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Disruption of T cell signaling networks and development by Grb2 haploid insufficiency

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The developmental processes of positive and negative selection in the thymus shape the T cell antigen receptor (TCR) repertoire and require the integration of multiple signaling networks. These networks involve the efficient assembly of macromolecular complexes and are mediated by multimodular adaptor proteins that permit the functional integration of distinct signaling molecules. We show here that decreased expression of the adaptor protein Grb2 in *Grb2*$^{+/-}$ mice weakens TCR-induced c-Jun N-terminal kinase (JNK) and p38, but not extracellular signal–regulated kinase (ERK), activation. In turn, this selective effect decreases the ability of thymocytes to undergo negative, but not positive, selection. We also show that there are differences in the signaling thresholds of the three mitogen-activated protein kinase (MAPK) families. These differences may provide a mechanism by which quantitative differences in signal strength can alter the balance of downstream signaling pathways to induce the qualitatively distinct biological outcomes of proliferation, differentiation or apoptosis.

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Adaptor or linker proteins play critical roles in linking the T cell receptor (TCR)-activated protein tyrosine kinases (PTKs) with enzymes that are required for the efficient generation of secondary messengers. Phosphorylation of two hematopoietic adaptor proteins, transmembrane linker for activation of T cells (LAT) and cytosolic SLP-76, by TCR-associated PTKs facilitates the relocalization of enzymes and the assembly of signaling complexes that are required for T cell function[1]. As LAT is preferentially localized to glycolipid-enriched microdomains within the plasma membrane, tyrosine phosphorylation of LAT provides a scaffold by which multiple signaling molecules[2,3], such as phosphatidyinositol 3-kinse (PI3K) and phospholipase Cγ1 (PLC-γ1), can be recruited to these specialized microdomains[4]. In addition to enzymes, tyrosine-phosphorylated LAT also binds multiple members of the Grb2 family of adaptor proteins, such as Grb2, Grap and Gads (also known as Mona, GrpL, Grf40 and Grap2), to facilitate the assembly of macromolecular signaling complexes that are required for efficient T cell activation[5–7].

Grb2 (which is a prototypic adaptor protein) consists of a central SH2 domain that is flanked by SH3 domains, both of which bind the Sos guanine nucleotide exchange factor (GEF). The interaction of tyrosine-phosphorylated LAT with Grb2 or its related family member, Grap, provides a mechanism by which Grb2 or Grap-associated Sos is recruited to the plasma membrane and potentially activates Ras[8–10]. Studies on Ras guanine nucleotide–releasing protein (Ras-Grp), a Ras activator with calcium-binding EF hands and a diacylglyercol-binding domain, demonstrate that this release factor also translocates to the membrane fraction following TCR engagement. It also enhances extracellular signal–regulated kinase (ERK) activation and interleukin 2 secretion when overexpressed in Jurkat T cells[11,12]. In addition, Ras-Grp–deficient mice show that there is a block in T cell development at the CD4$^+$CD8$^+$ stage and that these CD4$^+$CD8$^+$ TCR^{lo} thymocytes are unable to activate Ras or ERK after TCR cross-linking[13]. Hence, the role of Grb2 in Ras activation in T cells remains unclear.

Activation of Ras further triggers a cascade of the protein kinases Raf, mitogen-activated kinase (MAPK) kinase (MEK) and ERK to induce the transcription of immediate early genes. Expression of dominant-negative forms of Ras inhibits Raf, MEK and ERK activation and, correspondingly, expression of dominant-negative forms of Raf, MEK or ERK inhibits T cell function[14,15]. Studies suggest that Ras can also regulate the activation of Rho GTPases, which, in turn, activate other MAPK families, such as JNK[16–19]. In light of this, Grb2 may be positioned to play a central role in the activation multiple MAPK family members.

In T cells, the fate of a developing thymocyte is critically determined by the interaction between the TCR and its ligands[20–22]. Studies support both the qualitative and quantitative aspects of TCR activation that guide a T cell to either proliferate and differentiate or undergo apoptosis. In the quantitative model, "strong" activation signals result in cell death (negative selection) and "moderate" activation signals result in survival (positive selection). Although the signaling requirement for regulating strong *versus* weak signals from the TCR remains largely unknown, the three families of MAPKs appear to play

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qualitatively distinct roles: ERK regulating positive selection and JNK and p38 regulating negative selection. Inhibition of ERK activation results in defective positive, but not negative, selection[23–27]. Conversely, attenuation of JNK or p38 function results in defective negative, but not positive, selection[28–32]. These studies pose a paradox between the quantitative (strength of signal) and qualitative (MAPK) models of positive and negative selection. In light of the potential central role of Grb2 in the regulation the MAPK-activated signaling networks, we assessed whether Grb2 expression may differentially regulate the different MAPKs in T cell development and function. We found that *Grb2* haploid insufficiency selectively attenuates JNK and p38, but not ERK, activation. In turn, these biochemical alterations are associated with a reduced ability in thymocytes to undergo negative, but not positive, selection.

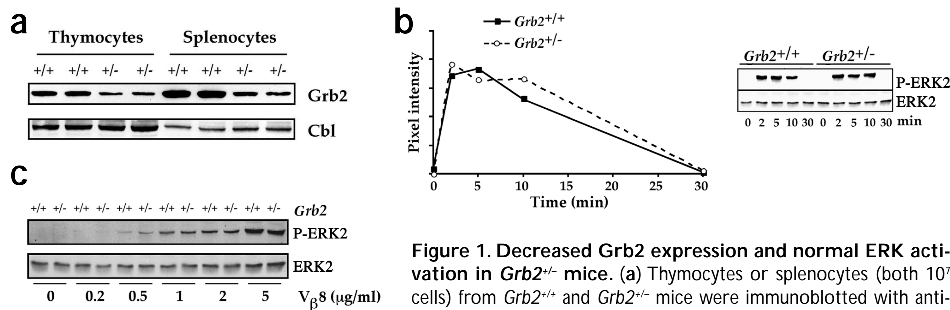

Figure 1. Decreased Grb2 expression and normal ERK activation in *Grb2*$^{+/-}$ mice. (**a**) Thymocytes or splenocytes (both 10^7 cells) from *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ mice were immunoblotted with antisera Grb2 or Cbl. (Results from two mice for each genotype are shown.) (**b**) *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ thymocytes (10^7 cells/lane) were stimulated with an anti-TCR (2C11) for the indicated times. MAPK activation was determined by immunoblotting with antibodies specific for bi-phosphorylated ERK2 (P-ERK2). Immunoblotting for total ERK2 (ERK2) confirmed comparable loading of protein in each lane. ERK2 activation was quantified with UN-SCAN-IT software. (Data are representative of eight independent experiments.) (**c**) Thymocytes from *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ mice were analyzed for ERK2 activation as in **b**, but were treated with increasing doses of anti-TCRβ ($V_\beta 8$). (Data is representative of two independent experiments.)

