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Molecular Characterization of the Gerbil C5a Receptor and Identification of a Transmembrane Domain V Amino Acid That Is Crucial for Small Molecule Antagonist Interaction*

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Stephen M. Waters[‡1], Robbin M. Brodbeck[‡], Jeremy Steflik[‡], Jianying Yu[‡], Carolyn Baltazar[‡], Amy E. Peck[‡], Daniel Severance[§], Lu Yan Zhang[¶], Kevin Currie[¶], Bertrand L. Chenard[¶], Alan J. Hutchison[¶], George Maynard[¶], and James E. Krause[‡]

From the Departments of [‡]Biochemistry and Molecular Biology, [§]Biophysical Chemistry, and [¶]Medicinal Chemistry, Neurogen Corporation, Branford, Connecticut 06405

Anaphylatoxin C5a is a potent inflammatory mediator associated with pathogenesis and progression of several inflammation-associated disorders. Small molecule C5a receptor (C5aR) antagonist development is hampered by species-specific receptor biology and the associated inability to use standard rat and mouse *in vivo* models. Gerbil is one rodent species reportedly responsive to small molecule C5aR antagonists with human C5aR affinity. We report the identification of the gerbil C5aR cDNA using a degenerate primer PCR cloning strategy. The nucleotide sequence revealed an open reading frame encoding a 347-amino acid protein. The cloned receptor (expressed in Sf9 cells) bound recombinant human C5a with nanomolar affinity. Alignment of the gerbil C5aR sequence with those from other species showed that a Trp residue in transmembrane domain V is the only transmembrane domain amino acid unique to small molecule C5aR antagonist-responsive species (*i.e.* gerbil, human, and non-human primate). Site-directed mutagenesis was used to generate human and mouse C5aRs with a residue exchange of this Trp residue. Mutation of Trp to Leu in human C5aR completely eliminated small molecule antagonist-receptor interaction. In contrast, mutation of Leu to Trp in mouse C5aR enabled small molecule antagonist-receptor interaction. This crucial Trp residue is located deeper within transmembrane domain V than residues reportedly involved in C5a- and cyclic peptide C5a antagonist-receptor interaction, suggesting a novel interaction site(s) for small molecule antagonists. These data provide insight into the basis for small molecule antagonist species selectivity and further define sites critical for C5aR activation and function.

Anaphylatoxin C5a is generated during complement system activation via the classical, alternative, or mannose-binding lectin pathway. C5a potently mediates inflammatory responses by increasing vascular permeability (1, 2), mast cell degranulation (3), smooth muscle contraction (4), neutrophil chemotaxis (5), and cytokine release (6, 7). Excessive production of C5a is implicated in several inflammatory disorders, including rheumatoid arthritis (8), asthma (9), cystic fibrosis (10), sepsis (11), psoriasis (12), and atherosclerosis (13). The effects of C5a are mediated through binding to its G-protein-coupled receptor (C5aR)[2] and the subsequent activation of pertussis toxin-sensitive G-proteins (14), mitogen-activated protein kinase (15, 16), and phospholipase C (17) and calcium mobilization. Inhibition of C5a activity has been sought through generation and use of anti-C5a peptide antibodies (18, 19), anti-C5aR antibodies (20), anti-C5 convertase antibodies (21), and C5aR antagonists (22–25). However, no C5a activity-modifying agent is currently approved for clinical use.

To date, C5aR has been cloned from human (14), rat (26), mouse (27), dog (28), rabbit (29), guinea pig (30), pig (partial; GenBank™ accession number AF284498), sheep (partial; GenBank™ accession number AF284499), and several non-human primates (partial) (31). Interestingly, C5aR sequence homology across these various species is unusually divergent. Overall C5aR sequence homology is >95% between human and non-human primate. Conversely, between human and non-primate C5aRs, homology is only 65–75%. These differences are unusual for G-protein-coupled receptors, which are typically 85–95% homologous across species. All full-length, recombinant, and natively expressed C5aRs, except rat (26), bind human C5a with high affinity (14, 27–30), suggesting relative conservation of C5a ligand-binding domains. However, cyclic peptide (32) and small molecule (24) C5aR antagonists demonstrate a greater degree of species selectivity. This suggests different C5aR binding and activation determinants for C5a peptide and small molecule antagonists.

A small molecule C5aR antagonist (W-54011) inhibits C5a-mediated responses in human, cynomolgus monkey, and gerbil neutrophils, but not in mouse, rat, guinea pig, rabbit, or dog neutrophils (24). Because of this observed small molecule antagonist species selectivity, we sought to clone gerbil C5aR and to identify amino acid residues potentially responsible for the observed species-selective pharmacology. In this study, we have identified the gerbil C5aR sequence and a key Trp residue in transmembrane domain (TM) V associated with small molecule antagonist binding and function. The location of this amino acid, in relation to those previously identified as important for C5a and cyclic peptide antagonist binding, suggests that the small molecule antagonists tested interact deeper within the transmembrane domains to affect C5aR signaling and function.

EXPERIMENTAL PROCEDURES

Materials—AcPhe[L-Orn-Pro-D-cyclohexylalanine-Trp-Arg] (AcF-[OPdChaWR]) was purchased from Biomolecules Midwest, Inc.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY220494 and AY220495.

[1] To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Neurogen Corp., 35 NE Industrial Rd., Branford, CT 06405. Tel.: 203-488-8201 (ext. 5320); Fax: 203-483-8317; E-mail: swaters@nrgn.com.

