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Down-Regulation of the SWI/SNF Chromatin Remodeling Activity by TCR Signaling Is Required for Proper Thymocyte Maturation

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The process of thymocyte development requires an exquisite regulation of many genes via transcription factors and chromatin remodeling activities. Even though the SWI/SNF chromatin remodeling complex has been thought to play important roles during thymocyte development, its known function is very limited. In this study, we show that the SWI/SNF chromatin remodeling activity is finely regulated during thymocyte maturation process, especially during thymocyte selections. We found that TCR signaling directly down-regulates mBRG1 and SWI3-related gene, the core components of murine SWI/SNF complex, during thymocyte maturation. Constitutive expression of SWI3-related gene in developing thymocytes attenuated the down-regulation of the SWI/SNF complex and resulted in a change in the expression of genes such as linker for activation of T cells and casitas B lineage lymphoma, which affected the TCR-mediated intracellular signaling pathway. The defects in TCR signaling resulted in the disruption of both positive and negative selections in specific TCR transgenic mice systems. Our results state, for the first time, that the chromatin remodeling activity needs to be finely controlled for proper thymocyte selection and maturation processes. The Journal of Immunology, 2007, 178: 7088–7096.

Lymphocytes are produced through complicated developmental stages in the thymus from their bone marrow-derived precursors. Thymocyte maturation processes are controlled by a complex set of molecules, including cell surface proteins such as receptors, signal transducers, and transcription factors. Induction of specific transcription regulators and changes in chromatin structure of genes by external signals are of importance in controlling the processes.

Several transcription factors are known to be involved in thymocyte maturation. Mice deficient of E2A, a helix-loop-helix transcription factor, displayed enhanced positive selection (1). However, mice deficient of Id3, an inhibitor of E protein and induced by TCR stimulation of double-positive (DP)4 thymocytes, showed reduced positive selection (2). Nur77, E2F1, and cKrox are involved in thymocyte differentiation and lineage decision (6–10). Although relevance of many genes to thymocyte development has been identified, the exact regulatory mechanisms for differential gene expression are still unclear.

Recent studies have provided many pieces of evidence supporting the role of chromatin remodeling complexes in thymocyte maturation process. There are three major families of ATP-dependent chromatin remodeling complexes, which are SWI/SNF, ISWI, and Mi-2 families. The ISWI complex is implicated in TCR gene accessibility and Mi-2 complex in CD4 activation (11, 12). The SWI/SNF complex has been shown to play a critical role in the regulation of gene expression during thymocyte development, especially lineage commitment. BAF57, a DNA-binding subunit of the SWI/SNF complex, was reported to regulate CD4 gene expression (13). In addition, thymus-specific deletion of BRG1, the ATPase subunit of the SWI/SNF complex, led to derepression of CD4 at the double-negative (DN) stage and a developmental block at the DN to DP transition (14, 15). However, due to the block in the generation of DP cells in these systems, the roles of the SWI/SNF complex during thymocyte maturation remain yet unresolved.

SWI3-related gene (SRG3), a murine homolog of human BAFl55, is a core component of the murine SWI/SNF complex (16). Previously, we have reported that the expression level of SRG3 is down-regulated after positive selection, and this is critical for conferring the glucocorticoid (GC) resistance to mature thymocytes (16–18). The E2A/HEB protein complex maintains the expression of SRG3 high in immature DP thymocytes, and TCR signaling down-regulates the expression of SRG3 via the induction of Id3, which inhibits the activity of E2A/HEB protein complex (19, 20). Furthermore, Notch1-mediated signaling down-regulates the expression of SRG3 by blocking the recruitment of p300 to the E2A/HEB protein complex bound to the Srgr3 promoter (21, 22).
These results indicate that SRG3 is tightly regulated during thymocyte maturation by TCR and Notch signaling and that this may be important for normal thymocyte maturation.

Although BRG1 is the most essential component of SWI/SNF complex by harboring the ATPase activity, the in vitro chromatin remodeling activity of the recombinant BRG1 alone seems to vary depending on the assay system (23, 24). Recombinant BRG1 alone is reported to have chromatin remodeling activity in various in vitro assays (23), however, it was also reported that BRG1 alone cannot remodel chromatin structure in the specific in vitro experimental system, where the existence of the core component BAF155 is essential for the chromatin remodeling function of BRG1 (24). The exact role of BAF155 in such experimental system has not been resolved, but it has been shown that BAF155 and BAF170 regulate the steady-state protein level of BAF57 by controlling its proteasomal degradation (25). We have recently reported the similar role of SRG3 as controlling the proteasomal degradation of the major components of the SWI/SNF complex, BRG1, SNF5, and Baf60a, both in vitro and in vivo (26). Moreover, SRG3 functions as a scaffold protein of the SWI/SNF complex by directly interacting with BRG1, SNF5, and Baf60a. Taken together, these results show the critical role of BAF155/BAF170/SRG3 in maintaining the stability of the SWI/SNF complex.