Results

Decreased Grb2 expression in *Grb2*$^{+/-}$ mice

As Grb2 is required for endoderm differentiation and formation of the epiblast, we were unable to evaluate the function of Grb2 in the immune system of *Grb2*$^{-/-}$ mice[33]. This fundamental defect precluded the use of the recombination activating gene 2–deficient (*Rag-2*$^{-/-}$) blastocyst complementation assay to analyze the function of Grb2 in the immune system. To evaluate the potential role of Grb2 in T cell function, we analyzed mice that were heterozygous for a null mutation in *Grb2*. Grb2 expression in thymocytes and splenocytes isolated from *Grb2*$^{+/-}$ mice was ~40% of that detected in cells derived from *Grb2*$^{+/+}$ mice (**Fig. 1a**). In contrast, expression of a number of other signaling proteins, including the Cbl proto-oncogene and the Grb2-associated Sos GEF, was comparable between *Grb2*$^{+/-}$ and *Grb2*$^{+/+}$ thymocytes (**Fig. 1a** and data not shown).

Normal ERK activation in *Grb2*$^{+/-}$ thymocytes

To analyze the downstream effects of Grb2 haploid insufficiency, we compared the activation of ERK2 upon cross-linking of the TCR in *Grb2*$^{+/+}$ or *Grb2*$^{+/-}$ thymocytes. The degree and kinetics of ERK2 activation were similar in *Grb2*$^{+/+}$ or *Grb2*$^{+/-}$ thymocytes (**Fig. 1b**). In addition, no difference in ERK2 activation was observed with the wide range of antibodies that were used for receptor stimulation (**Fig. 1c**). Finally, up-regulation of CD69, an ERK-dependent function[34], was also comparable in *Grb2*$^{+/+}$ or *Grb2*$^{+/-}$ thymocytes over a wide range of anti-CD3 doses used (data not shown). Hence, ERK activation and its downstream functions were comparable between *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ thymocytes.

Attenuated JNK and p38 activation in *Grb2*$^{+/-}$ thymocytes

We next compared the ability of *Grb2*$^{+/+}$ or *Grb2*$^{+/-}$ thymocytes to activate the JNK1 and JNK2 (JNK1/2) and p38 members of the MAPKs. Although ERK activation was similar between *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ thymocytes, activation of the p46 and p54 isoforms of JNK1/2 was attenuated by 40–70% in *Grb2*$^{+/-}$ thymocytes (**Fig. 2a,b**). This attenuation of JNK1/2 occurred at all time points analyzed and was

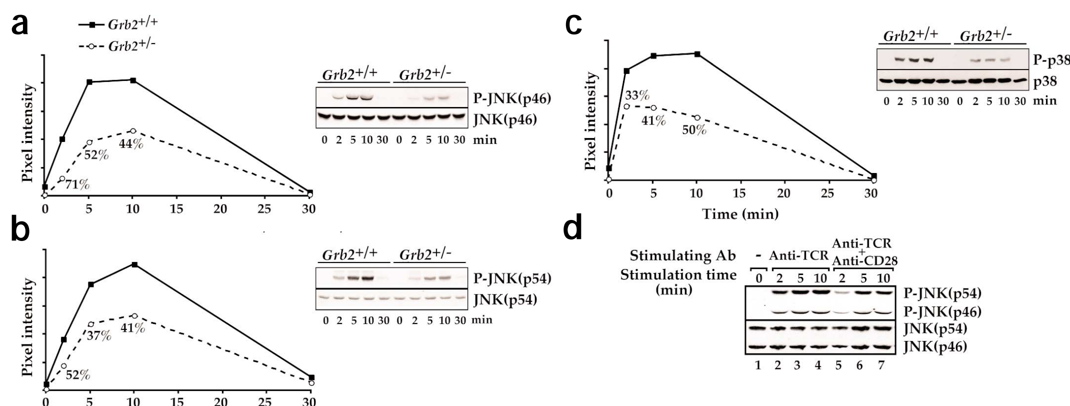

Figure 2. Attenuated JNK and p38 activation in *Grb2*$^{+/-}$ thymocytes. (**a–c**) *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ thymocytes (10^7 cells/lane) were analyzed as described for Fig. 1b but blotted for (**a**) the p46 isoform of JNK1/2 (**b**) the p54 isoform of JNK1/2 or (**c**) p38. The top panels of each gel show immunoblotting with antibodies specific for bi-phosphorylated MAPK (P-JNK and P-p38) and the bottom panels show blotting with a pan-MAPK antibody (JNK and p38). Data were quantified, as in Fig. 1b, and are shown in graph form. (Each panel is representative of eight independent experiments.) (**d**) Thymocytes from DO11.10 Tg$^+$ mice were treated as in **a–c** except cells were stimulated for the indicated time periods and the KJ anti-clonotypic was used instead of anti-CD3 (2C11, lanes 2–4). In lanes 5–7, cells were treated with the combination of anti-KJ and anti-CD28 (10 μg/ml) for the indicated time periods (lane 1 shows the control). The top panel of the gel was immunoblotted with anti-pJNK and the bottom was immunoblotted with anti-pan JNK. The lower amount of P-JNK, observed in lane 5, that was obtained with co-cross-linking of anti-CD3 and anti-CD28 was not reproducible in other experiments.

