeats; transcription

ABBREVIATIONS

MHC	major histocompatibility complex
CIITA	class II transactivator
LRR	leucine-rich repeats
LMB	leptomycin B

INTRODUCTION

Constitutive and inducible expression of class II major histocompatibility complex (MHC), and related genes, is under the exquisite transcriptional control of a complex promoter. Consensus elements within the promoter [W(S), X, and Y] have been known for some time. Interestingly, the transcription factors that bind the promoter are expressed ubiquitously and, thus, fail to account for the observed tissue restricted endogenous and widespread interferon-gamma (IFN-γ) inducible expression of MHC genes. This dilemma was surmounted by the discovery of the class II transactivator (CIITA) [1]. This 145 kD non-DNA binding protein parallels the expression pattern of class II MHC, is responsive to IFN-γ, and is transcriptionally downregulated by a number of processes known to negatively modulate class MHC expression [2–6].

Structure/function studies of CIITA have elucidated a number of important functional domains (reviewed in [7]). The N-terminal acidic domain is sufficient for transcriptional activation [8, 9] and interacts with components of the basal transcription machinery [10–13].

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The adjacent proline-serine-threonine-rich region, although necessary for function, remains enigmatic [14]. CIITA binds GTP via a central GTP-binding domain and requires GTP-binding for successful nuclear import [7]. CIITA lacks intrinsic GTPase activity and forcing GTPase activity renders CIITA cytoplasmic and less effective at transactivation [7]. CIITA contains at least two nuclear localization sequences; one near the C-terminus and another within the proline-serine-threonine-rich region [15, 16]. The first of these constitutes the defect in a known bare lymphocyte (BLS) patient [1, 15]. CIITA has been observed to have leucine rich sequences at its c-terminus [1, 17, 18]. Recently these have been recognized as consensus leucine rich repeats (LRR) [14, 18, 19].

Leucine rich repeats are found in a diverse array of proteins ranging from bacteria and plants to yeast and humans [19]. These sequences are involved in a broad range of biologic activities, including RNAse inhibition [20], activation of GTPases [21], protein–protein interactions [22], signal transduction [23], and transcription [24]. CIITA mutants lacking the C-terminus where the LRR reside are non-functional, and some have dominant negative properties, which suggests an important role for this region of CIITA [25, 26]. Multiple point mutations in CIITA residues predicted to participate in protein–protein interactions abolish its transactivator function due to a loss of nuclear translocation [18]. This prior study focused exclusively on the β-strand/loop region defined by the interaction between RNase inhibitor and RNase in solution. However, no information is available on the α-helices of CIITA and only a paucity of functional information exists for the α-helices of other LRR-containing proteins. Additionally, very little has been published regarding the contribution of the landmark leucine residues of LRRs.

In this study, we present evidence that conserved leucine residues at motif defining positions in the C-terminal LRR in CIITA participate in regulating the rate of CIITA nuclear translocation. Point mutations at conserved leucines within putative α-helical or β-sheet regions of the LRR have variable effects on transactivation of a class II MHC promoter, ranging from full activity to a null phenotype (hereafter referred to as functional and non-functional mutants, respectively). Despite the variation in transactivation function, these mutants all have a pronounced effect on nuclear localization, resulting in apparent cytoplasmic accumulation. This paradox is resolved when we use leptomycin B to inhibit nuclear export and actinomycin D to inhibit the synthesis of new CIITA. Under these conditions, nuclear accumulation of functional mutants does occur, although the accumulation is less than wild-type CIITA. Non-functional mutants do not accumulate in the nucleus. A titration study was performed where lower DNA concentrations of functional mutants do reveal impaired transactivation function. These data demonstrate the importance of the leucine residues in the α-helices of CIITA.

MATERIALS AND METHODS

Generation of CIITA Mutants

Site-directed mutagenesis of p3FgCIITA [17], encoding N-terminally FLAG-tagged wild-type CIITA, was performed using Quick Change (Stratagene, La Jolla, CA, USA) site-directed mutagenesis. Briefly, site-specific primers were designed that would mutate single codon changes to either alanine and/or proline. Following DNA amplification of plasmid template with mutagenic primers using PfuTurbo, parental plasmid DNA was digested with DpnI to remove all methylated copies of the plasmid. The remaining, unmethylated copies were visualized with ethidium bromide after electrophoresis in 0.8% agarose. DH10α competent cells were transformed with 1 μl of the digest. Colonies containing putative mutants were cultured and the presence of the desired mutation (and absence of deleterious changes) was determined by nucleotide sequencing.

Cell Lines and Transfection

COS7 cells were cultured in DMEM supplemented with 10% FCS, 2-mM L-glutamine, 100-U/ml penicillin, and 100-μg/ml streptomycin (complete medium). Transient cotransfection was performed using FuGene6 (Roche, Indianapolis, IN, USA). Briefly, 2×10^5 cells/well were plated in 6-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), p3FgCIITA, or CIITA mutant DNA [15]. The transfected cells were lysed 16–18 hours post-transfection in 1× Reporter Lysis Buffer (Promega, Madison, WI, USA) and luciferase assays were performed as previously described [27]. Each transfection was performed in triplicate and luciferase data normalized to total protein.

Western Blotting

Following transfection of cells with 1 μg of DNA, cells were lysed in 1× Reporter Lysis Buffer (Promega) containing 1× complete (Roche) protease inhibitors. Protein concentrations were normalized and 5 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8%) and transferred to nitrocellulose. Blotting for the FLAG epitope was performed as described [28].

