Notch1 confers a resistance to glucocorticoid-induced apoptosis on developing thymocytes by down-regulating SRG3 expression

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We previously have reported that SRG3 is required for glucocorticoid (GC)-induced apoptosis in the S49.1 thymoma cell line. Activation of Notch1 was shown to induce GC resistance in thymocytes. However, the specific downstream target of Notch1 that confers GC resistance on thymocytes is currently unknown. We found that the expression level of SRG3 was critical in determining GC sensitivity in developing thymocytes. The expression of SRG3 also was down-regulated by the activated form of Notch1 (NotchIC). The promoter activity of the SRG3 gene also was down-regulated by NotchIC. Expression of transgenic SRG3 resulted in the restoration of GC sensitivity in thymocytes expressing transgenic Notch1. These results suggest that *SRG3* is the downstream target of Notch1 in regulating GC sensitivity of thymocytes.

In the thymus, thymocytes develop from their precursors to become mature T cells through ordered processes. The interaction between T cell receptor (TCR) expressed in thymocytes and self-MHC plus peptide antigen in thymic stromal cells appears to be one major determining factor in the survival (positive selection) or death (negative selection) of developing thymocytes (1, 2). $CD4^+CD8^+$ double positive (DP) thymocytes bearing TCRs with subthreshold avidity are removed by programmed cell death (death by neglect), probably because of insufficient TCR-mediated signals necessary to survive (3, 4).

Glucocorticoids (GCs), known to play complex roles during thymocyte development, have been thought to be important in the removal of unselected thymocytes (5). This hypothesis is based on the observations that DP thymocytes are sensitive to GC-induced apoptosis, whereas mature single positive (SP) thymocytes are relatively resistant (6), and that GCs also are produced in thymus (7). DP thymocytes acquire resistance to GCs during differentiation into mature $CD4^+$ or $CD8^+$ SP thymocytes (6). It seems that positive selection may confer on developing thymocytes a resistance to surrounding GCs in thymus. However, the molecular basis for the difference in GC sensitivity between DP thymocytes and mature SP thymocytes and the mechanism of how GC resistance is acquired by developing thymocytes are not known.

Notch1 has been reported to affect GC-induced apoptosis of thymocytes. Expression of the intracellular domain of Notch1 (NotchIC) inhibited GC-induced apoptosis in a thymic lymphoma line (AKR1010) and a T cell hybridoma (2B4.11) without affecting GC receptor (GR) and known GC-regulated genes (8). Thymocytes from transgenic mice expressing NotchIC showed relative resistance to GC-induced apoptosis. Expression of NotchIC induces other events associated with the maturation of DP thymocytes into SP thymocytes (9). However, the downstream target of Notch1 signaling, conferring a GC resistance on thymocytes, remains unclear. Bcl2 was not always up-regulated by the NotchIC in T cell lines (8). Thus, there may be other general downstream targets involved in Notch1 signaling. In addition, Notch1 signaling may play some roles in positive selection and the following CD8/CD4 lineage commitment (8, 10). Notch1 signaling, conferring GC resistance on DP thymocytes, may prolong their survival during their differentiation into $CD4^+$ SP and $CD8^+$ SP thymocytes (8). This prolonged survival of DP thymocytes may affect the CD8/CD4 lineage determination. However, this possibility has not been directly tested yet.

SRG3, a mouse homolog of yeast SWI3 and human BAF155, initially was isolated as a gene expressed highly in thymus but at a low level in periphery (11). It is a core component of mouse SWI/SNF complex, a chromatin-remodeling complex required for the regulation of transcriptional processes associated with development, cellular differentiation, and proliferation (12, 13). Interestingly, the expression of antisense RNA to *SRG3* in a thymoma cell line decreased the apoptosis induced by GCs, suggesting that this molecule is involved in GC-induced apoptosis during T cell development (11). Here, we show that the expression level of SRG3 determines GC sensitivity of thymocytes. The expression of SRG3 is down-regulated after positive selection. We also found that SRG3 is the downstream target of Notch1 signaling in conferring GC resistance on thymocytes.

Materials and Methods

Mice. C57BL/6 mice were purchased from The Jackson Laboratory, and FVB mice were purchased from B&K Universal (Sollentuna, Sweden). Mice were maintained in our animal facility (Seoul National University, Korea). NotchIC-9 mice in C57BL/6 background were kindly provided by B. J. Fowlkes (National Institutes of Health, Bethesda, MD) (10) and mated with C57BL/6 more than five times in our facility. Transgenic mice expressing SRG3 mRNA in reverse orientation (lck-αSRG3$^+$ transgenic mice) and overexpressing SRG3 (CD2-SRG3$^+$ transgenic mice) were produced and maintained in FVB background. Double transgenic mice expressing both CD2-SRG3 and NotchIC were generated by crossing CD2-SRG3$^+$ transgenic mice (FVB) with NotchIC-9$^+$ transgenic mice (C57BL/6). Transgenic mice were identified by PCR. Primers for NotchIC are 5′-GCA TTG GGC GGC CGC GGT GA-3′ and 5′-GTG CCA CCC AGG GCA GTG CC-3′. Primers for CD2-SRG3 are 5′-GAC TAG ACC AAA CAT CTA CCT C-3′ and 5′-GTC AAC TGA GCG ACT TGG ATC-3′. Three- to 4-week-

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Abbreviations: TCR, T cell receptor; GC, glucocorticoid; GR, GC receptor; DP, double positive; SP, single positive; DEX, dexamethasone; RT, reverse transcriptase.

