WATER SOLUBLE MEMBRANE PROTEINS AND METHODS FOR THE PREPARATION AND USE THEREOF

RELATED APPLICATION(S)

This application claims the benefit of U.S. Provisional Application No. 61/445,740, filed on February 23, 2011. The entire teachings of the above application(s) are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Membrane proteins play vital roles in all living systems. Approximately ~30% of all genes in almost all sequenced genomes, code for membrane proteins. However, our detailed understanding of their structure and function lags far behind that of soluble proteins. As of February 2012, there are over 79,500 structures in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do), however, there are 952 membrane protein structures with 320 unique structures including 8 G-protein coupled receptors. Although there are about 400 functional olfactory receptors in human, not a single olfactory receptor has been determined.

There are several bottlenecks in elucidating the structure and function of olfactory receptors and their recognition and odorant-binding properties although they are of great interest. The most critical and challenging task is that it is extremely difficult to produce milligrams quantities of soluble and stable receptors. Inexpensive large-scale production methods are desperately needed, and have thus been the focus of extensive research. It is only possible to conduct detailed structural studies once these preliminary obstacles have been surmounted. Therefore, there is a need in the art for improved methods of studying G-protein coupled receptors, including olfactory receptors.

SUMMARY OF THE INVENTION

The present invention is directed to water-soluble membrane peptides, compositions comprising said peptides, methods for the preparation thereof and methods of use thereof.

The invention encompasses a water-soluble polypeptide comprising a modified α-helical domain, wherein the modified α-helical domain comprises an amino acid sequence in

which one or more hydrophobic amino acid residues within a α-helical domain of a native membrane protein is replaced with one or more hydrophilic amino acid residues. The invention also encompasses a method of preparing a water-soluble polypeptide comprising replacing one or more hydrophobic amino acid residues within the α-helical domain of a native membrane protein with one or more hydrophilic amino acid residues. The invention additionally encompasses a polypeptide prepared by replacing one or more hydrophobic amino acid residues within the α-helical domain of a native membrane protein with one or more hydrophilic amino acid residues.

The invention further encompasses a method of treatment for a disorder or disease that is mediated by the activity a membrane protein in a subject in need thereof, comprising administering to said subject an effective amount of a water-soluble polypeptide comprising a modified α-helical domain, wherein the modified α-helical domain comprises an amino acid sequence in which one or more hydrophobic amino acid residues within a α-helical domain of the membrane protein is replaced with one or more hydrophilic amino acid residues. In certain aspects, the water-soluble polypeptide retains the ligand-binding activity of the membrane protein. Examples of disorders and diseases that can be treated by administering a water-soluble peptide of the invention include, but are not limited to, cancer (such as, small cell lung cancer, melanoma, triple negative breast cancer), Parkinson's disease, cardiovascular disease, hypertension, and bronchial asthma.

The invention also encompasses a pharmaceutical composition comprising a water-soluble polypeptide of the invention and pharmaceutically acceptable carrier or diluent.

In some aspects, the α-helical domain is a 7-transmembrane α-helical domain. In an additional embodiment, the native membrane protein is a G-protein coupled receptor (GPCR). In some aspects of this embodiment, the GPCR is selected from the group comprising purinergic receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$), M_1 and M_3 muscarinic acetylcholine receptors, receptors for thrombin [protease-activated receptor (PAR)-1, PAR-2], thromboxane (TXA_2), sphingosine 1-phosphate ($S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$), lysophosphatidic acid (LPA_1, LPA_2, LPA_3), angiotensin II (AT_1), serotonin ($5\text{-}HT_{2c}$ and $5\text{-}HT_4$), somatostatin (sst_5), endothelin (ET_A and ET_B), cholecystokinin (CCK_1), V_{1a} vasopressin receptors, D_5 dopamine receptors, fMLP formyl peptide receptors, GAL_2 galanin receptors, EP_3 prostanoid receptors, A_1 adenosine receptors, α_1 adrenergic receptors, BB_2 bombesin receptors, B_2 bradykinin receptors, calcium-sensing receptors, chemokine

receptors, KSHV-ORF74 chemokine receptors, NK_1 tachykinin receptors, thyroid-stimulating hormone (TSH) receptors, protease-activated receptors, neuropeptide receptors, adenosine A2B receptors, P2Y purinoceptors, metabolic glutamate receptors, GRK5, GPCR-30, and CXCR4. In yet an additional embodiment, the native membrane protein or membrane protein is an integral membrane protein. In a further aspect, the native membrane protein is a mammalian protein. In yet a further aspect, the native membrane protein is an olfactory receptor. In additional embodiments, the olfactory receptor is mOR103-15.

In some aspects, the hydrophilic residues (which replace one or more hydrophobic residues in the α-helical domain of a native membrane protein) are selected from the group consisting of glutamine (Q), threonine (T), tyrosine (Y) and any combination thereof. In additional aspects, one or more hydrophobic residues selected from leucine (L), isoleucine (I), valine (V) and phenylalanine (F) are replaced.

