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Cohesin is a multiprotein, ringed complex that is most well-known for its role in stabilizing the association of sister chromatids between S phase and M. More recently, cohesin was found to be associated with transcriptional insulators, elements that are associated with the organization of chromatin into regulatory domains. The human MHC class II (MHC-II) locus contains 10 intergenic elements, termed MHC-II insulators, which bind the transcriptional insulator protein CCCTC-binding factor. MHC-II insulators interact with each other, forming a base architecture of discrete loops and potential regulatory domains. When MHC-II genes are expressed, their proximal promoter regulatory regions reorganize to the foci established by the interacting MHC-II insulators. MHC-II insulators also bind cohesin, but the functional role of cohesin in regulating this system is not known. In this article, we show that the binding of cohesin to MHC-II insulators occurred irrespective of MHC-II expression but was required for optimal expression of the HLA-DR and HLA-DQ genes. In a DNA-dependent manner, cohesin subunits interacted with CCCTC-binding factor and the MHC-II–specific transcription factors regulatory factor X and CIITA. Intriguingly, cohesin subunits were important for DNA looping interactions between the HLA-DRA promoter region and a 5′ MHC-II insulator but were not required for interactions between the MHC-II insulators themselves. This latter observation introduces cohesin as a regulator of MHC-II expression by initiating or stabilizing MHC-II promoter regulatory element interactions with the MHC-II insulator elements, events that are required for maximal MHC-II transcription. The Journal of Immunology, 2011, 187: 000–000.

The cohesin complex is composed of Smc1, Smc3, Rad21/Sccl, and Scc3/SA1 proteins (21). Cohesin is best known for its role in maintaining the pairing of sister chromatids after S phase of the cell cycle. It is also known for its role in stabilizing sister chromatid cohesion throughout mitosis and meiosis (12). Cohesin is a multiprotein, ringed complex that is most well-known for its role in stabilizing the association of sister chromatids between S phase and M. More recently, cohesin was found to be associated with transcriptional insulators, elements that are associated with the organization of chromatin into regulatory domains. The human MHC class II (MHC-II) locus contains 10 intergenic elements, termed MHC-II insulators, which bind the transcriptional insulator protein CCCTC-binding factor. MHC-II insulators interact with each other, forming a base architecture of discrete loops and potential regulatory domains. When MHC-II genes are expressed, their proximal promoter regulatory regions reorganize to the foci established by the interacting MHC-II insulators. MHC-II insulators also bind cohesin, but the functional role of cohesin in regulating this system is not known. In this article, we show that the binding of cohesin to MHC-II insulators occurred irrespective of MHC-II expression but was required for optimal expression of the HLA-DR and HLA-DQ genes. In a DNA-dependent manner, cohesin subunits interacted with CCCTC-binding factor and the MHC-II–specific transcription factors regulatory factor X and CIITA. Intriguingly, cohesin subunits were important for DNA looping interactions between the HLA-DRA promoter region and a 5′ MHC-II insulator but were not required for interactions between the MHC-II insulators themselves. This latter observation introduces cohesin as a regulator of MHC-II expression by initiating or stabilizing MHC-II promoter regulatory element interactions with the MHC-II insulator elements, events that are required for maximal MHC-II transcription. The Journal of Immunology, 2011, 187: 000–000.

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cell cycle (22–24) and in repair of dsDNA breaks (25–29). However, over the last few years, the function of this multisubunit, ringed complex has diversified considerably (30–34). Genome-wide chromatin immunoprecipitation (ChIP)-sequencing and ChIP-chip analyses found that the majority of the CTCF binding sites (35, 36) are also bound/associated with the cohesin complex (37–39). Transient knockdown of a cohesin subunit resulted in a loss of CTCF-bound enhancer-blocking activity (40). This finding and the fact that cohesin was expressed and bound to chromatin in interphase nuclei suggest that cohesin may play a functional role with CTCF transcriptional insulators. More recent ChIP-sequencing studies found that cohesin subunits were associated with sites that also bound the mediator coactivator complex of RNA polymerase II, suggesting that cohesin also played a role in the transcriptional regulation of some genes (41). We recently showed that the cohesin subunits Rad21 and Smc3 colocalized with the MHC-II insulator sites identified within the MHC-II locus (19); however, the role of these cohesin subunits in this system was not investigated.

In this study, the role of cohesin in regulating human MHC-II genes was examined using the C1 MHC-II insulator CTFC binding element, which lies 24 kb upstream of the HLA-DRA transcription start site and is the 5′ of the MHC-II insulators (Fig. 1). In the transcriptionally inactive state, C1 interacts with the MHC-II insulator XL9, which is located 3′ to the HLA-DRβ1 gene (19). This interaction forms a large chromatin loop that encompasses the HLA-DR subregion. In the transcriptionally active state, C1 interacts directly with the HLA-DRA gene and is the predominant MHC-II insulator controlling HLA-DRA gene expression (19).

