Gasp, a Grb2-associating protein, is critical for positive selection of thymocytes

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T cells develop in the thymus through positive and negative selection, which are responsible for shaping the T cell receptor (TCR) repertoire. To elucidate the molecular mechanisms involved in selection remains an area of intense interest. Here, we identified and characterized a gene product Gasp (Grb2-associating protein, also called Themis) that is critically required for positive selection. Gasp is a cytosolic protein with no known functional motifs that is expressed only in T cells, especially immature CD4/CD8 double positive (DP) thymocytes. In the absence of Gasp, differentiation of both CD4 and CD8 single positive cells in the thymus was severely inhibited, whereas all other TCR-induced events such as β-selection, negative selection, peripheral activation, and homeostatic proliferation were unaffected. We found that Gasp constitutively associates with Grb2 via its N-terminal Src homology 3 domain, suggesting that Gasp acts as a thymocyte-specific adaptor for Grb2 or regulates Ras signaling in DP thymocytes. Collectively, we have described a gene called Gasp that is critical for positive selection.

differentiation | signal transduction | T cell receptor | thymus

Development of conventional T cell receptor (TCR)-$\alpha\beta$ T cells in the thymus requires multiple stages defined by the expression pattern of CD4 and CD8 coreceptor molecules. The most immature $CD4^-CD8^-$ [double negative (DN)] thymocytes differentiate to the $CD4^+CD8^+$ [double positive (DP)] stage through the first selection process called β-selection (pre-TCR selection). These DP thymocytes are subjected to both positive and negative selection to become either class II MHC-restricted helper $CD4^+CD8^-$ [CD4-single positive (CD4-SP)] or class I MHC-restricted cytotoxic $CD4^-CD8^+$ (CD8-SP) cells (1). After receiving positive selection signals, DP thymocytes go through an intermediate $CD4^+CD8^{lo}$ stage, irrespective of their lineage decision (2). The fate of individual DP thymocytes is determined by the strength of affinity and longevity of interaction between their TCR and peptide:MHC ligand (3). Although it is known that strong TCR/ligand interaction leads to negative selection and weak association results in positive selection (4), how this quantitative difference of TCR interaction can be converted to the qualitative difference is not known. Therefore, it is important to investigate the difference in molecular mechanisms of positive and negative selection.

One of the widely accepted models for explaining the difference between positive and negative selection is differential MAPK activation (5). Initially, differential requirements for ERK in positive selection and JNK/p38 in negative selection were focused on (6). The guanine nucleotide exchange factor (GEF) Sos has dual activity for Ras and Rac, therefore it can activate both the ERK and JNK/p38 pathways. Recently, RasGRP, which is another GEF for Ras, was shown to be critical for positive but not negative selection (7). Furthermore, mice heterozygous for Grb2, which constitutively associates with Sos, showed inefficient JNK/p38 activation, but normal ERK activation (8). From these results, positive selection signals were thought to induce the RasGRP/Ras/ERK pathway, and negative selection signals were thought to induce the Grb2-Sos/Rac/JNK p38 pathway. The model that activation through RasGRP results in weak sustained ERK activation to induce positive selection, whereas activation through Sos induces strong temporary ERK activation leading to negative selection is still widely accepted (9). Recently, Daniels et al. (10) elegantly showed that positive selection signals induced subcellular compartmentalization of RasGRP/Ras/ERK to the Golgi membrane, whereas negative selection signals induced localization of Grb2-Sos/Ras/ERK to the plasma membrane. Furthermore, positive selector-induced ERK activation lasted longer in Golgi than in the plasma membrane. Therefore, subcellular compartmentalization of Ras-GEF and Ras upon TCR stimulation is now widely accepted to be the branch point of positive and negative selection (11).

To find novel genes involved in the positive selection of thymocytes, we tried to isolate unknown genes whose expression is highly restricted to the thymus, the site where selection takes place. We used EST databases and performed in silico cloning, the strategy successfully used for isolating various novel tissue or cell type-specific genes. We selected several "thymus-specific genes" by our own computer algorithm based on their thymus-restricted expression. Among these thymus-specific genes, we focused on one gene E430004N04Rik (GeneID 210757), mainly because of its exclusive expression in immature DP thymocytes. Because we found that this protein constitutively associates with Grb2, we called the gene Gasp (Grb2-associating protein). Gasp contains no known protein motifs or homology domain and has no known function, although it has well conserved orthologs in multiple vertebrates from fish to human. To elucidate the function of Gasp, we established *Gasp*-deficient mice and found that the gene is critical for positive selection but not for other TCR-mediated signaling events.

Results

Expression of Gasp Protein. To identify novel genes specifically expressed in the thymus, we used information from the National Center for Biotechnology Information Unigene database of expressed sequence tags (ESTs). Each Unigene cluster contains information about the number of EST clones from a tissue source. We searched Unigene clusters based on the proportion of clones derived from thymus and total number of clones from the thymus. Finally, we selected five genes as thymus-specific genes. The gene Gasp (E430004N04Rik, Themis, Tsepa) was one of these thymus-specific genes. We first examined the expression of Gasp in various

