THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 22, Issue of May 28, pp. 15757–15765, 1999

Printed in U.S.A.

C5a Receptor Activation

GENETIC IDENTIFICATION OF CRITICAL RESIDUES IN FOUR TRANSMEMBRANE HELICES*

(Received for publication, December 1, 1998, and in revised form, February 17, 1999)

Thomas J. Baranski‡§, Paul Herzmark‡, Olivier Lichtarge‡¶, Basil O. Gerber‡‖, Joshua Trueheart, Elaine C. Meng‡, Taroh Iiri‡ ‡‡, Søren P. Sheikh‡ §§, and Henry R. Bourne‡ ¶¶**

*From the ‡Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94143 and **Cadus Pharmaceutical Corporation, Tarrytown, New York 10591-6705*

Hormones and sensory stimuli activate serpentine receptors, transmembrane switches that relay signals to heterotrimeric guanine nucleotide-binding proteins (G proteins). To understand the switch mechanism, we subjected 93 amino acids in transmembrane helices III, V, VI, and VII of the human chemoattractant C5a receptor to random saturation mutagenesis. A yeast selection identified 121 functioning mutant receptors, containing a total of 523 amino acid substitutions. Conserved hydrophobic residues are located on helix surfaces that face other helices in a modeled seven-helix bundle (Baldwin, J. M., Schertler, G. F., and Unger, V. M. (1997) *J. Mol. Biol.* 272, 144–164), whereas surfaces predicted to contact the surrounding lipid tolerate many substitutions. Our analysis identified 25 amino acid positions resistant to nonconservative substitutions. These appear to comprise two distinct components of the receptor switch, a surface at or near the extracellular membrane interface and a core cluster in the cytoplasmic half of the bundle. Twenty-one of the 121 mutant receptors exhibit constitutive activity. Amino acids substitutions in these activated receptors predominate in helices III and VI; other activating mutations truncate the receptor near the extracellular end of helix VI. These results identify key elements of a general mechanism for the serpentine receptor switch.

Serpentine receptors serve as ligand-activated molecular switches, relaying signals from extracellular ligands to heterotrimeric ($\alpha\beta\gamma$) G proteins on the cytoplasmic face of the plasma membrane. These receptors catalyze ligand-dependent exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the α subunit of the heterotrimer, causing dissociation of α·GTP from the $\beta\gamma$ dimer; α·GTP and free $\beta\gamma$ subsequently activate effector enzymes and ion channels (1, 2). More than 1,000 serpentine receptors of mammals share with their counterparts in yeast and plants a conserved three-dimensional architecture, comprising seven α-helices in a transmembrane bundle (3–6). The switch mechanism is also conserved, as indicated by the abilities of mammalian receptors to activate G protein trimers in yeast (7–9). The switch clearly resides in the seven-helix bundle: swapping of extra- or intracellular loops preserves the ability of ligands to activate G proteins while transferring specificity of ligand binding or G protein activation, respectively, from one receptor to another (10–13).

A static model of the three-dimensional structure of the helix bundle is beginning to take shape. A low resolution (6 Å) electron cryomicroscopic structure of rhodopsin (14), the retinal light receptor, reveals relative positions and tilts of seven transmembrane helices in the plane of the membrane. Based on many mutations, the rhodopsin structure, and an analysis of the primary structures of more than 500 rhodopsin-like serpentine receptors, Baldwin and co-workers (15) constructed an α-carbon template of the helix bundle, hereafter termed the Baldwin model. In this model, transmembrane helices I-VII bundle together in clockwise order as viewed from the cytoplasm. The probable arrangement of helices and the positions of specific amino acids in the model are inferred from patterns of conserved hydrophobic and hydrophilic residues in many receptors. The Baldwin model specifies which helix corresponds to which density in the electron projection map, approximate orientations of cognate amino acids around the helical axes, and the cytoplasmic and extracellular limits of each transmembrane sequence (16).

How does the switch work? It is difficult to infer a conserved switch mechanism from the functional effects of site-directed mutations reported in a large number of different receptors, because relatively few positions have been mutated in any one receptor (17, 18). Accordingly, we undertook a systematic genetic analysis of a single serpentine receptor, with the goal of identifying functionally important residues and sites of helix-helix interactions that relay the ligand signal to G protein activation. We selected functional receptors after random saturation mutagenesis of the four transmembrane helices (III, V, VI, and VII) most consistently implicated in ligand binding or G protein activation by various serpentine receptors (3–6, 17, 18). This comprehensive approach determines the relative importance of side chains in each α-helix by identifying those that

* The research was supported in part by National Institutes of Health Grant GM-27800 (to H. R. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ A Howard Hughes Medical Institute Physician Postdoctoral Fellow. Present address: Depts. of Medicine and Molecular Biology and Pharmacology, Washington University School of Medicine, Box 8103, 660 S. Euclid Ave., St. Louis, MO 63110.

¶ Supported by funds from the American Heart Association. Present address: Dept. of Molecular and Human Genetics, Baylor College of Medicine, 1 Baylor Plaza, Rm. T921, Houston, TX 77030.

‖ An Early Career Fellow of the Swiss National Science Foundation.

‖ ‖ Supported by a Julius H. Comroe Jr. Award by the Cardiovascular Research Institute. Present address: Fourth Department of Internal Medicine, University of Tokyo School of Medicine, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112, Japan.

§§ Supported by the Danish Heart Association and by funds from Daiichi. Present address: Laboratry of Molecular Cardiology, University of Copenhagen, Rigshospitalet 9312, Juliane Mariesvej 20, DK-2100 Copenhagen, Denmark.

¶¶ To whom correspondence should be addressed: Dept. of Cellular and Molecular Pharmacology, University of California, Box 0450, 513 Parnassus Ave., San Francisco, CA 94143. Tel.: 415-476-8161; Fax: 415-476-5292; E-mail: h_bourne@quickmail.ucsf.edu.

cannot be altered in mutated receptors selected for maintenance of function. The results suggest a hypothesis to explain the conserved switch mechanism of serpentine receptors.

EXPERIMENTAL PROCEDURES

Library Construction—To construct mutagenized C5a receptors, we engineered restriction endonuclease sites (which did not alter the amino acid sequence) into the C5a receptor gene at the approximate boundaries of helices III (*Sph*I and *Pst*I), V (*Bss*HII), VI (*Bgl*II and *Xho*I), or VII (*Hin*dIII). A *Nco*I site at the start methionine introduced an aspartic acid in place of an asparagine at amino acid position 2. To ensure efficient library construction and eliminate the possibility of contamination by wild-type receptor DNA, for each helix library we created a subcloning vector that contained a piece of nonreceptor DNA substituted between the flanking restriction sites. For example, in the Helix III subcloning vector, a 383-base pair fragment of $G\alpha_{12}$ digested with *Sph*I and *Pst*I was subcloned into the C5a receptor DNA containing silent *Sph*I and *Pst*I sites. The following oligonucleotides were used (Genemed, S. San Francisco, CA; underlines denote bases doped with 20% nonwild-type nucleotides): Helix III, 5′-CCCGCATGCTCTATTCTACCATCTCTAATTCTACTAAACATGTACGCTTCTATTCTACTACTAGCTACTATTTCTGCAGAA-3′; Helix V, 5′-TTTGCGCGCTGTTGCTATTGTTAGACTAGTTCTAGGTTTCCTATGGCCTCTACTAACTCTAACTATTTGTTACACTTTCATTCTGCTCCGGAGC-3′; Helix VI (two oligonucleotides); 5′-ATAAGATCTACCAAAACACTCAAAGTTGTTGTTGCAGTTGTTGCAAGCTTCTTTATCTTCTGGTTA-3′, and 5′-AAACTCGAGAAAAGACATCATTATCCCCGTCACCTGGTATGGTAACCAGAAGAT-3′; Helix VII, 5′-CCAAAGCTTGATTCTCTATCTGTTTCTTTTGCTTACATTAATTGTTGTATTAATCCAATTATTTACGTTGTGGCCGGCCA-3′. Oligonucleotides encoding Helices III, V, or VII were mutually primed via extended palindromic sequences at their 3′ termini (19, 20), and the complementary strand synthesized using Klenow DNA polymerase. The products were digested with the appropriate restriction enzymes and subcloned into the C5a receptor gene in pBS-SK Bluescript vector. Because of technical difficulties in synthesizing doped oligonucleotides greater than 100 base pairs, we annealed two oligonucleotides encoding either the N- or C-terminal half of helix VI via a 12-base pair overlap at their 3′ termini, synthesized the complementary strand using Klenow polymerase, and subcloned the *Bgl*II and *Xho*I digested products into the C5a receptor gene in pBS-SK Bluescript vector. After determining the complexity and the quality of the library (by DNA sequencing and restriction mapping), we subcloned *Nco*I and *Fse*I fragments of each mutated helix library into the yeast shuttle vector, p1303ADE2 (derived from p1303, Cadus Pharmaceuticals, New Jersey). The number of recombinants obtained equaled or exceeded the initial size of the mutated helix library.

