RasGRP1 Transduces Low-Grade TCR Signals which Are Critical for T Cell Development, Homeostasis, and Differentiation

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Summary

Two important Ras-guanyl nucleotide exchange factors, Sos and RasGRP1, control Ras activation in thymocytes. However, the relative contribution of these two exchange factors to Ras/ERK activation and their resulting impact on positive and negative selection is unclear. We have produced two lines of RasGRP1$^{-/-}$ TCR transgenic mice to determine the effect of RasGRP1 in T cell development under conditions of defined TCR signaling. Our results demonstrate that RasGRP1 is crucial for thymocytes expressing weakly selecting TCRs whereas those that express stronger selecting TCRs are more effective at utilizing RasGRP1-independent mechanisms for ERK activation and positive selection. Analysis of RasGRP1$^{-/-}$ peripheral T cells also revealed hitherto unidentified functions of RasGRP1 in regulating T cell homeostasis and sustaining antigen-induced developmental programming.

Introduction

The thymus is responsible for generating mature T cells that express a diverse TCR repertoire and, thus, ensuring the capacity to react with a vast number of pathogen-associated foreign peptides in the context of self-MHC. In addition to its function in controlling antigen (Ag) responsiveness in mature T cells, the TCR also plays critical roles in the development of immature thymocytes. Upon entry from the bone marrow, T cell precursors undergo an ordered progression of signal-dependent maturation processes within the thymic microarchitecture. The expression of the pre-TCR by CD4$^-$CD8$^-$ (DN) thymocytes results in ligand-independent signaling and differentiation into CD4$^+$CD8$^+$ (DP) cells (Kruisbeek et al., 2000). Accompanying the rearrangement and expression of the α chain, TCR signaling becomes contingent on the recognition of self-peptides/self-MHC molecules (Hogquist, 2001). The intensity of this interaction between the TCR and its ligands on thymic cortical epithelial cells and bone marrow-derived cells determines the fate of the developing thymocyte. The strength of signal hypothesis model predicts that a lack of signaling through the TCR results in death by neglect, a strong signaling results in negative selection, and a weak-moderate signaling results in positive selection and differentiation into either mature CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ (SP) T cells. However, the molecular mechanism for distinguishing signals that lead to positive and negative selection remains to be defined.

The small GTPase Ras, originally identified as an oncogene capable of inducing cell transformation, has been implicated in TCR signal transduction (Downward et al., 1990; Genot and Cantrell, 2000). Recent work has established that Ras acts as a molecular switch, turning on activation, proliferation, and differentiation programs in various cell types (Genot and Cantrell, 2000). By associating with different effector proteins, activated Ras can initiate signaling through multiple downstream pathways and, hence, influence gene expression. During thymocyte differentiation, both qualitative and quantitative aspects of TCR signaling have been implicated in determining whether a cell is positively or negatively selected. The findings that Ras acts at a branch point for multiple signaling pathways and that it has been demonstrated to partake in both of the aforementioned processes suggests a central role for Ras in this cell fate decision.

Ras activity cycles between a GDP-bound off and a GTP-bound on conformation proximal to the inner surface of the plasma membrane. It can be inactivated by either its own intrinsic GTPase activity or by pairing with GTPase-activating proteins (Ras GAPs). Conversely, guanine nucleotide exchange factors (Ras GEFs) control the activation of Ras by catalyzing GDP release from Ras and, hence, facilitating its association with GTP. In thymocytes, Ras activation is mediated by at least two Ras GEFs, the well-characterized Sos (Gong et al., 2001) and the recently identified RasGRP1 (Dower et al., 2000). Regulation of these two Ras GEFs is mediated by their recruitment to the plasma membrane after cellular activation (Roose and Weiss, 2000). TCR stimulation and subsequent LAT (linker for activated T cells) phosphorylation results in the adaptor protein Grb2 complexed with Sos being targeted toward the membrane via Grb2's SH2 domain. In addition, phospho-LAT also recruits phospholipase Cγ1 (PLCγ1). The action of activated PLCγ1, converting phosphotidylinositol 4,5 bisphosphate (PIP2) into inositol 3,4,5 triphosphate (IP3) and diacylglycerol (DAG), results in membrane localization for RasGRP1 through its DAG binding C1 domain (Ebinu et al., 1998; Tognon et al., 1998). The recruitment and subsequent activity of RasGRP1 accounts for the well-documented activation of Ras in T cells following treatment with DAG analogs such as PMA (Dower et al., 2000).

Ras signaling can result in the activation of three families of mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38. These MAPKs have been implicated in playing qualitatively and quantitatively distinct roles in thymic selection (Sebzda et al., 1999). The Ras-ERK pathway that activates Raf-1, MAPK kinase (MEK1), and ERK has been determined to play an important role in the formation of mature thymocytes (Alber-

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ola-Ila et al., 1995; Delgado et al., 2000; Sharp et al., 1997; Sugawara et al., 1998; Werlen et al., 2000). Overexpression of dominant-negative forms of Ras (Swan et al., 1995), Raf-1 (O'Shea et al., 1996), and MEK1 (Alberola-Ila et al., 1995) in transgenic mice results in defective positive but normal negative selection. Conversely, decreases in JNK and p38 activation have correlated with defective negative but not positive selection (Dong et al., 1998; Rincon et al., 1998; Sabapathy et al., 1999; Sugawara et al., 1998). More recent studies using Grb2$^{+/-}$ mice yielded results that are also consistent with this hypothesis (Gong et al., 2001). Grb2 haploinsufficiency led to decreased Ras activation, resulting in reduced JNK and p38 activation and impaired negative selection. ERK activation and positive selection were unaffected in Grb2 heterozygotes. These studies demonstrated that the activation threshold for ERK was lower than that for JNK and p38, explaining why halving of the amount of Grb2/Sos did not affect ERK signaling. Together, these observations suggest that a relatively weak TCR signal results in only ERK activation and positive selection whereas a strong TCR signal results in the activation of the full range of MAPKs and negative selection. However, ERK activation has recently been implicated in negative selection as well (Bommhardt et al., 2000; Mariathasan et al., 2000). Perhaps, the strength and/or duration of ERK signaling may determine whether a thymocyte is programmed to survive or die.

RasGRP1-deficient mice display a block at the DP stage of thymocyte development suggesting a defect in thymic selection. Associated with this defective developmental program is an inability to activate ERK. This observation led to the hypothesis that the principal mechanism for distinguishing between positive and negative selection signals is coupled to the utilization of the RasGRP1 and Grb2/Sos pathways and, hence, the MAPKs that get activated (Yun and Bevan, 2001). According to this model, thymocytes that express low affinity TCR for their selecting ligand(s) will activate Ras and ERK via RasGRP1 leading to positive selection. Thymocytes expressing high-affinity TCR for the selecting ligand(s) will activate Ras, ERK, JNK, and p38 via the Grb2/Sos pathway leading to negative selection. To investigate this hypothesis, we generated two lines of RasGRP1$^{-/-}$ TCR transgenic (Tg) mice, expressing either a weakly or strongly selecting TCR. Our findings indicate that a weakly selecting TCR is critically dependent on RasGRP1 for its positive selection. However, ERK activation and positive selection of thymocytes expressing a strongly selecting TCR are much less dependent on RasGRP1. By contrast, RasGRP1 deficiency has no effect on JNK and p38 MAPK activation or negative selection.

