nal precursors and could be used to steer neuronal precursors to specific locations of the adult brain to replace neurons lost by disease or injury.

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18. DiI crystals were placed in the olfactory peduncle after removal of the olfactory bulb, or in the SVZ of the rostral part of the lateral ventricle in brains fixed in 3% paraformaldehyde. Brains were incubated for 30 days at 39°C in 3% paraformaldehyde in phosphate-buffered saline. Sections were cut in a Vibratome (50 μm) and mounted with glycerol. In these cases, cells in the RMS were not labeled by DiI, confirming that the DiI-labeled chains observed in the RMS after DiI injection in vivo were due to cell migration.
19. Sections were processed for immunocytochemistry as in (*15*) with mouse monoclonal antibodies to GFAP (Sigma, dilution 1:600) and PSA–N-CAM (dilution 1:150).
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25. The RMS was sectioned serially in the frontal plane. Semithin (1.5-μm) sections were alternated with seven ultrathin (40- to 50-nm) sections for up to 26 μm. Several chains were photographed in one out of every seven ultrathin sections.
26. Adult CD-1 mice (n = 4) were perfused with 2% paraformaldehyde and 0.2% glutaraldehyde, and their brains were cut into 200-μm sagittal sections. Sections were incubated overnight in 100 mM NH_4Cl, postfixed for 30 min in 0.25% OsO_4, dehydrated and embedded in LRwhite resin (London Resin Company, London), and cut into 60-nm sections. Ultrathin sections were collected onto Formvar-coated nickel grids and processed for immunocytochemistry with antibodies to GFAP (Sigma, dilution 1:2500). Secondary antibodies (Amersham, dilution 1:20) were coupled to 10-nm colloidal gold particles. Sections were then stained with 2% uranyl acetate and examined with a Jeol 100CX electron microscope.
27. C. Lois, J.-M. García-Verdugo, A. Alvarez-Buylla, data not shown.
28. CD-1 male mice (n = 2) received stereotaxic microinjections of [^{3}H]thymidine as described (*10*). Three days after [^{3}H]thymidine administration, the animals were killed and their brains processed for EM as in (*22*). The 2-μm semithin sections were processed for autoradiography and stained with Giemsa, and [^{3}H]thymidine-labeled cells were photographed with a light microscope. Semithin sections were reembedded and cut into 60-nm-thick sections. Ultrathin sections were stained with lead citrate and examined with a Jeol 100CX electron microscope.
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Role of Rho in Chemoattractant-Activated Leukocyte Adhesion Through Integrins

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Heterotrimeric guanine nucleotide binding protein (G protein)–linked receptors of the chemoattractant subfamily can trigger adhesion through leukocyte integrins, and in this role they are thought to regulate immune cell-cell interactions and trafficking. In lymphoid cells transfected with formyl peptide or interleukin-8 receptors, agonist stimulation activated nucleotide exchange on the small guanosine triphosphate–binding protein RhoA in seconds. Inactivation of Rho by C3 transferase exoenzyme blocked agonist-induced lymphocyte α4β1 adhesion to vascular cell adhesion molecule–1 and neutrophil β2 integrin adhesion to fibrinogen. These findings suggest that Rho participates in signaling from chemoattractant receptors to trigger rapid adhesion in leukocytes.

Regulated leukocyte adhesion is critical to immunity and inflammation and controls cellular positioning, cell-cell interactions, and immune cell responses. For example, rapid triggering of integrin-mediated adhesion is required for the arrest of blood-borne lymphocytes and neutrophils at sites of leukocyte recruitment from the blood. This extremely rapid and robust adhesion, triggered within a few seconds during leukocyte "rolling" along endothelium, is initiated by pertussis toxin–sensitive $G\alpha_i$-linked receptors of the rhodopsin-related seven transmembrane family (*1*). β2 integrin–mediated arrest of neutrophils, for example, can be triggered through stimulation of the formyl peptide, leukotriene B4, or interleukin-8 (IL-8) chemoattractant receptors in vivo (*1*). The regulation of integrin adhesion through chemoattractant receptors is likely important in cellular locomotion and cell-cell interactions within tissues as well. Intracellular signaling pathways that mediate chemoattractant modulation of calcium flux, neutrophil respiratory burst, and adenyl cyclase activity have been identified (*2*). However, signaling events that control rapid chemoattractant activation of leukocyte integrin adhesion have not been defined.