Yeast Strains—Strain BY1142, engineered by standard yeast strategies, has the genotype *far1Δ1442 tbt1–1 FUS1-HIS3 can1 ste14::trp1::LYS2 ste3Δ1156 gpa1(41)*-$G\alpha_{i2}$ *lys2 ura3 leu2 trp1 his3 ade2*. BY1142 expresses a yeast/human Gα chimera in which the N-terminal 41 residues of yeast GPA1 (a region predicted to interact with Gβγ based on the crystal structure of the G_i heterotrimer (21)) replaces the first 33 residues of human $G\alpha_{i2}$. A *Sac*I restriction site introduces a point mutation that substitutes leucine for valine at position 34 of $G\alpha_{i2}$. The chimera is designed for optimal ability to sequester yeast Gβγ and release it upon receptor activation, thereby triggering the mating response pathway. Expression of the FUS1/HIS3 reporter enzyme, stimulated by the mating response pathway, allows BY1142 cells (*his3*) to grow in histidine-deficient medium. The *far1* deletion blocks cell cycle arrest that is induced by pheromone. The *tbt1–1* mutation increases transformation efficiency by electroporation and *ste14*, a carboxymethylase deletion, reduces basal signaling of the pheromone response pathway. Inclusion of an *ADE2* gene in the C5a receptor plasmid, p1303 (p1303ADE2, *PGK*-hC5a*RADE2 REP3* 2 μm-ori AmpR f1ori), allowed us easily to infer from the color of a colony whether its growth on histidine-deficient medium depends on the C5a receptor (red colonies lack *ADE2* and, by implication, the receptor plasmid). A separate plasmid, p1297 (*ADH1-mfα1*-hC5a*URA3 REP3* 2μm-ori AmpR f1ori, Cadus Pharmaceuticals), allowed autocrine expression of the C5a ligand as an α-factor prepro/C5a ligand fusion protein; autocrine expression was necessary because C5a cannot traverse the yeast cell wall.

Yeast Transformation and Receptor Selection—Using electroporation, we cotransformed BY1142 yeast cells with plasmid libraries of mutated C5a receptors and the C5a ligand plasmid, p1297. Colonies bearing functioning receptors were selected by replica-plating on histidine-deficient medium containing 1 mM 3-aminotriazole (AT)[1] (Sigma). We recovered the plasmids encoding functioning C5a receptors and confirmed the phenotypes by retransforming each candidate C5a receptor plasmid and the C5a ligand plasmid (or without C5a ligand, to assess constitutive signaling) into the parental yeast strain, BY1142. Growth after 3 days at 30 °C in the presence of different concentrations of AT (0, 0.5, 1, 2, 5, and 10 mM) served to quantitate the relative intensities of signals mediated by mutant receptors. Each mutant receptor was tested at least twice and the results were averaged. In each case, the wild-type C5a receptor expressed in parallel yeast served as a positive control. The results of ten independent transformations of wild-type C5a receptor were growth at 5, 5, 5, 5, 5, 5, 2, 5, 2, and 5 mM AT. The mutant receptor sequences were obtained by DNA sequencing with Thermosequenase cycle sequencing (Amersham Pharmacia Biotech).

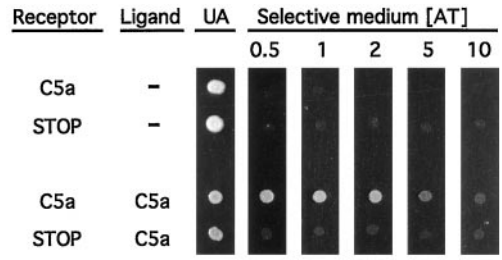

FIG. 1. **C5a and the C5a receptor allow growth of yeast strain BY1142.** Yeast BY1142 cells (11) were transformed with *ADE2* plasmid DNA encoding the C5a receptor (*rows 1* and *3*) or, as a negative control, a C5a receptor containing a stop codon in helix III (*STOP*, *rows 2* and *4*), and *URA3* plasmid DNA encoding vector alone (*rows 1* and *2*) or C5a ligand (*rows 3* and *4*). After selection on uracil- and adenine-deficient medium (*UA*), colonies were grown to near stationary phase and aliquots spotted onto nonselective UA medium or histidine-deficient medium containing the indicated concentrations of the HIS3 inhibitor, AT, and incubated at 30 °C for 3 days.

RESULTS AND DISCUSSION

Selection of Functional Receptors—We studied the human chemoattractant C5a receptor, a member of the rhodopsin family of serpentine receptors. This receptor activates G_i and mediates chemotaxis of neutrophils toward the C5a ligand, a 74-residue polypeptide (22, 23). To select functioning receptors from populations of mutant receptors we took advantage of the G protein-mediated response of *Saccharomyces cerevisiae* to mating pheromones (24–26). We constructed a yeast strain, BY1142, in which activation of the C5a receptor induces expression of the *HIS3* gene, thereby allowing cells with functioning receptors to survive and proliferate in growth medium lacking histidine. In this selection procedure, the relative intensities of signals mediated by mutant receptors can be quantitated by assessing growth in the presence of different concentrations of AT, a competitive inhibitor of the HIS3 gene product (27). Expression of both C5a and the C5a receptor allows BY1142 cells to grow in selective medium lacking histidine and containing up to 5 mM AT (Fig. 1).

Helix by helix, we subjected stretches of amino acid sequence to random saturation mutagenesis, for a total of 93 positions in helices III, V, VI and VII. Quality of the libraries was assessed in two ways. First, DNA sequencing of 15–20 independent recombinants from each library before pooling revealed oligonucleotide mutagenesis rates between 19–22%, resulting in amino substitution rates of 36–42% (Table I). Second, we determined the substitution rate at the third nucleotide position of degenerate codons for amino acid positions at which few substitutions were observed in functioning receptors (see below). The observed substitution rate of 17% closely matches the predicted rate of 20%, demonstrating that little or no bias occurred in the randomization of the libraries.

[1] The abbreviation used is: AT, 3-aminotriazole.

TABLE I
Characteristics of mutated C5a receptors

Size, number of individual recombinants in each helix library; AA, amino acids; AAΔ, amino acid substitution rate; avg, average per scanned transmembrane segment.

Libraries				Selected receptors			
Helix	Size ($\times 10^{-3}$)	AA scanned	AAΔ avg	Total	AAΔ avg	Constitutive	Truncated
			%		%		
III	200	21	8.0 (38)	30	4.4 (21)	5	0
V	1,000	25	10.5 (42)	40	4.7 (19)	0	0
VI	50	24	9.9 (38)	25	8.1 (31)	16	9
VII	1,000	21	7.6 (36)	28	3.2 (15)	0	0

