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The class II *trans*-activator CIITA interacts with the TBP-associated factor $TAF_{II}32$

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ABSTRACT

The class II *trans*-activator (CIITA) is the main transcriptional co-activator for the expression of MHC class II proteins. Its N-terminal 125 amino acids function as an independent transcriptional activation domain. Analyses of the primary amino acid sequence of the activation domain predict the presence of three α-helices, each with a high proportion of acidic residues. Using site-directed mutagenesis, we found that two of these predicted α-helices are required for full transcriptional activation by CIITA. Moreover, a CIITA protein in which both functional α-helices have been deleted displays a dominant negative phenotype. This activation domain of CIITA interacts with the 32 kDa subunit of the general transcription complex TFIID, $TAF_{II}32$. Decreased transcriptional activation by N-terminal deletions of CIITA is correlated directly with their reduced binding to $TAF_{II}32$. We conclude that interactions between $TAF_{II}32$ and CIITA are responsible for activation of class II genes.

INTRODUCTION

Cell type-specific and IFNγ-inducible expression of genes in the class II major histocompatibility complex (class II) is required for normal function of the immune system. Congenital lack of class II determinants results in the severe combined immunodeficiency known as type II bare lymphocyte syndrome (BLS II) (1,2). In contrast, aberrant or over-expression of class II genes has been associated with a variety of autoimmune diseases (3).

Expression of class II determinants is regulated at the level of transcriptional initiation (reviewed in 2,4–7) and requires the class II *trans*-activator (CIITA) (8). CIITA was isolated by genetic complementation of a class II-negative BLS II cell line (8). Induction of class II genes by IFNγ requires *de novo* synthesis of CIITA (9,10). CIITA is expressed at high levels in mature B cells and its extinction in terminally differentiated plasma cells is correlated with the absence of class II determinants (11). By activating expression of the invariant chain, HLA-DMA and HLA-DMB, CIITA is also associated with other aspects of antigen processing and presentation (12,13). In the mouse, disruption of the CIITA gene resulted in loss of class II gene expression in all tissues except for isolated thymic epithelial cells (14). Normal development of $CD4^+$ T cells was severely impaired and other hallmarks of the BLS II phenotype were faithfully reproduced (14). Thus, CIITA functions as the 'master switch' for expression of class II genes in all cells examined to date.

Like many proteins involved in transcriptional regulation, the 1130 amino acid CIITA protein has a modular structure. The N-terminus is rich in acidic amino acids, which suggests that this region may function as an activation domain (8). Riley *et al.* demonstrated that amino acids from position 1 to 125 could activate transcription via the DNA binding domain of Gal4 at levels similar to those observed with the full-length protein in B cells (15). Additional experiments demonstrated that a third of this activity remained with amino acids from position 37 to 125 (15). Similarly, Zhou *et al.* mapped the activation domain of CIITA using LexA fusion proteins and demonstrated that amino acids from position 1 to 114, which contain ~80% of the acidic amino acid region, produced near wild-type levels of transcriptional activation in yeast (16). Moreover, when the N-terminal 300 amino acids of CIITA were replaced with the activation domain of VP16 from herpes simplex virus 1 (HSV1) the chimera activated expression of class II genes to levels ~25% that of the wild-type CIITA (16). These data indicate that the N-terminal activation domain shares functional homology with VP16 and that the C-terminal 830 amino acids of CIITA are involved in targeting the protein to class II promoters.

Using acidic activators as models, we investigated the structure–function relationships of the N-terminal activation domain of CIITA. Analysis of the primary amino acid sequence of the N-terminal region of CIITA yielded three putative α-helices. Each of these α-helices contains ~40% acidic residues. These predicted acidic α-helices provided a reasonable starting point for structural analyses of the activation domain of CIITA. Mutational analyses of these structures demonstrated that two of these α-helices are required for the transcriptional activity of CIITA. A CIITA protein in which these two active α-helices were deleted displayed a dominant negative phenotype in co-transfection experiments with wild-type CIITA. *In vitro* binding studies revealed that the N-terminus of CIITA binds to the 32 kDa subunit of TFIID ($TAF_{II}32$) and that the loss of transcriptional activation by deletion of the two active α-helices from CIITA correlates directly with loss of its binding to $TAF_{II}32$. We conclude that the interaction between CIITA and $TAF_{II}32$ is a critical event in the

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transcriptional activation of class II genes. This is the first demonstration of a functional interaction between a cellular co-activator and a TAF.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

