Inhibition of G-protein-coupled Receptor Function by Disruption of Transmembrane Domain Interactions*

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G-protein-coupled receptors (GPCR) represent a superfamily of proteins that mediate the function of neurotransmitters and peptide hormones and are involved in viral entry and perception of light, smell, and taste. GPCRs are characterized by the presence of seven transmembrane domains (TMs). We demonstrate here that structural analogs of individual TMs of GPCRs can serve as potent and specific receptor antagonists. Peptides derived from the transmembrane regions of CXCR4 and CCR5 chemokine receptors specifically inhibited receptor signaling and the in vitro replication of human immunodeficiency virus-1 (HIV-1) at concentrations as low as 0.2 μM. Similarly, peptides mimicking the TMs of cholecystokinin receptor A, were found to abolish ligand binding and signaling through the receptor. Negative charges positioned at the extracellular termini of peptide antagonists appeared to be important for correct spontaneous insertion of the compounds into the cell membrane and for their activity. Targeting of the specific interactions between transmembrane domains of GPCRs is suggested as a general sequence-based method to disrupt receptor function for application in drug design and for structure-function studies of the receptors.

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§ To the abbreviations used are: GPCR, G-protein-coupled receptor; TM, transmembrane domain; CXCR4, CXC chemokine receptor 4; CCR5, CC chemokine receptor 5; CCKAR, cholecystokinin receptor type A; CCK-8, cholecystokinin octapeptide; SDF-1α, stromal cell-derived factor-1α; Rhod, rhodamine; HPLC, high performance liquid chromatography; CHO, Chinese hamster ovary; HIV-1, human immunodeficiency virus-1; RG, rhodamine green.

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EXPERIMENTAL PROCEDURES

Peptide Synthesis—The peptides were synthesized by solid phase peptide synthesis on 432A or 433A Applied Biosystems peptide synthesizers equipped with conductivity monitoring units utilizing Fmoc-protected triisopropylsilylethoxycarbonyl (9-fluorenyl)methoxycarbonyl amino acid derivatives. The synthesis was performed with conditional blocking of unreacted amino groups with acetic anhydride for easier purification of the resulting peptides. To overcome aggregation that frequently occurs during the synthesis of hydrophobic peptides and leads to the blocking of the growing peptide chain, NovaSyn TGA resins (Nova Biochrem, San Diego, CA) were used. The peptides were purified by reverse phase HPLC on a C18 column in a gradient of 0.05% trifluoroacetic acid, water and acetonitrile. The purity of the peptides was assessed by reverse phase HPLC on C18 and C4 columns, and the structures were confirmed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry as described (14).

Assessment of [Ca2+]i—The measurements were carried out in Fura-2/AM-treated cells utilizing the Attofluor digital imaging system as described (15). Stock solution of antagonists (5–10 μM) in dimethyl sulfoxide were diluted in phosphate-buffered saline and added to the cells preloaded with Fura-2/AM. Stimulation with an agonist was carried within seconds after application of an antagonist. CXCR4-positive cells were stimulated with 50 nM stromal cell-derived factor-1 (SDF-1α) in a gradient of 0.05% trifluoroacetic acid, water and acetonitrile. The purity of the peptides was assessed by reverse phase HPLC on C18 and C4 columns, and the structures were confirmed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry as described. The minimal concentration required for complete inhibition of signaling was determined by consecutive 2-fold dilutions. Further validation of the mini-
Transmembrane Antagonists of GPCRs

FIG. 1. The hypothetical mechanism of transmembrane antagonists action.

Results and Discussion

The hypothesis for this research was that an externally added peptide corresponding to one of the putative transmembrane domains of a GPCR was able to disrupt the function of the receptor by interfering with the proper association of the TM domains. To test this hypothesis, we first synthesized peptides corresponding to all seven predicted transmembrane regions of CXCR4 chemokine receptor 4 (CXCR4) (17) and tested their ability to inhibit signaling through the receptor. In the antagonist nomenclature used throughout the paper, the first number after the receptor name refers to the transmembrane domain from which the peptide was derived, and the second number corresponds to the order in which the peptides were synthesized. Potential antagonist activity of the peptides was evaluated by measuring the inhibition of the ligand-induced intracellular Ca\(^{2+}\) release in cells stably expressing CXCR4; SDF-1α was used as a ligand at a concentration of 50 nM (K_d = 6 nM). In the preliminary screen, peptides corresponding to the second and sixth TM domains were found to abolish SDF-1α-induced signaling through CXCR4 receptor (Table I).

