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A Comprehensive Structure-Function Map of the Intracellular Surface of the Human C5a Receptor

*II. ELUCIDATION OF G PROTEIN SPECIFICITY DETERMINANTS**

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Marissa L. Matsumoto[‡], Kirk Narzinski[‡], Gregory V. Nikiforovich[§], and Thomas J. Baranski[‡1]

From the [‡]Departments of Medicine and Molecular Biology & Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110 and the [§]Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Within any given cell many G protein-coupled receptors are expressed in the presence of multiple G proteins, yet most receptors couple to a specific subset of G proteins to elicit their programmed response. Numerous studies demonstrate that the carboxyl-terminal five amino acids of the Gα subunits are a major determinant of specificity, however the receptor determinants of specificity are less clear. We have used a collection of 133 functional mutants of the C5a receptor obtained in a mutagenesis screen targeting the intracellular loops and the carboxyl terminus (Matsumoto, M. L., Narzinski, K., Kiser, P. D., Nikiforovich, G. V., and Baranski, T. J. (2007) *J. Biol. Chem.* 282, 3105–3121) to investigate how specificity is encoded. Each mutant, originally selected for its ability to signal through a nearly full-length $G\alpha_i$ in yeast, was tested to see whether it could activate three versions of chimeric Gα subunits consisting of Gpa1 fused to the carboxyl-terminal five amino acids of $G\alpha_i$, $G\alpha_q$, or $G\alpha_s$ in yeast. Surprisingly the carboxyl-terminal tail of the C5a receptor is the most important specificity determinant in that nearly all mutants in this region showed a gain in coupling to $G\alpha_q$ and/or $G\alpha_s$. More than half of the receptors mutated in the second intracellular loop also demonstrated broadened G protein coupling. Given a lack of selective advantage for this broadened signaling in the initial screen, we propose a model in which the carboxyl-terminal tail acts together with the intracellular loops to generate a specificity filter for receptor-G protein interactions that functions primarily to restrict access of incorrect G proteins to the receptor.

G protein-coupled receptors (GPCRs)[2] are seven transmembrane-spanning receptors that constitute one of the largest families of proteins, encoded by more than 1% of human genes (1). GPCRs are necessary for transmission of signals across cell membranes to orchestrate essential processes ranging from cell fusion in yeast to the ability to mobilize leukocytes to sites of infection in humans. The central role of GPCRs in nearly all physiologic processes is underscored by the fact that they are targets of ~30% of all available pharmaceutical drugs (1).

GPCRs are named for their ability to bind and activate heterotrimeric G proteins made up of a Gα, Gβ, and Gγ subunit. Activation occurs by transmitting a conformational change in the receptor, triggered by ligand binding, to the G protein. This catalyzes the release of GDP from Gα allowing GTP to bind and trigger dissociation of this subunit from Gβγ. Gα and Gβγ are then free to signal to their downstream targets to begin the signaling response. In humans there are 17 Gα, 5 Gβ, and 12 Gγ subunits (2). The Gα subunits are classified by the type of downstream effectors that they signal to and the responses they evoke and can divided into four families: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (3, 4). $G\alpha_s$ subunits stimulate adenylyl cyclase, whereas $G\alpha_i$ subunits inhibit adenylyl cyclase. $G\alpha_q$ subunits signal through phospholipase C, and $G\alpha_{12/13}$ activates various Rho guanine nucleotide exchange factors. Given that a single cell may express many different G proteins and GPCRs, the G proteins to which the receptors couple to must be tightly controlled in order for a GPCR to activate the correct downstream response. Each GPCR signals through only a subset of Gα subunits creating a specificity profile for the receptor. For example, the GPCR that we study, the complement-derived C5a receptor (C5aR), couples to $G\alpha_i$ and the promiscuous $G\alpha_{16}$ subunit of the $G\alpha_q$ family, but not $G\alpha_q$ itself, nor $G\alpha_s$. Despite the ability of many receptors to couple to the same G protein, there is little homology among their intracellular loops, making it difficult to predict which G protein(s) a receptor will signal through based upon primary sequence alone.

The nature of G protein specificity has been previously investigated using chimeric GPCRs created by swapping intracellular loop regions between receptors that couple to different Gα subunits. These experiments demonstrate that the second (5–7) and third (5, 8–13) intracellular loops (IC2 and IC3) generally are the most important in determining specificity. Exchanging the carboxyl terminus (CT) alone does not lead to a

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[1] To whom correspondence should be addressed: Depts. of Medicine and Molecular Biology & Pharmacology, Washington University School of Medicine, Campus Box 8127, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-747-3997; Fax: 314-362-7641; E-mail: baranski@wustl.edu.

[2] The abbreviations used are: GPCR, G protein-coupled receptor; 3AT, 3-aminotriazole; C5a, complement factor 5a; C5aR, complement factor 5a receptor; CT, carboxyl terminus; CT1, first half of the carboxyl terminus; CT2, second half of the carboxyl terminus; CT2stop323, truncated C5aR with a stop codon at position 323; Endo-H_f, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; IC1, intracellular loop 1; IC2, intracellular loop 2; IC3, intracellular loop 3; IP_3, inositol 1,4,5-triphosphate; r.m.s., root mean square deviation; RSM, random saturation mutagenesis; W5Cha, hexapeptide agonist of the C5a receptor.

change in the coupling profiles of most receptors (5, 12, 14, 15); however, when swapped in combination with IC2 and/or IC3, activity of the hybrids can be enhanced (13, 16–18). Individual loop regions in various receptors have also been targeted by mutagenesis to investigate specificity (19–24); however, to our knowledge, no comprehensive mutational study has been carried out on all intracellular loops of a single receptor.

Specific points of contact between the G protein and receptor have been identified, and these include the amino terminus (residues 8–23), the $\alpha4$–$\beta6$ loop (residues 311–328), and the last 11 amino acids (residues 340–450) of the Gα subunit (25–28). The receptor has also been demonstrated to interact with the carboxyl-terminal tail of the Gγ subunit (29–31). Although several points of contact on the receptor-G protein interface have been identified, only the last five amino acids of the Gα subunit have been shown to play an integral role in G protein specificity. Exchange of these extreme carboxyl-terminal residues confers G protein specificity in both mammalian cells and yeast (32, 33).

We have used a collection of 133 functional C5aR mutants obtained by random saturation mutagenesis (RSM) of each intracellular region (Matsumoto *et al.*, Ref. 67) to identify residues that interact with the last five amino acids of Gα to confer specificity. We found that all of the carboxyl-terminal mutants and more than half of the IC2 mutants demonstrated a gain in coupling to Gα_q and/or Gα_s chimeras. Based on the wide variety of mutations and truncations observed that allowed coupling to multiple chimeric Gα subunits, we propose that the carboxyl terminus and the intracellular loops create a specificity filter that acts to restrict access of incorrect G proteins to the receptor.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains BY1142 (Gα_{i3}), BY1173 (Gα_{i3}), BY1172 (Gα_q), BY1401 (Gα_s), and BY1404 (Gα_s) have been previously described (33, 34). Briefly, BY1142 has the genotype *MATα far1Δ1442 tbt1-1 fus1Δ::P_{FUS1}-HIS3 can1 ste14::trp1::LYS2 ste3Δ1156 gpa1* (41)-Gα_{i3} *lys2 ura3 leu2 trp1 his3 ade2*. BY1142 contains a fusion of the amino-terminal 41 amino acids of the yeast Gα protein, Gpa1, followed by residues 34–354 of the human Gα_{i3}. The BY1143 strain was created by transforming the BY1142 strain with a *URA3* plasmid encoding C5a (pBN444) as previously described (34, 35). BY1173 has the genotype *MATa ura3 leu2 trp1 his3 can1 gpa1Δ::ade2Δ::3XHA far1Δ::ura3Δ fus1Δ::P_{FUS1}-HIS3 LEU2::P_{FUS1}-lacZ sst2Δ::ura3Δ ste2Δ::G418R trp1::GPA1/*Gα_{i3}. BY1172 has the genotype *MATa ura3 leu2 trp1 his3 can1 gpa1Δ::ade2Δ::3XHA far1Δ::ura3Δ fus1Δ::P_{FUS1}-HIS3 LEU2::P_{FUS1}-lacZ sst2Δ::ura3Δ ste2Δ::G418R trp1::GPA1/*Gα_q. BY1401 has the genotype *MATa ura3 leu2 trp1 his3 can1 gpa1Δ::ade2Δ::3XHA far1Δ::ura3Δ fus1Δ::P_{FUS1}-HIS sst2Δ::ura Δtrp1::GPA1/*Gα_s. BY1404 has the genotype *MATa ura3 leu2 trp1 his3 can1 gpa1Δ::ade2Δ::3XHA far1Δ::ura3Δ fus1Δ::P_{FUS1}-HIS3 LEU2::P_{FUS1}-lacZ sst2Δ::ura3Δ ste2ΔG418R lys2Δ Δtrp1::GPA1/*Gα_s. BY1173, BY1172, BY1401, and BY1404 contain a fusion of amino acids 1–467 of the yeast Gα protein, Gpa1, followed by the last 5 amino acids of human Gα_{i3}, Gα_q, Gα_s, and Gα_s, respectively. These strains were transformed with either a *URA3* plasmid expressing C5a (pBN444) or an empty *URA3* vector (pBN443) and an *ADE2* plasmid expressing the C5aR that were previously described (34, 35). Activation of the C5aR expressed from a plasmid in all strains used leads to signaling through the mitogen-activated protein kinase cascade and expression of the P_{FUS1}-*HIS3* reporter gene, allowing the yeast to grow in the absence of histidine. In addition, BY1173, BY1172, and BY1404 contain a P_{FUS1}-β-galactosidase reporter gene. The CT2stop323 truncation was generated by mutating codon 323 of the C5aR to a stop codon using Pfu turbo mutagenesis (Stratagene). The *RGS4* plasmid was a gift from Dr. Maurine Linder. Mutants M1–M4 were made by designing complementary oligonucleotides encoding the desired mutation(s), and a two-step PCR strategy was used to introduce the mutation(s) into the wild-type C5aR coding sequence in a pcDNA3.1(+) (Invitrogen) mammalian expression vector. All mutations were verified by sequencing at the Washington University Protein and Nucleic Acid Chemistry Laboratory.

