THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 19, pp. 13471–13481, May 9, 2008

Unconventional Association of the Polycomb Group Proteins with Cytokine Genes in Differentiated T Helper Cells*

Received for publication, December 4, 2007, and in revised form, January 22, 2008 Published, JBC Papers in Press, February 19, 2008, DOI 10.1074/jbc.M709886200

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The cytokine transcription profiles of developing T helper 1 and T helper 2 cells are imprinted and induced appropriately following stimulation of differentiated cells. Epigenetic regulation combines several mechanisms to ensure the inheritance of transcriptional programs. We found that the expression of the polycomb group proteins, whose role in maintaining gene silencing is well documented, was induced during development in both T helper lineages. Nevertheless, the polycomb proteins, YY1, Mel-18, Ring1A, Ezh2, and Eed, bound to the *Il4* and *Ifng* loci in a differential pattern. In contrast to the prevailing dogma, the binding activity of the polycomb proteins in differentiated T helper cells was associated with cytokine transcription. The polycomb proteins bound to the cytokine genes under resting conditions, and their binding was induced dynamically following stimulation. The recruitment of the polycomb proteins Mel-18 and Ezh2 to the cytokine promoters was inhibited in the presence of cyclosporine A, suggesting the involvement of NFAT. Considering their binding pattern at the cytokine genes and their known function in higher order folding of regulatory elements, we propose a model whereby the polycomb proteins, in some contexts, positively regulate gene expression by mediating long-distance chromosomal interactions.

When naive T helper (Th)[3] cells ($CD4^+$) encounter antigen in the periphery, they can differentiate into several lineages distinguished by their cytokine production. The most studied are the Th1 and Th2 lineages. The hallmark cytokine of Th1 cells is IFNγ while Th2 cells transcribe IL-4, IL-5, and IL-13. IFNγ exerts protective functions in microbial infections and is observed clinically in cases of autoimmune disease. In contrast, IL-4 is strongly apparent during parasitic infections and is associated with deleterious allergic reactions. Lineage polarization can be achieved *in vitro* by manipulating the cytokine milieu: IL-12 and IL-4 strongly potentiate Th1 and Th2 differentiation, respectively, via the transcription factors STAT4 and STAT6. The lineage-specific transcription factors T-bet and GATA3 are critical for Th1 and Th2 differentiation, respectively; when ectopically expressed these proteins not only promote transcription of the relevant cytokines but also suppress expression of the inappropriate cytokines (1, 2).

The antigen-inducible transcription factor NFAT1 is important for the expression of both Th1 and Th2 cytokine genes (3). Although NFAT1 is activated and enters the nucleus equivalently in both lineages, we have previously shown that NFAT1 binds the cytokine genes, *in vivo*, in a restricted manner: NFAT1 binds the *Ifng* regulatory elements only in stimulated Th1 cells and the *Il4* regulatory elements only in stimulated Th2 cells (4). Looking for an explanation, we and others found that the presence of the polarizing cytokines, IL-12 and IL-4, as well as their downstream transcription factors is necessary to establish a differential pattern of histone acetylation at the cytokine genes (5–8). The hyperacetylated status of the cytokine genes correlates with the selective binding of NFAT1, and therefore we suggested that the differentiation process of Th cells reinforces chromatin structural changes, that facilitate differential accessibility to acute transcription factors (5).

The polarizing cytokines and downstream transcription factors are also crucial for the establishment of other epigenetic features of the cytokine genes in developing Th cells (1, 2). However, even in the absence of the polarizing cytokines, differentiated Th1 and Th2 cells memorize their previous cytokine expression profiles and transiently transcribe the appropriate cytokines upon T cell receptor (TCR) stimulation. The activity of STATs is inducible; therefore it is unlikely that they maintain constitutively the poised transcriptional status of the cytokine genes in memory cells (7, 9). The simplest and most plausible idea is that lineage-specific transcription factors downstream of the STATs sustain the open chromatin configuration. The question of whether GATA3 is necessary for *Il4* transcription in differentiated Th2 cells was studied by several groups using conditional ablation approaches (10–12). In all of these cases, a substantial percentage (11, 12), or even all (10) of the IL-4-producing differentiated Th2 cells maintain their ability to produce IL-4 after *in vitro* deletion of Gata3 (although the expression levels were decreased (10)). In parallel, T-bet (13), which is important for the establishment of the permissive chromatin configuration of the *Ifng* gene in developing Th1 cells (5, 6, 14–18), may be dispensable once the cells have firmly committed to the Th1 lineage (17). Together, these data suggest the involvement of factors other than the lineage-specific tran-

* This work was supported in part by grants from the Israel Science Foundation (Grant No. 986/04), the State of Lower Saxony, the Volkswagen Foundation Hannover Germany, and the Rappaport Family Institute for Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[3] The abbreviations used are: Th, T helper; PcG, Polycomb group; TrxG, Trithorax group; DH, DNase I hypersensitivity; PRC, PcG repressive complex; HDACs, histone deacetylases; HMTase, histone methyltransferase; DNMT, DNA methyltransferase; CsA, cyclosporine A; HATs, histone acetyltransferases; CNS, conserved non-coding sequence; PI, propidium iodide; ChIP, chromatin immunoprecipitation assay; IFN, interferon; IL, interleukin; TCR, T cell receptor; STAT, signal transducer and activator of transcription; NFAT, nuclear factor of activated T cells.

scription factors in the epigenetic inheritance of *Ifng* and *Il4* transcriptional status in differentiated cells.

T-bet and GATA3 may induce the expression of downstream Th1- and Th2-specific transcription factors, which, in turn, bind regulatory elements within the cytokine genes, and maintain their accessibility to acute transcription factors. Alternatively, but not mutually exclusive, the lineage-specific transcription factors could facilitate restricted binding of general maintenance machinery to the cytokine genes. A precedent is provided by the Polycomb group (PcG) and Trithorax group (TrxG) of proteins that maintain previously established, silenced, or active gene expression, respectively, throughout embryonic development into adulthood (19–27). Recent searches for targets of PcG proteins by genome-wide profiling approaches revealed that most of the target genes are regulators of differentiation (28–33). It was proposed that the PcG proteins keep stem cells in a pluripotent state by silencing these genes (24, 27). The PcG proteins also play a role during hematopoiesis in the establishment and proliferation of the lymphoid and myeloid lineages (34). Ezh2, a PcG protein, is found in association with the DNase I hypersensitivity (DH) sites IV and HSS3, regulatory elements of the *Il4* gene. However, the binding is non selective and occurs in naive Th1 and Th2 cells (35). Deficiency of another PcG protein, Mel-18, impairs Th2 differentiation (36), and the TrxG protein, MLL, is essential for Th2 memory cells to express the Th2 cytokines (37).

The PcG proteins form multimeric complexes that can be classified as either PcG repressive complex 1 (PRC1), with the core proteins M33, Bmi-1, Mel-18, Ring1A, and Ring1B, or PRC2 with the core proteins Suz12, Ezh2, and Eed. However, other combinations have also been reported, and the content of the PcG complexes depends on the cell type and the developmental stage (24, 27, 38). The mechanisms underlying the functions of the PcG proteins in gene silencing are not clearly understood, but probably entail the modification of histones: the PcG proteins interact with histone deacetylases (HDACs) and histone methyltransferase (HMTase) (38, 39). Moreover, Ring1B is a histone H2A ubiquitin E3 ligase (40, 41), and Ezh2 is an HMTase that preferentially methylates histone 3 on lysine 27 (H_3-K27), thus generating a binding site for the PRC1 (42–45). Ezh2 also recruits DNA methyltransferase (DNMT) (46). Whether these modifications are the cause or the consequence of the transcriptional maintenance program is unclear as yet (24).

