MOLECULAR AND CELLULAR BIOLOGY, May 2002, p. 3230–3236 Vol. 22, No. 10
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.10.3230–3236.2002
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# Grap Negatively Regulates T-Cell Receptor-Elicited Lymphocyte Proliferation and Interleukin-2 Induction

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Received 28 November 2001/Returned for modification 24 January 2002/Accepted 21 February 2002

**Grb-2-related adaptor protein (Grap) is a Grb2-like SH3-SH2-SH3 adaptor protein with expression restricted to lymphoid tissues. Grap$^{-/-}$ lymphocytes isolated from targeted Grap-deficient mice exhibited enhanced proliferation, interleukin-2 production, and c-*fos* induction in response to mitogenic T-cell receptor (TCR) stimulation, compared to wild-type cells. Ectopic expression of Grap led to a suppression of Elk-1-directed transcription induced by the Ras/Erk pathway, without having effects on gene expression mediated by Jnk and p38 mitogen-activated protein kinases. Together, these data suggest that Grap, unlike Grb2, acts as a negative regulator of TCR-stimulated intracellular signaling by downregulating signal relay through the Ras/Erk pathway.**

Antigen stimulation of the T-cell receptor (TCR) elicits multiple intracellular signaling pathways that culminate in the upregulation of transcription of specific genes, leading to lymphocyte activation and proliferation (9). Among the earliest events following TCR engagement is the sequential activation of Src family tyrosine kinases, Lck and Fyn, and ZAP-70 or Syk kinases (10, 33, 37), which results in downstream information flow via two major routes. One prominent event is the phosphorylation and activation of phospholipase C-γ1 (PLC-γ1) (27, 46). This leads to the production of phosphoinositide-derived second messengers, elevation of cytosolic calcium levels, and induction of protein kinase C (7). Increased calcium activates calcineurin, a serine/threonine phosphatase, that in turn dephosphorylates and activates the latent cytoplasmic transcription factor, nuclear factor of activated T cells (6, 19, 40). Another important signal relay proceeds through the Ras/mitogen-activated protein (MAP) kinase pathway (14). The MAP kinases are composed of three families, Erk, Jnk, and p38, each of which is specifically activated and apparently plays different roles in cell signaling (12). However, it is not fully understood how the Ras/MAP kinase cascades are induced and tightly controlled at multiple levels.

Recent work from a number of laboratories suggests that adaptor/scaffold proteins, such as Shc, Grb2, Grb-2-related adaptor protein (Grap), Slp-76, LAT, and Cbl, play critical roles in lymphocyte signaling by assembling a variety of enzymes into specific multiprotein complexes (32, 36). Without catalytic activities, these proteins are comprised entirely of one or more modular domains, such as the SH2, SH3, PTB, PH, and WW domains, which mediate protein-protein or protein-lipid interactions (31). These proteins are either ubiquitously expressed, such as Grb2 and Shc, or are predominantly restricted to lymphocytes and leukocytes, including Grap, Slp-76, and LAT (29). LAT is a transmembrane protein that upon tyrosine phosphorylation provides docking sites for the SH2-containing Grb2, PLC-γ1, and phosphatidylinositol 3-kinase (49). Slp-76 is apparently a major target of tyrosine kinases, such as ZAP-70 (29, 45). Recent gene-targeting experiments revealed an essential signaling role of LAT and Slp-76 adaptor proteins in normal T-cell development, since no mature T lymphocytes were detected in LAT$^{-/-}$ or Slp-76$^{-/-}$ mice (11, 50).

Researchers have previously reported the identification of a Grb2-like molecule, Grap, which is specifically expressed in lymphocytes (13). Grap is complexed with p36/38 (LAT), Shc, Sos, PLC-γ1, and Fyn upon T-cell activation (44). More recently, several groups identified another small molecule with similar architecture, variously called Gads, Grap-2, GrpL, or Graf40 (3, 23, 26, 34). Like Grb2 and Grap, Gads contains a central SH2 domain flanked by two SH3 domains but is distinguished from Grb2/Grap by also having a central proline-rich region. Biochemical analyses suggest the participation of Grap and Gads in signal relay in lymphocytes through interaction with other signaling molecules, such as LAT and Slp-76 (25, 44). A targeted deletion of the *gads* gene in mice resulted in a severe defect in the proliferation of CD4$^-$ CD8$^-$ thymocytes, and Gads$^{-/-}$ thymocytes failed to respond to CD3 stimulation and were impaired in positive and negative selection. Thus, Gads is required for T-cell development as a signal linker between LAT and Slp-76 (48). However, the physiological function of Grap in signaling pathways that regulate lymphocyte development, proliferation, and functions is unknown.

By homologous recombination, we created a Grap-deficient mutant mouse model and detected an augmented mitogenic response of lymphocytes in the absence of Grap. Additionally, ectopic expression of Grap leads to an interruption of signal

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transmission from the Ras-Erk pathway into the nucleus. Together, the genetic and molecular data suggest a negative regulatory role of Grap in mediating mitogenic responses of lymphocytes, by specifically limiting the signal transmission through the Ras-Erk pathway.

