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Characterization of the Coca Chemokine Receptor Four Agonist Activity of Ubiquitin

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LOYOLA UNIVERSITY CHICAGO

CHARACTERIZATION OF THE CXC CHEMOKINE RECEPTOR FOUR AGONIST ACTIVITY OF UBIQUITIN

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN MOLECULAR BIOLOGY

BY

DANIEL M. STAREN

CHICAGO, IL

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

LIST OF FIGURES vi

ABSTRACT viii

CHAPTER ONE: INTRODUCTION 1
G-protein coupled receptors 1
Chemokine receptors 3
CXC Chemokine receptor 4 (CXCR4) 4
CXCR4 ligands 5
CXCR4 signaling 8
Function of CXC chemokine receptor 4 10
Rationale of study 12

CHAPTER TWO: HYPOTHESIS AND SPECIFIC AIMS 13

CHAPTER THREE: MATERIALS AND METHODS 14
Materials 14
Antibodies 17
Cells 18
Experimental Methods 18
PBMC isolation 18
Trypan blue cell viability assay 19
Indirect competitive ubiquitin ELISA 19
Protein kinase phosphorylation array 20
Preparation of cell lysates 21
Protein quantification 21
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 21
Western blot 22
Filter migration assay 23
Statistics 24

CHAPTER FOUR: RESULTS 25
Specific Aim 1: To assess and compare the activation of important intracellular signal transduction pathways upon CXCR4 activation with SDF-1α and ubiquitin 25
Aim 1A. To screen the phosphorylation status of multiple MAPKs after CXCR4 activation with ubiquitin and SDF-1α utilizing a human phospho-MAPK array 26
Aim 1B. To confirm positive signals from MAPK array by Western blotting 29
Aim 1C. To define and compare the time course of phosphorylation of the identified serine/threonine protein kinases after CXCR4 activation with SDF-1α and ubiquitin 31
Specific Aim 2: To determine the effects of ubiquitin and SDF-1α on chemotaxis
  Aim 2A. To establish and optimize a chemotaxis assay 33
  Aim 2B. To determine and compare chemotactic effects of ubiquitin and SDF-1α 38
  Aim 2C. To evaluate the signaling events leading to chemotaxis induced by ubiquitin and SDF-1α 41

CHAPTER FIVE: DISCUSSION 44

REFERENCE LIST 50

VITA 62
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GPCRs can signal through two distinct signaling pathways</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>Two site binding model of the chemokine – receptor interaction</td>
<td>4</td>
</tr>
<tr>
<td>3.</td>
<td>Structural requirements of SDF-1α for CXCR4 binding and activation</td>
<td>6</td>
</tr>
<tr>
<td>4.</td>
<td>Molecular basis of the CXCR4 agonist activity of ubiquitin</td>
<td>7</td>
</tr>
<tr>
<td>5.</td>
<td>Structural requirements of ubiquitin for CXCR4 binding and activation</td>
<td>8</td>
</tr>
<tr>
<td>6.</td>
<td>G-protein coupled receptor mediated serine/threonine protein kinase activation</td>
<td>10</td>
</tr>
<tr>
<td>7.</td>
<td>Cell number and percent cell viability of THP-1 cells during culture</td>
<td>25</td>
</tr>
<tr>
<td>8.</td>
<td>Ubiquitin concentrations in THP-1 cell culture supernatants supplemented with or without serum</td>
<td>26</td>
</tr>
<tr>
<td>9.</td>
<td>Protein kinase phosphorylation after stimulation of THP-1 cells with ubiquitin and SDF-1α</td>
<td>27/28</td>
</tr>
<tr>
<td>10.</td>
<td>CXCR4 induced protein kinase phosphorylation</td>
<td>30</td>
</tr>
<tr>
<td>11.</td>
<td>Time course of CXCR4 induced protein kinase phosphorylation</td>
<td>32</td>
</tr>
<tr>
<td>12.</td>
<td>SDF-1α/Ubiquitin induced chemotaxis</td>
<td>33</td>
</tr>
<tr>
<td>13.</td>
<td>CXCR4 mediated chemotaxis</td>
<td>35</td>
</tr>
<tr>
<td>14.</td>
<td>Filter migration assay-filter plate pore size optimization</td>
<td>36</td>
</tr>
<tr>
<td>15.</td>
<td>Filter migration assay-cell incubation time optimization</td>
<td>37</td>
</tr>
<tr>
<td>16.</td>
<td>Filter migration assay-cell number optimization</td>
<td>38</td>
</tr>
<tr>
<td>17.</td>
<td>Filter migration assays performed under optimized conditions</td>
<td>39</td>
</tr>
</tbody>
</table>
18. Filter plate pore size optimization of migration assays with freshly isolated leukocytes 40

19. Filter migration assays with PBMCs 41

20. Intracellular signals responsible for CXCR4 mediated chemotaxis THP-1 cell 42

21. Intracellular signals responsible for CXCR4 mediated chemotaxis PBMC. 43

22. Working hypothesis for differential signaling through CXCR4 activation with ubiquitin and SDF-1α. 49
ABSTRACT

Ubiquitin has previously been identified as another natural agonist of CXC chemokine receptor 4 (CXCR4). In addition, recent evidence suggests that ubiquitin may activate CXCR4 through a binding site on the receptor, which is distinct from the binding site for the cognate ligand stromal cell-derived factor (SDF)-1α. The cellular consequences of ubiquitin induced CXCR4 activation, however, are still poorly defined and a side-by-side comparison of CXCR4 mediated functions after activation with SDF-1α and ubiquitin is lacking. Such information will be instrumental to better understand the physiological function of CXCR4 and to further define its role as a therapeutic target in various disease processes. Accordingly, the aim of this study was to determine and compare CXCR4 mediated effects on important signal transduction pathways and chemotaxis, a key function of CXCR4, upon receptor activation with ubiquitin and SDF-1α. Utilizing a MAPK array in combination with Western blot experiments, it is shown that activation of CXCR4 with ubiquitin and SDF-1α in THP-1 cells leads to increased phosphorylation of extracellular signal-related kinase (ERK) 1/2, ribosomal S6 kinase (RSK) 1, and protein kinase B (Akt). Analyses of the time progression of the MAPK phosphorylation revealed that both ligands induced a comparable degree of MAPK phosphorylation, which occurred transiently after activation of CXCR4 with ubiquitin and was sustained for at least 30 minutes with SDF-1α. To assess CXCR4 mediated chemotaxis, a filter migration assay was established and optimized. It is shown that THP-1 cells and human peripheral blood mononuclear cells
migrate dose dependently towards ubiquitin and SDF-1α. Under optimized conditions, ubiquitin was 4-5 times less efficacious than SDF-1α in promoting chemotaxis, but of similar potency. Pharmacological inhibition of signal transduction molecules that are known to be involved in the regulation of CXCR4 mediated chemotaxis resulted in similar effects on ubiquitin and SDF-1α induced chemotaxis in THP1 cells and PBMCs. This suggests that both ligands rely on a similar pattern of intracellular signaling to induce chemotaxis. In conclusion, activation of CXCR4 with ubiquitin and SDF-1α results in similar intracellular signaling events and functional consequences. With activation of CXCR4 by ubiquitin, however, serine/threonine protein kinase phosphorylations occurred more transiently, which could account for its weaker chemotactic activity, as compared with SDF-1α. Thus, ubiquitin appears to be a weaker CXCR4 agonist than SDF-1α, which may correspond to differential CXCR4 signaling mediated via distinct ligand binding sites on the receptor.
CHAPTER ONE
INTRODUCTION

G-protein coupled receptors

G-protein coupled receptors (GPCRs) are a large superfamily of transmembrane spanning receptor proteins found in the human genome and are present in all eukaryotes (1-3). The superfamily is further divided into three main sub-families: the most prominent being rhodopsin related (type A), receptors related to secretin/calcitonin (type B) and receptors related to the metabotropic receptors (type C) (4). All GPCRs have an extracellular N-terminal segment, a seven transmembrane spanning region, which form the transmembrane core, three extra cellular loops, three cytoplasmic loops, and an intracellular C-terminal segment. Ligand binding to the extracellular region causes a receptor conformational change (5,6) and GPCRs fluctuate between an active and inactive conformation (11). In the active state, they utilize several molecular mechanisms to induce a cellular response through two distinct signaling pathways (Figure 1-1): G-protein mediated signaling, the predominant signaling pathway, and G-protein independent signaling (7-9).