Yeast Transformation and Receptor Signaling Assays—Yeast transformations were done according to standard lithium acetate protocols. Relative signaling abilities of mutant receptors were assayed by restreaking three transformants of each mutant onto histidine-deficient medium containing varying amounts of 3-amino-1,2,4-triazole (3AT) (Sigma) (0, 1, 5, 10, 20, and 50 mM). Signaling levels were compared with wild-type C5aR expressed from an *ADE2* plasmid, pBN482, and a nonfunctional mutant C5aR containing a stop codon in transmembrane helix 3, pBN483, which were previously described (34, 36). Growth in the absence of histidine was inferred to be dependent on C5aR signaling based on colony color (red colonies lack the C5aR *ADE2* plasmid). β-Galactosidase assays were done by treating BY1173, BY1172, and BY1404 transformed with pBN482 with a range of the C5aR hexapeptide agonist W5Cha (GenScript) from 10^{-10} M to 10^{-5} M, and the assay was carried out as previously described (35).

Endo-β-N-acetylglucosaminidase Treatment and Western Blots—RSM receptors were subcloned into a pIRES vector (Clontech) with Gα_q (University of Missouri-Rolla cDNA Resource Center) and transiently transfected into HEK293 cells by standard calcium phosphate methods. Cells were lysed 2 days after transfection in 250 μl of a 1× sample buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol) supplemented with 2% β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 500 μM phenylmethylsulfonyl fluoride by shearing through a 27G½ syringe. Lysates were heated for 5 min at 50 °C. 27 μl of each lysate was treated with 1000 units of endo-β-*N*-acetylglucosaminidase H-maltose-binding protein fusion (Endo-H_β, New England Biolabs) at 37 °C for 3 h. Samples were heated for 5 min at 50 °C, resolved on a 12% SDS-PAGE gel, transferred to polyvinylidene difluoride, and immunoblotted with a rabbit polyclonal anti-C5aR antibody raised against residues 9–29 of the amino terminus. Western blots of yeast lysates were done as previously described.[3]

Inositol 1,4,5-Triphosphate Accumulation—For Gα_q signaling, the RSM receptors with Gα_q in the pIRES vector were transiently transfected into HEK293 cells by standard calcium

[3] M. L. Matsumoto, K. Narzinski, P. D. Kiser, G. V. Nikiforovich, and T. J. Baranski, unpublished observation.

phosphate methods. The m1 muscarinic acetylcholine receptor (University of Missouri-Rolla cDNA Resource Center) was used as a positive control. For $G\alpha_{16}$ signaling, RSM receptors and $G\alpha_{16}$ were subcloned into pcDNA3.1(+) (Invitrogen) and transiently co-transfected into HEK293 cells. IP_3 levels were measured as previously described (35) where cells expressing the C5aR were treated with 1 μM W5Cha (GenScript) and cells expressing the m1 muscarinic acetylcholine receptor were treated with 10^{-4} M carbachol (Sigma).

Molecular Modeling—Molecular modeling procedures for restoring low energy backbone conformations of the intracellular loops in the mutant C5a receptors were exactly as those described earlier for the WT, including employment of the ECEPP/2 force field, and mounting the loops on the three-dimensional structure of the transmembrane region of C5aR (67). All possible low energy conformations for IC1 (fragment 63–71), IC2-(138–150), IC3-(224–236), and the C-terminal fragment 300–310, as well as for the "package" of IC1+IC2+IC3+fragment 300–310 were determined by subsequent application of geometrical sampling followed by energy minimization (67). Final selection of low energy conformations for the package was based on the energy cut-off of 30 kcal/mol and yielded 56 low energy structures for WT (see Matsumoto *et al.*, Ref. 67), 22 for R35, 27 for R91, 33 for R89, 35 for R40, 28 for R58, 44 for R111, 50 for R52, 45 for M1, 47 for M2, 46 for M3, and 28 for M4. The obtained low energy structures for different mutant receptors were compared with each other by overlapping spatial positions of residues comprising the stems of transmembrane helices (residues 135–138 and 150–153 for IC2) and calculating the so-called "global" r.m.s. values for all heavy backbone atoms of the IC2 loop (residues 139–149).

RESULTS

Determining G Protein Coupling of Intracellular Loop Mutants—The 133 functional intracellular loop C5aR mutants were originally selected by their ability to signal in the yeast strain BY1143, which contains a chimera of residues 1–41 of the yeast Gα, Gpa1, followed by residues 34–354 of human $G\alpha_{i3}$ (67). All of these mutants were then tested for signaling in three different strains carrying chimeras of amino acids 1–467 of Gpa1 followed by the last five amino acids of human $G\alpha_{i3}$ (strain BY1173), $G\alpha_q$ (BY1172), or $G\alpha_s$ (BY1401) (33) to elucidate receptor determinants of specificity (Fig. 1*a*). The ability to express a single G protein chimera of interest in the presence of a single human GPCR provides a powerful tool for studying G protein-coupling specificity. In the isolated environment of the yeast cell, a single human GPCR can be studied in the absence of other receptors competing for G protein binding, and the readout of receptor activation is clear, because only a single G protein chimera is expressed. All yeast strains used in this study have been engineered so that receptor signaling leads to activation of the yeast-mating pathway resulting in expression of a P_{FUS1}-*HIS3* or P_{FUS1}-β-galactosidase reporter gene (33). Thus, if a receptor activates the particular G protein expressed, it confers the ability of the yeast to grow on histidine-deficient medium or express the β-galactosidase enzyme. To quantify the relative signaling strength of mutant receptors a β-galactosidase assay can be performed or the yeast can be

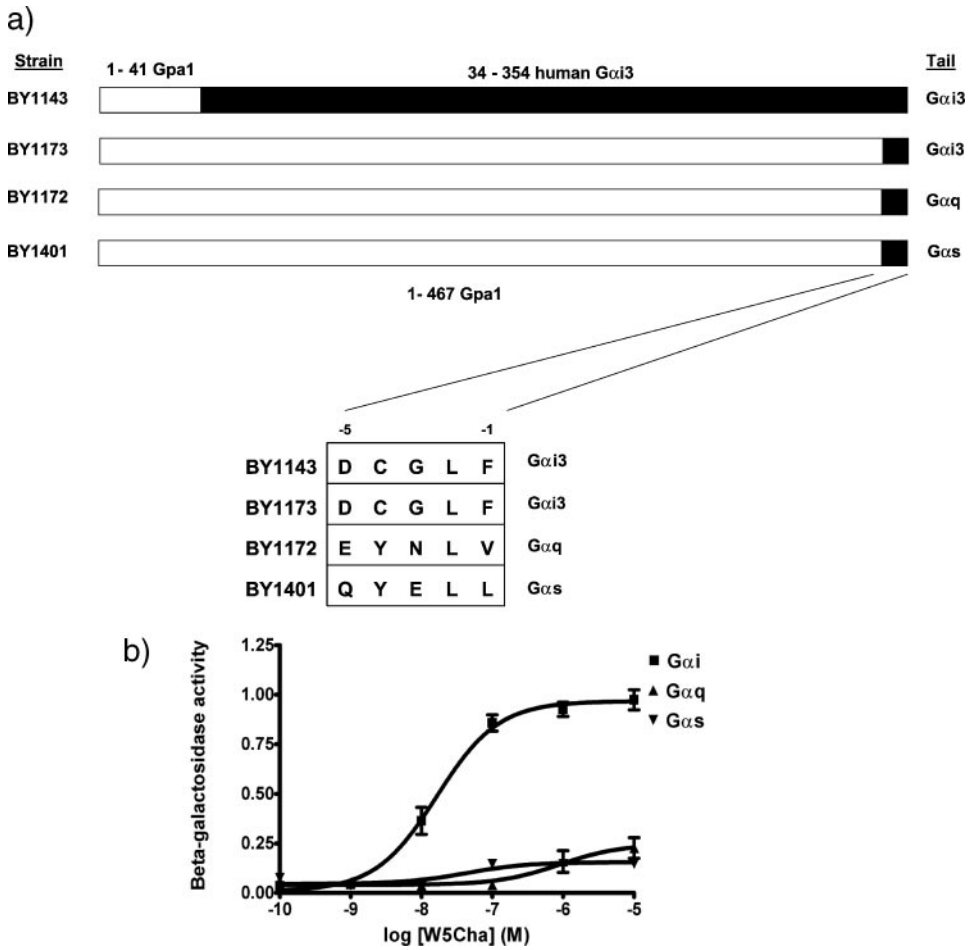

FIGURE 1. **Schematics of the C5a receptor and Gα subunits.** *a*, a schematic of the Gα subunits used in the yeast strains BY1143, BY1173, BY1172, and BY1401 are shown from the amino terminus to the carboxyl terminus, from *left* to *right*. Yeast Gpa1 residues are represented by an *open bar*, and human $G\alpha_{i3}$, $G\alpha_q$, or $G\alpha_s$ residues are *shaded black*. The Gα of BY1143 contains residues 1–41 of yeast Gpa1 followed by residues 34–354 of human $G\alpha_{i3}$. The Gα of BY1173 contains residues 1–467 of yeast Gpa1 followed by the last five residues of human $G\alpha_{i3}$. The Gα of BY1172 contains residues 1–467 of yeast Gpa1 followed by the last five residues of human $G\alpha_q$. The Gα of BY1401 contains residues 1–467 of yeast Gpa1 followed by the last five residues of human $G\alpha_s$. An alignment of the last five residues of the chimeras is shown. *b*, yeast strains BY1173, BY1172, and BY1404 expressing the chimeric Gα subunits consisting of residues 1–467 of Gpa1 and the last five amino acids of human $G\alpha_{i3}$, $G\alpha_q$, or $G\alpha_s$, respectively, were transformed with the wild-type C5aR. Cells were treated with increasing amounts of the C5aR hexapeptide agonist W5Cha, and signaling was assayed by induction of a β-galactosidase reporter gene. The mean of each experiment from three independent transformants is shown, ± S.D.