## MATERIALS AND METHODS

**Grap gene targeting.** Mouse Grap genomic DNA of the 129/Sv strain was isolated and characterized by restriction mapping and sequence analysis. A targeting construct was engineered by inserting 1.3- and 8.3-kb fragments into the pLoxPneo-TK vector as the left and right arms, respectively. The linearized targeting construct was electroporated into R1 embryonic stem (ES) cells (28), and colonies resistant to G418 and ganciclovir were isolated. Of 1,000 double-resistant clones, 7 had undergone homologous recombination as assessed by Southern blot analysis. Four targeted ES cell clones were used to generate chimeric animals using the morula aggregation method (28), and germ line transmission was obtained for chimeras derived from three independent ES clones. Heterozygous offspring were mated to generate wild-type (+/+), heterozygous (+/−), and homozygous Grap mutant (−/−) littermates. Some of the male heterozygous offspring were then backcrossed to C57BL/6 females for five generations to derive a more genetically homogeneous line. Sex- and age-matched (usually between 7 and 12 weeks old) $Grap^{+/+}$ or $Grap^{+/-}$ and $Grap^{-/-}$ mice were used for experiments. Genotyping of littermates was accomplished by Southern blotting and specific PCR analysis of tail DNA. Detection of the wild-type allele was analyzed by a pair of primers (Grap 9, 5′ GTG ATT GGT CCT GGT TAT AGC CAG TG 3′; and Grap 10, 5′ TGC TGC CTC CAA AGG CCA CTG ACT ATT CTG 3′). The *grap* mutant allele was identified by another pair of primers that recognize the neomycin gene in the targeting vector (Neo 5S, 5′ ATG GGA TCG ATT GAA CAA GAT G 3′; and Neo 3AS, 5′ TCA GAA GAA CTC GTC AAG AAG GCG 3′).

**Analyses of lymphocyte development and functions.** Thymocytes and erythrocyte-depleted splenocytes were prepared from isolated thymi and spleens of 7- to 10-week-old mice. Cell viability was determined by trypan blue exclusion assay. Peripheral lymph node cells and thymocytes did not undergo red blood cell lysis prior to either proliferation assays or fluorescence-activated cell sorter (FACS) analysis. For flow cytometry analysis, isolated lymphocytes ($10^6$) were stained with the following anti-mouse monoclonal antibody (MAb) conjugates: TCR beta (TCR-β)-fluorescein isothiocyanate (FITC) (H57-597), CD4-FITC (H129.19), CD25-FITC (interleukin-2 [IL-2] α receptor chain, 7D4), CD3ε-FITC (145-2C11), CD3ε-cytochrome (145-2C11), CD8-phycoerythrin (PE) (53-6.7), CD62L (L-selectin, MEL-14), anti-immunoglobulin M (αIgM)–FITC, and αIgD-FITC (Pharmingen). The collected FACS data were analyzed with Cell Quest software (Becton Dickinson). Th1 and Th2 cell differentiation was assessed on erythrocyte-lysed splenocytes by measuring IL-4 and gamma interferon secretion as described previously (21). For cell proliferation assays, lymphocytes ($10^5$) were prepared and seeded in 96-well plates, each containing a specific mitogen in RPMI medium supplemented with 10% fetal calf serum. A time course of 1 to 5 days was used and 1 μCi of [$^3$H]thymidine (ICN)/well was added for a DNA incorporation assay. TCR stimulations were accomplished by precoating the plates overnight with αCD3 MAb (2C11) in phosphate-buffered saline (PBS).

**Luciferase reporter assay.** HEK293 or COS-7 cell lines grown to 60 to 70% confluency were transiently transfected using the calcium phosphate method (Gibco-BRL) with 0.2 μg of oncogene construct, 0.125 μg of GAL4BD-ELK, and 2.5 μg of 5× GAL-luciferase and either 3 μg of pcDNA3 hemagglutinin (HA)-Grb2, pcDNA3 HA-Grap, or empty pcDNA3 expression vector. Activated versions of oncogenes included pSR-vAbl, pEXV-vSrc, pBp-RasV12, pEXV-Raf-CaaX, and pcDNA3-Mek-EE. To monitor the transfection efficiency and to normalize the luciferase data, a Rous sarcoma virus β-galactosidase construct (1 μg) was included as an internal control reporter. After overnight transfection, the medium was changed to serum-free Dulbecco's modified Eagle's medium for another 24 h. Cell lysates (0.2 ml of Luciferase Assay buffer; Promega) were prepared from each transfection replicate and were then aliquoted to determine both luciferase (Luciferase Assay Kit; Promega) and β-galactosidase activity (Galactolight; Tropix). The results are expressed as arbitrary units whereby the luciferase values are divided by the corresponding β-galactosidase values from each replicate. Relative expression levels of HA-Grb2 and HA-Grap were determined by immunoblotting with anti-HA MAb (Roche). The Jnk reporter used an assembly of GAL4BD-Jun, while the p38 reporter utilized a GAL4BD-Chop fusion (Pathdetect; Stratagene).

Jurkat cells ($20 \times 10^6$) were transfected with 5 μg of Gal4-Luc reporter vector, 1 μg of Gal4-Elk, and 300 ng of pRL-0 vector (Promega) (2). Besides, 10 μg of vector control or Grap or Grb2 expression constructs was cotransfected. After stimulation with αCD3ε MAb (American Type Culture Collection) for 6 h, luciferase activity was measured as described above.

