An Activation Switch in the Ligand Binding Pocket of the C5a Receptor

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Running Title
Ligand Binding to the C5a Receptor
Although agonists are thought to occupy binding pockets within the seven-helix core of serpentine receptors, the topography of these binding pockets and the conformational changes responsible for receptor activation are poorly understood. To identify the ligand binding pocket in the receptor for complement factor 5a (C5aR), we assessed binding affinities of hexapeptide ligands, each mutated at a single position, for seven mutant C5aRs, each mutated at a single position in the putative ligand binding site. In ChaW (an antagonist) and W5Cha (an agonist), the side chains at position 5 are tryptophan and cyclohexylalanine, respectively. Comparisons of binding affinities indicated that the hexapeptide residue at this position interacts with two C5aR residues, I116 (helix III) and V286 (helix VII); in a C5aR model these two side chains point toward one another. Both the I116A and the V286A mutations markedly increased binding affinity of W5Cha, but not that of ChaW. Moreover, ChaW, the antagonist hexapeptide, acted as a full agonist on the I116A mutant. These results argue that C5aR residues I116 and V286 interact with the side chain at position 5 of the hexapeptide ligand to form an activation switch. Based on this and previous work, we present a docking model for the hexapeptide within the C5aR binding pocket. We propose that agonists induce a small change in the relative orientations of helices III and VII, and that these helices work together to allow movement of helix VI away from the receptor core, thereby triggering G protein activation.
Serpentine receptors transmit a diverse array of extracellular stimuli to heterotrimeric G proteins located on the cytoplasmic face of the plasma membrane. These receptors promote exchange of GTP for GDP bound to the α subunit of the heterotrimer, allowing the α and βγ subunits to disengage from one another and activate intracellular effectors (1). Of several serpentine receptor families (2), the rhodopsin-like family is the largest (3). Low-resolution models of the 3D structure of the seven-helix bundle in the serpentine receptor core were based on patterns of conserved primary structure, biochemical observations with many receptors, and a low-resolution electron cryomicroscopy structure of rhodopsin (4). Three such models, constructed independently (3, 5, 6), predict 3D structures of the transmembrane helices that are remarkably similar to one another and to a recent 3D crystal structure of rhodopsin at atomic resolution (7). These similarities make the crystal structure a promising platform for designing and interpreting experiments aimed at elucidating structure and molecular mechanisms of other members of the rhodopsin-like family of serpentine receptors.

The mechanism of receptor activation, which is probably highly conserved also, resides in the transmembrane helices (8), where most of the evolutionarily conserved residues are located. Indeed, extra- or intracellular loops and termini can be exchanged between different receptors, leaving intact the receptors’ capacity to be activated, while swapping ligand- or G protein specificity (9). How is this conserved activation switch activated by an enormously diverse set of agonist ligands, widely differing in size and chemical character, which must occupy similarly diverse binding pockets in the receptors? Small agonists, including biogenic amines and chromophores, are thought to bind exclusively to a transmembrane receptor pocket; larger ligands, such as oligopeptides and proteins, interact in addition with extracellular domains of their receptors (2, 10, 11). Progress beyond this generalization has proved difficult; binding interactions have been studied in biochemical detail only for relatively small ligands,
such as adrenergic amines and retinal, the chromophore of the visual pigment, rhodopsin (reviewed in (10)).

The receptor for complement factor 5a (C5a), a 74 amino acid protein, furnishes an instructive experimental model for studying how larger peptides bind to and activate serpentine receptors (reviewed in (12)). Deletion mutants of the C5a receptor (C5aR) indicate that C5a interacts both with the receptor’s N-terminus and with the transmembrane bundle; the latter interaction is required for activation of the C5aR (13, 14). Molecular details of the C5a-C5aR interaction have been elucidated by pharmacological characterization of C5a mutants and peptides derived from the C-terminal amino acid sequence of C5a (13, 15-23). In addition, 3D structures have been determined for C5a (24) and a hexapeptide antagonist (22) Me-F-K-P-dCha-W-dR (hereafter termed ChaW; dCha = D-cyclohexylalanine). Taken together, these studies show that receptor activation is mediated by interaction of C-terminal residues of C5a with the receptor’s transmembrane helix bundle; hexapeptide ligands analogous to the C-terminal eight residues of C5a interact exclusively with the transmembrane pocket (13). One residue in transmembrane helix V of the receptor, R206, is essential for receptor activation by a hexapeptide agonist; the guanidinium group of R206 interacts with the terminal carboxylate of this agonist, as shown by characterization of simultaneously altered ligand and receptor mutants (21). Unlike its C-terminal residues, remaining regions of C5a sequence interact with the N-terminus of the C5aR (14); this interaction enhances ligand binding affinity, but is not required for receptor activation.