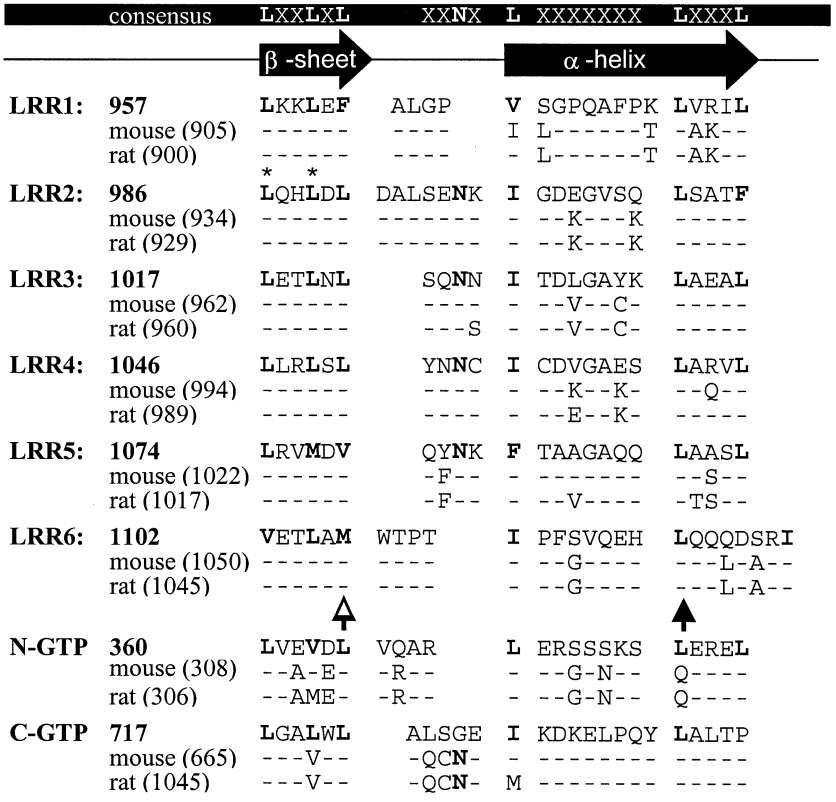

FIGURE 1 CIITA contains at least five, possibly six, conserved C-terminal leucine-rich repeats, and potentially two others flanking the GTP-binding domain (designated N-GTP and C-GTP). In the consensus an L indicates any of the hydrophobic residues (F, I, L, V, or M). The structure of the regions based on the crystal structure of porcine ribonuclease inhibitor is illustrated. Arrows indicate residues targeted for mutagenesis to alanine (open arrow) or alanine and proline (closed arrow). Numbering is based on CIITA form III for human (in boldface type), mouse, and rat. The sites of additional point mutants are indicated by an asterisk (*) above the mutated residue (see text).

Immunofluorescence Microscopy

Immunofluorescent staining of transiently transfected COS7 cells was performed as previously described [15]. Briefly, 5 to 8×10^4 cells were plated on 2-well chamber slides (Nunc, Roskilde, Denmark), incubated overnight at 37 °C and transfected with 1 to 1.5 μg of plasmid DNA using FuGene6. After a second overnight incubation, the cells were washed with 1× PBS, blocked in 1× PBS, 1% BSA, 10% goat serum, stained with anti-FLAG(M5) (Sigma Chemical Co., St. Louis, MO, USA), and goat anti-mouse IgG-FITC (Southern Biotech, Birmingham, AL, USA). 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. In experiments using leptomycin B (LMB; kindly provided by Dr. Barbara Wolffe) and actinomycinD, LMB was added at 10 nM and actinomycin D was added at 2.5 μg/ml in DMSO. LMB was included 2 hours post-transfection and actinomycin D was added 4 hours prior to staining. DMSO only controls were performed in parallel and no staining pattern alterations were observed (data not shown).

RESULTS

Identification of LRR Sequences in CIITA

Although the presence of four conserved LRR motifs in CIITA had been previously reported [18, 19], other reports demonstrating variability in the motif led us to consider the possibility that additional motifs might exist in CIITA. LRR are commonly defined based on the motif LxxLxLxx(N/C)xL(x)$_7$LxxxL (L indicates a leucine or other hydrophobic residue and x indicates any residue) where a β-sheet precedes the α-helical region of the repeat (see Figure 1). The residues and spacing defining the LRR motif display some variability frequently noted in descriptions of LRR containing proteins [29–31]. The most notable exception is the absence of the conserved cysteine or asparagine in the loop region between the β-sheet and α-helix (see Figure 1). These LRRs have

been referred to as non-Kobe A/B motifs as RNase inhibitor has only the cysteine and asparagine containing varieties. Other hydrophobic residues are common substitutes for the leucines that define the LRR motif, although charged, aromatic, and proline residues have been observed. The more drastic departures from leucine and other similar hydrophobic residues usually is limited to the first and last "leucine" residues of the motif. Variation in the spacing between LRR leucines also occurs, but seems largely limited to the loop, the span between the fourth and fifth leucine, with fewer differences involving spacing differences between the first and second, and between the fifth and sixth leucines. Spacing differences are typically only one or two residues in length. With these points in mind, examination of the amino acid sequence of CIITA reveals the presence of at least five conserved C-terminal tandem LRR (Figure 1) with homology to a variety of LRR-containing proteins including ribonuclease inhibitor [32], rna1p [29], and Nod1 [30]. These begin at residue 957 and continue until residue 1096. These criteria also suggest that CIITA has two other LRR-like sequences that flank the GTP-binding domain. The N-GTP LRR sequence matches the motif almost exactly with the only exception being the lack of cysteine/asparagine in the loop. The C-GTP LRR sequence is also very close to the motif, but also lacks cysteine/asparagine, ends with a proline residue, and has one additional residue between the fourth and fifth leucine. In the mouse and rat sequences an asparagine is present in the loop (Figure 1). LRR1 is a similar case, but the final residue is a leucine, thus matching the "consensus" motif better than C-GTP. LRR6 occurs in the last 25 residues of CIITA, lacks the cysteine/asparagine, and terminates with an aspartate. Although we have not observed aspartate specifically at this position in other published LRR, glutamate has been observed as have other bulky residues. Interestingly, the sequences from mouse and rat have a leucine that gives LRR6 an atypical, but not unique, spacing of two residues between the fifth and sixth leucines instead of the more typical three residues.

The crystal structure of LRRs in ribonuclease inhibitor reveals a "horseshoe" structure of 16 alternating β-sheets and α-helices [32]. If the LRRs of CIITA adopt a similar structure, the "horseshoe" will likely be about one-third of this size. Structural predictions generated by the 3D-PSSM algorithm [33, 34] suggest that residues from 957-1130 can form LRR-like structures consistent with the structure of RNAse inhibitor (Figure 2A) and Ran-GAP (Figure 2B). Both of these models predict six iterations of the motif based on a high degree of sequence similarity with ribonuclease inhibitor and Ran-GAP (see Figure 2), thus supporting the likely presence not only of LRR1, but also the less conventional LRR6 (Figure 1).

Sequences immediately N-terminal to the five consensus LRR are also leucine rich. Some sequences in this region conform very closely to the motif (especially if all of the published variations are taken into account), suggesting that they may represent non-conventional LRR or byproducts of gene-duplication events that led to generation of the LRR themselves. The current understanding of the molecular evolution of LRRs [19] supports the latter possibility.