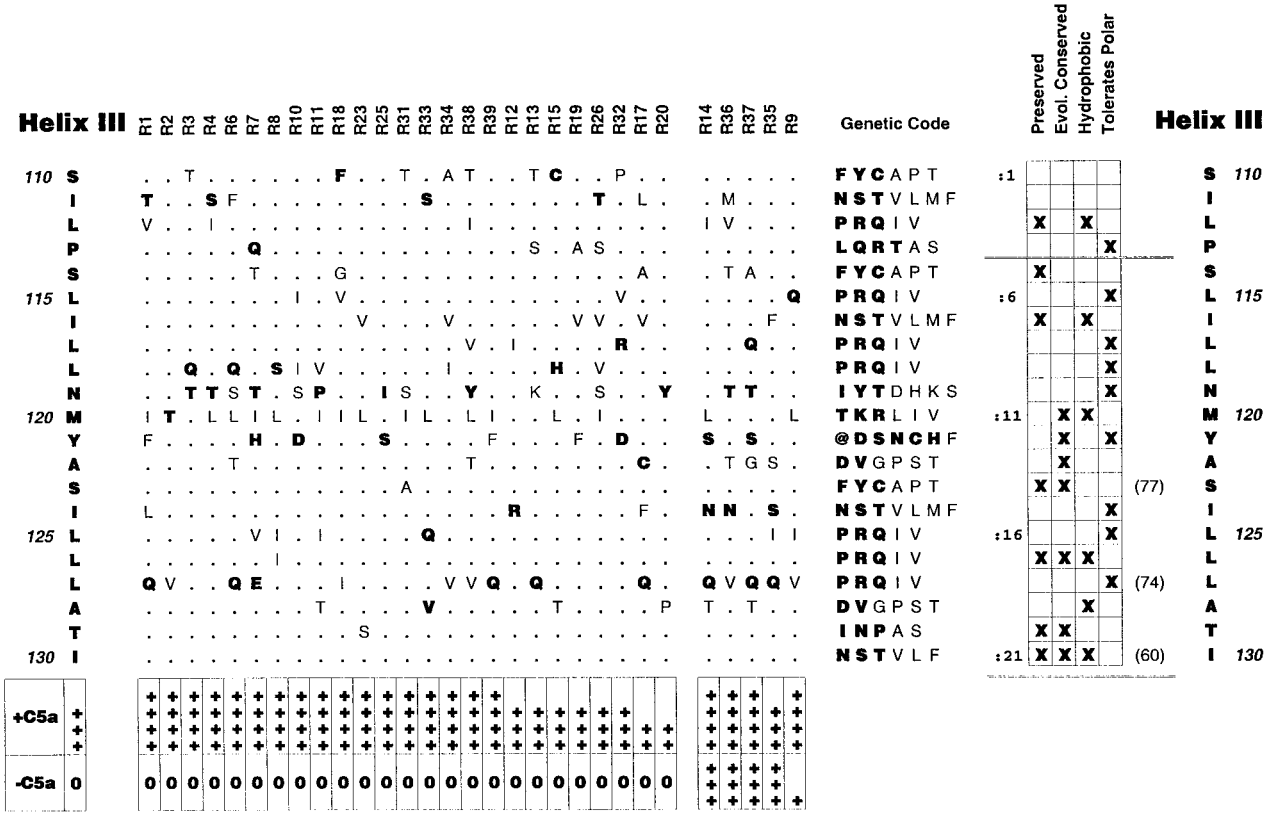

FIG. 2. **Functioning C5a receptors selected from a library containing random substitutions in helix III.** Mutated C5a receptors and the C5a ligand were expressed in BY1142 yeast and functioning receptors selected by the ability of yeast to grow in the absence of histidine and in 1 mM AT. C5a receptor sequences are shown from the extracellular (*top*) to the cytoplasmic surface (*bottom*). Wild-type sequence of the C5a receptor and the amino acid position numbers are indicated at the *left* and *right* of each figure. Position numbers in the notation of Baldwin (15, 16) are indicated at the *left* of the *boxed* columns. *Horizontal lines* indicate proposed borders of the lipid bilayer (16). Columns representing individual mutant receptors (each designated by *R* and a *number*, *top*) display their individual sequences (*dot* = unchanged from wild type). Receptor signaling strength, indicated below each mutant receptor sequence, was quantitated by growth on histidine-deficient media in the presence of AT. ++++, growth on 10 mM AT; +++, 5 mM AT; ++, 2 mM AT; +, 0.5 or 1 mM AT; 0, no growth on 0.5 mM AT. Constitutively active receptors are grouped separately. *Genetic code* refers to amino acid substitutions that were possible at the corresponding position by substituting only a single nucleotide base. Standard single-letter abbreviations indicate amino acids at each position; *bold letters* indicate amino acids that are not conserved with respect to the wild-type C5a receptor sequence. Characteristics of mutated residues in functional receptors (indicated by **X** in the boxed columns) are presented in four classes, as follows: *Preserved*, amino acid positions where side chain character is preserved (see text); *Evol. Conserved,* residues identified by the evolutionary trace method (38); *Hydrophobic*, positions at which only hydrophobic amino acids are observed (see text); *Tolerates Polar,* aspartate, glutamate, asparagine, glutamine, lysine, or arginine tolerated; @, stop codons observed. Numbers in parentheses indicate percent identity in 199 rhodopsin-family serpentine receptors (16).

We then selected recombinants from four separate libraries of mutant receptors, each library containing mutations in a single helix, for ability to support growth of BY1142 cells in the absence of histidine. At an average mutation rate of 8–10 amino acid substitutions per helix, fewer than one in 10^3 BY1142 cells transformed with a mutated C5a receptor survived the selection.[2] In the four scanned helices, the yeast selection identified 121 functioning mutant receptors (at least 25 from each helix library, Figs. 2, 3, 4, and 5), providing a data set of 523 amino acid substitutions (summarized in Table I). The receptor helices tolerate an average of 4.3 amino acid substitutions per mutated helix. We assume that most amino acid substitutions occur at less important parts of the receptor switch; some mutations, however, may compensate for functional defects caused by other mutations in the same helix.

Patterns of Preserved Residues Confirm the Structural Model—We analyzed the mutant C5a receptors, amino acid position by position, looking for physical properties of side chain character and hydrophobicity that were present or "preserved" in every functional receptor. For this analysis, we de-

[2] T. J. Baranski and H. R. Bourne, unpublished data.

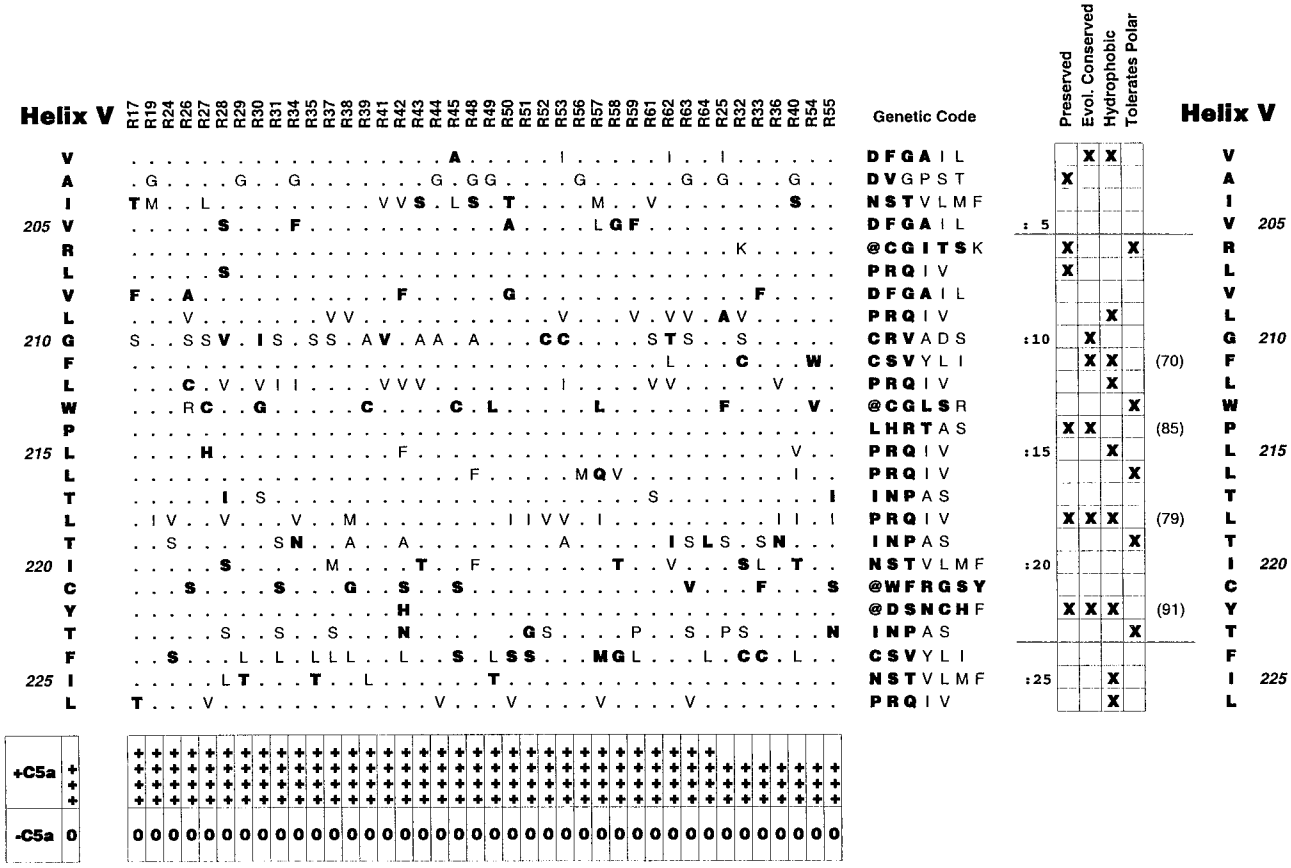

FIG. 3. **Functioning C5a receptors selected from a library containing random substitutions in helix V.** Mutated C5a receptors and the C5a ligand were expressed in BY1142 yeast and functioning receptors selected by the ability of yeast to grow in the absence of histidine and in 1 mM AT. For details, please refer to Fig. 2 legend.