The positive selection of thymocytes expressing a strongly selecting TCR lacking RasGRP1 offered the unique opportunity to investigate the role of RasGRP1 in peripheral T cell homeostasis and function. Our studies suggest that RasGRP1 is important for transducing low-grade TCR signals necessary for survival and differentiation while strong TCR signals, responsible for negative selection and the induction of Ag-driven growth, are RasGRP1 independent.
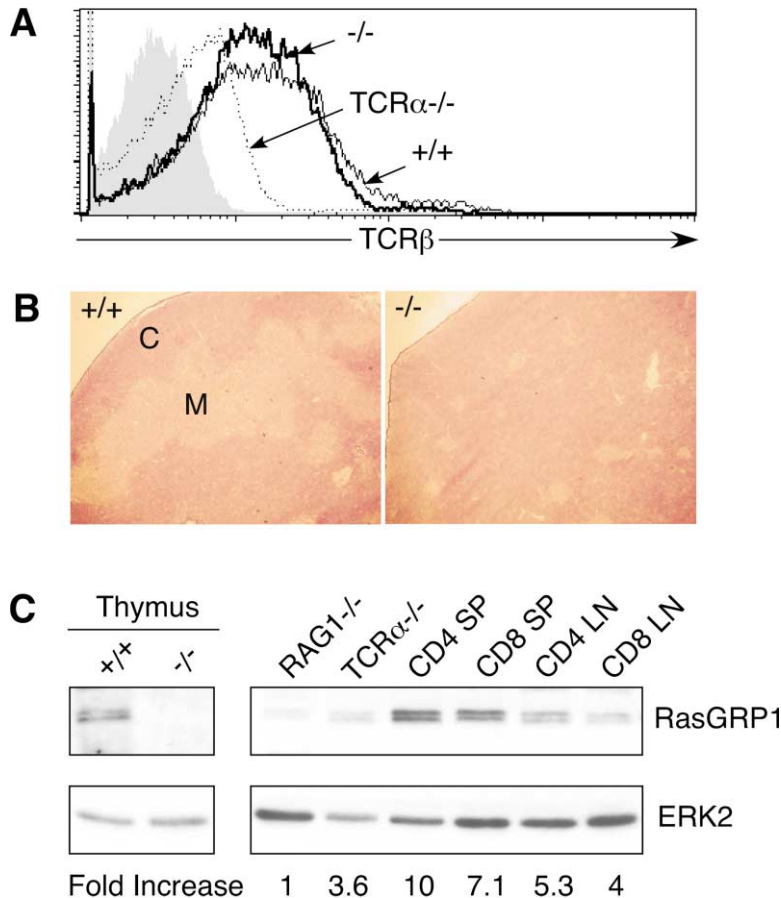
Results

Developing Thymocytes Require RasGRP1 for the Efficient Generation of Mature Single-Positive Cells

Mice bearing a systemic loss of RasGRP1 display a paucity of mature single-positive thymocytes and peripheral T cells demonstrating an important role in thymopoiesis (Dower et al., 2000). The fact that RasGRP1$^{-/-}$ thymi are near normal in size and possess wild-type-like numbers of both double-negative and double-positive subpopulations indicates that a critical role for RasGRP1 exists at a late stage of T cell development. Corroborating this conclusion, mutant and wild-type DP thymocytes expressed similar levels of the TCRβ chain (Figure 1A). Analyses of histological sections revealed that although mutant thymi possessed normal-sized cortical regions, their medullary compartments appeared shrunken and rudimentary (Figure 1B). As a deficiency in SP thymocytes could result from anatomical changes in the thymic microenvironment, we verified the cell-intrinsic nature of the phenotype by infusing RAG1$^{-/-}$ host mice with either wild-type or null bone marrow (data not shown). RAG1-deficient hosts receiving mutant bone marrow display a large reduction in the proportion and cell number of SP thymic subpopulations. Therefore, altered thymic architecture in mutant animals is likely a secondary effect, resulting from the shortage of mature thymocytes. To examine RasGRP1 expression, thymocytes possessing well-defined developmental blocks (RAG1$^{-/-}$, DN-arrested [Mombaerts et al., 1992]; TCRα$^{-/-}$, DP-arrested [Philpott et al., 1992]) and purified subpopulations from wild-type animals were immunoblotted with anti-RasGRP1 antibody (Figure 1C). These results indicate that the RasGRP1 protein is most highly expressed in mature SP thymocytes. Collectively, these data support the hypothesis that RasGRP1 plays a critical cell-autonomous role in the transition of immature DP thymocytes into mature SP thymocytes.

Differential Requirement for RasGRP1 in the Positive Selection of T Cells

The small amount of SP thymocytes present in the RasGRP1 null mouse suggests that some positive selection occurs via a RasGRP1-independent mechanism (Dower et al., 2000). It is possible that DP thymocytes expressing TCRs that are positively selected with differing efficiency might display differential dependence on RasGRP1-mediated signaling for their development. To investigate this hypothesis, we crossed the RasGRP1 mutation onto two TCR Tg lines to determine the impact on thymocyte selection. The H-Y TCR and the 2C TCR are both MHC class I-restricted TCRs and used as defined models for a weakly and a strongly selecting TCR, respectively (Lee et al., 1992; Robey et al., 1992). The strength of selection is likely determined by the overall avidity of the TCR for thymic self-peptides/MHC, a product of the affinity of the TCR for its selecting ligand(s) and the relative abundance of these ligands in the thymus. The expectation was that a weakly selecting TCR would show greater dependence on RasGRP1 for positive selection. Consistent with this prediction, we found that the positive selection of the H-Y TCR in female

Figure 1. RasGRP1 Deficiency Results in a Severe Block at the Double-Positive Stage of Thymocyte Development

(A) Flow cytometric analyses of TCRβ expression on wild-type (thin line), RasGRP1- (bold line), and TCRα-deficient (dotted line) thymocytes. The shaded histogram represents the fluorescence of unlabeled thymocytes.

(B) Hematoxylin and eosin staining of mutant thymi revealed rudimentary medullary compartments (40×, magnification). Positions of the cortical, C, and medullary, M, compartments are denoted in the wild-type thymus.

(C) Protein expression levels of RasGRP1 detected among various thymic subpopulations. The ratio, density of the RasGRP1 band/density of ERK2 band, denotes relative RasGRP1 expression levels. RasGRP1 expression in RAG1$^{-/-}$ thymocytes was arbitrarily given a value of 1.

mice is dramatically reduced in the absence of RasGRP1 (Figure 2A). Although CD8 SP T cells are still formed in null animals, their proportion and total cell number are decreased approximately 4-fold (Figure 2B). In contrast, RasGRP1 deficiency has a much milder effect on the positive selection of the 2C TCR. In this case, the number of CD8 SP T cells is almost identical between wild-type and mutant thymi (Figure 2B).