A genetic screen of C5aR mutants expressed in *S. cerevisiae* (25), designed to assess the functional importance of amino acids in receptor helices III, V, VI and VII, identified a cluster of residues situated at extracellular ends of the transmembrane helices that were required for C5aR signaling but that are not evolutionarily conserved. These residues are located at positions cognate to positions of residues that are thought
to interact with ligands for the β2-adrenoreceptor, rhodopsin and other serpentine receptors, suggesting that structurally different agonists may activate their receptors by a common mechanism, which involves similarly located elements of the receptors ligand binding pockets. To address this hypothesis, we characterized interactions of C5a and other ligands with C5aR mutants in which alanine or other residues replace natural residues at sites in the putative ligand binding pocket. These experiments identified a transmembrane binding pocket for the antagonist ChaW, as well as an apparent activation switch that involves ligand interaction with adjacent residues in helices III and VII. On this basis we propose a model of the complex of the C5aR with the synthetic antagonist, ChaW. The model and our biochemical observations point to a site for ligand-dependent activation that is probably conserved in many serpentine receptors.
EXPERIMENTAL PROCEDURES

Hexapeptide (ant)agonists ChaW and its single site derivatives F1L, dCha4dL, W5Cha, and dR6dH were from the Daiichi Research Center, University of California at San Francisco, San Francisco, CA. The peptides were synthesized by standard solid phase peptide chemistry, purified by high pressure liquid chromatography, and analyzed by mass spectroscopy.

Construction of Receptor Mutants and Yeast Assays Mutated C5aRs were created as described (25). Analysis of receptor signaling in yeast was performed by replica plating onto different concentrations of aminotriazole (AT), as previously described (25). For COS-7 cell assays, the various C5aR sequences were subcloned into plasmid pDM8 (Invitrogen, Carlsbad, CA).

Mammalian Cell Culture and Transfection COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, and 10 μg/ml gentamycin. Transfections of WT and mutant receptors were performed using a DEAE-dextran/adenovirus method, as described (26).

Membrane Preparations Membranes of COS-7 cells transfected with WT or mutant C5aR were prepared by a modification of a previously described method (26). Cells were harvested and lysed in PBS pH 7.4 with 10 mM EDTA, 2 mM DTT, and protease inhibitors (phenylmethylsulfonyl fluoride, bacitracin and leupeptin), and homogenized by passing 10 times through a 27-gauge needle. The supernatant fraction of two successive centrifugations at 900 X g for 10 min was centrifuged at 100,000 X g for 30 min, and the particulate membrane fractions resuspended in lysis solution and stored at -70 °C.

Binding Assays Membrane preparations expressing WT or mutant C5aR (1-10 μg total protein) were incubated at 37 °C for 1 h with 25 pM (NQ) or 100 pM (all others) [125I]-C5a (2200 Ci/mmol, NEN, Boston, MA) in 250 μl binding buffer (Hank’s balanced
salt solution supplemented with 25 mM HEPES pH 7.4 and 0.1% (wt/vol) bovine serum albumin). Recombinant, non-radioactive C5a (Sigma, St Louis, MO) was added to the indicated concentrations. Nonspecific binding was defined as the amount of radioactivity bound in the presence of 100 nM non-radioactive C5a. Incubations were terminated by vacuum filtration through presoaked GF/C filters (Whatman, Clifton, NJ) and rapid washing with 6 ml ice-cold binding buffer. Binding data were analyzed by nonlinear regression analysis using Prism 2.0 (GraphPad Software, San Diego, CA). For competition binding experiments, 1 nM [125I]-C5a (400 Ci/mmol) was incubated with the indicated concentrations of ChaW and analogs.

**Structure determination of ChaW by NMR** NMR experiments were performed on a 500 MHz Bruker Avance instrument. All experiments were carried out at 20°C with a 1 mM sample of ChaW dissolved in deuterated DMSO. Resonance assignments and structural constraints were obtained from two-dimensional 1H,1H-TOCSY and 1H,1H-NOESY experiments. HNHα coupling constants were obtained from one-dimensional slices along the acquisition dimension of the NOESY experiment with the help of the program INFIT (27). NMR data processing and analysis were carried out using the programs PROSA (28) and XEASY (29). Structure calculations were performed with the software package DYANA (30) using 84 NOE distance constraints and 2 HNHα coupling constants for the Trp and d-Cha residues.

