cCXCR1 is a receptor for cIL8 (9E3/cCAF) and its N- and C-terminal peptides and is also activated by hIL8 (CXCL8)

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Abstract: Chemokines are chemotactic cytokines that play important roles in immune responses and wound healing, as well as in pathological conditions such as chronic inflammation and tumorigenesis. The chemokines and their receptors are highly conserved and maintain similar functions in different species. One noteworthy exception is the chemokine interleukin (IL)8/CXC ligand 8 and its specific receptor CXCR1, which are found in humans but are not found in the traditional model organisms, mice and rats. As a consequence, we are using model organisms other than mice to study the functions of IL-8 and CXCR1, as well as the mechanisms involved in receptor activation by IL-8. Toward this goal, we have isolated and characterized a new receptor that is highly homologous to human (h)CXCR1, which we named chicken (c)CXCR1. To determine whether this receptor is activated by cIL-8 and its N- and C-terminal peptides and whether it responds to hIL-8, we expressed cCXCR1 in NIH3T3 cells, which naturally lack this receptor, and used single-cell Ca^{2+} imaging to detect increases in intracellular Ca^{2+} and immunoblot analysis to detect extracellular signal-regulated kinase 1/2 phosphorylation. We show that cIL-8, its N and C peptides, and hIL-8 activate cCXCR1. We further show that cIL-8 and hIL-8 stimulate chemotaxis of chicken embryonic fibroblasts, cells that express cCXCR1, and that this effect is specific for each chemokine and this receptor. These results strongly suggest that cCXCR1 is the ortholog for hCXCR1 and that chickens can be used as an effective model system to study the functions of IL-8, its terminal peptides, and its specific receptor CXCR1. J. Leukoc. Biol. 77: 000–000; 2005.

Key Words: chemokine signaling · signal transduction · Ca^{2+} release · MAPK · stress response

INTRODUCTION

The chemokine family is a group of low molecular weight, multifunctional cytokines, which are primarily known for their roles in inflammatory responses, particularly in the recruitment of leukocytes to the area of interest [1–5]. These molecules have additional roles in wound healing and tumorigenesis [6–11]. Chemokines are grouped into classes based on the position of the first two cysteine residues, thus forming the CC, CXC, CX3C, and XC classes; these chemokines bind to the CC chemokine receptor, CXC chemokine receptor (CXCR), CX3C chemokine receptor, and XC chemokine receptor seven-transmembrane G protein-coupled receptors, respectively.

In the last decade, many chemokines and chemokine receptors, with diverse functions, have been discovered and characterized. This has led to a greater understanding of chemokine activities, including leukocyte chemotaxis and activation, angiogenesis, wound healing, lymphocyte development, various pathological, inflammatory conditions, and tumor angiogenesis and metastasis [12–17]. As chemokines play important roles in pathological inflammation and tumorigenesis and as certain chemokine receptors can facilitate the entry of the human immunodeficiency virus into T cells and macrophages, chemokine and chemokine receptor antagonists are gaining popularity as a potential therapeutic agent [12, 18]. A greater understanding of the multiple functions of various chemokines, whether previously or newly discovered, may provide insight into the mechanisms used by these molecules to promote disease pathogenesis, which will aid in the development of such therapeutic agents. In addition, increased knowledge of the mechanisms, whereby the chemokines activate their receptors, may be useful in designing receptor antagonists. Such increased knowledge of the chemokines and their receptors will likely arise from the study of their activities in vitro and more importantly, in model organisms. However, more study is needed to identify and characterize chemokines found in model organisms, which are homologous to those found in humans, to more fully understand their physiological and pathological functions. Fortunately, the chemokines and their receptors are highly conserved and maintain similar functions in different species. One noteworthy exception is the chemokine interleukin (IL)8/CXC chemokine ligand 8 and its more specific receptor CXCR1, which are found in humans, rabbits, and chickens, among others, but are not found in the more traditional model organisms, mice or rats. Although IL8 and CXCR1 are not present in all organisms, IL8 is an important chemokine in many normal and pathological processes in humans. Thus, our laboratory is using model organisms other than mice to study...
the functions of IL-8 and CXCR1, as well as the mechanisms involved in receptor activation by IL-8. One of the model systems we use involves the chicken homologs of human (h)IL-8 and hCXCR1, chicken (c)IL-8, and cCXCR1, respectively.