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old transgene positive and negative littermates produced by each mating were used throughout the study.

Antibodies. Anti-Flag M2 mAb and streptavidin-phycoerythrin were purchased from Sigma. Anti-GR (BuGR2) Ab was purchased from Affinity Bioreagents (Neshanic Station, NJ). Anti-Lck Ab, anti-CD69-biotin, and anti-CD4-phycoerythrin (GK1.5) were purchased from PharMingen. Antiserum against SRG3 was raised from rabbits in our laboratory. Antiserum against hBRG1 was a generous gift from H. Kwon (Dankook University, Seoul, Korea).

Western Blotting. For the analysis of SRG3, mBRG1, GR, and Lck expression, total cell lysates of 10^7 DP thymocytes from mice were separated by SDS/PAGE and analyzed as described (11). For separation of DP thymocytes, thymocytes were stained with anti-$\alpha\beta$ TCR mAb and streptavidin-magnetic bead and then separated with MACS separation columns (Miltenyi Biotec, Auburn, CA). Separated cells were reanalyzed for purity by using flow cytometry. Densitometric band intensity was determined by using GEL-PRO ANALYZER software (Media Cybernetics, Silver Spring, MD).

Flow Cytometry, Cell Sorting, and Semiquantitative Reverse Transcriptase (RT)–PCR. Single cell suspensions of thymocytes were prepared from 3- to 4-week-old mice. Stained cells were collected on a FACStar^{plus} flow cytometer (Becton Dickinson). For cell sorting, single cell suspensions of thymocytes were stained with anti-CD3ε and anti-CD69 mAb. Cells (3×10^4) of the CD3^{lo} CD69$^-$ and CD3^{hi} CD69$^+$ populations were sorted. Purity of the sorted populations was confirmed by reanalyzing the cells. RT-PCR was carried out with purified RNA from sorted cells. The 413-bp DNA product corresponded to nucleotides 2002–2414 of *SRG3* cDNA. For competitive PCR, a plasmid containing the mutant *SRG3* cDNA was produced by internal deletion of the 198-bp *Hin*cII/*Hin*dIII fragment. For the control, a plasmid containing the mutant β-actin gene was constructed by adding 186 bp of the pBluescriptII *Eco*RV/*Pvu*II fragment into the *Msc*I site in a 245-bp *Kpn*I/*Xba*I fragment of the β-actin gene. Primers for SRG3 were 5′-CGT ACT CAG GAC GAA TGC ATC C-3′ and 5′-GCT GCT GAC CAT CAG GAT CTG T-3′. Primers for β-actin were 5′-CTC TAG ACT TCG AGC AGG AGA TGG-3′ and 5′-CCA GAC AAC ACT GTG TTG GCA TAG-3′.

Cell Culture, Transfection, Site-Directed Mutagenesis, and Luciferase Assay. S49.1, a mouse thymoma cell line, was maintained with DMEM supplemented with 10% FBS (HyClone). The *Eco*RI/*Acc*I fragment in the *SRG3* promoter region (−1140 bp to −1) was cloned into the pGL3-Basic vector (Promega) for the luciferase assay. E-box sequence in the *SRG3* promoter region (−480 to −475) was converted into a *Pst*I restriction enzyme site (C<u>ATCT</u>G into C<u>TGCA</u>G) by introducing mutations with PCR primers (5′-TAA GCA GAA ACC TGC AGA TGT GGT TCG-3′ and 5′-CGA GCC GCG TCT GCA GGT TTC TGC TTA G-3′). For transfection of NotchIC, a plasmid DNA (Notch DE6MT) that can express NotchIC by using the cytomegalovirus promoter was transfected into S49.1 as described (14). The amount of transfected DNA was kept constant by supplementation with a pcDNA3 vector. Reporter gene activity was determined with the luciferase assay system (Promega). β-galactosidase activity was measured to normalize transfection efficiency.

Generation and Identification of lck-αSRG3 Transgenic Mice. cDNA encoding mouse *SRG3* was cloned previously in our laboratory (11). First, the 2.9-kb *Xba*I fragment of *SRG3* cDNA was cloned into pBluescriptII. The 2.8-kb *Bam*HI fragment of *SRG3* cDNA in pBluescriptII was cloned into p1017 in the reverse orientation (15). The 8.2-kb transgenic cassette was excised with *Not*I and *Apa*I, gel-purified, dialyzed, and microinjected into FVB-fertilized eggs by using standard procedures (16). Transgene integration was determined by Southern blot analysis and PCR with tail DNAs, and three founders were established. PCR primers used for screening lck-αSRG3 were 5′-GCA TCT GCA TGA ACA TAC TTC TTG-3′ and 5′-CTA GCT CAG CGT GTG CTC ATC TG-3′.

