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Allelic Exclusion of IgH through Inhibition of E2A in a VDJ Recombination Complex

Jannek Hauser, Christine Grundström, and Thomas Grundström

A key feature of the immune system is the paradigm that one lymphocyte has only one Ag specificity that can be selected for or against. This requires that only one of the alleles of genes for AgR chains is made functional. However, the molecular mechanism of this allelic exclusion has been an enigma. In this study, we show that B lymphocytes with E2A that cannot be inhibited by calmodulin are dramatically defective in allelic exclusion of the IgH locus. Furthermore, we provide data supporting that E2A, PAX5, and the RAGs are in a VDJ recombination complex bound to key sequences on the IgH gene. We show that pre-BCR activation releases the VDJ recombination complex through calmodulin binding to E2A. We also show that pre-BCR signaling downregulates several components of the recombination machinery, including RAG1, RAG2, and PAX5, through calmodulin inhibition of E2A. *The Journal of Immunology*, 2014, 192: 2460–2470.

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de diversity of AgRs is generated by each B and T cell precursor assembling one variable (V), one joining (J), and, on some receptor genes, a diversity (D) gene segment from vast arrays of these segments. This V(D)J recombination of Ig genes starts with IgH. Most IgHs do not become functional because of the random nature of the fusion of gene segments in V(D)J recombination. Therefore, the expression of a pre-BCR with membrane-bound IgH associated with surrogate L chain proteins is a critical checkpoint that monitors for functional IgH rearrangement. Later in the development, the functional rearrangement of Ig L chain gene segments is verified by the complete Ab of the BCR. V(D)J recombination are initiated by introduction of dsDNA breaks by the recombination activating gene enzymes RAG1 and RAG2 at recombination signal sequences (RSSs). The RAG1/RAG2 complex binds sequence specifically to RSS sequences, and RAG2 specifically recognizes histone H3 trimethylated at lysine 4 (H3K4me3). Recognition of RSSs and H3K4me3 and accessibility of the chromatin are all necessary for V(D)J recombination (1–4). DNA elements regulating the accessibility include promoters and enhancers, and some transcription factors were shown to play a role (1–4). The strongiest link with a transcription factor is for E2A, which has the two splice forms E12 and E47 (3). These bind to “E-box” sequences in Ig genes and are required for both RAG expression and Ig recombination. E-boxes in the Igκ L chain intronic enhancer are crucial for activation of V-J rearrangement at Igκ loci (5), and E12 and E47 regulate the sequential rearrangement of the Ig L chain loci (6). Ectopic expression of E2A, together with RAG1/RAG2, induces D to J recombination at Igκ loci in cells that normally never undergo V(D)J recombination (7). The V segments of the Igκ locus have binding sites for several transcription factors, including E2A and PAX5 (8, 9), and PAX5 is essential in inducing and orchestrating IgH recombination in the V region (10–14). Embryonic kidney cells show V to DJ recombination at the Igκ loci exclusively when RAG1/RAG2 and E2A are ectopically coexpressed with PAX5 (14). Therefore, RAG1, RAG2, PAX5, and E2A are all indispensable for VDJ recombination of the Igκ locus.

A key feature of the immune system is the paradigm that one lymphocyte has only one Ag specificity that can be selected for or against to enable targeting of the pathogen while avoiding collateral damage and wasted resources. The nonspecificity requires that only one allele of genes for AgR chains is made functional. This allelic exclusion requires that only one allele at a time undergoes complete V(D)J recombination. Multiple mechanisms most likely contribute to allelic exclusion, but the relative role of the proposed models remains controversial (15–17). The allelic-exclusion system ensures that successful VDJ recombination on one allele shuts off recombination on the other allele by feedback inhibition to prevent a second successful rearrangement (15–17). This feedback inhibition from AgRs has an established major role in allelic exclusion, but little is known about the molecular mechanism(s) by which it is achieved (15–17). However, expression of RAG1 and RAG2 is lower in pre-B cells expressing the pre-BCR than in the preceding and succeeding developmental stages, suggesting negative feedback of these key recombination proteins (18), but the molecular mechanism(s) of the allelic exclusion by feedback inhibition has remained largely enigmatic.

When the main Ca²⁺-sensor protein calmodulin (CaM) is Ca²⁺-loaded, it can bind to and inhibit transcriptional activities of E2A (19–21). In this study, we analyzed expression of the two Igκ alleles in B cells expressing CaM-resistant (CaM[R]) E2A and show that they are dramatically defective in allelic exclusion. One mechanism of the dependence of allelic exclusion on CaM inhibition of E2A was shown to be that pre-BCR activation downregulates several components of the recombination machinery, including RAG1, RAG2, and PAX5, through CaM inhibition of E2A. Furthermore, data support that E2A, PAX5, and the RAGs are in a complex bound to key sequences on the Igκ gene, and they instead became bound to CaM after pre-BCR activation. The recombination complex is directly released from the key Igκ sites through CaM inhibition of E2A.

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Abbreviations used in this article: B6, C57BL/6; CaM, calmodulin; CaM[R], CaM resistant; ChIP, chromatin immunoprecipitation; H3K4me3, histone H3 trimethylated at lysine 4; PLA, proximity ligation assay; RSS, recombination signal sequence; shRNA, short hairpin RNA; TdT, terminal deoxynucleotidyl transferase; WT, wild-type.

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Materials and Methods

**Mice and allelic-exclusion analysis**

C57BL/6 (B6) male mice heterozygous for E2A knockout (E2A<sup>+</sup>) (22) were crossed with BALB/c females (Taomnic, Ry, Denmark). Total bone marrow was isolated from resulting E2A<sup>+</sup> F1 mice at age 6–8 wk and immediately injected with retrovirus expressing wild-type (WT) or CaM<sup>Δ</sup> mutant E12 in culture with complete RPMI 1640 medium, IL-7 (10 ng/ml; Peprotech), and poly(C) (10 µg/ml). Twenty-four hours postinfection, bone marrow cells were supplemented with fresh total bone marrow (a quarter of the infected amount) from B6 × BALB/c F1 E2A<sup>+</sup> mice, and the mixture was transplanted into lethally irradiated (9.5 Gy) B6 × BALB/c F1 E2A<sup>+</sup> recipients at age 6–8 wk (up to 2 × 10<sup>6</sup> cells in 150–200 µl HBSS/mouse) by tail-vein injection. Recipient mouse spleen cells were collected 2–3 wk posttransplantation. The effect of E12 WT and mutant expression on allelic exclusion was analyzed by FACS using the IgH allotype-specific Abs IgM<sup>M</sup>-PE (specific for IgH of the BALB/c strain; clone DS-1) and IgM<sup>B</sup>-biotin (specific for IgH of the B6 strain; clone AF6-78), anti-CD19-allophycocyanin (clone ID3), and streptavidin–PerCP (all from BD Biosciences) or streptavidin–PerCP–Cy5.5 (eBioscience). All cells were treated with CD16/CD32 Fc Block before staining, and dead cells were excluded by propidium iodide (both from BD Biosciences) or streptavidin–PerCP–Cy5.5 (eBioscience). Flow cytometry was performed with a FACSCalibur, and cell sorting was done with a FACSaria III instrument (both from BD Biosciences). IgMA<sup>+</sup> IgMB<sup>+</sup> double-positive B cells, as displayed in Fig. 1A, were sorted at 4˚C directly into culture in 500 µl complete RPMI 1640 medium supplemented with 20% FCS in a 24-well culture plate containing a layer of S17 feeder cells, irradiated at 30 Gy. Approximately 20,000–50,000 double-positive cells were isolated from a total of 10–20 million splenocytes. After sorting, the medium was adjusted to 1 ml with 25 µg/ml LPS (Calbiochem) and 10% FCS, and the cells were cultivated for 3 d before reanalysis by FACS and immunostaining. All mouse research was carried out under a Swedish project license and was subject to local ethical review.

