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The zinc finger proteins ZXDA and ZXDC form a complex that binds CIITA and regulates MHC II gene transcription

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Summary

The transcription of major histocompatibility complex class II (MHC II) genes depends critically upon the activity of the class II trans-activator (CIITA) protein. We previously described a novel CIITA-binding protein named zinc finger X-linked duplicated family member C (ZXDC) that contributes to the activity of CIITA and the transcription of MHC II genes. In the present study, we examined the contribution of a closely related family member of ZXDC, the ZXDA protein, to MHC II gene transcription. ZXDA has a domain organization similar to ZXDC, containing ten zinc fingers and a transcriptional activation domain. Knockdown and overexpression of ZXDA demonstrated its importance in the transcriptional activation of MHC II genes. We found that ZXDA and ZXDC can self-associate, and also form a complex with each other. The regions of the two proteins that contain zinc fingers mediate these interactions. Importantly, we found that the ZXDA-ZXDC complex interacts with CIITA, rather than either protein alone. Given our additional finding that ZXDC is present at MHC II promoters in HeLa cells, prior to and after treatment with IFN-γ, it appears that ZXDA and ZXDC form an important regulatory complex for MHC II gene transcription.

Keywords

gene transcription; major histocompatibility complex; zinc finger; class II trans-activator; protein-protein interactions

Introduction

Major histocompatibility complex class II (MHC II) proteins found on the surface of antigen presenting cells present peptide antigen to $CD4^+$ T cells. The expression of MHC II genes is restricted primarily to professional antigen presenting cells and thymic epithelial cells, though expression of these genes can be induced on most cell types by cytokines, particularly IFN-γ [1].

MHC II gene expression is regulated primarily at the level of transcription, via a series of conserved upstream sequences named the S (also known as W), X1, X2 and Y-boxes found in the proximal promoters of these genes [2; 3; 4; 5]. Additional S-X-Y cassettes have been

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identified within the MHC II locus and likely function as locus control elements [6; 7]. Factors that interact with each of the elements, except for the S box, have been identified. The regulatory factor X (RFX) [8; 9; 10; 11] and nuclear factor Y (NF-Y) [12; 13] complexes, each with three subunits, bind the X1 and the Y boxes, respectively; the cAMP responsive binding protein (CREB) binds the X2 box [14; 15].

As noted above, the transcription of MHC II genes is strictly limited to specific cell types. However, the constituents of the RFX and NF-Y complex, as well as the CREB protein are found in essentially all cell types. Though these DNA binding proteins are necessary, they are not sufficient for MHC II gene transcription. A transcriptional co-activator, know as the class II trans-activator (CIITA) is also required for transcription of MHC II genes [16]. The CIITA protein is recruited to MHC II promoters by interacting with the CREB protein [14; 15] and subunits of the RFX and NF-Y complexes [17; 18; 19; 20]. Since the presence of CIITA in a cell, rather than the CREB protein or RFX and NF-Y complexes, is the determining factor for transcription of MHC II genes, CIITA has been referred to as the “master switch” of MHC II transcription.

A large catalog of proteins has been shown to interact with the CIITA protein. These fall into one of three groups: (i) the aforementioned DNA binding proteins, consisting of the CREB protein and constituents of the complexes RFX and NF-Y [14; 15; 17; 18; 19; 20] (ii) general transcription factors such as TFIID [21] and P-Tefb [22] and (iii) factors that modify histone proteins (histone deacetylases, histone acetyltransferases and methylase) or remodel chromatin [23]. We recently identified an additional CIITA-binding protein known as zinc finger X-linked duplicated family member C (ZXDC), which we isolated by yeast two-hybrid screen with the C-terminal region of the CIITA protein [24]. ZXDC contains ten zinc fingers and a relatively potent transcriptional activation domain. ZXDC interacts with the region of CIITA that contains leucine-rich repeats. Through different approaches, we were able to demonstrate a convincing role for the ZXDC protein in the activation of MHC II gene transcription by CIITA: over-expression of ZXDC resulted in super-activation, whereas silencing of ZXDC expression reduced the ability of CIITA to activate transcription [24].

We report here that not only is ZXDC involved in the function of CIITA, but that a ZXDC-family member known as ZXDA also plays a role in regulating MHC II transcription. We found that silencing expression of either ZXDA or ZXDC produced a significant decrease in the induction of MHC II gene transcription by IFN-γ, without altering the expression of CIITA. Interestingly, we discovered that the ZXDA and ZXDC proteins can self-associate as well as interact with each other. Significantly, we found that it was only the ZXDA-ZXDC complex, but not either protein by itself, which interacted with CIITA. The results presented here confirm and extend our original findings that the ZXD family proteins are novel regulators of CIITA function and MHC II transcription.

Results

The ZXD gene family

We previously reported the cloning of the ZXDC cDNA by yeast two-hybrid assay, and demonstrated that it coded for an interacting partner of the CIITA protein [24]. A BLAT search of the human genome with the nucleotide sequence of the ZXDC gene [30] identified two highly related genes known as ZXDA and ZXDB, that to date have no known function. ZXDA and ZXDB share 98.7% nucleotide identity over their coding regions, with 97% amino acid identity [31]. Comparison of amino acid sequences between ZXDC and ZXDA/B demonstrated regions of high homology, with maximal identity (85%) across the ten zinc fingers of the proteins (Figure 1A). There was also modest identity (~50%) just C-terminal of the zinc fingers, a region that we functionally defined in ZXDC as a transcriptional activation domain [24]. Significantly,

the ZXDA/B proteins lack approximately 60 amino acids on their C-termini that are present in ZXDC; this is the region of ZXDC we defined previously as important for ZXDC-CIITA association, though the zinc finger region of ZXDC also contributed significantly [24]. Interestingly, although ZXDC has ten exons, both ZXDA and ZXDB are single exon genes, suggesting that they may have originated from retrotransposition of ZXDC mRNA. Nucleotide sequence comparisons across species (employing BLAT [30]) revealed that while mouse and chimpanzee have ZXDA, ZXDB and ZXDC genes, chicken, *Xenopus* and zebrafish have orthologues to only the ZXDC gene. *C. elegans* and *Drosophila* do not have orthologues of the ZXD gene family.

ZXDA cooperates with CIITA to activate the HLA-DRA promoter

We previously demonstrated, using reporter assays, that over-expression of ZXDC produced a minor activation of the reporter plasmid pDRA-luc, which contains the promoter from the HLA-DRA gene (−150/+31) linked to the Firefly luciferase gene [24]. Moreover, ZXDC cooperated with CIITA to produce a super-activation of the reporter plasmid [24]. To determine if ZXDA behaved in a manner similar to ZXDC, we co-transfected an expression plasmid for ZXDA (pCMV-FL-ZXDA) with the reporter plasmid pDRA-luc, into HEK293 cells. We found that ZXDA produced a minor, though reproducible, activation of pDRA-luc (Figure 2). However, when ZXDA was co-transfected with CIITA, the result was a significant super-activation of the reporter plasmid (Figure 2). In fact, the magnitude of effect of ZXDA on CIITA-dependent activation of pDRA-luc was slightly greater than the effect of ZXDC (Figure 2). The greatest level of activation was seen when both ZXDA and ZXDC were co-expressed with CIITA, suggesting that perhaps both ZXDA and ZXDC are required for full activity of the promoter (Figure 2). Expression of the flag-tagged ZXDA, ZXDC and the HA-tagged CIITA proteins was confirmed by Western analysis (data not presented).

Silencing of ZXDA and ZXDC expression results in reduced MHC II activation by CIITA

The data presented above suggest that both ZXDA and ZXDC are involved in the regulation of MHC II gene transcription. However, to test this hypothesis in a more physiologically relevant system, we employed RNA silencing to knockdown the expression of ZXDA and ZXDC in HeLa cells. We then induced CIITA (and by extension MHC II) transcription by treating the cells with IFN-γ for 18 hours. The transcript levels for ZXDA, ZXDC, CIITA, HLA-DRA and beta actin were assayed by RT-PCR, employing fluorescently-labeled primers. The amplified products were detected and quantified, employing a Typhoon 9410 imaging system and ImageQuant TL software (GE Bioscience, Inc.).

