Suppression of thymic development by the dominant-negative form of Gads

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Abstract

Gads, a hematopoietic-lineage-specific Grb2 family member, is involved in the signaling mediated by the TCR through its interactions with SLP-76 and LAT. Here, we generated transgenic mice expressing Grf40-dSH2, an SH2-deleted dominant-negative form of Gads, which is driven by the lck proximal promoter. The total number of thymocytes was profoundly reduced in the transgenic mice, whereas in the double-negative (CD4–CD8–) thymocyte subset, in particular the CD25/CD44– pre-T cell population, it was significantly increased. However, CD5 expression, which is mediated by pre-TCR stimulation, was significantly suppressed on the CD4–CD8– thymocytes of the transgenic mice. Furthermore, the SLP-76-dependent signaling was markedly suppressed as well. These data suggest that Gads plays an important role in the pre-TCR as well as TCR signaling in thymocytes.

Introduction

Stimulation of the TCR results in the sequential activation of the Src family and Syk family protein tyrosine kinases (PTK), including Lck, Fyn and ZAP-70, which trigger the calcium-dependent and Ras-dependent signaling pathways (1–3). The TCR-induced calcium flux depends on the tyrosine phosphorylation and activation of phospholipase C (PLC)-γ1, while the activation of Ras is thought to be initiated by the recruitment of a Grb2–Sos complex to the membrane (4,5). Two adapter/linker molecules, SLP-76 and LAT, are indispensable for coupling the PTK that mediate TCR signals to their downstream signaling pathways (6–8). LAT is palmitoylated, which targets it to membrane lipid rafts (9), and it is tyrosine phosphorylated upon TCR stimulation. Several signaling molecules, such as Grb2, PLC-γ1 and the 85 kDa subunit of phosphoinositol-3-kinase, bind to phosphotyrosine residues of LAT through their SH2 domains (7). We and others independently isolated cDNA clones coding for the same novel CD25–CD44– (17). The pre-TCR complex, which consists of a successfully rearranged TCR β chain, pre-TCR α chain and components of the CD3 complex, is expressed at the CD25+CD44+ pre-T cell stage, and the pre-TCR signaling is necessary and sufficient for sustained survival, proliferation.
and progression of the DN T cells into the CD4+CD8+ double-positive (DP) T cells (18). Knockout mice lacking either SLP-76 or LAT show a complete block in thymic development, with the presence of the DN subset but the absence of the DP and CD4+CD8+ (CD4SP) or CD4-CD8+ single-positive (CD8SP) subsets, indicating that both SLP-76 and LAT are critically involved in the pre-TCR signaling (19–21).

To investigate the in vivo functional significance of Gads' interactions with SLP-76 and LAT, we generated transgenic mice expressing a dominant-negative form of Gads, Grf40-dSH2, in thymocytes, and demonstrated the possible involvement of Gads in thymocyte development at the pre-T cell stage.

Methods

Plasmids and transgenic mice
cDNA fragments of a human Gads mutant with its SH2 domain deleted, Grf40-dSH2, were amplified by PCR from the plasmid pMycGrf40-dSH2 (11). The Grf40-dSH2 cDNA was inserted into the BamHI sites of the p1017 vector, which contains the lck proximal promoter and the human growth hormone genomic fragment to provide efficient splicing and expression of the transgene (Fig. 1A) (22). To generate transgenic mice, the Grf40-dSH2 fragment (6.1 kb) was excised by digestion with NotI, purified and injected into C57BL/Cr fertilized eggs (Fig. 1A). Four founder mice were identified by PCR from tail DNA, using a forward primer, TGGGAGGCGAGAAGTG-GTGACTAA, which binds within the lck proximal promoter and a reverse primer, CTTGGTTACTTAAAATCT-TCAGAACATCTC, which binds to the N-terminal SH3 region of the Gads sequence. The resulting product is a 0.3-kb fragment. One of the founder mice died for unknown reasons, and three transgenic lines, dnGads-1, dnGads-2 and dnGads-3, were obtained. The dnGads-1 and dnGads-2 lines contained 60–80 copies of the transgene, and the dnGads-3 line contained three copies.

Purification of DN thymocytes
The DN thymocyte subset was purified by auto-MACS (Miltenyi Biotec, Auburn, CA). In brief, 9.0×10^8 total thymocytes were resuspended in 2.0 ml of ice-cold PBS containing 2.0% FCS, and 200 μl of MACS CD4 and CD8 MicroBeads (Miltenyi Biotec) was added. After incubation for 15 min at 4°C, magnetic separation with positive selection columns was performed twice and the negative cells that passed through the columns were used as DN cells. The purity of the DN subset was 95–99%, as determined by flow cytometry.

