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Self-association of Class II Transactivator Correlates with Its Intracellular Localization and Transactivation*

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Androniki Kretsovali‡§‖, Charalambos Spilianakis‡§¶, Andreas Dimakopoulos‡¶, Takis Makatounakis‡, and Joseph Papamatheakis‡¶‖

From the ‡Foundation for Research and Technology, Institute of Molecular Biology and Biotechnology and the ¶Department of Biology, University of Crete, Heraklion, 711 10 Crete, Greece

Class II transactivator (CIITA) is the master regulator of major histocompatibility complex class II genes that regulates both B lymphocyte-specific and interferon γ-inducible expression. Here we identify protein regions and examine mechanisms that determine the intracellular distribution of CIITA. We show that two separate regions of CIITA mediate nuclear export: amino acids 1–114 and 408–550. Both regions interact with the export receptor CRM-1. The CIITA region spanning amino acids 408–550 of CIITA also determines its ability for homotypic self-association as well as heterotypic interactions with other regions residing at the amino and carboxyl termini of the protein. These observations are in line with data demonstrating that co-expression of amino- and carboxyl-terminal parts of CIITA promote subcellular relocalization and, remarkably, rescue transcriptional activation by individually inert molecules. CIITA point mutations that impair nuclear import and abolish its activation function show reduced self-association. We propose that the concerted action of homo- and heterotypic interactions of CIITA determine proper protein configuration that in turn controls its nucleocytoplasmic trafficking.

MHC[1] class II genes are essential for the presentation of foreign antigens to T helper lymphocytes (1). These genes are specifically expressed in antigen-presenting cells and can be induced in various other cell types by cytokines such as interferon γ (2). In humans, the lack of MHC class II gene expression results in severe immunodeficiency, bare lymphocyte syndrome (3). Molecular analysis of the factors associated with this syndrome (RFX5, RFXAP, RFXANK, and CIITA) provided invaluable information about the regulation of MHC class II genes. Whereas the trimeric RFX and NFY complexes are ubiquitously expressed, expression of CIITA is cell type-restricted and correlates with constitutive or interferon γ-mediated MHC class II gene expression (4–6). CIITA is recruited on the class II promoter through multiple interactions with RFX, NFY, and possibly other factors (7–10). CIITA acts as a very potent transcriptional co-activator via its amino-terminal activation domain that can interact with components of the basal transcriptional machinery (11, 12) and also with the histone acetylases CBP (13, 14) and PCAF (15).

CIITA has two nuclear localization signals (NLSs), one of which is localized at its carboxyl terminus and resides within a region deleted in a case of BLS (16), hereafter named NLS1. A second bipartite NLS (NLS2) resides at the amino-terminal part of the protein. Lysines within NLS2 are acetylated by PCAF and CBP (15). Nuclear levels of CIITA are regulated through many distinct mechanisms such as GTP binding to cognate sites (17), acetylation within the amino-terminal NLS2 (15), and protein-protein interactions involving the carboxyl-terminal leucine-rich repeats (8).

Despite the two NLSs, CIITA is found both in the nucleus and cytoplasm (16, 17) because of active nuclear export (15). We show here that an amino-terminal region and a central region of CIITA that also contain the activation domain and the two GTP binding sites, respectively, determine nuclear export. We also demonstrate that although the presence of an intact NLS is critical for nuclear import of CIITA, access of the protein to the import machinery is further modified by its ability to associate with itself. Point mutations that destroy self-association have a profound effect in the nuclear import and transactivation properties of the protein.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HeLa, COS-1, and COS-7 cell lines were maintained in Dulbecco's modified Eagle's medium and transfected as described previously (13). Luciferase assays were performed 24 h after transfection.

Plasmids—The mouse class II promoter Ea (extending to the −2.1 kilobase) was fused to the Luciferase gene producing the Ea-Luc construct. FLAG-tagged full-length CIITA or its derivatives were expressed from pCDNA3 expression vector. Green or red fluorescent protein fusions were constructed in pEGFPC-1 or the dsred (CLONTECH), respectively. CIITA mutants were constructed with the Gene Editor *in vitro* site-directed mutagenesis system from Promega. The mutagenic primers were as follows: ML1, CAGCGATGCTGACCCCGCGTGCGCCTACCACTTCTATGA; ML2, CCCTGCTCCGCCCGGGGGGCGGCGGCCGGCCTTTTCC; ML3, CTGCTCCGAGGTTGCACCGCCGCCGCCACAGCCCGGCCCCGGGGC; and F961S, AAGAAACTGGAGTCTGCGCTGGGCCCT. All constructions were verified by sequencing. Protein expression was assayed by Western blotting and immunofluorescence.

In Vitro Protein-Protein Interaction Experiments—Fragments of CIITA were subcloned into pGEX vectors (Amersham Pharmacia Biotech) in frame with glutathione *S*-transferase. Approximately 1 μg of fusion proteins were immobilized to glutathione-Sepharose beads and incubated with *in vitro* translated and ^{35}S-labeled (TNT, Promega) full-length CIITA or derivatives in a buffer containing 150 mM KCl, 20 mM

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§ These authors contributed equally to this work.
‖ To whom correspondence should be addressed: Inst. of Molecular Biology and Biotechnology, P.O. Box 1527, Heraklion 711 10, Crete, Greece. Tel.: 30-81-391175; Fax: 30-81-391101; E-mail: papamath@imbb.forth.gr and kretsova@imbb.forth.gr.