An 80% reduction in the level of ZXDC mRNA was achieved with a ZXDC-specific siRNA, an effect not seen with control siRNA (Figure 3(a)). In cells where ZXDC was silenced, induction of HLA-DRA gene transcription by IFN-γ was significantly reduced, compared to cells transfected with a control siRNA (Figure 3(a) lanes 3 and 4). However, the silencing of ZXDC had no effect on the expression of either CIITA, ZXDA or beta actin, confirming the specificity of the siRNA and the effect of ZXDC silencing (Figure 3(a)). Similarly, knockdown of ZXDA mRNA led to reduced induction of HLA-DRA by IFN-γ (Figure 3(a) lanes 5 and 6). Silencing both ZXDA and ZXDC had no significant additional effect on the transcription of HLA-DRA, beyond that achieved by silencing either gene alone (Figure 3(a) lane 7). We confirmed these results in a HeLa cell line that stably expresses the CIITA cDNA. Again, silencing either ZXDA or ZXDC had a deleterious effect on the transcription of HLA-DRA, but not β-actin and silencing of both had no additional effect (Figure 3(b)). Taken together, these data demonstrate that both ZXDA and ZXDC contribute to the transcription of MHC II genes by CIITA. The fact that silencing ZXDA and ZXDC was not additive suggested that the two proteins might combine into a functional complex.

The RFX complex and CREB protein are present at MHC II promoters in HeLa cells prior to induction of CIITA and MHC II transcription by IFN-γ [28]. We have previously reported that ZXDC is present at the HLA-DRA promoter in Raji cells, a Burkitt's lymphoma cell line that constitutively expresses MHC II genes [24]. We performed chromatin immunoprecipitation (ChIP) with HeLa cells, to determine how or if the occupancy of MHC II promoters by ZXDC is altered by IFN-γ. Agreeing with previous reports [28], we found a modest level of promoter occupancy by RNA polymerase II that increased after IFN-γ treatment (Figure 3(c) top panel). RFX binding to the HLA-DRA promoter was independent of IFN-γ, though occupancy of the promoter by CIITA was detected only after treatment with IFN-γ (Figure 3(c) top panel). These observations are also in agreement with previous reports [28; 29]. ChIP with anti-ZXDC clearly demonstrated the presence of ZXDC protein at the HLA-DRA promoter, the extent of which was not altered by IFN-γ treatment (Figure 3(c) top panel). Identical results were obtained with anti- RFX5: HLA-DRA promoter occupancy independent of IFN-γ treatment (Figure 3(c) top panel). PCR with primers for the GAPDH promoter demonstrated the specificity of the antibodies employed in the ChIPs experiments (Figure 3(c) bottom panel). We obtained the same results with ChIP experiments employing the RJ2.2.5 cell line, a CIITA$^{-/-}$ derivative of Raji [32], i.e. the ZXDC protein was found to occupy the HLA-DRA promoter in the absence of CIITA (data not presented). These results suggest that although ZXDC associates with CIITA, ZXDC can occupy the HLA-DRA promoter independently of CIITA.

The ZXDA protein contains a transcriptional activation domain

Given the observations that ZXDA cooperated with CIITA to activate MHC II transcription, and the ~50% amino acid identity between the transcriptional activation domain of ZXDC and the corresponding region of ZXDA, we decided to determine whether ZXDA had a functional transcriptional activation domain. To test this, a series of fusion proteins between full length or portions of ZXDA protein and the Gal4 DNA binding domain were constructed (Figure 4 (a)). These constructs were co-transfected into HEK293 cells with the reporter plasmid pG5-luc, which contains five Gal4 binding sites linked to the Firefly luciferase gene [24].

The Gal4-ZXDA(1-799) fusion protein, containing the entire ZXDA protein, activated pG5-luc in a dose dependent manner (Figure 4(a)). This finding indicated that ZXDA does indeed have a transcriptional activation domain. Deletion of the N-terminal 270 amino acids of ZXDA [Gal4-ZXDA(271-799)], a glycine-rich region, had no impact on transcriptional activation of the reporter plasmid (Figure 4(a)). Similarly, extending the deletion past the region of ZXDA containing the zinc fingers [Gal4-ZXDA(572-799)] had no effect. However, when the deletion was extended to amino acid 699 of ZXDA [Gal4-ZXDA(699-799)] transcriptional activation of pG5-luc was lost (Figure 4(a)). The region between amino acids 572-699 of ZXDA, which was deleted in construct Gal4-ZXDA(699-799), corresponds to the transcriptional activation domain of ZXDC. All of the Gal4-ZXDA fusion proteins were expressed at similar levels, as determined by Western blot with anti-Gal4 (data not presented). Thus, although ZXDA and ZXDC proteins share only ~50% amino acid identity over the region C-terminal of the zinc fingers, in both ZXDA and ZXDC these regions function as transcriptional activation domains.

ZXDA associates with CIITA in cells

An amino acid sequence alignment between ZXDA and ZXDC demonstrated that ZXDA lacks similarity with the far C-terminus of ZXDC. We previously demonstrated this region to be important for ZXDC-CIITA interaction (Figure 1) [24]. Previously, we employed a mammalian two-hybrid strategy to demonstrate that ZXDC and CIITA associated in cells [24]. We used a similar strategy to determine if ZXDA and CIITA could also associate. We co-transfected HEK293 cells with expression plasmids for either the Gal4 DNA binding domain or Gal4-ZXDA, along with pCMV-CIITA and pG5-luc. The Gal4 DNA binding domain alone did not activate the reporter plasmid, nor did the expression of CIITA along with Gal4 have any effect

(Figure 4(b)). Gal4-ZXDA(1-799) activated pG5-luc as in the previous experiment, and most importantly, co-expression of CIITA with Gal4-ZXDA(1-799) produced a super-activation of the reporter plasmid (Figure 4(b)). This indicated that the CIITA protein, with has a potent transcriptional activation domain, was recruited to the reporter plasmid by interaction with the ZXDA protein.

We found that the association of CIITA with ZXDA was undiminished by the deletion of the first 270 amino acids of ZXDA (Figure 4(b)). In contrast, Gal4-ZXDA(572-799), which lacks the zinc fingers but retains the transcriptional activation domain, did not associate with CIITA (Figure 4(b)). The C-terminal 100 amino acids of ZXDA [Gal4-ZXDA(699-799)] were also incapable of associating with CIITA (Figure 4(b)). These results indicate that ZXDA can associate with CIITA in cells, and that the portion of the ZXDA protein containing the ten zinc fingers is required.

ZXDA and ZXDC interact with each other and with themselves

We wanted to clarify the functional and physical relationship amongst the ZXDA, ZXDC and CIITA proteins. We first determined whether ZXDA and ZXDC could bind each other. We co-transfected HEK293 cells with expression plasmids for HA-ZXDC and FLAG-ZXDA, carried out co-IP with anti-FLAG, and performed Western blot with anti-HA. We found that the HA-ZXDC protein co-immunoprecipitated with FLAG-ZXDA (Fig 5(a) top panel, lane 1). When plasmids expressing either HA-ZXDC or FLAG-ZXDA were omitted from the transfection, co-immunoprecipitation (co-IP) did not occur (Fig 5(a) top panel, lanes 2 and 3, respectively). Western analysis of cellular lysates demonstrated that all transfected plasmids were expressed (Figure 5(a) middle and bottom panels).

To determine if ZXDC self-associates, we co-transfected expression plasmids for FLAG-ZXDC and HA-ZXDC into HEK293 cells, followed by immunoprecipitation with anti-FLAG and Western analysis with anti-HA. We found that ZXDC did in fact self-associate (Figure 5 (b) lane 2) and that the co-IPs were specific, since an irrelevant antibody (anti-GST) produced a negative result (Fig 5(b) lane 3). Similar experiments with myc-ZXDA and FLAG-ZXDA demonstrated that like ZXDC, ZXDA also self-associates (Figure 5(c)). From these experiments we conclude that ZXDC and ZXDA interact with each other and with themselves. Although it seems likely that the proteins form dimers, from these experiments we cannot rule out the possibility that higher order complexes can form.

The zinc fingers of ZXDA are required for the association of the ZXDA and ZXDC proteins

Since ZXDA and ZXDC associate, we wanted to determine the region of ZXDA required for this binding. Although the zinc finger region of ZXDC contributed significantly to its interaction with CIITA [24], the far C-terminus of ZXDC is absolutely required. Since ZXDA does not have an amino acid sequence homologous to the far C-terminus of ZXDC, we hypothesized that ZXDA might be interacting indirectly with CIITA via ZXDC. For this model to be sustained, we would need to observe that the region of ZXDA required for association with CIITA was the same for ZXDA-ZXDC binding.

