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RasGRP1 and RasGRP3 Regulate B Cell Proliferation by Facilitating B Cell Receptor-Ras Signaling¹

Jason J. Coughlin,* Stacey L. Stang,* Nancy A. Dower,[†] and James C. Stone^{2*}

The RasGRPs are a family of Ras activators that possess diacylglycerol-binding C1 domains. In T cells, RasGRP1 links TCR signaling to Ras. B cells coexpress RasGRP1 and RasGRP3. Using *Rasgrp1* and *Rasgrp3* single and double null mutant mice, we analyzed the role of these proteins in signaling to Ras and Erk in B cells. RasGRP1 and RasGRP3 both contribute to BCR-induced Ras activation, although RasGRP3 alone is responsible for maintaining basal Ras-GTP levels in unstimulated cells. Surprisingly, RasGRP-mediated Ras activation is not essential for B cell development because this process occurs normally in double-mutant mice. However, RasGRP-deficient mice do exhibit humoral defects. Loss of RasGRP3 led to isotype-specific deficiencies in Ab induction in immunized young mice. As reported previously, older *Rasgrp1*^{-/-} mice develop splenomegaly and antinuclear Abs as a result of a T cell defect. We find that such mice have elevated serum Ig levels of several isotypes. In contrast, *Rasgrp3*^{-/-} mice exhibit hypogammaglobulinemia and show no signs of splenomegaly or autoimmunity. Double-mutant mice exhibit intermediate serum Ab titers, albeit higher than wild-type mice. Remarkably, double-mutant mice exhibit no signs of autoimmunity or splenomegaly. B cell proliferation induced by BCR ligation with or without IL-4 was found to be RasGRP1- and RasGRP3-dependent. However, the RasGRPs are not required for B cell proliferation per se, because LPS-induced proliferation is unaffected in double-mutant mice. *The Journal of Immunology*, 2005, 175: 7179–7184.

The recognition of Ag by immune receptors initiates the activation of mature T and B cells during the adaptive immune response. At various stages in the life of a lymphocyte, the effects of Ag receptor stimulation are modified in important ways by contingent signaling through other membrane receptor systems, including those that facilitate T cell-B cell communication (1–5). Depending on the nature of Ag-receptor engagement and additional contextual signaling cues, TCR and BCR signaling can be associated with cell division, survival, maturation, activation or tolerance. However, the molecular events downstream of immune receptor engagement have not been fully defined. Furthermore, the contributions of individual intracellular signaling events to the function of the immune system remain obscure.

Both TCR and BCR signaling have been linked with the engagement of similar sets of intracellular signal transducers including protein tyrosine kinases, tyrosine phosphorylated adaptors, and phospholipase C (PLC)³ (6). The latter enzyme cleaves membrane phosphatidylinositol to generate diacylglycerol (DAG) and calcium messengers. Traditionally, the effects of DAG signaling have been attributed to the activation of the protein kinase C (PKC) family members (7).

Another protein implicated in immune receptor signaling is Ras, a small membrane-bound GTPase. Conversion of Ras-GDP to Ras-GTP leads to stimulation of various downstream pathways such as the Raf-Mek-Erk kinase cascade, with ensuing changes in transcription and other cellular responses. Early studies suggested that Ras in lymphocytes was regulated by PKC-mediated down-regulation of Ras GTPase activating proteins (8), or by membrane recruitment of Sos (9–11), a Ras guanyl nucleotide exchange factor (Ras GEF). We proposed that upon TCR engagement, PLC- γ -1 activation and DAG accumulation led to activation of RasGRP1, a Ras GEF (12, 13). RasGRP activation is controlled both by membrane recruitment through its DAG binding C1 domain (14–17) and by DAG responsive kinases (18–21).

Strongly supporting this model, thymocytes from *Rasgrp1*^{-/-} mice are defective in TCR- and DAG-induced activation of Ras-Erk signaling (13). Furthermore, young mutant mice exhibit a defect in positive selection as evidenced by a deficiency of single-positive thymocytes and peripheral T cells (13, 22). Notably, however, aged mice that lack RasGRP1 develop a paradoxical autoimmune disorder typified by serum autoantibodies, including antinuclear Abs reminiscent of those seen in systemic lupus erythematosus (23). Hallmarks of this murine syndrome are pronounced splenomegaly and lymphoproliferation, bearing in mind that this latter term is used to denote an abnormal increase in lymphocyte populations that occurs by a combination of increased multiplication and/or decreased cell death. The relatively rare thymocytes that do develop into T cells in the absence of RasGRP1 are activated and self-reactive. These T cells were proposed to lead secondarily to the B cell dysfunction in aged *Rasgrp1*^{-/-} mice.

