Structure of the Complement Factor 5a Receptor-Ligand Complex Studied by Disulfide Trapping and Molecular Modeling

Received for publication, November 19, 2007, and in revised form, January 8, 2008 Published, JBC Papers in Press, January 14, 2008, DOI 10.1074/jbc.M709467200

Ian S. Hagemann§, Daniel L. Miller†, Jeffery M. Kclo†, Gregory V. Nikiforovich*, and Thomas J. Baranski†%1

From the Departments of †Medicine, §Molecular Biology and Pharmacology, and *Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Complement factor 5a (C5a) is an anaphylatoxin that acts by binding to a G protein-coupled receptor, the C5aR. The relative orientation of this ligand–receptor pair is investigated here using the novel technique of disulfide trapping by random mutagenesis (DTRM) and molecular modeling. In the DTRM technique, an unpaired cysteine is introduced in the ligand, and a library of randomly mutagenized receptors is screened to identify mutants that introduce a cysteine at a position in the receptor that allows functional interactions with the ligand. By repeating this analysis at six positions of C5a, we identify six unique sets of intermolecular interactions for the C5a–C5aR complex, which are then compared with an independently developed computational three-dimensional model of the complex. This analysis reveals that the interface of the receptor N terminus with the cysteine-containing ligand molecules is selected from a variety of possible receptor conformations that exist in dynamic equilibrium. In contrast, DTRM identifies a single position in the second extracellular loop of the receptor that interacts specifically with a cysteine probe placed in the C-terminal tail of the C5a ligand.

One of the most biologically important families of receptors is the G protein-coupled receptors (GPCRs),2 which eukaryotic cells use to sense signals as diverse as light, odorants, small molecules, and polypeptide hormones (1). The GPCRs, which number ~1000 in the human genome (2), have a shared topology made up of seven transmembrane domains (TMs) linked by intracellular and extracellular (EC) loops, along with an extracellular N terminus and intracellular C terminus (Fig. 1). These receptors also have a shared mechanism of action, whereby the activated receptor serves as a guanosine exchange factor on the α subunit of a heterotrimeric G protein, thus transmitting the signal to the interior of the cell. Many drugs are directed against GPCRs, including adrenergic, muscarinic, dopaminergic, serotonergic, GABAergic, and histaminergic receptors. Taken together, drugs acting on GPCRs make up an estimated 50% of the current pharmacopoeia (3).

The structural basis of receptor–ligand interaction is of great interest in both basic and applied pharmacology. In the case of GPCRs, a central question is how receptors with a single common topology can be activated by such a wide variety of ligands. A related question is how specificity is built into these receptors, so that each is activated only by the appropriate ligands.

Many GPCRs are activated by polypeptide ligands, including chemokines such as SDF-1 (CXCL12), physiologic modulators such as angiotensin II, glycoprotein hormones such as luteinizing hormone, and chemotactic factors such as complement factor 5a (C5a). These ligands are too large to fit entirely in an interhelical cleft of a GPCR, so their bulk must serve some adaptive function other than receptor activation per se. In some cases, the ligand is recruited by its affinity for the receptor extracellular domains, and a second discrete domain of the ligand then activates the switch mechanism. Alternatively, some large ligands activate their receptors by interacting with large extracellular domains (as in the case of glycoprotein hormone receptors) (4), or by serving as a protease to cleave the N terminus of the receptor (as in the case of the thrombin receptor) (5).

In this study we examine the nature of the interaction between C5a and the N-terminal segment of its receptor, C5aR. C5aR is a 74-amino acid polypeptide that is proteolytically elaborated by the serum complement cascade at sites of inflammation; it serves as a chemotactic factor for neutrophils, which express the C5aR (6). As a potent anaphylatoxin, C5a has been examined as a possible therapeutic target in sepsis, arthritis, and other inflammatory states (7).

The C5a/C5aR interaction has previously been described by a two-site model (8–10). The first interaction is between the N terminus of the C5aR and undetermined components of the C5a ligand; the C-terminal tail of C5a then enters an interhelical pocket of the C5aR to form the second-site interaction. As evidence for the role of the first-site interaction, truncation of the N terminus reduces the affinity of the receptor for full-length ligand, but preserves the efficacy of hexapeptide analogs of the C5a C-terminal tail (8). Conversely, the small molecule L-584,020 competes with full-length C5a but not with hexapeptides (8), suggesting that L-584,020 inhibits binding...
but not activation. Furthermore, NMR spectroscopy has shown that the structure of the isolated C5aR N terminus is altered by incubation with C5a, suggesting that the N terminus of the receptor interacts with the ligand (11). Because hexapeptide analogs such as W5Cha are full agonists for the C5aR, the first site is apparently not a key component of the switch mechanism (8, 11–14). Instead, the first site confers “address”-like specificity on the interaction.

The second site involves the C terminus of C5a and an interhelical pocket of the C5aR (15, 16). Hexapeptide analogs of the C5a C terminus can serve as full agonists or as antagonists for the receptor, albeit with decreased potency (17), and this activity can be modulated by making mutations in the interhelical pocket (15, 18), suggesting that the receptor switch mechanism is actuated by the “message” delivered as a consequence of the first-site interaction providing the correct address (19).

In a recent study, we used random saturation mutagenesis of the C5aR to demonstrate that no single residue of the N terminus is responsible for ligand affinity. Rather, the affinity is built from multiple individually weak interactions (12). In our random mutagenesis experiments, mutations were introduced into multiple positions within the receptor region of interest; receptors that retained signaling ability despite their multiple mutations were selected by a functional screen in Saccharomyces cerevisiae. Surprisingly, we found that positions 24–30 of the C5aR showed a marked propensity to change to cysteine in functional mutants. Because human C5a contains an unpaired cysteine that is solvent-accessible, we reasoned that the cysteine in the ligand could serve as a disulfide-trapping “bait” to identify interacting regions of the receptor, using a library of mutant receptors containing cysteines as “prey.”

We refer to this technique as “disulfide trapping by random mutagenesis,” or DTRM. In principle, it should be generally applicable to a wide range of protein-protein interactions. In the work described here, we use DTRM to study the interaction between C5a and the C5aR. Specifically, we introduce an unpaired cysteine at various positions in the C5a ligand, then identify regions of the receptor that are susceptible to forming disulfide bonds with the mutant ligand. The results provide a series of constraints on the receptor-ligand interaction, allowing it to be described at a new level of molecular detail.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—Yeast strain BY1142 (20) has the genotype MATα far1Δ1442 tbt-1 P_FUS1-HIS3 can1 ste14:trp1::LYS2 ste3Δ1156 gpa1(41)-Goi3 lys2 ura3 leu2 trp1 his3 ade2. This strain expresses a chimeric Gα subunit: the first 41 residues of the endogenous yeast Gα subunit Gpa1 are substituted for the first 33 residues of human Gα13. The result is a predominantly human Gα subunit that can be activated by mammalian receptors, but can also signal to downstream components of the yeast mitogen-activated protein kinase pathway (21). Furthermore, the mitogen-activated protein kinase mating cascade is engineered so that G protein activation causes transcription of the P_FUS1-HIS3 reporter. Thus, G protein or receptor activity allows the yeast to grow on histidine-deficient medium.