To map the region of ZXDA necessary for binding to ZXDC, we performed co-IP experiments with truncated and deleted forms of ZXDA and full length ZXDC proteins. We expressed FLAG-ZXDC with myc-ZXDA in HEK293 cells, and immunoprecipitated FLAG-ZXDC. We were able to detect myc-ZXDA in the immunoprecipitates, confirming the interaction between ZXDA and ZXDC proteins (Figure 6(a)). Deletion of the N-terminal 266 amino acids of ZXDA (myc-ZXDAΔN) resulted in a severe reduction in binding to ZXDC, though interaction was still detected (Figure 6, top panel). Next, we deleted amino acids 267-573, the region containing the zinc fingers, from ZXDA (myc-ZXDAΔZnF). Although we did detect robust expression

of the myc-ZXDAΔZnF protein (Figure 6(a) bottom panel), we were unable to demonstrate interaction with ZXDC by co-IP (Figure 6(a) top panel). Finally, the deletion of the C-terminal 226 amino acids of ZXDA (myc-ZXDAΔC), between the zinc fingers and the C-terminus, had no effect on the binding between ZXDA and ZXDC (Figure 6(a)). From these data we conclude that the zinc finger region of ZXDA is absolutely required for the observed ZXDA-ZXDC interaction, and that the N-terminus contributes to this binding.

Next, we mapped the region of ZXDC required for binding ZXDA (Figure 6(b)). We were unable to achieve expression of a ZXDC protein lacking both the N- and C-termini. However, deletion of either the N-terminus (amino acids 1 to 176) or the C-terminus (amino acids 472-858) of FLAG-ZXDC had no effect on its binding to myc-ZXDA. To demonstrate specificity of the interaction, we attempted co-IP between myc-RFX5 and FLAG-ZXDC, but clearly the two proteins did not interact (Figure 6(b)). From these data it appears that the interaction between ZXDA and ZXDC is mediated by their zinc finger regions, an observation consistent with a previously demonstrated activity of zinc finger motifs [33].

ZXDA, ZXDC and CIITA form a tripartite complex

The RNAi experiments (Figure 3) suggested that a ZXDA-ZXDC complex might be necessary for MHC II transcription. We also have shown that ZXDA and ZXDC associate with each other (Figure 5). In addition, we have demonstrated that the zinc finger regions of both proteins are required for their interaction (Fig 6) and for their robust association with CIITA (Figure 4 (b) and [24]). Thus, we wanted to test whether a complex of ZXDA and ZXDC is required to interact with CIITA.

To address this possibility, we first performed mammalian two-hybrid experiments in HEK293 cells. The bait was a fusion protein between the Gal4 DNA binding domain and amino acids 323-1130 from CIITA, which lacks the N-terminal transcriptional activation domain (Gal4-CIITAΔN). By itself, Gal4-CIITAΔN was unable to activate the reporter plasmid pG5-luc (Figure 7(a)). Confirming our earlier observation that ZXDA and CIITA proteins associate (Figure 4(b)) we found that expression of the FLAG-ZXDA protein along with Gal4-CIITAΔN led to a robust activation of the reporter plasmid (Figure 7(a)). To test if the ZXDC protein contributes to the ZXDA-CIITA association, we first knocked-down expression of ZXDC by RNAi, and then assayed for the interaction between Gal4-CIITAΔN and ZXDA. We achieved an approximately 80% reduction in ZXDC protein levels (data not presented). We found that with the reduction in ZXDC levels, there was a significant drop in the activation of the pG5-luc reporter plasmid, when both FLAG-ZXDA and CIITA were expressed, suggesting that ZXDC contributes to the ZXDA-CIITA interaction (Figure 7(a)). This decrease was not observed when a control siRNA was used (Figure 7(a)). By Western analysis, we found that neither the control nor the ZXDC-specific siRNA had an effect on Gal4-CIITAΔN or FLAG-ZXDA protein levels (data not presented). These findings are consistent with ZXDC contributing to the association between CIITA and ZXDA.

To test directly whether ZXDA, ZXDC and CIITA form a tripartite complex, we co-transfected HEK293 cells with expression plasmids for FLAG-ZXDA, HA-CIITA and/or ZXDC in various combinations, followed by immunoprecipitation with anti-FLAG monoclonal antibodies. When all three proteins were co-expressed, we clearly detected both CIITA and ZXDC in the anti-FLAG immunoprecipitates (Figure 7(b) lane 1). In the absence of FLAG-ZXDA, neither ZXDC nor CIITA was immunoprecipitated by anti-FLAG (Figure 7(b) lane 3). Note that the slower migrating band in the anti-ZXDC blot is a post-translationally modified form of the protein, detected with high expression levels of the protein and long exposure times of the blot (data not presented.). With CIITA left out of the transfection (Figure 7(b) lane 2), or when only FLAG-ZXDA was expressed (Figure 7(b) lane 6) we detect the previously identified interaction between ZXDA and ZXDC. In the latter case, FLAG-ZXDA co-

immunoprecipitated the endogenous ZXDC protein. Since HEK293 cells do not constitutively express CIITA protein, it is clear that ZXDA-ZXDC binding does not require CIITA. When FLAG-ZXDA and HA-CIITA were co-expressed, HA-CIITA was detected in the immunoprecipitates, and also the endogenous ZXDC protein (Figure 7(b) lane 4). This result indicates that a complex containing ZXDA, ZXDC and CIITA forms even in the absence of over-expressed ZXDC, suggesting that ZXDC and ZXDA are both required for efficient binding to CIITA.

A complex of ZXDA and ZXDC is required for interaction with CIITA

To test directly whether CIITA interacts only with the ZXDA-ZXDC complex rather than either individual protein (or homodimers of the proteins), we performed co-IP experiments with proteins generated by *in vitro* transcription/translation. This removes the confounding presence of endogenous ZXDA or ZXDC protein expressed in cells. The Western blots in Figure 8(a) show the protein input into each of the binding reactions. We combined myc-ZXDA, HA-CIITA and/or ZXDC proteins in binding reactions followed by co-IP with either anti-myc or anti-ZXDC. Western analysis was performed to detect the three proteins. In a binding reaction containing all three proteins, Western blot of anti-myc immunoprecipitates resulted in the detection of all three proteins, confirming the formation of a tripartite complex between ZXDA, ZXDC and CIITA (Figure 8(b) lane 1). However, when only myc-ZXDA and HA-CIITA were present in the binding reaction, HA-CIITA was not detected in the anti-myc immunoprecipitates (Figure 8(b) lane 2). We did observe the interaction between ZXDA and ZXDC, in the absence of CIITA protein (Figure 8(b) lane 4). Identical results were obtained when anti-ZXDC was employed in the co-IPs. The ZXDA-ZXDC-CIITA tripartite complex was detected as was the ZXDA-ZXDC complex (Figure 8(c) lanes 1 and 4, respectively). However, when ZXDC and HA-CIITA were combined in a binding reaction, interaction between the two was not observed (Figure 8(c), lane 3). Taken together, these data clearly demonstrate that for interaction with CIITA, both the ZXDA and ZXDC proteins must be present. That is, CIITA binds to the complex formed by ZXDA-ZXDC, but does not appear to bind directly to either of the individual proteins.

Discussion

MHC II gene transcription is activated by the “master regulator” CIITA, a protein that is constitutively expressed in professional antigen presenting cells and thymic epithelial cells and induced by IFN-γ in most other cell types [1]. CIITA is recruited to MHC II genes via interaction with DNA binding proteins that bind to conserved upstream sequences present in the proximal promoters of all MHC II genes [17; 18; 19; 20]. Studies on the mechanism by which CIITA activates transcription have focused extensively on interactions with components of the general transcriptional machinery, particularly with a multitude of factors that mediate changes in histone modification and chromatin structure [23]. We have identified a group of related zinc finger proteins coded for by the zinc finger x-linked duplicated (ZXD) gene family, which facilitate the function of CIITA.

The present study is an extension of our previous report where we identified ZXDC, one member of the ZXD family, by a yeast-two hybrid screen with the CIITA protein [24]. In that report, we demonstrated that ZXDC cooperated with CIITA to regulate MHC II gene transcription [24]. We report here that a complex of ZXDA and ZXDC is required for MHC II gene regulation. Our experiments revealed that ZXDA and ZXDC both self-associate, as well as interact with each other. Such combinatorial interactions amongst members of transcription factor families is a well established phenomenon, and is a means of generating functional complexity while minimizing genetic complexity. We have mapped the interacting regions of ZXDA and ZXDC to the zinc finger domains of the two proteins. The involvement of a subset

of zinc fingers in polydactyl proteins in protein dimerization, whilst others are involved in binding DNA or RNA, has been observed for numerous other proteins [34; 35; 36; 37].