B cells express both RasGRP1 and RasGRP3 (18–20, 24, 25). Experiments with chicken DT40 cells have shown that RasGRP3 plays a key role in linking BCR to Ras, whereas RasGRP1 and Sos proteins play insignificant roles in this process (25). To study RasGRP3 function in the context of the mammalian immune system, we generated *Rasgrp3*^{-/-} mice and studied their B cell phenotypes. *Rasgrp1*^{-/-} and *Rasgrp1*^{-/-}*Rasgrp3*^{-/-} mice were also studied.

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² Address correspondence and reprint requests to Dr. James C. Stone, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. E-mail address: jim.stone@ualberta.ca

³ Abbreviations used in this paper: PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; GEF, guanyl nucleotide exchange; KLH, keyhole limpet hemocyanin; TNP, 2,4,6, trinitrophenyl.

Materials and Methods

Mice

Rasgrp3 mutant mice were derived on a 129/J and C57BL/6J (B6) hybrid background essentially as previously described for *Rasgrp1* mutant mice (13). Briefly, a neo cassette was inserted into the *BstX1* site in the second exon of *Rasgrp3* in NX1 ES cells, interrupting the coding sequence downstream of codon 40 in the Ras exchange motif (REM box) of the catalytic region. *Rasgrp3* null mice were viable, fertile, and lacked any overt phenotype. We previously reported that homozygous *Rasgrp1* null mice displayed reduced viability in this background (13), but such an effect has not been evident in subsequent work. In crosses between *Rasgrp1*^{-/-} and *Rasgrp3*^{-/-} mice on a mixed background, single and double homozygous mice were observed in approximately expected Mendelian ratios. Age- and sex-matched siblings from multiple matings were compared to randomize the effect of genetic background and minimize the effect of environment. The genotypes of all homozygous null mutant mice were determined by PCR. Genotyping results were verified by immunoblotting protein extracts with RasGRP1 and RasGRP3 Abs. Mice were bred under viral Ab-free conditions. All animal studies were done according to protocols approved by The Health Sciences Animal Policy and Welfare Committee at the University of Alberta, in accordance with Canadian Council on Animal Care Guidelines.

Reagents and Abs

PMA was purchased from Sigma-Aldrich. LPS was purchased from Sigma-Aldrich. Goat anti-mouse anti-IgM was from Southern Biotechnology Associates, hamster anti-mouse CD40 (HM40-3) was from BD Biosciences, recombinant mouse IL-4 was from BioSource International, anti-phosphotyrosine (4G10) and pan-Ras Ab (RAS10) were from Upstate Biotechnology, and the phospho-Erk Ab (9101) was from Cell Signaling Technology. Abs directed against RasGRP1 and RasGRP3 have been previously described (20, 26). CFSE (no. C1157) was from Molecular Probes. The reagents 2,4,6-trinitrophenyl (TNP)-BSA, TNP-LPS, and TNP-keyhole limpet hemocyanin (KLH) were purchased from Biosearch Technologies.

Cell enrichment and immunofluorescence

B cells and T cells were enriched by negative selection of undesired splenocytes, using B cell or pan T cell isolation kits, respectively, as per manufacturer's instructions (Miltenyi Biotec). This resulted in cell purities of 90–98% as determined by flow cytometry. B cell isolation kits remove cells expressing CD43 (several non-B cell types, activated B cells, plasma B cells, B1 cells), CD4 (T cell subset), and Ter¹¹⁹ (erythrocytes). B cells isolated by this method were either IgM⁺ or IgM^{low}, and all were lacking CD69 plus CD138 expression (data not shown). Antinuclear Abs were detected on HEP-2 slides (Bio-Rad) using serum samples from 3- to 6-month-old mice at a 1/50 dilution.

Signaling assays

For signaling experiments, splenic B cells from 5- to 6-wk-old mice were incubated at 37°C for 30 min in serum-free RPMI 1640 after enrichment at 0–4°C. Equal numbers of cells were then treated for indicated times with either 100 nM PMA or 20 µg/ml anti-IgM. Cells were then recovered by centrifugation and lysed as described previously (20). The Ras activation assay has been described previously (20). Lysates were run on SDS-PAGE, transferred to a filter and probed with the indicated Abs.

Flow cytometry

Single-cell suspensions of splenocytes were stained with fluorescent-labeled Abs (BD Biosciences) and analyzed by flow cytometry using a BD Biosciences FACSCalibur or FACSCanto to acquire the data and then analyzed using Flowjo software (Tree Star).

