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NLRC5 Cooperates with the RFX Transcription Factor Complex To Induce MHC Class I Gene Expression

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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 nucleotide-binding domain, leucine-rich repeat (NLR) 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. In this article, 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. Coimmunoprecipitation 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 coactivators CBP/p300 and general control nonderepressible 5, 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. *The Journal of Immunology*, 2012, 188: 4951–4958.

Major histocompatibility complex class I and class II molecules are essential components of the mammalian adaptive immune system. MHC class I molecules present peptide Ags of intracellular origin, such as viral or tumor Ags, to CD8⁺ T cells, whereas MHC class II molecules present peptide Ags of extracellular sources, such as bacterial Ags, to CD4⁺ T cells (1, 2). MHC class I molecules are composed of MHC-encoded H chains and the nonpolymorphic subunit β 2-microglobulin (β 2M) (3). Humans have three classical MHC class Ia molecules (HLA-A, HLA-B, and HLA-C), as well as three nonclassical 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 large multifunctional protease (LMP)2 and LMP7 (6). Peptide loading onto MHC class I requires the peptide loading complex, which includes the MHC class I H chain, β 2M, tapasin, ERp57, calreticulin, and TAP1/TAP2, a transporter that translocates peptides from the cytoplasm into the endoplasmic reticulum (6, 7).

MHC class Ia is ubiquitously expressed in almost all nucleated cells, unlike MHC class II, which is found mainly in APCs (3, 8). Both MHC class I and class II, as well as β 2M 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 nucleotide-binding domain (NBD) leucine-rich repeat (NLR) 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 has a role in the trans-

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Abbreviations used in this article: BLS, bare lymphocyte syndrome; CITA, class I transactivator; CIITA, class II transactivator; HAT, histone acetyltransferase; ISRE, IFN-stimulated response element; LMP, large multifunctional protease; β 2M, β 2-microglobulin; NBD, nucleotide-binding domain; NLR, nucleotide binding-domain leucine-rich repeat; PEST, proline, glutamate, serine, threonine-rich; RFP, red fluorescent protein.

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activation of MHC class I genes, at least in vitro (8–10, 20, 30, 31). However, although mutations in 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 expressions 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). Similarly to CIITA, NLRC5 is IFN- γ inducible and can translocate into the nucleus as a result of its nuclear localization signal. 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, because it 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 β 2M, TAP1, and LMP2 genes, indicating that it is a key transcriptional regulator of genes involved in the MHC class I Ag-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. In this article, 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 act as a platform for recruitment of histone acetyltransferase (HAT) activities provided by the general coactivators 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), and ATU (CIITA-deficient), as well as the teratocarcinoma cell line Tera-2, were described previously (11, 40). HEK293T cells were purchased from American Type Culture Collection (CRL-11268). All cell lines were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin (Life Technologies). Recombinant human IFN- γ was acquired from BioLegend and used at a concentration of 100 U/ml.

Plasmid construction

Cloning of human GFP-NLRC5 and GFP-CIITA was 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'-atatgcatccGAGGCGCAGGGTGTAGCGGAG-3' (forward), 5'-atatgtcgacTCA-CATTGATGTTCTCGGAACTG-3' (reverse); RFX5, 5'-atatgtcgccgc-TGGCAGAAGATGAGCCTGATGC-3' (forward), 5'-atatctgattATGGG-GGTGTTGCTTTTGGGTC-3' (reverse); and RFXANK/B, 5'-atatgcatcc-GAGCTTACCCAGCCTGCAGAAAG-3' (forward), 5'-atatctcgagTCACT-CAGGGTCAGCGGGCACCAG-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- Δ C, 5'-atatctcgagTTAGTCGGCTTCGGTGGTGAGGTC-3' (reverse); RFXANK/B- Δ ANK, 5'-atatctcgagTTACTCGTCTGGCTTGTGACGAG-3' (reverse); and RFXANK/B- Δ PEST, 5'-atatgcatccCAGGCAGGCAGCTCC-

CTGAAG-3' (forward). All plasmids were verified by sequencing analysis (DFCI Molecular Biology Core facilities).

