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ZXDC, a novel zinc finger protein that binds CIITA and activates MHC gene transcription

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Abstract

The class II trans-activator (CIITA) is recognized as the master regulator of major histocompatibility complex (MHC) class II gene transcription and contributes to the transcription of MHC class I genes. To better understand the function of CIITA, we performed yeast two-hybrid with the C-terminal 807 amino acids of CIITA, and cloned a novel human cDNA named zinc finger, X-linked, duplicated family member C (ZXDC). The 858 amino acid ZXDC protein contains 10 zinc fingers and a transcriptional activation domain, and was found to interact with the region of CIITA containing leucine-rich repeats. Over-expression of ZXDC in human cell lines resulted in super-activation of MHC class I and class II promoters by CIITA. Conversely, silencing of ZXDC expression reduced the ability of CIITA to activate transcription of MHC class II genes. Given the specific interaction between the ZXDC and CIITA proteins, as well as the effect of ZXDC on MHC gene transcription, it appears that ZXDC is an important regulator of both MHC class I and class II transcription.

Keywords

MHC II; Transcriptional regulation; Zinc finger; Molecular biology

1. Introduction

Major histocompatibility complex (MHC) class II determinants are cell surface glycoproteins that present peptide antigen to CD4⁺ T cells. Given the important role of MHC class II antigen presentation in the adaptive immune response, MHC class II gene expression is tightly regulated. MHC class II genes are expressed primarily on professional antigen presenting cells and thymic epithelial cells, though expression can be induced in most cell types by IFN γ (van den Elsen et al., 2004).

The proximal promoters of MHC class II genes contain a series of conserved upstream sequences, labeled the W (also known as S), X1, X2 and Y boxes, that are required for constitutive and inducible transcription (Nekrep et al., 2003). A similar arrangement of W–X1–X2–Y regulatory elements are found in HLA-DO and HLA-DM genes, the invariant chain gene Ii and MHC class I genes (van den Elsen et al., 2004). These conserved boxes have very stringent spatial constraints with respect to each other, and function essentially as a single regulatory element. Recently, additional X–Y cassettes have been found within the MHC class II locus and may function as locus control elements (Gomez et al., 2005).

Even though the range of cell types that transcribe MHC class II genes is strictly limited, the constituents of the complexes that bind the X1, X2 and Y boxes appear to be ubiquitously

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expressed. That is, the three subunits of the X1-binding regulatory factor X (RFX) complex, the three subunits of the nuclear factor Y (NFY) complex that binds the Y box and the CREB protein that binds the X2 box are found in essentially all cell types (reviewed in Boss and Jensen, 2003; Nekrep et al., 2003; Ting and Trowsdale, 2002; van den Elsen et al., 2004). These DNA-binding proteins are necessary, but not sufficient, for MHC class II gene transcription. A transcriptional co-factor, known as the class II trans-activator (CIITA), is also required for transcription of MHC class II genes (Steimle et al., 1993). CIITA does not bind DNA, but rather is recruited to MHC class II promoters by interacting with the CREB protein and subunits of the RFX and NFY complexes (DeSandro et al., 2000; Hake et al., 2000; Scholl et al., 1997; Zhu et al., 2000). In addition, the role of the W box in recruiting CIITA to MHC class II promoters is critical, though the factor(s) that binds this element have not been identified (Brown et al., 1998; Muhlethaler-Mottet et al., 2004).

Extensive structure–function analyses have identified several important regions of the CIITA protein. The N-terminus of CIITA contains a transcriptional activation domain, which binds and recruits to MHC class II gene promoters factors involved in chromatin remodeling and modification, as well as components of the general transcriptional machinery (Fontes et al., 1997, 1999; Kanazawa et al., 2000; Kanazawa and Peterlin, 2001; Kretsovali et al., 1998; Mahanta et al., 1997; Mudhasani and Fontes, 2002; Spilianakis et al., 2000; Zika et al., 2003). These and other observations have strongly implicated CIITA in regulating chromatin structure as a mechanism of activating gene transcription (Zika and Ting, 2005).

A series of leucine-rich repeats (LRR) in the C-terminus of CIITA are of particular interest, since mutational analyses have demonstrated a role for these repeats in nuclear localization, self-association and promoter targeting of the CIITA protein (Brown et al., 1998; Camacho-Carvajal et al., 2004; Hake et al., 2000; Linhoff et al., 2001; Sisk et al., 2001; Towey and Kelly, 2002). However, little is known how the LRR mediate these activities, though LRR often are involved in protein–protein interactions (Kobe and Kajava, 2001). CIITA has been assigned to a family of proteins known as CATERPILLER, which is an acronym for the domains conserved amongst members: CARD domain, transcriptional enhancer, purine binding, pyrin domain and lots of leucine-rich repeats (Harton et al., 2002). The CATERPILLER proteins are divided into several subfamilies, and essentially all of these proteins have roles in inflammation, adaptive immunity and/or apoptosis (Ting and Davis, 2004; Ting and Williams, 2005).

