ELSEVIER

Biochemical Pharmacology 61 (2001) 1571–1579

Biochemical Pharmacology

Modulation of ligand selectivity by mutation of the first extracellular loop of the human C5a receptor

Stuart A. Cain[a], Trent M. Woodruff[b], Stephen M. Taylor[b], David P. Fairlie[c], Sam D. Sanderson[d], Peter N. Monk[a,*]

[a]*Section of Neurology, Division of Clinical Sciences, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, UK*
[b]*Department of Physiology and Pharmacology, University of Queensland, Brisbane, Queensland 4072, Australia*
[c]*Centre for Drug Design and Development, University of Queensland, Brisbane, Queensland 4072, Australia*
[d]*Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, USA*

Received 21 November 2000; accepted 16 January 2001

Abstract

The cyclic C5a receptor antagonist, phenylalanine [L-ornithine-proline-D-cyclohexylalanine-tryptophan-arginine] (F-[OPchaWR]), has ~1000-fold less affinity for the C5a receptor (C5aR) on murine polymorphonuclear leukocytes than on human. Analysis of C5aR from different species shows that a possible cause of this difference is the variation in the sequence of the first extracellular loop of the receptor. The mouse receptor contains Y at a position analogous to P^{103} in the human receptor, and D at G^{105}. To test this hypothesis, we expressed human C5aR mutants ($P^{103}Y$, $G^{105}D$ and the double mutant, $P^{103}Y/G^{105}D$) in RBL-2H3 cells and investigated the effects of these mutations on binding affinity and receptor activation. All three mutant receptors had a higher affinity for human C5a than the wild-type receptor, but showed no significant difference in the ability of F-[OPchaWR] to inhibit human C5a binding. However, all of the mutant receptors had substantially lower affinities for the weak agonist, C5a des Arg^{74} ($C5adR^{74}$), and two altered receptors ($G^{105}D$ and $P^{103}Y/G^{105}D$) had much lower affinities for the C-terminal C5a agonist peptide analogue, L-tyrosine-serine-phenylalanine-lysine-proline-methionine-proline-leucine-D-alanine-arginine (YSFKPMPLaR). Although it is unlikely that differences at these residues are responsible for variations in the potency of F-[OPchaWR] across species, residues in the first extracellular loop are clearly involved in the recognition of both C5a and C5a agonists. The complex effects of mutating these residues on the affinity and response to C5a, $C5adR^{74}$, and the peptide analogues provide evidence of different binding modes for these ligands on the C5aR.

Keywords: Complement; C5a; Receptor; Antagonist; Mast cells

1. Introduction

The complement anaphylatoxin C5a is a potent activator of a wide variety of cell types, including mast cells, neutrophils, monocytes, and endothelial and epithelial cells. C5a, a 74-residue polypeptide, is involved in several stages of the inflammatory process, causing the chemotaxis and degranulation of leukocytes, enhancing vascular permeability, and stimulating cytokine production [1]. Peptide analogues of the C-terminal domain are full agonists at the C5a receptor (C5aR), suggesting that the C-terminus is solely responsible for receptor activation, the remainder of the molecule conferring high-affinity binding [2–4].

In vivo, C5a is rapidly degraded to $C5adR^{74}$ by serum carboxypeptidase [5]. The dR^{74} form of C5a has a different pattern of activity to intact C5a. For instance, human neutrophil chemotaxis is 10^1- to 10^2-fold less sensitive to

* Corresponding author. Tel.: +44 114 226 1312; fax: +44 114 276 0095.

E-mail address: p.monk@shef.ac.uk (P.N. Monk).

Abbreviations: C5aR, human complement fragment 5a receptor; WT, wild-type; $G^{105}D$, C5aR mutated to aspartate at $glycine^{105}$; $P^{103}Y$, C5aR mutated to tyrosine at $proline^{105}$; $P^{103}Y/G^{105}D$, C5aR containing both substitutions; $C5adR^{74}$, des arginated C5a; F-[OPchaWR], phenylalanine [L-ornithine-proline-D-cyclohexylalanine-tryptophan-arginine]; MeFK-PchaWr, *N*-methyl-L-phenylalanine-lysine-proline-D-cyclohexylalanine-tryptophan-D-arginine; PMN, polymorphonuclear leukocytes; PCR, polymerase chain reaction; YSFKPMPLaR, L-tyrosine-serine-phenylalanine-lysine-proline-methionine-proline-leucine-D-alanine-arginine; and YSFKD(MeNle)PIAR, L-tyrosine-serine-phenylalanine-lysine-aspartate-*N*-methyl-norleucine-proline-D-leucine-alanine-arginine.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00608-6

C5adR[74] than to C5a, whereas human monocytes show a much smaller difference between the ability of these ligands to cause chemotaxis and polarisation [6,7]. Similar variations in cellular responsiveness have also been observed with peptide analogues of the C-terminus of C5a. One such peptide, YSFKD(MeNle)PlAR, unlike the standard agonist YSFKPMPLaR, is capable of causing changes in blood pressure at doses that spare the activation of PMNs *in vivo* [8]. Recently, C5aR antagonists have been developed from C-terminal peptide analogues. A linear peptide, MeFK-PchaWr [9], and a cyclic peptide, F-[OPchaWR], have been shown to inhibit C5a binding and function at human and rat C5aRs [10]. Antagonists can also discriminate between C5aR on different cell types: F-[OPchaWR] is 30-fold more potent on human PMNs than the linear antagonist MeFK-PchaWr, but both compounds are equally potent on human umbilical artery macrophages [10].

The molecular basis for these cellular differences in the ability to discriminate between agonists, antagonists, and intact C5a/C5adR[74] has yet to be fully elucidated. A single receptor for C5a (hC5aR) has been cloned and is a member of the G-protein-coupled receptor superfamily [11,12]. Two of the extracellular loops (the second and third) and the N-terminal domain are essential for C5a binding [13]. The receptor N-terminus is required for high-affinity binding of C5a, but not for receptor activation by C5a or small peptide agonists derived from the C-terminus of C5a [14]. Studies with C5aR antagonists, using PMN from different species, have shown very large differences in receptor affinities, measured as competition for receptor binding with human C5a. For F-[OPchaWR], species may be placed in three groups: high affinity ($IC_{50} < 1$ μM; rat, dog, human), intermediate affinity (IC_{50} = 1–100 μM; guinea pig, sheep, rabbit, pig), and low affinity ($IC_{50} > 100$ μM; mouse) [15]. The rank order for MeFKPchaWr is similar to the cyclic antagonist, whereas the rank order of affinity for the peptide agonist, YSFKPMPLaR, is completely different: $IC_{50} < 1$ μM; rabbit, mouse, sheep, guinea pig, rat, $IC_{50} > 1$ μM; human, pig, dog [15]. In contrast, there were no significant differences in affinity for human C5a. It is likely that these differences are due to species-specific variations in the primary structure of C5aRs and may indicate that peptide agonists and antagonists have different binding sites on C5aR.