**Plasmids, viruses, cell culture, and transfections**

The human pre-B cell line Nalm-6 was maintained in RPMI 1640 medium supplemented with 5% FCS and antibiotics. The EBV-based pMEP4 shuttle vector expressing the mouse IgM<sup>Δ</sup> heavy chain, and the pMEP4 derivatives encoding WT E2A and the CaM<sup>Δ</sup> mutant m8N47 of E12 were described previously (21), and the MSCV-IRES-GFP–based retroviruses were described previously (21, 23, 24). Nalm-6 cells transfected to express pMEP4 derivatives were selected with Hygromycin B (Roche) for 5 d, as described previously (25). Their pre-BCR was prepped, as previously described (26), from the Nalm-6 human pre-B cell line, and EMSAs were performed with probe sequences (E2A-binding sites are underlined) from the mouse RAG1 promoter, 5′-AAGAGGCGAGTGCGACGCTGAC-3′; the RAG2 promoter, 5′-GGGAGGCCAGAGTTCTACC-3′; the RAG enhancer, 5′-AGAGGAGAGCTGGCTTCA-3′; and the TDT promoter, 5′-AGGGCGAGCTGCGAGCC-3′ (27–30). The control probe from the human GM-CSF promoter was described previously (25).

**Real-time RT-PCR**

RNA was extracted using TRIzol reagent (Invitrogen), and quantitative real-time RT-PCR analysis was performed, as previously described, using GAPDH as internal control (23). The specificity of the PCR amplifications was assessed by melt-curve analysis, as described by the manufacturer (Bio-Rad). Some of the primer pairs used were described previously (25, 31). The real-time RT-PCR primer pairs for mouse and human genes that had not been described previously were were: mouse Rag1: 5′-GACAAGAAGAAGTGAGGATG-3′ and 5′-AGGAAAGATTGACCAAGTAG-3′; mouse Rag2: 5′-TTCGGCCAATGTGTTGATG-3′ and 5′-ACTTGGTCTCTCCCTGAC-3′; mouse Tdt: 5′-GACATCCTGCTCATCG-3′ and 5′-TGGTTCTCTCCGGCAAG-3′; mouse Pax5: 5′-AGTCTCCAGCGCAATG-3′ and 5′-TCCTGGTGTTGGATAGATG-3′; mouse Ets1: 5′-CAACACAAACTTTCAAGTGC-3′ and 5′-GGGTTGATACAGCTCAAGTG-3′; human Rag1: 5′-CCGACGAAAAATGCCGAATA-3′ and 5′-TCAGCGCAAGATATGG-3′; human Rag2: 5′-GGAGCAATCTACAACAGC-3′ and 5′-ACATACAAAGTGGGCAA-3′; human TdT: 5′-GGGAAAGAAGGAGATTCTT-3′ and 5′-TCCTGTTTGAGGATAGATG-3′; mouse Fos: 5′-GAGGAGGAGGAGTGATG-3′ and 5′-AGTCTCCAGAATGACTCGCTTT-3′; human OcT2: 5′-AGTCTGTTGGAGAGGATG-3′ and 5′-TCCGCGGCA-TCTATCAACTTG-3′; and human Il6: 5′-GTCGAGTTGCTCCTCCCT-3′ and 5′-GCCCTTGGTGTCCCTTAC-3′.

**Proximity ligation assay**

Harvested mouse primary pre-B cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% FCS in PBS. Proximity ligation assay (PLA) (32–35) was performed with the Duolink system using in situ PLA kits from Olink Biosciences. In brief, cells were stained for intracellular proteins using Abs, as listed below. Secondary Abs conjugated with oligonucleotides (PLA probes) were subsequently used, according to the manufacturer’s protocol, to generate fluorescence signals only when the two PLA probes were in close proximity (<40 nm). The fluorescence signal from each detected pair of PLA probes was visualized as a distinct individual red dot in an epifluorescence microscope (Nikon 90i). The Abs were used at dilutions that yielded a low number of dots/cell to have a low risk for missing dots in the quantification. Nuclei were counterstained with DAPI dye. To enhance detection of infected cells, anti–GFP–FITC Ab (Novus Biologicals) was added during the PLA signal-detection step.

**Chromatin immunoprecipitation**

Infected primary mouse pre-B cells from E2A<sup>−/−</sup> B6 mice were cross-linked with 1% formaldehyde for 10 min at room temperature. Cross-linking was quenched with a 1:20 volume of 2.5 M glycine, and cells were washed with PBS. Cells were lysed on ice with 250 µl RIPA buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, and 5 mM EDTA) supplemented with protease inhibitor mixture (Roche Applied Science). The lysis was sonicated at 60% amplitude for 10 × 10 s on a Vibra-Cell instrument (Sonics) and clarified by centrifugation. The average size of sheared chromatin fragments was determined by agarose gel electrophoresis to be 180 bp. A total of 1 µl was saved as input, and the lysis was incubated overnight at 4°C with mouse anti–Flag-tag Ab (GenScript). Immunocomplexes were captured for 1 h by 20 µl magnetic Dynabeads Protein A (Invitrogen) at 4°C. Beads were washed three times with RIPA buffer and three times with Tris-EDTA (pH 8) on a magnet (Invitrogen). Cross-links were reversed with 1% SDS in Tris-EDTA (pH 8) at 65°C overnight, and samples were treated with Proteinase K (20 µg/ml) for 1 h at 45°C. DNA was phenol-chloroform extracted, ethanol precipitated, and analyzed by quantitative real-time PCR. Each sample was quantified relative to its input chromatin DNA. The following primers were used for the chromatin immunoprecipitation (ChiP) analysis of the mouse IgH gene: Eμ: 5′-TCAGAAGCAACGACACCTGCAGC-3′ and 5′-GGTGCCGGCGG-CAGACGAGTTCTACC-3′; 5′-AGGAAAGATTGACCAAGTAG-3′ and 5′-TCAGCGCAAGATATGGTGAGAGG-3′; mouse Pax5: 5′-CTCGGAGCTGCGAGCC-3′ and 5′-GGGTTCTCTCCCTGAC-3′; mouse Ets1: 5′-CCCTGCCCAATCTCCACAC-3′ and 5′-CAAGTACACCCCTCAAGAGAGTCTCTTCC-3′; human Fos: 5′-GAGGAGGAGGAGTGATG-3′ and 5′-TCCTGTTTGAGGATAGATG-3′; human OcT2: 5′-AGTCTGTTGGAGAGGATG-3′ and 5′-TCCGCGGCA-TCTATCAACTTG-3′; and human Il6: 5′-GTCGAGTTGCTCCTCCCT-3′ and 5′-GCCCTTGGTGTCCCTTAC-3′.