Transfection and luciferase assay

Cells were transiently transfected using polyethylenimine (1 mg/ml [pH 7.2]; Polysciences) at a DNA/polyethylenimine ratio 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 six wells, and a total of 3 μ g/well of DNA was used per transfection. Medium was changed the following day, and cell extracts were prepared 48 h post-transfection. For coimmunoprecipitation experiments, cells were transfected with expression vectors for NLRC5 and, on 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 1 d prior to transfection. Unless stated otherwise, cells were cotransfected with 50 ng of either GFP, or GFP-NLRC5, or GFP-CIITA expression plasmid together with 25 ng of the indicated luciferase reporter constructs; 25 ng/well of reporterless *Renilla* plasmid was included to allow for normalization of transfection efficiency. Cells were harvested 48 h posttransfection, and cell lysates were analyzed 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 \pm SD. The MHC class I reporter gene constructs, as well as the expression plasmids encoding ATF1, CBP, p300, GCN5, and PCAF, were described previously (11, 31, 40, 41).

SDS-PAGE and immunoblotting

Whole-cell extracts were prepared using $1 \times$ 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 the manufacturer's instructions (Bio-Rad). Cell extracts were subjected to SDS-PAGE using 4–12% gradient gels (Invitrogen). Gels were transferred to nitrocellulose membranes (Amersham HyBond ECL) for ≥ 2 h at 80 V. Membranes were blocked for 1 h in 5% BSA in TBS-Tween (50 mM Tris [pH 7.6], 150 mM NaCl, 0.1% Tween 20). The following Abs were used for protein detection: anti-HA (16B12; Covance), anti- β 2M (2M2; Bio-Legend), anti-GFP (JL-8; Clontech), anti- β -actin (I-19; Santa Cruz), and anti- α -tubulin (TU-02; Santa Cruz). The Ab against MHC class I H chain (3B10.7) is a kind gift of Dr. P. Cresswell (Yale University, New Haven, CT). The following HRP-conjugated secondary Abs 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 Super-Signal 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 h post-transfection using an anti-HA Ab (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 h. 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 samples were boiled for 10 min in 20 μ l loading buffer and subjected to SDS/PAGE and immunoblot analysis. Coimmunoprecipitated GFP-NLRC5 was detected by Western blot analysis using an anti-GFP Ab.

Quantitative real-time PCR 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 μ g 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), HLA-B, 5'-CTACCC-TGCGGAGATCA-3' (forward), 5'-ACAGCCAGGCCAGCAACA-3' (reverse), and GAPDH, 5'-GAAGGTGAAGGTCCGAGT-3' (forward), 5'-GAAG-ATGGTGTGGGATTTC-3' (reverse). For analysis of the transiently re-

constituted RFXANK/B-deficient cell line EBA, cells were sorted for GFP⁺, RFP⁺, or double-positive cell populations using a Beckman Coulter MoFlo FACS sorter (Dana-Farber Cancer Institute Core facilities).

Statistical analysis

All experiments were repeated at least twice, and data were subjected to the Student *t* test for analysis of statistical significance using Prism (GraphPad). Results are given as the mean \pm SD. A *p* value <0.05 was considered 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. 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 with that of mutant versions of the HLA-B promoter harboring mutations in the W/S, X1, or X2 box (Fig. 1A). As controls, CIITA and empty GFP expression vectors were transfected, and MHC class I promoter activity was compared. As shown in Fig. 1B, NLRC5 induces the transactivation of the wild-type HLA-B promoter to a similar extent as 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 could induce MHC class I expression, even in the absence of the Enhancer A and IFN-stimulated response element (ISRE) *cis*-regulatory elements, which are conserved in the proximal promoters of the classical MHC class I genes (Fig. 2, B190: Δ Enhancer A; B170: Δ Enhancer A, Δ ISRE). Again, deletion of the W/S box selectively abolished NLRC5-induced MHC class I induction, whereas CIITA-mediated activity remained intact (B140: Δ Enhancer A, Δ ISRE and Δ W/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 coactivators 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 coregulators and is able to recruit chromatin-modifying enzymes, such as HATs, to the MHC promoters. To compare the impact of NLRC5-mediated MHC class I expression with 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 with 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, coexpression of NLRC5 and the transcriptional coactivators CBP, p300, GCN5 (Fig. 3), and PCAF (data not shown) resulted in enhanced reporter

