# Functional Expression of the CXC-Chemokine Receptor-4/ Fusin on Mouse Microglial Cells and Astrocytes[1]

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**The mRNA for the seven-transmembrane-spanning G protein-coupled receptor fusin/CXCR-4 is expressed in primary mouse astrocyte cultures and the transformed mouse microglial cell line, N9. Cell surface expression of fusin in these cells was confirmed by staining with a polyclonal anti-fusin Ab. The functional capacity of this chemokine receptor was examined by evaluating the calcium responses following stimulation of glial cells with the CXC-chemokine, stromal-derived cell factor-1$\alpha$ (SDF-1$\alpha$). Both astrocytes and microglial cells mobilized calcium following stimulation with chemically synthesized SDF-1$\alpha$. SDF-1$\alpha$- and carbachol-mediated calcium responses of astrocytes were partially inhibited by treatment with pertussis toxin (PTx), suggesting receptor coupling to a combination of $G\alpha_i$ and other G proteins. In contrast, the calcium responses of microglial cells to SDF-1$\alpha$ were completely PTx sensitive, while responses to carbachol stimulation were PTx resistant. The ability of SDF-1$\alpha$ to induce glial cell migration was also examined. Synthetic SDF-1$\alpha$ was a potent chemoattractant for mouse microglial cells at ligand concentrations of 10 to 500 ng/ml; peak responses were noted at 100 ng/ml. In contrast, astrocytes did not migrate toward a gradient of SDF-1$\alpha$. The failure of SDF-1$\alpha$ to induce astrocyte migration was specific, as another chemokine, macrophage inflammatory protein-1$\alpha$, triggered astrocyte chemotaxis.** *The Journal of Immunology,* **1997, 159: 905–911.**

Recent studies have shown that the CXC-chemokine, stromal-derived cell factor-1$\alpha$ (SDF-1$\alpha$)[3] is a ligand for the receptor termed fusin (also referred to as CXC-chemokine receptor-4, CXCR-4) (1, 2). Fusin is a highly conserved seven-transmembrane-spanning G protein-coupled receptor (3–7). Interest in fusin was stimulated by the observation that human fusin/CXCR-4 serves as a coreceptor for T lymphocytotropic HIV-1 isolates (8). Coexpression of CD4 and fusin is required for the fusion and entry of HIV-1 into T cells (1, 2, 8, 9). Furthermore, SDF-1$\alpha$ blocks HIV-1 infection in vitro (1, 2). More recent studies demonstrate that fusin also serves as the primary receptor for HIV-2 infection, binding virus in the absence of CD4 (9).

To date, SDF-1$\alpha$ is the only physiologic ligand for fusin (1, 2). In vivo and in vitro, SDF-1$\alpha$ functions as a potent T cell and macrophage chemoattractant (10). In addition, SDF-1$\alpha$ stimulates chemotaxis of hemopoietic cell progenitors and proliferation of B cell progenitors (11, 12). Our knowledge about the biology of SDF-1 is largely restricted to the interactions of this CXC-chemokine with cells of hemopoietic lineage. Mice targeted for loss of SDF-1 expression provide evidence that SDF-1 also affects other organ systems; thus, SDF-1-deficient mice have defects in B cell and myeloid development as well as in heart development, implying important but poorly understood functions in non-hemopoietic cells (13).

Two alternately spliced forms of mouse fusin that differ by only two amino acids in the N-terminal ectodomain have been identified (14). The mRNA for both isoforms of murine fusin/CXCR-4 have been identified in a variety of organs and in non-hemopoietic cells (6, 7, 14), raising questions about the potential functions of these receptors on parenchymal cells. The current study examines the expression of fusin/CXCR-4 on mouse glial cells. Both astrocytes and microglia express this chemokine receptor. Furthermore, SDF-1$\alpha$ stimulates calcium mobilization in mouse glial cells, suggesting that the receptor is functional. The ability of SDF-1$\alpha$ to induce glial cell chemotaxis was also examined.

## Materials and Methods

### *Mice*

BALB/cHa mice were purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN), and were bred in our animal facilities. Mice were maintained in accordance with the guidelines of the committee on animals of the Harvard Medical School, the Society for Neuroscience, and those prepared by the committee on care and use of laboratory animals of the Institute of Laboratory Resources, National Research Council (Department of Health and Human Services, National Institutes of Health Publication 85–23, revised 1985, Bethesda, MD).

### *Reagents*

Mouse MIP-1$\alpha$ and MIP-2 were purchased from R&D Systems (Minneapolis, MN). FMLP was purchased from Sigma Chemical Co. (St. Louis, MO). Human SDF-1$\alpha$ (amino acids 1–67) was synthesized as described previously (15). SDF-1$\alpha$ is highly conserved between species, with only a single Ile to Val substitution at position 18 distinguishing human and mouse SDF-1$\alpha$. SDF-1$\alpha$ (amino acids 1–67) was generated using t-Boc

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Received for publication January 21, 1997. Accepted for publication April 11, 1997.

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[1] This work was supported by National Institutes of Health Grants NS31152 and CA67416 and a fellowship from the Deutsche Forschungsgemeinschaft (to M.H.).

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[3] Abbreviations used in this paper: SDF-1$\alpha$, stromal-derived cell factor-1$\alpha$; CXCR, CXC chemokine receptor; MIP-1$\alpha$, macrophage inflammatory protein-1$\alpha$; GFAP, glial fibrillary acidic protein; PTx, *Bordetella pertussis* toxin.