We show in this article that C1 is required for maximal HLA-DRA expression, encodes enhancer blocking activity, and that silencing RNA (siRNA) depletion of cohesin subunits correlates with a loss of the enhancer blocking of the C1 element and with the expression of HLA-DRA and other MHC-II genes. Immunoprecipitation assays showed that cohesin is in a complex with CTCF, RFX, and CIITA, but that such interactions were dependent on the presence of DNA in the extract. Cohesin subunit siRNA depletion experiments showed that cohesin binding to C1 was dependent on CTCF. Cohesin depletion also demonstrated that the interactions between C1 and the HLA-DRA proximal promoter region were dependent on cohesin subunits; but surprisingly, interactions between adjacent MHC-II insulators were not. These data therefore describe the novel role for cohesin in regulating MHC-II expression and separating cohesin function from that of CTCF.

Materials and Methods

Cell culture

The Burkitt’s lymphoma B cell line Raji (42) expresses MHC-II genes and was obtained from the American Type Culture Collection (ATCC). The cell line RJ2.2.5 (provided by R. Accola, University of Insubria, Varese, Italy) is a derivative of the Raji line that does not express MHC-II genes (43). The human wild-type and modified BAC DNAs (D) were purchased from BACPAC Resources Center at Children’s Hospital Oakland Research Institute (Oakland, CA). The human wild-type and modified BAC DNAs (D) were also used. These lines were grown in RPMI 1640 media as early supplemented with 10 and 20% FBS, respectively. Mouse A20 B cells are MHC-II− and were grown in RPMI 1640 medium with 2 mM l-glutamine and 10% FBS.

Bacterial artificial chromosome modification and plasmid constructions

The bacterial artificial chromosome (BAC) vector containing a section of MHC-II locus that included C1 and HLA-DRA (PR11-974L24; Fig. 1) was purchased from BACPAC Resources Center at Children’s Hospital Oakland Research Institute (Oakland, CA). This BAC was manipulated to delete C1 using a recA-mediated modification system protocol as described previously (46). A recombination shuttle vector (pLD535CA-E-B) was obtained from Dr. N. Heniz (The Rockefeller University, New York, NY). DNA sequences surrounding C1, representing the homologous targetting left and right arms, respectively (Chcr: 36, 41, 40, 37–39, 500 bp) were inserted into the restriction sites of AscI and PacI of the shuttle vector, resulting in pLD535CA-E-B-C1. The pLD535CA-E-B-CI was transformed into competent Escherichia coli bacteria harboring the BAC DNA by electroporation (Bio-Rad Gene Pulser) to allow for in vivo homologous recombination events. Bacteria were selected and grown in 1 ml Luria broth medium containing ampicillin (50 μg/ml) and chloramphenicol (15 μg/ml) overnight at 37°C. The culture was diluted 1000-fold and incubated at 37°C for another 16 h in the earlier selection medium. Bacteria were further diluted and spread onto Luria broth plates containing the above antibiotics and incubated at 37°C overnight. Colonies were analyzed for integration of the shuttle vector into the BAC by PCR. Bacteria containing cointegrated BAC/SHuttle vector were grown in Luria broth media supplemented with chloramphenicol (15 μg/ml) for 1 h at 37°C and spread onto plates containing chloramphenicol and 7% sucrose for resolution/excision of the recombined targeting vector sequences. After this step, bacterial colonies were screened by PCR for excision of the targeting vector sequences, and BAC DNA was analyzed by restriction enzyme digestion and Southern blotting to ensure that the recombination, integration, and resolution processes occurred precisely. Two independent cointegrated BACs were carried through the process, producing ΔC1a and ΔC1b that had no other detectable deletions but those in C1.

The minimal promoter firefly luciferase reporter plasmid, pGL3-promoter plasmid (Promega), was used as a base vector in transient transfection reporter assays to determine enhancer activity. The 520 bp encompassing C1 was cloned into pGL3-promoter using primers forward 5′-GATCTTGTGATATCTATCTATAG-3′ and reverse 5′-CTCCAGTCATGCAGCAACC-3′ to amplify the sequence. The 5′HS4 chicken β-globin insulator (17) and a fragment of a DNA pGL3-promoter vectors were described previously (47).

To assay the ability of C1 region to function as an enhancer blocker, we used the pGL3-promoter vector containing SV40 promoter upstream of the luciferase gene as the base plasmid vector (Promega). pGL3-5′HS4-SV40, derived from pGL3-promoter, was described previously (47) and contains the 5′HS4 SV40 enhancer upstream of the SV40 enhancer. C1 was placed between the SV40 enhancer and the SV40 promoter in pGL3-5′HS4-SV40 in a manner similar to that described for XL9 previously (47). The other enhancer blocking vectors were also described previously (47). In some constructions, the MHC-II insulator XL9 replaced HS4. The XL9 sequence was described previously (47, 48). Primers used to amplify this sequence were XL9: 5′-TGCTTCTTTTCAAGGTGCAAGGAGG-3′ and 5′-GGGCGAG CCCACAGGTAGTGGC-3′.

Transient transfection assays

The human wild-type and modified BAC DNAs (ΔC1a and ΔC1b) were transfected into the murine B cell line A20 by nucleofection using an Amaza Nucleoporation with transfection Kit V according to the manufacturer’s protocols (Lonza, Walkersville, MD). In each transfection, ~2 × 10^6 cells were seeded. Forty-eight hours after transfection, total RNA was isolated using RNAeasy kits (Qiagen), and the transcripts of human HLA-DRA gene, which was encoded in the human BAC, were analyzed by real-time RT-PCR as described previously (46). Transcripts were normalized to the levels of GAPDH mRNA. The data were averaged from three independent transfections.