In the G-protein mediated signaling pathway, a heterotrimeric G-protein complex comprised of αβγ subunits are in an inactive state at the receptor and the localization is a result of the Gα subunit being bound to GDP (46-48). The binding of an agonist induces an activating conformational receptor shift (49, 50). This shift enables GPCRs to act as a guanine nucleotide exchange factor, promoting the heterotrimeric G-protein activation resulting in GDP to GTP exchange at the Gα subunit (49,50) Subsequently, there is a conformational change in the heterotrimeric G-protein complex resulting in Gα and Gβγ
disassociation and activation of various second messengers leading to a functional cellular response (49, 50).

The G-protein independent signaling pathway is well established through β-arrestin mediated signaling (8, 45, 60-63). This pathway requires receptor phosphorylation, classically by G-protein-coupled receptor kinases (GRKs), but also by cAMP dependent kinase A (PKA) or PKC on the third intracellular loop of the GPCR enabling β-arrestin to target the receptor (8, 60). Upon receptor phosphorylation, arrestin-2 and/or arrestin-3 are then able to bind; preventing further G-protein mediated signaling (8,45,60-63). This pathway plays a role in regulating Gα interactions and is predominantly responsible for receptor desensitization, internalization, and degradation (29).

Figure 1-1: GPCRs can signal through two distinct signaling pathways: 1. G-protein (left), GPCR signals through G-protein activation. 2. β-arrestin (right), GPCR signals through β-arrestin.
Functional selectivity (biased agonism), a phenomenon that occurs when a ligand preferentially stabilizes an active conformation promoting only a subset of signaling effects (11-16), has gained particular attention with respect to GPCR mediated signaling. GPCRs are capable of adopting different active conformational states and this could lead to preferential (biased) G-protein dependent or independent signaling (10). While most GPCR-mediated biased signaling has been observed using synthetic agonists (14), C-C motif chemokine 19 and 21 (CCL19/21), natural agonists for GPCR C-C chemokine receptor 7 (CCR7), have been shown to produce G-protein signaling with equal potency. CCL19 alone causes receptor agonist-dependent phosphorylation and β-arrestin recruitment to terminate G-protein signaling (14). There is therapeutic promise to understanding GPCR mediated biased signaling because it broadens the scope of drug discovery tailored to receptor ligand specificity.

**Chemokine receptors**

Chemokine receptors are a member of the rhodopsin related (type A, subfamily A1 and A2) GPCR superfamily and are involved in immune system functions (17-19). The interaction of chemokine receptors with chemokines regulates cell migration for multiple cell types including leukocytes (100, 101). The receptors are classified on the basis of the ligand specificity for a particular chemokine class. The four main classes of chemokines are CC, CXC, CX3C and C, according to the number and spacing of conserved cysteines in the amino acid sequence (20, 21). Structure-function studies have shown that the chemokine-receptor interaction follows a 2-site binding model (Figure 1-2). In this model, the
extracellular N-terminal domain of the chemokine receptor (site I) initially interacts with a ligand for binding. The ligand then interacts with the chemokine receptor at a different receptor extracellular site (site II) to induce an activation signal (24, 43). It is known that there are 19 human chemokine receptors and over 50 distinct chemokines. As a result, chemokine receptors, like CCR7, are promiscuous in that they can bind more than one chemokine (14, 22, 23).

![Chemokine binding model](image)

**Figure 1-2:** Two site binding model of the chemokine – receptor interaction. Depicted is a model of the chemokine-chemokine receptor interaction. Chemokines binds to a site on the N-terminal domain of a chemokine receptor and then to a different site on the chemokine receptor to induce activation.

**CXC chemokine receptor 4 (CXCR4)**

CXC chemokine receptor 4 (CXCR4) was initially discovered as an orphan receptor leukocyte-derived seven-transmembrane domain protein (LESTR) (84) and is encoded by the CXCR4 gene (25, 26). Recently, small protein antagonists were used to determine the crystal structure of CXCR4 and it provided additional evidence CXCR4 is capable of existing as a homo- and hetero-dimer on the cell surface (102). The currently known endogenous CXCR4
ligands are stromal-cell derived factor 1 (SDF-1α), macrophage migration inhibitory factor (MIF) and ubiquitin (28-32). Site-directed mutagenesis experiments suggest CXCR4 may have 2 separate ligand binding sites for ubiquitin and SDF-1α (44).

**CXCR4 ligands**

*Stromal-cell derived factor-1 alpha (SDF-1α)*

Human stromal cell-derived factor 1 alpha (SDF-1α), commonly referred to as chemokine (C-X-C motif) ligand (CXCL12), is a small (8 kDa) protein that is ubiquitously expressed in many tissues such as the liver, pancreas, and spleen (105). The CXCL12 gene encodes different isoforms of SDF-1, SDF-1α and SDF-1β, and the two splice variants arise as a result of alternative splicing (103). SDF-1α exists as a monomer but can homo-dimerize under non-acidic pH conditions in the presence of stabilizing counterions and then displays partial agonist activity (104). While it has been well described that SDF-1α is the cognate ligand for CXCR4 and binds with high affinity (Kd: 1.5 – 24 nM) (106, 107), SDF-1α is promiscuous in that it has also been described as a CXCR7 agonist (33). Mutagenesis experiments to synthesize SDF-1α analogs and subsequent calcium flux and binding assays suggest SDF-1α utilizes the classical two-site binding mechanism of chemokine-receptor interactions (33-35, 36-39). The docking domain of SDF-1α interacts with the N-terminus of CXCR4 to bind to the receptor (site I) and the flexible N-terminus of SDF-1α interacts with the central binding pocket of CXCR4 (site II) to activate the receptor (40) (Figure 1-3).
**Figure 1-3: Structural requirements of SDF-1α for CXCR4 binding and activation:** Depicted is a ribbon diagram of the structure of SDF-1α. The blue box in the middle of the image depicts the RFFESH residues on SDF-1α that interact with CXCR4 for docking. The blue box to the left depicts the K1 and P2 residues on SDF-1α that interact with CXCR4 for receptor activation.

*Ubiquitin*

Ubiquitin is a small (8.5 kDa), heat stable protein present in every eukaryotic cell (41) encoded by 4 mammalian genes. Recent mutagenesis experiments suggest *UBA52* and *RPS27A* genes code for mono ubiquitin and *UBB* and *UBC* genes code for polyubiquitin precursor proteins (108). Ubiquitin is highly conserved and is most known for its intracellular functions in the ubiquitin-proteasome pathway of protein degradation (42). Ubiquitin is also present outside the cell and kinetic binding experiments suggest ubiquitin, like SDF-1α, binds to CXCR4 with a Kd of ~100 nM (28). Ubiquitin is distinct from SDF-1α, however, because it does not share CXCR7 as a receptor (43). Nuclear magnetic resonance (NMR) spectroscopy with the N-terminal domain of CXCR4, receptor binding and signaling studies in presence of antibodies directed against specific regions of CXCR4 suggesting ubiquitin, unlike SDF-1α, does not interact with the CXCR4 N-terminus for receptor docking (43).
Subsequent site-directed mutagenesis of CXCR4 followed by receptor binding experiments suggest there are distinct sites on the receptor important for ubiquitin-CXCR4 binding, but not for SDF-1α (43) (Figure 1-4).

Figure 1-4: Molecular basis of the CXCR4 agonist activity of ubiquitin. Depicted is a ubiquitin-CXCR4 interaction model. Ubiquitin does not follow the typical two-site binding model of chemokine receptor interactions because the receptor N-terminus is not required for ubiquitin binding to CXCR4.

Furthermore, experiments with ubiquitin point mutants suggest that the binding of ubiquitin to CXCR4 follows the two-site model of the structure function relationship of chemokines (44) (Figure 1-5).

Figure 1-5: Structural requirements of ubiquitin for CXCR4 binding and activation: Depicted is a ribbon diagram of the structure of ubiquitin. The 2 blue boxes correspond to the blue arrows and show residues F4 and V70 interact with CXCR4 and are responsible for receptor docking. The red arrow corresponds to the red box on the model and shows G75 and G76 interact with CXCR4 for receptor activation.
Macrophage Migration Inhibitory factor (MIF)

Macrophage migration inhibitory factor is a lymphokine that is encoded by the \textit{MIF} gene and is a trimer comprised of three identical subunits (109, 110). It has been suggested MIF is a regulator of innate immunity by promoting pro-inflammatory functions of immune cells, specifically macrophage migration (111). While it has been described MIF is a ligand for CXCR4, MIF is promiscuous in that it has also been shown to bind CD74 and CXCR2 (112). Information on the structure function relationship of the MIF-CXCR4 interaction is currently not available.