The positive selection of mutant thymocytes expressing the H-Y TCR is also less efficient compared to 2C-expressing thymocytes on the basis of clonotypic TCR expression and differentiation markers (Figure 2C). H-Y DP and CD8 SP thymocytes express much lower levels of the clonotypic TCR than do 2C cells. Mutant CD8 SP T cells expressing the H-Y TCR also display the greatest differences in the developmental markers CD69, HSA, and CD5 levels as compared to wild-type. However, despite their near normal numbers, 2C CD8 SP thymocytes lacking RasGRP1 are less mature than their wild-type counterpart as assessed by HSA and CD69 expression levels. To determine whether this phenotypic immaturity had an effect on their rate of export from the thymus to the periphery, intrathymic FITC injections were undertaken as described previously (Scollay et al., 1980). Eighteen hours postintrathymic labeling, spleens were harvested to quantitate FITC-labeled cells as a measure of thymic emigration rates. As expected, the paucity of SP in RasGRP1$^{-/-}$ animals is correlated with a dramatic decrease in thymic export (Figure 2D). Con-

sistent with previous work (Kelly et al., 1993), wild-type TCR Tg mice exhibit enhanced thymic emigration as compared to normal mice. However, despite a normal CD8 SP number, RasGRP1$^{-/-}$ 2C thymi export about 3-fold fewer CD8 T cells than do wild-type 2C mice. This finding suggests that RasGRP1 is also important for the maturation and efficient export of SP thymocytes expressing strongly selecting TCRs.

Negative Selection Is Unaffected by RasGRP1 Deficiency

To examine the effects of RasGRP1 deficiency on negative selection, we compared thymic development between wild-type and RasGRP1$^{-/-}$ H-Y male mice. The H-Y TCR Tg mice have been used extensively as a model for negative selection studies (Kisielow et al., 1988). Negative selection in this system is associated with a massive depletion of DP thymocytes and a corresponding decrease in the yield of thymocytes in Ag-expressing (male) mice. Consistent with the conclusion that RasGRP1 is not required for efficient negative selection, an equivalently low number of thymocytes were recovered from male H-2^b H-Y mice lacking RasGRP1 (Figure 3A). However, the mutant developmental profile possesses an increased proportion of DP cells (Figure 3B). These unusual DP cells express very low levels of H-Y TCR (Figure 3C). Perhaps, the increased proportion of DP thymocytes in RasGRP1$^{-/-}$ male thymi may arise from their lowered TCR expression, implying a role for
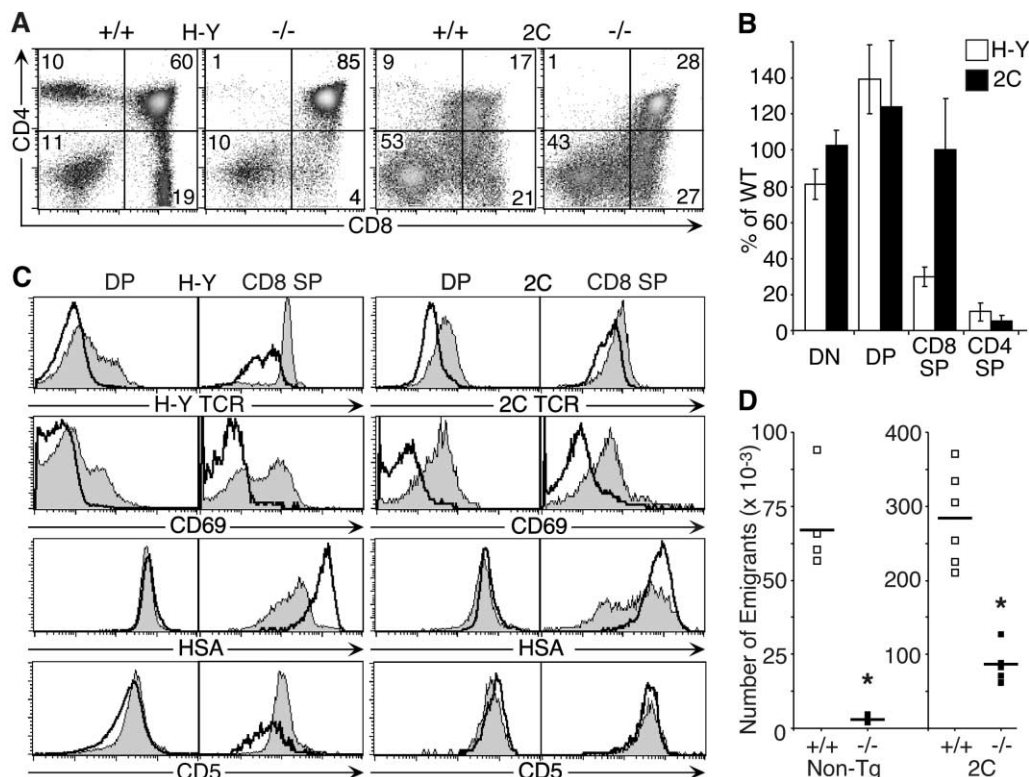
Figure 2. Delayed Positive Selection in RasGRP1$^{-/-}$ Mice Expressing Either H-Y or 2C TCR

(A) CD4/CD8 developmental profiles of wild-type and mutant H-Y and 2C TCR Tg mice.

(B) Cell numbers present within various thymic subpopulations expressed as percent of wild-type. Thymic cell recovery from wild-type and RasGRP1-deficient TCR Tg animals were as follows: wild-type 2C, $10.9 \pm 1.5 \times 10^6$ (n = 8); mutant 2C, $16.5 \pm 2.6 \times 10^6$ (n = 7); wild-type H-Y female, $95.6 \pm 14.3 \times 10^6$ (n = 10); and mutant H-Y female, $103.7 \pm 16.7 \times 10^6$ (n = 8). Error bars represent the SD.

(C) Flow cytometric analyses of the clonotypic TCR (T3.70 for H-Y; 1B2 for 2C) and developmental markers, CD69, HSA, and CD5, on both DP and CD8 SP subpopulations are shown. Wild-type and mutant subpopulations are represented by shaded thin-line and bold histograms, respectively.

(D) Decreased thymic emigration in non-Tg and 2C TCR Tg RasGRP1-deficient mice. Average age and number of animals used per group were as follows: +/+ non-Tg, 40.8 days, n = 4; −/− non-Tg, 39.5 days, n = 4; +/+ 2C, 50 days, n = 6; −/− 2C, 57.5 days, n = 5. The asterisk represents p values, using an unpaired, one-tailed Student's t test, calculated at p < 0.0003 for non-Tg and p < 0.0001 for 2C data. Bars represent the mean.