**Northern blot and immunoblot analysis.** Total RNA was isolated from lymphocytes, and Northern blot analysis was performed using the *c-Fos* or *TCR*α gene as a probe following standard procedures. For immunoblot analysis, cell lysates were separated on sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and blotted with primary antibodies as indicated. Specific signals were detected by enhanced chemiluminescence (ECL analysis kit; Amersham Corp.) following blotting with horseradish peroxidase-conjugated secondary antibodies. Antibody against Grap was produced by injection of rabbits with purified glutathione *S*-transferase fusion protein containing the SH3-C domain of Grap following standard procedure. Antibodies to phospho-Erk (αp-Erk), phospho-Jnk, phospho-p38, and phospho-p90Rsk were obtained from New England Biolabs, Inc.

## RESULTS

**Generation of Grap-deficient mice.** Grap-deficient mice were generated by homologous recombination using a standard gene-targeting approach in mouse ES cells. Disruption of the *grap* gene was confirmed by Southern blotting with a specific probe for *grap* as well as immunoblot analysis using a specific rabbit antiserum raised against the SH3-C domain of Grap (Fig. 1). From eight breeding pairs of $Grap^{+/-}$ parents, accumulative offspring numbers representing the three genotypes (+/+, +/−, and −/−) were in agreement with expected Mendelian ratios (46:100:47). There was also no bias in the male/female ratio between $Grap^{+/+}$ and $Grap^{-/-}$ animals. $Grap^{-/-}$ mice were fertile and did not exhibit obvious developmental abnormalities.

Since Grap is predominantly expressed in lymphoid tissue, we focused our attention on examining the development of lymphocytes in the phenotypic analysis of mutant mice. There was no obvious reduction or increase in the size of the thymus, spleen, and lymph nodes, and comparable cellularity was exhibited between $Grap^{+/+}$ and $Grap^{-/-}$ littermates under the C57BL/6 background. However, slightly increased lymphocyte numbers were detected in the thymus and spleen of $Grap^{-/-}$ mice with a CD1 × 129/Sv background (data not shown). Flow cytometry analysis of the thymus demonstrated normal T-cell development with a slight increase in the CD4-single-positive population of $Grap^{-/-}$ mice compared to that of control littermates (data not shown). However, peripheral T-cell populations in the spleens and lymph nodes of $Grap^{-/-}$ mice were indistinguishable from those of wild-type littermates. Likewise, FACS analyses of splenocytes for B220/IgM or B220/IgD markers demonstrated normal B-cell numbers in $Grap^{-/-}$ mice (data not shown). There was little difference in the B-cell proliferation between $Grap^{+/+}$ and $Grap^{-/-}$ siblings upon treatment with αIgM, $F(ab')_2$, or CD40 (data not shown).

**$Grap^{-/-}$ lymphocytes exhibit enhanced proliferative response to TCR stimuli.** To determine if Grap has a significant role in modulating mitogenic signaling of lymphocytes, we assessed cellular proliferation to αCD3 stimulation and to costimulations of αCD3 + αCD28 or αCD3 + IL-2 by measuring [$^3$H]thymidine incorporation. $Grap^{-/-}$ lymphocytes exhibited about a twofold-greater proliferation than $Grap^{+/+}$ cells on days 2, 3, and 4 poststimulation by αCD3 alone (Fig. 2A). In the presence of IL-2 (25 U/ml), $Grap^{-/-}$ cells but not $Grap^{+/+}$ cells responded to very low αCD3 concentrations (1.8 to 50 ng/ml) (Fig. 2B). Costimulation with either αCD28 or IL-2