**CXCR4 signaling**

CXCR4 signals through G-protein dependent and independent signaling upon ligand binding and signaling is regulated by three processes: receptor desensitization, receptor internalization, and receptor degradation (115). CXCR4 is a G\(_{\alpha_i}\) coupled receptor and pertussis toxin, a potent G\(_{\alpha_i}\) protein inhibitor, has been pivotal in elucidating the distinct signaling pathways (115). Through G-protein dependent signaling, it has been described CXCR4 can inhibit adenylyl cyclase, the enzyme that synthesizes cyclic adenosine monophosphate (cAMP) (115). Other resulting intracellular signals of G-protein mediated signaling are calcium fluxes and activation of focal adhesion components, protein kinase C (PKC), phospholipase C (PLC) as well as mitogen-activated protein kinases (MAPK p42/44) and phosphoinositide 3-kinase (PI3-K)/Protein Kinase B (PKB/Akt) activation (29, 50-52, 53-59).
As previously described, G-protein independent signaling is thought to be mainly responsible for receptor desensitization. There is evidence, however, that the β-arrestin pathway also results in other signal transduction cascades, leading to cellular responses such as serine/threonine specific protein kinase activation (45, 61-63).

Serine/Threonine specific protein kinases are cell-signaling enzymes that phosphorylate the hydroxyl group of serine and threonine protein residues (64). These kinases can respond to extracellular stimuli and ultimately regulate a variety of cellular functions (64). There are many sub-classifications of these kinase types including mitogen-activated protein kinase (MAPK) and protein kinase B (Akt).

Mitogen-activated protein kinases (MAPKs) are signaling molecules involved in migration and survival. Activation of CXCR4 leads to G-protein mediated phosphorylation of the MAPK extracellular signal-related kinase (ERK) 1/2 (65-67) and subsequently ERK 1/2 phosphorylates ribosomal S6 kinase (RSK) 1 (68) (Figure 1-6). It has also been described cell migration can be regulated by CXCR4 induced ERK1/2 activation (69,70).

Protein kinase B (Akt) is a signaling molecule that is also involved in cell survival and migration (71,72). Akt has been implicated to be activated through CXCR4 mediated G-protein signaling (66) (Figure 1-6). Akt also can regulate CXCR4 mediated cell migration (69,72).
Figure 1-6: G-protein coupled receptor mediated serine/threonine protein kinase activation: Left: G proteins function as guanine nucleotide exchange factors and act on Ras. Ras then binds Raf and initiates a phosphorylation cascade, acting on mitogen-activated protein kinase kinase MEK-1/2 and then activating ERK-1/2. Right: G proteins function as guanine nucleotide exchange factors and act on PI3-K. PI3-K then phosphorylates the 3-OH position of phosphatidylinositol (4,5)-bisphosphate (PIP2) resulting in phosphatidylinositol (3,4,5)-trisphosphate PIP3. PIP3 then acts as a lipid anchor for phosphoinositide-dependent kinase-1 (PDK1) and Akt. Akt is then phosphorylated by PDK1 and PDK2.

Function of CXC chemokine receptor 4

CXCR4 is abundant in leukocytes and in most human tissues and plays pleiotropic roles as an immune modulator (27). CXCR4 is involved in platelet aggregation and reduces the production of tumor necrosis factor-α (TNF-α) and enhances interleukin (IL) 10 production under inflammatory conditions (93-95). CXCR4 is crucial for normal development and CXCL12 (SDF-1α) and CXCR4 gene knockouts in mice models are embryonic lethal because the mice display defective vasculogenesis, neurogenesis, hematopoiesis, and cardiogenesis (73-77, 89, 90). It has also been described ubiquitin gene
knockouts are embryonic lethal but it is unknown if this is due to lack of ubiquitin-CXCR4 binding (108). There are many disease states associated with CXCR4 dysregulation:

When CXCR4 is in an unbound state, the receptor acts as a co receptor for human immunodeficiency virus (HIV-1) and has been described as a drug target for HIV-1 infection (78,79). The interaction between SDF-1α and CXCR4 has been shown to interfere with productive cellular entry of HIV-1 (34,35,80). This phenomenon does not occur when ubiquitin interacts with CXCR4 (43).

CXCR4 is highly expressed on 23 different cancer cell types (115) including prostate, gastric, lung, and breast (85-88). CXCR4 has previously been implicated as a drug target for cancer (73,78) given that the regulation of chemotaxis is regarded as a key function of the CXCR4 signaling axis. Chemotaxis is the directed movement of cells toward a chemical gradient that is dependent on cell type and environmental conditions (81). As a result, CXCR4 expression may correlate with metastases, which is the spread of cancer from one part of the body to another (115). Gene expression profiles and in vivo studies suggest there are multiple cancer cell types that metastasize to areas that express high levels of SDF-1α (116,117). While variation exists between cancer cell types, CXCR4 mediated chemotaxis is regulated by a set of intracellular signal transduction pathways including Gαi, PLC, PI3K/Akt and MAPK Erk1/2 activation (69,70,72,82,83).

Warts, hypogammaglobulinemia, infection, and myelokathexis (WHIM) syndrome is a dominant inherited primary immunodeficiency disorder of the CXCR4 gene (96). Further genetic evidence suggests a single amino acid substitution in the carboxy-terminus of CXCR4 can lead to truncation of the C-terminal tail and elimination of possible
phosphorylation sites. The C-terminal tail truncation may lead to a hyperactive receptor, because GRKs can not phosphorylate the receptor and induce desensitization. This ultimately results in an increased responsiveness to SDF-1α (97,99). The lack of receptor desensitization (113) results in enhanced cellular chemotaxis (113), enhanced calcium flux (114), and a decrease in SDF-1α promoted internalization (113,114). The name of the disease describes the significant clinical symptoms (98).

**Rationale of study**

Biased agonists result in biased intracellular signaling and different biological functions through the same receptor, but the mechanisms leading to these effects are largely unknown. It is known that ubiquitin and SDF-1α bind to CXCR4 at distinct ligand binding sites (43). They do not, however, share CXCR7 as a receptor and ubiquitin also does not interfere with HIV-1 entry (43). We therefore propose that distinct ligand binding sites for ubiquitin and SDF-1α on CXCR4 are a structural correlate for biased agonism and result in different biological responses. The cellular consequences of ubiquitin induced CXCR4 activation, however, are still poorly defined and a side-by-side comparison of CXCR4 mediated functions after activation with SDF-1α and ubiquitin is lacking. This information will be instrumental to better understand the physiological roles of CXCR4 and to further define its role as a therapeutic target in various disease processes.
CHAPTER TWO
HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

Based on the foregoing literature review, we proposed the hypothesis that distinct ligand binding sites for ubiquitin and SDF-1α on CXCR4 are a structural correlate for biased agonism and result in differential biological responses upon activation of CXCR4 with ubiquitin and SDF-1α.

To address this hypothesis, we developed the following specific aims:

Aim 1. To assess and compare the activation of important intracellular signal transduction pathways upon CXCR4 activation with SDF-1α and ubiquitin.

Aim 1A. To screen the phosphorylation status of multiple MAPKs after CXCR4 activation with ubiquitin and SDF-1α utilizing a human phospho-MAPK array

Aim 1B. To confirm positive signals from the MAPK array by Western blotting

Aim 1C. To define and compare the time course of phosphorylation of the identified MAPKs after CXCR4 activation with SDF-1α and ubiquitin

Aim 2. To determine and compare the effects of ubiquitin and SDF-1α on chemotaxis

Aim 2A. To establish and optimize a chemotaxis assay

Aim 2B. To determine and compare chemotactic effects of ubiquitin and SDF-1α

Aim 2C. To evaluate the signaling events leading to chemotaxis induced by ubiquitin and SDF-1α.
CHAPTER THREE
MATERIALS AND METHODS

Materials:

**Ubiquitin**: Ubiquitin was purchased from Boston Biochem (Cat # U-100) and suspended in 10 mM phosphate-buffered saline solution (PBS, containing 138 mM NaCl, 2.7 mM KCl, 2 mM KH$_2$PO$_4$, and 10 mM Na$_2$HPO$_4$, pH 7.4, Sigma-Aldrich (Cat # P3813)). The working concentration varied by experiment.

**N-Terminal Biotin labeled ubiquitin**: N-terminal biotin labeled ubiquitin was purchased from Boston Biochem (Cat # UB-560) and suspended in PBS.

**SDF-1α**: SDF-1α was purchased from Peprotech (Cat # 300-28A) and was suspended in PBS. The working concentration varied by experiment.

**Fetal Bovine Serum (FBS)**: FBS was purchased from Sigma-Aldrich (Cat # F6765).

**Bovine Serum Albumin (BSA)**: BSA was purchased from Sigma-Aldrich (Cat # 9048-46-8).

**Histopaque density gradient cell separation media (Histopaque)**: Histopaque was purchased from Sigma-Aldrich (Cat # 10771).

