Receptor Activation by Human C5a des Arg^{74} but Not Intact C5a Is Dependent on an Interaction between Glu^{199} of the Receptor and Lys^{68} of the Ligand†

Torsten Crass,‡ Wilfried Bautsch,‡ Stuart A. Cain,§ James E. Pease,|| and Peter N. Monk*,§

Department of Medical Microbiology, Medizinische Hochschule, Hannover, Germany, Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, U.K., and Department of Applied Pharmacology, Imperial College School of Medicine at the National Heart & Lung Institute, London, U.K.

Received January 21, 1999; Revised Manuscript Received May 12, 1999

ABSTRACT: Despite the expression of only one type of receptor, there is great variation in the ability of different cell types to discriminate between C5a and its more stable metabolite, C5a des Arg^{74}. The mechanism that underlies this phenomenon is not understood but presumably involves differences in the interaction with the C5a receptor. In this paper, we have analyzed the effects of a substitution mutation of the receptor (Glu^{199} → Lys^{199}) and the corresponding reciprocal mutants (Lys^{68} → Glu^{68}) of C5a, C5a des Arg^{74} and peptide analogues of the C-terminus of C5a on the ability of the C5a receptor to discriminate between ligands with and without Arg^{74}. The use of these mutants indicates that the Lys^{68}/Glu^{199} interaction is essential for activation of receptor by C5a des Arg^{74} but not for activation by intact C5a. The substitution of Asp for Arg^{74} of C5a [Lys^{68}] produces a ligand with equal potency on both the wild-type and mutant receptors, suggesting that it is the C-terminal carboxyl group rather than the side chain of Arg^{74} that controls the responsiveness of the receptor to Lys^{68}. In contrast, the mutation of Lys^{68} to Glu^{68} has little effect on the ability of either C5a or C5a des Arg^{74} to displace [^{125}I]C5a from the receptors, indicating that binding of ligand and receptor activation are distinct but interdependent events. C5a and the truncated ligand, C5a des Arg^{74}, appear to have different modes of interaction with the receptor and the ability of the human C5a receptor to discriminate between these ligands is at least partly dependent on an interaction with the receptor residue, Glu^{199}.

The complement anaphylatoxin C5a is a potent activator of a wide variety of cell types, including mast cells, neutrophils, monocytes, endothelial, and epithelial cells. C5a is involved in several stages of the inflammatory process, causing the chemotaxis and degranulation of leukocytes, enhancing vascular permeability, and stimulating cytokine production (*1*); production of an effective inhibitor of C5a activity is therefore a high priority. The structure of C5a has been elucidated by NMR studies and consists of a helical N-terminus, a disulfide-linked core, and a loop-helix C-terminal domain (*2, 3*). Mutational studies have indicated that certain residues are essential for C5a function: His^{15} and Lys^{19,20} between the N-terminus and the core region; Arg^{37,40,46} in the disulfide-linked core; and Lys^{68}, Leu^{72}, and Arg^{74} of the C-terminus (*4*−*6*). These residues have been postulated to interact with the cell surface receptor for C5a, although the specific sites of interaction have not been fully elucidated. In vivo, C5a is rapidly degraded to C5a des Arg^{74} by serum carboxypeptidase (*7*). The des Arg^{74} form of C5a has a different pattern of activity to intact C5a, having little spasmogenic or anaphylactic activity and being generally less active in other assays, but this is dependent on the cell type and response under investigation. For instance, human neutrophil chemotaxis is 10^1-10^2-fold less sensitive to C5a des Arg^{74} than to C5a (*8, 9*); in contrast, human monocytes show a much smaller difference between the ability of these ligands to cause chemotaxis and polarization. In binding assays, the des Arg^{74} form was clearly less active than C5a (*8, 9*) although these authors disagree over the extent of the disparity between binding and biologic activity. C5a des Arg^{74} is 1 order of magnitude less potent than C5a on the stimulation of degranulation in resting human basophils whereas basophils primed with interleukins 3 or 5 respond equally well to both (*10*). Histamine release from subsets of mast cells which express the C5a receptor, (isolated from skin and heart and the mast cell line, HMC-1, but not lung, uterus or tonsil) is 10^3-10^4-fold less sensitive to C5a des Arg^{74} than to C5a (*11*−*13*). C5a and C5a des Arg^{74} are equipotent chemoattractants for human skin fibroblasts (*14*). The glycosylation status of C5a also affects the potency of the des Arg^{74} form. Human C5a is heavily glycosylated (M_r = 3000) on Asn^{64}, near the C-terminus. Removal of polysaccharide has been shown to have no affect on the response of neutrophils to intact C5a, but to increase the activity of C5a des Arg^{74} by almost 10-fold (*15*).