This study started with the aim of identifying epigenetic regulators that are expressed similarly in both Th1 and Th2 cells, but differentially bind the cytokine genes. While the PcG proteins meet these criteria, their bulk binding activity was found, unexpectedly, to correlate with the competence to transcribe the cytokine gene and even stronger with active transcription. The expression of the PcG proteins was induced during the development of Th1 and Th2 cells. Their binding activity in the differentiated cells was dynamically modified following stimulation and was inhibited in the presence of cyclosporine A (CsA). We suggest that the known function of the PcG proteins in pairing DNA elements (19, 22, 23, 25–27, 47), is not necessarily associated with gene repression, but rather, under some circumstances, it may support active gene expression or maintain transcriptional status by mediating long-distance chromosomal interactions (2).

EXPERIMENTAL PROCEDURES

Mice—3–4-week-old female BALB/c mice were purchased from Harlan Biotech, Israel and maintained under pathogen-free conditions in the animal facility of the Faculty of Medicine, Technion-Israel Institute of Technology.

In Vitro Th Cell Differentiation—Th cell differentiation was carried out as previously described (5). Briefly, $CD4^+$ T cells were purified from the spleen and lymph nodes of 3–4-week-old mice with magnetic beads (Dynal). For Th differentiation, cells were stimulated with 1 μg/ml anti-CD3ϵ antibodies and 1 μg/ml anti-CD28 antibodies (145.2C11 and 37.51, respectively, Pharmingen) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine, penicillin-streptomycin, nonessential amino acids, sodium pyruvate, vitamins, HEPES, and 2-mercaptoethanol in a flask coated with 0.3 mg/ml goat anti-hamster antibodies (ICN). For Th1 differentiation, the cells were stimulated in the presence of 10 ng/ml recombinant mouse IL-12 (R&D systems) and 10 μg/ml purified anti-IL-4 antibodies (11B11). For Th2 differentiation, cells were stimulated in the presence of 1000 units/ml mouse IL-4 (added as a supernatant of the 13L6 cell line), 5 μg/ml purified anti-IFNγ antibodies (XMG1.2), and 3 μg/ml purified anti-IL-12 antibodies (C178). After 2 days, the medium was expanded (4-fold) in the absence of anti-TCR and anti-CD28 antibodies, but in the continued (although reduced) presence of cytokines and antibodies, which included 12 units/ml IL-2. The medium was then expanded every other day. After 8 days, differentiated Th cells were left unstimulated or were stimulated with PMA (15 nM) and ionomycin (0.75 μM). When indicated, 2 μM CsA was added 0.5 h before stimulation.

Chromatin Immunoprecipitation (ChIP)—ChIP analysis was based on the described protocol (48), with several modifications. Briefly, cells ($10–24 \times 10^7$) were cross-linked on ice for 20 min, by adding a one-tenth volume of 11% formaldehyde solution (0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES, pH 8.0) directly to the media. Glycine was then added to a final concentration of 0.125 M. Following incubation in 100 mM Tris-HCl, pH 9.4, 10 mM dithiothreitol, the cells were washed and resuspended in 0.5 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 25 μg/ml leupeptin, and 10 mM iodoacetamide) and sonicated three times with 1 min intervals at 4 °C. The samples were centrifuged at 14,000 rpm at 12 °C, and the cleared supernatants were diluted with equal amounts of dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1, and protease inhibitors). Aliquots containing $\sim 3 \times 10^7$ cells were stored at −80 °C. The samples were thawed in 1 ml of dilution buffer and precleared with 45 μl of a slurry of salmon sperm DNA-coated protein A- or G-Sepharose beads (ssDNA-beads) for 2 h at 4 °C (the beads were incubated first with 100 μg/ml ssDNA). After 10 min of high speed centrifugation, the cleared samples were incubated overnight at 4 °C with 10 μg of specific antibody followed by a 3-h incubation with ssDNA-beads. Specific antibodies used were: anti-Mel-18 (Santa Cruz Biotechnology; sc-8905 or

sc-10744), anti-ENX-1 (Ezh2, Santa Cruz Biotechnology; sc-25383), anti-YY1 (Santa Cruz Biotechnology; sc-1703), anti-RING1 (Santa Cruz Biotechnology; sc-28736), and anti-EED (Santa Cruz Biotechnology; sc-28701). After immunoprecipitation, washes, and reverse cross-linking, the samples were extracted twice with phenol/chloroform, once with chloroform and ethanol precipitated in the presence of 30 μg of glycogen. 20 μl of the resulting 160-μl samples were used in a PCR reaction with 28–30 cycles (1 min at 95 °C, 1 min at 48 °C, and 1 min at 72 °C, completed by 10 min at 72 °C). The following primers were used: *Il-4* P, 5′-TTGGTCTGATTTCACAGG-3′ and 5′-ATCAATAGCTCTGTGCCG-3′ (240-bp product); *Il-4* enhancer (VA), 5′-AGGGCACTTAAACATTGC-3′ and 5′-ACGCCTAAGCACAATTCC-3′ (239-bp product); *Il-4* DH site IV, 5′-CTCTTCTTCCCTTGATCG-3′ and 5′-GCACTTGGTATATGAGGC-3′ (219-bp product); *Il-4* Site II, 5′-GGGTGTGAATAAGCCATATTG-3′ and 5′-CCCAGCGTTTACATG AGC-3′ (175-bp product); *Il-4* LCR RHS7/RAD50-C, 5′-CCACACACTGGGATGTGTAGCTCA-3′ and 5′-AGACCCAGCTCCTCAGAAGGTAGT (250-bp product); *Ifng* P, 5′-GCTCTGTGGATGAGAAAT-3′ and 5′-AAGATGGTGACAGATAGG-3′ (250-bp product); *Ifng* CNS-1, 5′-CTTTGAAGGATACCATGG-3′ and 5′-AGGTTTCCTCTTAAGGGC-3′ (224-bp product). As controls, PCR using *Ifng* promoter or VA primers was performed directly on input DNA purified from chromatin before immunoprecipitation. Selected input samples were also amplified with each pair of specific primers. PCR products were resolved on 3% NuSieve/agarose gels and visualized with ethidium bromide.

Quantitative PCR was performed using Absolute Blue SYBR-Green ROX mix (Thermo Scientific, ABgene), according to the manufacturer's instructions, and an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Dissociation curves after amplification showed that all primer pairs generated single products. The amount of PCR product amplified was calculated relative to a standard curve of the input. The value of control immunoprecipitation was subtracted from the specific immunoprecipitation.

The following primers were used: *Ifng* P 5′-GAGAATCCCACAAGAATGGCA-3′ and 5′-CAGCTATGGTTTTGTGGCATGT-3′ (105-bp product); *Il-4* P 5′-CTCATTTTCCCTTGGTTTCAGC-3′ and 5′-CAATAGCTCTGTGCCGTCAGTG-3′ (123-bp product).