**Trypan blue**: Trypan blue was purchased from Lonza (Cat # 17-942E)

**ELISA plate strips**: ELISA plate strips were purchased from Greiner Bio-One (Cat # 762061).
**3,3’,5’5, Tetramethylbenzidine (TMB):** TMB was purchased from Sigma-Aldrich (Cat # T0440).

**Super Signal West Dura Extended Duration Substrate (Substrate detection solution):** Substrate detection solution was purchased from Thermo Scientific (Cat # 34075).

**Radioimmunoprecipitation assay (RIPA) Lysis and Extraction Buffer (RIPA buffer):** RIPA buffer was purchased from Thermo Scientific (Cat # 89900).

**Sodium orthovanadate (Vanadate):** Vanadate was purchased from New England BioLabs (Cat # P0758L) and suspended in double distilled water (ddH2O) to a stock concentration of 100 mM. The working concentration used was 1 mM.

**Phenylmethylsulfonyl Fluoride (PMSF):** PMSF was purchased from Santa Cruz Biotechnologies (Cat # SC-3597) and suspended in methanol to a stock concentration of 10 mM. The working concentration used was 500 µM.

**Precision Plus Protein Dual Color standard (Protein standard):** Protein standards were purchased from Bio Rad (Cat # 161-0374).

**Laemmli sample buffer:** Laemmli sample buffer was purchased from Bio Rad (Cat # 161-0737).

**Polyvinylidene fluoride (PVDF) Membranes (PVDF membranes):** PVDF membranes were purchased from Bio Rad (Cat # 162-0174).

**Accustain Wright-Giemsa stain:** Wright-Giemsa stain was purchased from Sigma-Aldrich (Cat # WG128).

**Pertussis Toxin (catalyzes the ADP-ribosylation of α subunit of heterotrimeric guanine nucleotide regulatory proteins; Gαi proteins remain in GDP-bound “inactive” state):** Pertussis Toxin (from *Bordetella Pertussis*) was purchased from Sigma-Aldrich (Cat
# P7208) and suspended in 500 µL ddH2O to a stock concentration of 0.1 mg/ml. The working concentration used for cell treatments was 100 ng/mL and the incubation time was 2 hours, followed by a wash with PBS before experimentation.

**U73312 (Inhibits the coupling of G protein-PLC activation, remaining unaffected by production of cAMP):** U73312 was purchased from Sigma-Aldrich (Cat # U6756) and was suspended in dimethyl sulfoxide (DMSO) from Sigma-Aldrich, (Cat # 67-68-5) to a stock concentration of 10 mM. The working concentration used for cell treatments was 5 µM, 0.1% DMSO and the incubation time was 30 minutes, followed by a wash with PBS before experimentation.

**U73343 (Inactive analog of U73312; used as a negative control):** U73343 was purchased from Sigma-Aldrich, (Cat # U6881) and was suspended in DMSO to a stock concentration of 10 mM. The working concentration used for cell treatment was 5 µM, 0.1% DMSO and the incubation time was 30 minutes, followed by a wash with PBS before experimentation.

**U0126 (Selectively binds to and inhibits MEK 1/2 activity thereby preventing ERK 1/2 phosphorylation and kinase activity):** U0126 was purchased from Cell Signaling (Cat # 9903) and suspended in DMSO to a stock concentration of 10 mM. The working concentration used for the cell treatment was 10 µM, 0.1% DMSO and the incubation time was 30 minutes, followed by a wash with PBS before experimentation.

**LY294002 (Selectively binds to and inhibits PI3 Kinase thereby preventing Akt phosphorylation and kinase activity):** LY294002 was purchased from Cell Signaling (Cat # 9901S) and was suspended in DMSO to a stock concentration of 10 mM. The working
concentration used for cell treatments was 50 µM, 0.1% DMSO and the incubation time was 1 hour, followed by a wash with PBS before experimentation.

**AMD3100 (Highly selective CXCR4 antagonist):** AMD3100 was purchased from Sigma-Aldrich (Cat # A5602) and suspended in PBS to a stock concentration of 50 mM. The working concentration used for all cell treatments was 10 µM, followed by a wash with PBS before experimentation.

**Trichostatin A [(TSA), Histone deacetylase inhibitor]:** TSA was purchased from Selleck Chem (Cat # S1045) and suspended in DMSO to a stock concentration of 1 mM. The working concentration used for cell treatments was 20 µM, 0.1% DMSO and the incubation time was 18 hours, followed by a wash with PBS before experimentation.

**Antibodies:**

The primary antibodies used for Western blots were anti-phospho p90 ribosomal S6 kinase (RSK1) (Ser-380) rabbit IgG, anti-phospho-ERK1 (Thr-202/Tyr-204)/ERK2 (Thr-185/Tyr-187) rabbit-IgG and anti-phospho-Akt pan (Ser-473) rabbit IgG (all from R&D Systems), each diluted 1:2000 in PBS-1% Casein blocker solution (BioRad). The secondary antibody used was anti-rabbit IgG horseradish peroxidase (HRP)-linked whole antibody (Amersham Biosciences) and was diluted 1:5000 in PBS-1% Casein blocker solution.

To control for protein loading, 1:1000 anti- Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse (Applied Biosystems) was used. The secondary antibody used was anti-mouse IgG HRP-linked whole antibody (Amersham Biosciences) diluted 1:5000 in PBS-1% Casein blocker solution.
The antibodies used for the competitive ubiquitin enzyme-linked immunosorbent assay (ELISA) were anti-ubiquitin and anti-biotin peroxidase conjugate both purchased from Sigma-Aldrich (Cat # U5379 and Cat # A4541, respectively) diluted 1:500 and 1:1000 in PBS-1% Casein blocker solution, respectively.

Cells:

**THP-1:** THP-1 cells are a human acute monocytic leukemia cell line obtained from American Type culture collection depository (ATCC) (Cat # TIB-202). Cells were cultured in Roswell Park Memorial Institute medium from Sigma-Aldrich (RPMI 1640), (Cat # R0883) supplemented with 10% FBS and 2% Penicillin and Streptomycin from Sigma-Aldrich (Cat # P4333). Cells were grown in a 37°C sterile, 95% humidified incubator supplemented with 5% CO2. Cell were split into two parts and incubated in fresh media every 2-3 days.

**Peripheral blood mononuclear cell (PBMC):** PBMCs, from human peripheral venous blood, that were drawn from healthy human donors of both sexes were isolated according to an IRB approved protocol (118).

Experimental Methods:

**PBMC isolation:** Histopaque solution was allowed to equilibrate to room temperature. Three milliliters of the histopaque solution were pipetted into 15-ml centrifuge tubes. Then, 3 mL of freshly drawn blood were pipetted into each centrifuge tube with 3 mL histopaque, ensuring the blood rested on the top of the histopaque solution. The centrifuge tubes were then centrifuged at 1400 revolutions per minute (RPM) for 30 minutes. After centrifugation, the top layer of serum was aspirated as not to disturb the middle white layer
of PBMCs. The PBMCs were then aspirated and placed into separate centrifuge tubes and washed with 10 mL of PBS. The tubes were then spun at 1100 RPM for 10 minutes. After centrifugation, the PBS solution was aspirated as not to disrupt the cell pellet. The cells were then washed again with 5 mL PBS and combined into a larger 50 mL conical tube. The 50 mL conical tube was spun at 1100 RPM for 10 minutes. The PBS was aspirated and the PBMCs were suspended in 1 mL PBS; cells were then counted on a hematocytometer and diluted as required.

**Trypan Blue Cell Viability assay:** The trypan blue viability assay was performed according to manufacturer recommendations (Lonza). The culture samples were prepared at a ratio of 1 part 0.4% trypan blue stain: 1 part culture sample; samples were then incubated at room temperature for 15 minutes. The cells were then counted under a microscope at 10x magnification on a hematocytometer. Non-viable cells stained blue and viable cells remained unstained. The percentage of viable cells is approximated by dividing the total number of viable cells by the total number of cells and then by multiplying by 100.

**Indirect Competitive Ubiquitin ELISA:** The indirect competitive ubiquitin ELISA was performed as previously described (119). High Binding ELISA plate strips were coated with anti-ubiquitin and incubated for 18 hours at 4°C. The plates were washed three times with 200 µL PBS per well and were incubated with blocking buffer (1% BSA-PBS) for 1 hour. After washing three times, 60 µL of 2 µg/mL ubiquitin standard solution or 60 µL of samples were mixed with 60 µL of 0.2 ng/mL biotinylated ubiquitin and placed in the plates. Each sample was tested in eight dilutions. Dilutions for the standard curve and test samples were prepared in blocking buffer. After incubation for 2 hours, the plates were washed with 200 µL PBS, 0.01% Tween-20 (PBST) per well. Then, 100 µL of anti-biotin peroxidase
conjugate was added to each well. After one-hour incubation, the plates were washed again with PBST and 100 µL of TMB was added to each well. The reaction was stopped after 15 minutes with 100 µL of 2N hydrochloric acid (HCl) added to each well. Optical densities were measured using a micro plate Biotek Synergy2 plate reader (test filter, 450 nm; reference filter, 540 nm). The ubiquitin concentration in the test sample was calculated with the Gen5 software for Windows program, Version 1.05.11 (Bio-Tek Instruments, Inc.), from a four-parameter logistic fit using ubiquitin as the standard (0-2000 ng/mL). The correlation coefficients for each standard curve were 0.998-1.