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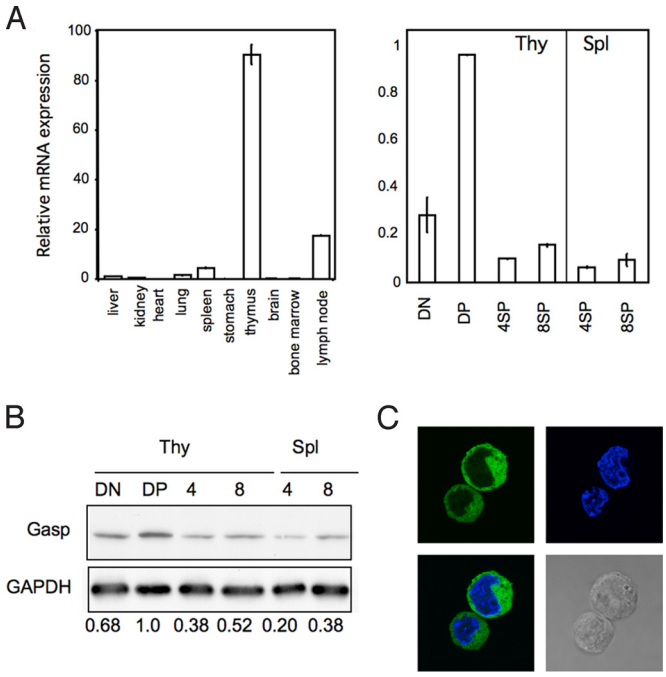

Fig. 1. Thymus-specific expression of Gasp. (*A*) Expression of Gasp mRNA was analyzed by real-time PCR from C57BL/6 mouse tissues and cells. RNA expression was normalized relative to β-actin. Error bars are the SD (n = 2 and 3). (*B*) Expression of Gasp protein in sorted thymic or splenic T cell subpopulations. Results are representative of two independent experiments. (*C*) Representative micrograph showing subcellular localization of Gasp. GFP-fused Gasp was exogenously introduced in DPK cell line (green) and costained with DAPI (blue). (Magnification: ×1,000.)

tissues and cells. As expected, mRNA expression of Gasp is highly restricted to lymphoid organs, especially in the thymus and T cells (Fig. 1*A*). Among T cell subsets, expression is highest in DP thymocytes, and its expression decreases along with maturation. The same pattern of protein expression was confirmed by Western blot analysis using Gasp-specific antibody (Fig. 1*B*). Expression of the gene in CD8-SP cells is slightly higher than in CD4-SP cells in both the thymus and spleen. Expression of Gasp in Treg cells was lower than in conventional CD4 T cells, consistent with a previous report describing Gasp as one of the most reduced genes in Tregs by comprehensive microarray analysis (12). Transcription of Gasp was not affected upon stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Analysis by confocal microscopy of exogenously introduced GFP-fusion Gasp protein (both N- and C-terminal fusion protein) in a DP thymoma line (DPK) (13) showed homogeneous distribution in cytosol and exclusion from the nucleus (Fig. 1*C*). We did not observe a change in the distribution of GFP-fusion Gasp protein upon TCR stimulation.

Gasp Is Required for Positive Selection but Not for Negative Selection and β-Selection. To elucidate the precise function of Gasp, we generated *Gasp*-deficient mice by replacing the first exon of *Gasp* with a LacZ and Neo-expressing cassette (Fig. 2*A*). Protein expression of Gasp in $Gasp^{+/+}$, $Gasp^{+/-}$, and $Gasp^{-/-}$ thymocytes is shown in Fig. 2*B*, confirming the absence of Gasp protein in the deficient mice and reduced protein expression in $Gasp^{+/-}$ thymocytes. In thymi of $Gasp^{-/-}$ mice, total cell number was not significantly altered, and proportions of DN1 through DN4 in DN cells were normal, indicating that β-selection was unaffected in the mice (Table S1). However, we observed a marked decrease of CD4-SP and CD8-SP cells in the thymus (Fig. 2*C*). The effect of Gasp on positive selection is dose-dependent, because the phenotype of $Gasp^{+/-}$ mice was intermediate to that of $Gasp^{+/+}$ and $Gasp^{-/-}$ mice. Reduction of CD4-SP cells in the thymus can be observed even in the neonate, suggesting that the defect is a relatively early event in positive selection (Fig. 2*D*). To confirm the defect in positive selection, we next examined the developmental fate of thymocytes expressing three different fixed TCRs. DP thymocytes expressing class II-MHC restricted OT-II TCR transgenic (Tg) mice on a *RAG* null background did not differentiate into CD4-SP cells at all in the absence of Gasp (Fig. 2*E Top*). The class I-specific female HY-TCR Tg (14) $RAG^{-/-}$ thymocytes and OT-I TCR Tg thymocytes did not differentiate into CD8-SP cells either (Fig. 2*E Middle* and *Bottom*). Therefore, Gasp is critically required for positive selection of both thymocytes expressing class I- and class II-restricted TCR. We extensively analyzed various surface markers (e.g., CD2, CD5, HSA, CD25, class I MHC, etc.) of each stage of thymocyte development, but we did not observe significant differences between $Gasp^{+/+}$ and $Gasp^{-/-}$ thymocytes. The only difference we found was in the expression of CCR9 and CD62L (Fig. S1). Down-regulation of CCR9 on CD4-SP cells was not observed in $Gasp^{-/-}$ thymocytes, and the expression of CD62L on $Gasp^{-/-}$ CD4-SP cells was significantly decreased (Fig. S1). Differentiation of γδ-T cells in $Gasp^{-/-}$ mice was not altered (Table S1). By hematoxylin and eosin staining of thymus sections, no significant difference was observed in cortical and medullary architecture.