	57			60					65					70		72	Gi	Gq	Gs
IC1	**L**	**V**	**V**	**W**	**V**	**T**	**A**	**F**	**E**	**A**	**K**	**R**	**T**	**I**	**N**	**A**	50	0	0
R3	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	50	20	0
R7	.	.	.	.	A	S	.	L	.	G	.	.	K	V	S	.	50	20	0
R9	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	50	20	0
R11	W	.	.	.	.	N	.	.	.	.	.	K	.	.	.	.	50	20	0
R25	F	.	.	C	.	.	.	L	.	.	.	T	.	.	.	.	50	20	0
R28	.	.	.	L	.	S	Y	.	.	K	.	.	K	.	.	.	50	20	0
R17	.	.	.	.	D	.	.	L	D	.	.	.	.	.	.	.	50	10	0
R24	.	.	.	.	D	.	.	Y	.	V	.	.	N	.	.	.	50	10	0
R14	.	.	A	L	D	.	.	.	A	P	R	.	P	V	.	.	50	5	0
R1	S	.	G	.	.	.	.	.	.	K	P	.	P	V	.	.	50	0	0
R2	.	.	.	R	I	.	.	.	.	V	.	I	.	.	.	.	50	0	0
R4	.	A	.	L	.	S	.	.	.	V	.	.	A	.	.	.	50	0	0
R5	.	.	.	.	D	.	.	S	.	V	.	G	.	.	.	.	50	0	0
R6	F	.	.	L	D	.	V	.	.	T	.	.	.	.	Y	.	50	0	0
R10	F	.	.	R	.	.	R	.	.	.	.	K	.	.	.	.	50	0	0
R12	.	.	G	.	.	.	V	I	.	.	.	K	I	V	T	S	50	0	0
R13	.	.	.	R	.	.	.	W	.	V	N	S	.	.	.	.	50	0	0
R15	F	.	F	C	.	.	.	V	.	.	R	I	.	.	.	.	50	0	0
R16	.	.	L	.	.	.	.	V	Q	N	.	.	.	.	.	S	50	0	0
R19	.	.	.	.	D	.	.	C	.	G	.	.	.	.	.	.	50	0	0
R20	.	.	A	.	.	.	.	L	.	R	.	.	.	.	.	.	50	0	0
R21	.	.	.	L	.	.	.	.	.	Q	I	.	S	.	.	.	50	0	0
R22	.	.	.	.	D	.	T	.	.	.	.	.	.	.	.	.	50	0	0
R23	.	.	.	L	.	.	.	C	G	V	.	.	.	.	T	S	50	0	0
R26	.	.	I	.	.	S	.	.	.	.	.	K	.	.	.	.	50	0	0
R27	.	.	I	.	N	N	.	.	.	G	.	.	N	.	.	.	50	0	0
R29	.	A	F	.	.	S	.	.	K	P	.	.	.	.	T	S	50	0	0
R30	.	.	F	.	R	.	.	C	D	.	R	.	N	.	H	.	50	0	0

FIGURE 2. **Signaling of IC1 mutants via $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ chimeras.** The wild-type sequence of the region targeted for mutagenesis in IC1 is given (*top* in *bold*) with the residue numbers marked. The amino acid sequences of the functional mutant receptors obtained (designated *R* and *numbered*, *left*) are indicated (*dots*, unchanged amino acid compared with wild-type). Receptors are sorted according to their ability to signal through the various $G\alpha$ subunits. $G\alpha_i$ denotes strain BY1173, $G\alpha_q$ denotes strain BY1172, and $G\alpha_s$ denotes strain BY1401. Signaling strength is scored as the ability to grow on histidine-deficient medium in the presence of up to 1, 5, 10, 20, or 50 mM 3AT. The maximal amount of 3AT that supports growth is indicated for each receptor. A score of 0 indicates no growth in the presence of 1 mM 3AT.

grown in the presence of increasing amounts of 3AT, a competitive inhibitor of His3.

The wild-type C5aR receptor was tested for signaling in the BY1173 ($G\alpha_{i3}$), BY1172 ($G\alpha_q$), and BY1404 ($G\alpha_s$) strains by β-galactosidase assay (Fig. 1*b*). The wild-type C5aR demonstrated dose-dependent β-galactosidase activity when treated with increasing amounts of the C5aR hexapeptide agonist W5Cha in the presence of the $G\alpha_i$ chimera but not in the presence of the $G\alpha_q$ or $G\alpha_s$ chimeras. This specificity profile was confirmed by the growth assay. BY1173 ($G\alpha_{i3}$), BY1172 ($G\alpha_q$), and BY1401 ($G\alpha_s$) transformants of wild-type C5aR in the presence of the C5a ligand were assayed for signaling by testing their ability to grow on histidine-deficient medium in the presence of 0, 1, 5, 10, 20, and 50 mM 3AT. The wild-type receptor demonstrated signaling in the presence of up to 50 mM 3AT with the $G\alpha_i$ chimera but showed no signaling with the $G\alpha_q$ or $G\alpha_s$ chimeras (Figs. 2–6). Thus, the chimeric $G\alpha$ subunits demonstrate the natural specificity profile of the C5aR in mammalian cells. Each of the 133 receptors in our functional mutant collection were tested for the ability to signal through the chimeric $G\alpha$ subunits in the presence of the C5a ligand in strains BY1173 ($G\alpha_{i3}$), BY1172 ($G\alpha_q$), and BY1401 ($G\alpha_s$) using the growth assay. All 133 mutants that were originally selected for signaling in BY1143 signaled as well as the wild-type C5aR in the BY1173 strain (Figs. 2–6). Thus, there was no difference in the ability of the mutants to couple to either a more human $G\alpha_{i3}$-like (BY1143) or a more Gpa1-like $G\alpha$ subunit (BY1173).

Twenty-eight intracellular loop 1 (IC1) mutants were tested, and only nine of these coupled to the $G\alpha_q$ chimera, whereas none of the mutants gained the ability to couple to the $G\alpha_s$ chimera (Fig. 2). Of note, the IC1 mutants that show signaling through the $G\alpha_q$ chimera do not signal as strongly as many of the second intracellular loop (IC2) or CT mutants that showed a gain in coupling to the $G\alpha_q$ chimera (see below). Comparison of the sequences of mutant receptors that are able to couple to the $G\alpha_q$ chimera does not reveal any trend in amino acid substitutions associated with a gain in coupling.

Of the 30 IC2 mutant receptors tested, 20 were able to signal through $G\alpha_q$, and three of these were also able to signal through $G\alpha_s$ (Fig. 3). Analysis of the sequences of mutant IC2 receptors that show broadened coupling reveals that a single point mutation, Q145R, is sufficient to allow signaling through $G\alpha_q$ in yeast (Fig. 3). Comparison of IC2 mutant sequences that couple to $G\alpha_q$ show that nine of these receptors contain the Q145R mutation, whereas this mutation is not observed in any of the receptors that couple exclusively to $G\alpha_i$. Interestingly, a positive charge at this position is not sufficient to allow $G\alpha_q$ coupling, because several receptors that cannot signal through $G\alpha_q$, such as R40, R56, and R82, contain a lysine or a histidine at this same position.

The G protein specificity profiles of the IC3 mutants were very similar to that of IC1. Only 7 of the 18 receptors were able to activate $G\alpha_q$, and none were able to activate $G\alpha_s$ (Fig. 4). In addition, most of the mutants that gained coupling to the $G\alpha_q$ chimera did not signal as strongly as many of the IC2 or CT mutants (see below). As with IC1 there was no trend in the types of amino acid substitutions observed to explain why a receptor can or cannot signal through $G\alpha_q$.

The results from the carboxyl terminus are quite different from those obtained for the rest of the receptor. All mutants in the first half of the carboxyl terminus (CT1) were able to signal strongly via $G\alpha_q$ (Fig. 5). This includes truncated receptors with as many as 40 amino acids missing from the carboxyl-terminal tail. In addition, two truncated CT1 mutants could also signal via $G\alpha_s$, indicating that the CT is not essential for activation of either the $G\alpha_i$, $G\alpha_q$, or $G\alpha_s$ chimeras. All 30 of the mutants in the second half of the

	133		135					140					145				149	Gi	Gq	Gs
IC2	**D**	**R**	**F**	**L**	**L**	**V**	**F**	**K**	**P**	**I**	**W**	**C**	**Q**	**N**	**F**	**R**	**G**	50	0	0
R89	.	.	.	.	.	.	.	.	.	.	.	Y	R	I	M	.	.	50	50	50
R104	.	.	C	.	.	.	.	.	.	.	.	.	R	.	G	H	R	50	50	50
R105	.	.	.	.	.	.	I	I	.	F	.	.	R	.	.	.	A	50	50	50
R32	.	.	L	.	.	.	Y	.	.	.	.	.	L	.	I	.	.	50	50	0
R35	.	.	.	.	.	.	.	Q	.	.	.	.	R	.	.	.	.	50	50	0
R52	.	.	.	.	.	I	.	I	.	.	R	Y	R	.	.	.	.	50	50	0
R54	.	.	.	.	.	.	.	R	.	.	.	Y	K	.	.	.	.	50	50	0
R58	.	.	.	.	.	.	.	.	.	.	S	Y	H	.	.	.	.	50	50	0
R74	.	.	A	.	Q	.	.	.	.	.	.	.	R	K	.	H	R	50	50	0
R78	.	.	.	.	.	.	L	.	.	.	.	.	R	.	.	.	.	50	50	0
R81	.	.	.	.	.	I	L	.	.	.	.	.	R	.	V	.	R	50	50	0
R91	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	50	50	0
R111	.	.	.	.	I	.	.	.	.	F	R	Y	.	.	.	.	.	50	50	0
R34	.	.	C	.	.	.	.	.	.	M	A	Y	V	.	L	.	.	50	20	0
R39	.	.	C	I	.	.	M	.	.	.	.	.	.	K	.	.	V	50	20	0
R53	.	.	.	.	.	.	.	.	.	.	.	.	.	K	S	.	.	50	1	0
R72	.	.	.	.	.	.	.	.	.	.	.	.	K	.	Y	.	.	50	1	0
R77	E	.	C	.	R	.	.	.	.	.	.	.	H	.	Y	.	.	50	1	0
R83	.	.	.	.	.	.	.	.	.	.	.	G	N	M	E	H	R	50	1	0
R114	.	.	.	.	.	.	.	.	.	.	.	W	K	.	L	C	.	50	1	0
R31	.	.	.	.	R	.	I	.	.	.	.	Y	.	K	I	.	R	50	0	0
R40	.	.	C	.	.	I	L	.	.	.	C	Y	.	.	V	.	.	50	0	0
R56	.	.	.	.	.	I	C	R	A	.	.	Y	K	K	.	.	R	50	0	0
R59	.	.	.	.	Q	.	.	.	.	.	.	.	L	.	.	.	.	50	0	0
R82	.	.	.	.	Q	.	I	.	.	.	.	.	H	.	.	.	V	50	0	0
R85	.	.	.	.	.	.	V	.	.	.	.	.	.	.	H	H	.	50	0	0
R86	E	.	.	.	R	.	.	Q	.	.	.	F	.	.	K	.	V	50	0	0
R102	.	.	.	.	.	.	T	.	.	.	.	.	H	.	Y	.	.	50	0	0
R107	.	.	.	.	.	.	C	R	.	.	.	.	H	.	.	.	.	50	0	0
R108	E	.	.	.	.	.	.	.	.	.	.	.	L	.	.	L	.	50	0	0

FIGURE 3. **Signaling of IC2 mutants via $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ chimeras.** The wild-type sequence of the region targeted for mutagenesis in IC2 is given (*top* in *bold*) with the residue numbers marked. The amino acid sequences of the functional mutant receptors obtained (designated *R* and *numbered*, *left*) are indicated (*dots*, unchanged amino acid compared with wild-type) and annotated as in Fig. 2.

