Published in final edited form as:
J Immunol. 2012 May 15; 188(10): 4951–4958. doi:10.4049/jimmunol.1103160.

NLRC5 cooperates with the RFX transcription factor complex to induce MHC class I gene expression[1]

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Abstract

Tight regulation of MHC class I gene expression is critical for CD8 T cell activation and host adaptive immune responses. The promoters of MHC class I genes contain a well-conserved core module, the W/S-X-Y motif, which assembles a nucleoprotein complex termed MHC-enhanceosome. A member of the NLR (nucleotide binding domain, leucin-rich repeat) protein family, NLRC5, is a newly identified transcriptional regulator of MHC class I genes. NLRC5 associates with and transactivates the proximal promoters of MHC class I genes, although the molecular mechanism of transactivation has not been understood. Here, we show that NLRC5-mediated MHC class I gene induction requires the W/S and X1, X2 *cis*-regulatory elements. The transcription factors RFX5, RFXAP and RFXANK/B, which compose the RFX protein complex and associate with the X1-box, cooperate with NLRC5 for MHC class I expression. Co-immunoprecipitation experiments revealed that NLRC5 specifically interacts with the RFX subunit RFXANK/B via its ankyrin repeats. In addition, we show that NLRC5 can cooperate with ATF1 and the transcriptional co-activators CBP/p300 and GCN5, which display histone acetyltransferase activity. Taken together, our data suggest that NLRC5 participates in an MHC class I specific enhanceosome, which assembles on the conserved W/S-X-Y core module of the MHC class I proximal promoters, including the RFX factor components and CREB/ATF1 family transcription factors to promote MHC class I gene expression.

Keywords

MHC class I; enhanceosome; CITA

[1]This work was supported by grants from the NIH and the Crohn's and Colitis Foundation of America. K.S.K. is a recipient of the Investigator Award from the Cancer Research Institute and the Claudia Adams Barr Award. T.B.M. has been a recipient of the EMBO Long Term fellowship and A.B. is a recipient of Crohn's Colitis Foundation of America fellowship.

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Disclosures
The authors have no financial conflict of interest.

Introduction

Major histocompatibility complex (MHC) class I and class II molecules are essential components of the mammalian adaptive immune system. MHC class I molecules present peptide antigens of intracellular origin such as viral or tumor antigens to CD8+ T cells, whereas MHC class II molecules present peptide antigens of extracellular sources such as bacterial antigens to CD4+ T cells (1, 2). MHC class I molecules are composed of MHC-encoded heavy chains and the non-polymorphic subunit β2-microglobulin (β2M) (3). Humans have three classical MHC class Ia molecules (HLA-A, HLA-B and HLA-C), as well as three non-classical MHC class Ib molecules (HLA-E, HLA-F and HLA-G), which have immune regulatory functions (4, 5). MHC class I peptides are mostly produced from the degradation of cytoplasmic proteins by the specialized "immunoproteasome" containing several IFN-γ-inducible subunits, such as LMP2 and LMP7 (6). Peptide loading onto MHC class I requires the peptide loading complex (PLC), which includes the MHC class I heavy chain, β2M, tapasin, ERp57, calreticulin and TAP1/TAP2, a transporter that translocates peptides from the cytoplasm into the ER (6, 7).

MHC class Ia is ubiquitously expressed in almost all nucleated cells, unlike MHC class II, which is found mainly in antigen-presenting cells (3, 8). Both MHC class I and class II, as well as β2M genes, are highly inducible by IFN-γ and share similar *cis*-regulatory elements in their promoters, termed W/S, X1, X2 and Y-box motifs, which are occupied by similar transcription factor complexes and are critical for transactivation of MHC class I and II genes (9–12). These transcription factors include the X1-box binding trimeric RFX protein complex (composed of RFX5, RFXAP and RFXANK/RFXB) (13–16), members of the X2-box binding CREB/ATF1 family of transcription factors (11, 17), and the Y-box binding NF-Y protein (composed of NF-YA, NF-YB and NF-YC) (18–20). Together, they form a macromolecular nucleoprotein complex called the MHC-enhanceosome (21).

Although the transcription factors directly associated with the W/S-X-Y motif of MHC gene promoters are critical, the formation of an active enhanceosome requires additional transactivators such as the class II transactivator (CIITA). CIITA, a member of the NLR or nucleotide binding domain (NBD), leucine rich repeat (LRR) family of proteins (22, 23), regulates the transcription of MHC class II by associating with the MHC-enhanceosome (21, 24). The expression of CIITA is induced in B cells and dendritic cells as a function of developmental stage and is inducible by IFN-γ or upon activation such as in human T cells (25–29). Therefore, CIITA is important for both constitutive and inducible expression of MHC class II and is referred to as a master regulator of MHC class II genes. In addition to MHC class II genes, CIITA also has a role in the transactivation of MHC class I genes at least *in vitro* (8–10, 20, 30, 31). However, while mutations in either CIITA, RFX5, RFXAP or RFXANK (or RFX-B) genes cause bare lymphocyte syndrome (BLS), an immunodeficiency characterized by the lack of MHC class II expression, BLS patients with mutations in CIITA but not in RFX genes retain MHC class I expression (32, 33). Similarly, in mice deficient for CIITA, both constitutive and IFN-γ-induced expression of MHC class I molecules are intact, indicating that there should be another mechanism for the activation of MHC class I *in vivo* (34–36). This niche has been largely filled by the recent finding that another NLR protein, NLRC5, can act as a transactivator of MHC class I genes (37). Similar to CIITA, NLRC5 is IFN-γ-inducible and can translocate into the nucleus due to its nuclear localization signal (NLS). NLRC5 associates with and transactivates MHC class I promoters (37). The expression of NLRC5, or class I transactivator (CITA), as opposed to CIITA, specifically upregulates the expression of MHC class I but not MHC class II genes (37). The NBD is a critical domain for the function of NLRC5, as NBD is required for both nuclear import and transactivation of MHC class I genes (38). In addition to MHC class I, NLRC5 induces the expression of β2M, TAP1 and LMP2 genes, indicating that NLRC5 is a key

transcriptional regulator of genes involved in the MHC class I antigen presentation pathway (37, 39).

Although the discovery of NLRC5 as an MHC class I transactivator and its similar function to CIITA are striking, the molecular mechanism by which NLRC5 transactivates MHC class I gene promoters has not been investigated. Here, we demonstrate that NLRC5 cooperates with components of the RFX factor complex, which assembles on the W/S-X-Y motif, to induce MHC class I gene expression. We show that NLRC5-mediated class I gene transactivation requires the X1 *cis*-regulatory element, the well-known RFX protein complex binding site. We also find that NLRC5 associates with the RFX subunit RFXANK/B through its ankyrin repeats. Furthermore, like CIITA, NLRC5 may also act as a platform for recruitment of histone acetyltransferase activities provided by the general co-activators CBP/p300, GCN5 and PCAF.

Materials and Methods

Cell lines and reagents

The SV40-transformed BLS patient fibroblast cell lines WSI (wild-type), ABI (RFXAP-deficient), OSE (RFX5-deficient), EBA (RFXANK/B-deficient), ATU (CIITA-deficient) as well as the teratocarinoma cell line Tera-2 were described previously (11, 38). Human embryonic kidney 293 (HEK293T) were purchased from ATCC (CRL-11268). All cell lines were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/ streptomycin (Gibco). Recombinant human IFN-γ was acquired from BioLegend and used at a concentration of 100 Units/ml.

Plasmid construction

Cloning of human GFP-NLRC5 and GFP-CIITA has been described previously (37). The cDNAs of the human RFX factors, RFX5, RFXANK/B, and RFXAP were subcloned into a modified pcDNA3.1 vector (Invitrogen) encoding for an N-terminal HA or RFP tag using standard molecular cloning techniques; The following primers were used: RFXAP, 5’-atatggatccGAGGCGCAGGGTGTAGCGGAG-3’ (forward), 5’-atatgtcgacTCACATTGATGTTCCTGGAAACTG-3’ (reverse); RFX5, 5’-atatatgcggccgcTGGCAGAAGATGAGCCTGATGC-3’ (forward), 5’-atattctagattATGGGGGTGTTGCTTTTGGGTC-3’ (reverse), RFXANK/B, 5’-atatggatccGAGCTTACCCAGCCTGCAGAAG-3’ (forward), 5’-atatctcgagTCACTCAGGGTCAGCGGGCACCAG-3’ (reverse).

