

Biology of Chemokine and Classical Chemoattractant Receptors: Differential Requirements for Adhesion-triggering versus Chemotactic Responses in Lymphoid Cells

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Abstract. Several chemoattractant receptors can support agonist-induced, integrin-dependent arrest of rolling neutrophils in inflamed venules in vivo, as well as subsequent crawling into tissues. It has been hypothesized that receptors of the G α_i -linked chemoattractant subfamilies, especially receptors for chemokines, may mediate parallel activation-dependent arrest of homing lymphocyte subsets. However, although several chemokines can attract subsets of B or T cells, robust chemoattractant triggering of resting lymphocyte adhesion to vascular ligands has not been observed. To study the biology of individual leukocyte chemoattractant receptors in a defined lymphoid environment, mouse L1/2 pre-B cells and/or human Jurkat T cells were transfected with α (IL-8 receptor A) or β (MIP-1 α /CC-CKR-1) chemokine receptors, or with the classical chemoattractant C5a (C5aR) or formyl peptide receptors (fPR). All receptors supported robust agonist-dependent α 4 β 1 integrin-mediated adhesion of lymphocytes to VCAM-1. L1/2 cells cotransfected with fPR and β 7 integrin were also induced to bind MAdCAM-1, suggesting common mechanisms coupling chemoattractant receptors to activation of distinct integrins. Adhe-

sion was rapid but transient, with spontaneous reversion to unstimulated levels within 5 min after peak binding. When observed under flow conditions, α 4 β 1-mediated arrest occurred within seconds after initiation of contact and rolling of IL-8RA transfectants on a VCAM-1/IL-8 co-coated surface; and arrest reversed spontaneously after a mean of 5 min with a return to rolling behavior. Each of the receptors also conferred agonist-specific chemotaxis; however, whereas strong adhesion required simultaneous occupancy of many receptors with maximal responses above the K_d , chemotaxis in each case was suppressed at high agonist concentrations. The findings indicate that α and β chemokine as well as classical chemoattractant receptors can trigger robust adhesion as well as directed migration of lymphoid cells, but that the requirements for and kinetics of adhesion triggering and chemotaxis are distinct, thus permitting their independent regulation. They suggest that the discordance between proadhesive and chemoattractant responses of circulating lymphocytes to many chemokines may reflect quantitative aspects of receptor expression and/or coupling rather than qualitative differences in receptor signaling.

RHODOPSIN-related G α_i -linked 7 transmembrane "serpentine" receptors of the chemoattractant subfamily have been implicated in many aspects of leukocyte behavior, notably including the regulation of leukocyte adhesion, locomotion, and chemotaxis. This subfamily includes classical chemoattractant receptors for formyl peptides and C5a, receptors for the emerging family of small

polypeptide chemoattractant cytokines, the chemokines, as well as a number of orphan receptors whose agonists and functions have yet to be defined (24, 33). Only a few of the receptors for the more than 30 known chemokines have been identified as yet, and the patterns of expression and cellular responses to different leukocyte receptors are only now being explored. It is already apparent, however, that the leukocyte responses to stimulation through chemoattractant receptors can be diverse. Stimulation of myeloid cells through classical chemoattractant or through IL-8 receptors triggers rapid activation of leukocyte inte-

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grins leading to firm adhesion (3, 10). During neutrophil trafficking, this is reflected in rapid arrest of neutrophils rolling along inflamed venules (46, 47). The same agonists, when applied experimentally to the extravascular space, can also support subsequent chemotaxis and diapedesis of the adherent polymorphonuclear leukocyte (17, 41). Physiologic studies of lymphocyte homing suggest that lymphocytes can also be stimulated through pertussis toxin-(PTX)-sensitive receptors to adhere rapidly to α 4 and β 2 integrin ligands (4, 5). However, although several chemokines are chemotactic for subsets of circulating T and B cells, the proadhesive activity of known chemokines on resting lymphocytes is minimal and robust integrin activation with rapid kinetics, comparable to those reported for myeloid cells (which can occur within seconds), has not been observed. For example, subsets of lymphocytes migrate in response to the α chemokines interleukin 8 (2, 26, 38) and IP-10 (44); and to the β chemokines RANTES (38, 43, 44), MIP-1 α (37, 43), MIP-1 β (37, 42, 43), MCP-1 (11, 30, 45), and MCP-2 and -3 (30, 45), and several chemokines can initiate uropod formation and polarization of lymphocytes adherent on integrin substrates (13), yet these chemokines fail to trigger rapid (within seconds) binding of resting peripheral blood leukocytes (PBL) to integrin substrates (29, 42-44). On the other hand, recent studies have shown that lymphoid cells do have the necessary machinery to respond to chemoattractant stimulation with robust adhesion to VCAM-1, when stimulated through transfected formyl peptide receptors (fPR) (22). Thus the discordance between chemokine chemoattractant and proadhesive activities for lymphocytes might reflect a unique biology of the chemokine receptors, or of their expression. Direct analyses of the biology of chemoattractant receptors is difficult in normal leukocyte populations, however, because of the potential for cell type-specific variations in responsiveness, differences in receptor levels, overlapping receptor expression, and promiscuity of receptor-agonist interactions (28, 34).

The present study was designed to overcome these difficulties by expressing α and β chemokine receptors along with classical chemoattractant receptors in a common lymphoid cell environment. Our findings demonstrate common features in the biology of these diverse receptors when expressed at similar levels in lymphocytes, and highlight several potential mechanisms for the differential regulation of proadhesive versus chemoattractant responses during lymphocyte migration and positioning *in vivo*.

Materials and Methods

Engineering Signal and FLAG Peptides onto the Amino Terminus of G_{α_i} -linked Chemoattractant Receptors

The DNA sequences for the human CD5 signal peptide (to enhance surface expression (1, 19) and the "FLAG" (IBI-A Kodak Co., New Haven, CT) octapeptide were added to the 5' end of G_{α_i} -linked chemoattractant receptors by two sequential rounds of PCR amplification. The template cDNA was reverse transcribed from human PBL. The first round of PCR used a 5' primer of ~70 bases, which included ~1/3 of the CD5 signal peptide, all of the FLAG sequence, and 17 bp of overlap with the gene of interest

(including the codon for the native start methionine). The second round of PCR amplification used a 5' primer of ~95 bases, which included a NOT-1 restriction site, followed by a translational start consensus sequence (25), followed by the remaining 2/3 of the signal peptide DNA sequence including 17 bases of overlap with the 5' end of the first round PCR product. The 3' primer for the first round was selected within the 3' untranslated region of the target cDNA, and the 3' primer for the second round was nested within that of the first round 3' primer and included an XBA-1 restriction site. The products of the second round of PCR were directionally cloned into the expression vector pRc/CMV (Invitrogen, San Diego, CA). The primers were designed using the sequences from the following Genbank submissions: hufPR (M60626), huIL-8RA (M68932), huCC-CKR-1 (L09320) and hu C5aR (X58674). The FLAG octapeptide is only recognizable by the anti-FLAG mAb M1 (see below) if it is present on the absolute amino terminus of the protein chain (i.e., the mAb will not recognize the FLAG if it is preceded by even a single amino acid [19]). Thus, if a transfected cell line is recognized by the mAb M1, it indicates that the receptors have reached the cell surface and cleaved the signal peptide.

Stable Transfection of L1/2 and Jurkat Cells

Cells were transfected with linearized plasmid by electroporation as previously described (22). Cells transfected with "FLAGGED" receptor or hu β 7 (15) in pRc/CMV were selected with 1 mg/ml G-418 (GIBCO BRL, Gaithersburg, MD) in complete culture medium (RPMI-1640, 10% iron-supplemented bovine serum). HuFPR/hu β 7 double transfectants, were derived by further transfecting L1/2 cells previously transfected with hufPR in pMRB-101 (an expression vector selectable by mycophenolic acid) (22) with hu β 7 in pRc/CMV as above. The drug-selected cells were stained with fluorescent antibodies to the FLAG or hu α 4 β 7 surface proteins (see below) and sorted for high expression by flow cytometry on a FACScan with software (Consort-30; Becton-Dickinson, San Jose, CA).