For the luciferase reporter transient transfection assays, a constitutively expressing vector (Renilla luciferase) in DNA expression vector (pRLTK) was cotransfected into Raji cells along with the test or control firefly luciferase vectors described earlier. All transfections were carried out by nucleofection as described earlier. After 48 h, cell lysates were prepared and luciferase


activity for both *firefly*, and *Renilla* was determined with a luminometer as instructed in the manufacturer’s protocol (dual-luciferase reporter assay system; Promega). The value of measured activity of firefly luciferase for each construct was normalized to the activity of the cotransfected *Renilla* luciferase reporter. All assays were carried out at least three times, and the data are presented as the average with the SEM.

**siRNA treatment**

SMART pool siRNAs purchased (Dharmacon, Thermo Scientific) specific for CTCF, Rad21, and SMC3 were transfected into 4 × 10⁵ cells using a nucleofection apparatus and transfection reagents (Kit V) from Amaxa Biosystems to knock down the expression of these genes as previously described (19). As a negative control, siRNA against GFP was also transfected. Transfected cells were grown for 3 d and then assessed by either Western blotting or quantitative RT-PCR to determine the efficiency of the knockdown and for use in the assays described.

**Coimmunoprecipitations and Western blots**

Coimmunoprecipitations were carried out essentially as described previously (20). A total of 1.5 × 10⁷ M-280 sheep anti-rabbit and anti-mouse Ab-coupled magnetic beads (Invitrogen) were used overnight to 3 μg of the appropriate Ab for each immunoprecipitation. Ab-bound beads were mixed with 200 μg nuclear extract, which was prepared as previously described (20), and the volume was adjusted to 300 μl by the addition of lysis buffer (50 mM Tris [pH 8], 150 mM NaCl, and 1% Nonidet P-40) plus protease inhibitors. Immunoprecipitation reactions were continued overnight at 4°C with gentle rotation. Immune complex-bound beads were washed three times with the lysis buffer. Washed beads were resuspended in SDS-PAGE sample buffer. Precipitates were eluted from the beads in a boiling water bath for 5 min, separated by 6 or 7.5% SDS-PAGE, and analyzed by Western blotting. Western blots were performed according to standard protocols with Immobilon-P transfer membranes (Millipore) coupled with ECL detection kits (GE Healthcare). Anti-CTCF and β-actin were purchased from Millipore and Chemicon, respectively.

In some reactions, ethidium bromide (50 μg/ml) was added to the nuclear extract and incubated on ice for 30 min as described previously (20). After incubation, the nuclear extract was cleared by centrifugation and used in the coimmunoprecipitation assay described earlier. For some immunoprecipitation reactions, samples were treated with DNease I as described previously (20). For these, the coimmunoprecipitation reaction was performed first; then 30 U/ml DNease I (Roche Applied Science) was added for a 5-min incubation at room temperature (20). The protein-bound beads were washed with lysis buffer and analyzed as described earlier.

**FIGURE 1.** Optimal HLA-DRA gene expression is dependent on the MHC-II insulator C1, which is constitutively occupied by CTCF and cohesin. A, A relative schematic of the HLA-DR, HLA-DQ region with the MHC-II insulators C1, X9, and C2 is shown. Wild-type and mutant human BAC clones derived from PR11-974L24, containing the C1-HLA-DRA region are represented. The exact DNA sequence coordinates of PR11-974L24 from the UCSC Genome Browser are Chr6:32,341,463 to 32,456,230. B, Wild-type and two independently derived ΔC1 deleted mutant BACs (ΔC1a and ΔC1b) were transiently transfected into the murine B cell line (A20) by nucleofection. Forty-eight hours post-transfection, RNA was purified and analyzed for the presence of HLA-DRA gene transcripts by real-time RT-PCR using a primer set specific for the human HLA-DRA gene. Results were normalized with respect to the level of GAPDH mRNA expression and the average plotted as relative expression. C, Quantitative real-time PCR-based ChIP assays were performed using chromatin prepared from the indicated cells to determine the in vivo occupancy of CTCF, Rad21, and SMC3 at C1. A431 cells (∼10⁵ IFNγ) were also used in this ChIP assay. Antisera specific to CTCF, Rad21, and SMC3, and a nonspecific IgG control antiserum were used as indicated. Primers used to amplify the C1 and negative control C15 (19) region are provided in Supplemental Fig. 1. These results represent an average of at least three independent experiments.