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Protein expression and binding assay

The cDNAs for full-length murine PAX5 and amino acids 431-652 of murine E12 were obtained from Genscript and subcloned into pET21b(+)载体，a derivative of pET21b(+) (Novagen)，in which the N-terminal T7-tag is replaced with the pelB leader from pET20b+(Novagen). The His-tagged proteins were expressed in Escherichia coli BL21(D3) pLysS. E12-expressing cells were centrifuged and lysed by sonication in 50 mM Tris-HCl (pH 8), 1 M KCl, 1 mM EDTA, 1 mM PMSF. E12 was purified from the soluble fraction by Ni-NTA agarose chromatography (QIAGEN) under native conditions, according to the manufacturer’s instructions, followed by purification on Heparin-Sepharose. E12 was eluted with 20 mM HEPES (pH 8), 0.01% Triton X-100, 0.5 M NaCl, 10% glycerol, and 2 mM DTT. PAX5-expressing cells were centrifuged and the pellet was solubilized and lysed by sonication in 100 mM NaH2PO4, 10 mM Tris (pH 8), and 8 M urea and centrifuged. The PAX5-expressing supernatant was purified by Ni-NTA agarose chromatography, according to the manufacturer’s instructions. Purified PAX5 was renatured by dialysis against 10 mM Tris (pH 8), 1 mM EDTA, and 5 mM DTT, with gradually decreasing concentrations of urea; 0.4 M arginine was added in the last dialysis without urea.

Purified PAX5 dialyzed against 1 M potassium acetate (pH 8.3) was coupled to CNBr-activated Sepharose 4B (GE Healthcare), following the manufacturer’s instructions, and using 1 M potassium phosphate (pH 8.3) as coupling buffer. Whole-cell extract from 1.5 million mouse B cells or purified WT or CaM E12 or ETS1 (0.2 μg) was incubated with 10 μl PAX5-Sepharose or control Sepharose beads in a binding buffer containing 50 mM Tris-HCl (pH 8), 500 mM NaCl, 1.5% Triton X-100, 1 mM DTT, and protease inhibitor mixture tablet without EDTA (Roche) and either 0.1 mM CaCl2 or 0.1 mM EGTA with rotation for 30 min at room temperature. The PAX5-Sepharose and control Sepharose beads were washed twice with binding buffer, and bound proteins were eluted by boiling in Laemmli loading buffer. Samples were separated by 12% SDS-PAGE and analyzed by Western blot using TetraHis Ab (QIAGEN) for E. coli-produced E12; ETS1/ETS2 Ab (C-275) for E. coli-produced ETS1; and β-actin Ab (ACTBD11B7), CD19 Ab (E-20), a mixture of E2A Abs (V-18, H-208, and Yae) (all from Santa Cruz Biotechnology), and α-tubulin Ab (B-5-1-2; Sigma) for proteins from whole-cell extract. Pixel intensities of 12 bands were quantified by ImageJ software and plotted as percentage of E12 bound to PAX5.

Statistical analysis

The data are expressed as means ± SD. The results were analyzed using the Student t test with two-tailed distribution, with the exception that data in Fig. 1E were analyzed using the Pearson χ2 test.

Results

Defective allelic exclusion in B cells expressing CaMβ E2A

To analyze whether inhibition of E2A by CaM was relevant for allelic exclusion, we analyzed double expression of the IgH alleles in mice. C57BL/6 (referred to as B6) male mice heterozygous for E2A knockout (E2A−/−) were crossed with BALB/c females, and total bone marrow was isolated from resulting E2A−/− mice and infected with retrovirus expressing either WT or CaMβ mutant of the E12 splice form of E2A together with GFP. Infected bone marrow cells were transplanted into lethally irradiated B6 x BALB/c F1 mice and E2A−/− recipients. Recipient mouse spleen B cells were analyzed by FACS for BALB/c (IgMβ) and B6 (IgMβ) IgH allotype expression. The frequency of live-gated mouse splenic CD19+ B cells expressing both IgH alleles was 1–1.5% for the GFP−(i.e., noninfected) control cells from infection with virus expressing WT E2A (Fig. 1A, 1B, Supplemental Fig. 1A), as previously reported for FACS analysis of double expression of IgH (40). Importantly, in sharp contrast, both IgH alleles were detected in 7.17 ± 0.53% of the cells infected with virus expressing CaMβ mutant E2A in the first experiment and in 6.33 ± 0.64% of the cells in the second experiment (Fig. 1A, 1B, Supplemental Fig. 1A). This dramatic increase in double-expressing cells also was in sharp contrast to the cells infected with virus expressing WT E2A: 1.81 ± 0.35% and 2.50 ± 0.37%, respectively, appeared to double express the IgH alleles (Fig. 1A, 1B, Supplemental Fig. 1A). Compared with GFP− cells from the infection with virus expressing WT E2A, apparently double-expressing cells were slightly more frequent both
among cells infected with virus expressing WT E2A and among GFP− cells from infections with virus expressing CaMR E2A (Fig. 1A, 1B, Supplemental Fig. 1A). These slight increases might be due to a slightly less efficient inhibition of E2A in some cells that slightly overexpress WT E2A and due to a slight expression of CaMR E2A in some cells selected as GFP− after the infections with virus expressing CaMR E2A. Nevertheless, the p values for the difference in the double-expression of the two IgH alleles between the GFP+ cells of the infections with virus expressing WT and CaMR E2A were 0.0003 and 0.00002, respectively, in the two experiments (Fig. 1B), and the p values for the differences in double expression between cells expressing the CaMR E2A and the two GFP− controls were even lower.

The increased frequency of apparent double expression of the IgH alleles in the mutant implies that CaMR E2A either dramatically increases allelic inclusion of IgH or, much less likely, through an unknown mechanism radically increases the background of cells that appear double positive in the FACS analysis but are not true double expressors. To discriminate between the alternatives, sorted apparently double-positive cells were cultivated for 3 d and reanalyzed. Most apparently double-positive cells in FACS analysis are background, because they do not show double expression in the second FACS analysis (40), and this also was the case with the B cells infected with virus expressing WT E2A (Fig. 1C, 1D). Importantly, most IgH double-positive cells with CaMR E2A in sharp contrast showed double expression also in the second FACS analysis of IgH expression (Fig. 1C, 1D). Furthermore, we also analyzed the sorted double-positive cells, cultivated for 3 d, that remained apparently double positive in the second FACS analysis, by intracellular immunostaining after the second sorting. Most apparently double-positive cells after this second sorting of WT E2A–infected cells were background because they were not double stained, whereas most resorted double-positive cells with CaMR E2A instead showed both IgMA and IgMB immunostaining (Supplemental Fig. 1B). Thus, the mutation of E2A increased true double expression and not the background. To further discriminate between the two alternatives, VDJ recombination of the IgH locus of sorted GFP+ (i.e., infected) apparently double-positive cells, selected as shown in Fig. 1A and Supplemental Fig. 1D, was analyzed by single-cell PCR. Approximately two thirds of cells with both alleles detected that were infected with virus expressing WT E2A, and the control cells, had only one VDJ recombined allele, whereas the other allele was DJ recombined (Fig. 1E, Supplemental Fig. 1C). In contrast, 75% of the cells expressing CaMR E2A had both alleles VDJ recombined (Fig. 1E, Supplemental Fig. 1C). The p values for this difference between the mutant and the WTs were ≤0.00016. Thus, CaM resistance of E2A dramatically increases allelic inclusion of IgH.

Next, we analyzed whether CaMR E2A increases allelic inclusion only when overexpressed or whether it also inhibits the allelic exclusion at normal expression levels. E2A−/− mice have 50% reduced expression of E2A (22). FACS analysis showed increased E2A expression level with increased GFP fluorescence level (Supplemental Fig. 1F). Interestingly, significantly increased allelic inclusion was seen in the CaMR mutant over a broad range of expression levels, and the peak level of allelic inclusion in the mutant was around the normal WT level of E2A (Supplemental Fig. 1E, 1F). Thus, CaMR E2A strongly increases allelic inclusion of IgH at normal E2A expression levels. We also noted that strong overexpression of either WT or CaMR E2A distorted the control of VDJ recombination, leading to a high level of cells not expressing IgMA or IgMB (Supplemental Fig. 1F), which further supports that E2A has a critical role in the control of VDJ recombination. These strongly GFP-fluorescent and heavily E2A-overexpressing cells were excluded from the analyses in Fig. 1 and Supplemental Fig. 1B, 1C (as shown in Supplemental Fig. 1D). These cells were present 2 wk posttransplantation (Supplemental Fig. 1D–F) but were almost completely absent 1 wk later (Fig. 1B, left panel, Supplemental Fig. 1A), indicating that cells heavily overexpressing E2A are rapidly selected away.