Fluorescent Antibody Staining

Cells were stained for anti-FLAG or anti-hu α 4 β 7 by resuspending 10^6 cells in 200 μ l of 5 μ g/ml mAbs M1 (IBI-A Kodak Co.) or ACT-1 (27), respectively, in staining medium (SM = 1 \times PBS, 2% BSA, 1 mM CaCl₂, 1 mM MgCl₂, and 0.1% NaN₃). After incubation on ice for 30 min, the cells were washed and resuspended in the same volume of a 1:50 dilution of phycoerythrin-conjugated goat anti-mouse IgG H&L (F(ab)₂ fragment; JacksonImmuno Research Laboratories, West Grove, PA). Isotype controls were included for each cell type in each experiment. For anti-mu α 4, primary staining with PS/2 (32) was essentially as above, but second stage antibody was a phycoerythrin-conjugated goat anti-rat IgG H&L (F(ab)₂ fragment; JacksonImmunoResearch Laboratories). Stained cells were analyzed using a FACScan driven by software (Cell Quest; Becton-Dickinson).

Ligand Binding Studies

The ligand binding properties of FLAGGED fPR were assayed using a fluorescein-conjugated formyl peptide as described previously (22), and were found indistinguishable from those of the unflagged receptor (Sklar, L., personal communication). The binding of IL-8 was carried out in HBSS supplemented with 0.5% BSA and 0.1% sodium azide. Receptor transfectants were resuspended to 10^7 /ml and aliquots of 50 μ l (5×10^5) cells were added into Eppendorf tubes, incubated first with unlabeled IL-8 of various concentrations, then 0.1 nM ¹²⁵I-labeled IL-8 (Amersham Corp., Arlington Heights, IL). The final reaction vol was 200 μ l. Total binding was carried out in the absence of unlabeled chemokines and non-specific binding was determined by incubating cells with radio-labeled IL-8 in the presence of 125 nM of "cold" IL-8. After 30 min at 37°C, the cells were overlaid on 800 μ l of 20% sucrose and spun at 3,000 rpm to separate unbound isotope. The tubes were snap-frozen on dry-ice/ethanol and the tips of the tubes containing the cell pellets were cut off and counted. MIP-1 α binding to CC-CKR-1 transfectants was measured as above, with slight modifications. The binding buffer consisted of 50 mM Hepes, 1 mM CaCl₂, 5 mM MgCl₂, and 0.5% BSA. After incubation with radio-labeled and unlabeled MIP-1 α , cells were washed three times in binding buffer plus 0.5 M NaCl. The cell pellets were transferred into fresh tubes and counted in a gamma counter. All experiments were carried out using duplicates and repeated at least three times. Scatchard plots were calculated by Microsoft Excel using linear curve fit. The brightness of anti-FLAG staining was found to be proportional to the number of receptors per cell,

1. Abbreviations used in this paper: fPR, formyl peptide receptors; fMLP, synthetic *N*-formyl-Met-Leu-Phe; PTX, pertussin toxin.

and for some determinations of receptor density, various FLAGGED-transfectant subclones with known densities were used to create a standard curve to convert FLAG-staining brightness to receptors per cell.

Assay for Inducible Adhesion to VCAM-1 or MAdCAM-1

Native mouse VCAM-1 and recombinant mouse MAdCAM-1 were affinity purified and coated onto 5-mm-diam wells of glass slides as previously described (7, 22). The coated wells were blocked with 20 μ l bovine serum for 15 min at 37°C in a humidified chamber. The serum was aspirated and 10^5 cells added in 16 μ l complete culture medium (equilibrated at 37°C in 8% CO₂) and the cells were allowed to settle for 5 min. At various time points, 4 μ l of 5 \times agonist or control was added at the 12 o'clock position of the well. At the appropriate time point, the slide was dipped twice (once in each direction) in ice-cold HBSS/10 mM Hepes to remove nonadherent cells, then fixed in ice-cold HBSS/10 mM Hepes with 1.5% glutaraldehyde for 1 h. The 0.2-mm² field closest to the site of addition was counted using video imaging software (NIH Image 5.0) and the percentage of starting cells bound per field was calculated. Each experiment was performed in triplicate for each time point and the SEM calculated. Synthetic *N*-formyl-Met-Leu-Phe (fMLP) and recombinant human C5a were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human IL-8 and MIP-1 α were obtained from R & D Systems Inc. (Minneapolis, MN).

Determination of the Dose Response of Agonist-induced VCAM-1 Binding

VCAM-1-coated slides were prepared as above, but blocked with serum at 0°C instead of 37°C. Slides were kept at 0°C by placing them on an ice-cold metal block. Cells were chilled to 0°C in a 0.5-ml tube and mixed with 5 \times agonist to a final concentration of 10^5 cells/20 μ l. Cells and ligand were incubated together on ice for 15 min. The serum was aspirated from the ice-cold slides and replaced with 20 μ l cells + agonist, which were allowed to settle for 5 min. After settling, the slides were warmed rapidly by moving them from the 0°C metal block to a 37°C block, and incubated at 37°C for 2 min before washing and fixing as above (2 min was determined to be the time of peak adhesion in this assay; not shown). Triplicate wells were performed for each experiment, and four fields (0.2 mm²) counted per well. No adhesion was observed when identical slides were kept at 0°C for the entire experiment (not shown).

Interaction of IL-8RA Transfectants with VCAM-1 + IL-8 under Flow

The inside of 100- μ l "microcap" capillary tubes (Drummond Scientific Co., Broomall, PA) were coated with muVCAM-1 and the density estimated at 100 sites/ μ m² by radioimmunoassay as described (6). Some areas of the tube were left uncoated as a control. After coating, the unbound VCAM-1 was washed away and the entire inside of the capillary blocked with 100% BCS at room temperature for 30 min. To co-coat the VCAM-1 areas with immobilized IL-8 (or control), 20 μ l of 1 μ M IL-8 (in complete medium) was added through the downstream end of the capillary and coaxed into the same position as the VCAM-1-coated area. Great care was taken that no IL-8 touched the capillary upstream of the VCAM-1. The tube was incubated for 5 min at room temperature. Just before the experiment, the unbound IL-8 was washed out through the downstream end of the tube by infusing 5 ml of warm, complete medium into the upstream end. Washing in this direction prevented IL-8 from contacting the upstream areas. The amount of IL-8 bound to the capillary by this procedure was estimated using ¹²⁵I-IL-8 (Amersham Corp.) of known specific activity, and found to be coated to an approximate density of 3×10^3 molecules per μ m². The presence of VCAM-1 did not affect the amount of IL-8 bound, and perfusion of 20 μ l warm, complete medium through the capillary did not significantly reduce the amount of IL-8 bound (not shown).

Cells were passed through the capillary in warm, complete medium at a density of 2×10^6 cells/ml. The rate of flow was controlled by a "Harvard 33" syringe pump (Harvard Apparatus, South Natick, MA). The reported experiments were performed at a flow rate of 1,700 μ l/min, which creates a wall shear rate of ~ 2.7 dynes/cm² for a capillary of 1.025 mm inner diam as calculated from Poiseuille's law for Newtonian fluids, with viscosity 0.01 *P*. (WSS in dynes/cm² = mean flow velocity (mm/s) \times [8 / tube diam (mm)] \times viscosity (*P*)). The interactions of cells with the coated areas were recorded on video tape, and the behavior of individual cells analyzed frame-by-frame.

PTX Treatment

L1/2 IL-8RA-FLAG transfectants were suspended at a density of 2×10^6 cells/ml in complete medium (RPMI 1640/10% BCS) and treated with intact PTX (List Biological Laboratories, Inc., Campbell, CA) or mutant PTX (31, 35) at 100 ng/ml for 2 h at 37°C in an 8% CO₂ incubator.

Migration Assay

Cells were spun down and washed in migration medium (MM = RPMI 1640, 0.5% BSA (fraction V; Sigma Chemical Co.), equilibrated overnight in 8% CO₂ at 37°C) and resuspended at 10^7 cells/ml in MM (or in MM + agonist for the checkerboard assays). Tissue-culture inserts (Collaborative Biomedical Products, Bedford, MA) were placed in each of the wells of 24-well tissue-culture plates, forming an upper and lower chamber separated by a polyethylene terephthalate membrane bearing 3- μ m-diam pores. The wells were set up ahead of time with 600 μ l of warm, equilibrated dilutions of agonist or controls in MM. One million cells in 100 μ l were added to the upper chamber immediately after it was placed in the well and the cells were allowed to migrate through to the lower chamber in an 8% CO₂, 37°C incubator for 4–5 h. The inserts were removed after migration and the cells in the bottom well were transferred to chilled 12 \times 75 mm test tubes. L1/2 and Jurkat cells were not adherent to the 24-well plates, and were easily resuspended by gentle pipetting. The cells were counted by mixing a predetermined number of polystyrene beads (15 μ m; Polysciences, Inc., Warrington, PA) with the cells and running the mixture through the FACscan. The beads and cells were easily distinguishable on a plot of side-scatter vs. forward scatter, allowing us to calculate the ratio of beads to cells and thereby calculate the total number of cells migrated to the bottom well. This counting method was confirmed to accurately represent the number of cells present by testing it with known numbers of cells and beads (not shown).