[1] The abbreviations used are: MHC, major histocompatibility complex; CIITA, class II transactivator; NLS, nuclear localization signal; BLS, bare lymphocyte syndrome; PAGE, polyacrylamide gel electrophoresis; N/C, nuclear to cytoplasmic ratio; NER, nuclear export region; GFP, green fluorescent protein; GST, glutathione *S*-transferase; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor.

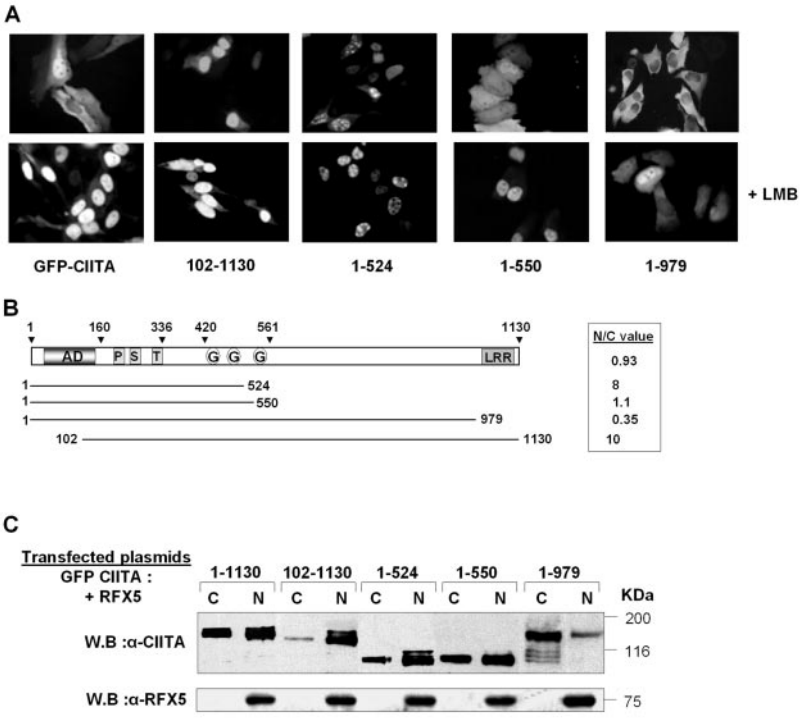

FIG. 1. **Opposing effects of CIITA truncations on nuclear import and LMB mediated nuclear retention.** *A*, GFP fusions of the indicated regions of CIITA were transfected into HeLa cells. GFP-CIITA is the full-length (amino acids 1–1130) protein. Green fluorescence was observed 24 h later either without treatment or following a 2-h treatment with 20 nM leptomycin B (+*LMB*). *B*, schematic view of CIITA and truncation end points. The activation domain (*AD*), GTP binding motifs (*GTP*), nuclear import signals (*NLS*), and the leucine-rich region (*LRR*) are indicated. Shown on the *right* are the corresponding nuclear/cytoplasmic ratios obtained by the Scion Beta 4 image analysis program, and the values represent averages from at least 50 cells. *C*, cytoplasmic (*C*) and nuclear (*N*) fractions were prepared from HeLa cells transfected with 5 μg of the indicated GFP CIITA derivatives and 1.5 μg of an RFX5 expressing plasmid. Western blotting was performed using either α-CIITA or α-RFX5 antibodies.

Hepes, pH 7.9, 0.5% Nonidet P-40, 5 mM $MgCl_2$, 0.2% bovine serum albumin and supplemented with protease inhibitors. the reactions were carried out at 4 °C for 5 h and washed three times in the same buffer without bovine serum albumin. Bound proteins were subjected to SDS-PAGE and detected by autoradiography.

Immunoprecipitation and Western Blot—For *in vivo* protein-protein interactions, COS-1 cells in 100-mm diameter were transfected with 5–10 μg of each plasmid using the calcium phosphate method. Whole cell extracts were prepared in lysis buffer containing 10 mM Tris-HCl, pH 8, 170 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitors. Extracts were incubated for 16 h at 4 °C with anti-FLAG M2 agarose (Sigma). The immunoprecipitated samples were washed four times with lysis buffer containing 250 mM NaCl, and subjected to SDS-PAGE. Western blotting analysis was performed using monoclonal anti-FLAG (Sigma) or anti-GFP (CLONTECH) antibodies.

For cell fractionation experiments, HeLa cells were transfected with GFP-CIITA derivatives and RFX5 and lysed in hypotonic buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol) with three strokes using a Dounce homogenizer, pestle type B. The post-centrifugation supernatant was kept as cytoplasmic fraction. Pelleted nuclei were further washed with hypotonic buffer with two more strokes using a Dounce homogenizer with a loose pestle and following centrifugation nuclei were extracted with buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). Fractions were subjected to SDS-PAGE and immunoblotted with α-CIITA (a polyclonal antiserum raised against the amino-terminal 330 amino acids of CIITA) and α-RFX5 (Rockland) antibodies.