Mutation of Similarly Positioned Leucines in the LRR α-Helices Have Disparate Effects on CIITA Function

Mutational analyses of LRR-containing proteins have focused on the conserved asparagine and nearby residues in the "loop" between the β-sheet and α-helix of the repeat and adjacent residues (*e.g.*, see [18, 21, 35]). This focus has resulted largely from crystallographic studies of RNase inhibitor reporting that the analogous residues are involved in a contact-face with RNaseA [20]. The role of motif-defining leucines within the five C-terminal LRRs of CIITA (LRR1-5) that most closely match the consensus motif were studied. Leucines centrally located within the predicted α-helical portion of the helix were selected for mutational analysis (indicated by a filled arrow in Figure 1). We reasoned that mutation of leucine to alanine would preserve the helical nature of the repeat, whereas proline substitution would likely disrupt the helix, revealing whether specific leucines or local structures are most important. Mutation of leucines 976 and 1035 to alanine had no detrimental effect on the ability of CIITA to activate transcription from a class II MHC promoter (Figure 3, top panel). Surprisingly, both of these mutations have a slight positive effect on activation. However, mutation of leucines 1007, 1064, or 1092 to alanine completely disrupted transactivation by CIITA. This demonstrates that these leucines are important for CIITA function and suggest that a fundamental difference may exist between the helices containing leucines 976 and 1035 compared with those containing leucines 1007, 1064, and 1092. Mutation of any of these residues to proline completely abrogates transactivation function, suggesting that the helical nature of the motifs is most important (Figure 3, bottom panel).

Mutation of Similarly Positioned Leucines in the LRR β-Sheets Have Little Effect on CIITA Transactivator Function

As mutations at conserved leucines in the α-helical portion of the LRR had effects on CIITA function, it was of interest to examine conserved hydrophobic residues ("leucines") in or near the β-sheet. Unlike the α-helix mutants, mutation of residues F962, L991, L1022, L1051, or V1079 (marked by an open arrow in Figure 1) to alanine

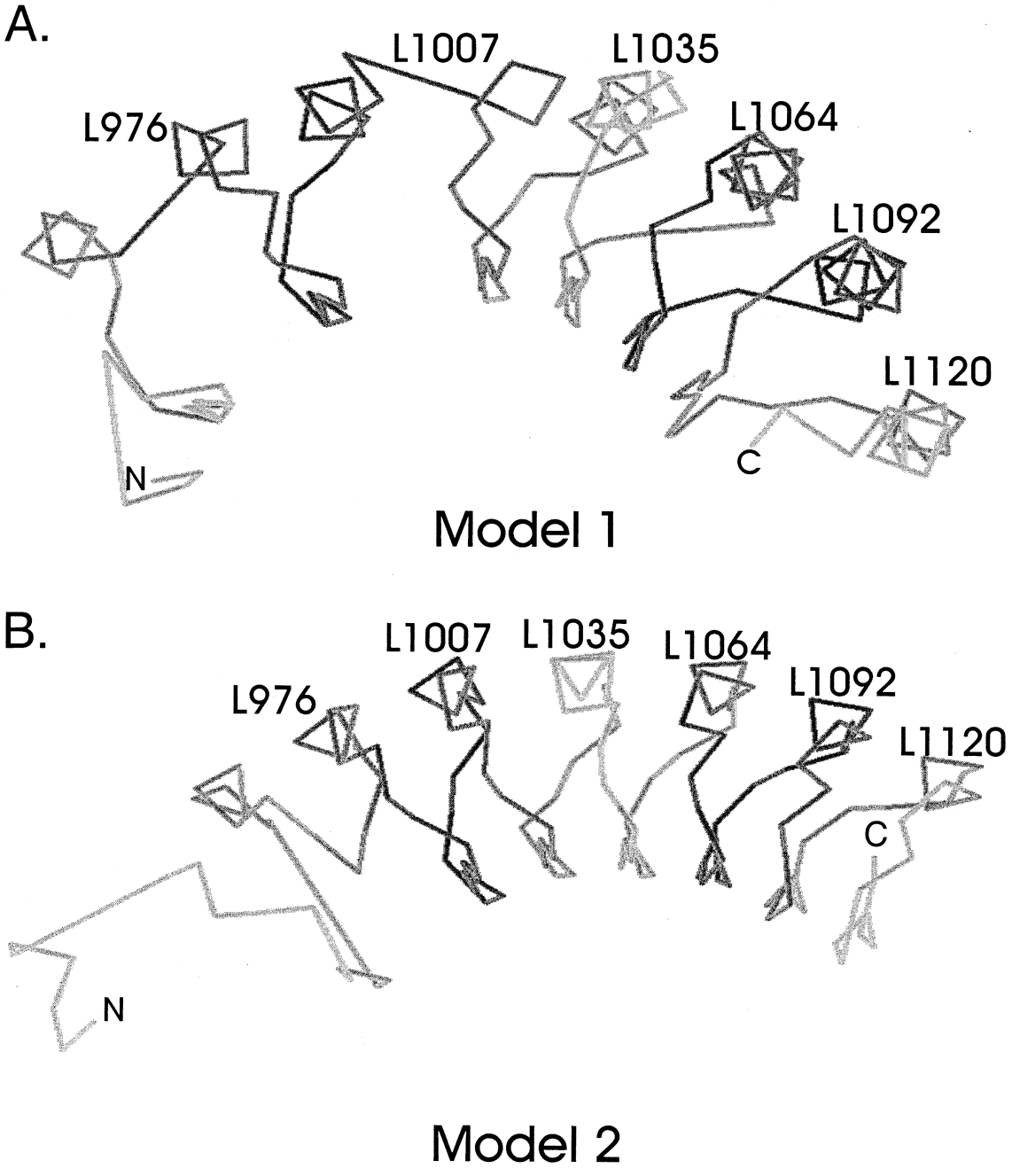

FIGURE 2 Computer modeling of the C-terminal LRR of CIITA. Amino acid sequences 957-1130 comprising the C-terminal LRR motifs of CIITA were submitted to the 3D-PSSM server at the Imperial Cancer Research Fund (London, United Kingdom) [32, 33]. Based on sequence homology and predicted secondary structure similarities with RNAse inhibitor and Ran-GAP (two LRR-containing proteins with known crystal structures) two high confidence models were generated. Top panel: model 1 based on RNAse inhibitor; bottom panel: model 2 based on Ran-GAP. The approximate positions of leucines in the α-helices are illustrated.

did not diminish CIITA transactivation function (Figure 4). Transactivation function for F962A and L991A is indistinguishable from wild-type CIITA. With the exception of these two, a consistent increase over wild-type CIITA was noticed with these constructs. Despite the lack of a detectable effect for these mutants in the β-sheet regions, not all leucines in this region are dispensable. For example, within LRR2, a single-point mutant, L986A, was completely non-functional. Similarly, whereas L991A is functional, adding a second mutation, L989A abolishes function.

