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*J Immunol* 2000; 164:4720-4729; doi: 10.4049/jimmunol.164.9.4720

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RGS Molecule Expression in Murine B Lymphocytes and Ability to Down-Regulate Chemotaxis to Lymphoid Chemokines\textsuperscript{1,2}

Karin Reif and Jason G. Cyster\textsuperscript{3}

Ag-mediated changes in B lymphocyte migration are important for normal immune function, yet the mechanisms by which these changes occur are poorly defined. Because chemokines direct many lymphocyte movements, molecules that regulate signaling by G protein-coupled chemokine receptors are likely to participate in Ag receptor-induced changes in cell migration. In this study, we have investigated the expression pattern and activity in murine B cells of members of the regulators of G protein signaling (RGS) family of molecules. We present the sequence of mouse RGS1 and describe a novel short isoform of RGS3 that we term RGS3s. Following in vivo activation by Ag, B cells rapidly up-regulate expression of RGS1 and RGS2 while simultaneously decreasing expression of RGS3 and RGS14. Anergic hen egg lysozyme autoantigen-binding B cells are also shown to have slightly elevated RGS1 and RGS2 expression. CD40 signaling, by contrast, fails to cause rapid up-regulation of RGS1 or RGS2. Using a transient transfection approach in a mature B cell line, 2PK3, we demonstrate that RGS1 and RGS3s are effective inhibitors of chemotaxis toward the lymphoid tissue chemokines stromal cell-derived factor-1, B lymphocyte chemotactrant, and EBV-induced molecule 1 ligand chemokine, whereas RGS2 has a minimal effect on migration to these chemokines. Together these findings support the conclusion that Ag-mediated changes in RGS molecule expression are part of the mechanism by which Ag receptor signaling regulates B cell migration within lymphoid tissues. The findings also suggest important roles for additional G protein-mediated events in B cell activation and tolerance. The Journal of Immunology, 2000, 164: 4720–4729.

Migration of resting and Ag-engaged B lymphocytes to specific compartments within lymphoid organs is important for recirculation and for mounting immune responses (1). Exclusion of autoreactive B lymphocytes from lymphoid follicles also plays a role in B cell tolerance (2). Recent progress has been made in identifying chemokines and chemokine receptors that are responsible for lymphocyte homing in secondary lymphoid organs. The chemokine receptor CXCR5 (formerly Burkitt’s lymphoma receptor 1) is expressed by mature B cells and is required for B cell migration into lymphoid follicles (3). Its ligand, a CXC chemokine termed B lymphocyte chemotactic (BLC)\textsuperscript{4} or B cell-attracting chemokine-I, is expressed in the follicular regions of secondary lymphoid organs (4, 5). Predominant chemokines present in the T zones of lymphoid organs are the CCR7 ligands, secondary lymphoid tissue chemokine (SLC)/6Ckine, and EBV-induced molecule 1 ligand chemokine, whereas RGS2 has a minimal effect on migration to these chemokines. Together these findings support the conclusion that Ag-mediated changes in RGS molecule expression are part of the mechanism by which Ag receptor signaling regulates B cell migration within lymphoid tissues. The findings also suggest important roles for additional G protein-mediated events in B cell activation and tolerance.

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\textsuperscript{2} The sequences presented in this paper have been submitted to GenBank/EMBL/ DDBJ under accession numbers AF215667 (mRGS1), AF215668 (mRGS2), AF215669 (mRGS3s), and AF215670 (mRGS3).

\textsuperscript{3} K.R. was supported by a European Molecular Biology Organization fellowship and is presently supported by a Human Frontier Science Program fellowship. J.G.C. is a Pew Scholar in the biomedical sciences. This work was supported by a grant from the National Institutes of Health (AI40098).

\textsuperscript{4} Abbreviations used in this paper: BLC, B lymphocyte chemotactrant; BCR, B cell receptor; ELIC, EBV-induced molecule 1 ligand chemokine; ERK, extracellular signal-regulated kinase; EST, expressed sequence tag; GAP, GTPase-activating protein; GEF, nucleotide exchange factor; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HEL, hen egg lysozyme; RAG, recombination-activating gene; RGS, regulator of G protein signaling; SDF-1, stromal cell-derived factor-1; SLC, secondary lymphoid tissue chemokine.
human RGS1, RGS3, RGS4, and RGS16 predominantly interact with Gai subunits, RGS2 with Goq, RGSZ1 with Gaq, and p115RhoGEF with Go12/13. These relationships may not be rigid, however, as some studies have indicated that RGS1 and RGS3 can inhibit signaling via Goq-coupled receptors (16–18), while RGS2 may antagonize some Gai-coupled receptors (13). Evidence is accumulating that the specificity of RGS proteins can also be regulated through interactions with the GPCRs themselves (16, 19, 20). Further work is therefore needed to understand how individual RGS proteins regulate individual GPCRs.