We used an in vitro model to study the intracellular mechanisms that trigger lymphocyte adhesion through chemoattractant receptors. The mouse L1/2 B lymphoid cell line was transfected with the human formyl peptide receptor (fPR) or with the human IL-8 receptor type A (IL-8RA). Agonist stimulation of these transfectants triggers robust and rapid α4β1-dependent adhesion to purified vascular cell adhesion molecule–1 (VCAM-1) (*3*).

Because chemoattractants including

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formyl-Met-Leu-Phe (fMLP, a synthetic ligand for human fPR) stimulate a cascade of second messengers, leading to activation of protein kinase C (PKC) (*4*), we investigated the role of PKC in triggered adhesion. Calphostin C, a powerful and specific inhibitor of diacylglycerol and Ca^{2+}-dependent isoforms of PKC (*5*), had no effect on fMLP-triggered or IL-8–triggered adhesion to VCAM-1 (Fig. 1, A and B). In contrast, adhesion stimulated by phorbol myristate acetate (PMA), a direct PKC activator, was blocked efficiently by calphostin C, as expected (Fig. 1C).

We next focused on the Rho subfamily of small guanosine triphosphate (GTP)–binding proteins. These cytosolic proteins, which are in an inactive state when bound to guanosine diphosphate (GDP) and in an active state when bound to GTP (6), regulate the assembly of focal adhesion complexes and actin stress fibers in fibroblasts (*7*) and PMA-induced integrin-dependent aggregation in lymphocytes (*8*); these processes involve relatively slow responses that are typically assayed over periods from 30 min to several hours. The role of Rho proteins in chemoattractant receptor signaling to integrins in leukocytes has not been examined. Transfectants were treated with recombinant *Clostridium botulinum* C3 transferase, which specifically inhibits Rho by adenosine diphosphate ribosylation on Asp^{41} (*9*) in a region believed (by analogy with Ras) to be required for interaction with downstream targets (*10*). Adhesion induced by chemoattractants or by PMA stimulation was blocked in a dose-dependent manner by C3 transferase (Fig. 1). Cellular viability, expression of α4β1 integrin, and the increase of intracellular calcium triggered by fMLP or

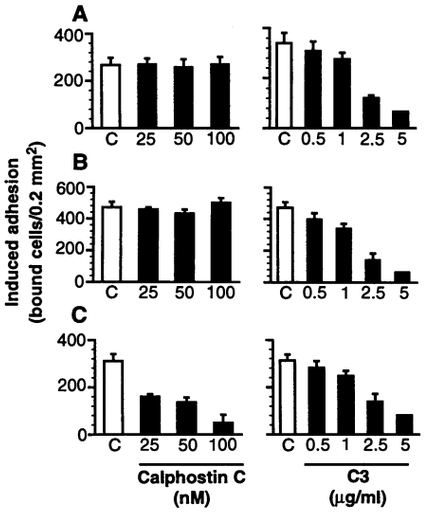

Fig. 1. Inhibition of Rho, but not of PKC, blocks chemoattractant-induced lymphocyte adhesion to VCAM-1. Human fPR or human IL-8RA transfectants were treated for 10 min at 37°C in RPMI 1640 with DMSO (C, control) or with the indicated concentrations of calphostin C (LC Laboratories, Woburn, Massachusetts) and then stimulated for 3 min with 100 nM fMLP (**A**), for 3 min with IL-8 (100 ng/ml) (**B**), or for 10 min with PMA (100 ng/ml) (**C**). Because of the reported light dependency (*15*), both the treatment and the adhesion assay with calphostin C were performed under cool-white fluorescent light. Cells were treated with the indicated amount of recombinant C3 transferase (C3) (UBI, Lake Placid, New York) during culture for 24 hours in RPMI 1640 containing 10% FBS. Values are the mean counts of bound cells in 5 to 11 experiments; error bars are SDs (*16*). Background binding in the absence of agonist was minimal (*16*) and was subtracted in this and subsequent figures.

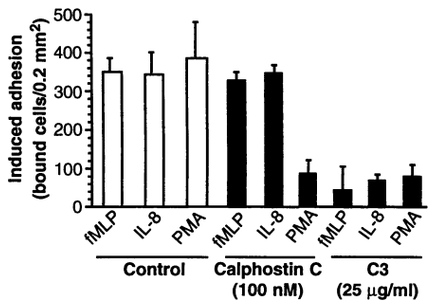

Fig. 2. Effects of C3 transferase and calphostin C on chemoattractant-induced polymorphonuclear neutrophil adhesion to fibrinogen. Human blood neutrophils were treated with calphostin C and then stimulated with 100 nM fMLP, IL-8 (100 ng/ml), or PMA (100 ng/ml) as in Fig. 1. The treatment with C3 transferase was for 20 min at 37°C after electropermeabilization (*17*).