To further understand the structural requirements for a successful antagonist, structure-activity studies were conducted on the peptides corresponding to the second TM domain of CXCR4 (Table II). The most potent antagonist thus far obtained, a 24-amino acid residue peptide CXCR4-2-2, completely blocked signal transduction at 0.2 μM. Terminal anionic residues appeared to be important for antagonist activity, as elimination of two C-terminal Asp residues (CXCR4-2-1) decreased antagonist potency more than 10-fold, and substitution of Asp residues with positively charged Lys residues (CXCR4-2-3) resulted in a 100-fold decrease in antagonist activity. It is assumed that the charge distribution provides for a proper orientation of the peptides during penetration into the cellular membrane (18). The results obtained from structure-activity studies with TM2 were applied to other domains and allowed the identification of antagonists derived from all but the first, third, and fifth TM domains (Table III, Fig. 2). Peptides derived from the first and the fifth domains imposed significant synthetic difficulties because of aggregation and turned out to be very poorly soluble. In the case of the peptides corresponding to the fourth and seventh TM domain, the positioning of the negatively charged residue at the intracellular terminus of the peptide instead of extracellular terminus or substitution of extracellular Asp with Lys residues abolished the antagonist activity, as had been observed with TM2.

The antagonist potency of CXCR4-2-2 did not depend on the concentration of SDF-1α in a range between 50 and 300 nM. The activity of the compound was tested in U87 cells stably transfected with CXCR4, in HeLa cells that naturally express the receptor, and in HeLa cells stably expressing CXCR4 tagged with green fluorescent protein (25). All tests produced basically the same results, suggesting that variations in the density of receptor molecules that are within the natural levels of receptor expression do not affect the potency of the antagonist.

The specificity of the TM domain interaction was demonstrated by the fact that all peptides derived from CXCR4 appeared to be selective for that receptor and had no influence on signaling through CCR5, which is the other major chemokine receptor involved in HIV-1 entry. Similarly, a peptide derived from the second TM of CCR5, CCR5-2-1 (Table III) completely abolished agonist (RANTES (regulated on activation normal T cell expressed))-induced CCR5 signaling in U87 cells at 0.5 μM but had no effect on signaling of CXCR4.

Interestingly, an equimolar mixture of two peptides CXCR4-6-1 and CXCR4-7-3 was an order of magnitude more potent than the most active of the two and completely inhibited signaling at 1 μM concentration (Table III). Other pairs of peptides failed to show improved potency compared with single components, likely due to nonoptimal combinations and/or nonoptimal structures. Nevertheless, we hypothesized that the synergistic effect produced by a pair of TM derivatives may be a general phenomenon. Consequently, overcoming the entropy factor by linking two TM analogs may result in significantly increased affinity, and thus, appropriate pairs of TM analogs may produce very potent antagonists.

To further generalize the approach, we have synthesized the peptides derived from the TMs of the rat CCKAR. CCKAR was selected for this study because the pharmacology, signaling, and trafficking characteristics of the receptor are well documented (19). Although CCKAR belongs to the same rhodopsin family of GPCRs as CXCR4, its sequence is only 15% identical to that of CXCR4. The degree of identity in transmembrane parts is only 27%. None of CCKAR-derived peptides served as

### Table I

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration required for complete inhibition of [Ca(^{2+})]_i release</th>
</tr>
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<tbody>
<tr>
<td>CXCR4-1-5: DDIFLPTYSIFLTGV-NH(_2)</td>
<td>&gt;30 μM</td>
</tr>
<tr>
<td>CXCR4-2-1: LLFVTLLPFWADAVANYFGN-OH</td>
<td>5 ± 1 μM</td>
</tr>
<tr>
<td>CXCR4-3-1: KAVHVTVTNLYSSLALIFSL-NH(_2)</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>CXCR4-4-1: KYYVGVWIPALLTPDIF-OH</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>CXCR4-5-1: HINVGLILGIVLCSYLH-NH(_2)</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>CXCR4-6-1: VILALAFFACWLPSYYHSID-OH</td>
<td>10 ± 1 μM</td>
</tr>
<tr>
<td>CXCR4-7-1: ALAFFFHCLNLPIYAFGLAK-NH(_2)</td>
<td>&gt;100 μM</td>
</tr>
</tbody>
</table>