We describe here a new CIITA-binding protein, identified by yeast two-hybrid, named zinc finger, X-linked, duplicated family member C (ZXDC). ZXDC was named based upon sequence homology with the previously cloned ZXDA and ZXDB genes (38). ZXDC is a zinc finger protein that we show here to contain a transcriptional activation domain, and a specific domain that interacts with the LRR containing region of CIITA. Over-expression of ZXDC activated transcription of both MHC class I and MHC class II promoters modestly, and leads to much greater activation of these genes by CIITA. We found also that silencing of endogenous ZXDC expression resulted in a dramatic decrease in the ability of CIITA to activate MHC class II genes. Interestingly, the activation domain and CIITA-binding region of ZXDC are necessary and sufficient to achieve significant cooperation with CIITA in activating MHC class II genes, though the zinc fingers of ZXDC are necessary for full activity. These observations are consistent with an important role for ZXDC in regulating MHC class II gene transcription, in conjunction with CIITA.

2. Materials and methods

2.1. Yeast two-hybrid assay

Yeast two-hybrid was performed with the MATCHMAKER kit (Clontech Inc., Palo Alto, CA, USA) according to the manufacturer's protocol. CIITA cDNA (GenBank accession no. NM 000246) from nucleotide position 1108 to 3531 coding for amino acids 323–1130 was subcloned into the bait plasmid pGBKT7. The prey cDNA library, purchased from a commercial supplier, was constructed in plasmid pGADT7, and derived from human spleen mRNA (Clontech Inc.). Following sequencing of the ZXDC clone isolated by yeast two-hybrid screen, a full length ZXDC cDNA in plasmid pCMVSPORT6 was obtained (GenBank accession no. AL553476; Invitrogen Inc., Carlsbad, CA, USA), and sequenced on both strands. The sequence we obtained for ZXDC was deposited in GenBank (GenBank accession no. AY936556).

2.1.1. Western and Northern blot—Western and Northern analyses were performed essentially as described (Mudhasani and Fontes, 2002) except that Northern hybridizations were performed in Ultrahyb solution (Ambion Inc., Austin, TX, USA). A Northern blot containing poly-A⁺ mRNA from human tissues was purchased from Origene Technologies (Rockville, MD, USA) and hybridized with a probe made from exons 8 to 10 of the ZXDC cDNA, stripped and reprobbed with human beta-actin. ZXDC antiserum was generated and affinity purified by Bethyl Laboratories Inc. (Montgomery, TX, USA). The immunizing peptide represented the first 20 amino acids of ZXDC: MDLPALLPAP-TARGGQHGGG.

2.2. Cell culture and transfection

The human Burkitt's lymphoma cell line Raji (ATCC CCL-86) and its CIITA-negative derivative RJ2.2.5 (Accolla, 1983) were maintained in RPMI-1640 media supplemented with 10% fetal calf serum, 100 units/ml penicillin and 500 µg/ml streptomycin. The human embryonic kidney cell line 293 (HEK293; ATCC: CRL-1573) was maintained in DMEM supplemented as above. HEK293 cells were transfected with a total of 800 ng of plasmid DNA in 24-well plates, with Lipofectamine 2000 (Invitrogen Inc.) per manufacturer's instructions. RJ2.2.5 cells were transfected with a total of 40 µg of plasmid DNA by electroporation (10⁷ cells, 300 V, 960 µF, 0.4 cm gap, in total volume of 0.8 ml RPMI-1640).

2.3. Plasmid DNA constructs

Reporter plasmid pDRA-luc and expression plasmid pCMV-CIITA were previously described (Mudhasani and Fontes, 2002). pG5luc was constructed by subcloning the five UASg sites and TATA box from pG5E1bCAT (Lillie and Green, 1989) into the luciferase reporter plasmid pGL3basic (Promega, Madison, WI, USA). Plasmid pHLAB-luc was constructed by PCR amplification of the HLAB1 gene promoter from −284 to +1, which was then cloned into plasmid pGL3basic. For the Gal4 DNA-binding domain fusion proteins, the cDNA coding for the Gal4 DNA-binding domain was subcloned from plasmid pSG424 (Sadowski and Ptashne, 1989) into pCDNA3.1 (Invitrogen) to create pCGal4. The appropriate regions of ZXDC or CIITA were amplified by PCR, and subcloned into pCGal4, in frame with the Gal4 DNA-binding domain. The cDNA coding for the VP16 activation domain (Cress and Triezenberg, 1991) was subcloned into pCDNA3.1 (plasmid pCVP), to create the VP16 fusions with CIITA. The appropriate regions of CIITA were amplified by PCR and subcloned into pCVP. ZXDC deletion mutants were generated by PCR amplification of the desired region, followed by subcloning into plasmid pCHA (pCDNA3.1 modified to contain the coding sequence for the hemagglutinin epitope tag at the 5' end of the multiple cloning site of the vector). All plasmids containing PCR-amplified cDNAs were sequenced to check for inadvertent mutations.

2.3.1. RNA silencing—HEK293 cells were transfected with SMARTpool siRNA directed against the ZXDC cDNA (catalog number MQ-023561-00; Dharmacon Inc., Lafayette, CO, USA) using RNAifect (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Cells were transfected twice with siRNA, 24 h apart, followed by transfection with plasmid DNA 6 h after the second siRNA transfection. Twenty-four hours after plasmid DNA transfection, RNA was purified from the cells for gene expression analysis by quantitative real-time reverse transcriptase PCR (see below).