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chemistry on an Applied Biosystems 430A peptide synthesizer (Foster City, CA). Proteins were folded by air oxidation and purified using at least two steps of reverse phase HPLC (15). The molecular mass was determined by electrospray mass spectrometry on a API-3 triple quadrupole mass spectrometer (SCIEX, Thornhill, Ontario, Canada). The average mass ± SD was 7831.63 ± 0.76 Da.

Carbamylcholine chloride (carbachol) and thrombin were purchased from Sigma Chemical Co. The above reagents were treated with Detoxi-Gel (Pierce Chemical Co., Rockford, IL) to eliminate endotoxin before use in all functional assays.

### *Astrocyte isolation*

Astrocytes were prepared from neonatal (<24 h) mouse brains, as described previously (16). Briefly, after removal of the meninges, the brains were separated into single-cell suspensions by passage through nylon mesh (112 μm; Tetko, Inc., Briarcliff Manor, NY). The primary glial cell cultures were maintained in MEM (Sigma Chemical Co.) supplemented with 10% FCS (Sigma Chemical Co.), 2 mM glutamine, 2 mg/ml glucose, 5 μg/ml bovine pancreas insulin (Sigma Chemical Co.), 2.2 mg/ml $NaHCO_3$, 50 U/ml penicillin, and 50 μg/ml streptomycin (referred to as complete medium) in a moist 10% $CO_2$ atmosphere at 37°C. After 10 and 13 days, the flasks were agitated on an orbital shaker (L.E.D. Orbit-Shaker, Lab Line Instruments, Inc., Melrose Park, IL) for 2 h at 250 rpm at 37°C, and the nonadherent oligodendrocyte and microglial cells were removed. After 13 days, the astrocytes were trypsinized and expanded at a 1:6 ratio in complete medium. One day after expansion, the flasks were agitated as described above, and the medium was changed. The purity of the astrocyte population was >95%, as determined by indirect immunofluorescence assays with anti-Mac-1 to detect microglial cells, anti-galactocerebroside to detect oligodendrocyte contamination, and anti-glial fibrillary acidic protein (GFAP) Abs to identify astrocytes (16).

All astrocytes were cultured for a total of 20 to 26 days before use. To remove residual oligodendrocytes and microglial cells, the flasks were agitated for 1 h, as described above, before harvest. Thereafter, the cells were trypsinized for 10 min at 37°C and washed three times. The cells were allowed to recover from trypsinization by incubation in complete medium for 90 min at 37°C before use in all assays.

### *Microglial cell isolation*

Primary microglial cells were prepared from 13-day mixed glial cell cultures, as described above. Briefly, 13-day mixed glial cell cultures were agitated on an orbital shaker for 4 h at 250 rpm at 37°C. The supernatant was collected, and microglia were filtered through a 32-μm nylon mesh (16). Microglia were incubated on uncoated petri dishes in a moist 10% $CO_2$ atmosphere for 20 min at 37°C to allow microglial cells to attach. The unattached cells were removed, and the adherent microglial cells were collected with a cell scraper. The purity of microglia obtained by this procedure was >95% as determined by indirect immunofluorescence with anti-Mac-1 Abs (16).

The mouse embryonic microglial cell line N9 was a gift from Dr. P. Ricciardi-Castagnoli (Milan, Italy). The preparation of these v-*myc*-transformed cells was previously described (17). The N9 cells are $F4/80^+$, $FcR^+$, Mac-$1^+$ and lack astroglial ($GFAP^-$), bipotential glial precursor ($A2B5^-$), and oligodendroglial (galactocerebroside$^-$) markers (16). The N9 cell line was cultured in RPMI 1640 supplemented with 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin in a moist 7% $CO_2$ atmosphere at 37°C.

### *Screening of glial cell cDNA for expression of CXCR-4*

Total RNA and cDNA were prepared from $8 \times 10^7$ astrocytes, N9 microglial cells, or L929 mouse fibroblasts using methods described previously (6). Briefly, total RNA was isolated by the method of Chomczynski and Sacchi (18). Before cDNA synthesis, the RNA was treated with 1 U of DNase-I (bovine pancreas; Sigma Chemical Co.) for 15 min at room temperature in 10 μl of 20 mM Tris-HCl (pH 8.4) containing 2 mM $MgCl_2$ and 50 mM KCl, which was then inactivated by incubation with 2.5 mM EDTA at 65°C for 10 min. Single-stranded cDNA was synthesized from 1.5 μg of total RNA incubated in a 20-μl reaction containing 50 ng of random hexamers, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 10 mM 1,4-DTT, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), and 200 U of reverse transcriptase (Superscript Preamplification System for First Strand cDNA Synthesis, Life Technologies, Gaithersburg, MD) for 10 min at 25°C, followed by 50 min at 42°C. The sample was then incubated with 1 μl of RNase $H^-$ for 20 min at 37°C. To control for the possibility of contaminating genomic DNA, RNA samples were included that were not subjected to reverse transcriptase. Gene-specific primers for mouse fusin/CXCR-4 were: sense, GGCTGTAGAGC GAGTGTTGC; and antisense, GTAGAGGTTGACAGTGTAGAT. PCR amplification was conducted in a reaction mixture containing 2 mM $MgCl_2$, 0.5 μM primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 4 U/70 μl Amplitaq DNA Polymerase (Perkin-Elmer, Modesto, CA). The PCR program was as follows: preincubation at 94°C for 1 min and then 85°C while enzyme was added, and 27 cycles of PCR at 94°C for 45 s plus 45-s annealing and 1-min 72°C extension. The annealing temperatures used were 52°C for CXCR-4 and 55°C for β-glucuronidase. Six microliters of the PCR mixtures were visualized on a 1.5% agarose gel.