Strain BY1173 has the genotype MATα ura3 leu2 trp1 his3 can1 gpa1Δ ade2Δ:3xHA far1Δ:ura3Δ fus1::P_FUS1-HIS3 LEU2::P_FUS1-LacZ sst2Δ:ura3Δ ste2Δ:G4188 trp1::GPA11/Gα13 (22); in this strain, the final 5 residues of Gpa1 are replaced by the corresponding residues of human Gα11, again to provide a signaling assay for mammalian receptors. The integrated P_FUS1-LacZ reporter causes G protein activation to elicit β-galactosidase expression. Yeast were transformed by the standard lithium acetate procedure and grown at 30 °C.

**Construction of Mutant Ligands**—Point mutations were introduced into the wild-type C5aR ligand by site-directed mutagenesis using Pfu Turbo polymerase (Stratagene). Plasmid construction was verified by sequencing (Protein and Nucleic Acid Chemistry Laboratory, Washington University School of Medicine, St. Louis, MO).

**β-Galactosidase Assay**—Yeast BY1173 transformed with wild-type C5aR and ligand plasmids or empty vector was grown in suspension at 30 °C under selective conditions. To assess yeast density after overnight growth, the A_600 was measured using a Spectronic-20 spectrophotometer (Bausch & Lomb). All cultures were assayed at A_600 of ~0.15. For each culture, a standard quantity of yeast (15 μl divided by...
the \( A_{\text{opt}} \) was seeded into a 96-well microtiter plate containing yeast growth medium (to 100 \( \mu l \)) and lysis/substrate buffer containing the final concentrations of 0.5% Triton X-100, 1 mg/ml chlorophenol red \( \beta \)-d-galactopyranoside, and 25 mm PIPES, pH 6.8. The quantity of yeast was sufficiently small that there was no turbidity visible in the wells. The assay plate was sealed and incubated at 37 °C. After 1 h, color development was halted by addition of \( Na_2CO_3 \) to 0.2 M, and \( A_{570} \) was measured on a Bio-Rad Model 680 microplate reader.

**Library Construction and Screening**—Libraries of mutant C5aRs were generated as described previously (12, 23). Briefly, the nucleotide sequence of the region of interest (NT or EC2) was flanked by silent restriction sites, then replaced by a non-coding “stuffer” sequence to prevent wild-type contamination. This region was flanked by silent restriction sites, then replaced by a non-coding sequence to prevent wild-type contamination. For the NT library, residues 2–32 of the C5aR were mutagenized; for the EC2 library, the mutations spanned residues 175–199.

We screened the libraries for receptors whose behavior suggested that they made disulfide interactions with cysteine-containing ligands. To accomplish this, yeast BY1142 was cotransformed with a receptor library (ADE2 vector) and with a C5a mutant ligand plasmid (URA3 vector), then grown under Ade− Ura− selection. After 2 days of growth, colonies were replica-plated onto His− Ura− plates with 1 mm Ade to assay them for receptor signaling. Functional receptors conferred histidine prototrophy and allowed growth on this medium; furthermore, the strength of signaling was assessed by replica plating on media containing increasing concentrations of 3-amino-1,2,4-triazole, a competitive inhibitor of the HIS3 gene product. Growth on 3-amino-1,2,4-triazole was considered to be receptor-dependent if colonies maintained their white color, indicating continued adenine prototrophy. Colonies that lost their receptor plasmid became reliant on scarce environmental adenine, therefore either failing to grow or producing a red pigment that was cause for exclusion from our screen.

Plasmids conferring ligand-dependent growth on 3-amino-1,2,4-triazole were selected for further analysis. The mutant receptor plasmids were isolated from these colonies by plasmid rescue, sequenced, and resubjected to the growth assay to confirm their phenotypes. Receptors were also assayed for their ability to be activated by C5a C27R. Those receptors that were activated by the cysteine-containing ligand but not by the “Cys-less” ligand C5a C27R were considered to be hits in our screen.

**Statistical Methods**—The number of cysteines expected at each position under the null hypothesis (absence of any selective pressure favoring cysteine) was calculated as described in the supplementary materials. Expected and actual values were compared by the two-tailed Fisher’s exact test (GraphPad Software) with expectation values rounded to the nearest integer.

**Molecular Modeling**—The building of the three-dimensional model of the complex between C5aR and C5a, which followed general procedures developed earlier (24–26), is described in detail elsewhere. Briefly, energy calculations were performed by minimizing the energy of atom-atom interactions between the molecular fragments, such as fragments of C5a or C5aR, involved in each calculation. During minimization, the fragments were allowed to move as rigid bodies in six dimensions each (three rotations and three translations). The dihedral angles of the side chains (but not of the backbone) were also involved in minimization. Also, at each step of minimization (several times during the process), the side chains were repacked to yield optimal positions before starting minimization itself.

C5aR—A three-dimensional model of the TM region of human C5aR was developed earlier (26) using the x-ray structure of rhodopsin as a template with the transmembrane helices defined as Ile38–Ala63 (TM1), Asn74–Glu98 (TM2), Ala107–Val138 (TM3), Ala150–Phe172 (TM4), Glu199–Phe224 (TM5), Arg236–Phe267 (TM6), and Leu281–Tyr300 (TM7). All realistic low-energy conformations of the EC loops were restored independently according to a modeling procedure developed earlier (25). The EC loops were defined as EC1 (His99–Gly106), EC2 (Leu173–Arg198), and EC3 (Leu268–Lys280); a total of 29 low-energy options for the conformers of the “package” EC1 + EC2 + EC3 were found. The package was mounted onto the TM region of C5aR and the “most open” conformation of the loops (that with the largest distance between the “tips” of EC2 (Cα177) and EC3 (Cα269)) was selected for the three-dimensional model of the TM region + EC loops of C5aR used in further docking of C5a and its fragments.

C5a-(59–74) and Docking—First, fragment C5a-(65–69) was docked within an opening in the three-dimensional model that allowed access to Glu199, Arg206, and Asp208, which are the residues suggested as possible contacts to C5a by site-directed mutagenesis (see e.g., Ref. 7). The backbone conformation of C5a-(65–69) was extracted from the NMR/calculation data on YSF/KPMPLbR, a known agonist of C5aR (27, 28). Docking was performed by a systematic search on the six-dimensional grid (three translations and three rotations) that covered a total of 2,268 starting orientations of C5a-(65–69) within the TM region of C5aR. Twenty low-energy orientations of C5a-(65–69) without steric clashes with the EC loops (Cα–Cα distances ≥ 4 Å) were selected for further consideration. Then, all low-energy conformations of fragment C5a-(59–74) were obtained assuming that (i) backbone conformations of C5a-(65–69) remained the same; (ii) the backbone conformation of C5a-(59–62) was the same as revealed by NMR data for C5a (the PDB entry 1KJS (29, 30)); (iii) the backbone conformations of C5a-(63–65) and -(70–74) might vary, but the distance between Ca59 and Ca74 was kept greater than about 22 Å (otherwise it would be impossible to extend the structure to the entire C5a). The obtained conformations of C5a-(59–74) were checked as to possible steric clashes with C5aR for all selected low-energy orientations of C5a-(65–69) and, in turn, subjected to energy minimization. Four types of low-energy orientations of C5a-(59–74) possessed no steric clashes with C5aR and were used for placing the entire C5a within C5aR.