The expression vectors pREP4-RFX5, pREP4-RFXANK/B, and pREP4-RFXAP were used as templates (11). For the construction of RFXANK/B deletion mutants the following primers were used: RFXANK/B-ΔC, 5’-atatctcgagTTAGTCGGCTTCGGTGGTGAGGTC-3’ (reverse); RFXANK/B-ΔANK, 5’-atatctcgagTTACTCGTCTGGCTTGTTGACGAG-3’ (reverse); RFXANK/B-ΔPEST, 5’-atatggatccCAGGCAGGCAGCTCCCTGAAG-3’ (forward). All plasmids were verified by sequencing analysis (DFCI molecular biology core facilities).

Transfection and luciferase assay

Cells were transiently transfected using PEI (1 mg/ml, pH 7.2 polyethylenimine, Polysciences, Inc.) at a ratio of DNA:PEI of 1:3–4, or using FuGENE 6 Transfection Reagent (Roche) in serum-free media, following the manufacturer’s instructions. For Western blot and immunoprecipitation experiments 5×10^5 cells were seeded in 2 ml medium into 6 wells, and a total of 3 μg/well of DNA was used per transfection. Medium was changed the following day and cell extracts were prepared 48 hrs post transfection. For

co-immunoprecipitation experiments, cells were first transfected with expression vectors for NLRC5 and the following day with plasmids encoding components of the RFX factor complex, to allow for similar expression levels.

For luciferase assays, cells were split at a density of 2×10^4 / 0.5 ml into 24-wells one day prior to transfection. Unless stated otherwise, cells were co-transfected with 50 ng of either GFP, GFP-NLRC5, GFP-CIITA expression plasmid and 25 ng of the indicated luciferase reporter constructs. 25 ng per well of reporterless Renilla plasmid was included to allow for normalization of transfection efficiency. Cells were harvested 48 hrs post transfection, and cell lysates were analysed using the dual-luciferase reporter assay system (Promega), following the manufacturer's protocol. Unless stated otherwise, experiments were performed in duplicates, repeated at least twice, and results are given as the mean ± SD. The MHC class I reporter gene constructs as well as the expression plasmids encoding ATF1, CBP, p300, GCN5, and PCAF have been described previously (11, 31, 38, 39).

SDS-PAGE and immunoblotting

Whole cell extracts were prepared using 1x Cell Lysis Buffer (Cell Signaling) supplemented with 1 mM DTT, and 1 mM PMSF, prior to extraction and centrifugation of whole cell lysates. Protein concentration was determined using the Bradford protein assay according to manufacturer's instructions (Bio-Rad). Cell extracts were subjected to SDS-polyacrylamide gel electrophoresis using 4–12% gradient gels (Invitrogen). Gels were transferred to nitrocellulose membranes (Amersham HyBond ECL) for at least 2 hrs at 80V. Membranes were blocked for 1 hour in 5% BSA in Tris-buffered saline–Tween (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20). The following antibodies were used for protein detection: anti-HA (16B12, Covance), anti-β2M (2M2, BioLegend), anti-GFP (JL-8 Clontech), anti-β-actin (I-19, Santa Cruz), and anti-α-Tubulin (TU-02, Santa Cruz). The antibody against MHC class I heavy chain (3B10.7) is a kind gift of Dr. P. Cresswell (Yale University). The following horseradish peroxidase (HRP)-conjugated secondary antibodies were used: anti-mouse IgG and anti-rabbit IgG (GE Healthcare), anti-goat IgG (Santa Cruz) and anti-rat IgG2a (Alpha Diagnostics). Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and imaged using the Molecular Imager ChemiDoc XRS+ System (Bio-Rad) or exposed to autoradiography film (Denville). Quantification was performed using ImageQuant (Molecular Dynamics).

Immunoprecipitation

Immunoprecipitation of the HA-tagged RFX subunits and RFXANK/B deletion mutants was performed on HEK293T cell lysates 48 hrs post transfection using an anti-HA antibody (16B12, Covance). After rotating samples at 4°C overnight, Protein A/G UltraLink Resin (Thermo Scientific, Rockford, IL) was added to each tube, and rotated at 4°C for 3 hrs. The beads were washed three times sequentially in cell lysis buffer (Cell Signaling) and washing buffer (20 mM Tris-HCl (pH 7.4), 0.1% Nonidet P-40) and subsequently samples were boiled for 10 min in 20 μl of loading buffer and subjected to SDS/PAGE and immunoblot analysis. Co-immunoprecipitated GFP-NLRC5 was detected by Western blot analysis using an anti-GFP antibody.

Quantitative real-time PCR (qPCR) analysis

RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The integrity of isolated RNA was verified by 1% agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μg RNA using the qScript Flex cDNA synthesis kit (Quanta Biosciences), and RNA expression was quantified on the 7300 Real-Time PCR System (Applied Biosystems) using the PerfeCTa SYBR Green SuperMix with ROX (Quanta Biosciences). The following primers were used for amplification: HLA-A, 5'-

AAAAGGAGGGAGTTACACTCAGG-3' (forward), 5'-GCTGTGAGGGACACATCAGAG-3' (reverse); GAPDH, 5'-GAAGGTGAAGGTCGGAGT-3' (forward), 5'-GAAGATGGTGATGGGATTTC-3' (reverse). For analysis of the transiently reconstituted RFXANK/B-deficient cell line EBA, cells were sorted for GFP+, RFP+ or double positive cell populations using a Beckman Coulter MoFlo FACS sorter (DFCI Core facilities).

Statistical Analysis

All experiments were repeated twice or more and data were subjected to Student's *t* test for analysis of statistical significance using Prism (GraphPad). Results are given as the mean ± SD. A *P*-value of < 0.05 was considered to be significant.

Results

A functional W/S-X-Y motif is required for NLRC5-mediated MHC class I transactivation

The proximal promoter region of MHC class I genes contains a well-characterized core module consisting of the W/S-X-Y motif. In order to determine which *cis*-regulatory elements are required for NLRC5-mediated MHC class I gene transactivation, we performed promoter assays using luciferase reporter constructs driven by the HLA-B promoter. An expression vector for NLRC5 was transfected into HEK293T cells together with a reporter gene construct containing the immediate upstream region of the HLA-B gene, and reporter gene activity was compared to that of mutant versions of the HLA-B promoter harboring mutations in the W/S, the X1, or the X2 box (Fig 1*A*). As control, CIITA and empty GFP expression vectors were transfected and MHC class I promoter activity was compared. As shown in Fig. 1*B*, NLRC5 induces the transactivation of the wild-type HLA-B promoter to a similar extent to that of CIITA, as previously shown (37). Interestingly, mutation of the W/S box *cis*-regulatory element (mW/S) specifically abrogated NLRC5-induced HLA-B promoter activity but left CIITA-driven MHC class I transactivation intact. In contrast, mutations of either half of the X box (mX1, mX2), which are known to bind to the trimeric RFX factor complex (for X1 box) and the CREB/ATF1 family transcription factors (for X2 box), abolished both NLRC5 and CIITA-induced HLA-B gene transactivation.

In a different set of experiments, we used deletion mutants of the HLA-B promoter and observed that NLRC5 can induce MHC class I expression even in the absence of the Enhancer A and ISRE *cis*-regulatory elements, which are conserved in the proximal promoters of the classical MHC class I genes (Fig. 2, B190: ΔEnhancer A; B170: ΔEnhancer A, ΔISRE). Again, deletion of the W/S box selectively abolished NLRC5-induced MHC class I induction, whereas CIITA-mediated activity remained intact (B140: ΔEnhancer A, ΔISRE and ΔW/S). Taken together, NLRC5-mediated MHC class I gene activation requires the conserved X1 and X2 box, as well as the W/S motif, which is dispensable for CIITA-mediated MHC class I gene activation.

