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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 Chemokines¹,²

Karin Reif and Jason G. Cyster³

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 chemoattractant, 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.

M igration 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 chemoattractant (BLC)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 (ELC)/macrophage-inflammatory protein-3β (6) (reviewed in Refs. 1 and 6). In agreement with a role in T zone organization, SLC and ELC strongly attract naive T lymphocytes and weakly attract B cells (7, 8). Another candidate for positioning B and T lymphocytes within secondary lymphoid organs is stromal cell-derived factor-1 (SDF-1), the ligand for CXCR4 (9).

Chemokine receptors are coupled to heterotrimeric G proteins, which consist of α, β, and γ subunits. Four families of Gα subunits can be distinguished based on their function and amino acid sequence homology, and are termed Gαs, Gαi, Gq, and Gα12. In vitro studies suggest that Gαi family members are essential for mediating chemoattractant responses (10). For Gα subunits to function, they must switch between an inactive GDP-bound and an active GTP-bound conformation. Essential elements in this GDP/GTP cycling are positive regulators that accelerate the nucleotide exchange on Gα and negative regulators that increase the intrinsic GTPase activity of Gα. While activated G protein-coupled receptors (GPCRs) themselves act as nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) for heterotrimeric G proteins have only recently been recognized and were named regulators of G protein signaling (RGS) molecules (11–14). Studies on the specificity of RGS molecules for different G proteins to date have indicated that

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human RGS1, RGS3, RGS4, and RGS16 predominantly interact with Gαi subunits, RGS2 with Gαq, RGS21 with Gαz, and p115RhoGEF with Gα12/13. These relationships may not be rigid, however, as some studies have indicated that RGS1 and RGS3 can inhibit signaling via Gαq-coupled receptors (16–18), while RGS2 may antagonize some Gαi-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 is also highly expressed in spleen as well as in brain and lung (26). Recently, it has also been 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, Sunnyvale, CA). Reagents

Cell culture and transient transfection

The murine B cell lines were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM t-glutamine, 50 μg/ml streptomycin, 50 μg/ml penicillin, 100 units of penicillin, 2 mM l-glutamine, 50 μg/ml streptomycin, and 50 μg/ml gentamicin. Mouse RGS1, RGS2, and RGS3 were expressed in COS-1 cells by electroporation with the following amounts of plasmid DNA: 10 μg pEGFP-C1, 30 μg pEGFP-C1-mRGS1, 40 μg pEGFP-C1-mRGS1T60, 40 μg pEGFP-C1-mRGS2, and 30 μg pEGFP-C1-mRGS3. Cells (1–1.5 × 10^6 in 0.4 ml) were pulsed at 250 V and 960 μF. Assays were started 10–14 h after transfection.

Cell culture and flow cytometry

Mouse lymphocyte cell suspensions were obtained from spleen or lymph node by passing teased tissues through a 70-μm cell strainer (Falcon; Becton Dickinson, Franklin Lakes, NJ). B cells were purified from spleen cell suspensions by staining non-B cells with anti-IgM and anti-CD11c biotin (PharMingen, San Diego, CA) and depletion using streptavidin-PE. T cells were stained with anti-CD4 and anti-CD8 (clone FKG2) (29) which was a gift from Antonius Rolink (Basel Institute for Immunology, Basel, Switzerland). Hen egg lysozyme (HEL) was purchased from Sigma (St. Louis, MO).

Mouse and animal challenge

C57BL/6 MD4 Ig transgenic (Ig<sup>HEL</sup>) mice carry transgenes encoding IgM<sup>HEL</sup> and IgD<sup>HEL</sup> 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 metallothionine promoter and contains HEL at 10–30 ng/ml in serum (30). C57BL/6 MD4 and MLS transgenic mice were mated to obtain double-transgenic (Ig<sup>HEL</sup>/HEL) mice. Transgenic mice were subjected to gel electrophoresis and transferred to a Hybond-N membrane. 2PK3 and 300-19 cells were transfected by electroporation with the following amounts of plasmid DNA: 10 μg pEGFP-C1, 30 μg pEGFP-C1-mRGS1, 40 μg pEGFP-C1-mRGS1T60, 40 μg pEGFP-C1-mRGS2, and 30 μg pEGFP-C1-mRGS3. Cells (1–1.5 × 10^6 in 0.4 ml) were pulsed at 250 V and 960 μF. Assays were started 10–14 h after transfection.