† This work is supported by a fellowship grant from the Arthritis and Rheumatism Campaign M0543 (to P.N.M.), the British Heart foundation project Grant PG95119 (to P.N.M.) and a grant from the Niedersachsische Vorab der Volkswagenstiftung (to W.B.).

* To whom correspondence should be addressed: Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Sheffield, S10 2UH. Phone: +44 114 2224188. Fax: +44 114 2728697. E-mail: p.monk@shef.ac.uk

‡ Department of Medical Microbiology.

§ Krebs Institute for Biomolecular Research.

|| Department of Applied Pharmacology.

10.1021/bi990139q CCC: $18.00
Published on Web 07/02/1999

The molecular basis for these differences in the ability to discriminate between intact C5a and C5a des Arg^{74} is unknown. A single receptor for C5a (hC5aR)[1] has been cloned and found to be a member of the G protein-coupled receptor superfamily, containing seven transmembrane domains connected by intra- and extracellular loops. The intracellular loops and the C-terminus are involved with G protein binding and activation whereas two of the extracellular loops (the second and third) and the N-terminal domain appear to be essential for ligand binding (*16*−*18*). Deletion of 22 amino acids of the N-terminus inhibits high-affinity binding of C5a but does not inhibit receptor activation, either by C5a or by peptide mimics of the C-terminus of C5a (*19*, *20*). In confirmation of this, antibodies raised against the receptor N-terminus can block high-affinity binding of C5a, but do not prevent receptor activation by peptide mimics (*21*, *22*). Therefore, it appears that distinct sites on the receptor are necessary for ligand binding and for receptor activation. We have previously shown that mutation of Glu^{199} to Gln in the second extracellular loop increases the ED_{50} of C5a and a peptide analogue of the C-terminus of C5a (peptide 1: TyrPheLysAlaChaChaLeuDPheArg) acting on receptors transfected into a model cell line, rat basophilic leukaemia cells (*23*). It has also been proposed that Arg^{206}, in the fourth transmembrane domain, has a role in ligand binding possibly as a counterion for the carboxylate group of Arg^{74} (*24*, *25*). However, no receptor mutations have yet been reported which discriminate between intact C5a and the truncated ligand, C5a des Arg^{74}.

In this paper, we have investigated the nature of the interaction between Lys^{68} of C5a and Glu^{199} of C5aR by constructing reciprocal mutants, in which Glu^{199} is mutated to Lys and the Lys^{68} analogue is changed to Glu (peptide 5: TyrPhe**Glu**AlaChaChaLeuDPheArg; C5a[Glu^{68}]; C5a[Glu^{68}] des Arg^{74}). We show that the Lys^{68}/Glu^{199} interaction is essential for receptor activation by C5a des Arg^{74} but not by intact C5a. However, this effect on receptor activation is unrelated to the affinity of the receptor for C5a des Arg^{74}, which remains largely unchanged. This is the first report of a mutation in the C5aR which has dramatically different effects on the responses to C5a des Arg^{74} and intact C5a and suggests that these ligands have distinct modes of receptor activation.