Because the above data show that Gasp is crucial in positive selection, we next investigated the effect of Gasp on negative selection, the other important TCR signal-initiated event. Contrary to the defect in positive selection, Gasp deficiency does not affect negative selection as the generation of DP was not recovered in the absence of Gasp in male HY-TCR Tg background (Fig. 2*F*).

***Gasp*$^{-/-}$ T Cells Expand in Periphery.** In contrast to severe impairment of positive selection in the thymus of $Gasp^{-/-}$ mice, the reduction of mature T cells in the periphery was much milder. In particular, reduction of CD8-SP cells was less significant and thus the reduction of CD4-SP cells is always severer than that in CD8-SP cells in $Gasp^{-/-}$ mice. It is noteworthy that the proportion of CD8-SP cells was even higher in $Gasp^{-/-}$ mesenteric lymph node (mLN) and inguinal lymph node (iLN) (Fig. 3*A*) compared with wild type. Because the total numbers of lymph node and spleen cells of $Gasp^{-/-}$ mice were fewer than wild type, absolute numbers of both CD4-SP and CD8-SP cells in spleen and lymph nodes were always less than in wild-type controls (Fig. 3*B*).

The less severe phenotype in number of peripheral T cells could be explained by homeostatic expansion of differentiated T cells. Therefore, we evaluated memory/activated phenotype of peripheral cells. As shown in Fig. 3*C*, CD4-SP cells in $Gasp^{-/-}$ mice contained many more memory/activated ($CD44^{hi}$ $CD62L^{-}$) phenotype cells than wild-type controls. CD8-SP cells also express CD44 at high levels, but somehow expression of CD62L was not reduced (Fig. 3*C*). Consistent with these activated phenotypes, both CD4-SP and CD8-SP cells in $Gasp^{-/-}$ mice showed significantly increased BrdU uptake in vivo, indicating that peripheral CD4-SP and CD8-SP cells in $Gasp^{-/-}$ mice proliferate without stimulation (Fig. 3*D*). Although such proliferation could be caused by autoreactive T cells, we did not see any signs of autoimmune disease in the deficient mice. We observed very few CD8-SP cells in the periphery of female HY-TCR Tg $RAG^{-/-}$, $Gasp^{-/-}$mice. Because it was reported that homeostatic proliferation does not occur in the periphery of female HY TCR-Tg mice (15), the results also support the model that the increased number of peripheral T cells in $Gasp^{-/-}$ mice was caused by homeostatic proliferation. From these results, we conclude that positive selection of CD4-SP and CD8-SP cells are both blocked in $Gasp^{-/-}$ mice, but the number of T cells in periphery is increased by homeostatic expansion.

Normal Activation of Mature *Gasp*$^{-/-}$ T Cells. We next investigated functions of peripheral T cells. Purified splenic CD4-SP cells ($CD4^{+}$, $CD8^{-}$, and $Mac1^{-}$ cells) were stimulated with immobilized