	229						235					240					245					249	Gi	Gq	Gs
IC3	**T**	**W**	**S**	**R**	**R**	**A**	**T**	**R**	**S**	**T**	**K**	**T**	**L**	**K**	**V**	**V**	**V**	**A**	**V**	**V**	**A**	50	0	0
R36	I	R	.	S	T	R	I	.	.	.	.	.	R	.	.	G	.	.	.	.	.	50	50	0
R2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	S	50	20	0
R14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	50	20	0
R17	.	.	.	.	K	.	.	.	.	S	.	.	W	.	.	S	.	.	.	.	.	50	20	0
R42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	50	20	0
R46	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	A	.	.	.	.	.	50	20	0
R47	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	F	.	.	50	20	0
R4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	.	50	0	0
R6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	F	T	A	.	.	50	0	0
R21	.	.	C	.	.	.	.	.	.	I	.	.	.	Q	.	S	.	.	.	.	.	50	0	0
R25	.	.	.	.	.	.	.	.	.	.	.	A	F	.	.	S	F	.	I	.	.	50	0	0
R29	.	.	R	.	.	.	.	.	.	V	.	A	.	.	.	.	.	G	.	.	.	50	0	0
R34	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	50	0	0
R37	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	S	50	0	0
R38	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	50	0	0
R39	.	.	T	.	.	.	.	.	.	.	.	.	.	.	F	S	.	.	F	.	.	50	0	0
R40	.	.	.	.	.	.	.	.	.	P	.	.	F	.	F	.	.	.	F	.	.	50	0	0
R45	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	A	.	T	.	.	.	50	0	0

FIGURE 4. **Signaling of IC3 mutants via $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ chimeras.** The wild-type sequence of the region targeted for mutagenesis in IC3 is given (*top* in *bold*) with the residue numbers marked. The amino acid sequences of the functional mutant receptors obtained (designated *R* and *numbered*, *left*) are indicated (*dots*, unchanged amino acid compared with wild-type) and annotated as in Fig. 2.

carboxyl terminus (CT2) signaled strongly through $G\alpha_q$, and all except four mutants also signaled strongly through $G\alpha_s$ (Fig. 6). This also included many truncated receptors like in CT1. Comparison of CT1 or CT2 mutant sequences reveals that substitutions at every position from residue 305 to 349 are tolerated and that these mutations result in coupling to the chimeric $G\alpha_q$ or $G\alpha_s$ subunits. Not only does mutation broaden the G protein coupling ability of the receptor but so does truncation. In addition, there was no trend in the types of mutations observed at any one position. Thus, inserting nearly any combination of mutations in the CT seems to change the $G\alpha$ subunit to which the receptor can couple.

Based upon the number of mutant receptors in each region that show a gain in coupling and the number of different $G\alpha$ subunits to which they broaden their specificity, we can rank the relative importance of the intracellular loop regions in the specificity of interaction with the $G\alpha$ tail. CT2 makes the largest contribution, followed by CT1, IC2, and lastly IC1 and IC3.

Truncation of the Carboxyl Terminus—Analysis of the mutants obtained in the RSM screen show that a stop codon in the carboxyl terminus can be tolerated as amino-terminal as residue 311 (Fig. 5). This was surprising, because in mammalian cells truncation of the C5aR at codon 305 leads to a non-functional receptor by preventing cell surface expression (37). Even more unexpected was the broadened G protein specificity of the truncations (Figs. 5 and 6). To test whether truncation alone permits signaling and specificity broadening, a premature stop codon was introduced into the context of the wild-type receptor at position 323, which is at the beginning of CT2. The CT2stop323 truncation was tested for signaling in the BY1142 strain by the growth assay (Table 1). The CT2stop323 truncation actually signaled better than wild-type (growth on 50 mM and 5 mM 3AT, respectively) in a ligand-dependent manner, despite being expressed at lower levels (Fig. 7). This indicates that the carboxyl terminus of the C5aR is not required for signaling in the yeast system.

To determine whether the specificity of G protein coupling was altered in the CT2stop323 truncation, we expressed the receptor in BY1173 ($G\alpha_{i3}$), BY1172 ($G\alpha_q$), and BY1401 ($G\alpha_s$) (Table 1). Although the wild-type receptor coupled only to the $G\alpha_i$ chimera, the CT2 truncation gained the ability to couple to the $G\alpha_q$ but not the $G\alpha_s$ chimera. This indicates that, although the carboxyl-terminal tail of the receptor is not required for signaling, it plays an important role in determining the specificity of interaction with the $G\alpha$ carboxyl terminus.

		305					310					315			318	Gi	Gq	Gs
	CT1	Q	G	F	Q	G	R	L	R	K	S	L	P	S	L	50	0	0
Full-Length Receptors	R2	.	.	.	.	.	.	.	.	M	.	.	.	P	W	50	50	0
	R8	R	.	I	.	.	S	.	.	.	F	.	.	P	.	50	50	0
	R10	.	.	.	.	.	.	.	.	.	.	.	.	T	W	50	50	0
	R13	.	.	.	L	R	.	.	.	.	.	.	.	P	.	50	50	0
	R37	P	.	.	K	D	.	.	.	.	A	M	.	.	.	50	50	0
	R40	P	.	.	L	P	.	.	.	.	.	.	.	.	.	50	50	0
	R44	P	S	.	.	.	.	.	K	.	L	F	.	.	W	50	50	0
	R45	.	.	.	.	E	.	.	.	.	T	.	R	.	.	50	50	0
	R26	P	.	.	.	V	.	.	.	Q	Y	V	.	.	.	50	20	0
	R28	P	.	N	L	.	.	.	S	.	L	.	.	N	F	50	20	0
	R43	.	Y	G	.	R	.	W	.	.	C	.	.	.	W	50	20	0
	R9	P	.	.	.	.	.	.	.	Q	.	V	R	Y	.	50	10	0
	R22	P	.	.	.	.	.	.	.	.	.	.	Q	.	M	50	10	0
	R31	P	.	.	.	S	.	M	.	.	A	Y	H	.	V	50	10	0
		305					310					315			318	Gi	Gq	Gs
	WT	Q	G	F	Q	G	R	L	R	K	S	L	P	S	L	50	0	0
Truncated Receptors	R27	R	.	.	.	N	.	.	.	.	.	.	Q	@		50	50	50
	R29	.	.	.	H	R	.	.	.	.	.	.	.	.	@	50	50	50
	R15	.	.	.	.	.	.	.	K	.	L	F	Q	T	@	50	50	0
	R18	.	.	.	.	.	I	.	.	.	.	.	A	.	@	50	50	0
	R19	.	.	R	.	.	I	.	.	.	.	F	@			50	50	0
	R24	.	.	S	L	A	.	.	.	.	.	V	.	.	@	50	50	0
	R25	P	.	L	R	S	.	.	K	N	.	.	L	.	@	50	50	0
	R32	R	.	Y	H	.	.	.	.	E	.	@				50	50	0
	R33	P	.	L	K	R	.	V	G	@						50	50	0
	R35	.	R	.	.	R	K	@								50	50	0
	R36	P	.	.	H	.	.	.	.	.	.	.	Q	F	@	50	50	0
	R38	.	.	S	H	.	.	.	.	.	F	.	.	.	@	50	50	0
	R39	.	.	P	L	A	.	.	.	E	F	.	.	.	@	50	50	0

FIGURE 5. **Signaling of CT1 mutants via $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ chimeras.** The wild-type sequence of the region targeted for mutagenesis in CT1 is given (*top* in *bold*) with the residue numbers marked. The amino acid sequences of the functional mutant receptors obtained (designated *R* and *numbered*, *left*) are indicated (*dots*, unchanged amino acid compared with wild-type) and annotated as in Fig. 2. *@*, stop codon.

Signaling of the C5aR in yeast leads to cell growth through the action of free $G\beta\gamma$ that has been released from $G\alpha$ upon GTP binding (38). Thus, a mutant receptor may permit cell growth by binding and sequestering $G\alpha$ without actually catalyzing GTP exchange, leaving $G\beta\gamma$ free to signal. To evaluate this possibility, we expressed the CT2stop323 truncation in the BY1142 strain in the presence and absence of the mammalian GTPase-activating protein, RGS4 (Table 1). Signaling of both the wild-type C5aR and the CT2stop323 truncation was sensitive to RGS4 expression indicating that these receptors likely catalyze $G\alpha$-GDP turnover.

Signaling of Mutants in Cell Culture—To determine whether mutants that show broadened G protein coupling with the chimeric $G\alpha$ subunits can also couple to wild-type full-length human G proteins, selected carboxyl-terminal mutants were tested in mammalian cells. Various CT1 (R13, R40, and R45) and CT2 (R12, R19, R34, R42, R63, and the CT2stop323 truncation) mutants, both full-length and truncated, were transiently transfected into HEK293 cells, and lysates were subjected to Endo-H_f treatment. Endo-H_f cannot cleave complex *N*-linked oligosaccharides that are formed in the Golgi but can remove the high mannose sugars added in the ER. An improperly folded receptor will be retained in the ER by the quality control system of the cell and will be Endo-H_f-sensitive. Therefore, Endo-H_f resistance indicates that the receptor exited the ER, passed through the Golgi and likely made it to the cell surface. All CT1 and CT2 mutants tested were Endo-H_f-sensitive, indicating failure to exit the ER.[3] Expression of the mutated receptors in COS7 cells failed to rescue the ER retention of the receptors.[3] The improper trafficking of the truncated C5aRs confirms the role of the carboxyl-terminal tail of the C5aR in receptor folding and exit from the ER as previously suggested (37). This is consistent with other studies that have implicated GPCR carboxyl-terminal tails in folding and receptor trafficking (39–46).