**ChIP assay**

ChIP assays were performed as described previously (20, 49). In this study, cells were cross-linked in 1% formaldehyde for 10 min, and a chromatin lysate was prepared and sonicated to generate fragments averaging 500 bp in length. For immunoprecipitations, 5–10 μg anti-CTCF (catalog no. 06-917; Upstate), anti-cohesin (anti-Rad21; catalog no. 05-988; Upstate), (anti-SMC3; catalog no. ab8263; Abcam), anti-RFX5 (50), anti-CIITA (49), or anti-TCR (nonspecific control) Abs were used. The immunoprecipitated DNA was quantitated by real-time PCR using a five-point genomic DNA standard curve and an I-cycler (BioRad Laboratories). Quantitative PCR (qPCR) reactions contained 5% DMSO, 1X SYBR green (Bio Whittaker Molecular Applications), 0.04% gelatin, 0.3% Tween 20, 50 mM KCl, and 20 mM Tris [pH 8.3], 3 mM MgCl₂, 0.2 mM dNTP, and 100 nM of each primer. Sequences for all primers used in the ChIP real-time PCR assays are listed in Supplemental Fig. 1. All ChIP experiments were performed at least three times from independent preparations of chromatin. The data were averaged and plotted with respect to the input chromatin. Under the earlier conditions of cross-linking we did not detect RFX5 or CIITA at the MHC-II insulators, nor did we detect CTCF or cohesin at MHC-II proximal regulatory regions.

**Quantitative chromatin conformation capture assay**

A modified chromatin conformation capture (3C) assay protocol was used as described previously (19, 20). In these 3C assays, 1 × 10⁵ cells were used and cross-linked with formaldehyde to a final concentration of 1% and incubated for 10 min at room temperature. Prepared nuclei were digested with EcoRI. After heat inactivation of the enzyme, the samples were diluted into ligation buffer at a ratio of ~40:1 and then ligated overnight with T4 DNA ligase at 16°C. The 3C DNA was purified and quantified by real-time PCR using a five-point standard curve as described previously (19). Standard curve templates for the 3C products were generated in vitro by restriction enzyme cleaving and religating a BAC containing the region being studied. The BACs RP11-974L24, RP11-257P24, and RP11-54H13, which cover the MHC-II region from C1 through C2, were used to generate all 3C product templates and standard curves. All primer combinations listed in Supplemental Fig. 1 were tested previously (19) in the 3C assay to determine whether they could efficiently amplify a single product on EcoRI cleaved/religated BAC DNA. All primers had >90% PCR efficiency and produced a single amplicon on cleaved/religated BAC DNA. All sites used were previously shown to be equally cleavable by EcoRI in these assays (19). Data are presented as relative cross-linked frequency and represent an average derived from three independent biological replicates. Relative cross-link frequency was calculated as the qPCR value for the 3C sample × 100, divided by the qPCR value for the input (49), or anti-TCR (nonspecific control) Abs were used. The immunoprecipitated DNA was quantitated by real-time PCR using a five-point genomic DNA standard curve and an I-cycler (BioRad Laboratories). Quantitative PCR (qPCR) reactions contained 5% DMSO, 1X SYBR green (Bio Whittaker Molecular Applications), 0.04% gelatin, 0.3% Tween 20, 50 mM KCl, and 20 mM Tris [pH 8.3], 3 mM MgCl₂, 0.2 mM dNTP, and 100 nM of each primer. Sequences for all primers used in the ChIP real-time PCR assays are listed in Supplemental Fig. 1. All ChIP experiments were performed at least three times from independent preparations of chromatin. The data were averaged and plotted with respect to the input chromatin. Under the earlier conditions of cross-linking we did not detect RFX5 or CIITA at the MHC-II insulators, nor did we detect CTCF or cohesin at MHC-II proximal regulatory regions.

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value for that potential 3C junction represented in a pool of all potential 3C combinations generated using EcoRI cleaved and religated BAC DNA to the regions assayed.

**Statistical analyses**

All quantitative experiments were reproduced at least three times from independent biological samples. The data are presented as an average with SEM. The Student t test was used to determine statistical significance between samples, and the results of such tests are indicated in the figures or legends.

**Results**

**C1 is required for maximal expression of the HLA-DRA gene**

Ten strong CTCF binding regions were identified in the human MHC-II locus (19). C1 represents the MHC-II insulator that is closest to and interacts with the *HLA-DRA* gene and was chosen in this study as the model element to study the role of cohesin (Fig. 1A). One critical feature of MHC-II insulators that was not previously determined was whether deletion of the insulator region would affect MHC-II gene expression. Although a targeted disruption of the gene in human cells is not practical, the manipulation of a human BAC construct, followed by transfection into murine cells, can be used to demonstrate a role for a *cis*-acting element such as C1 (46). The RP11-974L24 BAC encodes C1 and the *HLA-DRA* gene (Fig. 1A). BAC recombineering, which uses targeted homologous recombination vectors to alter the BAC DNA, was used to create a 520-bp deletion encompassing the C1 MHC-II insulator. Two independent recombineered clones, termed ΔC1a and ΔC1b, were isolated with identical mutations. Restriction enzyme digestion, PCR, and Southern blot analyses (data not shown) demonstrated that the mutant BAC DNAs contained only the C1 deletion. Wild-type and ΔC1 mutant BAC DNAs were transfected into the murine B cell line A20, and 48 h post-transfection, RNA was isolated and analyzed. Real-time RT-PCR for *HLA-DRA* gene expression showed that it was expressed from the wild-type BAC. In contrast, the two mutant ΔC1 BACs showed ∼50% reduction in the level of *HLA-DRA* mRNA (Fig. 1B). These results suggest that as a *cis*-element, C1 is required for maximal expression of *HLA-DRA*.