Immunofluorescence Microscopy—Cells were fixed onto poly-L-lysine slides (Sigma) in 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.5% Triton X-100 for 5 min on ice. The cells were washed three times with phosphate-buffered saline, and nonspecific binding was blocked by incubation in 5% donkey serum (0.5% Nonidet P-40, phosphate-buffered saline) for 30 min at room temperature. Then the cells were incubated with anti-PcG antibodies (4 μg/ml, 5% donkey serum in phosphate-buffered saline) for 1 h at room temperature, followed by Cy-2-conjugated donkey anti-goat antibodies or rabbit IgG (7.5 μg/ml, Jackson Laboratories). For controls, the primary antibody was omitted. The DNA was counterstained with Vectashield Mounting Medium with propidium iodide (PI) (Vector, Burlingame). Images were recorded with a Zeiss LSM Meta confocal (Zeiss, Oberkochen, Germany) hooked to an inverted motorized microscope Zeiss Axiovert 200 m using a Zeiss EC Plan-NEOFLUAR 40×/1.3 oil DIC M27 objective, zoom 2. For fluorescence detection, 488-nm and 561-nm lasers were used. Images were acquired using LSM 510 version 4.2 and processed by LSM Image Browser; they represent one middle Z-stack of the cells (1 μm).

RT-PCR—Total RNA was extracted, reverse-transcribed, and amplified with the following primer sets: *Il4*, 5′-CATCGGCATTTTGAACGAGGTCA-3′ and 5′-CTTATCGATGAATCCAGGCATCG-3′ (Genomic: 4,610-bp product, cDNA: 240-bp product); *Ifng*, 5′-CATTGAAAGCCTAGAAAGTCTG-3′ and 5′-CTCATGAATGCATCCTTTTTCG-3′ (Genomic: 1,548-bp product, cDNA: 267-bp product); *Gata3*, 5′-GAACACTGAGCTGCCTGGCGCCGT-3′ and 5′-CTTTGCGGGATAGTTTAGCAA-3′ (Genomic: 812-bp product, cDNA: 391-bp product); *T-bet* 5′-GCTACCCGCCCGTGGATGG-3′ and 5′-CCGGTGTTGGGGGAGTCTGG-3′ (Genomic: 13,279-bp product, cDNA: 384-bp product).

RESULTS

YY1 Binds to the Cytokine Genes in a Differential Manner—Most PcG proteins do not possess a DNA binding domain. YY1 is a generally expressed PcG protein that interacts with both PRC1 and PRC2 and has sequence-specific DNA binding activity (49–51). Therefore, YY1 might play a role in the recruitment of PcG complexes to DNA. YY1 has been found in association with key chromatin remodeling factors, among them HATs, HDACs, and DNMTs (39, 49–51). Depending on the context not typically documented for other PcG proteins, YY1 can either activate or repress the transcription of many genes. Targeted deletion of YY1 in mice is lethal (52).

In vitro, YY1 binds to the *Il4* promoter, and transient co-transfection assays show its ability to enhance *Il4* transcription (53). YY1 also inhibits or enhances *Ifng* promoter activity, depending on the cell type (54–56). We used the ChIP assay to assess the binding activity of YY1, *in vivo*, to selected regulatory elements and DH sites at the *Ifng* and *Il4* loci in 8-day-differentiated Th1 and Th2 cells (Fig. 1*A*). For the *Ifng* gene, we tested the promoter and the conserved non-coding sequence (CNS)1 enhancer that binds NFAT1 and T-bet and that is located 5-kb upstream of the transcription start site (57). At the *Il4* locus, we tested the silencer DH site IV (58, 59) that appears in naive Th1 and Th2 cells and binds T-bet and Runx3 (60). We also tested the Th2-specific DH sites VA, site II, and RHS7/RAD50-C. VA is an *Il4* enhancer that binds GATA3 and NFAT1 (5, 58, 61), and its accessibility to restriction enzymes correlates with IL-4 production in stimulated Th2 cells (62). RHS7/RAD50-C is a core element among several DH sites that comprise the Th2 cytokine locus control region (LCR) at *Rad50*, upstream of *Il4* (63–65) (reviewed in Ref. 2). Our results showed that YY1 binds to the cytokine genes in a differential way; the *Ifng* promoter and CNS1 were only bound in Th1 cells whereas the *Il4* regulatory elements, the promoter, VA enhancer, site II, site IV, and RHS7/RAD50-C were bound strikingly more strongly in Th2 cells (Fig. 1*B*). YY1 binds to the regulatory elements even under resting conditions, but at most sites the binding is increased upon stimulation.

The binding of YY1 to the cytokine genes was dynamic with induction in the binding activity 30 min after stimulation fol-