As SRG3 expression is exquisitely regulated during thymocyte maturation and SRG3 is essential in maintaining the stability of the SWI/SNF complex, we hypothesized that the chromatin remodeling activity of the SWI/SNF complex may be regulated by SRG3 and such changes will affect the thymocyte development. To test this hypothesis, we used various SRG3 transgenic models and showed that the down-regulation of the SWI/SNF chromatin remodeling activity by reduced SRG3 expression was required for normal thymocyte maturation.

Materials and Methods

Reagents and Abs

PerCP-conjugated streptavidin and PE-conjugated anti-CD3ε, anti-CD3e-biotin, anti-CD4-PE, anti-CD4-biotin, FITC-conjugated anti-CD8, anti-CD69-biotin, anti-TCR β-biotin, and anti-SNF5 Abs were purchased from BD Biosciences Pharmingen; anti-phospholipase Cε (PLCε) Ab from BD Transduction Laboratories; anti-LAT Ab from Cell Signaling Technology; anti-β-actin (AC-15) Ab from Sigma-Aldrich; anti-phosphotyrosine (4G10) and anti-phospho-ERK Abs from Upstate; and anti-mouse IgG-FITC and anti-mouse IgG-PE from BD Biosciences; and data were analyzed by using FACSDiva software (Tree Star).

Intracellular staining for phospho-ERK

For phospho-ERK staining, single-cell suspension of thymocytes was rapidly prepared in 2% formaldehyde and then incubated for 20 min at room temperature. The fixed cells were permeabilized in 100% methanol on ice and then stained with phospho-ERK-specific Ab, followed by staining with anti-mouse IgG-FITC. Cell events were collected by using a FACSARia (BD Biosciences), and data were analyzed by using FACSDiva software (BD Biosciences).

Flow cytometry

Stained cells were analyzed or collected on a FACSARia flow cytometer (BD Biosciences). For cell sorting by surface expression of CD3 and CD69, single-cell suspension of thymocytes was stained with anti-CD3ε-PE and anti-CD69-biotin Abs and followed by streptavidin-PerCP conjugation. Cells (2 × 106) of the CD3ε+CD69+, CD3ε+CD69−, and CD3ε−CD69− populations were sorted. For cell sorting of DP or CD4 single-positive (SP) thymocytes, anti-CD8-FITC and anti-CD4-PE Abs were used. The purity of the populations was confirmed by reanalyzing the sorted cells and was >99%.

Western blot analysis

For immunoblot analysis of total protein extracts, cells were lysed in modified radioimmunoprecipitation assay buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM DTT, 1 mM Na3VO4, and 10 mM NaF) in the presence of protease inhibitors. Proteins were resolved on SDS-PAGE gels (7.5 or 10%) and transferred to Immobilon-P membrane (Millipore). For preparation of nuclear extracts, 1 × 106 cells were resuspended in 200 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, and 0.1 mM EDTA) in the presence of protease inhibitors. Proteins were resolved on SDS-PAGE gels (7.5 or 10%) and transferred to Immobilon-P membrane (Millipore). For preparation of nuclear extracts, 1 × 106 cells were resuspended in 200 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, and 0.1 mM EDTA) in the presence of protease inhibitors and incubated on ice for 20 min. Two-hundred microliters of buffer A plus 0.5% Nonidet P-40 was added to the above mixture and gently mixed. After incubation on ice for 20 min, the nuclei were spun down by centrifuging at 5000 × g for 2 min. The nuclei pellet was resuspended in 50 μl of buffer C (10 mM HEPES (pH 7.9), 400 mM NaCl, 1.5 mM MgCl2, 1 mM DTT, and 1 mM EDTA) and vortexed vigorously. After incubating on ice for at least 1 h, the supernatant was collected as nuclear extract after centrifugation at full speed for 10 min at 4°C.

Reverse transcription and semiquantitative PCR

RNA was purified from thymocytes using TRIzol (Invitrogen Life Technologies) reagent, according to the manufacturer’s instructions. One microgram of RNA was used for each reverse transcription by SuperScriptIII (Invitrogen Life Technologies). Semiquantitative PCR was performed inAccepted Manuscript

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µg/ml anti-CD4-biotin Ab and washed in medium, and the bound Ab was cross-linked by an addition of 40 µg/ml streptavidin. Stimulation was then terminated by placement of cells on ice.

Endonuclease accessibility assay

For analysis of restriction endonuclease accessibility, nuclei of thymocytes treated with dexamethasone (Dex; Sigma-Aldrich) or vehicle were isolated as described previously (27). Nuclei were digested with DpnI or Alul (20–100 U) restriction enzymes. Genomic DNA was purified by phenol-chloroform extraction and then subjected to PCR for the Hsd11B2 promoter. A 311-bp region of Hsd11b2 promoter was amplified using following primers: forward, 5′-ATGCACTTGGGACCTTGGTC3′, and reverse, 5′-GCTTCCCTCACCCTATCTTTAGCAC3′. The 3′ untranslated region (UTR) region of p21, which lacks both DpnI and Alul restriction enzyme sites, was used as a control. Primers used to amplify the 3′ UTR region of p21 were 5′-GTCCTTGTTTGGTGGACAG3′ (forward) and 5′-CT TAGCCCCAAGACCAT3′ (reverse).