Results

Transfection of Lymphocyte Lines with Serpentine Receptors

To compare the biology of individual G α_i -linked chemoattractant receptors within a consistent cellular context, a panel of stably transfected L1/2 cells was created, each line expressing a different human receptor. L1/2 cells are an Abelson-transformed murine pre-B cell clone bearing functional levels of cell surface $\alpha 4\beta 1$ integrin (22), a ligand for the vascular cell adhesion molecule (VCAM-1). The chemoattractant receptors chosen represent members of the classical chemoattractant (fPR [8] and C5aR [18]), the α chemokine (IL-8RA [21]) and β chemokine (CC-CKR-1 [34]) receptor families. L1/2 cells were suitable for transfection with these receptors, as untransfected cells did not respond to agonists by calcium flux, suggesting that these receptors were not extant on the parent line (unpublished data). Additionally, untransfected cells did not respond above background to any of these agonists in any of the functional assays discussed below. The constructs for transfection were engineered so that the mature receptor protein bears an amino-terminal peptide "FLAG," recognizable by a monoclonal antibody, which allowed transfectants to be sorted by flow cytometry. Fig. 1 shows example L1/2 transfectants for each of the "FLAGGED" receptors stained with anti-FLAG mAb-M1.

Ligand affinity was assayed on "FLAGGED" transfectants using ¹²⁵I-human IL-8 for IL-8RA, ¹²⁵I-human MIP-1 α for CC-CKR-1, and fluoresceinated formyl-Nle-Leu-Phe-Nle-Tyr-Lys for fPR (this peptide has an ~ 30 -fold higher affinity than fMLP for fPR; Sklar, L., personal communication), and the *K_d*s were found to be 0.2, 10.3, and 0.4 nM, respectively. Thus, these FLAGGED receptors have ligand

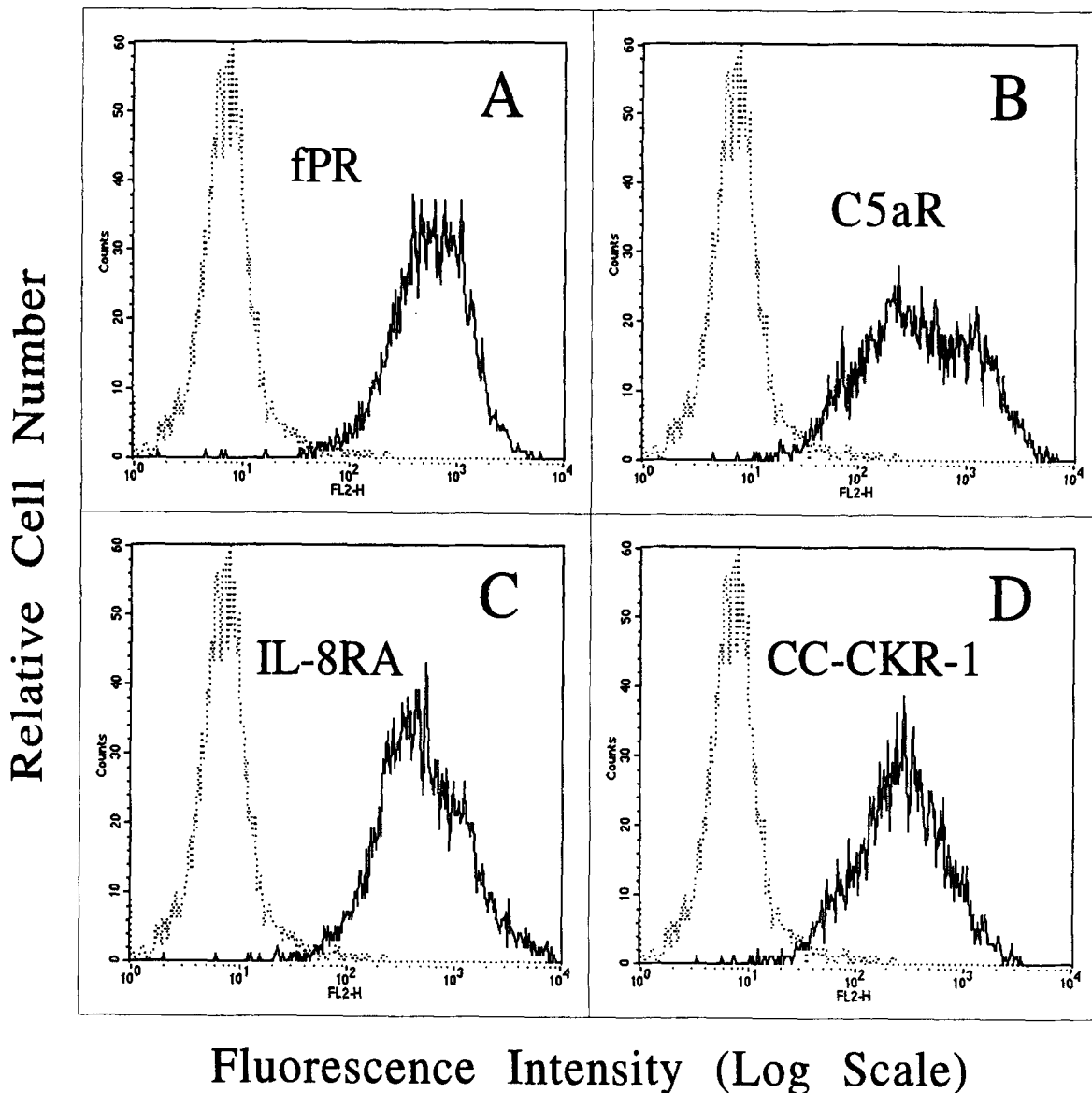


Figure 1. Anti-FLAG staining of example L1/2 cells transfected with cDNA for various FLAGGED G_{α_i} -linked receptors. After electroporation, cells that grew in selective medium were sorted for high cell surface FLAG expression. The cell lines shown were routinely sorted to maintain approximately 4×10^4 to 1×10^5 receptors per cell. Staining: α -FLAG mAb followed by phycoerythrin-conjugated α -mouse Ig second stage. Dotted lines, control vector-transfected L1/2 cells. Solid lines, receptor transfectants. A, fPR; B, C5aR; C, IL-8RA; D, CC-CKR-1.

affinities close to those reported for the unmodified receptors, indicating that the presence of the FLAG sequence did not prevent specific ligand binding (not shown). The observed K_d CC-CKR-1 (10.3 nM) is slightly higher than previously reported (5.1 nM) (34). This small difference (if real) may be due to expression in different cell types (i.e., lymphoid cells in our study vs. kidney cells in [34]), or due to the presence of the FLAG interfering slightly with ligand binding. C5a receptor ligand binding was not determined. The receptor expression of transfected lines sometimes drifted in culture, so they were routinely sorted to maintain between 4×10^4 and 1×10^5 mean receptors per cell (see Materials and Methods).