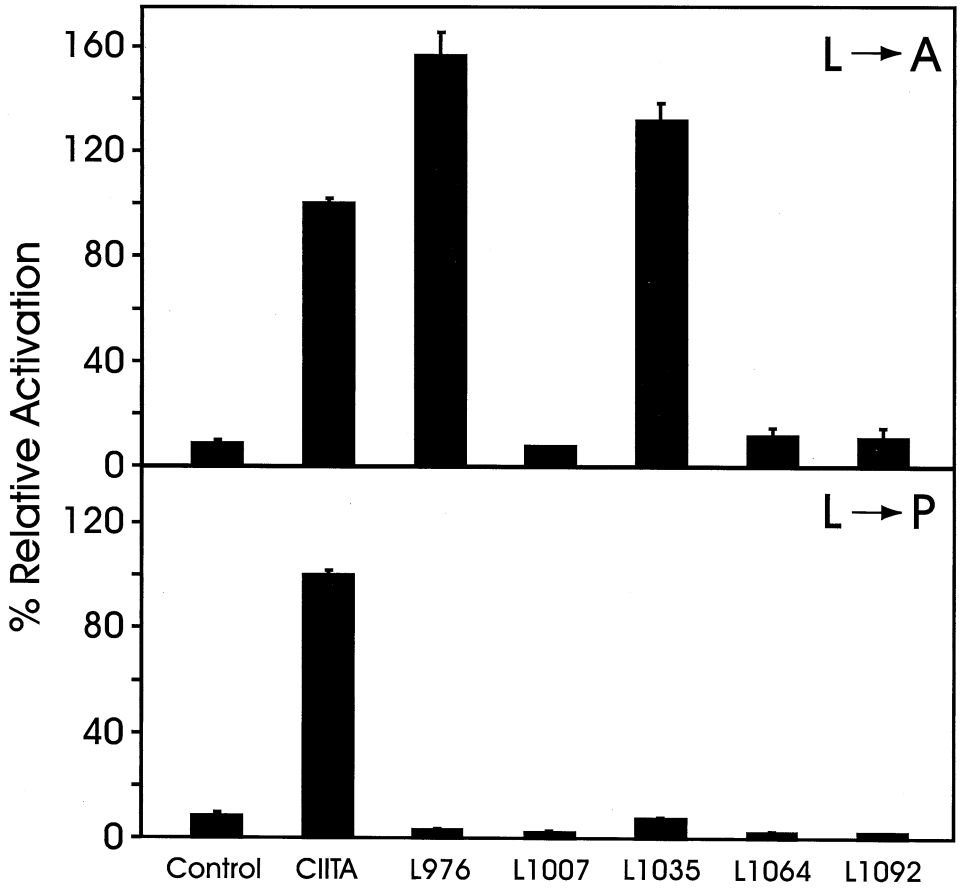

FIGURE 3 Mutations in the LRR α-helices of CIITA have disparate effects on transactivation. COS7 cells were transfected with 1 μg of the class II major histocompatibility complex human leukocyte antigen -DRαa promoter/luciferase reporter, DRA300Luc, and 1 μg of the indicated CIITA construct. Empty pcDNA3 vector was used as a negative control. Luciferase activity was assayed 18 hours after transfection. Results are expressed as percent activity relative to wild-type CIITA (set at 100%). Upper panel, leucines centrally located in the α-helical portion of the LRR were mutated to alanine. Lower panel, the same leucines residues were mutated to proline. The mean ± the standard error of the mean (SEM) is illustrated and represents three or more experiments performed in triplicate. Similar results were obtained in the CIITA negative G3A human fibroblastoid sarcoma cell line (not shown).

LRR Mutations Alter Nuclear Import of CIITA

Expression of LRR mutants was comparable to wild-type CIITA (Figure 5), suggesting that functional differences are not due to differential expression. We have previously reported that defects in CIITA's ability to activate transcription often correlate with failed nuclear translocation, such as defects in GTP-binding [7] and defects that delete the C-terminal nuclear localization sequence (NLS) [15]. Thus, we examined the effects of LRR mutations on nuclear localization of CIITA by immunofluorescent staining. Surprisingly, all of the alanine mutations in the α-helices led to a loss of nuclear expression (Figure 6A), despite the essentially normal transactivation function observed for L976A and L1035A, supporting the importance of these leucines for import of CIITA into the nucleus. Not unexpectedly, the proline mutations in the helices (L976P, L1007P, L1035P, L1064P, and L1092P) were uniformly cytoplasmic (Figure 6B). The β-sheet alanine mutations F962A, L991A, L1022A, L1051A, and V1079A were also uniformly cytoplasmic (Figure 6C), suggesting that leucines in the β-sheets are involved in nuclear import. Paradoxically, like L976A and L1035A, the β-sheet mutants retained normal function (Figures 3 and 4). These data demonstrate that motif sequences in both the α-helices and β-sheets of CIITA's LRR are important for nuclear localization of CIITA, but the discrepancy with the transactivation data necessitated further investigation.

LRR of CIITA Control Nuclear Accumulation

At face value these data present a seeming paradox, that detectable nuclear localization of CIITA is not necessary for function. It is generally accepted that CIITA works in the nucleus [14]. Because we observed complete function