Measurement of intracellular calcium concentration

A total of 5 × 106 thymocytes was labeled in RPMI 1640 medium containing 4 µg/ml Fluo-4 (Molecular Probes) in the presence of 0.2% Pluronic (Molecular Probes) for 30 min at 37°C. Cells were washed in RPMI 1640 medium twice. After adding anti-CD3ε-biotin Ab, cells were incubated on ice for 20 min, washed with RPMI 1640 medium twice, and resuspended in 1× HBS solution. Cells were collected on a FACSARia (BD Biosciences) for 30 s to establish a baseline for unstimulated cells. Prewarmed streptavidin (50 µg/ml; Sigma-Aldrich) was added to the cells, and the samples in the FL1 was monitored for the following 5 min. Changes in intracellular Ca2+ levels were analyzed by using FlowJo software (Tree Star).

For phospho-ERK staining, single-cell suspension of thymocytes was rapidly prepared in 2% formaldehyde and then incubated for 20 min at room temperature. The fixed cells were permeabilized in 100% methanol on ice and then stained with phospho-ERK-specific Ab, followed by staining with anti-mouse IgG-FITC. Cell events were collected by using a FACSARia (BD Biosciences), and data were analyzed by using FACSDiva software (BD Biosciences).

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Reverse transcription and semiquantitative PCR

RNA was purified from thymocytes using TRIzol (Invitrogen Life Technologies) reagent, according to the manufacturer’s instructions. One microgram of RNA was used for each reverse transcription by SuperScriptIII (Invitrogen Life Technologies). Semiquantitative PCR was performed in

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premni PCR kit (ELPIS Biotech). Amplifications were conducted in an Eppendorf Master Cycler (Eppendorf) under the following conditions: denaturation for 5 min at 94°C, followed by 25 cycles of 30 s for denaturation at 94°C, 30 s for annealing at 60°C, and 30 s for elongation at 72°C. Specific primers used for PCR were as follows: Brg1 (forward, 5′-AG CAGCAGTGGACGTCAG-3′; reverse, 5′-TTCCTCAGCCACTCTTC-3′), Srg3 (forward, 5′-TTAGCTTGCCGTCGACG-3′; reverse, 5′-CGG GAAGAAGAAGAGATGGAACAGCA-3′), Snf5 (forward, 5′-CCGCTGCAGTGAGATG-3′; reverse, 5′-TCACATCGGGATCCATGCTG-3′), and Baf60α (forward, 5′-ACCCAGACGTCCAGTG-3′; reverse, 5′-CGGGTATCCACCCACAC-3′).

Real-time PCR

Real-time PCR was performed using ABI Prism 7700 (Applied Biosystems) with 2× SybrGreen MasterMix (Applied Biosystems). Template cDNA (4 μl) was amplified in 25 μl of PCR containing 0.25 μM of the respective primers under the following condition: denaturation for 5 min at 94°C, followed by 40 cycles of 15 s for denaturation at 94°C, and 1 min for annealing and extension at 60°C. Specific primers used for PCR were as follows: p21 (forward, 5′-CCCTGGTTCTACCTTGAGCACTC-3′; reverse, 5′-GCTGGAGTGCAGACCCCATATAAGC-3′), Hsd11β2 (forward, 5′-ACTACATTGGACGACGTCACGG-3′; reverse, 5′-GAAGGG TGTGCTTAAGGACAGGAC-3′), Srg3 (forward, 5′-GGAAGGG TGTGCTTAAGGACAGGAC-3′; reverse, 5′-CCGGGAGTCATGAGGGAAGA-3′), Brg1 (forward, 5′-GGAGCAAG GGAAATGTA-3′; reverse, 5′-CAACCGACGCAAAGGAGGA-3′).

Results

SRG3 and BRG1 are down-regulated during positive selection

SRG3 is down-regulated during positive selection of developing thymocytes (21) and down-regulated by TCR signaling at both RNA and protein levels in a thymoma cell line (20). To further investigate how the expression of the components of the SWI/SNF complex is regulated in normal thymocytes, we quantified their expression in normal thymocyte populations and CD3αβCD69+ (prepositive selection) and CD3αβCD69- (postpositive selection) cells using semiquantitative RT-PCR (Fig. 1A). The expression levels of Srg3 transcript (0.58) was reduced in the CD3αβCD69+ thymocyte population as reported previously (21). The expression level of Brg1 transcript (0.62) was also reduced after positive selection, but those of Snf5 and Baf60α transcripts (1.10 and 0.93, respectively) remained unchanged.

Down-regulation of BRG1 expression during thymocyte maturation was further confirmed by real-time PCR (Fig. 1B). The expression level of Brg1 transcript was reduced to 60% after receiving TCR signaling and further reduced to 42% as the thymic development progressed. Stimulation of thymocytes with PMA and ionomycin also resulted in down-regulation of mRNA level of the Brg1 gene (Fig. 1C, lane 6 vs lane 1). Therefore, it is concluded that BRG1, as well as SRG3, are regulated at the transcriptional level by TCR signaling during thymocyte development.