We investigated this ligand selectivity by introducing mutations into the human C5aR to test the hypothesis that the first extracellular loop contains critical residues that determine antagonist affinity/potency. We found that mutant human C5aRs had identical affinities to wild-type receptor for the antagonist F-[OPchaWR]. However, the mutated receptors showed a complicated pattern of affinities and responsiveness to C5a, C5adR[74], and the peptide analogues of the C5a C-terminus, suggesting that residues in the first extracellular loop are involved in the formation of the ligand-binding site. This is the first report of a role for this loop in C5aR activation and ligand binding.

2. Materials and methods

2.1. Cloning and partial sequencing of sheep and pig C5aR

The total RNA was isolated from the white cell fraction using Ultraspec total RNA reagent (Biotecx) as described in the manufacturer's instructions. First-strand cDNA synthesis was primed using an oligo(dT) primer (100 pmol) and translated using Superscript II (GIBCO BRL). The cDNA was used as a template for PCR using primers designed on the conserved regions of the C5aR, taken from sequence alignment of the known species. The primers, which map to the transmembrane helices I and VII, had the following sequences: sense 5′-CAATGCCCTGGTGGTCTGGGT-GAC-3′, antisense 5′-GGGTT(G/A/T)A(C/T)(G/A)CAG-CA(A/G)TTGATG TA(G/A)GC. The PCR was performed at 94° for 30 sec, 50° for 30 sec, and 72° for 1 min for 30 cycles. The resulting PCR products were isolated from an agarose using a NucleoSpin Extract kit (Macherey-Nagel) and ligated into the pCRII vector of the TA cloning kit (Invitrogen). For each receptor, inserts from 5 different colonies were sequenced using ABI Big Dye terminator cycle sequencing kit. Sheep and Pig C5a receptor DNA sequences have been deposited with GenBank. Accession numbers: Sheep C5a receptor, AF284499; pig C5a receptor, AF284498.

2.2. Construction of human C5aR mutants

The mutant C5aRs, $P^{103}Y$, $G^{105}D$, and the double mutant were constructed by overlap extension mutagenesis as described previously [16]. The mutagenesis primers for the $P^{103}Y$ mutant were: sense 5′-ACCACTGG**TA**CTTTG-GCGG-3′, antisense 5′-CCGCCAAAG**TA**CCAGTGGT-3′; for the $G^{105}D$ mutant: sense 5′-GCCCTTTG**A**CGGGGCC-3′, antisense 5′-GGCCCCG**T**CAAAGGGC-3′, and for the double mutant: sense 5′-CCACTGG**TA**CTTTG**A**CGGGGCC-3′, antisense 5′-GGCCCCG**T**CAAAG**TA**CCAGTGG-3′. The mutated nucleotide sequence is shown in bold. The start and end primers for C5aR had the sequences of sense 5′-GCGC*AAGCTT*GCCGCCACCATGAACTCCTTCAA-TTATACCACCC-3′ and antisense 5′-GCGCG*AATTC*TTATTACTACACTGCCT-GGGTCTTCTGG-3′, the added *Hind* III and *Eco*R I restriction sites are shown respectively in *italics*, and the Kozak sequence used is shown underlined. PCR reactions were performed in 100-μL reactions containing 10 ng of template, 2 mM $MgCl_2$, 250 μM of each dNTP, 50 pmol of each primer, and reaction buffer supplied by the manufacturer (Boeringher Mannheim). The PCR was performed at 94° for 45 sec, 45° for 30 sec, and 72° for 1 min for 30 cycles, followed by incubation at 72° for 6 min. The half-reactions and full-length second-round PCR products were purified from agarose gels using a NucleoSpin Extract kit (Macherey-Nagel). The full-length PCR products were di-

gested with EcoR I and Hind III (Boeringher Mannheim) and ligated into the expression vector pEE6. The C5aR mutant clones were sequenced using ABI Big Dye terminator cycle sequencing kit, and the correct constructs were purified using Nucleobond Kit PC500 (Macherey-Nagel).

2.3. Transfection and cell culture

RBL-2H3 cells were routinely cultured in Dulbecco's modified Eagle's medium + 10% (v/v) fetal bovine serum, which was supplemented with 400 mg/L G-418 for transfected cells, at 37°, 5% CO_2. RBL-2H3 cells were transfected by electroporation, as previously described [16]. A monoclonal antibody (S5/1; Serotec) that recognises the entire N-terminal sequence of the C5aR was used to sort the highest 50% of transfected cells on a Becton Dickinson Vantage flow cytometer in two rounds of fluorescence-activated cell sorting.

2.4. Production of peptides and recombinant C5a and C5adR[74]

Wild-type C5a and C5adR[74] were produced in *Escherichia coli* and purified by the methods described in [17]. Agonist and antagonist peptides were synthesised as described previously [10].

2.5. Measurement of receptor activation of RBL cells

Receptor activation was measured as the release of β-hexosaminidase from intracellular granules, as described [17]. The percentage of β-hexosaminidase release was calculated as a percentage of the maximal release (in response to 250 nM C5a, Fig. 2). Total β-hexosaminidase content was determined following cell lysis with 0.1% Nonidet P40. Assay of the antagonist activity was performed as described above, except that the antagonists were added at varying concentrations for 15 min before the addition of C5a at a final concentration of 100 nM. IC_{50}, EC_{50}, and standard error values were obtained by iterative curve fitting using GraphPad Prism 2.0.

2.6. ^{125}I-C5a binding assay

Binding assays using 50 pM ^{125}I-C5a were performed on adherent C5aR-transfected RBL cells in 96-well microtitre plates as described previously [16]. Binding levels are shown in Fig. 2. IC_{50} and standard error values were obtained by iterative curve fitting using GraphPad Prism 2.0.