3 Nikiforovich, G. V., Marshall, G. R., and Baranski, T. J. (2008) Biochemistry, in press.
Entire Complex of C5a and C5aR—Independently, energy calculations were performed for the N-terminal segment 8–41 of C5aR (NT 8–41) resulting in 185 low-energy conformations of the peptide backbone. (The N-terminal segment 1–7 was not included due to difficulties in modeling the carbohydrate moiety at the N-glycosylated site.) When mounted onto the three-dimensional model of C5aR (onto the N-terminal residues 38–41 of TM1), only 44 conformations of NT 8–41 possessed no steric clashes with C5aR. Then, possible orientations of the entire structure of C5a were restored by overlapping the rigid core C5a-(1–62) from PDB entry 1KJS with each of the four selected orientations of C5a-(59–74) within C5aR. It was found that only one specific orientation of C5a satisfied the requirements of spatial proximity of positions 15, 18, 19, 20, 27, and 46 in C5a to specific residues in the N-terminal segment 8–41 of C5aR, as was suggested by available site-directed mutagenesis data (31, 32). Seventeen conformations of NT 8–41 (out of 185) and 11 conformations of the EC1 + EC2 + EC3 loop package (out of 29) did not show steric clashes with this particular orientation of C5a; all these conformations together with the selected orientation of C5a and the TM region of C5aR make up the final model of the C5a-C5aR complex.

RESULTS

Generation of Mutant C5a Ligands with Unpaired Sulfhydryls—The wild-type C5a ligand contains seven cysteine residues, of which six (Cys-21, -22, -34, -47, -54, and -55) are involved in intramolecular disulfide bonds that stabilize an α-helical bundle (Fig. 2A). The seventh cysteine (Cys27) has an outwardly directed side chain (29, 30) that was previously used for disulfide trapping analysis (12). The data in our earlier study showed that in the receptor-ligand complex, Cys27 of C5a is positioned close to residues 24–30 of the C5aR. To apply DTRM to this system, we moved the unpaired cysteine to eight positions in C5a (Fig. 2, B and C). In each of these bait mutants, Cys27 was replaced with Arg; this residue was chosen because it replaces Cys27 in rodent C5a sequences, which are otherwise highly conserved relative to human. The sites in the C5a ligand were chosen based on their surface accessibility, previous data demonstrating potential roles in receptor binding, and inspection of our initial models of the docked C5a ligand (12). To verify that the ligands were functional, we coexpressed them with the C5aR in a S. cerevisiae expression system (denoted by letters from “A” to “E”), where changes had been randomly introduced into receptor regions of interest. We hypothesized that some randomly mutated receptors would contain cysteine side chains at positions suitable for forming a disulfide bond with the mutant ligand, thereby complementing the affinity lost via receptor mutagenesis.
Use of C5a C27R/D24C to Trap Interactions with the C5aR NT—As an initial test of the DTRM technique, we screened a library of N-terminal mutant C5aRs with the ligand C5a C27R/D24C (Fig. 3). Relative to our earlier work with the wild-type ligand (12), we predicted that moving the unpaired cysteine by a small number of amino acid positions would cause a correspondingly small displacement in the receptor positions where a disulfide cross-link could form. When the N-terminal library was screened against ligand C27R/D24C in yeast BY1142 (an engineered yeast strain in which C5a receptor signaling allows growth on selective media; see “Experimental Procedures”), we identified 11 receptors meeting our screening criteria, in that they were activated by C5a C27R/D24C but were much less strongly activated by C5a C27R (Fig. 3, the A series of receptors). We noted that all of the A receptors, with the exception of A20, contained at least one and usually two cysteine residues. These cysteines were concentrated in two bands within the N terminus: positions 10–12 and 23–27 (Fig. 3). We wished to determine whether these cysteines exceeded the number that would have arisen by chance in the absence of selective pressure favoring cysteine insertion. To this end, we calculated the probability of incorporating a cysteine at each position, and multi-

Use of C5a C27R/D24C to Trap Interactions with the C5aR NT—As an initial test of the DTRM technique, we screened a library of N-terminal mutant C5aRs with the ligand C5a C27R/D24C (Fig. 3). Relative to our earlier work with the wild-type ligand (12), we predicted that moving the unpaired cysteine by a small number of amino acid positions would cause a correspondingly small displacement in the receptor positions where a disulfide cross-link could form. When the N-terminal library was screened against ligand C27R/D24C in yeast BY1142 (an engineered yeast strain in which C5a receptor signaling allows growth on selective media; see “Experimental Procedures”), we identified 11 receptors meeting our screening criteria, in that they were activated by C5a C27R/D24C but were much less strongly activated by C5a C27R (Fig. 3, the A series of receptors). We interpret these receptors as being those whose ligand affinity depends upon the presence of a cysteine in the ligand. We noted that all of the A receptors, with the exception of A20, contained at least one and usually two cysteine residues. These cysteines were concentrated in two bands within the N terminus: positions 10–12 and 23–27 (Fig. 3). We wished to determine whether these cysteines exceeded the number that would have arisen by chance in the absence of selective pressure favoring cysteine insertion. To this end, we calculated the probability of incorporating a cysteine at each position, and multi-

Use of C5a C27R/D24C to Trap Interactions with the C5aR NT—As an initial test of the DTRM technique, we screened a library of N-terminal mutant C5aRs with the ligand C5a C27R/D24C (Fig. 3). Relative to our earlier work with the wild-type ligand (12), we predicted that moving the unpaired cysteine by a small number of amino acid positions would cause a correspondingly small displacement in the receptor positions where a disulfide cross-link could form. When the N-terminal library was screened against ligand C27R/D24C in yeast BY1142 (an engineered yeast strain in which C5a receptor signaling allows growth on selective media; see “Experimental Procedures”), we identified 11 receptors meeting our screening criteria, in that they were activated by C5a C27R/D24C but were much less strongly activated by C5a C27R (Fig. 3, the A series of receptors). We interpret these receptors as being those whose ligand affinity depends upon the presence of a cysteine in the ligand. We noted that all of the A receptors, with the exception of A20, contained at least one and usually two cysteine residues. These cysteines were concentrated in two bands within the N terminus: positions 10–12 and 23–27 (Fig. 3). We wished to determine whether these cysteines exceeded the number that would have arisen by chance in the absence of selective pressure favoring cysteine insertion. To this end, we calculated the probability of incorporating a cysteine at each position, and multi-
plied this by the number of selected receptors to estimate the expected number of cysteines in the set as a whole.