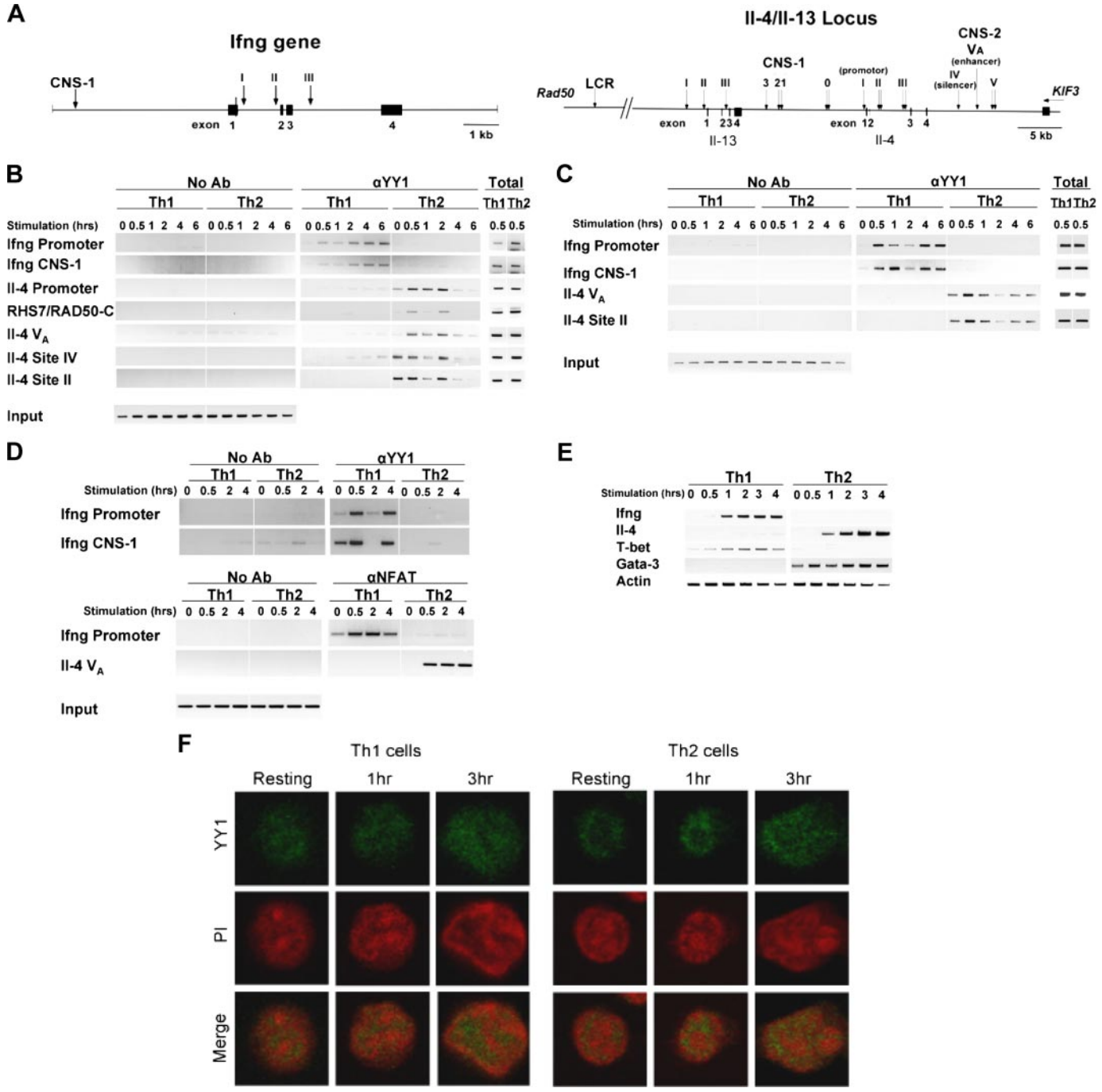

FIGURE 1. **YY1 binds to the cytokine genes selectively and dynamically.** *A*, diagram depicting the *Il4/Il13* and the *Ifng* loci. The last exon of the *KIF3* gene is shown downstream of, and the *Rad50* gene upstream of, the *Il4/Il13* locus. *Arrows* indicate the location of Th1 and Th2 DH sites, and *black rectangles* represent exons. *B*, ChIP assay assessing the binding of YY1 to the *Il4* and *Ifng* regulatory regions shown in *A* at the indicated time points under resting and stimulated conditions. Chromatin complexes were immunoprecipitated with anti-YY1 antibodies or without antibodies (*No Ab*), and PCR primers specific for the *Ifng* promoter or CNS1, or the *Il4* promoter, enhancer (V_A), DH site IV, DH site II, or RHS7/RAD50-C (LCR) were used to amplify the precipitated DNA. As a control, the PCR was performed directly on input DNA purified from non-precipitated chromatin from Th1 and Th2 cells stimulated for 0.5 h (*Total*; *right columns*). The *lowest panel* shows the PCR products of the complete input using PCR primers specific for the *Ifng* promoter. *C*, independent experiment similar to the one shown in *B*. *D*, *upper panel*, independent experiment similar to the one shown in *B*. *Lower panel*, ChIP assay assessing the binding of NFAT1 to the *Ifng* promoter and *Il4* enhancer using the same chromatin samples as in the *upper panel*. *E*, RT-PCR with RNA extracted from resting and stimulated Th1 and Th2 cells. *F*, immunofluorescence staining with anti-YY1 antibodies of resting and stimulated Th1 and Th2 cells at the indicated time points.

lowed by a temporary reduction after 1–2 h. Although this phenomenon was consistent, the strength and the kinetics of binding showed some variability among experiments (compare Fig. 1, *B*, *C*, and *D*, *upper panel*). A control ChIP assay with the same chromatin preparation as in the *upper panel* of Fig. 1*D*, showed that NFAT1 bound to the *Ifng* promoter and to the *Il4* enhancer VA with the expected pattern as regards to differentiation and induction, and this result confirms that the cells were adequately differentiated and stimulated (Fig. 1*D*, *lower panel*). However, we did not detect any dynamic binding activity of NFAT1. The binding activity of several PcG proteins is dynamic during the cell cycle (24, 66). In Chinese hamster ovary and

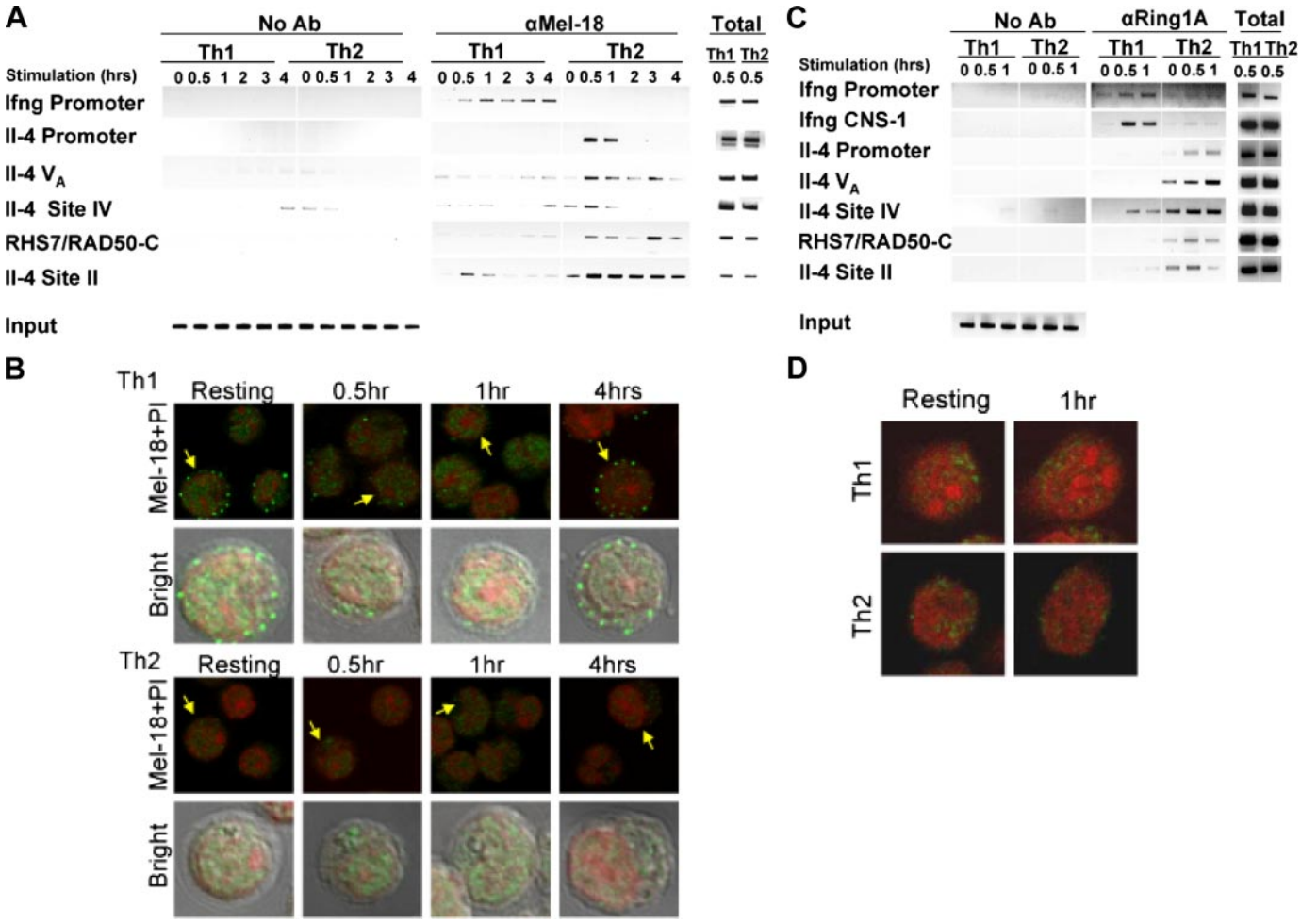

FIGURE 2. **Mel-18 and Ring1A are associated with the cytokine genes in a differential and inducible manner.** *A*, ChIP assay assessing the binding of Mel-18 to the *Il4* and *Ifng* regulatory elements at the indicated time points in resting and stimulated cells as in Fig. 1*B*. *B*, immunofluorescence staining with anti-Mel-18 antibodies (sc-8905) of resting and 1-h-stimulated Th1 and Th2 cells. The panel denoted *Bright* shows more highly magnified images of the cells that are marked in the *upper panel* with an *arrow*. *C*, ChIP assay assessing the binding of Ring1A to the *Il4* and *Ifng* regulatory elements as in Fig. 1*B*. *D*, immunofluorescence staining with anti-Ring1A antibodies of resting and 1-h-stimulated Th1 and Th2 cells. Images show merged data of antibody and PI staining.