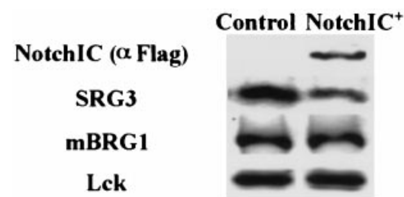

Fig. 1. Western blot analysis of SRG3, mBRG1, and Lck expression in DP thymocytes from NotchIC$^+$ transgenic mice and NotchIC$^-$ littermate control. The whole-cell extracts of sorted DP thymocytes from NotchIC$^+$ and NotchIC$^-$ littermate mice were separated by SDS/PAGE and analyzed by Western blotting. Densitometric analysis of band intensity shows about a 4-fold reduction in SRG3 expression in NotchIC$^+$ transgenic mice compared with control littermates. Three repeated experiments gave similar results.

Northern Blot Analysis. Total RNAs from indicated organs were purified as described (17). Twenty micrograms of each RNA sample was hybridized with a specific probe. Single-stranded PCR product of a 2.9-kb *Xba*I fragment of *SRG3* cDNA in pBluescriptII KS(−) generated with the T3 primer was used as a probe. 28S and 18S ribosomal RNAs were visualized by using ethidium bromide staining to confirm equal loading.

Analysis of Thymocyte Sensitivity to GCs. Thymocytes were incubated in medium alone or medium with dexamethasone (DEX). Cells were harvested and stained with anti-CD4-phycoerythrin and anti CD8-FITC mAb, and 40,000 cells were analyzed by FACS. A viable cell gate was established based on forward scatter and side scatter, and the number of CD4$^+$CD8$^+$ events falling in the viable gate was used to quantitate the number of viable DP cells. The percentage of relative survival was calculated according to the following formula previously described (8):

Relative survival of DP thymocytes (%) = $100 \times A/B$, where A = survival rate of DP thymocytes (%) in medium supplemented with DEX and B = survival rate of DP thymocytes (%) in medium alone.

Results

Activated Notch1 Reduces SRG3 Protein Expression in DP Thymocytes. Because the expression of NotchIC reduced GC sensitivity of thymocytes (8), and lowering of the SRG3 expression level also decreased GC sensitivity in a thymoma cell line S49.1 (11), we speculated that Notch1 and SRG3 proteins are related in some way in regulating GC sensitivity of DP thymocytes. To test that possibility, we first analyzed the expression level of the SRG3 protein in the thymus of NotchIC-9 transgenic mice. Because the thymus of the NotchIC-9 transgenic mice is skewed toward CD8 SP subsets compared with control littermates (10), thymocytes populations expressing TCR at low level (TCR^{lo}) were prepared by removing cells expressing TCR at high level (TCR^{hi}) by using mAb against $\alpha\beta$-TCR (H57.597-biotin) and streptavidin conjugated with magnetic beads. The isolated thymocytes from the NotchIC$^+$ transgenic mice and NotchIC$^-$ control littermates were mostly DP ($\geq$95%) when analyzed with FACS (data not shown). Cell extracts prepared with the isolated DP thymocytes (10^7 cells) from NotchIC$^+$ transgenic and control mice were analyzed for the expression of Flag-NotchIC, SRG3, mouse homolog of human BRG1 (mBRG1), and Lck proteins (Fig. 1).

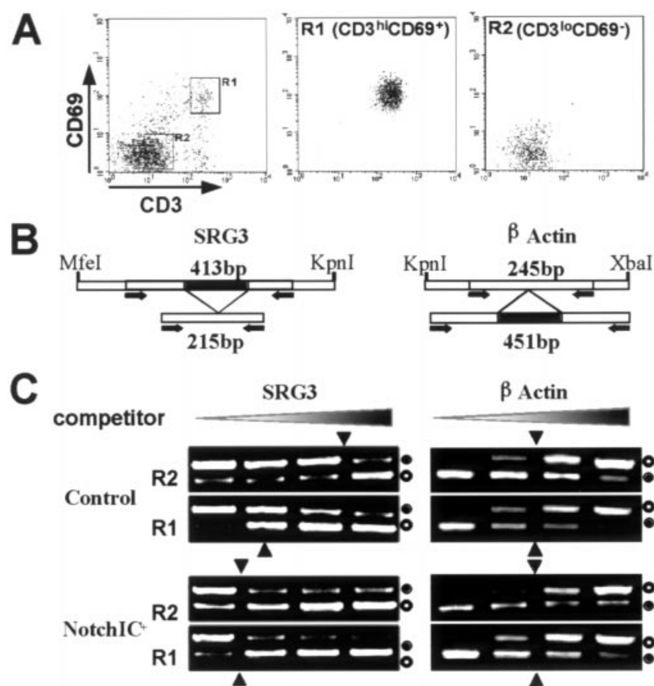

Fig. 2. SRG3 expression is down-regulated after positive selection of thymocytes and also because of expression of NotchIC in thymocytes. (*A*) $CD3^{lo}CD69^{-}$ and $CD3^{hi}CD69^{+}$ thymocytes were sorted by the gates shown (R1, R2), and the sorted populations were reanalyzed to confirm the purity (>98%). (*B*) Competitors were prepared for semiquantitative competitive RT-PCR by deleting a 198-bp internal fragment of *SRG3* and inserting a 206-bp fragment into the β-actin. Black arrows indicate PCR primers. (*C*) The *SRG3* expression pattern was analyzed by semiquantitative competitive RT-PCR. Total RNA purified from 3×10^4 cells of each population was reverse-transcribed. After one-twentieth of the RT product was mixed with 25 fg, 50 fg, 100 fg, and 200 fg of competitors in each reaction, the mixtures were amplified by PCR. Arrowheads indicate the points at which equilibrium was reached. R1 represents $CD3^{hi}CD69^{+}$ population, and R2 represents $CD3^{lo}CD69^{-}$ population. ● denote bands amplified from RT products, and ○ denote bands amplified from added competitors. One representative PCR profile of three independent experiments with similar results is shown.