We have characterized many of the functions of cIL-8 in vivo and have isolated and characterized a receptor in chickens that is highly homologous to hCXCR1 [19]. We have called this receptor cCXCR1. In addition, we have shown that the N- and C-terminal peptides of cIL-8 function in vivo [11, 20]. However, it is not yet known whether cIL-8 and its peptides are ligands for cCXCR1 and whether this receptor responds to cIL-8.

To investigate this possibility, we used a tetracycline-regulated retroviral system to express cCXCR1 in NIH3T3 cells, which being murine cells, lack endogenous CXCR1. In these cells, the activation of cCXCR1 by hIL-8, cIL-8, or the cIL-8 peptides was determined by two assays. In the first assay, we used single-cell calcium imaging to detect increases in intracellular Ca\(^{2+}\) after application of the chemokines or peptides to the CXCR1-expressing cells; in the second assay, we used immunoblot analysis to detect extra- cellular signal-regulated kinase (ERK)1/2 phosphorylation and thus, their activation. In addition, we used inhibitors specific for certain signal transduction molecules to partially elucidate the signal transduction pathway upstream of ERK1/2. We also examined the activity of the N- and C-terminal peptides of cIL-8 in chemotaxis assays using chicken embryonic fibroblasts (CEFIs), which normally express cCXCR1 and respond to cIL-8. We find that cIL-8, hIL-8, and the cIL-8 terminal peptides can activate cCXCR1, albeit at different concentrations of the respective ligands. The findings reported here contribute to furthering our knowledge of chemokines and their receptors, have the potential to advance understanding of the multifunctionality exhibited by chemokines under various physiological and pathological conditions, reveal a new mode of functional regulation, and may serve as the basis for therapeutic tar-

**MATERIALS AND METHODS**

**Material**

We generated a polyclonal antibody against cCXCR1 by immunizing rabbits with its N-terminal peptide conjugated to keyhole limpet hemocyanin. Anti-hCXCR1 was a gift from Genentech (San Francisco, CA). We used the Tet ON RetroTet-ART vector system [21] to express cCXCR1 in NIH3T3 cells. The cell line GP2-293, a pantropic retroviral expression system (Clontech, Palo Alto, CA), was used to package and produce the virus containing the cCXCR1 gene. Doxycycline (Dox), a tetracycline-derived antibiotic, and polybrene were purchased from Sigma Chemical Co. (St. Louis, MO). cIL-8 was synthesized by Gryphon Inc. (South San Francisco, CA). cIL-8 N peptide [1–15] amino acids (aa); LGQGRTLVKMGNELR] was synthesized by SynPep and C peptide (56–83 aa: CLLDPTAPWQLVKALMAKAQLNSDAPL), by Gryphon Sciences. The inhibitors, pertussis toxin (PTX), PI908959, calphostin C, genestein, AG1517, and herbimycin were purchased from Calbiochem (San Diego, CA).

**Vector construction, retrovirus production, and NIH3T3 cell infection**

The HRSpuro-GUS vector in the RetroTet-ART system served as a parental vector for cloning. The full-length gene for cCXCR1 (1478–2971 bp genomic sequence in GenBank, Access #AF227061) was excised from pBlueScript-cCXCR1 and cloned into SaI and NotI sites to replace the GUS cDNA in HRSpuro-GUS to generate HRSpuro-cCXCR1. The Clontech pantropic retroviral expression system was used to package all three vectors: HRSpuro-cCXCR1; RetroTet RTA(+) (58), the transcription activator for the tetracycline response element; and RetroTet RTG(–), the transcription repressor for the same elements to quench background expression. To produce the retroviruses in GP2-293 cells, we performed transient transfection as described previously [22]. The cells were incubated at 37°C and 5% CO\(_2\) for 24 h. The medium was filtered, and the virus was collected to infect NIH3T3 cells. One day prior to being infected and just before infection, the medium was removed and replaced with concentrated viral supernatant. The cells were then incubated for 4 h at 37°C and 5% CO\(_2\) before adding fresh media. After 2 days, Dox was added to stimulate receptor expression. The protocol of infection was done in the following sequence: The cells were first infected with virus containing HRSpuro-cCXCR1. When they reached confluence, they were passaged and infected with virus containing RTA(+) (58). When they reached confluence, they were passaged and infected with virus containing RTG(–).