Lymphoid tissues and cells have been found to express multiple RGS molecules (21). Human RGS1 (BL34/1R20) was first identified through its expression in activated B cells (22, 23), and RGS3 was isolated by screening a B cell cDNA library with an RGS domain probe (13). RGS2 (GOS8) was first isolated as a gene induced by treatment of human blood mononuclear cells with the T cell mitogen, Con A (24). Further work has shown that expression of human RGS1 is induced in B cells by treatment with anti-IgM Abs as well as by IL-4, cAMP, or platelet-activating factor (13, 22, 23). RGS1 is strongly inducible by phorbol esters, whereas RGS2 is induced more strongly by calcium ionophore (25). RGS14 (13, 22, 23) is expressed in activated T cells and RGS3 is expressed in both mice and humans. Mouse RGS1 (BL34/1R20) was first identified through its expression in activated B cells (22, 23), and recent work has also reported that RGS16 is expressed in some lymphoid cell types (27).

To explore whether RGS molecules might function downstream of B cell surface receptors in regulating B cell responsiveness to lymphoid chemokines, we have characterized the expression pattern and activity of several mouse RGS proteins. We report the sequence of mouse RGS1 and describe a novel short isoform of RGS3, RGS3s, present in both mice and humans. Mouse RGS1 and RGS3 are expressed at high levels in spleen and lymph nodes. The expression patterns of mouse RGS2 and RGS14 are also examined, and RGS1, RGS2, RGS3, and RGS14 transcript levels are shown to be rapidly modulated in B lymphocytes in response to Ag challenge in vivo. Using cell lines transfected with RGS1-, RGS2-, and RGS3-GFP fusion proteins, we demonstrate that RGS1 and RGS3 can attenuate migration to the lymphoid chemokines BLC, ELC, and SDF-1. These findings suggest an important role for RGS molecules in helping regulate cell positioning in lymphoid organs during the immune response.

Materials and Methods

Reagents

Mouse rELC (macrophage-inflammatory protein-3β) was purchased from R&D Systems (Minneapolis, MN). Mouse rBLC was isolated as a HIS-tagged protein, as described (28). Human SDF-1α (N33A) was synthesized by chemical ligation and was a gift from M. Siani (Gryphon Sciences, South San Francisco, CA). Note human and mouse SDF-1 only differ in 1 aa, and SDF-1α (N33A) has identical activity to native human and mouse SDF-1. The anti-CXCR5 Abs (clone FGK) (29) was a gift from Anthony Rolink (Basel Institute for Immunology, Basel, Switzerland). Hen egg lysosome (HEL) was purchased from Sigma (St. Louis, MO).

Mice and animal challenge

C57BL/6 MD4 Ig transgenic (IgbHEL) mice carry transgenes encoding IgMζ and Igδ heavy and light chains specific for HEL (30). C57BL/6 HEL transgenic mice were of the Mls line, which carries a transgene encoding HEL under the metallothionein promoter and contains HEL at 10–30 ng/ml in serum (30). C57BL/6 MD4 and MLD5 transgenic mice were mated to obtain double-transgenic (IgbHEL/IgbHEL) mice. Transgenic mice were obtained from Dr. Bolognesi-Pialzi (Department of Biochemistry and Molecular Biology, European Institute of Oncology, Milan, Italy) and back-crossed to C57BL/6 for 10 generations before use. The expression pattern of mouse RGS proteins is shown in Table I. Using this system, we have characterized the expression patterns of mouse RGS proteins in vivo.

Clone identification and sequence analysis

Advanced BLAST searches of the NCBI mouse expressed sequence tag (EST) database using TBLASTN (32) with the respective template, retrieved mouse ESTs for RGS1, RGS2, RGS3s, RGS3p, and RGS14, as indicated in the result section. The indicated mouse EST clones were obtained from Genome Systems (St. Louis, MO) as EcoRI-Nol inserts in the pT7T3-Pac vector, and sequenced. Similarity scores were calculated using Clustalw with the Blossum matrix. To obtain a probe for detecting RGS14 expression, the database was searched with the mouse cDNA sequence that had previously been deposited in GenBank (Accession U850505). One EST clone was identified (AA981480) containing the 3′-untranslated region and sequence encoding the C-terminal 5 aa of mouse RGS14.

Expression of murine RGS proteins

GFP-RGS fusion proteins were generated by cloning cDNA fragments encoding the open reading frame of RGS1, RGS2, or RGS3 into the pEGFP-C1 plasmid (Clontech, Palo Alto, CA). To adapt the respective cDNA in frame to the sequence encoding EGFP, compatible restriction sites were introduced by PCR at the 5′ and 3′ end of the coding region in the RGS cDNAs. In addition, a truncated version of RGS1 was designed that retained the N-terminal 60 aa, but lacks the RGS domain. Primers were used as follows (written 5′-3′): RGS1 forward, CAGATCTTCCAGAATGTTCTTTCGTCAGCCAAAAAG; RGS1 reverse, CGCGGATCCACATGCTGTCAGCCAGGAATCACTCACTTTAAAGT; RGS1T60 forward, GTTCTGCAGTACCTAGCCCATGCCTTGCAGTCC, and reverse, CGCCGATCCTGGGCCAGTCC.