Flow cytometry
Thymocytes of 3- to 4-week-old mice were stained with the following mAb for flow cytometry analysis: anti-CD4–phycoerythrin (PE), anti-CD4–FITC, anti-CD8–FITC, anti-CD5–PE, anti-CD44–CyChrome, anti-Mac-1–FITC, anti-NK1.1–FITC, anti-B220–FITC, anti-TER-119 (PharMingen, San Diego, CA) and anti-CD25–biotin (23). The anti-TER-119 antibody was used after labeling with FITC. When necessary, cells were further stained with streptavidin–allophycocyanin.

Fig. 1. Construction of the transgene, expression of the transgene and thymocyte numbers of the Grf40-dSH2 transgenic mice. (A) The Grf40-dSH2 transgene (0.7 kb) was subcloned into the BamHI site of the p1017 expression vector. The cDNA fragment bearing the transgene was the NotI fragment of p1017-Grf40-dSH2 (6.1 kb). The transgene construct contained introns and exons of the human growth hormone gene (hGH gene). (B) Thymocytes were prepared from the wild-type (+/−), heterozygous (+/+) and homozygous (+/+) transgenic mice from the three lines (dnGads-1, dnGads-2 and dnGads-3). Their lysates were immunoblotted with the anti-Gads antibody. The endogenous wild-type Gads (Gads) and the transgene product (Grf40-dSH2) are indicated with arrowheads. (C) Total thymocyte numbers were calculated for the wild-type (wild) and transgenic mice (dnGads-1+/−, dnGads-1+/+ and dnGads-2+/+) at 3–4 and 8–9 weeks of age. Mean values are indicated by the filled bars. The average dnGads-1+/− thymocyte number was ~50% of the wild-type thymocyte number in both age groups (P < 0.0001, by Student’s t-test). The average dnGads-1+/− thymocyte number was 10% of the wild-type thymocyte number in 3- to 4-week-old mice (P < 0.0001). The dnGads-2+/− thymocyte number was also reduced in both 3- to 4- and 8- to 9-week-old mice (P = 0.0002).
Involvement of Gads in pre-T cell development

Fig. 2. Suppression of thymocyte development in Grf40-dSH2 transgenic mice. (A) The percentage of thymocytes belonging to different thymocyte subsets in wild-type ($n = 14$), $dnGads^{-1}/- (n = 6)$ and $dnGads^{-1}/- (n = 9)$ mice was assessed using anti-CD4-PE and anti-CD8-FITC mAb. The average percentage (mean ± SEM) of the total is shown for each subset. (B) All the thymocyte subsets except for the DN subset were reduced in the transgenic mice. The absolute cell numbers of wild-type (solid) or $dnGads^{-1}/- (n = 10)$ mice were calculated based on the percentages obtained after CD4 and CD8 staining, and the total thymocyte numbers of 3- to 4-week-old mice given in (A). Mean values are indicated by the filled bars. $P$ values are shown, where significance was indicated by Student’s $t$-test. (C) The subpopulations of the DN thymocyte subset were detected with anti-CD25-biotin, streptavidin–allophycocyanin and anti-CD44–CyChrome mAb. Thymocytes were prepared from wild-type ($n = 12$), $dnGads^{-1}/- (n = 9)$ and $dnGads^{-1}/- (n = 7)$ mice. The average percentage (mean ± SEM) of the total is shown for each subset. In the CD25/CD44 analysis, cells expressing any one of the CD8, CD4, B220, NK1.1, TER-119, Mac-1 or Gr-1 markers were excluded from the analysis. (D) The absolute cell numbers of each subpopulation of wild-type (solid) or $dnGads^{-1}/- (n = 6)$ DN thymocyte were calculated based on the numbers of DN thymocytes given in (B). Mean values are indicated by the filled bars.
The cells were then analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

**Thymocyte stimulation and immunoblot analysis**

Thymocytes \(1 \times 10^7\) were suspended in 300 µl of ice-cold PBS containing 10 µg/ml biotinylated anti-mouse CD3ε (PharMingen) and incubated on ice for 20 min. They were washed with ice-cold PBS and cross-linked with 100 µl of pre-warmed PBS containing 20 µg/ml streptavidin (Wako Pure Chemical, Osaka, Japan) at 37°C for the indicated times. Immunoblotting was carried out as described previously (24).