Cell culture and flow cytometry

Cell purification and flow cytometry

Mouse lymphocyte cell suspensions were obtained from spleen or lymph node by passing teased tissues through a 70-μm cell strainer (Falcon; Becton Dickinson, Franklin Lakes, NJ). B cells were purified from spleen cell suspensions by staining non-B cells with anti-IgM and anti-CD11c biotin (PharMingen, San Diego, CA) and depletion using streptavidin-PE. T cells were stained with anti-CD4 and anti-CD8 (clone FKG2) (29) which was a gift from Antonius Rolink (Basel Institute for Immunology, Basel, Switzerland). Hen egg lysozyme (HEL) was purchased from Sigma (St. Louis, MO).

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-Vol inserts in the pITT73-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 U85055). 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 encoding EGFP, compatible restriction sites were introduced by PCR at the 5′ end of the coding region in the RGS cDNAs. In addition, a truncated version of RGS1 was designed that retains the N-terminal 60 aa, but lacks the RGS domain. Primers used were also tested for the induction of CD69 using anti-CD69 FITC (PharMingen). For integrin and CXCR5 expression analysis, the following Abs were used: anti-CD11a (LFA-1, Mac-1) (Caltag Laboratories, Burlingame, CA), anti-CD11c, and Gr-1 (PharMingen), followed by depletion by MACS. Flow cytometry analysis on a FACSscan (Becton Dickinson, San Jose, CA) confirmed that purity of the isolated B cells was >92% B220<sup>−</sup> cells, and of isolated T cells >93% CD4<sup>+</sup> or CD8<sup>+</sup> cells. When mice were challenged with HEL or anti-CD40 mAb before isolation of B cells, purified B cells were depleted for the induction of CD69 using anti-CD69 FITC (PharMingen). For integrin and CXCR5 expression analysis, the following Abs were used: anti-CD11a (LFA-1 α chain) biotin (PharMingen), followed by streptavidin-PE (Caltag); anti-CD29 (β, integrin chain, followed by anti-hamster IgG PE (PharMingen); and anti-CXCR5 (Burkitt’s lymphoma receptor 1) (33), followed by anti-rabbit IgG biotin (PharMingen) and streptavidin PE.

Northern blot analysis

A total of 10–15 μg of RNA from mouse tissues or purified cells was subjected to gel electrophoresis and transferred to a Hybond-N<sup>+</sup> membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Blots were probed successively with random-primed 32P-labeled mouse DNA probes (>2 × 10<sup>6</sup> cpm/ml) at 65°C for 1–4 h in ExpressHyb solution (Clontech). Membranes were washed according to the manufacturer’s instructions, exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA), and quantitated by PhosphorImager analysis. The cDNA fragments used as probes were from the mouse EST clones in the pITT73-Pac vector, as follows: RGS1 Xhol-PvuII (809 bp); RGS2 XbaI-BamHI (605 bp); RGS3 XhoI-HindIII (359 bp).
the XhoI site is in the vector polylinker; the fragment encodes aa 222–316 of the predicted full-length mouse RGS3 protein; RGS3s AvrHI-Amal (197 bp; this probe includes 145 bp specific to RGS3s); and RGS14 EcoRl-Ndel (480 bp; the EcoRl 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 media) at a concentration of 7.5 × 10⁶/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 existence of this variant RGS3 sequence in both mouse and human indicates a conserved role and leads us to name this form of RGS3 as RGS3s. Finally, we isolated and sequenced not shown). The existence of this variant RGS3 sequence in both mouse and human indicates a conserved role and leads us to name this form of RGS3 as RGS3s.

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, AA119350, AA288401, AA200913, 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, AA80930, and AA789696 that contained a 3′-untranslated region and nucleotides encoding for 219 aa homologous to the C terminus of human RGS3 (Fig. 1A). The high nucleotide identity (76%) and amino acid identity (87%) of this sequence with human RGS3 (Fig. 1A) support its designation as murine RGS3. The search for mouse RGS3 led to identification of EST clones W49391, AA80930, and AA789696 that contained a 3′-untranslated region and nucleotides encoding for 219 aa homologous to the C terminus of human RGS3. This partial mouse RGS3 cDNA showed 90% amino acid identity to human RGS3 (aa 222–519 of human RGS3) and contains an alternative 5′-untranslated region (nt 591–2638 of human RGS3). We also identified three ESTs (AA278130, AA717912, and A529892) encoding a novel variant of RGS3 that lacks the N-terminal 326 aa encoded by exon 3 in human RGS3 (34) and contains an alternative 5′-untranslated region encoding 21 aa, giving rise to a predicted protein of 192 aa (Fig. 1B). A search of the human EST database revealed two ESTs (H08458 and N87218) encoding an identical NH2-terminal region to that identified in the short form of mouse RGS3 and also containing highly similar 5′-untranslated sequence (data not shown). The existence of this variant RGS3 sequence in both mouse and human indicates a conserved role and leads us to name this form of RGS3 as RGS3s. Finally, we isolated and sequenced mouse RGS2 from the EST clone AA221794. The sequence was found to differ from the previously reported mouse RGS2 cDNA (35) by 4 nt in the coding region. The EST cDNA contained the nucleotides GG instead of CA at position 137/138 and GA instead of GC at position 232/233.

