Regulation of Chemotactic and Proadhesive Responses to Chemoattractant Receptors by RGS (Regulator of G-protein Signaling) Family Members*

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Serpentine Goi-linked receptors support rapid adhesion and directed migration of leukocytes and other cell types. The intracellular mechanisms mediating and regulating chemoattractant-directed adhesion and locomotion are only now beginning to be explored. RGS (regulator of G-protein signaling) proteins are a recently described family that regulate Goi-stimulated pathways by acting as GTPase-activating proteins. Little is known about the GTPase activity of the Goi proteins involved in adhesion and chemotaxis, or the significance of their regulation to these responses. Using transiently transfected lymphoid cells as a model system, we show that expression of RGS1, RGS3, and RGS4 inhibits chemoattractant-induced migration. In contrast, RGS2, a regulator of Goi activity, had no effect on cell migration to any chemoattractant. RGS1, RGS3, and RGS4 also reduced rapid chemoattractant-triggered adhesion, although the proadhesive response appears quantitatively less sensitive to RGS action than chemotaxis. The results suggest that the duration of the Goi signal may be a particularly important parameter in the chemotactic responses of leukocytes, and demonstrate the potential for RGS family members to regulate cellular adhesive and migratory behaviors.

Cell migration is a required behavior in the development and maintenance of multicellular organisms. During embryonic development, epithelial cell migration occurs at the gastrulation stage, and neural crest cell migration is required to form the developing nervous system. The axonal growth cones of elongating nerves detect and migrate in response to various chemotactants and chemorepellents in the surrounding environment as they are directed toward their precise target. In the adult, movement of fibroblasts into areas of injury plays an important role in wound repair, and the migration of endothelial cells performs a paramount role during angiogenesis. Leukocytes are recruited to sites of inflammation, and during normal immune surveillance, lymphocytes migrate from the blood into various microenvironments of lymphatic organs whereupon they encounter antigen presenting cells that stimulate the lymphocytes’ effector functions and promote a more potent immune response. Although much is known concerning the motor that drives cell migration (actin assembly and disassembly) (1, 2), the cellular signal transduction pathways that coordinate these processes in the context of cell movement are largely undefined.

In addition to pro-migratory stimuli (chemokinesis), chemoattractants also provide a directional signal to cells leading to migration toward the source of the chemoattractant (chemotaxis). In the case of leukocytes, chemoattractants direct migration from the bloodstream into organized lymphoid organs and into inflamed tissues (3–6). Subsequently, they may target lymphocytes into specialized microenvironmental niches within lymphoid organs (4). Similarly, myeloid cells in inflamed tissues can be directed toward the invading organism via the interaction of host-derived and target-derived chemoattractants (7–9). Chemoattractants also can direct the rapid, integrin-dependent adhesion of leukocytes to various cell-associated or extracellular proteins if the corresponding chemoattractant receptor is expressed at high levels (10). The number of identified leukocyte chemoattractants has exploded in the past years with the addition of many newly cloned chemokines (chemoattractant cytokines) to the list of “classical” chemoattractants such as formyl peptides, C5a, and leukotriene B4.

Most leukocyte chemoattractants mediate their activity by binding and stimulating specific Goi-coupled receptors (11, 12). Chemoattractant binding to receptor facilitates the receptor-coupled Goi subunit exchange of bound GDP for GTP, and subsequent dissociation of Goi from the receptor and the βγ heterodimer. The active GTP-bound Goi subunit and the released βγ heterodimer can then stimulate their respective effector pathways. Recent studies suggest that βγ-mediated signaling is critical to the chemotactic response (13, 14). The Goi subunit has an intrinsic GTPase activity that converts the active GTP-bound form back to the inactive GDP-bound form. GDP-bound Goi reassociates with the βγ heterodimer to complete the cycle and turn off the G-protein-stimulated signal(s) (15). Importantly, little is known as yet about the duration of...
the stimulated G-protein response to chemoattractants (16), let alone about the importance of this to proadhesive versus migratory behaviors of the cell.

Sophisticated mechanisms exist, however, to modify cellular responses through G-protein coupled receptors. Treatment with a chemoattractant can render the cell less responsive to subsequent stimulation by the same agonist, a process to which homologous receptor phosphorylation and desensitization can contribute (17). Treatment with some attractants can also render a cell less responsive to subsequent treatment with agonists that bind different receptors, a process that can be mediated by heterologous receptor desensitization (17), heterologous receptor sequestration (18), or, potentially, inhibition of downstream machinery. Both homologous and heterologous cross-talk between agonists and their receptors can help control and target leukocyte migration to chemoattractants (7–9).