We next expressed IC2 RSM receptors R35, R52, R58, R89, R91, and R111 in HEK293 cells. To test whether these mutants exit the ER, lysates of transiently transfected cells were tested for Endo-H_f resistance (Fig. 8*a*). All six of the IC2 mutants were able to exit the ER, and the expression level and the amount of receptor-containing complex *N*-linked oligosaccharides were comparable to the wild-type receptor. All six of the IC2 mutants were also able to signal through the promiscuous $G\alpha_{16}$ subunit at levels comparable to the wild-type C5aR, indicating proper folding and cell surface expression (Fig. 8*b*). These mutants were then tested for a gain in coupling to $G\alpha_q$. All mutants demonstrated low basal activity, but only R89 showed robust IP_3 accumulation upon ligand stimulation when co-expressed with $G\alpha_q$ (Fig. 8*c*). Mutant R89 demonstrated a 6-fold increase in IP_3 levels when stimulated with 1 μM of the C5aR small molecule agonist W5Cha. This is similar to the m1 muscarinic acetylcholine receptor, a true $G\alpha_q$-coupled receptor, which showed a 7.5-fold increase in IP_3 levels when stimulated with 100 μM carbachol.[3]

R89 contains only four amino acid substitutions: C144Y, Q145R, N146I, and F147M. The lack of signaling of R91 indicates that the Q145R point mutation alone is not sufficient to confer $G\alpha_q$ signaling in mammalian cells. The discrepancy between $G\alpha_q$ signaling in mammalian cells and yeast likely reflects the differences in the heterotrimeric G protein and/or the relative sensitivities of the signaling pathways. The yeast system contains the Gpa1-$G\alpha_q$ chimera containing only the last five amino acids of $G\alpha_q$, whereas the mammalian assays were done in the presence of full-length human $G\alpha_q$. In addition, the yeast system contains the yeast $G\beta\gamma$ complex of Ste4/Ste18, whereas the HEK293 cells contain mammalian $G\beta\gamma$ complexes.

To further investigate the minimal requirements of R89 for signaling through $G\alpha_q$ in mammalian cells we designed

		321			325					330				335					340					345				349	Gi	Gq	Gs
	CT2	N	V	L	T	E	E	S	V	V	R	E	S	K	S	F	T	R	S	T	V	D	T	M	A	Q	K	T	Q	A	50	0	0
Full-Length Receptors	R4	.	.	.	P	.	.	H	.	G	.	.	F	.	Y	.	A	.	.	S	.	.	.	.	.	H	N	.	Y	G	50	50	50
	R8	.	A	V	S	D	.	.	.	F	G	.	F	.	.	Y	.	K	.	.	.	.	.	V	.	.	.	S	H	.	50	50	50
	R9	K	.	.	P	.	P	F	.	.	S	.	.	.	.	.	.	S	Y	.	.	V	.	T	S	.	.	N	.	.	50	50	50
	R19	.	.	.	P	.	.	.	.	.	.	.	A	.	.	.	.	I	.	.	.	.	.	K	V	K	.	.	R	.	50	50	50
	R20	.	G	.	.	V	V	P	.	.	W	.	.	I	A	.	S	.	.	.	.	G	.	K	.	.	.	.	.	G	50	50	50
	R21	.	L	.	.	A	.	.	.	F	.	V	.	F	.	S	I	G	A	S	.	K	N	K	.	.	.	S	.	.	50	50	50
	R34	.	.	F	S	K	.	.	.	.	.	.	.	R	.	Y	.	.	P	Y	.	.	S	.	.	P	R	.	H	.	50	50	50
	R37	.	.	W	.	G	.	F	.	A	.	.	N	.	.	.	.	I	.	S	I	.	.	.	.	H	.	.	L	.	50	50	50
	R38	.	G	.	.	.	.	.	.	.	.	.	W	N	F	.	A	I	.	N	.	.	.	.	.	.	.	.	.	.	50	50	50
	R49	K	.	.	.	Q	L	.	.	G	S	.	P	Q	T	L	.	I	C	S	F	.	.	L	.	.	T	.	.	D	50	50	50
	R52	K	.	.	P	K	.	.	.	.	.	.	.	Y	.	.	S	.	.	.	.	G	.	.	.	L	.	S	R	S	50	50	50
	R56	K	G	.	.	.	Y	.	.	E	.	V	.	.	.	.	R	.	.	.	D	.	.	K	S	.	Q	H	.	.	50	50	50
	R62	.	F	R	.	G	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	N	R	.	.	N	.	.	.	50	50	50

		321			325					330				335					340					345				349	Gi	Gq	Gs
	CT2	N	V	L	T	E	E	S	V	V	R	E	S	K	S	F	T	R	S	T	V	D	T	M	A	Q	K	T	Q	A	50	0	0
Truncated Receptors	R12	K	D	.	.	.	.	G	.	.	.	A	F	.	.	@															50	50	50
	R13	.	.	I	S	Q	.	.	.	.	T	.	.	.	F	.	S	@													50	50	50
	R14	.	.	S	P	.	K	.	.	.	I	.	A	I	.	V	N	.	T	N	.	.	.	.	.	.	@				50	50	50
	R33	K	.	Q	.	R	.	.	I	.	@																				50	50	50
	R41	.	D	.	.	.	.	.	.	F	K	.	Y	@																	50	50	50
	R42	.	G	S	.	K	.	.	.	.	.	.	.	.	F	R	.	.	.	S	F	.	A	.	.	.	@				50	50	50
	R55	K	.	S	.	.	@																								50	50	50
	R58	.	G	K	.	.	.	.	.	.	M	A	.	.	.	.	A	@													50	50	50
	R60	K	.	F	E	@																									50	50	50
	R63	.	.	S	.	.	A	F	.	.	.	.	.	N	.	@															50	50	50
	R66	.	G	.	.	.	.	F	.	.	.	@																			50	50	50
	R67	.	.	S	.	G	K	F	.	.	.	.	.	.	@																50	50	50
	R70	.	.	S	.	D	G	.	G	G	K	.	.	.	.	.	.	@													50	50	50
	R2	.	.	.	S	V	.	.	.	.	.	.	.	@																	50	50	0
	R7	.	.	.	P	D	D	A	I	.	S	.	F	@																	50	50	0
	R11	K	.	F	.	.	@																								50	50	0
	R40	.	.	Q	.	@																									50	50	0

FIGURE 6. **Signaling of CT2 mutants via $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ chimeras.** The wild-type sequence of the region targeted for mutagenesis in CT2 is given (*top* in *bold*) with the residue numbers marked. The amino acid sequences of the functional mutant receptors obtained (designated *R* and *numbered*, *left*) are indicated (*dots*, unchanged amino acid compared with wild-type) and annotated as in Fig. 2. @, stop codon.

TABLE 1

Signaling of the CT2stop323 truncation

The receptors indicated below were transformed into strains BY1142, BY1173, BY1172, and BY1401 expressing the C5a ligand (+C5a) or carrying an empty vector (−C5a). Signaling was assayed on histidine-deficient medium with varying concentrations of 3AT for three independent transformants: +++++, growth on 50 mM 3AT; ++++, growth on 20 mM 3AT; +++, growth on 10 mM 3AT; ++, growth on 5 mM 3AT; +, growth on 1 mM 3AT; 0, no growth on 1 mM 3AT. Signaling in BY1142 was tested in the presence (+RGS4) and absence (−RGS4) of the GTPase activating protein, RGS4.

	BY1142			BY1173		BY1172		BY1401	
	−C5a, −RGS4	+C5a, −RGS4	+C5a, +RGS4	−C5a	+C5a	−C5a	+C5a	−C5a	+C5a
WT[a]	0	++	0	0	+++++	0	0	0	0
TM3 trunc	0	0	0	0	0	0	0	0	0
CT2stop323 trunc	0	+++++	0	0	+++++	0	+++++	0	0

[a] WT, wild-type C5aR; TM3 trunc, truncation within transmembrane helix 3 that is non-functional; CT2stop323 trunc, truncation with a stop codon at position 323.

four site-directed mutants with replacements in positions 144–146, namely M1 (C144Y), M2 (C144Y and Q145R), M3 (C144Y, Q145R, and N146A), and M4 (C144Y, Q145R, and N146L) (Fig. 9*a*). The single mutation Q145R in R91 was not sufficient to evoke signaling as discussed above. The mutation of the cysteine side chain in position 144 to tyrosine (M1) alone or coupled with the Q145R mutation (M2) was designed to investigate whether it would be sufficient to change side chains at these two positions. Mutants M3 and M4 targeted the asparagine side chain in position 146, the former effectively eliminating it, and the latter replacing it by a hydrophobic side chain similar to asparagine by volume. The four mutant receptors were then expressed in HEK293 cells and tested for exiting the ER by Endo-H_f treatment (Fig. 9*b*). Mutants 1–4 were able to exit the ER, and the expression level and the amount of receptor-containing complex *N*-linked oligosaccharides were comparable to the wild-type and R89 receptors. The mutants were then tested for signaling through $G\alpha_q$ in HEK293 cells by measuring IP_3 accumulation (Fig. 9*c*). M1 does not show any activation of $G\alpha_q$, whereas M2 and M3 show weak activation upon stimulation with 1 μM W5Cha. M4, however, is able to signal as well as R89 indicating that replacements in three positions (144–146) represent the minimal requirement for signaling through $G\alpha_q$, in mammalian cells.

In addition to signaling through the $G\alpha_q$ chimera in yeast, R89 was also able to signal through the $G\alpha_s$ chimera. To test whether R89 could activate full-length $G\alpha_s$ in mammalian cells, R89 and human $G\alpha_s$ were co-expressed in HEK293 cells, and cAMP levels were measured. Upon treatment with 1 μM W5Cha, cAMP levels were unchanged indicating that R89 was unable to activate full-length $G\alpha_s$.[3]

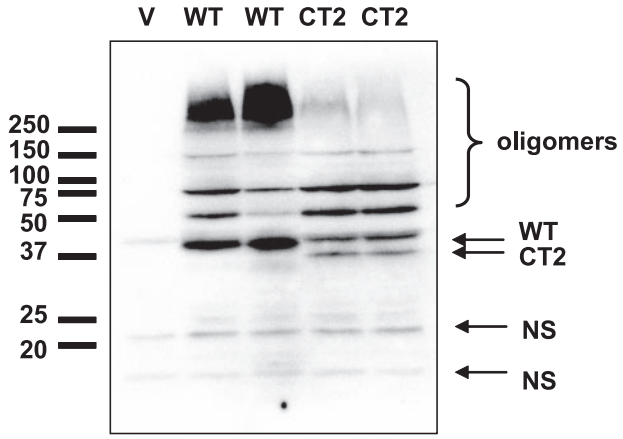

FIGURE 7. **Expression level of the CT2stop323 truncation in yeast.** Lysates from BY1143 yeast expressing no receptor (*V*), wild type (*WT*), or the CT2stop323 truncation (*CT2*) were analyzed by immunoblotting for C5aR. Two transformants of each are shown. WT C5aR migrates at ~40 kDa, and CT2 migrates at ~35 kDa. Nonspecific bands (*NS*) and oligomers are indicated.

