Published in final edited form as:

Methods Enzymol. 2009 ; 460: 315–330. doi:10.1016/S0076-6879(09)05215-X.

Characterization of Chemokine Receptor CXCR2 Interacting Proteins Using a Proteomics Approach to Define the CXCR2 “Chemosynapse”

Dayanidhi Raman^{*,‡,1}, Nicole F. Neel^{*,1}, Jiqing Sai^{*,†}, Raymond L. Mernaugh[†], Amy-Joan L. Ham[†], and Ann J. Richmond^{*,‡}

^{*} Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[†] Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[‡] Veterans Affairs Medical Center, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Abstract

Chemokine-receptor signaling is initiated upon ligand binding to the receptor and continues through the process of endocytic trafficking by the association of a variety of adaptor proteins with the chemokine receptor. In order to define the adaptor proteins that associate with CXCR2 before and after ligand activation, a protocol was developed using differentiated HL-60 cells transfected to express CXCR2 stimulated or not stimulated with ligand for one minute. CXCR2-associating proteins were isolated by immunoprecipitation with CXCR2 antibody and the eluted proteins were electrophoretically run into the separating gel directly without a stacking gel. The stained single band was subjected to in-gel trypsin digestion. The tryptic peptides were subjected to, LC/MS/MS proteomic analysis. Proteins identified in a minimum of three of four separate experiments with multiple peptides were then validated as CXCR2 adaptor proteins by coimmunoprecipitation, GST pull-down studies, and immunocytochemical CXCR2-colocalization experiments using dHL-60-CXCR2 cells. Subsequently, a functional analysis of the interaction between CXCR2 and CXCR2 interacting proteins was performed. This approach can be used to characterize chemokine receptor–associating proteins over time both before and after ligand stimulation, allowing definition of the dynamic spatial and temporal formation of a “chemosynapse.”

1. Introduction

Chemotactic cytokines or chemokines bind to and activate their cognate G-protein–coupled receptors (GPCRs) and this event results in a variety of biological responses (Thelen and Stein, 2008). The varied biological response is due in part to different repertoires of adaptor proteins binding to chemokine GPCRs at various spatiotemporal points. This differential coupling dictates subsequent biological response and fine tuning of the chemokine GPCR signaling. The assembly of proteins bound to the chemokine GPCRs is analogous to the immunological or neurosynapse wherein the protein–protein interactions initiate, maintain, and regulate a particular biological activity. We are defining this dynamic spatial and temporal assembly of adaptor/signaling proteins on the chemokine receptor as the

“chemosynapse.” When chemokines activate their chemokine GPCRs, the active receptors not only signal at the plasma membrane but also continue to signal at the endosome level. This is possible due to the binding of different adaptor proteins as the chemokine receptor traverses through its vesicular trafficking route. The phosphorylation status of the chemokine GPCR also enables the assembly of different ensembles of adaptor proteins bound to the receptor, and thus will be very different from the basal unphosphorylated or dephosphorylated states of the receptor. Thus, the activity status of the chemokine receptor dictates in part the type of adaptor protein that may bind the chemokine receptor at a particular time.

In some cases, there is a genetic alteration of chemokine receptors such as in WHIM syndrome (warts, hypogammaglobulinemia, infections, and myelokathexis) where CXCR4 is genetically altered at its C-terminus, resulting in a combined immunodeficiency disease (Hernandez *et al.*, 2003). Patients with this mutation exhibit altered response to CXCL12, suggesting that loss of ability to form an appropriate chemosynapse can result in serious disease (Gulino *et al.*, 2004) (Balabanian *et al.*, 2005; Lagane *et al.*, 2008). Expression of a similarly truncated CXCR4 in MCF-7 breast cancer cells *in vitro* led to an epithelial to mesenchymal-like transition (EMT) in these cells (Ueda *et al.*, 2006). Expression of C-terminally truncated CXCR2 with additional mutations in AP-2 binding motif in mouse keratinocytes in a transgenic mouse under the control of K14 promoter resulted in the loss of the mouse tail and extensive skin lesions (Yu *et al.*, 2008). Thus, the carboxyl-terminal domain and possibly intracellular cytoplasmic loops of a chemokine receptor provide a structural landing for components of the chemosynapse and are functionally important for orchestration of the normal physiological responses of cells and tissues.

1.1. The CXCR2 chemosynapse

To characterize the CXCR2 chemosynapse, a proteomic approach was pursued to identify novel CXCR2-interacting proteins in response to ligand activation of the receptor. We then characterized the role of these proteins in modulation of the CXCR2 receptor function (Fig. 15.1) (Neel, 2008; Neel *et al.*, 2009).