2.4. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed with the EZ-ChIP kit (Upstate Inc., Charlottesville, VA, USA) according to manufacturer's instructions. For immunoprecipitations, the following antisera were used: 25 µl of affinity purified anti-ZXDC, 5 µl of anti-NFYA (FL-207; Santa Cruz Biotech, Santa Cruz, CA, USA), 1 µl anti-RNA polymerase II or 1 µl non-specific serum provided with the EZ-ChIP kit. Detection of co-immunoprecipitated DNA was by quantitative real-time PCR. Five microliter of eluted DNA and primers directed against the HLA-DRA promoter (Spilianakis et al., 2003) or the GAPDH promoter (provided in the EZ-ChIP kit) were combined with Reaction Mix (QuantiTect SYBR Green PCR kit; Qiagen Inc.). Data were normalized to the signal detected with non-specific antiserum.

2.5. Quantitative real-time reverse transcriptase PCR

Three micrograms of total RNA was reverse transcribed with the Thermoscript RT-PCR system according to the manufacturer's protocol (Invitrogen). For real-time quantitative PCR, 2 µl of the reverse transcription mix was combined with primers for HLA-DRA (Masternak et al., 2003) or beta-actin, and Reaction Mix (QuantiTect SYBR Green PCR kit; Qiagen Inc.). The reactions were carried out in an Opticon 2 real-time thermal cycler (MJ Research Inc., Reno, NV, USA). Data were analyzed as described (Livak and Schmittgen, 2001).

2.6. Luciferase assay

Luciferase assay was performed with the DLR-Dual Luciferase kit (Promega Inc.) as previously described (Mudhasani and Fontes, 2002). All transfections for luciferase assay included an expression plasmid for *Renilla* luciferase. The firefly luciferase activity was normalized to *Renilla* luciferase activity, to account for variation in transfection efficiency.

3. Results

3.1. Cloning and sequence analysis of ZXDC cDNA

To identify proteins that interact with the C-terminal region of CIITA, we performed yeast two-hybrid with a bait protein consisting of amino acids 323–1130 of CIITA fused to the Gal4 DNA-binding domain. This segment of CIITA lacks the N-terminal transcriptional activation domain, and did not activate the reporter construct by itself (data not presented). Following extensive screening of a human spleen cDNA library we identified a number of cDNAs that expressed proteins capable of binding to CIITA. One cDNA expressed a 78 amino acid peptide that did not share homology with any previously characterized proteins. BLAST analysis of this sequence demonstrated that it represented the final 78 amino acids of the cDNA for the gene zinc finger, X-linked, duplicated, family member C (Gen-Bank accession no. NM 025112). However, the sequences for ZXDC present in online databases did not represent the full length cDNA. We identified and obtained an EST cDNA that is full length (GenBank accession no. AL553476) which codes for a protein with an additional 371 amino acids on the N-terminus, compared to NM 025112. The cDNA insert is 3412 nucleotides in length

(including 58 adenosine residues on the 3' end) with an open reading frame coding for 858 amino acids and a predicted mass of 90 kDa (Fig. 1; GenBank accession no. AY936556).

The ZXDC gene, which is present on chromosome three, was named based upon significant sequence homology with the X-chromosome genes ZXDA and ZXDB (Greig et al., 1993). The amino acid sequences of ZXDA and ZXDB are 97% identical, and ZXDC has 73% amino acid identity with ZXDA and ZXDB over a roughly 500 amino acid region (Fig. 1). The region of homology does not include the portion of ZXDC that binds CIITA, though the zinc finger sequences are conserved almost perfectly amongst the three proteins. ZXDC contains 10 C₂H₂-type zinc fingers, suggesting that ZXDC is a DNA-binding protein, though zinc fingers can also mediate protein–protein, protein–RNA and protein–lipid interactions (Matthews and Sunde, 2002). There are presently no published reports describing the function of the ZXDC family proteins.

3.2. Expression of the ZXDC gene

To characterize ZXDC gene transcription, we probed a Northern blot containing mRNA from multiple human tissues. The probe consisted of exons 8–10 of ZXDC, outside the region of homology with ZXDA/B. Two transcripts were detected in most tissues: ~4 and ~5 kbp (Fig. 2A). Expression of the 4 kbp transcript was highest in general, and present at relatively high levels in testis, kidney and liver, to the apparent exclusion of the 5 kbp transcript. Low levels of both transcripts were seen in lung, muscle and placenta, with modest levels in spleen, colon and small intestine.

We had antiserum produced, employing a peptide that contained the first 20 amino acids of ZXDC (Bethyl Labs). This peptide is unique to ZXDC, based upon BLAT search of the human genome (Karolchik et al., 2003; Kent, 2002). To determine if our cDNA expressed the predicted ZXDC protein, we transfected HEK293 cells with an expression plasmid containing the ZXDC cDNA (pCMV-ZXDC) and performed Western analysis with our antibody. We detected three bands with molecular mass of 149, 91 and 72 kDa (Fig. 2B); the 91 kDa band is in good agreement with the predicted molecular mass of 89.9 kDa. The endogenous protein is not visible on the blot in Fig. 2B, due to short exposure time. Three bands with the same molecular masses were observed when N-terminal hemagglutinin (HA)-tagged ZXDC was transfected into HEK293 followed by anti-HA Western blot (data not presented). The endogenous ZXDC protein present in HEK293 cell extracts was visualized by using more protein extract, and performing relatively long exposures of the blot. Two bands of 149 and 91 kDa were visualized, identical in molecular mass to the two largest bands seen when the ZXDC cDNA was over-expressed (Fig. 2C). We also performed Western analysis of in vitro transcribed and translated ZXDC cDNA, with the anti-ZXDC serum. In this instance, a single band of 91 kDa was detected (Fig. 2D). Detection of all the bands was blocked when the primary antibody was incubated in the presence of immunizing peptide (data not presented).