Further work is therefore needed to understand how individual RGS proteins regulate individual GPCRs.
the XhoI site is in the vector polylinker; the fragment encodes aa 222–316 of the predicted full-length mouse RGS3 protein); RGS3s AvrII-Xmal (197 bp; this probe includes 145 bp specific to RGS3s); and RGS14 EcoRI-Ndel (480 bp; the EcoRI site is in the vector polylinker). To control for loading and RNA integrity, membranes were reprobed with a mouse elongation factor-1α probe.

Chemotaxis assay

Chemotaxis assays were performed in 5-μm Transwell plates (Coming Costar, Cambridge, MA). Transfected cells were collected and resuspended in RPMI 1640 containing 0.5% BSA and 10 mM HEPES (migration medium) at a concentration of 7.5 × 10^6/ml (live cells). Chemokines were resuspended in migration media to the indicated concentration, and 600 μl was aliquoted into 24-well plates forming the bottom chamber during the assay. The 5-μm-pore polycarbonate Transwell inserts were transferred to the wells containing media alone or plus chemokine, 100 μl of cells were added into the Transwell insert (top chamber), and cells were allowed to migrate through the porous bottom for 3 h at 37°C. Cells that migrated to the bottom chamber were enumerated, and GFP-positive cells were identified by collecting events for a fixed time (60 s) on a FACScan. By counting a 1/5 dilution of input cells in the same manner, the absolute number of cells that migrated to the bottom chamber could be determined. The percentage of migrated cells was calculated by dividing the number of transmigrated GFP-negative, GFP-dull, and GFP-bright cells by the number of input cells expressing the same GFP levels. Each data point is the mean of duplicate wells. To simplify the comparison between the different transfected cell populations, the GFP-negative fractions of each GFP-RGS-transfected population were normalized to the GFP-negative fraction of the control GFP-transfected cells. This was achieved by first determining the percentage of input GFP-negative cells that migrated in each GFP-RGS-transfected population and then dividing this number by the percentage of input GFP-negative cells that migrated in each control GFP-RGS-transfected population. The percentage of cells in each of the GFP-negative, dull, and bright gates that migrated was then multiplied by this number. The data presented for the different GFP-RGS-transfected populations in the GFP-dull and GFP-bright compartment are the mean from duplicate wells normalized to the migration of GFP-negative cells in the same wells. Dead cells identified by their decreased forward scatter were not included in the analysis.

Results

Identification of murine RGS1 and 3 and a novel RGS3 variant

To study the expression and function of murine RGS1 and RGS3, we searched the mouse NCBI EST database by using human RGS1 (22, 23), or human RGS3 (13) as a template. Twelve mouse ESTs homologous to human RGS1 were identified (AA154742, AA110076, AA120409, AA118893, AA199350, AA289401, AA200917, AA547074, AA608105, AA561820, AA915687, and AA684124) that could be aligned into a contig, and sequence analysis of clone AA154742 revealed a cDNA of 1274 bp encoding a predicted protein of 196 aa (Fig. 1A). The high nucleotide identity (76%) and amino acid identity (87%) of this sequence with human RGS1 (Fig. 1A) support its designation as murine RGS1. The search for mouse RGS3 led to identification of EST clones W49391, AA880930, and AA789696 that contained a 3′-untranslated region and nucleotides encoding for 297 aa homologous to the C terminus of human RGS3. This partial mouse RGS3 cDNA showed 90% amino acid identity to human RGS3 (aa 327–519, encoded by exons 4–8) were aligned with corresponding human RGS sequences.