**Cohesin subunits associate with C1 irrespective of MHC-II gene expression**

Using ChIP with Raji cells, we found the cohesin subunits Rad21 and Smc3 to be associated with each of the 10 MHC-II insulators, including C1 (19). To determine whether the binding of cohesin subunits was correlated with MHC-II gene expression, we examined the binding of Rad21 and Smc3 to C1 in MHC-II+ and MHC-II− cells by ChIP. Chromatin was prepared and assayed from several MHC-II+ cell lines: RJ2.2.5 (CITA-deficient, MHC-II+, B cell line) (43, 44), SJO (RFX5-deficient, MHC-II−, B cell line) (45), A431 (MHC-II− epithelial cell line), H929 (MHC-II− plasma cell line; CRL-9068; ATCC), CCRF (MHC-II− T cell leukemic cell line) (51), and Kasumi-1 (MHC-II− acute myeloid leukemic cell line) (52). In addition to Raji cells, which are constitutive for MHC-II, chromatin was also isolated and examined from A431 epithelial cells treated with IFN-γ to induce *CITA* and MHC-II gene expression (49). Although there is some variability in the strength of the signal observed, the results showed that C1 significantly bound CTCF. Rad21, and Smc3 in all cell types/lines examined (Fig. 1C). The levels of binding were comparable with the other MHC-II insulators as previously reported for Raji cells (19). Serving as a CTCF+ site for these ChIP assays, C15 exhibited 6- to 18-fold lower levels of binding of CTCF and the cohesin subunits depending on the cell type. Student t test analyses showed that binding of CTCF, Smc3, or Rad21 was not significantly different in A431 cells treated with or without IFN-γ (Fig. 1C). Thus, irrespective of MHC-II gene expression.
expression, CTCF and cohesin are associated with the C1 CTCF site in multiple cell types.

Rad21/cohesin is required for full expression of the MHC-II genes

The binding of cohesin to the MHC-II insulator sites suggests that cohesin may also have an effect on MHC-II gene expression. To test this hypothesis, we used Rad21 and SMC3-specific SMART pool siRNA oligonucleotides (Dharmacon) to deplete transiently Rad21 or SMC3 from Raji cells. This treatment did not affect the overall growth or viability of these cells during the 48 h of the assay (data not shown). Immunoblotting showed that each of the factors was reduced to ∼20–30% of the untreated or irrelevant siRNA-treated cells (Fig. 2A). Importantly, the levels of Smc3, another component of cohesin, and β-actin were unchanged during the course of the assay (Fig. 2A). RNA purified from the earlier cell groups was analyzed by real-time RT-PCR. The results showed that CIITA and RFX5 mRNA levels were unchanged irrespective of treatment (Fig. 2B). As expected, the steady-state levels of Rad21 and SMC3 mRNA were substantially reduced by their respective and specific siRNA treatments. After either Rad21 or SMC3 siRNA depletion, the levels of HLA-DRA mRNA were reduced to ∼40–50% of the control-treated cells (Fig. 2B). These results demonstrated that at least two components of the cohesin complex were required for maximal HLA-DRA gene expression.

To define more broadly whether cohesin played a role in other MHC-II genes, we examined the expression of HLA-DRB1, HLA-DQA1, and HLA-DQB1 (Fig. 2C). These genes represent the major human MHC-II isotypes. Like HLA-DRA, the expression of these genes was also reduced significantly when either Rad21 or SMC3 was depleted from the system. These data suggest that cohesin is critical for maximal expression of MHC-II genes.

Cohesin is important for the function of C1

One experimental property initially observed for the MHC-II insulator XL9 was its ability to block the activity of an upstream enhancer from acting on a downstream promoter. Such a property may exist for C1 and may be dependent on cohesin for this function. To assess this characteristic, we defined the function of C1 as an element. The 520-bp region encompassing C1 was cloned 5′ to a minimal promoter luciferase reporter gene and assayed for enhancer activity after transient transfection into Raji cells. Similar to the chicken β-globin HS4 CTCF insulator (17, 20) and a control DNA from λ phage, C1 contains no enhancer activity (Fig. 3A). This result was also similar to that observed for the MHC-II insulator XL9 (47).

C1 was placed between an SV40 enhancer and a promoter, transfected into cells, and the ability of the enhancer to drive the luciferase reporter gene was assayed to determine whether C1 encoded an enhancer-blocking activity. An additional HS4 insulator was placed on the 5′ side of the enhancer to prevent the enhancer from operating from the other side of the circular plasmid DNA. C1 was as effective as the HS4 insulator at blocking the activity of SV40 enhancer in driving transcription of the reporter gene (Fig. 3B). The control λ DNA exhibited no enhancer-blocking activity. To ensure that the HS4 insulator provided no additional activities, we replaced it with XL9 in the enhancer-blocking constructs. XL9 is an MHC-II insulator that is normally located between the HLA-DRB1 and HLA-DQA1 genes (47, 48). After transfection and assessment of the activities of these reporters, similar data were collected indicating that the HS4 insulator was not providing additional enhancer-blocking activities to the construct (Fig. 3C). Thus, C1 has no intrinsic enhancer activity but can block the activity of an enhancer.