**Calcium imaging**

Activation of cCXCR1 by cIL-8 was measured by changes in intracellular Ca\(^{2+}\) concentration as described previously [23]. Briefly, NIH3T3 cells expressing the cCXCR1 were cultured on glass coverslips and monitored in the fluorescence microscope using the Ca\(^{2+}\) indicator fluo-3 ( Molecular Probes, Eugene, OR). Cells were loaded with the indicator by incubation at 37°C for 30 min with the acetoxymethyl (AM) ester of fluo-3 (flu-3 AM; 10 µg/ml; Molecular Probes). Solubilization of fluo-3 AM in aqueous medium was accomplished by adding the Ca\(^{2+}\) indicator pluronics F-127 (0.025%; Molecular Probes). The dye was allowed to de-esterify for 30 min at room temperature (RT) or 37°C. Fluor-3 emission was visualized with a 40×/1.3 oil immersion S Fluor objective and wide-field epifluorescence illumination and was detected with an intensified charge-coupled device camera (IC-500, Photon Technology International, Lawrenceville, NJ), which was attached to a Nikon TE300 inverted microscope. All experiments were performed at RT (20–23°C) with the cells placed in a solution containing 10 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), and 5–20 mM glucose at pH 7.4. Images were captured every 5 s, five times before the ligand to be tested was added by pressure ejection with a glass micropipette (Picospritzer II; Parker Instrumentation, Cleveland, OH) throughout the experiment (275 s, if otherwise not specified). To ensure the efficiency of fluo-3 de-esterification, we stimulated the cells with the Ca\(^{2+}\) ionophore, 4BraA23187 (10 µM), at the end of the experiment. Only the cells with dF/Fo greater than 100% after 4BraA23187 treatment were counted for data analysis (dF=F−Fo, where Fo is the average fluorescence intensity for each cell at rest, and dF is the change of fluorescence intensity for each cell upon ligand addition). Data are expressed as means ± SEM. Only when dF/Fo was greater than average ± 3 × SD in two consecutive images were the cells counted as able to respond.

**Mitogen-activated protein kinase (MAPK) activation assay**

cCXCR1-expressing NIH3T3 cells cultured in the presence or absence of Dox were incubated for 18 h in conditioned medium before chemokine treatment. After treatment with various inhibitors of signal transduction pathways, the cells were lysed with I× radioimmunoprecipitation assay (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% Nonidet P-40, 1% NaDOC, 0.1% sodium dodecyl sulfate, pH 7.5), containing protease and phosphatase inhibitors (cocktails I and II, Sigma Chemical Co.). Immunoblot analysis was performed using a monoclonal antibody to phosphorylated p42 and p44 MAPK (Thr202 and Tyr204; E10, Cell Signaling Technology, Beverly, MA), horseradish peroxidase-conjugated goat anti-mouse secondary antibody, and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). The antibodies were then stripped, and the membranes were reprobed with a monoclonal anti-ERK2 antibody (Upstate Biotechnology, Lake Placid, NY) to ensure equal loading of protein in all wells.

Chemotaxis assays

Chemotaxis assays were performed using CEFs. Transwell polycarbonate membrane with 8 μm pore size (BD Bioscience, San Jose, CA) was coated on both sides with 50 ng/ml type I collagen (Sigma Chemical Co.) by incubation at 37°C for 6 h. Fibroblasts (1×10^5) in 100 μl secondary medium were seeded on the underside of the transwell membrane with the transwell inverted, and the cells were allowed to adhere to the membrane. Thirty minutes after the incubation, each transwell was turned right-side up and inserted into a 24-well plate with the seeded cells facing down into the lower chamber. Medium was added to upper (100 μl) and lower chambers (1000 μl). The agent to be tested was added to the upper chamber at the concentrations indicated in the figures. The cells subjected to different treatments were incubated at 37°C for 3 h. At the end of the experiments, the cells remaining on the underside of the membrane were removed with a cotton swab, and the membranes were fixed and stained with 2% toluidine blue in 4% paraformaldehyde. The number of cells on the upper side of the membrane was counted in 10 fields at 10X magnification and averaged.