For the His-CRM1 pull-down experiment, His-tagged CRM-1 was immobilized on nickel-nitrilotriacetic acid-agarose beads (Qiagen). The beads were washed three times with lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 60 mM imidazole, 0.5% Triton X-100, 5% glycerol, and protease inhibitors mix). One more wash was done with EBC buffer without EDTA (10 mM Tris, pH 8.0, 170 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors mix). Following 1 h of blocking with 5 mg/ml bovine serum albumin and two washes with EBC buffer, COS cell extracts (precleared with nickel-nitrilotriacetic acid-agarose beads) containing GFP CIITA derivatives were added. Following a 4-h interaction at 4 °C and three washes with NETN buffer, the CRM1-retained proteins were resolved in SDS-PAGE (10% for the left part and 8% for the right part) and detected by immunoblotting using anti-GFP antibody.

Fluorescent Protein Analysis—Localization of transfected proteins was analyzed with an Olympus IMT2 fluorescence microscope on living or fixed (phosphate-buffered saline/acetone 2:3) cells with or without further staining by an anti-FLAG monoclonal antibody and the appropriate tetrarhodamine isothiocyanate-conjugated anti-mouse antibody. Quantitation of nuclear and cytoplasmic content of the expressed CIITA proteins was done with fluorescence image analysis by the Scion Image Beta 4 program (www.scioncorp.com). Quantitative protein expression was determined by Western blotting analysis with an anti-GFP monoclonal antibody (CLONTECH).

RESULTS

Multiple Regions Determine the Nucleocytoplasmic Distribution of CIITA—We have shown recently that CIITA is the first co-activator that is actively exported from the nucleus (15). In agreement with this and other observations (16) we found CIITA to be evenly distributed between the nucleus and cytoplasm with nuclear to cytoplasmic ratio (N/C) of 0.93. To delineate regions that mediate export, we generated amino- and carboxyl-terminal deletions of CIITA fused to the GFP (Fig. 1*B*). A deletion of the first 102 amino acids (Fig. 1*A*, *102–1130*) rendered CIITA nuclear (N/C = 10), indicating that this region regulates nuclear export. Thus, sequences within the transcription activation domain of the protein determine nuclear export. All carboxyl-terminal deletions of CIITA that retain the amino-terminal NLS2 (15) and extend up to amino acid 524 are mainly nuclear. As shown in Fig. 1*A* CIITA 1–524 shows high nuclear levels (N/C = 8). In contrast, a CIITA molecule that extends to amino acid 550 showed equal distribution in both compartments (N/C = 1.1) and was retained in the nucleus when LMB was added. These distribution patterns are consistent with the presence of additional sequences between amino acids 525 and 550 that are involved in nuclear export. We designate regions 1–102 and 525–550 as nuclear export regions (NERs) 1 and 2, respectively. A CIITA deletion that extends to amino acid 979 (Fig. 1*A*, *1–979*) and contains both NLSs (15, 16) showed cytoplasmic distribution (N/C = 0.35) and was partially responsive to LMB. Thus, in agreement with a recent report (8), our results show that additional information residing in the carboxyl-terminal part of the protein that includes the earlier described leucine-rich region is required for nucleocytoplasmic transport. Fractionation experiments of HeLa cells co-transfected with the same set of constructs shown in

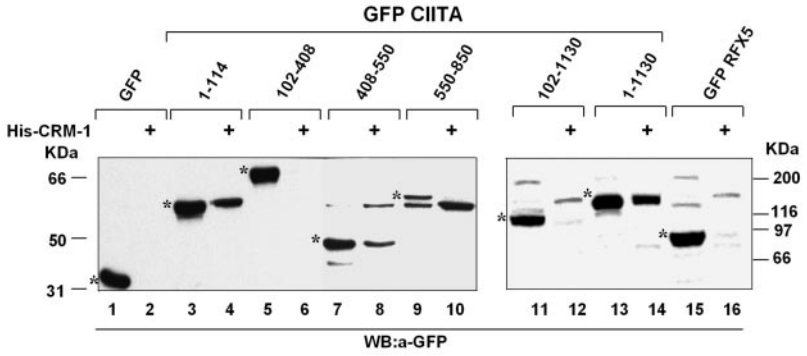

FIG. 2. **Distinct regions of CIITA interact with the CRM1 nuclear exporter.** Extracts from COS-1 cells transfected with GFP fusions of the indicated CIITA segments (amino acid end points are indicated) or a specificity control RFX5 were passed through a column of His-tagged CRM1 1 immobilized on a nickel-nitrilotriacetic acid-agarose (+). Retained proteins (*even-numbered lanes*) in comparison with their input (*odd-numbered lanes*) were detected by immunoblotting with an anti-GFP antibody. Specific protein bands are indicated with *asterisks*.