3.3. Regulation of MHC gene transcription by ZXDC

To determine the affect of ZXDC on MHC class II gene transcription, we performed transfection experiments with HEK293 cells. HEK293 cells do not constitutively express MHC class II genes, though introduction of CIITA into these cells results in MHC class II transcription (Fig. 3). We co-transfected the reporter plasmid pDRA-luc which contains the HLA-DRA proximal promoter linked to the luciferase gene, along with expression plasmids for ZXDC and/or CIITA (plasmids pCMV-ZXDC and pCMV-CIITA, respectively). We observed that ZXDC alone produced a small (approximately two-fold) but consistent, activation of the HLA-DRA promoter (Fig. 3A). The relatively small amounts of pCMV-CIITA transfected led to a modest three-fold increase in luciferase expression. However, ZXDC cooperated with CIITA to super-activate the HLA-DRA promoter, in a dose-dependent manner

(Fig. 3A). Together, ZXDC and CIITA produced an almost 12-fold activation. It is important to note that ZXDC over-expression did not activate expression of the endogenous CIITA gene in HEK293 cells, nor did it affect the expression of a luciferase gene transcribed from the CMV or RSV promoters (data not presented). We obtained similar results with the cell line RJ2.2.5, a CIITA^{-/-} derivative of the Burkitt's lymphoma line Raji. In RJ2.2.5 cells, ZXDC alone resulted in a modest activation, and cooperated with CIITA to super-activate the HLA-DRA promoter (Fig. 3B).

We confirmed the ability of ZXDC to regulate MHC class II transcription by examining the transcription of the HLA-DRA gene. We transfected RJ2.2.5 cells with pCMV-ZXDC and/or pCMV-CIITA and measured HLA-DRA mRNA by quantitative real-time reverse transcriptase PCR. We observed that over-expression of ZXDC resulted in a modest increase in HLA-DRA transcription and that ZXDC again cooperated with CIITA to super-activate HLA-DRA (Fig. 3C). Expression of the beta-actin gene, used as an internal control to normalize the measured HLA-DRA expression, was very consistent regardless of the expression of ZXDC or CIITA, indicating that neither affected gene transcription non-specifically (data not presented). Taken together, these data demonstrate a role for ZXDC in regulating MHC class II transcription, in particular by increasing gene activation by CIITA.

MHC class I genes contain a W-X-Y cassette within their proximal promoters, and are regulated by CIITA (Gobin et al., 2001; Martin et al., 1997). To determine if ZXDC regulates MHC class I genes, we performed co-transfection experiments in HEK293 cells. We constructed a reporter plasmid containing the promoter of the HLA-B gene (nucleotide position -284/+1) linked to the luciferase gene (plasmid pHLAB-luc). We found that co-transfection of pHLAB-luc with ZXDC alone resulted in a minor, but consistent, activation of the HLA-B promoter (Fig. 3D). CIITA activated the HLA-B promoter modestly, approximately four-fold. However, when co-expressed, ZXDC and CIITA super-activated the HLA-B1 promoter (Fig. 3D) consistent with our findings with the HLA-DRA gene. Thus, it appears that the ability of ZXDC to augment the function of CIITA is not limited to MHC class II genes, but extends to MHC class I genes as well.

To demonstrate that the endogenous ZXDC protein present in cells, and not just over-expressed ZXDC, contributes to CIITA function and MHC gene transcription, we knocked-down ZXDC expression by RNA silencing. We transfected siRNA oligos directed against ZXDC (Dharmacon Inc.) or an siRNA with random nucleotide sequence, into HEK293 cells. Subsequently, expression plasmids for ZXDC and/or CIITA were transfected into the cells, followed by quantitation of HLA-DRA gene expression by quantitative real-time RT-PCR. We found that ZXDC protein levels were drastically reduced in cells transfected with the ZXDC siRNA (Fig. 4B). Consistent with this, the silencing of ZXDC resulted in an approximately 60% reduction in the induction of the DRA promoter by CIITA (Fig. 4A). The random siRNA had no effect on either ZXDC protein or CIITA function. The super-activation we observed when both ZXDC and CIITA were over-expressed was also reversed by silencing ZXDC, but not by the random siRNA (Fig. 4A). Taken together, these data indicate that ZXDC is an important regulator of MHC class II gene transcription.

3.4. Protein-protein interactions between CIITA and ZXDC

To further characterize the binding between CIITA and ZXDC proteins, we performed co-immunoprecipitation experiments. We over-expressed HA-tagged CIITA in HEK293 cells and immunoprecipitated ZXDC with our anti-ZXDC antiserum. We performed Western analysis with anti-HA on the immunoprecipitate and the crude lysate. CIITA co-immunoprecipitated with the endogenous ZXDC protein (Fig. 5A). ZXDC protein was immunoprecipitated, in the presence and absence of CIITA (Fig. 5B). Pre-immune serum did not co-immunoprecipitate CIITA (data not presented).

