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A Functional IFN-γ-Inducible Protein-10/CXCL10-Specific Receptor Expressed by Epithelial and Endothelial Cells That Is Neither CXCR3 Nor Glycosaminoglycan[1]

Kenzo Soejima and Barrett J. Rollins[2]

Interferon-γ-inducible protein-10 (IP-10)/CXCL10 is a CXC chemokine that attracts T lymphocytes and NK cells through activation of CXCR3, the only chemokine receptor identified to date that binds IP-10/CXCL10. We have found that several nonhemopoietic cell types, including epithelial and endothelial cells, have abundant levels of a receptor that binds IP-10/CXCL10 with a K_d of 1–6 nM. Surprisingly, these cells expressed no detectable CXCR3 mRNA. Furthermore, no cell surface expression of CXCR3 was detectable by flow cytometry, and the binding of ^{125}I-labeled IP-10/CXCL10 to these cells was not competed by the other high affinity ligands for CXCR3, monokine induced by IFN-γ/CXCL9, and I-TAC/CXCL11. Although IP-10/CXCL10 binds to cell surface heparan sulfate glycosaminoglycan (GAG), the receptor expressed by these cells is not GAG, since the affinity of IP-10/CXCL10 for this receptor is much higher than it is for GAG, its binding is not competed by platelet factor 4/CXCL4, and it is present on cells that are genetically incapable of synthesizing GAG. Furthermore, in contrast to IP-10/CXCL10 binding to GAG, IP-10/CXCL10 binding to these cells induces new gene expression and chemotaxis, indicating the ability of this receptor to transduce a signal. These high affinity IP-10/CXCL10-specific receptors on epithelial cells may be involved in cell migration and, perhaps, in the spread of metastatic cells as they exit from the vasculature. (All of the lung cancer cells we examined also expressed CXCR4, which has been shown to play a role in breast cancer metastasis.) CXCR3-negative endothelial cells may also use this receptor to mediate the angiostatic activity of IP-10/CXCL10, which is also expressed by these cells in an autocrine manner. *The Journal of Immunology,* **2001, 167: 6576–6582.**

Chemokines are low m.w. proteins that were first identified by their ability to act as chemoattractants for specific leukocyte subsets (1). Examples include IL-8/CXCL8, which attracts neutrophils, and monocyte chemoattractant protein-1/CCL2, which attracts monocytes. Other chemokines have higher degrees of specificity, such as the ability to attract functional subsets of T lymphocytes (2, 3). Target cell specificity is primarily determined by restricted expression of chemokine receptors. To date, all functional chemokine receptors are members of the seven-transmembrane-spanning, G protein-coupled receptor family (4). The physiology of this system is complicated by the fact that in vitro analyses show that most chemokines bind to several receptors with equivalently high affinities, and most receptors bind several chemokines. Nonetheless, receptor promiscuity appears not to translate into functional redundancy in vivo since knockout mice deficient for a single chemokine or a single chemokine receptor have uniquely abnormal phenotypes (5–14).

Leukocytes are not the only cell types that respond to chemokines. For example, although the CXC chemokine IFN-γ-inducible protein-10 (IP-10)[3]/CXCL10 was initially characterized as a chemoattractant for T lymphocytes (15, 16), it also has antiangiogenic activities that appear to be mediated by its direct effects on endothelial cells (17–19). The only known functional receptor of IP-10/CXCL10 is CXCR3 (20), which also binds the CXC chemokines Mig (monokine induced by IFN-γ)/CXCL9 (20) and I-TAC/CXCL11 (21) and the murine CC chemokine SLC/CCL21 (22, 23) with equal affinities. But, even though IP-10/CXCL10 has direct effects on endothelial cell preparations such as HUVECs, some endothelial cells express no detectable CXCR3 (24–26), suggesting that there may be other functional IP-10/CXCL10 receptors. IP-10/CXCL10 can also bind heparan sulfate glycosaminoglycan (GAG), but this interaction does not result in signal transduction (18).

In addition to endothelial cells, chemokine receptors have also been found on epithelial cells, although their functional significance is not clear (27–30). Recently, it has been suggested that these receptors may be involved in patterns of metastatic spread (31, 32). In a search for chemokine receptor expression by normal lung epithelial and lung cancer cells, we tested several normal and malignant cells for their ability to bind chemokines and to express chemokine receptors. To our surprise, we found that all of these cells, as well as a wide variety of other cell types including endothelial cells, display high affinity binding sites for IP-10/CXCL10. Furthermore, binding is due neither to CXCR3 nor to GAG. Engagement of this apparently novel receptor results in signal transduction, which suggests that it may be involved in IP-10/CXCL10-mediated effects that could range from tumor cell migration or metastasis to regulation of angiogenesis.

Materials and Methods

Cells and reagents

PBMC were isolated from heparinized human blood using Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Normal human bronchial epithelial (NHBE) cells and HUVECs were obtained from Clonetics (Walkersville, MD).

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[3] Abbreviations used in this paper: IP-10, IFN-γ-inducible protein-10; BEGM, bronchial epithelial cell growth medium; CHO, Chinese hamster ovary; GAG, glycosaminoglycan; IP-10, IFN-γ-inducible protein-10; Mig, monokine induced by IFN-γ; NHBE, normal human bronchial epithelial; NSCLC, nonsmall cell lung cancer; PF4, platelet factor 4; RPA, RNase protection assay; SDF-1α, stromal cell-derived factor-1α.