Recently, a distinct molecular mechanism with the potential to modulate G-protein responses has been discovered. Genetic evidence from mutants in Saccharomyces cerevisiae (19), Caenorhabditis elegans (20), and the filamentous fungus Aspergillus nidulans (21) suggested that a group of proteins with a common domain, the RGS1 (for regulator of G-protein signaling) domain (20, 22), can directly control aspects of G-protein-stimulated signaling pathways. RGS proteins appear to enhance the endogenous GTPase activity of G-proteins, thus decreasing the half-life of the active GTP-bound state and limiting the duration of Goi signaling. At least 19 mammalian RGS members have been identified (23). Many RGS members have been shown to bind to Go, proteins and activate the Go, subunit’s intrinsic GTPase activity in in vitro biochemical assays. Although only few in vivo studies have been performed in mammalian cells, these studies have suggested that RGS proteins can regulate G-protein-mediated events such as G-protein-gated inward rectifier K+ (GIK) channels (24, 25) as well as Goi-stimulated responses. Inhibition of βγ signaling by RGS proteins may reflect GDP-bound Goi reassociation with and hence sequestration of free active βγ heterodimers.

Based on these observations, we hypothesized that RGS proteins might also regulate cellular migratory and proadhesive responses to chemoattractants. It is not apparent, however, whether to expect that RGS proteins would stimulate or inhibit chemotaxis and adhesion responses. RGS4 and RGS8 have unexpectedly been shown to stimulate rather than inhibit the opening of GIK channels in Xenopus oocytes following the addition of acetylcholine or dopamine. Subsequent to the increased conductance through these channels, RGS4 and RGS8 also stimulated the rapid closure of the same channels following agonist removal (24, 25). In addition, recent studies of light-stimulated rhodopsin/transducin signaling clearly indicate that certain cellular responses (e.g., the amplitude of light-induced membrane depolarization) are regulated not by the duration of the G-protein signal, but rather by the rate of receptor desensitization, a G-protein-independent process (26). Thus, if and how RGS proteins regulate proadhesive and migratory responses must depend on the dynamics of the signaling pathways involved, and must be assessed experimentally.

This study was undertaken to address this issue using lymphoid cell responses to classical chemoattractants and chemokines as a model. The results suggest that regulation of the GTPase activity can significantly influence the migratory and adhesive response to chemoattractants and demonstrate the potential for RGS control of chemoattractant-directed cell motility.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cDNAs for human FLAGged formyl peptide receptor (FPR) (27), human FLAAGed CXCR1 (CXCR1) (28), human FLAAGed C5a receptor (C5aR) (29, 30) and human FLAAGed CCR-2B (31) encoded within pRC-CMV (Invitrogen, Carlsbad, CA) have been described previously (10). "FLAG" epitope addition to the N terminus of these receptors does not affect the binding of their corresponding agonist nor their subsequent responses (10). The L1/2.2 line was derived by transient transfection of the parental L1–2 cells with a chemoattractant receptor and selecting for the cells that migrated to ligand. The migrated cells were expanded (losing receptor expression in the process) and the cycle repeated once more to generate the L1/2.2 line. The L1/2/CXCR1/CCR-2B cell line was developed as follows. L1–2 cells were stably transfected with the FLAAGed CXCR1 as described in Ref. 10. FACS sorted for high expression using the anti-FLAG monoclonal antibody M1, and selected for functional expression of the receptor by chemotaxis to interleukin-8 (IL-8) (32, 33). Subsequently, the CXCR1–stabilized cells were stable transfected with FLAAGed CCR-2B and cells with functional levels of receptor obtained by chemotaxis of the double transfecants to the CCR-2B ligand, monocye chemoattractant protein (MCP-1) (34). The L1/2/FP/CXCR1 cell line was developed in an analogous way as the L1/2/CXCR1/CCR-2B cell line. The cDNAs for RGS1, RGS2, and RGS3 encoded within pRC-CMV and RGS4 encoded within pCR3 (Invitrogen) have been described previously (22). The RGS4N88S mutant was made by annealing primers containing the appropriate mutation (AAT to AGT) to the pCR3 vector containing RGS4, performing 12 cycles of PCR and then digesting the starting vector with DpnI according to manufacturer’s instructions enclosed in the QuiChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Clones were isolated that had lost the unique Sphi site in RGS4 following mutagenesis and were sequenced to confirm that there were no additional mutations. pGreen Lantern was obtained from Life Technologies, Inc. and contains a “humanized” GFP gene with a S65T mutation. FMLP and recombinant human C5a was purchased from Sigma. IL-8 was a generous gift from Sandoz Forschungsinstitut (Vienna, Austria), and MCP-1 was obtained from R&D Systems (Minneapolis, MN).