Murine RGS1, RGS2, and RGS3 protein sequences and alignment with corresponding human RGS sequences. A. Alignment of mouse RGS1 (mRGS1) protein sequence with those of human RGS1 (hRGS1), mouse RGS2 (mRGS2), and human RGS2 (hRGS2). Residues shaded in gray refer to the changes in the amino acid sequence found between mRGS2 and the previously published mRGS2 cDNA (U67187). B. Alignment of RGS3 protein sequences. The short isoform of mouse RGS3 (mRGS3s) and the C-terminal portion of full-length human RGS3 (hRGS3) (aa 327–519, encoded by exons 4–8) were aligned to the partial cDNA clone of murine RGS3 (mRGS3p). The boxed sequence refers to the unique region present in RGS3. Identical amino acids to mRGS1 (A) or mRGS3p (B) are shown as hyphens, and dots represent gaps inserted for optimal alignment. The line above the amino acid sequences indicates the region of the RGS domain. Numbering is with respect to the first amino acid of the full-length protein. These sequence data are available from GenBank/EMBL/DDBJ under accession numbers AF215667 (mRGS1), AF215668 (mRGS2), AF215669 (mRGS3), and AF215670 (mRGS3).
expression of RGS1 was also detected in thymus, lung, peritoneal cells, white blood cells, and bone marrow cells (Fig. 2A). 300-19 pre-B cells expressed detectable levels of RGS2, whereas the 2PK3 cell line differed from mature B cells in lacking measurable RGS2 (Fig. 2A). Analysis of full-length (~3.5-kb) mouse RGS3 transcripts revealed their presence in most tissues, with lymphoid tissues typically expressing high levels (Fig. 2, A and B). B and T lymphocytes also appeared to make only a small contribution to total splenic RGS3 based on expression levels in RAG1<sup>−/−</sup> spleen, although RGS3 expression was evident in both subsets of lymphocytes (Figs. 2, A and B, and 3B). The 300-19 and 2PK3 cell lines expressed little mouse RGS3 (Fig. 2, A and B). Mouse RGS3s (~1.8 kb) was also expressed in most lymphoid tissues, but typically at lower levels than full-length RGS3 (Fig. 2B). High expression of mouse RGS3s was detected in heart, brain, and lung (Fig. 2B). Finally, RGS14 expression was examined because of a report indicating it was highly expressed in rat spleen (26). Mouse RGS14 was constitutively present at high levels in most lymphoid tissues and cells, including thymus, spleen, lymph node, peritoneal cells, white blood cells, and bone marrow cells. High basal expression was detected in freshly purified B and T lymphocytes, and consistent with this, the level of RGS14 was reduced in total RNA from RAG1<sup>−/−</sup> spleen compared with wild-type spleen (Fig. 2A). Low expression of RGS14 was detected in Peyer’s patches, lung, heart, skeletal muscle, intestine, and the 2PK3 and 300-19 cell lines (Fig. 2A).

**RGS expression is dynamically regulated in in vivo activated B cells**

To analyze the expression pattern of RGS1, RGS2, RGS3, and RGS14 during Ag stimulation of B lymphocytes, we used Ig<sub>H</sub>HEL transgenic mice carrying Ig heavy and light chain transgenes encoding Ig<sub>H</sub> and Ig<sub>D</sub> specific for the protein Ag HEL (30). Consistent with previous studies reporting spontaneous changes in RGS1 and RGS2 in human PBMC following in vitro culture (25), we found that expression of RGS1, 2, and 14 underwent rapid changes in murine B cells upon in vitro culture (data not shown). To circumvent this problem, B cells were activated in vivo by i.p. challenge of Ig<sub>H</sub>HEL transgenic mice with HEL, and splenic B cells were then purified at various time points and used directly for RNA preparation. Intraperitoneal injection of HEL Ag was effective in activating splenic B cells in an Ag-specific manner, as established by the increase in expression of the activation marker CD69 on B cells in Ig<sub>H</sub>HEL transgenic mice, but not in nontransgenic mice (Fig. 3A, left panels). Northern blot analysis of total RNA from the isolated B cell populations revealed marked differences in the pattern of RGS molecule expression (Fig. 3, B and C). RGS1 transcripts were expressed at very low levels in B cells from unchallenged mice, but were rapidly induced to high levels after in vivo exposure of Ig<sub>H</sub>HEL transgenic B cells to HEL Ag (Fig. 3, B and C) and persisting at elevated levels for at least 6 h (Fig. 3, B and C). The time course of RGS2 induction in HEL-stimulated Ig<sub>H</sub>HEL B cells was similar, except RGS2 mRNA levels became elevated only ~2-fold over quiescent levels compared with about a 40-fold increase in RGS1 transcript levels (Fig. 3C). In marked contrast, RGS3 and RGS14 transcripts were present in naive B...
cells at substantial levels and HEL stimulation suppressed expression (Fig. 3, B and C). Although RGS3 mRNA was only moderately down-regulated in response to HEL activation, to about half basal levels, RGS14 transcripts showed a more substantial 6-fold decrease. Thus, the RGS1 and RGS2 genes vs the RGS3 and RGS14 genes exhibit reciprocal patterns of expression in naive and Ag-stimulated B cells in vivo.