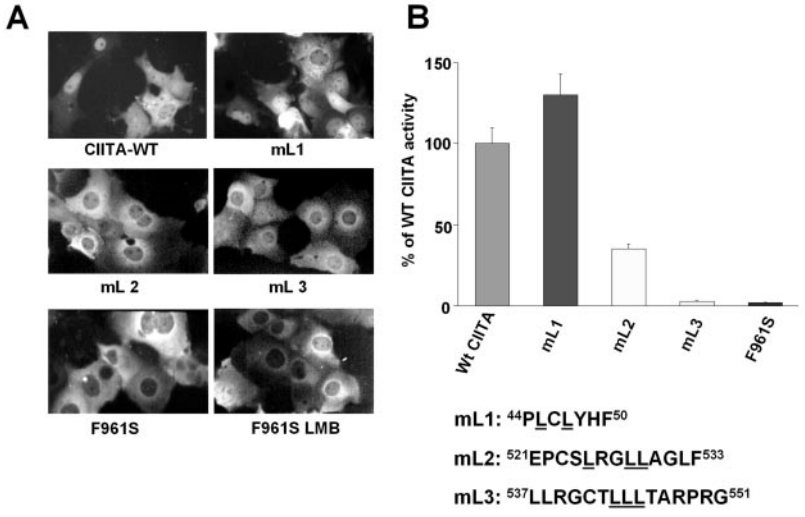

FIG. 3. **Defective nuclear import and activity of CIITA point mutants.** *A*, the subcellular distribution of the indicated wild type (*WT*) and mutant CIITA molecules fused to GFP was analyzed following expression in COS-7 cells. *B*, transactivation of a class II-Luc reporter (1 μg) by the indicated wild type and mutant CIITA expressing plasmids (100 ng) was evaluated following transfection of 1.5×10^5 HeLa cells. The results using either FLAG- or GFP-CIITA plasmids were comparable and are averaged values from six experiments presented as percentages relative to the Luciferase activity produced by the wild type CIITA expressing plasmid (*Wt CIITA*), whose activity is referred to as 100. The alanine substituted clustered leucine residues in ml1, ml2, and ml3 are *underlined*.

Fig. 1*B* along with a construct coding for RFX5 showed that the distribution of proteins in the nuclear and cytoplasmic fractions (Fig. 1*C*) is in agreement with results of the visual analysis. RFX5 that is exclusively nuclear[2] serves as a purification control.

To investigate the role of CIITA regions in controlling nuclear export, we performed experiments to evaluate the binding of various CIITA parts to the export receptor CRM1 (18, 19). Whole cell extracts prepared from COS-1 cells expressing various CIITA derivatives fused to GFP were passed through a CRM1 affinity column. Specific protein retention on the CRM1 column was assayed by immunoblotting. Fig. 2 shows that full-length CIITA was specifically retained on the column (Fig. 2, *lane 14*), whereas RFX5 a nuclear protein was not (*lane 16*).[2] CIITA fragments 1–114 and 408–550 that contain the NER1 and NER2 elements described previously did bind to the CRM1 (*lanes 4* and *8*, respectively), whereas fragments spanning the regions 102–408 and 550–850 did not (*lanes 6* and *10*). A deletion that removes the first 102 amino acids of the protein (amino acids 102–1130) that is exclusively nuclear did not bind to CRM1 (*lane 12*), indicating that although it contains sequences that promote nuclear export, these sequences were not accessible to the export machinery in this context.

Both NERs contain closely spaced leucines, although they do not match perfectly to the consensus for nuclear export signals (20). To investigate the possibility that these leucines may be required for CIITA export, we generated the mutants shown in Fig. 3. The intracellular distributions and activities of GFP-tagged proteins were next studied. The distribution of ml1 was similar to the mixed pattern of wild type protein, whereas mutant proteins ml2 and ml3 were mainly cytoplasmic in the great majority of cells (Fig. 3*A*). Therefore leucines mutated in ml2 and ml3 are involved in the nuclear localization of CIITA. We also generated a naturally occurring point mutation that substitutes amino acid 961 (F961S) recently reported in a case of late onset BLS, which inactivates CIITA (21). This point mutation also rendered the protein cytoplasmic (Fig. 3*A*). Treatment with LMB did not influence the distribution of ml2, ml3 (not shown), or F961S (Fig. 3*A*), thus suggesting that these mutants are defective in nuclear import. Transient transfection assays (Fig. 3*B*), using an MHC class II promoter-Luc fusion plasmid further showed that ml1 had activity slightly higher than the wild type. Mutant ml2 retained about 30% of activity, in agreement with an earlier report (22) and ml3 or BLS are practically inactive (Fig. 3*B*). Overall, these results suggest that mutations that do not directly destroy individual import signals abolish the nuclear import and activity of CIITA.

CIITA Self-associates—Impaired nuclear import of CIITA mutations that do not directly affect the NLS raise the possibility that import may require a proper protein configuration that is disrupted in the above mutants. Such a configuration could be acquired by protein-protein interactions. The sequence homology of CIITA with the caspase activator Nod1 that is able to oligomerize (23) led us to investigate whether CIITA also self-associates.

Fig. 4*A* shows that *in vitro* translated full-length CIITA interacted strongly with the region extending from amino acids 408 to 550 (*lane 6*) and with the carboxyl-terminal region (amino acids 650–1130 and 408–1130; *lanes 8* and *12*, respectively). The same probe could associate to a lesser extend with the amino-terminal deletion of amino acids 102–1130 (*lane 11*). A probe of amino acids 408–550 (Fig. 4*B*) could interact with itself (*lane 6*), with the amino-terminal amino acids 1–114 (*lane 2*) and 114–408 (*lane 3*) and less efficiently with the carboxyl-

[2] A. Dimakopoulos and J. Papamatheakis, unpublished data.