CIITA cDNA from plasmid pCDNAI/CIITA (10) was ligated into the expression vector pSVSPORT-1 (Life Technologies Inc., Gaithersburgh, MD) at the *Eco*RI and *Xba*I sites. Deletion of the amino acids from position 1 to 161 in pSVCIITAΔN was achieved by amplification of CIITA cDNA from position 599 to 3512 by PCR. The PCR product was ligated into pSVSPORT-1 using *Eco*RI and *Xba*I sites in the PCR primers. Deletions of α-helices (pSVCIITAΔah1, pSVCIITAΔah2, pSVCIITAΔah3 and pSVCIITAΔah12) and alanine to proline [pSVCIITA(A94P)] glycine to proline [pSVCIITA(G58P)] and acidic amino acid substitutions [pSVCIITA(Δah2DE-N) and pSVCIITA(Δah2DE-NC)] were generated in pSVCIITA using the Transformer site-directed mutagenesis kit from Clontech (Palo Alto, CA) as per the manufacturer's instructions. pGal4C(1–143) was constructed by amplifying CIITA cDNA from base pair 116 to 429 by PCR, followed by ligating the PCR product into the Gal4 DNA binding domain fusion vector pSG424 (17) using *Eco*RI and *Xba*I sites in the PCR primers. pGal4CΔah1, pGal4CΔah2, pGal4CΔah3 and pGal4CΔah12 were constructed in the same manner as pGal4C(44–143), except that the templates for the PCR reactions were the appropriate mutated CIITA cDNA. Plasmids expressing the GST fusion proteins GSTCIITA(1–143) and GSTCIITA (1–143)Δah12 were constructed by amplifying CIITA cDNA from base pair 116 to 429 by PCR, using pSVCIITA and pSVCIITAΔah12 respectively as template. The PCR products were ligated into the vector pCR3 (InVitrogen Inc., San Diego, CA), excised with *Bgl*II (in the 5′ PCR primer) and *Eco*RI and then ligated into the prokaryotic expression vector pGEX3X (Pharmacia, Upsala, Sweden) at the *Bam*HI and *Eco*RI sites. pDRASCAT has been described (18). The GST–$TAF_{II}32$ prokaryotic expression vector pGEX2TK/$TAF_{II}32$ (19) was a gift from Robert Tjian.

Cell culture, transfection and CAT assay

HeLa and COS cells were maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine. Transfection of HeLa (1 μg total plasmid DNA) and COS cells (2 μg total plasmid DNA) was performed with lipofectamine (Life Technologies Inc., Gaithersburgh, MD) according to the manufacturer's instructions. The CAT assay was performed as described (20). Transfections were performed in duplicate, a minimum of three times. Duplicate values were averaged and the SEM calculated. A human chorionic gonadotropin expression plasmid was included in all transfections as a transfection efficiency control, as described (20).

Production of recombinant proteins in *Escherichia coli*

Recombinant proteins GSTCIITA(1–143), GSTCIITA (1–143) Δah12 and GST–$TAF_{II}32$ were all produced essentially as described (21). *Escherichia coli* strain DH5α was transformed with the expression plasmid and a 50 ml overnight culture from a single colony was grown in LB broth containing 100 μg/ml ampicillin. The overnight culture was used to inoculate 500 ml LB broth containing 100 μg/ml ampicillin and this culture was grown to an OD_{590} of ~0.5, followed by addition of isopropyl-β-D-thiogalactoside (Sigma, St Louis, MO) to a final concentration of 0.1 mM. The cultures were incubated for a further 3 h, the bacteria were collected by centrifugation and lysed in 4.0 ml EBC-D buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, 5 mM DTT). The lysate was cleared by centrifugation at 25 000 *g* for 30 min and stored at –70°C until used in binding reactions.

Nuclear extract preparation

Nuclear extract was prepared from human B cells, collected by leukaphoresis from a patient with B cell leukemia. Freshly collected cells were washed five times in phosphate-buffered saline and then nuclear extract prepared exactly as described (22). Protein concentration was 18 μg/ml, determined by Bradford assay (BioRad Inc., Hercules, CA).

In vitro protein interaction assays

Recombinant GST fusion proteins were purified by combining 100 μl cleared lysate from *E.coli*, 350 μl EBC-D buffer and 50 μl glutathione–Sepharose beads (Pharmacia, Uppsala, Sweden) pre-washed in EBC-D buffer. The reactions were incubated at 4°C with inversion for 60 min and the Sepharose beads collected by centrifugation. The beads were washed five times in EBC-DS (EBC-D buffer containing 0.075% SDS) and twice in TGEM200 (20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 5% glycerol, 200 mM NaCl). The beads were resuspended in 600 μl TGEM200 for use in the binding reactions. For binding of $TAF_{II}32$ to GSTCIITA(1–144) or GSTCIITA(1–144)Δah12, 50 μl B cell nuclear extract were added to the glutathione–Sepharose beads bound with the appropriate GST fusion protein. The reaction was incubated for 2 h at 4°C with inversion and the beads collected by centrifugation. The beads were washed four times with TGEM200 and twice with TGEM500 (TGEM200 with the concentration of NaCl increased to 500 mM). The beads were resuspended in 50 μl glutathione elution buffer (80 mM glutathione, 50 mM Tris–HCl, pH 8.5, 5 mM DTT) and incubated at room temperature for 30 min with occasional vortexing. The supernatant containing eluted GSTCIITA(1–144) or GSTCIITA (1–144)Δah12 and any interacting protein was collected by centrifugation of the beads. The eluates were divided equally and run on two SDS–PAGE gels, followed by transfer onto nitrocellulose membranes (Amersham Inc., Arlington Heights, IL.). Western blot analysis using anti-$TAF_{II}32$ anti-serum (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-GST anti-serum (Pharmacia, Uppsala, Sweden) was performed as described (20). The binding of full-length wild-type or mutated CIITA to GST–$TAF_{II}32$ was carried out identically, except that *in vitro* transcribed/translated (IVT) CIITA or mutant CIITA protein was included in the binding reactions instead of nuclear extract. The CIITA proteins were produced using the TnT Coupled *In Vitro* Transcription/Translation kit (Promega, Madison, WI) with 5 μCi [^{35}S]methionine according to the manufacturer's instructions.