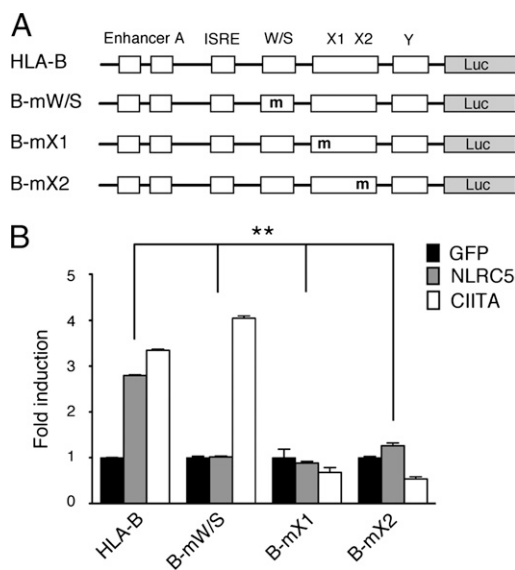


FIGURE 1. NLRC5-mediated transactivation of the HLA-B promoter requires the W/S and X box *cis*-regulatory elements. **(A)** Schematic representation of the HLA-B promoter constructs and the indicated mutant versions used in this study. Mutated *cis*-regulatory elements are marked (m). **(B)** Reporter gene analysis of the HLA-B promoter. HEK293T cells were transiently transfected with expression vectors for GFP (black bar), GFP-NLRC5 (gray bar), or GFP-CIITA (white bar), along with the indicated HLA-B luciferase reporter constructs. Cell lysates were analyzed 48 h posttransfection 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. $**p < 0.01$.

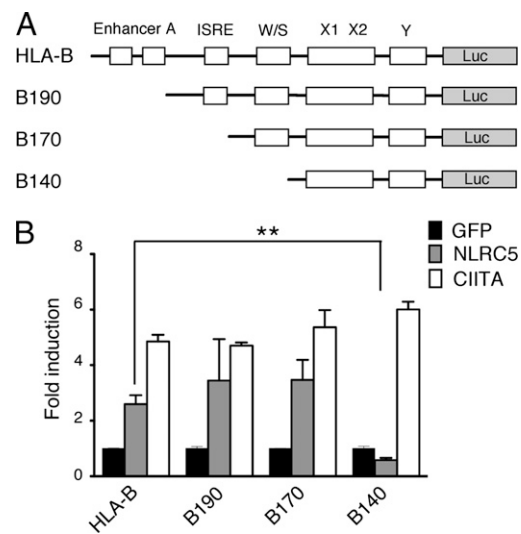


FIGURE 2. HLA-B transactivation by NLRC5 occurs in the absence of Enhancer A and ISRE. **(A)** Schematic representation of the HLA-B promoter-deletion mutants used in this study. B190 (Δ Enhancer A), B170 (Δ Enhancer A, Δ ISRE), B140 (Δ Enhancer A, Δ ISRE, Δ W/S). **(B)** NLRC5-mediated transactivation of the HLA-B promoter and the indicated promoter mutants (gray bar) compared with empty vector control (GFP, black bar), and CIITA (white bar). Transiently transfected HEK293T cells were analyzed 48 h posttransfection by dual-luciferase assay. The data were 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 represent SD. $**p < 0.01$.