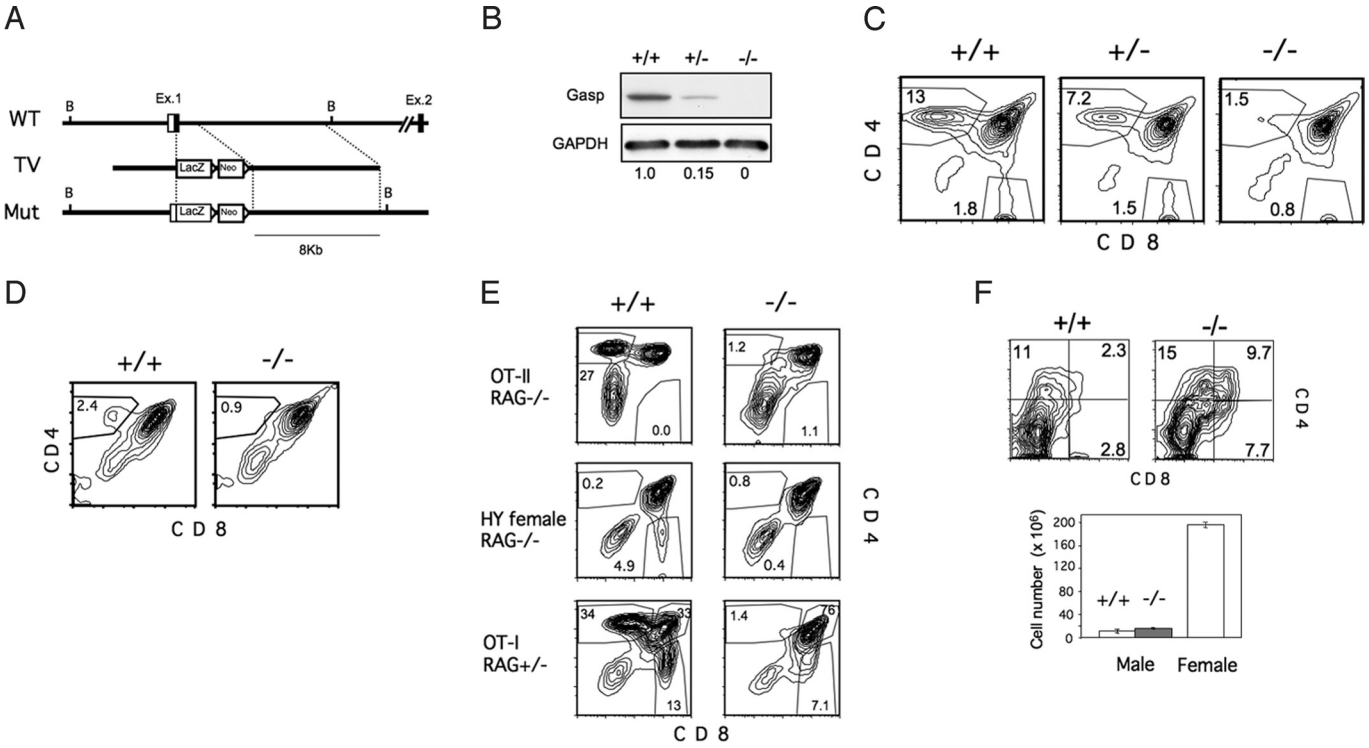

Fig. 2. Generation and analysis of *Gasp*$^{-/-}$mice. (*A*) A gene-targeting strategy was used to insert a lacZ/neo cassette into exon 1 of the Gasp gene. (*B*) Western blot analysis of DP thymocytes with anti-Gasp-specific antisera. Results are representative of two independent experiments. (*C*) CD4 and CD8 profile of *Gasp*$^{+/+}$, *Gasp*$^{+/-}$, and *Gasp*$^{-/-}$ thymocytes. (*D*) CD4 and CD8 expression of neonatal thymocytes. (*E*) CD4 and CD8 profile of thymocytes from OT-I, OT-II Tg *RAG*$^{-/-}$, and female HY Tg *RAG*$^{-/-}$mice. (*F*) CD4 and CD8 profile and number of thymocytes from male HY Tg *RAG*$^{-/-}$mice. In *C–F*, data are representative of more than three independent experiments.

anti-CD3 and CD28 antibodies in vitro, then measured for growth and IL-2 production. As shown in Fig. 4 *A* and *B*, TCR-dependent cell growth evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and production of IL-2 in supernatant was comparable between *Gasp*$^{-/-}$and *Gasp*$^{+/+}$ mice. We established alloantigen (H-2^{d})-specific CD4-SP and CD8-SP T cell lines from wild-type and *Gasp*$^{-/-}$splenocytes. Upon TCR stimulation, *Gasp*$^{-/-}$CD4-SP helper T cell lines produced amounts of IL-4 comparable to wild-type lines (Fig. 4*C Left*), CD8-SP CTL T cell lines produced the same amount of IFN-γ upon stimulation as wild types (Fig. 4*C Right*). We also measured CTL activity of these CD8-SP clones and found that specific killing of CD8-SP CTL clones against allogenic H-2^{d} MHC was not changed in the absence of Gasp (Fig. 4*D*). From these results, we conclude that activation of mature T cells does not require Gasp.

Phenotypes of *Gasp*$^{-/-}$ Thymocytes. We noticed that the phenotype of *Gasp*$^{-/-}$ mice was quite similar to *thid* (LEC) mutant rat (16). Those reports showed reduced numbers of CD4-SP cells, but not CD8-SP cells in lymph nodes (16), and lower expression of CD62L in CD4-SP cells (17), which are exactly the same phenotypes as *Gasp*$^{-/-}$mice. The responsible gene for *thid* mutation was recently reported as *PTPRK* (protein tyrosine phosphatase receptor K) (18, 19), and the gene is only 100 Kb apart from the *Gasp* gene locus. To exclude the possibility that the disturbance of expression of the adjacent *PTPRK* gene was responsible for the phenotype of *Gasp*$^{-/-}$ mice, we analyzed expression of PTPRK mRNA in *Gasp*$^{-/-}$ thymocytes by real-time RT-PCR analysis. The expression of PTPRK is not reduced but rather increased in *Gasp*$^{-/-}$thymocytes (Fig. 5*A*). Therefore, the phenotype of *Gasp*$^{-/-}$ mice is likely independent of PTPRK.

We next performed bone marrow chimera experiments to investigate whether the developmental defect in the thymus was caused by an intrinsic thymocyte defect or a defect in the thymic microenvironment. As shown in Fig. 5*B*, thymocytes from wild-type bone marrow developed normally in irradiated *Gasp*$^{-/-}$ mice, whereas wild-type mice reconstituted with *Gasp*$^{-/-}$ bone marrow showed almost identical phenotypes as *Gasp*$^{-/-}$ mice. Therefore, the defect is thymocyte intrinsic, which is consistent with the specific expression of Gasp in thymocytes (Fig. 1*A*).

We next analyzed the expression of TCR on thymocytes. Although TCR-β expression on CD4-SP cells in the thymus is consistently lower than wild type, expression of TCR on preselected DP and peripheral mature T cells (Fig. S2 *A* and *B*) were comparable to the wild type. Expression of TCR on DP thymocytes of TCR-Tg mice was also not significantly changed (Fig. S2*C*). Furthermore, spontaneous and TCR-induced cell death evaluated by AnnexinV staining was not significantly different in the absence of Gasp (Fig. S3).

