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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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 Gα 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 Gαo, Gαq, or Gαi 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 Gαo and/or Gαi. 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: Gα12, Gα13, Gαq, and Gα11, and Gαq, Gα12/13 (3, 4). Gα12 subunits stimulate adenyl cyclase, whereas Gα13 subunits inhibit adenyl cyclase. Gαq subunits signal through phospholipase C, and Gα12/13 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 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 Gα13 and the promiscuous Gα12 subunit of the Gαq family, but not Gα11 itself, nor Gα12. 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

IC3, intracellular loop 3; IP3, inositol 1,4,5-triphosphate; r.m.s., root mean square deviation; RSM, random saturation mutagenesis; W5Cha, hexapeptide agonist of the C5a receptor.

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The abbreviations used are: GPCR, G protein-coupled receptor; 3AT, 3-amino-triatrazole; C5a, complement fragment 5a; C5αR, complement fragment 5a receptor; CT, carboxyl terminus; CT1, first half of the carboxyl terminus; CT2, second half of the carboxyl terminus; CT2stop323, truncated C5αR with a stop codon at position 323; Endo-Hf, endo-β-N-acetylglucosaminidase H; ER, endoplasmic reticulum; IC1, intracellular loop 1; IC2, intracellular loop 2;
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 α4–β6 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 Gβ 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 carboxyterminal 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 carboxy-terminal mutants and more than half of the IC2 mutants demonstrated a gain in specificity. We found that all of the carboxy-terminal mutants and more than half of the IC2 mutants demonstrated a gain in specificity. We found that all of the carboxy-terminal mutants and more than half of the IC2 mutants demonstrated a gain in specificity. We found that all of the carboxy-terminal mutants and more than half of the IC2 mutants demonstrated a gain in specificity.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—Yeast strains BY1142 (Gαq), BY1173 (Gαq), BY1172 (Gαq), and BY1404 (Gαq) have been previously described (33, 34). Briefly, BY1142 has the genotype MATa ura3 leu2 trp1 GPA1::URA3 H9251, BY1401 (Gαq), and BY1404 (Gαq) have been previously described (33, 34). Briefly, BY1142 has the genotype MATa ura3 leu2 trp1 GPA1::URA3 H9251, BY1401 (Gαq), and BY1404 (Gαq) have been previously described (33, 34). Briefly, BY1142 has the genotype MATa ura3 leu2 trp1 GPA1::URA3 H9251, BY1401 (Gαq), and BY1404 (Gαq) have been previously described (33, 34). Briefly, BY1142 has the genotype MATa ura3 leu2 trp1 GPA1::URA3 H9251, BY1401 (Gαq), and BY1404 (Gαq) have been previously described (33, 34). Briefly, BY1142 has the genotype MATa ura3 leu2 trp1 GPA1::URA3 H9251, BY1401 (Gαq), and BY1404 (Gαq) have been previously described (33, 34). Briefly, BY1142 has the genotype MATa ura3 leu2 trp1 GPA1::URA3 H9251, BY1401 (Gαq), and BY1404 (Gαq) have been previously described (33, 34).

BY1173 (Gαq), BY1403 (Gαq), and BY1404 contain a fusion of amino acids 41–467 of the yeast Gα protein, Gpa1, followed by the last 11 amino acids (residues 340–450) of the Gα subunit and the intracellular loops create a specificity filter that acts to restrict access of incorrect G proteins to the receptor.

**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, 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 Gαq (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 27G1s 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-Hp, 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.

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

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phosphate methods. The m1 muscarinic acetylcholine receptor (University of Missouri-Rolla cDNA Resource Center) was used as a positive control. For Ga16 signaling, RSM receptors and Ga16 were subcloned into pcDNA3.1(+) (Invitrogen) and transiently co-transfected into HEK293 cells. IP3 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 Ga, Gpa1, followed by residues 34–354 of human Ga13 (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 Ga13 (strain BY1173), Gaq (BY1172), or Gs (BY1401) (33) to elucidate receptor determinants of specificity (Fig. 1a).