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Nonsmall cell lung cancer (NSCLC) cell lines, COS cells, and pgsA-745 Chinese hamster ovary (CHO) cells (33) were purchased from American Type Culture Collection (ATCC, Manassas, VA). NHBE cells were cultured in bronchial epithelial cell growth medium (BEGM; Clonetics, San Diego, CA) supplemented with 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 ng/ml human recombinant epidermal growth factor, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, and 6.5 ng/ml triiodothyronine. HUVECs were cultured in microvascular endothelial cell growth medium (Clonetics) supplemented with 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 12 μg/ml bovine brain extract, and 2% FBS. All NSCLC cells and COS cells were maintained in DMEM with 10% FCS (Sigma, St. Louis, MO). PgsA-745 cells were grown in Ham's F12K medium with 10% FCS. Recombinant human stromal cell-derived factor-1α (SDF-1α)/CXCL12, IP-10/CXCL10, and Mig/CXCL9 were obtained from R&D Systems (Minneapolis, MN). Plasmids containing cDNA clones of CXCR3 and orphan receptors Apj, DEZ, GPR1, and GPR15 were kindly provided by C. Gerard, Children's Hospital (Boston, MA). The plasmid, pBR322-pc-*fos*-[human]-1, was purchased from ATCC, and a *Xho*I/*Nco*I fragment was used as a probe for Northern blot analysis of c-*fos* expression.

RNase protection assay (RPA)

Total cellular RNA was isolated using RNeasy kits (Qiagen, Hilden, Germany). Fifty micrograms of total RNA were treated with 5 U RNase-free DNase I (Life Technologies, Gaithersburg, MD) in the presence of RNasin (Promega, Madison, WI) for 15 min at 37°C. Multiprobe template sets hCR5 and hCR6 were purchased from BD PharMingen (San Jose, CA). DNA templates were used to synthesize probes incorporating [α-^{32}P]UTP (3000 Ci/mmol; Life Science Products, Boston, MA) using T7 RNA polymerase (Promega). Hybridization with 15 μg of each target RNA was performed overnight, followed by digestion with RNase A and T1 (Boehringer Mannheim, Mannheim, Germany), according to the BD PharMingen protocol. The samples were treated with a proteinase K-SDS mixture, extracted with phenol:chloroform:isoamyl alcohol (50:49:1), and then precipitated with ethanol in the presence of ammonium acetate. Protected RNA was loaded on a 5% acrylamide-urea sequencing gel next to the labeled probes, and electrophoresed at 50 W in 0.5× Tris-borate/EDTA electrophoresis buffer.

Reverse-transcription PCR

Total RNA was isolated and treated with DNase I, as described above. Reverse transcription was performed by using a GeneAmp RNA PCR kit (Perkin-Elmer, Branchburg, NJ). PCR was performed on cDNA samples, using intron-straddling primers. Primer sequences for CXCR3 were 5′-AACCACAAGCACCAAAGCAG-3′ (forward) and 5′-TGATGTTGAAGAGGGCACCT-3′ (reverse); for CXCR4, 5′-ATCTGGAGAACCAGCGGTTA-3′ (forward) and 5′-GGAAGTTCCCAAAGTACCAG-3′ (reverse); and for CCR5, 5′-GAAGAGCTGAGACATCCGTT-3′ (forward) and 5′-CGATTGTCAGGAGGATGATG-3′ (reverse). PCR conditions were as follows: 95°C for 5 min; 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min (40 cycles); and 72°C for 7 min. Amplified products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Flow cytometry

Cell staining was performed using mouse anti-human chemokine receptor mAbs, followed by FITC-conjugated, affinity-purified, multiply adsorbed, polyclonal goat anti-mouse Abs (BD PharMingen). The mAbs used in this study were directed against CXCR2 (6C6, IgG1), CXCR3 (1C6, IgG1), CCR2 (5A11, IgG1), and CCR5 (2D7, IgG1), all generous gifts from Millennium Pharmaceuticals (Cambridge, MA), and CXCR4 (12G5, IgG2a; BD PharMingen). The isotype control Abs were purchased from BD PharMingen. Samples were analyzed by FACScan (BD Biosciences, Mountain View, CA).

Chemokine-binding assay

Binding assays were performed using 1–2 × 10^5 NSCLC cells, NHBE cells, or COS cells transfected with receptor expression plasmids using a standard calcium phosphate transfection procedure (34). Cells were plated in 24-well culture plates and incubated overnight in standard medium. For the binding assay, cells were washed twice with washing buffer (0.5 M NaCl, 50 mM HEPES, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% BSA, pH 7.2), once with binding buffer (washing buffer without NaCl), and then incubated in duplicate with a constant concentration (85 pM) of ^{125}I-labeled chemokines (Life Science Products) in the presence of increasing concentrations (up to 800-fold molar excess) of unlabeled chemokines. Incubations took place in 200 μl binding buffer. After incubation at room temperature for 90 min, binding buffer was aspirated, and cells were washed three times with the washing buffer and then were lysed in 0.5 ml 1 N NaOH. Radioactivity was determined using a gamma counter. Data were analyzed using MacLigand software (35). These salt conditions have been optimized for chemokine binding and minimize the potential for chemokine self-association (36). The low salt-binding conditions did not induce osmotic lysis.