[2] The abbreviations used are: C5aR, C5a receptor; TM, transmembrane domain; AcF[OPdChaWR], AcPhe[L-Orn-Pro-D-cyclohexylalanine-Trp-Arg]; RACE, rapid amplification of cDNA ends; hW213L, human W213L; mL214W, mouse L214W; rhC5a, recombinant human C5a; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

(Waterloo, IL). NDT9520492 and W-54011 were synthesized at Neurogen Corp. Unless specified otherwise, all biochemical reagents were purchased from Sigma.

Molecular Cloning and Mutagenesis of C5aRs—Full-length coding regions of gerbil, human, and mouse C5aRs were cloned by PCR and individually subcloned into a baculoviral expression vector. Specifically, gerbil C5aR was cloned from Mongolian gerbil lung (Harlan Bioproducts for Science, Inc., Indianapolis, IN). Lung total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was generated using random primers and Powerscript™ reverse transcriptase (Clontech) according to the manufacturer's instructions. Gerbil cDNA was initially PCR-amplified using degenerate oligonucleotides based on TMI (5′-primer, 5′-TGCCCTGGTGGTSTGGGTGAC-3′) and TMVII (3′-primer, 5′-CGTAGATGATGGGGTTRAYRCAGC-3′) of previously published C5aRs (14, 26–31). Based on the resultant cDNA sequence (determined using an ABI PRISM 310 genetic analyzer, PerkinElmer Life Sciences), primers were generated to obtain gerbil C5aR 5′- and 3′-sequence by rapid amplification of cDNA ends (RACE; FirstChoice™ RLM-RACE kit, Ambion, Inc., Austin, TX). The 5′-fragment was generated using the gene-specific reverse primers 5′-CACCAGCAGGAAACGGTCG-3′ (outer) and 5′-CGTGCAGGCCAGGGAGCTGAAG-3′ (inner). The 3′-fragment was generated using the gene-specific forward primers 5′-GTAGCGGCCGTGGTGAGCTG-3′ (outer) and 5′-CTGGCTGCCCTCGGCCTCGC-3′ (inner). The full-length gerbil C5aR sequence was identified and generated by PCR using gerbil lung cDNA and primers 5′-CCCCACCATGGACCTCATCGACGAC-3′ (5′-primer, containing optimal Kozak sequence) and 5′-GTCCTGATGCCTCCTCTACAC-3′ (3′-primer). The resultant cDNA (GenBank™ accession number AY220495) was subcloned into pBacPAK8 (Clontech).

C57BL/6J mouse C5aR was generated from lung cDNA using primers 5′-CCACCATGGACCCCATAGATAACAG-3′ (5′-primer, containing optimal Kozak sequence) and 5′-CTACACCGCCTGACTCTTCC-3′ (3′-primer). The resultant cDNA (GenBank™ accession number AY220494) was subcloned into pBacPAK9 (Clontech). Of note, the C57BL/6J C5aR sequence we obtained differed from a previously published BALB/c mouse sequence (27) by nine nucleotides and seven deduced amino acids. The nucleotide sequence was verified from multiple clones from multiple cDNA synthesis reactions.

Human C5aR was generated from a fetal brain cDNA library using primers 5′-CCACCATGAACTCCTTCAATTATAC-3′ (5′-primer, containing optimal Kozak sequence) and 5′-CTACACTGCCTGGGTCTTCTG-3′ (3′-primer). The resultant cDNA sequence was identical to that obtained in previous studies (14, 33) and was subcloned into pBacPAK9.

Human W213L (hW213L) and mouse L214W (mL214W) C5aR mutants were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Specifically, hW213L C5aR was generated using human wild-type C5aR in pBacPAK9 template with primers 5′-GGGCTTCCTGTTGCCTCTAC-3′ (forward) and 5′-GTAGAGGCCACAGGAAGC CC-3′ (reverse). mL214W C5aR was generated using mouse wild-type C5aR in pBacPAK9 template with primers 5′-GGTTTTGTGTGGCCTCTGCTC-3′ (forward) and 5′-GAGCAGAGGCCACACAAAACC-3′ (reverse). The resultant cDNAs were sequence-verified to contain the appropriate mutations.

Recombinant Expression of C5aRs in Baculovirus-infected Sf9 Cells—Specific C5aR- and G-protein subunit-containing baculoviral expression vectors were cotransfected along with BaculoGold DNA (Pharmingen) into Sf9 cells. The Sf9 cell culture supernatant was harvested 3 days after transfection. The recombinant virus-containing supernatant was serially diluted in Hink's TNM-FH insect medium (JRH Biosciences, Inc., Lenexa, KS) supplemented with Grace's salts, 4.1 mM L-Gln, 3.3 g/liter lactalbumin hydrolysate, 3.3 g/liter ultrafiltered yeastolate, and 10% heat-inactivated fetal bovine serum (hereinafter referred to as "insect medium") and plaque-assayed for recombinant plaques. Recombinant baculoviral clones were amplified, and passage 3 baculoviral stocks were titered via plaque assay. A multiplicity of infection and incubation time course experiment was performed to determine optimal receptor expression conditions. The results from the receptor optimization experiment showed that a multiplicity of infection of 0.1 and a 72-h incubation were ideal infection parameters to achieve optimal C5aR expression in up to 1-liter Sf9 cell infection cultures.