To explore the specificity of RGS induction in B cells during the response to Ag challenge, we also tested the effect of in vivo stimulation by CD40 using an agonistic anti-CD40 Ab (29). In contrast to the initial follicular exclusion of B cells in response to B cell receptor (BCR) signaling, CD40 signaling is associated with B cell migration into follicles and formation of germinal centers (39). Consistent with previous studies using anti-CD40 in vivo (40), we found that treatment with anti-CD40 Ab, but not with a control IgG Ab, was effective in causing activation of splenic B cells (left panel, bottom). The effect of anti-CD40 mAb was also specific and did not occur after challenge with a control rat mAb (right panel, bottom). Mice were challenged for 6 h (bottom panels) or the indicated times (top panels). B–F, Northern blot analyses showing levels of RGS1, RGS2, RGS3, and RGS14 mRNA in quiescent and activated B cells isolated from spleen tissues of the indicated mice. As a control for RNA loading, the blots were hybridized with an EF1-α probe. B, Time course of RGS gene expression in IgHEL B cells before (0 h) or after 2, 6, 12, or 24 h of in vivo exposure to HEL. C, Quantification of relative RGS mRNA levels at the 0-, 2-, and 6-h time points from the Northern blot shown in B and additional blots. Relative RGS mRNA levels were determined by PhosphorImager analysis of the Northern blots and were normalized to the value obtained for the EF1-α mRNA level. Data show the fold change in RGS mRNA level relative to the mean value of the unstimulated (0-h) cells. Data for individual mice are shown as white dots and means as black bars. n, Indicates the number of individual mice. Statistical analysis by Student’s t test; comparing activated cells to the untreated controls was as follows: RGS1 at 2 and 6 h (p < 0.005); RGS2 at 2 h (p < 0.01) and 6 h (p < 0.005); and RGS14 at 2 and 6 h (p < 0.001). The decrease in RGS3 expression at 2 and 6 h was not statistically significant (p > 0.5). D, Expression of RGS genes in B cells after in vivo challenge of nontransgenic mice with anti-CD40 mAb for the indicated times. Two independent experiments are shown. E, Northern blot analysis of RGS expression in B lymphocytes freshly isolated from spleens of IgHEL transgenic or IgHEL/HEL double (Db1)-transgenic mice. Numbers refer to individual mice analyzed. F, Relative RGS mRNA levels of the Northern blot shown in E and similar blots. Quantification was as in C.
cell line (42), the IgG$^+$ mature B cell lines A20 (43) and 2PK3 (37), the surface Ig-negative M12 B cell line (43), and the J558L plasmacytoma cell line (44). Using 5-μm-pore Transwell chemotaxis chambers, we found that the 2PK3 cell line responded well to all three lymphoid chemokines, whereas 300-19 pre-B cells responded only weakly to SDF-1 and ELC and failed to respond to BLC. Among the other cell lines, A20 and J558L cells showed little ability to migrate to any of the chemokines, M12 cells exhibited high background migration but were able to respond to SDF-1 and BLC, and WEHI231 cells responded to SDF-1 and weakly to BLC and ELC (data not shown). 2PK3 cells were therefore chosen for most of our experiments, although, because of their previous characterization, 300-19 cells were also tested in some cases. To be able to measure quantitatively levels of transfected RGS protein, we tagged the N terminus of mouse RGS1, mouse RGS2, and mouse RGS3s with green fluorescent protein (GFP). 2PK3 cells were transiently transfected with the chimeric GFP-RGS constructs or as controls with GFP or GFPmRGS1T60, which does not contain the RGS domain (Fig. 4A). The chemotactic response of transfected cells to BLC, ELC, or SDF-1 was measured as described for 2PK3 cells in B. The results in B are representative of three or four independent experiment, and data points represent the mean of assay points performed in duplicate; and results in C are the mean of seven experiments with data points performed in duplicate.

FIGURE 4. RGS1 and RGS3 negatively regulate chemotaxis of 2PK3 cells and 300-19 cells to lymphoid chemokines. A, Flow cytometry of GFP levels in 2PK3 cells transiently transfected with either GFP, GFPmRGS1, GFPmRGS1T60, GFPmRGS2, or GFPmRGS3s expression vectors. Relative GFP expression is indicated by N, D, and B, which refers to GFP-negative, GFP-dull, or GFP-bright gates. The GFP-negative population encompassed 63–81% of total live cells, the GFP-dull gate 13–17%, and the GFP-bright gate 11–16%, except for the GFPmRGS2-bright population that contained ~4% of total live cells. The expression levels shown are from the input cell populations of the ELC experiment in B. Similar expression levels were observed in every experiment. B, Regulation of chemotactic activity by RGS proteins in transiently transfected 2PK3 cells. Cells were transiently transfected with pEGFP-C1 containing no insert (GFP alone) (○), mouse RGS1 (■), mouse RGS2 (▲), mouse RGS3s (●), or mouse RGS1T60, which does not contain the RGS domain (□). Transfected cell populations were subjected to chemotaxis through 5-μm-pore Transwell filters. The number of input and migrated cells in each GFP gate, as outlined in A, was determined by flow cytometry. Results are expressed as the percentage of input cells in each GFP gate that migrated to the bottom chamber. The data were normalized to the GFP-negative populations, as described in Materials and Methods. C. Effects of RGS1 and RGS1T60 on 300-19 cell chemotaxis to SDF-1. 300-19 cells were transiently transfected with GFP, GFPmRGS1, or GFPmRGS1T60, and chemotaxis was measured as described for 2PK3 cells in B. The results in B are representative of three or four independent experiment, and data points represent the mean of seven experiments with data points performed in duplicate.
FIGURE 5. RGS1 and RGS3s do not alter surface integrin or CXCR5 expression. Cell surface expression of LFA-1, β1 integrin, and CXCR5 by 2PK3 cells transiently transfected with GFPmRGS1 and GFPmRGS3s, as indicated, 13 h after transfection. GFPmRGS1- or GFPmRGS3s-transfected 2PK3 cells were gated on the GFP-negative (dotted line) or GFP-bright (thick solid line) populations, as described in Fig. 4A to compare RGS-negative vs RGS-expressing cells. Control staining without primary Ab (for β1 integrin or CXCR5 staining) or with a control biotinylated rat IgG (for LFA-1 staining) in the GFP-negative (thin solid line) or the GFP-bright (dashed line) gate shows the background level of fluorescence.