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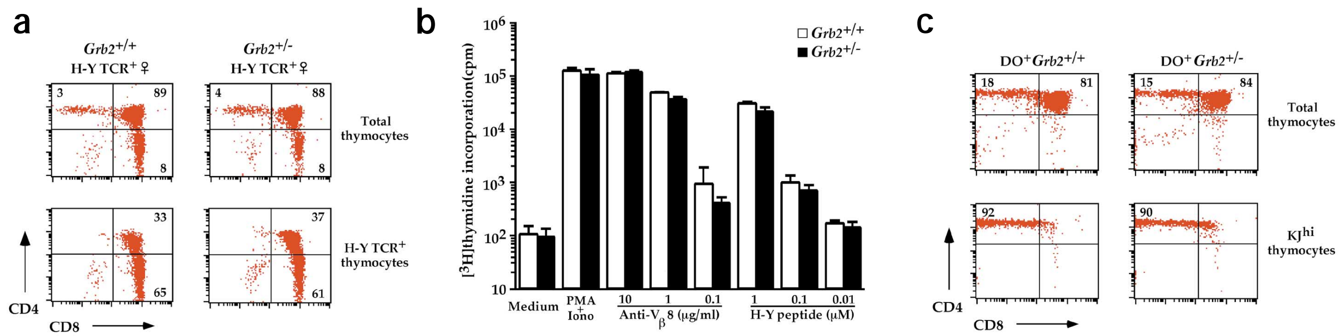

Figure 3. Normal positive selection in *Grb2*$^{+/–}$ mice. (**a**) Normal thymocyte development in H-Y TCR$^+$ *Grb2*$^{+/–}$ female mice. Total and H-Y TCR$^+$ thymocytes from *Grb2*$^{+/+}$ and *Grb2*$^{+/–}$ female mice were stained for CD4 and CD8. Cell recoveries from the thymi of *Grb2*$^{+/+}$ and *Grb2*$^{+/–}$ female mice were 1.92±0.24×10^8 and 1.89±0.16×10^8 cells, respectively. Data was collected using a FACS Calibur and analyzed. All data was collected from live cells within the lymphocyte gate, as defined by forward and side-scatter values. (Data are representative of eight independent pairs of 4 to 6-week-old mice.) (**b**) Normal proliferation of H-Y TCR$^+$ thymocytes. Thymocytes (2×10^5 cells) from *Grb2*$^{+/+}$ and *Grb2*$^{+/–}$ female mice were incubated overnight with medium, PMA + ionomycin (5 ng/ml + 500 ng/ml), anti-V$_\beta$8 or H-Y peptide in the presence of irradiated antigen presenting cells. [^{3}H]thymidine (1 μCi) was added at 72 h and its incorporation measured at 88 h. (**c**) Total and DO11.10 TCR$^+$ (DO) thymocytes were stained in *Grb2*$^{+/+}$ and *Grb2*$^{+/–}$ thymocytes. Surface staining of CD4, CD8 and CD3 was determined by FACS analysis as described in **a** except that gating on DO11.10 TCR^{hi} thymocytes was done using the KJ anti-DO11.10 clonotypic TCR. (Data are representative of ten independent pairs of 4 to 6-week-old mice.)

observed in thymocytes stimulated with anti-clonotypic TCR (in H-Y TCR$^+$ *Grb2*$^{+/–}$ and *Grb2*$^{+/+}$ thymocytes), anti-CD3ε or mitogen (**Fig. 2a,b** and data not shown). Analysis of p38 activation revealed a similar degree of attenuation in *Grb2*$^{+/–}$ thymocytes (**Fig. 2c**). The attenuated JNK activation observed in *Grb2*$^{+/–}$ thymocytes was specific for signals activated through the TCR because JNK activation that was induced, by anisomycin or ultraviolet irradiation, in *Grb2*$^{+/–}$ thymocytes was comparable to JNK activation in *Grb2*$^{+/+}$ thymocytes (data not shown).

As coengagement of the TCR with the CD28 coreceptor is required for efficient JNK activation in peripheral T cells[35,36], we analyzed whether coreceptor engagement was required for JNK activation in thymocytes. Treatment of *Grb2*$^{+/+}$ thymocytes with an anti-TCR (10 μg/ml) was sufficient to activate JNK (**Fig. 2d,** lanes 2–4). The additional co-cross-linking with an anti-CD28 in the presence of an optimal TCR stimulus did not further enhance JNK activation (lanes 5–7). In addition, co-cross-linking of CD28 with the TCR did not rescue the attenuated JNK activation that was observed in *Grb2*$^{+/–}$ thymocytes (data not shown). Hence, the attenuated JNK activation observed in *Grb2*$^{+/–}$ mice could be attributed to TCR-CD3 signaling.

Normal positive selection in *Grb2*$^{+/–}$ mice

Because the three MAPKs exert different effects on thymic selection, we next addressed whether the differences in MAPK activation that were observed in *Grb2*$^{+/–}$ mice influenced this biological outcome. *Grb2*$^{+/+}$ and *Grb2*$^{+/–}$ mice were crossed to mice that express a major histocompatibility complex (MHC) class I–restricted transgenic TCR specific for the H-Y male antigen[37]. The numbers and profiles of all developmental subsets of thymocytes and splenocytes in the H-Y TCR$^+$ *Grb2*$^{+/+}$ and H-Y TCR$^+$ *Grb2*$^{+/–}$ female mice were comparable (**Fig. 3a** and data not shown). In response to different doses of anti-TCR, H-Y antigen or mitogen, we found that T cell activation (as measured by CD69 up-regulation and cellular proliferation) was indistinguishable between *Grb2*$^{+/+}$ and *Grb2*$^{+/–}$ thymocytes (**Fig. 3b,** data not shown). Similar data were also obtained for *Grb2*$^{+/+}$ and *Grb2*$^{+/–}$ mice that expressed a MHC class II–restricted DO11.10 transgenic TCR specific for ovalbumin (**Fig. 3c**)[38]. Hence, no differences in positive selection or proliferative capacity were detected between *Grb2*$^{+/+}$ and *Grb2*$^{+/–}$ thymocytes.

Decreased negative selection in *Grb2*$^{+/–}$ mice

Although reduced expression of Grb2 showed no detectable effect on positive selection, it did have an effect on negative selection. In male mice with the H-Y antigen, the engagement of H-Y TCR$^+$ thymocytes induces apoptosis and results in a reduction in the absolute number of thymocytes[39]. H-Y TCR$^+$ *Grb2*$^{+/–}$ male mice, however, showed a 50% increase in total thymocyte number and a 100% increase in CD4$^+$CD8$^+$ double-positive and CD8$^+$ single-positive thymocytes (**Fig. 4a,b**). Conversely, the development of CD4$^+$ thymocytes was comparable between *Grb2*$^{+/–}$ and *Grb2*$^{+/+}$ mice. A corresponding increase in H-Y TCR$^+$CD8$^+$ T cells was also observed in the spleen (mean±s.e was 29.0±6.2% in *Grb2*$^{+/+}$ cells *versus* 46.1±5.7% in *Grb2*$^{+/–}$cells, n=7, P<0.001) and lymph nodes (48.1±2.2% in *Grb2*$^{+/+}$ cells *versus* 61.3±1.7% in *Grb2*$^{+/–}$ cells, n=4, P<0.001) of *Grb2*$^{+/–}$ mice. Consistent with the notion that these CD8$^+$ cells had undergone selection, the CD8 mean fluorescent intensity (MFI) was also higher in *Grb2*$^{+/–}$ as compared to *Grb2*$^{+/+}$ CD8$^+$ single-positive thymocytes and peripheral T cells (**Fig. 4a,** lower panels and data not shown) and these cells were capable of up-regulating the CD69 T cell activation marker in response to receptor engagement (data not shown)[39]. Together, these data suggest that the reduced Grb2 expression is associated with increased numbers and decreased negative selection of H-Y TCR$^+$ T cells.

