Site-specific Disulfide Capture of Agonist and Antagonist Peptides on the C5a Receptor*

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Elizabeth Buck, Henry Bourne, and James A. Wells†
From the Sunesis Pharmaceuticals Inc., South San Francisco, California 94080 and the Department of Molecular Pharmacology, University of California at San Francisco, California 94143

The manner by which peptidic ligands bind and activate their corresponding G-protein-coupled receptors is not well understood. One of the better characterized peptidic ligands is the chemotactic cytokine complement factor 5a (C5a), a 74-amino acid helical bundle. Previous studies showed 6-mer peptide analogs derived from the C terminus of the C5a ligand can bind to C5aR (Kd values ~0.1–1 μM) and either agonize or antagonize the receptor (Gerber, B. O., Meng, E. C., Dotsch, V., Baranski, T. J., and Bourne, H. R. (2001) J. Biol. Chem. 276, 3394–3400). Here, we provide direct biochemical data using disulfide trapping to support a model that these peptides bind within a transmembrane helical triad formed by α-helices III, VI, and VII. We show that the three amino acids on the C terminus of the peptide analogs bind too weakly to exert a functional effect themselves. However, when a cysteine residue is placed on their N terminus they can be trapped by disulfide interchange to specific cysteines in helix III and VI and not to other cysteines, engineered into the C5aR. The trapped peptides function as agonists or partial antagonists, similar to the non-covalent parents from which they were derived. These data help to further refine the binding mode for C5a to the C5aR and suggest an approach and a binding site that may be applicable to studying other peptide binding receptors.

Receptors that couple to heterotrimeric G-proteins (GPCRs) represent a major class of cell surface receptors (1, 2). Currently, more than 600 GPCRs have been identified, and members of this receptor family are involved in nearly all facets of cellular signal transduction. To date the only crystal structure reported from this class of receptors is the bovine retinal photoreceptor rhodopsin (3), and our understanding of the molecular mechanism underlying ligand binding and receptor activation for GPCRs has been largely guided by biochemical approaches including mutagenesis and peptide analog studies (4–11).

GPCRs can be activated by a diverse collection of ligands (12–15). Despite this diversity, the receptors may be activated by a common triggering mechanism located in the transmembrane region (2, 4). Even for peptidic chemokines and cytokines data suggest part of the binding and activation mechanism is contained in the transmembrane domain of the receptor (16–20). For example, a six-amino acid peptide (Cha-Cha, Table I) derived from the C terminus of the C5a cytokine can bind and fully activate the C5aR (10, 21). Slight alterations in the residue at position 5 of these peptides (Cha to Trp) results in a peptide (Cha-Trp) that retains binding but is converted to a partial antagonist. Based on these studies and mutational data it was proposed that the peptides occupy a site formed by helices III, V, VI, and VII.

To further evaluate the binding hypothesis for these peptides we employed a disulfide trapping approach. Deletion of the first three amino acids from the 6-mer peptides dramatically reduced their binding affinity and retained the penultimate residue that switched the 6-mers from agonists to partial antagonists. Cysteines were appended to the N terminus of these 3-mers and then allowed to undergo thiol-disulfide interchange with different cysteines designed into helices II, III, and VI of the C5aR. The peptides could be captured from the designed cysteines that were most consistent with the previously proposed binding model. The trapped 3-mer peptides exerted the same agonist or partial agonist effects as the non-covalent parents from which they were derived, and their effects were fully reversed by reduction of the disulfide bond. Tethered peptides derived from the protein ligand provide orthogonal support for this binding model and may be generally useful for localizing ligand binding sites and activation switches in other peptide binding receptors.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant C5a was from Sigma. 125I-Labeled C5a and myo-[3H]inositol were from PerkinElmer Life Sciences. All peptides were from the Tufts University Core Facility.

Transfection of COS-7 Cells—COS-7 cells were transfected with a plasmid carrying the cDNA for C5aR using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. COS-7 cells were cultured to 80% confluence prior to transfection and harvested 2 days (for IP3 accumulation) or 3 days (for preparation of membranes) post-transfection.

Preparation of Membranes from COS-7 Cells—Cells were suspended into hypotonic lysis buffer and lysed by passage through a 22-gauge needle. Cell lysate was layered on a sucrose cushion and centrifuged at 45,000 rpm for 1 h at 4 °C. Membrane pellets were resuspended into hypotonic lysis buffer, frozen in liquid nitrogen, and stored at −80 °C.