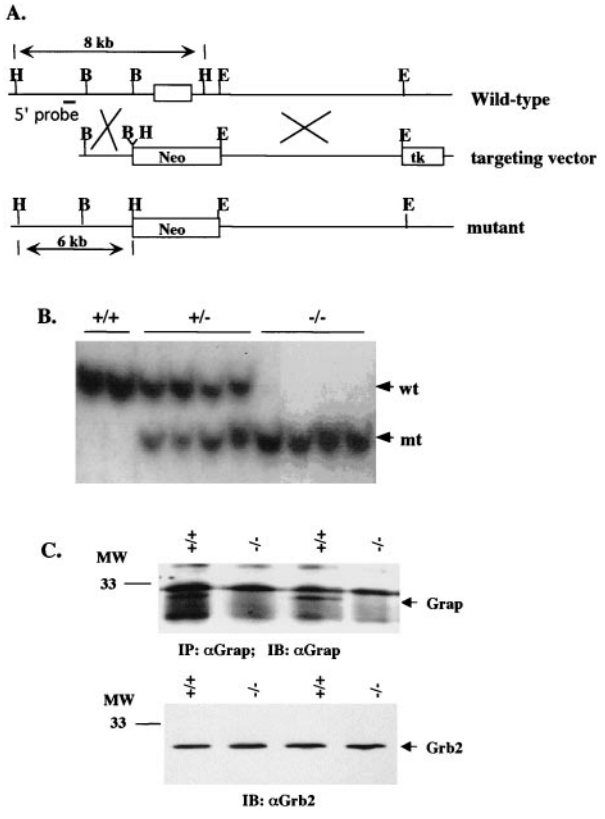


FIG. 1. Generation of Grap-deficient mice. (A) Gene-targeting strategy. The targeting vector was designed to delete an exon encoding amino acids 160 to 217 of Grap. Only relevant restriction sites are indicated: B, *Bam*HI; E, *Eco*RI; and H, *Hin*dIII. The 5′ external probe used to distinguish the wild-type and mutant allele is indicated below the wild-type restriction map. (B) Southern blot analysis. Tail DNA samples were prepared and digested with *Hin*dIII for Southern blot analysis using a 5′ probe as shown in panel A, which detects an 8-kb fragment for the wild-type (wt) allele and a 6-kb fragment for the targeted mutant (mt) allele. (C) Grap and Grb2 expression. Grap was immunoprecipitated (IP) from thymocyte extract (1 mg of total proteins) and immunoblotted (IB) with an anti-Grap antibody raised against the SH3-C domain of Grap. The same cell lysates (50 μg) were immunoblotted with an anti-Grb2 antibody, showing similar Grb2 expression in $Grap^{+/+}$ and $Grap^{-/-}$ thymocytes. We also purchased from Santa Cruz Biotechnology an antibody to the SH3-N domain of Grap (sc-6101) but failed to detect Grap in either the immunoprecipitation or immunoblotting experiment with the antibody. Thus, it is not clear whether a truncated protein of Grap is made or not in $Grap^{-/-}$ lymphocytes. MW, molecular weight (in thousands).

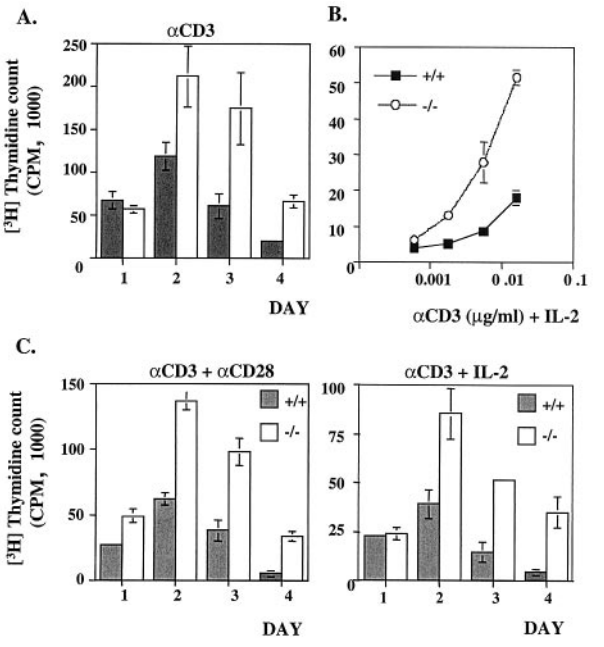


FIG. 2. Enhanced proliferation of $Grap^{-/-}$ lymphocytes upon αCD3 stimulation. (A) Lymphocyte proliferation stimulated by αCD3. T cells were specifically stimulated through the TCR by αCD3ε (2C11 MAb, plate bound after being coated overnight in PBS) for 1, 2, 3, and 4 days, and cells were labeled with [$^3$H]thymidine for the last 12 h of culture. (B) Dosage effect of costimulation by αCD3 + IL-2. Lymphocytes ($10^5$ cells/well) were stimulated with various dosages of plate-bound αCD3 (1.8, 5.5, 16.6, and 50 ng/ml) in combination with IL-2 (25 U/ml) for 72 h. Cells were incubated with [$^3$H]thymidine for the last 12 h. (C) Lymphocyte proliferation upon costimulation by αCD3 + αCD28 or αCD3 + IL-2. A low αCD3 concentration (0.5 μg/ml) was used to precoat wells for αCD3 costimulation with αCD28 coreceptor (50 ng/well; Pharmingen) or soluble IL-2 (25 U/well; Roche) for the times as indicated.

induced an amplified proliferative response from both genotypes, but the greater proliferative ability of $Grap^{-/-}$ than of $Grap^{+/+}$ lymphocytes was still witnessed under these conditions (Fig. 2C).

There are several plausible explanations why $Grap^{-/-}$ lymphocytes respond with increased cell proliferation. First, $Grap^{-/-}$ lymphocytes might be in a "preactivated" state and thus predisposed to immediate response. A few gene-targeting studies have described cases of preactivated lymphocytes that have downregulated CD62L and/or elevated CD69 and CD25 levels (17, 22). To explore this possibility, FACS analysis of $Grap^{+/+}$ and $Grap^{-/-}$ lymphocytes was first performed for CD62L, which is highly expressed on resting cells. CD62L on wild-type and mutant cells was comparably high prior to costimulation by αCD3 + αCD28 and was similarly downregulated 24 h poststimulation (data not shown). Similarly, there were very low levels of CD25 expression on the surface of resting cells, and after 24 h of costimulation by αCD3 + αCD28, the increase in CD25 expression appears to be equivalent for $Grap^{+/+}$ and $Grap^{-/-}$ T cells (data not shown). Thus, $Grap^{-/-}$ T cells are not preactivated, and their increased proliferation is unlikely due to premature upregulation of the IL-2 receptor. Another possibility is that decreased activation-induced cell death could also contribute to the elevated thymidine incorporation during αCD3 stimulation (20). We thus examined the activation-induced cell death of activated lymphocytes after restimulation by αCD3. The results showed that approximately 80% of both $Grap^{+/+}$ and $Grap^{-/-}$ lymphocytes underwent programmed cell death. Control cells from both groups with PBS treatment displayed only about 20% spontaneous apoptosis under the same culture condition. To determine if the increased proliferation had any effect on T-helper-cell differentiation, we also evaluated Th1 and Th2 cell differentiation upon stimulation of $CD4^+$ T cells with αCD3 activation in the presence of the cytokine IL-12 or IL-4. IL-4 production by Th2 cells and gamma interferon secretion from Th1 cells were found to be similar for $Grap^{+/+}$ and $Grap^{-/-}$