Most critically, we found that CIITA does not bind either ZXDA or ZXDC proteins individually, but rather CIITA interacts with a complex of ZXDA and ZXDC. Although there is significant similarity between the primary amino acid sequences of ZXDA and ZXDC, there are also regions with little homology. It seems likely that these unique amino acid sequences combine to form an interacting surface for CIITA, which would not be present in homodimers of ZXDA or ZXDC. We originally mapped the region of ZXDC necessary for binding CIITA by mammalian one-hybrid. In these experiments, we found that deleting the zinc finger region of ZXDC led to an approximately 50% reduction in ZXDC-CIITA interaction [24]. This is consistent with our present observation that ZXDA and ZXDC interact via their zinc finger regions, i.e. deletion of this region within ZXDC led to loss of ZXDA-ZXDC complex formation and therefore full interaction with CIITA. Given that the two-hybrid assay is far more sensitive than co-immunoprecipitation in detecting weak or transient interactions, our results suggest that the far C-terminus of ZXDC can interact independently with CIITA, though in a much less efficient manner than the binding of the ZXDA-ZXDC complex with CIITA. This interpretation is also consistent with the fact that ZXDC was cloned as a CIITA-binding protein in yeast two-hybrid assay, which seemingly contradicts the requirement of both ZXDA and ZXDC for CIITA binding that we demonstrate here.

The necessity of a ZXDA-ZXDC complex to bind CIITA also suggests a potential mode of regulating CIITA function. We observed that though ZXDA and ZXDC can self-associate, the apparent homodimers are incapable of interacting with CIITA. Therefore, if the degree to which ZXDA and ZXDC hetero- and homodimerize can be modulated, for example by post-translational modification, the levels of ZXDA-ZXDC heterodimers available for interacting with CIITA could be altered. As such, the extent of formation of the pro-transcriptional ZXDA-ZXDC-CIITA complex could be modulated. The relative levels of ZXDC and ZXDA in a given cell type, as well as the relative affinities the proteins have for each other and for themselves, would also be a contributing factor to the efficiency and extent of tripartite complex formation and by extension MHC II transcription. With respect to this model, over-expression of either ZXDA or ZXDC with CIITA, in reporter plasmid experiments, led to an increase in HLA-DRA promoter activity. These findings suggest that ZXDA and ZXDC might preferentially self-associate, in complexes incapable of activating MHC II genes, and that over-expression of either protein promotes the formation of active hetero-duplexes.

All of the functional studies presented here indicate very clearly that ZXDA, in cooperation with ZXDC, have a positive effect on MHC II gene transcription. Knockdown of either ZXDC or ZXDA led to a significant reduction in the induction of the HLA-DRA gene in HeLa cells by IFN-γ and in a HeLa cell line stably expressing the CIITA cDNA. We observed no further reduction in MHC II transcription when both ZXDA and ZXDC were silenced, compared to when either of the genes was silenced singly. This finding is consistent with ZXDA and ZXDC regulating MHC II transcription as a complex, since reducing one necessary component would have the same effect as reducing both components on the efficiency of complex formation. These observations, made in the more physiological setting of IFN-γ induction of the HLA-DRA gene (as opposed to reporter plasmid assays in HEK293 cells), strongly support the notion that ZXDA and ZXDC are *bone fide* regulators of MHC II genes.

The mechanism by which ZXDA and ZXDC regulate MHC II genes is still a matter for further study. While both proteins have transcriptional activation domains, their mechanism of action may not be restricted to simply contributing these domains towards the assembly of the pre-initiation complex. Of particular interest is the role of the zinc fingers present in the ZXDA and ZXDC proteins. We have determined that the region of the ZXDA protein containing its

ten zinc fingers is required for association with ZXDC. However, given that the ZXDA-ZXDC complex, necessary for binding CIITA, has a total of twenty zinc fingers, it is difficult to imagine that DNA binding by these zinc fingers does not contribute somehow to the function of the complex. In addition, other polydactyl zinc finger proteins, such as TFIIIA, WT-1 and TRA-1, are capable of binding both DNA and RNA sequences [38], and we cannot rule out this possibility for the ZXD proteins.

We have made extensive efforts to demonstrate binding of ZXDA and ZXDC to specific DNA sequences conserved amongst MHC II promoters, particularly the S box, but have so far been unable to do so. Chromatin immunoprecipitation (ChIP) has demonstrated that ZXDC is present at MHC II promoters of Raji cells, which constitutively express MHC II genes [24]. In addition, in ChIP experiments in the present study, ZXDC was present at the HLA-DRA promoter in HeLa cells, prior to and unaffected by IFN-γ treatment of the cells. This finding is significant, as the other DNA binding proteins at MHC II promoters appear to be present prior to IFN-γ treatment [28] suggesting that ZXDA-ZXDC may also function in a similar manner. Whether ZXDA-ZXDC functions as part of the over-all enhanceosome with RFX, NF-Y and CREB remains to be established. In particular, how the ZXDA-ZXDC complex might affect interaction of CIITA with its other binding partners is an open, but critical question. It is important to note that the primer pair used to detect HLA-DRA promoter DNA in these experiments, amplifies a region from −175/+68 of the HLA-DRA gene. This includes sequences well outside the S-X-Y “cassette” (nucleotide position −114/−44). As such, the ZXDA-ZXDC complex may bind to sequences upstream or downstream of this region. The binding of at least ZXDC to MHC II promoters, in the absence of MHC II transcription, is reminiscent of the behavior of the RFX complex and the CREB protein [28; 29].

In terms of mechanism by which the ZXD complex regulates MHC II gene transcription, the findings of promoter occupancy are intriguing. We found that over-expression of ZXDC could slightly activate HLA-DRA gene transcription amount in RJ2.2.5 cells [24] but with far less efficiency than one might expect given the presence of a potent transcriptional activation domain in the protein. However, several of the proteins that bind the MHC II promoter in the absence of CIITA, such as CREB and the constituents of the NF-Y complex do not activate MHC II genes without CIITA present, though they have been implicated in direct activation of other genes. The NF-Y complex for example, activates transcription of genes other than MHC II, most likely via binding and recruiting general transcription factors to target promoters [39; 40; 41; 42]. However, NF-Y cannot by itself activate MHC II genes. A likely explanation is that the recruitment of CIITA is the limiting step in a crucial change in chromatin structure, that allows for subsequent contribution of the other factors present at MHC II promoters to pre-initiation complex formation. Additional work is needed to clarify how the ZXDA-ZXDC complex contributes to the recruitment and/or subsequent activity of the CIITA protein.

The critical roles of the RFX complex and CIITA protein in MHC II transcription is unquestioned, given the genetics of the bare lymphocyte syndrome [5; 43]. In this syndrome, genes coding for one of the subunits of RFX or for CIITA are defective, such that functional protein is not made. The result is the complete absence of MHC II gene transcription, resulting in severe combined immunodeficiency. Defining the role of the NF-Y complex and CREB protein in enhanceosome formation have relied primarily on biochemical rather than genetic evidence, but their critical importance is none-the-less established [2]. While there are still many unanswered questions, our data support the notion that the ZXD proteins are regulators of CIITA function and MHC II gene transcription. Further study will be necessary to establish the ZXD family as regulators with similarly critical roles as the RFX, NFY and CREB proteins.

Materials and Methods

Plasmid constructs

The plasmids pDRA-luc, pCMV-CIITA and pG5-luc were previously described [24; 25]. The ZXDA cDNA in plasmid pBluescript (Genbank accession number **BC059356**) [26] was obtained via the I.M.A.G.E. consortium and was sequenced by the Cleveland State University core facility to confirm its identity. pCMV-FL-ZXDA was constructed by subcloning the ZXDA cDNA from pBluescript-ZXDA into pCMV-FLAG5a (which adds the coding sequence of the FLAG epitope to the 5′ end of the subcloned cDNA) via EcoRI and BamHI restriction sites. The vector pCMV-myc was constructed by subclonic oligos coding for the myc-epitope, into plasmid pcDNA3.1 (InVitrogen, Inc.) via HindIII and BamHI restriction sites. pCMV-myc-ZXDA was constructed by amplifying the protein coding sequence of the ZXDA cDNA, followed by subcloning the amplified product into pCMV-myc via BamHI and EcoRI sites. Similarly, pCMV-myc-ZXDAΔN (deletion of amino acids 1–266) and pCMV-myc-ZXDAΔC (deletion of amino acids 574–799) were constructed by amplification of the appropriate portions of the ZXDA cDNA followed by cloning the amplified products into pCMV-myc. pCMV-myc-ZXDAΔZnF, which lacks the coding sequence for the ten zinc fingers (amino acids 267–573) was constructed by deleting this region with the Quikchange II XL-Site Directed Mutagenesis kit (Stratagene, Inc., La Jolla, CA) from plasmid pCMV-myc-ZXDA. pGAL4-ZXDA (1–799), (271–799), (572–799) and (699–799) were constructed by PCR amplification of the appropriate regions of the ZXDA cDNA, followed by cloning the PCR products into plasmid pcDNA3.1-Gal4 [24] via BamH1 and XbaI restriction sites.

pCMV-FL-ZXDC was constructed by amplifying the entire protein coding region of the ZXDC cDNA and subcloning the PCR product into plasmid pFLAG-CMV-5a via HindIII and BamH1 restriction sites. pCMV-HA-ZXDC was generated by sub-cloning the full-length ZXDC cDNA from pcDNA3-Gal4-ZXDC into the plasmid pcDNA3-HA [24] via BamHI and XbaI restriction sites. pCMV-FL-ZXDCΔN (deletion of amino acids 1–176) and pCMV-FL-ZXDCΔC (deletion of amino acids 472–858) were constructed by PCR amplification of the appropriate regions of the ZXDC cDNA and subcloned the products into the plasmid pcDNA3-3XFLAG (an N-terminal fusion vector containing three copies of the FLAG epitope) via BamHI and XbaI restriction sites.