In certain embodiments, one or more phenylalanine residues of the α-helical domain of the protein are replaced with tyrosine. In certain additional embodiments, one or more isoleucine and/or valine residues of the α-helical domain of the protein are replaced with threonine. In yet additional aspects, one or more phenylalanine residues of the α-helical domain of the protein are replaced with tyrosine and one or more isoleucine and/or valine residues of the α-helical domain of the protein are replaced with threonine. In additional embodiments, one or more leucine residues of the α-helical domain of the protein are replaced with glutamine. In yet additional embodiments, one or more leucine residues of the α-helical domain of the protein are replaced with glutamine and one or more isoleucine and/or valine residues of the protein are replaced with threonine. In further embodiments, one or more leucine residues of the α-helical domain of the protein are replaced with glutamine and one or more phenylalanine residues of the α-helical domain of the protein are replaced with tyrosine. In yet additional aspects, one or more leucine residues of the α-helical domain of the protein are replaced with glutamine, one or more phenylalanine residues of the α-helical domain of the protein are replaced with tyrosine, and one or more isoleucine and/or valine residues of the α-helical domain of the protein are replaced with threonine.

In additional embodiments, the water-soluble polypeptide retains at least some of the biological activity of the native membrane protein. In an aspect of this embodiment, the water-soluble polypeptide retains the ability to bind the ligand which normally binds to the

native membrane protein. In another embodiment, one or more amino acids within potential ligand binding sites of the native membrane protein are not replaced. In an aspect of this embodiment, examples of native membrane proteins with one or more amino acids not replaced within potential ligand-binding sites are purinergic receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$), M_1 and M_3 muscarinic acetylcholine receptors, receptors for thrombin [protease-activated receptor (PAR)-1, PAR-2], thromboxane (TXA_2), sphingosine 1-phosphate ($S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$), lysophosphatidic acid (LPA_1, LPA_2, LPA_3), angiotensin II (AT_1), serotonin ($5\text{-}HT_{2c}$ and $5\text{-}HT_4$), somatostatin (sst_5), endothelin (ET_A and ET_B), cholecystokinin (CCK_1), V_{1a} vasopressin receptors, D_5 dopamine receptors, fMLP formyl peptide receptors, GAL_2 galanin receptors, EP_3 prostanoid receptors, A_1 adenosine receptors, α_1 adrenergic receptors, BB_2 bombesin receptors, B_2 bradykinin receptors, calcium-sensing receptors, chemokine receptors, KSHV-ORF74 chemokine receptors, NK_1 tachykinin receptors, thyroid-stimulating hormone (TSH) receptors, protease-activated receptors, neuropeptide receptors, adenosine A2B receptors, P2Y purinoceptors, metabolic glutamate receptors, GRK5, GPCR-30, and CXCR4.

In another embodiment, one or more amino acids within potential odorant binding sites of the native membrane protein are not replaced.

In one embodiment, water-soluble polypeptide comprising a modified α-helical domain comprises the amino acid sequence of MERRNHTGRV SEFVLLGFPA PAPQRALQFF QSLQAYVQTL TENIQTITAI RNHPTLHKPM YYFLANMSFYL ETWYTTVTTP KMQAGYIGSE ENHGQLISFE ACMTQLYFFQ GLGCTECTLL AVMAYDRYVA TCHPLHYPVI VSSRQCVQMA AGSWAGGFGT SMTVKVYQISR LSYCGPNTIN HFFCDVSPLL NLSCTDMSTA ELTDFILAIF ILLGPLSVTG ASYMAITGAV MRIPSAAGRH KAFSTCASHL TTVITYYAAS IYTYARPKAL SAFDTNKLVS VLYAVIVPLL NPIIYCLRNQ EVKKALRRTL HLAQGDANT KKSSRDGGSS GTETSQVAPA (SEQ ID NO: 2). In yet an additional embodiment, the water-soluble polypeptide comprising a modified 7-transmembrane α-helical domain comprises one or more of the following amino acid sequences:

a. PQRALQFFQSLQAYVQTLTENIQTITAI R (SEQ ID NO: 3)
b. M YYFLANMSFYLETWYTTVTTPKMQAGYI (SEQ ID NO: 4)
c. CMTQLYFFQGLGCTECTLLAVMAYDRYVA TC (SEQ ID NO: 5)
d. RQCVQMAAGSWAGGFGTSMTVKVYQ (SEQ ID NO: 6)

e. LTDFILAIFILLGPLSVTGASYMAITGAV (SEQ ID NO: 7)

f. HKAFSTCASHLTTVITYYAAS IYTY (SEQ ID NO: 8)

g. TNKLVSVLYAVIVPLLNPIIYCLRN (SEQ ID NO: 9)

In certain aspects of the invention, the secondary structure of the water-soluble peptide is determined. In some embodiments, the secondary structure is determined using circular dichroism.

In certain embodiments, ligand binding to the water-soluble polypeptide is measured. In some aspects, ligand binding affinity of the water-soluble polypeptide is compared to that of the native protein. In additional aspects, ligand binding is measured using microscale thermophoresis, calcium influx assay or any combination thereof.

In yet an additional embodiment, the invention encompasses a cell transfected with a water-soluble peptide comprising a modified α-helical domain. In certain embodiments, the cell is a mammalian cell. One example of a mammalian cell that can be transfected is a HEK293 cell.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

FIG. 1 shows the amino acid sequences of native mOR103-15 and mutated mOR103-15 using glutamine, threonine and tyrosine (QTY) replacements. Use of QTY replacements to systematically mutate key residues on the 7-transmembrane α-helices to convert a water-insoluble olfactory receptor into a water-soluble one. We only change the positions of *b, c, f* with the more water-soluble residues Q, T, Y. These positions are on the hydrophilic face of the helices. We maintain the positions *a, d, e, g* that are on the hydrophobic face. It is likely that these changes will maintain the individual α -helices. The mutations are labeled in capital blue letters on top of the receptor sequence. The small letters, *abcdefg*, are helical wheel positions. The underlines are the locations of 7-transmembrane α -helices. The numbers (8, 7, 3, 5, 4, 5, 4) are mutations in each α -helix. There are 36-residue changes, ~10.5% of the total 340 residues.