Decreased apoptosis of *Grb2*$^{+/–}$ thymocytes to anti-CD3

As the numbers of CD4$^+$CD8$^+$ and CD8$^+$ thymocytes in the H-Y TCR$^+$ transgenic male mice represent developing, dying and emigrating T cells, we next analyzed the ability of thymocytes to undergo apoptosis using several additional models of negative selection. Because negative selection occurs at the TCR^{lo}CD4$^+$CD8$^+$ stage in the thymic cortex as well as early in the TCR^{hi}CD4$^+$CD8$^{–/lo}$HSA^{hi} stage in the thymic medulla, we examined deletion of TCR^{lo}CD4$^+$CD8$^+$ thymocytes induced by cross-linking of CD3 *in vivo*[40]. Deletion of the CD4$^+$CD8$^+$ thymocytes was substantially reduced, by up to 90%, in the *Grb2*$^{+/–}$ mice (**Fig. 4c,d**), and was present at both 48 and 96 h after anti-CD3 injection (data not shown). Increased resistance to apoptosis was also observed in *Grb2*$^{+/–}$ mice that expressed the transgenic DO11.10 TCR after injection of the KJ1-26 anti-clonotypic DO11.10 TCR (data not shown)[38].

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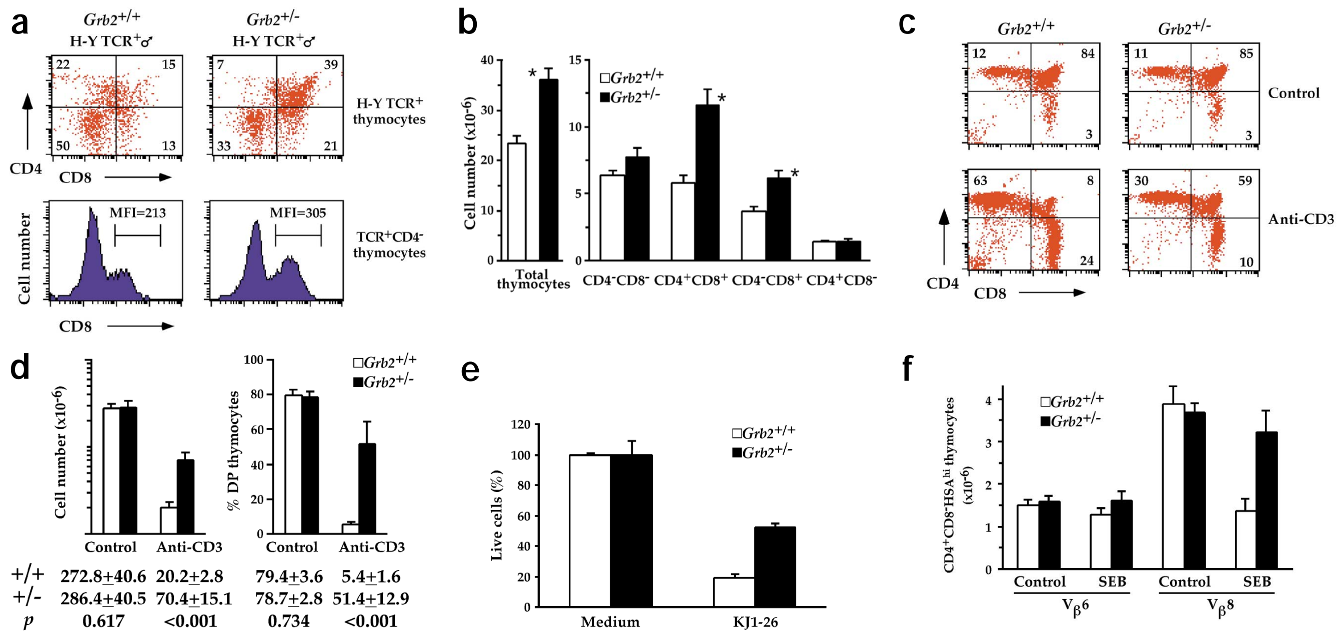

Figure 4. Haploid insufficiency effect of Grb2 on negative selection. (**a**) H-Y TCR$^+$ thymocytes from *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ male mice were stained for CD4 and CD8. (Data are representative of 13 independent pairs of 4- to 6-week-old mice. Single histogram analyses for CD8 expression and the MFIs are shown in the lower panels.) (**b**) The total number of thymocytes and T cell subsets of *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ male mice are shown. (Mean±s.e. values for H-Y TCR$^+$ *Grb2*$^{+/-}$ and H-Y TCR$^+$ *Grb2*$^{+/+}$ male mice are shown. Data were analyzed using the Student's *t*-test, n=13 and *P<0.001.) (**c**) *In vivo* deletion of CD4$^+$CD8$^+$ thymocytes. *Grb2*$^{+/+}$ or *Grb2*$^{+/-}$ mice were injected with anti-CD3 (2C11, 50 μg) and thymocytes stained for CD4 and CD8 54 h after injection. In addition to the decreased percentages of CD4$^+$CD8$^+$ thymocytes, the total numbers of thymocytes were also reduced. (**d**) The total number of thymocytes and percentage of CD4$^+$CD8$^+$ thymocytes from **c** (data for five *Grb2*$^{+/+}$ or *Grb2*$^{+/-}$ littermates are shown). (**e**) DO11.10 thymocytes were incubated with medium or anti-KJ1-26 (1 μg/ml) for 18 h. The percentage of live cells was quantified by propidium iodide (PI) and annexin V staining (see Methods). (**f**) Fas-independent deletion of TCR^{hi}CD4$^+$HSA^{hi} thymocytes by SEB. SEB (2 μg) was injected intraperitoneally into *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ mice and the numbers of V$_\beta$6$^+$ or V$_\beta$8$^+$ TCR^{hi}CD4$^+$HSA^{hi} thymocytes quantified at time 0 and 48 h after injection.