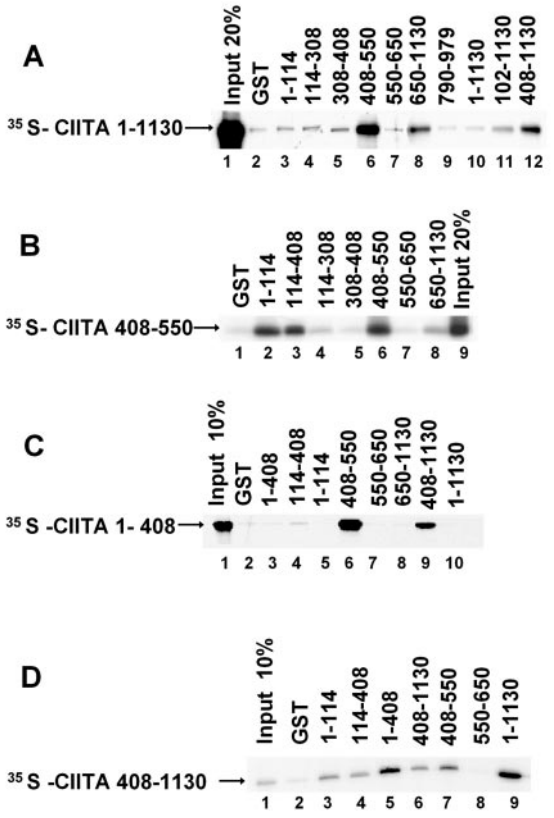

FIG. 4. **Homotypic and heterotypic self-interactions by distinct regions of CIITA *in vitro*.** GST pull-down assays were performed with equal amounts (1 μg) of GST fusions of the indicated CIITA regions and *in vitro* translated and ^{35}S labeled CIITA, full-length (*A*), 408–550 (*B*), 1–408 (*C*), and 408–1130 (*D*).

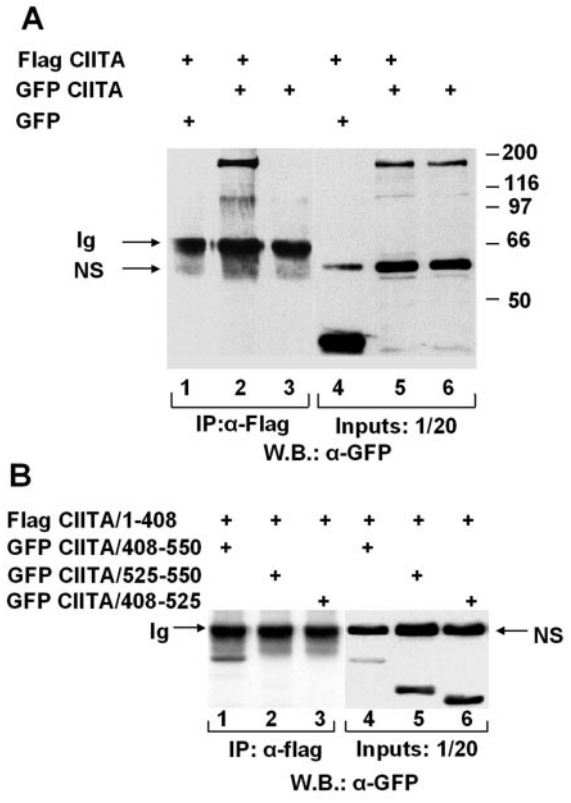

FIG. 5. **CIITA self-interaction *in vivo*.** Whole cell extracts from COS-1 cells transfected with the indicated (+) GFP- or FLAG-tagged proteins were immunoprecipitated by anti-FLAG antibody run on SDS-PAGE and then immunoblotted with anti-GFP antibody. The indicated GFP proteins were detected either before (*lanes 4–6*) or after immunoprecipitation (*IP*) by anti FLAG antibody (*lanes 1–3*). FLAG-tagged proteins were FLAG CIITA (*A*) and FLAG CIITA/1–408 (*B*). Mouse Ig and nonspecifically (*NS*) reacting proteins are indicated.

terminal amino acids 650–1130 (*lane 8*). In agreement with this, a fragment spanning the first 408 amino acids was efficiently retained by GST-CIITA/408–550 (Fig. 4*C*, *lane 6*) but had no ability of homotypic interaction (Fig. 4*C*, *lanes 3–5*). The carboxyl-terminal amino acids 408–1130 could bind to the amino-terminal amino acids 1–408 (Fig. 4*D*, *lane 5*) and less efficiently to itself (Fig. 4*D*, *lane 6*). Remarkably, an intact CIITA molecule was incapable of binding to itself (Fig. 4*A*, *lane 10*) or to the amino-terminal 408 amino acids (Fig. 4, *A*, *lanes 3–5* and *C*, *lane 10*), whereas it could bind to the carboxyl-terminal amino acids 408–1130 (Fig. 4, *A*, *lane 12* and *D*, *lane 9*). Therefore the amino-terminal region might interfere with the self-interaction of CIITA *in vitro*.