**Transfection of L1/2 Cells—**L1/2, L1/2/CXCR1, L1/2/CCR-2B, and L1/2/CXCR1/CCR-2B cells grown in RPMI 1640 containing 10% iron-supplemented bovine calf serum, 3 mM sodium pyruvate, penicillin-streptomycin, 2 mM 1-glutamine, and 500 μM β-mercaptoethanol were harvested by centrifugation after reaching 3 × 106 cells/ml (post-logarithmic growth). Cells were resuspended in RPMI 1640 at 1.0–1.5 × 107/ml and 0.8 ml of cells transferred to an Electrophor (0.4 cm gap, Life Technologies, Inc.). The DNAs to be co-transfected were mixed in a sterile microcentrifuge tube, brought to 137 mM NaCl, and 500 μg of yeast tRNA (Boehringer Mannheim) in phosphate-buffered saline added. The DNA mixture was added to vette, mixed by inversion, and allowed to stand at room temperature for 10 min. The cells were electroporated at 310 V/1180 microfarads using a Cell-PORATOR (Life Technologies, Inc.). After electroporation, cells were allowed to stand at room temperature for 10 min and then diluted into 5–7 ml of Growth Medium and incubated at 37 °C and 8% CO2 for 5–6 h. Cells were harvested by centrifugation, resuspended in 0.7 ml of Growth Medium, and layered on top of 0.7 ml of Histopaque 1119 (Sigma) in a microcentrifuge tube. The cells were centrifuged at 400 × g for 30 min at room temperature. Viable cells were harvested from the interface, washed in Growth Medium, and incubated overnight in Growth Medium containing 2 mM sodium butyrate (Sigma) to maximize the expression of proteins transcribed from the cytomegalovirus promoter (35) and to increase the cells’ intrinsic migratory capacity if they were to be used for most chemotaxis assays. Cells were not incubated with sodium butyrate if they were transfected with any of the GFP-RGS chimerae since the GFP intensity was a direct measure of RGS expression and there was no need to maximize the expression of the GFP-RGS chimerae.

**FACS Analysis of Transfected Cells—**5 × 106 cells were stained with the mouse anti-FLAG M1 monoclonal antibody (IBI, New Haven, CT) or isotype control antibody (MOPC-141, Sigma) at 3 μg/ml for 1 h on ice in 1× phosphate-buffered saline containing 2% bovine serum albumin (Fraction V, Sigma), 1 mM CaCl2, 1 mM MgCl2, and 0.1% NaN3. Cells were washed and stained with phycoerythrin-conjugated goat anti-
mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h on ice at the concentrations indicated in the figure legends. Cells were analyzed on a FACSScan using Cellquest software (Becton Dickinson, San Jose, CA).

**Agonist-stimulated Chemotaxis of Receptor-transfected L1/2 Cells—** L1/2.2 cells were co-transfected with 25 μg of pRC-CMV FLAGged CXCR1 and either 12.5 μg of empty pRC-CMV (panels a and b) or 12.5 μg of pGreen Lantern (panels c and d) and grown as described under "Experimental Procedures." The cells were stained with 3 μg/ml of either isotype control MOPC-141 (panels a and c) or M1 monoclonal antibody (panels b and d) followed by phycoerythrin-conjugated goat-anti-mouse IgG (1:800 dilution). The M1 antibody recognizes the FLAG epitope engineered onto the N-terminus of CXCR1 and can used to visualize receptor expression on the y axis. Similar results were obtained in three separate experiments.

**Quantitative and Qualitative Western Blot Analysis—** For quantitative analysis, 15 μg of whole cell lysate and increasing amounts of His6-RGS2 (kindly provided by David Siderovski, Amgen Institute, Torrance, CA) or glutathione S-transferase-RGS3 (GST-RGS3) were separated on an 11% SDS-PAGE gel, transferred to polyvinylidene difluoride membranes, and blocked overnight in WB buffer (25 mM Tris, pH 7.4, 300 mM NaCl, 1 mM CaCl2) containing 5% nonfat dried milk. The blots were washed twice for 10 min each with WB buffer containing 0.05% Tween 20 (WBT), incubated with rabbit polyclonal anti-RGS2 (1:400) or anti-washed twice for 10 min each with WB buffer containing 0.05% Tween 20 (WBT), incubated with rabbit polyclonal anti-RGS2 (1:400) or anti-GFP antiserum (CLONTECH) at a 1:2000 dilution. The blots were then washed extensively in WBT three to four times for 10 min each, then washed twice with 25 mM diethanolamine, pH 9.5, 300 mM NaCl, 1 mM CaCl2, 1 mM MgCl2 for 10 min each. Bound antibodies were detected using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate or the chemiluminescent substrate CDP-Star (NEN Life Science Products) and visualized with Hyperfilm-ECL (Amersham Pharmacia Biotech). Quantification of immunoreactive band intensity was performed by comparison to known quantities of His6-RGS2 and glutathione S-transferase-RGS3 probed side by side. The quantification was performed on a LAS-BP or a Bio-Rad UltraScan XL enhanced laser densitometer using GelScan XL software (Pharmacia LKB Biotechnology, Uppsala, Sweden).