To demonstrate the presence of ZXDC at MHC class II promoters, we performed chromatin immunoprecipitation experiments. Antiserum against ZXDC, NFYA and RNA polymerase II or non-specific antiserum was used to precipitate chromatin from Raji cells. Following isolation of co-immunoprecipitated DNA, quantitative real-time PCR was performed to determine the presence of the HLA-DRA promoter DNA or GAPDH promoter DNA. Anti-ZXDC was as effective as anti-NFYA in co-precipitating HLA-DRA promoter DNA, indicating that ZXDC is present at the HLA-DRA promoter in Raji cells (Fig. 5C). The interaction of ZXDC with the HLA-DRA promoter was shown to be specific, since GAPDH promoter DNA was not brought down by anti-ZXDC (Fig. 5C).

In addition to the co-immunoprecipitation, we demonstrated CIITA–ZXDC association by mammalian two-hybrid. We constructed a mammalian expression plasmid to express Gal4 DNA-binding domain-CIITA fusion protein (Gal4–CIITAΔN) which contains amino acids 323–1130 from CIITA. We co-transfected HEK293 cells with Gal4–CIITAΔN and a reporter plasmid containing one Gal4-binding sites linked to the luciferase gene (pG5-luc). Since Gal4–CIITAΔN lacks the transcriptional activation domain of CIITA, it did not activate pG5-luc (Fig. 6A). However, when we co-transfected pCMV-ZXDC along with Gal4–CIITAΔN, activation of pG5-luc rose in a dose-dependant manner, confirming the association between CIITA and ZXDC proteins (Fig. 6A). Note that the ZXDC protein expressed from pCMV-ZXDC is not a fusion protein, but still activated the reporter plasmid when bound to Gal4–CIITAΔN. This result indicated that the ZXDC protein has a transcriptional activation domain.

The presence of an activation domain in the ZXDC protein was confirmed when we expressed a Gal4–ZXDC fusion with pG5-luc. By itself, Gal4–ZXDC activated expression threefold (Fig. 6B). When we included CIITA in the transfection, a dose-dependent increase in expression from pG5-luc was observed, demonstrating again ZXDC–CIITA binding (Fig. 6B). We mapped the activation domain of ZXDC by generating N-terminal truncations of the Gal4–ZXDC fusion protein (Fig. 6C). Activation levels were much greater in this experiment than in Fig. 6B, since the ratio of Gal4–ZXDC plasmid to reporter plasmid used in Fig. 6C was much higher. The activation domain of ZXDC was found to reside in amino acids 578–688, which are relatively rich in proline, serine, leucine and acidic residues. This region is immediately N-terminal of the CIITA-binding region of ZXDC (see below).

3.5. ZXDC binds to the region of CIITA containing leucine-rich repeats

To identify the region of CIITA to which ZXDC binds, we performed mammalian two-hybrid in HEK293 cells, with the Gal4–ZXDC fusion protein. We created the expression plasmid pVP-CIITA, containing amino acids 323–1130 of CIITA fused to the herpesvirus VP16 transcriptional activation domain (Cress and Triezenberg, 1991). The VP-CIITA fusion protein interacted with Gal4–ZXDC in mammalian two-hybrid assay, resulting in the activation of the reporter plasmid pG5-luc (Fig. 7B). We next subdivided the C-terminus of the CIITA protein into three regions, and made separate fusion proteins with VP16. The first region of CIITA consisted of amino acids 336–702, which contains the GTP-binding motifs (VP-GTP), and two of the four LXXL motifs present in CIITA (Boss and Jensen, 2003). The second region consisted of amino acids 708–955, containing two LXXL motifs [VP-(708/955)], and the third region amino acids 956–1130, which contains the C-terminal leucine-rich repeats of CIITA (VP-LRR).

Neither the VP-GTP nor VP-(708/955) fusion proteins activated the reporter plasmid, when expressed by themselves or with the Gal4–ZXDC fusion protein (Fig. 7B). Expression of both VP-GTP and VP-(708/955) proteins was confirmed by Western analysis (Fig. 7C). These results indicated that ZXDC does not interact with either of these two regions of CIITA protein. In contrast, we found that the VP-LRR fusion protein activated the reporter plasmid when co-expressed with Gal4–ZXDC. The activation was ~70% of that seen with VP-CIITA (Fig. 7B).

From this result, we conclude that ZXDC binds to the region of CIITA protein containing the leucine-rich repeats.