Immunizations and ELISA

To determine immune responses, mice were immunized by i.p. injection with 100 µg of TNP-KLH (T cell-dependent) or 20 µg of TNP-LPS (T cell-independent type I) in 100 µl of PBS on days 0 and 14. Mice were bled before the first immunization (day 0) as well as on days 7, 14 (before secondary immunization), 21, and 28. For aged basal serum Ab titers, 3- to 6-month-old mice were bled. IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 were quantified using SBA Clonotyping System/HRP (Southern Biotechnology Associates) according to the manufacturer's instructions. IgE was quantified using BD OptEIA mouse IgE ELISA set according to the manufacturer's instructions. For total Ab levels, plates were coated with goat anti-mouse Ig capture Ab, whereas for TNP-specific Ab levels, plates were

coated with 10 µg/ml TNP-BSA. Values were obtained by serially diluting samples and using dilutions that fell within a standard curve using isotype-specific controls (Southern Biotechnology Associates).

Proliferation assays

For CFSE-labeling, B cells, or CD4⁺ T cells from the spleens of 6-wk-old mice, were labeled according to the originally published method (27). B cells were cultured at 10⁵ cells in 1 ml of medium with various combinations of the following: 20 µg/ml anti-IgM, 10 ng/ml IL-4, 10 µg/ml anti-CD40, and 20 µg/ml LPS. Following 4 day treatments, cells were analyzed by flow cytometry. For all CFSE-labeling experiments, equal numbers of cells were analyzed, although flow cytometry histograms are shown with variable axes because of the reduced viability of double-knockout B cells. Average number of cell divisions and the percentage of divided cells were calculated by analyzing flow cytometry results using the Flowjo proliferation platform.

BrdU labeling was used to detect S phase cells. Splenic B cells were allowed to proliferate in medium alone or in combination with the indicated stimulus. After 3 days incubation, cells were exposed for 1 h to BrdU in culture medium. Cells were then fixed, permeabilized, and stained with anti-BrdU and 7-aminoactinomycin D following the manufacturer's instructions (BD Biosciences). Cells were then analyzed by flow cytometry.

Results

RasGRP1 and RasGRP3 link BCR to Ras-Erk

We first performed immunoblotting to determine whether both RasGRP1 and RasGRP3 are expressed in splenic B cells, as they are in several B cell lines (20, 24, 25). As expected, wild-type B cells express both RasGRP1 and RasGRP3 (Fig. 1A). In contrast, splenic T cells express only RasGRP1. These species are missing in extracts from the corresponding null mutant mice. Furthermore, the expression level of one RasGRP species is not affected by the status of the other. Notably, T cells express substantially more RasGRP1 than do B cells. RasGRP2 is a Rap activator whereas RasGRP4 expression has been documented only in mast and myeloid tumor cells (28–31), so these family members were not studied.

Because *Rasgrp1*^{-/-} mice develop a late onset autoimmune disorder associated with splenomegaly (23), we used splenic B cells from mice <6 wk of age to assess the contribution of RasGRP1 and RasGRP3 to Ras-Erk signaling. Treatment of wild-type B cells with the DAG analog PMA resulted in rapid accumulation of Ras-GTP and phosphorylated Erk (Fig. 1B). Splenic B cells from either *Rasgrp1*^{-/-} or *Rasgrp3*^{-/-} mice also exhibited robust activation of Ras-Erk signaling in response to PMA. In contrast, B cells from double-mutant mice displayed no Ras activation and a weaker phospho-Erk signal in response to PMA stimulation. Thus, both RasGRP species function as DAG-responsive Ras activators in splenic B cells.

In parallel cultures, treatment with soluble anti-IgM Abs was used to simulate Ag-BCR ligation. Compared with PMA-treated cells, activation of Ras and Erk was weaker (Fig. 1B). Relative to wild-type cells, *Rasgrp1*^{-/-} B cells showed slightly weaker Ras and Erk activation. Interestingly, *Rasgrp3*^{-/-} B cells exhibited undetectable basal Ras-GTP levels as well as defective BCR-induced Ras activation. At later time points, the activation of Erk was intermediate in each mutant compared with wild-type cells and totally absent in the double-mutant cells. Taken together these results indicate that, although each RasGRP species studied has a distinct role in regulating Ras in splenic B cells, there is also some functional redundancy.

We examined the level of total cell protein tyrosine phosphorylation as a proxy for early BCR signaling events. Numerous phosphorylated proteins were observed after BCR stimulation but these were not significantly affected by genotype (Fig. 1C). These results indicate that early BCR signaling events are not influenced by RasGRP activity. They support the argument that the defects in