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_FUSTR-HIS3 or P_FUSTR-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 Gaq, Gαo, or Gαs residues are shaded black. The Ga of BY1143 contains residues 1–41 of yeast Gpa1 followed by residues 34–354 of human Gaq. The Ga of BY1172 contains residues 1–467 of yeast Gpa1 followed by the last five residues of human Gaq. An alignment of the last five residues of the chimeras is shown. b, yeast strains BY1143, BY1173, BY1172, and BY1401 expressing the chimeric Gα subunits consisting of residues 1–467 of Gpa1 and the last five amino acids of human Gaq, Gαo, or Gαs, 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.](image-url)
TABLE 1

| IC1 | 57 | 60 | 65 | 70 | 72 | Gi | Gq | Go |
|-----|----|----|----|----|----|----|----|----|
| R3  | . . | M  | .  | .  | .  | 50 | 20 | 0  |
| R7  | . . | A  | S  | L  | G  | 50 | 20 | 0  |
| R9  | . . | L  | .  | .  | .  | 50 | 20 | 0  |
| R11 | W  | .  | N  | .  | .  | 50 | 20 | 0  |
| R25 | F  | .  | C  | .  | .  | 50 | 20 | 0  |
| R28 | . . | L  | S  | Y  | K  | 50 | 20 | 0  |
| R17 | . . | D  | L  | D  | .  | 50 | 10 | 0  |
| R24 | . . | .  | D  | Y  | V  | .  | 50 | 10 | 0  |
| R14 | A  | L  | D  | A  | P  | R  | P  | V  | 50 | 5  | 0  |
| R1  | S  | G  | .  | .  | .  | .  | .  | .  | 50 | 0  | 0  |
| R2  | .  | .  | R  | 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  | .  | .  | .  | .  | 50 | 0  | 0  |
| R12 | G  | .  | .  | V  | I  | K  | I  | V  | T  | 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  | N  | .  | Q  | N  | .  | 50 | 0  | 0  |
| R18 | .  | .  | D  | C  | G  | .  | .  | .  | 50 | 0  | 0  |
| R20 | A  | .  | L  | A  | .  | .  | .  | .  | 50 | 0  | 0  |
| R21 | L  | .  | 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  | .  | .  | .  | .  | .  | 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 Goq, Gσq, and Gσi chimera. 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 Go subunits. Goq denotes strain BY1173, Gσq denotes strain BY1172, and Gσi 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.

The wild-type C5aR receptor was tested for signaling in the BY1173 (Gσ13), BY1172 (Gσq), and BY1404 (Gσi) strains by β-galactosidase assay (Fig. 1b). 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 Gq ligand. The wild-type receptor demonstrated signaling in the BY1173 strain (Fig. 2–6). Twenty-eight intracellular loop 1 (IC1) mutants were tested, and only nine of these coupled to the Gσq chimera, whereas none of the mutants gained the ability to couple to the Gσi chimera (Fig. 2). Of note, the IC1 mutants that show signaling through the Gσq 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 Gσi chimera (see below). Comparison of the sequences of mutant receptors that are able to couple to the Gσq 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 Gσq, and three of these were also able to signal through Gσi (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 Gσq in yeast (Fig. 3). Comparison of IC2 mutant sequences that couple to Gσq 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 Gσi. Interestingly, a positive charge at this position is not sufficient to allow Gσq coupling, because several receptors that cannot signal through Gσq, 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 Gσq and none were able to activate Gσi (Fig. 4). In addition, most of the mutants that gained coupling to the Gσq 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 Gσq.

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 Gσq (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 Gσi, indicating that the CT is not essential for activation of either the Gσq, Gσ13, or Gσi chimera. All 30 of the mutants in the second half of the
carboxyl terminus (CT2) signaled strongly through Gq/H9251q, and all except four mutants also signaled strongly through Gq/H9251s (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 Gq/H9251q or Gq/H9251s 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 Go 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 Go 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 Go tail. CT2 makes the largest contribution, followed by CT1, 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 (Gq/H9251i3), BY1172 (Gq/H9251i), and BY1401 (Gq/H9251s) (Table 1). Although the wild-type receptor coupled only to the Gq chimera, the CT2 truncation gained the ability to couple to the Goq but not the Goi 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 Go carboxyl terminus.
BL-bailase specificity of the C5aR

Signaling of the C5aR in yeast leads to cell growth through the action of free Gβγ that has been released from Gα upon GTP binding (38). Thus, a mutant receptor may permit cell growth by binding and sequestering Gα without actually catalyzing GTP exchange, leaving Gβγ free to signal. To evaluate this possibility, we expressed the CT2stop323 truncation in the BY1142 strain in the presence and absence of the mammalian system contains the yeast Gβγ-q complex, Gpa1-Gαq complex, and the yeast Gαq complex, respectively.