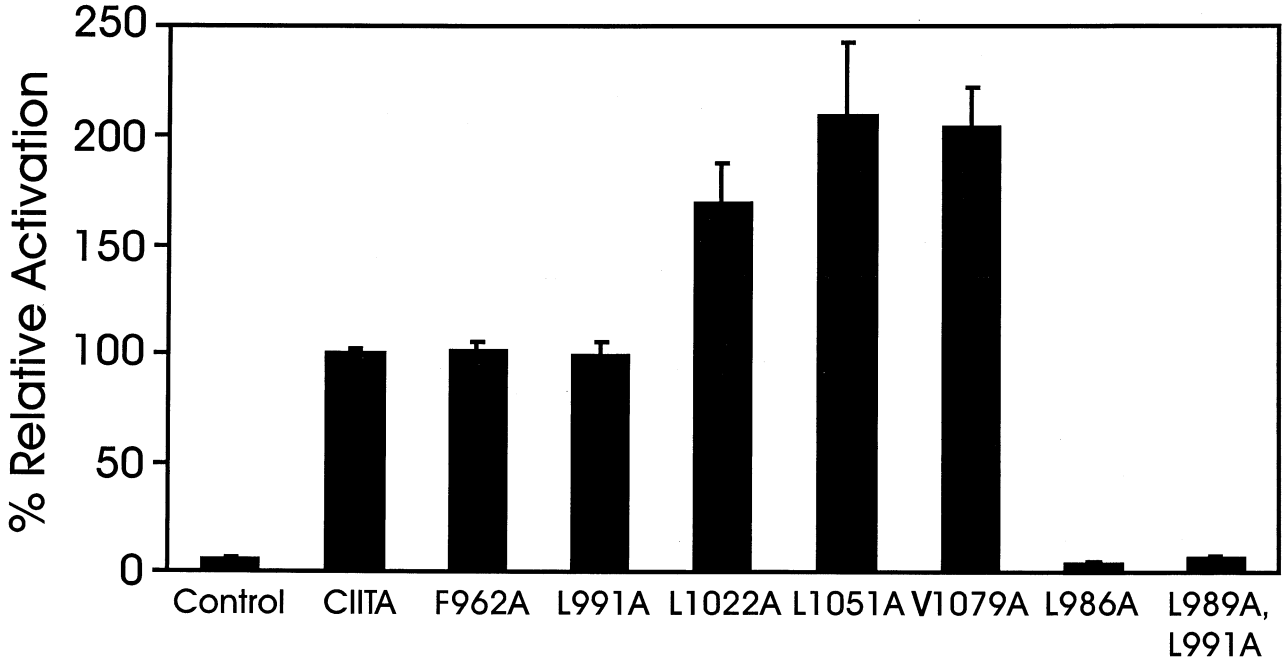

FIGURE 4 Mutations in the LRR β-sheets of CIITA have little effect on transactivation. Conserved hydrophobic residues in the β-sheet portion of the LRR were mutated to alanine, and transactivator function was tested as Figure 2. With the exception of L986A and L989A/L991A, which were tested at least twice in triplicate, the mean ± SEM represents at least three experiments performed in triplicate. Similar results were obtained in the G3A fibroblastoid sarcoma cell line (not shown).

in the seeming absence of nuclear localization, we considered the possibility that the functional mutants were entering the nucleus, but that nuclear accumulation was reduced. Nuclear accumulation of CIITA represents the balance of nuclear import, nuclear export, and *de novo* synthesis of CIITA molecules. To examine nuclear localization in the absence of the confounding effects of nuclear export and import of new CIITA, we performed the following experiment. The functional leucine to alanine mutants that displayed cytoplasmic localization (L976A, L1035A, and L1051A) were transfected into COS7 cells and treated with either leptomycin B (LMB), an irreversible inhibitor of nuclear export [36, 37], or LMB together with actinomycin D to inhibit new CIITA production. The cells were then examined by immunofluorescent staining. Treatment with LMB leads to the nearly complete concentration of wild-type CIITA in the nucleus compared with the untreated control (Figure 7). Because LMB blocks export, nuclear accumulation of a protein with decreased translocation or nuclear accumulation would be greater than that in untreated samples and thus easier to detect. Indeed, LMB treatment of COS cells expressing the functional mutants permits some accumulation of L976A, L1035A, and L1051A, indicating that they do enter the nucleus, but perhaps at a slower rate. Nuclear expression is more readily observed for L1051A and L976A than L1035A using LMB. The addition of LMB and actinomycin D together simplifies further the visualization of a limited pre-existing pool of protein as *de novo* RNA synthesis is blocked. With both LMB and actinomycin D, wild-type CIITA expression appears almost completely nuclear, reflecting the rate of

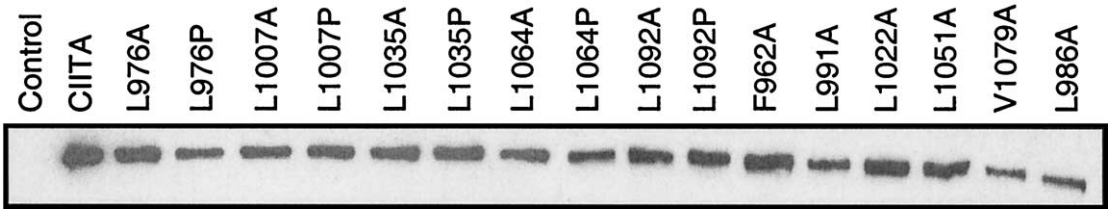

FIGURE 5 Expression of wild-type class II transactivator (CIITA) and leucine-rich repeats (LRR) mutants. COS7 cells were transfected with 1 μg of the indicated construct, lysed, subjected to SDS-PAGE (8%), transferred to nitrocellulose, and blotted with anti-FLAG(M5) followed by goat anti-mouse IgG-horseradish peroxidase. Then 5 μg of whole cell lysate was loaded per lane.