Signal Transduction in *Gasp*$^{-/-}$ DP Cells. We next focused on the DP stage when positive selection takes place. We observed a significant reduction in the earliest postselected DP (CD69^{hi} TCR^{hi} DP) in *Gasp*$^{-/-}$mice (Fig. 5*C*), suggesting that the defect lies in a relatively early phase of the selection process. We next investigated TCR-stimulated signal transduction of DP thymocytes. Stimulation with plate-coated anti-CD3 plus CD28 antibody successfully induced CD69 up-regulation on *Gasp*$^{-/-}$DP thymocytes (Fig. 5*D*), indicating that the signaling pathway leading to CD69 transcription was not affected. In accordance with that, anti-CD3 mAb induced Ca^{2+} influx in DP thymocytes was not significantly disturbed (Fig. 5*E*). Furthermore, anti-CD3 mAb induced phosphorylation of ERK, phospholipase C γ (PLC-γ), and SLP76, all were unaltered in *Gasp*-deficient DP thymocytes (Fig. 5*F*).

Gasp Constitutively Associates with Grb2. To figure out the function of Gasp further, we attempted to identify proteins associated with

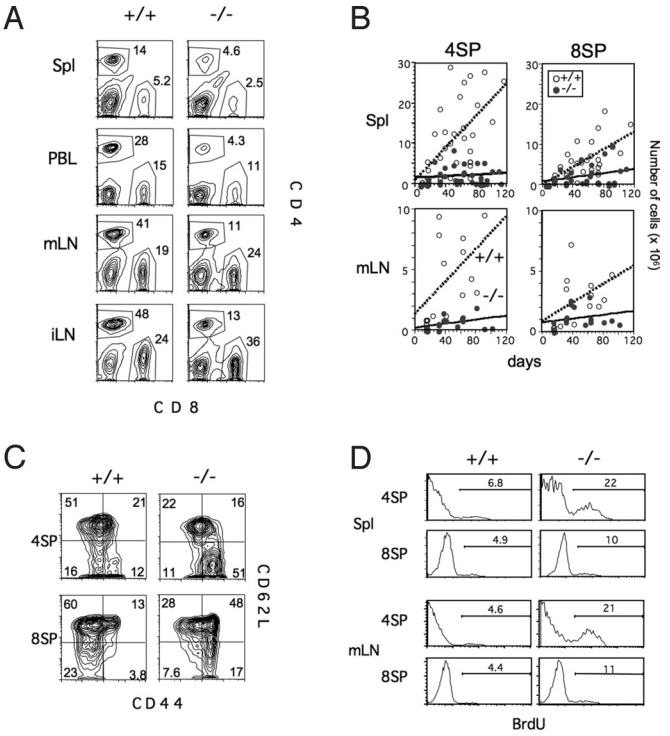

Fig. 3. Phenotype of peripheral T cells in *Gasp*$^{-/-}$mice. (*A*) CD4 and CD8 profile of the cells from spleen, peripheral blood (PBL), mLN, and iLN of *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ mice. Results are representative of more than five independent experiments. (*B*) Absolute number of CD4-SP and CD8-SP cells of Spl and mLN of *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ mice. Each dot represents individual mice at day of age. Solid (−/−) and dashed (+/+) lines show the linear regression correlation between age and absolute number of cells. (*C*) CD44 and CD62L profile of splenic CD4-SP and CD8-SP cells from *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ mice. (*D*) BrdU uptake of CD4-SP and CD8-SP cells from spleen and mLN of *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ mice. After mice were fed with BrdU for 5 days with drinking water, the cells were stained with anti-BrdU antibody. In *C* and *D*, results are representative of more than three independent experiments.

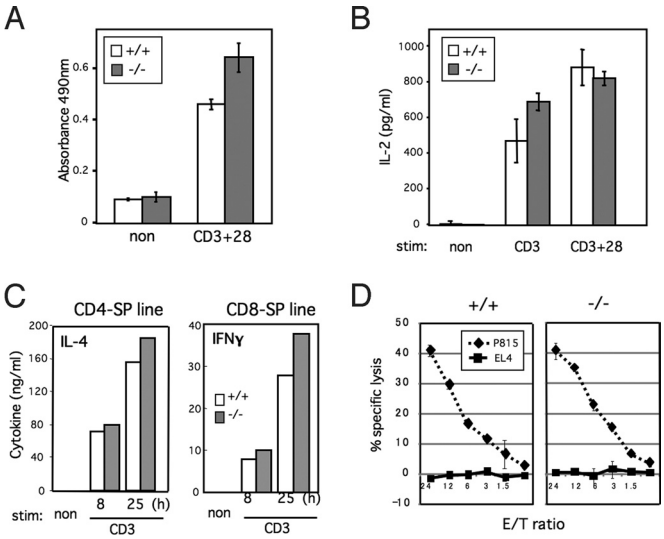

Fig. 4. Activation of peripheral CD4 and CD8 mature T cells. (*A*) MTT assay was applied for TCR-stimulated sorted splenic CD4-SP cells of *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ mice after 3 days of culture. (*B*) Primary CD4-SP T cells from *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ mice were stimulated with plate-bound anti-CD3 or CD3^{+} 28 mAb for 24 h, and IL-2 concentration in supernatants was measured by ELISA. (*C*) TCR-dependent production of IL4 and IFN-γ from cell line established from *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ splenocyte. (*D*) H-2^{d}-specific allo-CTL lines were established from splenic CD8-SP cells, and CTL assay was analyzed for CTL function by using specific (P815) and nonspecific (EL4). Results are representative of more than two independent experiments.