Rapid Triggering of Integrin-mediated Cell Adhesion

The panel of receptor transfectants was tested for agonist-mediated adhesion in a VCAM-1 binding assay. Briefly, cells were allowed to settle on murine VCAM-1-coated glass and incubated with agonist for various times, after which unbound cells were washed away. Bound cells were then fixed and counted. As previously demonstrated for unflagged fPR transfectants (22), L1/2^{fPR-FLAG} cells were induced to bind VCAM-1 within a very short time span (Fig. 2 A). All of the other receptors tested were capable of mediating similarly rapid and robust binding responses in L1/2 cells when exposed to their respective ligands (Fig. 2, B–D). The response was quite rapid in all cases, with sig-

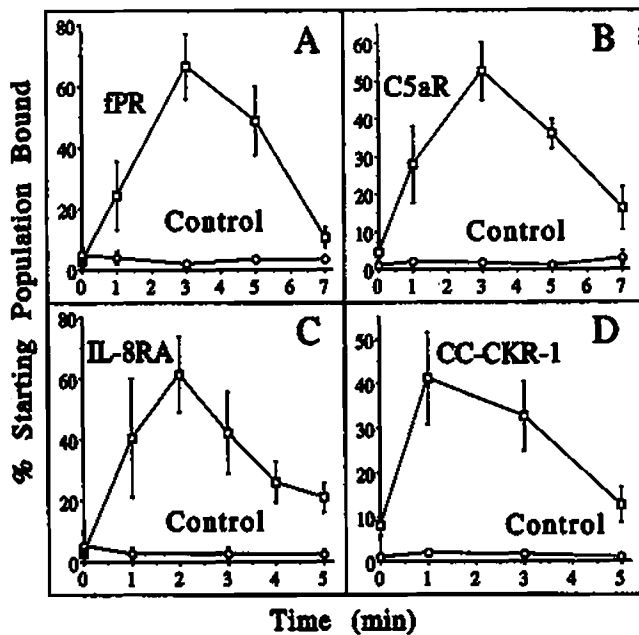


Figure 2. Rapid and reversible agonist-induced adhesion of L1/2 transfectants to purified μ VCAM-1. Transfectants (open squares) or vector controls (open circles) were allowed to settle on a VCAM-1-coated surface, and 100 nM agonist was added. The slides were washed vigorously at the indicated time points to remove nonadherent cells. The adherent cells were then fixed and counted. Transfectants: A, fPR; B, C5aR; C, IL-8RA; D, CC-CKR-1. Agonists: A, fMLP; B, C5a; C, IL-8; D, MIP-1 α . Error bars show the standard error of three wells done in parallel.

nificant increases in binding at the earliest time points examined. The CC-CKR-1 and IL-8RA transfectants appeared to reach their maximal binding somewhat more rapidly than C5aR and fPR transfectants. However, characteristic of many G protein-mediated effects, all responses were transient, with the number of adherent cells declining to baseline within 5 min after the peak. Control transfectant cells and unstimulated receptor transfectants displayed little background binding (Fig. 2, circles). In this system, no binding was induced in the absence of VCAM-1, and induced adhesion was blocked by mAbs to $\alpha 4$ integrin or VCAM-1 (not shown). Responses were inhibitable by PTX treatment, indicating that the signal is transduced through a member of the $G\alpha_i$ family (20, 40) (not shown).

Stimulation of fPR Induces Both $\alpha 4\beta 1$ - and $\alpha 4\beta 7$ -mediated Adhesion

To determine if stimulation through a chemoattractant receptor would also trigger the integrin $\alpha 4\beta 7$, L1/2^{fPR} cells were cotransfected with cDNA encoding the human $\beta 7$ integrin chain (15). Staining with the anti- $\alpha 4$ mAb PS/2 (32) demonstrated that the hu $\beta 7$ transfectants maintained a level of $\alpha 4$ expression comparable to that of the fPR-only transfectants (Fig. 3 B). The hu $\beta 7$ transfectants bound the mAb Act-1 (27), which recognizes human $\beta 7$ in the context of $\alpha 4$ (39), suggesting the formation of $\mu\alpha 4$ -hu $\beta 7$ complexes (Fig. 3 A). MADCAM-1 is a ligand for $\alpha 4\beta 7$ integrin but not for $\alpha 4\beta 1$ (7). The presence hu $\beta 7$ on these cells allowed them to respond to fMLP by binding much

more avidly to MADCAM-1 than cells transfected with fPR only (Fig. 3 C). The increased adhesion to MADCAM-1 was accompanied by a corresponding decrease in binding to VCAM-1 (Fig. 3 D). As $\alpha 4\beta 1$ determines VCAM-1 binding in many settings (7, 12), this result may indicate that the levels of $\alpha 4\beta 1$ were reduced due to displacement of $\mu\beta 1$ with expressed hu $\beta 7$, and that $\alpha 4\beta 7$ was not as efficient as $\alpha 4\beta 1$ at binding VCAM-1. However, the decrease in VCAM-1 binding should be interpreted with caution, as it could be an artifact of the xeno-heterodimer.

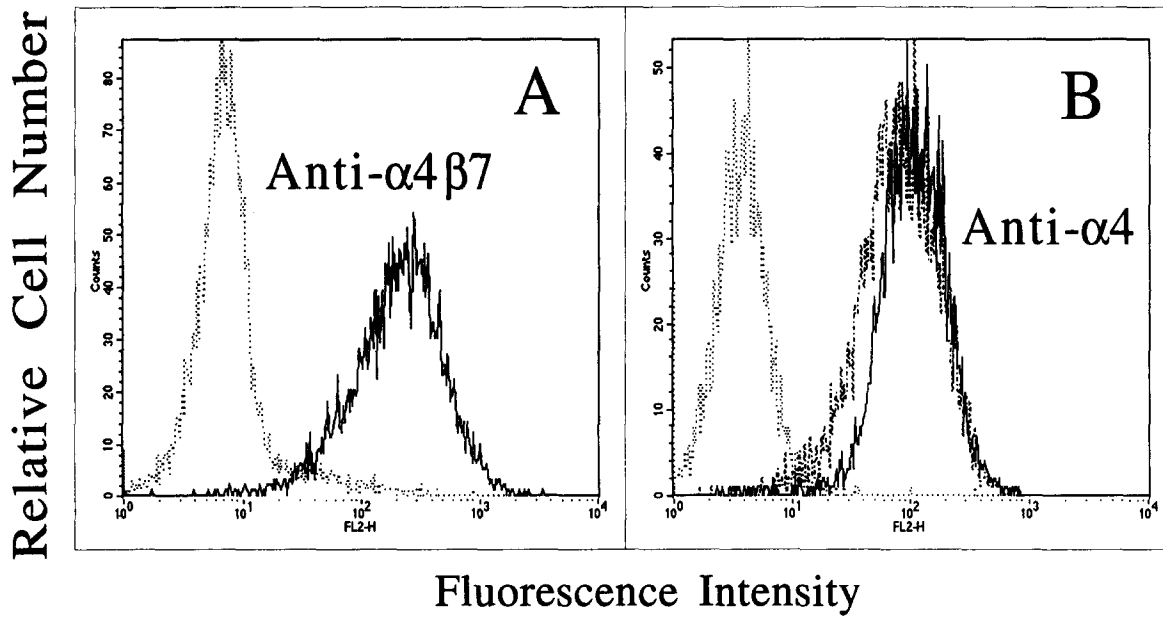
Dose-Response of Ligand-induced Adhesion

The binding response of receptor transfectants to various concentrations of agonist was analyzed in detail. In this assay, cells were mixed with agonist on ice and then allowed to settle on ice-cold VCAM-1-coated slides for several min. The slides were then rapidly warmed to 37°C and incubated for 2 min before washing and fixing the bound cells. 2 min was chosen because although the binding response was more prolonged at lower agonist concentrations for IL-8RA transfectants, it was the time point of peak binding at all agonist concentrations tested (not shown). Induction of maximal VCAM-1 binding was achieved at agonist concentrations above the K_d for each receptor, and reached a plateau at higher concentrations (Fig. 4).

Real-Time Analysis of IL-8-Induced Integrin Triggering in an In Vitro Flow Assay

To assess more accurately the time course of agonist-induced integrin activation, an in vitro flow assay was developed to observe individual cells from the first moment they encountered agonist. The inside walls of glass capillary tubes (Fig. 5) were coated with VCAM-1, cells were passed through the tube in warm medium, and their behavior was recorded on video tape through an inverted microscope. We have previously shown that L1/2 cells under similar conditions were able to initiate interaction with VCAM-1 through surface $\alpha 4\beta 1$ integrin and to begin rolling slowly on this substrate (6). When passed through the tube at a shear rate of 2.7 dynes/cm², tumbling cells (i.e., those noninteracting cells in the focal plane of the uncoated inner tube wall) traveled at a mean velocity of $\sim 2,000 \mu\text{m/s}$ ($2,094 \pm 92 \mu\text{m/s}$, SD = 92, $n = 10$ in a representative experiment). At this same shear rate, cells interacting with VCAM-1 (coated to a density of ~ 200 sites/ μm^2) rolled at a mean of $\sim 3 \mu\text{m/s}$ ($3.2 \pm 0.5 \mu\text{m/s}$, SD = 0.5, $n = 19$ in a representative experiment), which is a >600 -fold reduction in speed (Fig. 5 A). This system was used to assess the effect of agonist-induced $G\alpha_i$ -mediated signaling on rolling cells. IL-8 was chosen as the agonist in this model because of its ability to immobilize on solid substrates (36). To observe IL-8-induced arrest, IL-8 was co-coated on areas of the capillary previously coated with VCAM-1. The immobilization of IL-8 on the glass was not dependent on the presence of VCAM-1, and IL-8 was not washed off by medium passed through the tube at high shear (see Materials and Methods). When L1/2^(IL-8RA-FLAG) cells were passed through a co-coated capillary tube (Fig. 5 B), the number of interacting cells and the time required