Log-phase Sf9 cells were infected with recombinant baculoviral stock, followed by culture at 27 °C in insect medium. Infections were performed with C5aR in combination with G-protein-encoding viral stocks obtained from BioSignal Inc. (Montreal, Canada). Heterotrimeric G-protein subunit-expressing viral stocks were as follows: 1) rat $G\alpha_{i2}$ (BioSignal catalog no. V5J008), 2) bovine $G\beta_1$ (BioSignal catalog no. V5H012) and 3) human $G\gamma_2$ (BioSignal catalog no. V6B003). Infections were performed at a multiplicity of infection of 0.1:1.0:0.5:0.5 for C5aR, $G\alpha_{i2}$, $G\beta_1$, and $G\gamma_2$, respectively, followed by harvesting 72 h post-infection. A cell suspension sample was analyzed for viability by trypan blue dye exclusion, and the remaining Sf9 cells were harvested via centrifugation at 3000 rpm for 10 min at 4 °C.

^{125}I-Labeled Human C5a Affinity Binding—Sf9 cell pellets expressing C5aR, $G\alpha_{i2}$, $G\beta_1$, and $G\gamma_2$ were resuspended in homogenization buffer (10 mM HEPES, 250 mM sucrose, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 200 μM phenylmethylsulfonyl fluoride, and 2.5 mM EDTA (pH 7.4)) and homogenized. The homogenate was centrifuged at 536 × *g* for 10 min at 4 °C to pellet nuclei. The supernatant containing isolated membranes was centrifuged at 48,000 × *g* for 30 min at 4 °C and resuspended in 30 ml of homogenization buffer. This centrifugation and resuspension step was repeated twice. The final pellet was resuspended in ice-cold Dulbecco's phosphate-buffered saline containing 5 mM EDTA and stored in frozen aliquots at −80 °C until needed. The protein concentration of the resulting membrane preparation was determined using the Bradford protein assay (Bio-Rad). Purified P2 membranes were resuspended by Dounce homogenization in binding buffer (50 mM HEPES (pH 7.6), 120 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1% bovine serum albumin (pH 7.4), 0.1 mM bacitracin, and 100 kallikrein-inactivating units/ml aprotinin).

For saturation binding analysis, membranes (5 μg) were added to polypropylene tubes containing 0.005–0.500 nM ^{125}I-labeled recombinant human C5a (rhC5a; PerkinElmer Life Sciences) and binding buffer with the final concentrations of additives as indicated above. Nonspecific binding was determined in the presence of 300 nM rhC5a (Sigma) and accounted for <10% of total binding.

For competition analysis, Sf9 membranes were added to polypropylene tubes containing 0.030 nM ^{125}I-labeled rhC5a. For displacement binding studies, non-radiolabeled ligands were added at concentrations ranging from 10^{-10} to 10^{-5} M to yield a final volume of 0.250 ml. After a 2-h incubation at room temperature, reactions were terminated by rapid vacuum filtration. Samples were filtered over Whatman GF/C filters (presoaked in 1.0% polyethyleneimine for 2 h prior to use) and rinsed two times with 5 ml of cold binding buffer without bovine serum albumin, bacitracin, and aprotinin. The remaining bound radioactivity was quantified by γ-spectrometry. K_i and Hill coefficients were determined by plotting data as a log-logit function, and the 50% inhibition point was determined by using the linear portion of the data plot. Hill