As anti-CD3–induced cell death involves both Fas-dependent and Fas-independent contributions, we also analyzed the effects of Fas-independent doses of anti-CD3–induced apoptosis (induced with 2 μg of 2C11 per mouse)[41]. Although 44±3% (n=3) of *Grb2*$^{+/+}$ CD4$^+$CD8$^+$ thymocytes undergo apoptosis following treatment with Fas-independent doses of anti-CD3, 18±1% (n=3, P<0.001) cell death was observed in *Grb2*$^{+/-}$ CD4$^+$CD8$^+$ thymocytes. Hence, *Grb2* haploid insufficiency decreases the ability of cells to undergo apoptosis.

To determine whether increased *Grb2*$^{+/-}$ thymocyte resistance to apoptosis was intrinsic to the T cell, we examined the ability of CD4$^+$CD8$^+$ thymocytes to undergo TCR-induced apoptosis *in vitro* by incubating DO11.10 TCR$^+$ *Grb2*$^{+/+}$ or *Grb2*$^{+/-}$ thymocytes with the KJ1-26 monoclonal antibody. Consistent with the *in vivo* data, CD4$^+$CD8$^+$ *Grb2*$^{+/-}$ thymocytes showed increased survival, as compared to CD4$^+$CD8$^+$ *Grb2*$^{+/+}$ thymocytes, after receptor engagement (**Fig. 4e**). Hence, the decreased cell death observed in *Grb2*$^{+/-}$ mice represents an intrinsic T cell defect.

Decreased apoptosis of *Grb2*$^{+/-}$ thymocytes to SEB

We next examined whether negative selection of the medullary TCR^{hi}CD4$^+$CD8$^{-/lo}$HSA^{hi} population by staphylococcal enterotoxin B (SEB) was also influenced by Grb2 expression. Similar to studies with anti-CD3, SEB-induced cell death uses both Fas-dependent and independent pathways[41,42]. Administration of Fas-independent doses of SEB (2 μg/mouse) in *Grb2*$^{+/+}$ mice resulted in 65±6% (n=5) deletion of V$_\beta$8$^+$CD4$^+$CD8$^-$HSA^{hi} thymocytes

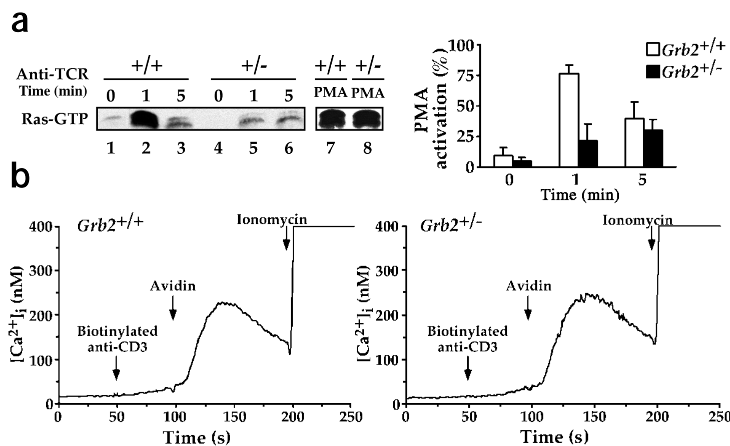

Figure 5. Decreased Ras activation and normal calcium increases in *Grb2*$^{+/-}$ mice. (**a**) (Left panel) Ras activation was measured for *Grb2*$^{+/+}$ (lanes 1–3) or *Grb2*$^{+/-}$ (lanes 4–6) thymocytes under resting conditions (lanes 1 and 4) or after TCR activation (2C11, 10 μg/ml) for different time periods as described in Methods. GTP-bound Ras was analyzed by immunoblotting with an anti-Ras (Ab-3). PMA-induced Ras activation was comparable in *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ mice (lanes 7–8). Total Ras and Sos expression were comparable in *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ thymocytes (data not shown). (The data from three representative experiments were quantified using UN-SCAN-IT software). (Right panel) Quantification of three independent experiments measured at time 0, 1 or 5 min following TCR cross-linking are shown. (**b**) Thymocytes from *Grb2*$^{+/+}$ (left) or *Grb2*$^{+/-}$ (right) mice (6×10^6 cells) were loaded with Fura-2 and treated with biotinylated anti-CD3ε, avidin and ionomycin at the indicated times (see Methods.)

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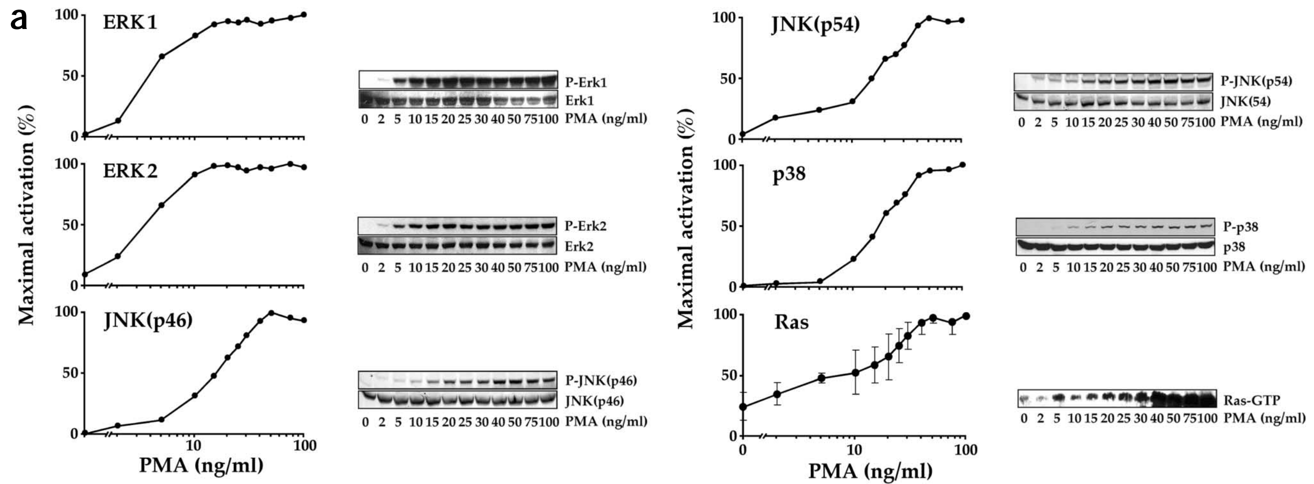