HeLa cells, the subcellular distribution of YY1 changes from mainly cytoplasmic at G_1 to mainly nuclear at early and middle S phase (67). Under our experimental conditions, the percentage of unstimulated Th cells in the S plus M phases ranged between 10 and 25% in different experiments, but stayed almost the same 4 h following stimulation, as monitored by flow cytometry (data not shown). The RNA extracted from differentiated cells as a routine, verified their ability to express the expected pattern of tissue-specific transcription factors and cytokines (Fig. 1*E*). RNA levels of the cytokines increased following stimulation, but after that, at the tested intervals, we did not monitor any further changes. Immunostaining experiments showed that YY1 is expressed similarly in both Th1 and Th2 cells as diffuse nuclear staining and without obvious changes between the resting and stimulated condition (Fig. 1*F*). In control assays, where the primary antibody was omitted, the labeling was negligible. These results indicated that, although YY1 was expressed in both Th lineages, the binding activity correlated selectively with poised and overt gene transcription.

The PRC1 Proteins, Mel-18 and Ring1A, Are Also Associated with the Cytokine Genes in a Restricted Manner—Because YY1 is involved in both gene repression and gene activation, we wanted to test the binding pattern of the core proteins in the PcG complexes. Mel-18, a member of the PRC1, negatively regulates the self-renewal activity of hematopoietic stem cells (68). Mel-18-deficient mice have severe proliferative defects in lymphoid cells and as a result have splenic and thymic hypoplasia (34, 69, 70). The differentiation of Th2 cells derived from Mel-18-deficient mice is impaired; *Il4* gene expression is reduced whereas expression of the *Ifng* gene is enhanced (36). However, because the expression of GATA3 is also lower in these mice, T cells from Mel-18-deficient mice might not be differentiated properly considering the known importance of GATA3 for T cell development (71). Indeed, these mice have only 5–10% of the thymocytes found in control mice (36, 70). The results of our ChIP experiments revealed a differential recruitment pattern of Mel-18 to the cytokine genes, which correlated with gene activity; Mel-18 binds to the *Ifng* promoter in Th1 cells and to the *Il4* regulatory elements, promoter, VA enhancer, site IV, RHS7/RAD50-C, and site II, much more strongly in Th2 cells, in a similar way to YY1 (Fig. 2*A*). The binding activity of Mel-18 was higher in stimulated cells in comparison to resting cells, and the binding pattern was dynamic. The kinetics was variable between experiments, but

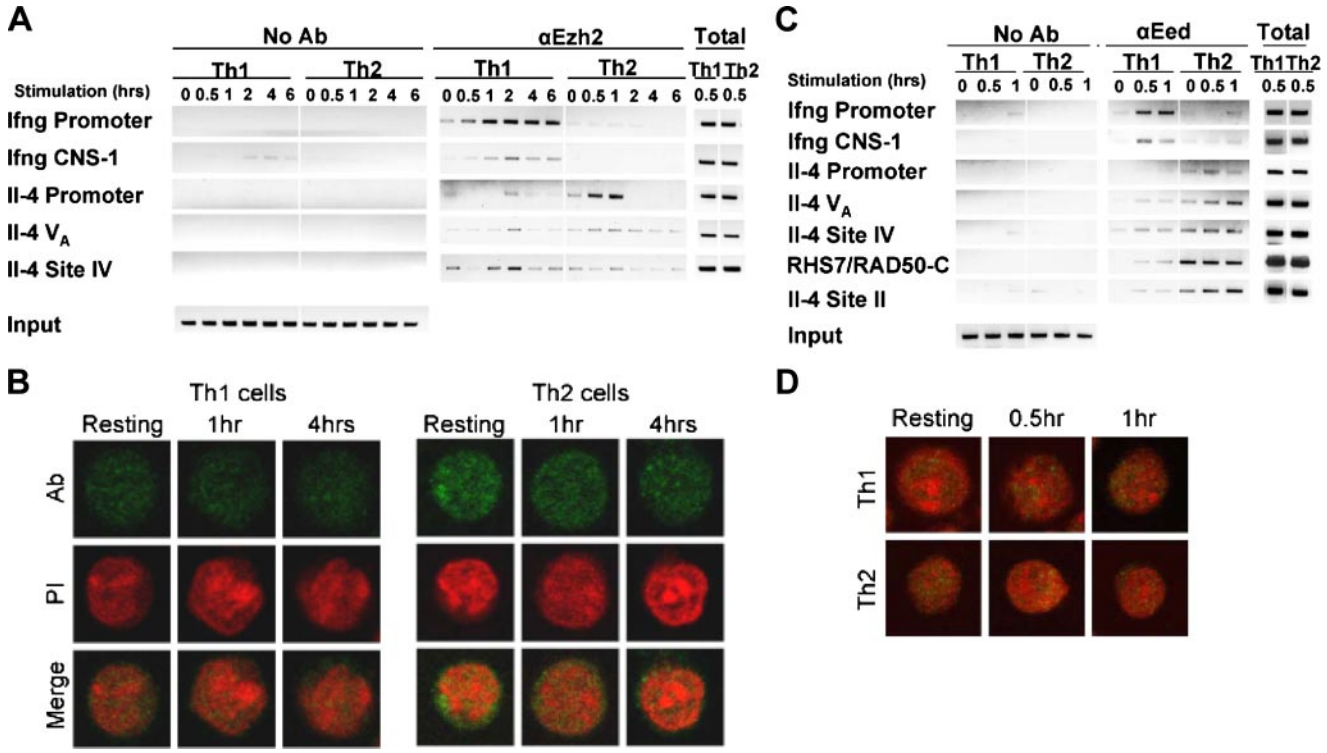

FIGURE 3. **The recruitment of Ezh2 and Eed to the cytokine genes is partially restricted.** *A*, ChIP assay assessing the binding of Ezh2 to the *Il4* and the *Ifng* regulatory regions in resting and stimulated cells at the indicated time points as in Fig. 1*B*. *B*, immunofluorescence staining with anti-Ezh2 antibodies of resting and stimulated Th1 and Th2 cells at the indicated time points. *C*, ChIP assay assessing the binding of Eed to the *Il4* and the *Ifng* regulatory regions. *D*, immunofluorescence staining with anti-Eed antibodies of resting and stimulated Th1 and Th2 cells at the indicated time points. Images show merged data of antibody and PI staining.

consistently demonstrated binding of short duration to the *Il4* promoter and prolonged binding to the *Il4* site II. The results of the immunostaining experiments detected two types of distribution of Mel-18 in both Th1 and Th2 cells (Fig. 2*B*): (i) a diffuse distribution throughout the nucleoplasm, which was less bright 4 h after stimulation in comparison to earlier time points, and (ii) distinct protein aggregations in the cytoplasm of resting cells, which were more visible in Th1 cells but did also exist in Th2 cells. Their number was reduced 0.5 h following stimulation, and then gradually increased 2 h later (Fig. 2*B* and data not shown). Further studies are necessary to address the potential relationship between the spatial distribution of Mel-18 and its dynamic binding pattern on the cytokine genes.