At receptor positions 10–12, most of the cysteines were able to arise via a single nucleotide mutation, making them somewhat likely to occur even by chance. In the absence of selective pressure favoring cysteines, a set of 11 randomly selected receptors would have been expected to contain 0.59 cysteines at positions 10–12; the A set, however, contained 4 cysteines in this range (p = 0.36). All of the cysteines at positions 23–27 required two nucleotide changes, so they were much less likely to arise by chance. The expected number of cysteines in this range for a set of 11 receptors was 0.15, but the observed number was 10 (p = 0.0012). Therefore, the data reflected stronger selective pressure for incorporating cysteines at these positions. The remaining positions of the C5aR NT (outside of the ranges 10–12 and 23–27) were expected to contain 2.9 cysteines in a set of 11 receptors, and in fact, 3 cysteines were observed at these positions. Therefore, consistent with our hypothesis, moving the ligand cysteine from C5a position 27 to 24 caused a corresponding small displacement in the major interacting region of the C5aR NT (from 24–30 to 23–27) and made an additional minor interacting region available (at 10–12). The specific contacts are summarized in Table 1.

**Use of C5a C27R/H15C to Trap Interactions with the C5aR NT**—The observations with the C5a C27R/D24C ligand suggested that the ligand cysteine was able to identify interacting sites on the receptor. To probe a different region of the ligand and identify its possible sites of interaction with the receptor, we moved the unpaired cysteine to position 15. This position of the ligand was previously identified as being a probable interactor with the receptor (9). When the N-terminal mutant C5aR library was screened against C5a C27R/H15C, we identified 18 receptors that were activated specifically by the cysteine-containing ligand (Fig. 4). Again, these receptors showed a remarkable preference for incorporating cysteines at a specific location of the N terminus; however, the “hot spot” for cysteines had moved to positions 11–15. These were positions where the wild-type codon only needed to undergo one nucleotide change.

### Specific C5a-C5aR contacts identified by DTRM

| C5a residue | Interacting C5aR residues |
|-------------|--------------------------|
| Cys27       | Thr23, Val26, Asp25, Thr29, Ser30 (Ref. 12) |
| Asp24       | Asp13, Thr29, Val26, Asp27 (Fig. 3) |
| His15       | Gly32, Tyr14 (Fig. 4) |
| Arg41       | Gly32, Asp15, Thr29, Val26 (Fig. 5) |
| Arg46       | Ser3, Tyr (Fig. 6) |
| Ser66       | His32 (Fig. 7) |

| N term/H15C | Genetic Code | P(cysteine) | E(cysteine) |
|-------------|--------------|-------------|-------------|
| 2 D . E . G | V A E G      | 0.0000      | 0.0000      |
| 3 S . C . O | Y V A A P A A | 0.0435      | 0.7821      |
| 4 F . I . Y | L Y L S       | 0.0435      | 0.7821      |
| 5 N . C . D | R K Q K K A   | 0.0032      | 0.0576      |
| 6 Y . S . N | S N N C       | 0.0032      | 0.0576      |
| 7 R . T . S | S             | 0.0032      | 0.0576      |
| 8 T . A . K | N A A R       | 0.0032      | 0.0576      |
| 9 P . Q . R | Q R T A       | 0.0004      | 0.0079      |
| 10 D H N H Y | Q N V A E P A Y| 0.0032      | 0.0576      |
| 11 Y S C N   | A             | 0.0032      | 0.0576      |
| 12 G R G H   | C D C C C R C C | 0.0435      | 0.7821      |
| 13 H E G N C | D D C F       | 0.0435      | 0.7821      |
| 14 D G C A V | V Y N Y E G   | 0.0032      | 0.0576      |
| 15 G D G E   | E K S Y H G Q N Y Q | 0.0032 | 0.0576 |
| 16 K L Q N S | T T N L       | 0.0032      | 0.0576      |
| 17 D Y V N V | A N N R A I E | 0.0032      | 0.0576      |
| 18 T . A . N | S             | 0.0032      | 0.0576      |
| 19 L I N    | V             | 0.0004      | 0.0079      |
| 20 D N Y H N | N E A E . . Y | 0.0032      | 0.0576      |
| 21 L I V Q   | R V P         | 0.0004      | 0.0079      |
| 22 N K H K T | D K V K       | 0.0032      | 0.0576      |
| 23 P A T S L | A L Q S R     | 0.0032      | 0.0576      |
| 24 V . A I I | G D L         | 0.0032      | 0.0576      |
| 25 D K Q R G | I T           | 0.0032      | 0.0576      |
| 26 T . N . S | S S S S       | 0.0032      | 0.0576      |
| 27 S T Y A P | H A           | 0.0032      | 0.0576      |
| 28 N T D D D | D D D K K H   | 0.0032      | 0.0576      |
| 29 T S T I S | M N A S S     | 0.0032      | 0.0576      |
| 30 +C5a C27R | +H15C        | 0.0343      | 0.7821      |
| 31 +C5a C27R | +C5a C27R    | 0.0032      | 0.0576      |

**FIGURE 4.** Sequences of the disulfide-dependent C5aR mutants identified by using C5a C27R/H15C to screen the NT C5aR library (the B series of receptors). Data are reported as described in the legend to Fig. 3.
to transition to cysteine; it is, nonetheless, remarkable that every identified receptor reflected such a transition. We calculated that the 18 receptors would have been expected to contain 1.73 cysteines between positions 11 and 15, in the absence of selective pressure; instead, we observed 13 cysteines in this domain (p = 0.0053). The expected number of cysteines outside of positions 11–15 was 4.14, and the observed number was 5. The most likely explanation of this specific selective pressure is that positions 11–15 of the receptor lie in proximity to position 15 of the ligand in the C5a-C5aR complex, so that a disulfide bond can rescue the receptor-ligand interaction (see Table 1).

Use of C5a C27R/R40C to Trap Interactions with the C5aR NT—C5a Arg40 was also previously identified as a likely receptor-interacting residue (9, 32). We therefore made C27R/R40C mutations in C5a and used this ligand, which was able to activate the wild-type receptor (Figs. 2B and 5), to screen the N-terminal C5aR library. We identified 19 receptors that were strongly activated by C5a C27R/R40C and were less strongly activated by C5a C27R (Fig. 5, the “C” series of receptors). These receptors were relatively dependent upon the ligand cysteine at position 40 for signaling, in the sense that the mutation R40C allowed them to function as super-agonists relative to the C27R ligand. Eighteen of the C receptors contained a cysteine at some position, chiefly distributed across two bands of positions (C5aR positions 6–15 and 21–28). Based upon our earlier reasoning, these cysteines were presumably able to form disulfide bonds with the unpaired sulfhydryl of the ligand. In the range 6–15, the expected number of cysteines was 2.95, and the observed number was 10 (p = 0.087); in the range 21–28, 0.38 cysteines were expected, but 8 were observed (p = 0.0071). Positions 12, 15, 24, and 26 exhibited a particularly strong preference for transitioning to cysteine, relative to the expectation value (see Fig. 5 and Table 1).