For qualitative analysis, whole cell lysate was separated on a 10% SDS-PAGE gel, transferred, and blocked as described above. The blot was incubated with rabbit polyclonal anti-GFP antiserum (CLONTECH) at a 1:2000 dilution. The blot was developed as described above. Activation-stimulated Adhesion of Receptor-transfected L1/2 Cells to VCAM-1—Agonist-activated adhesion was performed as described in Ref. 10 with the following modifications. 16 μl of cells were allowed to settle onto the VCAM-1-coated well for 7 min. Following addition of 4 μl of 500 mM FMLP and incubation for 4 min, the slides were inverted into a coupling jar with ice-cold HBSS, 10 mM Hepes, pH 7.4. After allowing the non-adherent cells to fall off the slide for 3 min, the slide was removed and re-inverted onto a cold metal block to prevent drying while other slides in the group were being handled. 20 μl of HBSS (without divalent cations), 10 mM Hepes, pH 7.4, 5 mM EDTA, 5 mM EGTA, 0.5% BSA (Fraction V) (Recovery Medium) containing approximately 10,000 polylysine beads (15 μm, Polysciences, Inc., Warrington, PA) and analyzing the ratio of cells to beads on the FACSScan as described previously (10). Dead cells, displaying decreased forward scatter and propidium iodide uptake, were eliminated from all analysis.

Quantitative and Qualitative Western Blot Analysis—For quantitative analysis, 15 μg of whole cell lysate and increasing amounts of His6-RGS2 (kindly provided by David Siderovski, Amgen Institute, Torrance, CA) or glutathione S-transferase-RGS3 (GST-RGS3) were separated on an 11% SDS-PAGE gel, transferred to polyvinylidene difluoride membranes, and blocked overnight in WB buffer (25 mM Tris, pH 7.4, 300 mM NaCl, 1 mM CaCl2) containing 5% nonfat dried milk. The blots were washed twice for 10 min each with WB buffer containing 0.05% Tween 20 (WBT), incubated with rabbit polyclonal anti-RGS2 (1:400) or anti-GFP antiserum (CLONTECH) at a 1:2000 dilution. The blots were then washed extensively in WBT three to four times for 10 min each, then washed twice with 25 mM diethanolamine, pH 9.5, 300 mM NaCl, 1 mM CaCl2, 1 mM MgCl2 for 10 min each. Bound antibodies were detected using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate or the chemiluminescent substrate CDP-Star (NEN Life Science Products) and visualized with Hyperfilm-ECL (Amersham Pharmacia Biotech). Quantification of immunoreactive band intensity was performed by comparison to known quantities of His6-RGS2 and glutathione S-transferase-RGS3 probed side by side. The quantification was performed on a LAS-BP or a Bio-Rad UltraScan XL enhanced laser densitometer using GelScan XL software (Pharmacia LKB Biotechnology, Uppsala, Sweden).

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**Analysis of Subcellular Distribution of RGS Family Members by Confocal Microscopy—** RGS family members were tagged by fusing them by their N termini with an enhanced GFP mutant. Fusion of sequences to the N termini of RGS members have previously been shown not to inhibit RGS function (36–46). The full-length sequence of different RGS members were PCR-amplified using Pfu DNA polymerase (Stratagene, La Jolla, CA) and primers containing a KpnI site. The resulting PCR products were cut using KpnI and cloned non-directionally into the KpnI site of pEGFP-C1 (CLONTECH) by standard techniques. Vector clones with the correct orientation were tested for their ability to regulate L1/2 migration to chemoattractants. Selected clones were linearized with ApaLI, transfected into L1/2/FPR (47) using the transfection method detailed above, and placed under G418 selection to obtain stable transfectants. Individual clones of L1/2 cells stably transfected with GFP, GFP-RGS1, GFP-RGS2, and GFP-RGS3 were obtained by limiting dilution. Clones with similar GFP expression levels were selected for further confocal microscopy analysis. The subcellular distribution of the different GFP-tagged RGS members was analyzed using a Molecular Dynamics MultiProbe 2010 laser scanning confocal microscope, a 60× oil-immersion lens, and ImageSpace software. The displayed slices were obtained from the middle of the cells. The nucleus was localized by incubating cells for 1.5 h in 5 μl SYTO-59 (Molecular Probes, Eugene, OR) and washing.
there is a positive correlation between the expression of the two transfected genes; thus, the level of GFP expression can be used as an indicator of the relative expression from a co-transfected vector.