EXPERIMENTAL PROCEDURES

Tissue Culture. RBL-2H3 cells were routinely cultured in Dulbecco's modified Eagle's medium + 10% (*v*/*v*) fetal calf serum, which was supplemented with 400 mg/L G-418 for transfected cells, at 37 °C, 5% CO_2.

Peptide Synthesis. Agonistic peptide analogues of the C-terminus of hC5a (peptide 1, TyrPhe**Lys**AlaChaChaLeuDPheArg (*26*); peptide 2, TyrPhe**Glu**AlaChaChaLeuDPheArg) were synthesized on a Milligen 9050 peptide synthesizer, using standard *N*-(9-fluorenyl)methoxycarbonyl amino acid derivatives and analyzed by mass spectrometry.

Transfection and DNA Manipulation. Stable transfection of RBL-2H3 cells was achieved by electroporation, as previously described (*27*), using the pEE6hCMV.neo vector containing either a wild-type or a mutant hC5aR cDNA insert in which the glutamate residue at position 199 was changed to lysine (Lys^{199}) by the overlap extension method as described previously (*27*). A rabbit antiserum, raised against a peptide analogue of the entire N-terminal sequence of the C5a receptor, was used to sort the highest 5% of transfected cells on a Becton-Dickinson Vantage flow cytometer in two rounds of FACS. Cytometric analysis of the final expression levels of each receptor gave median channel numbers of 2.29 for wtGlu^{199}R and 0.59 for Lys^{199}R, a 3.9-fold difference.

Production of Recombinant C5a and C5a des Arg^{74}. Wild-type and mutant C5a and C5a des Arg^{74} were produced in *Escherichia coli* and purified by the methods described in ref *5*.

Measurement of Release of Glucuronidase from U937 Cells. U937 cells were grown in RPMI 1640 medium with glutamax-I, supplemented with 5% FCS, 50 units/mL penicillin and 50 μg/mL streptomycin, at 37 °C and 5% CO^2 in air to a cell density of 2×10^5/mL. Subsequently, the cells were differentiated by adding 1 mM Bt_2cAMP and incubating for 3 days. The test itself was performed in microtiter plates. Into each well, 30 μL of C5a dilution and 10 μL of 20 μg/mL cytochalasin B was pipetted (in quadruplicate). The test was launched by adding 75 μL of cell suspension (2×10^6/mL, preincubated for 20 min at 37 °C) and incubation at 37 °C for 3 min. Immediately after incubation, the cells were centrifuged at 500*g* at 4 °C for 3 min and 75 μL of the supernatant of each well was pipetted into another microtiter plate containing 100 μL of substrate solution (8 mM *p*-nitrophenyl-*N*-acetyl-β-glucosamine in 40 mM sodium-acetate buffer, pH 4.5) per well. The plate was incubated at 37 °C for 1 h and developed by adding 75 μL of 0.4 M glycine/NaOH buffer, pH 10.4, per well. The OD was measured using a microtiter plate reader at 405 nm. The ED_{50} values and standard errors from a minimum of three separate experiments was determined by iterative curve fitting using Sigma Plot for Windows with a four-parameter logistic equation as fit type.

Measurement of 5-Hydroxy[^{3}H]Tryptamine Release. Secretion was measued as the release of [^{3}H]5-HT from intracellular granules, as described previously (*27*). Release was calculated as a percentage of total cell associated radioactivity [measured following cell lysis in 0.5% (v/v) Triton X-100 in BSS]. In response to 250 nM rhC5a, wtGlu199R, and Lys199R cells typically released 40 and 66% of total radioactivity, respectively. ED_{50} values were obtained by iterative curve fitting using GraphPad Prism 2.0.

Measurement of C5a Binding. Cells were harvested with nonenzymatic cell dissociation solution and binding assays performed as described previously (*27*) using [^{125}I]rhC5a.Log IC_{50} values were obtained by iterative curve fitting using GraphPad Prism 2.0.