Use of C5a C27R/R40C to Trap Interactions with the C5aR NT—C5a Arg40 was also previously identified as a likely receptor-interacting residue (9, 32). We therefore made C27R/R40C mutations in C5a and used this ligand, which was able to activate the wild-type receptor (Figs. 2B and 5), to screen the N-terminal C5aR library. We identified 19 receptors that were strongly activated by C5a C27R/R40C and were less strongly activated by C5a C27R (Fig. 5, the “C” series of receptors). These receptors were relatively dependent upon the ligand cysteine at position 40 for signaling, in the sense that the mutation R40C allowed them to function as super-agonists relative to the C27R ligand. Eighteen of the C receptors contained a cysteine at some position, chiefly distributed across two bands of positions (C5aR positions 6–15 and 21–28). Based upon our earlier reasoning, these cysteines were presumably able to form disulfide bonds with the unpaired sulfhydryl of the ligand. In the range 6–15, the expected number of cysteines was 2.95, and the observed number was 10 (p = 0.087); in the range 21–28, 0.38 cysteines were expected, but 8 were observed (p = 0.0071). Positions 12, 15, 24, and 26 exhibited a particularly strong preference for transitioning to cysteine, relative to the expectation value (see Fig. 5 and Table 1).

Use of C5a C27R/R40C to Trap Interactions with the C5aR NT—C5a Arg40 was also previously identified as a likely receptor-interacting residue (9, 32). We therefore made C27R/R40C mutations in C5a and used this ligand, which was able to activate the wild-type receptor (Figs. 2B and 5), to screen the N-terminal C5aR library. We identified 19 receptors that were strongly activated by C5a C27R/R40C and were less strongly activated by C5a C27R (Fig. 5, the “C” series of receptors). These receptors were relatively dependent upon the ligand cysteine at position 40 for signaling, in the sense that the mutation R40C allowed them to function as super-agonists relative to the C27R ligand. Eighteen of the C receptors contained a cysteine at some position, chiefly distributed across two bands of positions (C5aR positions 6–15 and 21–28). Based upon our earlier reasoning, these cysteines were presumably able to form disulfide bonds with the unpaired sulfhydryl of the ligand. In the range 6–15, the expected number of cysteines was 2.95, and the observed number was 10 (p = 0.087); in the range 21–28, 0.38 cysteines were expected, but 8 were observed (p = 0.0071). Positions 12, 15, 24, and 26 exhibited a particularly strong preference for transitioning to cysteine, relative to the expectation value (see Fig. 5 and Table 1).

The “C” mutants all contained cysteine residues, with the exception of mutant C7, but on the other hand, they did not strictly require an unpaired cysteine in the ligand to be functional (Fig. 5). In contrast, our screens using C5a C27R/H15C and C27R/D24C showed a much stronger cysteine dependence of the functional receptors. The incomplete cysteine dependence of the C receptors suggests that the unpaired C5a sulfhydryl was only contributing part of the N-terminal receptor binding energy, and that there was additional selective pressure to retain endogenous binding elements. Consistent with this
hypothesis, we note that the residues believed to contribute most of the binding energy, C5aR positions 20–30 (11, 12), were relatively highly conserved in the C receptors, as compared with the rest of the N terminus (Fig. 5).

Use of C5a C27R/R46C to Trap Interactions with NT or C5aR—

In previous work, point mutations at C5a Arg46 reduced ligand binding and downstream receptor effects, suggesting that this residue was involved in the C5a/C5aR interaction. This effect was synergistic with mutations at other positions, including His15 (32). Using DTRM to identify Arg46-interacting residues of the C5aR, we introduced an unpaired sulfhydryl at C5a position 46 and used this ligand to screen the N-terminal mutant C5aR library. As in the other screens, we selected for mutant receptors that were activated by C5a C27R/R46C, but not by C5a C27R. The screen identified 18 receptors (Fig. 6), all of which had at least one cysteine residue introduced in the N terminus. These introduced cysteines were preferentially located between positions 3 and 8 of the N terminus (expected 2.46 cysteines, observed 15; p = 0.0016), with a second band at positions 11–14 (expected 1.73 cysteines, observed 5; p = 0.44).

Outside of these bands, we expected 1.74 cysteines and observed 1. The overall picture is of selective pressure favoring the incorporation of cysteines specifically at C5aR positions 3–8, implying contact between these regions of the receptor and position 46 of the ligand (see Table 1).

Use of C5a C27R/S66C to Trap Interactions with EC2 of C5aR—

The constraints at C5a positions 27, 24, 15, 40, and 46 provided possible points of contact between the ligand and the N terminus of the receptor. We next asked whether DTRM could be used to identify contacts between the ligand and other regions of the receptor. Based on inspection of our earlier docked model (12), we identified C5a residue Ser66 as possibly making contact with the second extracellular loop (EC2) of C5aR. A cysteine was introduced at this position (C5a C27R/S66C) and the mutant ligand was used to screen a library of C5aRs with mutations randomly introduced in EC2 (23).

Two mutant receptors were identified as being dependent on ligand for activation and were furthermore dependent on the unpaired cysteine at C5a position 66 (Fig. 7A). Receptor E1 was isolated in duplicate (two identical clones) and E5 in triplicate, suggesting that the library had been screened to saturation. Both of these E receptors contained a cysteine at position 194. Because the expected number of cysteines at this position was 0.011, yet the observed number was 2, we infer that significant selective pressure favoring cysteine was present, although the number of observations was too small to allow statistical

| Nterm/R46C | C5a C27R/ R46C |
|------------|----------------|
| 2 D C C C C T P C C C C Y F T |
| 5 N Y D N C N C G L F C Y |
| 10 Y S L C C D I |
| 15 D G Y N N H K E H |
| 20 L G F V R |
| 25 P I |
| 30 S C A N Y V Y |

FIGURE 6. Sequences of the disulfide-dependent C5aR mutants identified by using C5a C27R/R46C to screen the NT C5aR library (the “D” series of receptors). Data are reported as described in the legend to Fig. 3.

| Genetic Code | P(cysteine) | E(cysteine) |
|-------------|-------------|-------------|
| V A E G     | 0.0000      | 0.0000      |
| F Y C A P T | 0.0435      | 0.7821      |
| C S V Y L I | 0.0435      | 0.7821      |
| I Y T D H K S | 0.0032   | 0.0576      |
| G D S N C H F | 0.0435   | 0.7821      |
| I N P A S | 0.0032      | 0.0576      |
| I N P A S | 0.0032      | 0.0576      |
| L Q R T A S | 0.0004      | 0.0079      |
| Y V A E N G H | 0.0032     | 0.0576      |
| G D S N C H F | 0.0435   | 0.7821      |
| C R V A D S | 0.0435      | 0.7821      |
| L P Y Q N R D | 0.0032    | 0.0576      |
| G D S N C H F | 0.0435   | 0.7821      |
| Y V A E N G H | 0.0032     | 0.0576      |
| Y V A E N G H | 0.0032     | 0.0576      |
| G E M T R N Q | 0.0004    | 0.0079      |
| Y V A E N G H | 0.0032     | 0.0576      |
| I N P A S | 0.0032      | 0.0576      |
| P R Q I V | 0.0004      | 0.0079      |
| I Y T D H K S | 0.0032   | 0.0576      |
| I N P A S | 0.0032      | 0.0576      |
| L Q R T A S | 0.0032      | 0.0576      |
| D F G A I L | 0.0032      | 0.0576      |
| Y V A E N G H | 0.0032     | 0.0576      |
| G E M T R N Q | 0.0004    | 0.0079      |
| I N P A S | 0.0032      | 0.0576      |
| F Y C A P T | 0.0435      | 0.7821      |
| I Y T D H K S | 0.0032   | 0.0576      |
| I N P A S | 0.0032      | 0.0576      |
hypothesis testing. Outside of position 194, the expected number of cysteines was 1.62 and the observed number was 2 (as the endogenous Cys188 was preserved in both \(E_\text{1}\) and \(E_\text{5}\)). Besides the cysteine at 194, these receptors had relatively few mutations, but those that did occur preferentially targeted cationic residues of EC2.