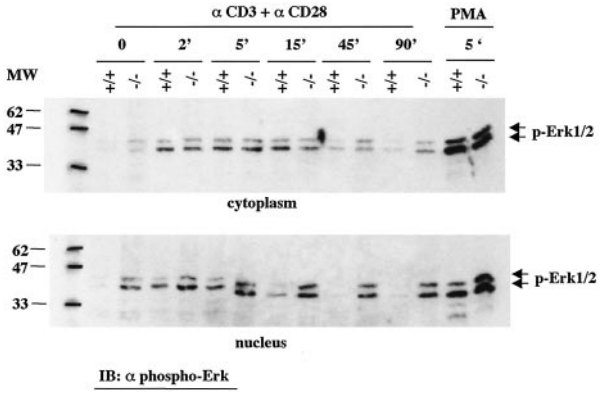


FIG. 3. Enhanced Erk activation in Grap$^{-/-}$ lymphocytes. Erk activity assay. Lymphocytes were treated with αCD3 and αCD28 antibodies for the indicated times (given in minutes). Cell lysates were separated into cytoplasmic and nuclear fractions using a kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents) from Pierce. Immunoblot (IB) analysis of the separated cell lysates was done using αp-Erk. MW, molecular weight (in thousands). PMA, phorbol myristate acetate.

animals (data not shown). Taken together, these observations strongly suggest that Grap deficiency leads to an alteration of an intracellular signaling process that modulates T-cell proliferation, rather than effector differentiation or apoptosis.

**Grap has a negative effect on signal relay through the Ras/Erk pathway.** To explore the biochemical mechanism for the apparently negative effect of Grap in the control of lymphocyte proliferation, we examined Erk activation in response to TCR stimulation. T lymphocytes were treated with αCD3 and αCD28 antibodies for 0, 2, 5, 15, 45, and 90 min, and cell lysates were further separated into cytoplasmic and nuclear fractions. Erk activation in the two fractions was assessed by immunoblot analysis using αp-Erk. As shown in Fig. 3, the basal levels of Erk activity were slightly increased in both the cytoplasm and the nucleus in Grap$^{-/-}$ cells compared to in wild-type cells. After TCR stimulation, Erk activation appears to last longer in the absence of Grap, particularly in the nucleus. In response to phorbol myristate acetate stimulation, there was also a slight increase in the Erk activation in Grap-deficient cells compared to in wild-type cells. Therefore, Grap deficiency leads to an enhanced and more sustained activation of Erk, pointing to a negative role of Grap in modulation of signals emanating from TCRs.

To corroborate this data in a separate system, we transfected HA-tagged Grap and Grb2 expression constructs into HEK293 cells and compared their influence on Ras/Erk-dependent luciferase reporters, using a bipartite Gal4BD-Elk fusion protein and a Gal-luciferase reporter. Transcriptional activation of the Gal-luciferase reporter requires phosphorylation of the Elk domain by Erk1/2. Cotransfection of the oncogene v-*abl*, an upstream Ras pathway initiator, resulted in a 10-fold increase of luciferase activity. As expected, Grb2 coexpression had a minor enhancing effect in this assay. However, coexpression of Grap caused a dramatic suppression to about 5% of the empty vector control (Fig. 4A). Grap expression also resulted in suppression of RasV12-induced Elk activation in the luciferase reporter assay (Fig. 4B), suggesting that the acting point of Grap must be further downstream of or in parallel to Ras. Consistent with the notion that Grb2 acts upstream of Ras, Grb2 dominant negative mutants had no effect on RasV12-induced Elk reporter activity. The repression shown by Grap is not restricted to the Gal4-Elk system, as the use of AP-1 reporter, also dependent on the Ras/Erk pathway for activation, illustrated the same inhibitory phenomenon (data not shown). However, Grap transfection had no influence on two other parallel MAP kinase signaling cascades, p38 and Jnk. Transfection of Grb2 or Grap had minimal effects on GAL4BD-Chop luciferase reporter induced by Mek3EE, a specific activator of p38, or on Gal4BD-Jun reporter levels induced by Mek4, which activates Jnk (Fig. 4C and D).

To confirm that Grap functions similarly in 293 and T cells, we transfected Grap and Grb2 into Jurkat lymphoid cells and measured the luciferase reporter activity in response to αCD3 stimulation. As shown in Fig. 4E, we observed a similar opposite effect of Grap and Grb2 in mediating the activation of Elk-1 activity, with Grap acting as a signal suppressor. These results correlate well with the data from Grap-deficient mice and support the notion that Grap has a specific negative effect in modulating the signal strength of the Erk pathway but not of the Jnk and p38 routes. In the absence of Grap, Erk activation was prolonged and active Erk stayed longer in the nucleus. In contrast, overexpression of Grap resulted in suppression of Elk-1 transcription activity, a target of Erk in the nucleus.