NLRC5 cooperates with ATF1 and the transcriptional co-activators CBP, p300, and GCN5

Next, we examined whether NLRC5 cooperates with other transcriptional regulators involved in MHC class I transactivation. It has been well established that CIITA interacts with other transcriptional co-regulators and is able to recruit chromatin modifying enzymes, such as histone acetyltransferases, to the MHC promoters. In order to compare the impact of NLRC5-mediated MHC class I expression to previously reported data for CIITA (11), we used the well characterized teratocarcinoma cell line Tera-2. Both NLRC5 and CIITA activated the HLA-B promoter in this cell line to a similar extent (Fig. 3). In agreement to what has been reported for CIITA(11), we observed a synergistic induction of the HLA-B promoter with NLRC5 and the X2 box binding protein ATF1, a member of the CREB

family of transcriptional activators. Similarly, co-expression of NLRC5 and the transcriptional co-activators CBP (CREB-binding protein), p300, GCN5 (Fig. 3) and PCAF (data not shown) resulted in enhanced reporter activity, comparable to that seen for CIITA (Fig. 3). Overexpression of the individual co-factors had no significant impact on the HLA-B promoter, probably because they are constitutively expressed in most cell types. In summary, similar to CIITA, NLRC5 cooperates with the X2-box binding factor ATF1 and the transcriptional co-activators CBP/p300, GCN5 and PCAF, which all display histone acetyltransferase activity, to transactivate MHC class I genes.

NLRC5 cooperates with the RFX factor complex to promote MHC class I expression

We have previously demonstrated by chromatin immunoprecipitation experiments that NLRC5 specifically binds to the proximal promoter of MHC class I genes (37). The observation that NLRC5-mediated induction of the HLA-B promoter required the X1 box, the binding site for the RFX factor complex, prompted us to investigate the role of the RFX factor components in this process. In order to dissect the role of each RFX transcription factor for NLRC5-mediated MHC class I transactivation, we used wild-type (WSI), RFX5-deficient (OSE), RFXAP-deficient (ABI) and RFXANK/B-deficient (EBA) fibroblasts derived from BLS patients. These cell lines have previously been characterized for their levels of constitutive and inducible MHC class I and β2M expression (40–42). We confirmed reduced MHC class I expression by qPCR analysis using primers specific for HLA-A and on the protein level by Western blot analysis using a pan-HLA(Hc) antibody (Supplemental Fig. 1*A* and *B*). Our results confirm previous data that MHC class I expression is reduced in all three cell lines investigated, OSE, ABI, EBA, when compared to wild-type cells (WSI). Next, we assessed whether NLRC5-mediated MHC class I induction requires individual components of the RFX complex using these cell lines. In line with previous observations (11), reconstitution of the RFXAP-deficient cell line (ABI) with an expression plasmid encoding RFXAP resulted in increased reporter gene activity of the HLA-B promoter (Fig. 4A). Similarly, transfection of NLRC5 into the same cell line induced the HLA-B promoter, although only to a moderate extent. RFXAP/NLRC5 co-transfection into the RFXAP-deficient cell line, however, resulted in a synergistic activation of the reporter gene construct. This synergy was also detected on the protein level by Western blot for MHC class I and β2M (Fig. 4*B*). While RFXAP transfection into the ABI cell line alone did not change MHC class I expression levels to a noticeable extent, co-transfection of RFXAP together with NLRC5 significantly increased MHC class I and β2M protein levels. A similar synergy was also observed when CIITA was co-transfected with RFXAP into the ABI cell line (Fig. 4*B*).

Next, we examined whether the RFX component RFX5 is essential for NLRC5-induced MHC class I expression by reconstituting the RFX5-deficient cell line OSE with RFX5 in the presence or absence of NLRC5. Again, we observed a synergistic activation of the HLA-B reporter when both components were transfected into the OSE cell line together with the HLA-B reporter gene construct (Fig. 4*C*) and on the protein level using anti-MHC class I (Hc) and anti-β2M antibodies (Fig. 4*D*). Yet still, both NLRC5 as well as CIITA can induce MHC class I expression even in the absence of RFX5 (Fig. 4C, D), suggesting a possible redundancy among the RFX factor components.

A similar set of experiments using the RFXANK/B-deficient cell line (EBA) revealed that efficient induction of NLRC5-mediated MHC class I expression requires the presence of the RFX factor component RFXANK/B (Fig. 5). We observed a strong synergy between NLRC5 and RFXANK/B in the HLA-B reporter gene assay while expression of RFXANK/B or NLRC5 alone activated the MHC class I promoter poorly (Fig. 5*A*). Only upon co-transfection of RFXANK/B together with NLRC5 or CIITA did we observe an efficient MHC class I promoter activation (Fig. 5*A,B*). Cooperative induction of MHC class I genes

by RFXANK/B and NLRC5 was confirmed both at the RNA level by qPCR for HLA-A and HLA-B, and at the protein level by Western blot analysis using the anti-MHC class I (Hc) antibody (Fig 5*C, D*). Together, these results support the view that the RFX component RFXANK/B is important for efficient NLRC5-induced transactivation of MHC class I genes.

NLRC5 specifically binds to the RFX component RFXANK/B via its ankyrin repeats

The requirement for the X1 box in NLRC5-induced MHC class I induction and synergy between NLRC5 and the RFX proteins prompted us to investigate whether NLRC5 can directly associate with RFX factor components. An expression vector for GFP-NLRC5 was co-transfected with expression vectors for HA-tagged RFX5, RFXAP or RFXANK/B into HEK293T cells. RFX proteins were immunoprecipitated with an anti-HA-antibody and associated GFP-NLRC5 was detected by Western blot. Interestingly, we only observed an enrichment of GFP-NLRC5 above background levels in the immunoprecipitates of HA-RFXANK/B, not in the immunoprecipitates of RFX5 and RFXAP (Fig. 6).

To characterize which domain of RFXANK/B is required for binding to NLRC5, we performed co-immunoprecipitation experiments with RFXANK/B deletion mutants lacking the following domains: RFXANK/B-ΔPEST (aa 62–260, lacking the N-terminal PEST domain), RFXANK/B-ΔANK (aa 1–122, C-terminal truncation of the ankyrin repeats) and RFXANK/B-ΔC (aa 1–221, lacking the very C-terminus) (Fig. 7*A*). The HA-tagged RFXANK/B deletion mutants were co-expressed with GFP-tagged NLRC5 in HEK293T cells. Binding of NLRC5 was retained even in the absence of the very C-terminal portion of RFXANK/B and did not require the presence of the N-terminal PEST (Proline, Glutamate, Serine, Threonine-rich) domain. In contrast, deletion of the ankyrin repeats (ΔANK), which comprise well-known protein-protein interaction modules, resulted in loss of NLRC5 binding (Fig. 7*B*). These results support a role of the ankyrin-repeats of RFXANK/B in recruiting NLRC5 to the RFX factor complex that assembles on the X1 *cis*-regulatory element in the MHC-class I gene promoters.

Discussion

Although CIITA can transactivate both MHC class I and class II promoters in *in vitro* experiments, the contribution of CIITA to the regulation of MHC class I gene expression *in vivo* remained unclear. It has been observed previously that loss of CIITA in BLS patients and CIITA-deficient mice does not seem to significantly affect the expression levels of MHC class I genes (32–36). The recent identification of NLRC5 as an MHC class I transactivator (CITA) significantly improved our understanding of the regulation of MHC class I genes (37). The molecular mechanism how NLRC5 transactivates the promoters of MHC class I and functionally related genes, however, had not been investigated in greater detail. Similar to CIITA, NLRC5 is lacking a known DNA-binding domain (37). NLRC5 will thus rely on other transcription factors, which directly associate with MHC class I promoters to perform its function as an MHC class I transactivator. Regulation of both MHC class I and class II genes depends on the W/S-X-Y motif, which contains conserved *cis*–regulatory elements found in their proximal promoters (9, 10). These *cis*-regulatory elements are constitutively occupied by the RFX transcription factor complex, members of the CREB/ATF1 family and the NF-Y transcription factor complex. Together, they form a stable DNA-protein complex, which remains inactive without the expression of an additional transactivator such as CIITA (43). In this study, we demonstrate that NLRC5 also requires a functional W/S-X-Y motif for an efficient transactivation of MHC class I genes. Reporter gene assays showed that the W/S, X1 and X2 box are essential for NLRC5-mediated MHC class I transactivation. In line with this observation, NLRC5 can cooperate with the X1 box binding transcription factor RFX and the X2 box binding transcription factor ATF1 in MHC

class I promoter activation. In addition, we demonstrate that NLRC5 specifically associates with the RFX subunit RFXANK/B via its ankyrin repeats. Taken together, our findings suggest that the requirement of NLRC5 for the X-box found in the MHC class I promoters is similar to that of CIITA. Both molecules cooperate with RFX proteins and ATF1/CREB family members bound to the X1 and X2 box to transactivate MHC class I genes.