3. Results

3.1. Partial sequencing of sheep and pig C5aR cDNA

Sheep and pig C5aR cDNA was cloned using PCR primers designed to be complementary to sequences in the highly conserved transmembrane helices I and VII. The protein sequences are shown in Fig. 1, aligned with C5aR from human, mouse, rat, rabbit, cow, guinea pig, and dog. The sequences were analysed for differences in extracellular or transmembrane domains consistent with the variations in affinity for the cyclic peptide antagonist, F-[OPchaWR], across species. In particular, differences between mouse and rat receptors would be interesting, because the rank order of affinity for F-[OPchaWR] shows that mouse PMNs are the least sensitive and rat PMNs the most [15]. In extracellular loop 2, S^{193} is conserved in the three high-affinity species (human, rat and dog), but is G in the other species. However, this position can be either G or S in the non-human primate C5aR sequences [18] and thus was not considered important. Other positions showing differences between these two species did not vary consistently across all species, but two positions in extracellular loop 1 sequences that showed the greatest consistency were selected. P^{103} in hC5aR is conserved in the high-potency group (human, rat, dog, and non-human primates), but is either S or Y in all of the intermediate-affinity species (sheep, pig, cow, and guinea pig) except rabbit. The lowest-affinity species, mouse, has a C5aR that is unique in having Y^{103} and also D instead of G^{105}.

3.2. Expression of mutant human C5aR in RBL-2H3 cells

We examined the possibility that residues in loop 1 could influence the response to F-[OPchaWR] by mutating human C5aR P^{103} to Y (P^{103}Y) and G^{105} to D (G^{105}D) individually and together (P^{103}Y/G^{105}D), i.e. making the human receptor resemble mouse at these positions. WT and mutant C5aR were transfected into RBL-2H3 cells, and stable transfectants obtained by selection with G-418. Homogenous populations of cells were collected by two rounds of fluorescence-activated cell sorting, selecting for the top 50% of expressing cells using an anti-C5aR monoclonal antibody. Receptor expression levels were measured as the specific binding of 50 pM ^{125}I-C5a and by immunofluorescence (Fig. 2). By these criteria, receptor expression was similar on all cell lines. Degranulation of cell lines in response to a high dose of C5a (250 nM) was also assessed, and was found to vary widely between cell lines (Fig. 2).

3.3. The effects of loop 1 mutations on receptor affinity

The affinity of WT and mutant C5aR for C5a, the antagonist F-[OPchaWR], the partial agonist C5adR[74], and the C-terminal agonist YSFKPMPLaR was measured by pretreating transfected RBL cells with these agents, then incubating with 50 pM ^{125}I-C5a (Table 1). All of the mutant receptors appeared to have a 2–3 fold higher affinity for C5a than WTC5aR (Table 1). The double mutant had a similar affinity to the single mutant receptors, indicating that the effect is not additive. In contrast, affinity for C5adR[74] was significantly lowered by all the mutations (Table 1).

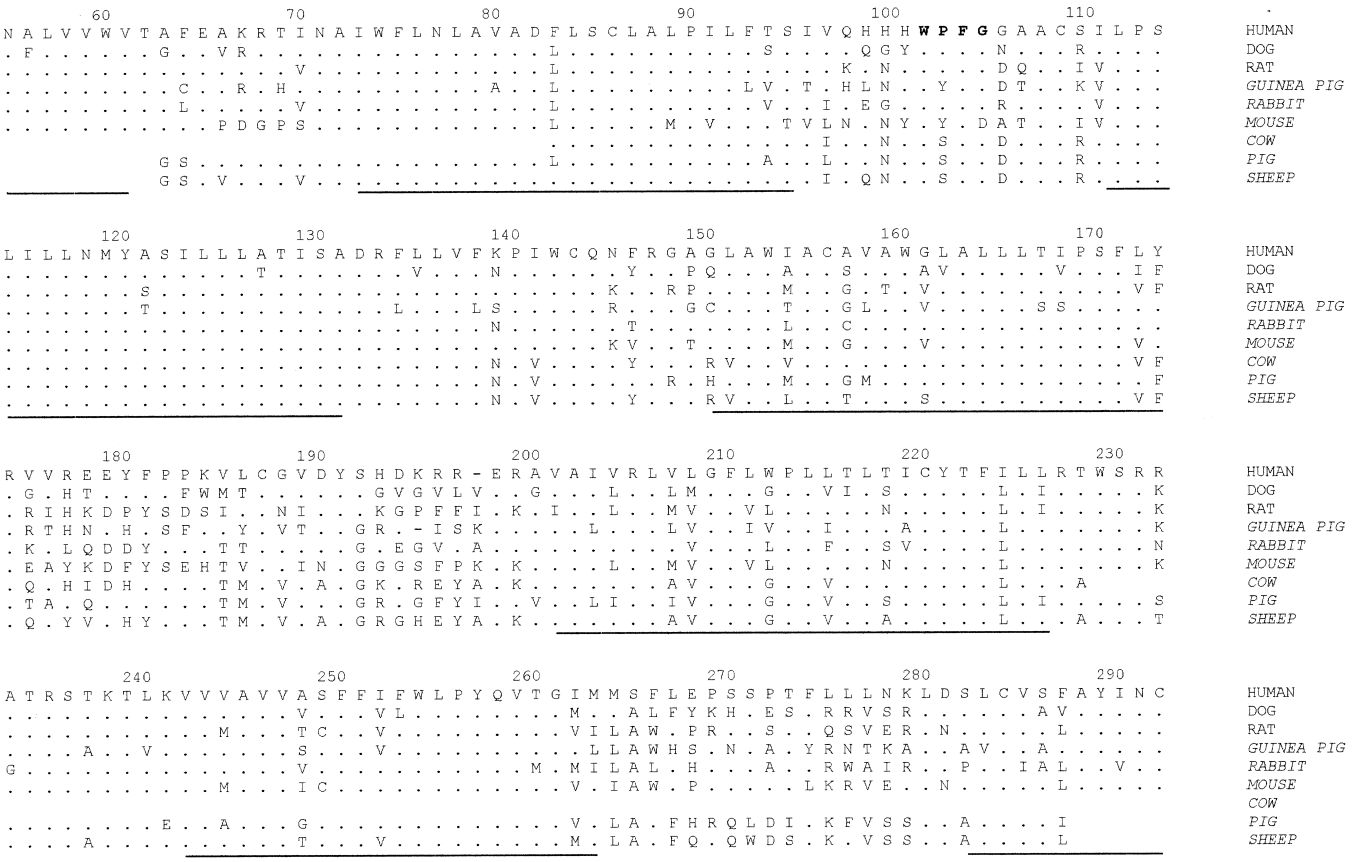

Fig. 1. Sequences of C5aRs. The partial sequences of human [11,12], dog [20], rat [21,22], guinea pig [23], rabbit [24], mouse [25], cow [20], pig, and sheep are shown. Species with neutrophils that have an intermediate or low affinity for the peptidic antagonist F-[OPchaWR] are shown in italics. Putative transmembrane domains are underlined. Residues 102–105 (WPFG) of the human receptor are shown in bold. Accession numbers: rat C5aR, P97520; dog C5aR, S27357; human C5aR, A37963; rabbit, AAF13030; guinea pig, AAC40074.