The ability of the HL-60 human promyelocytic leukemia cells (Gallagher *et al.*, 1979) to differentiate into neutrophil-like cells was exploited here to generate cells for proteomic analysis of CXCR2-associated proteins. These cells express very little CXCR2, so a CXCR2 retroviral construct was transduced into these cells and cells expressing CXCR2 at levels comparable to human neutrophils were selected based on G418 resistance and by fluorescence activated cell sorting (FACS) for CXCR2 expression (Sai *et al.*, 2006). The HL-60-CXCR2 cells were differentiated into neutrophil-like cells (dHL-60 cells) by incubation with 1.3% dimethyl sulfoxide (DMSO) for 6 to 7 days, followed by stimulation with CXCL8. Since the signaling in neutrophil-like dHL-60 cells occurs rapidly, and most intracellular signaling molecules such as Akt, Rac2, and Cdc42 were activated maximally by 1 min (Sai *et al.*, 2006), we studied the CXCL8-stimulated molecular repertoire of CXCR2 chemosynapse at the 1-min time point. Cell lysates were made of differentiated CXCR2 HL-60 cells not treated with CXCL8 and cells treated with CXCL8 for 1 min. CXCR2 and CXCR2-associated proteins were immunoprecipitated from these lysates and characterized by proteomic analysis (Neel, 2008). This protocol offers advantages over identification of CXCR2 interacting proteins by the yeast two-hybrid system since, it is possible to uncover ligand-dependent association of CXCR2 interacting proteins over time. CXCL8 binding to CXCR2 activates the heterotrimeric G-proteins ($G_{\alpha\beta\gamma}$) and the GRKs that rapidly phosphorylate the carboxyl-terminal domain (CTD) of CXCR2 on serine residues. The phosphorylated CTD can serve as a nexus for the binding of a number of adaptor proteins to facilitate the movement of CXCR2 into clathrin-coated pits and endosomal compartments.

1.2. Proteomic screen for the CXCR2 chemosynapse adaptor proteins

The protocols used for this analysis are those developed and described by Neel and colleagues (Neel, 2008; Neel *et al.*, 2009). HL-60 cells stably expressing human CXCR2 were grown in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 3 mM L-glutamine and penicillin (50 units/ml)/Streptomycin (50 µg/ml) (Mediatech, Herndon, VA). The pH of the RPMI-1640 medium was stabilized with 25 mM HEPES, pH 7.4. The initial seeding density of the cells was set at 1×10^5 cells/ml in order to keep them robustly healthy, and they were subcultured every 3rd day for routine maintenance. In order to differentiate into neutrophil-like cells, HL-60 cells were seeded at a density of 1×10^5 cells/ml in antibiotic-free RPMI-1640 containing 10% heat-inactivated, fetal bovine serum, and 1.3% endotoxin-free DMSO (Sigma, St. Louis, MO) for 6 to 7 days (Sai *et al.*, 2006).

Differentiated HL-60 cells stably expressing CXCR2 (dHL-60 CXCR2) were washed with serum-free RPMI-1640 and stimulated with vehicle (0.1% bovine serum albumin in phosphate buffered saline [PBS]) or CXCL8 100 ng/ml for 1 min. The cell pellet was lysed in 50 mM Tris-HCl, pH 7.5, 0.05% Triton X-100, 300 mM NaCl with mammalian protease inhibitor cocktail (AEBSEF; [4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride], aprotinin, leupeptin hemisulfate salt, E-64 [(N-trans(epoxysuccinyl)-L-leucine 4-guanidinobutylamide], bestatin hydrochloride, and pepstatin A) and phosphatase inhibitor cocktail I (microcystin LR, cantharidin, and bromotetramisole, cat. no. P 2850) and II (sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole, cat. no. P 5726) (all were from Sigma, St. Louis, MO) by nutation for 10 min at 4 °C. After clarification by centrifugation, the lysates were precleared with normal rabbit IgG (Jackson ImmunoResearch, West Grove, PA) conjugated to N-hydroxysuccinimide (NHS)-activated sepharose beads (GE Healthcare, Piscataway, NJ). Following preclearing, lysates from untreated and CXCL8-treated dHL-60 cells were nutated with either normal rabbit IgG (mock) or with anti-CXCR2 rabbit antibody conjugated to NHS-activated sepharose beads for 1 h at 4 °C. The beads were centrifuged and washed thrice with lysis buffer and the proteins were eluted with Laemmli sample buffer at 60 °C for 10 min. The samples were loaded directly onto a 10% resolving sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel (no stacking gel) and ran 1 cm into the gel and stained with colloidal blue stain (Invitrogen, Carlsbad, CA). The single stained band was excised and an in-gel trypsin digestion was performed. The tryptic peptides were analyzed by LC/MS/MS using a Thermo Finnigan LTQ ion trap mass spectrometer equipped with Thermo MicroAS autosampler, Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur 1.4 instrument control. Analysis and protein identification protocols were followed as described by Neel and colleagues (Neel, 2008; Neel *et al.*, 2009). Proteins were identified using the Sequest search algorithm and were filtered for confident identifications using a database program called Complete Hierarchical Integration of Protein Searches (CHIPS). Subsequent validation was performed on proteins that were identified in at least three of the four experiments with multiple peptides for the same protein (Neel, 2008; Neel *et al.*, 2009). We identified 7 proteins that uniquely associate with CXCR2 after ligand stimulation, 11 proteins that associate with CXCR2 in the ligand-unstimulated state, and 6 proteins that associate with CXCR2 under both ligand-stimulated and -unstimulated conditions. These proteins can be grouped into four types: proteins involved in organization of the actin cytoskeleton, proteins involved in receptor trafficking, proteins involved in scaffolding and signaling, and other proteins (Fig. 15.2) (Neel, 2008).