**Protein Kinase Phosphorylation Array**: Screening of the phosphorylation status of various protein kinases after ubiquitin and SDF-1α stimulation was performed in THP-1 cells according to manufacturer’s instructions using a human phospho mitogen activated protein kinase (MAPK) antibody array from R&D Systems. 10⁷ THP-1 cells were stimulated with 1 µM of ubiquitin or SDF-1α for 10 minutes at 37°C. Unstimulated cells served as controls in parallel experiments. Cells were lysed in 1 mL of lysis buffer on ice for 30 minutes, centrifuged (1,400 x g for 5 minutes) and the supernatant (=lysate) collected. 250 uL of the cell lysate was incubated with a pre-wet array membrane for 15 hours at 4°C. After three washing steps at room temperature, array membranes were incubated with the diluted antibody cocktail for 2 hours at room temperature, washed and incubated with strepavidin-HRP solution for 30 minutes at room temperature. After washing of the array membranes, substrate detection solution (prepared at a ratio of 1 luminol/enhancer solution: 1 stable peroxide buffer) was added for 5 minutes. Images of array membranes were then captured with a Chemi-doc XRS Imager [Quantity One V4.5.2 software (Bio-Rad)] for appropriate exposure times.
**Preparation of cell lysates:** Cells were washed with ice-cold PBS and centrifuged at 1400 RPM for 5 minutes. After centrifugation, the supernatant was aspirated and the cell pellet remained on ice. Cells were lysed in RIPA buffer supplemented with 1 mM vanadate and 500 μM PMSF. Lysates were vortexed immediately for 10 seconds and then placed on ice. Samples were then sonicated with a sonic demembrator, model 100 from Fisher-Scientific, 2 times for 20-second intervals on ice. Samples remained on ice for 15 minutes and were then centrifuged for an additional 15 minutes, at 14000 RPM, 4°C. The sample supernatant was then collected and placed in a clean centrifuge tube.

**Protein Quantification:** Protein concentration was determined using a modified Bradford Bio-Rad DC Protein Assay Kit II (Cat # 500-0112). A BSA standard curve was generated with concentrations ranging from 0 mg/ml to 1.42 mg/ml. 5 uL of each standard was pipetted in duplicate into a 12-well clear flat bottom ELISA strip. 5 ul of each cell extract was pipetted in duplicate into a 12-well clear flat bottom ELISA strip. Then, 25 μL of reagent A, an alkaline copper tartrate solution, and then 200 μL of reagent B, a dilute Folin Reagent, were added to each well containing a standard or sample. The plate was then incubated on a shaker for 15 minutes at room temperature. The micro plate was then placed into a micro plate Biotek Synergy2 plate reader and the absorbance was read at 750 nm. The BSA protein standard absorbance was then plotted against the BSA protein standard concentration and a standard curve was generated by linear regression analysis. Protein concentration of an unknown sample was then calculated from the standard linear regression curve.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):** SDS-PAGE was carried out using the Mini-Format Vertical Electrophoresis system from Bio-Rad,
and mini-protean TGX precast gels (8.6 cm x 6.7 cm, 0.1 cm thickness) of 4-20% polacrylamide. Then, 2-10 µg/mL of the protein samples were suspended in Laemmli sample buffer and then loaded into each lane of the mini-cast gel. A protein standard was loaded in a lane of the mini-cast gel for all SDS-PAGE experiments. The polyacrylamide gels were run at 150V in 1X Tris/Glycine/SDS running buffer from BioRad for 45 minutes or until bands reached the bottom of the gel.

**Western Blot:** PVDF membranes were presoaked for 5 seconds in 100% MeOH, and were then washed and equilibrated in transfer buffer (Milli-Q water (Millipore-Q Water Purification System, Millipore), 25 mM Tris/HCl, 192 mM glycine, 20% MeOH, pH 7.4). Extra thick mini blot size papers from BioRad were simultaneously equilibrated in transfer buffer. A transfer “sandwich” was then assembled according to standard protocol: Blot size paper, PVDF membrane, gel, blot size paper. The semi-dry transfer with transfer buffer was run at 25V for 48 minutes at room temperature. The PVDF membranes were then briefly rinsed in milli-Q water, then PBST. The membranes were then blocked in PBS-1% Casein blocker solution for 60 minutes. After blocking, the membranes were incubated with the respective primary antibodies in PBS-1% Casein blocker solution overnight at 4°C. The membranes were washed 6 times in PBST for 6 minutes and incubated with horseradish peroxidase (HRP)-linked secondary antibody diluted 1:5000 in PBS-1% Casein blocker solution for 1 hour at room temperature. The membranes were washed again, 6 times in PBST for 6 minutes per wash. After the final wash, the membranes were incubated with the substrate detection solution (prepared 1 part luminol/enhancer solution to 1 part stable peroxide buffer) for 5 minutes. Membranes were then captured with a Chemi-doc XRS.
Imager (Quantity One V4.5.2 software, Bio-Rad) for 0-60 second exposure time. Band densities were quantified using the densitometry analysis tool on the Quantity One program.

**Filter Migration Assay:** Chemotaxis was assessed using the ChemoTx 96-well cell migration system, manufactured by Neuroprobe. Each well of the lower portion of the micro plate apparatus was filled with 29 uL of the respective test solutions. The micro plate was then covered with the ChemoTx® filter. The cells were washed once with PBS, centrifuged at 1200 RPM and then the cells were re suspended in PBS to a concentration of 0-200,000 cells per 25 uL. Then 25 uL of the cell solution was placed on the filter pore for each respective well. The plate was then covered and allowed to incubate at 37ºC for 0-6 hours. The plate was removed and the top of the filter was rinsed with PBS. The filter was then removed from the micro plate and the filter was allowed to dry. Then, ice cold 100% methanol was used to rinse the lower portion of the filter plate for 20 seconds. The methanol was poured off and then the filter was allowed to dry. A 6 mL mixture of 1 part ddH2O: 1 part Wright-Giemsa stain was then prepared. The stain mixture was added to the lower portion of the filter plate. The plate was incubated at room temperature for 10-15 minutes, shaking every 2 minutes. The stain was rinsed off and the entire plate was then submerged in cool water twice using fresh water each wash. The filter plate was then allowed to dry. Each well on the lower portion of the filter plate was counted under a microscope at 400x magnification (average cell count of 3 non-overlapping fields of vision). The chemotactic index (CI), the ratio of cells that transmigrated through the filter in the presence versus the absence (=PBS/control) of the test solutions, was then calculated.
**Statistics:**

Data are expressed as mean ± standard error of the mean (SEM) of n independent experiments that were performed on different days. Differences between assays were analyzed with repeated measures analysis of variance (ANOVA) with Dunnett's multiple comparison tests as appropriate, to control for multiple testing. A two-tailed p < 0.05 was considered significant. Data were analyzed using the GraphPad-Prism 5 software.
CHAPTER FOUR

RESULTS

Specific Aim 1: To assess and compare the activation of important intracellular signal transduction pathways upon CXCR4 activation with SDF-1α and ubiquitin.

To exclude that changes of the ubiquitin concentration in the THP-1 cell culture supernatant would interfere with subsequent experiments, we measured cell viability, total cell number, and the ubiquitin concentration in the culture supernatant when cells were cultured in the presence and absence of serum for 0-72 hours. Fig. 4-1 (left panel) shows that the cell viability remained above 95% and the cell number increased when cells were cultured in the presence of serum. The cell viability decreased and the total cell number remained constant when cells were cultured without serum (Fig. 4-1 right panel).

Figure 4-1. Cell number and percent cell viability of THP-1 cells during culture. THP-1 cells were cultured for various time points, Cell number (■) and percent viability (▲) (n=3). Left: Cell number and percent cell viability when cells are cultured in the presence of serum. Right: Cell number and percent cell viability when cells are cultured without serum.
Fig. 4-2 shows that the ubiquitin concentration in RPMI media alone at the beginning of cell culture was 15±3.0 ng/mL and 40±1.28 ng/mL after supplementation with serum. There was not an observable change of the ubiquitin concentration in RPMI media during cell culture with or without serum. These results suggest that for the chosen culture conditions, changes in the ubiquitin concentration of the culture supernatant in the presence or absence of serum will not interfere with subsequent experiments.