To confirm that such self-interactions also occur *in vivo* we used co-immunoprecipitation of CIITA proteins tagged either with FLAG epitope or with GFP. Fig. 5*A* shows that FLAG CIITA efficiently co-immunoprecipitated GFP-CIITA (*lane 2*) but not GFP (*lane 1*). No GFP-CIITA alone could bind to the anti-FLAG column (*lane 3*). Therefore intact CIITA molecules are able to self-oligomerize *in vivo* but not *in vitro*, indicating the involvement of additional factors or protein modifications. Similar co-immunoprecipitation experiments confirmed that the interaction of the amino-terminal amino acids 1–408 to the 408–550 region of CIITA also occurs *in vivo* (Fig. 5*B*, *lane 1*) and requires the intact central region because fragments thereof did not interact (Fig. 5*B*, *lanes 2* and *3*). These experiments show that CIITA undergoes multiple homotypic and heterotypic interactions that may lead to intramolecular and/or bimolecular self-associations.

We next tested the ability of mutant proteins ml2, ml3, and F961S to self-associate *in vitro* and *in vivo*. Fig. 6*A* shows that mutants ml2 and ml3 had reduced binding to a GST-CIITA amino acids 408–550, and mutant F961S shows practically no interaction. *In vivo* co-immunoprecipitation experiments showed that all three mutants had reduced ability for self-association (Fig. 6*B*, *lanes 2–4*) as compared with the wild type protein (Fig. 6*B*, *lane 1*). Overall these data favor the possibility that these point mutations lead to altered protein topology and defective function by affecting the ability for self-association.

Self-association of CIITA Leads to Cellular Redistribution and Functional Trans-complementation—To examine whether the self-association properties of CIITA may influence its subcellular localization, we co-transfected various GFP- and FLAG-CIITA expressing plasmids into COS-7 cells and compared their subcellular distribution. Fig. 7 shows that a FLAG CIITA/1–408 is mainly nuclear (N/C>10) (*panel a*) and deletion 408–1130 that lacks NLS2 is cytoplasmic (N/C = 0.33) (*panel b*). However, upon co-transfection, the 408–1130 molecule showed increased nuclear levels (compare *panels b* and *d*) in cells that also express CIITA/1–408 (compare *panels c* and *d*). In such experiments the average N/C of 408–1130 estimated from cells co-expressing the two molecules increased from 0.33 to 0.83, thus approaching the wild type CIITA levels. The same approach was employed to study the interaction between two different intact CIITA molecules within cells. For this reason the effect of a red fluorescent CIITA (dsred-CIITA) (Fig. 7*e*) on the localization of a GFP-CIITA that alone was rendered nuclear (Fig. 7*f*) because of the action of an exogenously supplemented SV40 NLS (GFP-NLSCIITA) was studied. dsred-CIITA led to increased cytoplasmic content of the co-expressed GFP-NLS-CIITA (compare *panels h–f*), indicating that in this context, a single S40 NLS present in dimers formed with native CIITA is not sufficient to overcome the action of the

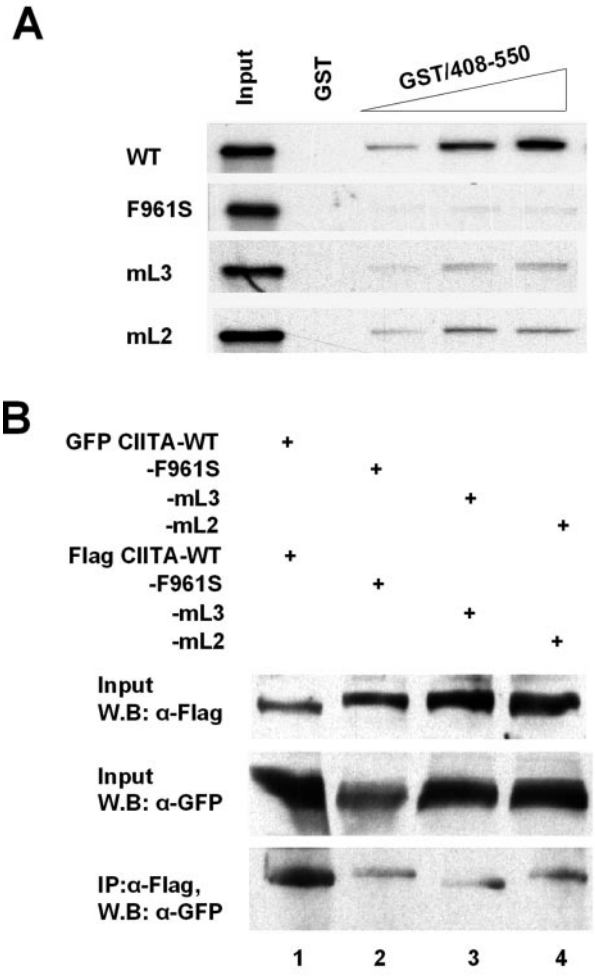

FIG. 6. **Cytoplasmic CIITA mutants show reduced ability for self-association.** *A*, retention of increasing amounts (0.25, 1, or 2 μg) of wild type (*WT*) and the indicated CIITA mutants by GST-CIITA/408–550. *B*, extracts of COS-1 cells co-transfected with the indicated (+) pairs of FLAG-and GFP-wt or mutant CIITA expressing plasmids were used for co-immunoprecipitation using anti-FLAG antibody followed by immunoblotting using anti-GFP antibody. *W.B.*, Western blot.