Computational Modeling of the Intracellular Loops in the C5aR Mutants—To explore structural mechanisms for the role of IC2 in G protein specificity, we performed modeling studies to determine the possible low energy conformations of the peptide backbone of the IC2 loop for each receptor. The IC2 loop in R89 possessed a specific conformation of the peptide backbone that was geometrically similar to a conformation of the IC2 loop in M4 (according to the r.m.s. cut-off of 2 Å) but was different from all low energy conformations of IC2 available for M1, M2, and M3. Fig. 9*d* depicts the corresponding low energy structure of the intracellular loops in M4 and R89, as well as the structures most similar to this one in WT, M1, M2, and M3. One can see that only M4 adopts the same unique IC2 conformation as R89, which is characterized by the specific spatial arrangement of the Tyr-144 and Arg-145 side chains. As to the side chain in position 146, comparison of the IC2 structures of M3 and M4, which differ only by an alanine or leucine substitution of asparagine 146, respectively, reveals that mutation from alanine to leucine dramatically changes the conformation of the Tyr-144 and Arg-145 side chains potentially interacting with the G protein. Importantly, the IC2 conformation in question was also not similar (according to the r.m.s. cut-off of 2 Å) to any of the low energy IC2 conformations of the receptors that did not signal through full-length human $G\alpha_q$, namely wild-type, R35, R52, R58, and R91, the only exception being R111 (Fig. 9*e*). However, the crucial side chains of Tyr-144 and Arg-145 are oriented differently in the similar backbone conformations of R89 and R111.[4] In our view, the above results strongly suggest that the specific conformation of IC2 in R89 and M4 that places the side chains of Tyr-144 and Arg-145 in positions depicted in Fig. 9*d* may allow for an initial interaction with full-length human $G\alpha_q$ or with other receptor structures such as the carboxyl tail that facilitate signaling.

DISCUSSION

Receptor-G protein specificity is one example of how nature constructs a protein-protein interface. A receptor must be able to locate the correct G protein and bind to it in the presence of other competing G proteins. This is a general protein-protein

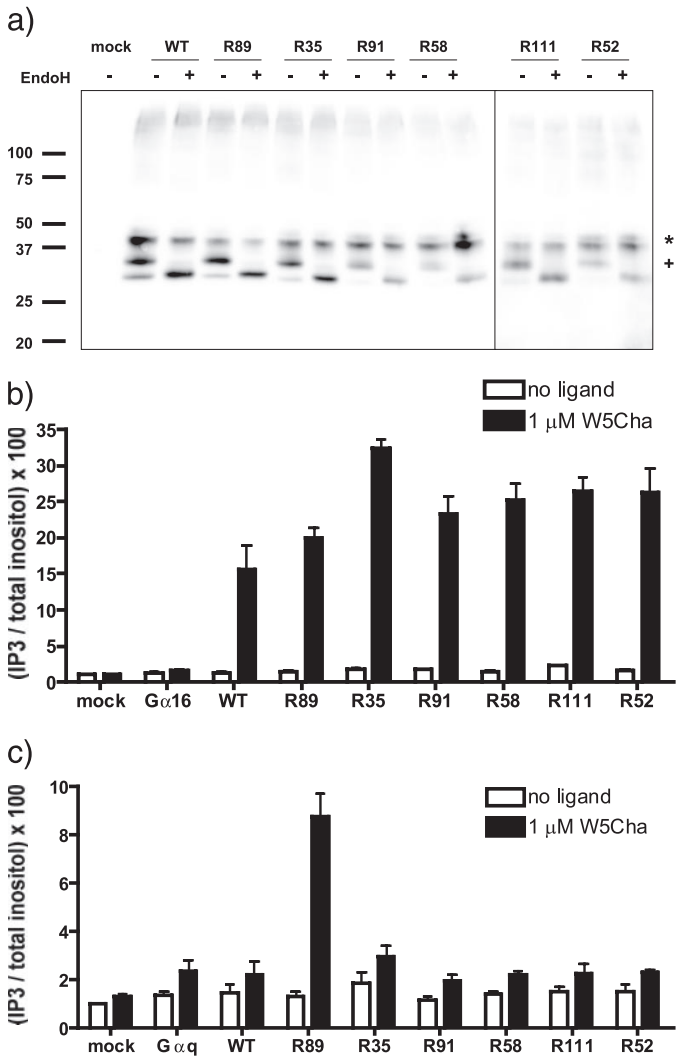

FIGURE 8. **G protein specificity in mammalian cells.** *a*, lysates of HEK293 cells transiently transfected with wild-type C5aR (*WT*) or IC2 mutants R89, R35, R91, R58, R11, or R52 were treated with (+) or without (−) Endo-H_f and analyzed by immunoblotting for C5aR. Endo-H_f-resistant receptors containing complex *N*-linked oligosaccharides (*) and Endo-H_f-sensitive high-mannose oligosaccharides (+) are shown. *b*, HEK293 cells transiently transfected with $G\alpha_{16}$ or $G\alpha_{16}$ plus wild-type C5aR (*WT*) or IC2 mutants R89, R35, R91, R58, R111, or R52 were treated with 1 μM W5Cha or no ligand and assayed for IP_3 accumulation. Values are normalized to the mock transfection without ligand. Each *bar* represents the mean of three independent trials, ± S.D. *c*, HEK293 cells transiently transfected with $G\alpha_q$ or $G\alpha_q$ plus wild-type C5aR (*WT*) or IC2 mutants R89, R35, R91, R58, R111, or R52 were treated with 1 μM W5Cha or no ligand and assayed for IP_3 accumulation. Values are normalized to the mock transfection without ligand. Each *bar* represents the mean of three independent trials, ± S.D.

interaction problem that occurs in the crowded environment of the cell. For a protein to bind to the correct partner, two factors must be taken into consideration: stability and specificity. Stability employs positive design, which maximizes favorable interactions with the correct binding partner. Specificity derives from negative design to maximize unfavorable interactions with incorrect binding partners. Computational protein design has demonstrated that the combination of stability and specificity allows proteins to bind to their partners with more fidelity than when stability alone is maximized (47).

The most striking finding of the current study is that the majority of the mutated C5aRs gained the ability to activate