fined side chain character as preserved if we observed at that position no more than one amino acid substitution or if all substitutions at that position involved closely related amino acids (log odds score of 1.0 or greater in the PAM 250 scoring matrix (28)). Single amino acid substitutions were included to allow for the rare mutation that might have occurred at a critical position, but only in the setting of other compensatory substitutions. For example, the Tyr-222 position is conserved in 91% of serpentine receptors (15) and is preserved in 39 of the 40 mutated receptors in our study. The single Y222H substitution obtained in Arg-42 occurs in the setting of substitutions in the two neighboring residues, C221S and T223N (Fig. 3). Hydrophobicity is considered preserved at positions where only hydrophobic amino acids with free energy transfer$_{octanol}$ of $>$0.1 kcal/mol (29) are observed. The functioning mutant receptors preserve side chain character at 25 of the 93 positions mutated (Figs. 2–5). Thirty of the 93 mutated positions preserve their hydrophobic character in functioning receptors; this group includes 11 of the 25 positions at which side chain character was preserved (Figs. 2–5).

We expected that in functioning mutant receptors the side chains that face other helices would tolerate fewer mutations than side chains that face the surrounding lipid, because association of transmembrane helices with one another depends upon preservation of complementary shapes that maximize van der Waals interactions (30). Moreover, the established role of hydrophobic interactions in stabilizing the tertiary structure of soluble proteins (31, 32) suggests that hydrophobicity should be preserved at positions that point toward other helices.

The results of our analysis meet both expectations; the patterns of preserved side chain character and hydrophobicity strongly confirm the helix orientations specified in the Baldwin model. In helical wheel plots based on the Baldwin model, both sets of residues (character-preserving and preserved hydrophobics) point toward other helices or toward the center of the helix bundle, rather than toward the surrounding lipid (Fig. 6). The argument is circular, however, because the Baldwin model itself (15, 16) is based on patterns of side chain character and hydrophobicity conserved through evolution. Nonetheless, the patterns observed in mutant receptors constitute experimental validation of the model. A quite different approach, based on substituting cysteines at many positions in helices of the D_2-dopamine receptor, also furnishes a comprehensive confirmation of the helix orientations specified by the Baldwin model. In these experiments, cysteines that proved accessible to a water-soluble chemical probe were located on the inner faces of the four helices tested (III, IV, VI, and VII) (33–36). In contrast to regions of the transmembrane helices involved in helix-helix interactions, hydrophobicity is not strictly preserved at positions predicted by the Baldwin model to face the surrounding lipid. 12 of the 29 mutated positions that tolerate substitution of a polar residue are predicted to contact the lipid. This striking tolerance of polar side chains in lipid-contacting faces of transmembrane helices confirms a previous analysis of random mutations in three transmembrane helices of a bacterial diacylglycerol kinase (37).

To assess the reliability of the mutation and selection procedures for identifying side chains important for receptor function, we tested the effect of a single amino acid substitution at many of the preserved positions (Table II). Fourteen of 17 such point mutations tested do impair receptor function (0 or + growth *versus* +++ for the unmutated C5a receptor; see Fig. 2

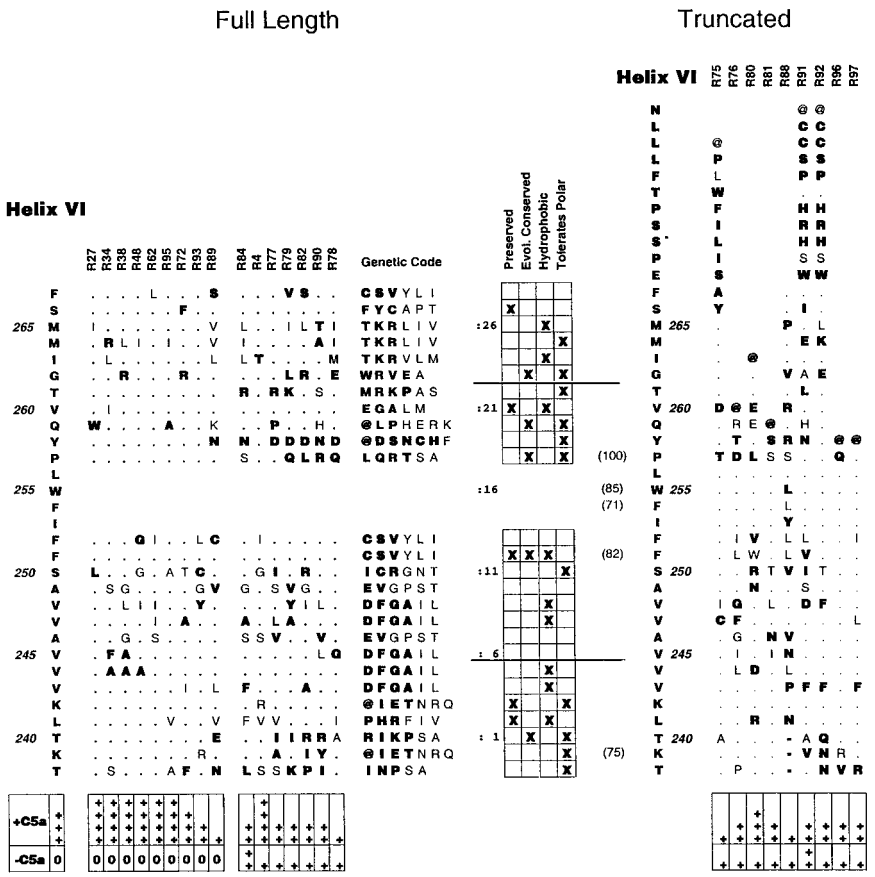

FIG. 4. **Functioning C5a receptors selected from a library containing random substitutions in helix VI.** Mutated C5a receptors and the C5a ligand were expressed in BY1142 yeast and functioning receptors selected by the ability of yeast to grow in the absence of histidine and in 1 mM AT. For details, please refer to Fig. 2 legend. @, stop codons observed. Note that four positions in helix VI (Ile-253, Phe-254, Trp-255, Leu-256) were not subjected to mutation; the corresponding nucleotides were kept constant to anneal two sets of randomized oligonucleotides.

legend), indicating functional importance of the corresponding preserved residue. Three of the 17 preserved positions tolerate amino acid substitution; side chains at these positions are presumably important in a context of multiple additional mutations but are not necessary for function in an otherwise wild-type receptor.

Preserved Residues Identify Working Parts of the Switch—The first clues to specific roles of preserved residues came from comparing positions preserved in the functioning mutant C5a receptors with positions deemed evolutionarily important among serpentine receptors. We applied an evolutionary trace analysis method (38) to 62 serpentine receptors closely related to the human C5a receptor. The evolutionary trace method assesses patterns of sequence conservation in alignments of genes for related proteins and maps the conserved positions onto a shared backbone structure. The method successfully identified contact surfaces in SH2 and SH3 domains (38) and Gα surfaces that interact with $\beta\gamma$ and the serpentine receptors (39).[3] Figs. 2–5 indicate the top ranked positions (labeled *Evol. Conserved*). As expected, the evolutionary trace approach identifies many of the same residues found in these helices by Baldwin's analysis of 199 unique serpentine receptor sequences (16). The latter analysis identified 16 positions at which residues are conserved in at least 60% of the receptors (Figs. 2–5); 12 of these positions appear in the evolutionary trace.