FIG. 2 shows molecular models of a QTY Replacement olfactory receptor mOR103-15. A total 36 mutations have been made (~10.5%) in the 7-transmembrane helical segments. These mutations do not change the charged residues, so the variant receptor mass and pI remain largely unchanged. The molecular shapes and sizes of amino acids, Q, T, and Y are very similar to L, V/I and Y, so there are minimal overall local shape changes. A segment of 20 amino acids at the C-terminus are not modeled for clarity.

FIGs. 3A-3C A) Top view of the QTY replacements and B) side view of the QTY replacements. Note the mutations are only on one side of the helices. The native receptor without mutations has a folded structure similar to $\alpha_1$adrenergic receptor, whereas after mutation, the structure is similar to the $\beta_2$adrenergic receptor. C) Simulated structures of superimposed native mOR103-15 (red) and designed QTY mutation of mOR103-15 (blue). The overall structural difference is ~0.8Å average.

FIG. 4 Circular dichroism spectrum of CXCR4 and designed QTY mutation of CXCR4-QTY.

FIG. 5 SDS Gel showing comparison of molecular weight between native CXCR4 and CXCR4 with QTY mutations (SEQID NO:10: CXCR4 QTY).

FIG. 6 Use of QTY replacements to systematically mutate key residues on the 7-transmembrane alpha-helices and few other hydrophobic residues to convert the water-insoluble membrane form CXCR4 into a water-soluble form. **A**) We have changed positions *b, c, f* with the more water-soluble residues Q, T, Y. We do not change the positions *a, d, e, g*. These positions are believed to maintain the specific clustering of individual alpha-helices. **B**). The superimpositions of membrane form CXCR4 (red) and QTY water-soluble CRCR4 (blue). **C**) The native residues are labeled in red letters and **D**) mutations are labeled in blue letters in the sequence. A total of 29 QTY mutations among 352 residues have been made (about8.2%) in the seven transmembrane helical segments. These mutations do not change the charged residues, so the variant receptor mass and pI remain largely unchanged. The molecular shapes and sizes of amino acids, Q, T and Y are very similar to L, V/I and Y, so there are minimal overall, local shape changes.

DETAILED DESCRIPTION OF THE INVENTION

A description of preferred embodiments of the invention follows.

The words "a" or "an" are meant to encompass one or more, unless otherwise specified.

In some aspects, the invention is directed to the use of the QTY (Glutamine, threonine and tyrosine) replacement method to systematically change the 7-transmembrane α-helix hydrophobic residues leucine (L), isoleucine (I), valine (V), and phenylalanine (F) of a native protein to the hydrophilic residues glutamine (Q), threonine (T) and tyrosine (Y). This invention will convert the native membrane protein from a water-insoluble one to a water-soluble counterpart.

Another innovation of the invention is to convert the water-insoluble olfactory receptor mOR103-15 into a water-soluble one with about 10.5% specific residues changes (36aa/340aa). This will be accomplished by systematically and selectively changing key residues at the α-helical positions *b, c, f* that usually face the hydrophilic surface, while maintaining the hydrophobic residues at α-helical positions *a, d, e, g*. The synthetic biology design method is general and broadly applicable to the study of other olfactory receptors and G-protein coupled receptors. This strategy has the potential to overcome the bottleneck of crystallizing olfactory receptors, as well as additional GPCRs and other membrane proteins.

We used synthetic biology methods to convert a water-insoluble olfactory receptor into a water-soluble one with ~10.5% of the residues changes (36aa/340aa) (FIG. 1 and 2). We have systematically and selectively changed key residues at the α-helical positions *b, c, f* (which usually form the hydrophilic surface), but maintained the hydrophobic residues at α-helical positions *a, d, e, g* (FIG. 1). Our synthetic biology design method is general in nature, thus it is broadly applicable to the study other olfactory receptors, chemokine CXCR4 as well as other G-protein coupled receptors (GPCRs) and other membrane proteins. This simple strategy may partly overcome the bottleneck of structural studies of olfactory receptors, GPCRs, and other membrane proteins if the converted water-soluble membrane proteins remain biologically functional.

In order to facilitate the study of the structural aspects of olfactory receptors and their binding properties, we will use the QTY replacement method to design a water-soluble 7-bundle helical olfactory receptor mOR103-15 (FIGs. 1-3). It is known that seven amino acids have α-helical forming tendencies (32): leucine (L) (1.30), glutamine (Q) (1.27),

phenylalanine (F) (1.07), tyrosine (Y) (0.72), isoleucine (I) (0.97), valine (V) (0.91) and threonine (T) (0.82). We also know that side chains of Q, Y and T can all form hydrogen bonds with water: Q can form 4 H-bonds (2 H-donors from $-NH_2$, 2 H-acceptors from C=O), and T and Y can form 3 H-bonds each (-OH, 1-H donor from –H and 2 acceptors from -O). The Q, T, Y residues are more water-soluble than L, F, I, or V, which cannot form any hydrogen bonds with their side chains. The proposed substitutions will not have any positive- or negative-charges changes. Furthermore, the molecular shapes and sizes are very similar for the pairs: leucine/glutamine, phenylalanine/tyrosine, valine/threonine, and isoleucine/threonine (33-34). The proposed changes should thus increase the solubility of 7-transmembrane α--helices while maintaining the overall helical structure (FIG. 3C).