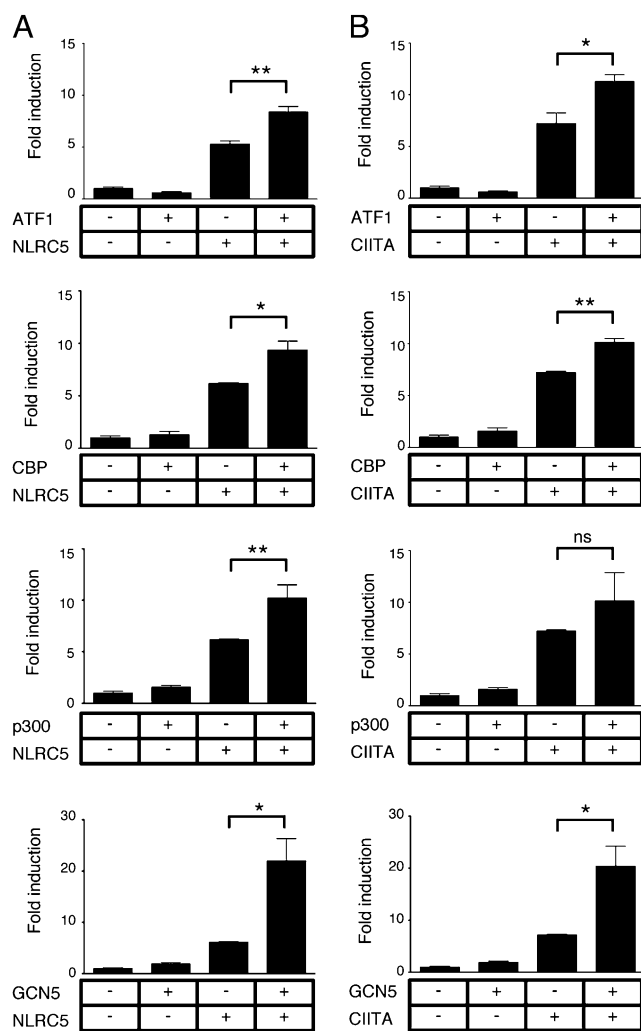


FIGURE 3. NLRC5 cooperates with ATF1 and the transcriptional coactivators CBP/p300 and GCN5. Transactivation of the HLA-B promoter by NLRC5 (**A**) or CIITA (**B**), with coexpression of ATF1 and the transcriptional coactivators CBP, p300, and GCN5, was examined. Tera-2 cells were transiently transfected with either empty control vector or the indicated expression plasmids, and cells were harvested 48 h 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. * $p < 0.05$, ** $p < 0.01$.

activity, comparable to that seen for CIITA (Fig. 3). Overexpression of the individual cofactors had no significant impact on the HLA-B promoter, probably because they are constitutively expressed in most cell types. In summary, similarly to CIITA, NLRC5 cooperates with the X2 box-binding factor ATF1 and the transcriptional coactivators CBP/p300, GCN5, and PCAF, which all display HAT activity, to transactivate MHC class I genes.

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

We 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. 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 been characterized for their levels of constitutive and inducible MHC class I and β 2M expression (42–44). We confirmed reduced MHC class I expression by quantitative real-time PCR analysis using primers specific for HLA-A and on the protein level by Western blot analysis using a pan-HLA(Hc) Ab (Supplemental Fig. 1A, 1B). Our results confirm previous data that MHC class I expression is reduced in all three cell lines investigated (OSE, ABI, and EBA) compared with 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. However, RFXAP/NLRC5 cotransfection into the RFXAP-deficient cell line 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 β 2M (Fig. 4B). Although RFXAP transfection into the ABI cell line alone did not change MHC class I expression levels to a noticeable extent, cotransfection of RFXAP, together with NLRC5, significantly increased MHC class I and β 2M protein levels. A similar synergy was also observed when CIITA was cotransfected with RFXAP into the ABI cell line (Fig. 4B).