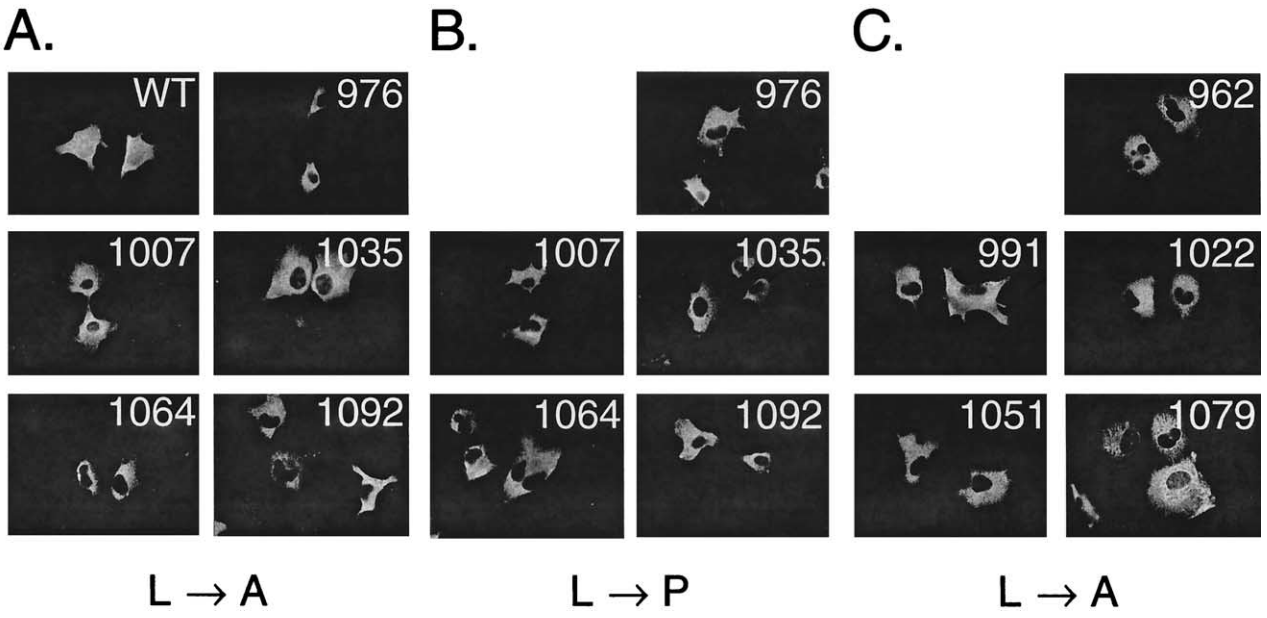

FIGURE 6 Mutations in the LRR of CIITA uniformly disrupt nuclear import. COS7 cells were transfected with 1 μg of the indicated construct and stained with anti-FLAG(M5) and FITC-conjugated goat anti-mouse IgG (GAM-FITC) as described, and examined by immunofluorescence microscopy. GAM-FITC-stained control cells revealed negligible staining (not shown). (A) Alanine substitutions in the LRR α-helices. (B) Proline substitutions. (C) Alanine substitutions in the LRR β-sheets. Individual photomicrographs are representative of the predominant pattern of localization in at least three independent experiments.

nuclear accumulation. Combined LMB and actinomycin D treatment also increased nuclear accumulation for the leucine to alanine mutants. The level of nuclear expression for these mutants approaches that of untreated transfectants expressing wild-type CIITA. This demonstrates that the LRR of CIITA likely perform an important role in CIITA function through optimization of nuclear translocation and/or accumulation. In contrast to the functional mutants, L1035P revealed no nuclear accumulation with LMB or LMB plus actinomycin D, indicating that this mutation completely prevents nuclear accumulation. This suggests that disruption of the helical structure completely disrupts nuclear import and is congruent with its lack of transactivator function (Figure 3). Interestingly, this is the only mutation in the LRR that affected the physical self-association of CIITA in another study [28].

The above experiment suggests that the amount of CIITA that localizes to the nucleus is down-modulated in these three leucine to alanine LRR mutants. However, in a previous experiment (Figures 3 and 4) these same mutants were revealed to transactivate the DRA promoter as efficiently as wild-type CIITA. We reasoned that when using 1 μg of transfected CIITA DNA, a small but sufficient quantity of LRR mutant CIITA could enter the nucleus within the timeframe of the transactivation assay (16–20 hours), potentially explaining the discrepancy between function and nuclear translocation described above. We have previously observed that CIITA is highly active even when as little as 10 ng of CIITA is transfected [38]. Therefore, reducing the amount of transfected DNA should better reveal if a mutant CIITA exhibits reduced activity because less protein would be available for nuclear import in the same amount of time. To test this possibility we transfected cells with 10 ng, 100 ng, or 1 μg of wild-type CIITA or the functional leucine to alanine mutants, and compared their ability to activate transcription (Figure 8). Using 10 ng, 100 ng, or 1 μg, wild-type CIITA transactivates the DR promoter efficiently (50× to 100× over control), whereas at 10 ng and 100 ng L1035A and L1035P are now indistinguishable from the negative control. At 100 ng, L976A and L1051A have lost approximately 40%–50% of their activity compared with 1 μg, but are still comparable to wild-type CIITA. At 10 ng, these two mutants are approximately 50% less active than wild-type. The transactivation ability of the functional mutants was dramatically different at 10 ng and 100 ng compared with experiments using 1 μg where L976A, L1035A, and L1051A all have transactivating activities exceeding wild-type. This result correlates well with the nuclear expression patterns observed in Figure 7 where, in the presence of LMB and actinomycin D, nuclear expression of L1035A is less than that of L976A and L1051A. These data are consistent with the conclusions of the localization experiments and support the hypoth-