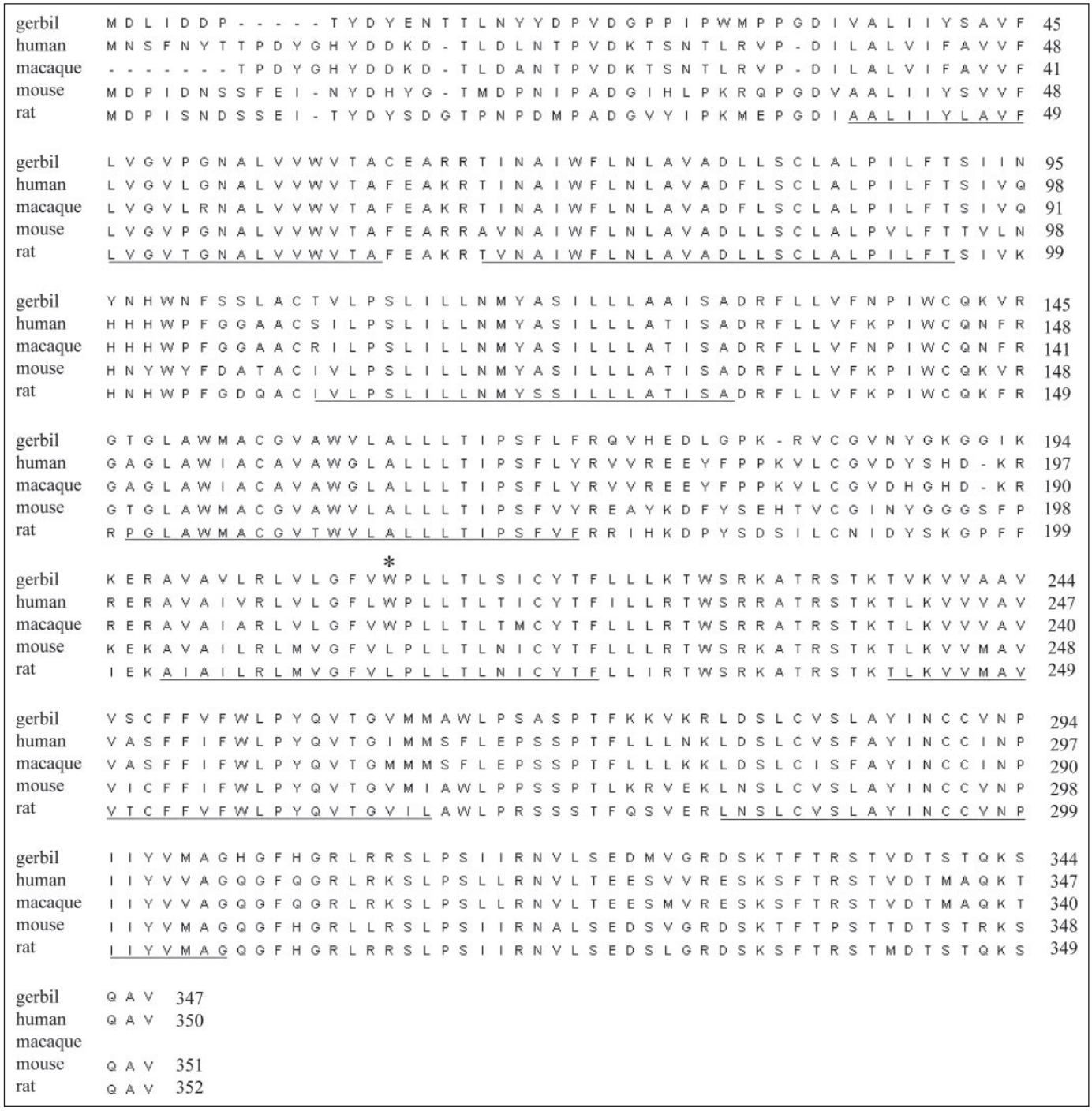

FIGURE 1. **Amino acid sequence comparison of gerbil, human, macaque, mouse, and rat C5aRs.** Putative transmembrane domains (53) are indicated by *underlined*. The *asterisk* indicates the TMV Trp residue crucial for small molecule antagonist-C5aR interaction. The nucleic acid sequences of gerbil (accession number AY220495) and C57BL/6J mouse (accession number AY220494) C5aRs were deposited in the GenBank™ Data Bank.

values were determined as the slope factor of the resultant linear transformed data.

[^{35}S]GTPγS Binding Assays—[^{35}S]GTPγS binding activity was measured using a modification of a previously described method (34). Sf9 cells recombinantly expressing C5aR and G-protein subunits $G\alpha_{i2}$, $G\beta_1$, and $G\gamma_2$ were used to prepare P2 membranes as described for affinity binding. Thawed membrane homogenates were resuspended in [^{35}S]GTPγS binding assay buffer (50 mM Tris (pH 7.0), 120 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 0.1% bovine serum albumin, 0.1 mM bacitracin, 100 kallikrein-inactivating units/ml aprotinin, and 5 μM GDP) and added to reaction tubes at a concentration of 30 μg/reaction tube. After adding test compounds at concentrations ranging from 0.01 nM to 10 μM, reactions were initiated by the simultaneous addition of 100 pM [^{35}S]GTPγS and rhC5a ranging in concentration from 0.001 nM to 10 μM (final assay volume of 0.250 ml). After a 60-min incubation at room temperature, reactions were terminated by vacuum filtration over Whatman GF/C filters (presoaked in wash buffer (50 mM Tris (pH 7.0) and 120 mM NaCl) containing 0.1% bovine serum albumin) with ice-cold wash buffer. Bound [^{35}S]GTPγS was determined by liquid scintillation spectrometry.

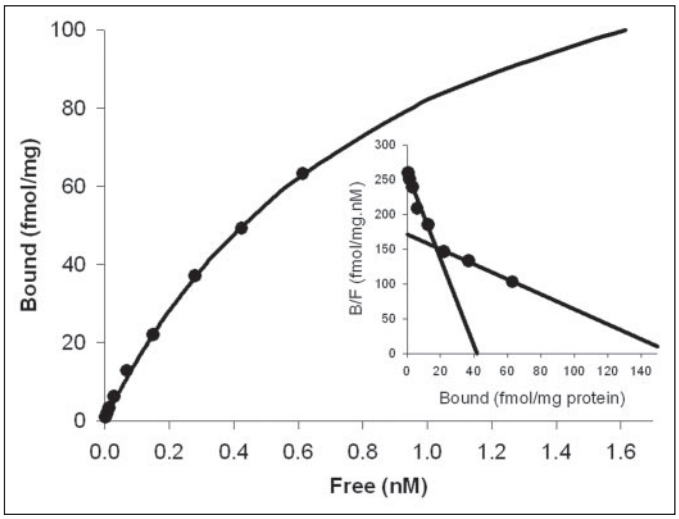

FIGURE 2. **Representative saturation binding curve for ^{125}I-labeled rhC5a binding to cloned gerbil C5aR.** Each of eight ^{125}I-labeled rhC5a concentrations was tested in duplicate. The K_d and B_{max} values were determined by analysis of the saturation isotherm data with SigmaPlot (Jandel Scientific). The data demonstrate a two-affinity state fit, with K_{d1} = 0.22 nM and K_{d2} = 0.91 nM and B_{max1} = 54 fmol/mg and B_{max2} = 157 fmol/mg, respectively. The *inset* shows the corresponding linear Rosenthal plot. *B/F*, bound/free.

TABLE ONE

Saturation binding analysis of wild-type and mutant C5aR-containing Sf9 cell membranes with ^{125}I-labeled rhC5a

P2 membranes prepared from Sf9 cells recombinantly expressing C5aR and G-protein subunits $G\alpha_{i2}$, $G\beta_1$, and $G\gamma_2$ were used to perform saturation radioligand binding assays as described under "Experimental Procedures." K_d and B_{max} values were determined by analysis of the saturation isotherm data with SigmaPlot.