RESULTS

To test the activation of cCXCR1 by cIL8 and the N and C peptides, we took advantage of the fact that CXCR1 is a seven-transmembrane receptor [19]. It is well known that these types of receptors, when activated, trigger the recruitment of trimeric G proteins, followed by activation of phospholipase C- and Ca^{2+} influx from the endoplasmic reticulum into the cytosol [24, 25]. Therefore, we used Ca^{2+} mobilization from intracellular stores to determine whether cCXCR1 responds to cIL8 and its N-and C-terminal peptides. To achieve these goals, we used NIH3T3 cells (murine), which do not express an endogenous CXCR1 receptor. The cCXCR1 gene was effectively incorporated into the NIH3T3 cell genome (Fig. 1A), and its expression was tightly regulated by Dox treatment (Fig. 1B). Without Dox addition, cCXCR1 expression levels in infected cells were indistinguishable from the background, whereas in the presence of Dox, expression was increased, and this was dependent on the duration of Dox treatment (Fig. 1B). The full-length cIL8 and the peptide ligands used in this study are depicted in Figure 1C.

**cIL8 and its N and C peptides activate cCXCR1**

To determine whether cIL8 and its terminal peptides activate cCXCR1, we monitored intracellular Ca^{2+} levels after adding each ligand to the cells expressing cCXCR1. For these experiments, we cultured cCXCR1-expressing cells on glass coverslips and loaded them with the Ca^{2+} indicator fluo-3 for 30 min. A field of cells was chosen for examination and to establish the baseline fluorescence (Fig. 2A, left panel). The ligand was then applied onto the cells by pressure ejection from a glass micropipette positioned directly above the cells. This allowed the ligand to be delivered rapidly, avoided the problem of receptor desensitization, and allowed us to measure the threshold response for each ligand. Under these conditions, all cells responded to cIL8 (Fig. 2A, right panel). Statistical analysis of the responses in many cells showed that cIL8 activates cCXCR1 in a typical chemokine-receptor response of Ca^{2+} mobilization (Fig. 2, B and C). These effects are specific; infected NIH3T3 cells without Dox treatment did not show an increase in Ca^{2+} levels when exposed to cIL8 (Fig. 2, B and C). Although activation of cCXCR1 by cIL8 is PTX-sensi-
tive, indicating that signaling is G\textsubscript{i}/G\textsubscript{o}-dependent, the increase in Ca\textsuperscript{2+} levels caused by cIL8 is not completely abrogated by this toxin (Fig. 2, B and C).

Similar experiments were also performed with the N and C peptides. cIL8 N peptide activates cCXCR1 effectively, although it required a higher concentration than the full-length cIL8, and the response is not as robust (compare Fig. 3, A–C, with Fig. 2, A–C). Much like the full-length molecule, this Ca\textsuperscript{2+} response is dose-dependent and PTX-sensitive, although this inhibitor was much less efficient in eliminating the response than in the case of the full-length molecule, suggesting that a considerable part of the N peptide-activating pathways is G\textsubscript{i}/G\textsubscript{o}-independent. cIL8 C peptide activates cCXCR1 at approximately the same concentration as the N peptide and is also only partially inhibited by PTX (Fig. 3, D and E).

We further examined the activation of cCXCR1 by cIL8 and its peptides, by analyzing ERK1/2 activation, using immunoblot analysis and specific antibodies to their phosphorylated forms. Activation of these kinases depends on the phosphorylation of Thr202 and Tyr204 of the protein and is completely inhibited by a specific inhibitor of MAPK kinase (MEK)1, the kinase that activates ERK1/2 [26, 27]. At doses comparable with those used for Ca\textsuperscript{2+} mobilization, this chemokine specifically stimulates the activation of ERK1/2, particularly that of ERK1, albeit to a lesser extent than the phorbol ester-positive control (Fig. 4A). cIL8-induced ERK1/2 phosphorylation/activation is dose-dependent (Fig. 4B), but only ERK2 is time-dependent; after initiation of exposure to the ligand, it increases steadily until 5 min, when it appears to saturate (Fig. 4, B and C).