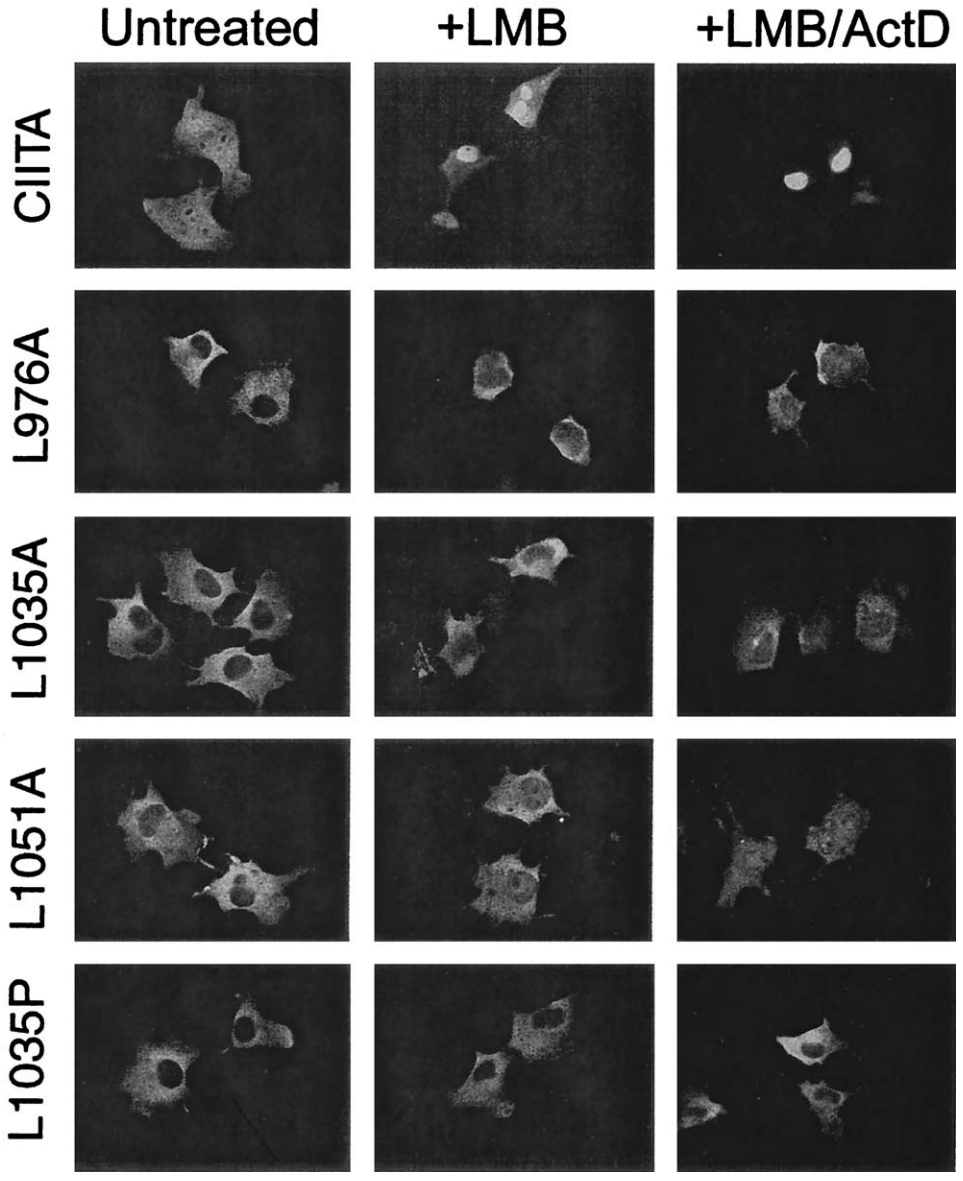

FIGURE 7 LRR mutants alter the efficiency of nuclear import. Immunofluorescence microscopy was performed as in Figure 5, with the addition of either leptomycin B (LMB) alone, or LMB together with actinomycin D (ActD). A panel of alanine substitution mutants representing both α-helical and β-sheet mutants is shown. The proline mutant, L1035P is included as a negative control (no appreciable nuclear staining is observed even with addition of LMB and ActD). Individual photomicrographs are representative of the predominant pattern of localization in at least three independent experiments.

esis that certain LRR mutants can be divided into at least two groups [1]: those that are non-functional and do not enter the nucleus (*e.g.*, L1035P), and those that are functionally competent but enter the nucleus at a reduced rate (*e.g.*, L976A, L1035A, and L1051A).

DISCUSSION

It is generally accepted that LRRs play a role in mediating protein–protein interaction, and perform a wide range of functions (*e.g.*, inhibition of ribonuclease, GTPase activating protein, signal receptor, transcription factor). Because leucines are often important in protein–protein interactions, we explored the function of the LRR of CIITA by mutating conserved leucine residues that comprise the LRR motif. Some leucines were necessary for successful CIITA import, but others slowed down import as evidenced by their ability to activate transcription of a class II MHC reporter. Our data suggests a regulatory role for these LRR in CIITA nuclear import.

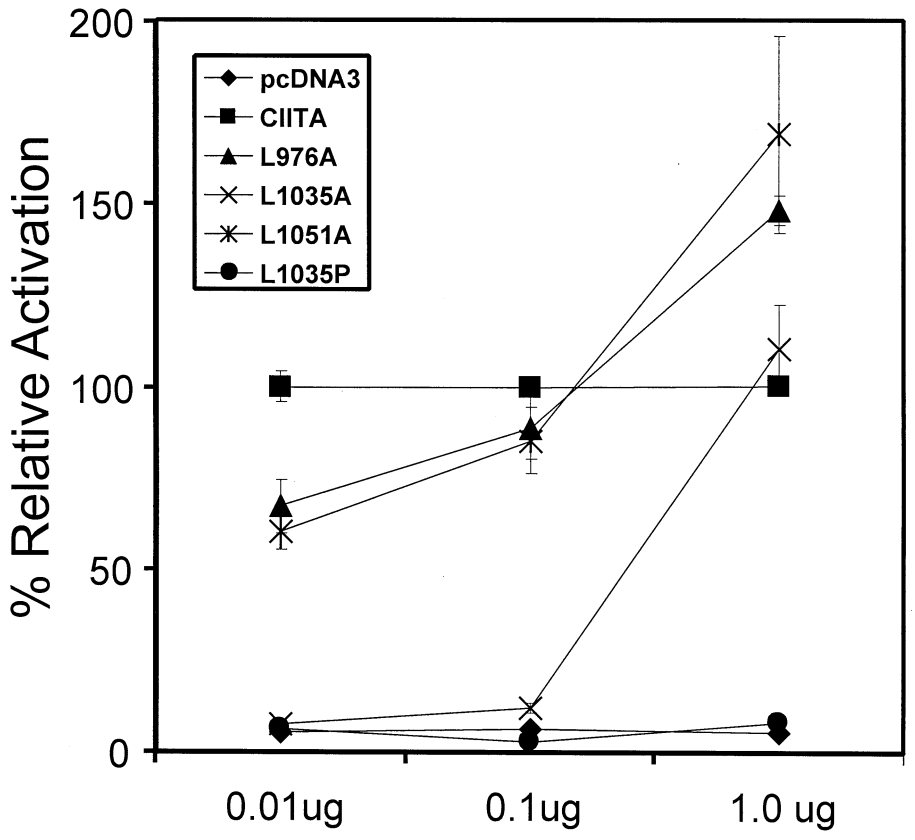

FIGURE 8 Import defects correlate with reduced activation. Transactivation assays using the indicated mutants were performed as in Figure 2, except that varying amounts of transfected CIITA constructs (10 ng, 100 ng, or 1 μg) were cotransfected with reporter. Empty vector (pcDNA3) was added to 1 μg where 10 ng or 100 ng were used. For each concentration of DNA used CIITA was set to 100%. At 100 ng of CIITA, wild-type CIITA activity is approximately 200% relative to 1 μg of CIITA. For each point $n \geq 6$ and the means ± SEM are illustrated.