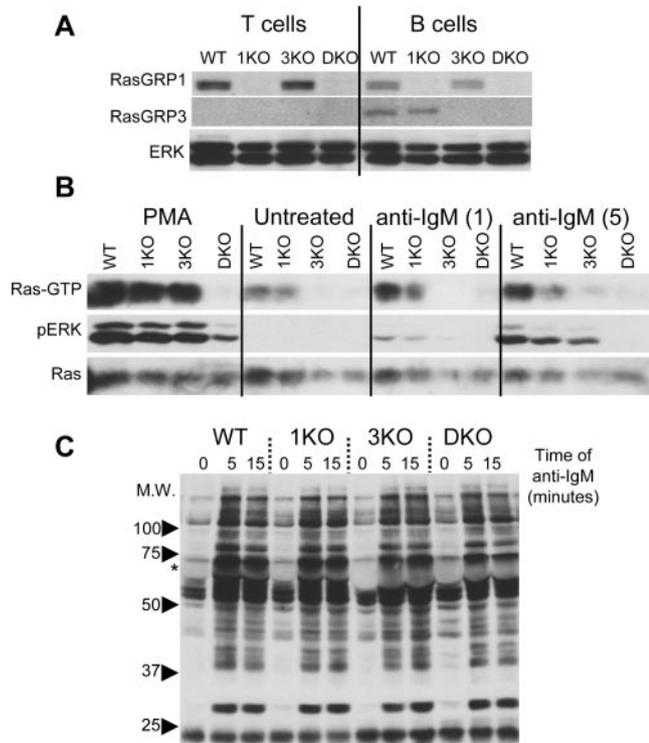


FIGURE 1. Role of RasGRP1 and RasGRP3 in BCR signaling. *A*, Splenic T and B cells were prepared from mice of the indicated genotypes, and total cell lysates were subjected to immunoblotting with anti-RasGRP1, anti-RasGRP3, and anti-Erk (loading control) Abs. Genotypes were as follows: wild-type (WT), *Rasgrp1*^{-/-} (1KO), *Rasgrp3*^{-/-} (3KO), and *Rasgrp1*^{-/-}*Rasgrp3*^{-/-} double mutant (DKO). *B*, Enriched splenic B cells from 6-wk-old mice of indicated genotype were left untreated, treated with PMA for 10 min or with anti-IgM for 1 or 5 min, as indicated. Cell lysates were assayed for Ras-GTP, phospho-ERK, and total Ras. Results are representative of three experiments. *C*, Purified splenic B cells from 6-wk-old mice of indicated genotype were left untreated, or treated with anti-IgM, as indicated. Cell lysates were probed for phosphotyrosine. Molecular mass markers (M.W.) are shown on left. A zone of distortion (*) in the gel that arose from BSA contained in the B cell isolation buffer is indicated. Results are representative of three experiments.

Ras and Erk activation as we previously referred to are specific, direct effects of reduced Ras GEF activity. Furthermore, from the equivalent phosphotyrosine responses, one might argue that these cell populations derived from the four different genotypes are functionally comparable in terms of early BCR signaling events.

RasGRP3 deficiency decreases humoral function and suppresses *Rasgrp1*^{-/-} autoimmunity

To determine the biological consequences of decreased Ras activation due to RasGRP1 and RasGRP3 loss, we examined several immunological parameters in mice of various genotypes and ages. As reported previously (23), *Rasgrp1*^{-/-} mice develop splenomegaly at 2–3 mo of age (Fig. 2*A*). This phenotype apparently arises from a defect in T cell development, which leads to lymphoproliferation and autoimmunity. Splenomegaly was never seen with *Rasgrp3*^{-/-} mice. Surprisingly, splenomegaly was also never observed in aged double-mutant mice. The suppression of the *Rasgrp1*^{-/-} lymphoproliferative phenotype by the *Rasgrp3* null mutant was also apparent from the numbers of cells recovered per spleen (Fig. 2*B*).

Serum Ig levels were studied to assess the B cell function in aged mice of each genotype. As expected from previous studies,