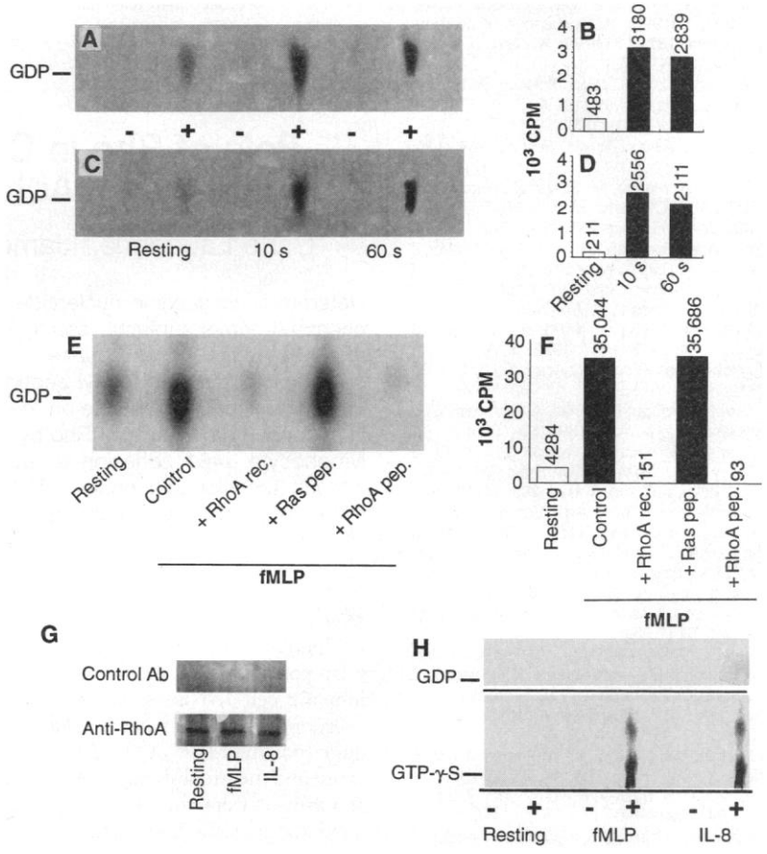

Fig. 3. Stimulated nucleotide exchange on RhoA in cells transfected with chemoattractant receptors. (**A** through **D**) Agonist stimulation induces association of radioactive GDP with RhoA. Transfected cells were labeled with [^{32}P]orthophosphate for 2 hours and were then treated with 100 nM fMLP (A and B) or IL-8 (100 ng/ml) (C and D) for the indicated times at 37°C. Resting cells were treated with DMSO or buffer. Lysates were then immunoprecipitated with rabbit antibody to mouse immunoglobulin (–) or to RhoA (+). The radioactivity bound to immunoprecipitated RhoA migrated with the GDP standard; GTP was undetectable. (**E** and **F**) Recombinant RhoA (RhoA rec.) or RhoA peptide (pep.), but not Ras peptide, prevented detection of GDP, which shows the specificity of the immunoprecipitation. In this experiment, transfected cells were labeled with [^{32}P]orthophosphate for 20 hours and then stimulated with 100 nM fMLP for 1 min at 37°C. (**G**) Equal amounts of total RhoA were precipitated from resting and agonist-stimulated transfectants. Shown are protein immunoblots of anti-RhoA or control antibody (Ab) immunoprecipitates from lysates of transfectants stimulated with buffer (resting), 100 nM fMLP, or IL-8 (100 ng/ml) for 1 min at 37°C. (**H**) Agonist-dependent association of GTP-γ-[^{35}S] with RhoA. Transfected cells were loaded with GTP-γ-[^{35}S] and then stimulated with agonist for 1 min at 37°C. The radioactivity bound to immunoprecipitated RhoA migrated with the GTP-γ-S standard; GDP was undetectable (*18*).

IL-8 were not affected (*11*). C3 transferase also blocked chemoattractant-stimulated neutrophil adhesion (Fig. 2).