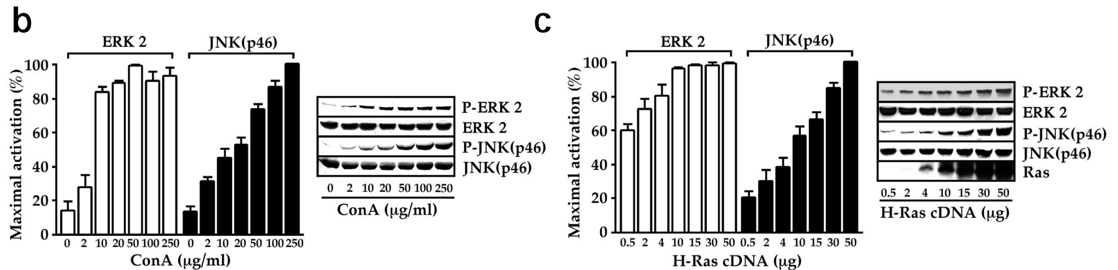

Figure 6. Distinct thresholds for activation of MAPKs. (**a**) Thymocytes (5×10^6 cells/lane) were incubated with different doses of PMA for 2 min and activation of ERK1, ERK2, JNK (p46), JNK (p54) or p38 was measured as described in Fig. 2a. Cell lysates were also analyzed for Ras activation as measured by the ability of Ras-GTP to bind a Raf fusion protein (see last panel). The EC_{50}s were ERK1, 4.4±0.6 ng/ml (n=4); ERK2, 4.5±0.8 ng/ml (n=4); JNK (p46) 18.2±4.5 ng/ml (n=4); JNK (p54) 20.2±9.9 ng/ml (n=4); p38, 16.7±3.2 ng/ml (n=4); and Ras, 14.4±5.7 ng/ml (n=3). (Data represent four independent experiments.) (**b**) Thymocytes (5×10^6 cells/lane) were activated with the indicated amounts of ConA for 2 min at 37 °C. (Right panel) Cells were lysed and immunoblotted for P-ERK2, total ERK2, P-JNK and total JNK. (Left panel) Data, quantified as described in Fig. 1b,d, is shown. (Data are representative of three independent experiments.) (**c**) 293 cells were transfected using a standard calcium phosphate protocol with the indicated amounts of H-Ras–encoding cDNA. (Right panel) Cells were lysed and immunoblotted for P-ERK2, total ERK2, P-JNK, total JNK and Ras. (Left panel) Representative data, quantified as described in Fig. 1b, from one of three independent experiments is shown.

(**Fig. 4f**)[41,42]. In contrast, *Grb2*$^{+/-}$ mice showed an 80% reduction in deletion of $V_{\beta}8^+CD4^+CD8^-HSA^{hi}$ thymocytes (12±2%, n=5, P<0.001). This SEB-induced deletion was antigen receptor–specific because the number of $V_{\beta}6^+CD4^+CD8^-HSA^{hi}$ thymocytes was not affected by SEB.

Decreased Ras activation in *Grb2*$^{+/-}$ T cells

Because Grb2 has been implicated in Ras activation[43], we analyzed Ras activation in *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ thymocytes, as determined by the binding of Ras-GTP (but not Ras-GDP) to a Raf fusion protein[44]. Concomitant with decreased Grb2 expression in *Grb2*$^{+/-}$ mice, Ras activation was also decreased in *Grb2*$^{+/-}$ thymocytes after TCR cross-linking (**Fig. 5a**, lanes 1–6). The decrease was most profound 1 min after TCR cross-linking (71.4±17.5%, n=3). The reduction in Ras activation was not due to an intrinsic defect in the ability of *Grb2*$^{+/-}$ cells to activate Ras because treatment of cells with PMA, which activates Ras in a PTK-independent fashion, induced comparable Ras activation in *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ thymocytes (lanes 7 and 8). A reduction in TCR-induced Ras-activation was also observed in purified $CD4^+CD8^+$ thymocytes and in peripheral T cells (50.6±6.9%, n=3 and 53.3±15.8%, n=3, respectively). In contrast to the decreased Ras activation, *Grb2*$^{+/-}$ thymocytes showed increases in free cytoplasmic calcium (**Fig. 5b**) and induction of tyrosine-phosphorylated proteins (data not shown) that were comparable to those induced in *Grb2*$^{+/+}$ thymocytes. Together, these data provide evidence that Grb2 regulates Ras activation after TCR engagement.

Distinct activation thresholds for MAPK activation

These observations raised the question of how Grb2 selectively affects JNK and p38, but not ERK, activation. One possible explanation is that JNK and p38 activation may be regulated through a Grb2-dependent pathway, whereas ERK is regulated through a Grb2-independent mechanism (such as Ras-Grp). However, a decrease in Ras activation was observed in *Grb2*$^{+/-}$ thymocytes and a large body of evidence implicates a central role for Ras in ERK activation[45]. A second possible explanation is that JNK and p38 may exhibit greater sensitivity to Grb2 expression. As Grb2 can interact with multiple proteins aside from Sos, activation of ERK may exhibit less dependence on Grb2-Ras–regulated signaling pathways compared to JNK and p38. Multiple signaling pathways contribute to ERK activation and studies in Jurkat T cells indicate that ERK can be regulated through Ras and calcium-dependent, as well as Ras and calcium-independent, signaling pathways[11,14,46]. Additionally, JNK and p38 may exhibit different activation thresholds as compared to ERK. In turn, different intrinsic activation thresholds combined with the presence of multiple pathways for MAPK activation would give T cells the ability to tightly control the kinetics and duration of MAPK activation.