The PRC1 complex also contains the proteins Ring1A and Ring1B. Ring1B catalyzes the ubiquitylation of histone H2A on K119 (H2A-K119) (41), although both Ring1A and Ring1B contribute to this E3 ligase activity *in vivo* (40, 41, 72). Ring1B is highly expressed and essential during early development (40, 41, 72–74), whereas Ring1A is more associated with differentiated cells and has a role in the maintenance of ubiquitylation of the inactive X chromosome (40, 75, 76). In our experiments, as shown in Fig. 2*C*, Ring1A bound to the cytokine genes in a differential pattern, as did YY1 and the other PRC1 member, Mel-18; it bound to the *Ifng* promoter and CNS1 more strongly in Th1 cells and to the *Il4* promoter, VA enhancer, site IV, site II, and RHS7/RAD50-C more strongly in Th2 cells. The binding was increased following stimulation. As can be seen in Fig. 2*D*, Ring1A was localized to small dots in the nucleus and the cytoplasm of both Th1 and Th2 cells (Fig. 2*D*). The correlation of the PRC1 binding pattern with gene expression strengthened the idea that PcG proteins positively regulate the transcription of the cytokine genes *Ifng* in Th1 cells and *Il4* in Th2 cells.

The Binding Activity of the PRC2 Proteins, Ezh2 and Eed, Is Partially Differential—Next, we assessed the binding activity of Ezh2 to monitor the recruitment of the PRC2 complex. As mentioned before, Ezh2 was found in association with two regulatory elements in the *Il4* gene, site IV and HSS3, in both Th1 and Th2 cells (35). We also observed a less differential binding pattern of site IV and as well of *Il4* enhancer VA, in comparison to the former restricted binding activity of the PcG proteins. However, we clearly saw that Ezh2 binds to the cytokine gene promoters selectively: it bound to the *Ifng* promoter in Th1 cells and to the *Il4* promoter in Th2 cells (Fig. 3*A*). Ezh2 also bound to the *Ifng* CNS1 only in Th1 cells. The binding to the *Ifng* promoter increased gradually following stimulation, whereas the binding to the *Il4* promoter increased upon stimulation, reached a peak 1 h later and dramatically decreased (Fig. 3*A*). The results of the immunostaining experiments demonstrated that Ezh2 was nuclear and cytoplasmic in both Th lineages (Fig. 3*B*).

Another member of the PRC2 proteins, Eed, cooperates with Ezh2. Cells carrying a null allele of Eed do not localize Ezh2 to the inactive X chromosome and do not maintain X inactivation (77). Eed isoforms differentially target the Ezh2-containing complexes toward histone H3-K27 or H1-K26 (78). As with Ezh2, we found that Eed bound to the *Ifng* promoter and CNS1 in Th1 cells, and to the *Il4* promoter in Th2 cells. The binding activity to other *Il4* regulatory elements, the VA enhancer, site

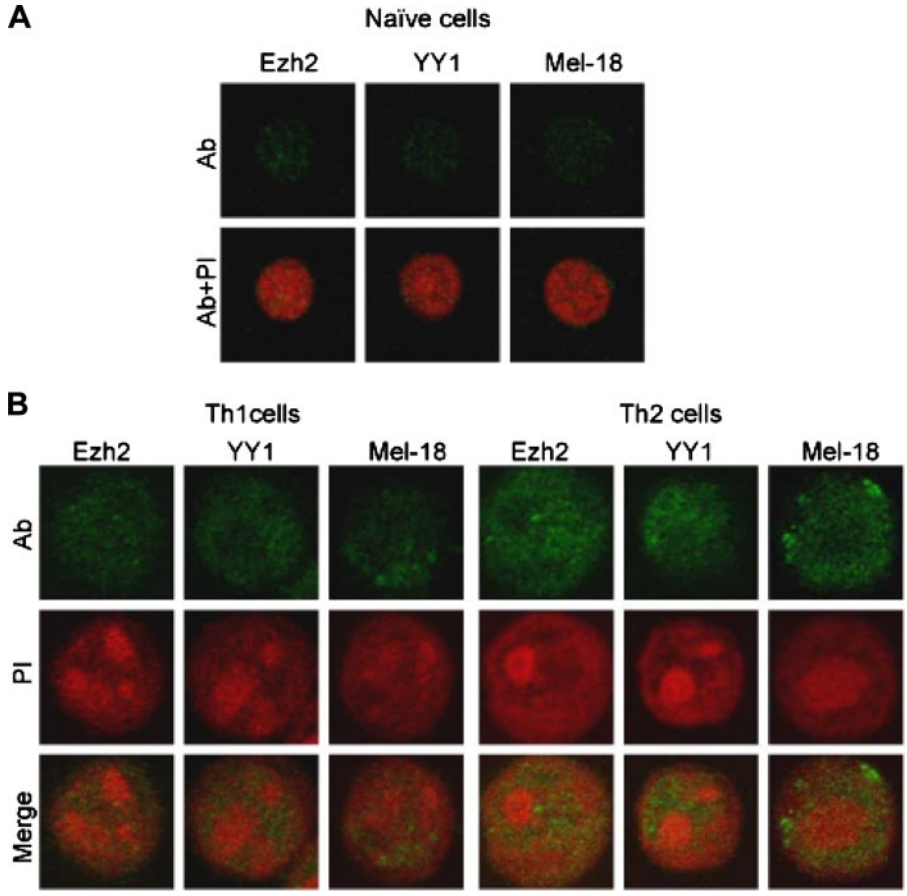

FIGURE 4. **The expression of YY1, Mel-18, and Ezh2 is induced during Th cell development.** *A*, immunofluorescence staining with anti-Ezh2, anti-YY1, and anti-Mel-18 (sc-10744) antibodies of freshly isolated CD4+ cells (naive) from 3-week-old mice. *B*, immunofluorescence staining as in *A* of CD4+ T cells incubated for 48 h under polarizing Th1 or Th2 conditions.

IV, RHS7/RAD50-C and site II, was less selective, but clearly higher in Th2 cells (Fig. 3*C*). Eed was localized mainly to the nucleus in both Th lineages, although it was also seen in the cytoplasm (Fig. 3*D*). The partially selective binding pattern of the PRC2 proteins might suggest that the activity of the PcG proteins is imposed by binding to selected key regulatory elements, and that the context dictates the function.

The Expression of PcG Proteins from PRC1 and PRC2 Is Induced in Developing Th1 and Th2 Cells—To test the expression pattern of the PcG proteins during Th cell development, we stained freshly purified CD4+ Th cells (naive) and 48-h differentiating Th1 and Th2 cells with anti-YY1, anti-Mel-18, and anti-Ezh2 antibodies. The staining intensity of naive cells was low (Fig. 4*A*). However, 48 h later, under both polarizing conditions, the expression levels of the PcG proteins were augmented significantly (Fig. 4*B*). The induction of expression of the PcG proteins during development in both lineages, and their similar expression pattern in the differentiated cells suggest that the PcG proteins perform a similar function in Th1 and Th2 cells. The target genes, however, are distinct and appropriate to the required cytokine expression programs.

The Binding Activity of Mel-18 and Ezh2 at the Cytokine Promoters Is Calcineurin-dependent—The differential and inducible binding activity of the PcG proteins is reminiscent of the binding pattern of NFAT1. CsA impairs the dephosphorylation of NFAT by calcineurin and as a consequence prevents its nuclear translocation. Stimulation of differentiated Th cells in the presence of CsA impaired the recruitment of Mel-18 to the *Ifng* promoter in Th1 cells and to the *Il4* promoter in Th2 cells (Fig. 5, *A* and *B*). Similar results were obtained with Ezh2 (Fig. 5, *C* and *D*). These results strongly implicated NFAT in the recruitment of the PcG proteins to the cytokine genes, although the exact mechanism is unclear as yet.