FIGURE 1. CaMR E2A leads to allelic inclusion of IgH. B6 male mice heterozygous for E2A knockout (E2A+/−) were crossed with BALB/c females, and total bone marrow was isolated from resulting E2A−/− F1 mice and infected with retrovirus coding for either WT or CaMR mutant E12 splice form of E2A, together with GFP, which resulted in 5–10% of infected GFP-expressing cells (data not shown). Twenty-four hours postinfection, the GFP-expressing retrovirus-infected cells (GFP+, lower panels) of B6 × BALB/c F1 E2A−/− mice, and each cell mixture was transplanted into a lethally irradiated B6 × BALB/c F1 E2A−/− mouse. (A) Recipient spleen B cells were analyzed by single-cell PCR from infections with virus expressing CaMR E2A. Nevertheless, the p values for the difference in the double-expression of the two IgH alleles between the GFP+ cells of the infections with virus expressing WT and CaMR E2A were 0.0003 and 0.00002, respectively, in the two experiments (Fig. 1B), and the p values for the differences in double expression between cells expressing the CaMR E2A and the two GFP− controls were even lower.

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Inhibition of expression of components of the recombination machinery upon pre-BCR activation dependent on CaM sensitivity of E2A

A possible reason for the defect in allelic exclusion when E2A is CaM\(^{\text{R}}\) is that sensitivity of E2A to CaM is needed for the signal from the pre-BCR that arrests further recombination. To test this hypothesis, we characterized the effects of pre-BCR signaling on the expression of proteins relevant for the recombination. We used mouse bone marrow–derived pre-B cells grown on limiting numbers of stromal cells. We showed previously that the pre-BCR is not signaling constitutively at these conditions and is inducible upon activation by cross-linking with Ab against the \(\mu\) H chain (25, 41).

The pre-BCR stimulation efficiently induced increased intracellular Ca\(^{2+}\) concentration within \(<\)1 min (Supplemental Fig. 2), and several of the effects of pre-BCR stimulation on gene expression are through Ca\(^{2+}\) signaling (25, 41). Quantitative RT-PCR showed that the pre-BCR stimulation reduced the mRNA levels of RAG1, RAG2, and TdT, which adds nucleotides at the RAG-generated DNA ends, as well as those of the PAX5 and ETS1 transcription factors, between 2- and 4-fold within 2 h (Fig. 2A). The levels of the proteins also were reduced in most cases, but with a delay. These reductions were \(>\)2-fold within 5–8 h (Fig. 2A, Supplemental Fig. 3A).

However, the level of E2A mRNA was reduced to a much lesser extent, and the E2A protein level was not reduced by the pre-BCR stimulation (Fig. 2A, Supplemental Fig. 3A). To study whether Ca\(^{2+}\) signaling is important for the identified downregulation, we inhibited all Ca\(^{2+}\) signaling with the Ca\(^{2+}\) chelator BAPTA-AM and blocked \(I_{\text{P}}\)_1 receptor Ca\(^{2+}\) channels with TMB-8 and/or blocked L-type Ca\(^{2+}\) channels with nifedipine. For RAG1, RAG2, TdT, PAX5, and ETS1, the reduction in mRNA level after pre-BCR stimulation was partially or completely blocked by TMB-8 or nifedipine, and it was completely blocked by the Ca\(^{2+}\) chelator or by combining the channel blockers (Fig. 2B). Thus, the regulation of these proteins by the pre-BCR is dependent on Ca\(^{2+}\) signaling. The Ca\(^{2+}\)-dependent downregulation of RAG1, RAG2, and TdT is not species specific, because we obtained similar results using the human pre-B cell line Nalm-6 (Supplemental Fig. 3B, 3C).

Inhibitors of other signaling pathways from AgRs (PD98059, a MEK1/2 inhibitor; bisindolylmaleimide, PKC inhibitor) showed that Ca\(^{2+}\) plays a dominant, but not exclusive, role in pre-BCR regulation of these genes. PAX5 and ETS1 mRNAs could not be analyzed in this way, because their levels remained unchanged upon pre-BCR stimulation as a result of unknown causes, perhaps coupled to the fact that Nalm-6 is a tumor cell line.

We determined whether activation of the pre-BCR affected the amount of protein that could bind to E2A binding sites in the RAG1, RAG2, and \(TDT\) promoters, as well as the RAG enhancer \(E\text{rag}\), by EMSA of nuclear extracts in the absence of Ca\(^{2+}\). No reduction in the amount of any of the E2A–DNA complexes was seen during 5 h of anti-\(\mu\) treatment (Fig. 2C). This was expected, because pre-BCR stimulation did not have much effect on the expression of E2A mRNA or protein in primary pre-B cells (Fig. 2A) or in Nalm-6 cells (25) (data not shown). The shifted bands were identified as E2A–DNA complexes using neutralizing Abs (Supplemental Fig. 3D).

The binding of E2A to the RAG1, RAG2, \(TDT\), and \(E\text{rag}\) probes, but not binding of other transcription factors to a control probe, was much reduced under conditions that enable Ca\(^{2+}\) loading of CaM (Fig. 2C). Id proteins are inhibitors of DNA binding of E2A and other E-proteins. However, induction of Id protein did not contribute to the rapid inhibition of recombination-coupled genes and proteins upon pre-BCR stimulation, because the number of E2A–DNA complex with either of the RAG/TdT sites was not reduced upon pre-BCR stimulation in the absence of Ca\(^{2+}\) (Fig. 2C).

Furthermore, none of the Id mRNA or protein levels shows an increase after pre-BCR stimulation either in primary pre-B cells or Nalm-6 cells (25, 41) that can account for the relatively fast reductions in the mRNAs and proteins in this study.

To examine whether Ca\(^{2+}\) signaling from the pre-BCR inhibited the expression of recombination components through CaM inhibition of E2A, we first used an EBV-based shuttle vector that stably directs synthesis of an shRNA interfering with human E2A mRNA. This shRNA plasmid reduced the E2A mRNA and protein levels in Nalm-6 cells by \(>\)85%, as previously reported (25). The shRNA expression plasmid reduced the expression of RAG1, RAG2, and TdT mRNAs by 70–80% (Fig. 2D), which confirmed that the expression of these genes is E2A dependent. These reductions were specific, because the shRNA expression plasmid did not affect the expression of the pre-BCR–regulated genes \(FOS\), \(OCT2\), and \(IL6\) (Fig. 2D). Importantly, stimulation of the pre-BCR did not inhibit RAG1, RAG2, or TdT expression further in the presence of the shRNA against E2A (Fig. 2D), indicating that the inhibitions by pre-BCR engagement were entirely dependent on inhibition of E2A. The reduction in human E2A mRNA could be complemented by cotransfection with the expression plasmid for the E12 isoform of mouse E2A with which the shRNA does not interfere. This complementation fully restored the expression levels of RAG1, RAG2, and TdT mRNA and their sensitivity to pre-BCR stimulation (Fig. 2D). Importantly, anti-\(\mu\) had no effect on any of these genes when complementation was performed instead with the CaM\(^{R}\) mutant of E12 (Fig. 2D). This loss of effect of pre-BCR engagement was specific, because the mutation had no effect on the inhibition of the control genes (Fig. 2D).