The level of the SRG3 protein in DP thymocytes of the $NotchIC^{+}$ transgenic mice was reduced to about one-fourth compared with $NotchIC^{-}$ control mice. However, the expression levels of Lck and mBRG1 did not change significantly. Therefore, activated Notch1 appears to down-regulate the expression of SRG3 protein in the thymus.

***SRG3* mRNA Expression Is Reduced in Positively Selected Thymocytes.** We next investigated how the expression of SRG3 is regulated during thymocyte development, especially during the positive selection process. Based on observations that thymocytes are induced to express CD69 and increase their TCR/CD3 expression after positive selection (18, 19), two thymocyte populations, $CD3^{lo}CD69^{-}$ (presumably before the positive selection) and $CD3^{hi}CD69^{+}$ (after the positive selection) cells, were isolated from thymi of C57BL/6 mice by using flow cytometry (Fig. 2*A*). The purity of the populations was confirmed by reanalyzing the sorted cells and was over 98%. The $CD3^{lo}CD69^{-}$ cells in region R2 (Fig. 2*A*) were mostly (95 ± 3%) $CD4^{+}CD8^{+}$ immature DP T cells.

To estimate the expression levels of SRG3 in the two populations, semiquantitative competitive RT-PCR was performed following a method described by Kim *et al.* (20), with total RNAs isolated from 3×10^4 cells of each sorted population. A plasmid containing a 198-base deletion in the middle region of the *SRG3* was used as a competitor (Fig. 2*B*). As a control, the expression levels of β-actin mRNA in the two populations were tested by the same method using a plasmid containing a 206-bp insertion in the middle of the gene as a competitor. The expression level of the *SRG3* gene was reduced to one-third in the $CD3^{hi}CD69^{+}$ thymocyte population, where about 50 fg of the competitor was required to reach equilibrium (Fig. 2*C*). On the other hand, about 150 fg of the competitor was required for equilibrium for the $CD3^{lo}CD69^{-}$ thymocyte population. This level of reduction in SRG3 expression after positive selection is similar to that observed in the mature T cell (11). In contrast, the expression levels of the β-actin gene were similar between two populations (Fig. 2*C*).

The same analysis was applied to thymocytes of the $NotchIC^{+}$ transgenic mice. In $NotchIC^{+}$ transgenic mice, essentially the same results as normal mice were obtained for the expression of the β-actin gene. However, about 30–35 fg of the competitor was required to reach equilibrium for both R1 and R2 populations. Therefore, *SRG3* in the $CD3^{lo}CD69^{-}$ population of the $NotchIC^{+}$ transgenic mice is expressed at a level that is even lower than that in the $CD3^{hi}CD69^{+}$ thymocyte population of normal mice. These results confirm that thymocytes from $NotchIC^{+}$ transgenic mice express a reduced level of *SRG3* compared with those from normal mice.

NotchIC Down-Regulates the *SRG3* Promoter Activity. We subsequently tested whether NotchIC directly affects the *SRG3* promoter activity. We isolated a 1.2-kb DNA fragment containing the promoter region of the *SRG3* gene by genomic library screening. The major transcription start site was mapped ≈80 bp upstream from the ATG sequence. Minimal promoter activity was found ≈14–120 bp upstream from the transcription start site (data not shown). We inserted a 1.2-kb *SRG3* promoter sequence into the pGL3-Basic plasmid for luciferase assay (Fig. 3*A*). A thymoma cell line, S49.1, was cotransfected with the reporter construct and Notch DE6MT. As the amount of NotchIC expression vector DNA was increased, the *SRG3* promoter activity was progressively reduced (Fig. 3*B*). Furthermore, DNA sequence analysis of the 1.2-kb fragment revealed five E-box-like sequences, which are potential binding sites for Hes1, a downstream effector molecule of Notch1 (21). Introduction of mutations (from CATCTG to CTGCAG) in one of the E-box-like sequences (located at −480 to −475) abolished the down-regulation effect of NotchIC on the SRG3 promoter activity (Fig. 3*C*). These results strongly indicate that NotchIC suppresses the promoter activity of *SRG3* through the E-box sequence.