Fig. 7 compares the evolutionary trace to the pattern of preserved residues in mutant functioning C5a receptors, as mapped onto the α-carbon template of the Baldwin model (15). Positions preserved in mutant C5a receptors form two distinct clusters in the three-dimensional model, as indicated by the red and yellow balls in Fig. 7. Red indicates positions at which residues are both preserved in mutant receptors and identified by the evolutionary trace method; yellow indicates preserved positions that are not highly conserved throughout evolution. (Blue indicates positions that are deemed evolutionarily important but are not preserved in our genetic screen, see below.) Yellow positions are located at or near the extracellular face of the receptor, whereas red positions cluster tightly in the cytoplasmic half of the transmembrane helices (Fig. 7). The distributions indicate different functions for these important residues.

We propose that the cluster of yellow residues disposed toward the extracellular fluid defines a receptor surface that interacts with the C5a ligand (Fig. 8*A*). Mutations at two of these positions (Arg-206 in helix V and Asp-282 in helix VII) alter binding affinity for C5a (40). In accord with the two-site binding model proposed for C5a binding (41–44), the carboxyl-terminal tail of C5a probably inserts into the interhelical crevice depicted in Fig. 8*A*, whereas other portions of the C5a ligand interact with the amino terminus of the C5a receptor. Moreover, as shown in Fig. 8*A*, positions of five of the six preserved residues near the extracellular termini of helices III and V coincide precisely with positions assigned by the Baldwin model to residues that bind ligands in receptors for biogenic amines (45–47). Glu-113 of rhodopsin, which corresponds to the preserved Leu-112 of the C5a receptor, serves as a counterion for the Schiff's base formed between retinal and Lys-296 in helix VII (48–50). Thus small amines, a retinal chromophore, and a 74-residue polypeptide probably interact with amino acids at many of the same positions in the helix bundle (albeit with different side chains) to activate serpentine receptors. This suggests that serpentine receptors that bind very different agonists share a common activation mechanism.

In the Baldwin model, the α-carbons of all ten clustered red

[3] A more complete analysis of the evolutionary trace in lineages of serpentine receptors is in preparation, O. Lichtarge, H. R. Bourne, and F. E. Cohen.

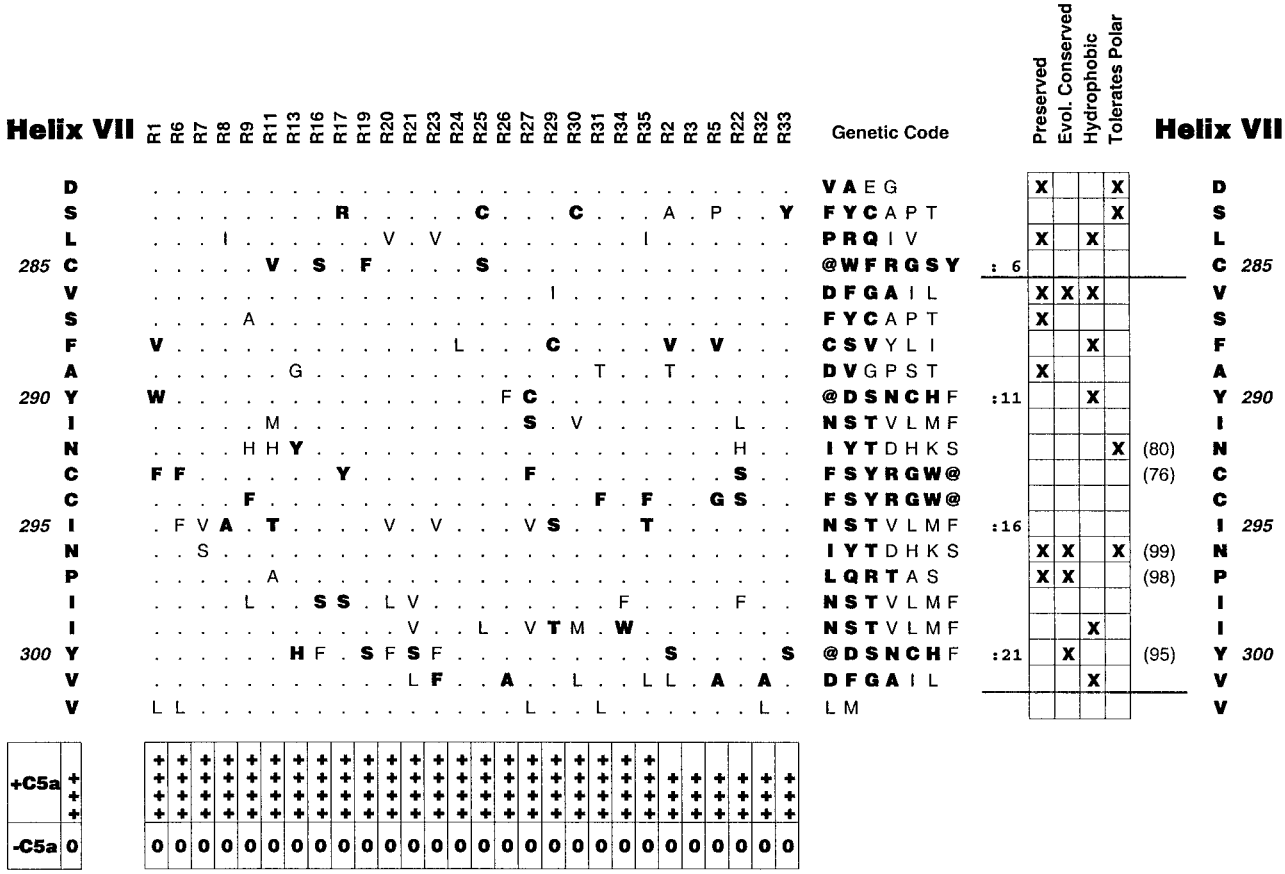

FIG. 5. **Functioning C5a receptors selected from a library containing random substitutions in helix VII.** Mutated C5a receptors and the C5a ligand were expressed in BY1142 yeast and functioning receptors selected by the ability of yeast to grow in the absence of histidine and in 1 mM AT. For details, please refer to Fig. 2 legend.

positions in Fig. 7 are located within 10 Å of the α-carbon of at least one other highly conserved residue. (Some of these are conserved residues in helices I, II, and IV; conserved is defined as identical in at least 60% of 199 serpentine receptors assessed by Baldwin *et al.* (15).) This cluster, highly preserved both in evolution and in our genetic selection for functional C5a receptors, is positioned to form an interacting network that is apparently crucial for structure and function of serpentine receptors. We focused our mutations on components of a transmembrane switch, rather than on residues implicated in interactions with G proteins; for this reason the red positions do not overlap significantly with regions in other receptors that are thought to bind G proteins (51–54) (cyan in Fig. 8*B*). Instead, we propose that these positions form a structural core that allows conformational changes induced by ligand binding to be transmitted to the G protein interaction surface.

Some positions (blue in Fig. 7) that are highly important in many serpentine receptors throughout evolution are nonetheless sites of frequent substitution in functioning C5a receptor mutants. They may mediate a specific function subject to a selective pressure in evolution that was absent in our yeast screen. One possibility is that these blue residues, located in the extracellular half of the helix bundle, participate in interhelical interactions responsible for maintaining the receptor switch in the off state in the absence of ligand. We have not tested whether single mutations at the blue positions systematically produce constitutive activation of the receptor.