In this soluble olfactory receptor design, we have performed the following substitutions: phenylalanine to tyrosine (F->Y), isoleucine/valine to threonine (I/V->T), and leucine to glutamine (L->Q). The secondary structure of the water-soluble olfactory receptor, as well as measure its odorant-binding capabilities can be examined. If odorant-binding is detected with the QTY replacements, then it is likely that we have preserved important components of the original structure. The secondary structure and binding of the designed water-soluble olfactory receptor with the native olfactory receptor can be prepared. Milligram quantities of the water-soluble receptor can be produced and crystal screens can be set up with and without odorants.

In one embodiment, the native membrane protein is a G-protein coupled receptor (GPCR). In yet another embodiment, the native membrane protein is an olfactory receptor. In some embodiments, the olfactory receptor is a mammalian receptor. In yet another embodiment, the olfactory receptor is mOR103-15. In certain aspects, the water-soluble polypeptide retains at least some of the biological activity of the native membrane protein. In yet another aspect, the membrane protein is a membrane receptor that mediates a disease or condition.

In a further embodiment, the native membrane protein is a GPCR selected from the group comprising purinergic receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$), M_1 and M_3 muscarinic acetylcholine receptors, receptors for thrombin [protease-activated receptor (PAR)-1, PAR-2], thromboxane (TXA_2), sphingosine 1-phosphate ($S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$), lysophosphatidic acid (LPA_1, LPA_2, LPA_3), angiotensin II (AT_1), serotonin ($5\text{-}HT_{2c}$ and $5\text{-}HT_4$), somatostatin (sst_5), endothelin (ET_A and ET_B), cholecystokinin (CCK_1), V_{1a}

vasopressin receptors, D_5 dopamine receptors, fMLP formyl peptide receptors, GAL_2 galanin receptors, EP_3 prostanoid receptors, A_1 adenosine receptors, α_1 adrenergic receptors, BB_2 bombesin receptors, B_2 bradykinin receptors, calcium-sensing receptors, chemokine receptors, KSHV-ORF74 chemokine receptors, NK_1 tachykinin receptors, thyroid-stimulating hormone (TSH) receptors, protease-activated receptors, neuropeptide receptors, adenosine A2B receptors, P2Y purinoceptors, metabolic glutamate receptors, GRK5, GPCR-30, and CXCR4. In a further embodiment, the invention is directed to a pharmaceutical composition or method of treatment described herein wherein the native membrane protein is a GPCR selected from the group comprising purinergic receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$), M_1 and M_3 muscarinic acetylcholine receptors, receptors for thrombin [protease-activated receptor (PAR)-1, PAR-2], thromboxane (TXA_2), sphingosine 1-phosphate ($S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$), lysophosphatidic acid (LPA_1, LPA_2, LPA_3), angiotensin II (AT_1), serotonin ($5\text{-}HT_{2c}$ and $5\text{-}HT_4$), somatostatin (sst_5), endothelin (ET_A and ET_B), cholecystokinin (CCK_1), V_{1a} vasopressin receptors, D_5 dopamine receptors, fMLP formyl peptide receptors, GAL_2 galanin receptors, EP_3 prostanoid receptors, A_1 adenosine receptors, α_1 adrenergic receptors, BB_2 bombesin receptors, B_2 bradykinin receptors, calcium-sensing receptors, chemokine receptors, KSHV-ORF74 chemokine receptors, NK_1 tachykinin receptors, thyroid-stimulating hormone (TSH) receptors, protease-activated receptors, neuropeptide receptors, adenosine A2B receptors, P2Y purinoceptors, metabolic glutamate receptors, GRK5, GPCR-30, and CXCR4

In another embodiment, the water-soluble polypeptide retains the at least some of the ligand-binding activity of the membrane protein. In some embodiments, the GPCRs are mammalian receptors.

In a further embodiment, one or more amino acids within potential ligand binding sites of the native membrane protein are not replaced. In an aspect of this embodiment, examples of native membrane proteins with potential ligand-binding sites having one or more amino acids not replaced include purinergic receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$), M_1 and M_3 muscarinic acetylcholine receptors, receptors for thrombin [protease-activated receptor (PAR)-1, PAR-2], thromboxane (TXA_2), sphingosine 1-phosphate ($S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$), lysophosphatidic acid (LPA_1, LPA_2, LPA_3), angiotensin II (AT_1), serotonin ($5\text{-}HT_{2c}$ and $5\text{-}HT_4$), somatostatin (sst_5), endothelin (ET_A and ET_B), cholecystokinin (CCK_1), V_{1a} vasopressin receptors, D_5 dopamine receptors, fMLP formyl peptide receptors, GAL_2 galanin

receptors, EP_3 prostanoid receptors, A_1 adenosine receptors, α_1 adrenergic receptors, BB_2 bombesin receptors, B_2 bradykinin receptors, calcium-sensing receptors, chemokine receptors, KSHV-ORF74 chemokine receptors, NK_1 tachykinin receptors, thyroid-stimulating hormone (TSH) receptors, protease-activated receptors, neuropeptide receptors, adenosine A2B receptors, P2Y purinoceptors, metabolic glutamate receptors, GRK5, GPCR-30, and CXCR4.