Thus, CaM resistance of E2A specifically causes the expression of the genes for the components of the recombination process to be unaffected by pre-BCR stimulation. To further examine the importance of CaM inhibition of E2A, we infected primary pre-B cells with retroviruses encoding WT or CaM\(^{R}\) E12, together with GFP. It was not possible to study the effects of overexpression of CaM, because no infected mouse pre-B cells with substantial overexpression of this protein were obtained (data not shown), presumably because CaM overexpression affects viability and/or differentiation of pre-B cells. Infection with both WT and mutant E12 resulted in, on average, only a slight increase in total E2A protein compared with vector-infected cells (detected by FACS with E2A Ab) in this system, as previously reported (25). Stimulation of the pre-BCR of the primary pre-B cells infected with retrovirus expressing WT E12 or with vector control for 7 h resulted in significant \((p \leq 0.01)\) ~2-fold inhibition of RAG1, RAG2, TdT, and PAX5 protein expression (Fig. 2E). Importantly, in cells infected with virus expressing CaM\(^{R}\) E12, the protein levels became completely resistant to pre-BCR stimulation, implying that the inhibition of their expression is through CaM inhibition of E2A.

Inducible in vivo proximities of the recombination-coupled components E2A, RAG1, RAG2, and PAX5 with CaM and loss of proximities with H3K4me3 upon pre-BCR stimulation

The downregulation of recombination-coupled components was relatively modest and took many hours, which suggested the presence of another effect of pre-BCR stimulation to achieve efficient arrest of \(I\text{gh}\) recombination. To further analyze the regulation of the recombination, we performed PLAs, which enable visualization and quantification of protein interactions. Primary mouse pre-B cells were stained with Abs against different combinations of two target proteins and subsequently with secondary Abs conjugated with oligonucleotides.
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FIGURE 2. Downregulation of key components of the recombination process after pre-BCR stimulation dependent on CaM sensitivity of E2A. (A) Levels of RAG1, RAG2, TdT, PAX5, E2A, and ETS1 mRNA and protein after stimulation of the pre-BCR of primary bone marrow–derived mouse pre-B cells by anti-μ were determined by quantitative real-time RT-PCR and Western blotting, respectively. Levels before addition of anti-μ were set as 100%. Results are mean ± SD (n = 3). With the exception of E2A, the reductions were significant for all mRNA after 1 h of anti-μ (p < 0.012) and for all proteins after 5 h of anti-μ (p < 0.047). (B) Calcium signaling–dependent downregulation of the indicated genes after pre-BCR activation. Anti-μ was added to one half of BAPTA-AM–treated (15 μM), TMB-8–treated (50 μM), nifedipine–treated (50 μM), or TMB-8 plus nifedipine–treated or untreated primary pre-B cells for 3 h, followed by quantitative real-time RT-PCR. Values represent normalized mRNA levels with anti-μ as the percentage of the levels without anti-μ. Results are mean ± SD (n = 3). With the exception of E2A, the reductions were significant for all mRNA after 1 h of anti-μ (p < 0.012) and for all proteins after 5 h of anti-μ (p < 0.047). (C) No change in DNA binding activity of E2A in the absence of Ca2+ after pre-BCR activation with anti-μ (left panel) and inhibition of formation of the E2A–DNA complex in the presence of Ca2+ (right panel). Amounts of E2A–DNA complexes (indicated by arrow) using nuclear extracts (NE) from the Nalm-6 human pre-B cell line and probes containing E2A-binding sequences from the human RAG1 promoter, RAG2 promoter, the RAG enhancer Erag, the TdT promoter, and a control probe from the human GM-CSF promoter were analyzed by EMSA in the presence of the chelator EDTA (0.5 mM) or Ca2+ (0.5 mM CaCl2). (D) Effects of a shuttle vector that expresses shRNA targeting E2A, RAG1, RAG2, and PAX5 against human E2A mRNA or the empty shuttle vector or with the shRNA expression vector plus the corresponding pMEP4 vector expressing WT or CaM-M mutant of mouse E12, all together with CMV-EBNA expression plasmid. Where indicated (shaded bars), cells were treated with anti-μ for 5 h before the harvest. Levels of mRNA were determined by quantitative real-time RT-PCR. Results are mean ± SD (n = 3). (E) Loss of anti-μ sensitivity of RAG1, RAG2, TdT, and PAX5 expression by expression of CaM-M E12. Changes are shown in the indicated protein levels upon pre-BCR stimulation with anti-μ for 7 h in mouse primary pre-B cells grown with IL-7 on S17 stromal cells and infected with retrovirus encoding WT E12 or CaM-M mutant of E12, together with GFP. Proteins were quantified by intracellular immunostaining and flow cytometry. Cells with a large increase in GFP fluorescence were considered retrovirus infected and were used to calculate average protein levels. The expression levels are calculated as the percentage of the level of the corresponding cells not treated with anti-μ. Data are mean ± SD (n = 4). *p < 0.05, **p < 0.01.

In vivo regulation of the proximities of E2A, PAX5, and the RAG proteins through CaM inhibition of E2A

To further study the regulation of the suggested recombination complex, we performed PLA of primary pre-B cells infected with mRNA. Nalm-6 cells were transfected with pMEP4 vector expressing shRNA against human E2A mRNA or the empty shuttle vector or with the shRNA expression vector plus the corresponding pMEP4 vector expressing WT or CaM-M mutant of mouse E12, all together with CMV-EBNA expression plasmid. Where indicated (shaded bars), cells were treated with anti-μ for 5 h before the harvest. Levels of mRNA were determined by quantitative real-time RT-PCR. Results are mean ± SD (n = 3). (F) No change in DNA binding activity of E2A in the absence of Ca2+ after pre-BCR activation with anti-μ (left panel) and inhibition of formation of the E2A–DNA complex in the presence of Ca2+ (right panel). Amounts of E2A–DNA complexes (indicated by arrow) using nuclear extracts (NE) from the Nalm-6 human pre-B cell line and probes containing E2A-binding sequences from the human RAG1 promoter, RAG2 promoter, the RAG enhancer Erag, the TdT promoter, and a control probe from the human GM-CSF promoter were analyzed by EMSA in the presence of the chelator EDTA (0.5 mM) or Ca2+ (0.5 mM CaCl2). (G) Effects of a shuttle vector that expresses shRNA targeting E2A, RAG1, RAG2, and PAX5 against human E2A mRNA or the empty shuttle vector or with the shRNA expression vector plus the corresponding pMEP4 vector expressing WT or CaM-M mutant of mouse E12, all together with CMV-EBNA expression plasmid. Where indicated (shaded bars), cells were treated with anti-μ for 5 h before the harvest. Levels of mRNA were determined by quantitative real-time RT-PCR. Results are mean ± SD (n = 3). (H) Loss of anti-μ sensitivity of RAG1, RAG2, TdT, and PAX5 expression by expression of CaM-M E12. Changes are shown in the indicated protein levels upon pre-BCR stimulation with anti-μ for 7 h in mouse primary pre-B cells grown with IL-7 on S17 stromal cells and infected with retrovirus encoding WT E12 or CaM-M mutant of E12, together with GFP. Proteins were quantified by intracellular immunostaining and flow cytometry. Cells with a large increase in GFP fluorescence were considered retrovirus infected and were used to calculate average protein levels. The expression levels are calculated as the percentage of the level of the corresponding cells not treated with anti-μ. Data are mean ± SD (n = 4). *p < 0.05, **p < 0.01.
retrovirus expressing either WT or CaMR E12, together with GFP. As for noninfected cells, the proximity between CaM and each of E2A, RAG1, RAG2, and PAX5 with CaM and rapid reduction of the proximities with H3K4me3 upon pre-BCR stimulation. The detection of the proximal location of each protein pair analyzed (visualized as red dots) was done by in situ PLA (original magnification ×60). The controls are shown in Supplemental Fig. 4. (B) Summary of PLA results on regulation of proximities of the recombination-coupled components E2A, RAG1, RAG2, and PAX5 with CaM, with H3K4me3, and with each other from (A) and Supplemental Fig. 4A–C. Different Abs against the same protein are distinguished by superscript numbers.