Northern blot analysis

NSCLC cells were grown to subconfluence in DMEM with 10% FCS, and then medium was changed to DMEM without FCS for 24 h. Cells were treated with 100 ng/ml chemokine or 0.1% BSA-PBS for 30 min, followed by RNA extraction, as described above. Since BEGM does not contain serum, NHBE cells were grown to subconfluence in BEGM and directly treated with chemokines. Fifteen micrograms of total RNA were electrophoresed through a 1.2% agarose/2.2 M formaldehyde gel with 3-[*N*-morpholino]propane sulfonic acid/EDTA buffer and transferred to nylon filters (Nytran; Schleicher & Schuell, Dassel, Germany), in 10× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7), using a TurboBlotter (Schleicher & Schuell). RNA was covalently fixed to the membrane by UV cross-linking using a Stratalinker (Stratagene, La Jolla, CA). A c-*fos* cDNA probe was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol; Life Science Products) by random primer labeling (High Prime; Boehringer Mannheim). Hybridization was conducted in ExpressHyb hybridization solution (Clontech Laboratories, Palo Alto, CA) under stringent conditions.

F-actin staining

Cells (1 × 10^6) were incubated in a Chamber Slide (Nunc, Naperville, IL) at 37°C in FCS-free DMEM with 100 ng/ml SDF-1α/CXCL12 or IP-10/CXCL10 for 2, 4, 8, 15, 30, or 60 min. A negative control with 0.1% BSA in DMEM was analyzed in the same manner. After incubation and washing with PBS, cells were fixed, permeabilized, and stained in a single step by addition of 0.5 ml 40% paraformaldehyde-PBS containing 0.2 μM Oregon Green 488 phalloidin (Molecular Probes, Eugene, OR) and 100 μg/ml lysophosphatidylcholine (Sigma), and the mixture was incubated for 20 min at 4°C. Cells were photographed using an Eclipse E800 camera (Nikon, Tokyo, Japan) with a ×40 water immersion lens.

Chemotaxis

Cells were washed with PBS, then washed with chemotaxis buffer (DMEM containing 12 mM HEPES (pH 7.4) and 0.1% BSA) and resuspended in the same buffer. A total of 3 × 10^4 cells was placed in the upper wells of a 48-well chemotaxis chamber (Neuroprobe, Cabin John, MD). Lower wells contained varying amounts of IP-10/CXCL10 in the same buffer. Lower and upper wells were separated by polycarbonate filter with 8-μm pores that had been precoated with fibronectin. After incubation at 37°C for 6 h, the filter was removed, cells were scraped from the upper surface, and cells that remained adherent to the lower surface were fixed in methanol and stained with Diff Quik (Baxter, McGaw Park, IL). Cells were counted in four high power fields in each of two duplicate wells.

Results

Chemokine receptor mRNA expression in NHBE and NSCLC cells

In an attempt to identify chemokine receptors on epithelial cells, we screened 11 NSCLC cell lines and NHBE cells for their ability to bind radiolabeled chemokines. While several chemokines showed no binding activity, IP-10/CXCL10 bound with high affinity to all examined cells. Fig. 1 shows a typical example of a displacement curve generated by this analysis, and the results from all of the binding experiments are collected in Table I. All 11 NSCLC cell lines as well as NHBE cells had a single class of high affinity binding sites for IP-10/CXCL10 (K_d ranging from 1.2 nM to 5.6 nM) present at 40,000–600,000 sites/cell.

Although in all cases the displacement data were clearly consistent with a single site binding model, it should be noted that four cell types, namely NHBE, Calu-3, SK-MES-1, and NCI-H520, generated IP-10/CXCL10 displacement curves that statistically fit a two-site binding model more closely than a one-site model ($p < 0.05$ using the F-test). The high affinity site K_d were 0.1–1.3 nM, and the low affinity site K_d were 30–80 nM. While the high affinity sites were present at reasonably abundant levels (≈20–100,000

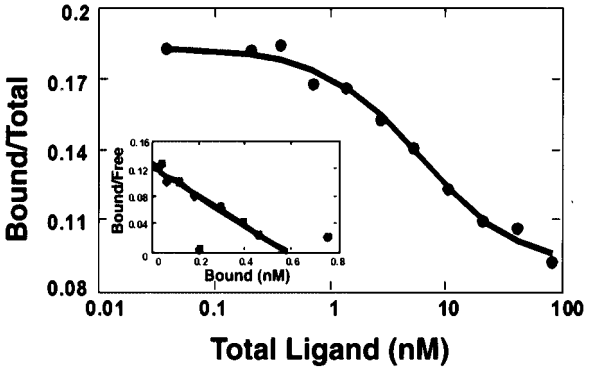


FIGURE 1. IP-10/CXCL10 binding to the A549 NSCLC cell line. ^{125}I-labeled IP-10 ([^{125}I]IP-10)/CXCL10 was bound to A549 cells in the presence of increasing amounts of nonradiolabeled IP-10/CXCL10, as described in *Materials and Methods*. Displacement curve and Scatchard transformation (*inset*) are shown for this representative experiment.

sites/cell), the two-site model implied the existence of more than 2,000,000 low affinity sites/cell, suggesting that this model was biologically inaccurate.