Thymocytes from lck-αSRG3 Transgenic Mice Become Less Sensitive to GC-Induced Apoptosis. To test whether developing thymocytes become resistant to GC by lowering the expression of SRG3, we generated transgenic ($lck\text{-}\alpha SRG3^{+}$) mice expressing *SRG3* mRNA in reverse orientation under the *lck* proximal promoter (Fig. 4*A*). We have shown previously that the 2.9-kb *Xba*I fragment of SRG3 cDNA can be expressed in reverse orientation to block GC-induced apoptosis in S49.1 (11). The cDNA fragment was used for DNA construction to produce lck-αSRG3 transgenic mice. We established three lines of transgenic mice and chose the one that expressed the highest level of the transgene. The transgene was expressed only in thymus (Fig. 4*B*). The total number of thymocytes of the 3- to 4-week-old transgenic mice increased by 5–10% compared with that of control littermate mice (data not shown). The CD8/CD4 profile of thymocytes, however, was similar to that of control littermates. The level of SRG3 protein reduced by half in the thymus of transgenic mice compared with control littermates (Fig. 4*C*). However, we found no significant difference in the levels of GR and Lck between these mice. DP thymocytes from these mice

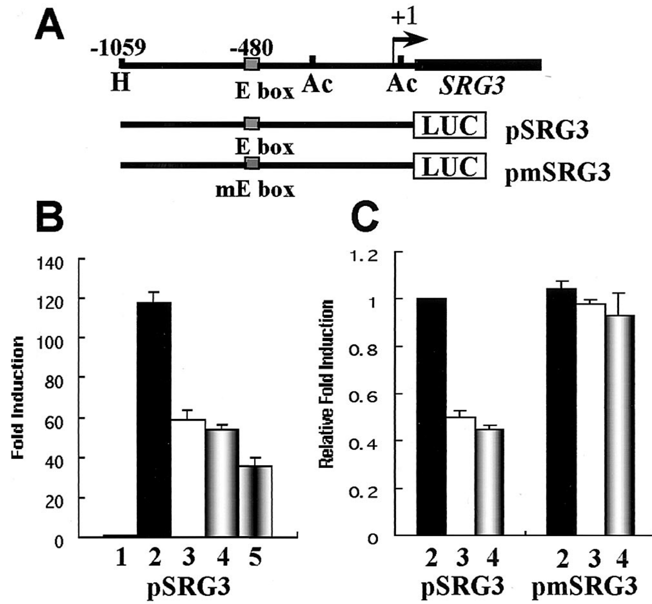

Fig. 3. NotchIC down-regulates the promoter activity of *SRG3*. (*A*) Reporter constructs to assess *SRG3* promoter activity. *SRG3* promoter is shown with transcription initiation site (+1) and a potential E box. H, *Hin*dIII; Ac, *Acc*I; pSRG3, normal *SRG3* promoter linked to a reporter in pGL3; pmSRG3, *SRG3* promoter with mutated E box (mE box) linked to a reporter in pGL3; LUC, luciferase. (*B*) The effect of NotchIC on the *SRG3* promoter activity. pSRG3 was transfected with Notch DE6MT into S49.1 cells. The luciferase activity measured when pGL3 Basic plasmid alone was transfected into S49.1 was set as 1-fold. β-galactosidase (β-gal) activity was also measured to normalize the transfection efficiency. Lane 1: pGL3 Basic + 0.5 μg β-gal; lane 2: 4 μg pSRG3 + 0.5 μg β-gal; lane 3: 4 μg pSRG3 + 2 μg Notch DE6MT + 0.5 μg β-gal; lane 4: 4 μg pSRG3 + 4 μg Notch DE6MT + 0.5 μg β-gal; and lane 5: 4 μg pSRG3 + 8 μg Notch DE6MT + 0.5 μg β-gal). Bars = SE. (*C*) The effect of putative E box (−480 to −475) on the *SRG3* promoter activity. pSRG3 or pmSRG3 was transfected in the same way as in *B*, and the luciferase activity was shown as relative value against the one obtained when pSRG3 only was transfected into S49.1 (lanes 2–4 are the same as in *B*). Bars = SE.