FIGURE 3. Inducible in vivo proximities of the recombination-coupled components E2A, RAG1, RAG2, and PAX5 with CaM and rapid reduction of the proximities with H3K4me3 upon pre-BCR stimulation. (A) The pre-BCR of mouse primary pre-B cells grown with IL-7 on S17 stromal cells was stimulated with anti-μ for the indicated times. The detection of the proximal location of each protein pair analyzed (visualized as red dots) was done by in situ PLA (original magnification ×60). The controls are shown in Supplemental Fig. 4. (B) Summary of PLA results on regulation of proximities of the recombination-coupled components E2A, RAG1, RAG2, and PAX5 with CaM, with H3K4me3, and with each other from (A) and Supplemental Fig. 4A–C. Different Abs against the same protein are distinguished by superscript numbers.

retrovirus expressing either WT or CaM E12, together with GFP. As for noninfected cells, the proximity between CaM and each of E2A, RAG1, RAG2, and PAX5 was induced upon pre-BCR stimulation for 30 min in the cells infected with virus expressing WT E12 (Fig. 4A). Importantly, all of these inductions were completely lost in cells infected with virus expressing the CaMR E12 mutant (Fig. 4A). Proximity between H3K4me3 and each of E2A, RAG1, RAG2, and PAX5 was inhibited upon pre-BCR stimulation for 30 min in the cells infected with virus expressing WT E12 (Fig. 4A), as shown for noninfected cells. The smaller reductions in the infected cells compared with noninfected ones might reflect that the higher level of E2A, and, thereby, less CaM relative to E2A, could reduce the efficiency of the inhibition. In contrast to cells infected to express WT E2A, no reduction in the proximity between H3K4me3 and E2A, RAG1, RAG2, or PAX5 was seen after pre-BCR stimulation in the cells infected with virus expressing CaM E12 (Fig. 4A). These data support that CaM binds to E2A in a complex of E2A, RAG1, RAG2, and PAX5 after pre-BCR stimulation, thus inhibiting the DNA binding of E2A and, thereby, making the recombination complex leave the H3K4me3-modified chromatin.

FIGURE 4. CaM E12 blocks pre-BCR stimulation–induced changes of in vivo proximity of E2A, PAX5, and RAG proteins with CaM and H3K4me3, and E12 interacts directly with PAX5. (A) Mouse pre-B cells were infected with retrovirus expressing WT or CaM mutant of E12, as in Fig. 2E. Infected cells were identified by their GFP fluorescence in microscopy and used to quantify the numbers of proximities (dots/cell). The Abs were used at dilutions that yielded a low number of dots/cell to have a low risk for missing dots in the quantifications. The number of dots/cell in pre-B cell samples before stimulation of the pre-BCR were for all pairs—E2A-CaM (4.4 dots/cell for WT and 9.5 dots/cell for CaM), RAG1-CaM (4.0 dots/cell for WT E2A and 11.0 dots/cell for CaM E2A), RAG2-CaM (8.7 dots/cell for WT E2A and 20.2 dots/cell for CaM E2A), PAX5-CaM (11.1 dots/cell for WT E2A and 24.9 dots/cell for CaM E2A)—set as 100%. The results are mean ± SD (n = 3). (B) E12 binds directly to PAX5. His6-tagged full-length PAX5 was produced in E. coli, and the purified PAX5 was immobilized on cyagenon bromide–activated Sepharose 4B. Binding to the PAX5-Sepharose is shown for proteins from whole-cell extracts of mouse B cells (upper panels) and for purified proteins (middle and lower panels). The whole-cell extract from 1.5 million cells or 0.2 mg of purified His6-tagged WT or CaM E12 (aa 431–652) or ETS1 from expression in E. coli was allowed to interact with 10 μl of PAX5-Sepharose for 30 min. The incubations of whole-cell extract were in the presence of 0.1 mM EGTA, and those of purified proteins were in the presence of either 0.1 mM EGTA or 0.1 mM CaCl2. After washing, the bound proteins were detected by Western blot against the protein or, in the case of E. coli–produced E12 proteins, against the His6-tags. Preloads were 10% of the amount of protein extract, E12, or ETS1 added to the binding reactions. Negative controls were with empty Sepharose (Seph.) and Protein-A Sepharose (Prot.A-Seph.) and without addition of protein or with addition of ETS1 instead of E12 to PAX5-Sepharose (PAX5-Sephr.). Quantification of the E12 bands from three to nine experiments were plotted as the percentage of E12 bound to PAX5 (bottom panel). Results are mean ± SD. The multiple bands in the panel with E2A from cell extract are due to alternative splicing and multiple phosphorylation sites in E2A, and the lower band in the panels with E. coli–produced E12 is due to partial protease cleavage N-terminal to the bHLH domain during E. coli production. *p < 0.05, **p < 0.01, ***p < 0.001.
The interactions in vivo between the RAGs, PAX5, E2A, and CaM could all be direct; alternatively, one or more interactions could be indirect through another protein in the suggested complex. Zhang et al. (14) reported a direct interaction between the RAG1/RAG2 complex and PAX5, and we (19–21) reported and characterized the direct interaction between E2A and CaM. However, no interaction between PAX5 and E2A has been reported. To analyze whether PAX5 and E2A can interact directly with each other, we recombinantly expressed full-length PAX5, the E12 splice form of E2A (C-terminal 222 aa), and ETS1 in E. coli, purified the proteins, and determined the ability of E12 and the control protein to bind to PAX5. We also analyzed the binding of E2A and other proteins from a mouse B cell extract to the PAX5 Sepharose. E2A from the whole-cell extract showed very efficient binding to the PAX5 Sepharose, which was in sharp contrast to the poor binding of β-actin, α-tubulin, and CD19 control proteins (Fig. 4B). The E. coli–produced E12 bound to PAX5 Sepharose beads but not to control Sepharose beads with Protein A or without coupled protein, and E12 bound PAX5 Sepharose much more than did ETS1 control protein (Fig. 4B). The binding of E12 to PAX5 was equally efficient in the presence of Ca2+ and when Ca2+ was absent, because of the presence of the Ca2+ chelator EGTA, and the CaMR mutation of CaM could all be direct; alternatively, one or more interactions could be indirect through another protein in the suggested complex. The fraction of anti-Flag immunoprecipitated DNA varied for the analyzed probe sites for the different Flag-labeled proteins (Fig. 5). This probably occurs, at least in part, because of the detection of the interaction of the suggested complex with the chromatinized Igh DNA varies because it can bind with any of its DNA-binding domains and the H3K4me3-binding domain of RAG2. The levels of cross-linking in ChIP depend on the specific amino acid side chain that is located at different points of close contact of the protein with a reactive group at the base at the DNA. Thus, the efficiency of ChIP can change when either the nucleotide sequence at the site and/or the protein(s) binding there is changed or has a changed conformation. Furthermore, in this case, the accessibility for the Ab, and thereby, the efficiency of the ChIP, might be different when the Tag is on different proteins in the suggested complex. In addition, all protein