Gasp by using liquid chromatography-based electrospray tandem mass spectrometry (20). Human embryonic kidney cells were transfected with Flag-tagged human Gasp, and lysates from these cells were immunoprecipitated with anti-Flag antibody. Immunoprecipitated proteins were subjected to proteolysis followed by liquid chromatography-based electrospray tandem mass spectrometry. The analysis gave us several candidate Gasp-associating proteins. Among them, the most frequently detected amino acid sequences were derived from Grb2. Grb2 is an adaptor protein constitutively associated with Sos, having two Src homology 3 (SH3) domain (N and C terminus) and one SH2 domain in the middle (21). Therefore, we next performed coimmunoprecipitation experiments using Flag-tagged Gasp and myc-tagged Grb2 and their mutants. As shown in Fig. 6, myc-Grb2 could successfully pull down Flag-Gasp, and Flag-Gasp also coprecipitated myc-Grb2. Although a Grb2 mutant lacking the entire SH2 region (Grb2-ΔSH2) could associate with Gasp, an SH3 mutant (Grb2–42L/203R) in which both the N- and C-terminal SH3 domains were mutated (22) could not (Fig. 6). Because Grb2–203R in which only the C-terminal SH3 domain was mutated could associate with Gasp (Fig. 6*B*), Gasp likely associates with Grb2 via its N-terminal SH3 region, which is the same binding site for Sos (23). We noticed that Gasp contains a proline-rich sequence (^{555}PPPRPPKHP) in its C terminus. Although it is different from the consensus Grb2 SH3 domain binding sequence (PVPPPVPPR), we made a deletion mutant of this proline-rich sequence (HA-Gasp-ΔPro) and tested its interaction with Grb2. As shown in Fig. S4, we found that the association was independent of this proline-rich region. We also found Gasp was not tyrosine-phosphorylated upon TCR stimulation or PMA+ionomycin stimulation (Fig. S5), consistent with the observation that treatment with pervanadate did not augment the association of Gasp and Grb2.

Discussion

Although it is well known that both positive selection and negative selection are evoked by stimulation of TCRs of different affinities, the molecular basis of these selection processes is poorly understood. The molecules exclusively required for either selection process will give us a hint to figure out the molecular mechanism of these two selections. At present, ERK (24), Calcineurin (25), TCR-αcpm (26), and RasGRP (7) are described to be required only for positive selection but not for negative selection, whereas bim (26), MINK (27), and nur77 (28) are required only for negative selection. We have now added another gene to the list of players required for positive selection.

Gasp, which is preferentially expressed in immature DP thymocytes, has quite unique characteristics. Most of the proteins reported to be required for positive selection are involved in TCR-induced signal transduction. As a result, other TCR-related signaling events such as peripheral activation and homeostatic expansion are also affected. Unlike other positive selection-deficient mutant mice, *Gasp*$^{-/-}$ mice showed a defect only in positive selection among all of the TCR-induced signaling events. However, it should be noted that the male HY-TCR Tg system may not be appropriate for assessment of physiological negative selection because developmental arrest occurs before the DP stage (29).

In the periphery of *Gasp*$^{-/-}$ mice, the number of CD8-SP cells in lymph nodes was more than CD4-SP cells , suggesting some effect of Gasp on CD4/CD8 lineage choice. However, analysis of three independent TCR Tg mice clearly showed there is no lineage conversion nor incomplete block in positive selection of class I-restricted TCR.