In preliminary experiments to assess the effects of RGS proteins on chemotaxis, L1/2.2 cells were co-transfected with an RGS3-encoding vector and vectors encoding FLAâ€‘gged FPR, C5aR, or CXCR1. RGS3 is a RGS family member originally isolated from a B-cell cDNA library (22) and up-regulated in PMA-stimulated tonsilar B and T cells. Importantly, the levels of chemokine receptor expression were not influenced by RGS co-transfection (data not shown). L1/2.2 cells receiving chemokine receptor-encoding vectors (L1/2.2â€‘CxR) migrated robustly toward specific agonist, there was negligible migration in the absence of agonist, and checkerboard analysis revealed that the majority of the migration was chemotactic and not chemokinetic. Co-transfection of RGS3 potently inhibited L1/2.2â€‘CxR cell migration to FMLP, C5a, or IL-8 (data not shown). In contrast, transfection of RGS3 had no effect on L1/2 cell adhesion to VCAM-1 stimulated by phorbol esters, a Gâ€‘independent event (50).

**Effects of RGS Proteins on Chemotaxis of Cells Stably Expressing CXCR1 and CCR-2B**—To study the effects of different RGS proteins and the importance of relative expression levels in an otherwise constant cellular environment, we next transfected RGS-encoding or control vectors along with a vector encoding GFP into L1/2 cells stably transfected with CXCR1 and CCR-2B (L1/2CxCR1/CCR-2B). A typical histogram of GFP expression by each of the four RGS transfectants is depicted in Fig. 2a. The L1/2CxCR1/CCR-2B cell line averages 30% transient transfection efficiency and there is little or no difference in the GFP expression among the various transfectants (Fig. 2a). In this model, transfected cells express homogeneous levels of CXCR1 and CCR-2B independent of the co-transfected RGS member (as assessed by immunofluorescence staining and flow cytometric analyses); thus, all cells have the potential to respond to chemokinactant. However, the RGS super-transfected cells can be distinguished since they co-express GFP.

The response to chemokinactant by two of these transfectants is illustrated in Fig. 2b. L1/2CxCR1/CCR-2B cells were co-transfected with a vector encoding GFP and either a vector encoding RGS3 or a control empty vector. These two transfected populations, which expressed similar amounts of GFP (Fig. 2a), were allowed to migrate through a transwell filter toward 1 nM IL-8. After migration, GFP-positive cells were significantly depleted from the RGS3-transfected population (Fig. 2b, dark line) but not from the control empty vector-transfected population (Fig. 2b, light line). Moreover, GFP-high cells were depleted to a greater extent than GFP-intermediate cells. Since the relative amounts of RGS expression within a population can be inferred from the intensity of GFP expression (see Fig. 1), we conclude that inhibition of migration correlates with levels of RGS3 expression.

The assay was repeated to compare the effects of different RGS proteins on chemotaxis to IL-8 and MCP-1. As illustrated in Fig. 3 (a and d), the GFP-negative (and by inference RGS-negative) population of cells behaved identically in each case indicating that the transfection procedure was consistent and without significant nongenetic toxicity. Maximal chemotaxis occurred at 1 nM IL-8 and 3–10 nM MCP-1 with 20% and 7% of GFP-negative cells migrating, respectively. This efficiency of chemotaxis is similar to that exhibited by untransfected L1/2CxCR1/CCR-2B cells in terms of both the magnitude and concentration dependence of migration (data not shown). Among the GFP-intermediate (and by inference RGS intermediate) populations, RGS3 (Fig. 3b) and RGS4 (data not shown, but see below) substantially inhibited IL-8-stimulated chemotaxis, as expected. RGS3 (Fig. 3e) and RGS4 (data not shown) also inhibited cell migration to MCP-1. However, there was no or little effect of RGS1 or RGS2 on IL-8- or MCP-1-stimulated chemotaxis of the GFP-negative transfected cells (Fig. 3, panels b and e, respectively). Gating on the GFP-high (and by inference RGS high) population, however, revealed not only a more potent inhibition by RGS3 (Fig. 3, panels c and f) and RGS4 (data not shown, but see below) on L1/2CxCR1/CCR-2B chemotaxis to IL-8 and MCP-1, but also demonstrated a significant inhibitory effect of RGS1. In this population, RGS1 was a potent inhibitor of MCP-1-stimulated chemotaxis at all MCP-1 concentrations. RGS1 also inhibited chemotaxis to suboptimal and optimal (1 nM) concentrations of IL-8. There was no shift in the concentration dependence of chemotaxis to MCP-1 or IL-8 following

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RGS1 or RGS3 expression (Fig. 3, b, c, e, and f). There was no evidence, however, that any of the RGSs inhibited random migration in the absence of chemotactant; “background” migration through 8-μm pores over 8 h was not diminished by RGS expression (data not shown).

Most interestingly, RGS2 did not inhibit migration to IL-8 or MCP-1 even when assessed under an experimental design that focused upon the most highly expressing (GFP^{intermediate}) cells (Fig. 3, c and f). The vector encoding RGS2 was sequenced to confirm the lack of RGS2 action was not due to a mutation in the RGS2 coding sequence.