### *Measurement of intracellular calcium concentration*

Astrocytes ($5 \times 10^5$/ml) and N9 microglial cells ($5 \times 10^5$/ml) were preincubated with 2.5 μM fura-2/AM (Molecular Probes, Eugene, OR) in HBSS containing 1% BSA and 1.25 mM $CaCl_2$ for 60 min at 37°C. Subsequently, the cells were washed twice and resuspended in a light-shielded tube at room temperature until use. Fluorescence measurements were performed at excitations of 340 and 380 nm with a fluorescence emission at 510 nm in a fluorospectrophotometer (Hitachi F-4500, Tokyo, Japan) while stirring 2 ml of cell suspension ($2 \times 10^6$ cells/ml) at 37°C. The data are presented as the relative ratio of fluorescence at 340 and 380 nm. From this ratio, the level of intracellular calcium was calculated. To calibrate intracellular calcium levels 0.1 mM digitonin was used to release the indicator dye, and 4 mM EDTA was used to clamp intracellular calcium levels. Calcium concentrations were calculated according to the manufacturer's protocol, using a $K_d$ ($Ca^{2+}$) of 224 nM.

### *Cell migration assay*

Cell migration was evaluated in 48-well Boyden microchambers (Neuroprobe, Cabin John, MD) as described previously (19). Astrocytes ($4 \times 10^6$ cells/ml), N9 microglial cells ($4 \times 10^6$ cells/ml), and primary microglial cells ($1 \times 10^6$ cells/ml) were washed and resuspended in 1% BSA-containing MEM (for astrocytes and microglial cells) or 1% BSA-containing RPMI 1640 (for the microglial cell line). Fifty microliters of cells were added to the upper well of the Boyden chamber. Thirty microliters of cells, containing the indicated concentration of endotoxin-depleted chemokine, were placed in the lower microchamber. For astrocyte migration the wells were separated by a 14-μm pore size polycarbonate filter (Poretics Corp., Livermore, CA). A 5-μm pore size polycarbonate filter was used for primary microglia and N9 cells. All responses were assayed in triplicate. The chambers were incubated for 4 h (astrocytes) or 80 min (microglial cells) at 37°C in a moist 10% $CO_2$ atmosphere for primary astrocytes and microglial cells or in a moist 7% $CO_2$ atmosphere for N9 microglial cells. After incubation, the upper surfaces of the filters were scraped to remove nonmigrating cells. Filters were subsequently fixed in methanol and stained with Diff-Quik (Baxter, McGaw Park, IL). The number of migrating cells per well (astrocytes) or in 10 high power fields (microglial cells) was determined microscopically at ×400 magnification. Since the background (without chemokine) migration varied among experiments, the data are normalized as a migration index (number of migrating cells in the experimental group/number of migrating cells in control groups that lacked chemokine).

### *Purified rabbit anti-fusin Abs*

New Zealand White rabbits were immunized four or five times with 1 mg of a branched multiple antigenic peptide vaccine in CFA. The multiple antigenic peptide structure was constructed with eight branches on a lysine core (20). Human fusin peptide (residues 9–32) SDNY*T*EE*M*GSGDY DS*M*KEPCFR*E*E(K) was synthesized using an automated model 430A peptide synthesizer (Applied Biosystems, Foster City, CA). This peptide sequence is conserved among species; the four italicized residues are replaced by S, V, N, and D, respectively, in mouse fusin (6, 7). Immune serum was collected 1 wk after the last immunization.

To purify the anti-peptide Abs, 2 mg/ml fusin peptide (residues 9–32) were coupled to 1 ml of cyanogen bromide-activated Sepharose beads (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol. After extensive washing, 0.5 ml of rabbit anti-fusin peptide antiserum was incubated with peptide-coupled beads for 2 h at room temperature. After extensive washing with 20 mM Tris-HCl containing 1 M NaCl and 1% Triton X-100 (pH 7.5) followed by washing with 20 mM Tris-HCl containing 0.15 M NaCl (pH 7.5) and, finally, washing with 0.15 M NaCl, the polyclonal Abs were eluted with 0.1 M glycine-HCl buffer (pH 2.5). The Ab eluate was immediately neutralized with 1 M Tris, dialyzed against PBS (pH 7.2), and stored at −20°C.

### *Flow cytometry*

Samples containing $1 \times 10^6$ cells were incubated on ice for 30 min with 20 to 50 μl containing 1 to 2 μg Ab and washed two or three times with PBS

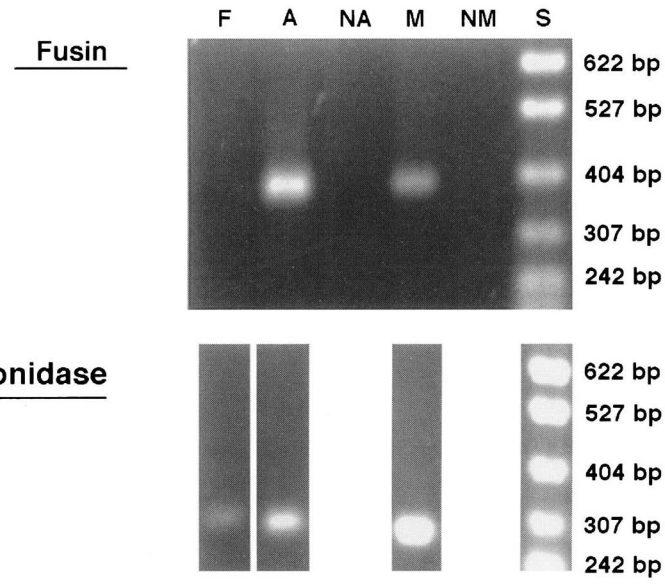


**FIGURE 1.** Representative reverse transcriptase-PCR results using murine fusin/CXCR-4 (390 bp) and β-glucuronidase (301-bp) primers. PCR products were run with RNA from: A, astrocytes; NA, astrocytes with no reverse transcriptase added; M, N9 microglial cells; NM, N9 microglial cells with no RT added; or F, L929 fibroblasts.