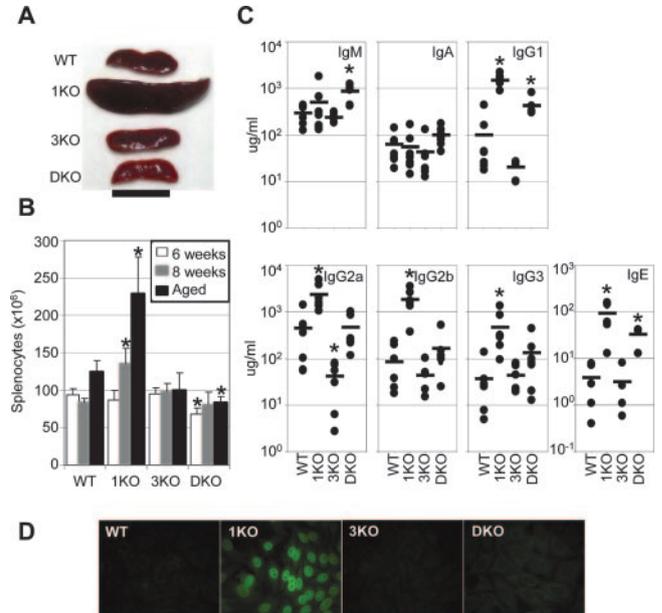


FIGURE 2. Loss of RasGRP3 expression results in isotype-selective hypogammaglobulinemia, but suppresses autoimmunity associated with *Rasgrp1*^{-/-}. *A*, Spleens from 4-mo-old mice are compared. Bar indicates 1 cm. *B*, Single-cell suspensions were prepared from the spleens of 6-wk-old, 8-wk-old, and 3- to 6-mo-old (“aged”) mice, and cells were counted. *C*, Sera were collected from aged mice, and Ab isotype concentrations were determined. Each individual mouse (●) is represented and a bar represents the mean. *D*, Sera from representative mice in *C* were tested for antinuclear Abs using HEp-2 cells. Data are representative of five experiments. Genotypes are indicated as in Fig. 1. Statistical difference (*, $p < 0.05$) is determined from wild-type mice using the Student’s *t* test.

aged *Rasgrp1*^{-/-} mice exhibited elevated titers of most Ig isotypes (Fig. 2*C*). These Abs included species that gave a strong antinuclear staining pattern (Fig. 2*D*). In contrast, sera from *Rasgrp3*^{-/-} mice exhibited notably lower titers of IgG1 and IgG2a. Furthermore, antinuclear Abs were not evident. Double-mutant mice also lacked antinuclear Abs, consistent with the hypothesis that *Rasgrp3*^{-/-} acts to suppress the *Rasgrp1*^{-/-} lymphoproliferative and autoimmune phenotype. Intriguingly, Ig titers of double-mutant mice were higher than those of *Rasgrp3*^{-/-} single mutants (Fig. 2*C*).

B cell development does not require RasGRP1 or RasGRP3

We used surface marker analysis to determine whether a lack of BCR-induced Ras activation in double-mutant mice caused a defect in B cell development. Previously, Nagaoka et al. (32) demonstrated that expression of an inhibitory form of Ras during early B cell development negatively influenced cell survival. In the spleen, immature B cells (T1) pass through an intermediate stage (T2) before becoming mature cells (M). We found that peripheral B cell development occurs largely normally in the absence of RasGRP1 and/or RasGRP3, both in terms of total B cells per spleen and the relative ratios of B cells in T1, T2, and M stages (Fig. 3, *A* and *B*), with the variability between genotypes being less than that found within a given genotype. One consistent variation was an increase in the proportion of otherwise rare CD138⁺ cells in *Rasgrp1*^{-/-} mice, indicating an expanded population of plasma cells (Fig. 3*C*). Double-mutant mice always had relatively lower levels of these cells, further confirming that *Rasgrp3*^{-/-} can act to suppress the syndrome associated with aged *Rasgrp1*^{-/-} mice. The increased CD138⁺ population was generally observed in

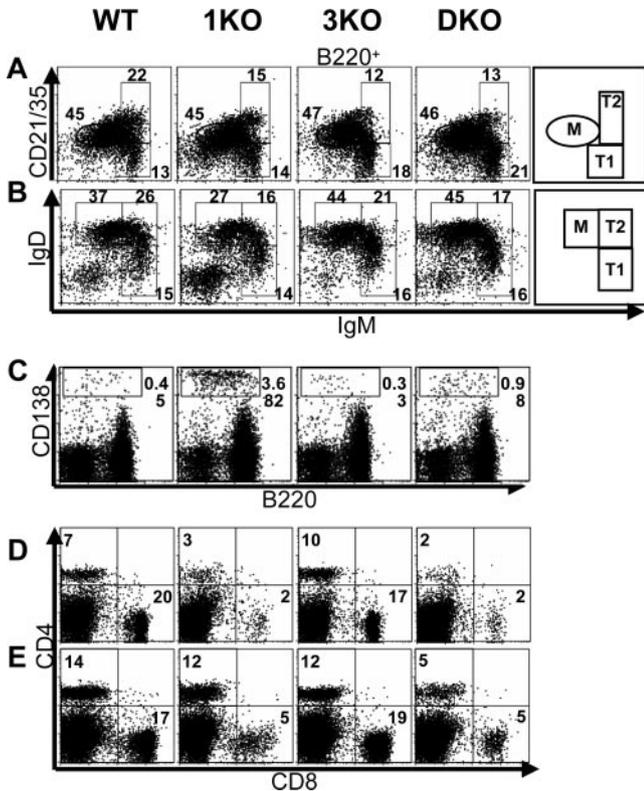


FIGURE 3. B cell development is unaffected by the absence of RasGRP1 and/or RasGRP3. Total splenocytes from 6-wk-old (A–D) or 4-mo-old (E) mice were stained with Abs as indicated. A and B, Results were obtained by gating on live B220⁺ cells. Populations are identified according to the standard B cell classification scheme (see *Materials and Methods*). C–E, Results of gating total live splenocytes are plotted. Values (*inset*) indicate the percentage of total cells displayed in a particular region. Values (*bottom*, C) indicate the total number of cells per spleen $\times 10^5$. Genotypes are indicated as described in Fig. 1.