In order to determine whether the inhibitory action of the transfected RGSs was due to their GAP activity toward G-proteins, the effect of an N88S mutation on RGS4’s ability to regulate chemotaxis was investigated. Crystal structure data of RGS4 complexed with G_{i1} revealed that Asn-88 of RGS4 directly contacts G_{i1} (44). Mutation of RGS4’s Asn-88 (N88S) essentially abolished RGS4 binding to G_{i1} in vitro and greatly diminished RGS4’s activity in vivo (51). Transfection of RGS4 into L1/2*CXCR1/CCR-2B cells inhibited the migration to IL-8 when expressed at both the GFP^{intermediate} and GFP^{high} level (Fig. 4). RGS4N88S was totally ineffective at modulating chemotaxis when expressed at the GFP^{intermediate} level and was only marginally effective when expressed at the highest levels achievable in this system. Western blot probing of lysates with an anti-RGS4 antibody revealed that there were similar levels of wild type and mutant RGS4 expressed following transfection (data not shown). We conclude that the inhibitory effect of the “active” RGSs on chemotaxis is due to their ability to bind G_{i1} subunits and stimulate the conversion of the active GTP-bound form back to the inactive GDP-bound form.

In order to assess whether RGS2 expression levels might contribute to the lack of chemotaxis inhibition, quantitative Western blot analysis was performed on whole cell lysates of L1/2.2 cells transfected with RGS2 or with the most potent RGS protein, RGS3, under the conditions described above. As demonstrated in Fig. 5, transfected RGS2 and RGS3 are expressed as doublets in L1/2 cells, perhaps due to differential post-translational modification or to degradation following cell lysis. Comparison of the intensity of immunoreactive staining of the transfected protein with a standard curve of purified RGS2 and RGS3 provided a quantitative measure of expression in the transfected population overall. Averaging five independent pairs of transfections, RGS2 transfection yielded 50 ± 20 fmol of RGS2/15 μg of protein (mean ± S.E.), whereas RGS3 transfection yielded 300 ± 50 fmol of RGS3/15 μg of protein (mean ± S.E.). It should be noted that the reported expression levels of RGS2 and RGS3 are an average of the whole population with many cells expressing less and a few cells expressing more than the reported values. The frequency of transfection, assessed by co-transfected receptor expression, was similar between the groups. Therefore, we estimate ≈6-fold more RGS3 than RGS2 is expressed on average. However, taking into account that the gates used to distinguish GFP^{intermediate} and GFP^{high} separate cells that express 10-fold differences in the co-transfected vector-encoded proteins, RGS2/GFP^{high} cells should express RGS levels similar to or greater than those of RGS3/GFP^{intermediate} transfectants. Despite this, the RGS2/ GFP^{high} transfectants migrated normally with no detectable reduction in efficiency, whereas the RGS3/GFP^{intermediate} population was markedly inhibited. Thus, RGS2 is relatively impotent in modulating the chemotactic responses studied here.
shown) fusion proteins were very potent inhibitors of L1/2 migration. The resulting migrated population was assessed as in Fig. 3. As seen, the L1/2 cells containing stably transfected CXCR1 and supertransfected with the different GFP-RGS members were subjected to a chemotaxis assay and the GFP intensity of each fusion protein, and negligible proteolysis products active bands of the appropriate molecular weight were obtained for each fusion protein, and negligible proteolysis products were seen. The L1/2 cells containing stably transfected CXCR1 and supertransfected with the different GFP-RGS members were subjected to a chemotaxis assay and the GFP intensity of the resulting migration population was assessed as in Fig. 3. As can be seen from Fig. 6B, RGS1, RGS3, and RGS4 (data not shown) fusion proteins were very potent inhibitors of L1/2 migration to IL-8 when expressed at high (GFPbright) levels. Consistent with the results in Fig. 3, RGS2 expressed at the same levels was unable to modulate chemoattractant-induced migration.

Effects of RGSs on Adhesion Triggered through Co-transfected Chemoattractant Receptors—Signals in the high endothelial venules of lymph nodes and on inflamed endothelium cause the rapid shear-resistant integrin-dependent adhesion of leukocytes (reviewed in Ref. 4). The signal(s) that promote integrin-dependent adhesion in vivo are pertussis toxin-inhibitable, suggesting that Goi-coupled receptors are involved (52), and this rapid adhesion event can be modeled by expressing chemoattractant receptors and stimulating the transfected receptor in the presence of the appropriate cell adhesion molecules (e.g., VCAM-1) (10). Therefore, L1/2*IPR/CXCR1 cells transfected with GFP or any of the “active” GFP-RGS chimeras were stimulated by FMLP addition to bind VCAM-1-coated wells via their endogenous αβ integrins. In the absence of agonist, there is low background adhesion to VCAM-1 (2% of cells bind spontaneously), but after FMLP addition there is a rapid increase in the total cells bound (20% of input cells bind after 4 min of stimulation with 100 nM FMLP). As seen in Fig. 7, expression of bright levels of GFP-RGS1, GFP-RGS3, and GFP-RGS4 inhibited the adhesion signal and decreased the recovery of those cells from the VCAM-1 coated wells. In contrast, none of the GFP-RGS chimeras inhibited PMA-stimulated L1/2 cell adhesion to VCAM-1, a Goi-independent event (data not shown).