![Graph showing ubiquitin concentrations](image)

**Figure 4-2. Ubiquitin concentrations in THP-1 cell culture supernatants supplemented with or without serum.** THP-1 cells were cultured for various time points and ubiquitin concentrations in the supernatant were measured with an indirect competitive ubiquitin ELISA (n=3). *Left:* Ubiquitin concentration of culture supernatant when cells are cultured in the presence of serum. *Right:* Ubiquitin concentration of culture supernatant when cells are cultured in the absence of serum.

**Aim 1A. To screen the phosphorylation status of multiple MAPKs after CXCR4 activation with ubiquitin and SDF-1α utilizing a human phospho-MAPK array**

To screen the phosphorylation status of multiple protein kinases after stimulating cells with ubiquitin and SDF-1α, THP-1 cells were incubated with 0 or 1 μM of ubiquitin or SDF-1α for 10 minutes at 37°C. Whole cell lysates were then probed for protein kinase phosphorylation utilizing a human phospho MAPK array. Fig. 4-3 shows representative
images of the array membranes from experiments when cells were treated with vehicle (control), ubiquitin, or SDF-1α. The numbers on the array membrane correspond to the spot positions for phosphorylated ERK1 (1), ERK2 (2), RSK 1 (3), Akt 1 (4), Akt 2 (5) and Akt pan (6). The chemiluminescence signal of each spot corresponds to the degree of phosphorylation of each protein kinase. Although there was considerable variation among the individual experiments for some of the protein kinases, cells stimulated with ubiquitin and/or SDF-1α consistently showed an increase in the densities of the spots that correspond to phosphorylated ERK1/2, RSK1, and Akt, as compared with untreated cells.

**Figure 4-3. Protein kinase phosphorylation after stimulation of THP-1 cells with ubiquitin and SDF-1α.** A human phospho MAPK array was used to screen the phosphorylation status of multiple MAPKs. Typical images of the proteome array membranes after vehicle (control), ubiquitin, and SDF-1α treatment. The numbers on the array membrane correspond to the spot positions for phosphorylated ERK1 (1), ERK2 (2), RSK 1 (3), Akt 1 (4), Akt 2 (5) and Akt pan (6).
Fig. 4-4 shows the densitometric quantification of the spot densities that correspond to each phosphorylated protein kinase after treatment of THP-1 cells with ubiquitin and SDF-1α from 4 independent experiments; the spot densities are given as normalized pixel densities (1=control, dashed line). The floating bars (white: SDF-1α treatment; grey: ubiquitin treatment) extend from the minimum to the maximum and the horizontal line shows the mean. The chemiluminescence signals that correspond to ERK1/2, RSK1, and Akt, suggest that stimulation of THP-1 cells with ubiquitin and SDF-1α result in phosphorylation of the MAPKs, as compared to the unstimulated cells.

**Figure 4-4.** Protein kinase phosphorylation after stimulation of THP-1 cells with ubiquitin and SDF-1α. A human phospho MAPK array was used to screen the phosphorylation status of multiple MAPKs. Densitometric quantification of spot densities, as in Fig. 4-3 (n=4). Spot densities are given as normalized pixel densities (1= unstimulated cells, dashed line). The bars (white: SDF-1α treatment; grey: ubiquitin treatment) extend from the minimum to the maximum; the horizontal line shows the mean.
Aim 1B. **To confirm positive signals from the MAPK array by Western blotting**

To confirm the results from the proteome array that ERK1/2, Akt, and RSK1 are phosphorylated after THP-1 cells are stimulated with ubiquitin and SDF-1α, we treated THP-1 cells with 1 µM ubiquitin or SDF-1α for 10 minutes and performed Western blot experiments. Fig. 4-5 shows representative images from Western blots performed with antibodies directed against phosphoERK1/2, phosphoAkt, and phosphoRSK1. The bands correspond to a phosphorylated serine/threonine protein kinase. Upon stimulation with ubiquitin or SDF-1α, there was an increase in the band density of phosphoERK1/2, phosphoAkt, and phosphoRSK1 as compared to cells that were not treated. Pretreatment of the THP-1 cells with the selective CXCR4 antagonist AMD3100 prevented the observed increase of the band densities that correspond to phosphoERK1/2, phosphoAkt, and phosphoRSK1 after cell stimulation with ubiquitin and SDF-1α. These results confirm that ERK1/2, Akt, and RSK1 are phosphorylated after THP-1 cells are stimulated with ubiquitin or SDF-1α and suggest that these effects are mediated through CXCR4.
Figure 4-5. **CXCR4 induced protein kinase phosphorylation.** Western blot analyses of MAPK phosphorylation after stimulation of THP-1 cells with ubiquitin and SDF-1α. *Top* (phosphoERK 1/2), *Center* (phosphoRSK1), *Bottom* (phosphoAkt). Cells were pre-treated with or without AMD3100 and stimulated with 1 μM of ubiquitin or SDF-1α for 10 minutes at 37°C, as indicated.
**Aim 1C. To define and compare the time course of phosphorylation of the identified serine/threonine protein kinases after CXCR4 activation with SDF-1α and ubiquitin**

To define the time course of the respective serine/threonine protein kinase phosphorylation, we stimulated THP-1 cells with 1μM of ubiquitin or SDF-1α for 0, 5, 10, 15, or 30 minutes and performed Western blot experiments with antibodies directed against phosphoERK1/2, phosphoAkt, and phosphoRSK1. The left panels of Fig. 4-6 show representative images from the Western blots. Upon stimulation with ubiquitin or SDF-1α, there were time dependent increases in the band densities corresponding to phosphoERK1/2, phosphoAkt, and phosphoRSK1, as compared to untreated control. The right panels of Fig. 4-6 show the densitometric quantifications of chemiluminescence signals of the bands after cell stimulation with ubiquitin or SDF-1α from 5-10 independent experiments.

Measurements of the time progression of the phosphorylation status of these protein kinases confirm that cell activation with both ligands result in a comparable increase in protein kinase phosphorylation. With ubiquitin activation, the increase of phosphorylation occurred transiently and declined within 30 minutes. In contrast, the increase of phosphorylation was sustained for 30 minutes with SDF-1α activation.
Figure 4-6. Time course of CXCR4 induced protein kinase phosphorylation. Western blot analyses of MAPK phosphorylation after stimulation of THP-1 cells with ubiquitin and SDF-1α. Left panel: Top (pERK1/2), Center (pRSK1), Bottom (pAkt), after stimulation of cells with 1 µM of ubiquitin or SDF-1α for 0-30 minutes. Right panel: Quantification of the chemiluminescence signals after stimulation as on the left. White Bars: SDF-1α stimulation. Grey Bars: Ubiquitin stimulation. N=5-10. *: p<0.05 vs. unstimulated cells.
Specific Aim 2: To determine and compare the effects of ubiquitin and SDF-1α on chemotaxis.

Aim 2A. To establish and optimize a chemotaxis assay

The regulation of cell trafficking is considered as a key function of the SDF-1α/CXCR4 axis. Whether ubiquitin also has chemotactic activity, however, is unknown. Therefore, we used the chemotactic response of THP-1 cells as a functional read-out for CXCR4 agonist activity of ubiquitin and SDF-1α. Fig. 4-7 shows the average chemotactic indices (CI) from 7 independent filter migration experiments in which the concentration dependency of the chemotactic activity of ubiquitin and SDF-1α were tested. When compared with SDF-1α, cell migration towards ubiquitin was detectable at similar concentrations. However, the CI at concentrations that induced maximal cell migration was lower with ubiquitin (CI: 4.2±0.9 with ubiquitin vs. 7.1±1.4 with SDF-1α; p<0.05) (Fig. 4-7).