**Activation Assays** 5 x 10^5 COS-7 cells cotransfected with 0.25 µg plasmid encoding Ga16 and with 0.25 µg plasmid encoding WT or mutant C5a receptors were incubated overnight with 2 µCi [3H]-inositol (21 Ci/mmol, NEN, Boston, MA), washed with assay medium (RPMI-1640 supplemented with 20 mM HEPES pH 7.4 and 5 mM LiCl), incubated with or without 10 nM C5a for 1 h at 37 ºC, aspirated and incubated with 750 µl 20 mM cold formic acid at 4 ºC for 30 min, and adjusted with 100 µl of solution I (6 ml concentrated ammonium hydroxide/l). Poly-Prep chromatography columns (BIO-RAD, Hercules, CA) with 1 ml AG 1-X8 resin 100-200 mesh (BIO-RAD,
Hercules, CA) were equilibrated with 10 ml of solution II (4 M ammonium formate, 0.2 M formic acid), followed by 5 ml solution III (10-fold dilution of solution II). Samples were loaded, columns eluted with 1 ml solution III and the resulting inositol fractions collected. Columns were washed with 4 ml solution IV (40 mM ammonium formate, 0.1 M formic acid), eluted with 1 ml of solution II and the resulting inositol phosphate (IP) fractions collected. Inositol phosphate accumulation was reported as the fraction of total inositol used ([IP] / ([IP] + [total inositol]) X 100%).

Molecular Modeling. The rhodopsin crystal structure (7) was the starting point for our model of the C5aR. Only the transmembrane helix portions were used; the side chains corresponding to the human C5aR sequence were substituted for the rhodopsin side chains with the program SCWRL (31), which uses a backbone-dependent rotamer library. Only the side chains of positions which differ between bovine rhodopsin and the C5aR were replaced; otherwise, the original coordinates were retained. The NMR structure of ChaW (see above) was used for manual docking to the C5aR. Consistent with the observed flexibility, torsions of the sidechains and backbone (termini only) of ChaW were adjusted. Sidechains of the C5aR model were also rotated during manual docking: I116, L117, Y121, R206, and Y290. The C5aR complex was relaxed with the following energy-minimization protocol: In SYBYL (SYBYL Release 6.5; Tripos, Inc., 1699 S. Hanley Rd, Suite 303, St. Louis, MO 63144-2913; http://www.tripos.com), essential hydrogens were added to the receptor and all hydrogens were added to ChaW. Kollman united-atom charges were loaded for the receptor and Gasteiger-Marsili charges were computed for ChaW. Energy minimization of only the ligand and its immediate surroundings was performed with the "Minimize Subset" option, specifying the entire ligand as the subset. Other than the charge sets mentioned above and an increase in the number of iterations to 500, default parameters were used, including the Tripos force field. Interactive docking and molecular graphics figures were done in MidasPlus (32) (Computer Graphics Laboratory, University of California,
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RESULTS AND DISCUSSION

_Intramolecular epistasis points to a binding pocket for C5a_. Residues at 12 positions located near extracellular ends of the transmembrane helices were consistently preserved in functioning mutant C5aRs selected in our yeast screen (25); these are colored orange and yellow in Figure 1. Of these, we chose seven (orange in the Figure) for further analysis, based on two criteria: (a) a strict requirement for receptor function (the corresponding single site mutants show no detectable activity in our yeast assay; see Table I and (25)); (b) localization on a helical face pointing into the transmembrane pocket between helices III, V, VI, and VII.

These criteria are compatible with the idea that these seven residues form part of the C5a binding pocket. If so, amino acid substitutions at these positions, which block C5a-triggered signals, should not affect signaling by a constitutively active receptor whose activity is independent of the agonist. In an intramolecular epistasis experiment, we therefore combined each of the seven single site substitutions (L112A, I116A in helix III; A203V, R206H, L207A in helix V; V286A, S287A in helix VII) with the previously documented (25) activating double mutation I124N/L127Q (NQ; cyan in Figure 1). The NQ double mutation involves residues located in helix III, two helical turns farther from the extracellular space than residues in the putative ligand binding pocket. Thus direct compensatory interactions between mutated NQ residues and substituted residues in the ligand pocket are unlikely. The signaling phenotypes of six of the seven combination mutants were similar to that of NQ itself. Based on this epistasis analysis of single site substitutions (Table I), we infer that these six residues are important for ligand-dependent signaling but not for constitutive signaling, and therefore that they are likely to comprise a transmembrane binding pocket for C5a. One combination mutant, NQ/R206H, was inactive; this negative result does not rule out a role for R206 in binding ligand, however, because the R206H mutation may impair folding or expression of the mutant C5aR.
Mutant cycle analysis identifies a receptor switch in the ligand-binding pocket. Amino acid substitutions for residues that directly interact with the ligand should alter ligand binding affinity. Accordingly, we assessed affinities of the seven putative binding pocket mutants for binding $^{[125]}$I-C5a; binding assays were conducted with C5aR mutants expressed in COS-7 cells, because in our hands binding assays were not reproducible in the heterologous yeast system. To our surprise, the binding affinities of mutant receptors for C5a, were indistinguishable from that of the wild type (WT) C5aR (Table II). We imagine that the strong contribution of the receptor’s N-terminus to its binding affinity for C5a (13) overshadows more subtle contributions from individual residues in the transmembrane binding site, which interacts with a different part of C5a (see above). Indeed, replacement of R206 by alanine (instead of histidine, as in our experiment) similarly failed to alter affinity for C5a (21).