To test whether there are intrinsic biochemical differences in the activation thresholds of the three MAPKs, we first analyzed the ability of different doses of phorbol esters, which activates Ras through Grb2-independent mechanisms, to regulate the different MAPKs. The EC_{50}s

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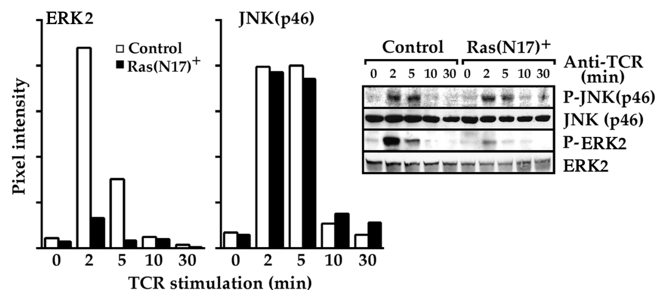

Figure 7. Inhibition of ERK but not of JNK activation in mice that express Ras(N17). (Right panel) Thymocytes (10^7 cells/lane) from mice that express a Ras(N17) transgene or nontransgenic littermates (control) were analyzed, as described in Fig. 2a, after stimulation with anti-TCR (2C11) for the indicated time periods. (Left panel) Data that represent two independent experiments are shown.

for activation of ERK1 and ERK2 by phorbol 12-myristate 13-acetate (PMA) in thymocytes were 4.4±0.6 ng/ml and 4.5±0.8 ng/ml, respectively. Both forms were maximally activated by 15 ng/ml of PMA (**Fig. 6a**). In contrast, EC_{50}s for JNK and p38 were fourfold higher and required 50 ng/ml of PMA to elicit maximal activation. Differences between ERK1 and ERK2 *versus* JNK and p38 were independent of the kinetics of activation as these differences were observed at all time points analyzed and independent of the addition of other stimuli, including ionomycin (data not shown). Mitogen activation of the TCR revealed similar differences in the activation of ERK2 and JNK (**Fig. 6b**). Although maximal ERK2 activation was achieved by treatment of thymocytes with 50 µg/ml of concanavalin A (ConA), maximal JNK activation required 250 µg/ml of ConA. In addition, the EC_{50} for ERK2 activation was 4.5±0.5 µg/ml ConA (n=3) as compared to 17.6±5.0 µg/ml for JNK (n=3).

Taken together, these results predict that the degree of Ras expression may also exert quantitatively different effects on ERK and on JNK and p38 activation. Due to technical limitations in regulating the expression of Ras in thymocytes and differences in the coreceptor requirements of JNK activation in thymocytes as compared to T cell lines, we examined the effects of expressing increasing amounts of Harvey Ras (H-Ras) on ERK and JNK activation in 293 cells. As with the different thresholds of activation observed above (**Fig. 6a,b**), maximal ERK2 activation was induced with expression of ~10 µg of H-Ras cDNA, whereas maximal JNK required >50 µg of H-Ras cDNA (**Fig. 6c**). Similarly, the EC_{50}s for ERK2 and JNK activation were <0.5 µg and 7.7±1.9 µg of H-Ras cDNA, respectively (n=3).

Effects of N17(Ras) in thymocytes

Expression of Ras(N17), a dominant-negative form of mutant Ras, in which Gly[17] is mutated to Asn, inhibits ERK as well as JNK activation in Jurkat T cells[14,47]. Our results, therefore, would appear to be in conflict with observations in mice expressing a Ras(N17) transgene, in which positive, but not negative, selection is affected[25]. Because the relative inhibitory effect of Ras(N17) on ERK and JNK activation had not been characterized, we compared ERK and JNK activation following TCR cross-linking in mice that were expressing Ras(N17). Mice that were expressing Ras(N17) showed an 85% reduction in ERK activation following TCR cross-linking (**Fig. 7**)[25]. However, expression of Ras(N17) had no effect on JNK activation. Thus, the normal negative selection in the Ras(N17) transgenic mice is in precise agreement with the normal JNK activation observed in these mice.

Discussion

Although the effects of haploid insufficiency have been described for Grb2 in the induction of mammary carcinomas in polyomavirus middle T antigen transgenic mice and in Drosophila[33,48], a coordinated examination of the biochemical and biological effects has not been carried out mainly because of the lack of a system for such an analysis. Our study shows that *Grb2* haploid insufficiency in thymocytes results in normal ERK, but decreased JNK and p38, activation. Because the different MAPK members play qualitatively distinct roles in T cell development, we analyzed how the biochemical alterations observed in *Grb2*$^{+/-}$ thymocytes affected T cell development. Consistent with normal ERK activation, *Grb2*$^{+/-}$ mice bred onto two different transgenic TCR backgrounds showed normal positive selection. Conversely, the attenuated JNK and p38 activation observed in *Grb2*$^{+/-}$ thymocytes is associated with decreased cell death and negative selection.

Based on models of transmembrane receptor tyrosine kinases, recruitment of the Grb2 adaptor protein, which is associated with Sos GEF, to the plasma membrane has been established as a mechanism for Ras and ERK activation. Despite observations that have identified a central role for Ras in ERK activation, the *Grb2*$^{+/-}$ mutation had no apparent impact on ERK activity in thymocytes. We have also shown that ERK has an intrinsically lower threshold of activation, in comparison to the thresholds of JNK and p38, in both thymocytes and fibroblasts. In turn, the reduced induction of Ras-GTP that is caused by *Grb2* haploid insufficiency would selectively attenuate JNK and p38 MAPKs, with higher intrinsic thresholds of activation, and conversely exert reduced effects on ERK activation.

In view of the intrinsic differences in the activation thresholds for each of the different MAPKs, we propose that extremely "low" signals might not trigger ERK and do not lead to positive selection. A "moderate" amount of signaling would lead to ERK, but not JNK, activation and then lead to positive selection (**Fig. 8**). Only with very high signals would ERK, JNK and p38 be activated and contribute to negative selection. Thymocytes that express TCRs that are associated with mutated ζ subunits, which contain less than three immunoreceptor tyrosine-based activation motifs (ITAMs), or thymocytes that lack the Itk and/or Rlk members of the Tec-family of PTKs show lowered signaling abilities and decreased positive and negative selection[22,49,50]. Our studies, by revealing different activation thresholds for each MAPK family member, extend these previous observations by providing a mechanism by

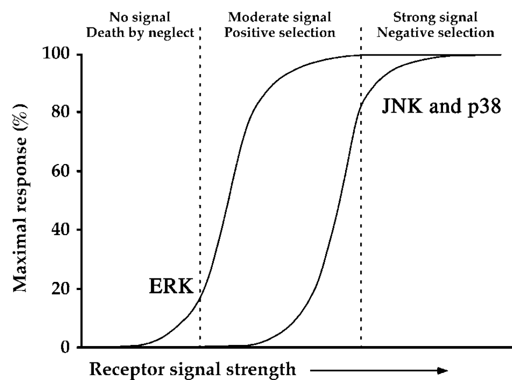

Figure 8. Relationship between signal strength, MAPK activation and biological outcome. The ERK, JNK and p38 MAPKs show intrinsically distinct activation thresholds. These intrinsic differences in MAPK activation may translate different receptor-induced signal strengths into distinct biological outcomes for a given cell.