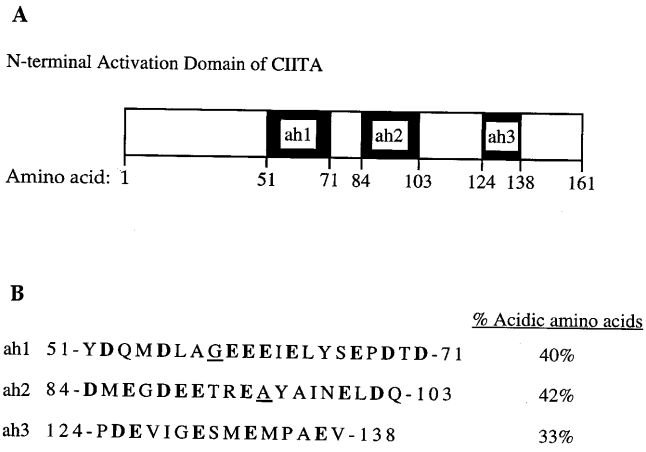

Figure 1. Predicted structure and acidic amino acid content of the activation domain of CIITA. (**A**) The N-terminal 161 amino acids of CIITA function as a transcriptional activation domain. There are three predicted α-helices present in this region at amino acid positions 51–71 (ah1), 84–103 (ah2) and 124–138 (ah3). (**B**) The primary amino acid sequences of the three α-helices present in the activation domain of CIITA. Acidic residues (glutamic acid and aspartic acid) are in bold type; the underlined glycine (at position 58) and alanine (at position 94) are targets for point mutation to proline.

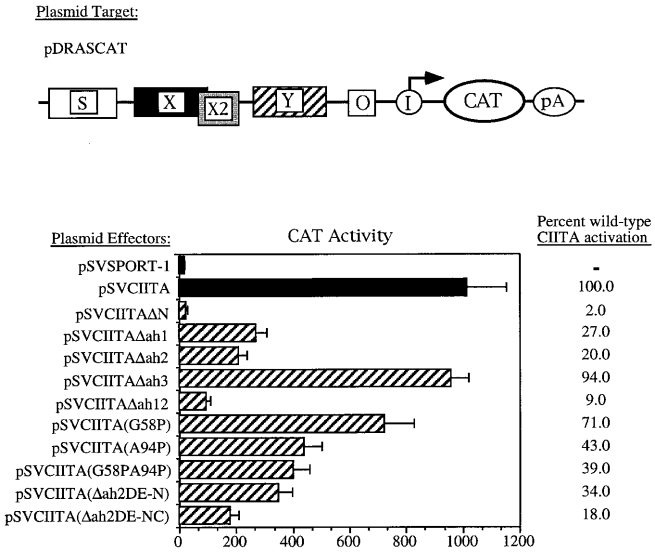

Figure 2. The predicted α-helices in the activation domain of CIITA are required to activate the DRA promoter in HeLa cells. The plasmid target pDRASCAT (0.25 μg) (18), containing the DRA promoter from position –150 to +31 upstream of the *CAT* gene was co-transfected with the indicated plasmid effector (0.75 μg) into HeLa cells. pSVSPORT-1 is the parental plasmid effector, containing the SV40 early promoter and bovine growth hormone polyadenylation sequence. pSVCIITA contains full-length wild-type CIITA cDNA (from nucleotide position +1 to +3081). pSVCIITAΔN contains the CIITA cDNA from nucleotide position +483 to +3081. pSVCIITAΔah1, ah1 deleted; pSVCIITAΔah2, ah2 deleted; pSVCIITAΔah3, ah3 deleted; pSVCIITAΔah12, ah1 and ah2 deleted; pSVCIITA(G58P), mutation of glycine to proline at amino acid position 58; pSVCIITA(A94P), mutation of alanine to proline at amino acid position 94; pSVCIITA(Δah2DE-N), mutation of aspartic acid and glutamic acid residues at amino acid positions 84 and 86 respectively; pSVCIITA(Δah2DE-NC), the same mutations as pSVCIITA(Δah2DE-N) with additional mutations of aspartic acid at amino acid position 88 and glutamic acid residues at amino acid positions 89 and 90; S, S box of the DRA promoter; X, X box; X2, X2 box; Y, Y box; O, octamer binding site; I, initiator element. CAT assay was performed 48 h after transfection. Data shown are means of duplicate transfections ± SEM.