In brief, the stimulated thymocytes were lysed with lysis buffer (150 mM NaCl, 2 mM EDTA, 1.0% NP-40, 40 mM Tris-HCl, pH 7.6, 0.1% DMSO, 1.0% aprotinin, 1 mM PMSF and 1 mM Na3VO4). The lysates (10 µg of protein per lane) were fractionated by SDS-PAGE then transferred to PVDF filters (Millipore, Bedford, MA). After incubation in TBS containing 3.0% BSA or 5.0% skim milk and 0.1% Tween 20, the filters were probed with the indicated antibodies and visualized using the ECL detection system (Amersham Pharmacia Biotech, Rainham, UK). The antibodies used were anti-Gads polyclonal rabbit antibody (anti-Grf40), anti-phosphotyrosine Ab (4G10), anti-SLP-76 Ab (Upstate Biotechnology, Lake Placid, NY), anti-PLC-γ-1 mixed mAb (Upstate Biotechnology), anti-ZAP-70 Ab (LR) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-PLC-γ-1 mAb (Biosource, Camarillo, CA), anti-phospho-p44/42 MAPK (ERK1/2) mAb and anti-p44/42 MAPK (ERK1/2) mAb (New England Biolabs, Boston, MA).

**Results**

**Generation of transgenic mice expressing a dominant-negative form of Gads**

A human dominant-negative Gads mutant, Grf40-dSH2, was inserted into the p1017 expression vector, which contains the thymocyte-specific lck proximal promoter (22) (Fig. 1A). Using this vector, we established three lines of Grf40-dSH2 transgenic mice, dnGads-1, dnGads-2, and dnGads-3, which expressed Grf40-dSH2 as well as the endogenous wild-type Gads. Thymocytes from dnGads-1/+, dnGads-1+/− and dnGads-2+/− mice expressed Grf40-dSH2 at levels that were 270 ± 14 (n = 4) and 177 ± 13 (n = 7) respectively (P = 0.0015).

**Developmental defects of DN thymocytes in dnGads mice**

To investigate the effect of the Grf40-dSH2 transgene on thymic development, we first examined the absolute thymocyte numbers of the transgenic mice. The average thymocyte numbers (mean ± SEM) of the wild-type, dnGads-1+/−, dnGads-1+/− and dnGads-2+/− mice were 18.5 ± 1.1×10⁷, 8.0 ± 0.8×10⁷, 2.0 ± 0.4×10⁷ and 11.2 ± 0.6×10⁷ respectively at 3–4 weeks of age. At 8–9 weeks of age, the average thymocyte numbers of the wild-type, dnGads-1+/− and dnGads-2+/− mice were 22.1 ± 1.5×10⁷, 8.3 ± 0.4×10⁷ and 9.1 ± 0.5×10⁷ respectively (Fig. 1C). The expression level of Grf40-dSH2 was the highest in dnGads-dSH2 mice, compared with the dnGads-1+/− and dnGads-2+/− mice (Fig. 1B), the severity of thymocyte reduction seemed to depend on the amount of the transgene expressed. We thus demonstrated that one characteristic of the Grf40-dSH2 transgenic mice is a disturbance of thymocyte development.

To analyze more precisely the reduction of thymocyte numbers in the transgenic mice, the T cell subset profile was examined using CD4 and CD8 surface markers. The percentage of DN subpopulation was increased >5- and >2-fold in dnGads-1+/− and dnGads-1+/− mice respectively (Fig. 2A), an increase that correlated with the amounts of the transgene expressed (Fig. 1B). The absolute numbers of cells in the thymocyte subsets including the DN, DP, CD4SP and CD8SP subsets were calculated based on the data from the FACS analyses, and all the subsets except for the DN subset were significantly decreased (Fig. 2B).
The percentage of CD25<sup>+</sup>CD4<sup>+</sup> pre-T cell population of the DN subset was significantly increased in <sub>dnGads-1<sup>+/−</sup></sub> and <sub>dnGads-1<sup>+/−</sup></sub> mice, compared with wild-type mice (Fig. 2C). A similar increase in the same thymocyte population was also observed in the <sub>dnGads-2<sup>+/−</sup></sub> mice (data not shown). We further estimated the absolute cell number of each subpopulation of DN thymocytes (Fig. 2D). Only the CD25<sup>−</sup>CD44<sup>−</sup> subpopulations of the DN subset was significantly increased in the <sub>dnGads-1<sup>+/−</sup></sub> mice as compared with the wild-type mice. We also confirmed the expression of Grf40-dSH2 in the DN thymocytes of the <sub>dnGads-1<sup>+/−</sup></sub> and <sub>dnGads-2<sup>+/−</sup></sub> mice along with the expression of the wild-type Gads and Grb2 (Fig. 3A). These results suggest that the expression of the dominant-negative Gads mutant, Grf40-dSH2, induces suppression of thymic development at the CD25<sup>−</sup>CD44<sup>−</sup> pre-T cell stage of the DN thymocytes.