The invention further encompasses a method of treatment for a disorder or disease that is mediated by the activity of a membrane protein, comprising the use of a water-soluble polypeptide to treat said disorders and diseases, wherein said water-soluble polypeptide comprises a modified α-helical domain, and wherein said water-soluble polypeptide retains the ligand-binding activity of the native membrane protein. Examples of such disorders and diseases include, but are not limited to, cancer, small cell lung cancer, melanoma, breast cancer, Parkinson's disease, cardiovascular disease, hypertension, and asthma.

As described herein, the water-soluble peptides described herein can be used for the treatment of conditions or diseases mediated by the activity of a membrane protein. In certain aspects, the water-soluble peptides can act as "decoys" for the membrane receptor and bind to the ligand that activates the membrane receptor. As such, the water-soluble peptides described herein can be used to reduce the activity of a membrane protein. These water-soluble peptides can remain in the circulation and bind to specific ligands, thereby reducing the activity of membrane bound receptors. For example, the GPCR CXCR4 is over-expressed in small cell lung cancer and facilitates metastasis of tumor cells. Binding of this ligand by a water-soluble peptide such as that described herein may significantly reduce metastasis.

The chemokine receptor, CXCR4, is known in viral research as a major coreceptor for the entry of T cell line-tropic HIV (Feng, et al. (1996) Science 272: 872-877; Davis, et al. (1997) *J Exp Med 186*: 1793-1798; Zaitseva, et al. (1997) Nat Med 3: 1369-1375; Sanchez, et al. (1997) *J Biol Chem 272*: 27529-27531). T Stromal cell derived factor 1 (SDF-1) is a chemokine that interacts specifically with CXCR4. When SDF-1 binds to CXCR4, CXCR4 activates Gαi protein-mediated signaling (pertussis toxin-sensitive) (Chen, et al. (1998) *Mol Pharmacol 53*: 177-181), including downstream kinase pathways such as Ras/MAP Kinases and phosphatidylinositol 3-kinase (PI3K)/Akt in lymphocyte, megakaryocytes, and hematopoietic stem cells (Bleul, et al. (1996) *Nature 382*: 829-833; Deng, et al. (1997) *Nature 388*: 296-300; Kijowski, et al. (2001) *Stem Cells 19*: 453-466; Majka, et al. (2001)

Folia. Histochem. Cytobiol. 39: 235-244; Sotsios, et al. (1999) *J. Immunol. 163*: 5954-5963; Vlahakis, et al. (2002*) J. Immunol. 169:* 5546-5554). In mice transplanted with human lymph nodes, SDF-1 induces CXCR4-positive cell migration into the transplanted lymph node (Blades, et al. (2002) *J. Immunol. 168*: 4308-4317).

Recently, studies have shown that CXCR4 interactions may regulate the migration of metastatic cells. Hypoxia, a reduction in partial oxygen pressure, is a microenvironmental change that occurs in most solid tumors and is a major inducer of tumor angiogenesis and therapeutic resistance. Hypoxia increases CXCR4 levels (Staller, et al. (2003) *Nature 425*: 307-311). Microarray analysis on a sub-population of cells from a bone metastatic model with elevated metastatic activity showed that one of the genes increased in the metastatic phenotype was CXCR4. Furthermore, overexpression CXCR4 in isolated cells significantly increased the metastatic activity (Kang, et al. (2003) *Cancer Cell 3*: 537-549). In samples collected from various breast cancer patients, Muller et al. (Muller, et al. (2001) *Nature 410*: 50-56) found that CXCR4 expression level is higher in primary tumors relative to normal mammary gland or epithelial cells. Moreover, CXCR4 antibody treatment has been shown to inhibit metastasis to regional lymph nodes when compared to control isotypes that all metastasized to lymph nodes and lungs (Muller, et al. (2001)). As such a decoy therapy model is suitable for treating CXCR4 mediated diseases and disorders.

In another embodiment of the invention relates to the treatment of a disease or disorder involving CXCR4-dependent chemotaxis, wherein the disease is associated with aberrant leukocyte recruitment or activation. The disease is selected from the group consisting of arthritis, psoriasis, multiple sclerosis, ulcerative colitis, Crohn's disease, allergy, asthma, AIDS associated encephalitis, AIDS related maculopapular skin eruption, AIDS related interstitial pneumonia, AIDS related enteropathy, AIDS related periportal hepatic inflammation and AIDS related glomerulo nephritis.

In another aspect, the invention relates to the treatment of a disease or disorder selected from arthritis, lymphoma, non-small lung cancer, lung cancer, breast cancer, prostate cancer, multiple sclerosis, central nervous system developmental disease, dementia, Parkinson's disease, Alzheimer's disease, tumor, fibroma, astrocytoma, myeloma, glioblastoma, an inflammatory disease, an organ transplantation rejection, AIDS, HIV-infection or angiogenesis.

The invention also encompasses a pharmaceutical composition comprising said water-soluble polypeptide and a pharmaceutically acceptable carrier or diluent.

The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the pharmacologic agent or composition. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like. Pharmaceutical compositions can also include large, slowly metabolized macromolecules such as proteins, polysaccharides such as chitosan, polylactic acids, polyglycolic acids and copolymers (such as latex functionalized SEPHAROSE™, agarose, cellulose, and the like), polymeric amino acids, amino acid copolymers, and lipid aggregates (such as oil droplets or liposomes).