Small GTP-binding proteins can act as triggered amplifiers of signal transduction cascades. However, the effect of C3 transferase could also be explained if constitutive Rho activity were required for maintenance of one or more components of the stimulated signaling pathway. To assess whether Rho was rapidly activated by chemoattractants, we analyzed accumulation of radioactive nucleotides on RhoA, the predominant Rho isoform in lymphocytes (*8*). Stimulation of cells with fMLP caused an increase in RhoA-bound radioactive GDP after 10 and 60 s; the maximum amount was about seven times that in unstimulated cells (Fig. 3, A and B). Stimulation with IL-8 caused a similar pattern of increase in RhoA-bound radioactive GDP; the maximum amount was 12 times that in unstimulated cells (Fig. 3, C and D). Recombinant unlabeled RhoA and the RhoA peptide recognized by an antibody to RhoA (anti-RhoA), but not a control peptide from Ras, prevented detection of GDP present in the immunoprecipitates (Fig. 3, E and F). The total amount of RhoA immunoprecipitated was not influenced by stimulation (Fig. 3G). The low amount of radioactive GDP on RhoA from unstimulated cells labeled for 2 hours with [^{32}P]orthophosphate (Fig. 3B) suggests that RhoA normally undergoes minimal nucleotide exchange. The amount of radioactive GDP on RhoA in resting cells was greater after prolonged incubation with [^{32}P]orthophosphate, but even after 20 hours of labeling, stimulation with fMLP still induced a ninefold increase in the amount of radioactive GDP bound (Fig. 3, E and F). RhoA-bound radioactive GTP was undetectable. The absence of GTP in our immunoprecipitates could reflect a high guanosine triphosphatase (GTPase) activity of RhoA that led to rapid GTP hydrolysis in vivo, consistent with the transient nature of the induced adhesion [which normally lasts no longer than 3 to 5 min (*3*)], or it could reflect hydrolysis of GTP to GDP during the processing of immunoprecipitates, which takes hours (*12*).

To further analyze stimulated nucleotide exchange, we loaded the cells with GTP-γ-[^{35}S], a hydrolysis-resistant radioactive analog of GTP (*13*). Treatment of cells with either fMLP or IL-8 stimulated binding of GTP-γ-[^{35}S] to RhoA (Fig. 3H). In contrast, RhoA did not bind GTP-γ-[^{35}S] in nonstimulated cells; this finding further demonstrated the low basal rate of nucleotide exchange on RhoA in the absence of stimulation. The low rate of spontaneous RhoA nucleotide exchange may be important in maintaining the normal low degree of adhesiveness of unstimulated lymphocytes. The increase in radioactive GDP content on RhoA and the association with GTP-γ-[^{35}S] after agonist treatment indicate that chemoattractants activate RhoA by increasing its guanine nucleotide exchange activity. Nucleotide exchange is extremely rapid, consistent with a role for RhoA in adhesion triggering, which in L1/2 cells occurs within seconds (*3*).

Our results indicate a critical role for Rho GTP-binding proteins in coupling of G protein–linked chemoattractant receptors to integrin-mediated adhesion in leukocytes. In contrast to the adhesion-triggering pathway defined here, chemoattractant-induced calcium flux and the respiratory burst are insensitive to C3 transferase (*14*), which implies that independent regulation of chemoattractant-stimulated adhesion and effector functions may be possible. The activity of Rho-related GTP-binding proteins may be an important target for pharmacological modulation of adhesive functions of lymphocytes and other leukocytes in pathologic inflammation.

