N-3

Determination of Survival and Death during T-cell Development

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Abstract

Immature, double positive thymocytes are sensitive to glucocorticoid(GC)-induced apoptosis, while mature single positive T cells are relatively resistant. These double positive thymocytes seem to acquire resistance to GCs during differentiation into mature $CD4^+$ and $CD8^+$ single positive thymocytes. However, detailed knowledge concerning what determines the sensitivity of thymocytes to GCs and how GC-sensitivity is regulated in thymocytes during development is lacking. We found that the expression level of a SRG3 protein determines the GC-sensitivity of T cells in mice. SRG3 associates with the glucocorticoid receptor(GR) only in the thymus but not in the periphery. In addition, blocking the formation of the SRG3-GR complex with a dominant negative mutant form of SRG3 decreases GC sensitivity in the T cells. Furthermore, transgenic overexpression of the SRG3 protein in peripheral T cells induces the formation of the complex and renders the cells to become sensitive to GC-induced apoptosis. The expression of SRG3 is down-regulated after positive selection of thymocytes during differentiation. We found that the SRG3 expression is regulated by signals transduced through T-cell receptor (TCR) and also by Notch1 activation. Specifically, Ras/MEK/ERK and PI3K pathway mediated the downregulation of SRG3 by TCR ligation. Activation of Notch1 was shown to induce GC resistance in thymocytes. Expression of transgenic SRG3 resulted in the restoration of GC sensitivity in thymocytes expressing transgenic Notch1. The promoter activity of the SRG3 gene also was downregulated by activated form Notch1, suggesting that SRG3 is the downstream target of Notch1 in regulating GC sensitivity of thymocytes.

These results suggest that the expression level of the SRG3 is a key regulator for the sensitivity of developing thymocytes to GCs, and its expression is finely regulated during T cell development.

1. SRG3 controls the glucocorticoid-induced apoptosis in developing thymocytes

To examine the amount of SRG3-GR complex correlates with the expression level of SRG3 protein and this formation of SRG3-GR complex determines the sensitivity to GC-induced apoptosis, we analyzed transgenic mice that overexpress SRG3 in T lineage cells (Fig. 1A) by a heterologous promoter. Western blot analysis revealed that transgenic mice express ~2 times more SRG3 protein than control littermates in both thymus and periphery (Fig. 1B). Immunoprecipitation of lymph node extracts with BuGR-2 (anti murine GR antibody) showed an increased formation of the complex containing SRG3 and GR in peripheral T cells (Fig. 1C). These results suggest that the formation of SRG3-GR complex is dependent on the expression level of the SRG3 protein in T cell. We then examined whether the lymph node T cells, overexpressing SRG3 protein and now containing the SRG3-GR complex, became sensitive to GC-induced apoptosis. As shown in Fig. 2, mature T cells became more sensitive to GC-induced apoptosis by increased expression of SRG3. These results suggest that the expression level of SRG3 is important for the formation of SRG3-GR and also in determining the sensitivity of T cells to GC-induced apoptosis.

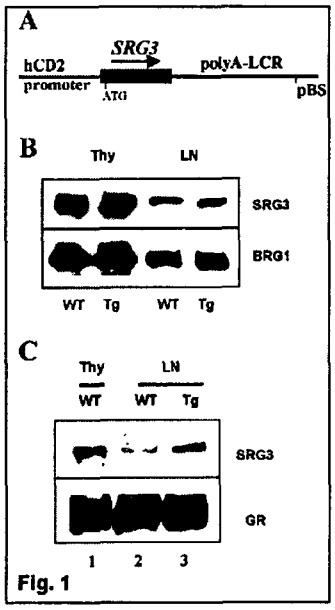

Fig. 1

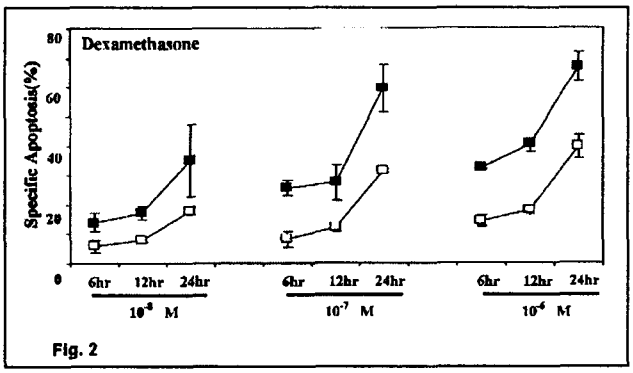

Fig. 2

We have found that an 89-aa fragment from aa 854922 of SRG3 has a dominant negative mutant effect against SRG3. The *in vitro* translated 89-aa fragment of SRG3 was coimmunoprecipitated with GR (Fig. 3A). This result indicates that the 89-aa fragment of SRG3 may act as a dominant negative mutant of SRG3 by blocking the SRG3-GR association. To test the above possibility, we examined whether this fragment would block GR-mediated transcription (Fig. 3B). The pGRE-LUC plasmid containing two GREs was transfected into S49.1 cells. When these cells were treated with DEX after transfection, luciferase activity showed up to a ~30-fold increase compared with that