FIGURE 1. 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). 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 with corresponding human RGS sequences.
Expression pattern in mouse tissues of RGS1, RGS2, RGS3, and RGS14. Northern blot analysis of total RNA showing levels of RGS1, RGS2, RGS3, and RGS14 mRNA (A) and RGS3 and RGS3s mRNA (B) in the indicated cells and tissues. As a control for RNA loading, the blots were hybridized with an EF1-α probe. BM, bone marrow; mLN, mesenteric lymph nodes; PP, Peyer’s patches; Spl, spleen; skel, skeletal. The tissue from which the B or T cells were isolated is indicated in parentheses.

Tissue distribution of murine RGS1, RGS2, RGS3, and RGS14 transcripts

Human or rat molecules of RGS1, RGS2, RGS3, and RGS14 have been reported to be expressed in lymphoid tissues (22–24, 26). To examine the expression pattern of these RGS members in murine lymphoid tissues, Northern blot analyses were performed (Fig. 2). Human or rat molecules of RGS1, RGS2, RGS3, and RGS14 were highly conserved, with 11 aa changes between mouse RGS1 and human RGS1, 7 between mouse RGS3 and human RGS3, and only 1 difference between mouse RGS2 and human RGS2.

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 IgHEL transgenic mice carrying Ig heavy and light chain transgenes encoding IgM+ and IgD+ 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 IgHEL 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 IgHEL 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. 3A, 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 IgHEL transgenic B cells to HEL Ag (Fig. 3B and C) and persisting at elevated levels for at least 6 h (Fig. 3B and C). The time course of RGS2 induction in HEL-stimulated IgHEL 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 and the IgG+ mature B cell line, 2PK3 (37), revealed a striking difference in RGS1 levels, with strong expression in the 300-19 cells, but no detectable expression in 2PK3 cells (Fig. 2A). RGS2 was found to be strongly expressed in brain, heart, lung, spleen, white blood cells, and bone marrow cells (Fig. 2A) in accordance with previous studies (35). Again strong expression was detected in RAG1−/− spleen and expression was low in purified lymphocytes. However, in contrast to RGS1, basal RGS2 expression was detected in freshly isolated B cells, and this level was typically higher than in T 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−/− 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−/− 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).

FIGURE 2. Expression pattern in mouse tissues of RGS1, RGS2, RGS3, and RGS14. Northern blot analysis of total RNA showing levels of RGS1, RGS2, RGS3, and RGS14 mRNAs (A) and RGS3 and RGS3s mRNAs (B) in the indicated cells and tissues. As a control for RNA loading, the blots were hybridized with an EF1-α probe. BM, bone marrow; mLN, mesenteric lymph nodes; PP, Peyer’s patches; Spl, spleen; skel, skeletal. The tissue from which the B or T cells were isolated is indicated in parentheses.
Ab, was effective in causing activation of splenic B cells (Fig. 3). We found that treatment with anti-CD40 Ab, but not with a control IgG, caused changes in cell migration and formation of germinal centers. Consistent with previous studies using anti-CD40 in vivo (40), we observed up-regulation of CD40 on B cells. In contrast to the effects of activation by Ag, chronic exposure of B cells to self Ag leads to a state of anergy and causes changes in cell migration and survival (2). To examine the effect of chronic self Ag exposure on the expression levels of RGS mRNA in B cells, B lymphocytes were isolated from spleens of Ig HEL transgenic or Ig HEL/HEL double (Dbl)-transgenic mice. Numbers refer to individual mice analyzed. E, Northern blot analysis of RGS expression in B lymphocytes freshly isolated from spleens of Ig HEL transgenic or Ig HEL/HEL double (Dbl)-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.

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 (Fig. 3A, right panels). In striking contrast to the effects of activation by Ag, challenge of mice with anti-CD40 mAb for various times did not change RGS1 expression in B cells and caused a modest decrease in RGS2 mRNA levels (Fig. 3D). RGS3 and RGS14 transcripts appeared to be transiently reduced in response to CD40 stimulation, as observed for BCR-activated B cells.