We also used pathway-specific inhibitors to study the signal transduction events from cCXCR1 activation to ERK2 phosphorylation (Fig. 5). PD98059, a specific inhibitor of MEK1, completely blocked activation of ERK1/2 (Fig. 5A), and much as we observed with the Ca\textsuperscript{2+} imaging, PTX partially inhibited cIL8-induced ERK phosphorylation. It is interesting that ERK1 was more strongly inhibited by PTX than was ERK2. Similar results were obtained with calphostin C, a PKC inhibitor, and with a general tyrosine kinase inhibitor, genestein (Fig. 5A), which completely inhibited ERK1 but only partially inhibited ERK2. Because of the strong effect of genestein, we used more specific tyrosine kinase inhibitors to determine whether we could identify tyrosine kinases pertinent to cIL8 activation of its receptor (Fig. 5B). In many cases, Src tyrosine kinase activation is sufficient and necessary for G protein-coupled, receptor-initiated MAPK activation [28]. Therefore, we tested the possibility of involvement of Src in the cCXCR1-ERK1/2 activation by cIL8. Indeed, the general inhibitor for the Src family of kinases, herbimycin A, completely blocked the activation of both ERKs (Fig. 5B). In addition, we tested for inhibition of the EGFR tyrosine kinase, as it has been reported that in ovary tumor cells, hCXCR1 and -2 activate the MAPK cascade through the EGFR [29]. cIL8 treatment in the presence of AG1517, a specific EGFR tyrosine kinase

**Fig. 2.** cIL8 is a functional ligand for cCXCR1. NIH3T3 cells expressing cCXCR1 were cultured on plastic coverslips in the presence of Dox for 2 days and then loaded with Fura-3 Ca\textsuperscript{2+} indicator. cIL8 was applied directly onto the cells by pressure ejection to avoid receptor desensitization. Images were acquired every 5 s. (A) Representative images of cells at rest (left panel) and with peak Ca\textsuperscript{2+} response (right panel). (B) Average changes in Ca\textsuperscript{2+} concentration in the cytosol for cells imaged as in A. dF/Fo averages of the selected cells are plotted against time. cIL8 induced rapid (within 15 s), strong, and transient Ca\textsuperscript{2+} mobilization (signal returns to initial levels ~2 min after stimulation). The straight, solid line below the curves indicates the period of exposure to cIL8. Treatment with PTX reduced peak height by a factor of 7. (C) Dose-dependence of peak level of Ca\textsuperscript{2+} response in cells treated as in B. Error bars: SEM; n = number of cells counted in the experimental group.
inhibitor, did not significantly affect ERK1/2 activation by this chemokine (Fig. 5B). Similar experiments were performed with the N and C peptides. cIL8 N peptide was able to target ERK2 phosphorylation much more effectively than ERK1, whereas the cIL8 C peptide targeted ERK1 and 2 (Fig. 5C). These results, taken together, indicate that cIL8 and its N and C peptides can stimulate cCXCR1, leading to intracellular Ca\textsuperscript{2+} release and MAPK activation.

![Fig. 4. cIL8-cCXCR1 interaction results in ERK1/2 activation. Cells treated as for Ca\textsuperscript{2+} imaging were also prepared to test for the level of ERK1/2 phosphorylation on Thr202 and Tyr204, which are the markers for activation of these two enzymes. (A) NIH3T3 cells have low background for ERK activation in the absence of cIL8 or expression of cCXCR1 (see also Fig. 3). With cCXCR1 expression, cIL8 induces ERK1 phosphorylation (P-ERK1) and dramatically induces ERK2 phosphorylation (P-ERK2). The phorbol ester phorbol 12,13-dibutyrate (PDBu) served as positive control. (B) cIL8 stimulates ERK1/2 activation in a dose-dependent manner. Samples were collected after 5 min treatment at 37°C. (C) cIL8 (42 nM) stimulates ERK2 activation in a time-dependent manner that saturates after ~5 min. (A–C) Lower panels show equal loading of the proteins.](image-url)
The cIL8 and its N and C peptides are functional ligands for cCXCR1