RESULTS

Response of Cells Transfected with C5a Receptor Mutants to Peptide Analogues of the C5a C-Terminus. The role of the interaction between Lys^{68} of rhC5a and Glu^{199} of hC5aR was examined using a mutant hC5aR in which Lys was substituted for Glu^{199} (Lys^{199}R). RBL cells transfected with Lys^{199}R or wtGlu^{199}R were then treated with peptide 1

[1] Abbreviations: hC5aR, human complement fragment 5a receptor; 5-HT, 5-hydroxytryptamine; wtGlu^{199}R, wild-type hC5aR; Lys^{199}R, substitution of Lys for Glu^{199} of hC5aR; Bt_2-cAMP, dibutyryl cyclicAMP.

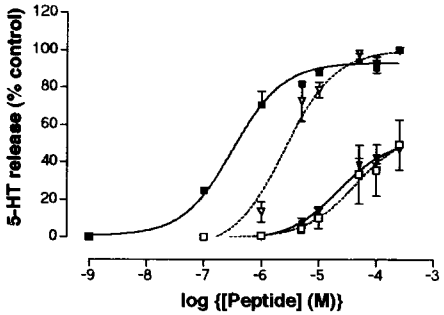

FIGURE 1: The secretory response of RBL cells transfected with the wild-type or mutant human C5a receptors to peptide analogues of the C5a C-terminus. RBL cells transfected with either wild-type (Glu^{199}, closed symbols) or mutant C5a receptor (Lys^{199}, open symbols) were labeled with [^{3}H]5-HT prior to stimulation with Peptides 1 (Lys^{68}, squares) and 5 (Glu^{68}, triangles). The results are the means ± SE of three separate experiments performed in duplicate and are expressed as a percentage of the maximal response to 250 μM peptide 1 (for wtGlu^{199}R) or peptide 5 (for Lys^{199}R).

(containing an analogue of Lys^{68}) or peptide 5 (containing Glu instead of Lys), and the release of [^{3}H]5-HT was measured. wtGlu^{199}R responded well to peptide 1 (ED_{50} = 0.32μM) (Figure 1). However, Lys^{199}R responded poorly to this peptide (ED_{50} = 43.7μM); this was very similar to the response of wtGlu^{199}R to peptide 5 (ED_{50} = 22.4μM). In contrast, Lys^{199}R showed a strong response to peptide 5 (ED_{50} = 2.57μM) (Figure 1), showing that reciprocal mutations in receptor and peptide sequences can rescue the responsiveness of the transfected RBL cells.

Response of Cells Transfected with the C5a Receptor Mutant to C5a Mutated at Position 68. The reciprocity of responsiveness to peptide analogues was repeated when rhC5a [Lys^{68}] and rhC5a [Glu^{68}] were used as stimuli although the differences in the responses were much smaller (Figure 2, panels A and B). wtGlu^{199}R and Lys^{199}R responded reciprocally with ED_{50} values of 5.50 and 17.4 nM for the Lys^{68} form of C5a and 27.5 and 11.2 nM for [Glu^{68}], respectively. In contrast, rhC5a [Met^{68}] did not appear to discriminate between the different receptors and was identical in activity to wild-type C5a on Lys^{199}R and rhC5a [Glu^{68}] on wtGlu^{199}R (Figure 2C). This suggests that the overall conformation of the receptor is not changed significantly when residue 199 is mutated and that the differences in responsiveness are due to altered interactions with ligand residue 68.

Response of Cells Transfected with C5a Receptor Mutants to C5a des Arg^{74} Mutated at Position 68 and to C5a [Asp^{74}]. rhC5a des Arg^{74} treatment showed the most interesting differences between the receptor variants. Somewhat surprisingly, recombinant hC5a [Lys^{68}] des Arg^{74} was as active as intact recombinant hC5a on wtGlu^{199}R (ED_{50} = 5.50 nM) but hC5a des Arg^{74} purified from human serum was some 10-fold less active (result not shown). The difference between recombinant and purified C5a des Arg^{74} is presumably due to the lack of glycosylation of the bacterially expressed form. No response to either form of C5a [Lys^{68}] des Arg^{74} was observed with Lys^{199}R at concentrations up to 30μM (Figure 3A and data not shown).