The negative effect of Grap in signaling Elk-1 activation would predict the upregulation of its target genes in Grap$^{-/-}$ lymphocytes. To test this possibility, we assessed the expression of c-*fos*, an immediate early response gene, in response to TCR stimulation. Northern blot analysis demonstrated a significant increase in c-*fos* mRNA levels in Grap$^{-/-}$ lymphocytes compared to in Grap$^{+/+}$ lymphocytes 20 and 40 min after costimulation by αCD3 and αCD28 (Fig. 5).

To determine whether this increase in c-*fos* expression has any physiological consequences, we monitored IL-2 production, which is activated by AP-1. As shown in Fig. 6, two- to threefold higher amounts of IL-2 secretion were detected in the supernatants from Grap$^{-/-}$ lymphocytes than in those from Grap$^{+/+}$ cells 9 and 24 h post-costimulation by αCD3 and αCD28. The enhanced IL-2 production correlates with and may be partly responsible for the increased proliferation of Grap$^{-/-}$ lymphocytes upon stimulation by αCD3 and αCD28. Thus, we have identified Grap, a small SH3-SH2-SH3 adaptor molecule, as a negative regulator in mitogenic signaling of lymphocytes, which is opposite to the stimulatory activity of Grb2.

## DISCUSSION

Mice carrying a targeted deletion at the *grap* locus were generated, and homozygous mutants (Grap$^{-/-}$) were viable, fertile, and healthy. There were no severe developmental defects in T and B lymphocytes in which Grap was expressed. However, a statistically significant increase in the mitogenic response to TCR stimulation was observed when it was evaluated for cell proliferation and IL-2 secretion. Furthermore, αCD3-stimulated Erk activity and expression of c-*fos* were higher in Grap$^{-/-}$ than in Grap$^{+/+}$ lymphocytes. These observations point to a negative regulatory role of Grap in a mito-