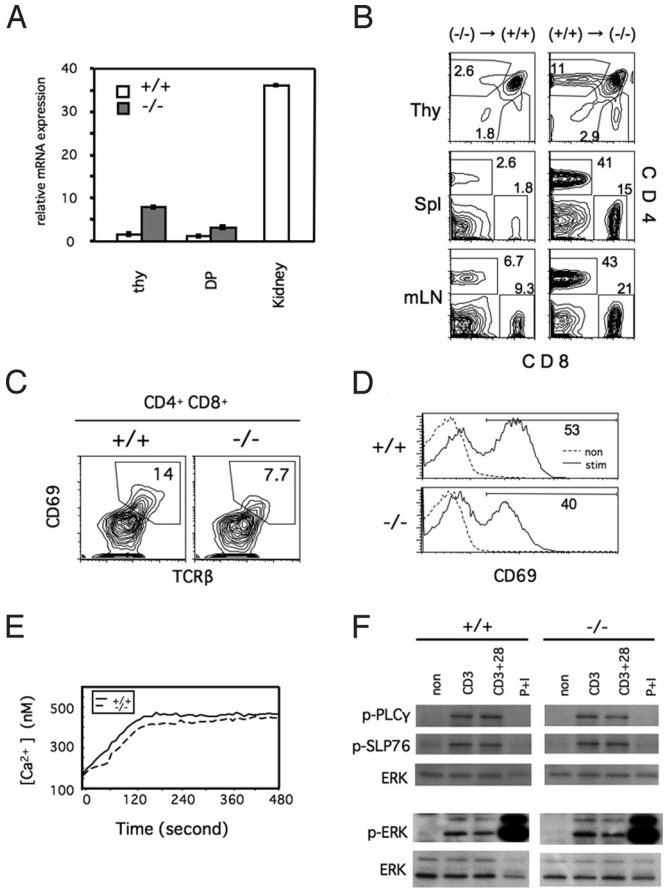

Fig. 5. Characteristics of Gasp deficiency. (*A*) Defects in *Gasp*$^{-/-}$ mice are independent of PTPRK. Expression of PTPRK mRNA in *Gasp*$^{-/-}$ thymocytes was determined by real-time RT-PCR. Error bars are the SD (n = 2 and 3). (*B*) Developmental defects in *Gasp*$^{-/-}$ mice are thymocyte intrinsic. Bone marrow cells from CD45.1 *Gasp*$^{+/+}$ mice were injected into lethally irradiated CD45.2 *Gasp*$^{-/-}$ mice or vice versa. After 2 months, cells from indicated organs were stained with CD4, 8, 45.1, and 45.2. Results are representative of more than two independent mice. (*C*) Proportion of post-selected CD69^{+} TCR^{hi} DP cells in *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ mice. Results are representative of four independent experiments. (*D*) Sorted DP thymocytes from *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ mice were activated with plate-bound CD3 + 28 Ab overnight, then stained with CD69. Results are representative of more than three independent experiments. (*E*) DP cells from *Gasp*$^{-/-}$ mice were stimulated with anti-CD3 mAb followed by anti-hamster IgG, then Ca^{2+} concentration was measured by using Fura2-AM. Results are representative of three independent experiments. (*F*) DP thymocytes were activated by the indicated stimuli (2 min for anti-CD3 and 3 + 28, 5 min for PMA+Iono), then Western blotted with phosphorylated-ERK-, SLP76-, and PLCγ-specific antibody. Results are representative of more than three independent experiments.

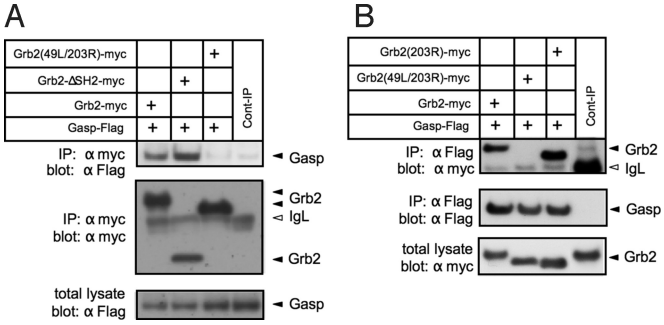

Fig. 6. Gasp associates with Grb2. (*A*) HEK293T cells were transfected with myc-tagged Grb2, SH2-deleted Grb2 (Grb2-SH2-myc), N-/C-terminal SH3 mutant (Grb2–49L/203R-myc), and Flag-tagged Gasp. Lysates were immunoprecipitated with anti-Flag mAb then blotted with the indicated Abs. (*B*) myc-Grb2, Grb2–49L/203R, C-terminal SH3 mutant (Grb2–203R-myc) ,and Flag-Gasp were transfected and immunoprecipitated with anti-myc mAb then blotted with the indicated Abs. Results are representative of more than seven independent experiments.

Reduction of CD69^{+}TCR^{hi}DP in *Gasp*$^{-/-}$mice and the disappearance of CD4^{+}CD8^{lo} post-selected cells (2) in OT-I TCR Tg thymocytes also indicate that the defect in positive selection is relatively early and affects both CD4 and CD8 lineages. We found that a large part of peripheral *Gasp*$^{-/-}$ T cells are actively proliferating without antigenic stimulation, and that is why the peripheral phenotype of *Gasp*$^{-/-}$ mice is much milder than that in the thymus. Because we do not observe any signs of autoimmune symptom in even >1-year-old *Gasp*$^{-/-}$mice, these proliferating T cells are not autoreactive, but expand by homeostatic proliferation. We always observed severer reductions in CD4-SP cells than CD8-SP cells in the periphery of *Gasp*$^{-/-}$mice. Furthermore, higher expression of CD62L in Gasp$^{-/-}$ CD8-SP cells could explain preferential migration of CD8-SP cells into lymph nodes.

Our finding that Gasp constitutively associated with Grb2 is quite intriguing and provides some possible links to hypothesize its function in positive selection, because Grb2 is an important adaptor for Sos in the TCR-mediated signal transduction pathway. According to the currently well-accepted model (5, 9–11, 30), Grb2/Sos activation is involved only in negative selection. Because Gasp could compete with Sos for binding to Grb2, existence of Gasp would likely inhibit negative selection, which does not explain the current phenotype well. However, there is no direct evidence that Sos is required for thymic selection, because Sos1 deletion is embryonic lethal (31), and Sos2-deficient mice showed no phenotype (32). Although an earlier study using Grb2$^{+/-}$ mice suggested Grb2 is involved in negative selection but not in positive selection (8), recent results from lck-Cre driven Grb2 conditional deficient mice suggests that Grb2 is required for positive selection. According to these facts, Gasp is more likely to function as an adaptor for Grb2, bringing some unknown molecule required for positive selection to the LAT signalosome complex. If Gasp functions in TCR-mediated signal transduction, it is surprising that *Gasp*$^{-/-}$ DP thymocytes did not show any defect in signal transduction induced by various dose ranges (5–0.1 μg/mL) of anti-TCR antibody. However, it is still possible that *Gasp*$^{-/-}$ thymocytes have signaling defects when stimulated by weaker signals or physiological MHC/peptide ligands.

In conclusion, we found a Grb2-associating protein that is specifically expressed in the thymus and is critical in positive selection but not in other TCR-related signal transduction events. Detailed function of the protein in positive selection should be studied further.

We would like to note that, during the review process of this article, several other groups' reports describing the same gene under the name "*Themis*" were published (33–35, 40).