**Binding of E12, RAG, and PAX5 at the Igh locus is sensitive to CaM inhibition of E2A**

We used retrovirus-infected primary pre-B cells and performed ChIP to analyze the effects of CaM resistance of E12 on the localization of E12, RAG, and PAX5 in the Igh locus before and after pre-BCR stimulation. We added a C-terminal sequence encoding a tandem Flag-epitope to the full-length E12 (E12-Flag) in the retrovirus. Lysates of infected pre-B cells were subjected to ChIP against the Flag-epitope. The recovered DNA was quantified by PCR using primer pairs amplifying regions of the Igh locus containing binding sites for E2A (E-box sequences), RAG1/RAG2 (RSS), RAG2 (H3K4me3-rich sequences), and PAX5. E2A has a strong preference for E-boxes with the sequence CAGCTG, but there are also preferences for the surrounding nucleotides, and there are only a few E2A sites/million bp in the genome (42). As expected, we observed ChIP signals for the E-box–containing Igh enhancers Eμ and hs3b (36) in cells expressing WT E12-Flag over the background of cells infected with empty vector (Fig. 5A). Importantly, anti-Flag signals for E12-Flag also were above the vector background for most amplicons containing RAG1, RAG2, or PAX5 binding sites but not for control sequences having acetylated (i.e., active) chromatin without a binding site for E2A, RAG, or PAX5 (Fig. 5A). Thus, E2A binds not only to E2A sites but also to many RAG and PAX5 sites. Next, we added a C-terminal tandem Flag-epitope to RAG2 and PAX5 in retroviruses. E12 remained without the Flag-tag, thereby allowing expression of both E12 and either flagged RAG2 or flagged PAX5 from the same virus. We did not construct the corresponding retrovirus for RAG1 because of the large size of its cDNA. As expected, cells infected with virus expressing RAG2-Flag plus E12 gave anti-Flag signals over the background of empty virus in the ChIP for most of the RAG1 and RAG2 binding sites (Fig. 5B). Importantly, signals also were strong for the E-box–containing hs3b, as well as three of five PAX5 binding sites (Fig. 5B). Finally, cells infected with PAX5-Flag plus E12 gave anti-Flag signals much over the vector control for all five PAX5 binding sites (Fig. 5C). Importantly, specific signals also were obtained for the E-box–containing Eμ and hs3b, as well as all of the

**FIGURE 5. ChIP of E2A, PAX5, and RAG proteins at the Igh locus.** Mouse pre-B cells were infected with empty retrovirus (vector) or viruses expressing E12-Flag (A), E12 plus RAG2-Flag (B), or E12 plus PAX5-Flag (C) on the same MSCV-IRES virus and subjected to ChIP using the Flag-epitope of the tagged protein. The recovered DNA was analyzed by quantitative real-time PCR using primer pairs amplifying regions of the Igh locus containing binding sites for E2A (E-box sequences), RAG1/RAG2 (RSS), RAG2 (H3K4me3-rich sequences), and PAX5 (PAX5-consensus sequences) or acetylated active chromatin. The E2A and PAX5 sites were confirmed by ChIP (8, 14, 36). The amplified VαA1, Vα10, and Vh1558.47(2) segments contain the RSS where V and DJ segments are known to join after cleavage by bound RAG1/RAG2 and, thus, are known RAG binding sites, and RAG binding to the DQ52 and JH1–JH2 sites was verified by ChIP (38). The identical amount of DNA, in mass, was used in the analysis of all samples from the same mouse, all samples were treated identically, and all quantifications were from the early log phase of the PCR. Three mice were analyzed, and all PCR samples from these three mice were measured in triplicate. The figure shows the amount of PCR product obtained for each site after immunoprecipitation with anti-Flag (expressed as % of the amount of PCR product before immunoprecipitation). All data are mean ± SD.
contacts in a complex will not be cross-linked when precipitating a large complex of proteins on DNA; therefore, differential stability of noncross-linked protein interactions in the complex, in vivo or during the ChIP procedure, also could affect the strength of the ChIP signals. Nevertheless, it is notable that E\textsubscript{m} did not precipitate significantly more than the vector control in the RAG-Flag ChIP (Fig. 5B). This could be a result of the technical reasons described above that the combination of Flag on RAG and the E-box site of E\textsubscript{m} gives weak signals for the complex. However, it is also possible that there is less RAG protein at this site compared with sites where RAG-Flag gives significant ChIP signals. Our data suggesting that E2A, PAX5, and RAG are together in a complex at many sites on the Igh locus does not necessarily imply that all of these proteins are present in stoichiometric amounts at all of the sites. In that case, it would make sense if less RAG is located at E\textsubscript{m}, or if the binding of RAG is less stable during the ChIP procedure at E\textsubscript{m}, compared with DNA segments that have RAG binding sites (RSS and/or H3K4me3) and are RAG cleavage sites.

To analyze the effects of pre-BCR stimulation and the role of CaM inhibition of E2A, we infected the pre-B cells with retrovirus expressing either WT or CaMR E12 and performed anti-Flag ChIP before and after pre-BCR stimulation. We analyzed those Igh DNA sequences that resulted in signals clearly over background in Fig. 5. In cells infected with the retrovirus expressing WT E12-Flag, 30 min of pre-BCR stimulation reduced the ChIP signal with anti-Flag between 2- and 4-fold for all E2A binding sites, RSS sites (RAG1/RAG2 binding sites), H3K4me3 sites (RAG2 binding sites), and PAX5 binding sites (Fig. 6). These reductions were statistically significant ($p < 0.035$ in all cases) and remained approximately equally large after 2 h. Thus, pre-BCR stimulation rapidly reduces the binding of E2A to the Igh locus both on E2A binding sites and on RAG and PAX5 sites where the localization of E2A must be indirect. Importantly, the reductions in anti-Flag ChIP signals were abolished both on the E2A binding sites and on sites for RAG1, RAG2, and PAX5 by the expression of CaMR E12-Flag. All of the differences between WT and mutant E12 were statistically significant after 30 min of pre-BCR stimulation (Fig. 6). These results suggest that the recombination complex leaves the Igh locus upon pre-BCR stimulation through CaM inhibition of E2A. All of the reductions in ChIP signal after pre-BCR stimulation when expressing E12 plus RAG2-Flag were 2–10-fold and were highly statistically significant (after 30 min all $p < 0.025$; after 2 h all $p \leq 0.014$), with the exception of the hs3b site where no significant reduction was obtained (Fig. 6). A possible reason for this exception is that other proteins binding together with E2A at this DNase-hypersensitive site might retain RAG2 there when inhibition by CaM reduces the DNA binding of E2A. Importantly, all reductions in ChIP signal after pre-BCR stimulation when expressing E12 plus PAX5-Flag resulted, similarly to E2A-Flag and RAG1-Flag, in $\approx$2-fold reductions for the WT E12 that were

![E12-Flag](image1)

![E12+RAG2-Flag](image2)

![E12+PAX5-Flag](image3)

**FIGURE 6.** The binding of E2A, PAX5, and RAG proteins to the Igh locus is sensitive to CaM inhibition of E2A. Mouse pre-B cells were infected with retrovirus constructs, as in Fig. 5, using either WT or CaMR E12. The pre-BCR of infected cells was stimulated with anti-\(\mu\) for the indicated times and then subjected to ChIP using the Flag-epitope of the tagged protein and analyzed by quantitative real-time PCR, as in Fig. 5. The figure shows the amount of PCR product obtained for each site after immunoprecipitation with anti-Flag compared with the amount of PCR product before immunoprecipitation. These values for anti-Flag–immunoprecipitated DNA are expressed relative to the immunoprecipitated DNA from the corresponding unstimulated cells expressing either WT or CaMR E12 that were set as 100% (bars not shown). All data are mean $\pm$ SD ($n = 3$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 

