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Functional studies of a chimeric CXCR2/G_{αi2} protein

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Abstract:

As transmembrane proteins, chemokine receptors possess a large hydrophobic portion that is unstable in water and does not fold into the proper conformation in an aqueous environment. Consequently, it is difficult to isolate these receptor proteins in order to perform structural studies on them. However, it is believed that a fusion protein of the receptor connected to the alpha subunit of the G protein would stabilize the C-terminus side of the receptor since the G-protein is soluble in water. Similarly, the addition of ligand would do the same on the N-terminus side of the protein. This would increase the solubility of the receptor protein and hopefully confer enough stability to secure it in its natural confirmation. Here, a fusion protein of the receptor and alpha subunit is produced. Using transfected HEK 293 cells, the fusion protein is shown to be expressed on the cell surface.

Introduction:

Chemokines are small, pro-inflammatory protein signals that induce rapid, directed chemotaxis in phagocytes and T cells toward sites of physical damage by bacteria, viruses, and other destructive agents⁴. In addition to increased cell motility, interaction of chemokines with receptor cells produces changes in cell shape, generation of superoxide anions, and release of enzymes³. The result of this is a general inflammatory response from phagocytic cells followed by a specific immune response from T cells⁴.

The large superfamily of chemokines, approximately 40 proteins, is subdivided into four groups: C-, CC-, CXC-, and CX₃C-^{3,4}. These divisions are based on differences in the number and arrangement of the first four conserved cysteine residues in the amino acid sequence³. For example, the C-group has only one pair of cysteine residues, while the other three groups have two pairs⁵. Furthermore, the CXC- and CX₃C-groups have cysteine pairs separated by one and three variable amino acids, respectively⁵.

The activity of chemokines is mediated by their receptors, which are membrane-spanning G-protein coupled receptors (GPCR). Like other transmembrane proteins, they have seven alpha-helical transmembrane domains connected by six loops, three intracellular and three extracellular². The N-terminus is extracellular, binds ligand, is slightly acidic, and has N-linked glycosylation sites³. Conversely, the C-terminus is intracellular, interacts with heterotrimeric G-proteins, and has regulatory phosphorylation sites on threonine and serine residues³. Extracellular loops 1 and 2 are connected by a disulfide bond between conserved cysteine residues, and intracellular loop 3 may also interact with the G-proteins^{2,3}.

Upon binding of ligand, a conformational change in the receptor induces an exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) by the alpha subunit of the G-protein, leading to dissociation of the alpha subunit from the beta-gamma subunit complex². Exchange of GDP for GTP can be eliminated through a mutation that replaces an arginine residue with a histidine¹. The beta subunit then activates phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol-4,5-diphosphate (PIP₂) to inositol 1,4,5-triphosphate (IP₃) and diacyl-glycerol (DAG)³. IP₃ leads to the release of calcium ions from intracellular stores which then interact with

DAG to activate protein kinase C (PKC)³. Activation of PKC leads to phosphorylation of other proteins and further cellular responses³. Simultaneously, the activated alpha subunit leads to MAP kinase activation and down-regulation of the receptor by phosphorylation of C-terminus serine and threonine residues³. Following this, the chemokine is degraded intracellularly and the activated receptor is returned to the plasma membrane³. This desensitization is important in maintaining the ability to sense a chemical gradient³.

CXCR2 is a chemokine receptor found on granulocytes, monocytes, and mast cells and on some T cells and natural killer cells³. Its ligands include interleukin-8 (IL-8), one of the most studied chemokines, and all CXC-group chemokines with the N-terminal Glu-Leu-Arg (ELR) motif^{3,4}. It is associated with the inhibitory form of the G-protein alpha subunit ($G_{\alpha i2}$)². Expression of CXCR2 on neutrophils can be down-regulated by tumor necrosis factor and lipopolysaccharide and increased by granulocyte-colony-stimulating factor³.