The high affinity of C5a for all mutant receptors allowed us to characterize other ligands in competition binding experiments and to perform a mutant cycle analysis, described below. To do so, we assessed binding affinities of hexapeptide ligands, including the antagonist ChaW and four derivatives, each of which differs from ChaW by substitution of a single amino acid residue (Figure 2A). Because the hexapeptide ligands interact exclusively with the transmembrane pocket of the C5aR (13), their binding affinities unlike that of C5a do not depend on interaction with the receptor’s N-terminus. A second advantage of these ligands is that ChaW shows an ordered structure in solution (see below and (22)), which allowed us to model the putative ligand-receptor binding complex. Finally, replacement of the tryptophan at position 5 of ChaW by cyclohexylalanine converts the antagonist into an agonist (W5Cha) (20). Thus, as described below, a receptor site found to interact with the side chain at position 5 of the ligand is likely to comprise part of the elusive ligand-receptor activation switch.
Mutant cycle analysis, which has successfully identified amino acids involved in intermolecular protein-protein interactions (33-36), is based on the following intuitive principle: If residue $a$ of the protein ligand (ChaW or its derivatives) interacts with residue $b$ of the C5aR, the effect of mutating $a$ on the ligand’s binding affinity should depend on whether receptor residue $b$ is mutated as well. In practice, we simply compared the relative changes of binding affinities by dividing the respective inhibition constants ($K_I$; see the legend of Table II). We assessed binding affinities of ChaW and four singly substituted derivatives to the WT C5aR and C5aR mutants with substitutions at each of the seven residues in the putative ligand binding pocket. Results of these experiments are summarized in Table II and Figure 2B, and illustrated in Figure 3A-F.

Comparing the relative degrees of cooperation between each of four hexapeptide residues and each of the seven genetically conserved receptor positions (Figure 2B) clearly indicates two direct and specific interactions: the side chain at position 5 of the ligand (tryptophan in ChaW, cyclohexylalanine in the agonist W5Cha) apparently interacts with two C5aR residues, I116 in helix III and V286 in helix VII. This analysis failed to show interaction of the other three ChaW positions tested (F1, dCha4, or dR6) with any of the seven mutated receptor side chains. The apparent specificities of the I116A and V286A mutations for cooperating with side chains at position 5 of the ligand make it unlikely that these receptor mutations act indirectly to alter ligand affinity; otherwise, they would be expected to alter apparent cooperation with other side chains in the ligand as well.

The I116A C5aR mutation converts ChaW into an agonist. The fact that substitutions at position 5 in this series of hexapeptide ligands determine their capacity to act as agonists (20) suggests that receptor residues that interact with this side chain of the ligand form part of the C5aR activation switch, which distinguishes agonists from antagonists. In keeping with this prediction, replacement of I116 by alanine in the
receptor converted ChaW from an antagonist (or weak partial agonist) into a very strong agonist (Figure 2C; Figure 3G-I). As assessed by measuring inositol phosphate (IP) accumulation in response to the hexapeptides, the WT and all the mutant C5a receptors (Figure 2C; Figure 3G-I), expressed in COS-7 cells, showed robust responses to W5Cha, as well as C5a. In cells expressing the I116A receptor mutant, ChaW at best a weak partial agonist for the WT C5aR induced IP accumulation comparable to responses observed with W5Cha acting on the WT or any mutant C5aR (Figure 2C; Figure 3G-I). This surprisingly robust functional cooperation in combination with the highly cooperative effects of the I116A mutation on ligand binding affinity argues strongly for a direct interaction between the side chain at this position and position 5 of the hexapeptide ligand. The V286A receptor mutant C5aR, carrying a substitution for the other side chain that appears to interact with the residue at position 5 of the hexapeptide, did not recognize ChaW as an agonist; with this receptor mutant the molar potencies of both C5a and W5Cha were slightly lower than those observed with the WT and I116A receptors (Figure 3G-I). Similar to ChaW, the three other ChaW-derivatives F1L, dCha4dL, and dR6dH, induced a response with the I116A mutant only (data not shown).