Activating Mutations and Truncations—Although selected in yeast cells expressing the C5a ligand, a substantial number of functioning mutant receptors remains active when expressed

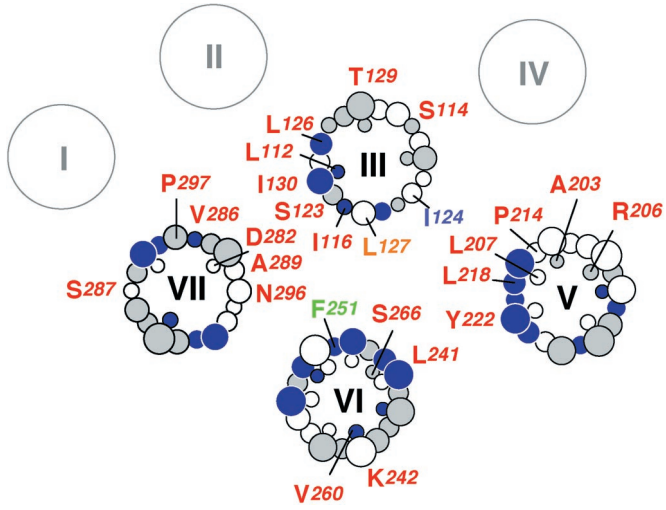

FIG. 6. **Side chains are preserved on faces of helices that point toward other helices in the Baldwin model.** Helical wheel representations are based on the Baldwin model (16). Helices are presented as viewed from the cytoplasm and in positions that correspond to their relative positions at the middle of the bilayer (16). *Larger circles* indicate residues closer to the cytoplasm. *Red letters* and *numbers* indicate residues and positions at which side chain character is preserved in mutant functional receptors (see text). *Dark blue circles* indicate positions at which hydrophobicity is preserved; *white* indicates positions that tolerate polar substitutions (as defined in the legend of Fig. 2); other positions are *gray*. *Blue*, *orange*, and *green letters* indicate positions (Ile-124, Leu-127, and Phe-251, respectively) at which substitutions caused constitutive activation of the receptor (see text and Table II).

TABLE II

Site-directed point mutations of key residues identified by random saturation mutagenesis

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

III			V			VI			VII		
	+C5a	−C5a		+C5a	−C5a		+C5a	−C5a		+C5a	−C5a
Wild type	+++	0									
L112A	0	0	A203V	0	0	S266A	+	0	D282A	0	0
S123A	++++	0	R206H	0	0	V260A	+	0	V286A	0	0
L126A	0	0	L207A	+	0	F251A	+++	++	V286L	0	0
T129A	+	0	L207I	+	0	K242A	+++	0	S287A	0	0
I130A	+	0	Y222A	0	0				N296A	0	0
I124N, L127Q	++++	+++									
I124N	++++	+									
L127Q	++++	+									

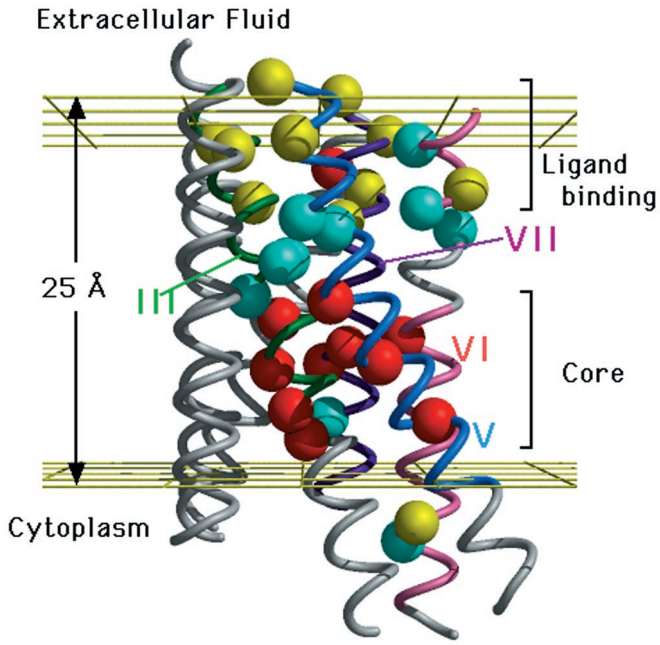

FIG. 7. **Comparison of the locations of preserved amino acids in the mutant functional C5a receptors *versus* highly conserved residues in evolution.** *Colored tubes* represent the α-carbon backbones of the helices in the Baldwin model. Portions of helices III (*green*), V (*blue*), VI (*pink*), and VII (*purple*) were subjected to random saturation mutagenesis; *gray* indicates residues or helices not so tested. *Yellow* and *red spheres* indicate α-carbon positions at which side chain character is preserved in functioning C5a receptors; *blue* and *red spheres* indicate positions that are conserved, as assessed by the evolutionary trace method (38). *Red spheres* indicate positions that are both preserved in the genetic screen and also conserved in evolution of serpentine receptors. The *yellow grid* (lines separated by 10 Å) indicates proposed borders of the lipid core of the membrane (16).

in cells lacking C5a (Table I, Figs. 2 and 4); several of these activated receptors are truncated by stop codons in helix VI (Fig. 4). Taken together, these mutations and the truncated receptors point to helices III and VI as key elements of the turn-on switch and to a possible role for helix VII as a ligand-sensitive inhibitor of activation.

Ligand-independent receptor activity resulted from point mutations in helices III (five of 30 functional receptors) and VI (16 of 25 functional receptors) but not helices V and VII (Table I). We infer that receptor activation involves release of constraints on movement of helix VI relative to the rest of the transmembrane helix bundle. This inference is compatible with the tolerance of helix VI for amino acid substitutions (found in helix VI at almost twice the rates observed for the three other helices tested, Table I) and with its remarkable predilection for activating mutations. This susceptibility of helix VI to activating mutations appears to be shared by receptors for biogenic amines, luteinizing hormone, and thyrotropin (55, 56). The importance of helix VII for constraining mobility of helix VI and preventing constitutive activation was a principal conclusion of a computational modeling analysis of activating mutations (predominantly in helix VI) in the luteinizing hormone receptor (57).

A "hot spot" for activating mutations in helix III is also compatible with a relation between receptor activation and movement of helix VI in the helix bundle. The potential hot spot (Ile-124 and Leu-127) was identified by inspecting the sequences of constitutively active helix III mutants (Fig. 2); it is confirmed by the constitutively activated phenotype of a receptor with combined point mutations that substitute polar residues (Asn and Gln, respectively) for these two hydrophobic residues in helix III (Table II). Receptors containing individual substitutions of either I124N or L127Q show weak constitutive activity (Table II). Other amino acid substitutions in helix III may contribute to an activated phenotype; Arg-37 displays a constitutively activated phenotype despite only a single mutation at the Ile-124 and Leu-127 positions. In the Baldwin model, side chains at these positions face helix VI (Figs. 6 and 8), suggesting that introduction of polar groups may activate the receptor switch by decreasing hydrophobic interactions and releasing helix VI from a constraint that keeps it close to helix III. In contrast to helix III, C5a receptors mutated in helix VI do not display an obvious consensus for mutations that confer an activated phenotype. However, a point mutation in helix VI, F251A, did generate a constitutively active receptor (Table II). This was unexpected because the Phe-251 was preserved in all of the full-length, functioning C5a receptors from the helix VI screen (Fig. 4). Recently, a random saturation study of helix VI of the m5 muscarinic receptor demonstrated that mutation of the phenylalanine at the cognate position to Phe-251 caused constitutive activity (58). In the Baldwin model, the phenylalanine side chain potentially points toward the activating positions in helix III, Ile-124 and Leu-127. Thus mutations that decrease the hydrophobicity of closely adjacent surfaces of either III (I124N, L127Q) or VI (F251A) activate the receptor, supporting the hypothesis that hydrophobic interactions between these surfaces promote the inactive state of the receptor.