Molecular analysis of chemokine receptor expression in NSCLC and NHBE cells

Having documented high affinity binding sites for IP-10/CXCL10, we next sought evidence for expression of CXCR3, the only functional IP-10/CXCL10 receptor identified to date. A multiplex RPA was unable to detect CXCR3 mRNA expression by any of the NSCLC cell lines or NHBE cells (Fig. 2*A*). In contrast, other chemokine receptors were expressed, including CXCR4 by 8 of 10 NSCLC cell lines, CXCR2 by 2 cell lines, and both CCR1 and CCR2a by 1 line (Fig. 2, *A* and *B*).

To ensure that the apparent absence of CXCR3 mRNA expression was not due to the insensitivity of RPA, cellular mRNA was also analyzed by RT-PCR. Primers were chosen to straddle CXCR3's intron to distinguish between products arising from authentic CXCR3 mRNA and those arising from contaminating genomic DNA. Fig. 3 shows that only NCI-H661 cells expressed detectable CXCR3 mRNA. Thus, RT-PCR generally confirmed the RPA results, indicating that despite their ability to bind IP-10/CXCL10, all cell lines but one expressed no detectable CXCR3. Surface expression of CXCR3 was also not detected by fluorescence-activated cell sorting using anti-CXCR3 (data not shown).

Table I. *Biochemical characterization of IP-10/CXCL10 receptors on NSCLC cell lines and NHBE cells*[a]

Cell Line	K_d (nM)[b]	Binding Sites/Cell[b]
A427	2.8 ± 0.1	167,545 ± 28,245
Calu-1	3.3 ± 1.8	369,863 ± 203,197
Calu-3	5.6 ± 2.8	626,429 ± 208,429
Calu-6	3.0 ± 1.2	240,150 ± 102,500
SK-LU-1	2.9 ± 1.2	438,650 ± 41,350
SK-MES-1	4.2 ± 1.8	212,826 ± 77,174
SW900	1.9 ± 0.1	118,674 ± 31,327
NCI-H520	2.3 ± 0.5	43,659 ± 10,171
A549	4.4 ± 1.7	340,072 ± 143,219
NCI-H596	1.2 ± 0.3	357,273 ± 62,728
NCI-H661	4.7 ± 3.3	187,194 ± 54,562
NHBE	1.5 ± 0.6	97,800 ± 34,200

[a] IP-10/CXCL10 self-displacement experiments were performed on each of the indicated cell lines as described in *Materials and Methods*. Scatchard transformations were carried out to infer K_d and number of binding sites per cell (35).

[b] Mean ± SD for two to three independent analyses.

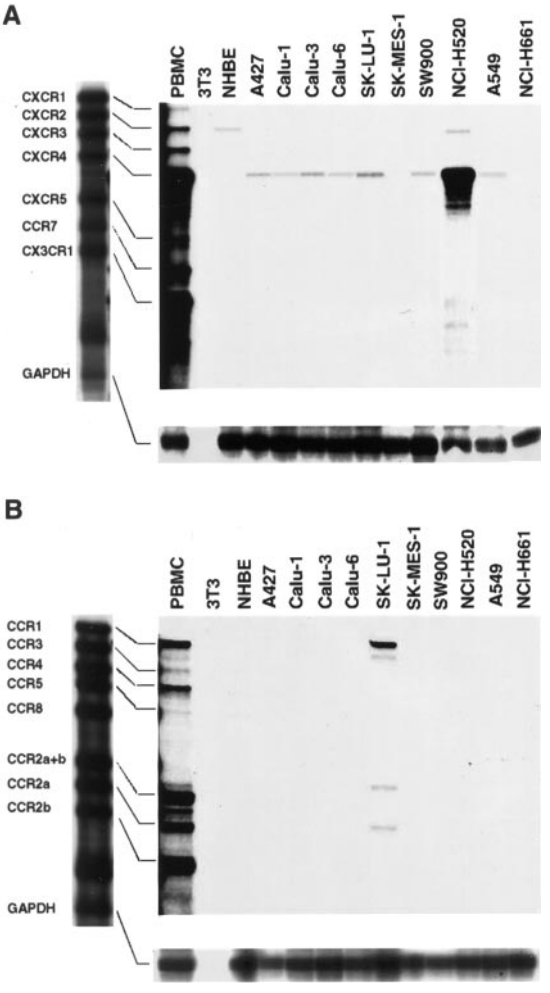


FIGURE 2. RPA using multiprobe template sets on RNA from NHBE cells and 10 NSCLC cell lines. *A*, Multiprobe template set for CXC receptors as well as CCR7 and CX_3CR1. CXCR4 is constitutively expressed by A-427, Calu-1, Calu-3, Calu-6, SK-LU-1, SW900, NCI-H520, and A549 cells. CXCR2 is expressed by NHBE and NCI-H520 cells. *B*, Multiprobe template set for CCR. CCR1 and CCR2a are expressed by SK-LU-1 cells. Human PBMCs were used as a positive control, murine 3T3 cells as a negative control. *Left lane*, Nondigested RNA probes representative of size markers. These results are representative of three experiments with similar outcomes.

The increased sensitivity of RT-PCR compared with RPA also revealed that all cell lines, rather than just 8 of 10, expressed CXCR4 mRNA (Fig. 3).