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16. L1/2 cells (mouse pre-B lymphocytes) were stably transfected with human fPR or with human IL-8RA as described (*3*). Eighteen-well glass slides were coated overnight at 4°C with a 1:50 dilution in phosphate-buffered saline (PBS) of purified mouse VCAM-1 (*3*) and blocked for 10 min with fetal bovine serum (FBS). Transfectant cells (8×10^4 per well; 4×10^6 per milliliter) in RPMI 1640) were added, incubated for 10 min at 37°C, and then stimulated by addition of agonist for 3 min [fMLP (100 nM) or IL-8 (100 ng/ml)] and 10 min [PMA (100 ng/ml)] before washing, fixation, and computer-assisted enumeration of bound cells (*3*). Background binding in the absence of added agonist was minimal [21 ± 8 cells per 0.2 mm^2 for control of calphostin C; 6 ± 4 cells per 0.2 mm^2 for C3 transferase treatments] and was subtracted from agonist-stimulated adhesion for data presentation.
17. Human blood neutrophils were isolated under endotoxin-free conditions as described [C. P. Nielson, R. E. Vestal, R. J. Sturm, R. Heaslip, *J. Allergy Clin. Immunol.* **86**, 801 (1990)]. Eighteen-well glass slides were coated for 60 min at 37°C with human fibrinogen (Sigma) (10 μg per well in LPS-free water). Neutrophils (5×10^4 per well; 2.5×10^6 per milliliter in RPMI 1640) were added and incubated for 10 min at 37°C and then stimulated by addition of the agonists for 3 min [fMLP (100 nM) or IL-8 (100 ng/ml)] or 10 min [PMA (100 ng/ml)]. For electropermeabilization, neutrophils (10^7 per milliliter) were resuspended in ice-cold LPS-free and calcium-free PBS, 10 mM glucose, 1 mM $MgCl_2$, 2 μM nicotinamide adenine dinucleotide (Sigma), and C3 transferase (25 μg/ml); 0.8 ml were pulsed at 1.3 kV/cm from a 25-μF capacitor in a Bio-Rad pulser. In control experiments, electroporation had no effect on spontaneous or agonist-induced adhesion responses in the absence of C3 transferase. Background (no agonist) binding for control, calphostin C–treated, and C3 transferase–treated neutrophils was 69 ± 11, 81 ± 11, and 10 ± 3 cells per 0.2 mm^2, respectively, and was subtracted.
18. L1/2 cell transfectants were kept overnight in phosphate-free RPMI 1640 containing dialyzed FBS (10%) and labeled with [^{32}P]orthophosphate (0.2 mCi/ml for either 2 or 20 hours) in the same medium. Cells were resuspended at 4×10^7/ml in PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and bovine serum albumin (BSA; 1 mg/ml) and stimulated with the appropriate agonist at 37°C while stirring for the indicated times. Cell suspensions (0.5 ml; 2×10^7 cells) were lysed on ice in 0.5 ml of 100 mM Hepes buffer (pH 7.4), 2% Triton X-100, 1% deoxycholate, 0.1% SDS, 300 mM NaCl, 10 mM $MgCl_2$, 2 mM EGTA, BSA (2 mg/ml), 20 mM benzamidine, leupeptin-pepstatin-aprotinin-soybean trypsin inhibitor (20 μg/ml), and 2 mM phenylmethylsulfonyl fluoride. Nuclei were sedimented and lysates were adjusted to 500 mM NaCl. After preclearing for 30 min with 10 μl of trisacryl–protein A beads (Pierce), the proteins were immunoprecipitated with 4 μg of rabbit polyclonal anti-RhoA, which recognizes the sequence Lys-Asp-Leu-Arg-Asn-Asp-Glu-His-Thr-Arg-Arg-Glu-Leu-Ala (119–132) (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit antibody to mouse immunoglobulin (Sigma) for 60 min at 4°C, followed by 4 μl of trisacryl–protein A beads (Pierce) for 90 min. For the competition experiments, the antibody was incubated for 30 min with RhoA(119–132) or H-Ras(96–118) peptides (20 μg/ml) or recombinant human RhoA (10 μg/ml) purified from GST fusion protein. The beads were washed eight times in 50 mM Hepes buffer (pH 7.4), 500 mM NaCl, 0.1% Triton X-100, and 0.005% SDS, and the nucleotides were eluted in 5 mM EDTA, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GTP, and 0.5 mM GDP for 20 min at 68°C [J. Downward, J. D. Graves, P. H. Warne, S. Rayter, D. A. Cantrell, *Nature* **346**, 719 (1990)]. Separation of eluted nucleotides was on PEI-cellulose plates (Sigma) run in 1 M KH_2PO_4 (pH 3.4) [B. Q. Li, D. Kaplan, H.-f. Kung, T. Kamata, *Science* **256**, 1456 (1992)]. Radioactive spots, determined by autoradiography with X-OMAT AR films (Kodak), were scraped off the plates and counted in a scintillation β counter. Alternatively, the cells were resuspended at 4×10^7 per milliliter in Ca^{2+}/Mg^{2+}-free PBS, 0.5% pluronic F-68 (Sigma), and GTP-γ-[^{35}S] (50 μCi/ml). The cells were syringe-loaded through a tuberculin syringe with a 33-gauge needle (*13*). After 14 passes through the needle, 0.5 to 1% of added radioactivity was incorporated into the cells. The cells were washed twice and, after 20 min recovery at 37°C, stimulated for 1 min and processed as above. After 3 days of exposure, radioactivity was detected with a Molecular Dynamics PhosphorImager 445 SI.

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