In contrast to the results in yeast (Table I), in COS-7 cells maximal IP responses to C5a and the agonist hexapeptide W5Cha were not affected by any of the seven receptor mutations tested (Figure 2C; Figure 3G-I). To account for this discrepancy, we imagine that the C5aR in yeast cells is a less robust signal transducer than in mammalian cells (owing, for example, to differences in protein expression, folding, stability, functional interactions with G proteins, etc.). Indeed, enhanced susceptibility of receptors in the heterologous yeast system to detrimental effects of point mutations appears to increase the sensitivity with which we can identify residues that play important functions, even though these functions are not absolutely required, under some conditions, for signaling of single site mutants expressed in mammalian cells.
In combination with previous work (21; see below), identification of I116 and V286 as sites for interaction with a specific residue of hexapeptide ligands furnishes stringent constraints for orienting ChaW relative to the C5a receptor binding site, provided that the ligand structure is known. We therefore solved the NMR structure of ChaW in DMSO (see Materials and Methods). Despite its small size, ChaW shows an ordered structure in solution, comprising an inverse γ-turn formed by the Lys-Pro-D-Cha residues and a distorted type II β-turn that includes the residues of the γ-turn and the tryptophan at position 5. This structure (Figure 4) appears to agree with an earlier NMR study that identified a well-defined backbone conformation composed of the same structural motifs (22). Because the authors of this analysis chose not to make their ChaW coordinates available to us, we cannot directly compare our structure with theirs.

Docking and modeling of a ChaW/C5aR ligand-receptor complex With the structure of ChaW in hand, we used intermolecular contacts as constraints in building a model of the receptor-antagonist complex. Two of these, detected by mutant cycle analysis (see above), are interactions of the W5 residue of ChaW with I116 in helix III and V286 in helix VII of the receptor. We also used constraints identified in a previous study (21) of the receptor’s interaction with the C-terminal dArg6 residue of ChaW. In this study, DeMartino and coworkers used an R206A receptor mutant and a series of ChaW derivatives to show that the side chain of R206 acts as a “gate-keeper,” allowing hexapeptide ligands to bind to the transmembrane receptor core only when the ligands present a C-terminal carboxylate, which appears to interact with the guanidinium group of R206. In addition to the carboxylate, the side chain of dArg6 was also required for receptor interaction, leading these investigators to suggest that it interacts with the transmembrane receptor pocket.

These constraints, together with the known backbone structure of ChaW, orient the hexapeptide in relation to the C5aR model. Figure 4 shows ChaW docked in the
receptor’s putative transmembrane ligand binding pocket (see Materials and Methods). The orientation of ChaW with respect to the ligand binding pocket correlates nicely with results of detailed pharmacological analyses of ChaW and its derivatives (23, 24). Thus K2 and P3 of the hexapeptide, sites at which substitutions do not affect binding affinity or antagonistic potential, point away from the putative ligand binding pocket of the C5aR model (Figure 4). In contrast, substitutions for F1 greatly decreased binding affinity of ChaW (23). The location of the F1 side chain close to the extreme extracellular end of helix V may also favor contacts with the N-terminal end of the receptor’s second extracellular loop, a region that appears to mediate high affinity binding of C5a: replacement of this region in a C5a/formyl peptide receptor chimera abolished C5a binding (37). Although the dCha4 side chain is close to that of L112 (helix III) in the receptor binding pocket (Figure 4), mutant cycle analysis did not indicate an energetically important interaction between the two side chains.

In our docking model the long arginine side chain at position 6 of ChaW penetrates deeply into the receptor’s helix bundle, between helices III, V, and VI (Figure 4C). Its guanidinium group, located one helical turn lower than any of the genetically conserved residues in the putative ligand binding pocket, is cradled in a pocket of aromatic, mostly conserved residues in helices V and VI (green in Figure 4C). Interaction of a positively charged side chain (arginine or lysine) with aromatic residues is not unlikely in protein structures; indeed, a recent analysis (38) of interactions between cationic groups and delocalized $\pi$-electron systems in proteins found such interactions to be much more frequent and energy-rich than previously assumed. This predicted location for the dR6 side chain of the ligand is especially intriguing in light of previous evidence (21) that this side chain is necessary for receptor activation.

*Trigger zone for receptor activation* Our results indicate that the side chain at position 5 of ligand hexapeptides is clasped by hydrophobic side chains of amino acids
in helix III (I116) and helix VII (V286). The role of position 5 in determining whether a hexapeptide acts as an agonist or an antagonist, combined with conversion of ChaW into an agonist by the I116A mutation, points to the cleft between helices III and VII as a potentially pivotal site at which the ligand may trigger C5aR activation of the C5aR.