The compositions can be administered parenterally such as, for example, by intravenous, intramuscular, intrathecal or subcutaneous injection. Parenteral administration can be accomplished by incorporating a composition into a solution or suspension. Such solutions or suspensions may also include sterile diluents such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents. Parenteral formulations may also include antibacterial agents such as, for example, benzyl alcohol or methyl parabens, antioxidants such as, for example, ascorbic acid or sodium bisulfite and chelating agents such as EDTA. Buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose may also be added. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Additionally, auxiliary substances, such as wetting or emulsifying agents, surfactants, pH buffering substances and the like can be present in compositions. Other components of pharmaceutical compositions are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

Injectable formulations can be prepared either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The preparation also can also be emulsified or encapsulated in liposomes or micro particles such as polylactide, polyglycolide, or copolymer for enhanced adjuvant effect, as discussed above. Langer, *Science* 249: 1527, 1990 and Hanes, *Advanced Drug Delivery Reviews 28*: 97-119, 1997. The compositions and pharmacologic agents described herein can be administered in the form of a depot injection or implant preparation which can be formulated in such a manner as to permit a sustained or pulsatile release of the active ingredient.

Transdermal administration includes percutaneous absorption of the composition through the skin. Transdermal formulations include patches, ointments, creams, gels, salves and the like. Transdermal delivery can be achieved using a skin patch or using transferosomes. [Paul et al., *Eur. J. Immunol. 25*: 3521-24, 1995; Cevc et al., *Biochem. Biophys. Acta* 1368: 201-15, 1998].

"Treating" or "treatment" includes preventing or delaying the onset of the symptoms, complications, or biochemical indicia of a disease, alleviating or ameliorating the symptoms or arresting or inhibiting further development of the disease, condition, or disorder. A "patient" is a human subject in need of treatment.

An "effective amount" refers to that amount of the therapeutic agent that is sufficient to ameliorate of one or more symptoms of a disorder and/or prevent advancement of a disorder, cause regression of the disorder and/or to achieve a desired effect.

The invention will be better understood in connection with the following example, which is intended as an illustration only and not limiting of the scope of the invention. Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art and such changes and may be made without departing from the spirit of the invention and the scope of the appended claims.

EXAMPLES

Example 1: Systematic analyses of the ligand-binding properties of olfactory receptors

The Q (Glutamine) T (Threonine) Y (Tyrosine) QTY replacement are used to convert a water-insoluble olfactory receptor to a water-soluble one for biochemical, biophysical and structural analyses. Our specific aims are to:

1) Use the QTY (Glutamine, threonine and tyrosine) replacement method to systematically change the 7-transmembrane α-helix hydrophobic residues leucine (L), isoleucine (I), valine (V), and phenylalanine (F) to the hydrophilic residues glutamine (Q), threonine (T) and tyrosine (Y). This method converts the protein from a water-insoluble olfactory receptor to a water-soluble one.

2) Produce and purify milligram quantities of native and bioengineered olfactory receptors using commercial cell-free *in vitro* translation systems (Invitrogen and Qiagen).

3) Determine the secondary structure of the purified olfactory receptors using circular dichroism (CD).

4) Determine the binding affinity of the native and bioengineered olfactory receptor variants using microscale thermophoresis.

5) Transfect the native and variant OR genes into HEK293 cells, and use calcium influx assays to measure odorant activation of the native and mutant olfactory receptors. These measurements will correlate the microscale thermophoresis binding data to functional responses within cells.

6) Systematically screen the native and bioengineered olfactory receptors for crystallizing conditions in the presence and absence of odorants and the presence and absence of detergent.

RESEARCH STRATEGY

Use QTY replacement to design a soluble 7-helical bundle olfactory receptor mOR103-15. An innovation of our study is to convert the water-insoluble olfactory receptor mORI03-15 into a water soluble one with about 10.5% specific residues changes (36aa/340aa). We have systematically and selectively changed key residues at the α-helical positions *b, c, f* that usually face the hydrophilic surface, while maintaining the hydrophobic residues at a-helical positions *a, d, e,* g. Our synthetic biology design method is general and broadly applicable to the study of other olfactory receptors and G-protein coupled receptors. This strategy has the potential to overcome the bottleneck of crystallizing olfactory receptors, as well as additional GPCRs and other membrane proteins. While our design to change the solubility of the sequence is focused on the *b,c,f* positions of the helical wheel, some further changes to other parts of the sequence can be made without significantly affecting the function or structure of the peptide, polypeptide or protein. For example conservative

mutations can be made.

The experimental approach

1) Use of QTY replacements to design a water-soluble 7-helical bundle olfactory receptor mOR103-15. We used synthetic biology methods to convert a water-insoluble olfactory receptor into a water-soluble one with ~10.5% of the residues changes (36aa/340aa) (FIGs. 1-3). We have systematically and selectively changed key residues at the α-helical positions *b,* c, *f* (which usually form the hydrophilic surface), but maintained the hydrophobic residues at α-helical positions *a, d, e, g* (FIG. 1). Our synthetic biology design method is general in nature, thus it is broadly applicable to the study other olfactory receptors as well as other G-protein coupled receptors (GPCRs). This simple strategy may partly overcome the bottleneck of structural studies of olfactory receptors, GPCRs, and other membrane proteins if the converted water-soluble membrane proteins remain biologically functional.