MAB Staining



fMLP-Induced Adhesion

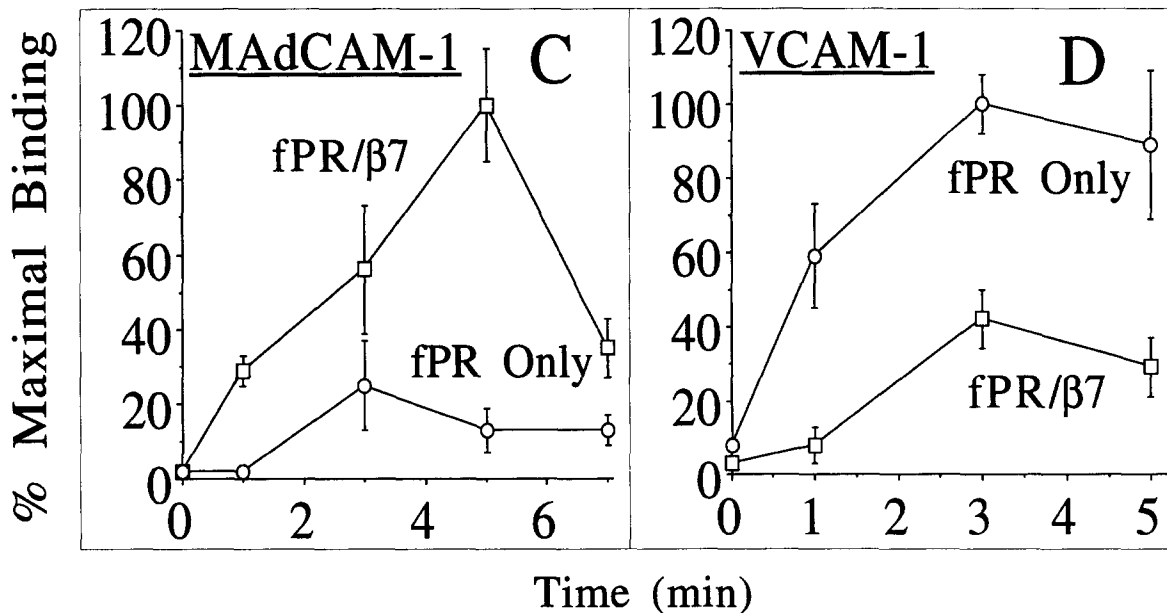


Figure 3. Integrin- $\alpha 4\beta 7$ heterodimer expression and agonist-dependent MAdCAM-1 binding in hu $\beta 7$ /fPR transfectants. (A) Anti- $\alpha 4\beta 7$ heterodimer (ACT-1) staining. Dotted line, L1/2 cells transfected with fPR only. Solid line, L1/2 cells transfected with fPR and hu $\beta 7$. (B) Anti- $\alpha 4$ chain (PS/2) staining. Dotted line, Isotype-matched control staining. Solid line, PS/2 staining of L1/2 cells transfected with fPR only. Hatched line, PS/2 staining of L1/2 cells transfected with fPR and hu $\beta 7$. (C and D) fMLP-induced binding of fPR-only transfectants (*open circles*) and fPR + hu $\beta 7$ transfectants (*open squares*) to purified muMAdCAM-1 (C) or purified muVCAM-1 (D). Procedure as in Fig 2. Maximum binding for MAdCAM-1 was 55 cells per field, and for VCAM-1 was 495 cells per field. Error bars show the range of duplicates done in parallel.

to achieve rolling speed was unaffected by the presence of IL-8 (i.e., the amount of time necessary for an interacting cell to reduce velocity from 1,000 $\mu\text{m/s}$ to 10 $\mu\text{m/s}$ was ~ 0.3 s whether or not IL-8 was present). However, after achieving rolling speed in the presence of IL-8, most of the

cells rapidly decelerated to a complete stop and remained at arrest for >1 min. Complete arrest was never seen in the absence of IL-8 (Fig. 5, A and C). The mean time for a rolling cell to come to complete stop was ~ 4 s (4.4 s, SD = 0.9, $n = 9$ in a representative experiment). During

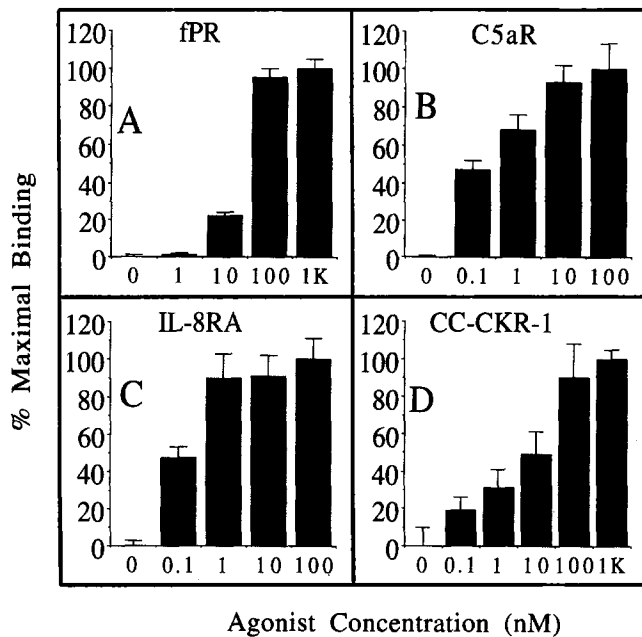


Figure 4. Agonist dose-response curves for VCAM-1 binding. Cells were mixed with the indicated concentration of agonist (on ice) and allowed to settle on VCAM-1 coated wells (on ice). The slides were then rapidly warmed to 37°C for 2 min and washed to remove nonadherent cells. The adherent cells were then fixed and counted (1K indicates 1,000 nM). Transfectants: A, fPR; B, C5aR; C, IL-8RA; D, CC-CKR-1. Agonists: A, fMLP; B, C5a; C, IL-8; D, MIP-1 α . Error bars show the standard error of four fields each from three wells. Background binding was subtracted. No significant induction over background was seen for any of these agonists in control transfectants analyzed in parallel (not shown).

this time, the cells traveled a mean distance of only 16 μ m (16 μ m, SD = 0.5, n = 9 in a representative experiment) or approximately a single L1/2 cell diameter. The results were similar if the IL-8 was added in soluble form, excluding the possibility that the effect was merely due to a mechanical interaction between cell surface receptor and immobilized IL-8 (not shown). No shear-resistant adhesion was ever seen in the absence of agonist and no rolling or sticking was seen in the absence of VCAM-1 or in the presence of blocking mAbs to VCAM-1 or α 4 integrin (not shown). The effects of IL-8 were inhibited by PTX treatment, but not by a mutant toxin lacking the G α_i -ADP ribosylation function (31, 35) (Fig. 5 C). PTX did not affect rolling (not shown).

As seen in the static VCAM-1 binding assays, IL-8-induced VCAM-1 binding was transient (Fig. 5 D). The duration of arrest was variable, ranging from a minimum of 2.3 min to >10 min, with a mean of ~5 min (5.0 min, SD = 1.3, n = 27 in a representative experiment). At the termination of arrest, the cells began rolling again, eventually reaching the mean rolling speed of cells observed in the absence of IL-8 (Fig. 5 B).

Ligand Induced Migration of Receptor Transfectants

Each G protein-linked receptor studied here was originally characterized as a chemoattractant receptor (33).