RESULTS

Prediction of the secondary structure in the N-terminal activation domain of CIITA

We wanted to determine if the N-terminal acidic activation domain of CIITA contained any predicted secondary structures which might provide insight into its function. Specifically, the presence of α-helices, which represent functional motifs in other acidic activators (23–25), could provide a starting point for mutagenesis of CIITA. The primary amino acid sequence of CIITA was analyzed using MacVector computer software (Eastman Kodak, New Haven, CT). Both the Chou–Fasman and Robson–Garnier methods were employed and three consensus α-helices were detected, which we labeled ah1 (amino acids 51–71) ah2 (amino acids 84–103) and ah3 (amino acids 124–138; Fig. 1A). Inspection of the primary sequence of each of these α-helices demonstrated that they were rich in acidic amino acid residues (Fig. 1B). Based on this analysis, these three acidic α-helices were chosen as the focus for a mutational analysis of the activation domain of CIITA.

Mutation of ah1 and ah2, but not ah3, results in loss of activation of the DRA promoter by CIITA

We wanted to determine if the three predicted acidic α-helices in the activation domain of CIITA were important for its ability to activate the DRA promoter, which is the prototypic class II promoter (18). Each of the three α-helices was deleted from full-length CIITA and activation of the DRA promoter by mutant and wild-type CIITA proteins was assayed in HeLa cells.

The plasmid target pDRASCAT contains the DRA promoter from position –150 to +31 linked to the chloramphenicol acetyltransferase (*CAT*) reporter gene (Fig. 2). Co-transfection of pSVCIITA, which directs expression of full-length CIITA from the SV40 early promoter, with pDRASCAT resulted in levels of expression which were 54-fold greater than those with pDRASCAT alone (Fig. 2). Deletion of the N-terminal 161 amino acids of CIITA (pSVCIITAΔN) resulted in complete loss of CIITA function (Fig. 2). When ah1 (pSVCIITAΔah1) or ah2 (pSVCIITAΔah2) were deleted, activation of pDRASCAT was reduced. pSVCIITAΔah1 activated pDRASCAT 14-fold, representing 27% of the wild-type CIITA activity (Fig. 2). The reduction in activity with pSVCIITAΔah2 was more severe. Expression of pDRASCAT was reduced to 20% of that with wild-type CIITA (Fig. 2). Deletion of both ah1 and ah2 (pSVCIITAΔah12) resulted in an essentially non-functional protein which activated transcription to levels only 9% of the wild-type CIITA (Fig. 2), similar to removal of the entire activation domain of CIITA. In sharp contrast, deletion of the C-terminal α-helix (ah3) was not detrimental to the function of CIITA. pSVCIITAΔah3 was able to activate pDRASCAT to nearly wild-type levels, i.e. 50-fold.

To test the structural predictions regarding the ah1 and ah2 regions, proline substitutions were made in the center of each of the two active α-helices, alone and in combination. Such insertions should disrupt formation of the α-helix. pSVCIITA(G58P), in which the glycine residue at position 58 of ah1 was converted to proline (Fig. 1B), activated pDRASCAT 38-fold, which represented

71% of the activation by wild-type CIITA. Disruption of ah2 by mutation of alanine at position 94 to proline [pSVCIITA(A94P)] had a more severe effect on CIITA function, following the trend of the larger deletions. pSVCIITA(A94P) activated pDRASCAT at levels 43% of those observed with wild-type CIITA (Fig. 2). When both alanine to proline mutations are present in CIITA [pSVCIITA(G58PA94P)], activation of pDRASCAT is reduced to 39% that of wild-type CIITA (Fig. 2). We conclude that ah1 and ah2 are critical for activation of the DRA promoter by CIITA and likely represent true acidic α-helices.

To determine if the acidic residues present in the activation domain of CIITA are indeed required to activate transcription, we made mutations in ah2, sequentially reducing the number of acidic residues. In plasmid pSVCIITA(Δah2DE-N) an aspartic acid residue at position 84 and a glutamic acid residue at position 86 were mutated to alanines and the resulting CIITA protein assayed for its ability to activate pDRASCAT. The loss of these two acidic residues resulted in a drop in activation to 34% that of wild-type CIITA. Three additional acidic amino acids were then mutated to alanine (aspartic acid at position 88 and two glutamic acids at positions 89 and 90) to produce plasmid pSVCIITA(Δah2DE-NC). This further reduction in net negative charge resulted in a severe reduction in activation, to 18% of wild-type CIITA. It should be noted that the loss of five of eight acidic residues in ah2 [plasmid pSVCIITA(Δah2DE-NC)] resulted in a CIITA protein which activated transcription at levels similar to that seen when the entire ah2 region was deleted. This indicates that the acidic nature of the ah2 region is indispensable for its ability to activate transcription.