125I-C5a Binding—COS-7 cell membranes were incubated with C5a ligand, peptides, or small molecule compounds in buffer (20 mM Hepes pH 8.0, 5 mM MgCl2, 1 mM CaCl2, 100 μM Pefabloc, 0.1% bacitracin, 0.5% bovine serum albumin) along with 0.1 μM 125I-C5a for 3 h at room temperature. Binding reactions were then filtered over Millipore Micronose 0.22-μm filters, and filters were washed three times with ice-cold binding buffer. Optiphase liquid scintillant was added directly to the filters, and counts were measured in a Wallac microbeta plate reader.

IP3 Accumulation—COS-7 cells are cultured and co-transfected with Gαqα and C5aR in 24-well culture dishes. 24 h post-transfection cell monolayers were washed 1× with PBS and growth medium was exchanged with isosolvent-free Dulbecco’s modified Eagle’s medium containing 10% dialyzed fetal bovine serum and 100 μM myo-[3H]inositol. Cells...
was assayed for IP₃ accumulation 24 h post-labeling as described previously (11).

RESULTS

Previous studies with hexapeptides, Cha-Cha and Cha-Trp (Table I), had indicated that the first three amino acid residues, especially the Phe at position 1, were important for receptor binding affinity (23). Truncated versions of the agonist (Cha-Cha) and antagonist (Cha-Trp) hexapeptides were prepared that contained cysteines at their N termini (Table I). We reasoned that truncating the first three residues of the hexapeptides would reduce binding affinity but retain some of the crucial determinants, including the penultimate residue, for receptor activation. The corresponding 3-mer peptides would likely not possess sufficient affinity to bind on their own; however, if a cysteine were appended to their N termini (Cys-Cha and Cys-Trp (Table I)) their low intrinsic affinity could be stabilized by specific cysteines engineered into the receptor and the truncated peptides could be trapped in the receptor-binding site.

A proposed computational model of the Cha-Trp antagonist hexapeptide in complex with a rhodopsin-based homology model of the C5a receptor guided our selection of receptor sites that might serve as cysteine anchors for the Cys peptides, Fig. 1A (11). Cysteines were introduced at four positions of C5aR: G262C on helix VI, P113C and L117C on helix III, and F93C on helix II (Fig. 1, B and C). Mutagenesis studies have shown these are not directly involved in ligand binding (5, 24). Assuming the actual binding configuration of the dCha-Cha-dArg peptide is the same as that predicted by the model it would be possible to form a disulfide with the N-terminal cysteine on the peptides (Cys-Cha and Cys-Trp) to cysteines engineered at residues 262, 113, and 117 but not 93 of the receptor.

All cysteine mutant forms of the receptor had binding affinities and efficacies for C5a and the Cha-Cha hexapeptide that were similar to that observed for the wild type receptor (Table II). As expected the hexapeptide Cha-Cha inhibited C5a binding to wild type with an IC₅₀ value of ∼235 nm, whereas the truncated cysteine-containing Cys-Cha and Cys-Trp peptides had little effect (Fig. 2A). Similar results were seen for the F93C receptor variant (Fig. 2B). However, when a cysteine anchor is introduced at position 262, the apparent affinities for both Cys-Cha and Cys-Trp were dramatically enhanced (Fig. 2C); both ligands could block binding of ¹²⁵I-C5a with apparent IC₅₀ values of 8 and 33 µM, respectively. A similar result was obtained for the cysteine engineered at the 113 position (Fig. 2D). The effect of the Cys-Cha peptide on L117C, located one helical turn below P113C (Fig. 1C), was severalfold weaker, 45 µM (Fig. 2E). The differential effects observed are consistent with the model and reflect a fairly narrow window for proper binding of the peptides.