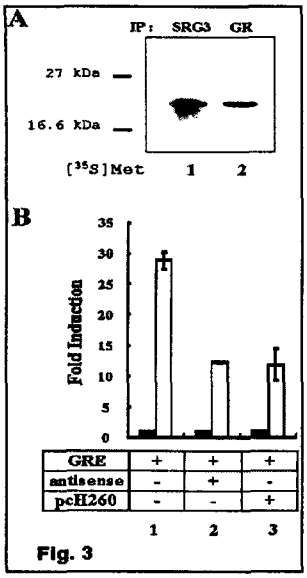

Fig. 3

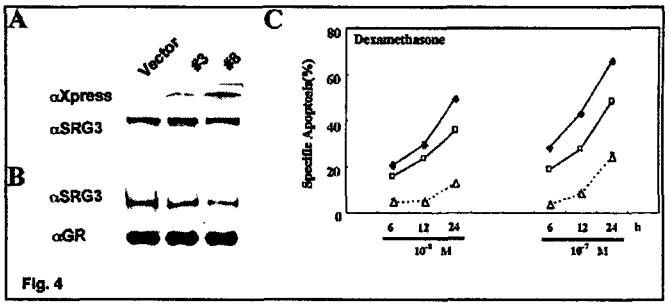

Fig. 4

of nontreated transfectants (Fig. 3B, lane 1). Cotransfection of this DNA together with the pRcASRG3 plasmid, expressing the antisense SRG3 gene under the control of a CMV promoter or the pcH260 plasmid DNA expressing the 89-aa fragment of SRG3 was cotransfected, blocked the enhancement of luciferase activity induced by DEX treatment (Fig. 3B, lane 2,3). These results suggest that SRG3 is required for GR-mediated transcription, and the 89-aa fragment can block this process.

To test whether the 89-aa fragment can disrupt the association between SRG3 and GR, pcH260 DNA was stably transfected into GC-sensitive S49.1 cells. Two clones (3 and 8) expressing the 89-aa fragment at different levels were isolated by selection with G418. Western blot analysis with cell extracts showed that clone 8 expressed the fragment at a significantly higher level (5 times higher) compared with clone 3, whereas the expression levels of the SRG3 protein were similar (Fig. 4*A*). SRG3-GR association was significantly reduced in clone 8 (58% reduction), which expressed higher levels of the fragment, than in clone 3 (14% reduction; Fig. 4*B*). We also found that clone 8 contained significantly less SRG3-GR complex than clone 3. As expected, clone 8 was less sensitive to DEX treatment than clone 3 and the vector transfectants (Fig. 4C). Our results show that the 89-aa fragment of SRG3 blocks the association of SRG3 with GR, GR-mediated transcription, and GC-induced apoptotic cell death. The results suggest that the level of SRG3-GR complex is a critical parameter determining the GC sensitivity in T cells, and the amount of SRG3-GR complex depends on the expression level of SRG3.

2. SRG3 is downregulated by TCR signaling

The SRG3 expression is downregulated by signal through TCR. The Ras/Raf/MAP kinase pathway was reported to be important for positive selection of developing thymocytes. To examine whether the TCR-mediated Ras activation would affect