Constitutive expression of SRG3 attenuates TCR-mediated down-regulation of the SWI/SNF complex

In the previously described transgenic mice expressing SRG3 constitutively in T lineage cells under the control of the human CD2 promoter (CD2-SRG3 transgenic mice), thymic SRG3 protein expression is up-regulated by 1.3- to 2-fold compared with that of normal littermate control (NLC) mice (17). Recently, we have shown that the protein levels of the other components of the SWI/SNF complex, such as BRG1, BAF60α, and SNF5, were also increased in the CD2-SRG3 transgenic mice (Ref. 26; Fig. 2A, lane 3 vs lane 1), without changes in the transcript levels (data not shown). This result was in agreement with the in vitro result of Sohn et al. (26) and suggested the protection effect of SRG3 against proteasomal degradation of the other components. With this transgenic model, we further investigated the effect of TCR stimulation on the protein levels of the SWI/SNF complex components. In the NLC mice, TCR cross-linking resulted in down-regulation of SRG3 and other components of the SWI/SNF complex (Fig. 2A, lane 2 vs lane 1). In CD2-SRG3 transgenic mice, the protein level of SRG3 was down-regulated to a lesser extent by TCR cross-linking (Fig. 2A, lane 4 vs lane 3) than in NLC mice (Fig. 2A, lane 2 vs lane 1), most likely due to the constitutive expression of SRG3. Moreover, the protein levels of other components of the SWI/SNF complex in CD2-SRG3 transgenic mice were also decreased to a lesser degree than in NLC mice. Such differences were not due to the different cell death ratio by TCR cross-linking between thymocytes in CD2-SRG3 transgenic mice and those in NLC mice because we did not observe any difference in cell death upon TCR cross-linking for 8 h between CD2-SRG3 transgenic and NLC mice (data not shown). Thus, the attenuated decrease of the protein levels of the other components of the SWI/SNF complex in CD2-SRG3 transgenic mice seems to be due to the protein stabilization of the other components by SRG3. In conclusion, our results suggest that exogenous SRG3 not only increases the protein levels of other components of the SWI/SNF complex but also attenuates the TCR-mediated down-regulation of the SWI/SNF complex.

FIGURE 1. SRG3 and BRG1 are down-regulated during positive selection. A, CD3αβCD69+ and CD3αβCD69- thymocyte populations were sorted from C57BL/6 mice, and the sorted cells were subjected to semiquantitative RT-PCR. The amount of template was increased by 2-fold in each lane (from left to right). Gapdh was used as an internal control. CD5 and Rag2 were used as controls for proper sorting process. The transcript levels were corrected with respect to that of Gapdh, and the ratio of band intensity in each corresponding lane (CD3αβCD69+/CD3αβCD69-) was calculated. Numbers are relative mean values to that observed in lane 1. B, CD3αβCD69+, CD3αβCD69-, CD3αβCD69+, and CD3αβCD69- thymocyte populations were sorted from C57BL/6 mice and subjected to real-time PCR using Brg1-specific primers. The transcript levels were corrected with respect to that of Gapdh. Error bars, SE in two independent experiments. C, Total thymocytes from C57BL/6 mice were treated with vehicle (lane 1), PMA alone (0.2 and 2.0 ng/ml for lanes 2 and 3), ionomycin alone (250 and 500 ng/ml for lanes 4 and 5), and 0.2 ng/ml PMA plus 250 ng/ml ionomycin for 3 h (lane 6). Total RNA (10 μg) was prepared and resolved on a 1.2% formaldehyde gel, followed by transferring to membrane. The membrane was probed with Brg1-specific probes. 28S rRNA was used as a loading control.
Constitutive expression of SRG3 enhances of the chromatin remodeling activity of the SWI/SNF complex

We next examined whether the chromatin remodeling activity of the SWI/SNF complex is actually up-regulated in CD2-SRG3 transgenic mice. According to previous studies, the SWI/SNF complex is required for the transactivation of GC-responsive genes, $\text{Hsd11}/\text{H9252}$ and $\text{p21}$ (28). Thus, we indirectly compared the chromatin remodeling activity of the SWI/SNF complex in CD2-SRG3 transgenic mice with that in NLC mice by examining the expression levels of these GC-responsive genes. For this, we performed semiquantitative RT-PCR (Fig. 2B, left panels) and real-time PCR (Fig. 2B, right panels) analyses with Dex-treated thymocytes from NLC and CD2-SRG3 transgenic mice. The expression levels of $\text{Hsd11}/\text{H9252}$ and $\text{p21}$ transcripts were increased to a higher extent upon Dex treatment in CD2-SRG3 transgenic mice (Fig. 2B, 4 h CD2-SRG3 vs 0 h CD2-SRG3) than in NLC mice (Fig. 2B, 4 h NLC vs 0 h NLC), while those of $\text{p53}$ transcript, a control, remained unchanged. Similar results were obtained with