WTC5aR bound C5adR^{74} with a 283-fold lower affinity than intact C5a; this ratio was 985, 1873, and 2423 for G^{105}D, P^{103}Y, and the double mutant, respectively. Despite these large differences, there was no significant effect of the mutations on the sensitivity to the antagonist, F-[OPchaWR] (Table 1). In contrast, affinity for the peptide agonist, YSFKPMPLaR, was decreased, but only by the G^{105}D mutation; a similar loss of affinity was also observed in the double mutant (Table 1).

3.4. The effects of loop 1 mutations on receptor activation

In contrast to the effects on binding affinity, the two single mutants did not increase the ability of the receptors to stimulate degranulation with intact C5a, and a small decrease in sensitivity (i.e. an increase in the EC_{50} value) was seen with the double mutant (Fig. 3, Table 2). The degranulation response to C5adR^{74} also differed from the binding results. The P^{103}Y and double mutants had decreased sensitivity to the truncated ligand, whereas G^{105}D was identical to WTC5aR (Fig. 3, Table 2). The maximal response was similarly affected by loop 1 mutations: C5adR^{74} stimulated 80% of the maximal response to C5a with WT and G^{105}D C5aR, but only 60% with P^{103}Y and the double mutant (i.e. is only a partial agonist; Fig. 3). With the peptide agonist YSFKPMPLaR, only the double mutant showed a significant decrease in sensitivity (Fig. 3, Table 2). However, the P^{103}Y and double mutations appeared to respond supramaximally to YSFKPMPLaR, with a 20% higher degranulation response compared to C5a (Fig. 3). This supra-maximal response was also seen with the macrophage-selective peptide agonist, YSFKD(MeNle)PlAR (Fig. 3). The EC_{50} values were always much higher for YSFKD(MeNle)PlAR than for YSFKPMPLaR (Table 2). Interestingly, receptors containing the G^{105}D mutation showed a significant increase in sensitivity to this peptide (Fig. 3, Table 2). This effect is more marked when shown relative to the sensitivity to YSFKPMPLaR: the ratio of EC_{50} values for these peptides was 43 and 71 for WT and P^{103}Y C5aR, but only 22 and 16 for G^{105}D and the double mutant receptors, respectively. The single mutants had opposite effects on antagonism by F-[OPchaWR]. The P^{103}Y receptor had a decreased IC_{50}, whereas the G^{105}D mutation significantly increased IC_{50} (Fig. 4, Table 2). The combination of both mutations negated these effects, giving a similar IC_{50} value for the double mutant and for WTC5aR (Fig. 4, Table 2). The linear

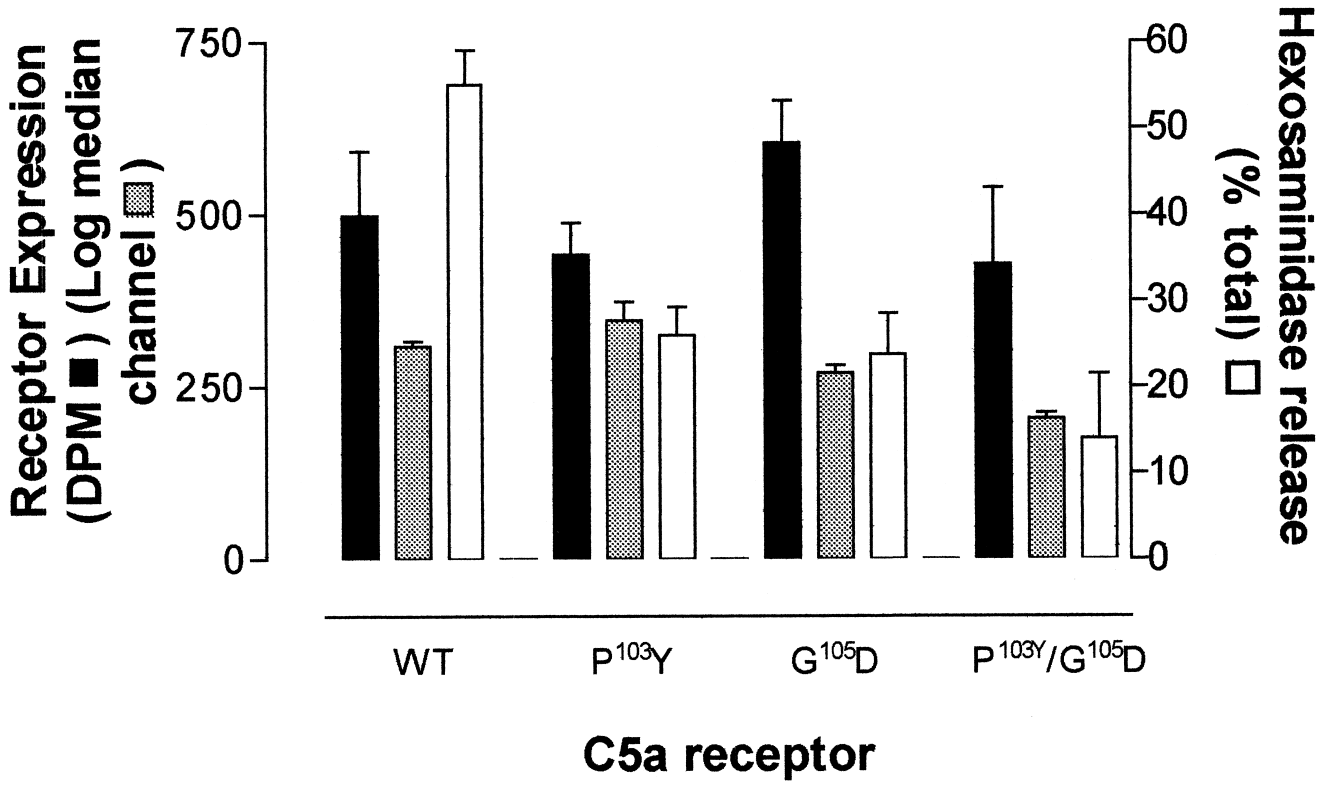

Fig. 2. Binding of C5a and degranulation by RBL cells transfected with WT and mutant C5aR. RBL cells transfected with WT, $P^{103}Y$, $G^{105}D$, or $P^{103}Y/G^{105}D$ C5aR were incubated with 50 pM ^{125}I-C5a to assess C5aR expression levels (black filled bars). The results show specific binding of ^{125}I-C5a as dpm/well to adherent cells in a 96-well microtitre plate, mean ± SD from 2–4 separate experiments performed in triplicate. Receptor expression levels were also measured by immunofluorescence (gray filled bars), and are shown as log median channel numbers, mean ± SD from a single experiment performed in duplicate. The maximal degranulation levels of these cell lines (open bars) in response to 250 nM C5a are shown as the percentage of total cellular β-hexosaminidase released into the supernatant, performed in duplicate.

antagonist MeFKPchaWr showed essentially the same pattern as F-[OPchaWR] (Fig. 4).