The ligand-binding profile of the IP-10/CXCL10 receptor on NSCLC and NHBE cells differs from that of CXCR3 and heparan sulfate proteoglycan

The absence of detectable CXCR3 mRNA expression by 10 cell lines suggested that the IP-10/CXCL10 receptor on these cells was not CXCR3. We tested this idea by attempting to compete radiolabeled IP-10/CXCL10 binding with unlabeled Mig/CXCL9, another non-ELR (glutamate-leucine-arginine)-containing CXC chemokine that binds to CXCR3 with the same affinity as IP-10/CXCL10 (20). Fig. 4*A* shows that Mig/CXCL9 was unable to displace radiolabeled IP-10/CXCL10 from A549 cells even when its concentration exceeded that of IP-10/CXCL10 by 800-fold. IP-10/CXCL10 efficiently displaced itself under the same conditions. As a control for Mig/CXCL9 activity, nonradiolabeled Mig/CXCL9 competed radiolabeled IP-10/CXCL10 from CXCR3

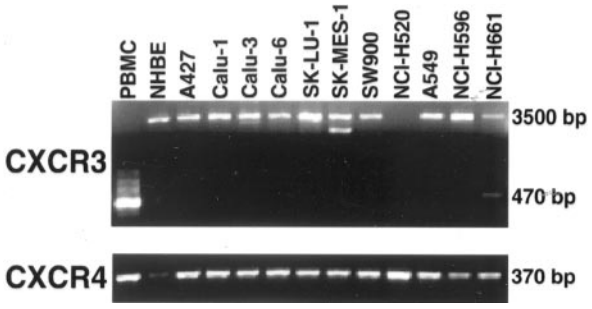


FIGURE 3. RT-PCR analysis of CXCR3 and CXCR4 RNA expression in NHBE cells and 11 NSCLC cell lines. RNA isolated from PBMCs was used as a positive control. The expected size of the mRNA-derived PCR product for CXCR3 is 470 bp, and that of the genomic DNA- or hnRNA-derived product is 3500 bp. Only PBMC and NCI-H661 cells have evidence for CXCR3 mRNA expression. All cell lines expressed CXCR4 mRNA.

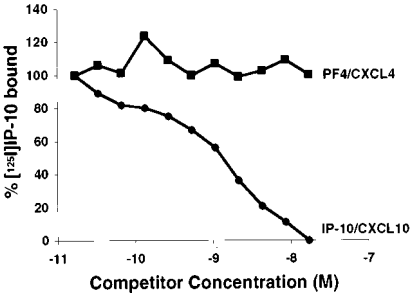


FIGURE 5. Inability to displace IP-10/CXCL10 from binding to A549 cells using PF4/CXCL4. [^{125}I]IP-10/CXCL10 was bound to A549 cells in the presence of increasing amounts of nonradiolabeled IP-10/CXCL10 (●) or PF4 (■), as described in *Materials and Methods*. At each concentration of nonradiolabeled ligand, the amount of [^{125}I]IP-10/CXCL10 still bound to cells is expressed as a percentage of the amount bound in the absence of competing ligand. Each point is the mean of duplicate measurements.

binding sites, but not non-CXCR3 sites, in transfected COS cells, as expected (data not shown). In addition, Mig/CXCL9 was able to displace only 20% of IP-10/CXCL10 from NCI-H661 cells (Fig. 4*B*), suggesting that CXCR3 comprised a minority of IP-10/CXCL10 binding sites on these cells. This low level of CXCR3 expression is consistent with our ability to detect it in NCI-H661 cells only by RT-PCR, and not by RPA or flow cytometry. Similar results were obtained using I-TAC/CXCL11, another ligand for CXCR3 (data not shown).

IP-10/CXCL10 also binds to cell surface heparan sulfate proteoglycan (18). However, the IP-10/CXCL10 receptor on NSCLC cells is unlikely to be heparan sulfate because platelet factor 4 (PF4)/CXCL4 did not compete for radiolabeled IP-10/CXCL10 binding (Fig. 5), as it does when heparan sulfate is the receptor. In addition, radiolabeled IP-10/CXCL10 bound to pgsA-745 cells, a CHO cell mutant deficient for a glycosyltransferase required for the production of GAG (33). In the absence of any cell surface GAG, these cells displayed 385,000 IP-10/CXCL10 receptors with K_d of 3.3 nM, similar to the receptor detected on NSCLC and NHBE cells. Since we saw comparable IP-10/CXCL10-binding characteristics using wild-type CHO cells, our binding conditions probably do not detect IP-10/CXCL10 binding to GAG.

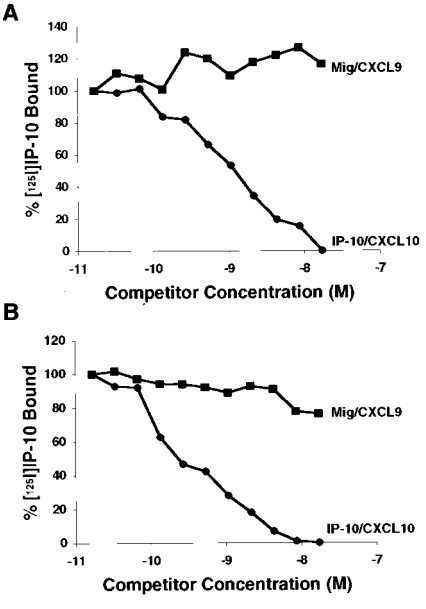


FIGURE 4. Inability to displace IP-10/CXCL10 from binding to NSCLC cell lines using Mig/CXCL9. [^{125}I]IP-10/CXCL10 was bound to A549 cells (*A*) or NCI-H661 cells (*B*) in the presence of increasing amounts of nonradiolabeled IP-10/CXCL10 (●) or Mig/CXCL9 (■), as described in *Materials and Methods*. At each concentration of nonradiolabeled ligand, the amount of [^{125}I]IP-10/CXCL10 still bound to cells is expressed as a percentage of the amount bound in the absence of competing ligand. Each point is the mean of duplicate measurements.