Changes in intracellular concentration of Ca\(^{2+}\) were studied in Fura-2/AM-treated U87 cells stably expressing CXCR4. The cells were stimulated by the addition of 50 nM SDF-1α in the presence of varying antagonists concentrations.
Transmembrane Antagonists of GPCRs

Structure-activity relationships in peptides derived from the second transmembrane domain of CXCR4:

HLSVADLLFVTLPFWAVANWNFGNFLCK (the predicted intramembrane portion is underlined)

The measurements were carried out in Fura-2/AM-treated U87 cell stably expressing CXCR4. Calcium release was induced by the addition of 50 nM SDF-1α. Ac, acetyl.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration required for complete inhibition of [Ca²⁺]ₗ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4-2-1: LLFVITLPFWAVANWNFGND</td>
<td>5 ± 1 μM</td>
</tr>
<tr>
<td>CXCR4-2-2: LLFVITLPFWAVANWNNFD-OH</td>
<td>0.2 ± 0.1 μM</td>
</tr>
<tr>
<td>CXCR4-2-3: LLFVITLPFWAVANWNFGanking-h</td>
<td>20 ± 2 μM</td>
</tr>
<tr>
<td>CXCR4-2-4: VITLPFWAVANWNFGNFLCK-KOH</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>CXCR4-2-5: LLFVITLPFWAVANWNNFK-OH</td>
<td>10 ± 1 μM</td>
</tr>
<tr>
<td>AcCXCR4-2-5: AcLLFVITLPFWAVANWNNFD-OH</td>
<td>10 ± 1 μM</td>
</tr>
<tr>
<td>CXCR4-2-6: LSVADLLFVTLPFWAVANND-OH</td>
<td>20 ± 2 μM</td>
</tr>
<tr>
<td>Rhod-CXCR4-2: AcLLFVITLPFWAVANWNNFDK (Rhod)D-OH</td>
<td>8 ± 1 μM</td>
</tr>
</tbody>
</table>

Table III

Biological activity of CXCR4 antagonists derived from different transmembrane domains

Antisignaling activity was determined in inhibition of intracellular calcium release induced by 50 nM SDF-1α in U87 cells stably expressing CXCR4. Anti-HIV-1 activity was assessed in a cytotoxicity assay utilizing CEM-SS cells infected with HIV-1₀₀₀, as described (27). ND, not determined.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration required for complete inhibition of signal transduction (μM)</th>
<th>EC₅₀ in anti-HIV-1 assay (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4-2-2: LLFVITLPFWAVANWNFGND</td>
<td>0.2 ± 0.1 μM</td>
<td>2.27 ± 0.2 μM</td>
</tr>
<tr>
<td>CXCR4-4-2: VYGVWIPALLTLDFIDIFAND-OH</td>
<td>5 ± 1 μM</td>
<td>0.3 ± 0.1 μM</td>
</tr>
<tr>
<td>CXCR4-6-1: VILLAFFACWLPYGGISID-OH</td>
<td>25 ± 1 μM</td>
<td>50 μM</td>
</tr>
<tr>
<td>CXCR4-7-3: DDEALAPFFICCLNPILYAFL-NH</td>
<td>25 ± 2 μM</td>
<td>3.27 ± 0.33 μM</td>
</tr>
<tr>
<td>CXCR4-6-1 + CXCR4-7-3 (equimolar mixture)</td>
<td>1 ± 0.2 μM</td>
<td>ND</td>
</tr>
<tr>
<td>CCR5-2-1 (CCR5 antagonist derived from TM2)</td>
<td>&gt;100 μM</td>
<td>1.16 ± 0.12 μM</td>
</tr>
</tbody>
</table>

*a*, Ac, acetyl.