RasGRP1 in modulating ligand-induced TCR upregulation. Nevertheless, it is clear that this unusual population of DP thymocytes is generated inefficiently since there is no increase in the total yield of thymocytes from mutant mice. By contrast, the expression of the H-Y receptor in DP of wild-type mice is bimodal, composed of H-Y TCR$^-$ and TCR$^+$ subpopulations. It has been previously shown that some DP cells survive by deleting the $\alpha\beta$ transgenes (Bluthmann et al., 1988) whereas the H-Y TCR$^+$ cells are likely undergoing apoptosis (data not shown).

To further address the role of RasGRP1 in negative selection, an in vitro thymocyte deletion assay, using DP thymocytes from H-Y females, was undertaken (Carlow et al., 1992). The H-Y TCR recognizes a male-specific peptide in the context of D^b (Markiewicz et al., 1998). Wild-type and mutant DP thymocytes were mixed with female splenic dendritic cells plus various concentrations of H-Y peptide. After 16 hr incubation, thymocytes were stained with CD4 and CD8 and the viability of DP determined using 7-AAD as described previously (Motyka and Teh, 1998). In agreement with the H-Y male

data, RasGRP1 does not appear to affect negative selection, as mutant DP were killed off as effectively as the wild-type cells (Figure 3D). These findings are particularly remarkable since female RasGRP1-deficient H-Y DP cells used in the assay express much lower levels of H-Y TCR (about 20% of wild-type MFI; Figure 2C). These in vitro studies indicate that RasGRP1 is not required for the efficient negative selection of autoreactive DP thymocytes.

Decreased ERK but Not JNK and P38 Activation

Previously, it was found that RasGRP1$^{-/-}$ thymocytes failed to activate ERK whether they were treated with DAG analogs or anti-TCR antibody (Dower et al., 2000). However, the inability to activate ERK upon TCR cross-linking may have been the result of the depletion of mature thymocytes, cells that are most responsive to this type of stimulation, in null animals. Consistent with these results, mutant thymocytes failed to activate ERK efficiently whether 5 or 25 ng/ml of PMA was used (Figure 4A). Interestingly, PMA-mediated activation of other MAPK family members, JNK and p38, were not affected
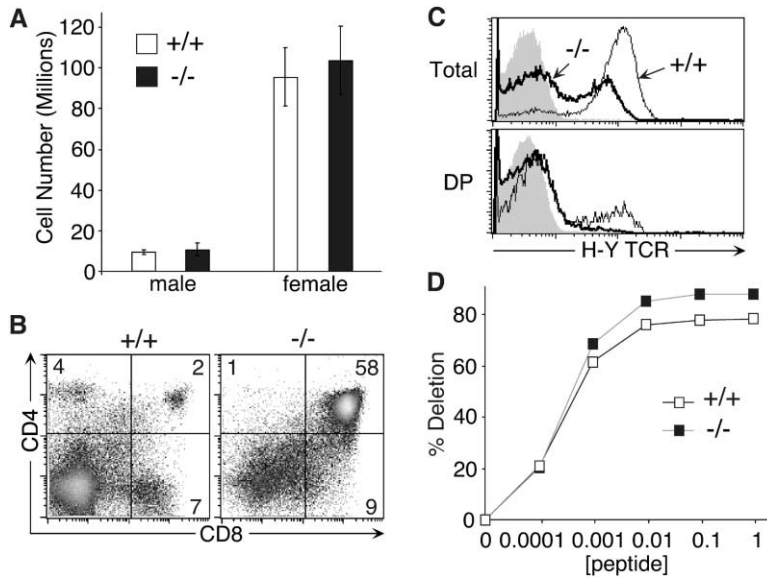
Figure 3. Negative Selection Functions Efficiently in the Absence of RasGRP1

(A) Thymic cell number comparisons between male and female H-Y TCR Tg mice. Error bars represent the SD.

(B) CD4/CD8 thymic developmental profiles of wild-type and mutant H-Y male mice.

(C) Expression of the clonotypic H-Y TCR by either total thymocytes (RasGRP1$^{+/+}$ or RasGRP1$^{-/-}$) or gated only on DP from thymocytes.

(D) In vitro thymocyte deletion assay. Thymocytes from female H-Y mice were incubated female splenic DCs plus various doses of the H-Y peptide (μM). The SD was smaller than the symbols used to represent each of the data points.

by the absence of RasGRP1. As the absence of RasGRP1 appears to selectively affect ERK activation, an event heavily implicated in positive selection (Sebzda et al., 1999), we sought to determine whether RasGRP1-independent positive selection was correlated with signaling through ERK. To compare levels of ERK activation between different thymic subpopulations, we adapted a flow cytometry-based method employing an anti-
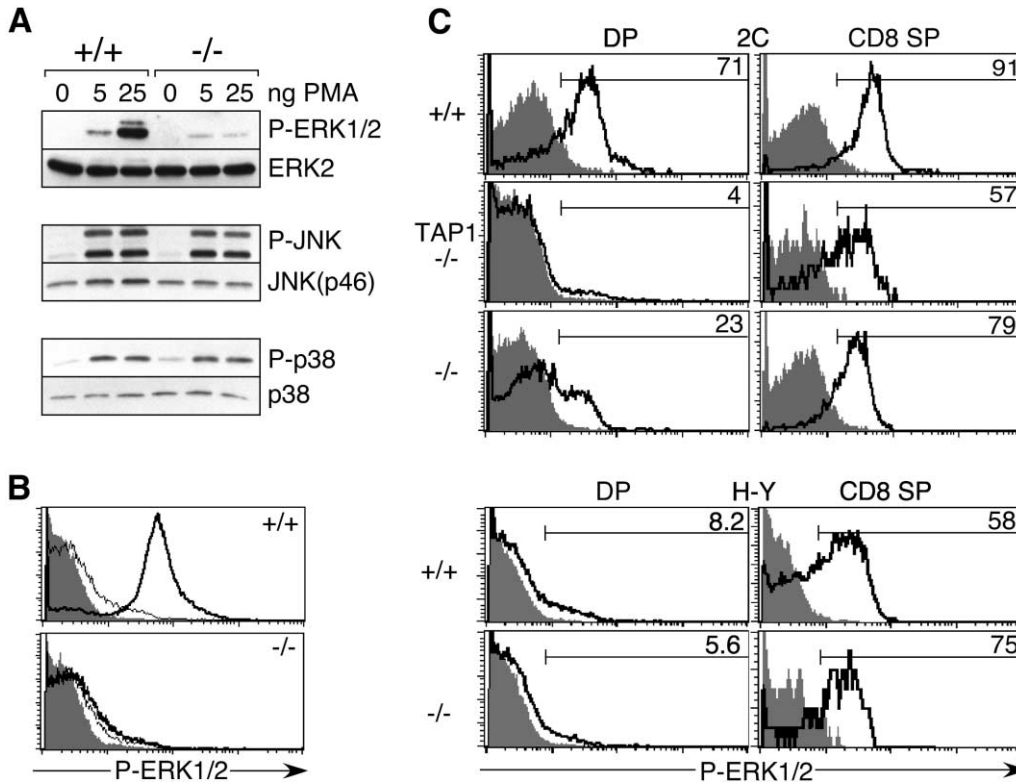

Figure 4. Decreased ERK Activation in Mutant Thymocytes

(A) Total thymocytes from wild-type and mutant non-TCR Tg mice were stimulated with either 0, 5, or 25 ng/ml of PMA for 2 min and assayed for the activation of ERK, JNK, and p38.