was a strong inhibitor of the migratory response to both ELC and BLC when expressed at sufficiently high levels (GFP-bright) (Fig. 4B), inhibiting migration by about 90% at all concentrations of BLC or ELC compared with migration of control cells expressing GFP alone (Fig. 4B). The inhibition was dose dependent, as cells expressing lower amounts of RGS1 molecules (GFP-dull) were less compromised in their response to ELC or BLC, demonstrating an inhibition of about 60% (Fig. 4B). The inhibitory effect observed was strongly dependent on the RGS domain, as the truncated GFPmRGS1T60 molecule had little effect (Fig. 4B). Similar to RGS1, expression of mouse RGS3s also inhibited migration to ELC and BLC, although the inhibition was less pronounced, being 20–23% at low RGS3s expression (GFP-dull) and about 70% at higher RGS3s expression levels (GFP-bright). Both RGS1 and RGS3s limited 2PK3 cell chemotaxis to SDF-1, but to a lesser extent than in response to ELC or BLC (Fig. 4B). In agreement with RGS1 and RGS3s inhibiting chemotaxis through effects on chemokine responsiveness rather than by causing changes in other molecules needed for the chemotactic response, flow-cytometric analysis showed that levels of LFA-1, β1 integrin, and CXCR5 in the transfected cells were unchanged (Fig. 5). In contrast with the effect of mouse RGS1 and mouse RGS3s, mouse RGS2 had weak effects on 2PK3 cell chemotaxis. Compared with the RGS domain-deficient GFPmRGS1T60 construct, GFPmRGS2 had no effect on the response to SDF-1 and only a small effect on the response to BLC and ELC (Fig. 4B). These findings for RGS2 are consistent with other studies suggesting RGS2 is relatively specific for Gαq family members and has little activity against Gαi proteins (16, 45). To confirm that the GFPmRGS2 protein was active as a GAP, we tested its ability to inhibit signaling by the Gαq-coupled M1 muscarinic receptor. Jurkat cells stably expressing the M1 receptor (46) were transiently transfected with GFPmRGS2 or GFP alone, and the GFP-bright cells were tested for ability to flux calcium in response to the M1 receptor agonist, carbachol. A substantial reduction in the calcium flux was observed in the RGS2-transfected cells compared with cells containing GFP alone (data not shown), providing strong evidence that the GFPmRGS2 protein was functional.

To test whether RGS1 could also down-modulate chemotaxis of 300-19 cells, we transiently transfected the cells with GFPmRGS1 or the control GFPmRGS1T60 construct, and tested the chemotactic response to SDF-1 (Fig. 4C). Notably, although expression of GFPmRGS1 had an inhibitory effect on 300-19 cell migration (Fig. 4C), the effect was not as strong as in 2PK3 cells, with about 30% inhibition for GFP-dull cells and 50% inhibition for GFP-bright cells. In this regard, it is important to note that 300-19 cells, in contrast to 2PK3 cells, express substantial levels of endogenous RGS1 mRNA (Fig. 2A). High endogenous RGS1 protein expression might cause this inhibitory pathway to be near saturation even without the introduction of further RGS1 by transfection. In agreement with this possibility was the very low chemotactic response to SDF-1 of untransfected 300-19 cells compared with 2PK3 cells. At optimal SDF-1 concentrations, only 1% of total 300-19 cells migrated (Fig. 4C) compared with 35% of total 2PK3 cells (Fig. 4B). Interestingly, transfection of 300-19 cells with the GFPmRGS1T60 mutant caused an increase in the migration to SDF-1 compared with cells transfected with GFP alone (Fig. 4C). This effect was not seen in the 2PK3 cells that do not express endogenous RGS1 (Fig. 4B). As RGS1T60 lacks the GAP activity of RGS1, but retains the unique NH2-terminal region, it may compete with endogenous RGS1 for receptor/Gαi association, while being unable to negatively regulate Gαi responses. Taken together, these findings provide evidence that the endogenous RGS1 gene can be expressed at sufficient levels to have effects on cell migration.

Discussion

The findings above establish that in vivo activation of B lymphocytes by Ag promotes rapid up-regulation of RGS1 and RGS2 and decreased expression of RGS3 and RGS14. Self-reactive B cells that have been chronically exposed to autoantigen have a small constitutive elevation in expression of RGS1 and RGS2. In transfection studies in a mature B cell line, RGS1 and RGS3s, but not RGS2, strongly inhibit the Gαi-dependent chemotactic response to lymphoid chemokines BLC, ELC, and SDF-1. These observations suggest that Ag receptor-induced changes in RGS molecule expression may function to regulate B cell responsiveness to lymphoid chemokines and thereby help direct cell-positioning events in lymphoid organs.