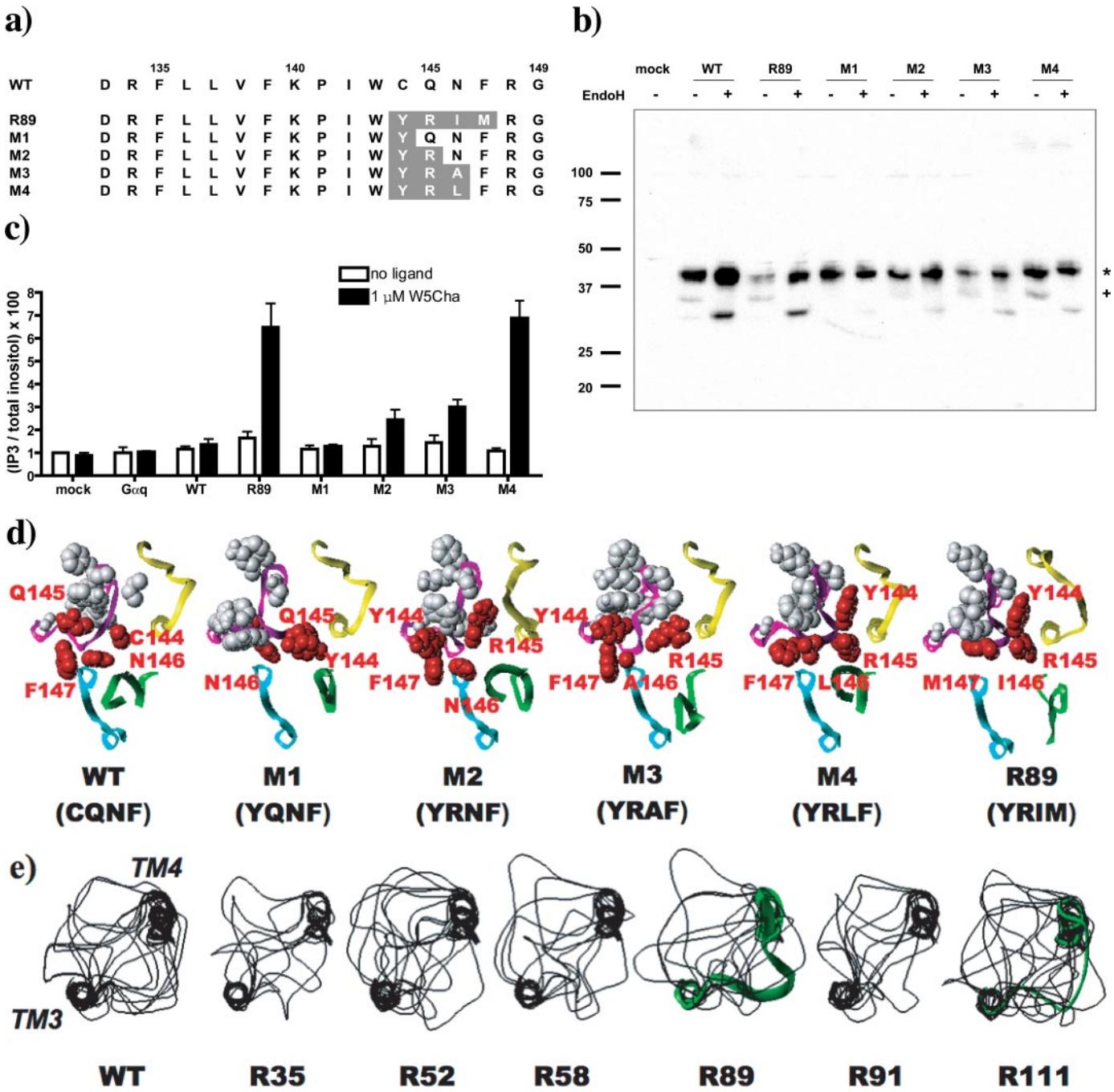

FIGURE 9. **IC2 design mutants.** *a*, the sequence of the wild-type (*WT*) IC2 region is aligned with the sequences of IC2 mutants R89 and design mutants 1–4 (*M1–M4*). Mutated residues are highlighted in *gray*. *b*, lysates of HEK293 cells transiently transfected with wild-type C5aR (*WT*), or IC2 mutants R89, M1, M2, M3, or M4 were treated with (+) or without (−) Endo-H_f and analyzed by immunoblotting for C5aR. Endo-H_f-resistant receptors containing complex *N*-linked oligosaccharides (*) and Endo-H_f-sensitive high-mannose oligosaccharides (+) are shown. *c*, HEK293 cells transiently transfected with $G\alpha_q$ or $G\alpha_q$ plus wild-type C5aR (*WT*), or IC2 mutants R89, M1, M2, M3, or M4 were treated with 1 μM W5Cha or no ligand and assayed for IP_3 accumulation. Values are normalized to the mock transfection without ligand. Each *bar* represents the mean of three independent trials, ± S.D. *d*, comparison of the low energy conformation common for mutants R89 and M4 and those most similar to this one in wild-type (*WT*), M1, M2, and M3. The intracellular fragments are shown as shaded ribbons in *cyan* (IC1), *magenta* (IC2), *yellow* (IC3), and *green* (fragment 300–310). Only side chains of fragment 139–149 (IC2) are shown as *space-filled models*. The side chains of residues in positions 144–147 are shown and labeled in *red*. Sequences 144–147 for each mutant are shown in *brackets*. *e*, comparison of the low energy backbone conformations found for wild-type (*WT*), R35, R52, R58, R91, and R111. Only conformations different from each other according to the r.m.s. cut-off of 2 Å are displayed. Conformations are shown as *black* one-line ribbons; the unique IC2 conformation in R89 is shown as a *shaded ribbon* in *green*, and the conformer similar to it in R111 is shown as a *green tube*.

$G\alpha_q$ and $G\alpha_s$ chimeras, despite no selective pressure to do so. The mutants used in this study were initially selected by their ability to couple to a $G\alpha_i$ chimera, and there was no selective pressure for or against coupling to the $G\alpha_q$ or $G\alpha_s$ chimeras, yet many of the mutants demonstrated broadened G protein coupling. The most direct interpretation of our results is that the loops and especially the carboxyl-terminal tail represent examples of negative design acting as selectivity filters to provide G protein specificity. We cannot rule out that, in the yeast, selective pressures for structural features that mediate functions not

obvious, such as receptor stability or receptor oligomerization, could also result in broadened specificity. Nonetheless, the most straightforward interpretation of the results is that the gain of coupling results from disruption of negative design elements present in the wild-type C5aR.

In the setting of a single $G\alpha$ subunit expressed in yeast, negative design features become dispensable and are therefore lost in the "forced evolution" that occurs with saturation mutagenesis. The strongest evidence of the presence of negative design is the broadened G protein activation that results from introducing a wide variety of mutations at every position of the carboxyl-terminal tail and even complete removal of the tail. Thus, we propose that the carboxyl terminus and intracellular loops provide a selectivity filter that acts as a barrier to incorrect G protein binding. The tail would provide a gatekeeper function to block any unwanted binding partners, and this function could be disrupted by either mutation of the loops and tail or truncation of the tail. With a negative design approach, there would be many ways in which to prevent binding of competing G proteins. This may explain why the intracellular regions of GPCRs that activate the same G proteins can be so divergent.

If negatively designed regions of a receptor are mutated, the selectivity filter would be disrupted and a broadening, but not a complete switch of G protein coupling should be observed such that the receptor retains coupling to the original G protein. This is what was seen for the mutant receptors obtained in our screens, because they were selected to retain coupling to $G\alpha_i$. Many instances of broadened specificity resulting from receptor mutation have been reported in the literature. For example, a mutagenized V_2 vasopressin receptor (24) or serotonin 5-HT_{1A} receptor (23) as well as hybrid receptors between the V_{1a} and V_2 vasopressin receptors (5) or the β_2-adrenergic receptor and the thrombin receptor (7) all show a gain in coupling while retaining signaling through the original G protein. The one major exception is when the IC3 loop of rhodopsin is replaced by that of the β_2-adrenergic receptor, a loss of $G\alpha_t$ activation is seen along with a gain of signaling through $G\alpha_s$ (18). The loss of $G\alpha_t$ signaling could be due to removal of residues that are essential for the activation of $G\alpha_t$ along with removal of residues necessary for prevention of $G\alpha_s$ binding. Similar to the C5aR (67), IC3 of rhodopsin has been shown to contain many residues essential for G protein activation (48–52); therefore, specificity determinants and essential residues may overlap in IC3. We would predict that, to change specificity of the C5aR, receptors would need to be selected under conditions that require $G\alpha_q$ or $G\alpha_s$ coupling for signaling but also actively select against signaling through $G\alpha_i$. In fact, functional receptors selected in the $G\alpha_s$ chimeric strain all retained the ability to signal through the $G\alpha_i$ chimera.[3] If all intracellular regions of the C5aR make some contribution to specificity, we would also predict that mutation of more than one region may be necessary to achieve a complete switch in specificity.

Very few examples exist of the carboxyl terminus participating in G protein specificity. Alternative splicing of the prostaglandin EP3 receptor subtype alters the G protein to which the receptor couples, however, the receptor containing the shortest carboxyl terminus coupled to the fewest number of G proteins (53). The parathyroid hormone receptor has also been shown to have a carboxyl terminus involved in G protein coupling (54). When the parathyroid hormone receptor is truncated the receptor is able to couple to $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$, whereas the wild-type receptor only couples to $G\alpha_s$. This result mimics what we see with the C5aR tail, and we propose that a specificity filter also exists in the parathyroid hormone receptor and potentially other GPCRs.

The role of the carboxyl-terminal tail and all three of the intracellular loops in determining specificity suggests that the tail interacts with the loops to create a structure that acts as a selectivity filter. Interaction of the tail and IC1 has been demonstrated for rhodopsin in the inactive state by crystal structure (55), disulfide trapping (56), and EPR (57–59). An interaction between the tail and IC3 has also been shown by EPR (59). Our data suggest that these may be functional interactions contributing to specificity and provide a testable hypothesis. If these interactions were disrupted we would predict that specificity might be broadened.

Although this study demonstrates roles for the intracellular loops of the C5aR in negative design, other elements of the loops most likely do contain structures that contribute to positive design (*i.e.* stabilize interactions with $G\alpha_i$). For example, the Q145R mutation in IC2 may allow for a positive interaction to occur with the $G\alpha_q$ chimera, because nearly all of our receptors that couple to the $G\alpha_q$ chimera contain this mutation. This convergence on arginine is underscored by the fact that the m1, m3, and m5 muscarinic acetylcholine receptors (all couple to $G\alpha_q$) contain this arginine, whereas neither the m2 nor m4 receptors do (both couple to $G\alpha_i$), and is supported by previous work demonstrating that this arginine is critical for $G\alpha_q$ activation (19). The role of the Q145R mutation in coupling to $G\alpha_q$ however is not clear. Our modeling studies demonstrate that the Q145R mutation does contribute to an overall change in the backbone conformation of IC2 and, in the various conformations of IC2, may interact with other receptor loops, as well as with the carboxyl tail of the receptor. If residue 145 in the "activated" IC2 conformation interacts with the tail then substitution of an arginine may disrupt this interaction, allowing the receptor to adopt a more open conformation that permits additional G proteins to bind. If the Q145R mutation confers $G\alpha_q$ chimera coupling by mediating a direct interaction with the carboxyl-terminal tail of the $G\alpha$ subunit, then one might predict there to be corresponding candidate residues for interaction in the $G\alpha_q$ tail *versus* $G\alpha_i$. Comparison of the $G\alpha_{i3}$ and $G\alpha_q$ tails shows that the most dramatic amino acid change is from a glycine at position −3 to an asparagine (Fig. 1*A*). If residue 145 of the receptor interacts with the $G\alpha$ tail, the Q145R mutation may allow better packing with the asparagine residue. The IC2 R89 receptor may also contain an element of positive design allowing it to activate full-length $G\alpha_q$ in mammalian cells. Comparison of the sequences of WT, M3, M4, and R89 indicates that simply removing Asn-146 and replacing it with alanine is not sufficient to allow M3 to couple strongly to $G\alpha_q$, rather the introduction of a large hydrophobic residue such as isoleucine in R89 or leucine in M4 is necessary to see robust activation of $G\alpha_q$. Thus it is not the loss of the asparagine but the gain of the bulky hydrophobic residue that promotes $G\alpha_q$ coupling.

The inability to unequivocally determine a direct contact between receptor residues such as Q145R and the last five amino acids of the Gα subunit may be due to a role for the Gα tail in receptor activation, rather than simply stabilization of binding of the G protein to the receptor. Notably, in previous membrane reconstitution experiments, we demonstrated that a truncated Gα subunit (lacking the nine carboxyl-terminal residues) acted as a competitive inhibitor for receptor activation, thus demonstrating that the carboxyl terminus is not required for receptor binding but is likely more important for receptor-catalyzed exchange of GTP for GDP on the G protein (60). Moreover, NMR studies of semi-synthetic $G\alpha_{i1}$ proteins containing isotope labels in the carboxyl terminus or of a ^{15}N-labeled Gα subunit demonstrate that the carboxyl-terminal tail undergoes a conformational change during activation (60–62). From a mechanistic standpoint, in this activated conformation, the carboxyl terminus of the Gα subunit may sterically interfere with binding to the receptor interface, thereby encouraging the release of the activated Gα subunit from the receptor. In this model, the negative design elements within the loops and tail of the receptor might restrict access of an inappropriate Gα tail to the inner regions of the receptor that would result in G protein activation.