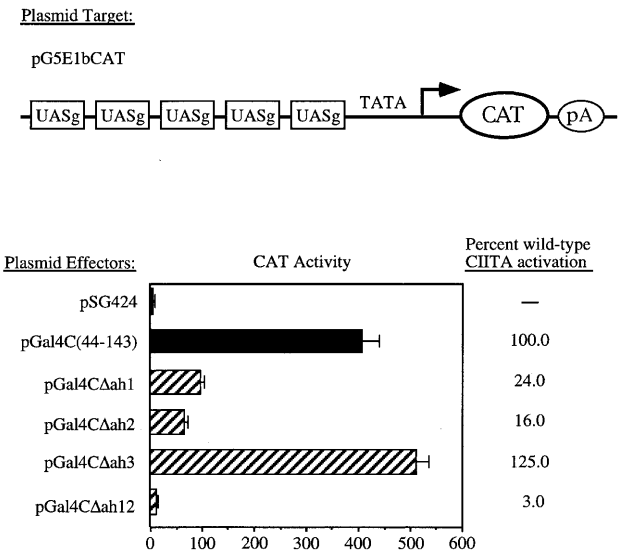

Figure 3. Deletions of predicted α-helices in the N-terminus of CIITA specifically effect function of the activation domain. The plasmid target pG5bCAT (0.5 μg) (1), containing five Gal4 binding sites and the TATA box from the adenovirus E1 gene upstream of the *CAT* gene, was co-transfected with the indicated plasmid effector (1.5 μg) into COS cells. The parental plasmid effector, pSG424 (17), expressed the Gal4 DNA binding domain from the SV40 early promoter. The remaining plasmid effectors express wild-type or α-helix-deleted CIITA from amino acid position 44 to 143, fused to the Gal4 DNA binding domain. pGal4C(44–143), wild-type CIITA; pGal4CΔah1, ah1 deleted; pGal4CΔah2, ah2 deleted; pGal4CΔah3, ah3 deleted; pGal4CΔah12, both ah1 and ah2 deleted; UASg, Gal4 binding site; TATA, TATA box; pA, polyadenylation sequence; GDB, Gal4 DNA binding domain. CAT assay was performed 48 h after transfection. Data shown are means of duplicate transfections ± SEM.

Deletion of ah1 and ah2 specifically affect function of the activation domain of CIITA

The reduction in activity of CIITA proteins bearing the deletion of ah1 and ah2 could be due to unrelated structural changes in the protein, rather than to specific alterations in function of its activation domain, even though CIITA protein expression levels were equivalent (data not presented). For example, a deletion of only 24 amino acids at the C-terminus of CIITA produced a completely non-functional protein in the BLSII cell line BLS2 (8). Since such small deletions can affect function or stability of the entire protein, we decided to test integrity of the activation domain independently of full-length CIITA. Therefore, fusion proteins between the 147 amino acid DNA binding domain of Gal4 and the region of CIITA containing the three α-helices (the 'three α-helix region') from wild-type and mutated CIITA were constructed.

The plasmid effector pGal4C(44–143) was constructed by inserting the cDNA coding for amino acids from position 44 to 143 of CIITA next to the DNA binding domain of Gal4. Following their co-transfection into COS cells, pGal4C(44–143) activated the plasmid target pG5bCAT, which contains five Gal4 binding sites upstream of the *CAT* reporter gene, 58-fold greater than the parental vector pSG424 (Fig. 3). Deletion of ah1 from pGal4C(44–143), to produce pGal4CΔah1, activated pG5bCAT only 14-fold over baseline levels (Fig. 3). This level of expression represents 24% of the activation observed with pGal4C(44–143). ah2, which was deleted in pGal4CΔah2, proved again to be the more active α-helix. Activation with pGal4CΔah2 was 10-fold greater than baseline levels, representing only 16% of the wild-type activity (Fig. 3). The double mutation pGal4CΔah12 was non-functional and activated transcription only 2-fold (Fig. 3). Interestingly, deletion of ah3 produced slightly increased activation of transcription. pGal4CΔah3 increased expression from pG5bCAT 73-fold, to levels which were 125% of those observed with pGal4C(44–143). This result may be due to the increased accessibility of ah1 and ah2 to the general transcriptional apparatus. We conclude that the three α-helix region, comprising amino acids from position 44 to 143, is sufficient for transcriptional activation by CIITA and that ah1 and ah2 are required for this activity.

CIITAΔah12 represents a dominant negative CIITA

The ah deletions in the N-terminal activation domain are not likely to effect function of the remainder of the protein. Since the C-terminal 830 amino acids of CIITA are sufficient to direct the protein to the DRA promoter (16), it is likely that the CIITAΔah12 mutant still interacts at the promoter. However, since CIITAΔah12 cannot activate transcription, it might function as a dominant negative CIITA protein. To test this possibility, pSVCIITA was co-transfected with pDRASCAT and increasing amounts of pSVCIITAΔah12 into COS cells.

Co-transfection of pDRASCAT with pSVCIITA resulted in levels of expression 17-fold greater than with pDRASCAT alone in COS cells. Addition of pSVCIITAΔah12 at a 2:1 ratio to pSVCIITA resulted in an ~50% reduction of expression from pDRASCAT (Fig. 4). At a ratio of 8:1, expression fell further, to 30% of levels observed with pSVCIITA alone (Fig. 4). In sharp