When the cysteine residues of the \(E\) receptors were reverted to histidine, these receptors were still expressed in yeast (Fig. 7C), but their activity was largely abolished (Fig. 7B). Furthermore, a cysteine in C5a at position 65 (rather than 66) was not able to support a receptor-ligand interaction (Fig. 7B). The most likely interpretation of these results is that C5a position 66 and C5aR position 194 are close to one another in the physiologically docked pair; thus, a disulfide bond between these positions can rescue the receptor-ligand interaction when it is disrupted by random receptor mutations.

**Structural Modeling of the C5a-C5aR Complex**

A model of the three-dimensional structure of the C5a-C5aR complex was obtained independently of the DTRM data using modeling procedures developed earlier (24—26). The most characteristic feature of this model was that it considered various conformational possibilities for the flexible EC loops and the N-terminal segment of C5aR. The obtained model combined a relatively rigid structure of the TM region of C5aR and a well defined orientation of C5a within this region with significant flexibility of the EC loops (11 possible conformations of the EC1 + EC2 + EC3 package) and the N-terminal segment of C5aR (17 possible conformations).

The modeling results generally confirmed the previously suggested two-site model for binding C5a to C5aR that included the first site interaction between the rigid core of C5a and the N-terminal segment of C5aR, and the second site involving interactions of the TM region of C5aR and the C-terminal fragment of C5a (8—10). Our modeling also demonstrated two very different modes of interaction at the two sites. The C-terminal fragment of C5a and the TM region of C5aR (the second site) interact through a well determined system of strong salt bridges and hydrogen bonding between specific side chains. This system of interactions is achieved by conformational adjustment of the flexible C-terminal fragment of the ligand to the rigid structure of the TM bundle of the receptor immobilized within the membrane. In contrast, interactions between the N-terminal segment of C5aR and the rigid core of C5a (the first site) are determined by fluctuating systems of residue/residue interactions that vary depending on conformations of the highly flexible N-terminal segment of C5aR interconverting into each other. Accordingly, the receptor conformations are adjustable to the rigid core of the ligand in this case.

The final model of the complex of C5a and C5aR is described in detail elsewhere. The model was successfully utilized for rationalizing available site-directed mutagenesis data in the N-terminal fragment of C5aR (mutations involving Asp\(^{15}\), Asp\(^{16}\), Asp\(^{21}\), and Asp\(^{27}\) (10, 14)) as well as in C5a (mutations involving His\(^{15}\), Val\(^{18}\), and Arg\(^{46}\) (32)). The model was also validated by comparison to the data on C5aR mutants E199K, D282R, R206A, and R175D (16, 18, 33—35).

---

**FIGURE 7.** A, sequences of the disulfide-dependent C5aR mutants identified by using C5a C27R/S66C to screen the EC2 C5aR library (the \(E\) series of receptors). Data are reported as described in the legend to Fig. 3. B, role of the unpaired receptor cysteine in activation by C5a C27R/S66C. Yeast BY1142 was transformed with the indicated receptor (ADE2 selection) and ligand (URA3 selection) and subjected to the growth assay. C, expression of mutant receptors was assessed by immunoblot of total yeast lysates.
We then compared the data obtained by DTRM scans to the docked model of C5a and C5aR. Specifically, the DTRM analysis predicts close proximity of residues Asn23, Thr24, Val26, and Asp27 of C5aR with Cys27 in C5a C27R/D24C (Fig. 3); Gly12 and Tyr14 of C5aR with Cys15 in C5a C27R/H15C (Fig. 4); Gly15, Asp15, Thr24, and Val26 of C5aR with Cys40 in C5a C27R/R40C (Fig. 5); Ser3 and Tyr3 of C5aR with Cys46 in C5a C27R/R46C (Fig. 6); and Thr24, Val26, Asp27, Thr26, and Ser26 of C5aR with Cys27 in the wild-type C5a (12). However, no single conformation of the N-terminal segment of C5aR found by independent modeling satisfied all listed requirements for mutual residue-residue proximity between C5aR and all C5a analogs (where proximity is defined as location of two residues at Ca-Ca distances ≤ 15 Å). At the same time, molecular modeling predicted that due to the high flexibility of the N-terminal segment, at least one conformation from the set of the N-terminal segment conformations found by modeling could satisfy all requirements for mutual residue-residue proximity between C5aR and each particular C5a ligand, as suggested by the DTRM experimental data. Fig. 8 presents sketches showing three-dimensional models of the C5a-C5aR complex compatible with the potential residue-residue interactions between the N-terminal segment of C5aR and the analogs C5a Xxx/C27R, Xxx being D24C (panel A), H15C (panel B), R40C (panel C), and R46C (panel D). Fig. 8 also shows the three-dimensional model compatible with experimental findings for the wild-type C5a Cys27 (panel E) (12) and the three-dimensional model showing all conformational possibilities for the N-terminal segment of C5aR found by modeling (panel F). One can see that, according to Fig. 8, interactions of C5aR with different C5a ligands may select different conformations of the N-terminal segment of C5aR out of the entire possible set of conformations predicted by modeling. Specifically, interactions of C5aR with C5a C27R/D24C, which were suggested by the DTRM results, are most compatible with 9 conformations of the N-terminal segment (out of 17), 2 conformations are most plausible for interactions with C5a C27R/H15C, 6 with C5a C27R/R40C, and only 1 with C5a C27R/R46C (see panels A–D in Fig. 8). For the wild-type C5a, 11 conformations are most compatible with the DTRM data (panel E in Fig. 8). The sets of conformations of the N-terminal segment of C5aR compatible with interactions with C5a C27R/D24C and with the wild-type C5a are similar to each other (compare panels A and E in Fig. 8), which might have been expected considering the close location of positions 24 and 27 in the peptide sequence of C5a. At the same time, the similarity of the conformations of the N-terminal segment in panels B (for C5a C27R/H15C) and D (for C5a C27R/R46C) is in line with the close spatial positions of His15 and Arg46 in the three-dimensional structure of C5a. Also, 8 conformations of the EC2 loop (out of 11 included in the final three-dimensional model of the C5a-C5aR complex) satisfied the requirement of mutual proximity of Ser66 in C5a and His194 in C5aR (see Fig. 7), with actual Cα-Cα distances ranging from 5.1 to 11.8 Å.