The earlier enhancer-blocking assay was performed in Rad21-depleted cells to determine whether C1’s enhancer-blocking activity was dependent on cohesin. The enhancer-blocking constructs were cotransfected with the Rad21 SMART pool siRNA oligonucleotides into Raji cells, and the luciferase activity of the reporter gene was measured at 48 h posttransfection (Fig. 3D). The HS4 chicken β-globin insulator (17) was also included to determine whether cohesin was also required for its activity in B cells. Constructs containing C1 or HS4 elements showed increased levels of luciferase expression in cells in which cohesin was depleted compared with control siRNA-treated cells. Rad21 depletion had no statistically significant effect on the SV40 enhancer-mediated expression in the control construct, which did not contain an insulator. Thus, Rad21 is required for the enhancer-blocking activity of the C1 and HS4 insulators.
Cohesin associates in a complex with CIITA and RFX5

The earlier experiments suggest a role for cohesin and CI in regulating HLA-DRA gene expression but do not provide a mechanism by which this may occur. Because cohesin proteins are not found at the MHC-II promoters, but together with CTCF are found at CI and the other MHC-II insulators, the ability of cohesin subunits to interact with MHC-II proximal promoter region transcription factors (RFX and/or CIITA) was hypothesized as a way in which these regions may interact. To determine the nature of interactions that may occur, we conducted coimmunoprecipitation and immunoblotting experiments in Raji cells, which are wild-type for all MHC-II transcription factors. Two other cell lines, which contain mutations in CIITA (RJ2.2.5) (43, 44) and RFX5 (SJO) (45), were used to dissect the interactions. As previously shown (20, 53), CIITA and RFX5 interact in the wild-type cells as indicated by the coimmunoprecipitation (Fig. 4A). Abs to Rad21 and Smc3 immunoprecipitated RFX5 in Raji cells (Fig. 4A), but not in cells lacking CIITA (RJ2.2.5) or the control RFX-deficient (SJO) cells. Reciprocally, RFX5 Abs precipitated Rad21 and Smc3, but only in Raji cells. Abs to CIITA immunoprecipitated Rad21 and Smc3 in Raji but not in RJ2.2.5 or SJO cell lysates. These data suggest that both RFX5 and CIITA form either direct or indirect interactions with cohesin subunits. Such interactions could be through a soluble complex or a complex assembled on DNA. To determine whether the coimmunoprecipitations were dependent on the presence of DNA, we added DNase1 or ethidium bromide, which prevents protein–DNA interactions (54), to the immunoprecipitation reactions. In the presence of ethidium bromide or DNaseI, coimmunoprecipitates between SMc3 and RFX5 or CIITA were no longer detected (Fig. 4B). Similarly, both ethidium bromide and DNaseI disrupted Rad21 coimmunoprecipitations with RFX5. Importantly, the interactions between CIITA and RFX5 were not affected, nor were the interactions between SMc3 and Rad21.

Cohesin interactions with CTCF require DNA

The earlier experiments question whether cohesin and CTCF interactions may also require DNA. Thus, Raji nuclear lysates were generated and coimmunoprecipitations in the presence and absence of either DNaseI or ethidium bromide were performed using anti-CTCF or -Rad21 Abs (Fig. 4C). Whereas coimmunoprecipitation interactions between Smc3 and Rad21 were not altered by the presence of DNaseI or ethidium bromide, these compounds disrupted the ability of Smc3 or Rad21 to interact with CTCF and form coimmunoprecipitates. These data argue that a DNA component is required for cohesin to interact with CTCF, RFX5, and/or CIITA. From our previous work, the association of CTCF with RFX5 or CIITA was not dependent on the presence of DNA (20).

CTCF is required to recruit cohesin to CI

CTCF and cohesin are colocalized at the CI MHC-II insulator. However, we do not know whether one of these factors is required for the other to associate with the MHC-II insulator. A set of ChIP assays was performed after RNA interference (RNAi) depletion of CTCF, SMc3, and Rad21 to examine the dependency of one factor for the other in associating with the CI MHC-II insulator. Depletion of CTCF resulted in the loss of CTCF binding (>80%) at CI and a 50–60% reduction in the association of SMc3 and Rad21 (Fig. 5). In sharp contrast, depletion of SMc3 and Rad21 did not alter the binding of CTCF to CI (Fig. 5, top left panel). SMc3 and Rad21 depletion by RNAi did, however, result in a reduction in their own association, as well as each other from CI. These observations suggest that CTCF is required to recruit cohesin to CI, and that CTCF binding/stability is independent of cohesin. The results also suggest that an intact cohesin complex is important for recruitment or stability of cohesin interactions at these sites.