Cell culture

The human embryonic kidney cell line 293 (HEK293; ATCC: CRL-1573) and the cervical adenocarcinoma cell line HeLa (ATCC: CRL-2) were maintained in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin and 500 μg/ml streptomycin. In experiments where HeLa cells were treated with IFN-γ, 100 units/ml was used.

Transfections, luciferase assay and RNAi

For plasmid DNA, HEK293 cells were transfected with a total of 1 μg of plasmid in 24 well plates, using Lipofectamine 2000 (InVitrogen Inc., Carlsbad, CA) per manufacturer's instructions. Luciferase assay was performed with the DLR-Dual Luciferase kit (Promega, Inc., Madison, WI) as previously described [25]. All transfections for luciferase assay included an expression plasmid for *Renilla* luciferase (pRL-TK; Promega, Inc.). The firefly luciferase activity was normalized to *Renilla* luciferase activity, to account for variation in transfection efficiency. For RNAi, HeLa cells were plated at a density of 10^5 cells per well in a 6-well plate, for transfection the following day. Cells were transfected twice with siRNA specific for ZXDC and/or ZXDA, or the appropriate control siRNA (see below) at twenty-four hours intervals. All transfections were performed with Lipofectamine RNAiMAX (InVitrogen, Inc.) according to the manufacturer's instructions. Briefly, 150 pmol siRNA was mixed with 250μl serum-free OPTI-MEM I (InVitrogen, Inc.) and then combined with 5μl Lipofectamine RNAiMAX

diluted in 250μl OPTI-MEM I, followed by incubation for 20 minutes. 500 μl of the transfection mix was added to the cells in 2.5 ml DMEM with 10% serum (without antibiotics). Cells were harvested twenty-four hours after the second transfection with siRNA.

Immunoprecipitation and Western blot

Cells were lysed in 1 ml lysis buffer [10 mM Tris-HCl, 300 mM sodium chloride, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 2 mM phenylmethylsulphonyl fluoride and Complete EDTA-free protease inhibitor cocktail (Roche Applied Science, Inc., Indianapolis, IN)]. Note that the lysis buffer does not contain EDTA, which has been shown to potentially create to non-physiological protein-protein interactions involving zinc finger proteins [27]. Cell lysates were sonicated and then centrifuged at 15,000 X g, 4°C for 15 minutes. 40 μl of lysate was reserved for Western analysis. For experiments employing co-IP of *in vitro* transcribed/ translated protein, equal amounts of each protein was diluted in 1 ml of lysis buffer; the subsequent IP procedure was identical to that for cell lysates. The cleared lysate was incubated with 5 μg of the appropriate antibody for three hours with inversion at 4°C. Protein A/G-plus agarose beads (Santa Cruz Biotech, Inc. Santa Cruz, CA) were added to the reaction and incubation continued for an additional two hours. The beads were collected by centrifugation and washed with lysis buffer a total of five times. The beads were mixed with 25 μl 2x Western loading buffer (100 mM Tris-HCl pH 6.8, 2% 2-mercaptoethanol, 4% SDS, 20% glycerol and 0.1% bromophenol blue) boiled for 5 minutes and resolved on an 8% SDS-PAGE. Western transfer was as previously described [25] except that fluorescent detection was performed with ECL-Plus reagent and a Typhoon 9410 imaging system (GE Healthcare, Piscataway, NJ).

Chromatin immunoprecipitation (ChIP)

ChIP was performed with the EZ-Chip kit (Upstate, Inc., Charlottesville, VA) according to manufacturer's instructions. Detection of co-immunoprecipitated DNA was by PCR. 5 μl of eluted DNA and primers for the HLA-DRA [28] or GAPDH (Upstate, Inc., Charlottesville, VA) promoter were combined with PCR reaction mix (Platimun PCR Supermix, InVitrogen, Inc.) and 0.1 μCi [α-^{32}P]dCTP per reaction. PCR products were resolved on 8% PAGE and detected by phosphoimager (Bio-Rad, Inc., Hercules, CA).

Oligonucleotides

siRNA (Stealth RNAi) was obtained from InVitrogen Inc. (Carlsbad, CA). The sequences were as follows: ZXDC, 5′GGGCTCAGCAAGAACTGATTACCGA; ZXDA, 5′ GAGCACAGCCCTGGACTATGAGTTT. Control non-silencing siRNAs were matched to the gene-specific siRNAs by GC-content. For ZXDC, the medium-GC control was used and for ZXDA the high-GC control was used (catalog numbers 12935-300 and 12935-400, respectively, InVitrogen, Inc.). The primers used for RT-PCR were as follows: ZXDA, 5′ GAGCACAGCCCTGGACTG and 5′CAATGTGTTCAAACCACCA; ZXDC, 5′ GTCTCCCTCAGGAGGGCTA and 5′CCACACTTGGTTTTGCCACA; CIITA, 5′ AAACCCTCAATCTGTCCCAGAA and 5′ATCCGTGAATCCTGTTGTTGC; HLA-DRA, 5′GCCCAACCTGGAAATCATGACA and 5′AGGGCTGTTGTGAGCACA; beta actin, 5′ CAAGGTGTGATGGTGGGAATGG and 5′CAGGATGGCGTGAGGGAGAGCA. The first beta actin primer of the pair was tagged on the 5′ end with Alexa Fluor 546. For all other primer pairs, the first listed for each gene was tagged on the 5′ end with Alexa Fluor 647.

Reverse-transcriptase PCR

Total RNA was isolated from cells with Trizol reagent according to the manufacturer's instructions (InVitrogen, Inc., Carlsbad, CA). 1 μg of total RNA was reverse transcribed using random hexamers and the SuperScript II kit (InVitrogen, Inc.) according to the manufacturer's instructions. 2 μl of the reverse-transcriptase reaction was used as template for PCR, with

primer pairs designed to detect the transcripts of interest. One primer in each pair was tagged with a fluorescent dye (see above). PCR was performed for 15–20 cycles, depending on the transcript being amplified, well within the linear range of amplification. PCR reactions were resolved on 8% PAGE gels, and the amplified products detected by fluorography with a Typhoon 9410 imaging system (GE Healthcare, Inc., Piscataway, NJ). Band intensity was quantified with Imagequant TL software form the same manufacturer.

In vitro transcription/translation

ZXDA, ZXDC and CIITA proteins were generated by *in vitro* transcription/translation of the appropriate plasmid, using the TNT Coupled Rabbit Reticulocyte lysate kit according to the manufacturer's instructions (Promega, Inc., Madison, WI).

Reagents and antibodies

Unless otherwise noted, chemical reagents were from Fisher Scientific (Hanover Park, IL). IFN-γ, anti-HA antibody (clone 12CA5) and anti-myc antibody (clone 9E10) were purchased from Roche Applied Science, Inc. (Indianapolis, IN). Anti-FLAG M2 antibody was from Sigma Chemical (St. Louis, MO). For ChIP the following antibodies were used: 25 μl of affinity purified anti-ZXDC [24], 5 μl of anti-RFX5 (E-17; Santa Cruz Biotech, Santa Cruz, CA), 10 μl of anti-CIITA (a generous gift of Jeremy Boss) [29], 1 μl anti-RNA polymerase II or 1 μl non-specific IgG provided with the EZ-Chip kit (Upstate, Inc., Charlottesville, VA).