(B) Flow cytometric determination of P-ERK1/2 in DP cells in non-TCR Tg thymocytes following PMA treatment.

(C) Single-cell suspensions from wild-type, RasGRP1$^{-/-}$, and TAP1$^{-/-}$ 2C TCR Tg mice were generated rapidly in serum-free media and immediately fixed. The percentage of P-ERK1/2 positive cells among the DP and CD8 SP subpopulations is indicated next to the marker. Shaded histograms indicate the level of staining with the secondary alone.

phospho-specific ERK1/2 antibody (Chow et al., 2001). To verify the specificity of our assay, we treated non-TCR Tg thymocytes with PMA for 2 min to determine whether we could recapitulate our Western blotting results by FACS analysis (Figure 4B). In agreement with the blotting data, wild-type cells showed a strong response to PMA stimulation whereas null thymocytes failed to activate ERK.

Sustained low-grade ERK activation has been suggested to be a critical factor in mediating positive selection (Mariathasan et al., 2001; Werlen et al., 2000). Using the phospho-ERK1/2 reactive antibody in the flow cytometric assay, we sought to determine whether we could detect ERK activity in 2C and H-Y thymic single-cell suspensions directly ex vivo and correlate these observations with the positive selection process. We found that there was considerably more ERK activity in wild-type 2C DP thymocytes compared to those from H-Y mice (Figure 4C). Furthermore, the finding that DP thymocytes from 2C mice deficient in class I MHC ligands (2C TAP1$^{-/-}$) fail to activate ERK demonstrates that this signaling is TCR mediated. These results also provide further support for the hypothesis that the efficiency of positive selection is determined in part by TCR signals and that stronger TCR signaling is associated with a higher degree of ERK activation. Furthermore, the differences in ERK activity between the two lines provide direct biochemical evidence in support of the hypothesis that the H-Y TCR signals less efficiently than the 2C TCR. Analysis of ERK activity in CD8 SP thymocytes (Figure 4C) indicated that, regardless of either the TCR expressed or the genotype, all CD8 SP thymocytes expressed a high degree of ERK activity. This same ERK activity is observed even for the few CD8 SP thymocytes from 2C TAP1$^{-/-}$ mice. Recently, it has been determined that the 2C TCR crossreacts with a nonclassical (class Ib) MHC class I ligand that is capable of mediating weak positive selection and the formation of CD8 SPs (Maurice et al., 2001). The fact that the scant number of CD8 SP thymocytes in TAP1$^{-/-}$ mice expresses active ERK indicates an intimate connection between this event and positive selection. Collectively, these data indicate that, regardless of the genotype or the mechanism by which positive selection occurs, ERK activity is a prerequisite for positive selection. They also reveal that ERK activation can occur via RasGRP1-dependent and -independent mechanisms.

Function of RasGRP1 in Mature T Cells

While it is clear that RasGRP1 plays an important role in the development of SP thymocytes, the role of RasGRP1 in the development and function of peripheral T cells has not been determined. To investigate the role of RasGRP1 in mature T cells, we examined the cell surface phenotype and functional responses of H-Y female and 2C CD8 T cells lacking RasGRP1. A reduction in the percentages of CD4$^-$CD8$^+$ T cells was found in both H-Y and 2C RasGRP1$^{-/-}$ mice (Figure 5A). The mutant mice also exhibited a similar CD8 T cell lymphopenia (percent of wild-type cell number for H-Y female and 2C mice, respectively: 31.4% ± 8.4 and 37.1% ± 12). However, correlated with the weaker signaling during positive selection, only CD8 T cells from H-Y mice dis-

played an immature cell surface phenotype, expressing higher levels of HSA and lower amounts of the CD8$\alpha\beta$ heterodimer (Figure 5B). We also noted that the vast majority of CD8 T cells from H-Y female mutant mice uniformly expressed high levels of the clonotypic H-Y TCR (Figure 5C). In contrast, only about one quarter of wild-type CD8 T cells express the clonotypic receptor (H-Y$^+$) whereas the other three quarters express endogenous TCRα chains paired with the Tg TCRβ chain (H-Y$^-$). Furthermore, when cell number comparisons are made between H-Y$^+$ and H-Y$^-$ cells, it becomes evident that the majority of CD8 T cell loss in mutant H-Y females results from the absence of the H-Y$^-$ cells (Figure 5D). In H-Y females, these clonotypic-negative cells are believed to be the product of peripheral expansion (Ernst et al., 1999; Rocha and von Boehmer, 1991). By contrast, H-Y TCR$^+$ CD8 T cells do not undergo homeostatic expansion in B6 female mice (Ernst et al., 1999). Thus, the deficiency of clonotypic-negative cells may be a direct consequence of these cells failing to undergo peripheral expansion. To investigate whether RasGRP1 plays a role in this process, we selected CD8 T cells expressing the 2C TCR since in B6 mice this receptor interacts with self-peptides/self-MHC with an appropriate avidity to undergo homeostasis-induced proliferation (Cho et al., 2000). Wild-type and mutant 2C CD8 T cells were labeled with the mitotic tracker CFSE, injected into sublethally irradiated recipient mice, and their proliferation was tracked 1 week later (Figure 5E). In stark contrast to wild-type cells, a large fraction of the adopted RasGRP1$^{-/-}$ T cells have failed to undergo a single division during this period. Collectively, these data suggest that the CD8 T cell lymphopenia in mutant H-Y and 2C animals is the result of both decreased peripheral expansion and reduced thymic production and/or export. Both of these processes are necessary to fully reconstitute the peripheral T cell pool (Scollay et al., 1980).