Figure 4-7. SDF-1α/Ubiquitin induced chemotaxis. Dose dependent migration of THP-1 cells toward a ubiquitin (●) and SDF-1α (■) gradient (n=7). *: p<0.05 vs. cells in the presence of PBS in lower compartment.
To provide additional evidence for chemotactic activity, we used ubiquitin, SDF-1α, and/or AMD3100 to disrupt the concentration gradients. Fig. 4-8 shows the migration of THP-1 cells in the presence or absence of ubiquitin, SDF-1α or AMD3100 (AMD, 10 µM) in the upper (top) and lower (bottom) compartment as indicated in the graph (n=4). Ubiquitin and SDF-1α were used at concentrations (2 experiments with 1 nM, 2 experiments with 10 nM) that showed maximal chemotactic activity, as determined in Fig. 4-7. Induction of cell migration by ubiquitin and SDF-1α required a concentration gradient; AMD3100 also prevented cell migration. A SDF-1α concentration gradient induced a chemotactic response in the presence of ubiquitin, whereas ubiquitin concentration gradients did not produce chemotactic movements in the presence of SDF- 1α. This is consistent with the weaker chemotactic activity of ubiquitin that we determined in the dose response experiments from Fig. 4-7. These results suggest that both molecules possess chemotactic activity, which is mediated through CXCR4.
**Figure 4-8. CXCR4 mediated chemotaxis.** Migration of THP-1 cells in the presence or absence of ubiquitin, SDF-1α or AMD3100 (AMD, 10 μM) in the upper (top) and lower (bottom) compartment, as indicated in the graph (n=4). Ubiquitin and SDF-1α were used at concentrations (2 experiments with 1 nM, 2 experiments with 10 nM) that showed maximal chemotactic activity, as determined in Fig. 4-7. *: p<0.05 vs. cells in the upper compartment and of ubiquitin in the lower compartment. #: p<0.05 vs. cells in the presence of PBS in the upper compartment and of ubiquitin in the lower compartment. ‡: p<0.05 vs. cells in the presence of PBS in the upper compartment and of SDF-1α in the lower compartment.

There was considerable variability among the previous chemotaxis assays. Therefore, we sought to optimize the assay. To determine if the 8-micron filter pore size is appropriate for filter migration assays with THP-1 cells, we performed experiments in parallel with filter plates with an 8 or 5-micron pore size, respectively. Fig. 4-9 shows the average CIs from 3 filter migration experiments performed in parallel on plates with 8-micron and with 5-micron pore sizes. Many of the CIs were higher on the 8-micron pore size plate compared to the 5-
micron pore size plate. Therefore, this suggests that filter plates with 5-micron pore size limit chemotactic movement of THP-1 cells. Thus, plates with 8-micron pore size were used in subsequent experiments.

Figure 4-9. Filter migration assay-filter plate pore size optimization. CIs from parallel filter migration assays performed with THP-1 cells on a 5 µm and 8 µm pore size plates (n=3). Cell migration of THP-1 cells we assessed toward a ubiquitin gradient (0.001-1000 nM).

We next evaluated the amount of time the THP-1 cells were allowed to incubate at 37°C and performed filter migration assays at various incubation times. Fig. 4-10 shows the average CIs from 3 independent experiments when cells were incubated for 0, 2, 3, 4, or 6 hours. The highest CI for ubiquitin, top, and SDF-1α, bottom, was observed after three hours. CI decreased when cells were allowed to incubate for more than 3 hours and decreased
near control CI values by 6 hours. This suggests that the optimal time to incubate the cells for filter migration assays is 3 hours.

Figure 4-10. Filter migration assay-cell incubation time optimization. Filter migration assays were performed with THP-1 cells and were allowed to incubate for 0-6 hours (n=3). Top: Ubiquitin and Bottom: SDF-1α, were used at a concentration (10 nM) that showed maximal chemotactic activity, as determined in Fig. 4-7.

Lastly, we optimized the cell number used for each well on the chemotaxis filter plate. Fig. 4-11 shows the average CIs from 6 independent filter migration experiments using an 8-micron pore size plate and 3 hour incubation time at 37°C with either 5 x 10⁴, 10 x 10⁴, or 20 x 10⁴ cells per well. Ubiquitin and SDF-1α were used at a concentration (10 nM) that
showed maximal chemotactic activity, as determined in Fig. 4-7. The highest CI for ubiquitin and SDF-1α was observed using 20 x 10⁴ cells per well.

**Figure 4-11. Filter migration assay-cell number optimization.** Cell migration assays using 5 x 10⁴, 10 x 10⁴, and 20 x 10⁴ THP-1 cells per well towards (Left): ubiquitin and (Right): SDF-1α (n=6). Ubiquitin and SDF-1α were used at a concentration (10 nM) that showed maximal chemotactic activity, as determined in Fig. 4-7.

**Aim 2B. To determine and compare chemotactic effects of ubiquitin and SDF-1α under optimized conditions**

To compare the chemotactic effects of ubiquitin and SDF-1α, we repeated the filter migration experiments under optimized conditions. Fig. 4-12 shows the average CIs from 6 independent cell migration experiments. THP-1 cells migrated dose dependently towards ubiquitin and SDF-1α, as was described before (Fig. 4-7). Under optimized conditions, ubiquitin is 4-5 times less efficacious than SDF-1α in promoting chemotaxis because the CI at concentrations that induced maximal cell migration was lower with ubiquitin (CI: 5.99±1.1 with ubiquitin vs. 19.89±3.28 with SDF-1α). SDF-1α and ubiquitin are of similarly potency because the concentrations that induced cell migration are similar for both ligands.
Figure 4-12. Filter migration assays performed under optimized conditions. Filter migration assays using $20 \times 10^4$ THP-1 cells toward a ubiquitin (■) and SDF-1α (▲) gradient (n=6).

It is known that SDF-1α directs migration of freshly isolated leukocytes, but it is unknown whether ubiquitin also induces migration. Therefore, to assess whether ubiquitin also directs cell migration of leukocytes, we isolated PBMCs from human venous blood and performed filter migration assays. We first performed experiments with 8 and 5-micron pore size filter plates, respectively, to assess whether the 8-micron filter pore size is appropriate for PBMCs. Fig. 4-13 shows the average CIs from 3 chemotaxis experiments performed in parallel on plates with a 5-micron pore size and on plates with 8-micron pore size. The correlation of the CIs from experiments done in parallel on plates with a 5 and 8-micron filter
pore size is linear. Therefore, either the 8 or 5-micron filter pore size plates are suitable and we used 8-micron filter pore size plates for experiments with PBMCs.

Figure 4-13. Filter plate pore size optimization of migration assays with freshly isolated leukocytes. CIs from experiments using PBMCs performed in parallel on plates with 5 and 8-micron pore size (n=3). Cell migration of PBMCs toward a ubiquitin gradient (0.001-1000 nM) ($r^2=0.1499$).

Fig. 4-14 shows the average CIs from 5 independent filter migration experiments using an 8-micron filter pore size plate under conditions described for THP-1 cells. PBMCs migrated dose dependently towards ubiquitin and SDF-1α. Compared with SDF-1α, cell migration towards ubiquitin was detectable at similar concentrations. However, the CI at concentrations that induced maximal cell migration was lower with ubiquitin (5±0.48) compared to SDF-1α (27±3.3). Similar to THP-1 cells (Fig. 4-12), ubiquitin is 4-5 times less efficacious than SDF-1α in promoting chemotaxis, but of similar potency.
Figure 4-14. Filter migration assays with PBMCs. Dose dependent migration of PBMCs toward a ubiquitin (●) and SDF-1α (■) gradient (0.001-1000 nM) (n=5).

**Aim 2C. To characterize the signaling events leading to chemotaxis induced by ubiquitin and SDF-1α**

The intracellular signals responsible for chemotaxis in response to SDF-1α have been previously characterized (69,70,72,82,83); whether these signals are responsible for chemotaxis in response to ubiquitin is unknown. Therefore, to characterize the intracellular signals that are responsible for chemotaxis after cells are stimulated with ubiquitin, we disrupted cognate CXCR4 signaling pathways with a variety of well-described pharmacological agents. Fig. 4-15 shows the CIs of THP-1 cells that were incubated with a pharmacological inhibitor, as compared to untreated control cells, from eight independent experiments. The bars on the graphs (left: ubiquitin treatment; right: SDF-1α treatment) correspond to the pharmacological agent used to disrupt the intracellular signals responsible for chemotaxis. AMD3100, Pertussis Toxin, U73122, U73343 LY294002 and U0126
significantly decreased chemotaxis of THP-1 cells for both ubiquitin and SDF-1α, as compared to control. Further, TSA (used as a non-specific control) did not significantly decrease the CIs of THP-1 cells for both ligands.

Figure 4-15. Intracellular signals responsible for CXCR4 mediated chemotaxis, THP-1 cells. Pharmacological agents as previously described were used to treat THP-1 cells and characterize the signaling pathways responsible for chemotaxis after ubiquitin and SDF-1α stimulation. The amount of chemotaxis compared to untreated cells (white) was measured. Ubiquitin (Left) and SDF-1α (Right) were used at a concentration (10 nM) that showed maximal chemotactic activity, as determined in Fig. 4-7 (n=8) *: p<0.05 vs. untreated control cells.

Next, we evaluated the effects of the pharmacological inhibitors on freshly isolated leukocytes to assess whether the intracellular signals that are responsible for cell migration in THP-1 cells are similar for PBMCs. Fig. 4-16 shows the CIs of PBMCs that were incubated with a pharmacological inhibitor compared to untreated control cells from six independent experiments. The bars on each graph (left: ubiquitin treatment; right: SDF-1α treatment) correspond to the pharmacological agent used to disrupt the intracellular signals responsible for chemotaxis in PBMCs. Similar to Fig. 4-15, AMD3100, Pertussis Toxin, U73122,
U73343, LY294002 and U0126 also significantly decreased the CIs of PBMCs for both ubiquitin and SDF-1α, as compared to control.