...proteins (22). Thus, the natural ligand, CCK-8, appeared to be able to compete out the binding of these two antagonists. However, the antisignaling potency of CCKAR-6–1 remained unchanged when the agonist concentration was increased 2 orders of magnitude.

The major purpose of the present study was to demonstrate the ability of externally added molecules to compete for the interactions between TM domains of GPCRs. Implicit in this idea was that the GPCR structure, even in properly folded molecules, is flexible enough to be disrupted in this fashion. A similar approach was used to inhibit coupling of GPCRs to intracellular signaling molecules, adenylate cyclase, (23) and G-proteins (24) by peptides corresponding to the intracellular loops of the receptors. Those studies were conducted primarily to provide an understanding of molecular mechanisms of receptor function and could not be applied directly for drug design because of the difficulties in intracellular delivery of the inhibitors. The hydrophobic nature of the transmembrane peptides makes their penetrations into the cell membrane bilayer highly probable, and orientation inside the membrane can be controlled by the addition of charged residues to the extracellular termini. Because the TM2 peptides of CXCR4 were the most effective antagonists identified, we next evaluated the intracellular distribution of these peptides using a fluorescent derivative of CXCR4–2–2 that was prepared by attachment of a rhodamine B moiety to the extracellular terminus. Rhod-CXCR4-2 was 40 times less potent than the parent CXCR4–2–2 peptide (see Table II) but still was able to disrupt receptor function. Localization of the peptide was characterized by confocal laser-scanning microscopy using HeLa cells that stably express a green fluorescent protein-labeled CXCR4 (see Fig. 5)
Rhod-CXCR4-2 was observed in the cellular membrane within minutes after application, and saturated endosomes and the endoplasmic reticulum were observed within 15 min of incubation. These observations confirm the ability of the new TM domain analogs to insert spontaneously into the cellular membranes, thus enabling interactions with the receptor in all cellular compartments where the receptor molecules are present. After the fluorescent antagonist was removed from the incubation medium, it was retained in the endoplasmic reticulum and in the endosomes for several hours. This observation suggests that the transmembrane peptides may be able to interact with newly synthesized receptor molecules in the endoplasmic reticulum and, thus, have prolonged inhibitory effects.

**Table IV**

The activity of CCKAR-derived TM peptides in the inhibition of CCK-8-induced intracellular calcium release and RG-CCK-8 binding

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration required for complete inhibition of signaling</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; in inhibition of RG-CCK-8 binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested with 10 nM CCK-8 Tested with 1 μM CCK-8</td>
<td></td>
</tr>
<tr>
<td>CCKAR-1-1</td>
<td>50 ± 5 μM &gt;100 μM</td>
<td>20 ± 2 μM</td>
</tr>
<tr>
<td>CCKAR-2-1</td>
<td>2 ± 0.5 μM &gt;100 μM</td>
<td>0.5 ± 0.12 μM</td>
</tr>
<tr>
<td>CCKAR-4-1</td>
<td>&gt;50 μM ND</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>CCKAR-5-1</td>
<td>&gt;50 μM ND</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>CCKAR-6-1</td>
<td>5 ± 1 μM 5 ± 1 μM</td>
<td>&gt;50 μM</td>
</tr>
</tbody>
</table>

**Fig. 3.** Displacement of RG-CCK-8 binding to CHO cells stably expressing rat CCKAR. CHO cells stably expressing CCKAR were incubated with RG-CCK-8 (10 nM) either alone or with indicated concentrations of antagonists. Binding was evaluated by quantitative confocal laser-scanning microscopy as described (15). Each value represents the mean ± S.E. from at least three experiments. ○, CCKAR-2-1; ●, CCKAR-1-1; ■, CCKAR-6-1.

**Fig. 4.** Positions of the sequences from which the peptides were derived in the primary structure of CCKAR (20). Transmembrane domains are underlined, and sequences used for peptide synthesis are in bold.

**Fig. 5.** Confocal laser-scanning microscopy image of HeLa cells stably expressing CXCR4-green fluorescent protein fusion protein (green) after 5 min treatment with 1 μM Rhod-CXCR4-2 (in red). A, red fluorescence of Rhod-CXCR4-2; B, green fluorescence of CXCR4-green fluorescent protein; C, overlay of A and B, colocalization of CXCR4 and Rhod-CXCR4-2 appears as yellow. D, Nomarski image. The microscopy was performed as described (15, 25).