Affinity state	K_d	B_{max}
	nM	*fmol/mg*
Gerbil wild-type receptor		
High	0.2	54
Low	0.9	157
Human wild-type receptor		
High	0.1	283
Low	1.2	1026
hW213L receptor		
High	0.2	198
Low	3.0	2381
Mouse wild-type receptor		
High	0.2	29
Low	2.3	238
mL214W receptor		
High	0.2	49
Low	2.9	298

Nonspecific binding was defined by 10 mM GTPγS and represented <5% of total binding.

RESULTS

Cloning and Sequence Analysis of Mongolian Gerbil C5aR—Gerbil C5aR was cloned based on the interspecies sequence homology of C5aR transmembrane domains. Using TMI and TMVII degenerate primers, a majority of the gerbil C5aR sequence was obtained. The 5′- and 3′-ends of the gerbil C5aR coding sequence were obtained by RACE, and a full-length cDNA was subsequently generated as described under "Experimental Procedures." An open reading frame of 1038 bp encoding a putative protein of 346 amino acids was identified (Fig. 1). The nucleotide sequence was verified from multiple clones from multiple cDNA synthesis reactions and deposited in the GenBank™ Data Bank with accession number AY220495. An overall homology comparison of gerbil, human, macaque, mouse, and rat proteins revealed that the gerbil C5aR sequence is more similar to the mouse and rat sequences (75%) than to the sequences from the other species (60–65%). The greatest homology between gerbil and other species was observed in the transmembrane domain regions (Fig. 1).

Characterization of Gerbil C5aR—Gerbil C5aR was coexpressed with $G\alpha_{i2}$, $G\beta_1$, and $G\gamma_2$ in Sf9 cells, and P2 membranes were assayed for ^{125}I-labeled rhC5a binding. As shown in Fig. 2, ^{125}I-labeled rhC5a bound gerbil C5aR in a saturable manner. Scatchard analysis of the saturation binding data indicated two high affinity states (Fig. 2, *inset*). The highest affinity state had a K_d of 0.22 nM and B_{max} of 54 fmol/mg of protein. The lower affinity state had a K_d of 0.91 nM and B_{max} of 157 fmol/mg of protein.

Peptide and Small Molecule Antagonist Competition Binding to Wild-type and Mutant C5aRs—C5a-induced Ca^{2+} mobilization is inhibited by a small molecule C5aR antagonist (W-54011) in gerbil, human, and non-human primate neutrophils, but not in the neutrophils of other species assayed (24). To determine which, if any, C5aR amino acid residue(s) are responsible for this species selectivity, the primary gerbil C5aR sequence was compared with all other reported C5aR sequences. A TMV Trp residue at position 210 of gerbil C5aR is the only transmembrane domain amino acid unique to gerbil, human, and non-human primate (Fig. 1). (Not all reported species are shown.) Other reported species contain Leu, Val, or Gly at this position. To evaluate the importance of this Trp residue, site-directed mutagenesis was used to generate both C57BL/6J mouse C5aR containing Trp at this position (mL214W) and the converse human C5aR mutant (hW213L). Saturation binding of wild-type and mutant C5aRs was determined using ^{125}I-labeled rhC5a. Displacement of ^{125}I-labeled rhC5a binding to wild-type and mutant C5aRs by peptide and small molecule C5aR antagonists was also determined.

The presence or exchange of the TMV Trp residue had no effect on ^{125}I-labeled rhC5a affinity binding. Each wild-type and mutant receptor demonstrated a two-affinity state fit, with comparable affinity observed for each receptor analyzed (TABLE ONE). However, the level of receptor expression for human and rodent receptors differed markedly (TABLE ONE). Human wild-type and mutant high affinity receptor sites were expressed 4–10-fold higher than gerbil or mouse receptor sites. Human wild-type and mutant low affinity receptor sites were expressed 10–15-fold higher than gerbil or mouse receptor sites.

Mutation of the TMV Trp residue had a minor effect on rhC5a displacement binding, as K_i values for wild-type and associated mutant C5aRs were slightly lower in receptors containing the TMV Trp residue (TABLE TWO). The cyclic peptide antagonist AcF[OPdChaWR] displaced ^{125}I-labeled rhC5a with the highest potency in human wild-type and hW213L C5aRs and with the lower potency in gerbil wild-type C5aR. No measurable AcF[OPdChaWR] displacement binding was detected in either mouse wild-type or mL214W C5aR. Therefore, peptide agonist (*i.e.* C5a) and peptide antagonist (*i.e.* AcF[OPdChaWR]) displacement binding was largely independent of the TMV Trp residue. In contrast, the TMV Trp residue was important for displacement binding of two unrelated small molecule antagonists. Both NDT9520492 (Fig. 3) and W-54011 showed displacement binding in human and gerbil wild-type C5aRs, with no measurable displacement binding in mouse wild-type C5aR (TABLE TWO). Substitution of the TMV Trp residue from human C5aR with leucine (hW213L) abolished measurable small molecule antagonist displacement binding. Mutation of the mouse TMV leucine residue at position 214 to Trp (mL214W) produced weak

TABLE TWO

Inhibition of ^{125}I-labeled rhC5a binding to wild-type and mutant C5aR-containing Sf9 cells by C5a or antagonist

P2 membranes from Sf9 cell membranes recombinantly expressing C5aR and G-protein subunits $G\alpha_{i2}$, $G\beta_1$, and $G\gamma_2$ were used to perform radioligand binding assays as described under "Experimental Procedures." K_i values are reported as means ± S.E. of four determinations.