**DISCUSSION**

We present here a genetic approach combined with independent molecular modeling as a method to study complex protein/protein interaction domains. In an earlier work (12) we described a random saturation mutagenesis analysis of the N terminus of the C5aR. In that study, an unpaired cysteine in the C5a ligand provided a fortuitous disulfide trap that was able to complement the affinity that was eliminated from the C5aR NT by random mutagenesis. This disulfide-based method for probing the receptor-ligand orientation is extended in the work described here, creating a disulfide-based map of ligand/receptor interactions. At five of the tested positions in C5a, positions 24, 15, 40, 46, and 66, DTRM identified regions of probable interaction on the receptor. These interactions (mediated by presumptive disulfide bonds) suggest that the corresponding regions of the wild-type receptor and ligand are close to one another in the physiologic docked conformation. The interactions we detected were between C5a 24 and C5aR fragments 10–12 and 23–27; between C5a 15 and C5aR fragments 11–15; between C5a 40 and C5aR fragments 6–15 and 21–28; between C5a 46 and C5aR fragments 3–8 and 11–14; and between C5a 66 and C5aR 194. The most strongly inferred contacts are listed in Table 1.

The ability to form a disulfide is a function of the proximity of two cysteine residues, the local flexibility of the protein, and the redox environment. Our DTRM analysis makes no assumptions regarding where cysteine might be introduced in the receptor. The unpaired cysteines act as a collisional probe to allow us to map potential interaction surfaces. In principle, any interaction between the C5a ligand and receptor could be trapped and in effect increase the local concentration of the ligand and receptor. Thus it is unclear whether all the conformations that we have identified for the mutated receptors approximate those for the wild-type receptor/ligand interaction. However, because this approach selects only for receptor/ligand interactions that are functional, it is likely that the majority if not all of the regional interactions also occur in the native binding events.

The positional requirements for cysteines in the receptor as a function of the location of the ligand cysteine provide further evidence that the mutant receptor-ligand orientation is specific and physiologic. This view is strongly supported by our independent molecular modeling that found multiple low-energy conformations for the flexible N-terminal segment of C5aR. The modeling results demonstrated that the sets of conformations of the N-terminal segment most plausible for interaction with different cysteine-containing ligands were different, and that no single set of conformations accounted for all the disulfide trapping results. Thus, the interface between the receptor and the ligand molecules is determined mostly by the ligand selecting the proper conformations of the receptor. A widely accepted notion presumes that ligand/receptor interactions require an “induced fit” of the flexible ligand to the more rigid receptor, which is the case for the second site of interaction between C5a and C5aR. However, our findings clearly demonstrate that both components of the receptor-ligand system are involved in a “mutually induced fit.”

The most direct interpretation of the DTRM results is that the cysteines observed in mutated C5a receptors were selected due to their ability to form covalent disulfide bonds with the unpaired cysteine probe in the C5a ligand. However, we have
FIGURE 8. Sketches showing stereoviews of conformations of the N-terminal segment of C5aR compatible with possible formation of disulfide bridges between residues of C5a (shown as red spheres) and C5aR (shown as blue spheres). Backbones of C5aR and C5a are shown as shaded ribbons in white and green, respectively. Only C5aR residues with enhanced preference for transitioning to cysteine (see the text) and located relatively close to cysteines in C5a analogs (Cα-Cα ≤ 15 Å) are shown, namely Asn23, Thr24, Val26, and Asp27 for C5a C27R/D24C (panel A); Gly12 and Tyr14 for C5a C27R/H15C (panel B); Gly12, Asp15, Thr24, and Val26 for C5a C27R/R40C (panel C); Thr8 for C5a C27R/R46C (panel D; C5aR locations of residues Ser3 and Tyr6 were not modeled; Thr8 is shown to mark a spatial position close to their possible location); Val26, Asp27, Thr29, and Ser30 for C5a (the wild type, panel E); and all the above residues are shown in panel F.
not yet succeeded in directly demonstrating that a disulfide mechanism was responsible for the behavior we observed in our screens. Attempts to coprecipitate C5a and C5aR, or to observe their coligation as a disulfide-linked complex on SDS-PAGE, have been unsuccessful. We suspect that the number of cross-linked pairs required for signaling is small and falls below the sensitivity of our immunoblotting technique; alternatively, the disulfide-linked receptor-ligand pair may be transient and rapidly degraded upon yeast lysis.

Although formation of disulfide bonds between receptor and ligand seems the most likely explanation of our data, it is in theory possible that the receptor cysteines each have some other function; for example, they may facilitate the surface expression of the receptors, possibly by interacting with chaperones in the yeast endoplasmic reticulum to delay the progress of the receptor through the secretory pathway until folding is complete. This scenario fails to explain why the positioning of the receptor cysteine should be so sensitive to the position of the ligand cysteine. Furthermore, our previous random saturation mutagenesis studies have not suggested that cysteines could promote C5aR expression in yeast (20, 23, 26, 36, 37).

The C5aR contains an endogenous extracellular disulfide bond, required for receptor stability, at Cys109–Cys188. The mutant receptor cysteines could therefore in theory be acting by promoting alternative disulfide bond formation. This might particularly be true for the EC2 mutants, in which the introduced cysteine at position 194 is in the vicinity of the Cys109–Cys188 disulfide. This scenario would not, however, explain why these receptors are active only when coexpressed with the cysteine-containing C5a C27R/S66C ligand, and not when coexpressed with the Cys-less ligand C5a C27R (Fig. 7A) or with C5a C27R/I65C (Fig. 7B). One would need to hypothesize some other positionally specific role for the unpaired ligand cysteine at position 66. This could be a disulfide formed by Cys66 of C5a, presumably with the remaining unpaired cysteine of the C5aR, but this would still amount to forming a receptor-ligand disulfide, so the implications would be quite similar.

Importantly, DTRM does not invariably give positive results (i.e. evidence of an interaction) for all positions of the ligand or receptor. For example, we screened the NT mutant C5aR library with C5a C27R/S8C, and an EC3 mutant C5aR library with C5a C27R/N64C, C27R/I65C, and C27R/S66C, all without identifying any cysteine-dependent functional mutants (data not shown). It is noteworthy that we have not found any evidence for the involvement of N-terminal residues 16–22 in binding, although we may simply not have probed the region of C5a with which they interact.