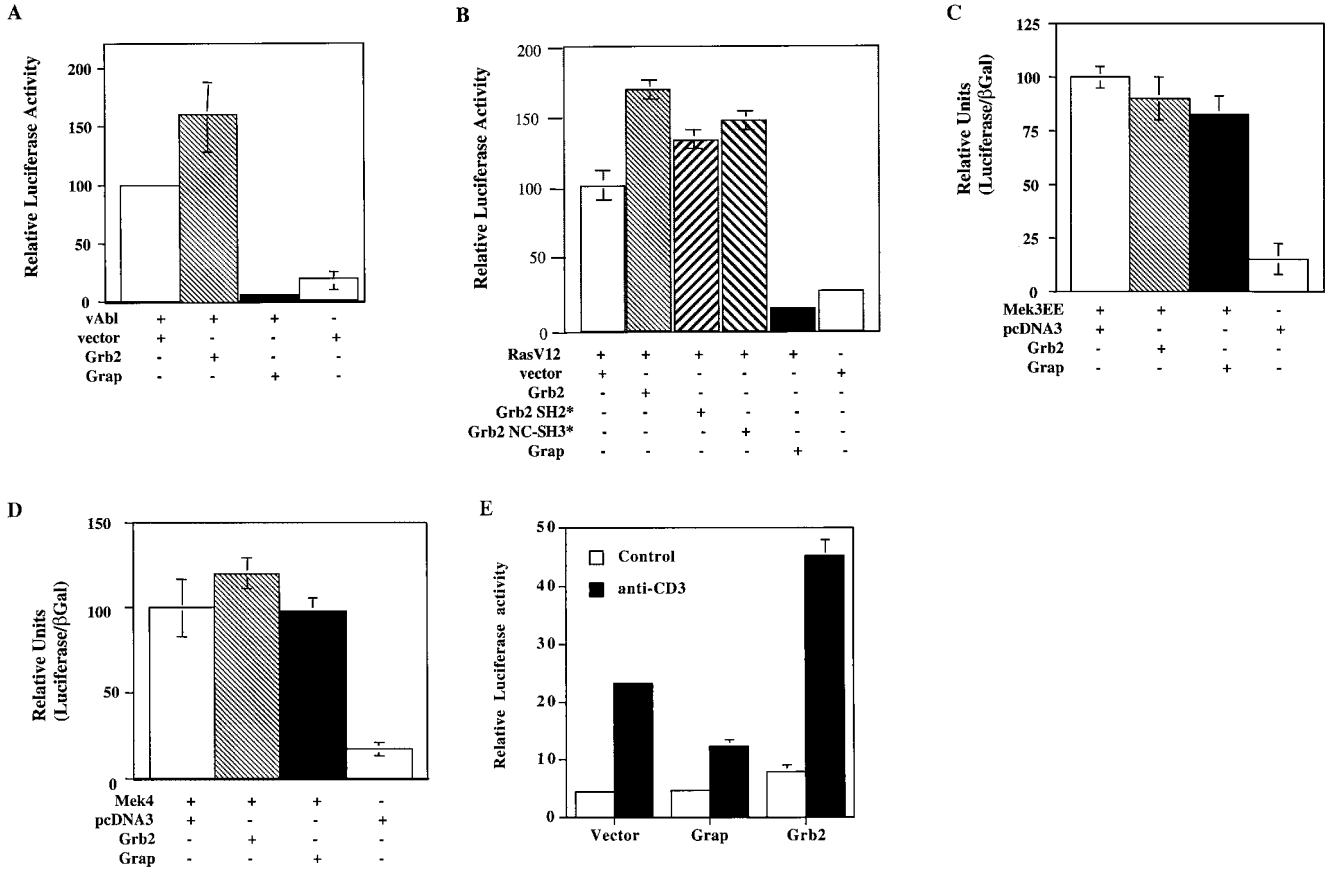


FIG. 4. Inhibitory effect of Grap on the Ras-Erk pathway. (A) Different effects of Grb2 and Grap on v-*abl*-induced Elk-1 activity. HEK293 cells were cotransfected with GAL4BD-Elk, 5× GAL-luciferase reporter, and other constructs as indicated. Results were averaged from five independent experiments (± standard error). Similar expression levels of HA-Grb2 and HA-Grap were detected by anti-HA immunoblotting. (B) Grap represses RasV12-induced reporter activity. HEK293 cells were cotransfected with constructs as indicated. The Grb2SH2*(R86K) and Grb2 NC-SH3* (W36K and W193K) mutants were described previously (43). Results were averaged from four independent experiments (± standard error). (C) Grap does not inhibit Mek3/p38-induced Elk-1 activation. Cells were transiently transfected with Gal4BD-Chop, 5× GAL-luciferase reporter, and Mek3EE, as well as with HA-Grap or HA-Grb2. Data were averaged from three independent experiments (± standard error). (D) Grap does not inhibit Mek4/Jnk-induced Elk-1 activity. Cells were transfected with the indicated pcDNA3 expression plasmids, GAL4BD-Jun, 5×GAL-luciferase reporter, and Mek4. Results were expressed as average ± standard error from four independent experiments. (E) Grap inhibits Elk-1 activity in lymphocytes. Jurkat cells ($20 \times 10^6$) were cotransfected with Gal4-Luc reporter vector, Gal4-Elk, and pRL-0 vector (Promega), as well as with vector control, Grap, or Grb2 expression constructs. Luciferase activity was measured 6 h after αCD3 stimulation. Experiments were repeated three times with reproducible results.

genic signaling pathway possibly mediated by Ras/Erk. Consistent with a promotion of Erk signaling in Grap$^{-/-}$ cells, overexpression of Grap in HEK293 cells and Jurkat cells had a suppressive effect on activation of Elk-1 by oncogenic Ras and αCD3, respectively, while Grb2 acted to enhance the signals in the same systems. On the other hand, activation of Jnk and p38 MAP kinases was indistinguishable between wild-type and Grap$^{-/-}$ cells. Consistently, transfection of Grap or Grb2 had similar effects on reporter activities for Jnk and p38 cascades. Taken together, Grap appears to be a negative regulator specific for the Erk pathway.

A growing number of lymphocyte-specific adaptor molecules have been identified and found to promote the TCR or B-cell receptor signaling by serving as substrates for tyrosine kinases and thereby coupling to downstream effectors. Accordingly, ablation of these genes resulted in defects in the lymphoid compartment in mice (11, 48, 50). We have now described a novel negative effect for Grap in T-lymphocyte signaling. Cell proliferation induced by αCD3 alone or in combination with either αCD28 or IL-2 was more profound in Grap$^{-/-}$ lymphocytes than in Grap$^{+/+}$ cells. This is apparently not caused by a relative increase in the percentage of mature T cells, which was similar between Grap$^{-/-}$ and Grap$^{+/+}$ siblings. Grap$^{-/-}$ lymphocytes are not in a preactivated state. CD62L expression levels were comparably high between Grap$^{-/-}$ and Grap$^{+/+}$ cells before αCD3 stimulation, and the amounts of CD25 expression were similarly low on both cell types in their resting states.

Further experiments demonstrated that the enhanced mitogenic response was associated with an increased secretion of IL-2 from Grap$^{-/-}$ cells, an autocrine growth factor critical for proliferation and differentiation of T and B cells. The increased IL-2 production correlates with c-*fos* expression in Grap$^{-/-}$ cells, which is downstream of Ras/Erk signaling. Thus, Grap functions as a downmodulator of signals from the TCR. Strong support of this genetic data was obtained from a

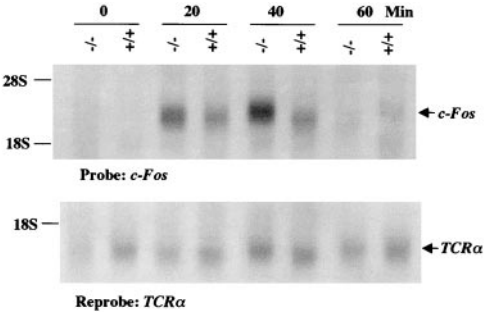


FIG. 5. Northern analysis of c-*fos* expression. Total RNA was extracted from Grap$^{+/+}$ and Grap$^{-/-}$ lymphocytes costimulated with αCD3 and αCD28 at different time points. RNA (5 μg) from each sample was loaded and hybridized to a $^{32}$P-labeled c-*fos* probe. The membrane was stripped and reprobed with TCR alpha (TCRα) probe for loading control. Densitometry showed 60 and 110% increases of c-*fos* mRNA levels in Grap$^{-/-}$ lymphocytes from those in Grap$^{+/+}$ lymphocytes, 20 and 40 min poststimulation, respectively.

series of biochemical assays showing that overexpression of Grap leads to a suppression of reporter gene expression induced by the Ras/Erk pathway. This highlights an intriguing issue in that Grap and Grb2, two adaptor molecules closely related in structure, have opposite effects in modulating the Ras pathway, which has been shown to be critical for efficient signal relay downstream of TCR (4, 5, 15, 18, 47).