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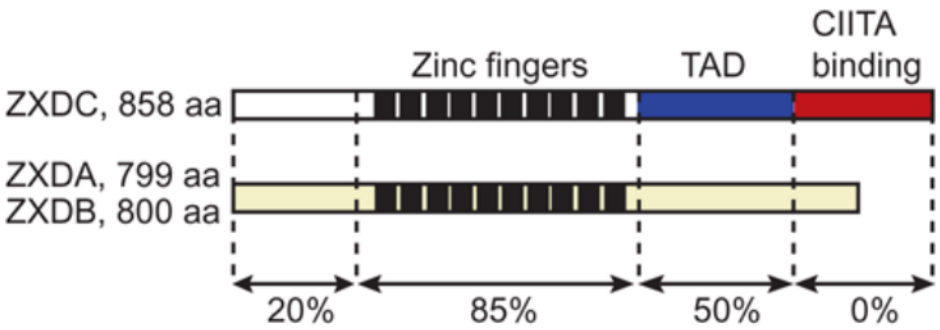

Figure 1.

Comparison of the primary amino acid sequences of ZXDA, ZXDB and ZXDC proteins. ZXDA and ZXDB have 97% amino acid identity over their entire lengths. The extent of amino acid sequence identity between ZXDA/B and ZXDC are indicated for the various regions of the proteins. *TAD*, transcriptional activation domain; *CIITA binding*, region of ZXDC required to retain binding between CIITA and ZXDC, though the zinc finger region of ZXDC is also important for ZXDC-CIITA association [24].

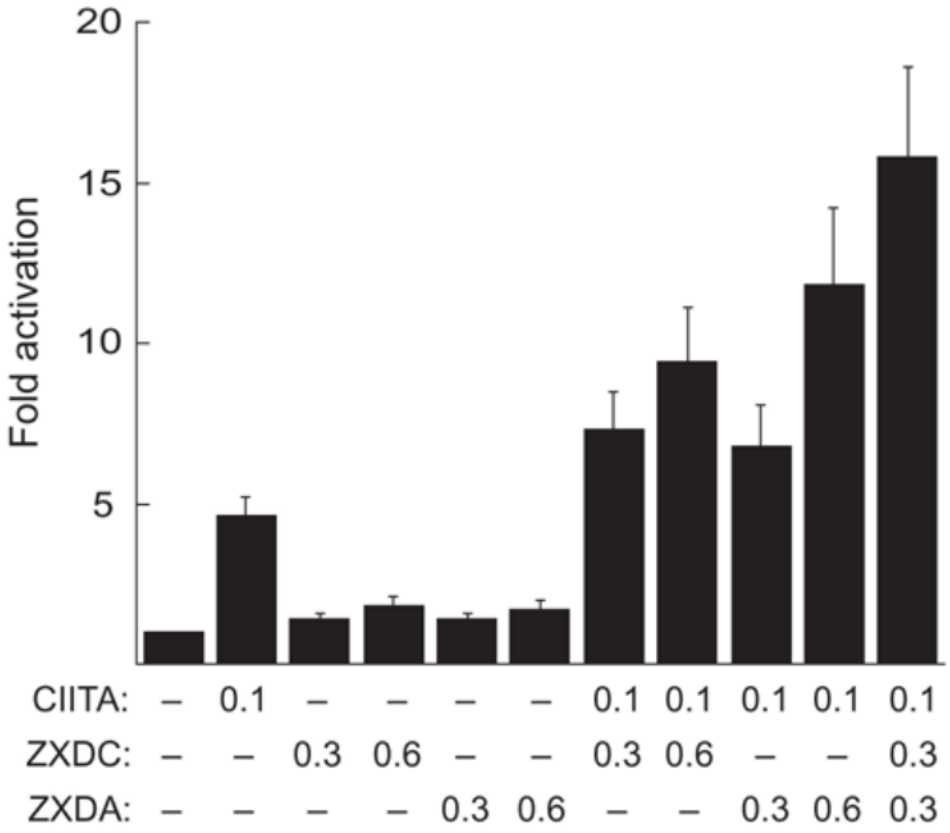

Figure 2.

ZXDA cooperates with CIITA and ZXDC to activate the HLA-DRA promoter. HEK293 cells were transfected with expression plasmids for the indicated proteins and the reporter plasmids pDRA-luc and pRL-TK (Promega, Inc., Madison, WI). Forty-eight hours post-transfection, cellular lysates were assayed for luciferase expression. Numerals indicate micrograms of plasmid DNA (total DNA per transfection was maintained at 0.8 μg). Data are presented as fold activation of the pDRA-luc plasmid transfected alone (normalized to *Renilla* expression). Error bars represent SEM of three independent experiments.

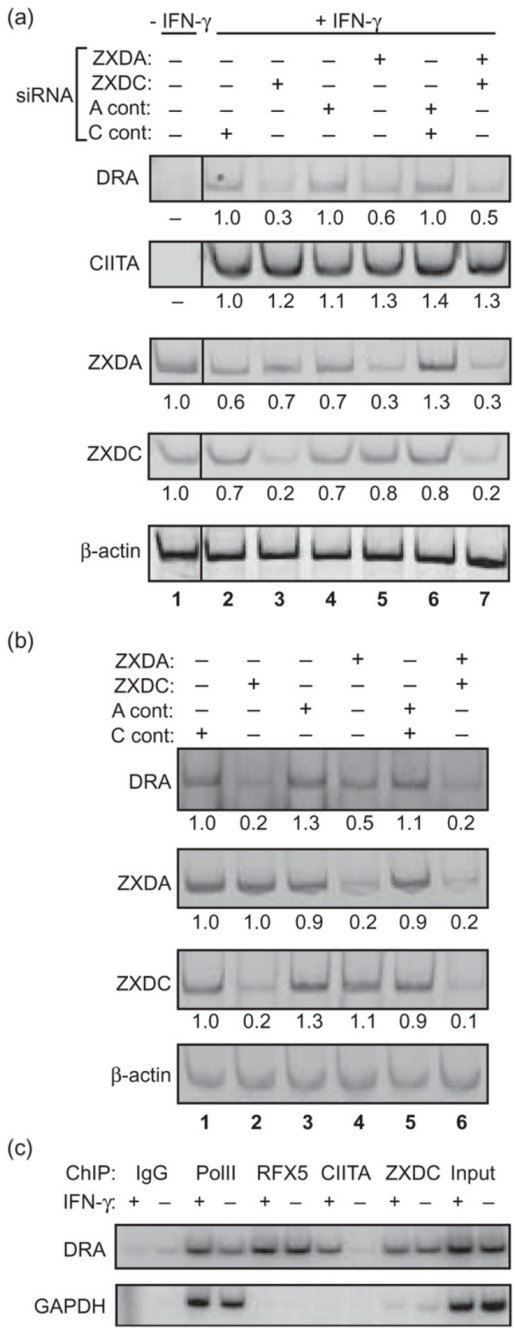

Figure 3.

ZXDA and ZXDC contribute to the transcription of MHC II genes by CIITA. (a) HeLa cells were transfected with the indicated siRNA and twenty-four hours later were treated with 100 u/ml IFN-γ for 14 hours. The GC content of control siRNAs were matched to the ZXDA (A cont.) and ZXDC (C cont.) specific siRNAs. RT-PCR reactions were carried out with fluorescently-labeled primers specific for the indicated transcripts. Detection of amplified products was performed with a Typhoon system and quantified with ImageQuant TL software (GE Healthcare, Inc., Piscataway, NJ). Quantified expression levels, normalized to beta actin, are indicated by numerals beneath each lane. Data presented are representative of at least three independent experiments. (b) Similar to the experiment in panel (a), except HeLa cells stably

expressing the CIITA cDNA were employed, and were not treated with IFN-γ. Data presented are representative of at least three independent experiments. (c) Chromatin immunoprecipitation (ChIP) from untreated HeLa cells, or cells treated with 100 units/ml IFN-γ for 14 hours. ChIP was performed with equal amounts of chromatin, using the indicated antibodies. Eluted DNA was detected by PCR incorporating [α-^{32}P]dCTP, employing primers to amplify either the HLA-DRA (top panel) or GAPDH (bottom panel) promoter.