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 resistance (Fig. 8a). 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 Gαq subunit at levels comparable to the wild-type C5aR.

Mutations in Cell Culture—To determine whether mutants that show broadened G protein coupling with the chimeric Gα 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 treatment. Endo-H 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-sensitive. Therefore, Endo-H 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 sensitive, indicating failure to exit the ER. Expression of the mutated receptors in COS7 cells failed to rescue the ER retention of the receptors. 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).

Though these mutants were then tested for a gain in coupling to Gαq, All mutants demonstrated low basal activity, but only R89 showed robust IP3 accumulation upon ligand stimulation when co-expressed with Gαq (Fig. 8c). Mutant R89 demonstrated a 6-fold increase in IP3 levels when stimulated with 1 μM of the C5aR small molecule agonist W5Cha. This is similar to the m1 muscarinic acetylcholine receptor, a true Gαq-coupled receptor, which showed a 7.5-fold increase in IP3 levels when stimulated with 100 μM carbachol.

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 Gαq signaling in mammalian cells. The discrepancy between Gαq 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-Gαq chimera containing only the last five amino acids of Gαq, whereas the mammalian assays were done in the presence of full-length human Gαq. In addition, the yeast system contains the yeast Gβγ complex of Ste4/Ste18, whereas the HEK293 cells contain mammalian Gβγ complexes.

To further investigate the minimal requirements of R89 for signaling through Gαq in mammalian cells we designed

| Full-Length Receptors | C5aR | Gγ | Gα |
|-----------------------|------|----|----|
| **CT1**              |      |    |    |
| R2                   |      |    |    |
| R8                   |      |    |    |
| R10                  |      |    |    |
| R13                  |      |    |    |
| R37                  |      |    |    |
| R40                  |      |    |    |
| R44                  |      |    |    |
| R45                  |      |    |    |
| R28                  |      |    |    |
| R29                  |      |    |    |
| R31                  |      |    |    |
| **CT2**              |      |    |    |
| R63                  |      |    |    |
| R89                  |      |    |    |
| R12                  |      |    |    |
| R13                  |      |    |    |
| R19                  |      |    |    |
| R21                  |      |    |    |
| R30                  |      |    |    |
| R31                  |      |    |    |

| Truncated Receptors | C5aR | Gγ | Gα |
|---------------------|------|----|----|
| **WT**              |      |    |    |
| R27                 |      |    |    |
| R29                 |      |    |    |
| R15                 |      |    |    |
| R18                 |      |    |    |
| R19                 |      |    |    |
| R24                 |      |    |    |
| R25                 |      |    |    |
| R32                 |      |    |    |
| R33                 |      |    |    |
| R35                 |      |    |    |
| R36                 |      |    |    |
| R38                 |      |    |    |
| R39                 |      |    |    |

FIGURE 5. Signaling of CT1 mutants via Gαq, Gγ, and Gα chimera. 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.

| 305 | 310 | 315 | 318 | Gγ | Gα |
|-----|-----|-----|-----|----|----|
| R2  |     |     |     | 50 | 50 | 0  |
| R8  |     |     |     | 50 | 50 | 0  |
| R10 |     |     |     | 50 | 50 | 0  |
| R13 |     |     |     | 50 | 50 | 0  |
| R37 |     |     |     | 50 | 50 | 0  |
| R40 |     |     |     | 50 | 50 | 0  |
| R44 |     |     |     | 50 | 50 | 0  |
| R45 |     |     |     | 50 | 50 | 0  |
| R28 |     |     |     | 50 | 50 | 0  |
| R29 |     |     |     | 50 | 50 | 0  |
| R31 |     |     |     | 50 | 50 | 0  |
| R63 |     |     |     | 50 | 50 | 0  |
| R89 |     |     |     | 50 | 50 | 0  |
| R12 |     |     |     | 50 | 50 | 0  |
| R13 |     |     |     | 50 | 50 | 0  |
| R19 |     |     |     | 50 | 50 | 0  |
| R24 |     |     |     | 50 | 50 | 0  |
| R25 |     |     |     | 50 | 50 | 0  |
| R32 |     |     |     | 50 | 50 | 0  |
| R33 |     |     |     | 50 | 50 | 0  |
| R35 |     |     |     | 50 | 50 | 0  |
| R36 |     |     |     | 50 | 50 | 0  |
| R38 |     |     |     | 50 | 50 | 0  |
| R39 |     |     |     | 50 | 50 | 0  |
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. 9a). 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-Hf treatment (Fig. 9b). 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\(_{i}/\)H\(_{9251}\)q in HEK293 cells by measuring IP\(_3\) accumulation (Fig. 9c). M1 does not show any activation of G\(_{i}/\)H\(_{9251}\)q, whereas M2 and M3 show weak activation upon stimulation with 1\%/M 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\(_{i}/\)H\(_{9251}\)q, in mammalian cells.