**Figure 4-16.** Intracellular signals responsible for CXCR4 mediated chemotaxis, PBMCs. Pharmacological agents as previously described were used to treat PBMCs and characterize the signaling pathways responsible for chemotaxis after ubiquitin and SDF-1α. The amount of chemotaxis compared to untreated cells was measured. Ubiquitin (*Left*) and SDF-1α (*Right*) were used at a concentration (10 nM) that showed maximal chemotactic activity, as determined in Fig. 4-7. (n=6) *: p<0.05 vs. untreated control cells.
CHAPTER FIVE

DISCUSSION

Ubiquitin was recently characterized as a CXCR4 agonist but the intracellular responses after CXCR4 activation with ubiquitin are largely unknown. In this study, we investigated whether the distinct ligand binding sites for ubiquitin and SDF-1α on CXCR4 resulted in differential biological responses. Our results suggest that activation of CXCR4 with ubiquitin and SDF-1α result in similar intracellular signaling events and functional consequences. Ubiquitin appears to be a weaker CXCR4 agonist than SDF-1α, however, and this may correspond to differential CXCR4 signaling mediated via distinct ligand binding sites on the receptor.

Our initial result provided evidence that we maintained healthy cells under our culture conditions, and that the ubiquitin concentration in the culture supernatant would not interfere with experiments to test our hypothesis. Our first finding that CXCR4 activation with ubiquitin appears to result in similar intracellular signaling properties compared to SDF-1α is supported by the MAPK array and subsequent Western blot experiments. Side-by-side comparisons of MAPK phosphorylations after ubiquitin and SDF-1α stimulation of THP-1 cells showed that both ligands produced similar patterns in the membrane array. We then used Western blot experiments and confirmed that ERK-1/2, RSK-1 and Akt are phosphorylated in response to CXCR4 activation after cell stimulation with ubiquitin and SDF-1α (53,120). Thus, the original thought was that the similar pattern of MAPK
phosphorylation, after CXCR4 activation with both ligands, argues against differential intracellular signaling properties (121). However, the time course Western blot experiments provided evidence that there are differential signaling properties; MAPK phosphorylation after CXCR4 activation with ubiquitin occurred more transiently compared to SDF-1α. It might be speculated that the transient effects of MAPK phosphorylation after cells were incubated with ubiquitin may be due to increased activity of phosphatases specific for ERK1/2, RSK-1, and Akt. However, it is unknown whether CXCR4 activation with ubiquitin or SDF-1α results in phosphatase over expression and/or activation.

Our finding that CXCR4 activation with ubiquitin appears to result in a similar functional response compared to SDF-1α is supported by our filter migration experiments. We used THP-1 cells and freshly isolated leukocytes and found that SDF-1α and ubiquitin displayed chemotactic activity at concentrations between 0.1 – 10 nM. This is in agreement with the wide range of SDF-1α concentrations that have been previously reported to induce chemotactic movements (130,131). Thus, it is reasonable to speculate that ubiquitin and SDF-1α induced maximal receptor activation at these optimized concentrations. These results also correspond to previous observations that intramuscular injection of ubiquitin led to the accumulation of large numbers of lymphocytes in the CXC12 myoblast cell line (132), which resembles the effects of SDF-1α after subcutaneous injection (122). Further, these findings are concurrent with the affinity of SDF-1α for CXCR4 (Kd: 1.5 – 24 nM), which has been reported with human peripheral blood monocytes, T-cells, and T-cell lines (32, 106, 107,133, 134). However, the range of concentrations observed that induced chemotactic responses towards ubiquitin were 10-1000-fold below the affinity of ubiquitin for CXCR4.
We can speculate that the lower chemotactic response is not due to the lower affinity of ubiquitin for CXCR4, as compared to SDF-1α, because CXCR4 is fully activated with ubiquitin under optimized conditions. Thus, it is possible that this phenomenon is a result of the alternative ubiquitin-binding site on CXCR4 (43). These results suggest, however, that only a small percentage of receptor occupancy by ubiquitin is necessary to elicit a cellular response. Such a dose-response relationship has been described for other GPCRs, showing half-maximal and maximal responses at receptor occupancies of 0.13% and of 0.8% (135, 136), respectively. We also optimized the filter migration assay (filter pore size, time cells were allowed to incubate, and cell number used per well) and confirmed and extended previously described results under optimized conditions (26, 122).

Our findings from filter migration experiments after we disrupted cognate CXCR4 signaling pathways show that CXCR4 activation with ubiquitin and SDF-1α result in similar intracellular signaling properties. The finding that THP-1 cells and PBMCs migrated toward ubiquitin or SDF-1α, that both ligands function through CXCR4 (133, 138), and cells rely on a similar pattern of intracellular signaling including Goi (32), PLC (138), PI3 kinase (139) and ERK1/2 (137, 140) to induce chemotaxis is in agreement with previous literature. It must be noted that pre-treatment of cells with U73122 had the strongest effects in both cell types and this may explain why there was a significant reduction in chemotaxis when cells were pre-treated with U73343.

Several studies show that CXCR4-β-arrestin signaling leads to phosphorylation of ERK1/2 and Akt, and may result in prolonged signal transduction events (123-125). There are also studies that show β-arrestin mediated signaling leads to prolonged ERK1/2 signaling.
and actin filament assembly at the leading edge of cells that is critical for GPCR mediated chemotactic responses (126-129). Thus, the transient MAPK phosphorylation after cell stimulation with ubiquitin and the lower efficacy of ubiquitin to induce a chemotactic response in THP-1 cells and PBMCs could suggest there is insufficient β-arrestin recruitment to CXCR4 after ubiquitin stimulation.

One limitation of our study is that our filter migration assays have thus far been conducted in vitro using THP-1 cells and PBMCs. There is evidence that multiple cancer cell types metastasize to SDF-1α (116, 117), and ubiquitin may have important therapeutic potential for cancer treatment. In order to confirm the results from our experiments, in vivo experiments are required. Another limitation is that, although Western blot experiments showed MAPK phosphorylation after cell stimulation with ubiquitin and SDF-1α, this was not indicative of the activity of the MAPKs and may not influence cellular function. Additionally, using PBMCs was a limitation for this study because PBMCs are not a homogenous cell population. The last limitation of note was while the pharmacological inhibitors used in the experiments have been well described, we would need to perform additional experiments to confirm that the intracellular signals are inactive.

**Working model for CXCR4 activation with ubiquitin and SDF-1α**

The results from the current study show that ubiquitin induces serine/threonine protein kinase activation in THP-1 cells and this appears to be specific through CXCR4. However, the response was more transient compared to SDF-1α induced CXCR4 activation. Additionally, the results demonstrate ubiquitin induces a chemotactic response specific through CXCR4 with THP-1 cells and PBMCs, but the response was less compared to SDF-
1α. However, the signaling events we tested that are involved in a chemotactic response were similar for ubiquitin and SDF-1α. In summary, the transient serine/threonine protein kinase activation and differences in chemotaxis may be explained by the amount of β-arrestin recruitment to the activated receptor. A working model is presented in Figure 5-1.

**Future directions for the investigation of functional selectivity through CXCR4 activation with ubiquitin and SDF-1α**

It has been described that β-arrestin is a crucial intracellular signal transducer and leads to functional responses. Therefore, a future study could investigate the magnitude of β-arrestin recruitment to CXCR4 upon stimulation with ubiquitin and SDF-1α. Additionally, GRKs phosphorylate CXCR4 at the third intracellular loop and this results in the active recruitment and increased affinity of β-arrestin to the receptor. Therefore a study of the interaction of GRKs with CXCR4 after activation with ubiquitin and SDF-1α, the phosphorylation status of the third intracellular loop of CXCR4, and finally the magnitude of β-arrestin recruitment to CXCR4 would provide additional evidence of differential receptor signaling.
Figure 5-1. Working hypothesis for differential signaling through CXCR4 activation with ubiquitin and SDF-1α. Our data demonstrate that ubiquitin induces serine/threonine protein kinase activation in THP-1 cells and this is specific through CXCR4. Our data also showed ubiquitin induces a chemotactic response specific through CXCR4 mediated intracellular signals. The serine/threonine kinase signals were more transient and chemotaxis was less with ubiquitin compared to SDF-1α. It is possible ubiquitin is a biased agonist and there is minimal or even no β-arrestin signaling with ubiquitin induced CXCR4 activation.
REFERENCES


VITA

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