To study the activity of RGS1 and RGS3 molecules in regulating chemokine responsiveness in murine lymphocytes, it was first necessary to isolate their mouse homologues. A single form of mouse RGS1 was identified in the EST database that was similar over its entire length to its human homologue. In addition to a conserved RGS domain, this included an ~60-aa amino-terminal domain that is unique to RGS1. Searches for mouse homologues of RGS3 led to identification of two forms of RGS3, a partial clone encoding an analogous short form of human RGS3 were also identified, and we have designated this new RGS3 variant as RGS3s. Human ESTs encoding an analogous short form of mouse RGS3 were also identified, and we have designated this new RGS3 variant as RGS3s. In this variant, the amino-terminal 348 aa encoded by exons 3 and 4 of human RGS3 (34) are replaced by a predicted 21-aa domain (Fig. 1B). The 5′ untranslated sequence of mouse and human RGS3s is also highly conserved and is distinct from sequence previously reported in human RGS3 exon 1, 2, or 3 (data not shown). The mechanisms leading to the expression of this new form of RGS3 are presently unclear, although they presumably involve splicing of one (or more) novel exon to the splice acceptor of what has previously been defined in human RGS3 as exon 5 (34). Future genomic sequence analysis should establish how many exons make up the unique region of RGS3s as well as determining their location with respect to the previously defined exons. Interestingly,
a truncated version of RGS3 (termed RGS3T) has previously been identified by 5′ race analysis (47). The untranslated sequence of RGS3T was found to begin within exon 3, and translation was suggested to start at either of three ATG codons within the 3′ end of exon 3. The RGS3s sequence defined in this study is distinct from the predicted RGS3T because RGS3s contains sequence (and putative exons) not present in the original RGS3, whereas the sequence encoded within RGS3T, although truncated, is identical to the original RGS3. Because RGS3s and RGS3T transcripts appear to be of similar size, they may both contribute to the prominent lower m.w. RGS3 hybridization bands seen on Northern blots of human heart, brain, lung, kidney, and liver RNA (13, 38). As discussed further below, the different amino-terminal regions may have significant effects on the function of RGS3. Thus, in future studies, it will be important to determine the relative amounts of the different RGS3 isoforms in B cells and other cell types.

By expressing GFP fusion proteins of RGS1, RGS2, and RGS3s in the 2PK3 B lymphoma cell line, we have been able to establish that RGS1 and RGS3s antagonize chemotactic responses mediated by SDF-1, BLC, and ELC, chemokines that signal through three different receptors (CXCR4, CXCR5, and CCR7, respectively). By contrast, RGS2 had only limited effects, causing slight reductions in the response to BLC and ELC and little or no reduction in SDF-1 responses. These findings are in close agreement with a recent report from Bowman et al., who found that human RGS1, 3, and 4, but not human RGS2, diminished the chemotactic response of transfected L1.2 pre-B cells to fMLP, IL-8, and monocyte chemotactic protein-1 (48). In another recent study, RGS3 was shown to antagonize the migratory response of intermedullary collecting duct kidney cells to lysophosphatidic acid (49). Interestingly, in the study of Bowman et al., RGS3 was more effective at inhibiting chemokine responses than RGS1 (48), whereas in our experiments, the RGS1 construct was more effective than the RGS3 construct (Fig. 4). These differences may reflect specific properties of the transfected cell lines (L1.2 vs 2PK3 and 300-19) or of the different chemokine receptors under study. However, it should be noted that the construct used by Bowman et al. contained the original large RGS3 isoform, whereas our construct was made with the shorter RGS3s variant. The NH2-terminal domain of RGS3 has recently been shown to translocate to cell membranes in response to increases in intracellular calcium (17), suggesting it may enhance the ability of RGS3 to inactivate membrane-associated G proteins. It therefore seems possible that the stronger inhibitory effect of RGS3 than RGS3s reflects intrinsic differences in the Gαi inhibitory properties of the two proteins. In addition to effects on chemotactic responses, RGS1, 2, and 3 have previously been shown to inhibit IL-8-induced activation of extracellular signal-regulated kinase (ERK) in transfected 293T cells (13). In biochemical studies, RGS1 and 3 functioned as GAPs for Gαi (50, 51), whereas RGS2 was more effective as a GAP for Gqα (16, 45). However, some studies have indicated that RGS1 and RGS3 can also have functional effects on Gqα family members (16–18, 47), and RGS2 may be able to antagonize some Gαq family members (13). Because the chemotactic response requires signaling by pertussis toxin-sensitive Gαi-coupled receptors, our findings support the conclusion that RGS1 and 3 can function as GAPs for Gαi molecules, whereas RGS2 is an inefficient GAP for this family of proteins.