DISCUSSION

The most surprising result of this work was that the binding activity of the PcG proteins in differentiated Th cells correlated with the competence to express the cytokine genes and even stronger with overt gene expression. This was unexpected considering the well documented functions of the PcG proteins in gene silencing. Fig. 6*A* schematizes the binding activities of the PcG proteins at the *Il4* and the *Ifng* promoters in Th cells shortly after stimulation.

Differential Binding Pattern of the PcG Proteins in Th Cells—The PcG proteins bind to the *Il4* and *Ifng* genes in a selective manner, but we did observe a low level of association of the PcG proteins with the repressed genes, especially for Eed, and partially non-selective binding of Ezh2. This binding might be the remnant of an earlier recruitment of the PcG proteins to the cytokine genes in naive cells, or more likely in developing Th cells, that might bind these complexes regardless of the cytokine milieu, in a similar way to the early non-differential histone hyperacetylation (5, 6). A few months ago, it was reported that Ezh2 is selectively associated with the *Ifng* promoter in 3-day-developing Th2 cells. Therefore, the involvement of Ezh2 in gene repression was suggested (79). Further investigation will determine whether some PcG proteins have a dual function during development and in differentiated cells.

Several studies demonstrated non-selective binding of PcG proteins to genes irrespective of their transcriptional status, and the context was proposed to restrict their activity (29, 80–83). The chromatin environment of the cytokine genes is totally different in differentiated Th1 and Th2 cells. *Il4* in Th2 cells has an accessible chromatin structure, and is bound by many lineage-specific and nonspecific transcription factors such as GATA3, STAT6, MAF, and NFAT, whereas under Th1 conditions *Il4* is associated with a more condensed chromatin structure and different proteins such as T-bet and Runx (1, 60). The content of the PcG complexes might also dictate their cat-

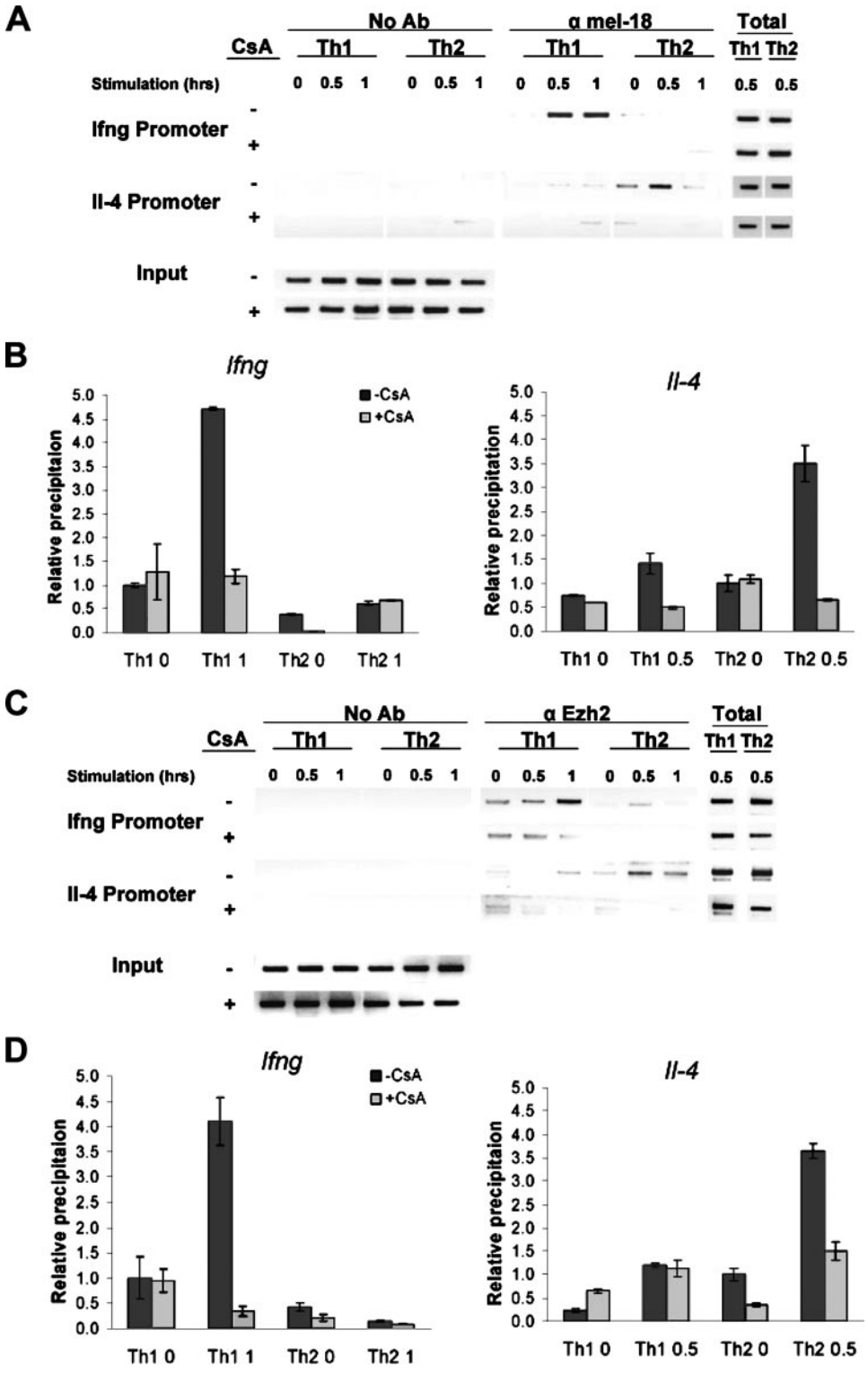

FIGURE 5. **The recruitment of Mel-18 and Ezh2 to the cytokine promoters is calcineurin-dependent.** ChIP assay assessing the binding of Mel-18 (*A* and *B*) and Ezh2 (*C* and *D*) to the *Ifng* and *Il4* promoters in resting and stimulated Th1 and Th2 cells at the indicated time points in the presence and absence of 2 μM CsA. *B* and *D*, real-time PCR was performed to quantify the binding of Mel-18 and Ezh2 to the *Ifng* and *Il4* promoters in similar experiments to *A* and *C*, respectively. The values for resting Th1 cells (Th1 0, *Ifng* primers) or resting Th2 cells (Th2 0, *Il4* primers), were arbitrary set to 1. Data represent the mean ± S.D. of duplicates.

alytic activity, *e.g.* Bmi-1 and Ring1A contribute to Ring1B activity and stability (40, 41, 72–74), whereas Mel-18, which is 70% identical to Bmi-1, has little effect on Ring1B E3 ligase activity (72). In a similar way, isoforms of Eed direct the enzymatic specificity of Ezh2 (78). In addition, the presence of a TrxG protein (37) can influence the function of the PcG proteins (82). Whereas distinct environments might explain the differential activity of PcG proteins with non-selective binding patterns, the majority of binding of the PcG proteins to the cytokine genes was restricted to the active genes. Therefore, this model system of *in vitro* differentiated Th cells might be a useful tool to dissect potential, as yet unknown, functions of the PcG proteins.