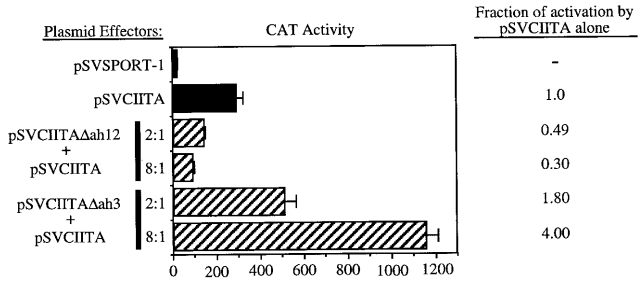

Figure 4. CIITAΔah12 functions as a dominant negative repressor of wild-type CIITA. The plasmid target pDRASCAT (0.1 µg) was co-transfected with pSVCIITA (0.1 µg) alone or with pSVCIITAΔah12 or pSVCIITAΔah3 (0.2 or 0.8 µg) into COS cells. Appropriate amounts of pSVSPORT-1 were included in each transfection to keep the total amount of plasmid DNA at 2 µg. CAT assay was performed 48 h after transfection. Data shown are means of duplicate transfections ± SEM.

contrast, addition of pSVCIITAΔah3 increased levels of expression from pDRASCAT. Levels increased 1.8- and 4.0-fold when pSVCIITAΔah3 was included in the transfection at ratios of 2:1 and 8:1 respectively with pSVCIITA (Fig. 4). These results confirm that deletion of ah1 and ah2 from CIITA does not inhibit the ability of the protein to interact with the DRA promoter, but rather only effects its ability to activate transcription.

ah1 and ah2 are required for full interaction of the activation domain of CIITA with the general transcription factor $TAF_{II}32$

Transcriptional activation by acidic activators has been correlated with their ability to interact with components of the general transcriptional machinery. VP16, for example, binds to several general transcription factors, including the 32 kDa subunit of the human TATA binding complex TFIID, $TAF_{II}32$ (19). Given its functional homology to VP16 (16), we examined whether CIITA might also bind to TFIID via the $TAF_{II}32$ subunit and whether ah1 and ah2 are important for this interaction. *In vitro* binding assays were performed with fusion proteins containing the activation domain of wild-type and mutant CIITA proteins fused to GST and nuclear extracts from human B cells. Proteins were analyzed by Western blotting, using an anti-$TAF_{II}32$ antiserum.

Two prokaryotic expression plasmids were constructed to express GST fusion proteins in *E.coli*. GSTCIITA(1–144) contained the first 144 amino acids of CIITA, which included the three α-helix region. The second fusion protein was similar, except that ah1 and ah2 were deleted to produce GSTCIITA (1–144)Δah12. The chimeras were incubated with nuclear extracts from human B cells and then purified using glutathione–Sepharose beads. After extensive washing, the GSTCIITA fusion proteins and any interacting proteins were eluted from the beads using glutathione and resolved on SDS–PAGE. Western blotting with the anti-$TAF_{II}32$ antiserum was performed to determine if $TAF_{II}32$ bound to either of these two fusion proteins. The 32 kDa $TAF_{II}32$ protein was easily detected in unfractionated nuclear extracts (Fig. 5, lane 1). $TAF_{II}32$ was not detected when the chimera deleted of ah1 and ah2 [GSTCIITA(1–144)Δah12] was used in the binding reaction (Fig. 5, lane 2). However, $TAF_{II}32$ was present in the eluate from the binding reaction containing GSTCIITA(1–144) (Fig. 5, lane 3). Probing identical blots with an anti-GST antiserum demonstrated that equal amounts of the

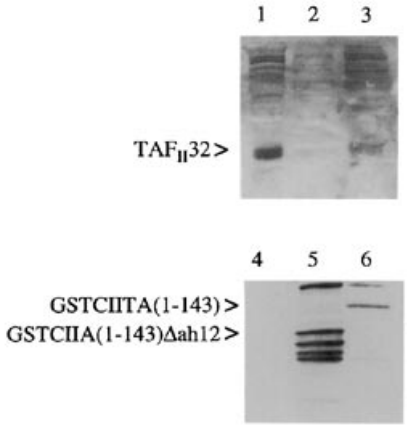

Figure 5. ah1 and ah2 are required for interaction of CIITA with the general transcription factor $TAF_{II}32$. Fusion proteins between GST and wild-type [GSTCIITA(1–144)] or ah1 and ah2 deleted [GSTCIITA(1–144)Δah12] CIITA from amino acid position 1 to 144 were expressed in *E.coli* and purified on glutathione–Sepharose beads. Glutathione eluates from binding reactions between GSTCIITA(1–144) or GSTCIITA(1–144)Δah12 and B cell nuclear extract were resolved on SDS–PAGE followed by Western transfer and immunodetection. (Top) Western blot with anti-$TAF_{II}32$ antiserum. Lane 1, detection of $TAF_{II}32$ in unfractionated B cell nuclear extract; lane 2, no $TAF_{II}32$ was present in eluate from the GSTCIITA(1–144)Δah12 binding reaction; lane 3, detection of $TAF_{II}32$ in eluate from the GSTCIITA(1–144) binding reaction. (Bottom) Western blot with anti-GST antiserum. Lane 4, nuclear extract; lane 5, GSTCIITA(1–144)Δah12; lane 6, GSTCIITA(1–144). Additional bands present in lane 5 are likely to be degradation products of GSTCIITA(1–144)Δah12.