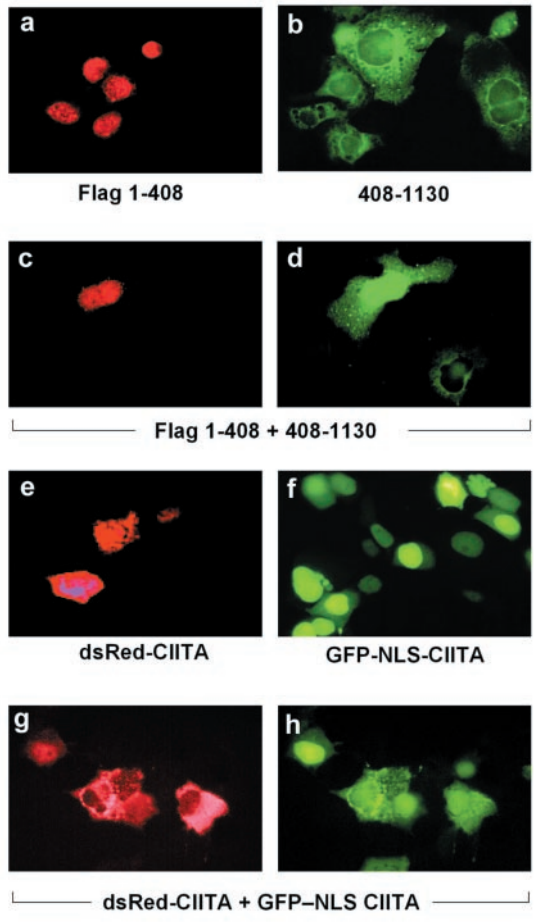

FIG. 7. **Co-expression of different CIITA molecules leads to intracellular relocation in cells.** The indicated FLAG-, GFP-, or dsred-CIITA derivatives were transfected alone or in combination into COS-7 cells, and their intracellular distribution was observed 24 h after transfection by direct fluorescence and/or staining using an tetrarhodamine isothiocyanate-conjugated anti-FLAG antibody.

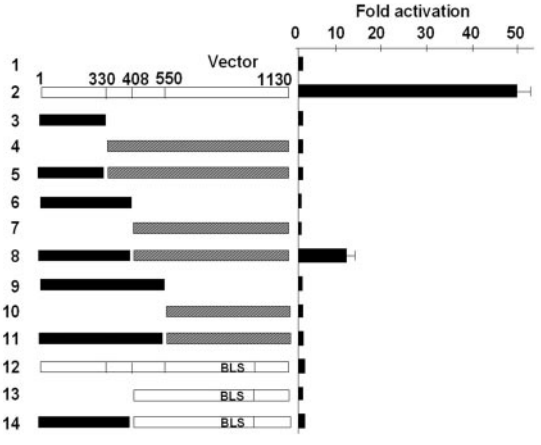

FIG. 8. **Trans-complementation of CIITA activity by individual amino- and carboxyl-termini of CIITA.** HeLa cells were co-transfected with equal amounts of amino or carboxyl terminus and/or vector stuffer DNA (0.3 μg) in the combinations shown, and their effect on the class II-Luciferase reporter was assayed in comparison with plasmids expressing full-length wild type CIITA. BLS denotes the F961S mutant. The results are presented as fold activation over the vector-transfected cells (taken as 1) and are the averages of four experiments.

export signals. Overall the bimolecular interaction of either intact CIITA molecules or fragments thereof influences their subcellular localization in a way that resembles that of the intact CIITA.

Finally, to test the functional consequences of the self-association of CIITA, we produced full-length complementing fragments of CIITA and co-transfected them into HeLa cells. Individually expressed amino- or carboxyl-terminal truncations of CIITA had no effect on a target MHC class II reporter (Fig. 8, *lines 3*, *4*, *6*, *7*, *9*, and *10*). Interestingly, co-expression of amino and carboxyl termini of CIITA separated at amino acid 408 (1–408 and 408–1130) showed significant trans-complementation activity (Fig. 8, *line 8*). Splitting the molecule at amino acid 330 or 550 did not rescue CIITA activity (Fig. 8, *lines 5* and *11*). Thus, a functional CIITA can be reconstituted by the assembly of two separate protein fragments derived by cleavage at position 408. On the contrary, the carboxyl-terminal parts (408–1130 amino acids) mutants BLS/F961S (line 14) or ml2 and ml3 (not shown), which are defective for self-association, were unable to rescue transcriptional activity by similar assays.

DISCUSSION

In this work we investigate mechanisms that control the nucleocytoplasmic trafficking of CIITA. We determine two separate regions, NER1 and NER2, that mediate the CRM1-dependent nuclear export of CIITA. Furthermore, we provide evidence that nuclear levels of CIITA correlate with its ability

for self-association. We show that CIITA can self-associate via homo- and heterotypic interactions. Based on the analysis of mutations that impair nuclear import, we suggest that structural constrains imposed by self-association affect recognition of CIITA by the import machinery.