3.6. CIITA binds to the C-terminus of the ZXDC protein

The ZXDC cDNA fragment we cloned by yeast two-hybrid coded for the C-terminal 78 amino acids of ZXDC, which are therefore sufficient for interaction between ZXDC and CIITA. However, we wanted to determine if additional regions of the ZXDC protein contribute to CIITA–ZXDC interaction. To map the region of ZXDC to which CIITA binds, we performed mammalian two-hybrid in HEK293 cells with Gal4–ZXDC and CIITA. By employing N-terminal truncations of ZXDC, we demonstrated that the most robust binding between CIITA and ZXDC required the final 170 amino acids of ZXDC (amino acid positions 689–858; Fig. 8A). Binding of this fragment of ZXDC to CIITA was as efficient as the interaction between CIITA and full length ZXDC. A Gal4–ZXDC fusion containing the amino acids of ZXDC that were present in the yeast two-hybrid clone (amino acid positions 781–858) led to a reduction of approximately 40% in activation (Fig. 8A). We performed similar experiments with C-terminal truncations of ZXDC. ZXDC–CIITA interaction was reduced modestly with the deletion of amino acids 781–858 of ZXDC (Fig. 8B). A further deletion of amino acids 689–858 completely prevented ZXDC–CIITA binding (Fig. 8B). Thus, the region to which CIITA binds is the final 170 amino acids of ZXDC, from amino acid position 689 to 858, just C-terminal of the transcriptional activation domain of ZXDC. No other region of the ZXDC protein appears to participate directly in the binding between CIITA and ZXDC.

3.7. Structure–function analysis of the ZXDC protein

To begin to address the mechanism by which ZXDC regulates MHC genes, we wanted to determine which regions of the ZXDC protein are required for the activation of MHC class II genes. We tested first whether a ZXDC protein lacking the CIITA-binding domain (amino acids 689–858), but containing the zinc fingers, could activate the HLA-DRA promoter. This truncated form of ZXDC did not cooperate with CIITA in activating the HLA-DRA promoter, when co-transfected with pDRA-luc (construct 1; Fig. 9). Next, an N-terminal truncation of ZXDC which removed the zinc finger region, had approximately 50% of the activity of wild-type ZXDC, in cooperating with CIITA (construct 2; Fig. 9). Similarly, a ZXDC protein containing only the transcriptional activation and CIITA-binding domains had half the activity of wild-type ZXDC (construct 3; Fig. 9). Expression of just the CIITA-binding region of ZXDC, which lacks both the zinc fingers and the transcriptional activation domain, was essentially incapable of affecting CIITA function (construct 4; Fig. 9). All forms of the ZXDC protein were expressed at similar levels, as determined by Western analysis (data not presented). Taken together, these data suggest that the CIITA-binding domain of ZXDC is necessary, though not sufficient, for cooperation between CIITA and ZXDC. In addition, a truncated ZXDC protein that contained the CIITA-binding domain and activation domain of ZXDC could mediate significant cooperation with CIITA, in the activation of MHC II genes. Finally, the zinc fingers appeared to be necessary for full activity of ZXDC.

4. Discussion

In this report, we described the cloning and characterization of a cDNA coding for a novel CIITA-binding protein, zinc finger, X-linked, duplicated family member C. The evidence we presented supports the notion that ZXDC is an important player in the activation of MHC class I and class II genes by CIITA.

Northern analysis of ZXDC gene transcription indicated that the gene is expressed in a broad range of tissues, similar to the constituents of the DNA-binding complexes RFX and NFY that occupy MHC class II promoters. This is in contrast to CIITA, the “master regulator” of MHC

class II gene transcription, whose presence in a cell results in MHC class II transcription and therefore is strictly limited in its range of expression (van den Elsen et al., 2004). Western analysis with an anti-ZXDC antibody demonstrated that two major species of the protein are present in HEK293 cells: 91 and 149 kDa. Expression of the ZXDC cDNA resulted in the production of proteins with similar molecular masses and additionally a 72 kDa protein was detected. We have evidence that the 149 kDa species is the result of post-translational modification (data not presented), though how the 72 kDa form is produced is currently unknown. Nevertheless, since expression of our cDNA in cells results in proteins that are similar in size to endogenous ZXDC, we are confident that we have the full length cDNA.

The affect of ZXDC on CIITA-activated gene transcription was striking. When ZXDC expression was reduced in cells by RNA silencing, there was a significant decrease in the ability of CIITA to activate MHC class II gene transcription. Conversely, over-expression of ZXDC along with CIITA led to super-activation of both MHC class I and MHC class II genes. The cooperation between CIITA and ZXDC during activation of MHC class II genes required the presence of the C-terminal 170 amino acids of ZXDC. This region of ZXDC was defined via mammalian two-hybrid experiments as the CIITA-binding domain, strongly arguing for the necessity of interaction between the CIITA and ZXDC proteins. Taken with the observation that over-expression of ZXDC did not alter transcription from the RSV and CMV promoters (data not presented), it is very unlikely that the action of ZXDC represents a non-specific effect on gene transcription.

The question arises as to the mechanism by which ZXDC facilitates CIITA function. We propose two non-exclusive models. Firstly, since ZXDC has a transcriptional activation domain it may be that it has a direct affect on transcriptional initiation, cooperating with the potent activation domain of CIITA. This would require recruitment of ZXDC to MHC class II promoters. Given that the zinc fingers of ZXDC are partially dispensable for ZXDC activity, this may require direct binding between CIITA and ZXDC. A second possible mechanism, however, is suggested by the presence of 10 zinc fingers in ZXDC, motifs that are most commonly associated with DNA binding. It is possible that ZXDC may contribute to the tethering of CIITA to MHC class II promoters, or perhaps participate some way in the overall assembly of the MHC class II enhanceosome. Again, since the deletion of the zinc fingers from ZXDC led to an approximately 50% reduction in the ability of ZXDC to cooperate with CIITA, a proposed role of DNA binding in ZXDC function seems reasonable.