The idea that receptor activation depends upon changes in the orientations of helices III and VI is supported by biochemical experiments based on fluorescence spectroscopy, chemical modification, and cross-linking the two helices (51, 59–63). An elegant study using site-directed spin labels in retinal rhodopsin showed that activation by light causes the cytoplasmic end

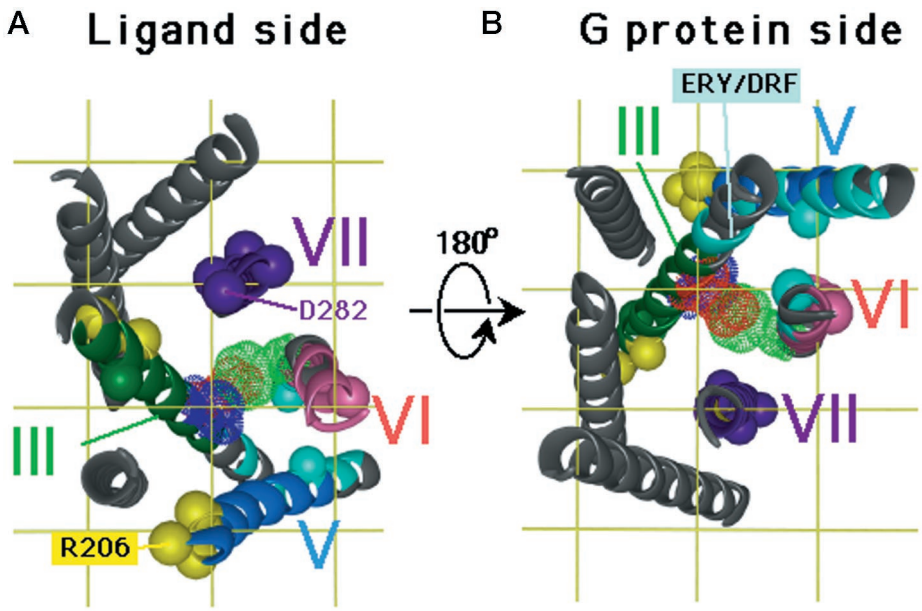

FIG. 8. **Positions of functionally important residues in the Baldwin model of serpentine receptors.** Positions at which side chain character is preserved are indicated by *spheres*. *A*, the ligand-binding pocket, as viewed from the extracellular fluid. *Yellow* indicates α-carbon positions of residues implicated in binding ligands for amine receptors and the retinal chromophore of rhodopsin. Thus the *yellow spheres* indicate α-carbon positions of residues preserved in functional mutant C5a receptors that coincide with ligand-binding residues in other receptors. *Green* or *purple spheres* indicate positions at which amino acid character is preserved (but which are not coincident with ligand-binding residues in amine receptors or rhodopsin). Together the spheres near the extracellular fluid constitute a probable ligand-binding pocket for C5a (see text). *Blue* and *orange* (van der Waals representation) indicate side chains of helix III at positions (Ile-124, Leu-127) subject to activating mutations; *light green* (van der Waals representation) indicates the side chain of Phe-251 in helix VI, where an alanine substitution caused constitutive activation (see Table II). The positions of these side chains are specified by their α-carbon positions in the Baldwin model (16) and rotamer side chain angles predicted by SCWRL program (73). *B*, putative G protein-interacting pocket, viewed from the cytoplasm. *Cyan* indicates α-carbon positions of residues (including the highly conserved ERY/DRF motif in helix III) that are implicated by biochemical and genetic evidence in other receptors as contact sites for G proteins. Many of these positions were not tested in the C5a receptor screen; partly for this reason, amino acid character is preserved at only two of the putative G protein-interacting positions (Tyr-222 and Leu-241, *cyan spheres* in helices V and VI, respectively). In *panels A* and *B*, the *yellow grids* (lines separated by 10 Å) indicate the proposed extracellular and cytoplasmic borders, respectively, of the lipid core of the membrane (16); the ribbon coloring scheme is the same as in Fig. 6 (see legend).

of helix VI to turn by 30° on its axis and to move away from helix III by ~12 Å (64). In addition, salt bridges (63) or disulfide links (64) connect these two helices in or near the cytoplasm block activation of the G protein.

Nine of the 21 constitutively activated C5a receptors are truncated in the extracellular half of helix VI (Fig. 4). These truncated receptors lack a significant fraction of the receptor polypeptide, including extracellular loop 3, helix VII, and the C-terminal cytoplasmic tail. Removal of DNA sequences 3′ to the stop codon did not alter the phenotypes of truncated C5a receptors, ruling out the possibility of suppressed stop codons.[2] To our knowledge, these mutants are the only serpentine receptors with fewer than seven transmembrane helices known to activate a G protein. Numerous studies describe truncated versions of serpentine receptors arising from alternative splicing; these include a GHRH receptor lacking helix VII, found in human pituitary adenomas (65); an opsin lacking helix VI, detected in human retina (66); and a thyrotropin receptor truncated in the middle of helix V (67). In general, most alternatively spliced receptors do not appear to be functional; however, a deletion of 14 amino acids in the cytoplasmic half of helix VII in an isoform of the calcitonin receptor alters ligand specificity and selectively abolishes coupling to phospholipase C (68). The apparent dispensability of the C-terminal tail of the C5a receptor in our experiments contrasts with evidence that the C-terminal tails of other receptors contact the G protein (perhaps the βγ subunit) (69, 70) and sometimes play key roles in determining receptor specificity for G protein (71). The contrast may be more apparent than real, however, because none of these functions was tested in our yeast selection procedure. Thus C-terminal tails of different receptors play different functions, but these probably are more or less dispensable in some receptors.

The truncated C5a receptors demonstrate that helix VII and the C-terminal tail of the receptor are not absolutely required for G protein activation. This conclusion is especially surprising because helix VII in our mutant screen tolerated the fewest average number of mutations per helix in functioning receptors (3.2%, Table I) and showed a high proportion of strongly preserved side chains (7 of 21 tested; Table I and Fig. 2). Moreover, helix VII mutations in functioning receptors do not truncate or cause constitutive activation. The dispensability of helix VII for activating the G protein can be reconciled with its resistance to mutations if we postulate that helix VII normally maintains the helix bundle in an inactive state. This interpretation is in keeping with the observation that mutations that interrupt a salt bridge between helices VII and III activate retinal rhodopsin (72). Helix VII probably plays a finely tuned structural role in the receptor, so that helix VII mutations that activate the receptor drastically reduce its stability (57); this might be anticipated from the Baldwin model, which places helix VII in close proximity to helices I, II, III, and VI.

In summary, we have performed a comprehensive genetic analysis of four of the seven helices in the C5a receptor. The results identify 25 residues, located in two clusters, that are critically required for C5a receptor function. One cluster includes residues, at or near the extracellular face of the receptor, that probably constitute a binding pocket for interaction with the C5a ligand; strikingly, this cluster shares a very similar "footprint" with residues in distantly related receptors that interact with rhodopsin and biogenic amines (Fig. 8*A*). The second cluster, at the core of the helix bundle, consists of

residues that are conserved in most serpentine receptors. Both clusters thus argue strongly for an activation mechanism that is conserved in all or most serpentine receptors. We propose that proper orientation of ligands —C5a, biogenic amines, and probably others —in the binding pocket (yellow residues in Fig. 6) induces a conformational change that is transmitted through the conserved core of the helix bundle to G proteins. At present we can only infer that locations of these critical residues indicate that they perform specific functions. To define the roles of these residues more precisely will require further biochemical and structural information.

Acknowledgments—We thank Fred E. Cohen, Shaun Coughlin, Mark von Zastrow, Ira Herskowitz, and members of our laboratories for helpful discussions and reading the manuscript, Drew Murphy and Lauren Silverman, from Cadus Pharmaceuticals, for the C5a ligand encoding plasmid, Ken Boakye for an *ADE2* disruption plasmid, and Helen Czerwonka for secretarial assistance.