Methods:

DNA encoding the genes for CXCR2 and $G_{\alpha i2}$ were amplified separately using polymerase chain reaction (PCR) with Ex Taq Polymerase. For CXCR2, the forward and reverse primers were hCXCR2 Not1 (For) (5'-GCGGCCGCATGGAAGATTTTAAACATGG-3') and hCXCR2-mut (Rev) (5'-CTCCCCGGGGAGAGTAGTGGAAGTGTGC-3'), and for $G_{\alpha i2}$, they were Sma1-Gia2 (Forward) (5'-CCCGGGATGGGCTGCACCGTGAGCGCCG-3' and Gia2-Apa1 (Reverse) (5'-GGGGCCCTCAGAAGAGGCCGCAGTCCTT-3'). The products were

run on a 1% agarose gel, and the bands corresponding to 1 kb were extracted using QIAquick Gel Extraction Kit.

Both CXCR2 and $G_{\alpha i2}$ DNA were then digested overnight at 37° C with *XmaI* restriction enzyme to create complimentary sticky ends at the end of CXCR2 and the beginning of $G_{\alpha i2}$. Each was cleaned up with QIAquick PCR Purification Kit, and equal amounts of DNA from each were ligated with T4 DNA ligase at room temperature overnight. PCR was then used to amplify the ligated DNA using the forward CXCR2 primer and the reverse $G_{\alpha i2}$ primer, and the band at 2 kb on a 1% agarose gel was extracted using QIAquick Gel Extraction Kit. This DNA was ligated into the pEXP5-CT/TOPO® vector using the Invitrogen TA Expression Kit and used to transform One Shot® TOP10 Competent *E. coli* cells. PCR screening confirmed the presence of the insert, and plasmid was isolated with Promega's Wizard *Plus* SV Minipreps DNA Purification System.

PCR was done on the fusion DNA to introduce a *HindIII* site at the beginning of CXCR2 and a *XbaI* site at the end of $G_{\alpha i2}$. The forward primer CXCR2 Hind III For was obtained from Dr. Tom Masi in the Sparer Lab, and the reverse primer was Gia2Xba2 (5'-CTAGTCTAGACTAGTCAGAAGAGG-3'). This DNA was ligated into the same vector as before and transformed One Shot® TOP10 Competent *E. coli* cells. Plasmids from colonies showing the insert through PCR screening were isolated as before and digested overnight at 37°C with *HindIII* and *XbaI*. Gel extraction isolated the insert, which was then ligated into the pRc/CMV vector by T4 DNA ligase with a 40:1 molar ratio of insert to vector. This reaction was incubated overnight in a water bath at 4°C. 1

μl of the ligation reaction was then used to transform DH5 α cells; colonies were screened by digestion with *HindIII* and *XbaI*.

This plasmid was used to transfect HEK 293 cells. A 6-well dish was plated with 2.0×10^5 cells per well two days before transfecting. GFP was used as a positive control, and untransfected HEK 293 cells were a negative control. The transfected cells were harvested from the wells and used for Fluorescent Activated Cell Sorting (FACS) by applying a fluorescent antibody specific for CXCR2. Some of the transfected cells were kept in T150 flasks in complete DMEM media with G418 antibiotic to create a stable line of transfectants. These stables were also analyzed by FACS one month after transfection, and some were frozen in 10% DMSO. Mutagenesis was also performed on the fusion DNA to introduce the R210H mutation in G_{ai2}. This was done using PCR with Pfu Ultra Polymerase and primers GaiR210H1 (5'- CAG CGG TCT GAG CAC AAG AAG TGG ATC -3') and GaiR210H2 (5'- GAT CCA CTT CTT GTG CTC AGA CCG CTG -3') to change the existing CGG codon to CAC. Both mutant and wild type fusion DNA were used to transfect HEK 293 cells to create stable cell lines. In collaboration with Dr. Becker's lab, the wild type CXCR2/G_{ai2} plasmid is being used to transform yeast cells.

Results:

Sequencing from the Molecular Biology Resource Facility confirmed the CXCR2/G_{ai2} insert ligated into the TOPO vector in the correct orientation with CXCR2 proceeding G_{ai2}. Since the insert was approximately 2 kb, a third primer obtained from Dr. Sparer's lab was used to sequence the region where the two ligated together. This confirmed neither CXCR2 nor G_{ai2} were mutated during ligation. Sequencing also confirmed the presence of the insert in the correct orientation in the pRc/CMV vector.