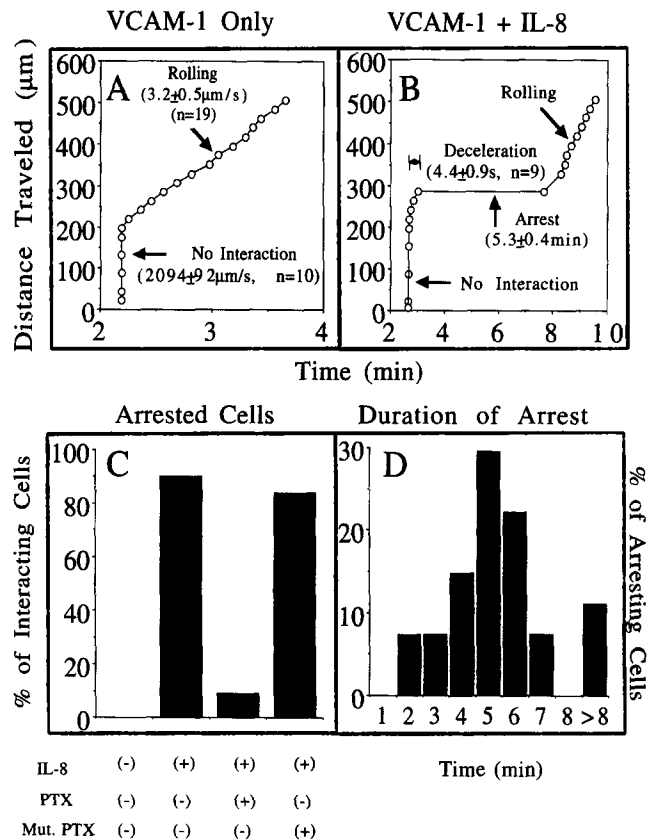


Figure 5. Immobilized IL-8 triggers rapid but transient arrest of IL-8RA transfectants rolling on VCAM-1 under flow. (A and B) Behavior plots of lymphocytes in the flow assay. The slope of the line is proportional to the velocity of the cell. Behavior of a representative cell is shown in each plot, with the mean and SD of each component (i.e., velocities for no interaction (tumbling), and rolling, and duration of deceleration and arrest phases) of the interaction for (n) cells in a representative experiment indicated in parentheses. (A) IL-8RA transfectants on VCAM-1 alone. (B) IL-8RA transfectants on VCAM-1 + immobilized IL-8. (C) Effect of PTX on IL-8-triggered adhesion. Bar graph shows the percent of interacting cells that arrested for longer than 1 min in a typical experiment. PTX inhibited arrest but did not reduce the number of interacting cells, while mutant PTX had no significant effect (n = 18–24 cells observed for each condition). Results are representative of four experiments performed at various shear forces. (D) Duration of arrest for arresting cells in the presence of immobilized IL-8 in a typical experiment (n = 27). All experiments shown were done at 2.7 dynes/cm², with VCAM-1 density estimated at 100 sites/ μ m².

This presented the opportunity to ascertain whether the L1/2 cell line contained all of the necessary intracellular machinery to respond to a chemotactic gradient through each of these receptors. Tissue culture inserts were placed in each of the wells of 24-well tissue culture plates, forming an upper and lower chamber separated by a tracked-etched polyethylene terephthalate membrane bearing 3 μ m-diam pores. Transfectants were placed in the upper wells, while chemoattractants were placed in the bottom wells at various concentrations. After 4–5 h incubation at 37°C, the cells that migrated through the 3- μ m pores into the bottom well were removed and counted. L1/2 cells are five times larger in diameter than the pores, and there was no

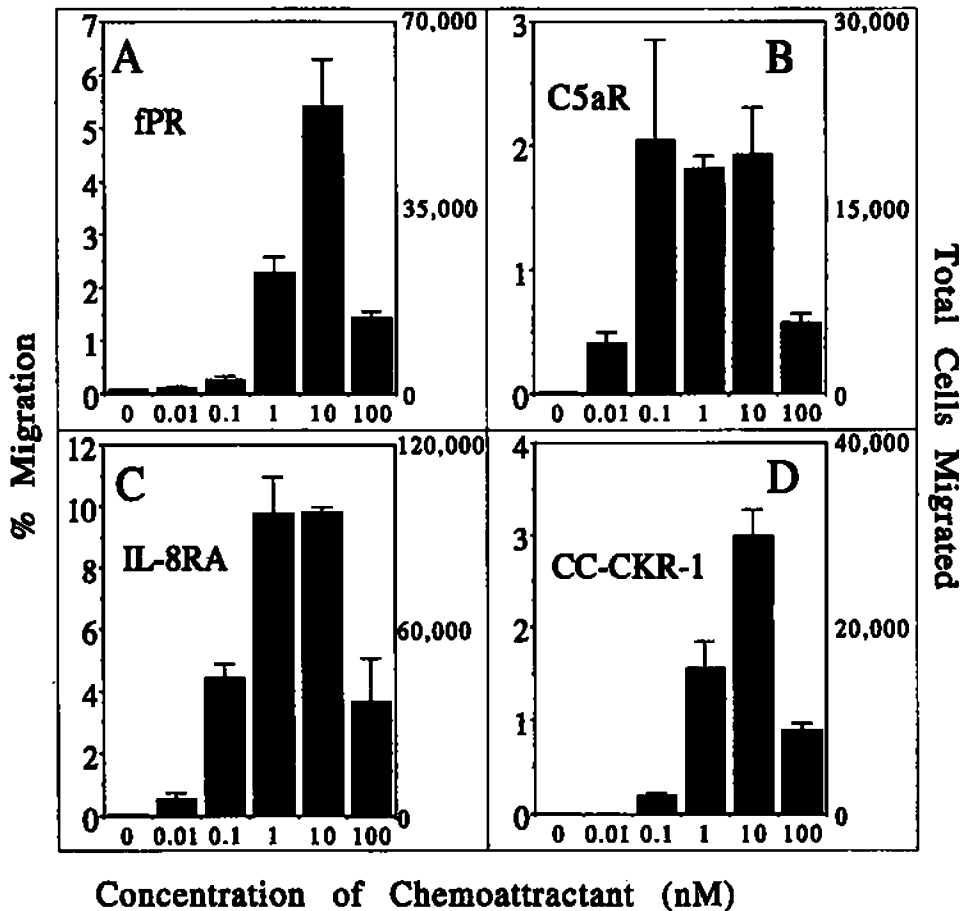


Figure 6. Migration of FLAGGED L1/2 receptor transfectants. Tissue-culture inserts were placed in each of the wells of 24-well tissue-culture plates, forming an upper and lower chamber separated by a membrane bearing 3- μ m pores. One million cells were placed in the upper chambers, while the indicated concentrations of chemoattractants were placed in the lower chambers. The number of cells migrated through the pores was calculated after 4–5 h incubation at 37°C. Transfectants: A, fPR; B, C5aR; C, IL-8RA; D, CC-CKR-1. Agonists: A, fMLP; B, C5a; C, IL-8; D, MIP-1 α . Error bars show the range of duplicate wells done in parallel. Left scale indicates the percent of the starting population that migrated. Right scale indicates the total number of cells that migrated into the bottom well. Results are representative of at least three experiments performed. No migration over background levels to any of these agonists was ever observed in wild-type, vector-transfected, or irrelevant receptor-transfected L1/2 cells (not shown).

significant migration in the absence of chemoattractant. All of the receptors in our panel were capable of mediating ligand-dependent migration of L1/2 cells (Fig. 6). Like the VCAM-1-binding responses, migration reached its peak near the K_d of the receptor. Unlike the binding response, migration was less efficient at higher concentrations, forming a bell-shaped chemotactic dose-response curve. In this system, migration was inhibited by PTX pretreatment, indicating that the chemotactic signals are also transduced through a member of the $G\alpha_i$ family (20, 40) (not shown).

Ligand-induced Migration Is Direction Dependent

Checkerboard analysis was performed on each of the transfectants in the panel, to distinguish directed migration from ligand-induced random motion. Briefly, a given concentration of agonist (10 nM) was placed in the upper, lower, neither, or both chambers. Again, no significant migration was observed in the absence of agonist. The largest responses were seen when agonist was placed in the lower well only, which allows formation of a chemotactic gradient. No migration was seen when ligand was placed in the top well only, showing that the cells could not migrate against a chemotactic gradient. However, when equal amounts of ligand were placed in the upper and lower wells, L1/2 cells expressing C5aR and IL-8RA did show

some degree of migration (Fig. 7, B and C). According to these experiments, $\sim 1/3$ of the migration mediated by these two receptors was direction independent at 10 nM. The responses of L1/2 cells expressing CC-CKR-1 or fPR were more direction dependent, showing little or no random migration at this concentration (Fig. 7, A and D).