Receptor	K_i			
	rhC5a	AcF[OPdChaWR]	W-54011	NDT9520492
	nM			
Gerbil wild-type	1.08 ± 0.03	456 ± 9.9	13.2 ± 0.09	108.8 ± 3.7
Human wild-type	0.35 ± 0.01	24.9 ± 1.2	4.4 ± 0.1	28.8 ± 1.5
hW213L	2.8 ± 0.03	49.3 ± 0.9	>10,000	>10,000
Mouse wild-type	5.8 ± 0.3	>10,000	>10,000	>10,000
mL214W	0.95 ± 0.04	>10,000	767 ± 16.0	>10,000

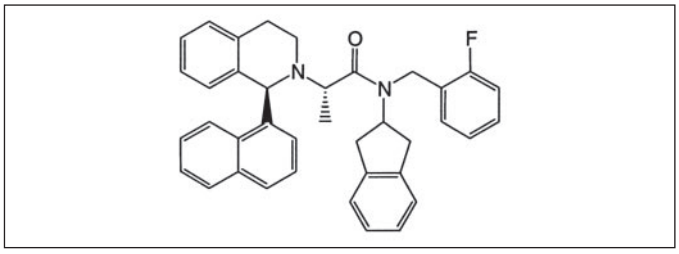

FIGURE 3. **Structure of NDT9520492.**

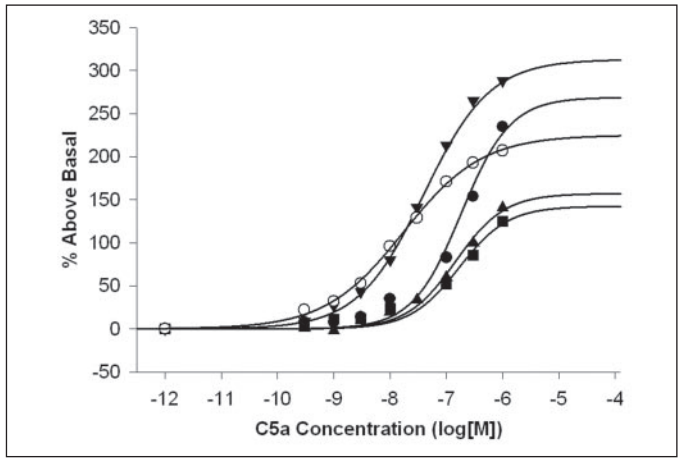

FIGURE 4. **Representative rhC5a-stimulated [^{35}S]GTPγS binding curves for gerbil wild-type C5aR (●), human wild-type C5aR (○), hW213L C5aR (▼), mouse wild-type C5aR (■), and mL214W C5aR (▲).** Each dose-response curve was tested in duplicate using eight concentrations of rhC5a. In each case, the indicated receptor was coexpressed along with the G-proteins $G\alpha_{i2}$, $G\beta_1$, and $G\gamma_2$. The EC_{50} and E_{max} values were determined by analysis of the stimulation curve data with SigmaPlot and are shown in TABLE THREE.

W-54011 displacement binding, whereas NDT9520492 remained unable to compete with ^{125}I-labeled rhC5a binding to this mouse mutant receptor.

Peptide and Small Molecule Antagonist Functional Activity for Wild-type and Mutant C5aRs—Because peptide and small molecule C5aR antagonist displacement binding showed differential dependence on the C5aR TMV Trp residue, we sought to examine compound functional activity for the various C5aRs using a [^{35}S]GTPγS binding assay. Each receptor analyzed produced a [^{35}S]GTPγS binding response in the presence of increasing concentrations of rhC5a (Fig. 4). The rhC5a EC_{50} and E_{max} values obtained for each receptor were not dependent on the presence or absence of the TMV Trp residue (TABLE THREE). rhC5a was most potent for human wild-type and hW213L mutant C5aRs, whereas the E_{max} values were similar for human wild-type, hW213L mutant, and gerbil wild-type C5aRs.

The cyclic peptide antagonist AcF[OPdChaWR] showed the greatest potency for human wild-type and hW213L C5aRs and lesser potency for gerbil wild-type C5aR (TABLE FOUR). As with displacement binding, no AcF[OPdChaWR] antagonist activity was observed in either mouse wild-type or mL214W C5aR. In contrast, inhibition of the [^{35}S]GTPγS binding activity of both small molecule antagonists was largely dependent on the TMV Trp residue. NDT9520492 and W-54011 potently inhibited [^{35}S]GTPγS binding to human wild-type, gerbil wild-type, and mL214W C5aRs. These compounds were relatively potent functional antagonists for mL214W despite having weak ^{125}I-labeled rhC5a displacement activity as described above. Both antagonists were inactive in mouse wild-type and hW213L C5aRs.

DISCUSSION

Inhibition of C5a function is believed to have therapeutic utility for several inflammatory disorders. Anti-C5a, anti-C5aR, and anti-C5 convertase antibodies and C5aR antagonists offer routes to block C5a action. Although anti-C5a and anti-C5 convertase antibody studies demonstrate the efficacy of this approach in relevant rat (19, 35) and mouse (21) models, use of these models for small molecule antagonist assessment is not possible due to species selectivity. The small molecule C5aR antagonist W-54011 inhibits C5a-induced Ca^{2+} mobilization in human, cynomolgus monkey, and gerbil neutrophils (24). Additionally, W-54011 dose-dependently reverses C5a-induced gerbil neutropenia (24), suggesting that gerbils may be an appropriate rodent model for *in vitro* and *in vivo* small molecule C5aR antagonist assessment. Although gerbil neutrophils serve as a C5aR source, recombinant receptor availability further facilitates *in vitro* characterization of receptor pharmacology.