containing 0.1% BSA and 0.1% $NaN_3$. These samples were then incubated for 30 min with 25 μl of FITC-conjugated anti-rabbit Ig reagent (Cooper Biomedicals, Organon Teknica, Malvern, PA). After the final incubation, samples were washed twice, fixed in 1% paraformaldehyde, and analyzed on a Profile II flow cytometer (Coulter Corp, Hialeah, FL).

### *Statistics*

Data are given as the mean stimulation index (number of cells migrating in experimental group/number of cells migrating in medium control group). Statistical analysis was performed with Student's $t$ test. $p < 0.05$ was considered significant.

## Results

### *CXCR-4 expression in astrocytes and N9 microglial cells*

To confirm that mouse glial cells express CXCR-4 (6, 14), RNA from astrocytes and N9 microglial cells was reverse transcribed and subjected to PCR amplification with a different pair of PCR primers. The mouse 390-bp CXCR-4 PCR products were detected in astrocytes and microglia (Fig. 1). Expression of fusin/CXCR-4 was confirmed by sequencing a PCR fragment from astrocytes. No PCR products were detected when reverse transcriptase was omitted (Fig. 1). Since primary astrocyte cultures may be contaminated with fibroblasts, mouse L929 fibroblasts were also tested for expression of fusin/CXCR-4; no PCR products were detectable (Fig. 1). As a control to exclude DNA contamination, β-glucuronidase PCR products were amplified from astrocytes and microglial cells; the only detectable PCR products had a size of 301 bp, consistent with the predicted product of the spliced cDNA (Fig. 1).

To establish that these cells also expressed cell surface receptors, anti-fusin Abs were used to stain glial cells (Fig. 2). Primary cultures of microglial cells were incubated with rabbit polyclonal anti-fusin peptide Abs and stained with FITC-labeled second Abs. Nearly all (95%) primary microglia expressed fusin (Fig. 2). Astrocytes and N9 microglial cells displayed weaker staining (42 and 43% positive, respectively). In contrast, L929 fibroblasts that failed to express CXCR-4 message (Fig. 1) also failed to stain with these Abs (Fig. 2).

### *SDF-1α induces calcium responses in astrocytes and microglia*

To examine whether the fusin protein is functional in microglia and astrocytes, calcium flux was measured after stimulation with ligand. SDF-1α treatment increased intracellular calcium levels in the microglial cell line (Fig. 3). The rapid calcium flux in microglial cells was noted within 5 s, and the intensity was dependent on the dose of SDF-1α. Stimulation with thrombin failed to trigger calcium responses in N9 microglial cells (Fig. 3*A*). Maximal SDF-1α responses were noted at 100 ng/ml, but detectable calcium mobilization was occasionally observed with as little as 0.1 ng/ml (Fig. 3*A*). SDF-1α also induced a rapid calcium flux in astrocytes, the magnitude of the intracellular calcium response in astrocytes was generally comparable to that in microglial cells (Fig. 3*B*). In contrast, another mouse α-chemokine, MIP-2, failed to stimulate calcium responses in astrocytes (Fig. 3*B*). The mesangial tumor cell line, MES-13, which does not express fusin/CXCR-4 mRNA (6), failed to respond to SDF-1α (Fig. 3*C*). A common feature of chemokines is the ability to induce homologous receptor desensitization. As expected, stimulation with 100 ng/ml SDF-1α completely desensitized primary astrocytes and N9 microglial cells for a subsequent challenge with the same dose of SDF-1α (Fig. 3, *D* and *E*).

### *SDF-1α-induced calcium responses are sensitive to pertussis toxin (PTx)*

Chemokine receptors belong to the family of seven-transmembrane-spanning molecules that couple to heterotrimeric G proteins. PTx pretreatment inhibits chemokine-induced calcium flux in most systems, suggesting that the receptors couple to $G\alpha_i$ subunits (11, 19, 21, 22). To determine whether SDF-1α-induced calcium mobilization is mediated by PTx-sensitive G proteins, astrocytes and N9 microglial cells were pretreated with varying doses of PTx before analysis. Cell viability, as assessed by carbachol responsiveness and trypan blue dye exclusion, was not affected by PTx treatment (Fig. 4 and data not shown). As illustrated in Figure 4, 100 to 1000 ng/ml PTx inhibited SDF-1α-induced calcium concentrations to background levels in microglia. However, after treatment of astrocytes with 100 or 1000 ng/ml PTx, inhibition of intracellular calcium remained incomplete (75%; Fig. 4). PTx treatment did not significantly block the calcium responses of microglial muscarinic receptors following stimulation with carbachol, but partially inhibited calcium flux in astrocytes, confirming the tissue specificity of PTx inhibition (Fig. 4). Muscarinic receptors are also G protein-coupled seven-transmembrane-spanning receptors, but are usually associated with PTx-resistant Gα proteins (21, 23). The results suggest that distinct combinations of G proteins are used in different glial populations. SDF-1α stimulates