The finding that the CD8 SP thymocytes from 2C RasGRP1$^{-/-}$ possess an immature phenotype raises the issue of whether these cells have gone through the positive selection process. A major criterion for positive selection is responsiveness to cognate Ag stimulation. To determine the functionality of these cells, we challenged equivalent numbers of 2C thymic subpopulations, DP and CD8 SP, as well as peripheral 2C CD8 T cells with cognate Ag in vitro. Previous studies have shown that the SIYRYYGL (SYRGL) peptide binds strongly to K^b and this complex is recognized with high affinity by the 2C TCR (Udaka et al., 1996). By varying the concentration of the SYRGL peptide, we can measure the proliferative response as a function of Ag concentration. These studies demonstrated that mutant 2C T cells failed to incorporate [^{3}H]-thymidine when pulsed at 72 hr poststimulation (Figure 6A). Furthermore, this failure to proliferate was observed over a broad range of Ag concentration. Similar results were also observed for RasGRP1$^{-/-}$ T cells expressing the H-Y TCR (data not shown). However, the addition of exogenous IL-2 partially rescued the proliferative defect in both mutant SP thymocytes and peripheral T cells (Figure 6B). The poorer recovery by RasGRP1$^{-/-}$ thymic SP thymocytes is likely a consequence of their immaturity (Figure 2C). Despite this incomplete recovery, these data nevertheless pro-
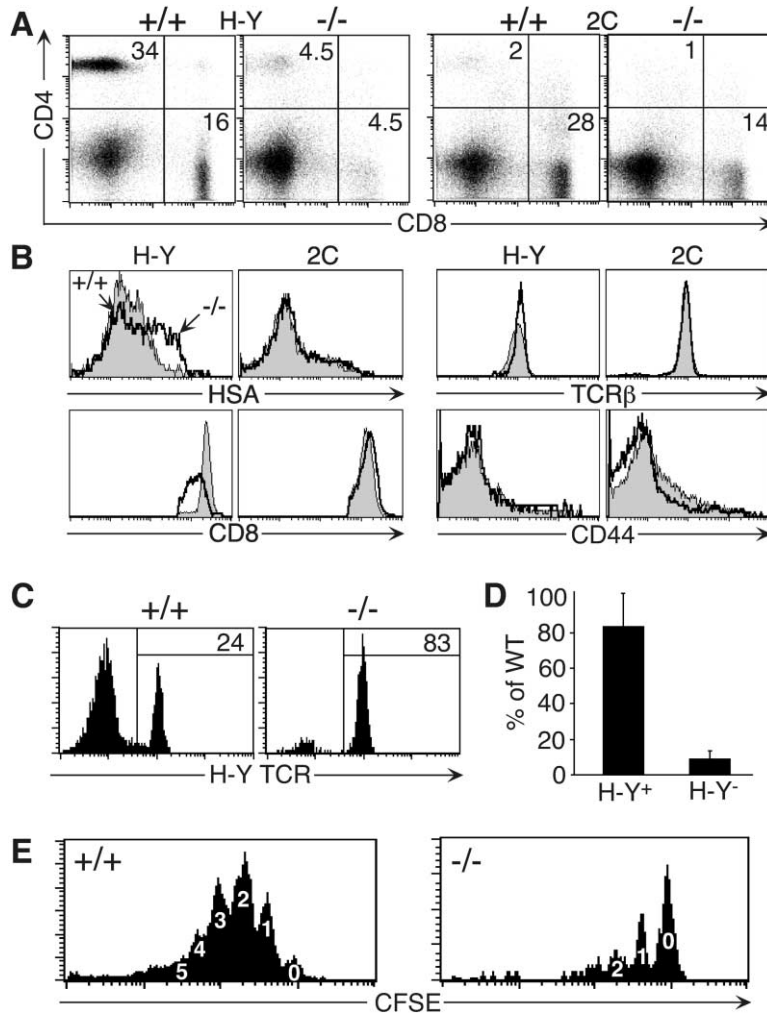
Figure 5. Decreased Numbers of Peripheral T Cells in RasGRP1 Mutant Mice Are Associated with a Defect in Peripheral Expansion

(A) Lymph node cells were stained with anti-CD4-PE and anti-CD8-TC and analyzed by flow cytometry.

(B) Expression of developmental markers on H-Y and 2C CD8$^+$ T cells that possess the clonotypic TCR.

(C) Expression of the clonotypic TCR on lymph node CD8 T cells from female H-Y TCR mice.

(D) Cell number comparisons of H-Y$^+$ and H-Y$^-$ CD8 T cells in mutant female H-Y TCR mice expressed as percent of wild-type. Error bars represent the SD.

(E) Defective homeostatic proliferation by 2C CD8 T cells lacking RasGRP1.

vide strong evidence that the SP thymocytes from RasGRP1$^{-/-}$ mice have undergone positive selection. As IL-2 does not completely restore Ag responsiveness, it suggests that RasGRP1 signaling may contribute to the production of this cytokine as well as other functions. Gain-of-function studies in the human Jurkat T cell line have implied that RasGRP1 signaling can lead to IL-2 production in peripheral T cells (Ebinu et al., 2000).

The acquisition of effector function by Ag-activated T cells is another stringent test for positive selection. Therefore, we determined whether Ag-stimulated SP thymocytes and T cells from RasGRP1$^{-/-}$ mice are able to differentiate into cytotoxic T cells. Purified SP thymocytes and peripheral T cells from mutant and wild-type 2C mice were activated with cognate Ag and IL-2 for 3 days and assessed for cytotoxicity against Ag-expressing (P815; L^{d+}) and syngeneic (EL-4; H-2^b) ^{51}Cr-labeled targets. Previous studies have shown that the 2C TCR recognizes allogeneic L^d molecules with high affinity (Udaka et al., 1993). We found that both wild-type and mutant Ag-activated T cells exhibited specific killer activity toward the P815 target (Figure 6C) but not toward the EL-4 line (data not shown). However, RasGRP1$^{-/-}$ thymic CD8 SP showed the greatest disparity with respect to its wild-type counterpart in this assay. Mutant

SP thymocytes were about 8-fold less efficient killers on a per cell basis than wild-type SP (20% specific lysis at 10:1 E/T ratio versus 1.25:1). In comparison, Ag-activated RasGRP1$^{-/-}$ peripheral T cells were 4 fold less efficient than wild-type (40% kill at 5:1 E/T ratio versus 1.25:1). These data provide further support for the conclusion that SP thymocytes and peripheral T cells from 2C RasGRP1$^{-/-}$ mice are the product of positive selection. They also demonstrate that RasGRP1 is necessary for the optimal proliferation and differentiation of Ag-activated CD8 T cells.

We next investigated whether RasGRP1-deficient T cells possessed the capacity to undergo normal early activation events upon TCR stimulation in the absence of exogenous IL-2. Rather than being refractory, mutant T cells blasted and upregulated the early activation markers CD69 and CD25 to a similar extent as wild-type (data not shown). To examine whether 2C CD8 T cells were also capable of proliferation in the absence of IL-2, we stimulated CFSE-labeled T cells with cognate Ag in vitro (Figure 6D). Samples were acquired on a FACScan for the same length of time, rather than an event count, under nonlimiting conditions and plotted on the same scale. These measures enable the CFSE profiles to also reflect cell numbers, rather than just cell division, pres-
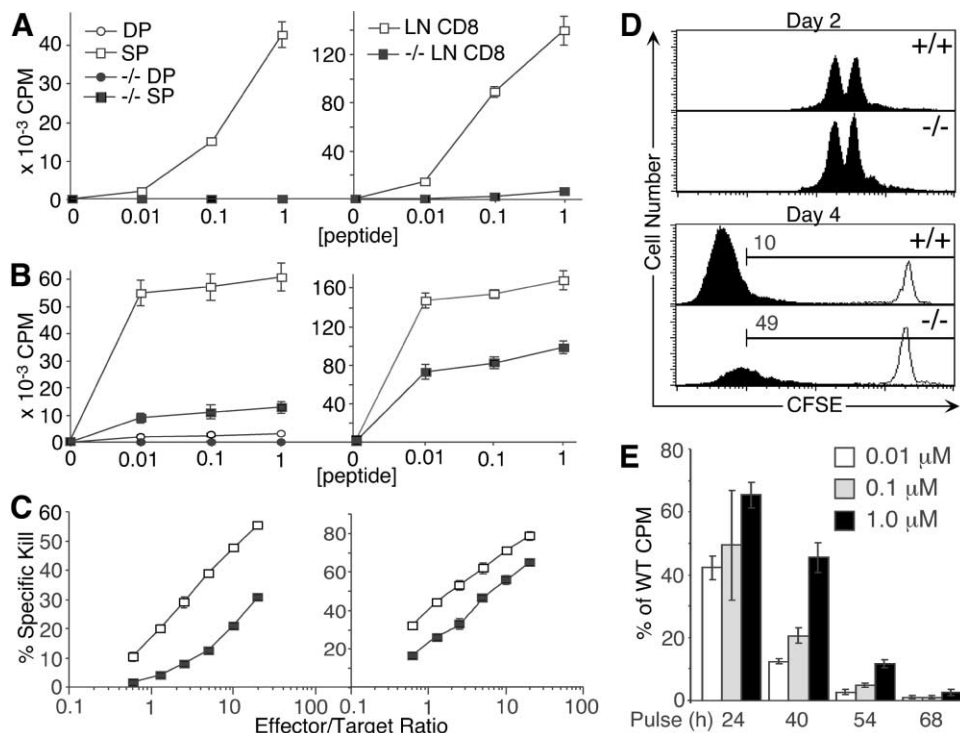
Figure 6. Ag-Stimulated Peripheral CD8 T Cells Lacking RasGRP1 Undergo Normal Early Activation Events and Acquire Cytolytic Function but Fail to Sustain Their Proliferation