In addition to signaling through the G\(_{i}/\)H\(_{9251}\)q chimera in yeast, R89 was also able to signal through the G\(_{s}/\)H\(_{9251}\)s chimera. To test whether R89 could activate full-length G\(_{s}/\)H\(_{9251}\)s in mammalian cells, R89 and human G\(_{s}/\)H\(_{9251}\)s were co-expressed in HEK293 cells, and cAMP levels were measured. Upon treatment with 1 \(\mu\)M W5Cha, cAMP levels were unchanged indicating that R89 was unable to activate full-length G\(_{s}/\)H\(_{9251}\)s.
**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. 9d 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. 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.

**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 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...
G Protein Specificity of the C5aR

The mutants used in this study were initially selected by their ability to couple to a G_{i} chimaera, and there was no selective pressure for or against coupling to the G_{q} or G_{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

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 and analyzed by immunoblotting for C5aR. Endo-H-resistant receptors containing complex N-linked oligosaccharides (*) and Endo-H-sensitive high-mannose oligosaccharides (+) are shown. c, HEK293 cells transiently transfected with G_{i} or G_{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 backbone conformations found for wild-type (WT), R35, R52, R58, R89, 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.
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 Go 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 Goq. Many instances of broadened specificity resulting from receptor mutation have been reported in the literature. For example, a mutagenized V1a vasopressin receptor (24) or serotonin 5-HT1A receptor (23) as well as hybrid receptors between the V1a and V2 vasopressin receptors (5) or the β2-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 β2-adrenergic receptor, a loss of Goq activation is seen along with a gain of signaling through Goq (18). The loss of Goq signaling could be due to removal of residues that are essential for the activation of Goq along with removal of residues necessary for prevention of Goq 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 Goq or Goq coupling for signaling but also actively select against signaling through Goq. In fact, functional receptors selected in the Goq chimeric strain all retained the ability to signal through the Goq 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 Goq, Goq′ and Goq″ whereas the wild-type receptor only couples to Goq. 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 Goq). For example, the Q145R mutation in IC2 may allow for a positive interaction to occur with the Goq chimera, because nearly all of our receptors that couple to the Goq 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 Goq) contain this arginine, whereas neither the m2 nor m4 receptors do (both couple to Goq), and is supported by previous work demonstrating that this arginine is critical for Goq activation (19). The role of the Q145R mutation in coupling to Goq 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 Goq chimera coupling by mediating a direct interaction with the carboxyl-terminal tail of the Go subunit, then one might predict there to be corresponding candidate residues for interaction in the Goq tail versus Goq. Comparison of the Goq and Goq′ tails shows that the most dramatic amino acid change is from a glycine at position −3 to an asparagine (Fig. 1A). If residue 145 of the receptor interacts with the Goq 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 Goq 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 Goq′, rather the introduction of a large hydrophobic residue such as isoleucine in R89 or leucine in M4 is necessary to see robust activation of Goq. Thus it is not the loss of the asparagine but the gain of the bulky hydrophobic residue that promotes Goq′ coupling.
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The inability to unequivocally determine a direct contact between receptor residues such as Q145R and the last five amino acids of the Go subunit may be due to a role for the Go 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 Go 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 Go1 proteins containing isotope labels in the carboxyl terminus or of a 15N-labeled Go 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 Go subunit may sterically interfere with binding to the receptor interface, thereby encouraging the release of the activated Go 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 Go tail to the interior regions of the receptor that would result in G protein activation.

Why did only one of the six IC2 mutants that signaled through the Goα chimera in yeast demonstrate Goα 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, N146L, 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 Goα 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 C5aR3 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 carboxy-terminal half of IC3 as well as their adjoining transmembrane helix helices, whereas the main determinants for G protein specificity were clustered in the carboxy-terminal tail and carboxy-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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