In this work, we isolated and expressed a cDNA encoding gerbil C5aR. Overall, gerbil C5aR is more closely related to mouse and rat C5aRs than to C5aRs from other species. Because, unlike other reported rodent species, gerbil C5aR demonstrates small molecule C5aR antagonist responsiveness (24), we sought to determine which, if any, amino acids distinguish human, non-human primate, and gerbil C5aR pharmacology from the pharmacology of other species. Using multispecies C5aR homology analysis and site-directed mutagenesis, we identified a TMV Trp residue crucial for small molecule antagonist-C5aR interaction. Interestingly, substitution at the TMV Trp position had differential effects on C5a peptide, peptide antagonist, and small molecule antagonist binding and functional activity.

Displacement binding analyses of human wild-type, hW213L, mouse wild-type, and mL214W C5aRs showed that C5a peptide and cyclic peptide antagonists were not dependent on the presence of the TMV Trp residue. However, the two small molecule antagonists tested (NDT9520492 and W-54011) lost apparent affinity when the human C5aR TMV Trp residue was replaced with Leu. Additionally, although NDT9520492 and W-54011 did not demonstrate displacement binding

TABLE THREE

rhC5a-stimulated [^{35}S]GTPγS binding using wild-type and mutant C5aR-containing Sf9 cell membranes

P2 membranes prepared from Sf9 cells recombinantly expressing C5aR and G-protein subunits $G\alpha_{i2}$, $G\beta_1$, and $G\gamma_2$ were used to perform rhC5a-stimulated [^{35}S]GTPγS binding assays as described under "Experimental Procedures." EC_{50} and E_{max} values were determined by analysis of the dose-response data with SigmaPlot.

Receptor	EC_{50}	E_{max}
	nM	*% above basal*
Gerbil wild-type	167 ± 17	263 ± 9
Human wild-type	18.5 ± 2	218 ± 11
hW213L	35.5 ± 4	279 ± 48
Mouse wild-type	173 ± 5	120 ± 31
mL214W	159 ± 13	127 ± 42

TABLE FOUR

Effect of peptide and small molecule antagonists on wild-type and mutant C5aR [^{35}S]GTPγS binding activity

P2 membranes from Sf9 cell membranes recombinantly expressing C5aR and G-protein subunits $G\alpha_{i2}$, $G\beta_1$, and $G\gamma_2$ were used to perform [^{35}S]GTPγS binding assays as described under "Experimental Procedures." K_i values are reported as means ± S.E. of four determinations.

Receptor	K_i		
	AcF[OPdChaWR]	W-54011	NDT9520492
	nM		
Gerbil wild-type	82.3 ± 1.3	1.8 ± 0.1	7.6 ± 0.3
Human wild-type	27.7 ± 2.2	13 ± 0.6	15.2 ± 4.8
hW213L	25.0 ± 2.0	>10,000	>10,000
Mouse wild-type	>10,000	>10,000	>10,000
mL214W	>10,000	8.5 ± 0.4	24.2 ± 1.1

in mouse wild-type C5aR, replacement of Leu-214 with Trp partially restored W-54011 displacement binding and did not affect NDT9520492 displacement binding. The lack of full displacement binding "recovery" for small molecule antagonists in mL214W may be a function of the proposed two-site C5a-binding model (36–38). The two-site model suggests that the C5a peptide core initially interacts with the C5aR N terminus, and subsequently, the C5a C terminus interacts with the C5aR transmembrane and extracellular loop domains (37, 38). Therefore, as NDT9520492 and W-54011 likely interact mainly within the hydrophobic C5aR transmembrane domains, it is possible that these compounds are unable to fully displace ^{125}I-labeled rhC5a from the mouse C5aR N terminus and thus give an incomplete displacement binding profile.

Using a functional [^{35}S]GTPγS binding assay, we more clearly defined the importance of the TMV Trp residue for C5aR and small molecule antagonist interaction. NDT9520492 and W-54011 effects on [^{35}S]GTPγS binding inhibition were completely dependent on the presence of the TMV Trp residue, whereas rhC5a and AcF[OPdChaWR] effects were not dependent on this residue. This suggests different interaction points within the transmembrane domains for peptide agonists, small molecule antagonists, and cyclic peptide antagonists of C5aR.

Previous reports have identified C5a-C5aR interaction sites critical for receptor binding and activation. Regions associated with C5a binding include the N terminus, the second and third extracellular loops (39), and transmembrane domain amino acids with close proximity to extracellular domains (40). Truncation of the first 22 amino acids of human C5aR greatly diminishes C5a binding (38), as does mutation of Glu-199 in the second extracellular loop (41). Mutation of amino acids believed to form a C5a-binding pocket also may affect C5aR binding due to decreased efficiency of G-protein coupling and a subsequent shift in affinity state. Several of these amino acids (*e.g.* Arg-175, Arg-206, and Asp-282) are positioned near the cytoplasmic surface and underlie the relevance of C5a interaction with C5aR amino acids accessible to the extracellular membrane (40, 42, 43).

Cyclic peptide C5aR antagonists, including AcF[OPdChaWR] and related analogs, were originally designed based on the C terminus of C5a (44, 45). This region of C5a contains sequence necessary for C5aR activation (37, 38, 46). As such, it has been hypothesized that these cyclic peptide antagonists and the C terminus of C5a interact with similar C5aR domains. Indeed, cyclic peptide antagonists such as AcF[OPdChaWR] are predicted to interact with Glu-199, Arg-206, and Asp-282 of human C5aR based on mutation and modeling analyses (47). Interestingly, AcF[OPdChaWR] has high affinity for gerbil and human C5aRs and human, canine, and rat polymorphonuclear leukocytes, but poor affinity for mouse C5aR and rat polymorphonuclear leukocytes (32). Although rat and mouse C5aRs are homologous at previously reported points of AcF[OPdChaWR] interaction, the fact that rat and mouse C5aRs are only 79% homologous overall suggests that additional amino acids are important for the species selectivity of this cyclic peptide antagonist.