(A and B) Purified 2C thymic subpopulations (left), DP and CD8 SP, and peripheral CD8 T cells (right) were stimulated with various doses of SYRGL peptide (μM) without (A) and with (B) the addition of exogenous IL-2.

(C) Mutant CD8 SP thymocytes and CD8 T cells possess reduced cytotoxic capacity.

(D) CFSE-labeled CD8 T cells stimulated with CD8-depleted B6 splenocytes in the presence of 0.1 μM SYRGL peptide. By gating on CD8$^+$ 2C TCR$^+$ cells (filled histogram), proliferation was tracked at 2 and 4 days poststimulation. Open scaled histogram represents fluorescence of nondividing cells (cultured in the absence of peptide).

(E) Ag-driven growth by mutant CD8 T cells ceases 68 hr poststimulation. Error bars represent the SD.

ent in the cultures. Surprisingly, RasGRP1$^{-/-}$ T cells underwent early proliferation and expansion almost identically to wild-type (Day 2; Figure 6D). However, analyses of mutant cultures at Day 4 revealed a smaller histogram and a large proportion of cells that have failed to completely lose their CFSE fluorescence, suggesting that proliferation may have ceased. To verify the conclusion from the CFSE experiment, parallel Ag-stimulated cultures, using unlabeled T cells, were pulsed for a 6 hr period at various times poststimulation (Figure 6E). Corroborating the CFSE data, RasGRP1-deficient T cells underwent proliferation at levels approaching wild-type, particularly at higher peptide concentrations, at early time points. However, regardless of peptide dose, mutant T cell proliferation almost completely ceased at 68 hr postchallenge with cognate Ag. The aborted proliferation observed in Ag-stimulated mutant CD8 T cells suggests for the first time that RasGRP1 plays a pivotal role in the differentiation of Ag-activated CD8 T cells.

Discussion

The molecular mechanisms that mediate T cell selection have been the subject of intensive investigation during the past decade. A central question in these studies is how the developing thymocyte chooses between a fate of cell survival or cell death, processes that are decided by interaction of the TCR with self-peptides/self-MHC ligands. Current experimental evidence favors the strength of signal hypothesis, which argues that weak signals lead to the selective activation of ERK MAPK and positive selection whereas strong signals lead to the activation of the full range of MAPK and negative selection. What is not clear from past studies is the mechanism by which ERK is activated during the positive selection process. By analyzing TCR Tg mice lacking RasGRP1, we have revealed important insights into its role in ERK activation and positive selection.

Two important regulators of Ras activation, RasGRP1 and Grb2/Sos, have been shown to function in developing thymocytes (Dower et al., 2000; Gong et al., 2001). Although these molecules may share some functional redundancy, the fact that reductions in Ras activation attributable to Sos and RasGRP1 have different functional consequences argues strongly that they have unique roles. In addition, there are a number of other RasGRP1-related molecules, RasGRP2 (Kawasaki et al., 1998), RasGRP3 (Lorenzo et al., 2001; Ohba et al., 2000), and RasGRP4 (Reuther et al., 2002), that might regulate Ras or Ras-like GTPases in thymocytes and T cells. All RasGRP family members share similar domain structures, implying that they are similarly regulated by DAG and calcium messenger. Since PMA-induced Ras activation is absent in RasGRP1$^{-/-}$ mice, this Ras GEF may

be the principal molecule of its type acting in thymocytes. Mice lacking RasGRP1 possess increased proportions of both DN and DP thymocytes (Dower et al., 2000). Since the most profound loss is of SP thymocytes, it demonstrates that the most critical role for RasGRP1 is during late thymic selection. Using weakly and strongly selecting TCRs to assess effects on thymic development, the present study revealed that weakly selecting TCRs display the greatest dependence on RasGRP1. In null animals, delayed positive selection was associated with a decrease in CD8 SP cell number and lowered levels of TCR and maturation markers. By contrast, the development of thymocytes expressing strongly selecting TCRs is much less dependent on RasGRP1. Although null thymi possessed normal CD8 SP numbers that express near wild-type levels of TCR, positive selection was still significantly delayed. Such CD8 SP cells appear immature with respect to cell surface expression of maturation markers, Ag responsiveness, and cytolytic capacity. This latter observation indicates that, while positive selection can occur via a RasGRP1-independent mechanism, it is less efficient.

Our analysis of ERK activation in RasGRP1$^{-/-}$ thymocytes revealed an obligatory role for ERK activation in positive selection. We noted in our studies that sustained ERK activation is associated with the formation of CD8 SP, regardless of whether positive selection is mediated by the H-Y or the 2C TCR or whether it occurs in RasGRP1-deficient or -sufficient animals. Therefore, these observations indicate that ERK can be activated by RasGRP1-dependent and -independent mechanisms. Our data are consistent with the hypothesis that relatively weak TCR signals are heavily dependent on RasGRP1 for ERK activation. These observations are also consistent with the view that stronger signaling may activate ERK via an alternative pathway, possibly involving Grb2/Sos. However, since Grb2/Sos also links TCR signals to JNK and p38 activation, leading to negative selection, this ERK activation pathway is potentially problematic for positive selection. It is conceivable that since ERK has a lowered threshold of activation (Gong et al., 2001), Grb2/Sos signaling could induce positive selection by selectively activating ERK but not JNK and p38 kinases. In non-TCR Tg RasGRP1$^{-/-}$ animals, SP thymocytes are produced in low numbers (Dower et al., 2000). It is likely that these rare SP cells are selected by mechanisms that are similar to the selection of 2C TCR$^+$ CD8 T cells, i.e., they utilize relatively strongly selecting TCRs and a RasGRP1-independent mechanism for their selection. More importantly, the rarity of SP thymocytes in RasGRP1$^{-/-}$ animals suggests that positive selection via RasGRP1-independent mechanism applies only to a small subset of T cells in normal mice. Interestingly, the formation of $\gamma\delta$ T cells in mutant mice does not appear to be affected. While 4- to 6-week-old mutant mice possess only about 10% of the $\alpha\beta$ T cell number of wild-type, their $\gamma\delta$ T cell numbers are modestly elevated. This phenomenon may be a result of such cells employing alternative signaling molecules and/or a reflection of the distinct mechanisms utilized for their development and peripheral maintenance (Hayday, 2000).