The second model poses an obvious question: where on MHC class II (and MHC class I) promoter DNA does ZXDC bind? Preliminary attempts to demonstrate binding of ZXDC to the W box, using both in vivo and in vitro techniques, were unsuccessful. Previous studies employing in vivo footprinting to characterize MHC class II gene promoter occupancy has generally shown protection of the X1, X2 and Y boxes within MHC class II promoters and a hypersensitive site at -150 of the HLA-DRA gene, upstream of the W box, though no direct protection of the W box itself (Kara and Glimcher, 1991, 1993; Wright and Ting, 1992). Interestingly, in RJ2.2.5 cells, a CIITA^{-/-} derivative of Raji cells, the hypersensitive site at -150 is missing, though the X1, X2 and Y box footprints are unchanged (Kara and Glimcher, 1991). It is tempting to speculate that when CIITA is not present at MHC class II promoters (as in RJ2.2.5 cells) ZXDC is also absent, resulting in the loss of the hypersensitive site at position -150 in RJ2.2.5 cells.

ZXDC binds to the region of CIITA that contains several leucine-rich repeats. These repeats mediate several important activities of CIITA, including nuclear localization, homodimerization and promoter targeting (Brown et al., 1998; Camacho-Carvajal et al., 2004; Hake et al., 2000; Linhoff et al., 2001; Sisk et al., 2001; Towey and Kelly, 2002). Preliminary studies in our lab have ruled out an affect of ZXDC on CIITA self-association and

nuclear accumulation (data not presented), though as mentioned above we propose that ZXDC may assist in tethering CIITA to MHC class II promoters. It has previously been reported that the leucine-rich repeats of CIITA were required for CIITA to co-immunoprecipitate a 33 kDa protein (Hake et al., 2000) though the identify of this protein has not yet been determined. Given the molecular mass of this unknown protein, it is almost certainly not ZXDC.

ZXDC is a novel co-factor for CIITA function. The presence of zinc fingers and a transcriptional activation domain in ZXDC suggests a model whereby ZXDC may assist in binding of CIITA to MHC class II promoters and/or may contribute its transcriptional activation domain towards activating MHC class II genes. ZXDC also regulates at least one other group of CIITA-activated genes, MHC class I, and may also regulate others. Based upon our findings, ZXDC is an important addition to the previously characterized X1, X2 and Y box-binding proteins that, along with CIITA, represent the critical components of the MHC class II enhanceosome.

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Fig. 1.

Schematic diagram of the 858 amino acid ZXDC protein. Open boxes, zinc fingers; black box, transcriptional activation domain (TAD); gray box, CIITA-binding domain. Region of amino acid homology with ZXDA and ZXDB (73% amino acid identity over approximately 500 amino acids) is indicated by dashed line. The amino acid sequences of the 10 zinc fingers in ZXDC are shown.

Fig. 2.

Expression of the ZXDC gene and cDNA. (A) Northern blot of poly-A⁺ mRNA, probed ZXDC cDNA exons 8–10, outside of the homology region with ZXDA and ZXDB (top panel) or with beta-actin (bottom panel). B, brain; C, colon; H, heart; K, kidney; Li, liver; Lu, lung; M, skeletal muscle; Pl, placenta; SI, small intestine; Sp, spleen; St, stomach; T, testis. (B) Western blot of HEK293 cell extracts from untransfected cells, or cells transfected or with pCMV-ZXDC. Blot was probed with affinity purified anti-ZXDC antiserum. Numerals represent molecular mass of bands, as determined by comparison with molecular mass standards. (C) Western blot of HEK293 cell extracts from untransfected cells with anti-ZXDC antiserum; longer exposure than in panel (B). (D) Western blot with anti-ZXDC antiserum of in vitro transcribed/translated ZXDC protein.

Fig. 3.

ZXDC cooperates with CIITA in activating MHC class I and class II transcription. (A) HEK293 cells were co-transfected with pCMV-CIITA and/or pCMV-ZXDC, along with the reporter plasmid pDRA-luc. pDRA-luc contains the promoter from the HLA-DRA gene (−150/+36) linked to the firefly luciferase gene. Numerals indicate nanograms of each plasmid DNA transfected (total plasmid DNA was kept constant). (B) Similar to (A) except RJ2.2.5 cells were transfected. Numerals indicate micrograms of each plasmid DNA transfected. (C) RJ2.2.5 cells were transfected with pCMV-CIITA and/or pCMV-ZXDC, and HLA-DRA gene transcription measured by quantitative real-time reverse transcriptase PCR. Numerals indicate micrograms of each plasmid DNA transfected. Data were normalized to beta-actin. (D)

Transfection was similar to (A) except that the reporter plasmid was pHLAB-luc, which contained the promoter from the HLA-B1 gene (−284/+1) linked to the firefly luciferase gene. Statistically significant differences in activation (by Student's *t*-test) are indicated. In all panels, error bars represent standard error of the mean of three independent experiments.

Fig. 4.