The negative effect of Grap in the Ras-Erk pathway is not simply interference with the Grb2 function in promoting Ras activation. Although a similar downregulatory effect on v-*abl* was observed for Grap and dominant negative mutants of Grb2, it is clear that Grb2 acts upstream of Ras, since these dominant negative mutants did not have any effects on RasV12 signaling. In contrast, Grap expression displayed a significant suppression on downstream signaling from constitutively active forms of Ras, Raf, and Mek (data not shown). Although Grap may act upstream of Erk to promote its activation, it seems likelier that the primary function of Grap is to restrict or downregulate the Erk activity in the nucleus. This is why more sustained phospho-Erk was detected in the nuclei of Grap$^{-/-}$ cells than in wild-type cells. Overexpression of Grap but not of Grb2 suppressed Elk-1 phosphorylation without having a significant effect on Erk phosphorylation (data not shown). It was

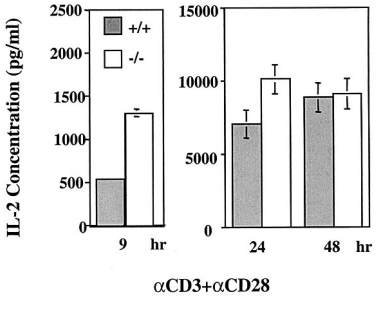


FIG. 6. Increased IL-2 production. Lymphocytes ($2 \times 10^6$ cells/well) were costimulated with αCD3 (0.5 μg/ml) and αCD28 (50 ng/well), and supernatant was harvested at 9, 24, and 48 h to assay for IL-2 secretion using ELISA 96-well plates (Immunosorp; Nunc) following standard procedures.

recently reported that HPK1 kinase seems to negatively regulate Erk2 and AP-1 activation by TCR stimulation, and a physical complex between Grap and HPK1 was detected in Jurkat cells (24). It will be interesting to know whether Grap and HPK1 work in concert in modulation of the Erk pathway.

The three MAP kinase pathways are known to play critical roles in mediating T-lymphocyte development. However, conflicting data exist in the literature regarding the involvement of Erk, Jnk, and p38 kinases in positive and/or negative selection of developing T cells. Experiments with dominant negative mutants of Ras and Mek1 or gain-of-function mutants of Erk2 and Mek1 in T lymphocytes suggested involvement of Erk in positive selection, without having effect on the negative selection (1, 39, 41, 42). Consistently, thymocyte maturation beyond the CD4$^+$ CD8$^+$ DP cell stage was reduced by half in Erk1$^{-/-}$ mice (30). On the other hand, the Jnk and p38 pathways were found to participate in the negative selection in thymocytes (35, 41). However, a more recent report described involvement of the Erk pathway in negative selection, and it was argued that the level of Erk activation may determine the physiological consequences in influencing positive or negative selection (8).

Results presented here suggest that, although activity of the Erk pathway is slightly enhanced, which leads to augmented IL-2 production and mitogenesis, T-lymphocyte development was normal in Grap$^{-/-}$ mice. Similar data were recently obtained for mice lacking the hematopoietic cell protein tyrosine phosphatase (HePTP) (16), which has been shown to negatively regulate the Erk pathway induced by TCR (38). Augmented activation of Erk but not of Jnk or p38 was observed in HePTP$^{-/-}$ lymphocytes, but HePTP deficiency does not cause a developmental problem in the lymphocyte compartment (16). Together, the data suggest that ablation of these negative effectors can be compromised by other molecules in lymphocyte development. It seems also likely that modulation of signal strength along the Erk pathway to variable extents might have different physiological consequences. The mitogenic response of lymphocytes appears to be more sensitive to alteration of Erk activity than the developmental program. Whether or not Grap has a role in positive or negative selection during T lymphopoiesis needs to be further addressed experimentally using TCR-transgenic mice. However, the downregulatory effect of Grap in IL-2 production and lymphocyte proliferation suggests a critical role of this small molecule in the regulation of immune responses. The Grap-deficient mice should serve as a model system in future genetic dissection of interactions between signaling molecules that controls lymphocyte development and function. A global view on immunoregulation can be obtained only upon elucidation of functions for positive as well as negative effectors, such as Grap.

## ACKNOWLEDGMENTS

We thank G. A. Koretzky, K.-L. Guan, M. Marshall, B. Mayer, and L. Quilliam for expression vectors and thank X. Lu, K. Pearle, and S. Reid for technical assistance.

This work was supported by grants from the American Cancer Society (RPG-98-273-01-TBE) and National Institutes of Health (HL66208) to G.-S.F., (AI45515) to M.H.K., and (AI40552) to T.M.

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