4. Discussion

The cyclic peptide, F-[OPchaWR], is a potent antagonist of C5a binding to PMNs. However, there are wide variations in affinity when using PMNs from other species. Mouse PMNs have ~1000-fold lower affinity for F-[OPchaWR] than rat or human PMNs, despite having a similar affinity for human C5a [15]. We hypothesised that this difference is likely due to the species-specific variations in sequence of the activation domains of the C5aR.

We cloned and partly sequenced the C5aR from sheep and pig PMN preparations, and compared these with other known C5aR sequences to detect sequence variations that correlated with the observed variations in F-[OPchaWR]

Table 1
Binding affinities of C5a receptor ligands

Receptor	WTC5aR			P^{103}YC5aR			G^{105}DC5aR			$P^{103}Y/G^{105}$DC5aR		
	pD_{21}[a] ± SE	IC_{50}[b] (nM)	N[c]	pD_{21} ± SE	IC_{50} (nM)	N	pD_{21} ± SE	IC_{50} (nM)	N	pD_{21} ± SE	IC_{50} (nM)	N
C5a	8.30 ± 0.05	5.01	4	8.83 ± 0.05***	1.48	2	8.64 ± 0.04**	2.29	2	8.84 ± 0.07***	1.44	3
C5adR[74]	5.84 ± 0.04	1450	4	5.56 ± 0.04**	2750	2	5.65 ± 0.04*	2240	2	5.48 ± 0.11*	3548	3
YSFKPMPLaR	5.77 ± 0.06	1700	4	5.83 ± 0.07	1480	2	5.43 ± 0.08*	3720	2	5.68 ± 0.09*	2900	3
F-[OPchaWR]	7.37 ± 0.06	42.7	4	7.48 ± 0.07	33.1	3	7.40 ± 0.03	39.8	3	7.70 ± 0.14	20.0	3

[a] $pD_{21} = -\log IC_{50}$.
[b] IC_{50} = concentration resulting in 50% inhibition of the maximum binding of 50 pM ^{125}I-C5a.
[c] N = number of separate experiments performed in triplicate.
Significantly different from WTC5aR: * <5%; ** <0.5%; *** <0.005% (two-tailed *t*-test).