Unfortunately, we do not know why cyclohexylalanine at ligand position 5 induces conformational changes required for receptor activation, but tryptophan at the same position does not. In our model complex (Figure 4), the tryptophan and cyclohexylalanine side chains appear to fit equally well into the cleft between I116 and V286. A previous pharmacological analysis of ChaW and derivatives altered at position 5 (20) provides a tantalizing clue, however, suggesting that contact between residues I116 and V286 and the agonist’s side chain at position 5 may pull helices III and VII closer to one another. These experiments (20) showed that agonist activity decreased with changes in the side chain at position 5 in the following order: Leu, Cha > Phe > naphthylalanine > Trp. Only the transition from a naphthyl to an indole side chain completely prevented C5aR activation, however. The principal chemical difference between the condensed aromatic rings of these groups, which are of similar size, is the presence of an hetero-aromatic nitrogen in the indole.

Thus, we speculate that the receptor is activated by insertion of a hydrophobic group (e.g., cyclohexylalanine) between helices III and VII, but that robust activation is not compatible with the greater separation of these helices that would be induced by insertion of the bulky indole group with its nitrogen which could further perturb an otherwise purely hydrophobic helix interface. While this explanation is severely limited by our lack of knowledge of the structure of the receptor’s active state, it is compatible with the revertant phenotype of the I116A mutation, in which replacement of isoleucine by alanine could compensate for the indole to allow agonism by ChaW, while the smaller size difference between valine and alanine in the V286A mutant furnishes too little extra room.
Even though we cannot provide a satisfying explanation of how ligand interaction with I116 and V286 activates the C5aR, it is likely that the same site in other receptors plays a role in triggering receptor activation, as suggested by studies of the β2-adrenoceptor (β2-AR) and retinal rhodopsin. A mutant β2-AR is activated by formation of a Zn(II) bridge between receptor residues at positions precisely cognate to I116 and V286 of the C5aR (39). In rhodopsin, the chromophore 11-cis-retinal forms a Schiff base with the side chain of K296 in helix VII (one turn below the position of V286 in the C5aR), which in turn participates in a salt bridge with E113 of helix III (one turn above I116 in the C5aR; rhodopsin is activated when this salt bridge is broken by light-triggered conversion of the chromophore to the all-trans conformation (reviewed in (40)). Moreover, constitutive activity of rhodopsin results from mutations that prevent formation of this helix III/VII salt bridge (that is, substitutions for either E113 or K296). We are thus left with strong hints that receptors share a common activation trigger located at about the same level of the helix III/VII interface, though none of the available evidence tells us how the trigger works.

**Perspective** Our model of the ChaW-C5aR complex (Fig. 4) is based on an NMR structure of the ligand, a homology model of the receptor helices, and interactions between specific side chains predicted from genetic analysis. The general location and orientation of ChaW in the predicted binding pocket within the receptor’s helix bundle are probably correct, because they explain a large array of pharmacological evidence from studies of mutant peptides and mutant C5aRs (see above). This docking model sets the stage for further experiments, including analysis of the interactions of the C5aR and C5a itself, as well as approaches to designing small-molecule antagonists for the C5aR.

We infer from our observations that C5a agonists activate the receptor by interacting with a trigger zone for activation, located between neighboring residues in helices III and VII. As summarized above, quite different experimental approaches in
other labs have identified pivotal sites for receptor activation at the same helix-helix contact site in two other serpentine receptors, the β2-adrenoceptor (39) and rhodopsin (40). Similarly placed activation triggers for three structurally different agonists—a 74-residue polypeptide, a biogenic amine, and a hydrophobic chromophoresuggest that this region plays a similar role in other serpentine receptors. This suggestion is in accord with other evidence suggesting that helix VII may move when receptors are activated. In rhodopsin, light activation exposes an epitope for monoclonal antibody located at the cytoplasmic end of helix VII, suggesting that activation induces a movement of this helix (41). In addition, truncating mutations that completely removed helix VII were found in C5aR mutants that signaled constitutively in yeast (25), suggesting that movement of this helix in the course of normal activation by ligands may relieve constraints that hold the receptor in an inactive conformation. Experiments in several laboratories (42, 43), including ours (44, 45), indicate that a movement of helix VI relative to helix III is necessary for activation of G proteins. We therefore speculate that a ligand-induced movement of helix VII relieves a constraint on helix VI, allowing helix VI to move away from helix III.