Mutation of LRR motif leucines to alanine has variable effects on CIITA's ability to activate transcription. Two of the α-helix mutants (L976A and L1035A) retained the ability to activate transcription with seemingly absent nuclear localization (see Table 1). The others (L1007A, L1064A, and L1092A) exhibited cytoplasmic localization, failed to activate transcription, and were comparable with proline substitution mutants. Leucine residues 1007, 1064, and 1092 are therefore critical, whereas 976 and 1035 are more tolerant to mutation, revealing a potentially important difference between residues 976/1035 and 1007/1064/1092. This result was unanticipated as sequence comparison between human, mouse, and rat CIITA (Figure 1) revealed a high degree of conservation between species with nearly all mutations occurring outside the motif-defining residues. Similar to the faces of leucine zippers [39], the most important leucines (1007/1064/1092) may serve as leucine side-chain-dependent contact points. One potential interaction partner is CIITA itself. In another study, we found that residues 939-1130 of CIITA (containing the LRR) could interact with residues 336-702 that contain the GTP-binding domain [28]. This self-interaction was eliminated by proline substitutions at 1035 and modestly reduced by a proline substitution at 1064, but was not negatively affected by any of the other mutations. Proline mutants at these positions are completely disruptive of function, both activation and localization, suggesting an important role for the helices themselves or for a general feature of local LRR architecture. It is tempting to speculate that contact-dependent events are disrupted by the loss of a helix or structures near the site of mutation. CIITA self-association experiments mentioned above support this notion in that only L1035P and L1064P exhibit decreased self-association. The other leucine to proline mutations may affect non-self interactions. Mutations of motif leucines to alanine in the LRR β-sheet are well tolerated in terms of transactivator functions, yet these mutations affect nuclear import as well, suggesting that slight perturbations in the LRR have a

TABLE 1 Summary of results

Construct		Transactivation 1 μg	Transactivation 0.1 μg	Localization	(+LMB/ActD)
CIITA		++	++	C/N	N
F962A	β	++	*	C	*
L976A	α	+++	+	C	C/N
L976P	α	−	*	C	*
L986A	β	−	*	C^{NS}	*
L991A	β	++	*	C^{NS}	*
L986A, L991A	β	−	*	C^{NS}	*
L1007A	α	−	*	C	*
L1007P	α	−	*	C	*
L1022A	β	+++	*	C	*
L1035A	α	++	−	C	C/N
L1035P	α	−	−	C	C
L1051A	β	++++	+	C	C/N
L1064A	α	−	*	C	*
L1064P	α	−	*	C	*
V1079A	β	++++	*	C	*
L1092A	α	−	*	C	*
L1092P	α	−	*	C	*

Abbreviations: β = beta-sheet; α = alpha-helix; C = cytoplasmic; N = nuclear; * = not tested; NS = not shown.

similar effect regardless of their location. Interestingly, another leucine to alanine mutation in the β-sheet (L986A) can completely disrupt function, suggesting that individual side chains in this region may also be involved. These data are consistent with point-mutant (F962S) isolated from a patient with markedly decreased expression of class II MHC and clinical symptoms similar to MHC class II deficiency [40].

Immunofluorescent microscopy studies utilizing LMB and actinomycin D revealed that a potential difference in the rate of nuclear accumulation accounts for the apparent lack of nuclear CIITA in a panel of functional mutants (L976A, L1035A, and L1051A, see Table 1 for summary). These mutants have much less nuclear expression than nuclear wild-type CIITA. With this in mind, it is of interest that activation of a class II MHC promoter by the functional mutants is as good as, if not better than, wild-type. We interpret this to be a result of the amount of CIITA transfected. Transfection of 1 μg of wild-type CIITA DNA generally gives excellent activation as do smaller amounts (*e.g.*, 100 ng; see Figure 8). When 100 ng of an "active" mutant is transfected, activity is substantially diminished (L976A, L1051A) if not almost completely absent (L1035A). We believe this indicates that when higher amounts of CIITA are available (1 μg of transfected DNA) sufficient protein for activation successfully transits to the nucleus. As activation only requires a sufficient amount of CIITA for the number of available promoters, activation by wild-type and the mutants is similar. Since import is slower for the mutants, when less DNA is transfected mutant CIITA is less active because it transits more slowly (*i.e.*, a sufficient number of molecules is not available in the given timeframe for efficient activation). This explanation is further supported by the observation that after transfection with 100 ng, allowing additional time for CIITA to accumulate in the nucleus (48 hours) results in restoration of activity, an effect not observed for L1035P (data not shown). Whereas further study is needed to understand the precise mechanisms used by the LRR to regulate nuclear expression, we speculate that the LRR may act in some way to mask accessibility to the nuclear import machinery.

CIITA also requires binding to GTP for successful nuclear translocation [7]. As mentioned above, L1035P in the context of residues 939-1130 had reduced interaction with the GTP-binding domain [28]. This dependence on both the GTP-binding domain and LRR for nuclear translocation of CIITA suggests a link between the two mechanisms. Studies of proteins with domain structures similar to CIITA, such as Apaf-1 and Nod1, two proteins involved in apoptosis [30, 41], and the plant disease resistance protein RPM1 [42] may inform further study of CIITA. These proteins are similar to CIITA in that they have nucleotide binding domains (NBD) and C-terminal repeat motifs (usually LRRs) that are important for function [7, 28, 30]. Studies of these proteins suggest the involvement of a cytoplasmic cofac-

tor. Alterations in nuclear translocation could be attributed to availability of GTP/cofactor and potentially altered affinity of the NLSs for importins.

An earlier study has examined the role of the LRR in CIITA function. Using a model of RNAse inhibitor interactions with RNaseA to target analogous residues in CIITA, they found that multiple-point mutants near the putative β-sheet regions and in the loop of the four consensus LRR had defective nuclear localization independent of function [18]. They also report that these mutations disrupt CIITA binding to a 33-kDa protein of unknown function. A number of single point mutations in this region disrupted transcriptional activation, but localization studies were not performed using these mutants. Interestingly, mutation of two asparagines (usually associated with the LRR motif residues) to alanine had no effect on function [18]. This observation is consistent with others indicating that asparagines and cysteine are not universally required in all LRRs [29, 31, 43], and supports our identification of additional LRRs in CIITA. Unlike the previous report, our study examines the contribution of the conserved leucines that define the LRR motif in both the α-helices and the β-sheets revealing that both regions impact nuclear localization. That individual leucine residues in the α-helices (and perhaps the helices themselves) are important for nuclear import in addition to some aspect of the β-sheets implies the involvement of both structural elements. Together with the requirement for specific leucines in some α-helices, this suggests that LRR-mediated events are likely complex and that leucines in the α-helices may be participating in an important interaction. For example, the differences between the effects of leucine to alanine mutations in the α-helical region are more dramatic in general than similar mutations in the β-sheet region. In addition, within the β-sheet region, different leucines in the motif have differential effects on nuclear accumulation and transactivation function. Finally, we present strong evidence that the LRR sequences are critical for the optimal rate of nuclear accumulation, best revealed when both nuclear export and de novo mRNA synthesis are eliminated as factors.

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