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stimulation), with the exception of JH2 (p = 0.054) and VHJ558 (p = 0.0074). The loss of inhibition after pre-BCR stimulation by the expression of CaM² E2A remained after 2 h (Fig. 6), including on the JH2 and VHJ558 sites, which supports the results after 30 min. The reductions with CaM-sensitive E2A within 30 min, which is much before any significant changes in levels for proteins strongly affected by AgR stimulation (41, 43), argue against the alternative that the reductions are indirect through changed expression of another unknown protein. Therefore, ChIP data strongly support the notion that E12, RAG2, and PAX5 interact in vivo in a complex on the Igh locus. The coimmunoprecipitation of the binding site of one protein with Ab against the Flag on the other protein implies a physical interaction between the Flagged protein and the protein that binds to the site. Furthermore, mutation of E12 to CaM resistance affects the level of this coimmunoprecipitation, even when RAG2 binding sites are analyzed with Flagged PAX5 and when PAX5 binding sites are analyzed with the Flag on RAG2, which strongly suggests that all three proteins are together in a complex on the Igh locus. Therefore, the results further support that the effect of CaM is through E2A that is together with PAX5 and RAG2 at PAX5, RAG2, and E2A sites of the Igh locus. In summary, ChIP analyses show that WT E12 allows the recombination complex to leave E2A, RAG, and PAX5 binding sites in the Igh locus upon pre-BCR stimulation, whereas CaM² E12 sequesters the complex on the Igh DNA.

Discussion

Feedback inhibition from AgRs has a well-established major role in allelic exclusion, but the molecular mechanism has remained largely enigmatic (15–17). In this study, we show that CaM resistance of E2A dramatically increases the normally very low frequency of double expression of the Igh alleles at the cell surface to ∼5–7%. This is close to the theoretical maximum in the absence of feedback inhibition that was estimated at ∼6.4%, because most VDJ recombinations do not lead to a functional IgH (15–17). At the genomic level, CaM resistance of E2A resulted in a remarkable ∼7-fold increase in the ratio between B cells VDJ recombinated on both alleles and B cells that had one allele VDJ recombinated and the other allele only DJ recombinated. Thus, the feedback inhibition is almost completely lost in B cells with E2A to which CaM cannot bind.

RAG1, RAG2, PAX5, and E2A are all indispensable for VDJ recombination at Igh (2–4, 10–14). In this study, signaling from the pre-BCR was shown to downregulate the expression of several components of the recombination machinery, including RAG1, RAG2, and PAX5, through inhibition of E2A by CaM. Data also were provided supporting the notion that E2A, PAX5, and RAG are in a complex bound together to E2A, PAX5, RAG1, and RAG2 sites on the Igh gene before pre-BCR activation. Upon activation of the pre-BCR, this complex instead bound to CaM and left these Igh sequences when E2A was CaM sensitive. What is the relative role in the allelic exclusion for the two negative effects on VDJ recombination? One argument that the latter is more important is that downregulation of expression of PAX5, RAG1, and RAG2 takes several hours, whereas the inhibition of the binding of the complex to the Igh gene was complete after 30 min, and Ca²⁺/CaM inhibits DNA binding of E2A in vitro in <5 min (44). Thus, CaM inhibition of the binding of the recombination complex could inhibit recombination of the second Igh allele much faster than the reductions in protein levels could. In addition, the reductions in PAX5, RAG1, and RAG2 were ∼2-fold, and the reductions in their pair-wise proximities with each other and with E2A also were relatively modest. Nevertheless, the identified downregulation of recombination-coupled components are likely to contribute to the allelic exclusion. The findings support that inhibition of binding of the complex to the key sites on Igh has a dramatic inhibitory effect on further recombination. The pre-BCR–induced reductions in binding of proteins in the complex to individual key sites in the Igh locus were 2–10-fold in ChIP analyses (Fig. 6). However, reductions in binding of the complex to important sites upon pre-BCR stimulation could be even larger for several reasons: 1) overexpression of E2A in a part of the infected cells could make inhibition of E2A by CaM appear less efficient, as indicated by the smaller effect of pre-BCR stimulation in infected cells than in noninfected cells in PLA (Figs. 3, 4, Supplemental Fig. 4A–C); 2) reductions in the joining of the many binding sites relevant for recombination through the complex could be much larger than reductions in protein binding to individual sites; and 3) the total number of PAX5, E2A, RAG1, and RAG2 sites in the locus is very high, and reductions in binding that are as large, or even larger, than those detected in this study might occur at some of the other sites.

Li et al. (45) reported a cyclin-dependent kinase site in RAG2 and that accumulation of RAG2 is regulated during the cell cycle, which raises the question of whether increased degradation of RAG2 could be relevant for the reduction in RAG2 after pre-BCR stimulation. However, the downregulation of proteins that we found was not limited to RAG2 and was preceded by reductions in the mRNAs that occurred much more rapidly and were of approximately the same magnitude. Thus, the reductions are through reductions in the mRNA and are not through induction of degradation of the proteins. The reductions in the mRNAs after pre-BCR stimulation also occur too rapidly to be through a shift in the cell cycle from G₁ to S and G₂/M, where degradation of RAG2 was reported to occur. Li et al. (45) showed that coupling of V(DJ) recombination to the cell cycle is not essential for enforcement of allelic exclusion. However, our data showing that CaM sensitivity of E2A is essential for allelic exclusion does not exclude the participation of other mechanisms. After the Ca²⁺ signal from the pre-BCR has terminated the VDJ recombination of Igh, something else enables initiation of the recombination of Igκ or Igλ. It should be noted that a Ca²⁺ signal is time limited, which is suitable for an inactivation that should be temporary. Because E2A also is needed in recombination of L chains (3, 5, 6), it is likely that the retargeting to a L chain gene happens after the Ca²⁺ signal from the pre-BCR has ended.

When Igh is accessible to the recombination machinery, chromosomal contraction and DNA looping enable recombination, whereas chromosomal expansion inhibits the recombination (46, 47). During VDJ recombination of Igh, the chromatin of the locus is organized in compartments containing clusters of loops separated by linkers, and the entire repertoire of V regions merges and juxtaposes to D elements (48, 49). Productive V(DJ) recombination at the Igh locus leads to reversal of locus compaction, and this decompaction was postulated to enforce allelic exclusion (47). The large-scale chromatin structures and the changes in them upon productive V–DJ recombination indicate the existence of protein complexes binding to multiple places in Igh and, thereby, enabling looping between the critical DNA elements during the VDJ recombination but not after feedback inhibition from the pre-BCR. The results presented in this study are consistent with PAX5, E2A, RAG1, and RAG2 being key participants in such protein complexes. The complexes also might contain CTCF, cohesin, YY1, and/or Ikaros, because studies (46, 50–52) support that these proteins contribute to the shaping of the conformation of the Igh locus.
The RAG proteins are essential for V(D)J recombination of all Ig and TCR loci. Also, E2A and E-boxes control many aspects of V(D)J recombination of Ig L chain and the TCR loci. Therefore, related protein complexes, as at the Igk locus, containing at least E2A, RAG1 and RAG2 might play corresponding roles on other AgR loci. Elucidation of this will be an interesting subject for future research.

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Disclosures
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References

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