6-wk-old *Rasgrp1*^{-/-} mice and therefore presages the development of the late-onset autoimmunity.

As reported previously, young *Rasgrp1*^{-/-} mice have very few T cells, reflecting defective positive-selection in the thymus (13). The small peripheral T cell population expands in older mice to form an autoreactive CD4⁺ T cell population that is implicated in the autoimmune disease (23). *Rasgrp3*^{-/-} mice have normal numbers of CD4⁺ and CD8⁺ splenic T cells, consistent with our observation that T cells do not express this protein (Fig. 3, D and E). Notably, older double-mutant mice have a lower proportion of CD4⁺ T cells than do the lymphoproliferative *Rasgrp1*^{-/-} mice of a similar age.

RasGRP deficiencies affect humoral responses to specific immunogens

We used the hapten-carrier system to determine the consequences of defective BCR-Ras signaling on Ab responses following immunization of our mutant mice. T cell-dependent Ags require T cell help to generate an effective B cell response. The immunogen is a hapten conjugated to a protein. T cell-independent type I Ags contain a polyclonal activator of B cells such as LPS conjugated to a hapten (33).

Compared with wild-type mice, *Rasgrp1*^{-/-} mice were able to generate similar levels of hapten-specific Abs in the T cell-dependent protocol (Fig. 4). Depending on the Ig isotype, *Rasgrp1*^{-/-} mice produced equal or even greater titers in the T cell-independent responses. In particular, IgG1 and IgG2a levels were higher in

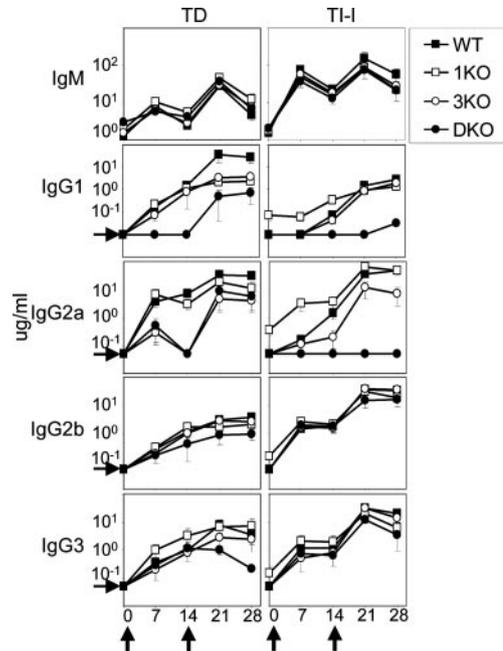


FIGURE 4. Impaired humoral immune responses in mutant mice. Six-week-old mice of the indicated genotype were immunized with either TNP-KLH (T cell-dependent; TD) or TNP-LPS (T cell-independent type I; TI-I) on days 0 and 14 (indicated with vertical arrows). Serum samples were collected on days indicated and tested for hapten-specific Abs of the given isotypes. Values are the mean of three to six mice, and error bars represent the SEM. Horizontal arrows represent value below level of detection. Genotypes are indicated as described in Fig. 1.

the *Rasgrp1*^{-/-} mutant, possibly reflecting a constitutive T cell influence that is not normally elicited by this injection protocol.

Rasgrp3^{-/-} mice were able to generate Ab responses in both immunization protocols, although they were not as strong as in wild-type mice in several cases. In particular, IgG2a titers were low in both protocols. As expected from our signaling results, double-mutant mice exhibited a more severe defect in Ab response compared with either single mutant, although the effect was again remarkably isotype specific. Notably, the double mutant exhibited the greatest defect in the T cell-independent type I induction of IgG1 and IgG2a. In contrast, the *Rasgrp1*^{-/-} single mutant showed an increased response in these latter two assays.

RasGRPs are required for BCR-induced proliferation *in vitro*

Proliferation of B cells is required for Ig class switching and differentiation into mature Ab secreting plasma cells (34). Furthermore, T cells that lack RasGRP1 exhibit reduced proliferative responses (22, 23). Accordingly, we examined the roles of RasGRP1 and RasGRP3 in B cell proliferation induced by various stimuli. B cells prepared from young mice were labeled with CFSE and then cultured with or without stimulation for 4 days. Flow cytometry was used to monitor dilution of cellular label, providing a measure of both the percentage of cells that had divided and the average number of cell divisions.