The data presented above show that cIL8 and its N and C peptides can activate cCXCR1 and lead to specific signaling mechanisms that stimulate the MAPK pathway. To determine whether cIL8 and its peptides stimulate specific cellular functions, we performed chemotaxis assays using primary fibroblasts (Fig. 6). The results showed that cIL8 induced directional cell migration and that this migratory behavior is mediated by cCXCR1; an antibody specific to this receptor abrogated the migratory effect (Fig. 6A). Similar assays using cIL8 peptides show that the N and C peptides are also able to stimulate the directional migration of fibroblasts (Fig. 6B) and do so in a dose-dependent manner (Fig. 6C). Inhibition of cCXCR1 with a specific antibody also abolished the chemotactic effect of each peptide (Fig. 6D). Furthermore, adding the peptide on upper and lower chambers at equal concentration to remove the peptide gradient across the membrane abrogated the directional migration (Fig. 6E), suggesting that this effect on migration is a result of chemotaxis. We further tested the specificity of the cCXCR1 receptor in mediating the chemotaxis induced by cIL8 and its peptides by pretreating the cells with an excessive amount of cIL8 or its peptides. Subsequent application of cIL8 at concentrations that stimulate chemotaxis failed to induce directional migration of the cells (Fig. 6F).

cCXCR1 is a functional receptor for hIL8

To determine whether hIL8 can activate the cCXCR1 receptor, we used NIH3T3 cells expressing cCXCR1, applied hIL8, and measured Ca²⁺ increase inside the cells as described above. The results show that hIL8-72 and hIL8-77 are functional ligands for cCXCR1 (Fig. 7, A and B, and data not shown). In this system, hIL8 stimulation of cCXCR1 also shows a sharp threshold response, but higher concentrations are necessary for activation when compared with cIL8, the natural ligand for cCXCR1. hIL8-72 and -77 stimulate ERK1/2 activation in a Dox-dependent and PTX-sensitive manner (Fig. 7C), much like that seen with cIL8 (Fig. 5A).

To test whether hIL8 can stimulate chemotaxis of chicken fibroblasts, as shown above for cIL8, we performed transwell migration assays using these cells. We found that hIL8-77 is efficient in chemoattracting chicken fibroblasts and that this effect is inhibited by antibodies specific for hIL8 or cCXCR1 (Fig. 8A). To determine whether hIL8 can stimulate biological functions in vivo, we tested hIL8 in chicken wounds, much like we did for cIL8 [11]. hIL8 accelerates wound closure in young chicks (Fig. 8B), albeit less efficiently than cIL8; higher concentrations of the human chemokine are needed to obtain similar effects. In addition, we have previously shown that expression of cIL8 is elevated in healing tissues, in particular, in areas where microvessels are abundant [10], and that this chemokine is angiogenic in the CAM assay by stimulating sprout formation [30]. Treatment of CAMs with hIL8 showed that this chemokine stimulates sprouting of the existing vessels of the CAM (Fig. 8, C and D). These findings strongly suggest that hIL8 activates cCXCR1 in vivo. Taken together, these results lead us to conclude that hIL8 is a ligand for cCXCR1, that this receptor is the functional homologue for hCXCR1 in chickens, and that avians can be a useful animal system for functional studies of hIL8 in vivo.
DISCUSSION

We have previously isolated and characterized a chicken chemokine receptor, which we called cCXCR1, as it is highly homologous to the human chemokine receptor hCXCR1. However, prior to this study, it was not known whether cCXCR1 is the receptor for cIL-8 and hence, serves as a functional homologue of hCXCR1. For the studies presented here, we used a cell line that does not express the cCXCR1 receptor and have expressed it in these cells in an inducible manner by treating with Dox. The cells respond to IL-8 in the presence of Dox treatment and do not respond in the absence of Dox. We used two criteria to measure this response: one, intracellular Ca$^{2+}$ release, and the other, MAPK activation. In addition to the experiments using single gene expression in a cell line, we used primary human microvascular endothelial cells in a chemotaxis assay to determine whether the same specificity exists in cells in which the receptor is naturally expressed. The data show that specific antibodies to IL-8 or cCXCR1 inhibit IL-8-induced chemotaxis. Furthermore, we also show that desensitization experiments and experiments in which the ligand is applied to both wells result in no response. These experiments show not only specific binding of the ligand to the receptor but also that the interaction results in signaling responses. In addition we show that the cIL8 N-terminal 15 aa and C-terminal 27 aa are effective ligands for cCXCR1; cCXCR1 activation is, in part, PTX-sensitive, but other signaling molecules are also involved in this process, such as the Src family of tyrosine kinases, which play a critical role in the downstream events leading to MAPK phosphorylation/activation; and all ligands activate cCXCR1 but do not activate the EGFR tyrosine kinase. These data also provide additional evidence that supports the well-known functions of these chemokine peptides in vivo.