**FIGURE 2.** Constitutive expression of SRG3 attenuates TCR-mediated down-regulation of the SWI/SNF complex. A, Total thymocytes of NLC and CD2-SRG3 transgenic mice were activated with coated anti-CD3 and anti-CD4 Abs as described in the Materials and Methods. Total extracts were prepared and subjected to immunoblot analysis. The protein levels were quantified and normalized to $\beta$-actin protein. Numbers are relative levels compared with that observed in NLC (0 h). Shown are the representative of three independent experiments, and the quantification results of the three experiments are displayed in the right graph. Error bars, SE of three independent experiments. B, Total thymocytes of NLC and CD2-SRG3 transgenic mice were treated with Dex ($10^{-7}$ M) or vehicle for 4 h. Total RNA was prepared, and semiquantitative RT-PCR (left) and real-time PCR (right) were performed. For semiquantitative RT-PCR analysis, the transcript levels of each gene were corrected with respect to that of Gapdh, and the ratio of band intensity in each corresponding lane was calculated. Numbers are relative values to that observed in NLC (0 h). One representative result of three independent experiments with similar results was shown. For real-time PCR analysis, the transcript levels were corrected with respect to the Gapdh. Error bars stand for SE of three independent experiments. 1) NLC mice, no treatment; 2) CD2-SRG3 mice, no treatment; 3) NLC mice, Dex treatment; and 4) CD2-SRG3 mice, Dex treatment. C, Schematic diagram of the proximal region of the $\text{Hsd11}/\text{H9252}$ promoter showing the restriction endonuclease cleavage sites ($\text{DpnI}$ and $\text{AluI}$), and the position of the primers used for PCR analysis. Arrow indicates position of PCR primer pairs. “+1” in the diagram indicates transcription initiation site of $\text{Hsd11}/\text{H9252}$ gene. Restriction endonuclease accessibility assay was performed on nuclei isolated from Dex-treated thymocytes of CD2-SRG3 transgenic (TG) and NLC (NLC) mice. Nuclei were digested with $\text{DpnI}$ or $\text{AluI}$ and then subjected to PCR analysis for $\text{Hsd11}/\text{H9252}$ promoter. The 3' UTR region of $\text{p21}$, which contains no $\text{DpnI}$ or $\text{AluI}$ site, was used as a control. The band intensity was quantified and corrected with corresponding those of $\text{p21}$. The functionality of $\text{DpnI}$ was confirmed by comparing the restriction enzyme accessibility assay results of the naked genomic DNA of $\text{Hsd11}/\text{H9252}$ (with $\text{DpnI}$ site) and $\text{p21}$ (without $\text{DpnI}$ site) in the presence or absence of $\text{DpnI}$ digestion. Three independent experiments were performed, and the mean values were calculated. Numbers are relative levels compared with that observed in NLC (0 h).
real-time PCR analysis (Fig. 2B, right panels). From these results, we postulated that the chromatin structure in the core promoter of Hsd11β2 gene was remodeled to a higher extent after Dex treatment in the thymocytes from CD2-SRG3 transgenic mice by enhanced chromatin remodeling activity. To further confirm this possibility, we performed restriction endonuclease accessibility assay with the core promoter regions of Hsd11β2 (Fig. 2C). Although the restriction site of DpnI (−280) in the promoter region of Hsd11β2 was not accessible after the Dex treatment, the restriction site of Alul (−150) was accessible after the treatment. Notably, enhanced Alul accessibility was seen more in CD2-SRG3 transgenic mice than in NLC mice. Taken together, these results demonstrate that the up-regulated expression of the protein components of the SWI/SNF complex in the CD2-SRG3 transgenic thymocytes yields enhanced chromatin remodeling activity.

Expression levels of Lat and Cbl genes are regulated by the activity of the SWI/SNF complex during thymocyte maturation

The SWI/SNF complex regulates expression of target genes by transcriptional activation or repression. We have shown here that constitutive expression of SRG3 attenuates down-regulation of the other components of the SWI/SNF complex by TCR signaling (Fig. 2A), and the chromatin remodeling activity of the SWI/SNF complex is increased in CD2-SRG3 transgenic mice (Fig. 2, B and C). These results suggest that the chromatin remodeling activity of the SWI/SNF complex is decreased to a lesser extent after receiving TCR signaling in CD2-SRG3 transgenic mice than in NLC mice, which may result in the change in the expression of genes involved in TCR signaling. Thus, we quantified the expression of genes involved in TCR signaling pathway by RT-PCR. Interestingly, the expression of linker for activation of T cells (LAT) was significantly down-regulated and that of CBL was up-regulated in thymocytes from CD2-SRG3 transgenic mice (Fig. 3A, left panels; for LAT, 0.43 ± 0.18 and for CBL, 2.39 ± 0.85), which was further confirmed by Northern blot analysis (Fig. 3A, right panels; for LAT, 0.76 ± 0.17 and for CBL, 1.80 ± 0.68).

We next investigated the expression pattern of Lat and Cbl genes during normal thymocyte maturation process, where the activity of the SWI/SNF complex is decreased upon TCR signaling. By performing real-time PCR with FACS-sorted CD3lo/CD69− (prepositive selection), CD3hi/CD69− (postpositive selection), and CD3hi/CD69+ thymocytes from NLC mice, we observed enhanced Lat and reduced Cbl gene expression in postpositive selection subset (Fig. 3B). However, in CD2-SRG3 transgenic mice, the expression levels of Lat and Cbl genes during thymocyte maturation were changed to a lesser degree than in NLC mice (Fig. 3C). Furthermore, in vitro reporter analysis showed that the down-regulation of the Lat promoter activity was dependent on the activity of the SWI/SNF complex (data not shown). Taken together, we conclude that the expression level of Lat and Cbl genes are regulated by the activity of the SWI/SNF complex during thymocyte maturation.