**Fig. 6.** Anti-HIV efficacy and toxicity of CXCR4-4-2 in cytoprotection assay. CEM-SS cells were infected with RF strain of HIV-1, which causes cell death in the absence of an effective inhibitor (27). Cell survival was assessed with the help of the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium) assay (27). Each value represents the mean ± S.E. from at least three experiments performed in triplicates.
effects on the treated cells. It is unlikely however, that the endoplasmic reticulum is the only site of action of the inhibitory peptides, since it is clear from the present data that fully assembled receptor molecules in the plasma membrane are also disrupted. The evidence for this comes from the complete inhibition of signaling within seconds after addition of the peptides (see “Experimental Procedures”).

The studies with CXCR4, CCR5, and CCKAR clearly demonstrate the ability of TM domain analogs of GPCRs to disrupt ligand binding and signaling through the receptor. However, our interest was to extend the observation to other biological functions of the receptors. CXCR4 and CCR5 are involved in the fusion of HIV-1 to host cells. Importantly, an indication that TM peptide analogs inhibit CXCR4-dependent functions might be applicable to drug design efforts. Given that CXCR4 represents one of the principal coreceptors for HIV-1 cell entry (reviewed in Ref. 26) and that the TM domain peptides of CXCR4 interfere with receptor functions, we tested TM peptide analogs (corresponding to TM domains 2, 4, 6, or 7) as possible inhibitors of HIV-1\textsubscript{gp} replication in a cell-based cytoprotection assay (27). As shown in Table III, the most potent peptide, CXCR4-4-2, inhibited infection at concentrations below 1 \textmu M (IC\textsubscript{50} \approx 300 nM) (see also Fig. 6), whereas peptides CXCR4-2-2 and CXCR4-7-3 inhibited in the 2–3 \textmu M range. None of CCKAR-derived peptides inhibited HIV-1 infection, and none of the peptides caused cell toxicity at concentrations of up to 100 \textmu M (higher concentration could not be tested due to limitations of solubility). These data clearly establish that TM peptide analogs of CXCR4 can inhibit HIV-1 replication, and this approach may be applied to CCR5 and other chemokine receptors that participate in HIV-1 entry into host cells.

We propose that the observed disruption of GPCR function by TM peptide analogs is due to competition of the peptides for interaction between intramembrane helices (Fig. 1). In the presence of an excess of the peptide the majority of receptor molecules will be inactive because the synthetic peptides occupying the place of the corresponding receptor domain lack the necessary extramembrane components. It should be noted that the antagonistic potency of the peptides may depend not only on the interaction with the target receptor but also on the efficiency of membrane insertion of externally added molecules. Thus, the poor activity of some peptides may be partially attributed to their weak ability to enter the plasma membrane. Indeed, different transmembrane domains of CCKAR were previously shown to have varying abilities to insert into membranes (28). The detailed characterization of the competence of different TM analogs to insert into the membranes and interact with each other in a specific manner as well as the participation of discrete residues of peptides to interact with receptor domains is ongoing in our laboratory. We suggest that information from these studies can be utilized to construct more efficient TM inhibitors. Screening of combinatorial libraries of peptides can also assist in selection of more potent antagonists. Peptidomimetics have been successfully applied for construction of analogs of helical peptides and, thus, can be used to obtain antagonists demonstrating enhanced pharmacokinetic properties (29).

In conclusion, we suggest that peptides, peptidomimetics, or even small molecules can be utilized as novel classes of antagonists to target the TM regions of GPCRs and disrupt receptor function. TM antagonists would not only be useful in the development of new drug candidates but would also facilitate the study of GPCR function and three-dimensional structures. In addition, effective antagonists might be designed based solely on amino acid sequences, and thus, the approach also should be useful for targeting orphan receptors. Finally, the TM-targeting approach need not be restricted to GPCR molecules but might also be applied to multimembrane-spanning proteins of other classes, since specific interactions between TM domains are vitally important for the functions of a diverse set of surface proteins.

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