Absence of IP-10/CXCL10 binding to candidate chemokine receptors

A variety of seven-transmembrane-spanning G protein-coupled receptors has been described that have no identified ligands, but do have sequence motifs shared by chemokine receptors. Several were tested for their ability to bind IP-10/CXCL10. Four receptor cDNAs were transfected into COS cells, and transfectants were tested for the presence of increased numbers of high affinity IP-10/CXCL10 binding sites. (Increased numbers of sites were monitored because untransfected COS cells express 119,000 IP-10/CXCL10 binding sites with a K_d of 1.7 nM.) While transient expression of CXCR3 resulted in a 4-fold increase in IP-10/CXCL10 binding sites, expression of Apj (37), DEZ (38), GPR1 (39), and GPR15 (40) did not. This suggests that the IP-10/CXCL10 receptor expressed by NSCLC cells is unlikely to be encoded by these orphan receptor cDNAs.

Functional activation of the non-CXCR3 IP-10/CXCL10 receptor

To determine whether the IP-10/CXCL10 receptors identified on NSCLC cells are functional, we examined all NSCLC cell lines and NHBE cells for physiological responses to IP-10/CXCL10. Although IP-10/CXCL10 treatment did not increase intracellular calcium concentration, it induced chemotactic responses in some cells. For example, Fig. 6 shows that IP-10/CXCL10 induced migration of SK-LU-1 cells with an EC_{50} between 0.1 and 1 nM. Checkerboard analysis showed that this movement was chemotactic rather than chemokinetic. (Similar results were observed with A549 cells.) Chemotaxis to IP-10/CXCL10 was inhibited by pretreating cells with pertussis toxin, indicating that this response involves receptor coupling to $G_{\alpha i}$. Consistent with the chemotactic response, IP-10/CXCL10 also induced F-actin redistribution in A549 and NCI-H661 cells, although not in others, and was observed as early as 2 min after adding IP-10/CXCL10 and lasted for

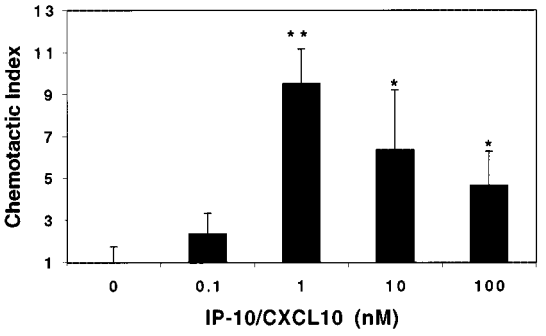


FIGURE 6. Chemotaxis of SK-LU-1 cells in response to IP-10/CXCL10. The migratory response of SK-LU-1 cells to various concentrations of IP-10/CXCL10 was tested using a modified Boyden chamber assay. The number of cells migrating in response to IP-10/CXCL10 was normalized to the number of cells migrating in the absence of IP-10/CXCL10 to derive the chemotactic index. **, $p < 0.001$; *, $p < 0.01$ compared with 0 nM IP-10/CXCL10.

30 min in A549 cells and 60 min in NCI-H661 cells (Fig. 7). Finally, since chemokines can modulate gene expression, IP-10/CXCL10 was tested for its ability to induce c-*fos* mRNA expression. IP-10/CXCL10 induced c-*fos* mRNA expression in SW900, A549, NCI-H520, and NCI-H661 cells (Fig. 8), and Calu-3 cells (not shown), but not in others. Protooncogene induction was not inhibited by pretreating cells with 500 nM pertussis toxin, suggesting that not all responses are coupled to $G_{\alpha i}$. Although all of the cells in this study expressed CXCR4 mRNA by RT-PCR, only two of them, NCI-H520 and A549, responded to SDF-1α/CXCL12 treatment with c-*fos* expression (Fig. 8).

IP-10/CXCL10 binding to endothelial and U937 cells in the absence of CXCR3

IP-10/CXCL10 is known to have antiangiogenic activity, and this may be effected through CXCR3 in endothelial cells that express this receptor (41–44). However, some endothelial cell preparations do not express CXCR3 (24–26). To determine whether these cells express another receptor through which IP-10/CXCL10 could exert direct effects, we performed a binding assay on HUVECs. Fig. 9*A* shows that HUVECs have a single class of high affinity binding sites for IP-10/CXCL10, characterized by a K_d of 3.1 nM and present at 166,000 sites/cell. In addition, like the majority of NSCLC cell lines examined above, HUVECs express no detectable CXCR3 mRNA (Fig. 9*B*). Notably, these cells express IP-10/CXCL10 constitutively, suggesting that the non-CXCR3 IP-10/CXCL10 receptor may be involved in an autocrine loop. However,