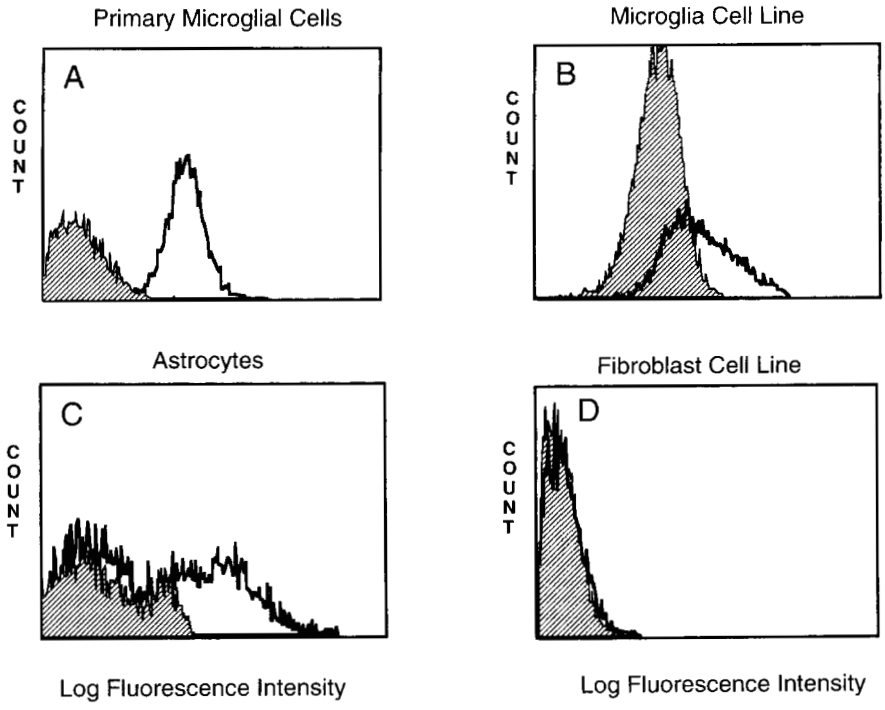


**FIGURE 2.** Flow cytometric analysis of CXCR-4 expression. Primary microglia (*A*), N9 microglia (*B*), primary astrocytes (*C*), or L929 fibroblasts (*D*) were incubated with either control rabbit Ig (cross-hatched) or affinity-purified anti-CXCR-4 peptide$_{9-32}$ Abs (thickened line) and stained with FITC-labeled anti-rabbit Ig.

astrocytes by a pathway that is partially dependent upon $G\alpha_i$ proteins; however, microglial responses to SDF-1$\alpha$ appear to be completely $G\alpha_i$ dependent.

*SDF-1$\alpha$ induces chemotaxis of microglia but not astrocytes*

Since SDF-1$\alpha$ functions as a chemoattractant of human and murine leukocytes (2, 10, 11), the chemoattractant effect of SDF-1$\alpha$ on glial cells was evaluated. The chemotaxis of primary microglial cells, primary astrocytes, and N9 microglial cells was tested in Boyden microchemotaxis chambers. Primary microglia showed migration toward SDF-1$\alpha$ at 10 to 1000 ng/ml, with a peak migration index of 5 at 100 ng/ml (Fig. 5*A*), and transformed N9 microglia cells showed a similar dose response (Fig. 5*B*). The mouse $\alpha$-chemokine MIP-2, a chemoattractant for mouse neutrophils, was used as a negative control for these chemotactic studies. Additional experiments demonstrated that anti-fusin Abs (1.7 $\mu$g/ml) inhibited SDF-1$\alpha$ chemoattractant activity (mean $\pm$ SD, 52 $\pm$ 9%), but failed to block migration to FMLP ($-2 \pm 16\%$).

In contrast, SDF-1$\alpha$ did not induce significant migration of astrocytes at any concentration tested (Fig. 5*C*). To demonstrate that astrocytes are capable of migration in this assay system, we tested the astrocyte response to another chemokine, MIP-1$\alpha$. Primary astrocytes and microglia migrate toward 1 to 100 ng/ml mouse MIP-1$\alpha$ (Fig. 5). To insure that astrocytes migrated in the assay, the membrane was stained with anti-GFAP; all migrating cells had the size and staining characteristics of astrocytes. Thus, these results demonstrate that SDF-1$\alpha$ is a potent chemoattractant for primary and transformed microglial cells but not for astrocytes.

To determine whether the microglial migratory responses were mediated by PTx-sensitive receptors, N9 cells were pretreated with 1 to 1000 ng/ml PTx before use in chemotaxis assays. Cell viability was not affected by PTx treatment. SDF-1$\alpha$ migratory responses were completely inhibited by treatment with 100 to 1000 ng/ml PTx and partially inhibited by 1 to 10 ng/ml PTx (Fig. 6).

## Discussion

Human and mouse fusin/CXCR-4 are broadly expressed in various tissues (4, 6, 7, 12, 24). A previous report identified fusin mRNA in murine lymphocytes, macrophages, neutrophils, N9 microglia, and cultured primary mouse astrocytes by Northern blot (6, 7, 12) and PCR (14). The staining of microglia and astrocytes with an anti-fusin peptide Abs confirmed the expression of fusin on these glial populations (Fig. 2). Two previous reports failed to detect fusin mRNA or protein on the human U87 cell line derived from a malignant glioma (8, 9). Although U87 cells are potentially of astrocyte origin, they may have lost gene expression in either the transformation or in vitro selection process. Our data are consistent with other analyses that demonstrate that fusin RNA is expressed in human and mouse brain (7, 12, 25, 26).