GST–CIITA fusion proteins were present in the binding reaction (Fig. 5 lanes 4–6). From these results we conclude that the first 144 amino acids of CIITA can interact with $TAF_{II}32$ and that this interaction requires the presence of both ah1 and ah2. Since ah1 and ah2 are necessary for transcriptional activation by CIITA, this result implies that binding of $TAF_{II}32$ is required for this activity.

Binding ability of CIITA and CIITA mutants to $TAF_{II}32$ corresponds with transcriptional activation

We demonstrated above that the first 144 amino acids of CIITA, which contain the three α-helix region, interact with $TAF_{II}32$. We therefore wanted to try to coordinate the ability of CIITA and mutations of CIITA to bind to $TAF_{II}32$ with their ability to activate the DRA promoter.

In vitro binding assays were performed using a GST–$TAF_{II}32$ fusion protein and wild-type and mutant CIITA proteins produced by *in vitro* transcription/translation of CIITA expression plasmids. Binding reactions and elutions were carried out as in Figure 5 and eluates were resolved on SDS–PAGE followed by autoradiography of the [^{35}S]methionine-labeled CIITA. Full-length wild-type CIITA and CIITAΔah3 bound to GST–$TAF_{II}32$ equivalently (Fig. 6, lanes 1 and 4 respectively). This result implies that fully functional CIITA proteins interact well with $TAF_{II}32$.

In contrast, CIITAΔah1 and CIITAΔah2 bound to $TAF_{II}32$ very poorly (Fig. 6, lanes 3 and 4 respectively), with CIITAΔah1 binding slightly more than CIITAΔah2, consistent with the results of the co-transfection experiments in Figure 2. With the double mutation CIITAΔah12 there was almost no detectable binding to $TAF_{II}32$ (Fig. 6, lane 5). One tenth of the input volume of *in vitro* transcribed/translated CIITA proteins were run on an SDS–PAGE gel and autoradiographed, to demonstrate that equal amounts of

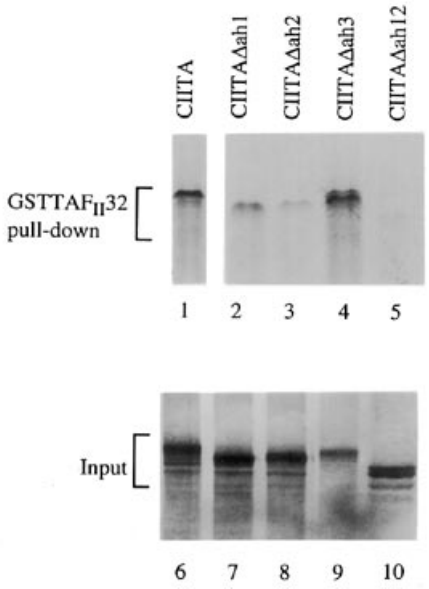

Figure 6. Binding of CIITA to the TBP-associated factor $TAF_{II}32$ requires both ah1 and ah2. A fusion protein between GST and human $TAF_{II}32$ was expressed in *E.coli* and purified on glutathione–Sepharose beads. GST–$TAF_{II}32$ was incubated with wild-type or mutated full-length CIITA proteins, produced by *in vitro* transcription/translation in the presence of [^{35}S]methionine. Eluates from the binding reactions were resolved on SDS–PAGE followed by autoradiography of the CIITA proteins. (Top) Autoradiograph of eluates from the GST–$TAF_{II}32$ binding reaction. Lane 1, wild-type CIITA; lane 2, CIITAΔah1, deletion of ah1; lane 3, CIITAΔah2, deletion of ah2; lane 4, CIITAΔah3, deletion of ah3; lane 5, CIITAΔah12, deletion of ah1 and ah2. (Bottom) One tenth the input volume of CIITA proteins from the *in vitro* transcription/translation reaction. Lane 6, wild-type CIITA; lane 7, CIITAΔah1, deletion of ah1; lane 8, CIITAΔah2, deletion of ah2; lane 9, CIITAΔah3, deletion of ah3; lane 10, CIITAΔah12, deletion of ah1 and ah2.

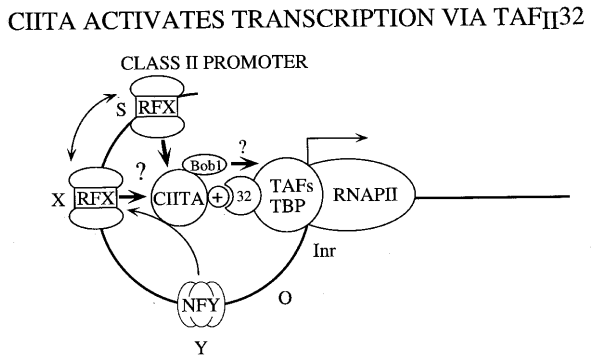

Figure 7. Protein–DNA and protein–protein interactions on the DRA promoter. NFY and RFX bind to conserved upstream sequences (S, X and Y boxes) of class II promoters. NFY stabilizes binding of RFX to the X box and might contribute to interactions with CIITA. Cooperative binding occurs between RFX complexes bound at the S and X boxes. Although CIITA requires minimally the X box, no direct binding between RFX and CIITA has been demonstrated. The B cell-specific co-activator Bob1 (OBF-1/OCA-B) interacts directly with CIITA, to increase transcription levels in B cells. The activation domain of CIITA interacts with TAFII32, which is a component of the TAF–TBP complex (TFIID). These protein–protein interactions increase rates of initiation of RNA polymerase II transcription.

protein were present in each binding reaction (Fig. 6, lanes 6–10). In all cases, the binding profiles of mutant CIITA proteins to GST–$TAF_{II}32$ corresponded with their ability to activate the DRA promoter in HeLa cells. We conclude that the interaction between CIITA and $TAF_{II}32$ is required for transcriptional activity of CIITA.