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which quantitative differences in signal strength could induce selective activation of MAPK family members. In turn, the balance between the MAPKs that are, or are not, activated by a given ligand may contribute to qualitative differences in thymocyte cell fate.

As Ras appears to play a pivotal role in regulating the balance of MAPK activation, the mechanisms that contribute to the strength and kinetics of Ras activation may represent critical factors in determining the biological outcome of a given stimulus. Our studies have shown that Grb2 contributes to Ras activation in both $CD4^+CD8^+$ thymocytes and in peripheral T cells. Because expression of an artificially membrane-localized Sos induces ERK activation in T cells[51], the translocation of Grb2-Sos complexes to the membrane is thought to play a critical role in localizing Sos GEF with Ras. The translocation of Grb2-Sos complexes to the membrane is mediated through phosphorylation of tyrosine residues within the cytoplasmic tail of the transmembrane LAT molecule. T cells that express a mutant LAT that does not bind Grb2 or Gads, but still retains its ability to bind PLC-γ1, show normal calcium responses but decreased ERK and nuclear factor AT (NFAT) activation[52]. Hence, the Grb2 and Gads proteins likely contribute to ERK activation, independent of the ability of LAT to bind PLC-γ1. Recent studies have also shown that PLCγ-dependent Ras-GRP GEF plays a critical role in Ras activation in T cells[11]. As *Grb2*$^{+/-}$ thymocytes show normal PLC-γ1–regulated calcium responses, Grb2 would appear to function independently of Ras-GRP. Hence, two independent pathways likely exist that potentially activate Ras in T cells and permit fine-tuning of the duration and strength of Ras activation during different stages of T cell development and/or antigenic stimuli. In this respect, the sum effects of these different signaling pathways may regulate the quantitative aspect of TCR signaling.

Although our studies show a role for Grb2 in JNK and p38 activation, the mechanism(s) by which Grb2 regulates JNK and p38 remain unclear. As Grb2 interacts with mitogen-activated protein–extracellular signal-regulated kinase kinase 1 (MEKK1), hematopoietic progenitor kinase 1 (HPK1), Cbl, dynamin, p125FAK and Sam68, we cannot exclude the possibility that Grb2 may regulate JNK activation through a Grb2-dependent, but Ras-independent, signaling pathway[53–57]. In ER22 cells, Grb2 regulates JNK through its interaction with MEKK1[53]. However, we have been unable to detect a Grb2-MEKK1 complex in thymocytes (data not shown). Conversely, several lines of experimentation support a role for Ras and Rac in JNK activation in T cells. Although expression of constitutively active Rac-1 augments JNK activation, expression of dominant-negative mutants of Rac-1 inhibits JNK activation in Jurkat T cells[18]. In addition, the induction of an AP-1–dependent (for Fos and Jun) reporter by Ras requires Rac-1 function[19] and overexpression of H-Ras in fibroblasts, PC12 cells and in thymocytes enhances ERK, JNK and p38 activation[58] (Q. Gong, W. Swat & A. Chan, unpublished data). Because *Grb2*$^{+/-}$ thymocytes show decreased Ras activation, we favor the interpretation that Grb2 regulates JNK and p38 through a Ras and Rac-dependent mechanism. Analysis of Rac-mediated signaling pathways in *Grb2*$^{+/-}$ thymocytes may reveal additional mechanistic insights.

Finally, the selective inhibition of ERK, but not JNK, by expression of Ras(N17) in the thymus was unexpected. It contrasts with the decreased JNK and p38 activation observed in *Grb2*$^{+/-}$ thymocytes as well as the inhibitory effects of Ras(N17) on ERK and JNK in Jurkat T cells[15,47]. Because Ras(N17) exerts its effects by binding Sos, Ras(N17) may inhibit a subset of Sos effectors in thymocytes that selectively affects ERK, but not JNK, activation[59]. Analysis of how Ras(N17) affects downstream targets may provide insights into mechanistic differences between decreased Ras activation and the effects of Ras(N17).

Our studies of Grb2 heterozygosity in thymic selection reveal how a small change in the expression of an adaptor protein may impinge on the fate of a developing thymocyte. Given that expression of many signaling components are regulated throughout T cell development, it is conceivable that even small but coordinated changes in the expression of signaling components may have significant consequences on the developing cells. One can also envision that slight alterations in adaptor function, caused by polymorphism or subtle mutations, may have a dramatic impact on the development of thymocytes that, in turn, affects the elimination of autoreactive lymphocytes and result in a predisposition to autoimmune disorders.

Methods

Antibodies. Antibodies used for cell staining, including antibodies to CD4, CD8, CD3, CD28, $V_\beta6$, $V_\beta8$, CD69 and heat-stable antigen (HSA), were from PharMingen (San Diego, CA). Antibodies used for immunoblotting for activated or total ERK, JNK and p38 were from Promega (Madison, WI) and NEB (Beverly, MA). Antisera to Grb2, Cbl and Sos were from Santa Cruz Biochem. (Santa, Cruz, CA). KJ1-26 anti-clonotypic Ti for the DO11.10 TCR was from K. Murphy (Washington University). T3.70 anti-clonotypic for the H-Y TCR was from H. S. Teh (University of British Columbia).

Calcium fluorimetry. Cells were loaded with Fura-2 (Molecular Probes, Eugene, OR) and increases in$[Ca^{2+}]_i$ monitored using a Hitachi F-2000 fluorimeter, according to manufacturer's recommendations.

Analysis of cell surface molecules. Cells were stained with the appropriate antibodies according to manufacturer's recommendations and analyzed using FACS Calibur and Cell Quest Analysis software (Becton Dickinson, San Jose, CA).

Cell activation studies. To stimulate thymocytes or T cells, cells were incubated with stimulating monoclonal antibodies to CD3, Ti or CD28 at 4 °C for 15 min, washed with cold PBS to remove nonbinding antibodies and incubated with warm PBS that contained a cross-linking antibody for the indicated time periods. Cells were quickly sedimented and cells lysed in 2×10^8 cells/ml of lysis buffer (10 mM Tris at pH 7.4, 150 mM NaCl and 1% NP-40) with protease and phosphatase inhibitors. Conditions for the Ras-GTP binding assay were as described[44].

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