2. Validation of the Interaction of Novel Proteins with CXCR2

Once the novel or known proteins that were previously not known to bind CXCR2 are identified by proteomics, each was validated for its authentic interaction with CXCR2 by

performing a coimmunoprecipitation experiment as well as colocalization in cells with and without CXCL8 stimulation.

2.1. Coimmunoprecipitation of CXCR2 and CXCR2-binding proteins

Coimmunoprecipitation was performed similar to that described above for proteomic identification except that after running the SDS-PAGE, the proteins were transferred to the nitrocellulose membrane, blocked with 5% milk in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 h at room temperature (RT) and the proteins that were coimmunoprecipitated with CXCR2 were probed with the primary antibody directed against the identified proteins in 1% milk in TBST (20 mM Tris-HCl, pH 7.5, 0.05% Tween-20) overnight at 4 °C. This was followed by incubation with either affinity-purified donkey, antirabbit secondary antibody tagged with IR dye 800 or with affinity-purified goat, antimouse antibody tagged with Alexa Fluor 680 for 1 h at RT, depending on the host species from which the primary antibody was derived. During this incubation, the blot was foil-wrapped to minimize any photobleaching of the IR 800 dye or Alexa Fluor 680. Following this, the blot was washed thrice with TBST. The CXCR2-binding protein bands were detected by scanning the wet, drained blot in an Odyssey detection system (LI-COR Biosciences, Lincoln, NE). The images were processed initially using LI-COR odyssey software followed by Photoshop computer program (Adobe Systems, San Jose, CA).

2.2. Colocalization CXCR2 and CXCR2-interacting proteins in dHL-60 cells

The colocalization of CXCR2 and CXCR2-interacting proteins in dHL-60 cells can be examined by either stimulating the cells with CXCL8 universally or by a chemokine gradient using a Zigmond chamber (Neuroprobe, Gaithersburg, MD).

2.2.1. Polarization in Zigmond chamber—Differentiated HL-60 cells stably expressing CXCR2 were seeded onto fibronectin-coated (100 µg/ml) glass coverslips for 10 min at 37 °C in tissue-culture incubator with 5% CO₂. The coverslip was inverted and placed face down on the Zigmond chamber so that the bridge of the chamber will be in the middle of the coverslip. The left chamber was filled with 90 µl of chemotaxis medium (serum-free RPMI-1640 with 0.1% BSA) and the right chamber with 90 µl of chemotaxis medium with 25 to 50 ng/ml of CXCL8. The loaded Zigmond chamber was incubated at 37 °C for 15 min in tissue-culture incubator with 5% CO₂. After this, the coverslip was gently rinsed in PBS (58 mM Na₂HPO₄, 17 mM NaH₂PO₄, and 68 mM NaCl, pH 7.5) (optional) and fixed in 4% paraformaldehyde for 10 min at RT.

2.2.2. Immunocytochemistry and confocal microscopy—The paraformaldehyde-fixed coverslips were washed thrice with PBST (PBS, pH 7.5, with 0.05% Tween-20). The polarized dHL-60 cells were permeabilized with 0.2% Triton X-100 for 5 min at RT. After aspiration of Triton X-100, any trace amount of Triton X-100 was rinsed out with PBST. The permeabilized cells were blocked with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 30 min to 1 h at RT. After rinsing in PBST, the coverslips were incubated with the primary antibodies directed against CXCR2 and the CXCR2-interacting proteins for 2 h at RT. If phosphorylated CXCR2-interacting proteins were to be detected, fixation with paraformaldehyde may destroy the phospho-CXCR2-binding protein signal. Alternatively, fixing in ice-cold methanol (cooled to −20 °C) allows detection of phosphorylated serines and threonines in a given protein but methanol does not stabilize F-actin. After washing thrice with PBST, the coverslips were incubated with either donkey antimouse or donkey antirabbit antibodies that are conjugated to the fluorophores cy2 or cy3 (Jackson ImmunoResearch, West Grove, PA). The filamentous actin (F-actin) was stained with rhodamine-conjugated phalloidin (Invitrogen, Carlsbad, CA). F-actin is a marker of the leading edge of the polarized cells and phalloidin is a toxin obtained from death cap