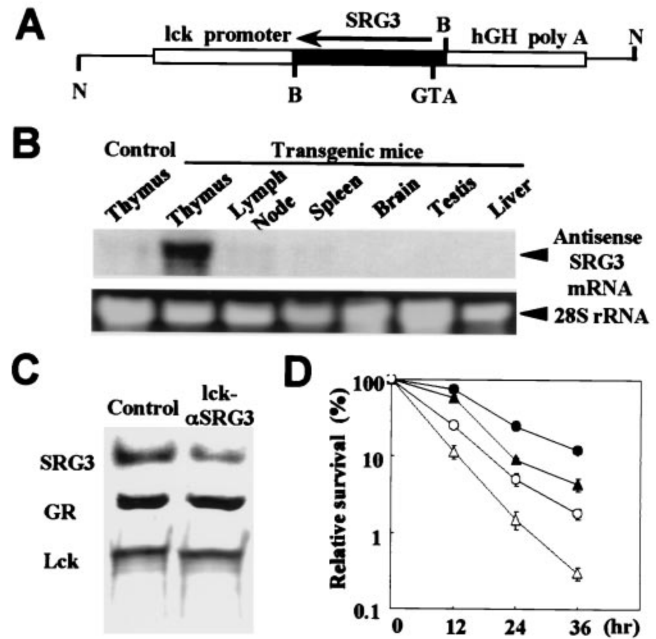

Fig. 4. Reduced expression of SRG3 confers GC resistance to thymocytes. (*A*) DNA construct for lck-αSRG3 transgenic mice. The 2.9-kb *Xba*I fragment of *SRG3* cDNA was placed in the reverse orientation under the *lck* promoter. N, *Not*I; B, *Bam*HI. (*B*) Northern blot of antisense *SRG3* mRNA expression in transgenic mice. Total RNAs were purified from indicated organs. As loading controls, 28S rRNAs are shown. (*C*) Western blot analysis of SRG3, GR, and Lck expressed in thymi from lck-αSRG3 transgenic and control littermate mice. (*D*) Thymocytes from lck-αSRG3 transgenic mice are relatively resistant to GC-induced apoptosis. Single-cell suspensions of thymocytes (10^7 cells) prepared from the transgenic mice and littermate controls were incubated with medium alone or medium with DEX (10^{-8} M, 10^{-7} M) for indicated time intervals. Cells then were analyzed for viability and CD4 and CD8 expression by using flow cytometry. The vertical axis shows relative survival rates of DP thymocytes. Circles: lck-αSRG3 transgenic mice; triangles: FVB littermate control mice. Filled symbols: 10^{-8} M DEX. Open symbols: 10^{-7} M DEX. Bars = SE.

were treated with 10^{-8} M and 10^{-7} M DEX, and relative survival rate was calculated by flow cytometry, as described (8). Thymocytes from the lck-αSRG3 transgenic mice were significantly less sensitive to GC-induced apoptosis than those from wild-type littermate control mice (Fig. 4*D*). These results suggest that the reduction of SRG3 protein confers on thymocytes a resistance to GC-induced apoptosis.

Increased SRG3 Expression Restores GC Sensitivity in DP Thymocytes Expressing NotchIC. We tested whether increasing the level of SRG3 expression in thymocytes of the NotchIC$^+$ transgenic mice could restore the GC sensitivity. We have produced transgenic mice (CD2-SRG3) overexpressing SRG3 in T lineage cells by using the human *CD2* (hCD2) promoter (30). The line with ≈10 copies of the transgene expressed the SRG3 protein about two times more than littermate controls both in the thymus and the periphery. We produced double transgenic mice expressing both transgenic SRG3 (FVB) under the *hCD2* promoter and NotchIC (C57BL/6) under the *lck* proximal promoter. Western blot analysis with thymic extracts prepared from F_1 littermates consistently showed that double transgenic mice expressed about two times more SRG3 protein than NotchIC$^+$ single transgenic mice (Fig. 5*A*). However, the expression level of SRG3 in the double transgenic mice was only half that of nontransgenic F_1 control (Fig. 5*A*). We could not produce any double transgenic mice with thymocytes expressing SRG3 at the level of the normal littermate control for unclear reasons. F_1 littermate mice expressing solely the *SRG3* transgene, however, expressed about two times a higher level of the SRG3 protein in the thymus compared with nontransgenic littermates. The expression levels of mBRG1 and Lck in the thymus were similar among those from double transgenic mice, transgenic mice expressing NotchIC only, and F_1 littermate control mice. To analyze the GC sensitivity of DP thymocytes from these mice, relative survival rate was calculated by flow cytometry after DEX treatment. DP thymocytes from littermate mice expressing NotchIC transgene only were much less sensitive to the DEX treatment than cells from control littermates as previously reported (Fig. 5*B*) (8). DP thymocytes from CD2-SRG3$^+$ and NotchIC$^+$ double transgenic mice became more sensitive to DEX treatment than cells from F_1 littermate mice expressing NotchIC only (Fig. 5*B*). GC sensitivity of DP thymocytes from the double transgenic mice was not fully restored to the level of littermate control, which might be because of incomplete restoration in the expression level of SRG3 (Fig. 5 *A* and *B*). These data show a clear correlation between the expression level of SRG3 and GC sensitivity in thymocytes and indicate that GC sensitivity can be restored by increasing the expression level of SRG3 in mice expressing the NotchIC transgene.

Differentiation of DP Thymocytes into CD4$^+$ and CD8$^+$ SP Thymocytes in NotchIC$^+$ and CD2-SRG3$^+$ Double Transgenic Mice. When we analyzed the CD8/CD4 profile of thymocytes from the CD2-