Transfection efficiency was done by analyzing GFP fluorescence one day after transfection. In each case, approximately 80% of the cells transfected showed GFP expression. For FACS analysis, a gate was set to include only cells which were a certain size so as to eliminate cell debris from being counted (Figure 1). The first 30,000 cells to fit this criterion were counted and analyzed. Using the untransfected HEK 293 as background, approximately 10% and 3% of the transient and stable wild-type transfectants, respectively, expressed more CXCR2 than normal (Figure 2). By analyzing fluorescence of antibodies bound to receptors on the cell surface, this confirmed not only the production of a fusion protein, but also its location on the plasma membrane. FACS analysis for the mutant CXCR2/G_{αi2} protein has not yet been performed.

Discussion:

The work presented here provides evidence that a chemokine receptor fused to its associated G-protein alpha subunit can be expressed on mammalian plasma membranes. However, more transient cells expressed the fusion protein than observed in the stable line. This is thought to occur because fusion of the receptor to the G-protein has created a constitutively active receptor that is down-regulated by the cell. In order to test this theory, the R210H mutation was made to inactivate the fusion G_{αi2} subunit, but data is not yet available to confirm or reject this hypothesis. The production of fusion CXCR2/G_{αi2} protein on cell surfaces provides opportunity for further experiments to test its functionality through binding and signaling assays with commercially available ligands such as IL-8 or gro-α. Comparison of fusion K_d and B_{max} values to those for wild-type CXCR2 will show if G_{αi2} in some way altered the ability of CXCR2 to bind its ligands. Observation of calcium flux or other downstream signaling effects, such as

phosphorylation of other proteins, will determine if the fusion protein can still participate in signal transduction.

For biophysical studies, large quantities of protein are needed, but since CXCR2 is a mammalian protein, this cannot be obtained using a bacterial system such as *E. coli*. At first, HEK 293 cells were to be used to isolate the protein, but these proved difficult to grow in suspension. Media without calcium was used to prevent attachment, but the cells died, became contaminated, or simply did not grow. Yeast cells, as a eukaryotic system, can express wild-type CXCR2, so they are to be used produce the fusion protein in large amounts. The correct plasmid, however, has yet to be produced from the in vivo ligation reaction of yeast.

References:

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Figure 1: Gated regions for countable cells outlined in black. (a) Transient transfectants
 (b) Stable transfectants

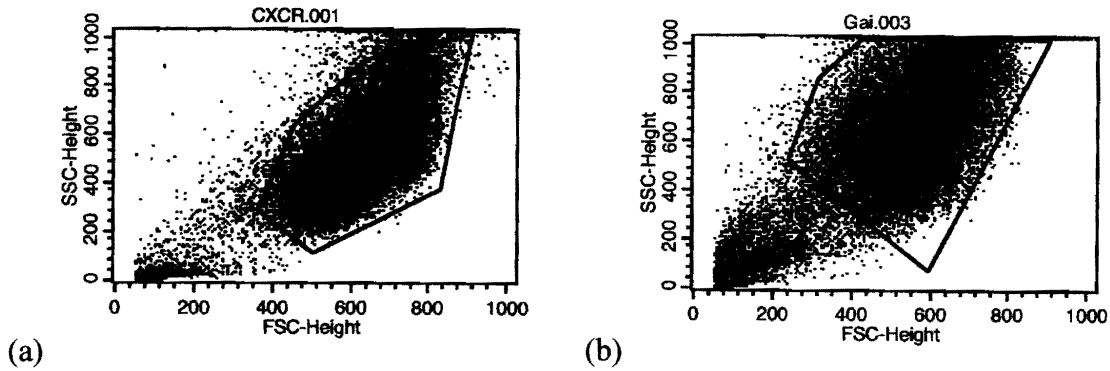


Figure 2: FACS data graphed as fluorescent counts versus number of cells. In both, the black line is background fluorescence from untransfected 293 cells. (a) Transient cells. Pink and Green lines are transfections done by Lori and Tom, respectively, at the same time. Approximately 10% of transfected cells show more expression than normal cells. (b) Stable cell fluorescence is red line. Only about 3% show more expression.