mushroom (*Amanita phalloides*) that binds to the polymerized actin filaments (F-actin) more avidly than the actin monomers. After washing thrice with PBST and once with PBS, the coverslips were mounted with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). The edges of the coverslip were sealed with nail polish. Confocal images of the polarized cells were acquired using a Zeiss Inverted LSM-510 meta laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY) with a 63× objective and 1.4 Plan-APOCHROMAT oil immersion lens. The images were processed by the Photoshop computer program (Adobe Systems, San Jose, CA).

2.3. Glutathione S-transferase pull-down studies

2.3.1. GST-CXCR2 C-tail constructs—For glutathione S-transferase (GST) pull-down studies, the gene encoding the full-length carboxyl terminus of CXCR2 (311-355) was inserted into BamH I and Xho I sites of the GST vector pGEX-6P-1 (GE Healthcare, Piscataway, NJ). The PCR primers for the construction of GST-CXCR2-311-355 plasmid follow: forward, 5'-CTCTAGGGATCCTTCATTGGCCAGAAGT-3', and reverse, 5'-CTAGCTCTCGAGTTAGAGAGTAGTGG-3'. The GST-CXCR2 C-tail construct was used as a template to generate the GST-CXCR2-311-330 construct (first half of the CXCR2 C-tail). To make this construct, a stop codon was introduced at position 331, converting a serine codon (AGC) to a stop codon (TGA). To accomplish this, the QuikChange mutagenesis kit (Stratagene, LaJolla, CA) was employed. The PCR primers follow: forward, 5'-CTAGCTATACATGGCTTGATCTGAAAGGACTCC-3', and reverse, 5'-GGGCAGGGAGTCCTTTTCAGATCAAGCCATGTAT-3'. To screen for the binding site for CXCR2 interacting proteins on the CXCR2 C-tail, the C-terminus was split in two (311-330 and 331-355) and were inserted into BamH I and Xho I sites of the vector pGEX-6P-1. One microliter of the restriction enzyme *Dpn I* was added to the PCR mixture and incubated at 37 °C for 1 h to digest the methylated parental strands of DNA leaving behind the intact nonmethylated PCR product. This removes any false-positive colonies showing up after transformation. After *Dpn I* digestion, about 10% (5 μ l out 50 μ l) of the PCR product was used to transform XL-1 blue competent cells supplied with the QuikChange mutagenesis kit. The nick in the PCR product will be repaired by the bacteria to produce a circular double-stranded plasmid. Following transformation, LB/Amp Petri plates were incubated overnight at 37 °C. Four colonies were picked up and 5-ml cultures were grown with ampicillin at 1 μ g/ml to isolate the plasmid using the GenElute HP plasmid mini-prep kit (Sigma, St. Louis, MO, cat. no. NA0160). The mini-prep plasmids from different colonies were verified for the correct coding or mutation or stop codon introduction by DNA sequencing using Big Dye Terminator chemistry at the Vanderbilt DNA sequencing core facility.

2.3.2. Production of GST-fusion proteins—After verification of the cDNA for the GST-fusion protein by sequencing, the constructs were transformed into BL 21 cells for production of GST-CXCR2 C-tail protein. Four clones were selected and 3-ml cultures were grown overnight at 37 °C in LB/ampicillin (1 μ g/ml) and the clones that express the optimal amount of protein were selected. For GST-fusion protein production to be used in pull-down studies, an overnight 20-ml culture with ampicillin (1 μ g/ml) was expanded to 200 ml with ampicillin (1 μ g/ml) and grown for an additional 2 h at 37 °C. The fusion protein expression was induced with 50 μ M of isopropyl β -D-thiogalactoside (IPTG) (Sigma, St. Louis, MO) for 2 h at 30 °C. The bacteria were pelleted by centrifugation at 5000 rpm for 10 min. The bacterial pellet was resuspended in PBS with 0.1% Triton and bacterial protease inhibitor cocktail (AEBSF, Bestatin HCl, E-64, EDTA [ethylenediaminetetraacetic acid] and pepstatin A; Sigma, St. Louis, MO, cat. no. P 8465). The bacteria were lysed on ice using a probe-tip sonicator (Branson Sonifier 250) (four cycles of 10 s each with in-between cooling on ice). The lysate was clarified by centrifugation at 13,000 rpm for 10 min, and the clear

supernatant was nutated with preswollen glutathione-agarose beads (Sigma, St. Louis, MO, cat no. G 4510) equilibrated with the lysis buffer for 1 h at 4 °C. The beads with bound GST-fusion proteins were washed thrice with the lysis buffer and once without any detergent in the lysis buffer. The protein on the beads was quantified by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and beads were aliquoted and stored at −80 °C.