Constitutive expression of SRG3 disrupts intracellular signaling pathway of thymocytes

LAT recruits GRB2-Sos complex and PLCγ1, which are involved in activating the Ras-ERK signaling pathway and the calcium signaling pathway, respectively (29). CBL protein has been known as a negative regulator of TCR signaling (30, 31). Thus, we examined whether the down-regulation of LAT and up-regulation of CBL shown in CD2-SRG3 transgenic mice might lead to the weakening of TCR signaling. Investigation of the early signaling events downstream of the TCR engagements showed enhanced CBL phosphorylation after TCR stimulation of thymocytes from CD2-

FIGURE 3. Expression level of Lat and Cbl genes are regulated by activity of the SWI/SNF complex during thymocyte maturation. A, left. Total extracts were prepared from total thymocytes of CD2-SRG3 transgenic and NLC mice and subjected to immunoblot analysis. β-Actin was shown as a control. The band intensity was quantified and normalized to β-actin protein. Three independent experiments were performed, and the mean values were calculated. Numbers are relative levels compared with that observed in NLC. Right, Total RNA was prepared from total thymocytes of CD2-SRG3 transgenic and NLC mice and subjected to Northern blot analysis. 28S rRNA was used as a control. The band intensity was quantified and normalized to 28S rRNA. Three independent experiments were performed, and the mean values were calculated. Numbers are relative levels compared with that observed in NLC mice. B, CD3lo/CD69−, CD3hi/CD69−, and CD3hi/CD69+ thymocyte populations were sorted from C57BL/6 mice, and the sorted cells were subjected to real-time PCR using LAT- or CBL-specific primers. The transcript levels were corrected with respect to that of Gapdh. Error bars, SE in two independent experiments. C, CD3lo and CD3hi thymocytes were sorted from NLC and CD2-SRG3 transgenic mice (TG), respectively. Real-time PCR was conducted with the sorted thymocytes using LAT- or CBL-specific primers. The transcript levels were corrected with respect to that of Gapdh. Error bars, SE in three independent experiments.

SRG3 transgenic mice as expected, though overall tyrosine phosphorylation pattern was similar (Fig. 4A).

To confirm whether TCR signaling is weakened in thymocytes from CD2-SRG3 transgenic mice, we analyzed calcium mobilization and Ras-ERK signaling in thymocytes after TCR stimulation. In contrast to normal Ca2+ mobilization in response to CD3e stimulation of thymocytes from NLC mice, thymocytes from CD2-SRG3 transgenic mice showed defects in
Ras-ERK signaling as well as calcium signaling has been known to be important in positive selection of thymocytes (32, 33). It is also known that ERK signaling may participate in negative selection (33). When we tested the pattern of ERK phosphorylation after stimulation of thymocytes with CD3ε and CD4 Abs, it was significantly decreased in CD2-SRG3 transgenic mice compared with NLC mice (Fig. 4C). Taken together, these results suggest that constitutive expression of SRG3, thus higher SWI/SNF chromatin remodeling activity, disrupted TCR-mediated intracellular signaling pathway of thymocytes.

**Constitutive expression of SRG3 disrupts normal positive selection of thymocytes**

It is generally accepted that the affinity/avidity of TCR for self-Ag/MHC determines the fate of immature DP thymocytes. Our results showed that constitutive maintenance of SWI/SNF chromatin remodeling activity results in weaker TCR signaling. Therefore, we tested whether this change in the TCR signaling strength observed in the CD2-SRG3 transgenic mice would result in changes in thymocyte maturation. Analysis of T cell profile showed that CD4/CD8 and CD3/CD69 profiles of thymocytes in CD2-SRG3 transgenic mice seemed to be normal (data not shown). Thus, we used several transgenic mice expressing TCR specific for class I or class II MHC proteins bound to antigenic peptides for these analyses.

**HY TCR recognizes male-specific Ag with H-2D^b** (class I MHC) so that thymocytes in female HY TCR transgenic mice develop into CD8 SP thymocytes through positive selection. Development of CD8 SP thymocytes was significantly blocked in female CD2-SRG3/HY TCR transgenic mice, in contrast to female HY TCR transgenic mice (Fig. 5A). In the lymph node, CD2-SRG3/HY TCR transgenic mice had less CD8 SP thymocytes than HY TCR transgenic mice (data not shown). The SRG3 expression in DP thymocytes of CD2-SRG3/HY TCR transgenic mice was increased by 2.1-fold than in HY TCR transgenic mice (Fig. 5B). We also found that constitutive expression of SRG3 increased BRG1/SRG3 complex as predicted from our previous results. To further clarify the defects in positive selection, we crossed CD2-SRG3 transgenic mice with HY TCR/RAG2^{-/-} mice. In HY TCR/RAG2^{-/-} mice, only HY TCR-positive thymocytes mature into CD8 SP stage as a result of the lack of TCR gene rearrangement. In these mice, a similar defect in positive selection was observed without significant difference in total thymocyte number (1.6 ± 0.17 × 10^8 total and 4.8 ± 0.5 × 10^6 CD8 SP thymocytes in HY TCR/RAG2^{-/-} vs 1.8 ± 0.35 × 10^8 total and 3.6 ± 0.33 × 10^6 CD8 SP thymocytes in CD2-SRG3/HY TCR/RAG2^{-/-} mice) (Fig. 5C).