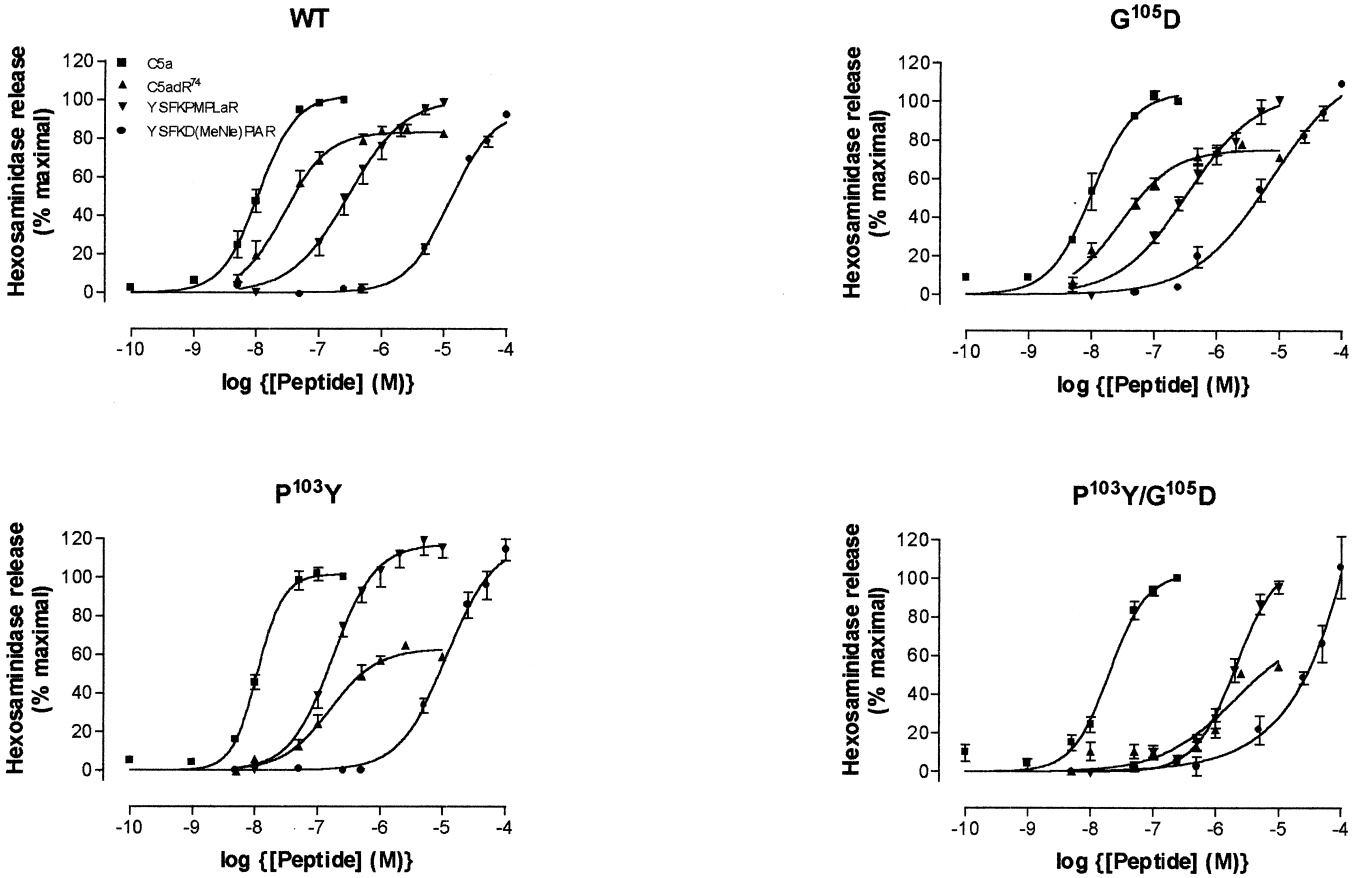

Fig. 3. The stimulation of degranulation in RBL cells transfected with WT and mutant C5aR. RBL cells transfected with WT, $P^{103}Y$, $G^{105}D$, or $P^{103}Y/G^{105}D$ C5aR were incubated with the stated concentrations of C5a, YSFKPMPLaR, YSFKD(MeNle)PlAR, or $C5adR^{74}$. Degranulation was assessed as the release of β-hexosaminidase, as described in Materials and Methods, and is shown as a percentage of degranulation in response to 250 nM C5a. Results are the means of at least three separate experiments (see Table 2) performed in triplicate ± SE.

affinity. It seemed most likely that receptor residues involved in F-[OPchaWR] binding would lie in the extracellular or transmembrane domains associated with receptor activation, rather than in the receptor N-terminus that appears to merely increase the affinity for C5a. The first extracellular loop 1 showed species-dependent sequence

Table 2
The effects of C5aR ligands on the degranulation of transfected RBL cells

Receptor	WTC5aR			P^{103}YC5aR			G^{105}DC5aR			$P^{103}Y/G^{105}$DC5aR		
	pD_{2E}[a] ± SE	EC_{50}[b] (nM)	N[c]	pD_{2E} ± SE	EC_{50} (nM)	N	pD_{2E} ± SE	EC_{50} (nM)	N	pD_{2E} ± SE	EC_{50} (nM)	N
C5a	7.97 ± 0.05	10.7	4	7.96 ± 0.03	11.0	3	8.00 ± 0.06	10.0	3	7.70 ± 0.06*	20.0	4
$C5adR^{74}$	7.55 ± 0.05	28.2	3	6.81 ± 0.08**	155	3	7.54 ± 0.06	28.8	3	5.87 ± 0.13***	1350	3
YSFKPMPLaR	6.54 ± 0.08	288	4	6.77 ± 0.05	170	4	6.47 ± 0.09	339	3	5.68 ± 0.09***	2090	4
YSFKD(MeNle) PlAR	4.91 ± 0.06	12300	6	4.92 ± 0.04	12000	4	5.13 ± 0.05*	7410	6	4.49 ± 0.06**	32400	4
	pD_{21} ± SE[d]	IC_{50}[e] (nM)	N	pD_{21} ± SE	IC_{50} (nM)	N	pD_{21} ± SE	IC_{50} (nM)	N	pD_{21} ± SE	IC_{50} (nM)	N
F-[OPchaWR]	7.87 ± 0.06	13.5	3	8.10 ± 0.03*	7.94	3	7.29 ± 0.16*	51.3	3	7.70 ± 0.14	20.0	3
MeFKPDchaWr	6.04 ± 0.13	912	5	6.85 ± 0.06*	141	2	4.75 ± 0.25**	17782	2	6.33 ± 0.14*	468	2

[a] $PD_{2E} = -\log EC_{50}$.
[b] EC_{50} = concentration resulting in 50% of maximal degranulation.
[c] N = number of separate experiments performed in triplicate.
[d] $PD_{21} = -\log IC_{50}$.
[e] IC_{50} = concentration resulting in 50% inhibition of the maximum degranulation.
Significantly different from WTC5aR: * <5%; ** <0.5%; *** <0.005% (two-tailed *t*-test).