2.3.3. GST pull-down experiment—Fifty to 100 μ g of the GST-CXCR2-311-355 (full-length C-tail), GST-CXCR2-311-330 (first half of the C-tail) and GST-CXCR2-331-355 (second half of the C-tail) were washed in the binding buffer (50 mM Tris-HCl, pH 7.5, 0.05% Triton X-100, 300 mM NaCl) and mixed with cell lysates expressing the CXCR2-binding proteins for 1 h at 4 °C. The bound proteins were separated from the unbound by washing the beads thrice by centrifugation at 1000 rpm for 30 s. The bound proteins were eluted by boiling in Laemmli sample buffer for 5 min, and then the eluent was analyzed by 10% SDS-PAGE followed by Western blotting and subsequent probing for the CXCR2-binding proteins with specific antibody. GST controls were included for comparison.

Alternatively, the binding of CXCR2-interacting proteins to GST-CXCR2 C-tail can be followed in a 96-well ELISA format assay. Briefly, GST-fusion proteins were isolated as described above and eluted with 50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione (GSH). A 96-well polyvinyl plate was coated with GST or GST-CXCR2 (at 3 μ g/ml) in triplicate followed by blocking in 0.5% Tween-20/0.5% Triton X-100 in PBS, pH 7.5. The coated plate was overlaid with varying amounts of His₆-CXCR2-binding protein for 1 h at RT. The plate was washed six times with PBST (PBS with 0.1% Tween-20). Bound CXCR2-binding protein was probed with His-probe-HRP (Pierce, Milwaukee, WI) and detected by colorimetry by incubating with peroxidase substrate solution consisting of 50 mM sodium citrate buffer, pH 4.2, 90 mM 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Sigma, St. Louis, MO) and 0.05 mM H₂O₂. The reaction was terminated by adding an equal volume of 1% (w/v) sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO). The color intensity was read at 405 nm with an ELX800_{NB} plate reader (Bio-Tek Instruments, Winooski, VT).

3. Mutational Analysis of Residues at Interactive Interface of CXCR2 and CXCR2-Binding Proteins

For any two bonafide interacting proteins, the functional significance behind their interaction can be inferred by mutating the contact surface residues on both proteins. In the CXCR2 chemosynapse model, the interaction of CXCR2 with its binding proteins may up- or down-regulate the function of CXCR2. If the interaction between CXCR2 and the CXCR2-binding proteins can be ablated by mutating amino acids either on the CXCR2 C-terminus or on the CXCR2-binding protein interactive surface, and then the functional outcome will point to the role played by the adaptor protein at a particular point in the cell within the context of the chemosynaptic network. To address this, the CXCR2 C-terminus fused to the GST protein was divided in two to examine the specific half or both halves of the CXCR2 C-terminus that support the binding of CXCR2-binding proteins. The construction of these cDNA constructs has been described in detail previously in the GST pull-down studies section. Once a small segment has been identified, then the binding capability of all of the amino acid residues in that segment can be verified by alanine scanning mutagenesis and performing a CXCR2-binding protein pull-down experiment. This will also yield information on biochemical nature of the interaction between CXCR2 C-tail and the CXCR2-binding proteins. Also, this same mutation can be substituted in the full-length CXCR2 and the pheno-type can be assessed in dHL-60 cells with regard to any chemotactic defects.

4. Radioactive Phosphorylation of CXCR2 Interacting Proteins

Cells were seeded onto 150 cm² dishes and allowed to attach and spread for 8 h. The cells were then washed with serum-free and phosphate-free DMEM (Invitrogen, Carlsbad, CA) twice and were starved overnight in the same medium. After 12 to 14 h, the medium was replaced with 9 ml of fresh serum and phosphate-free DMEM, and the ATP pool of the cell was metabolically radiolabeled with 300 μ Ci of ³²P-orthophosphate (Perkin Elmer Life and Analytical Sciences, Boston, MA) and the incubation continued for 4 h. Cells were stimulated with ligand for 1 min at 37 °C. The radioactive medium was carefully aspirated out and gently rinsed once in ice-cold, phosphate-buffered saline (PBS), pH 7.5. The cells were then lysed on ice using 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% IGEPAL CA-630 (NP-40) (Sigma, St. Louis, MO), 0.1% sodium deoxycholate (Sigma, St. Louis, MO), 5 mM EDTA supplemented with protease inhibitor cocktail, and phosphatase inhibitor cocktail I and II (all from Sigma, St. Louis, MO). These were nutated for 10 min at 4 °C. The lysates were clarified by centrifugation at 7000 for 7 min at 4 °C. The clarified lysate was precleared with 1 μ g of normal rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and 20 μ l of Protein A-Sepharose (Santa Cruz Biotech, Santa Cruz, CA). The precleared lysate was split in three and subjected to immunoprecipitation by normal rabbit IgG, and the antibody to the CXCR2 interacting protein for 1 h at 4 °C. The immune complexes were captured by 40 μ l of Protein A-Sepharose and were washed thrice in lysis buffer and once in 50 mM Tris, pH 7.5. The proteins were eluted by boiling for 5 min and then resolved by 10% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and the nitrocellulose filter is dried. Phosphorylated CXCR2 associating proteins were identified by autoradiography.