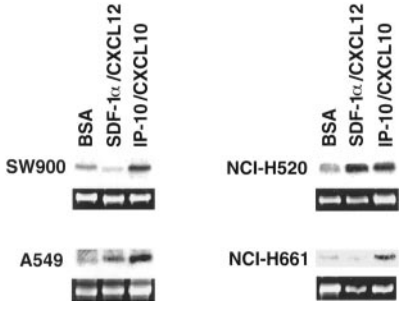


FIGURE 7. Induction of c-*fos* mRNA expression by SDF-1α/CXCL12 or IP-10/CXCL10. The indicated cell lines were cultured in the absence of serum for 24 h, then treated for 30 min with 100 ng/ml SDF-1α/CXCL12 or IP-10/CXCL10, or an equivalent volume of vehicle (0.1% BSA in PBS). RNA was extracted and analyzed for c-*fos* expression by Northern blotting, as described in *Materials and Methods*. The ethidium bromide-stained 18S rRNA band is shown to document the amount of RNA loaded per lane. The results are representative of two experiments with similar outcomes.

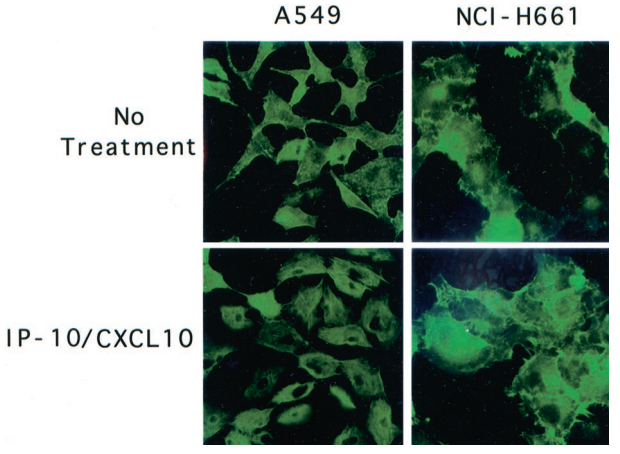
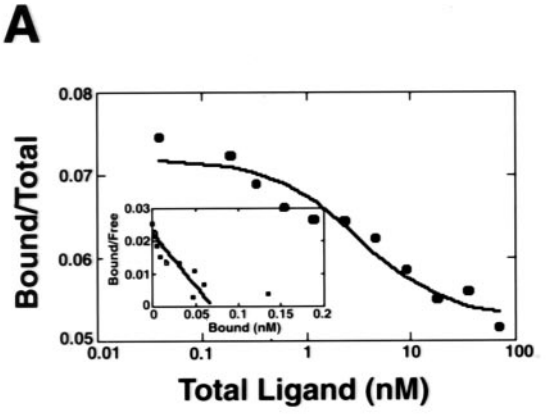


FIGURE 8. F-actin redistribution induced by IP-10/CXCL10 treatment. A549 and NCI-H661 cells were incubated with 100 ng/ml IP-10/CXCL10 for varying periods of time, then fixed and stained with Oregon Green 488 phalloidin, as described in *Materials and Methods*. Unstimulated cells demonstrated diffuse distribution of F-actin. A549 cells treated with IP-10/CXCL10 for 2 min and NCI-H661 cells treated with IP-10/CXCL10 for 30 min showed redistribution of F-actin to the cell periphery and polarization to the leading edge, resulting in the appearance of nuclear sparing.



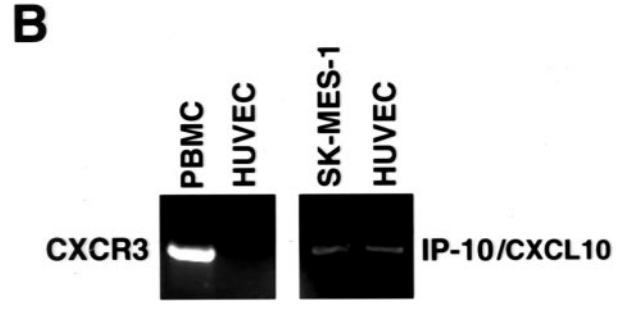


FIGURE 9. Presence of a non-CXCR3 IP-10/CXCL10 receptor on HUVECs. *A*, [^{125}I]IP-10/CXCL10 was bound to HUVECs in the presence of increasing concentrations of nonradiolabeled IP-10/CXCL10, as described in *Materials and Methods*. Displacement curve and Scatchard transformation (*inset*) are shown. *B*, RT-PCR analysis was performed on mRNA isolated from HUVECs. No CXCR3 mRNA-derived product was observed. SK-MES-1 NSCLC cells and HUVECs constitutively express IP-10/CXCL10 mRNA.

we have been unable to demonstrate IP-10/CXCL10-induced migration or c-*fos* expression in HUVECs, so the physiological consequences of receptor activation in these cells are unknown. Finally, we tested U937 myelomonocytic leukemia cells and found that they expressed 9000 receptors for IP-10/CXCL10 with a K_d of 2.2 nM. Like monocytes, U937 cells express no CXCR3 (20), and our findings may explain, in part, reports that monocytes respond to IP-10/CXCL10 (45).

Discussion

We have screened a variety of cell types for their ability to bind chemokines. Surprisingly, all cells examined in this survey expressed abundant high affinity IP-10/CXCL10 receptors having K_d of 1–6 nM. IP-10/CXCL10 treatment of several of these cells resulted in chemotaxis, cytoskeletal rearrangement, and new gene expression, indicating that the activated IP-10/CXCL10 receptor was able to transduce signals. Notably, however, in all cases but one, RT-PCR analysis detected no expression of mRNA encoding CXCR3, the only IP-10/CXCL10 receptor identified to date. Even in the exceptional cell line, NCI-H661, CXCR3 accounted for only 20% of the total IP-10/CXCL10 binding.