The data presented herein suggest that a TMV Trp residue in C5aR is crucial for non-peptide, small molecule antagonist interaction, whereas it has no effect on C5a or cyclic peptide antagonist interaction. The effect of small molecule antagonists on mL214W C5aR compared with the mouse wild-type receptor further illustrates the importance of this Trp residue for small molecule interaction. The TMV Trp residue present in gerbil, human, and non-human primate C5aRs lies approximately two α-helical turns below the Arg residue important for C5a and cyclic peptide antagonist interaction (Arg-206 in human as described above). In our hands, mutation of Arg-206 to Lys produced no effect on small molecule antagonism in [^{35}S]GTPγS binding (data not shown), supporting a relative lack of importance for the TMV Arg residue in small molecule antagonist function compared with the TMV Trp residue. That role of the TMV Trp residue was more evident in a functional assay than in displacement binding further suggests that this TMV amino acid is a critical site for small molecule antagonists to affect C5aR coupling to heterotrimeric G-proteins and activation.

The importance of human C5aR Trp-213 in the binding and functional efficacy of antagonists is interesting in light of evidence indicating that the C-terminal carboxylate of peptide C5aR agonists interacts directly with Arg-206 in the intrahelical crevice (42, 43). Because Trp-213 is located two helical turns below Arg-206, it is tempting to speculate that small molecule antagonists directly interact with Trp-213 deeper in the same binding pocket. However, receptor models based on the x-ray structure of rhodopsin (48) incorporate a bulge in TMV that would cause Trp-213 to be oriented away from the intrahelical crevice (*i.e.* facing the surrounding phospholipid membrane). In both rhodopsin and C5aR, a TMV bulge in the vicinity of residue 213 (residue 214 in rhodopsin) is made possible by the presence of a conserved Pro residue one amino acid toward the C-terminal end. However, twisting of TMV around Pro-215 appears to be facilitated in rhodopsin by the presence of a hydrogen bond network between Glu-122 in TMIII (49) and His-211 and between His-211 and Tyr-206 in TMV. In contrast, human C5aR has a Gly residue at position 211, eliminating the apparent driving force for twisting of TMV in this region. By analogy, TMI of rhodopsin, which contains comparably positioned residues Phe-45, Phe-52, and Pro-53, is observed in the x-ray structure to have Phe-45 and Phe-52 oriented on the same side of the helix. Another comparable group of residues (Ala-295, Asn-302, and Pro-303) exists in TMVII of rhodopsin. Again, Ala-295 and Asn-302 are positioned on the same side of the helix, although

with a slight helical twist. Taken together, the pivotal nature of Trp-213 in antagonist binding and functional efficacy and the lack of direct evidence for structural mimicry of TMV of C5aR with respect to the TMV bulge of rhodopsin suggest that Trp-213 is oriented toward the intrahelical space and on the same side of the helix as Arg-206.

It is worth noting that structural mimicry with respect to the TMV bulge of rhodopsin has been proposed for other G-protein-coupled receptors even though the His-211/Glu-122 pair is not conserved (50). Furthermore, it has been proposed that the flexibility at the top of TMV arising from Pro-215 in rhodopsin may be related to switching between active and inactive receptor conformations. For example, the dopamine D_2 receptor contains three Ser residues above Pro-201 that may form hydrogen bonds with backbone carbonyls from the preceding helical turn, causing a helical twist similar to the helical deformation seen in rhodopsin (51, 52). In C5aR, the Ser residues are replaced with Leu, Val, and Leu, again supporting an orientation of TMV that positions Trp-213 in the intrahelical crevice. In consideration of this analysis, it is possible that interaction of antagonists with Trp-213 prevents a conformational change around Pro-214 and, as a consequence, prevents reorientation of the top of TMV, thereby blocking receptor activation.

CONCLUSION

The discovery of orally bioavailable, small molecule C5aR antagonists has been hampered by medicinal chemistry barriers and species-specific receptor biology. The present identification of the gerbil C5aR sequence provides a reagent for *in vitro* compound assessment in a relevant *in vivo* model species. The discovery of a TMV Trp residue important for small molecule antagonist-C5aR interaction further clarifies the basis for species selectivity. Understanding this site of small molecule-receptor interaction may further define a target region for such organic molecules and enable refined structure-activity relationship analyses to generate more potent and selective antagonists for C5a-mediated disorders.

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ADDITIONS AND CORRECTIONS

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Regulation of the *Salmonella typhimurium* flavohemoglobin gene. A NEW PATHWAY FOR BACTERIAL GENE EXPRESSION IN RESPONSE TO NITRIC OXIDE.

Michael J. Crawford and Daniel E. Goldberg

Strain MCS38 has been found to have the *lacZ* integration in the *iroC* locus instead of the *hmp* locus and therefore reflects regulation of the *iroC* gene.

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PAGE 40622, LEFT COLUMN, LINE 45:

The last three words, "rat polymorphonuclear leukocytes," should be replaced with "mouse polymorphonuclear leukocytes."

We suggest that subscribers photocopy these corrections and insert the photocopies in the original publication at the location of the original article. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

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