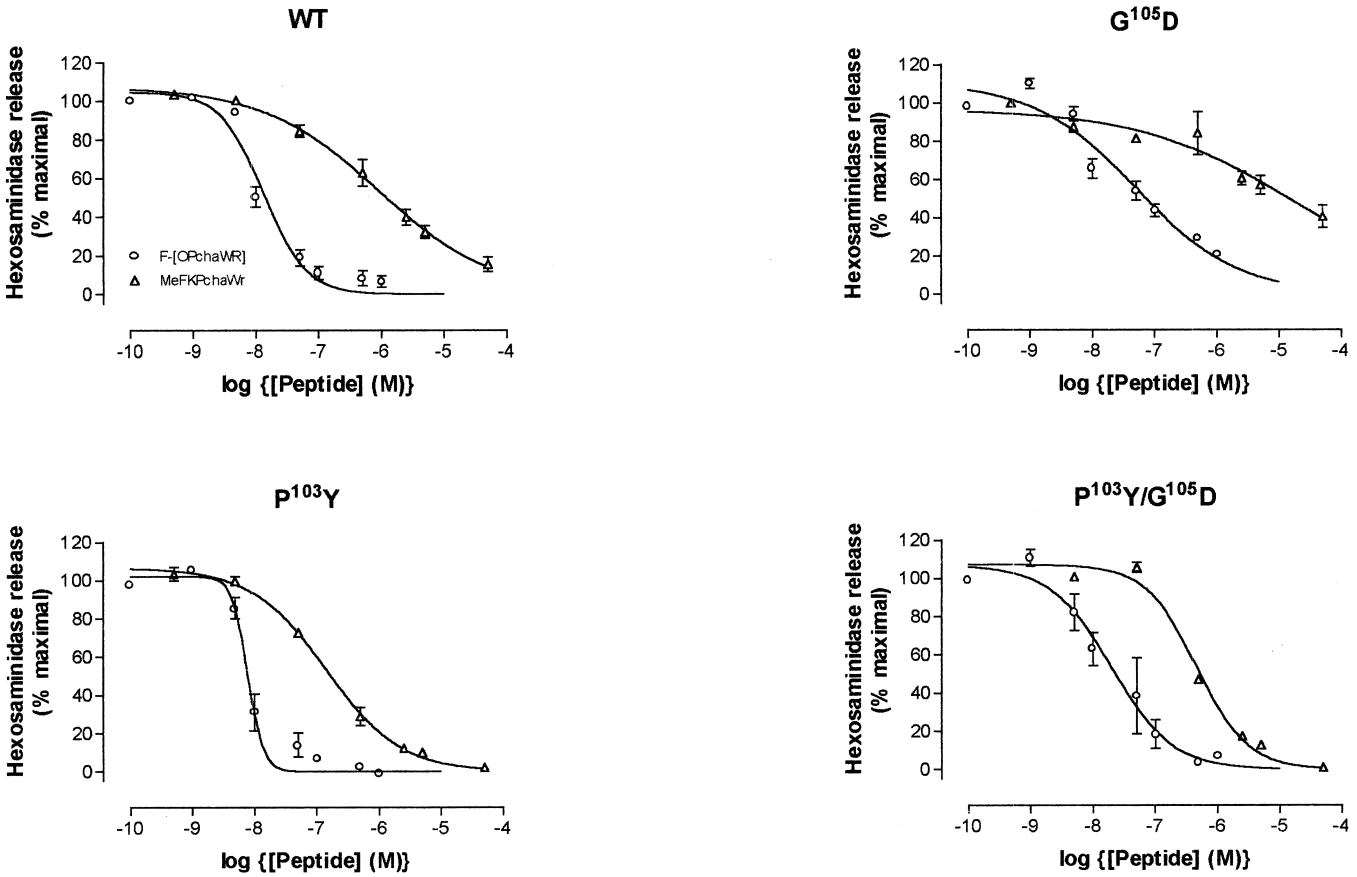

Fig. 4. Inhibition of C5a-stimulated degranulation in RBL cells transfected with WT and mutant C5aR. RBL cells transfected with WT, $P^{103}Y$, $G^{105}D$, or $P^{103}Y/G^{105}D$ C5aR were incubated with the C5aR antagonists F-[OPchaWR] or MeFKPchaWr for 15 min prior to the addition of 100 nM rhC5a. Degranulation was assessed as the release of β-hexosaminidase, as described in Materials and Methods. Results are the means of at least three separate experiments (see Table 2) performed in triplicate ± SE.

differences that appear to correlate with observed antagonist affinities. In the centre of the loop, a P residue (P^{103} of hC5aR) is either S or Y in all of the intermediate- and low-affinity receptors except rabbit. Near this, a G residue (G^{105} of hC5aR) is conserved in all species except mouse, suggesting that this region of loop 1 could be involved in the differential sensitivity to F-[OPchaWR]. To test this possibility, we mutated the human C5aR to give three mutants: $P^{103}Y$, $G^{105}D$, and a double mutant containing both mutations and expressed them in RBL-2H3 cells.

It is immediately obvious from the I^{125}-C5a binding data that these mutations had no effect on the affinity for F-[OPchaWR]. However, the affinity for other C5aR ligands was changed by the loop 1 mutations. $C5adR^{74}$ has previously been shown to bind to WTC5aR expressed in RBL cells with lower affinity than intact C5a [16]. The mutated receptors had a much lower affinity for $C5adR^{74}$ than WTC5aR, despite having a higher affinity for intact C5a, suggesting that loop 1 may be involved in ligand selectivity. In contrast, only $G^{105}D$ and the double mutant had decreased affinity for the C-terminal analog, YSFKPMPLaR. This suggests a specific involvement of this residue in the recognition of the peptide agonist. However, a study of mouse and human PMN C5a receptors has shown that although affinities for C5a and $C5adR^{74}$ are similar, the affinity of mouse PMNs for YSFKPMPLaR is significantly higher than that of human PMNs [15]. Thus the effects of $G^{105}D$ and $P^{103}Y$ on affinity for these ligands must be compensated by sequence variations for binding elsewhere in the mouse receptor.

The stimulation of degranulation in transfected RBL cells also indicates that the loop 1 mutations are not responsible for species-specific variations observed in the potency of F-[OPchaWR]. Indeed, $P^{103}Y$ actually increased the effectiveness of the antagonist slightly, although $G^{105}D$ caused a decrease and the double mutant showed no net change. The same pattern was seen with the linear peptide antagonist, MeFKPchaWR. The response to C5a was identical for WT and the single mutant receptors but slightly decreased in the double mutant, despite the higher binding affinity of all of these receptors for C5a. This is further evidence of the dissociation between binding affinity and receptor activation previously noted for this ligand [16], which is probably due to the physical separation of the

high-affinity binding and receptor activation sites [14]. The response to C5adR^{74} also shows this dissociation effect. The P^{103}Y and the double mutants showed significant decreases in sensitivity, in line with the binding affinity pattern observed for C5adR^{74}, whereas G^{105}D had no effect on activation by this ligand. In contrast, changes in sensitivity to receptor activation by agonist YSFKPMPLaR mirror the changes in receptor affinity. Interestingly, C5aR mutated at G^{105}D was more sensitive to the macrophage-selective analog YSFKD(MeNle)PlAR, suggesting an involvement of loop 1 in the ligand selectivity mechanism for peptide analogs, which bind only at the receptor activation site [14]. Receptors containing P^{103}Y exhibited a supra-maximal response to both agonist peptides. This is not due simply to a deleterious effect of this mutation on the C5a recognition site (P^{103}Y C5aR has a higher affinity for C5a than WTC5aR) and may be due to a specific effect on the receptor activation site that improves peptide access. Despite the increased maximal response to peptide, the P^{103}Y mutation diminished the maximal response to the partial agonist, C5adR^{74}. Taken together, these data suggest that extracellular loop 1 of the C5aR is involved in the formation of both ligand-binding and receptor activation sites. Peptide agonists, antagonists, and the C-termini of C5adR^{74} and C5a have different modes of interaction with C5aR that are differentially affected by mutations in loop 1.

In a previous study, a chimeric C5aR containing the first extracellular domain of the formyl peptide receptor bound intact C5a with an identical affinity to WTC5aR, and so this loop was not thought to be involved in the ligand interaction [13]. However, the formyl peptide receptor has an identical sequence to C5aR around P^{103} (WPFG), as do many other peptide-binding G-protein-coupled receptors. The conservation of loop 1 sequence in these receptors suggests that the structure of this region may be involved in receptor regulation or ligand binding. However, the present report is the first demonstration of a role for this loop in C5aR.

It is unclear from our data whether the mutation of P^{103} and G^{105} affects direct contacts with ligand or if they promote subtle changes to the conformation of the ligand-binding pocket of C5aR. However, the C-terminal of this loop, the transmembrane helix III, contains several residues that have been shown to be involved in ligand binding and receptor activation [19]. As there is no evidence for subtypes of the C5aR to explain cell type-specific responses to C5aR ligands, there must be an additional, perhaps conformational mechanism that controls the affinity of hC5aR. This additional mechanism, possibly acting through differential receptor glycosylation or G-protein coupling, operates for peptide antagonists, the cell type-selective peptide agonists, and the truncated ligand C5adR^{74}. The elucidation of this mechanism, which may involve the first extracellular loop, would greatly aid the design of cell type-specific antagonists for the C5aR.