The DTRM technique could in theory be ported to the exploration of other receptor-ligand pairs. The method requires the construction of mutant receptor libraries, but these are themselves of interest in deriving a functional receptor map. The method is applicable to both peptide and small-molecule ligands, because sulphydryls can be introduced in either of these by mutagenesis or chemical synthesis, respectively. DTRM may therefore become a useful general technique in structural biology. Similarly, molecular modeling procedures that account for conformational flexibility of the EC loops and the N-terminal segments could be applied to studies of many other GPCRs. The recently released crystal structure of the β2-adrenergic receptor (38) revealed a three-dimensional structure of the EC2 loop that was entirely different from that found earlier for rhodopsin, thus underlining the importance of considering flexibility of the non-TM regions of GPCRs. One may envision that a combination of the two independent approaches exemplified in the present study might be especially efficient in studies of the interaction of GPCRs with polypeptide ligands, such as chemokines, angiotensin II, oxytocin/vasopressin-related peptides, and opioid peptides, for which a mutual induced fit between the ligand and the receptor may also be expected.

REFERENCES

1. Bockaert, J., and Pin, J. P. (1999) *EMBO J.* 18, 1723–1729
2. Fredriksson, R., and Schioth, H. B. (2005) *Mol. Pharmacol.* 67, 1414–1425
3. Drews, J. (2000) *Science* 287, 1960–1964
4. Fan, Q. R., and Hendrickson, W. A. (2005) *Nature* 433, 269–277
5. Li, T. H., Grossmann, M., and Li, J. (1998) *J. Biol. Chem.* 273, 17299–17302
6. Gerard, N. P., and Gerard, C. (1991) *Nature* 349, 614–617
7. Allegritti, M., Moriconi, A., Beccari, A. R., Di Bitondo, R., Bizzarri, C., Bertini, R., and Colotta, F. (2005) *Curr. Med. Chem.* 12, 217–236
8. Siciliano, S. J., Rollins, T. E., DeMartino, J., Konteatis, Z., Malkowitz, L., Van Riper, G., Bondy, S., Rosen, H., and Springer, M. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 1214–1218
9. Mollison, K. W., Mandecki, W., Zuiderveld, E. R., Fayer, L., Fey, T. A., Krause, R. A., Conway, R. G., Miller, L., Edalji, R. P., Shallcross, M. A., Lane, B., Fox, J. L., Greer, J., and Carter, G. W. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 292–296
10. DeMartino, J. A., Van Riper, G., Siciliano, S. J., Molinexaux, C. J., Konteatis, Z. D., Rosen, H., and Springer, M. S. (1994) *J. Biol. Chem.* 269, 14446–14450
11. Chen, Z., Zhang, X., Gonnella, N. C., Pellase, T. C., Boyar, W. C., and Ni, F. (1998) *J. Biol. Chem.* 273, 10411–10419
12. Hagemann, I. S., Narzinski, K. D., Floyd, D. H., and Baranski, T. J. (2006) *J. Biol. Chem.* 281, 36783–36792
13. Mery, L., and Boulay, F. (1993) *Eur. J. Haematol.* 51, 282–287
14. Mery, L., and Boulay, F. (1994) *J. Biol. Chem.* 269, 3457–3463
15. Gerber, B. O., Meng, E. C., Dotsch, V., Baranski, T. J., and Bourne, H. R. (2001) *J. Biol. Chem.* 275, 3394–3400
16. Higginbottom, A., Cain, S. A., Woodruff, T. M., Proctor, L. M., Madala, P. K., Tyndall, J. I., Taylor, S. M., Fairlie, D. P., and Monk, P. N. (2005) *J. Biol. Chem.* 280, 17831–17840
17. Kawai, M., Quincy, D. A., Lane, B., Mollison, K. W., Or, Y. S., Luly, J. R., and Carter, G. W. (1992) *J. Med. Chem.* 35, 220–223
18. DeMartino, J. A., Konteatis, Z. D., Siciliano, S. J., Van Riper, G., Underwood, D. J., Fischer, P. A., and Springer, M. S. (1995) *J. Biol. Chem.* 270, 15966–15969
19. Kolakowski, L. F., Jr., Lu, B., Gerard, C., and Gerard, N. P. (1995) *J. Biol. Chem.* 270, 18077–18082
20. Baranski, T. J., Herzmark, P., Lichtarge, O., Gerber, B. O., Trueheart, J., Meng, E. C., Iiri, T., Sheikh, S. P., and Bourne, H. R. (1999) *J. Biol. Chem.* 274, 15757–15765
21. Dohlman, H. G., and Thorner, J. W. (2001) *Annu. Rev. Biochem.* 70, 703–754
22. Brown, A. J., Dyos, S. L., Whiteway, M. S., White, J. H., Watson, M. A., Marzioch, M., Clare, J. J., Cousens, D. J., Paddon, C., Plumpton, C., Romanos, M. A., and Dowell, S. J. (2000) *Science* 292, 296
23. Klco, J. M., Wiegand, C. B., Narzinski, K., and Baranski, T. J. (2005) *Mol. Pharmacol.* 67, 269–277
24. Nikiforovich, G. V., and Marshall, G. R. (2003) *Biochemistry* 42, 9110–9120
25. Matsumoto, M. L., Narzinski, K., Kiser, P. D., Nikiforovich, G. V., and Baranski, T. J. (2007) *J. Biol. Chem.* 282, 3105–3121
26. Finch, A. M., Vogen, S. M., Sherman, S. A., Kirmarsky, L., Taylor, S. M., and...
Structure of the C5a Receptor-Ligand Complex

28. Vogen, S. M., Finch, A. M., Wadi, S. K., Thatcher, J., Monk, P. N., Taylor, S. M., and Sanderson, S. D. (1999) J. Pept. Res. 53, 8–17
29. Zuiderweg, E. R., Nettesheim, D. G., Mollison, K. W., and Carter, G. W. (1989) Biochemistry 28, 172–185
30. Zhang, X., Boyar, W., Galakatos, N., and Gonnella, N. C. (1997) Protein Sci. 6, 65–72
31. Bubeck, P., Grotzinger, J., Winkler, M., Kohl, J., Wollmer, A., Klos, A., and Bautsch, W. (1994) Eur. J. Biochem. 219, 897–904
32. Toth, M. J., Huwyler, L., Boyar, W. C., Braunwalder, A. F., Yarwood, D., Hadala, J., Haston, W. O., Sills, M. A., Seligmann, B., and Galakatos, N. (1994) Protein Sci. 3, 1159–1168
33. Cain, S. A., Coughlan, T., and Monk, P. N. (2001) Biochemistry 40, 14047–14052
34. Cain, S. A., Higginbottom, A., and Monk, P. N. (2003) Biochem. Pharmacol. 66, 1833–1840
35. Raffetseder, U., Roper, D., Mery, L., Gietz, C., Klos, A., Grotzinger, J., Wollmer, A., Boulay, F., Kohl, J., and Bautsch, W. (1996) Eur. J. Biochem. 235, 82–90
36. Geva, A., Lassere, T. B., Lichtarge, O., Pollitt, S. K., and Baranski, T. J. (2000) J. Biol. Chem. 275, 35393–35401
37. Klco, J. M., Nikiforovich, G. V., and Baranski, T. J. (2006) J. Biol. Chem. 281, 12010–12019
38. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. J., Kuhn, P., Weis, W. I., Kobilka, B. K., and Stevens, R. C. (2007) Science 318, 1258–1265