Our studies, which reveal cIL-8 and cCXCR1 to be functional homologs of hIL-8 and hCXCR1, respectively, allow IL-8 and CXCR1 to be studied in the chicken, a model organism that normally expresses these molecules. The fact that hIL-8, cIL8, and their terminal peptides act on cCXCR1 is likely due to the high degree of sequence similarity between the two proteins.
like cIL-8, is capable of activating the chicken receptor strongly suggests that cIL-8 binding and activation of cCXCR1 may be similar to hIL-8 binding and activation of hCXCR1, providing further support for the use of this system to study the chemokine and its receptor. As neither the chemokine nor its receptor is found in rats or mice, the use of the chicken model system will be invaluable in the study of these molecules. In addition, this study highlights the highly conserved nature of chemokines and their receptors, as well as the mechanisms of receptor activation and downstream signaling events, as a human chemokine was able to bind and activate a chicken chemokine receptor. This conservation of the functions of the chemokines and their receptors further supports the use of model organisms in the study of these molecules, as knowledge gained from chemokine/chemokine receptor signaling events and cellular effects is likewise conserved.

In general, chemokine receptors are linked to heterotrimeric G proteins, usually G\(_{\alpha_i}\). After activation of the receptor by ligand binding, the G proteins are activated, leading to a variety of downstream responses. These responses are inhibited by PTX, which adenosine 5'-diphosphate ribosylates G\(_{\alpha_i}\), resulting in the inhibition of a guanosine 5'-triphosphate-guanosine 5'-diphosphate exchange. The response of cCXCR1 to hIL8, cIL8, and the peptides of cIL8 is partially inhibited by PTX, suggesting that this process is dependent on G\(_{\alpha_i}\). However, as PTX does not completely block Ca\(^{2+}\) mobilization and MAPK activation, it is likely that cCXCR1 signaling also requires PTX-insensitive G\(_{\alpha}\). The use of other G\(_{\alpha}\) proteins by chemokine receptors has been documented previously. For example, chemokine signaling in natural killer cells uses almost the entire spectrum of the G\(_{\alpha}\) family, with the exception of G\(_{\alpha_{12}}\) [25]. The ability of chemokine receptors to associate with multiple G\(_{\alpha}\) and G\(_{\beta\gamma}\) allows diversity of downstream effectors and, thus, cellular responses [31]. We are currently investigating the role of other G\(_{\alpha}\) proteins, in particular, G\(_{\alpha_i}\), as it has been shown that antibodies to G\(_{\alpha_i}\) block Ca\(^{2+}\) mobilization induced by the activation of CXCR3 and CXCR4 by interferon-inducible protein 10 and stromal cell-derived factor-1, respectively [32, 33].

Our data also show that Src kinases are critical for ERK1/2 activation upon cCXCR1 interaction with the various ligands (Figs. 5B and 6, B, C, E, and F). Previous studies have shown that upon stimulation, G\(_{\beta\gamma}\) subunits recruit Src tyrosine kinase, and this process is upstream of EGFR tyrosine kinase activation [34, 35]. This mechanism is supported by our data, as the activation of cCXCR1 by its ligands does not require EGFR activation but rather leads to MAPK phosphorylation through a Src-dependent pathway.