Materials and Methods

Mice. *Gasp*$^{-/-}$ mice (CDB0574K: www.cdb.riken.jp/arg/mutant%20mice%20list.html) were generated as described (36, 37). The first exon of *Gasp* was targeted by homologous recombination using vector backbone DT-A/LacZ/neo with 5′ and 3′ flanking arms of 4 and 8 kbp, respectively. Neomycin-selected ES cell lines were screened by PCR, and two independent mouse lines were established. Southern blot analysis confirmed target gene deletion. The two independent mouse lines showed identical phenotypes. OT-I, OT-II, and HY mice have been described (14). All mice were housed under specific pathogen-free conditions and used in accordance with International Medical Center of Japan institutional guidelines.

Real-Time RT-PCR. Total RNA was isolated from tissues or cells by using the RNeasy kit (Qiagen). cDNA generated by SuperScript III (Invitrogen) was analyzed by using primers for the indicated gene and the Platinum SYBR Green qPCR-UDG Supermix with ROX (Invitrogen). Results were normalized to β-actin expression levels. Primer sequences for Gasp, PTPRK, and β-actin are available on request.

Immunoprecipitations and Western Blot Analysis. Transfection and immunoprecipitation were performed as described (38) with the exception of using 0.05% Nonidet P-40 lysis buffer. Antibodies for Western blot analysis were against: pPLCγ-1, ERK, and pERK (Cell Signaling) and pSLP76 (BD Biosciences). Anti-Gasp-specific rabbit antiserum was generated by injection of recombinant full-length Gasp protein. Antibodies used for immunoprecipitations were: anti-myc (9E10), anti-HA (Roche), and anti-FLAG (M2, Sigma). Horseradish peroxidase-conjugated anti-IgG secondary antibodies against rabbit, rat, and mouse (GE Healthcare) were used with Lumiglo (Cell Signaling) substrate.

Plasmids and Recombinant DNAs. Full-length murine Gasp cDNA was PCR-cloned using IMAGE clone 40130002 (OpenBiosystems) as template into pcDNA3 vector to generate Gasp-HA. To generate Gasp-ΔPro-HA, the proline-rich sequence ^{555}PPPRPPKHP of Gasp was deleted by site-directed PCR mutagenesis. A BamHI and XbaI fragment from pSVEGrb49L or pSVEGrb49L/203R (kind gifts from Robert Weinberg, Whitehead Institute, Cambridge, MA) was cloned into to pcDNA3.1 to generate Grb2(203R)-myc and Grb2(49L/203R)-myc. Grb2-myc and Grb2-ΔSH2-myc were kind gifts from Kazuo Sugamura, Tohoku University, Sendai, Japan.

Establishment of CD8$^+$ and CD4$^+$ T Cell Lines. The CD8$^+$ or CD4$^+$ T cell lines were established in vitro by stimulating splenocytes from *Gasp*$^{+/+}$ and *Gasp*$^{-/-}$ mice after depleting CD4$^+$ or CD8$^+$ cells, with BALB/c (allogeneic) splenocytes or syngeneic splenocytes in the presence of 2C11 (1 μg/mL). T cell lines were maintained by biweekly stimulations in complete DMEM supplemented with 10% prescreened FCS and 5% conditioned medium that was prepared from culture supernatant of rat splenocytes stimulated with Con A for 48 h.

Cell-Mediated Lymphocytotoxicity Assay. Graded numbers of anti-H-2^d CD8$^+$ T cells were incubated with 5,000 ^{51}Cr-labeled P815 (H-2^d mastcytoma) or EL4 (H-2^b T lymphoma) for 4 h. The supernatants were harvested with the Skatron harvesting system, and radioactivities in the supernatants were measured with a gamma counter. Assays were performed in triplicate.

Cell Stimulation Assays. CD4CD8 DP thymocytes were sorted (FACSAria II; Becton Dickinson) and stimulated with soluble or plate-bound anti-CD3 (clone 2C11) and anti-CD28 antibody each at 5 μg/mL for the indicated times. After 24 h of stimulation IL-2 production was measured in supernatant by mouse IL-2 ELISA Ready-SET-Go! (Ebioscience). Analysis of pERK used CD3 or CD3+CD28 at 5 μg/mL and goat anti-hamster (GAH) Ab at 40 μg/mL.

Ca^{2+} Mobilization Measurements. Sorted CD4CD8 DP cells were labeled with 3 μM Fura2-AM (Molecular Probes/Invitrogen) for 1 h at 37 °C. Cells were washed with ice-cold PBS and resuspended in Ringer's solution. Five million cells were surface-labeled with CD3 or CD3 + 28 at 5 μg/mL on ice for 20 min. Labeled cells were washed and warmed up for 5 min in a cuvette, then cross-linked with 40 μg/mL GAH and analyzed with a fluorescence spectrophotometer (Hitachi F-2500) for fluorescence intensity every 10 s for up to 10 min.

BrdU Administration and FACS Analysis. Mice were fed 0.8 mg/mL BrdU (Nacalai Tesque) for 5 days in drinking water. Antibodies used for staining were phycoerythrin (PE)-antiCD4 (GK1.5; Ebioscience), allophycocyanin (APC)-antiCD8 (53–6.7; Biolegend). and FITC-antiBrdu (3D4; BD Pharmingen). Cells were isolated from spleens and mLN, surface-stained for CD4-PE and CD8-APC, and stained for BrdU incorporation as described (39).

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