In order to facilitate the study of the structural aspects of olfactory receptors and their binding properties, we can use the QTY replacement method to design a water-soluble 7-bundle helical olfactory receptor mORI03-15 (FIGs. 1-3). It is known that seven amino acids have α-helical forming tendencies (32): leucine (L) (1.30), glutamine (Q) (1.27), phenylalanine (F) (1.07), tyrosine (Y) (0.72), isoleucine (I) (0.97), valine (V) (0.91) and threonine (T) (0.82). We also know that side chains of Q, Y and T can all form hydrogen bonds with water: Q can form 4 H-bonds (2 H-donors from $-NH_2$, 2 H-acceptors from C=O), and T and Y can form 3 H-bonds each (-OH, I-H donor from -H and 2 acceptors from -0). The Q, T, Y residues are more water-soluble than L, F, I, or V, which cannot form any hydrogen bonds with their side chains. The substitutions will not have any positive- or negative-charges changes. Furthermore, the molecular shapes and sizes are very similar for the pairs: leucine/glutamine, phenylalaine/tyrosine, valine/threonine, and isoleucine/threonine. The changes increase the solubility of 7-transmembrane a-helices while maintaining the overall helical structure.

In this soluble olfactory receptor design, we have performed the following substitutions: leucine -> glutamine (L->Q), isoleucine/valine -> threonine (IN->T) and phenylalanine -> tyrosine (F->Y). In the study, we can examine the secondary structure of the water-soluble olfactory receptor, as well as measure its odorant-binding capabilities. If odorant-binding is measured with the QTY replacements, then it is likely that we have

preserved important components of the original structure. We can compare the secondary structure and binding of native olfactory receptor with the designed water-soluble olfactory receptor. We can also produce milligram quantities of the water-soluble receptor, and set up crystal screens with and without odorants.

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MERRNHTGRV SEFVLLGFPA PAPQRALQFF QSLQAYVQTL TENIQTITAI RNHPTLNKPM YYFLANMSYL ETWYTTVTTP
                       abcdefga bcdefgabcd efgabcdefg a        a bcdefgabcd efgabcdefg
KMQAGYIGSE ENHGQLISFE ACMTQLYFFQ GLGCTECTLL AVMAYDRYVA TCHPLHYPVI VSSRQCVQMA AGSWAGGFGT
abcdefg                abcdefgab cdefgabcde fgabcdefga bc            abcdefg abcdefgabc
SMTKVYQISR LSYCGPNTIN HFFCDVSPLL NLSCTDMSTA ELTDFIQAIY TLLGPLSTTG ASYMAITGAV MRIPSAAGRH
defgabcd                          abcdefgab cdefgabcde fgabcdefga          a
KAFSTCASHL TTVITYYAAS IYTYARPKAL SAFDTNKLVS VLYAVITPLQ NPITYCQRNQ EVKKALRRTL HLAQGQDANT
bcdefgabcd efgabcdefg abcd           abcdef gabcdefgab cdefgabc
KKSSRDGGSS GTETSQVAPA. (36aa mutations/340aa, ~10.5% mutations)
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2) Produce and purify milligram quantities of native and bioengineered variants of olfactory receptors. We can use commercial cell-free systems to produce milligrams of native and water-soluble mORI03-15. We can use the optimized protocols we have developed in our lab: this is the key advancement and innovation we have accomplished in the last few years. We can produce and purify the native and variant olfactory receptors in one day using immunoaffinity purification. Gel filtration can then be used to separate the monomeric and dimeric receptor forms.

3) Determine secondary structure using circular dichroism. We can use circular dichroism (CD) spectral analysis to measure the secondary structures of the purified receptors. CD is a very sensitive technique that is be able to detect any small structural changes between the native and mutant receptors. Specifically, CD analysis can be used to calculate the percentage of α-helices and β-sheets in a protein. If a proteins' structure is altered, it can be revealed in the CD analysis. In addition to determining whether specific mutations alter receptor structure, CD can also be used to measure any odorant-induced structural changes. See FIG. 4

4) Assay ligand-binding of olfactory receptors. Microscale thermophoresis are used to measure the binding affinity of the native and bioengineered proteins and their odorant ligands. The key advantages of this technique over SPR or other ligand binding technologies are that they are totally surface-free and label free. Thus, the receptors do not need to be modified. The measurements can be performed in solution using native tryptophan as a signal source. Additionally, small ligands (MW ~200 Daltons) can be reliably measured. Furthermore, each measurement needs 0.5J.t1 (lJlg/J.t1) of sample thus, save the precious receptor samples. These results show whether the mutant olfactory receptors are capable of

binding odorants as efficiently as the native protein.

5) Use calcium influx activation assay to measure olfactory receptor activation. We can use calcium influx assays to examine odorant-induced activation of the native and variant olfactory receptors in HEK293 cells. This data is be correlated to the microscale thermophoresis measurements. Microscale thermophoresis directly measures ligand binding, while calcium influx assays measure activation. Combined, these assays can verify whether specific mutations affect binding, activation, or both. Additionally, we can distinguish between agonist and antagonist ligands.

6) Systematic screen for crystallization conditions. We can systematically screen the native and bioengineered variant olfactory receptors for crystallizing conditions in the absence and presence of odorants. The technology for crystallization screening of water-soluble proteins is well developed. Commercial screens are available which supply a variety of precipitants, salts, buffers with fine tuned pH gradients, and a range of cationic and anionic substances. All of these variables are well known and will be used in crystallizing membrane proteins. An additional unique ingredient of membrane protein screens is the presence of one of more detergent molecules. However, precipitation techniques involving slow water removal from the hanging drop may continue to be effective. Although it is useful to form large crystals, the results of a crystal screen may yield smaller crystals.