To test the reversibility of these effects we reacted both the G262C and P113C mutants with the Cys peptides in the presence of increasing concentrations of the reducing agent β-ME (Fig. 3). The inhibition of ligand binding by the Cys peptides could be fully reversed by β-ME and supports that these peptides are exerting their effects via specific disulfide bonds with the engineered cysteines. The non-covalent 6-mer Cha-Cha peptide (Table I) can fully active the receptor as measured by IP₃ accumulation in COS-7 cells transfected with the C5aR (11). Here, the truncated Cys-Cha peptide was seen to activate the G262C and P113C variants (EC₅₀ values of 284 and 265 µM, respectively) and do so much more effectively than it can activate the wild-type receptor. This is consistent with the tighter binding affinity provided by forming the disulfide bond. The activation by Cys-Cha on the G262C variant was fully reversible with β-ME (Fig. 4B).

![Fig. 1. A homology model of the C5a receptor (gray) in complex with the Cha-Trp hexamer peptide (magenta) (11). B and C, homology models of the C5a receptor (gray) in complex with the Cys-Trp peptide. The cysteine of the Cys-Trp peptide is indicated in red. The cysteines introduced into the C5aR at positions 262 (blue), 113 (yellow), 117 (orange), and 93 (green) are highlighted. Distances from G262C and F93C to the cysteine within the Cys-Trp peptide is illustrated in B, and distances from P113C and L117C to the cysteine within the Cys-Trp peptide are illustrated in C. Models were rendered using PyMol (Delano Scientific).](http://www.jbc.org/)

| Binding affinity | Signal Transfer |
|------------------|----------------|
| Me Phe Lys Pro dCha Cha dArg COO = ChaCha | |
| Me Phe Lys Pro dCha Trp dArg COO = ChaTrp | |
| Cys dCha Cha dArg COO = CysCha | |
| Cys dCha Trp dArg COO = CysTrp | |

Table I

Sequences of parent 6-mer peptides (Cha-Cha and Cha-Trp) and truncated cysteine-containing 3-mer peptides (Cys-Cha and Cys-Trp) used in the following studies.

The Phe, implicated to be important for binding affinity, and the Cha, implicated to be important for binding and signal transfer, are indicated.
Summary of the apparent binding affinities and receptor activation for the C5a ligand and Cha-Cha peptide with the wild-type and cysteine-mutant forms (G262C, P113C, L117C, and F93C) of the C5aR.

For the IC$_{50}$ for C5a binding, S.D. = ±0.04–0.1 nm; for the IC$_{50}$ for Cha-Cha binding, S.D. = ±72–114 μM; for the EC$_{50}$ for C5a activation, S.D. = ±0.2–0.8 nm; ND, not determined.

|            | C5a binding | Cha-Cha binding | C5a activation, EC$_{50}$ |
|------------|-------------|-----------------|--------------------------|
| Wild type  | 0.25        | 235             | 0.8                      |
| G262C      | 0.15        | 113             | 2.5                      |
| P113C      | 0.48        | 263             | 1.5                      |
| L117C      | 0.25        | 151             | ND                       |
| F93C       | 0.18        | 482             | ND                       |

FIG. 2. Displacement of C5a from the C5aR by increasing concentrations of the 6-mer Cha-Cha peptide (open circles), Cys-Cha peptide (closed circles), and Cys-Trp peptide (closed squares) for the wild-type C5aR (A), the F93C (B), C5aR-G262C (C), C5aR-P113C (D), and C5aR-L117C (E). Results shown are representative of at least three independent experiments. The K_d value for binding of C5a to the C5aR is ~0.2 nm.

Whereas β-ME could reverse the effects of the Cys-Cha peptide on the G262C variant, it had no effect on the basal or C5a-stimulated activity of this mutant (data not shown).

Substitution of the penultimate residue Cha for Trp in the 6-mer peptide reverts it to a partial agonist (12). Although the truncated Cys-Trp peptide had no measurable effect on either basal or ligand activated receptor activity for the wild-type receptor or the G262C mutant, it behaved as a partial agonist for the P113C mutant form (Fig. 4C). This differential effect of Cys-Trp on G262C and P113C is likely the result of its 4-fold weaker binding affinity for the G262C mutant (Fig. 2, C and D). Cys-Trp could only stimulate P113C to ~30% of C5a stimulation with an EC$_{50}$ of 154 μM, and it inhibited C5a-stimulated activity with an IC$_{50}$ of 159 μM, indicating that it behaves as a partial antagonist (Fig. 4D).

FIG. 3. Effects of increasing concentrations of β-ME on the inhibition of C5a ligand binding by 30 μM of Cys-Cha peptide to G262C (A) and binding by 30 μM Cys-Cha or Cys-Trp to P113C (B). Results shown are representative of at least three independent experiments.