SDF-1$\alpha$ is the only known physiologic ligand for CXCR-4 (1, 2, 7, 24). The expression of SDF-1$\alpha$ RNA is constitutive in normal brain (12, 25, 27). The continuous expression of ligand implies that fusin/CXCR-4-bearing cells may normally be in a state of partial activation or perhaps desensitization depending on the level of SDF-1 protein expression. Changes in the level of SDF-1 expression could alter glial cell physiology, possibly affecting microglial locomotion or attachment of astrocytes with the cells responsible for maintenance of the blood-brain barrier (28, 29). Although changes in SDF-1 expression have not been reported, they could occur directly by regulation of SDF-1 gene expression or indirectly by entry of large numbers of fusin/CXCR-4-bearing leukocytes following breakdown of the blood-brain barrier.

The present studies compare astrocyte and microglial cell responses to the chemokine SDF-1$\alpha$ in vitro. Previous reports demonstrated that SDF-1$\alpha$ induces a rapid calcium flux in human leukocytes and fusin-transfected CHO cells (1, 2, 7, 11, 14). N9 microglial cells and astrocytes also mount a rapid calcium flux that can desensitize the SDF-1$\alpha$ receptor (Fig. 3).

The SDF-1$\alpha$-induced calcium responses of astrocytes are partially sensitive to 10 to 1000 ng/ml PTx. Under the same experimental conditions, SDF-1$\alpha$-induced calcium and chemotactic responses of microglial cells are completely sensitive to $\geq$100 ng/ml PTx. These findings suggest that SDF-1$\alpha$ receptors are coupled to different G proteins in astrocytes compared with microglia. The partial sensitivity of astrocytes to PTx suggests that while $G\alpha_i$