Acknowledgments

This work was supported by a fellowship grant (M0543) from the Arthritis Research Campaign and a Wellcome Trust project grant (007521) (both to P.M.N.) and an NHMRC Australian project grant (9937208) (to S.M.T./D.F.).

References

[1] Gallin JI, Goldstein IM, Snyderman R. Inflammation: basic principles and clinical correlates, 1st Edn. New York: Raven Press, 1992.
[2] Mollison KW, Mandecki W, Zuiderweg EP, Fayer L, Fey TA, Krause RA, Conway RG, Miller L, Edalji RP, Shallcross MA, Lane B, Fox JL, Greer J, Carter GW. Identification of receptor-binding residues in the inflammatory complement protein C5a by site-directed mutagenesis. Proc Natl Acad Sci USA 1989;86:292–6.
[3] Bubeck P, Grotzinger J, Winkler M, Kohl J, Wollmer A, Klos A, Bautsch W. Site-specific mutagenesis of residues in the human C5a anaphylatoxin which are involved in possible interaction with the C5a receptor. Eur J Biochem 1994;219:897–904.
[4] Toth MJ, Huwyler L, Boyar WC, Braunwalder AF, Yarwood D, Hadala J, Haston WO, Sills MA, Seligmann B, Galakatos N. The pharmacophore of the human C5a anaphylatoxin. Protein Sci 1994;3:1159–68.
[5] Bokisch VA, Muller-Eberhard HJ. Anaphylatoxin inactivator of human plasma: its isolation and characterization as a carboxypeptidase. J Clin Invest 1970;49:2427–34.
[6] Yancey KB, Lawley TJ, Dersookian M, Harvath L. Analysis of the interaction of human C5a and C5a des Arg with human monocytes and neutrophils: flow cytometric and chemotaxis studies. J Invest Dermatol 1989;92:184–9.
[7] Marder SR, Chenoweth DE, Goldstein IM, Perez HD. Chemotactic responses of human peripheral blood monocytes to the complement-derived peptides C5a and C5a des Arg. J Immunol 1985;134:3325–31.
[8] Short AJ, Paczkowski NJ, Vogen SM, Sanderson SD, Taylor SM. Response-selective C5a agonists: differential effects on neutropenia and hypotension in the rat. Br J Pharmacol 1999;128:511–4.
[9] Konteatis ZD, Siciliano SJ, Van Riper G, Molineaux CJ, Pandya S, Fischer P, Rosen H, Mumford RA, Springer MS. Development of C5a receptor antagonists. Differential loss of functional responses. J Immunol 1994;153:4200–5.
[10] Paczkowski NJ, Finch AM, Whitmore JB, Short AJ, Wong AK, Monk PN, Cain SA, Fairlie DP, Taylor SM. Pharmacological characterisation of antagonists of the C5a receptor. Br J Pharmacol 1999;128:1461–6.
[11] Gerard C, Gerard NP. The chemotactic receptor for human C5a anaphylatoxin. Nature 1991;349:614–7.
[12] Boulay F, Mery L, Tardif M, Brouchon L, Vignais P. Expression cloning of a receptor for C5a anaphylatoxin on differentiated HL-60 cells. Biochemistry 1991;30:2993–9.
[13] Pease JE, Burton DR, Barker MD. Generation of chimeric C5a/formyl peptide receptors: towards the identification of the human C5a receptor binding site. Eur J Immunol 1994;24:211–5.
[14] De Martino JA, Van Riper G, Siciliano SJ, Molineaux CJ, Konteatis ZD, Rosen H, Springer MS. The amino terminus of the human C5a receptor is required for high affinity C5a binding and for receptor activation by C5a but not C5a analogs. J Biol Chem 1994;269:14446–50.
[15] Woodruff TM, Strachan AJ, Sanderson SD, Monk PN, Wong AK, Fairlie DP, Taylor SM. Species variability in the binding affinities of new small molecule C5a receptor agonists and antagonists. Inflammation 2001;25:171–7.

[16] Crass T, Bautsch W, Cain SA, Pease JE, Monk PN. Receptor activation by human C5a des Arg74 but not intact C5a is dependent on an interaction between Glu199 of the receptor and Lys68 of the ligand. Biochemistry 1999;38:9712–7.
[17] Cain SA, Ratcliffe CF, Williams DM, Harris V, Monk PN. Analysis of receptor/ligand interactions using whole-molecule randomly-mutated ligand libraries. J Immunol Methods 2000;245:139–45.
[18] Alvarez V, Coto E, Setien F, Gonzalez-Roces S, Lopez-Larrea C. Molecular evolution of the N-formyl peptide and C5a receptors in non-human primates. Immunogenetics 1996;44:446–52.
[19] Baranski TJ, Herzmark P, Lichtarge O, Gerber BO, Trueheart J, Meng EC, Iiri T, Sheikh SP, Bourne HR. C5a receptor activation. Genetic identification of critical residues in four transmembrane helices. J Biol Chem 1999;274:15757–65.
[20] Perret JJ, Raspe E, Vassart G, Parmentier M. Cloning and functional expression of the canine anaphylatoxin C5a receptor. Evidence for high interspecies variability. Biochem J 1992;288:911–7.
[21] Rothermel E, Zwirner J, Vogt T, Rabini S, Gotze O. Molecular cloning and expression of the functional rat C5a receptor. Mol Immunol 1997;34:877–86.
[22] Akatsu H, Miwa T, Sakurada C, Fukuoka Y, Ember JA, Yamamoto T, Hugli TE, Okada H. cDNA cloning and characterization of rat C5a anaphylatoxin receptor. Microbiol Immunol 1997;41:575–80.
[23] Fukuoka Y, Ember JA, Yasui A, Hugli TE. Molecular cloning of two isoforms of the guinea pig C3a anaphylatoxin receptor: alternative splicing in the large extracellular loop. Int Immunol 1998;10:275–83.
[24] Bachvarov DR, Houle S, Bachvarova M, Bouthillier J, St-Pierre SA, Fukuoka Y, Ember JA, Marceau F. Cloning and preliminary pharmacological characterization of the anaphylatoxin C5a receptor in the rabbit. Br J Pharmacol 1999;128:321–6.
[25] Gerard C, Bao L, Orozco O, Pearson M, Kunz D, Gerard NP. Structural diversity in the extracellular faces of peptidergic G-protein-coupled receptors. Molecular cloning of the mouse C5a anaphylatoxin receptor. J Immunol 1992;149:2600–6.