The Inducible and Dynamic Recruitment of the PcG Proteins—The majority of PcG protein binding activity in Th cells was induced after stimulation, but the proteins were bound even under resting conditions. The selective pattern of histone acetylation and the three-dimensional structure of *Ifng* and *Il4* genes are apparent even in resting differentiated Th cells that do not actively transcribe the cytokine genes (1, 2). Based on the known YY1 ability to stimulate or repress gene expression depending on the context (49–51), it is tempting to speculate that YY1, and maybe other PcG proteins, serve as repressors under resting conditions, reduce the basal activity of the open loci and at the same time maintain the competence for gene expression. Following stimulation, YY1 might gain the ability to activate due to cooperation with acute transcription factors like NFAT1.

The mechanisms by which the PcG complexes are anchored to the chromatin are still poorly understood. YY1 possesses a DNA binding domain, but its binding sites alone do not make up polycomb response elements (PREs) in *Drosophila*, and other DNA elements are needed. The PRC2 locally trimethylates H3K27 and thereby creates binding sites for the PRC1 (42–45). However, the recruitment is probably more complicated and mediated also by sequence-specific DNA-binding proteins (23, 24). We did not observe substantial changes in the expression or the localization of the PcG proteins following stimulation, with the exception of Mel-18. Perhaps the resolution of the

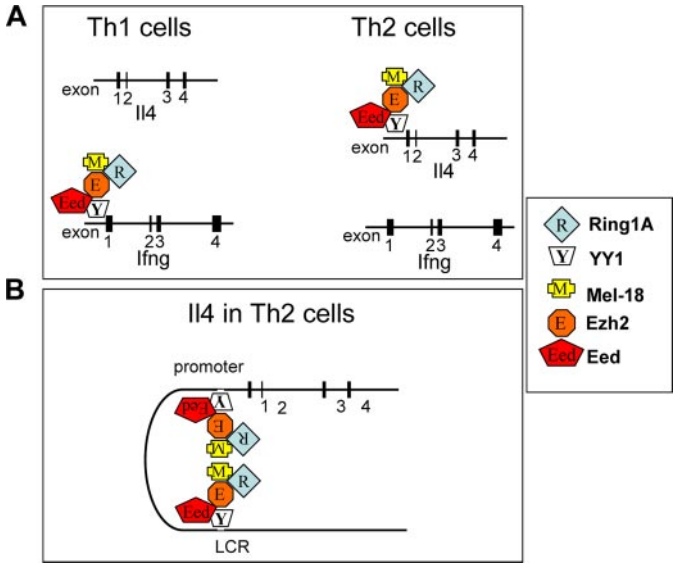

FIGURE 6. *A*, scheme summarizes the differential binding activity of the PcG proteins to the *Il4* and *Ifng* promoters ~0.5 h following stimulation. The spatial organization of the proteins inside the complexes is arbitrary. *B*, model depicting the potential involvement of the PcG proteins in long-distance interactions of the cytokine gene loci.

confocal microscope was not high enough, but alternatively the induction in the binding activity may result from post-translational modifications, alteration in the chromatin structure or active recruitment. We do not know yet whether the stimulation-induced reduction in the cytoplasmic aggregates of Mel-18 results from Mel-18 disappearance or spatial reorganization, but it may be related to the induction of binding. All of the PcG proteins showed nuclear localization, and with the exception of very faint cytoplasmic staining of YY1, they also appeared in the cytoplasm. There are several studies demonstrating cytoplasmic activity of PcG proteins in T cells: Ezh2 interacts with Vav (84) and is involved in actin polymerization (85), Ezh1 interacts with Zap-70 (86) and integrin receptors recruit Eed to the plasma membrane (87). Additional experiments are necessary to determine the potential cross-talk between these two PcG populations.

Our results suggest that NFAT is involved in the recruitment of the PcG complexes to the cytokine genes upon stimulation. Possibly, the mechanisms that dictate the selective recruitment of NFAT ultimately restrict the binding of the PcG proteins. T-bet and GATA3 may also be involved in the selective targeting of the PcG proteins by either the differential establishment of accessible chromatin at the cytokine loci or by the direct recruitment of these factors. In this matter, it is worth noting that the PcG protein Bmi-1 interacts with GATA3 and increases its stability in Th cells (88), and YY1 interacts with GATA1 at the α-globin enhancer (89).

The immunoprecipitation of YY1 and Mel-18 with the cytokine genes was dynamic, showing a window of lower binding activity. The observed binding activity of Mel-18 and Ezh2 at the *Il4* promoter was of short duration. Eed and Ring 1A were monitored only for a short period after stimulation. We can consider two scenarios to explain the dynamic activity. (i) The simplest idea is that the dynamic binding reflects a true release of the PcG proteins from the regulatory elements. Immunohistochemical and biochemical approaches in *Drosophila*, and human cells revealed that the bulk of the PcG proteins are depleted from metaphase chromosomes with different kinetics and to different extents and reassociate between anaphase and G_1 (67, 90–94). However, the results of our flow cytometry experiments indicated that the binding alterations occurred more quickly than changes in the cell cycle. Fluorescence bleaching studies on *Drosophila* PcG proteins in living embryos and larval tissues have demonstrated that PcG complexes exchange rapidly, within minutes, on their chromatin targets (95). Also, competition analysis in salivary gland nuclei revealed that PcG proteins bound to chromosomes exchange dynamically (24, 83). Therefore, the dynamic binding activity may result from intermittent recruitment that constantly samples the continuity of the TCR signaling. However, we cannot rule out the possibility that the PcG proteins are genuine repressors that reduce the expression from the open cytokine gene, but periodically leave the chromatin to allow regulated transcription. Regarding the last matter, we also cannot exclude the scenario that the PcG proteins only bind the cytokine genes in a subpopulation of cells that do not transcribe these genes. (ii) The reduction in signal observed in the ChIP experiments is misleading and reflects temporary epitope masking that is mediated by an alteration in the PcG protein accessibility as a result of either conformational change, accumulation of other proteins to form a large complex, or intrachromosomal interactions.

Potential Function of the PcG Proteins in Intra- and Interchromosomal Interactions—Considering that: (i) the PcG proteins have the ability to mediate higher order chromatin compaction and folding of regulatory elements (19, 22, 23, 25–27, 47); (ii) the recently documented long-distance intrachromosomal interactions in the *Il4* (65, 96, 97) and *Ifng* (98–100) loci, and the interchromosomal associations between both genes (100) (review in Ref. 2, 101); and (iii) our results in which we demonstrated the differential association of the PcG proteins in differentiated Th cells, we propose a model whereby the function of the PcG proteins is associated with the three-dimensional chromatin conformation of the cytokine genes (Fig. 6*B*). This might involve other nuclear factors such as nuclear matrix proteins for tethering the loops to the nuclear matrix (96, 99), or the RNAi machinery for the maintenance of long-range contacts (102, 103). The idea that the PcG proteins are involved in bringing distal chromosomal loci into close spatial proximity is supported by the role of Ezh2 and YY1 in the recombination of the immunoglobulin locus during B cell development (104, 105). Pro-B cells lacking YY1 have a defect in contraction of the locus (104), which is necessary for the recombination of the distal variable regions (106–108).

Further studies are necessary to elucidate the potential functions of the PcG proteins in inducible transcription and cellular memory. These studies will probably reveal additional layers of complexity and might be applicable to other models of differentiation.

Acknowledgments—We thank Ilana Drachsler for help with the tissue culture and Edith Suss-Toby from the interdepartmental facility for technical assistance with the confocal microscopy.

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