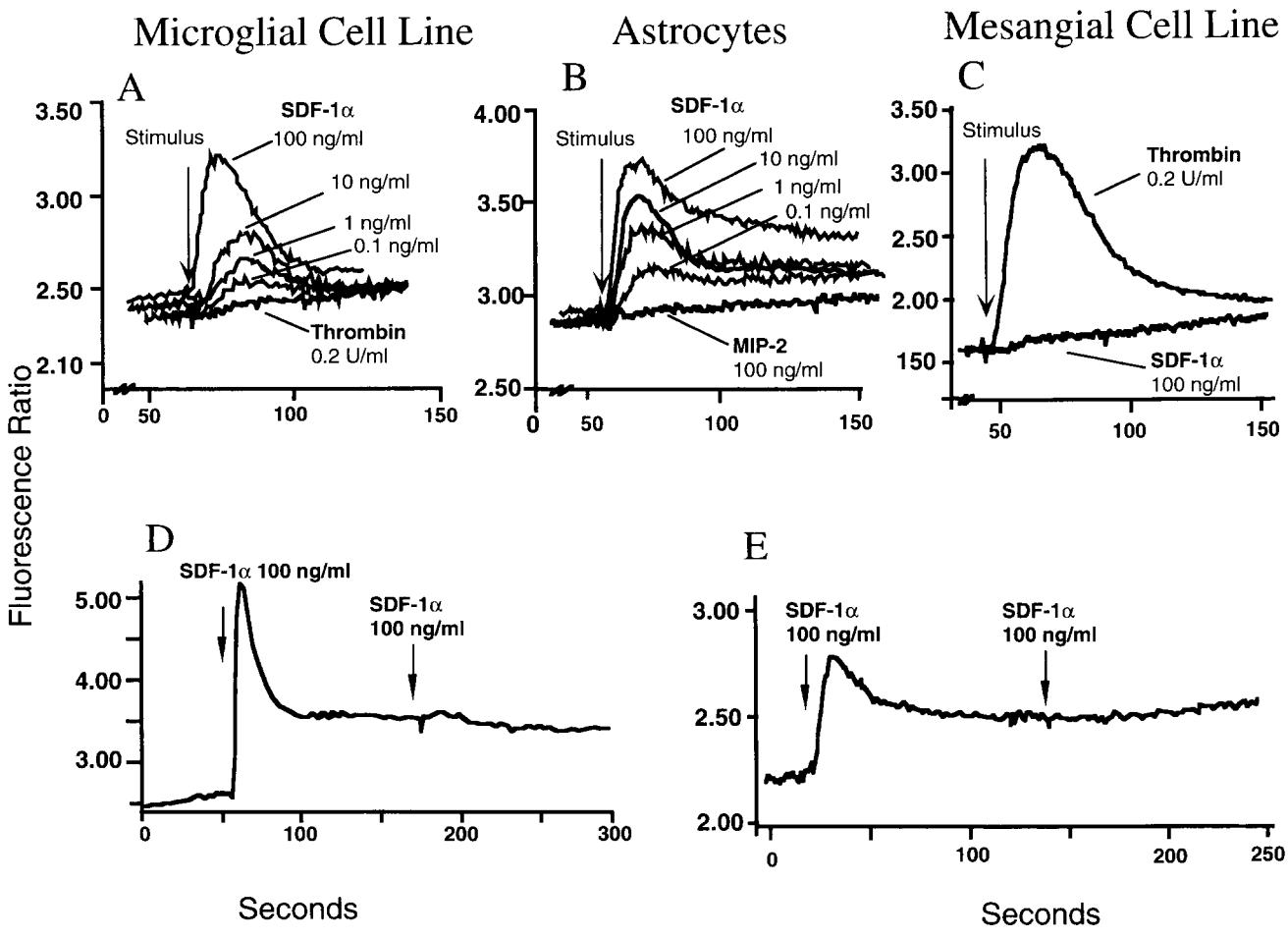


**FIGURE 3.** Calcium flux in astrocytes and microglial cells in response to SDF-1α. The N9 microglial cell line (*A* and *D*), primary astrocytes (*B* and *E*), or MES-13 mesangial cells (*C*) were loaded with fura-2 for 60 min at 37°C. After washing, $4 \times 10^6$ cells were incubated at 37°C in a fluorescence spectrometer with a continuously stirring cuvette for 1 to 1.5 min before addition of ligand. *A* through *C*, 100, 10, 1, and 0.1 ng/ml SDF-1α; 100 ng/ml MIP-2; or 0.2 U/ml thrombin were added to the cell suspensions (the time of chemokine addition is indicated by an arrow). Homologous desensitization with 100 ng/ml SDF-1α in N9 microglia (*D*) or astrocytes (*E*) is shown. The arrow indicates the time when glial cells were stimulated and challenged with SDF-1α. All data are expressed as the ratio of emission at 510 nm after excitation at 340 and 380 nm, respectively.

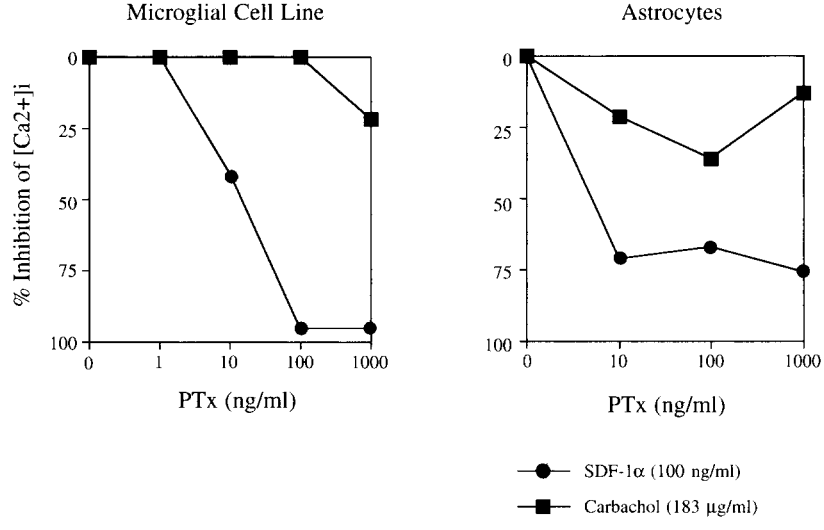


**FIGURE 4.** Effect of PTx pretreatment on SDF-1α-induced calcium mobilization of N9 microglial cells (*left*) or astrocytes (*right*). Glial cells ($5 \times 10^6$/ml) were incubated in serum-free medium with 0, 10, 100, or 1000 ng/ml PTx for 60 min at 37°C. After the incubation, cells were washed and resuspended in HBSS containing 1% BSA. The cell suspensions were loaded with fura-2 for 60 min at 37°C, washed twice, and adjusted to $2 \times 10^6$ cells/ml in HBSS containing 1% BSA. Both cell types were stimulated with 100 ng/ml SDF-1α (closed circles) or 183 μg/ml carbachol (closed squares). The data are expressed as the percentage of PTx-mediated inhibition of the SDF-1α-induced calcium response. The percent inhibition was calculated from the calculated intracellular calcium concentration. Data from one of two similar experiments are shown.

proteins are involved in SDF-1α-induced calcium responses, PTx-resistant G proteins also contribute to astrocyte responses to SDF-1α. Signaling through these alternative G proteins or downstream signaling differences may contribute to the cell type-specific failure of astrocytes to migrate toward SDF-1α. Multiple G protein interactions with human neutrophil IL-8 receptors have been demonstrated (30). PTx partially inhibited carbachol-induced calcium mobilization in astrocytes, although little or no blocking was observed with microglia