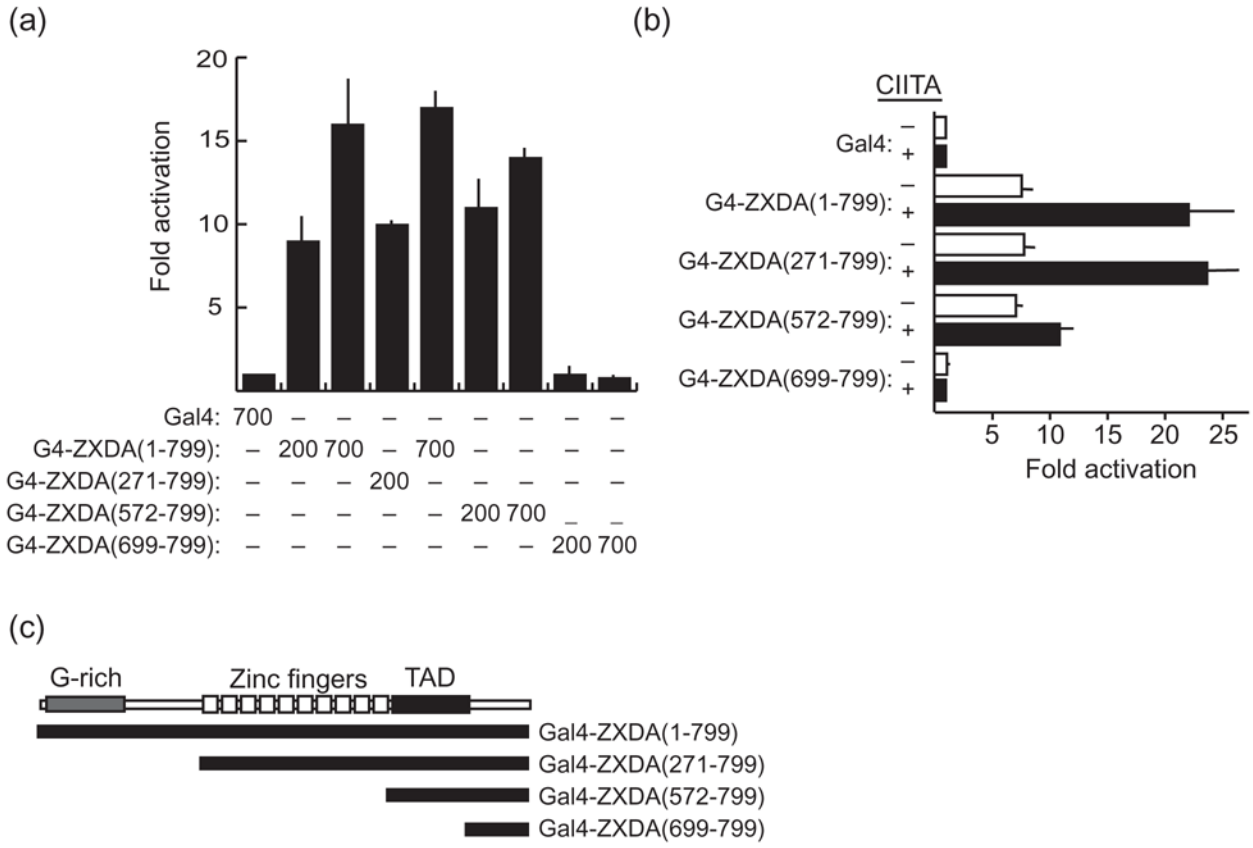

Figure 4.

ZXDA has a transcriptional activation domain, and associates with the CIITA protein in cells. (a) HEK293 cells were transfected with plasmids expressing either the Gal4 DNA binding domain alone or the indicated Gal4-ZXDA fusion proteins and the reporter plasmids pG5-luc and pRL-TK (Promega, Inc., Madison, WI). Numerals indicate nanograms of plasmid transfected. *TAD*, transcriptional activation domain; *G-rich*, glycine rich region of the ZXDA protein. (b) Similar to experiments in panel A, except that the expression plasmid pCMV-CIITA was included in the indicated transfections (filled bars) with the Gal4-ZXDA fusions. For both panels, forty-eight hours post-transfection cellular lysates were assayed for luciferase expression. Data are presented as fold activation of the pG5-luc plasmid transfected by itself (normalized to *Renilla* expression). Error bars represent SEM of three independent experiments. (c) Schematic diagram indicating the regions of ZXDA present in each Gal4-ZXDA construct.

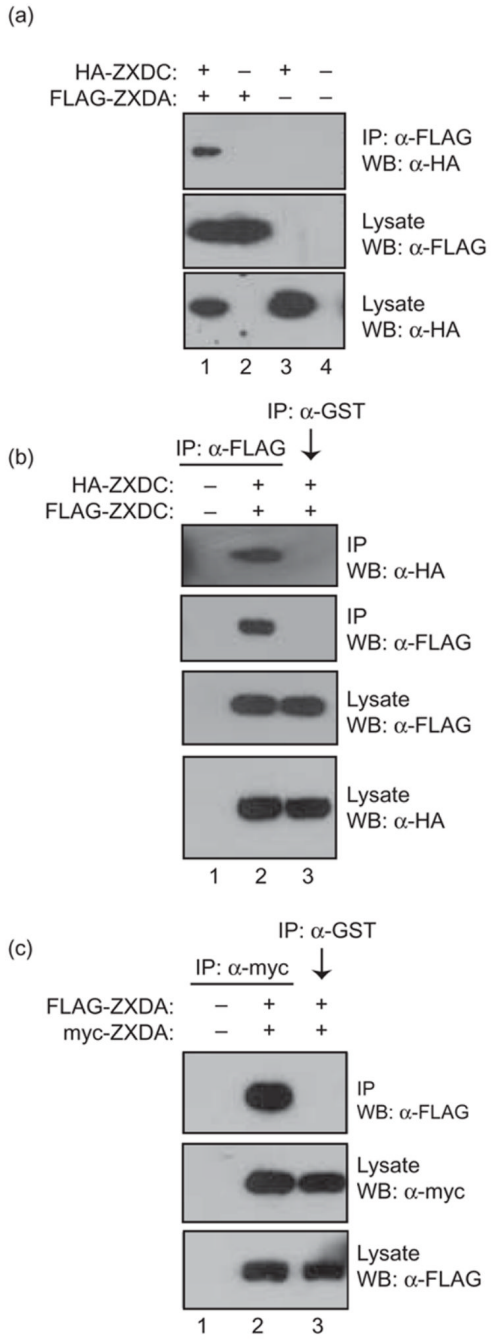

Figure 5.

ZXDA and ZXDC self-associate and associate with each other. HEK293 cells were transfected with expression plasmids for the indicated proteins. Cellular lysates were subjected to immunoprecipitation followed by Western analysis with the indicated antibodies. Cellular lysates were also subjected to Western analysis directly, to demonstrate the expression of the transfected plasmids. (a) Association of ZXDA and ZXDC was demonstrated by IP of FLAG-ZXDA followed by detection of HA-ZXDC. (b) Self-association of ZXDC was demonstrated by IP of HA-ZXDC followed by detection of FLAG-ZXDC. (c) Self-association of ZXDA was demonstrated by IP of FLAG-ZXDA followed by detection of myc-ZXDA. In panels (b) and (c), IP with anti-GST antiserum was performed to demonstrate specificity.

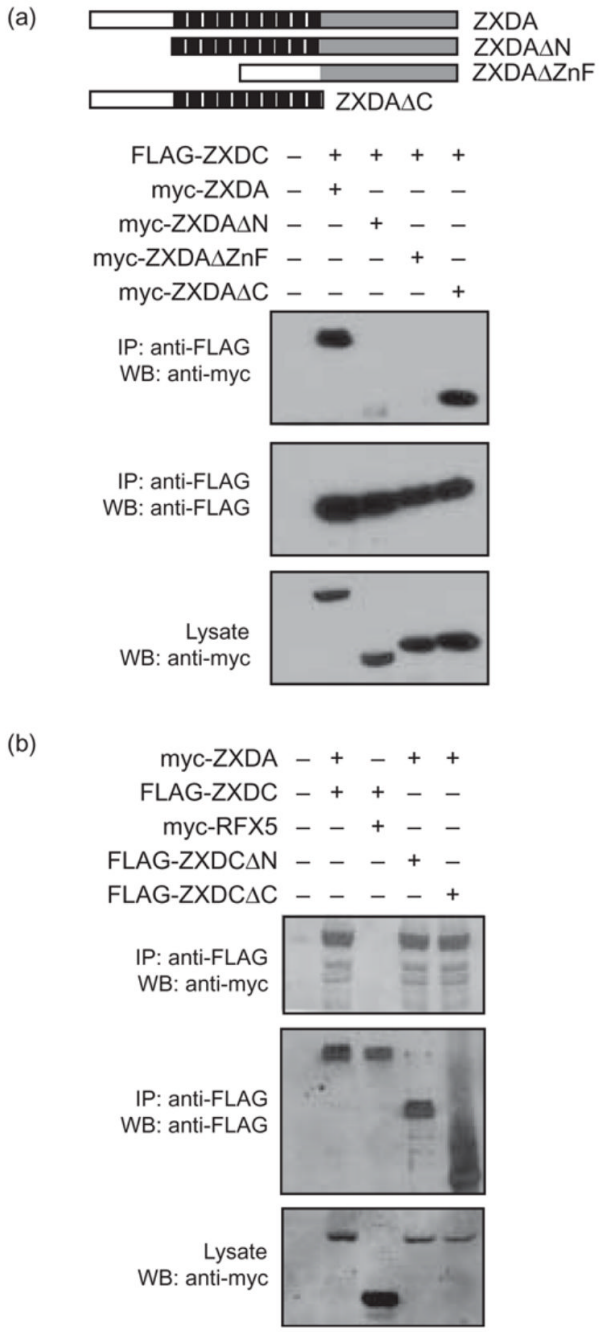

Figure 6.