Interestingly, we observed that NLRC5 interacts with the RFX component RFXANK/B but not with other RFX proteins, suggesting that RFXANK/B might assist in the recruitment of NLRC5 to the MHC class I promoter. Similarly, binding of CIITA to RFXANK/B through the ankyrin repeats has been reported previously (44). However, CIITA has been found to interact with all three components of the RFX complex, although CIITA can associate with the RFX complex more strongly when all three subunits exist (44–47). While it is still possible that NLRC5 may associate with all individual RFX proteins under less stringent condition, cooperative binding to the RFX factors is an alternative possibility; NLRC5 might be recruited and associate more tightly with the RFX complex in the presence of all three components, RFXANK/B, RFX5, and RFXAP together, as seen in the association between CIITA and the RFX complex. Curiously, we were unable to determine which domain of NLRC5 is interacting with RFXANK/B, as deletion of any domain of NLRC5 abolished binding to RFXANK/B (data not shown). These findings suggest that the overall conformational integrity of NLRC5 might be required for a robust interaction with the RFXANK/B subunit of the RFX complex.

Another interesting difference between NLRC5 and CIITA-induced MHC class I induction is that NLRC5 requires the W/S box for efficient transactivation (Fig. 1 and 2) (11). The role of the W-box, which inside contains the highly conserved S-box, is still not well understood compared to well-established roles of the X1-, X2- and Y-box. According to one study, the W/S-box might serve as a binding site for a second RFX complex (48). Alternatively the W/S-box has been suggested to represent a binding site for an as yet unidentified factor(s) (48, 49). It has previously been shown that recruitment of CIITA to the MHC-enhanceosome on MHC class II promoters requires both the conserved W/S box and a stringent spacing between the W/S and X boxes (49). By analogy, the W/S-box in MHC class I promoters may be important for the recruitment of NLRC5 to the MHC-enhanceosome on MHC class I promoters. Again, the fact that NLRC5 does not contain a discernable DNA binding domain makes it unlikely that NLRC5 directly binds to the W/S box, although this hypothesis has not been tested experimentally. Instead, it is more likely that NLRC5 will associate with the as yet unknown DNA-binding protein bound to the W/S-box and this interaction might prove pivotal for the recruitment and interaction of NLRC5 with the MHC-enhanceosome on the MHC class I promoter.

As previously shown and confirmed in this report, CIITA can transactivate MHC class I promoters at least *in vitro* (11, 30, 31, 37). It is an interesting question, therefore, whether NLRC5 requires the presence of CIITA to transactivate MHC class I promoters, possibly by forming a NLRC5-CIITA complex. However, our observation that NLRC5 alone can induce MHC class I expression in cell lines that do not express CIITA, such as HEK293T cells, suggests that this scenario is less likely (Fig. 1 and 2) (37). Moreover, expression of NLRC5 in a CIITA-deficient fibroblast cell line (ATU cells) can still activate MHC class I promoters and induce MHC class I gene expression (data not shown). These findings support the notion that NLRC5 acts as a CITA independently of CIITA to induce MHC class I gene expression.

We propose the following model describing the molecular composition of the NLRC5/CITA enhanceosome, which is required for the transactivation of MHC class I and related genes (Fig. 8). Similar to CIITA, NLRC5 relies on the presence of the X1-box-bound RFX factor

complex, which is composed of the RFX5, RFXAP and RFXANK/B subunits, for efficient MHC class I gene induction. NLRC5 also synergizes with additional transcription factors of the CREB/ATF1 family bound to the X2-box. Moreover, NLRC5 strictly requires the W/S-box, although the factor binding to the W/S box remains unknown. Additional *cis*-regulatory elements (Enhancer A, ISRE) in the MHC class I promoter also modulate MHC class I promoter activity. Once the CITA nhanceosome is established on the MHC class I proximal promoter, NLRC5 may recruit other transcriptional co-activators such as CBP/p300, GCN5 and PCAF to promote the transcription of MHC class I genes.

In summary, we have demonstrated that NLRC5 is a crucial component of an MHC class I enhanceosome, which assembles on the conserved W/S-X-Y motif. This CITA enhanceosome is specific to MHC class I gene promoters. Given the important roles of MHC class I genes in immunity, further characterization of the CITA enhanceosome may lead to the development of therapeutics beneficial in anti-viral treatment, tumor immunity and transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Peter Cresswell for providing reagents; Jonathan Kagan for helpful discussions; Andrea Dearth for technical assistance.

Abbreviations used in this paper

NLR proteins	nucleotide binding domain (NBD) leucine rich repeat (LRR) containing proteins
ER	endoplasmic reticulum
β2M	β2-microglobulin
LMP	large multifunctional protease
Ii	invariant chain
NLS	nuclear localization signal
RFX	regulatory factor X
ATF1	activating transcription factor 1
NF-Y	nuclear factor-Y
PCAF	P300/CBP-associated factor
GCN5	general control nonderepressible 5
HAT	histone acetyltransferase

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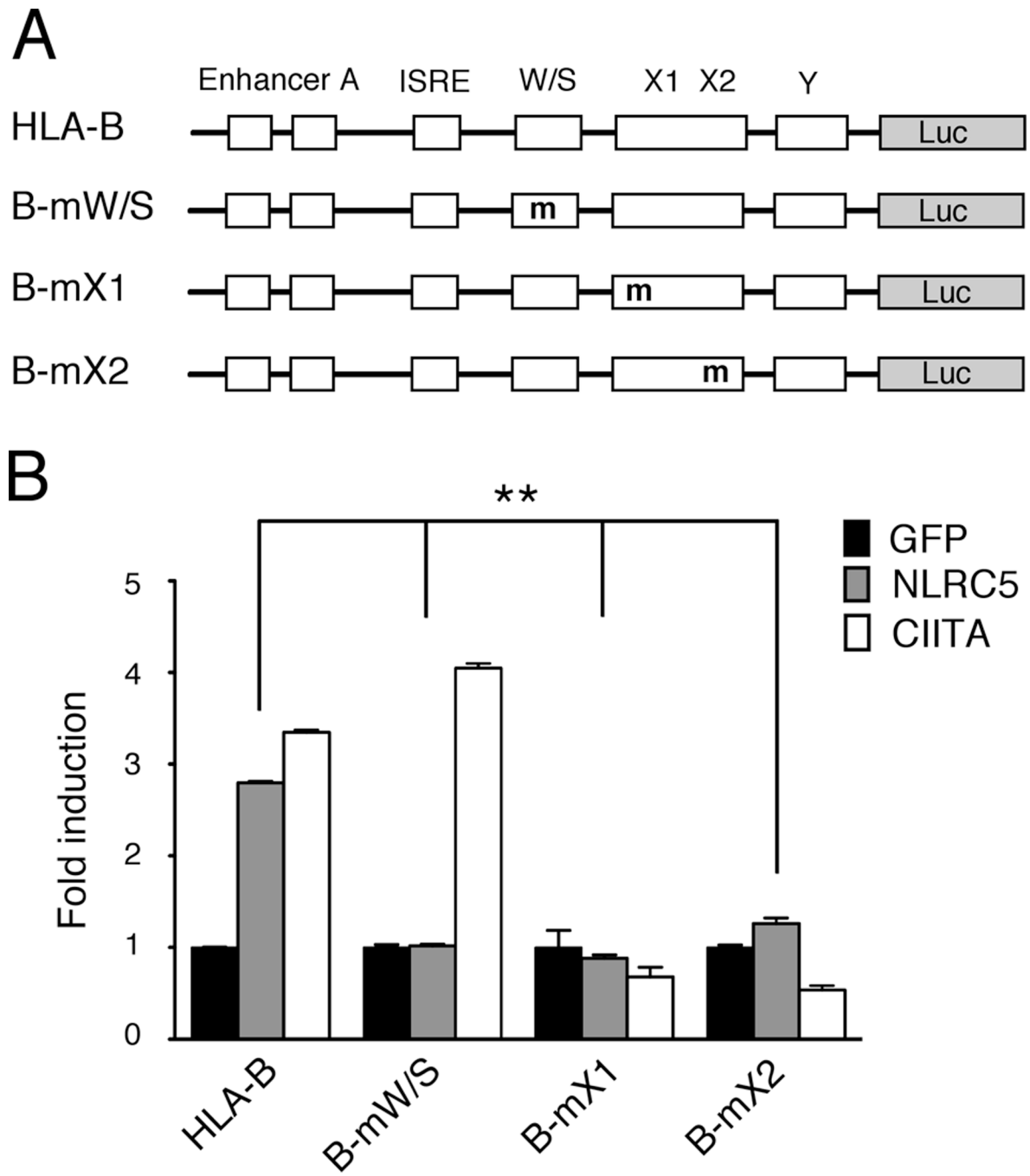