Silencing of ZXDC gene expression results in a reduction in MHC class II activation by CIITA. HEK293 cells were transfected with 1 mg of siRNA directed against ZXDC (or an siRNA with random sequence) followed by transfection with the indicated expression plasmids for ZXDC (700 ng) and/or CIITA (100 ng). (A) HLA-DRA gene expression, 24 h following transfection with plasmid DNA, measured by quantitative real-time RT-PCR. Error bars represent standard error of the mean of three independent experiments. (B) Western analysis with anti-ZXDC antiserum on protein lysates from HEK293 cells transfected with the indicated siRNA. Top panel was probed with anti-ZXDC and bottom panel was probed with anti-Hsp70 as a loading control.

Fig. 5.

Association between CIITA and ZXDC. (A) Co-immunoprecipitation of ZXDC and CIITA. HEK293 cells were transfected with pCMV-CIITA (which has an N-terminal HA-tag) or not transfected, and subjected to immunoprecipitation with anti-ZXDC (or pre-immune serum; data not presented). Western analysis was performed with anti-HA on immunoprecipitates (left panel) or lysates (right panel) to detect the presence of CIITA. (B) Western analysis was performed with anti-ZXDC on immunoprecipitates (left panel) or lysates (right panel) to detect the presence of ZXDC. (C) Chromatin immunoprecipitation with anti-ZXDC, anti-RNA polymerase II, anti-NFYA or non-specific antiserum. Co-immunoprecipitated HLA-DRA promoter DNA (filled bars) or GAPDH promoter DNA (open bars) was quantitated by real-time PCR. Error bars represent the standard error of the mean for three independent experiments.

Fig. 6.

Association of CIITA and ZXDC proteins. (A) Demonstration of ZXDC–CIITA association by mammalian two-hybrid assay. Gal4–CIITAΔN is a fusion between the Gal4 DNA-binding domain and amino acids 323–1130 of CIITA, which lacks the transcriptional activation domain of CIITA. Gal4–CIITAΔN was co-transfected with pG5-luc and increasing amounts of pCMV-ZXDC into HEK293 cells. (B) Gal4–ZXDC is a fusion of the Gal4 DNA-binding domain and the full length ZXDC cDNA. Gal4–ZXDC was co-transfected with pG5-luc and increasing amounts of pCMV-CIITA into HEK293 cells. Note that Gal4–ZXDC activates transcription in the absence of co-expressed CIITA, indicating the presence of a transcriptional activation domain in ZXDC. (C) The transcriptional activation domain of ZXDC was localized by

generating serial N-terminal truncations of ZXDC, and expressing them as fusion proteins with the Gal4 DNA-binding domain. The amino acids of ZXDC present in each fusion protein are indicated below the plasmid diagrams. The expression of the Gal4–ZXDC fusion proteins was confirmed by Western blot probed with anti-Gal4. The reporter plasmid for panels (B and C) was pG5-luc, containing five UASg sites linked to the firefly luciferase gene. In panels (B and C), error bars represent standard error of the mean of three independent experiments.

Fig. 7.

ZXDC binds to the region of CIITA containing leucine-rich repeats. (A) Schematic diagram of CIITA protein indicating conserved domains. AD, transcriptional activation domain; Walker-type GTP-binding motifs (black rectangles); LXXL motifs (open circles); LRR, leucine-rich repeats. Three fragments of the CIITA protein were expressed as fusion proteins with the transcriptional activation domain of herpesvirus VP16. Regions of CIITA protein fused to VP16 activation domain are indicated (see below). Diagram based upon Boss and Jensen (2003). (B) Mammalian two-hybrid transfection experiments in HEK293 cells to determine the ZXDC-binding region of CIITA. The VP-CIITA Δ N fusion protein contains amino acids 323–1130 of CIITA; VP-GTP consists of amino acids 336–702 of CIITA, which encompasses the GTP-binding motifs of CIITA; VP-(708–955) consists of amino acids 708–955 of CIITA, which lies between the GTP-binding domain and the leucine-rich repeats (LRR); VP-LRR consists of amino acids 956–1130 of CIITA, which contains the LRR. The reporter plasmid was pG5-luc, containing five UASg sites linked to the firefly luciferase gene. Numerals

indicate nanograms of each plasmid DNA transfected (total plasmid DNA was kept constant). The data are representative of four independent experiments. (C) Western analysis of transfected cells with anti-VP16 antiserum, to demonstrate expression of the VP16–CIITA fusion proteins.

Fig. 8.

CIITA binds to the C-terminal 170 amino acids of ZXDC. (A) N-terminal and (B) C-terminal truncations of ZXDC were created as Gal4 fusion proteins. Plasmids expressing the Gal4–ZXDC fusion proteins (100 ng) were co-transfected with, or without, pCMV-CIITA (600 ng) and the reporter plasmid pG5-luc (100 ng) in HEK293 cells. The amino acids of ZXDC present in each fusion protein are indicated below the plasmid diagrams. In both panels, error bars represent standard error of the mean of three independent experiments.

Fig. 9.

The zinc fingers of ZXDC are necessary for full cooperation between CIITA and ZXDC. Plasmids expressing the indicated fragments of ZXDC protein were co-transfected with, or without, pCMV-CIITA and the reporter plasmid pDRA-luc. On the ZXDC protein diagram, the black rectangle represents the 10 zinc fingers (ZnF), the hatched rectangle represents the transcriptional activation domain (AD) and the gray rectangle represents the CIITA-binding domain (CB). Error bars represent standard error of the mean of three independent experiments.