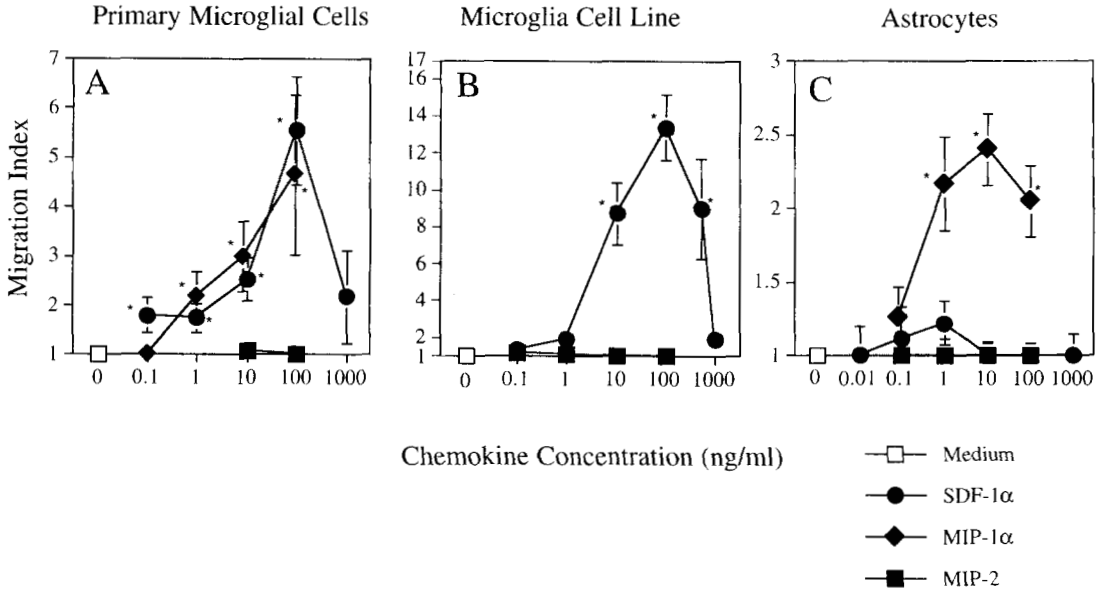


**FIGURE 5.** Chemotaxis of glial cells in response to various concentrations of SDF-1$\alpha$ (closed circle), MIP-1$\alpha$ (closed diamond), MIP-2 (closed square), or medium (open square). Target cells include the mouse N9 microglial cell line (*A*), primary microglial cells (*B*), and primary astrocytes (*C*). The number of cells migrating to SDF-1$\alpha$ per high power field was divided by the medium control for presentation as a migration index. The data represent a pool of three or more independent experiments and are expressed as the migration index ± SEM. An asterisk indicates a significant level of migration at the indicated chemokine concentration ($p < 0.05$) compared with that in medium alone.

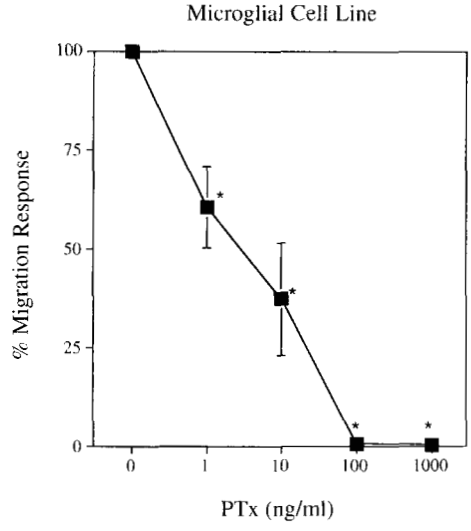


**FIGURE 6.** PTx sensitivity of SDF-1$\alpha$-induced chemotactic responses. N9 microglial cells were pretreated with the indicated PTx dose for 60 min at 37°C before addition to the chemotaxis chamber. The control migration index with 100 ng/ml SDF-1$\alpha$ was 8.1. The percent inhibition of migration ± SD is indicated. Compare results with those obtained with PTx-treated N9 microglial cells in calcium flux assays (Fig. 4).

(Fig. 4). Since carbachol receptors are also seven-transmembrane-spanning G protein-coupled receptors (23), these data are consistent with tissue-specific utilization of G proteins. Alternatively, cell type-specific accessory proteins can regulate the specificity of signal transduction by G protein-coupled receptors (31).

Although both glial populations mobilize calcium following SDF-1$\alpha$ stimulation, SDF-1$\alpha$ only triggers migration of microglia. SDF-1$\alpha$ induces migration of both primary and transformed microglial cells at nanomolar concentrations. The chemotactic responses display a typical bell-shaped dose-response curve, with peak migration at 100 ng/ml (Fig. 5). The weaker chemotactic responses at high SDF-1 concentrations may be due to receptor desensitization.

In contrast, astrocytes fail to migrate toward synthetic SDF-1$\alpha$, although this cell population migrates in response to other chemokines, including MIP-1$\alpha$ (Fig. 5), KC, and monocyte chemoattractant protein-1 (19). The possibility that distinct CXCR-4 isoforms are selectively expressed by these murine glial populations cannot be excluded, but microglia and astrocytes express both the CXCR-4A and CXCR-4B transcripts (14). The combined migration data and PTx results imply that distinct biochemical pathways operate for the same chemokine in different cell types. Other examples of distinct sorting of chemokine signals include the ability of monocyte chemoattractant protein-1 to preferentially stimulate basophil degranulation while stimulating only low levels of chemotaxis. In contrast, RANTES (regulated upon activation, normal T cell expressed and secreted) is a potent basophil chemoattractant, although it stimulates little degranulation (32, 33).

Fusin/CXCR-4 is a coreceptor required for the entry and fusion of T lymphocytotropic HIV-1 viruses (1, 2, 8) and can serve as a CD4-independent receptor for HIV-2 isolates (9). CD4-negative cells, including astrocytes, can become infected with HIV-1 (26, 34–36). Furthermore, gp120 binding can induce calcium mobilization and protein kinase C activity, increase glial fibrillary acidic protein expression, and up-regulate intracellular adhesion molecule-1 gene expression in astrocytes (37–41). Thus, the expression of CXCR-4, a receptor for HIV-1 and HIV-2 gp120, in glial cells has implications for HIV infection of the central nervous system and development of the neurologic complications of AIDS (26, 42, 43).

## Acknowledgments

We thank Dr. P. Ricciardi-Castagnoli for providing the microglial cell line N9, and Dr. J. C. Gutierrez-Ramos for helpful discussions and advice. We are very grateful to Mrs. N. Axelrod for her secretarial assistance.

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