FIGURE 1. NLRC5-mediated transactivation of the HLA-B promoter requires the W/S and X box *cis*-regulatory elements

A, Schematic representation of the HLA-B luciferase reporter construct and the indicated mutant versions used in this study. Mutated *cis*-regulatory elements are marked (m). *B*, Reporter gene analysis of the HLA-B promoter. HEK293T cells were transiently transfected with either expression vectors for GFP (black bar), GFP-NLRC5 (grey bar), or GFP-CIITA (white bar), along with the indicated HLA-B luciferase reporter constructs. Cell lysates were analyzed 48 hrs post transfection by dual-luciferase assay. Data are a representative of several independent experiments performed in duplicates and are plotted as fold induction with respect to the GFP control vector. Error bars represent ± SD. *$p < 0.05$; **$p < 0.01$.

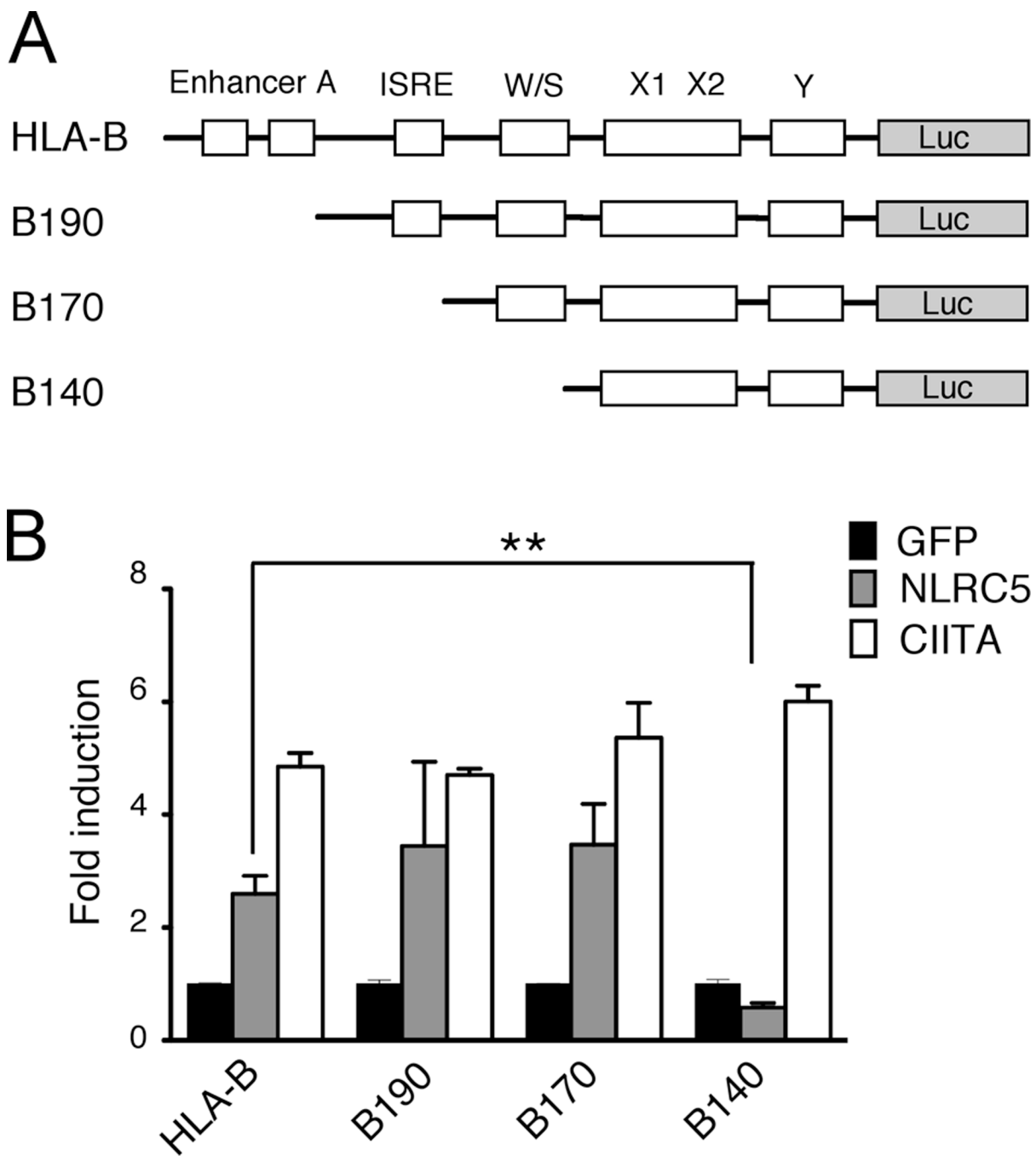

FIGURE 2. HLA-B transactivation by NLRC5 occurs in the absence of the Enhancer A and ISRE

A, Schematic representation of the HLA-B promoter deletion mutants used in this study. B190 (ΔEnhancer A), B170 (ΔEnhancer A, ΔISRE), B140 (ΔEnhancer A, ΔISRE, ΔW/S). *B*, NLRC5-mediated transactivation of the HLA-B promoter and the indicated promoter mutants (grey bar) is compared to empty vector control (GFP, black bar), and CIITA (white bar). Transiently transfected HEK293T cells were analyzed 48 hrs post transfection by dual-luciferase assay. The data was plotted as fold induction relative to the empty GFP expression vector control as the mean of duplicates from one representative experiment. The experiment was repeated twice. Error bars are given as ± SD. *$p < 0.05$; **$p < 0.01$.