The physiological relationship between BCR-induced changes in RGS molecule expression and changes in B cell responsiveness to chemokines is presently not understood. BCR triggering in B cells leads to a decrease in the magnitude of the response to the CXCR4 ligand, SDF-1, and an increase in the response to the CCR7 ligands, ELC and SLC (7, 52, 53). It seems possible that the rapid increase in RGS1 induced by BCR signaling works together with other processes, such as phosphorylation-mediated down-regulation of CXCR4 receptors (53), to decrease the chemotactic response to SDF-1. In this respect, it is noteworthy that germinal center B cells have high levels of RGS1 (22) and CXCR4, but fail to migrate to SDF-1 (52). Consistent with this is the low chemotactic response to SDF-1 of 300-19 pre-B cells, which express high constitutive levels of endogenous RGS1 (Figs. 2A and 4C). Likewise, the ability of the truncated form of RGS1 (RGS1T60) to augment the response of 300-19 pre-B cells to SDF-1 (Fig. 4C) indicates a possible physiological role for RGS1 in regulating CXCR4 responsiveness, because this deletion mutant might be expected to antagonize interactions needed for full-length RGS1 to function. 2PK3 cells did not have detectable endogenous RGS1 expression and, as predicted by this model, overexpression of RGS1T60 in these cells failed to augment the response to SDF-1. However, it remains to be established in which cell populations RGS1 regulates CXCR4 responsiveness in vivo.

Despite the increase in RGS1 message following BCR engagement and the ability of RGS1 to antagonize the response of transfected 2PK3 cells to ELC, chemotaxis of mature B cells to ELC is increased after BCR stimulation. This suggests that the action of RGS1 in inhibiting chemokine receptor-stimulated migration may be more selective in mature B cells than in the transformed 2PK3 cell line. RGS selectivity for responses mediated by particular chemokine receptors could arise at several levels, such as the G protein-coupling propensity of the chemokine receptor (54, 55), or through direct interaction of RGS molecules with chemokine receptors, as has been suggested in other GPCR systems (16, 19, 20). Alternatively, the levels of RGS1 induced in mature B cells by Ag may not be sufficient to inhibit the ELC response. Reciprocally, perhaps the small decrease in RGS3 expression that occurs in B cells activated in vivo by Ag contributes to the enhanced chemotactic response to ELC. The decreased expression of RGS14 might also be important in this enhancement, although it has yet to be shown whether RGS14 is an antagonist of chemokine-mediated chemotaxis. Alignment of the RGS14 GAP domain with other RGS molecules (data not shown) indicates is has highest similarity with RGS12, which can regulate Goi (19), consistent with a possible role for RGS14 in regulating chemotaxis.

Further to the changes in RGS expression in B cells activated acutely by Ag, small differences in RGS molecule expression were evident in chronically stimulated, anergic, B cells (Fig. 3). IgHEL/HEL double-transgenic mice contain fewer B cells than IgHEL transgenic mice, and the purity of the B cell preparations obtained from the double-transgenic animals is typically a few percentages lower than from IgHEL transgenic mice, making it possible that the differences seen in RGS molecule expression are due to differences in B cell purity. We think this unlikely, however, because if the elevation in RGS1 and 2 in anergic cells was attributable to contaminating cells, then it might be expected that RGS3 and 14 should also be elevated, as all four RGS molecules are expressed at high levels in total spleen cells (Fig. 2). Yet RGS3 and RGS14 levels were not elevated in the anergic cells and may even be reduced (Fig. 3). Previous studies in human PBMC have shown that RGS1 is strongly induced by phorbol esters, activators of the ERK pathway, whereas RGS2 is strongly up-regulated by treatment with ionomycin (25). Anergic B cells have a small constitutive elevation in ERK activity and in intracellular calcium (56), and these signals may therefore contribute to the elevated basal RGS1 and RGS2 expression. It seems possible that the differences in RGS1 molecule expression could contribute to the reduced competitiveness of anergic B cells for migrating into lymphoid follicles (57). The significance of the elevated RGS2 expression induced by
acute and chronic Ag exposure remains an intriguing question because the role of signaling by Gq family molecules in B cells is poorly defined. One group has suggested that GqQ molecules may regulate signaling via Btk’s tyrosine kinase (Btk) (58, 59), raising the possibility that RGS2 functions in activated and anergic B cells to limit the extent of Btk activity.

In summary, our studies together with others suggest that regulated expression of RGS1 and RGS3, and possibly RGS14, plays a role in changing B cell responsiveness to chemokines during the response of B cells to foreign or self Ags. Alterations in RGS2 expression during acute and chronic B cell activation appear more likely to regulate other, presently unknown, GPCR signals in B cells. In future studies, it will be important to characterize whether the additional RGS family members that have recently been identified in lymphoid tissues, including RGS12 (19, 26) and RGS16 (27), are also modulated during B lymphocyte activation. A more complete understanding of RGS function in regulating B cell migration, differentiation, and tolerance will also require greater knowledge of the interplay between these proteins and other molecules, such as GPCR kinases, protein kinase C, protein kinase As, and arrestins, that also regulate GPCR signaling.

Acknowledgments

We thank Paul Hyman for producing recombinant chemokines; A. Rolink for the anti-CD40 Ab; Cliff McArthur for calcium analysis on the MoFlo; and Mark Ansel, Eric Ekland, and Sanjiv Luther for comments on the manuscript.

References