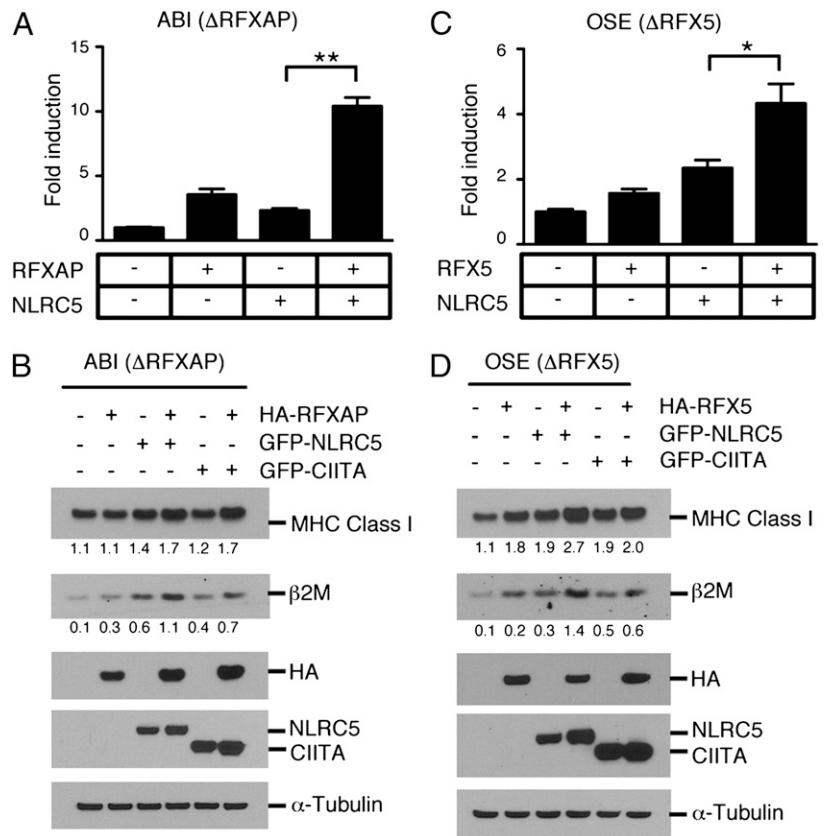
Next, we examined whether the RFX component RFX5 is essential for NLRC5-induced MHC class I expression by reconstituting the RFX5-deficient cell line OSE with RFX5 in the presence or absence of NLRC5. Again, we observed a synergistic activation of the HLA-B reporter when both components were transfected into the OSE cell line together with the HLA-B reporter gene construct (Fig. 4C) as well as on the protein level using anti-MHC class I (Hc) and anti- β 2M Abs (Fig. 4D). Yet still, NLRC5, as well as CIITA, could induce MHC class I expression, even in the absence of RFX5 (Fig. 4C, 4D), 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, whereas expression of RFXANK/B or NLRC5 alone activated the MHC class I promoter poorly (Fig. 5A). Only upon cotransfection of RFXANK/B with NLRC5 or CIITA did we observe an efficient MHC class I promoter activation (Fig. 5A, 5B). Cooperative induction of MHC class I genes by RFXANK/B and NLRC5 was confirmed both at the RNA level by quantitative real-time PCR for HLA-A and HLA-B and at the protein level by Western blot analysis using the anti-MHC class I (Hc) Ab (Fig. 5C, 5D). 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 cotransfected with expression vectors for HA-tagged RFX5, RFXAP, or RFXANK/B into HEK293T cells.

FIGURE 4. NLRC5 cooperates with the RFX components RFXAP and RFX5 for the induction of MHC class I. **(A and 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 cotransfected with the HLA-B promoter reporter gene construct, and luciferase activity was measured 48 h posttransfection using the dual-luciferase assay. The data were 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 represent SD. **(B and D)** Cell extracts of the indicated BLS patient cell line, transiently transfected with the specified expression plasmids, were examined for MHC class I and β 2M protein expression by immunoblotting 48 h posttransfection. Signal intensities were quantified using ImageQuant. Expression levels of α -tubulin are presented as a loading control. * $p < 0.05$, ** $p < 0.01$.



RFX proteins were immunoprecipitated with an anti-HA-Ab, and associated GFP-NLRC5 was detected by Western blot. Interestingly, we observed an enrichment of GFP-NLRC5 above background levels in the immunoprecipitates of HA-RFXANK/B but 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 coimmunoprecipitation experiments with RFXANK/B-deletion mutants lacking the following domains: RFXANK/B- Δ PEST (aa 62–260, lacking the N-terminal proline, glutamate, serine, threonine-rich [PEST] do-