The availability of phospho-specific antibodies for CXCR2 interacting proteins enables one to examine the relevance of a particular phosphorylation site in its interaction with CXCR2. Employment of specific mutants involving combinations of these phosphorylation sites can determine whether the specific phosphorylations were important for its binding to CXCR2. The phosphorylated residues on CXCR2 interacting proteins may directly participate in its binding to CXCR2 or it may induce allosteric conformational changes that may expose a new binding surface favoring its interaction to CXCR2.

5. Chemotaxis Assay

To determine the functional significance of a specific protein in the CXCR2 chemosynapse to CXCR2-mediated chemotaxis, the CXCR2-binding protein was knocked down in HL-60 cells by shRNA using a lentiviral delivery system (Kappes and Wu, 2001; Kappes *et al.*, 2003). shRNA clones against a particular CXCR2 interacting protein were selected from the GIPZ lentiviral shRNAmir library from Open Biosystems (Huntsville, AL). A nonsilencing construct in the same vector was chosen as the control. The lentiviruses containing shRNA or nonsilencing sequence were packaged in the 293-FT cell line (Invitrogen, Carlsbad, CA) by transfecting 6 μ g of shRNA construct of the CXCR2 interacting protein, 4 μ g of psPAX2, and 2 μ g of pMD2.G. The medium containing the viruses was collected 48 h and 72 h posttransfection and used to infect HL60-CXCR2 cells after concentration through an Amicon-50 Ultra filter (Millipore, Billerica, MA). Polyclonal stable cell lines were selected in 0.5 μ g/ml puromycin and the level of knock-down was determined by Western blot, and cells with at least 70% knock-down were tested in chemotaxis assays.

5.1. Chemotaxis and chemokinesis assays in modified Boyden chamber

The chemotaxis assay was performed with dHL-60-CXCR2 cells with a modified Boyden chamber (Neuroprobe, Gaithersburg, MD) as previously described (Sai *et al.*, 2006, 2008). Briefly, various concentrations of CXCL8 (Peprotech, Rocky Hill, NJ) were prepared in 1%

bovine serum albumin/phenol red-free RPMI (chemotaxis buffer), and 400 μ l of the chemotaxis medium containing different concentrations of CXCL8 are loaded into a 96-well plate in triplicates. A polycarbonate filter (3- μ m pore size) (Neuroprobe, Gaithersburg, MD) was placed above the wells and the chamber was assembled. dHL-60-CXCR2 cells were washed twice with the chemotaxis buffer and resuspended at a density of 5×10^5 cells/ml. Two hundred microliters (1×10^5 cells) of dHL-60-CXCR2 cells were loaded onto top wells for the series of chemokine concentrations and incubated for 1 h at 37 °C, 5% CO₂. After 1 h, the Boyden chamber was disassembled and the 96-well plate with transmigrated cells was centrifuged to pellet the cells. The cells were washed three times with modified Hank's buffer (mHBSS) (20 mM HEPES, pH 7.2, 150 mM NaCl, 4 mM KCl, 1.2 mM MgCl₂, and 10 mg/ml glucose) (Servant *et al.*, 1999), and finally resuspended in 100 μ l of mHBSS. The cells were lysed and a colorimetric reaction (60 μ l of NAG solution [4-nitrophenyl-N-acetyl- β -D-glucosaminide], Sigma, St. Louis, MO; 25 mM sodium citrate, 25 mM citric acid, and 0.25% Triton X-100, pH 5.0) was set up overnight at RT in the dark. One hundred microliters of the stop solution (50 mM glycine, 5 mM EDTA, pH 10.4) were added to each well, and the yellow color that develops was read at 405 nm using an ELISA plate reader. The number of transmigrated cells was calculated from the standard curve obtained from cells loaded at the bottom, ranging from 0 to 2500, 5000, 10,000, 20,000, and 40,000 cells. The chemotactic index was calculated by dividing the number of migrated cells in response to a particular concentration of the chemokine by the number of cells that transmigrated in response to buffer alone. Chemokinesis assay was performed similarly except that equal concentrations of the chemokine were added to both the top and the bottom wells.