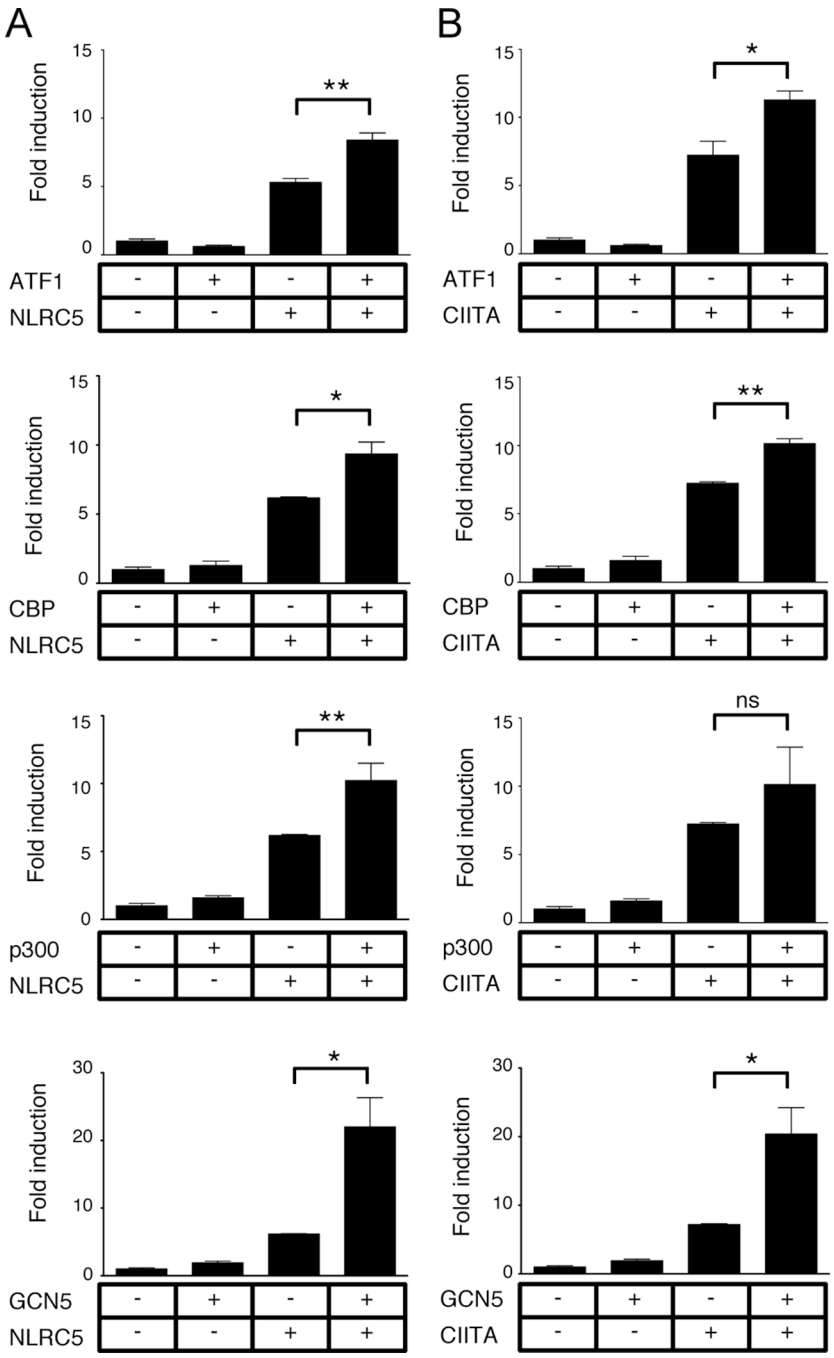

FIGURE 3. NLRC5 cooperates with ATF1 and the transcriptional co-activators CBP/p300, and GCN5

Transactivation of the HLA-B promoter by NLRC5 (*A*) or CIITA (*B*) with co-expression of ATF1 and the transcriptional co-activators CBP, p300 and GCN5 was examined. Tera-2 cells were transiently transfected with either empty control vector or the indicated expression plasmids and cells were harvested 48 hrs post transfection. Cell lysates were analyzed using the dual-luciferase assay and normalized against Renilla firefly activity. Data are a representative of several independent experiments performed in duplicates and are plotted as fold induction with respect to the control vector. Error bars represent ± SD. ns, not significant; *$p < 0.05$; **$p < 0.01$.

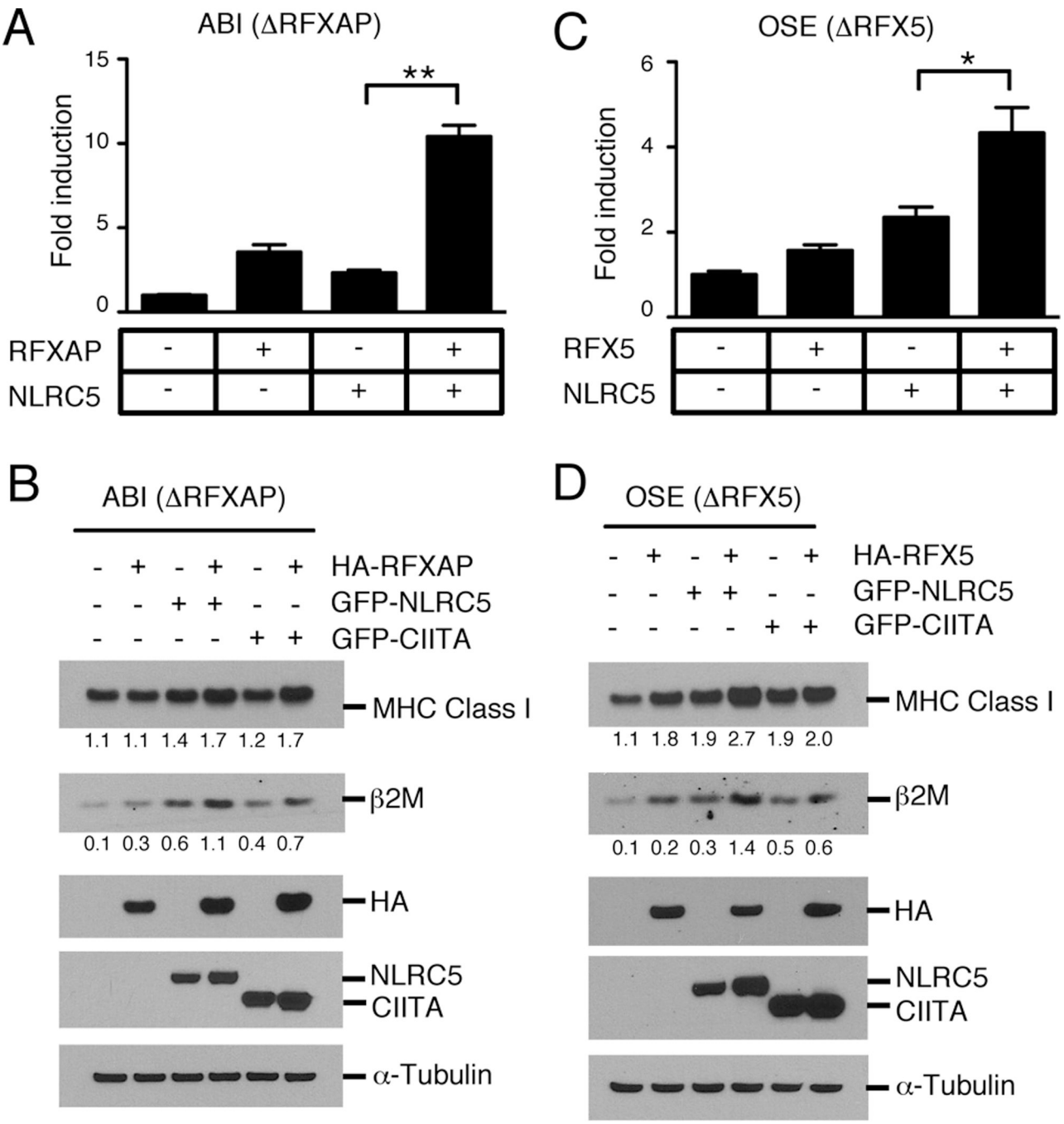

FIGURE 4. NLRC5 cooperates with the RFX components RFXAP and RFX5 for the induction of MHC class I

(*A, C*) Analysis of HLA-B reporter activity in human fibroblast cell lines derived from BLS patients. ABI (RFXAP deficient) or OSE (RFX5 deficient) fibroblasts were used in (A) and (C), respectively. The indicated expression plasmids were co-transfected with the HLA-B promoter reporter gene construct, and luciferase activity was measured 48 hrs post transfection using the dual-luciferase assay. The data was plotted as fold induction relative to the empty vector control as the mean of triplicates from one representative experiment out of several independent experiments. Error bars are given as ±SD. *$p < 0.05$; **$p < 0.01$. (*B, D*) Cell extracts of the indicated BLS patient cell line, transiently transfected with the

specified expression plasmids, were examined for MHC class I and β2M protein expression by immunoblotting 48 hrs post transfection. Signal intensities were quantified using ImageQuant. Expression levels of α-Tubulin levels are presented as a loading control.