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Figure Legends

FIG. 1. The transmembrane ligand binding pocket of the C5aR. Genetically conserved residues (25) presumed to form the transmembrane ligand-binding pocket of the C5aR (yellow and orange), viewed from the extracellular medium. The orange-colored residues were subjected to mutant cycle analysis, as described in the text. I124 and L127, which are substituted in the constitutively activating I124NL127Q (NQ) double mutant (see text), are cyan. Helices are designated by roman numerals, and key amino acid side chains are designated by amino acid (one-letter code) and number in the C5aR sequence.

FIG. 2. Identification of intermolecular contacts between ChaW and specific residues of the C5aR. A, amino acid sequences of the C-terminal octapeptide of C5a, a deleted form of C5a that acts as an related antagonist (17), the hexapeptide ChaW and ChaW derivatives used in these experiments. Mutated residues are shown in open and shaded representation. The two agonists (C5a and W5Cha) are marked by asterisks. B, summary of the mutant cycle analysis of binding affinities of all combinations of ChaW and its derivatives with the WT and all mutant C5a receptors. Bars indicating the interaction of W5 in ChaW with I116 and V286 in the C5aR are colored black. Refer to table II for calculations of cooperation values (Ω). C, agonist- (C5a, W5Cha) or antagonist- (ChaW) induced inositol phosphate production mediated by the WT or mutant C5a receptors. The front row indicates background (bck) levels in the absence of ligand. Activity is given as the percentage fraction of total inositol converted to inositol phosphate. The bar indicating ChaW-mediated activation of the I116A mutant is colored black.
FIG. 3. **Selected results illustrating or extending the mutant cycle analysis of the interaction of ChaW and its derivatives with the WT and mutant C5a receptors.** Results are shown for WT C5aR and the I116A and V286A mutant receptors and the C5a, ChaW and W5Cha ligands only. A-C, saturation binding experiments performed to determine the affinity of C5a (t) for the WT and the I116A and V286A mutant receptors. D-F, competition binding experiments performed to determine the affinities of ChaW (s) and W5Cha (n) for the WT and the I116A and V286A mutant receptors. G-I, stimulation of inositol phosphate accumulation by C5a (t), ChaW (s), and W5Cha (n). The ligand concentration (nM) required for half-maximal effect is indicated next to each respective curve. Panels show one of three or more independent experiments performed with duplicate or triplicate determinations (A-C) or the means ± S.E. of three independent experiments, each performed with duplicate determinations (D-I).

FIG. 4. **Model of the C5aR in complex with the antagonist ChaW.** A, top (extracellular) and B, side views of the docking of ChaW to the C5aR. Roman numerals designate transmembrane helices. Ligand backbone and side chains are cyan, except that the C-terminal carboxylate is red, the C-terminal arginine side chain dark blue, and the indole side chain at position five pink. Receptor residues identified as interacting with specific parts of the ligand (I116 in helix III, R206 in helix V, and V286 in helix VII) are orange; other receptor residues studied in our experiments, including L112 (helix III), A203 (helix V), L207 (helix V), and S287 (helix VII), are yellow. Aromatic side chains of the green receptor residues (Y121 in helix III, F211 in helix V, and F251, W255 and Y258 in helix VI) in C are postulated by the docking model to interact with the dR6 side chain of ChaW.
**Table I. Signaling strength of WT and mutant C5aR constructs in yeast**

Yeast strain BY1142 was cotransformed with a plasmid encoding the C5aR or the indicated mutants and with a plasmid encoding either vector alone (-C5a) or an a-factor prepro/C5a ligand (+C5a). Receptor signaling was assayed by growth of histidine deficient media in the presence of AT: ++++, growth on 10 mM AT; ++++, growth on 5 mM AT; ++, growth on 2 mM AT; +, growth on 1 mM AT; +, growth on 0.5 mM AT; 0, no growth on 0.5 mM AT.

|                | WTa-background | NQb-background |
|----------------|----------------|----------------|
|                | +C5a -C5a      | +C5a -C5a      |
| WT             | ++++ 0         | NQ ++++ ++++   |
| L112A          | 0 0            | NQL112A +++++++|
| I116A          | + 0            | NQI116A ++++++ |
| A203A          | 0 0            | NQA203V ++++   |
| R206H          | 0 0            | NQR206H 0 0    |
| L207A          | + 0            | NQL207A ++++   |
| V286A          | 0 0            | NQV286A ++++   |
| S287A          | 0 0            | NQS287A ++++   |

aWT: wildtype; bNQ: I124N/L127Q.
TABLE II. Analysis of saturation and inhibition binding for C5aR constructs