Maturation of thymocytes with class II MHC-restricted TCR was also defective in CD2-SRG3 transgenic mice. We used 5C.C7 TCR transgenic mice that express a Vα11/Vβ3 TCR transgenic specific for a pigeon cytochrome c peptide presented by the MHC class II E^b^ molecules (34). Positive selection of 5C.C7-positive thymocytes on an E^b^ background results in the appearance of mature CD4 SP thymocytes. Development of CD4 SP thymocytes was significantly impaired in CD2-SRG3/5C.C7 TCR/RAG2^{-/-} mice (1.62 ± 0.19 × 10^7 total and 4.86 ± 0.19 × 10^6 CD4 SP thymocytes in HY TCR/RAG2^{-/-} vs 1.8 ± 0.35 × 10^8 total and 3.6 ± 0.33 × 10^6 CD4 SP thymocytes in CD2-SRG3/5C.C7 TCR/RAG2^{-/-} mice) (Fig. 5D). We also crossed CD2-SRG3 transgenic mice to DO11.10 TCR transgenic mice. DO11.10 TCR is specific for chicken OVA in the context of I-A^d^. Positive selection of DO11.10-positive thymocytes on an I-A^d^ background results in the appearance of mature T cells that are virtually all CD4 SP.

initial peak of Ca^{2+} responses (Fig. 4B). In thymocytes, Ca^{2+} mobilization is controlled by the activity of PLCγ1. Consistent with the defective Ca^{2+} responses in CD2-SRG3 transgenic mice, mild defect in tyrosine phosphorylation of PLCγ1 after stimulation with CD3ε and CD4 Abs was observed (Fig. 4B; 0.71 ± 0.19).
thymocytes and express high amounts of transgenic TCR α and β polypeptides (35). We could also observe similar defects in CD2-SRG3/D011.10 TCR transgenic mice, showing clear difference between CD4 populations of CD2-SRG3/D011.10 TCR transgenic mice and DO11.10 TCR transgenic mice both in the thymus and periphery (Fig. 5E and data not shown). Taken together, these results indicate that constitutive expression of SRG3 disrupted normal positive selection of thymocytes in these TCR transgenic mice by enhanced SWI/SNF chromatin remodeling activity.

**Constitutive expression of SRG3 disrupts HY TCR-mediated but not endogenous superantigen-mediated negative selection**

To analyze the effect of constitutive expression of SRG3 on negative selection, we analyzed male CD2-SRG3/HY TCR transgenic mice. Interestingly, we could recognize the difference between thymi from male HY TCR transgenic mice and those from male CD2-SRG3/HY TCR transgenic mice with naked eyes (Fig. 6A). In male CD2-SRG3/HY TCR transgenic mice, a large number of immature DP and CD8 SP thymocytes were observed, whereas most of thymocytes in male HY TCR transgenic mice were DN cells (Fig. 6B). In addition, 35.1% of thymocytes expressing high level of HY TCR were CD8 SP cells in CD2-SRG3/HY TCR transgenic mice, suggesting that thymocytes expressing HY TCR were not removed by negative selection (Fig. 6C). These results show that HY TCR-mediated negative selection is defective in CD2-SRG3 transgenic mice.

However, we could not observe a significant change in endogenous superantigen-mediated negative selection in CD2-SRG3 transgenic mice (Table I). It has been known that Vβ3-, Vβ5-, and Vβ11-bearing T cells are deleted in BALB/c background (36, 37). CD2-SRG3 transgenic mice in C57BL/6 background were crossed with the MHC class II I-Ed mouse strain BALB/c and TCR β-chain usage was analyzed in C57BL/6 and F1 (C57BL/6 × BALB/c) backgrounds. Vβ3-, Vβ5-, and Vβ11-bearing T cells were not efficiently recovered in CD2-SRG3 transgenic mice compared with NLC in F1 background. These results suggest that endogenous superantigen-mediated negative selection is not significantly affected by the down-regulation of SWI/SNF remodeling activity.
sequences on its promoter. Sequence analysis of the TCR signaling down-regulated right after positive selection. The expression of SRG3 is finely regulated during thymocyte maturation (19, 21, 22). SRG3 is expressed at two to three times higher level in immature thymocytes than in mature thymocytes and is down-regulated right after positive selection. The expression of SRG3 is maintained at high level by transcription factor E2A/HEB in DP thymocytes (19). TCR signaling down-regulates the SRG3 expression by inducing Id3, which inhibits the binding of E2A to E-box sequences on its promoter. Sequence analysis of the Brg1 promoter region revealed existence of E-box sequences in proximal regions of promoter, suggesting that the expression of Brg1 may be regulated in a similar way to SRG3 by the TCR signaling.