In sharp contrast to mice hemizygous for Grb2, RasGRP1 deficiency did not influence negative selection either in vivo or in vitro. Correlated with normal negative

selection, the induction of JNK and p38 MAPKs were unaltered in null animals. To examine effects of RasGRP1 signaling, we treated thymocytes with PMA to selectively activate Ras through this Ras GEF. Despite the lack of Ras activity upon PMA treatment in RasGRP1-deficient thymocytes (Dower et al., 2000), activation of JNK and p38 occurs at levels resembling wild-type whereas ERK activation is virtually eliminated. Therefore, the activation of both JNK and p38 by PMA is independent of RasGRP1-mediated signaling and, most probably, Ras activation. The fact that RasGRP1 does not influence JNK and p38 activation implies that its actions are limited to positive selection. In contrast, TCR-mediated signaling in Grb2 heterozygotes is associated with decreases in Ras, JNK, and p38 activation (Gong et al., 2001). Therefore, the Grb2/Sos complex may pair Ras activity with different effectors than RasGRP1. Alternatively, Grb2's effect on JNK and p38 signaling may be Ras independent, resulting from either Grb2 interacting with a signaling molecule other than Sos or Sos interacting with some molecule other than Ras.

Analysis of mutant 2C peripheral T cells led to the discovery of hitherto unidentified functions of RasGRP1 in peripheral T cell homeostasis and naive CD8 T cell differentiation. Naive T cells mimic thymocytes in their need for contact with self-MHC molecules to generate low level TCR signaling and prevent death by neglect (Surh and Sprent, 2000). As the affinity/avidity of the ligands responsible for positive selection resembles the strength of ones that drive homeostatic proliferation of peripheral T cells (Surh and Sprent, 2000), it is conceivable that RasGRP1 may also be involved in this process. The finding that RasGRP1 null 2C CD8 T cells exhibit impaired homeostatic proliferation imply that RasGRP1 plays an important role in transducing low-grade TCR signals necessary for peripheral maintenance. Remarkably, RasGRP1 null CD8 T cells undergo early activation events and proliferation similar to wild-type T cells. However, this Ag-driven growth is completely aborted by 3 days poststimulation. Recent studies indicate that, after initial TCR engagement, naive CD8 T cells undergo an Ag-independent phase of a developmental program, composed of at least seven rounds of proliferation and differentiation into effector and memory T cells (Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2001). The fact that mutant T cells enter the cell cycle and proliferate upon initial Ag encounter demonstrates that these early events mediated by strong TCR signals are independent of RasGRP1. Therefore, RasGRP1 plays a fundamental role in the developmental programming of Ag-activated CD8 T cells by providing signals necessary for survival, sustaining proliferation, and differentiation of naive CD8 T cells, rather than being essential for the formation of effector T cells. It will be interesting to see whether other RasGRP family members serve a similar function, acting as a molecular cruise control by providing steady signals, in the differentiation of various cell types and/or tissues.

Experimental Procedures

Mice

Breeders for the H-2^b 2C TCR-Tg mice were kindly provided by Dr. Dennis Loh (then at Washington University, St. Louis). The H-Y and 2C TCR Tg mice were bred onto the C57BL/6 (H-2^b) background.

RasGRP1-deficient mice were bred onto C57BL/6 background at least six generations prior to mating with TCR Tg mice. C57BL/6-*Tcrα*$^{-/-}$, C57BL/6-*Tap1*$^{-/-}$, and C57BL/6-*RAG1*$^{-/-}$ mice were obtained from Jackson Laboratories. All studies followed guidelines set by the Canadian Council on Animal Care.

Flow Cytometry
Antibodies against H-Y TCR-PE, CD4-PE, CD44-PE, and CD69-PE were purchased from eBioscience except for CD8-Tricolor (Caltag). The 1B2 hybridoma, expressing the anti-2C TCR antibody, was obtained from Dr. Herman Eisen. For flow cytometric determination of ERK activation, a previously described method (Chow et al., 2001) was modified to allow labeling in 96-well format. In brief, cells were activated in a 200 μl volume, fixed by the addition of 50 μl of 10% formaldehyde, and incubated for 10 min at 37°C. After centrifugation, thymocytes were resuspended in 100 μl of ice-cold methanol and chilled for 30 min on ice prior to antibody processing. Permeabilized cells were incubated for 20 min at RT with anti-p-ERK1/2 (Cell Signaling Technology #9101). Bound antibody was detected with anti-rabbit Ig F(ab')$_2$-PE (Jackson ImmunoResearch Laboratories #711-116-152). After p-ERK1/2 staining, cell surface labeling with anti-CD4-FITC and -CD8-TC was carried out by incubation on ice for 20 min. Data was acquired using a FACScan and Cellquest software (BD Bisosciences) and analyzed with FCSPress software (www.fcspress.com).

In Vitro and In Vivo Assays of Mature T Cell Function
Thymic DP and SP cells were purified by cell sorting using a BD FACSVantage. Peripheral CD8 T cells were positively selected by magnetic separation using MiniMACS (Miltenyi Biotec). Ten thousand cells were stimulated with 1 × 10^6 B6 splenocytes and the indicated concentration of the SYRGL peptide. To assess proliferation, cultures stimulated with Ag for 72 hr were pulsed with 1 μCi of [^{3}H]-thymidine for a 6 hr period. To generate cytotoxic effectors, purified 2C thymic SP and peripheral CD8 T cells were stimulated for 3 days with 0.1 μM SYRGL-loaded syngeneic splenocytes in the presence of exogenous IL-2. CTL assays, CFSE labeling, and adoptive transfers were set up as previously described (Priatel et al., 2001).

Immunoblotting and Cell Signaling Studies
Cell sorting, using a FACSVantage (BD Biosciences), was used to purify SP thymocytes. Total RasGRP1 was detected using m199 monoclonal antibody (Santa Cruz Biotec). For activation, thymocytes were stimulated with indicated concentrations of PMA for 2 min. Cells were pelleted and lysed in 10 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.1% SDS, and protease and phosphatase inhibitors. Blots were developed using ECL system (Amersham). Antibodies used for immunoblotting that recognize activated and total ERK, JNK, and p38 were purchased from Cell Signaling Technologies.

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