Transfected IL-8RA Supports Adhesion and Chemotaxis in Jurkat Cells

Jurkat, a human T cell line, was also transfected with our "FLAGGED" IL-8 receptor construct (Fig. 8 A). In the absence of transfected receptor, Jurkat was not induced by IL-8 to bind VCAM-1 (Fig. 8 A, circles). In the transfectants, VCAM-1 binding was induced quite rapidly (Fig. 8 B, squares). However, the reversibility of adhesiveness was not as rapid as seen in L1/2 cells (Fig. 1 C). IL-8RA was also capable of mediating migration in Jurkat cells (Fig. 8 C), with a bell-shaped curve similar to that seen in L1/2 (Fig. 6 C). In Jurkat, there was no direction-independent component to IL-8-induced migration, suggesting that partial direction independence is not a general property of the IL-8 receptor, but depends on the cellular background. Like L1/2 cells, migration and adhesion induced through the IL-8 receptor were inhibited by PTX, indicating that the chemotactic signals are also transduced through a member of the $G\alpha_i$ family (not shown) (20, 40).

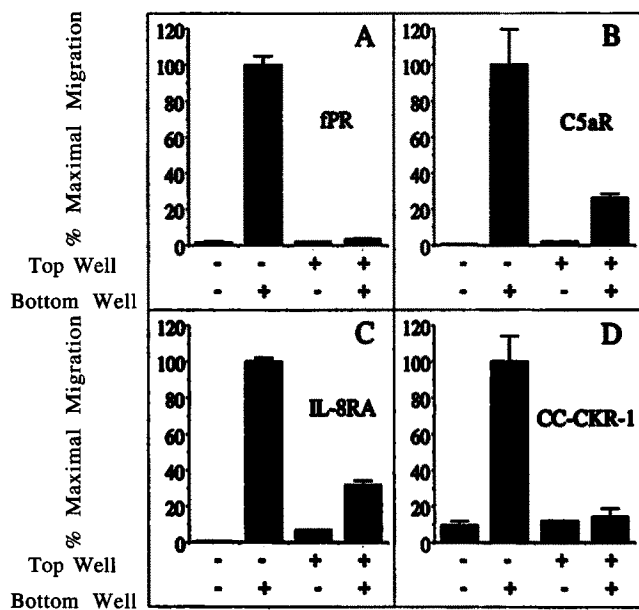


Figure 7. Checkerboard analysis of direction dependence of migration. Migration experiments were carried out as in Fig. 6, but 10 nM agonist was placed in the top, bottom, neither, or both chambers, as indicated. Transfectants: A, fPR; B, C5aR; C, IL-8RA; D, CC-CKR-1. Agonists: A, fMLP; B, C5a; C, IL-8; D, MIP-1 α . Error bars show the range of duplicate wells done in parallel. Results are representative of 2–4 experiments performed. The amount of induced random migration (i.e., in the top + / bottom + wells) among IL-8RA-transfected sublines was more variable than that of the other receptors, ranging from 10 to 50% of maximal migration (not shown).

Discussion

Comparison of the biological properties of different G α_i -linked receptors has been difficult because they are often expressed at different receptor densities per cell in vivo, making it impossible to distinguish qualitative from quantitative differences (14). Additionally, especially in the case of chemokines, agonists can bind more than one receptor, making it difficult to determine which receptor is involved in a given effect (28, 34). In this paper, we used a transfection system (in which known receptors were expressed individually and at comparable densities) to explore the biology of leukocyte serpentine chemoattractant receptors in a common cell host. Our findings demonstrate that the ability to trigger rapid, robust integrin activation in lymphocytes, as well as the ability to mediate chemotaxis, are shared properties of receptors representing both α and β chemokine, and classical chemoattractant receptor families. Although some of these G α_i -linked receptors are not normally expressed on lymphocytes, they are all capable of interacting with the signal transduction machinery present within transfected lymphoid cells, and of transducing productive signals.

We focused initially on proadhesive responses to receptor stimulation. Each of the transfected receptors was capable of mediating agonist-dependent integrin activation in static assays involving triggering of $\alpha 4\beta 1$ integrin binding to VCAM-1-coated surfaces. In general, significant binding was observed at very early time points, reaching a

peak 1–3 min after exposure to agonist, and dropping off to background at later time points. The doses of agonist required to trigger binding were near or above the K_d of each receptor, and the dose–response curve reached a plateau at higher concentrations. The responses were not restricted to $\alpha 4\beta 1$ integrin alone, as binding to MAdCAM-1 was also inducible when receptor transfectants were cotransfected with the $\beta 7$ integrin gene. The Jurkat human T cell line was also capable of agonist-dependent integrin-mediated VCAM binding when transfected with the IL-8 receptor A, demonstrating that the observed effects were not unique to the L1/2 background. High numbers of occupied receptors per cell (requiring high receptor expression levels more comparable to those of chemoattractant receptors on neutrophils rather than receptor expression levels for those of the known chemokines on lymphocytes) appear to be important for rapid integrin activation, as transfectants with <10,000 receptors did not undergo rapid adhesion in our assay, but were capable of chemotaxis (unpublished data).

To assess the rate of adhesion triggering more accurately, we turned to an in vitro flow assay. By comparing the behavior of cells encountering VCAM-1 alone vs. VCAM-1 + IL-8-coated surfaces, we were able to observe directly the consequences of adhesion triggering in real time. Upon interaction with VCAM-1, a typical L1/2 transfectant moving under shear and at high speed reduced its velocity >600-fold, after which it maintained a constant rolling speed indefinitely. In contrast, upon interaction of transfected L1/2 cells with a combination of IL-8 and VCAM-1, the same cells came to a complete stop, and remained at rest for several minutes. The response was extremely rapid, allowing the cell to roll a mean of only ~ 16 μ m (roughly one L1/2 cell diameter) before halting. The extremely rapid adhesion and arrest was not mediated by IL-8 receptor–ligand binding per se, but required signaling through the receptor, as PTX blocked arrest but not rolling. Moreover, similarly rapid adhesive triggering was observed when IL-8 was superfused over transfectants rolling on VCAM-1 (although in this case the measurement of time of exposure to agonist was less precise, not shown). This is the first demonstration that G protein-linked receptors can trigger lymphocyte $\alpha 4$ integrin-mediated arrest with strength and kinetics consistent with those observed during physiologic lymphocyte–EC interactions in vivo (4, 5).

A consistent feature of firm adhesion triggered through the transfected receptors was its reversibility over minutes. The mechanism for spontaneous reversion of integrin activation following triggering through chemoattractant receptors is unknown, although it may involve desensitization of the chemoattractant receptor and/or active mechanisms of downstream feedback with integrin deactivation. It is unlikely that receptor internalization is responsible for this rapid reversal, as most of the receptors still remain on the cell surface at the 5-min time point when most of the cells have already deadhered (Campbell, J., and E.C. Butcher, unpublished data). However, it is possible that receptor phosphorylation by GRKs may be involved in this rapid deactivation, as is the case in the $\beta 2$ -adrenergic receptor system (23). It seems likely that multiple intracellular mechanisms may enforce the transience of integrin activa-