Recent studies have revealed the critical role of BAF155, BAF170, and SRG3 in the maintenance of the SWI/SNF complex. Biochemically, BAF155 and BAF170 are known to regulate the steady-state protein level of BAF57 by controlling its proteosomal degradation (25). Likewise, SRG3 stabilizes major components of the SWI/SNF complex by attenuating their proteasomal degradation (26). Moreover, BAF155 interacts with BAF57 through the SWI3, ADA2, N-CoR, and TFIIB and leucine-zipper domains (25), while SRG3 interacts directly with SNF5 or BAF60a through the SWIRM domain and with BRG1 through the SANT domain of SRG3 (26). Therefore, it is suggested that SRG3 stabilizes other major components of the SWI/SNF complex by increasing the half-life of them through direct interaction. However, such a critical role of SRG3 has never been extended to examine its effect on the chromatin remodeling activity. In this study, we clearly showed for the first time that up-regulation of SRG3, which yielded up-regulation of the major components of the SWI/SNF complex (26), also resulted in enhanced chromatin remodeling activity of the SWI/SNF complex (Fig. 2, B and C).

In transgenic thymocytes expressing SRG3 constitutively, increased chromatin remodeling activity and attenuation of the down-regulation of the SWI/SNF complex have been shown, as predicted by the results showing stabilizing effects of SRG3 (Fig. 2). This disruption of normal down-regulation of the SWI/SNF complex resulted in the defects in both positive and negative selections in the TCR transgenic mice (Fig. 5 and 6). The down-regulation of the SWI/SNF complex during thymocyte maturation may lead to changes in gene expression, particularly of those involved in thymocyte maturation. We found that the expression of two genes, Lat and Cbl, was changed in CD2-SRG3 transgenic mice. The expression of LAT was significantly down-regulated in thymocytes of CD2-SRG3 transgenic mice. The protein level and the phosphorylation by CD3ε stimulation of CBL were increased in CD2-SRG3 transgenic mice compared with that in NLC mice. LAT is a substrate of the tyrosine kinases activated following TCR engagement and recruits PLCγ1 and the GRB2-Sos complex, which are involved in calcium signaling pathway and activating the Ras-ERK signaling pathway, respectively (29). CBL protein has been known as a negative regulator of TCR signaling (30, 31). In Cbl-deficient mice, DP thymocytes showed higher levels of TCR β and CD3 on the cell surface, possibly reflecting enhanced positive selection. And thymocytes from Cbl-deficient mice showed increased proliferation upon CD3ε stimulation. Thus, it is supposed that the decreased expression of LAT and the increased expression of CBL might result in the weakening of TCR signaling. Actually, we found that calcium mobilization and Ras-ERK signaling were defective in thymocytes from CD2-SRG3 transgenic mice (Fig. 4). These results are consistent with our observations, suggesting that TCR signaling may have become weakened by the constitutive expression of SRG3; immature DP and CD8 SP thymocyte populations were present in male CD2-SRG3/HY TCR transgenic mice.

Our results show that constitutive expression of SRG3 blocks HY TCR-mediated but not endogenous superantigen-mediated negative selection. Similar results have been reported in CD30- and death receptor 3-deficient mice (38, 39). Considering both CD30 and death receptor 3 are implicated in apoptosis, authors proposed that superantigen-mediated negative selection might have a different signaling requirement compared with MHC/peptide-mediated negative selection. In addition to these possibilities, in our experimental system, it is also possible that weakening the TCR signaling by the constitutive expression of SRG3 might not be enough to block negative selection mediated by endogenous superantigen, which is thought to deliver very strong signal inside thymocytes (40).

As expected from our results, it is interesting to find that the expression of Lat gene increases and that of Cbl gene decreases during normal thymocyte maturation (Fig. 3). It has been known that thymocytes become more sensitive to TCR stimulation after positive selection, which is reflected by the extent of IL-2 production or proliferation (41). Sensitization of thymocytes after positive selection has been thought to render mature T cells more competent in antigenic response in periphery (42, 43). MHC variant weak agonists, which can induce proliferation, mediate cytolyis, and induce CD69 up-regulation in mature T cells are unable to induce negative selection of thymocytes (43). It has been suggested that sensitization of thymocytes is achieved by up-regulation of surface TCR level (44). CD4-8low transitional thymocytes express 7-fold more surface TCR than DP thymocytes. In addition, maturation from the CD4+8low to CD4 SP stage involves more increase in
surface TCR level. Our results suggest that changes in the expression of related genes, including Lat and Cbl, as a result of down-regulation of the SWI/SNF complex may be an additional mechanism for the increased sensitization of mature thymocytes.

In this study, we have shown that the SWI/SNF chromatin remodeling complex is finely regulated during thymocyte maturation. The change in the expression of the SWI/SNF chromatin remodeling complex resulted in change in the chromatin remodeling activity, which in turn resulted in the change in the expression of a few genes involved in the TCR-mediated signaling in developing thymocytes. Attenuation of the down-regulation of the complex resulted in dramatic changes in the pattern of thymocyte selections. Therefore, our results suggest that thymocyte maturation process is subject to a fine regulation by several factors, including the SWI/SNF chromatin remodeling complex.

Disclosures
The authors have no financial conflict of interest.

References