Treatment with either anti-IgM Abs or IL-4 alone was ineffective at inducing proliferation, whereas costimulation with both agents induced substantial proliferation of wild-type B cells (Fig. 5A). This response was largely intact in RasGRP1 single-mutant B cells, more dramatically affected by the loss of RasGRP3, and totally absent in the double-mutant cells. The results directly parallel those of our biochemical analysis of BCR-Ras-Erk signaling.

When B cells were stimulated with anti-CD40 Abs, a marked defect was evident in *Rasgrp3*^{-/-} cells, while double-mutant cells

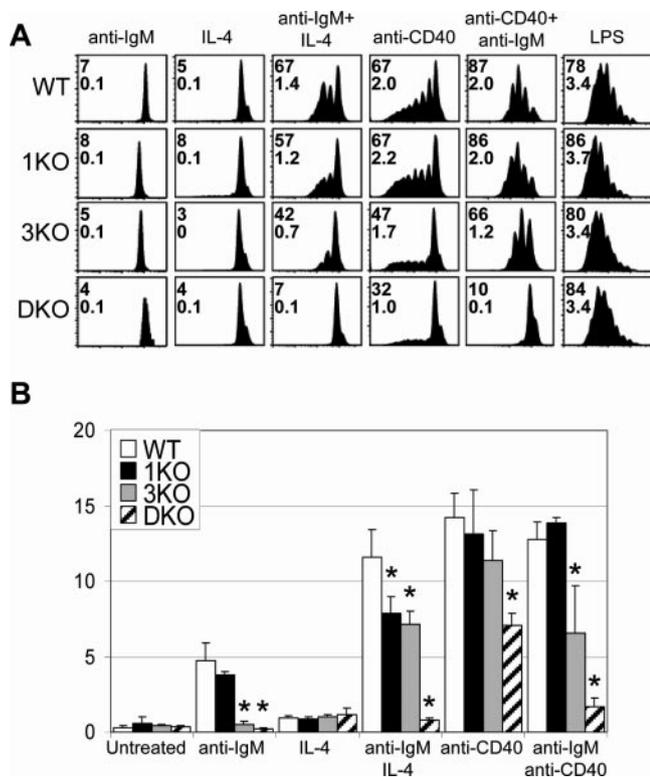


FIGURE 5. Impaired proliferation in the absence of RasGRP1 and RasGRP3 expression. *A*, B cells were labeled with CFSE and then treated for 4 days as indicated. Cells were then analyzed by flow cytometry. The value (*inset*) for the percentage of cells that divided at least once (*top left corner*) and the average number of cell divisions (*bottom left corner*) are indicated for each sample. B cell genotypes are indicated on left as described in Fig. 1. *B*, B cells were treated for 3 days as indicated, followed by BrdU incorporation, staining, and flow cytometry analysis. The percentages of B cells that were in S phase after various treatments were determined by flow cytometry. Error bars represent SEM. Statistical difference (*, $p < 0.05$) from wild-type cells using the Student's *t* test is indicated. Genotypes are indicated as described in Fig. 1.

were more severely affected (Fig. 5*A*). This protocol is thought to mimic direct stimulation of B cells by contact with T cells bearing CD40L. Unlike the situation with IL-4 treatment, significant mitogenic activity was seen without BCR costimulation. Indeed, costimulation with IgM decreased CD40-mediated cell division. When we examined responses to the B cell mitogen LPS, all four genotypes responded well. Thus, RasGRP1 and RasGRP3 are not required for B cell division per se.

The CFSE-labeling experiments revealed the net effect of proliferative stimulation over 4 days. To verify these findings with an independent method, cells were incubated for 1 h with BrdU to label the fraction of cells actively engaged in DNA replication at the end of the culturing period, followed by anti-BrdU Ab staining and flow cytometry analysis. Compared with wild-type cells, *Rasgrp1* and *Rasgrp3* single-mutant B cells retained some ability to respond to anti-IgM plus IL-4, but double-mutant B cells were virtually inactive in this assay (Fig. 5*B*). Overall, both sets of data support the hypothesis that RasGRP3 plays a more important role than RasGRP1 in BCR-induced proliferation, with partial redundancy evident in most circumstances.

Discussion

RasGRP1 and RasGRP3 are coexpressed in primary murine B cells. Although RasGRP1 is the only RasGRP family member implicated in Ras activation downstream of the TCR, both RasGRP1

and RasGRP3 are responsible for Ras-Erk signaling mediated by BCR ligation in B cells. In primary murine splenic B cells, RasGRP3 plays the major role in BCR-induced signaling and is solely responsible for basal Ras-GTP levels, which likely depend on tonic BCR signaling. B cells express less RasGRP1 than do T cells, which suggests that RasGRP1 plays a minor role in B cells. In double-knockout B cells, PMA-Ras signaling is absent despite significant Erk activation. One possible mechanism for this residual signaling to Erk in B cells is PKC-mediated Raf activation, which has previously been documented in other cell types (35, 36).