5.2. Chemotaxis assay in micro fluidic gradient device

Generation of a controllable and stable gradient of chemokine has been challenging for scientists to study chemotaxis *in vitro*. Jeon *et al.* (2002) demonstrated a microfluidic device that allows generation of a variety of stable gradients by keeping a constant flow of solution. The advantages of this device are (1) provides a stable gradient of chemokine, (2) generates different steepness of gradient as designed, and (3) different profiles of gradient, such as linear, exponential, bimodal, and so on. However, since the gradient is maintained by a constant flow of solution, the shear force applied on the cells and its impact on chemotaxis should be considered. Microfluidic gradient devices were made at the Vanderbilt Institute for Integrative Biosystems Research and Education (VIIBRE) as described previously (Fig. 15.3) (Walker *et al.*, 2005).

Devices were precoated with human fibronectin (100 μ g/ml) in modified Hank's buffer (mHBSS) (20 mM HEPES, pH 7.2, 150 mM NaCl, 4 mM KCl, 1.2 mM MgCl₂, and 10 mg/ml glucose) (Servant *et al.*, 1999) for 1 h at RT and washed briefly with mHBSS before use. Differentiated HL60-CXCR2 cells were washed and resuspended in serum-free RPMI/1640 medium at 4×10^6 cells/ml, and injected into the precoated device. Cells were seeded in the device for 5 min at 37 °C, 5% CO₂, and placed in a prewarmed temperature-controlled chamber of the inverted microscope (Axiovert 200 M, Carl Zeiss Microimage, Germany). The two input tubings of the device were connected to syringes with one filled with CXCL8 in serum-free RPMI/1640 medium containing 1% bovine serum albumin (BSA) and the other with RPMI/1640 only. The injection of the solutions from syringes into the device was driven by a syringe pump (Harvard PHD2000, Harvard Apparatus, Holliston, MA), first at 50 μ l/min to quickly fill the tubings with medium containing CXCL8 or just the medium and at 0.5 μ l/min to maintain a CXCL8 gradient in the main channel of the device. The live cell microscopic images were taken every 20 s for a period of 30 min by a CCD camera (Hamamatsu, Japan) controlled by the computer software Metamorph (Molecular Devices Corporation, Downingtown, PA). The data were analyzed by Metamorph to track the cell movements (Sai *et al.*, 2006, 2008).

6. Conclusions

The use of immunoprecipitation of chemokine-receptor and receptor-associating proteins from cells stimulated with chemokine ligand for varying periods of time, followed by LC/MS/MS proteomic analysis of the receptor-associating proteins reveals important information about the protein–protein interactions occurring over time in response to ligand activation of chemokine receptors. This methodology, when combined with careful GST-pull-down analysis of the interaction with purified proteins, immunofluorescence, and functional analysis of the receptor–receptor binding protein interacting sites will provide key information about the spatial and temporal dynamics of the chemosynapse.

Acknowledgments

This work was supported by grants from the National Cancer Institute (CA34590, A.R.), the Vanderbilt-Ingram Cancer Center (grant CA 68485), and Vanderbilt Multidisciplinary Basic Research Training in Cancer (grant T32CA09592). Support also came from the Department of Veterans Affairs through a VA Senior Research Career Scientist Award (A.R.) and through the Ingram family through an Ingram Professorship (A.R.).