The ability of the cIL8 and its terminal N and C peptides to stimulate cCXCR1, as shown by Ca\(^{2+}\) mobilization and MAPK activation, suggests that these regions of cIL8 can bind and activate cCXCR1 independently of each other. This hypothesis is supported by other studies, conducted in vitro and in vivo, which have indicated that chemokine-derived peptides are functional and that peptides derived from different regions of the same chemokine possess different activities. For example, the N-terminal pentapeptide of hIL8 induces apoptosis in various leukemic cell lines [36]. We have also shown that the N termini of cIL8 induce myofibroblast differentiation in vitro and in vivo and induce wound closure in vivo [11]. Furthermore, the C terminal α-helices of various chemokines exhibit multiple functions: The C terminus of hIL8 stimulates melanoma cell proliferation [37], that of cIL8 is angiogenic in vivo.
Fig. 8. hIL8 stimulates functions in chicken cells and in vivo. (A) Cells were plated as in Figure 6 and then treated with hIL8, which stimulated chemotaxis of chicken fibroblasts; this stimulation was specific, as it was inhibited by anti-hIL8, and it was cCXCR1-dependent, as it was inhibited by antibodies specific to this receptor. (B) Average percent wound closure in birds treated with cIL8 or with vehicle. Excision wounds were made on the underside of chick wings and then treated every other day with vehicle (H2O) or 1.5 μg hIL8. Areas of the wounds were measured on digital photographs using National Institutes of Health Image software. hIL8 accelerated wound closure. Percent wound closure was calculated by comparing the area of the closing wound to the area of the same wound on day 0. n = Six birds. *, P < 0.05, for days 3 and 5. (C) Methylcellulose pellet (P) containing 1 μg bovine serum albumin (BSA). Neither methylcellulose nor BSA caused changes in the structure of the chorioallantoic membrane (CAM) blood vessel. *, Tertiary blood vessels. (D) Methylcellulose pellet (P) containing 1 μg hIL8, which caused slight tortuosity of existing smaller blood vessels, stimulated extensive sprouting of new vessels (arrowheads point to representative sprouting), and induced the formation of interconnections between the tertiary vessels (arrows).
[30], and that of PF4 inhibits cell growth in some cells [38]. These results, taken together, strongly suggest that the termini of certain chemokines interact with their respective receptors independently of the full-length protein.

One explanation for this observation comes from the current information regarding chemokine-receptor interactions. X-ray crystallography and nuclear magnetic resonance analysis have led to the development of a number of structural models depicting chemokine-receptor interactions [39, 40]. These models, although lacking in detailed information, tend to implicate the N terminus of the receptor and the sequences of the chemokine between CXC/CC residues and C-terminal helix in chemokine-receptor binding. During this process, the N terminus of the receptor fits into a cleft in the chemokine C terminus, in which an incomplete barrel structure is formed from an N-loop and a triple-stranded, antiparallel β-sheet [39–41]. In this model, the N terminus of IL-8 occupies a position distant from the N terminus of the receptor, and the C-terminal α-helix is located behind the barrel structure; thus, the N and C termini of the molecule are free to interact with regions of the receptor other than its N terminus.

Mutagenesis studies also implicate regions of the chemokine receptors outside the N terminus in chemokine binding. For example, when various charged residues located in the extracellular domains of hCXCR1 and hCXCR2 were mutated, the receptors experienced decreased chemokine binding, leading to reduced cellular responses [42, 43]. Thus, the current body of information suggests the possibility that chemokines may interact with their receptors at the N terminus of the receptor and at various other extracellular regions. At this time, there is no direct evidence showing the interaction of the chemokine with the extracellular loops; however, molecular modeling of the interaction between hIL8 and hCXCR2 has implicated the extracellular domains 3 and 4 of hCXCR2 in hIL8 binding [44]. It is noteworthy that the critical residues located in the extracellular loops of hCXCR1 are highly conserved in cCXCR1, suggesting a conserved mechanism for chemokine receptor activation.

In conclusion, our results show that cCXCR1 is the functional homologue of hCXCR1 and can interact with hIL8 and with cIL8 and its peptides. The knowledge that chemokine-derived peptides can activate their receptors, leading to signaling much like that induced by their full-length counterparts, provides additional information regarding the mechanisms involved in ligand binding and receptor activation. The ability of separate regions of a chemokine to activate its receptor apart from the other chemokine regions may provide an explanation for the previously observed multifunctionality of these molecules. Furthermore, an increased understanding of individual chemokine domains may lead to the design of more effective chemokine-based therapeutic agents to treat inflammatory disorders and cancer.

ACKNOWLEDGMENTS

We thank Dr. H. Blau (Stanford University, CA) for providing the Tet-on retroviral system for CXCR1 expression and Dr. G. Nolan (Stanford University) for the protocols of retroviral work.

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cCXCR1 is a receptor for cIL8 (9E3/cCAF) and its N- and C-terminal peptides and is also activated by hIL8 (CXCL8)

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J Leukoc Biol published online December 2, 2004
Access the most recent version at doi:10.1189/jlb.0704398