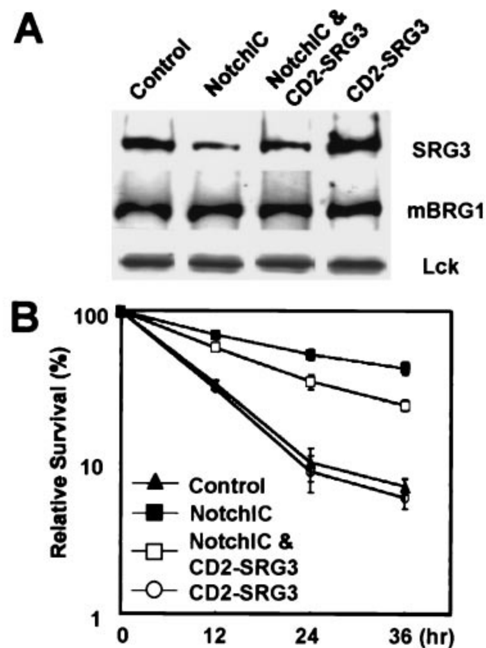

Fig. 5. Restoration of GC sensitivity in thymocytes expressing NotchIC by transgenic SRG3 expression. (*A*) Western blot analysis of SRG3, mBRG1, and Lck expression with thymocyte extracts prepared from littermates expressing no transgene, NotchIC transgene only, both CD2-SRG3 and NotchIC transgene, or CD2-SRG3 transgene only. SRG3 expression increased by about 2-fold in $NotchIC^+$ and $CD2\text{-}SRG3^+$ double transgenic mice than in $NotchIC^+$ transgenic littermates, but the expression level was about half that of normal littermate controls. The expression levels of mBRG1 and Lck did not change significantly. Mice used were all F_1 littermates produced by mating $CD2\text{-}SRG3^+$ transgenic mice (FVB) and $NotchIC^+$ transgenic mice (C57BL/6). (*B*) Thymocytes (10^7 cells) prepared from transgenic mice and littermate controls were incubated in medium alone or medium with DEX (10^{-8} M) for indicated times and analyzed by flow cytometry for viability and CD4 and CD8 expression. Results are shown as an average of three independent experiments. Bars = SE.

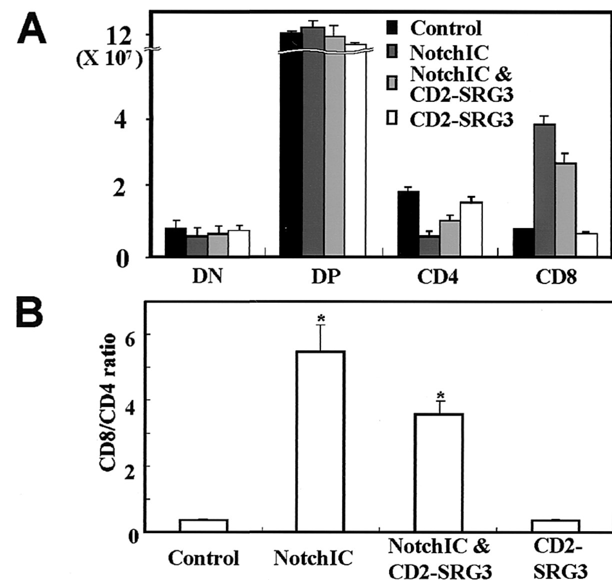

Fig. 6. Restoration of the CD8/CD4 ratio in mice expressing both NotchIC and CD2-SRG3 transgene. (*A*) Histogram shows absolute numbers of indicated thymocyte populations. All of the analyzed mice were 3–4 weeks old. The represented numbers are the averages of seven F_1 normal mice, 10 $NotchIC^+$ transgenic mice, 13 mice expressing both NotchIC and CD2-SRG3 transgenes, and six $CD2\text{-}SRG3^+$ transgenic mice. Mice used were all F_1 littermates produced by crossing $CD2\text{-}SRG3^+$ transgenic mice (FVB) and $NotchIC^+$ transgenic mice (C57BL/6). Bars = SE. (*B*) The effect of transgenic CD2-SRG3 expression on the CD8/CD4 ratio in mice expressing the NotchIC transgene. The CD8/CD4 ratios are the average of CD8/CD4 ratio obtained from the same mice as in *A*. The CD8/CD4 ratios are 5.5 in mice expressing the NotchIC alone and 3.6 in the double transgenic mice. *, $P = 0.0187$ (two-tailed Student's *t* test). Bars = SE.

$SRG3^+$ and $NotchIC^+$ double transgenic mice, an increase in the proportion of mature CD4 cells [6.4 (± 0.8) % vs. 3.4 (± 0.6) % in average (±SE)] and a decrease in mature CD8 cells [16.7 (± 3.6) % vs. 21.5 (± 1.9) %] were consistently observed when compared with F_1 littermate $NotchIC^+$ transgenic mice (Fig. 6*A*). The actual number of CD4 SP thymocytes also increased from 0.61 (± 0.11) × 10^7 cells in mice expressing NotchIC only to 1.06 (± 0.13) × 10^7 cells in the double transgenic mice [normal littermates: 1.92 (± 0.1) × 10^7]. In contrast, the number of CD8 SP thymocytes decreased from 3.85 (± 0.34) × 10^7 cells in NotchIC transgenic mice to 2.76 (± 0.3) × 10^7 cells in the double transgenic mice [normal littermates: 0.81 (± 0.04) × 10^7]. This resulted in the partial restoration of the distorted CD8/CD4 ratio observed in NotchIC transgenic littermate mice (Fig. 6*B*). We could not obtain complete restoration in the CD8/CD4 profile of thymocytes to a normal one in the double transgenic mice. This may be partly because the mice did not express transgenic SRG3 up to the level of normal mice. Transgenic mice overexpressing SRG3 only displayed a similar CD8/CD4 staining pattern to wild-type mice; however, these mice showed a reduced number of thymocytes (18% reduction in 3- to 4-week-old mice) compared with littermate controls. Our data suggest that the activation of Notch1 affects the differentiation process of DP thymocytes into $CD4^+$ and $CD8^+$ SP thymocytes, at least in part, by down-regulating SRG3 expression and, thereby, conferring GC resistance to developing thymocytes.