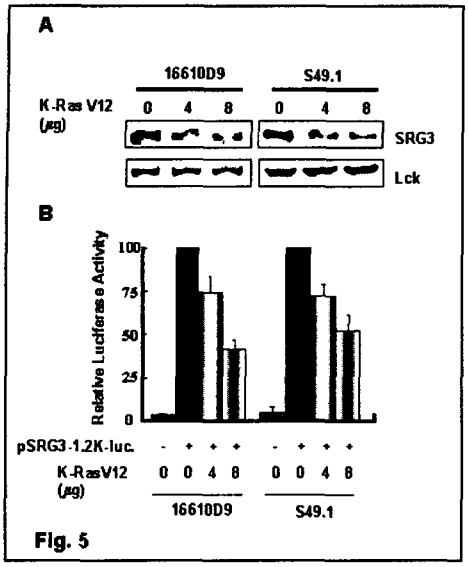

Fig. 5

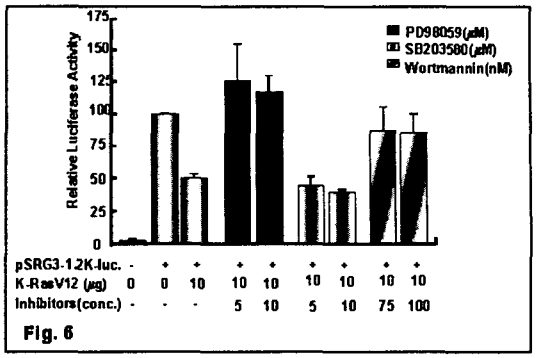

Fig. 6

SRG3 expression, the protein expression and the promoter activity of SRG3 were examined after transient transfection of constitutively active mutant of K-ras, K-RasV12 to the thymoma cell line, S49.1 and 16610D9 cell. As shown in Fig. 5, Ras signaling in a dose-dependent manner lowered the SRG3 protein expression and the promoter activity of SRG3.

Next, to see that what kind of Ras downstream molecules will be responsible for the SRG3 downregulation (MEK/ERK, PI3K or p38), we checked promoter activity using specific inhibitors to MEK (PD98059), PI3K (Wortmannin) and p38 kinase (SB203580). As shown in Fig. 6, PD98059 completely blocked downregulation of the SRG3 promoter activity by Ras. Wortmannin also inhibited repression of the SRG3 promoter activity by Ras, but SB203580 did not. These data suggest that inhibition of the SRG3 activity via Ras activation is largely mediated through the activation of ERK and/or PI3K pathway.

3. SRG3 is downregulated by Notch1 signaling

Previous results showed that thymocytes from Notch1 transgenic mice are resistant to apoptosis by glucocorticoids. And the protein expression of SRG3 and the promoter activity of the SRG3 gene were downregulated by the activated form of Notch1 (NotchIC). To examine whether the reduction of apoptosis shown in thymocytes from Notch1 transgenic mice correlates with the expression level of SRG3, we crossmated NotchIC transgenic mice with transgenic mice that overexpress SRG3. 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. 7A). 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. 7B). 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. 7B). These data show a correlation between the expression level of SRG3 and GC sensitivity in thymocytes and indicate that increasing the expression level of SRG3 in mice expressing the NotchIC transgene can restore GC sensitivity.

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. Minimal promoter activity was found ≈14-120 bp upstream from the transcription start site. We inserted a 1.2-kb SRG3 promoter sequence into the pGL3-Basic plasmid for luciferase assay (Fig. 8A). 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. 8B). Furthermore, DNA sequence analysis of the 1.2-kb fragment revealed five E-box-like sequences, which are potential binding sites for E proteins. 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. 8C). These results strongly indicate that NotchIC suppresses the promoter activity of SRG3 through the E-box sequence.

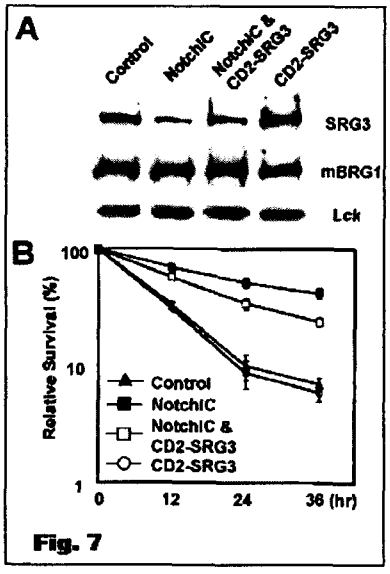

Fig. 7

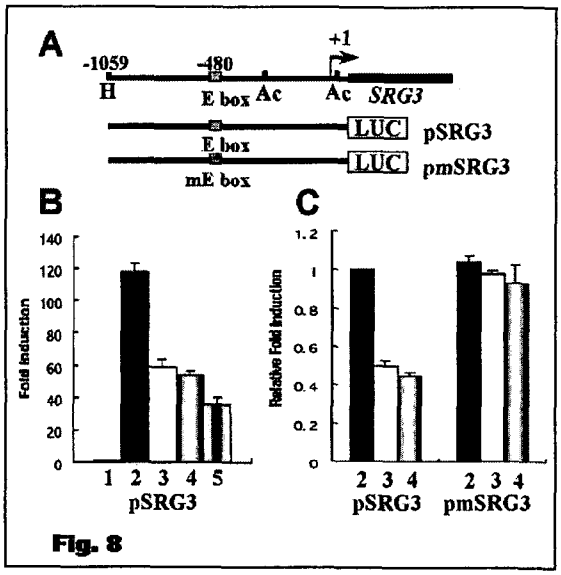

Fig. 8