Cohesin is required to form chromatin loops between CI and the HLA-DRA promoter region

Previously, we reported that CTCF is required for the chromatin interactions between MHC-II insulators and the proximal promoters of MHC-II genes (19). The earlier data suggest that cohesin could play a role in maintaining the chromatin architecture of the MHC-II locus or in the ability of the MHC-II insulators to interact with MHC-II promoters. To determine whether cohesin is...
required for similar chromatin interactions, we investigated whether interactions with C1 and XL9 were dependent on the cohesin subunit Rad21. In this study, 3C assays (19, 55) were used to assess the role that cohesin plays in the shaping of the architecture of the MHC-II region encompassing HLA-DRA. 3C assays use formaldehyde cross-linking to fix interactions between regulatory elements. The cross-linked chromatin is subjected to extensive restriction digestion and dilution, and is followed by religation of restriction fragments. Only restriction fragments that are in close spatial proximity to each other (because of the crosslinking) display a high frequency of ligation, which is measured by real-time PCR across the 3C generated novel ligation junction. As shown earlier (Figs. 3, 5), Rad21 depletion results in a ~70% reduction in Rad21 protein and occupancy at C1 in Raji cells. Using control and Rad21 siRNA-depleted Raji cells, we conducted 3C assays to measure the relative frequency of interactions between C1 and the HLA-DRA proximal regulatory region and several control restriction fragments (Fig. 6). Background levels of interactions were detected between C1 and the control restriction fragments (CL1, CL2, and P12). Such background interactions are common with this assay (19, 55, 56). In control cells, the relative cross-link frequency between C1 and the HLA-DRA proximal promoter were robust (Fig. 6) and recapitulate previous observations (19). Intriguingly, depletion of Rad21 from the system resulted in a significant reduction in C1/HLA-DRA interactions, suggesting that Rad21 was important for this interaction.

MHC-II insulators can also interact with each other (19, 20) in a manner that is independent of MHC-II gene expression. To determine whether cohesin was important for these interactions, 3C was performed using C1, XL9, and C2, the three MHC-II insulators that divide the HLA-DR and DQ subregions (Fig. 6). In these 3C reactions, C1 and XL9, and XL9 and C2 interactions were examined together with control fragments CL1, P12, CL4, and CL5. As previously observed (19), XL9 interacted with either C1 or C2 in control cells (Fig. 6). Surprisingly, Rad21 siRNA-treated cells showed no change in the ability of XL9 to interact with either C1 or C2 (Fig. 6). Thus, cohesin depletion by RNAi does not affect the ability of MHC-II insulators to interact with each other.

FIGURE 6. Cohesin controls the chromatin interactions between C1 and the proximal regulatory region of HLA-DRA. A, Schematic diagram of MHC-II insulators C1, XL9, and C2, the HLA-DRA, HLA-DRB1, HLA-DQB1 genes, the relative position of the EcoRI sites, and the positions of primer sets used to determine the relative frequency of chromatin interactions in the locus by 3C assay is shown. // indicates gaps in the map that are not shown to reduce the complexity of the illustration and its size. Relative primer positions for EcoRI sites are colored gray and represent 3C anchor sites. The other primer positions are colored as black. Primer sets are the same as described earlier (19). B, Quantitative 3C assays were performed in untreated Raji cells (cont) or Raji cells depleted for Rad21 by siRNA as described earlier. The 3C results determining the relative interaction frequencies between the indicated anchor sites and the indicated restriction fragment are plotted as the relative cross-link frequency with SE. Relative cross-link frequency represents the relative average amount of 3C product (from three independent chromatin preparations) determined from qPCR of the samples compared with a standard curve generated from BAC DNA that was fully digested with EcoRI and religated ×100. Cutting and religating the BAC DNA in this regard provides all possible combinations of EcoRI fragments. Asterisk represents the only Rad21-siRNA-treated cell sample that was significantly different from its control as determined by the Student t test (p < 0.05).

FIGURE 5. Recruitment of cohesin to C1 is CTCF dependent. Cross-linked chromatin was prepared from Raji cells after transfection with SMART pool siRNAs against CTCF, SMC3, Rad21, and GFP (nt) as indicated. In the case against siRNA to CTCF, negative HLA-DR cells were isolated as described earlier (19). ChIP assays were performed with Abs as indicated. Cont represents an IgG control ChIP. The average of three independent ChIP assays is shown with the data plotted with respect to the percentage of input chromatin in each reaction. Asterisks indicate samples that displayed p < 0.05 as determined by Student t tests.
Discussion

The MHC-II locus is organized architecturally through interactions across the series of CTCF binding sites that we have termed MHC-II insulators. Crucially, CTCF was found to be required for the full expression of MHC-II genes, identifying a novel mechanism of MHC-II gene expression (19). In 2008, genome-wide binding studies conducted by several groups found that subunits of cohesin were associated with many, but not all, CTCF binding sites (38, 39, 57). Cohesin was also found at each of the 10 MHC-II insulators, suggesting that cohesin may play a role in the expression of MHC-II genes (19). In this study, we chose to determine the role of cohesin on MHC-II gene expression and, for the most part, focused on one of the MHC-II insulators, C1. Cohesin binding to C1 varied slightly among the different cell lines/types but was always present irrespective of MHC-II gene expression, suggesting that it was part of the base architecture. Importantly, knockdown of cohesin subunits Rad21 or Smc3 showed that the HLA-DR and HLA-DQ genes were both dependent on cohesin for maximal expression. Thus, cohesin acting at MHC-II insulators is a novel and important component of MHC-II transcription regulation. To determine how it functioned, we examined some of the molecular features associated with insulator function. Like XL9 (47), C1 has no intrinsic enhancer activity but does encode an enhancer-blocking activity. This activity was dependent, in part, on the presence of the cohesin subunit Rad21. A similar observation was made for the chicken β-globin insulator (Fig. 3) (39), suggesting that cohesin may be required for this function at all enhancer-blocking insulators. The ability of MHC-II insulators to block enhancer activity in the artificial system suggests that these elements can do this in vivo and may serve to restrict the MHC-II proximal regulatory elements to single genes, or at least to genes between the MHC-II insulators. With MHC-II insulators surrounding each of the subregions (12, 19), including separating the HLA-DM genes from TAP1, PSMB9, and BRD2, this function may indeed be important.