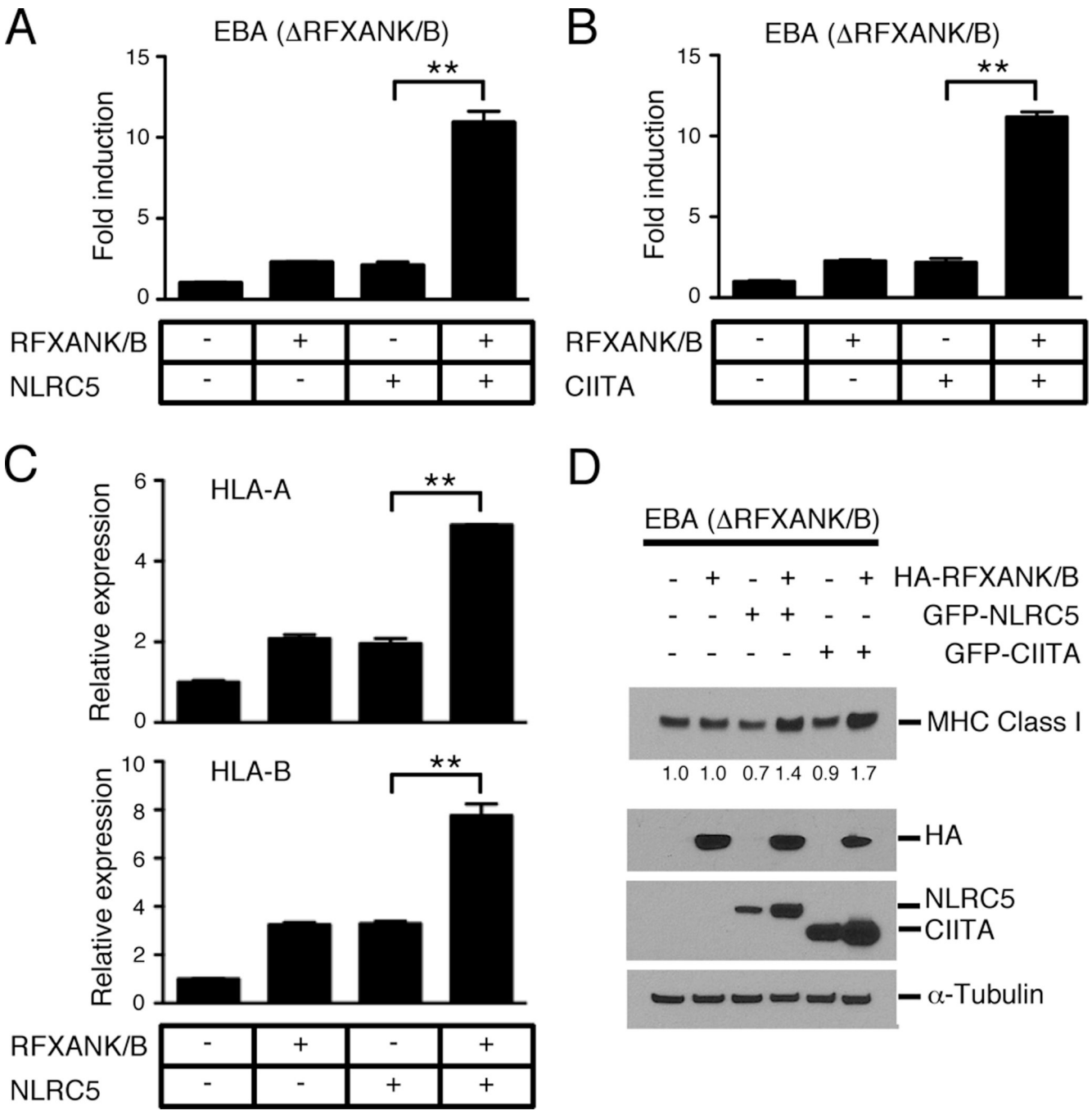

FIGURE 5. RFXANK/B is required for efficient MHC class I induction by NLRC5

HLA-B reporter gene activity induced by either NLRC5 (*A*) or CIITA (*B*) in the RFXANK/B-deficient cell line EBA. The indicated expression plasmids were co-transfected together with the HLA-B promoter reporter gene construct, and luciferase activity was determined 48 hrs post transfection using the dual-luciferase assay. Data are a representative of several independent experiments performed in triplicates, and are plotted as fold induction with respect to the empty vector control. Error bars represent ± SD. *$p < 0.05$; **$p < 0.01$. (*C*) Analysis of MHC class I gene expression in the RFXANK/B-deficient cell line EBA using gene specific primers for HLA-A and HLA-B. Cells were reconstituted with either RFP-RFXANK/B, GFP-NLRC5, or both expression plasmids and sorted for GFP+, RFP+ or

double positive cell population 48 hrs after transfection prior to RNA isolation. Error bars represent ± SD of duplicates. *$p < 0.05$; **$p < 0.01$. (*D*) MHC class I protein expression in RFXANK/B-deficient fibroblasts, transiently transfected with the indicated expression plasmids. Cell lysates were prepared 48 hrs post transfection and analysed by Western blotting. α-Tubulin levels are shown as a loading control.

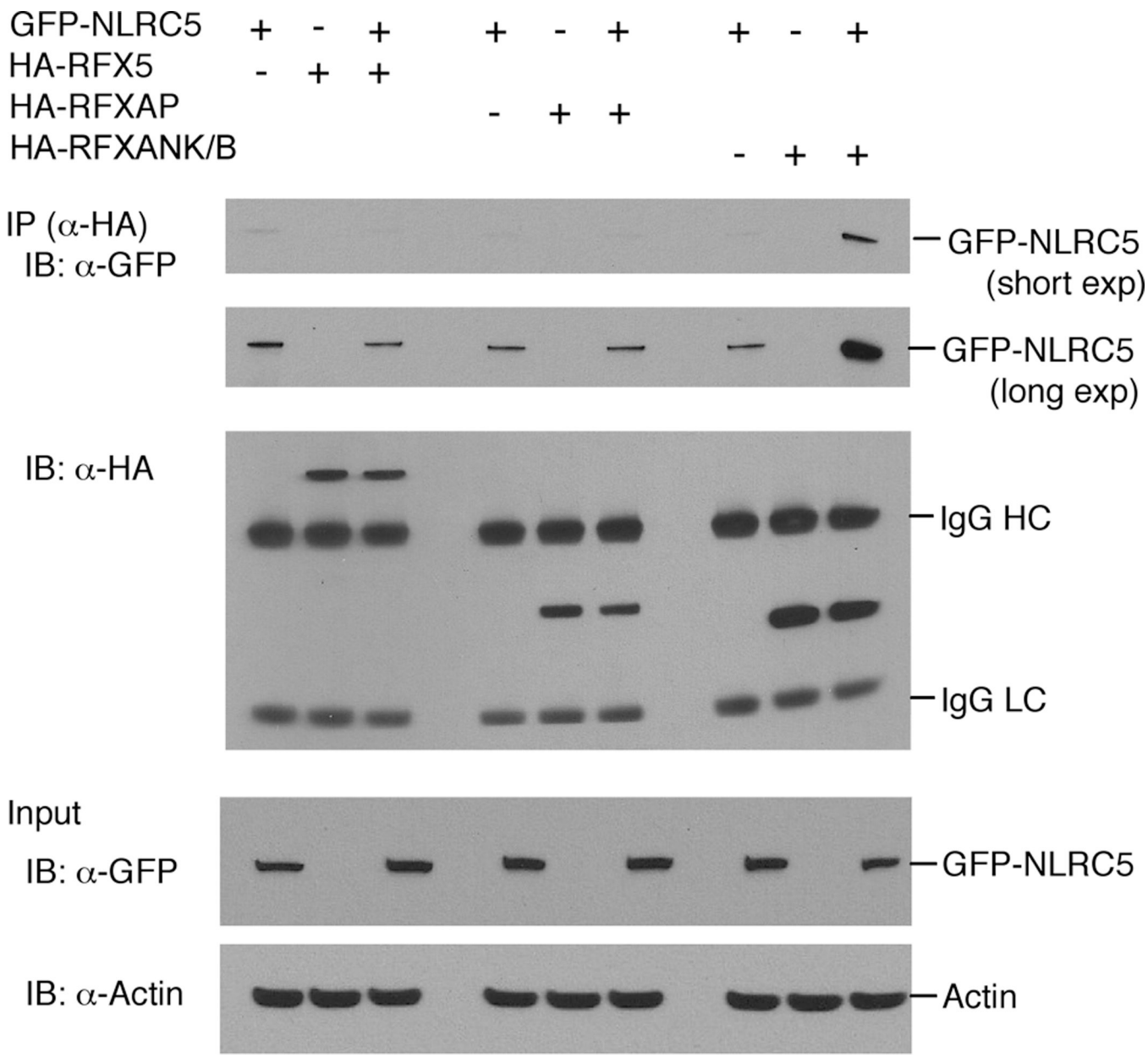

FIGURE 6. Specific binding of NLRC5 to the RFX component RFXANK/B

Association of NLRC5 with components of the RFX factor complex was analysed by co-immunoprecipitation. A GFP-NLRC5 expression plasmid was co-transfected with expression vectors for HA-tagged RFX5, RFXAP or RFXANK/B into HEK293T cells. Cell extracts were prepared 48 hrs post transfection. RFX proteins were immunoprecipitated using anti-HA antibody and associated GFP-NLRC5 was detected using an anti-GFP antibody. The blots shown are a representative of three independent experiments. Short exp; short exposure, long exp; long exposure.