Subcellular Distribution of RGS Proteins Assessed by Confocal Scanning Microscopy—GFP and the different GFP-RGS chimeras were expressed stably in L1/2*IPR cells and observed by confocal scanning microscopy. GFP is expressed in L1/2 cells at a fairly uniform concentration throughout the cell (Fig. 8c) as is GFP-RGS1 (Fig. 8b) and GFP-RGS4 (data not shown). However, a majority of the GFP-RGS2 is localized in a subcellular compartment away from the plasma membrane where RGS proteins would be expected to regulate receptor-coupled Goi-GTP levels (Fig. 8c). Staining with the cell-permeable DNA dye SYTO-59 identified this region of RGS2 localization as the nucleus (data not shown). Most of the remaining non-nuclear GFP-RGS2 is diffusely associated with the plasma membrane.
or a periplasma membrane region, with little in the cytoplasm in general. The same predominately nuclear localization was also observed when GFP-RGS2 was transfected into the adherent, non-lymphoid Balb/c 3T3 cell line (data not shown). Strikingly, the large GFP-RGS3 was cytosolic and totally excluded from the nucleus (Fig. 8d). Membrane association of RGS1, RGS3, and RGS4 (data not shown) was difficult to assess in the context of the intense diffuse cytoplasmic fluorescence of these proteins, but the GFP-RGS2 chimera was apparently on or near the plasma membrane due to the contrast with the largely negative cytoplasm. At the level of resolution available, no change in GFP-RGS distribution was apparent following FMLP stimulation (data not shown).

**DISCUSSION**

The RGS proteins are a recently identified family of molecules with the potential to regulate diverse heterotrimeric G-protein-mediated signaling events in vivo. In yeast, pheromones bind a G-protein-coupled receptor that signals downstream events that lead to mating preparation (19). These downstream events include changes in gene transcription, morphological and cytoskeletal changes, and growth arrest. If mating does not occur, however, the yeast becomes desensitized to the pheromone signal and recovers from the growth arrest (19). Mutational screens were used to isolate a protein (Sst2p) that, when expressed normally, promoted the recovery from pheromone-stimulated growth arrest. Genetic analysis suggested that Sst2p regulated the yeast Gα subunit (GPA1) and in an unidentified manner inhibited G-protein-mediated signaling (19). In independent studies, mutational screens to detect egg-laying defects in C. elegans revealed a protein (EGL-10) that modulated the G-protein-regulated egg laying behavior (20). Additionally, the flbA gene in A. nidulans antagonized a G-protein-dependent developmental change (21). All three genes modulated G-protein-mediated events, and all three contained a common domain. This domain has been named the RGS domain (for regulator of G-protein signaling (20, 22).

Recently, mammalian genes containing RGS domains have been isolated by a variety of techniques (22, 36, 38, 42, 53–58). Analysis of previously cloned mammalian genes revealed two human proteins containing RGS domains: BL34/1R20 (renamed RGS1), an early response/activation gene up-regulated during PMA-induced plasmacytoid differentiation (53) and enriched among B-cell specific genes (54); and GOS8 (renamed RGS2), an activation gene of mononuclear cells (55). RGS3 was...
subsequently isolated by homology from a B-cell cDNA library (22). RGS4 was obtained using a brain cDNA library to screen for genes that compensated for the yeast RGS mutant (22).

The RGS domain appears to mediate direct physical interaction with Go G-proteins (36). Genetic data from f/bA action suggested that the intrinsic GTPase activity of the G-protein was required for RGS-mediated inhibition of function (21), and in vitro assays using purified RGS members and G-proteins have confirmed these observations. RGS proteins had no effect on the G-protein’s ability to bind GTP or to release GDP, actions akin to GDS and GDI proteins on small GTP-binding proteins (59). Instead, RGSs enhance the intrinsic GTPase activity of the G-protein acting as GTPase-activating proteins (GAPs). Most RGS members tested modulate the activity of Go_{i} and/or Go_{o} G-proteins but do not modulate Go_{a} G-proteins (37–39, 60, 61). No selectivity toward the different members of the Go_{o} subfamily thought to mediate both the chemotactic and proadhesive activities of chemoattractant receptors has been reported.