The pre-T cells of the DN subset express the pre-TCR complex, the stimulation of which is indispensable for their further development into DP thymocytes (18). In addition, the expression of CD5 on DN thymocytes is known to be induced by pre-TCR stimulation (26). Hence, we compared CD5 expression on thymocytes between the transgenic and wild-type mice. We and others previously demonstrated that Gads associates SLP-76 and ZAP-70 is indicated with arrowheads. (B) The whole-cell lysates (10 µg protein per lane) were probed with anti-phospho-physically with SLP-76 and LAT, both of which are essential for TCR signaling (10–12). The two independent lines of Grf40-dSH2 transgenic mice that we describe here showed similar characteristics of suppressed thymic development at the CD25<sup>−</sup>CD44<sup>−</sup> pre-T cell stage. A developmental defect at the pre-T cell stage was also seen in knockout mice lacking either SLP-76 or LAT, and both SLP-76 and LAT are known to be involved in the pre-TCR pathway.

Failure of SLP-76-dependent signaling pathways in <sub>dnGads</sub> thymocytes

We and others previously demonstrated that Gads associates physically with SLP-76 and LAT, both of which are essential for TCR signaling (10–12). In addition, Grf40-dSH2 has been shown to act in a dominant-negative manner in TCR signaling in Jurkat cells (11). Hence we investigated the dominant-negative effect of Grf40-dSH2 on TCR signaling in thymocytes. Thymocytes stimulated with an anti-CD3 mAb were examined for tyrosine phosphorylation of SLP-76 and ZAP-70. The tyrosine phosphorylation of SLP-76 was significantly induced in <sub>dnGads-1<sup>+/−</sup></sub> thymocytes, but was markedly reduced to an undetectable level in <sub>dnGads-1<sup>+/−</sup></sub> thymocytes. However, the tyrosine phosphorylation of ZAP-70, although weakly induced in both <sub>dnGads-1<sup>+/−</sup></sub> and <sub>dnGads-1<sup>+/−</sup></sub> thymocytes, may indicate that ZAP-70 is normally activated by TCR stimulation in the thymocytes of the transgenic mice (Fig. 4A). Nevertheless, the subsequent tyrosine phosphorylation of SLP-76 is appreciably blocked by the expression of Grf40-dSH2. Because SLP-76 is involved in the downstream signaling of the TCR complex leading to activation of the p44/42 MAP kinase (ERK1/2) and PLC-γ1 (6), we further examined the phosphorylation of PLC-γ1 and ERK1/2 in thymocytes upon anti-CD3 stimulation. The phosphorylation levels of both PLC-γ1 and ERK1/2 were profoundly reduced in <sub>dnGads-1<sup>+/−</sup></sub> thymocytes as compared with <sub>dnGads-1<sup>+/−</sup></sub> thymocytes (Fig. 4B). These results suggest that the TCR signaling was suppressed in the thymocytes expressing Grf40-dSH2 by a block in the SLP-76-dependent pathway.

Fig. 4. Impairment of TCR signaling in the transgenic thymocytes. Thymocytes of <sub>dnGads-1<sup>+/−</sup></sub> and <sub>dnGads-1<sup>+/−</sup></sub> mice were stimulated for the indicated time with biotinylated anti-CD3e mAb (145-2C11) and streptavidin. (A) The whole-cell lysates (10 µg protein per lane) were fractionated by SDS-PAGE and subjected to Western blotting. The membrane was probed with the anti-phosphotyrosine mAb (4G10) (top panel), then reprobed with anti-SLP-76 (the second panel) and anti-ZAP-70 antibodies (bottom panel). The migration of SLP-76 and ZAP-70 is indicated with arrowheads. (B) The whole-cell lysates (10 µg protein per lane) were probed with anti-phospho-ERK1/2 (top panel) and anti-phospho-PLC-γ1 (third panel) antibodies, and reprobed with anti-ERK1/2 (second panel) and anti-PLC-γ1 (bottom panel) antibodies.