The region of ZXDA containing zinc fingers is required for the association of the ZXDA and ZXDC proteins. (a) HEK293 cells were transfected with the indicated plasmids, expressing FLAG-ZXDC and myc-ZXDA or myc-ZXDA with various regions deleted. Myc-ZXDAΔN lacks the N-terminal 266 amino acids of ZXDA; myc-ZXDAΔZnF lacks amino acids 267–573 and myc-ZXDAΔC lacks the C-terminal 226 amino acids of ZXDA. Black bars in the schematic diagram of ZXDA represent the zinc fingers of the protein. Immunoprecipitation was performed on cellular lysates with anti-FLAG, followed by Western analysis with anti-myc to detect co-immunoprecipitated myc-ZXDA (top panel) or anti-FLAG to detect immunoprecipitated FLAG-ZXDC (middle panel). Western analysis of cellular lysates with

anti-myc was performed to demonstrate the expression of the myc-ZXDA plasmids (bottom panel). (b) Similar to panel A, except that deletions of FLAG-ZXDC were expressed with full length myc-ZXDA. FLAG-ZXDC (full length protein), FLAG-ZXDCΔN (lacking amino acids 1–176) or FLAG-ZXDCΔC (lacking amino acids 472–858) was expressed with myc-ZXDA, followed by IP with anti-FLAG and Western blot with anti-FLAG. As a control for specificity of interaction between ZXDA and ZXDC, co-IP was performed with lysates from cells where myc-RFX5 was expressed with FLAG-ZXDC. Western analysis of cellular lysates with anti-myc was performed to demonstrate the expression of the myc-ZXDA and myc-RFX5 proteins (bottom panel).

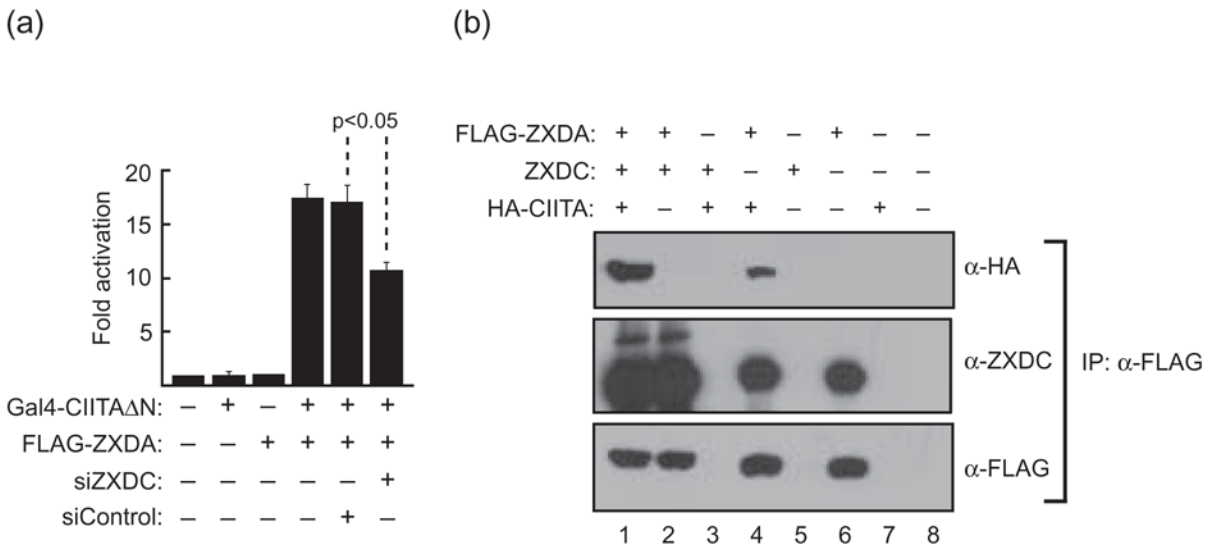

Figure 7.

ZXDA, ZXDC and CIITA form a tripartite complex. (a) Mammalian two-hybrid assay to demonstrate the role of ZXDC in ZXDA-CIITA association. HEK293 cells were transfected with expression plasmids for Gal4-CIITAΔN, with and without FLAG-ZXDA and the reporter plasmids G5-luc and pRL-TK (Promega, Inc., Madison, WI). To determine the contribution of ZXDC to the association of ZXDA and CIITA, cells were transfected with control siRNA or ZXDC-specific siRNA, twenty-four hours prior to transfection with the plasmids noted above. Data are presented as fold activation of the pG5-luc plasmid transfected by itself (normalized to *Renilla* expression). A statistically significant difference in activation (by student's T-test) is indicated. Error bars represent SEM of three independent experiments. (b) Co-immunoprecipitation of ZXDA, ZXDC and CIITA. HEK293 cells were transfected with the indicated plasmids, followed by immunoprecipitation with anti-FLAG. Western analysis with anti-FLAG, anti-HA or anti-ZXDC [24] was performed on immunoprecipitates. Note that in lanes 4 and 6, the endogenous cellular ZXDC protein was detected in the immunoprecipitates. The additional lower mobility band in lanes 1 and 2 of the anti-ZXDC blot represents a post-translationally modified form of ZXDC that is most apparent when ZXDC is highly over-expressed (data not presented).

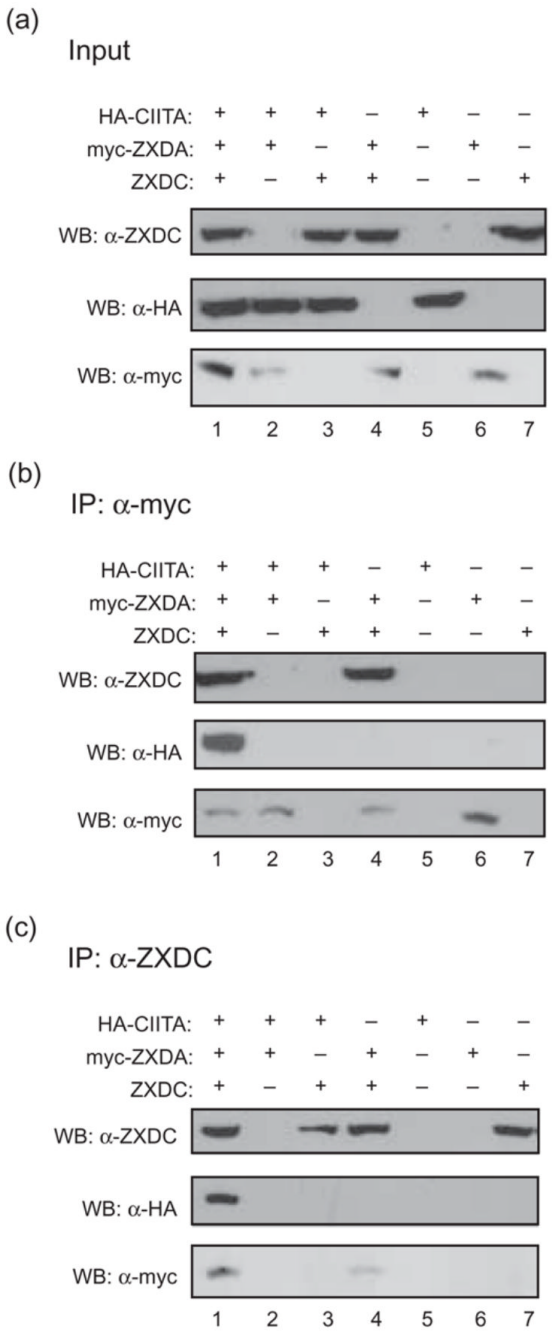

Figure 8.

A complex of ZXDA and ZXDC is required for interaction with CIITA *A,* Western analysis of HA-CIITA, myc-ZXDA and ZXDC proteins input into the binding reactions shown in panels (b) and (c). The proteins were produced by *in vitro* transcription/translation. The proteins indicated were combined, allowed to interact and then subject to immunoprecipitation by anti-myc (b) or with anti-ZXDC (c), followed by Western analysis with the indicated antibody. It is only when all three proteins are present in the binding reaction that a complex forms [lane 1 of panels (b) and (c)].