The present study was undertaken to assess the ability of RGS proteins to influence chemoattractant-stimulated cell migration and adhesion. We have demonstrated that some RGS members modulate the ability of transfected lymphoid cells to chemotax and adhere. Some but not all cellular responses to G-protein-linked receptors are regulated by the duration of G-protein signaling (26), and our results suggest strongly that chemoattractant-directed leukocyte migration and adhesion falls into this category. RGS1, RGS3, and RGS4 were potent inhibitors of LI/2 cell chemotaxis to each of the chemoattractants tested, including FMLP, C5a, IL-8, and MCP-1. RGS3 and RGS4 also inhibited chemotaxis to MIP-1α by LI/2*CXCR1/CCR-1 cells (not shown). RGS1 and RGS3 inhibited chemotaxis at all concentrations of chemoattractant with no apparent shift in the EC_{50} of the response; this is consistent with its action being downstream of the receptor. A decrease in response without a change in the EC_{50} of the agonist has been observed previously for RGS4 and RGS8 inhibition of βγ-activated GIRK channels stimulated by acetylcholine or dopamine (24, 25). The ability of these RGSs to modulate chemotaxis was dependent on their GAP activity since a mutant RGS4, shown previously to be impaired in its Go_{o} binding activity and subsequent GAP activity toward Go_{o} was much less effective at regulating chemotaxis than wild type RGS4. Although less dramatic than their effects on chemotaxis, RGS1, RGS3, and RGS4 also reduced the rapid, integrin-dependent adhesion stimulated through chemoattractant receptors as well, suggesting that the half-life of the activated G-protein subunit also plays a role in the intensity of the proadhesive signal, albeit quantitatively less significant than for chemotaxis.

RGS2 had no effect, under any experimental conditions, on Li/2 cell chemotaxis. Even when comparing populations normalized (by quantitative Western blot analysis or equal GFP fusion protein expression) for RGS levels at which RGS3 demonstrated dramatic inhibition of chemotaxis, no inhibition by RGS2 was detected. Although the unique, predominantly nuclear intracellular localization of GFP-RGS2 is of uncertain significance, it raises another possible correlation with the lack of RGS2 effect on chemotaxis. We conclude that RGS2 is relatively impotent as a modulator of the chemotactic response in lymphoid cells. An inability of RGS2 to regulate certain biological responses (I_{3} generation and K+ channels) that other RGSs modulate has been observed in other systems, as well (24, 62). RGS2 also differs from other RGS family members in its interaction with Go{ proteins. RGS4, RGS5, and RGS16 but not RGS2 bound well in vitro to Go_{o2} (the predominant Go_{i} protein in leukocytes (63, 64)), Go_{o}, and Go_{a} (42, 61). This observation has been extended very recently to show that RGS2 binds Go_{o}, and can modulate Go_{o}-stimulated IP_{3} generation in vitro (61). Thus, it is possible that in LI/2 cells, RGS2 can preferentially associate with Go_{o} versus Go_{i} proteins. The inability of RGS2 to inhibit chemotaxis may reflect this selectivity of Go interaction. However, these and our results do not rule out that RGS2 can act similar to RGS1, RGS3, and RGS4 in other cells; for example, all four RGSs inhibited IL-8-stimulated MAP kinase activity in 293T cells transfected with CXCR1 (22). If RGS2 itself localizes primarily to the nucleus in LI/2 cells (like the GFP-RGS2 chimera), it may be partially sequestered from the required site of action.

Our results confirm that RGS proteins can modify chemotactic and proadhesive responses, and suggest that differential and/or regulated expression of RGS members may allow fine tuning of leukocyte motility and adhesion in response to chemoattractant stimuli. Since RGS1 and RGS2 were first described as activation genes in leukocytes, and RGS3 has been demonstrated in PMA-stimulated tonsillar B and T cells, it is reasonable to hypothesize that RGS activity is modulated during lymphocyte activation and/or development, and it will be important to explore their differential regulation during leukocyte development and in response to proinflammatory or antigenic stimuli.

In conclusion, RGS1, RGS3, and RGS4 modulate the ability of motile lymphoid cells to migrate in a gradient of chemoattractant, presumably by decreasing the half-life of the activated G-protein. The results suggest that the duration of the Go_{o} active state is a major determinant of the chemotactic response, which requires coordination and integration of multiple downstream responses over a prolonged time period. In contrast, RGS2 was unable to regulate migration to any chemoattractant when it was expressed at any level achievable in our system. RGS1, RGS3, and RGS4 significantly inhibited rapid, chemoattractant triggered adhesion as well, although to a lesser extent than chemotaxis. We postulate that, within the intracellular environment, the regulated expression of RGS molecules in conjunction with Go_{o} specificities, regulatory interactions, and subcellular localization of RGS proteins may help control leukocyte migratory responses to chemoattractant stimuli, thus regulating the microenvironmental trafficking of leukocytes in vivo.

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