Why did only one of the six IC2 mutants that signaled through the $G\alpha_q$ chimera in yeast demonstrate $G\alpha_q$ coupling in mammalian cells? Of the six IC2 mutants tested, R89 is the receptor that contains the most drastic mutations with three non-conservative substitutions (C144Y, N146I, and F147M) according to a PAM250 matrix log-odds score of 1.0 or greater (63). The other five IC2 mutants tested contain two or fewer non-conservative substitutions. In addition to the role of Gα in specificity, Gβγ may also contain specificity determinants. Indeed this has been demonstrated for the α2-, β1-, and β2-adrenergic receptors (64–66). Thus, the difference between the yeast Ste4/Ste18 and the human Gβγ could also account for the difference in signaling in the yeast and mammalian systems.

In light of our map of the essential residues of the C5aR[3] and our map of specificity determinants presented here, we conclude that the receptor structures essential for G protein activation *versus* G protein specificity map to different regions of the intracellular face of the receptor. Residues identified as essential for signaling were found to be localized in the amino-terminal half of IC2 and the carboxyl-terminal half of IC3 as well as their adjoining transmembrane helix helices, whereas the main determinants for G protein specificity were clustered in the carboxyl-terminal tail and carboxyl-terminal half of IC2. This indicates that distinct regions of the intracellular face of the C5aR fulfill these two functions, which must be combined to achieve activation of the correct G protein.

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REFERENCES

1. Miller, K. J., Murphy, B. J., and Pelleymounter, M. A. (2004) *Curr. Drug Targets CNS Neurol. Disord.* **3,** 357–377
2. Wettschureck, N., and Offermanns, S. (2005) *Physiol. Rev.* **85,** 1159–1204
3. Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 639–650
4. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr. Rev.* **24,** 765–781
5. Liu, J., and Wess, J. (1996) *J. Biol. Chem.* **271,** 8772–8778
6. Nasman, J., Jansson, C. C., and Akerman, K. E. (1997) *J. Biol. Chem.* **272,** 9703–9708
7. Verrall, S., Ishii, M., Chen, M., Wang, L., Tram, T., and Coughlin, S. R. (1997) *J. Biol. Chem.* **272,** 6898–6902
8. Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G., and Lefkowitz, R. J. (1988) *Science* **240,** 1310–1316
9. Wess, J., Brann, M. R., and Bonner, T. I. (1989) *FEBS Lett.* **258,** 133–136
10. Wess, J., Bonner, T. I., Dorje, F., and Brann, M. R. (1990) *Mol. Pharmacol.* **38,** 517–523
11. Cotecchia, S., Ostrowski, J., Kjelsberg, M. A., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267,** 1633–1639
12. Olah, M. E. (1997) *J. Biol. Chem.* **272,** 337–344
13. Erlenbach, I., and Wess, J. (1998) *J. Biol. Chem.* **273,** 26549–26558
14. Wess, J., Bonner, T. I., and Brann, M. R. (1990) *Mol. Pharmacol.* **38,** 872–877
15. Takagi, Y., Ninomiya, H., Sakamoto, A., Miwa, S., and Masaki, T. (1995) *J. Biol. Chem.* **270,** 10072–10078
16. Liggett, S. B., Caron, M. G., Lefkowitz, R. J., and Hnatowich, M. (1991) *J. Biol. Chem.* **266,** 4816–4821
17. Pin, J. P., Joly, C., Heinemann, S. F., and Bockaert, J. (1994) *EMBO J.* **13,** 342–348
18. Kim, J. M., Hwa, J., Garriga, P., Reeves, P. J., RajBhandary, U. L., and Khorana, H. G. (2005) *Biochemistry* **44,** 2284–2292
19. Blin, N., Yun, J., and Wess, J. (1995) *J. Biol. Chem.* **270,** 17741–17748
20. Liu, J., Conklin, B. R., Blin, N., Yun, J., and Wess, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11642–11646
21. Kostenis, E., Conklin, B. R., and Wess, J. (1997) *Biochemistry* **36,** 1487–1495
22. Kostenis, E., Gomeza, J., Lerche, C., and Wess, J. (1997) *J. Biol. Chem.* **272,** 23675–23681
23. Malmberg, A., and Strange, P. G. (2000) *J. Neurochem.* **75,** 1283–1293
24. Erlenbach, I., Kostenis, E., Schmidt, C., Serradeil-Le Gal, C., Raufaste, D., Dumont, M. E., Pausch, M. H., and Wess, J. (2001) *J. Biol. Chem.* **276,** 29382–29392
25. Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koenig, B., and Hofmann, K. P. (1988) *Science* **241,** 832–835
26. Onrust, R., Herzmark, P., Chi, P., Garcia, P. D., Lichtarge, O., Kingsley, C., and Bourne, H. R. (1997) *Science* **275,** 381–384
27. Cai, K., Itoh, Y., and Khorana, H. G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4877–4882
28. Itoh, Y., Cai, K., and Khorana, H. G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4883–4887
29. Kisselev, O., and Gautam, N. (1993) *J. Biol. Chem.* **268,** 24519–24522
30. Kisselev, O. G., Ermolaeva, M. V., and Gautam, N. (1994) *J. Biol. Chem.* **269,** 21399–21402
31. Kisselev, O., Ermolaeva, M., and Gautam, N. (1995) *J. Biol. Chem.* **270,** 25356–25358
32. Conklin, B. R., Farfel, Z., Lustig, K. D., Julius, D., and Bourne, H. R. (1993) *Nature* **363,** 274–276
33. 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) *Yeast* **16,** 11–22
34. 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
35. Klco, J. M., Wiegand, C. B., Narzinski, K., and Baranski, T. J. (2005) *Nat. Struct. Mol. Biol.* **12,** 320–326
36. Geva, A., Lassere, T. B., Lichtarge, O., Pollitt, S. K., and Baranski, T. J. (2000) *J. Biol. Chem.* **275,** 35393–35401
37. Kolakowski, L. F., Jr., Lu, B., Gerard, C., and Gerard, N. P. (1995) *J. Biol. Chem.* **270,** 18077–18082
38. Spain, B. H., Koo, D., Ramakrishnan, M., Dzudzor, B., and Colicelli, J. (1995) *J. Biol. Chem.* **270,** 25435–25444

39. Schulein, R., Hermosilla, R., Oksche, A., Dehe, M., Wiesner, B., Krause, G., and Rosenthal, W. (1998) *Mol. Pharmacol.* **54,** 525–535
40. Krause, G., Hermosilla, R., Oksche, A., Rutz, C., Rosenthal, W., and Schulein, R. (2000) *Mol. Pharmacol.* **57,** 232–242
41. Bermak, J. C., Li, M., Bullock, C., and Zhou, Q. Y. (2001) *Nat. Cell Biol.* **3,** 492–498
42. Pankevych, H., Korkhov, V., Freissmuth, M., and Nanoff, C. (2003) *J. Biol. Chem.* **278,** 30283–30293
43. Duvernay, M. T., Zhou, F., and Wu, G. (2004) *J. Biol. Chem.* **279,** 30741–30750
44. Robert, J., Clauser, E., Petit, P. X., and Ventura, M. A. (2005) *J. Biol. Chem.* **280,** 2300–2308
45. Estall, J. L., Koehler, J. A., Yusta, B., and Drucker, D. J. (2005) *J. Biol. Chem.* **280,** 22124–22134
46. Thielen, A., Oueslati, M., Hermosilla, R., Krause, G., Oksche, A., Rosenthal, W., and Schulein, R. (2005) *FEBS Lett.* **579,** 5227–5235
47. Bolon, D. N., Grant, R. A., Baker, T. A., and Sauer, R. T. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 12724–12729
48. Franke, R. R., Sakmar, T. P., Oprian, D. D., and Khorana, H. G. (1988) *J. Biol. Chem.* **263,** 2119–2122
49. Franke, R. R., Sakmar, T. P., Graham, R. M., and Khorana, H. G. (1992) *J. Biol. Chem.* **267,** 14767–14774
50. Ernst, O. P., Hofmann, K. P., and Sakmar, T. P. (1995) *J. Biol. Chem.* **270,** 10580–10586
51. Yang, K., Farrens, D. L., Hubbell, W. L., and Khorana, H. G. (1996) *Biochemistry* **35,** 12464–12469
52. Natochin, M., Gasimov, K. G., Moussaif, M., and Artemyev, N. O. (2003) *J. Biol. Chem.* **278,** 37574–37581
53. Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., and Narumiya, S. (1993) *Nature* **365,** 166–170
54. Schneider, H., Feyen, J. H., and Seuwen, K. (1994) *FEBS Lett.* **351,** 281–285
55. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science* **289,** 739–745
56. Klein-Seetharaman, J., Hwa, J., Cai, K., Altenbach, C., Hubbell, W. L., and Khorana, H. G. (2001) *Biochemistry* **40,** 12472–12478
57. Altenbach, C., Klein-Seetharaman, J., Cai, K., Khorana, H. G., and Hubbell, W. L. (2001) *Biochemistry* **40,** 15493–15500
58. Altenbach, C., Cai, K., Klein-Seetharaman, J., Khorana, H. G., and Hubbell, W. L. (2001) *Biochemistry* **40,** 15483–15492
59. Cai, K., Klein-Seetharaman, J., Altenbach, C., Hubbell, W. L., and Khorana, H. G. (2001) *Biochemistry* **40,** 12479–12485
60. Anderson, L. L., Marshall, G. R., Crocker, E., Smith, S. O., and Baranski, T. J. (2005) *J. Biol. Chem.* **280,** 31019–31026
61. Abdulaev, N. G., Ngo, T., Zhang, C., Dinh, A., Brabazon, D. M., Ridge, K. D., and Marino, J. P. (2005) *J. Biol. Chem.* **280,** 38071–38080
62. Ridge, K. D., Abdulaev, N. G., Zhang, C., Ngo, T., Brabazon, D. M., and Marino, J. P. (2006) *J. Biol. Chem.* **281,** 7635–7648
63. Dayhoff, M. O., Schwartz, R. M., and Orcutt, B. C. (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., ed) pp. 345–352, National Biomedical Research Foundation, Washington, D. C.
64. Robillard, L., Ethier, N., Lachance, M., and Hebert, T. E. (2000) *Cell Signal.* **12,** 673–682
65. Kuhn, B., Christel, C., Wieland, T., Schultz, G., and Gudermann, T. (2002) *Naunyn-Schmiedebergs Arch. Pharmacol.* **365,** 231–241
66. Gibson, S. K., and Gilman, A. G. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 212–217
67. Matsumoto, M. L., Narzinski, K., Kiser, P. D., Nikiforovich, G. V., and Baranski, T. J. (2007) *J. Biol. Chem.* **282,** 3105–3121