Oh-hora et al. (25) generated RasGRP1 and RasGRP3 single and double deficient DT40 chicken B cells. In these cells, BCR-Ras signaling depends on RasGRP3 but not RasGRP1. These transformed chicken B cells did not display detectable tonic BCR-Ras signaling and, in the absence of both RasGRPs, there was residual Ras-Erk signaling upon BCR ligation. Thus, the mechanism of BCR-Ras signaling in DT40 cells is different from that of murine splenic B cells.

A major finding of our study is the dependence of *in vitro* BCR-induced proliferation on RasGRP1 and RasGRP3. In our assays, RasGRP3 plays a more important role than RasGRP1 although both RasGRPs are roughly equivalent in anti-IgM plus IL-4 costimulation assays. *In vivo*, B cell proliferation is important for controlling activation, differentiation, and class-switching (34). Therefore, we expected double-mutant mice to exhibit substantial disruption of the B cell compartment. Surprisingly, B cell development appears to proceed normally in the absence of both RasGRP1 and RasGRP3 as judged by surface marker analysis of splenocytes. Mice deficient in PLC- γ -2 have only a mild B cell developmental defect, which likely reflects a failure to activate RasGRPs as well as other molecules such as PKC (37, 38). These results are in contrast to the severe B cell developmental defects seen in mice expressing a dominant-negative Ras mutant under the control of a B cell-specific promoter (32, 39). However, the developmental defect in RasN17 transgenic mice occurs before the expression of the pre-BCR and is likely due to inhibition of IL-7R signaling. Possibly other GEFs, such as Sos, are the targets of the dominant-negative Ras in early stages of development.

We have established that RasGRP1 and RasGRP3 play important roles in Ig production by examining sera from both aged and young mice immunized by standard protocols. Compared with wild-type mice, aged *Rasgrp1*^{-/-} mice have elevated serum Ig levels, whereas *Rasgrp3*^{-/-} mice have reduced levels. Curiously, Ig levels in double-mutant mice are intermediate between those seen in either single mutant. In *Rasgrp1*^{-/-} mice the elevated Ig levels are likely a result of an indirect effect of autoimmune T cells, whereas the hypogammaglobulinemia in *Rasgrp3*^{-/-} mice probably arises from reduced BCR-Ras signaling. The intermediate values observed in double-mutant mice could reflect a balance of these two effects. Young *Rasgrp3*^{-/-} and double-mutant mice also have minor defects in the production of hapten-specific Abs.

Our analysis of serum Ig levels revealed some puzzling isotype-specific effects of RasGRP deficiency. Previous studies showed that CD4⁺ T cells from autoimmune *Rasgrp1*^{-/-} mice produce high amounts of IL-4 upon *in vitro* stimulation (23). This cytokine induces IgG1 and IgE synthesis (40), consistent with the elevated titer of these Ig isotypes that we observed in aged *Rasgrp1*^{-/-} mice. However, *Rasgrp1*^{-/-} T cells produced negligible IFN- γ *in vitro*, so it is unclear why these mice have elevated titers of IgG2a and IgG3. Additional cytokine abnormalities, possibly arising from non-T cells, may be influencing B cells in *Rasgrp1*^{-/-} mice. In addition, the isotype-specific differences that we observed in both our immunized and nonimmunized double-mutant mice are unexplained, because they do not match the deficiencies reported

for other mouse mutants with B cell proliferation defects. For example, PLC γ -2-deficient mice lack BCR-induced proliferation but exhibit decreased levels of IgM, IgG2a, and IgG3 in their sera and have normal T cell-dependent immune responses (37, 38). Likewise, loss of cyclin D₂, a likely effector of BCR-Erk signaling, results in decreased serum levels of IgG3 and IgA (41).

Mice that lack RasGRP1 expression in their T cells develop a late onset lymphoproliferative and autoimmune syndrome (23). Although this process complicates the analysis of RasGRP1 and RasGRP3 function in B cells from aged mice, our studies revealed that the *Rasgrp1*^{-/-} autoimmunity is suppressed in double-mutant mice. The loss of RasGRP1 expression in T cells has been shown to result in excess IL-4 production upon TCR stimulation in vitro (23). Although IL-4 plus anti-IgM induces proliferation of wild-type and *Rasgrp1*^{-/-} B cells, double-knockout B cells are not responsive to this treatment. We hypothesize that the lack of autoimmunity and splenomegaly in *Rasgrp1*-*Rasgrp3* double-mutant mice is due to the proliferation defect of B cells, which causes them to be less responsive to the signals originating from autoimmune T cells.

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Disclosures

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