Discussion

Grf40-dSH2, which contains the SH3 domains, is thought to have a dominant-negative effect on Grb2 as well as Gads, in the SLP-76-dependent signaling, since Gads and Grb2 are associated with both SLP-76 and LAT (10–12,27). The two independent lines of Grf40-dSH2 transgenic mice that we describe here showed similar characteristics of suppressed thymic development at the CD25<sup>−</sup>CD44<sup>−</sup> pre-T cell stage. A developmental defect at the pre-T cell stage was also seen in knockout mice lacking either SLP-76 or LAT, and both SLP-76 and LAT are known to be involved in the pre-TCR signaling as well as TCR signaling (19–21). Although we have not yet obtained direct evidence for the impairment of the pre-TCR signaling in the Grf40-dSH2 transgenic mice, we suspect that the accumulation of pre-T cells in the thymi of the Grf40-dSH2 transgenic mice results from a dysfunction in the pre-TCR signaling pathway caused by the dominant-negative effect of Grf40-dSH2. Actually, the SLP-76-dependent signaling pathway mediated by TCR stimulation was significantly impaired in the thymocytes of the transgenic mice. On the
other hand, it has been found that the decreased expression of Grb2 in Grb2 haploid insufficient (Grb2<sup>+/−</sup>) mice causes an impairment of negative selection of T cells, despite normal pre-T cell development (28). Furthermore, the TCR-induced activation of p38 MAP kinase and JNK, but not ERK, was attenuated in the Grb2<sup>+/−</sup> thymocytes, whereas the TCR-induced activation of ERK was significantly impaired in the thymocytes of our transgenic mice, suggesting a possible differential usage between Gads and Grb2 during the thymocyte development. Hence, it is possible that Gads is involved in the pre-TCR signaling more efficiently than Grb2. One may also consider the possibility that Grf40-dSH2 is implicated in the negative regulation for the expression of Rag1,2 and the pre-TCR components leading to a development defect at the pre-T cell stage. Furthermore, we are unable to rule out this possibility at present, but since Gads and Grb2 are associated with both SLP-76 and LAT, we expect that Grf40-dSH2 contributes to negative regulation of the pre-TCR signaling.

SLP-76-deficient mice showed a complete block in thymocyte development (19,20). Although the Grf40-dSH2 transgenic mice had a profound defect on SLP-76-dependent signaling as shown with the complete suppression of CD3-mediated phosphorylation of SLP-76, they showed an incomplete block of thymocyte development. These discrepant observations may be explained as follows. The expression levels of Grf40-dSH2 may be varied among the thymocyte populations and the amount of Grf40-dSH2 expressed in the CD25<sup>+</sup>CD44<sup>+</sup> pre-T cells of the DN population may be insufficient to induce a complete suppression of the pre-TCR signaling. Indeed, the expression level of Grf40-dSH2 in the total thymocyte populations was significantly higher than that in the DN thymocyte populations.

It has been demonstrated that the ERK and p38 MAP kinase pathways are differentially involved in positive and negative selection of T cells respectively (29,30). Grb2<sup>+/−</sup> mice are impaired in negative selection through disruption of p38 MAP kinase and JNK, but not ERK signaling. Interestingly, the CD3-mediated ERK phosphorylation was markedly decreased in <i>dnGads</i><sup>−/−</sup> thymocytes. In our preliminary experiments using HY-TCR<sup>+/−</sup>/dnGads<sup>−/−</sup> mice, we obtained evidence in support that Grf40-dSH2 induces suppressions of both positive and negative selection (unpublished data). It is possible that Gads is involved in the signaling for positive selection, while Grb2 is involved in the signaling for negative selection. We also speculate that the decreased negative selection in HY-TCR<sup>+/−</sup>/dnGads<sup>−/−</sup> mice might be caused by the suppressive effect of Grf40-dSH2 on the Grb2 function.

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Abbreviations

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<tr>
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<tr>
<td>DN</td>
<td>double negative</td>
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<tr>
<td>DP</td>
<td>double positive</td>
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<tr>
<td>Gads</td>
<td>Grb2-related adaptor downstream of Shc</td>
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<tr>
<td>Grf40</td>
<td>Grb2 family member of 40 kDa</td>
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<td>LAT</td>
<td>linker for activation of T cells</td>
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<td>SH2 domain-containing leukocyte protein of 76 kDa</td>
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<tr>
<td>SP</td>
<td>single positive</td>
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References

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replaces the requirement for linker for activation of T cells in T cell receptor signaling. J. Exp. Med. 192:1047.