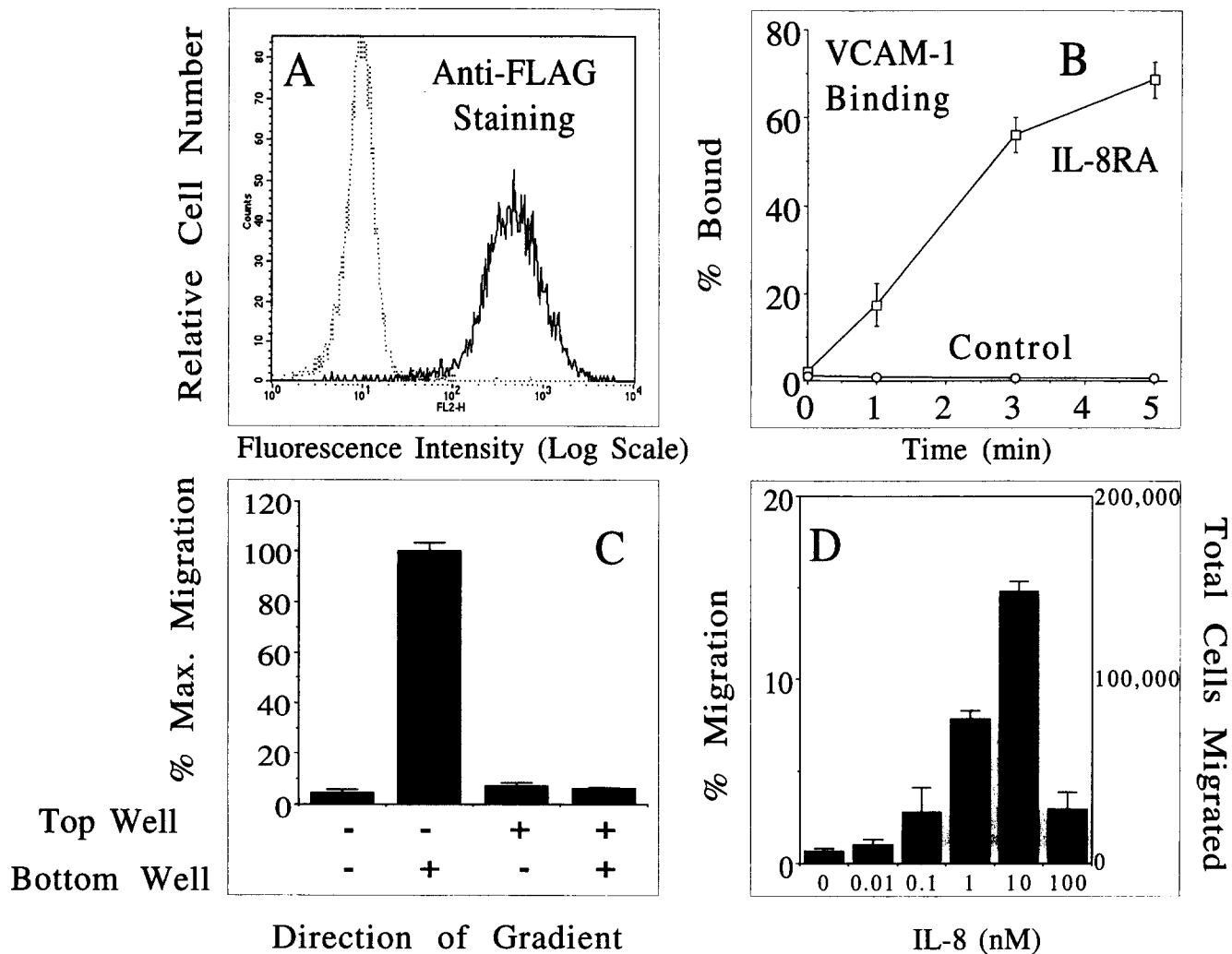


Figure 8. Jurkat cells transfected with the FLAGGED IL-8RA construct respond in VCAM-1 binding and migration assays. (A) Anti-FLAG staining as in Fig. 1. (B) IL-8-induced adhesion to VCAM-1 as in Fig. 2. (C) Checkerboard analysis of migration to 10 nM IL-8 as in Fig. 7. (D) Migration dose response as in Fig. 6. Untransfected Jurkat cells did not respond over background to IL-8 in any of these assays.

tion on lymphoid cells, to ensure their continued motility and responsiveness to changes in local microenvironmental stimuli.

Each of the transfected receptors also supported agonist-directed migration of L1/2 cells. The dose-response curve for migration reached a peak near or just above the reported K_d for receptor-ligand binding and fell off at higher and lower concentrations, forming a classical bell-shaped profile. Checkerboard analysis established that most of the migration was direction dependent. However, signals through C5aR and IL-8RA did trigger a significant level of random migration. IL-8RA-induced migration of Jurkat transfectants was purely direction dependent, indicating that the direction-independent component seen in L1/2 cells was not solely due to the IL-8RA, but also determined by the cellular context.

We were particularly interested in comparing and contrasting the requirements for robust adhesion vs. chemotaxis. The most obvious differences between these two processes are their distinct kinetics. The transience of

strong adhesion may insure that lymphocytes do not become irreversibly stuck to cells or matrix components expressing chemoattractants. In the context of leukocyte trafficking, for example, the transience of leukocyte arrest on endothelium, modeled here in the flow system, would argue that adherent lymphocytes would either be released eventually to return to the circulation, or would be freed to respond to chemoattractant or haptotactic gradients leading to diapedesis. Such a mechanism may ensure the integrity of the sequential, multi-step process of leukocyte-endothelial interaction and recruitment (9). After leaving the circulation, it seems unlikely that lymphocytes would often encounter the rapid exposure to high concentrations of soluble agonists required for strong adhesion in the context of cell-cell interactions within tissues. However, certain chemoattractants (e.g., the chemokines) may be presented at high concentrations on the surface of cells through association with specialized chemokine-binding glycosaminoglycans (42). Under such conditions, a crawling leukocyte could be triggered by localized agonist to ad-

here strongly to the presenting cell or matrix component. The effect of this would be to focus initial cellular interactions, but the transience of induced adhesion would ensure the requirement for additional mechanisms regulating continued interaction, or in the absence of such stimuli would allow subsequent further locomotion.

Another distinction emphasized by our results relates to the receptor occupancy requirements for adhesion vs. chemotactic responses. As mentioned previously, the efficiency of integrin triggering plateaued at agonist concentrations higher than the K_d for each of the receptors. In contrast, the efficiency of chemotaxis fell off sharply at agonist concentrations higher than that required for peak activity. The differences between these dose-response curves suggest a mechanism whereby triggered strong adhesive responses and locomotion may be regulated independently as a function of agonist concentration. This ability may be important for increasing potential combinatorial diversity and specificity in lymphocyte trafficking, by allowing independent control of vascular arrest and subsequent chemoattractant-dependent diapedesis. In support of this idea, preliminary data indicate that leukocytes bearing multiple chemoattractant receptors can respond to one chemoattractant in the presence of a high adhesion-triggering and subsequently desensitizing dose of another chemoattractant (Campbell, J.J., E.G. Flescher, and E.C. Butcher, unpublished data). This ability may also be important in controlling cell-cell interactions and positioning within the lymphoid tissue and inflammatory microenvironments.

Several chemokines, including IL-8 (2, 26, 38), MIP-1 α (37, 43), MIP-1 β (37, 42, 43), RANTES (38, 43, 44), IP-10 (44), MCP-1 (11, 30, 45), and MCP-2 and -3 (30, 45) can induce chemotaxis of human lymphocytes, and several of the above can trigger formation of uropod structures and cell polarization in lymphocytes adherent to integrin substrates (13). A subset of these chemokines also induce binding of lymphocytes to integrin ligands (e.g., MIP-1 α [29, 43], MIP-1 β [29, 42, 43], IP-10 [29, 44], and RANTES [29, 43, 44]). However, these binding responses were measured a minimum of 15 min (29, 42) to 1 h (43, 44) after stimulation, time points well beyond the rapid responses studied here. Such delayed adhesion may have different signaling requirements. Moreover, in most instances such *in vitro* chemokine-induced lymphocyte binding responses appear to require additional cellular activation, such as overnight treatment with cross-linked α CD3 (43, 44), suggesting that chemokine-stimulated adhesion of this type may be more relevant to the behavior of blasts rather than the majority of circulating lymphocytes. Thus, no chemokines have yet been demonstrated to trigger rapid adhesion of resting lymphocytes, and it therefore seems unlikely that known chemokines play the critical role in inducing the rapid arrest of circulating lymphocytes on HEV, or in vascular sites of inflammation observed *in vivo* (4, 5). Our results, however, suggest that the discordance between chemoattractant and proadhesive responses of circulating lymphocytes to chemokines may reflect quantitative aspects of receptor expression and/or coupling rather than qualitative differences in receptor-to-integrin signaling. The ability of each receptor studied here to support adhesion triggering as well as chemotaxis when ex-

pressed at high levels, however, leaves open the possibility that novel chemokines, perhaps acting through more highly expressed receptors, will fulfill the promise of regulating the rapid adhesion-triggering step of lymphocyte trafficking. Equally likely is the possibility that novel, nonchemokine chemoattractants may be involved, perhaps acting through highly expressed receptors of the chemoattractant receptor subfamily. Particularly intriguing in this context are recent reports of several orphan receptors, related to the known chemoattractant and chemokine receptors, that are expressed at high levels by subsets of B or T lymphocytes (16, 33).

We conclude that members of the α and β chemokine and classical chemoattractant subfamilies of G α_i -linked receptors can couple efficiently to integrin-activating signaling pathways in lymphocytes, as well as support chemotaxis. Most importantly, our results indicate that the distinctive receptor occupancy requirements and kinetics of adhesion and chemotaxis responses may permit independent control of these distinct events during lymphocyte trafficking and microenvironmental localization *in vivo*.

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