Discussion

Notch1 has been reported to affect GC-induced apoptosis of thymocytes (8). This finding suggests that activated Notch1 may protect thymocytes from GC-induced apoptosis and that Notch1 signaling may prevent developing thymocytes from death by neglect mediated by GCs. However, it is not clear which downstream target of Notch1 signaling is responsible for the induction of GC resistance in thymocytes. Our results indicate that SRG3 is responsible for determining the GC sensitivity of thymocytes and is a downstream target of Notch1 in conferring GC resistance on developing thymocytes. This conclusion is based on the following results. First, lowering the SRG3 level confers GC resistance on developing thymocytes. Second, SRG3 is down-regulated after positive selection in normal thymocytes. It is also down-regulated in $CD69^-CD3^{lo}$ thymocytes of transgenic mice expressing constitutively active NotchIC. Third, the promoter activity of *SRG3* is down-regulated by Notch1 through the putative E-box sequence. Finally, increased expression of SRG3 in DP thymocytes expressing NotchIC using a heterologous promoter restored the GC sensitivity.

It has been postulated that GCs may contribute to the process of death by neglect of DP thymocytes and that acquisition of the resistance to GCs is a key event that rescue thymocytes from apoptosis during positive selection (8, 22). Expression of Bcl2, an antiapoptotic protein, is considered to rescue thymocytes from GC-induced cell death. However, in transgenic T cells overexpressing SRG3, expression of Bcl2 did not change significantly (data not shown), suggesting that SRG3 and Bcl2 work independently in regulation of GC sensitivity. It has been shown that transgenic Notch3 expression induced antiapoptotic and proliferative pathways in late $CD4^-CD8^-$ thymocytes by activating NF-κB via IKKα-dependent degradation of IκBα (23). However, we did not observe any significant increase in the NF-κB binding activity in thymocytes from Notch1 transgenic mice compared

with those from control mice (data not shown). Therefore, gaining of GC resistance by NotchIC in thymocytes does not seem to be due to antiapoptotic effects by NF-κB activation.

Notch has been reported to be central in the immune cell-fate determination such as the lineage choice between the B and T cell (24) and the decision between $\alpha\beta$ and $\gamma\delta$ T cell lineages (25). It was also suggested that Notch signaling might regulate the determination of CD8/CD4 cell lineage. Mice constitutively expressing NotchIC have an excess number of $CD8^+$ SP thymocytes compared with normal mice (10). Therefore, it was hypothesized that NotchIC promotes DP thymocytes to develop into the CD8 lineage (10). However, results showing that NotchIC confers GC resistance on DP thymocytes suggest a plausible alternative explanation for the function of Notch1 during thymocyte maturation. It was suggested that NotchIC might prolong the survival of positively selected cells (8, 9). Then, cells that have an extremely low affinity interaction with self-MHC that would normally die by neglect may be rescued. This prolonged survival may promote the development of $CD8^+$ SP thymocytes because CD8 lineage commitment seems to require weaker signal from the TCR compared with the CD4 lineage commitment (26, 27). This presumption was further supported by results obtained from transgenic mice overexpressing Bcl2 in the thymus, in which excess $CD8^+$ SP thymocytes also were developed (28). In this report, we were able to restore GC sensitivity in thymocytes expressing NotchIC by introducing a *SRG3* transgene driven by a heterologous promoter. However, we were not able to produce double transgenic mice with thymocytes expressing the SRG3 at the level of normal mice. The *CD2* promoter used for transgenic SRG3 expression is not likely to be a reason for this because CD2 expression is not reduced in $NotchIC^+$ transgenic mice (data not shown). It is possible that NotchIC regulates the expression of SRG3 at a posttranscriptional level as well as at the transcriptional level. At any rate, results indicate that DP thymocytes from mice expressing both NotchIC and *SRG3* transgenes became more sensitive to GC than cells from NotchIC transgenic mice. In the thymus of the double transgenic mice, an increase in $CD4^+$ SP thymocytes and a decrease in $CD8^+$ SP thymocytes were consistently observed, resulting in an improvement in the CD8/CD4 ratio compared with littermate mice expressing the NotchIC transgene only. These data indicate that Notch1 signaling affects the CD8/CD4 lineage determination by conferring GC resistance on DP thymocytes. However, in transgenic mice expressing antisense *SRG3* mRNA, the CD8/CD4 profile was similar to that of normal mice, and we did not observe any significant increase in the $CD8^+$ SP population in thymus. Therefore, a change in GC sensitivity alone may not explain the excess production of $CD8^+$ SP thymocytes in $NotchIC^+$ transgenic mice.

Recently, Wolfer *et al.* (29) reported that Notch1-deficient thymocytes are normally sensitive to GC-induced apoptosis and that Notch1 does not appear to be essential in CD8/CD4 lineage commitment. We observed that stimulation of the TCR signaling pathway also down-regulated the SRG3 expression in DP thymoma cells (M.G., K.O., and J.J., unpublished work). Therefore, SRG3 expression appears to be regulated by TCR signaling as well as by Notch1 signaling pathways during thymocyte development. It will be interesting to see how the level of SRG3 expression is regulated during development of the Notch1-deficient thymocytes.

In summary, our results conclusively show that one of the important results of Notch1 activation is down-regulation of SRG3 expression in thymocytes so that they become GC resistant. This may be the basis for rescuing positively selected thymocytes from death by neglect, and the change in GC sensitivity also seems to affect, to some extent, the differentiation of DP thymocytes into SP thymocytes.

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