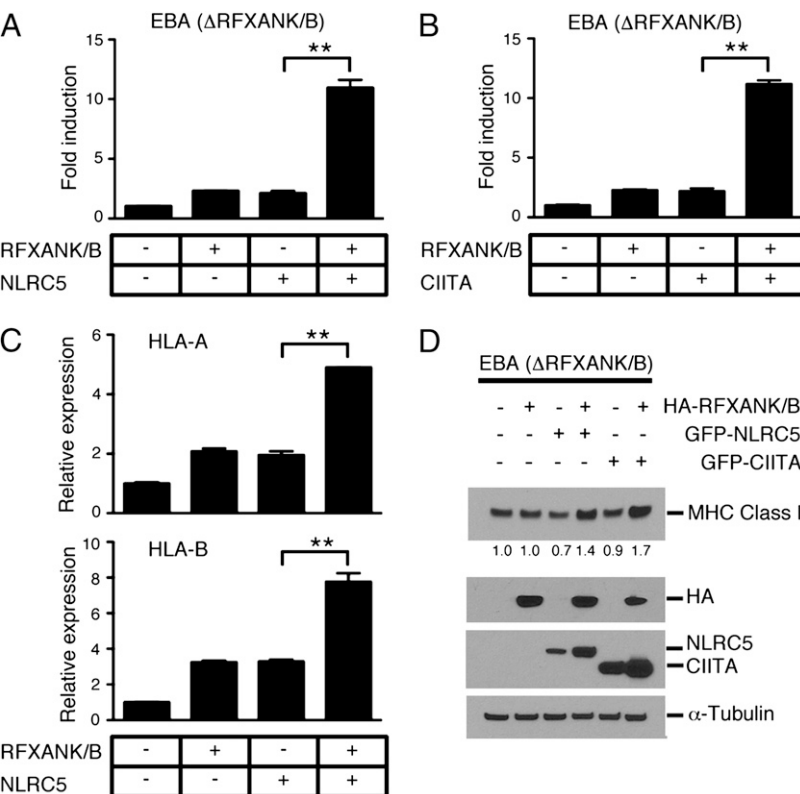


FIGURE 5. RFXANK/B is required for efficient MHC class I induction by NLRC5. HLA-B reporter gene activity induced by either NLRC5 **(A)** or CIITA **(B)** in the RFXANK/B-deficient cell line EBA. The indicated expression plasmids were cotransfected with the HLA-B promoter reporter gene construct, and luciferase activity was determined 48 h posttransfection 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. **(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 RFP-RFXANK/B, GFP-NLRC5, or both expression plasmids and sorted for GFP⁺, RFP⁺, or double-positive cell population 48 h after transfection prior to RNA isolation. Error bars represent SD of duplicates. **(D)** MHC class I protein expression in RFXANK/B-deficient fibroblasts, transiently transfected with the indicated expression plasmids. Cell lysates were prepared 48 h posttransfection and analyzed by Western blotting. α -Tubulin levels are shown as a loading control. ** $p < 0.01$.

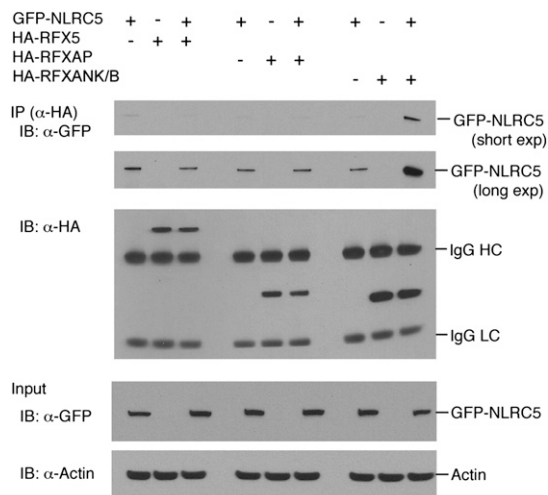


FIGURE 6. Specific binding of NLRC5 to the RFX component RFXANK/B. Association of NLRC5 with components of the RFX factor complex was analyzed by coimmunoprecipitation. A GFP-NLRC5 expression plasmid was cotransfected with expression vectors for HA-tagged RFX5, RFXAP, or RFXANK/B into HEK293T cells. Cell extracts were prepared 48 h posttransfection. RFX proteins were immunoprecipitated using anti-HA Ab, and associated GFP-NLRC5 was detected using an anti-GFP Ab. The blots shown are a representative of three independent experiments. long exp, Long exposure; short exp, short exposure.

main), RFXANK/B- Δ ANK (aa 1–122, C-terminal truncation of the ankyrin repeats), and RFXANK/B- Δ C (aa 1–221, lacking the very C terminus) (Fig. 7A). The HA-tagged RFXANK/B-deletion mutants were coexpressed with GFP-tagged NLRC5 in HEK293T cells. Binding of NLRC5 was retained, even in the absence of the