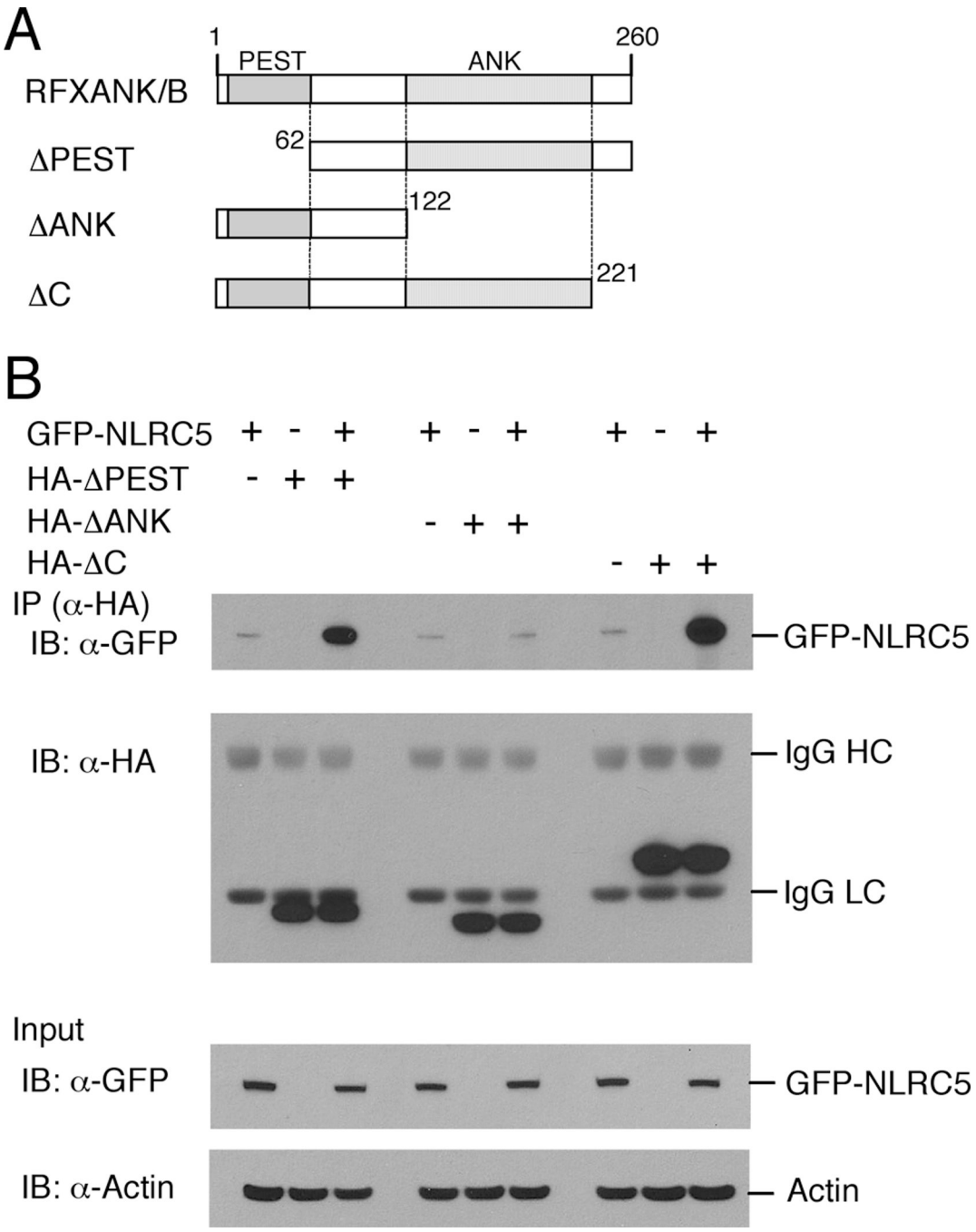

FIGURE 7. NLRC5 associates with RFXANK/B via its ankyrin repeats

A, Schematic representation of the domain structure of RFXANK/B and the indicated deletion mutants: RFXANK-ΔPEST (aa 62–260, lacking the N-terminal PEST domain), RFXANK-ΔANK (aa 1–122, C-terminal truncation of the ankyrin repeats) and RFXANK-ΔC (aa 1–221, lacking the very C-terminus).

B, Characterization of the NLRC5 binding domain using RFXANK/B deletion mutants. HEK293T cells were transiently transfected with the indicated expression plasmids, and cell extracts were prepared 48 hrs post transfection. RFXANK/B mutants were immunoprecipitated using an anti-HA antibody and associated GFP-NLRC5 was detected

by immunoblotting with an anti-GFP antibody. The blots shown are a representative of two independent experiments.

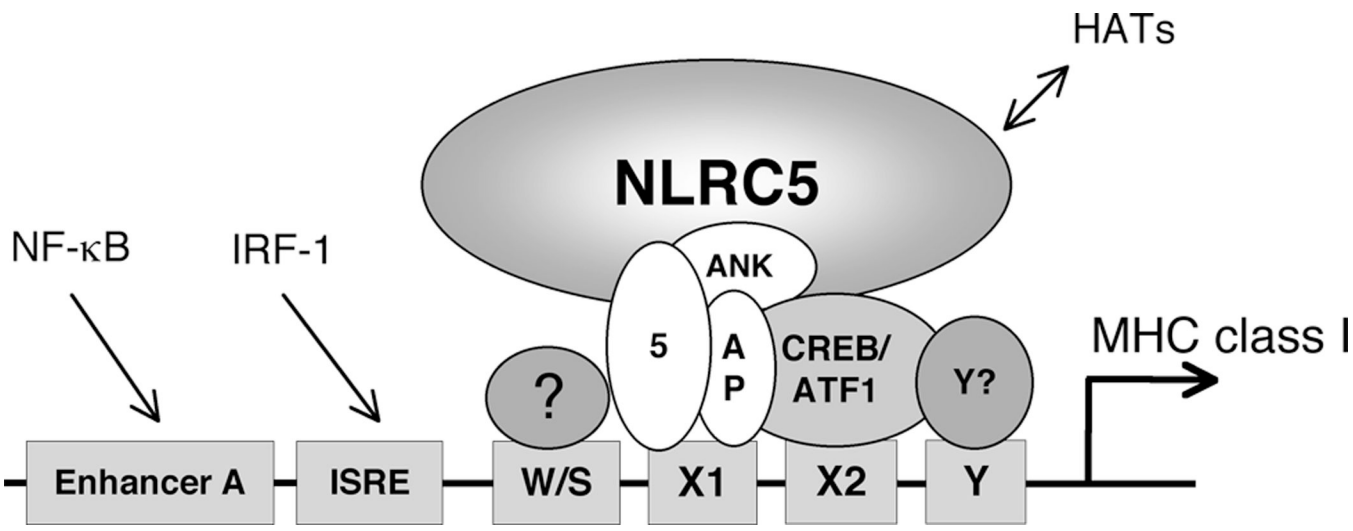

FIGURE 8. Model of the NLRC5 enhanceosome

Schematic representation of the NLRC5 (‘CITA’) enhanceosome. NLRC5 activates MHC class I genes by generating a nucleoprotein complex with other transcription factors bound to the conserved W/S-X-Y module found in the proximal promoter region of MHC class I genes. The trimeric RFX factor complex and the transcription factor ATF1/CREB are components of the CITA enhanceosome and bind to the X1 and X2 box *cis*-regulatory elements, respectively. The CITA enhanceosome requires the W/S motif, yet the factor binding to the W/S motif remains unknown. Additional *cis*-regulatory elements (Enhancer A, ISRE) in the MHC class I promoter also modulate the activity of MHC class I promoters. 5, RFX5; ANK, RFXANK/B; AP, RFXAP; Y, NF-Y; HAT, histone acetyltransferase.