Surface Plasmon Resonance analysis of CXCR4 QTY

Human CXCR4 and our CXCR4 QTY proteins obtained from cell-free production and purified with affinity beads were captured in different flow cells on a Biacore CM5 chip with immobilized 1D4 Antibody (Ab) in a Biacore 2000 instrument. Different concentrations of SDF1α, the native ligand for hCXCR4 receptor, were injected over the surface to allow interaction with the receptors.

HUMAN CXCR4 QTY (SEQ ID NO:10)

MEGISIYTSDNYTEEMGSGDYDSMKEPCFREENANYNKTFLPTIYSIIYQTGTVGNGL
VI
LVMGYQKKLRSMTDKYRLHLSTADLQFVTTLPYWATDATANWYFGNFLCKAVHVI
YTVNLYSSVLILAFISLDRYLAIVHATNSQRPRKLLAEKVVYVGVWTPAQLLTTPDY
TFANVSEADDRYICDRFYPNDLWVVVFQFQHIMVGLILPGIVILSCYCIIISKLSHSKG
HQKRKALKTTTTLIQAFFACWQPYYTGISIDSYILLEIIKQGCEFENTVHKWISTTEAQ

AFYHCCTNPTQYAYLGAKFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSS SFHS

Immobilization of 1D4 Antibody

Biacore CM5 chips were activated with 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride and N-hydroxysuccinimide according to the manufacturer's protocol prior to a 7 minute injection at 5µl/min of 1D4 Ab to flow cells 2-4 at 70µg/ml followed by deactivating of the surfaces in all the 4 flow cells with a short Ethanolamine pulse. The immobilization level of 1D4 Ab range from 8000-25000 Response units (RU).

Capture of GPCRs

CXCR4 and CXCR4 QTY mutant are captured by the 1D4 Ab on the CM5 chip by injecting a 0.1mg/ml sample of the protein to a single flow cell at 5µl/min during 15min with both sample and running buffer containing 0.2% Fos-Choline-14 detergent. The receptors were captured to a level of 800-3000 RU.

Interaction analyzis

SDF1α were injected over all flow cells to allow interaction with both the receptors and flow cell one is used as a reference cell without any immobilized protein. Injections were made at 0, 7.8nM, 15.6nM, 31.25nM, 62.5nM, 125nM, 250nM, 500nM, 1µM in triplicates, at 20ul/min for 2 minutes with 15 min waiting time to allow dissociation. HBST (50mM Hepes, pH 7.4, 150mM NaCl, 0.005% Tween-20) with the addition of 0.2% BSA and 0.2% Fos-Choline-14 was used as both running buffer and for dilution of the SDF1α samples.

Conclusion: The above described study shows ligand binding by CXCR4 QTY.

References

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While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

1. A water-soluble variant of a G-protein coupled receptor (GPCR), wherein in said variant, all 7-transmembrane α-helical hydrophobic residues Leucine (L), isoleucine (I), valine (V), and phenylalanine (F) in hydrophilic surface α-helical positions *b*, *c*, and *f* but not positions *a*, *d*, *e*, and *g* of the GPCR have been substituted by glutamine (Q), threonine (T), threonine (T), and tyrosine (Y), respectively.

2. The variant of claim 1, wherein the variant retains ligand-binding activity of the GPCR.

3. The variant of claim 1 or 2, wherein the pI of the variant is substantially the same as the pI of the GPCR.

4. The variant of any one of claims 1-3, wherein the GPCR is a mammalian receptor.

5. The variant of any one of claims 1-4, wherein the GPCR is selected from the group consisting of: purinergic receptors (P2Y1, P2Y2, P2Y4, P2Y6), M1 and M3 muscarinic acetylcholine receptors, receptors for thrombin [protease-activated receptor (PAR)-1, PAR-2], thromboxane (TXA2), sphingosine 1-phosphate (S1P2, S1P3, S1P4 and SIP5), lysophosphatidic acid (LPA1, LPA2, LPA3), angiotensin II (AT1), serotonin (5- HT2c and 5-HT4), somatostatin (sst5), endothelin (ETA and ETB), cholecystokinin (CCK1), V1a vasopressin receptors, D5 dopamine receptors, fMLP formyl peptide receptors, GAL2 galanin receptors, EP3 prostanoid receptors, A1 adenosine receptors, α1 adrenergic receptors, BB2 bombesin receptors, B2 bradykinin receptors, calcium- sensing receptors, chemokine receptors, KSHV-ORF74 chemokine receptors, NK1 tachykinin receptors, thyroid-stimulating hormone (TSH) receptors, protease-activated receptors, neuropeptide receptors, adenosine A2B receptors, P2Y purinoceptors, metabolic glutamate receptors, GRK5, GPCR-30, and CXCR4.

6. The variant of any one of claims 1-5, wherein the GPCR is a CXCR4.

7. The variant of claim 1, wherein at least one transmembrane domain has a sequence of SEQ ID NO: 3, 4, 5, 6, or 8.

8. A method of preparing a water-soluble variant of a G-protein coupled receptor (GPCR), the method comprising replacing all 7-transmembrane α-helical hydrophobic residues Leucine (L), isoleucine (I), valine (V), and phenylalanine (F) in hydrophilic surface α helical positions *b*, *c*, and *f* but not positions *a*, *d*, *e*, and *g* of the GPCR, with glutamine (Q), threonine (T), threonine (T), and tyrosine (Y), respectively.

9. The method of claim 8, further comprising measuring ligand binding.

10. The method of claim 8 or 9, wherein the GPCR is prepared using a cell- free system.

11. A cell comprising the variant of any one of claims 1-7.