Receptor expression values (B_max) and binding affinities were obtained from saturation (K_d) or inhibition (K_i) binding experiments with [125I]C5a. Values represent means ± S.E. from three independent experiments, each performed with duplicate or triplicate determinations. The cooperation value (Ω) for an intermolecular interaction between two residues (one on the receptor and one on the ligand, respectively) is defined as Ω = (K_i wtwt x K_i mtmt) / (K_i wtmt x K_i mtwt), where K_i wtwt is the inhibition constant of the WT C5aR with ChaW, K_i mtwt the inhibition constant of an individual C5aR mutant with ChaW, K_i wtmt the inhibition constant of the WT C5aR with an individual ChaW mutant, and K_i mtmt the inhibition constant of a mutant receptor with a ChaW mutant. For ease of comparison, the cooperation value of each pairing has been normalized by setting as = 1.0 the relative affinity change of ChaW vs. a substituted ligand for the WT C5aR. In addition, where the Ω for an interaction is less than 1.0, the value is reported as its reciprocal.

| construct | C5a B_max pmol/mg | W5Cha K_d nM | ChaW K_i nM | F1L K_i nM | dCha4dL K_i nM | R6H K_i nM | W5Cha Ω | F1L Ω | dCha4dL Ω | R6H Ω |
|-----------|-------------------|---------------|-----------|-----------|-----------|-----------|---------|-------|-----------|-------|
| WT        | 9.4 ± 2.9         | 1.05 ± 0.11   | 51 ± 15   | 62 ± 3    | 3300 ± 28 | 1054 ± 149 | 557 ± 87 | 1.0   | 1.0       | 1.0   |
| L112A     | 2.6 ± 0.3         | 0.99 ± 0.09   | 41 ± 9    | 96 ± 9    | 3100 ± 198| 719 ± 166  | 271 ± 74 | 2.0   | 1.6       | 2.3   | 3.2   |
| I116A     | 8.1 ± 0.5         | 0.88 ± 0.24   | 5.3 ± 0.3 | 267 ± 44  | 3275 ± 959| 739 ± 200  | 614 ± 147| 41    | 4.3       | 6.1   | 3.9   |
| A203V     | 2.4 ± 0.4         | 0.45 ± 0.11   | 1843 ± 19 | 623 ± 107 | 5015 ± 1061| 1011 ± 236| 638 ± 133| 3.6   | 6.6       | 10.4  | 8.7   |
| R206H     | 7.7 ± 2.6         | 1.44 ± 0.40   | 332 ± 60  | 136 ± 47  | 2135 ± 898| 560 ± 529  | 271 ± 77 | 3.0   | 3.4       | 4.1   | 4.5   |
| L207A     | 5.2 ± 1.2         | 0.96 ± 0.22   | 598 ± 198 | 108 ± 23  | 1860 ± 594| 434 ± 11   | 277 ± 74 | 6.7   | 3.1       | 4.2   | 3.5   |
| V286A     | 2.9 ± 1.1         | 1.13 ± 0.20   | 0.5 ± 0.2 | 44 ± 3    | 2405 ± 21 | 1390 ± 382 | 230 ± 8  | 76    | 1.0       | 1.9   | 1.7   |
| S287A     | 11.6 ± 4.1        | 1.13 ± 0.52   | 25 ± 5    | 156 ± 45  | 3350 ± 1004| 774 ± 238  | 400 ± 141| 5.1   | 2.5       | 3.4   | 3.5   |

W_T: wildtype.
Fig. 2

A

|        | H     | K     | D     | M     | Q     | L     | G     | R     |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|
| C5a*   |       |       |       |       |       |       |       |       |
| C5a-Q71C/Δ72-74H |       |       |       |       |       |       |       |       |
| ChaW   | Me    | F     | K     | P     | dCha  | W     | dR    |       |
| F1L    | Me    |       | K     | P     | dCha  | W     | dR    |       |
| dCha4dL | Me | F   | K   | P     |       |       |       |       |
| W5Cha* | Me    | F     | K     | P     | dCha  |       |       | Cha   |
| dR6dH  | Me    | F     | K     | P     | dCha  |       |       | W     |

B

Cooperation Value (Ω)

C

\[
\frac{[\text{IP}]}{([\text{IP}]+[\text{Ins}])} \times 100\%
\]
Fig. 3

(A) pmol Bound / mg Protein vs. nM \([^{125}\text{I}]C5a\]

(B) % Competition vs. log [ligand]

(C) % Competition vs. log [ligand]

(D) % Competition vs. log [ligand]

(E) % Competition vs. log [ligand]

(F) % Competition vs. log [ligand]

(G) % Competition vs. log [ligand]

(H) % Competition vs. log [ligand]

(I) % Competition vs. log [ligand]

WT, I116A, V286A
An Activation Switch in the Ligand Binding Pocket of the C5a Receptor
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