DISCUSSION

The N-terminal 125 amino acids of CIITA have been defined as an independent transcriptional activation domain (15). Computer analyses of the primary amino acid sequence of the CIITA activation domain predicted the presence of three α-helices rich in acidic amino acids. ah1 and ah2, the N-terminal and middle acidic α-helices, were found to be required for full activation of the class II DRA promoter. Deletion of ah1 and ah2 or amino acid substitutions which disrupted formation of the α-helices resulted in a severe reduction in function of the mutant CIITA proteins. Similarly, sequential reduction in net negative charge of ah2 resulted in concurrent reduction in the ability of the mutated CIITA proteins to activate transcription. In contrast, mutation of the C-terminal α-helix, ah3, was not detrimental to the activity of CIITA. Mutations which resulted in loss of transactivation of the DRA promoter by CIITA also resulted in proportional loss of binding to the 32 kDa subunit of the general transcription complex TFIID, $TAF_{II}32$. From these data we conclude that the functional motifs in the CIITA activation domain are the acidic α-helices ah1 and ah2 and that interaction between these α-helices and $TAF_{II}32$ is required for transcriptional activation by CIITA.

CIITA is an acidic transcriptional activator. This group of activators were first defined based on studies with the yeast proteins GAL4 and GCN4 (16,26). It was found that their ability to activate transcription correlated generally with the net negative charge of the acidic domain (16,26). Additional studies using random genomic DNA from *E.coli* (27) and artificially constructed sequences (25) demonstrated that the presence of an acidic amphipathic α-helix correlated with the ability of that sequence to activate transcription. Subsequent experiments, however, found that the C-terminal activation motif of GAL4 may not contain α-helices and that acidic residues were not critical for activator function (28,29). In contrast to what we have determined for CIITA, disruption of potential α-helices in VP16 by insertion of proline residues did not affect its ability to activate transcription (30). However, a recent report has identified a functional acidic α-helix (24) similar to what we have found with CIITA. In addition, the requirement of net negative charge has been firmly established for many activators (31–34), as well as the presence of bulky hydrophobic residues which are required for transcriptional activation (35–37). We have presented here strong evidence that CIITA contains functional acidic α-helices which are necessary for transcriptional activation.

To activate transcription of class II genes, assembly of protein–DNA complexes must first occur at the three conserved upstream sequences (CUS) present in all class II promoters, the S, X and Y boxes (Fig. 7). Following cooperative binding of factors to these elements (38–41), CIITA makes three important protein–protein contacts. The first is with the complexes bound at the S, X and Y boxes (Fig. 7). The nature of these contacts is unknown. Repeated attempts to demonstrate direct binding between CIITA and the X box binding complex RFX (42) have been unsuccessful (data not shown). Zhou *et al.* have presented evidence that the C-terminal 830 amino acids of CIITA are

sufficient to target CIITA to the promoter (16). That the mutant protein CIITAΔah12 could block activity of wild-type CIITA on the DRA promoter provides a further indication that the C-terminal portion of the molecule is involved in promoter targeting. The second protein–protein interaction is between the B cell-specific co-activator Bob1 (OBF-1, OCA-B) (43–45) and CIITA (46). The result of this interaction may be to increase expression of class II genes in B cell lines, by virtue of an additional activation domain being presented by CIITA.

The third protein–protein contact is between CIITA and the general transcriptional apparatus. We have shown here that this occurs at $TAF_{II}32$ (Fig. 7.). Identification of the two α-helices which are the precise contact points with $TAF_{II}32$ provides an extremely useful target for potential exogenous manipulation of class II gene expression. Aside from using the dominant negative CIITAΔah12, small molecules which mimic the structure of ah1 and/or ah2 could potentially block the function of CIITA specifically. In light of the fact that aberrant expression of class II molecules contributes to a variety of autoimmune inflammatory diseases (3), such reagents would be of great benefit in treating these conditions.

Our results to date do not rule out the possibility that CIITA may itself be functioning as a TAF and that its interaction with $TAF_{II}32$ represents the primary contact point between CIITA and TFIID. Recent reports identifying a B cell-specific TAF (47) and the observations that the large T antigen of SV40 virus (48) as well as the pX protein of HBV (49) may function as TAFs make this possibility intriguing. The presence of CIITA in a cell may potentially create 'class II-tropic' TFIID complexes, which would promote high levels of expression from target genes. In this way, large groups of genes responsible for most aspects of antigen presentation could be activated in a global manner.

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