Chronic exposure of B cells to self Ag leads to a state of anergy and causes changes in cell migration and survival (2). To examine the effect of chronic self Ag exposure on the expression levels of RGS mRNA in B cells, B lymphocytes were isolated from Ig HEL/HEL double-transgenic mice. These mice carry in addition to the Ig HEL transgenes a second transgene encoding soluble HEL as a circulating self Ag in a form and amount sufficient to trigger anergy, but not deletion (30). In chronically stimulated B cells isolated from Ig HEL/HEL double-transgenic mice, levels of RGS1 and RGS2 transcripts were elevated by about 2- or 3-fold, respectively (Fig. 3, E and F). Interestingly, the extent of up-regulation was reciprocal to that observed following acute activation: RGS2 transcript levels were relatively higher in chronically vs acutely activated B cells, whereas RGS1 levels were substantially lower in the chronically activated B cells. Both RGS3 and RGS14 appeared to be expressed at slightly reduced levels in chronically stimulated compared with naive B cells (Fig. 3, E and F), although the reductions appeared less marked than seen in acutely activated B cells.

RGS1 and RGS3 inhibit chemotaxis of transfected B cell lines to lymphoid chemokines

To test whether RGS1, RGS2, or RGS3 could regulate B cell chemotactic responses to lymphoid chemokines, we first sought to identify a transfectable B cell line that was responsive to BLC, ELC, and SDF-1. We compared the 300-19 pre-B cell line previously used in chemotaxis studies (41), with the IgM+ WEHI231 B cell line. Using the U937-HEL pre-B cell line derived from the WEHI231 cell line, we found that the U937-HEL line was responsive to BLC, ELC, and SDF-1, but not to HEL.

We next compared the effects of RGS1, RGS2, and RGS3 on the chemotaxis of the U937-HEL pre-B cell line. We used a Boyden chamber assay to measure the chemotaxis of U937-HEL cells in response to soluble HEL, BLC, ELC, and SDF-1. As shown in Fig. 4, A–D, RGS1 and RGS3 inhibited chemotaxis of U937-HEL cells to HEL, BLC, ELC, and SDF-1, whereas RGS2 had no significant effect on chemotaxis to HEL or BLC. These results suggest that RGS1 and RGS3 may play a role in regulating B cell chemotaxis to lymphoid chemokines.
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-

m

M-pore Transwell chemo-
taxis chambers, we found that the 2PK3 cell line responded well to

all three lymphoid chemokines, whereas 300-19 pre-B cells re-
sponded 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 ex-
hibited 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 there-
fore 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, a RGS1 chimera that lacks the RGS domain and hence the GAP
activity of the molecule (Fig. 4A). The chemotactic response of
transfected cells to BLC, ELC, or SDF-1 was measured in Trans-
well migration assays, and the migratory cells were divided into
those expressing intermediate (GFP-dull, second quadrant) or high
(GFP-bright, third quadrant) amounts of RGS-GFP fusion protein.

During our analysis, we found that each batch of transfected cells
showed differences in the overall migration levels, assessed by the
response of the GFP-negative cells in each population. To com-

pensate for this, the migration of each transfected population was
normalized so that the response of the GFP-negative cells was
matched (see Materials and Methods). We found that mouse RGS1

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 assay points performed in duplicate; and results in C are 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 ELC and BLC (Fig. 4B). These findings for RGS2 are consistent with other studies suggesting RGS2 is relatively specific for Gqq family members and has little activity against Gai proteins (16, 45). To confirm that the GFPmRGS2 protein was active as a GAP, we tested its ability to inhibit signaling by the Gqq-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/Gai association, while being unable to negatively regulate Gai 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 Gq-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 that appeared to correspond to full-length human RGS3, and several clones encoding a short form of mouse RGS3. Human ESTs encoding an analogous short form of human 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,
CCR7 ligands, ELC and SLC (7, 52, 53). It seems possible that cells leads to a decrease in the magnitude of the response to the chemokines is presently not understood. BCR triggering in B molecules, whereas RGS2 is an inefficient GAP for this family of proteins. However, some studies have indicated that RGS1 and RGS3 can 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 chemoattractant 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 Gq 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 Gq (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 Gqα family members (13). Because the chemotactic response requires signaling by pertussis toxin-sensitive Gqα-coupled receptors, our findings support the conclusion that RGS1 and 3 can function as GAPs for Gqα 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 Gqα (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/H double-transgenic mice contain fewer B cells than IgHEL/H 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/H 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 contamination, 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 Gzq family molecules in B cells is poorly defined. One group has suggested that Gzq molecules may regulate signaling via Bruton’s tyrosine kinase (Btk) (58, 59), raising the possibility that GRS2 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 GRS function in regulating B cell mobilization, differentiation, and tolerance will also require greater knowledge of the interplay between these proteins and other molecules, such as GPCR kinases, protein kinase Cs, protein kinases 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