References

- Balabanian K, Lagane B, Pablos JL, Laurent L, Planchenault T, Verola O, Lebbe C, Kerob D, Dupuy A, Hermine O, Nicolas JF, Latger-Cannard V, et al. WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. *Blood*. 2005; 105:2449–2457. [PubMed: 15536153]
- Gallagher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ting R, Ruscetti F, Gallo R. Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood*. 1979; 54:713–733. [PubMed: 288488]
- Gulino AV, Moratto D, Sozzani S, Cavadini P, Otero K, Tassone L, Imberti L, Pirovano S, Notarangelo LD, Soresina R, Mazzolari E, Nelson DL, et al. Altered leukocyte response to CXCL12 in patients with warts hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome. *Blood*. 2004; 104:444–452. [PubMed: 15026312]
- Hernandez PA, Gorlin RJ, Lukens JN, Taniuchi S, Bohinjec J, Francois F, Klotman ME, Diaz GA. Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. *Nat Genet*. 2003; 34:70–74. [PubMed: 12692554]
- Kappes JC, Wu X. Safety considerations in vector development. *Somat Cell Mol Genet*. 2001; 26:147–158. [PubMed: 12465466]
- Kappes JC, Wu X, Wakefield JK. Production of trans-lentiviral vector with predictable safety. *Methods Mol Med*. 2003; 76:449–465. [PubMed: 12526179]
- Lagane B, Chow KYC, Balabanian K, Levoe A, Harriague J, Planchenault T, Baleux F, Gunera-Saad N, Arenzana-Seisdedos F, Bachelier F. CXCR4 dimerization and {beta}-arrestin-mediated signaling account for the enhanced chemotaxis to CXCL12 in WHIM syndrome. *Blood*. 2008; 112:34–44. [PubMed: 18436740]
- Li Jeon N, Baskaran H, Dertinger SK, Whitesides GM, Van de Water L, Toner M. Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. *Nat Biotechnol*. 2002; 20:826–830. [PubMed: 12091913]
- Neel, NF. PhD diss. Vanderbilt University; 2008. Regulation of CXC chemokine receptor function through intracellular trafficking and novel receptor-interacting proteins.
- Neel NF, Barzik M, Raman D, Sobolik-Delmaire T, Sai J, Ham AJ, Mernaugh RL, Gertler FB, Richmond A. VASP is a CXCR2-interacting protein that regulates CXCR2-mediated polarization and chemotaxis. *J Cell Sci*. 2009 in press.
- Sai J, Raman D, Liu Y, Wikswo J, Richmond A. Parallel phosphatidylinositol 3-kinase (PI3K)-dependent and Src-dependent pathways lead to CXCL8-mediated Rac2 activation and chemotaxis. *J Biol Chem*. 2008; 283:26538–26547. [PubMed: 18662984]

- Sai J, Walker G, Wikswo J, Richmond A. The IL sequence in the LLKIL motif in CXCR2 is required for full ligand-induced activation of Erk, Akt, and chemotaxis in HL60 cells. *J Biol Chem*. 2006; 281:35931–35941. [PubMed: 16990258]
- Servant G, Weiner OD, Neptune ER, Sedat JW, Bourne HR. Dynamics of a chemoattractant receptor in living neutrophils during chemotaxis. *Mol Biol Cell*. 1999; 10:1163–1178. [PubMed: 10198064]
- Thelen M, Stein JV. How chemokines invite leukocytes to dance. *Nat Immunol*. 2008; 9:953–959. [PubMed: 18711432]
- Ueda Y, Neel NF, Schutyser E, Raman D, Richmond A. Deletion of the COOH-terminal domain of CXC chemokine receptor 4 leads to the down-regulation of cell-to-cell contact, enhanced motility and proliferation in breast carcinoma cells. *Cancer Res*. 2006; 66:5665–5675. [PubMed: 16740704]
- Walker GM, Sai J, Richmond A, Stremier M, Chung CY, Wikswo JP. Effects of flow and diffusion on chemotaxis studies in a microfabricated gradient generator. *Lab Chip*. 2005; 5:611–618. [PubMed: 15915253]
- Yu Y, Su Y, Opalenik SR, Sobolik-Delmaire T, Neel NF, Zaja-Milatovic S, Short ST, Sai J, Richmond A. Short tail with skin lesion phenotype occurs in transgenic mice with keratin-14 promoter-directed expression of mutant CXCR2. *J Leukoc Biol*. 2008; 84:406–419. [PubMed: 18505935]

Figure 15.1.

Depiction of CXCR2 chemosynapse. CXCR2 receptor signaling initiated upon CXCL8 binding to the receptor continues through the process of endocytic trafficking through the association of a variety of adaptor proteins to the chemokine receptor. The various signaling and adaptor proteins that are known to bind CXCR2 include $G_{i\alpha\beta\gamma}$, GRK2, β -arrestin 2, AP-2, clathrin, HIP, Rab11-FIP2, and novel proteins X and Y identified through the proteomic approach. *Italicized proteins: CXCR2-interacting proteins identified in Ann Richmond's laboratory.*

Figure 15.2.
Flow chart of the proteomic approach employed in the characterization of the CXCR2 chemosynapse.

Figure 15.3.

Gradient formation in microfluidic gradient device. FITC-dextran gradient was formed by a constant flow of 100 mg/ml FITC-dextran (MW 10,000) in Hank's buffer, and buffer alone driven by a syringe pump running at 1 ml/min. The fluorescent images were taken at various locations along the main channel. The intensity of the fluorescence was quantitated across the channel for each image and shown in the plots. (From Walker, G. M., Sai, J., Richmond, A., Stremmler, M., Chung, C.Y., and Wikswo, J. P. (2005). Effects of flow and diffusion on chemotaxis studies in a microfabricated gradient generator. *Lab Chip* 5, 611–618, with permission by The Royal Society of Chemistry, <http://dx.doi.org/10.1039/b417245k>.)