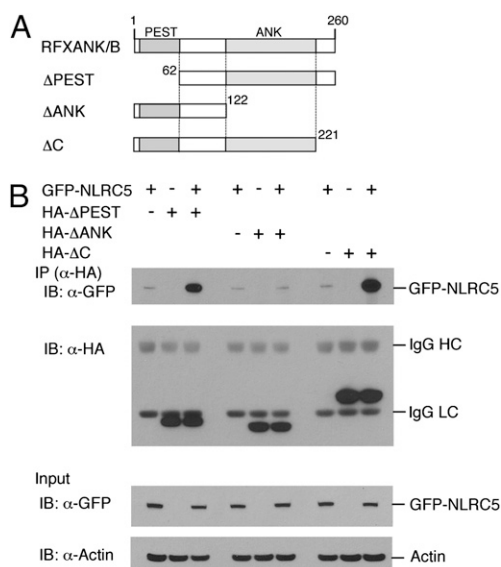


FIGURE 7. NLRC5 associates with RFXANK/B via its ankyrin repeats. (A) Schematic representation of the domain structure of RFXANK/B and the indicated deletion mutants: RFXANK- Δ PEST (aa 62–260, lacking the N-terminal PEST domain), RFXANK- Δ ANK (aa 1–122, C-terminal truncation of the ankyrin repeats), and RFXANK- Δ C (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 h posttransfection. RFXANK/B mutants were immunoprecipitated using an anti-HA Ab, and associated GFP-NLRC5 was detected by immunoblotting with an anti-GFP Ab. The blots shown are a representative of two independent experiments.

very C-terminal portion of RFXANK/B, and did not require the presence of the N-terminal PEST domain. In contrast, deletion of the ankyrin repeats (Δ ANK), which consist of well-known protein–protein interaction modules, resulted in loss of NLRC5 binding (Fig. 7B). These results support a role for 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 was 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 a CITA significantly improved our understanding of the regulation of MHC class I genes (37). However, the molecular mechanism by which NLRC5 transactivates the promoters of MHC class I and functionally related genes had not been investigated in greater detail. Similarly to CIITA, NLRC5 is lacking a known DNA-binding domain (37). Thus, NLRC5 must rely on other transcription factors that directly associate with MHC class I promoters to perform its function as an MHC CITA. 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 (45). 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 (Figs. 1, 2), which has been confirmed by an independent study with regards to the X1 box (46). 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

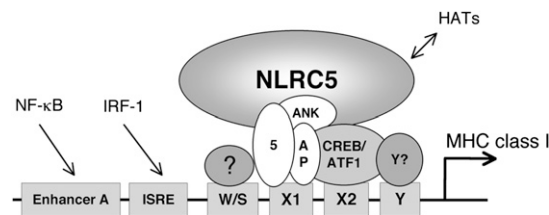


FIGURE 8. Model of the NLRC5 enhanceosome. Schematic representation of the NLRC5 (CITA) enhanceosome. NLRC5 activates MHC class I genes by generating a nucleoprotein complex with other transcription factors bound to the conserved W/S-X-Y module found in the proximal promoter region of MHC class I genes. 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. ANK, RFXANK/B; AP, RFXAP; Y, NF-Y; 5, RFX5.

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 was reported previously (47). However, CIITA was 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 (47–50). Although it is still possible that NLRC5 may associate with all individual RFX proteins under less stringent conditions, 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, because 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 (Figs. 1, 2) (11). The role of the W box, which inside contains the highly conserved S box, is still not well understood compared with well-established roles for 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 (51, 52). Alternatively, the W/S box was suggested to represent a binding site for an as-yet-unidentified factor(s) (50, 51). It was 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 (52). 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 associates 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). Therefore, it is an interesting question whether NLRC5 requires the presence of CIITA to transactivate MHC class I promoters, possibly by forming an 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 (Figs. 1, 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 functionally related genes (Fig. 8). Similarly 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 enhanceosome is established on the MHC class I proximal promoter, NLRC5 may recruit other transcriptional coactivators, such as CBP/p300, GCN5, and PCAF, to promote the transcription of MHC class I genes.

In summary, we 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 for MHC class I genes in immunity, further characterization of the CITA enhanceosome may lead to the development of therapeutics beneficial in antiviral treatment, tumor immunity, and transplantation.

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Disclosures

The authors have no financial conflicts of interest.

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