DISCUSSION

The refractory nature of GPCRs to direct structural analysis has restricted our ability to probe the regulatory mechanism(s) for these receptors. This is especially apparent for the peptide-ligand class of GPCRs where ligand interactions that evoke receptor activation have only begun to be described. Mutational studies provide important insights into residues that are critical for binding and activation of the receptor (4, 5, 7, 9, 24–26). Ligand analoging studies reveal structure activity relationships (SARs) that distinguish binding from activation (11, 21–23). We believe that ligand disulfide trapping provides an orthogonal approach that can tie these two databases together. Like mutagenesis it localizes the functional effects but does so within the context of known ligands and their SARs.

The key tools to facilitate this approach are weak binding cysteine-containing ligands, whose binding and functional effects are dependent upon the introduction of specific cysteine anchors. This provides the crucial controls to ensure selectivity. For example, the truncated 3-mer peptides do not bind tightly or affect the function of the wild-type receptor but only exert their effects in the context of specific cysteine mutants, where the disulfide functions to stabilize the low intrinsic affinity of the peptide. Even within the cysteine mutants there was a functional gradient in the ability of these to be affected by the cysteine-containing peptides in the following order: P113C > G262C > L117C >> F93C and wild-type. This was remarkably consistent with the previously reported model of the C5aR-peptide complex (11) (Fig. 1). The model predicts that the cysteine from the peptide can be modeled closest to P113C and G262C.

In the presence of a redox buffer, the process of disulfide exchange is an equilibrium process in which specific non-covalent interactions place the ligand in proximity to form the disulfide. In the absence of intrinsic binding affinity the ligands cannot be trapped because they are readily reduced by the redox buffer. This is the basis for Tethering®, a fragment-based ligand discovery tool that has been extensively used for discovering nucleating small ligands to nucleate the drug discovery process (for review, see Ref. 27). Numerous small molecule ligands have been captured by Tethering, and the structures of the covalent and non-covalent counterparts seen by x-ray crys-
tallography are virtually identical, suggesting the disulfide does not distort the binding of the Tethered versus free compound (28–30). A final advantage of the disulfide is that unlike mutagenesis, it is a reversible event, ruling out the possibility that the ligands exert their effects by nonspecific means.

The fact that the trapped peptides exhibited agonist or partial antagonistic effects, like the non-covalent parents they were derived from, provided important support that they are trapped in a manner that truly reflects the non-covalent mode of binding. Interaction between three amino acids of this peptide and the helical III-VI-VII triad are sufficient for maximal receptor activation. Ligand interactions with this helical triad likely trigger a conformational change that translates to receptor activation. This may involve the release of a transmembrane constraint, allowing the receptor to adopt its active conformation, as has been proposed for other members of the GPCR family (4).

Another site-directed method has been recently described using engineered metal binding sites to identify novel metal chelate structures which can bind and activate GPCRs (31–33). We believe ligand disulfide trapping provides an important, complementary, and orthogonal approach to localizing ligand binding sites in proteins. Combining this information with mutational and ligand SARs can provide strong support for binding sites in proteins. Combining this information with computational models for ligand binding sites. Ligand trapping methods can have a special impact on membrane proteins of pharmaceutical interest where there is often a mutational and ligand SARs can provide strong support for binding sites in proteins. Combining this information with computational models for ligand binding sites.

![Image](https://example.com/image.png)

**Fig. 4.** A, dose response for increasing concentrations of Cys-Cha peptide on activation of the wild-type C5aR (open circles) and two variant receptors, G262C (filled circles) or P113C (filled squares). B, dose response for increasing concentrations of the Cys-Cha peptide on the activation of the G262C receptor activation in the absence (closed circles) or in the presence of 0.5 mM β-ME (open circles) or 5.0 mM β-ME (open diamonds). C, activation of wild-type, G262C, or P113C receptors with 300 μM Cys-Trp peptide alone or in the presence of 10 nM C5a. Data are shown as percent of maximal stimulation by the C5a ligand. D, dose response for increasing concentrations of Cys-Trp peptide on activation of P113C alone (open circles) or in the presence of 2 nM C5a (closed circles). Results shown are a representative of three or more independent experiments.

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