REFERENCES

1. Clapham, D. E., and Neer, E. J. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37,** 167–203
2. Hamm, H. E. (1998) *J. Biol. Chem.* **273,** 669–672
3. Gether, U., and Kobilka, B. K. (1998) *J. Biol. Chem.* **273,** 17979–17982
4. Strader, C. D., Fong, T. M., Graziano, M. P., and Tota, M. R. (1995) *FASEB J.* **9,** 745–754
5. Wess, J. (1997) *FASEB J.* **11,** 346–354
6. Bourne, H. R. (1997) *Curr. Opin. Cell Biol.* **9,** 134–142
7. Erickson, J. R., Wu, J. J., Goddard, J. G., Tigyi, G., Kawanishi, K., Tomei, L. D., and Kiefer, M. C. (1998) *J. Biol. Chem.* **273,** 1506–1510
8. Price, L. A., Kajkowski, E. M., Hadcock, J. R., Ozenberger, B. A., and Pausch, M. H. (1995) *Mol. Cell. Biol.* **15,** 6188–6195
9. Price, L. A., Strnad, J., Pausch, M. H., and Hadcock, J. R. (1996) *Mol. Pharmacol.* **50,** 829–837
10. Cotecchia, S., Ostrowski, J., Kjelsberg, M. A., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267,** 1633–1639
11. Fong, T. M., Yu, H., and Strader, C. D. (1992) *J. Biol. Chem.* **267,** 25668–25671
12. Gether, U., Johansen, T. E., Snider, R. M., Lowe, J. D., Nakanishi, S., and Schwartz, T. W. (1993) *Nature* **362,** 345–348
13. Wong, S. K.-F., Parker, E. M., and Ross, E. M. (1990) *J. Biol. Chem.* **265,** 6219–6224
14. Unger, V. M., Hargrave, P. A., Baldwin, J. M., and Schertler, G. F. (1997) *Nature* **389,** 203–206
15. Baldwin, J. M., Schertler, G. F., and Unger, V. M. (1997) *J. Mol. Biol.* **272,** 144–164
16. Baldwin, J. M. (1994) *Curr. Opin. Cell Biol.* **6,** 180–190
17. Horn, F., Weare, J., Beukers, M. W., Horsch, S., Bairoch, A., Chen, W., Edvardsen, O., Campagne, F., and Vriend, G. (1998) *Nucleic Acids Res.* **26,** 275–279
18. van Rhee, A. M., and Jacobson, K. A. (1996) *Drug Dev. Res.* **37,** 1
19. Hill, D. E., Oliphant, A. R., and Struhl, K. (1987) *Methods Enzymol.* **155,** 558–568
20. Matteucci, M. D., and Heyneker, H. L. (1983) *Nucleic Acids Res.* **11,** 3113–3121
21. Wall, M. A., Coleman, D. E., Lee, E., Iñiguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* **83,** 1047–1058
22. Gerard, C., and Gerard, N. P. (1994) *Annu. Rev. Immunol.* **12,** 775–808
23. Zhang, X., Boyar, W., Toth, M. J., Wennogle, L., and Gonnella, N. C. (1997) *Proteins* **28,** 261–267
24. Herskowitz, I. (1995) *Cell* **80,** 187–197
25. Leberer, E., Thomas, D. Y., and Whiteway, M. (1997) *Curr. Opin. Genet. Dev.* **7,** 59–66
26. Wittenberg, C., and Reed, S. I. (1996) *Curr. Opin. Cell Biol.* **8,** 223–230
27. Stevenson, B. J., Rhodes, N., Errede, B., and Sprague, G. F., Jr. (1992) *Genes Dev.* **6,** 1293–1304
28. Dayhoff, M. O., Schwartz, R. M., and Orcutt, B. C. (1978) in *Atlas of Protein Sequence and Structure*. (Dayhoff, M. O., ed) Vol. 5, pp. 345–353, National Biomedical Research Foundation, Silver Spring, MD
29. Fauchere, J.-L., and Pliska, V. (1983) *Eur. J. Med. Chem.* **18,** 369–375
30. MacKenzie, K. R., Prestegard, J. H., and Engelman, D. M. (1997) *Science* **276,** 131–133
31. Chothia, C. (1984) *Annu. Rev. Biochem.* **53,** 537–572
32. Dill, K. A. (1990) *Biochemistry* **29,** 7133–7155
33. Fu, D., Ballesteros, J. A., Weinstein, H., Chen, J., and Javitch, J. A. (1996) *Biochemistry* **35,** 11278–11285
34. Javitch, J. A., Fu, D., Chen, J., and Karlin, A. (1995) *Neuron* **14,** 825–831
35. Javitch, J. A., Ballesteros, J. A., Weinstein, H., and Chen, J. (1998) *Biochemistry* **37,** 998–1006
36. Javitch, J. A., Fu, D., and Chen, J. (1995) *Biochemistry* **34,** 16433–16439
37. Wen, J., Chen, X., and Bowie, J. U. (1996) *Nat. Struct. Biol.* **3,** 141–148
38. Lichtarge, O., Bourne, H. R., and Cohen, F. E. (1996) *J. Mol. Biol.* **257,** 342–358
39. Lichtarge, O., Bourne, H. R., and Cohen, F. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7507–7511
40. 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
41. Chen, Z., Zhang, X., Gonnella, N. C., Pellas, T. C., Boyar, W. C., and Ni, F. (1998) *J. Biol. Chem.* **273,** 10411–10419
42. Gerard, C., Showell, H. J., Hoeprich, P. D., Jr., Hugli, T. E., and Stimler, N. P. (1985) *J. Biol. Chem.* **260,** 2613–2616
43. Mery, L., and Boulay, F. (1994) *J. Biol. Chem.* **269,** 3457–3463
44. Siciliano, S. J., Rollins, T. E., DeMartino, J., Konteatis, Z., Malkowitz, L., Van, R. G., Bondy, S., Rosen, H., and Springer, M. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1214–1218
45. Wess, J., Gdula, D., and Brann, M. R. (1991) *EMBO. J.* **10,** 3729–3734
46. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. F. (1994) *Annu. Rev. Biochem.* **63,** 101–132
47. Ho, B. Y., Karschin, A., Branchek, T., Davidson, N., and Lester, H. A. (1992) *FEBS. Lett.* **312,** 259–262
48. Nathans, J. (1990) *Biochemistry* **29,** 9746–9752
49. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 8309–8313
50. Zhukovsky, E. A., and Oprian, D. D. (1989) *Science* **246,** 928–930
51. Altenbach, C., Yang, K., Farrens, D. L., Khorana, H. G., and Hubbell, W. L. (1996) *Biochemistry* **35,** 12470–12478
52. Hill-Eubanks, D., Burstein, E. S., Spalding, T. A., Brauner-Osborne, H., and Brann, M. R. (1996) *J. Biol. Chem.* **271,** 3058–3065
53. Liu, J., Conklin, B. R., Blin, N., Yun, J., and Wess, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11642–11646
54. Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268,** 4625–4636
55. Paschke, R., and Ludgate, M. (1997) *N. Engl. J. Med.* **337,** 1675–1681
56. Scheer, A., and Cotecchia, S. (1997) *J. Recept. Signal Transduct. Res.* **17,** 57–73
57. Lin, Z., Shenker, A., and Pearlstein, R. (1997) *Protein Eng.* **10,** 501–510
58. Spalding, T. A., Burstein, E. S., Henderson, S. C., Ducote, K. R., and Brann, M. R. (1998) *J. Biol. Chem.* **273,** 21563–21568
59. Farahbakhsh, Z. T., Hideg, K., and Hubbell, W. L. (1993) *Science* **262,** 1416–1419
60. Farahbakhsh, Z. T., Ridge, K. D., Khorana, H. G., and Hubbell, W. L. (1995) *Biochemistry* **34,** 8812–8819
61. Gether, U., Lin, S., Ghanouni, P., Ballesteros, J. A., Weinstein, H., and Kobilka, B. K. (1997) *EMBO. J.* **16,** 6737–6747
62. Javitch, J. A., Fu, D., Liapakis, G., and Chen, J. (1997) *J. Biol. Chem.* **272,** 18546–18549
63. Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) *Nature* **383,** 347–350
64. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) *Science* **274,** 768–770
65. Hashimoto, K., Koga, M., Motomura, T., Kasayama, S., Kouhara, H., Ohnishi, T., Arita, N., Hayakawa, T., Sato, B., and Kishimoto, T. (1995) *J. Clin. Endocrinol. Metab.* **80,** 2933–2939
66. Jiang, M., Shen, D., Tao, L., Pandey, S., Heller, K., and Fong, H. K. (1995) *Exp. Eye Res.* **60,** 401–406
67. Silversides, D. W., Houde, A., Ethier, J. F., and Lussier, J. G. (1997) *J. Mol. Endocrinol.* **18,** 101–112
68. Shyu, J. F., Inoue, D., Baron, R., and Horne, W. C. (1996) *J. Biol. Chem.* **271,** 31127–31134
69. Phillips, W. J., and Cerione, R. A. (1994) *Biochem. J.* **299,** 351–357
70. Phillips, W. J., and Cerione, R. A. (1992) *J. Biol. Chem.* **267,** 17032–17039
71. Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., and Narumiya, S. (1993) *Nature* **365,** 166–170
72. Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., and Oprian, D. D. (1992) *Neuron* **9,** 719–725
73. Bower, M. J., Cohen, F. E., and Dunbrack, R. J. (1997) *J. Mol. Biol.* **267,** 1268–1282