To develop insight into how cohesin functions in this system, we conducted coimmunoprecipitation experiments between cohesin and the MHC-II specific transcription factors RFX5 and CIITA, and found that both Rad21 and Smc3 associated with these factors in extracts prepared from MHC-II–expressing B cells. The interactions were specific because cells that lacked functional RFX5 or CIITA showed no association. The interactions with RFX5 were either not sufficient or direct because no interaction was observed in R2J2.2.5 (CIITA−) cells. This would suggest that the interactions with RFX5 occurred through CIITA when it was bound to the MHC-II promoter regions. Intriguingly, the observed interaction was dependent on the presence of DNA in the extract because treatment with DNaseI or the intercalating agent ethidium bromide, which disrupts protein–DNA interactions, resulted in the loss of the coprecipitates. These data suggest that cohesin is interacting with an MHC-II specific multiprotein/DNA complex or that a specific DNA-dependent protein conformation is necessary. It is assumed that such interactions with DNA are occurring through specific interactions with WXY box DNA that is associated with the proteins in the lysates. However, it is possible that any DNA substrate would serve this purpose. As a point of reference, CTCF–CIITA interactions were not perturbed by the addition of DNaseI or ethidium bromide (20). A surprise was that the CTCF–cohesin interactions were also dependent on the presence of DNA. This observation was not previously reported and supports a novel mechanism of cohesin association with its target substrates. One possible substrate may be looped DNA or a pair of chromatized DNA helices that are in close proximity, which would occur because of insulator interactions or the pairing of sister chromatids, respectively. Coupled with these findings were experiments to determine the requirements for cohesin binding to MHC-II insulators. Using RNAi to deplete CTCF, Rad21, and Smc3, coupled with ChiP, we were able to determine that CTCF binding was independent of cohesin. This was not the case for either Rad21 or Smc3, which showed reduced binding when CTCF was depleted. In addition, Rad21 and Smc3 binding was dependent on each other, suggesting that these proteins are binding in a complex with each other.

Because cohesin was important for MHC-II gene expression and interacted at the MHC-II insulators, we initially postulated that siRNA depletion of Rad21 would disrupt the basal architecture associated with the MHC-II insulators. However, the 3C experiments indicated that Rad21 depletion had no effect on the ability of the C1, XL9, and C2 insulators to interact with each other. These insulators separate the HLA-DR and HLA-DQ subregions from each other. The data therefore suggest that the interactions between the MHC-II insulators, which are mediated by CTCF, are potentially sufficient for these interactions to occur. The fact that CTCF can form homodimers would suggest that CTCF dimer formation might be what holds MHC-II insulators together. In contrast, depletion of cohesin subunits disrupted the 3C interactions between the HLA-DRA promoter proximal regulatory and the C1 MHC-II insulator. Thus, the reason for loss of MHC-II gene expression on cohesin depletion was due to the loss of a chromatin loop between the MHC-II insulator and the promoter. This would suggest that the latter interactions are not stable, allowing for finer gene control, which, in this case, would be mediated by the level or functional activity of cohesin in the cell. The current data are in agreement with a model predicting that MHC-II insulators serve as focal points for the formation of a functional and efficient transcription complex at MHC-II gene promoters. We have previously shown that the loss of CTCF resulted in a decrease in the presence of transcriptionally active histone modifications at MHC-II gene promoters, suggesting that these interactions are either required for or are a consequence of CTCF-mediated activities and loop formation (20).

Although cohesin function in mitosis and DNA repair mechanisms is well established, it is clear that it is involved in many other aspects of genome structure. Kagey et al. (41) recently found cohesin bound to 43,687 sites in human embryonic stem cells, but only a fraction of these sites (24,741) overlapped with CTCF occupancy. The rest were located mostly at enhancers and core promoter regions of active genes and were associated with the binding of mediator complex subunits. Mediator is a transcriptional coactivator complex that associates with the C-terminal domain repeats of RNA polymerase II (58, 59). 3C experiments showed that these cohesin-bound regions interacted, bridging enhancer regions and core promoters (41). However, one difference observed between the CTCF/cohesin sites and the mediator sites was that the cohesin-loading factor Nip1 was mostly present at the mediator sites and not at the CTCF sites. Preliminary ChiP experiments did not find the mediator subunit Med12 at MHC-II insulators or core promoters (data not shown). Thus, MHC-II insulator interactions are not similar to cohesin binding regions that use mediator for their function.

In summary, we present a novel role for cohesin in regulating the expression of MHC-II genes by stabilizing interactions between distant CTCF-bound MHC-II insulators and MHC-II promoter proximal regulatory sequences when the genes are actively expressed. It is likely that cohesin carries out this function by encircling the DNAs associated with the loop that is formed between these regions.
Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1

Oligonucleotide Primers used in this study

**RT-PCR primers**

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**ChIP primers**

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**Quantitative 3C primers sets**

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