Regulated nuclear transport is a mechanism that rapidly converts a cell effector from an inactive into an active form and vice versa during various cell signaling, cell cycle progression, and differentiation pathways. Competence for transport through the nuclear membrane is achieved by diverse mechanisms. In some cases factor dimerization upon signal-dependent phosphorylation (STATs and IRF3) precedes nuclear import, although it is not determined whether it is a prerequisite for it. Interaction with other proteins is also a well established mechanism for the regulated nucleocytoplasmic transport. Binding to IκB inhibits nuclear import of p65 through masking of the NLS (24, 25). Conversely, binding to calcineurin inhibits the nuclear export of NFAT by masking the nuclear export signals (26). In addition, self-interaction may affect nucleocytoplasmic transport, as exemplified by tetramerization of p53s, which inhibits its nuclear export (27).

Although intracellular shuttling mostly characterizes transcription factors that are activated by post-translational mechanisms, CIITA, which is regulated at the transcriptional level, also shuttles between the nucleus and cytoplasm (15, 16). This behavior of CIITA is not typical for a co-activator, because there is only one other example of a co-activator that has been shown to move between nucleus and cytoplasm in response to different stimuli, namely the activating signal co-integrator 1 (28). The low expression level of the endogenous protein has not permitted thus far the elucidation of the role of CIITA nucleocytoplasmic shuttling in various physiological or developmental states. It is possible that the amount and/or timing of CIITA retained in the nucleus must be precisely adjusted. Low CIITA nuclear levels may be advantageous to avoid secondary effects such as inhibition of the activity of other factors as was shown for NFAT (29) and Fas ligand (30). Furthermore, the possibility of unknown functions of CIITA in the cytoplasm cannot be formerly excluded. In this line, CIITAs sequence similarity with the caspase activator Nod-1 (31) is very intriguing and needs further study to elucidate its role in apoptotic pathways.

Nod-1 is known to oligomerize via the nucleotide binding sequence (23). We demonstrate by biochemical, topological, and functional assays that CIITA is also able to oligomerize via sequences homologous to Nod-1. CIITA has the ability for multiple (homotypic and heterotypic) interactions, all of which involve the central part of the molecule that harbors the GTP motifs and the export region NER2. Intramolecular or bimolecular interactions can lead to a highly structured monomer or dimer in a nonmutually excusive manner. Neither type of configuration is ruled out by the present analysis.

The heterotypic, intramolecular interaction of amino-terminal sequences that include the NER1 with the central NER2 region limits the NER2-NER2 homotypic interaction and lowers the nuclear levels of CIITA. In accordance with this, elimination of NER1 shifts the equilibrium toward homotypic NER2-mediated interactions that result in an exclusively nuclear distribution observed with the 102–1130/GFP form of CIITA. Conversely, point mutations within NER2, which compromise the homotypic NER2-mediated dimerization, lead to the inability for nuclear import. This process is reminiscent of the IRF-3 protein, which is constitutively cytoplasmic because of an intramolecular association, which masks the NLS, whereas upon virus-mediated signaling and concomitant phosphorylation, it dimerizes and enters the nucleus (32). The balance of opposing import and export signals in determining the subcellular distribution of CIITA is demonstrated by the relocalization effects caused by the paired expression of amino- with carboxyl-terminally derived parts of CIITA or intact CIITA molecules, one of which is supplied with the SV40 NLS. In both cases co-expression of either pair of molecules moderates extreme nuclear or cytoplasmic protein distributions and almost restores the distribution pattern and the activity of native CIITA.

The interaction of CIITA with CRM1 depends on the presence of both NER1 and NER2. Because no significant binding to CRM-1 was detected using bacterial CIITA and *in vitro* translated CRM-1 in the presence or absence of Ran-GTP (not shown), the possibility that an intermediary factor is required for this interaction remains open for further investigation. CIITA derivatives lacking the amino terminus that harbors NER1 show a strong dominant negative effect (33–35). Thus, the negative effect of these proteins stems from their high nuclear localization (this study) in combination with their increased ability for promoter recruitment (9). The localization of NER1 within the transcriptional activation domain suggests that CBP and PCAF may compete with CRM1 for binding to CIITA in a way similar to calcineurin for NFAT (26). Interestingly, we have shown that PCAF increases nuclear levels of CIITA by two mechanisms: acetylation and direct binding (15). It is possible that the second mechanism involves masking of the CRM1 interaction surface of CIITA. Thus, CIITA molecules that are not engaged in transcription may undergo fast nucleocytoplasmic turnover. Nuclear retention via interaction with co-activators has also been reported for IRF-3 (36), HNF-4 (37), and ASC-1 (28).

It is intriguing that diverse mutations of CIITA such as the leucine substitutions and the BLS mutation reported here or mutations introduced in the GTP-binding site (17) or the leucine-rich region (8) result in defective nuclear import. A model consistent with these results could involve alterations of protein conformation that affect recognition of CIITA by the import machinery. Similar mechanisms might modulate the nucleocytoplasmic properties of other proteins as well. These results are in agreement with data reported on CIITA self-association by Linhoff and colleagues (38) and Sisk and colleagues (39).

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