There are several potential explanations for the presence of IP-10/CXCL10 receptors without detectable CXCR3 mRNA expression. First, IP-10/CXCL10 binding might be due to a functionally wild-type CXCR3 encoded by an alternatively spliced mRNA not detected by our RT-PCR primers. However, this is unlikely because we were unable to detect any portion of an authentic CXCR3 mRNA by RPA (Fig. 2*A*) or by Northern blotting using a full-length CXCR3 cDNA probe (data not shown). Furthermore, the *CXCR3* locus appears to be deleted in NCI-H520 cells (Fig. 3), which, nonetheless, display IP-10/CXCL10 binding sites. Second, our cell lines may have been infected with human herpesvirus 8, which encodes a G protein-coupled receptor that binds multiple chemokines, including IP-10/CXCL10 (46). We tested this possibility by performing an RT-PCR analysis to search for expression of this receptor's mRNA, but none was detected (data not shown). Third, IP-10/CXCL10 could be binding to cell surface heparan sulfate (18). However, this is unlikely to be the receptor we have identified, since the K_d for that interaction is 25 nM (5–20 times weaker than the interactions we detected), IP-10/CXCL10 binding to NSCLC cells results in signal transduction (which does not occur when IP-10/CXCL10 binds GAG), PF4/CXCL4 did not compete for IP-10/CXCL10 binding to NSCLC cells (Fig. 5), and IP-10/CXCL10 bound to mutant CHO cells that do not synthesize GAG. Fourth, the universal expression of CXCR4, at least at the mRNA level, parallels that of the IP-10/CXCL10 receptor (Figs. 2*A* and 3) and could theoretically be responsible for binding IP-10/CXCL10. However, CXCR4 has not been reported to bind IP-10/CXCL10, and only one cell line, NCI-H520, had sufficient surface expression of CXCR4 to be detected by SDF-1α/CXCL12 binding (data not shown), while all of the cells showed significant IP-10/CXCL10 binding.

The last possibility is that the IP-10/CXCL10 receptor identified in this study may be a novel chemokine receptor. In support of this notion is the observation that, unlike CXCR3 (21, 47), this receptor does not bind Mig/CXCL9 or I-TAC/CXCL11, and a receptor with this binding profile has not yet been described. Several G protein-coupled receptors without ligand assignments, so-called orphan receptors, have been described that have signature amino acid sequences found in chemokine receptors. None of the receptors we tested bound IP-10/CXCL10 with high affinity, suggesting that the potentially novel receptor we describe in this work is not one of these.

Regardless of the identity of this receptor, it appears to be functional; but what might its function be in epithelial cells? By analogy to chemokine receptors on leukocytes, activation of this receptor on NSCLC cells might control their trafficking during metastasis. Once a malignant cell has entered the vasculature, its subsequent metastatic invasion recapitulates the paradigm of the multistep model of leukocyte emigration. The circulating tumor cell must be tethered to the luminal surface of endothelial cells in the target organ, this interaction must be transformed into firm adhesion, and then the tumor cell must accomplish diapedesis into the subendothelium. If a tumor cell bearing IP-10/CXCL10 receptors comes into contact with IP-10/CXCL10 (or an as yet unidentified ligand for this receptor) displayed by endothelial cells, its activation may induce the tumor cell to stop and invade the tissue. Viewed in this light, metastases to lymph nodes, for example, might not reflect a passive filtration function of the node, but would rather be the result of an active cell migratory process by a cancer cell that has co-opted a system already in place for leukocyte trafficking. This function has recently been ascribed to CXCR4, which is expressed nearly universally by breast (31) and ovarian (32) carcinoma cells. It is worth noting that our data extend the ubiquity of CXCR4 expression to lung carcinomas, and that this does not exclude a similar role for an IP-10/CXCL10 receptor. It should also be noted that IP-10/CXCL10 did not elicit the same biological responses from all cells tested (nor did SDF-1α/CXCL12), indicating that the effects of IP-10/CXCL10 may not universally occur in all cells bearing this receptor.

Another cancer-related function for this receptor is suggested by its presence on endothelial cells. Considerable evidence indicates that IP-10/CXCL10 exerts its angiostatic effects directly on endothelial cells, including HUVECs (17–19). Although some endothelial cells express CXCR3 (41–44), we and others were unable to document the expression of CXCR3 by HUVECs, suggesting that the receptor we have identified biochemically might be the means by which IP-10/CXCL10 accomplishes this activity. Furthermore, the constitutive expression of IP-10/CXCL10 by these cells may indicate the presence of an angiostatic autocrine loop that would have to be interrupted in order for angiogenesis to occur. A variety of animal models can be used to test this hypothesis, but in the absence of reproducible in vitro correlates of antiangiogenic responses to IP-10/CXCL10, the role that this receptor might play in angiogenesis remains conjectural.

Regardless of its true function in normal physiology or disease, we have identified a widely expressed and functional receptor for IP-10/CXCL10. Although we have found extensive biochemical evidence that this is a novel, non-CXCR3 receptor, definitive characterization will require its cloning.

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