Whatever the basis for this difference between recombinant and purified C5a des Arg^{74}, rhC5a des Arg^{74} mutated at position 68 clearly discriminated between receptor types. wtGlu^{199}R did not respond to concentrations of rhC5a [Glu^{68}]

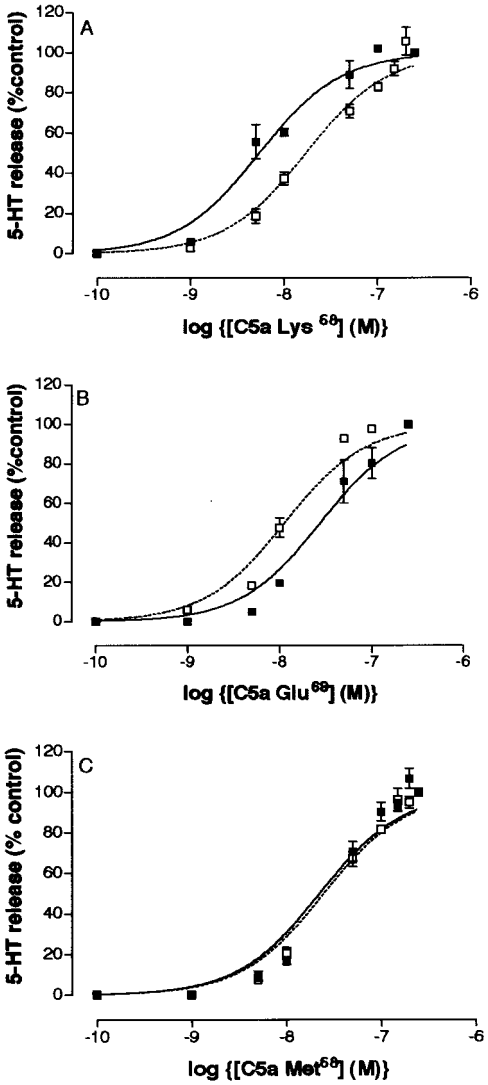

FIGURE 2: The secretory response of RBL cells transfected with wild-type and mutant human C5a receptors to mutant recombinant C5a. RBL cells transfected with either wild-type (Glu^{199}; closed squares) or mutant C5a receptor (Lys^{199}; open squares) were labeled with [^{3}H]5-HT and stimulated with wild-type rh C5a (Lys^{68}, panel A) or Lys68 substitution mutants (Glu^{68}, panel B; Met^{68}, panel C). The results are the means ± SE of three separate experiments performed in duplicate and are expressed as a percentage of the response to 250 nM rhC5a.

des Arg^{74} up to 30 μM. In contrast, Lys^{199}R responded moderately (ED_{50} = 1820 nM) to rhC5a [Glu^{68}] des Arg^{74} (Figure 3, panels A and B), indicating that some rescue of responsiveness occurs with the reciprocal mutations in ligand and receptor. In contrast, the addition of an Asp residue (rhC5a [Asp^{74}]) produces a ligand which acts similarly on both receptors (ED_{50} = 891 nM and 457 nM for wtGlu^{199}R and Lys^{199}R, respectively) although it is considerably less active on wtGlu^{199}R than either intact C5a [Lys^{68}] or rhC5a [Lys^{68}] des Arg^{74} (Figure 3c). This suggests that the presence of the C-terminal carboxyl group at position 74 can mask the effects of a Lys^{68}/Glu^{199} ligand/receptor interaction.

Glucosaminidase Release Assays on U937 Monocytic Cells. To ensure that the observed effects of mutating ligand residue 68 on C5a receptors expressed in rat cells did not reflect a species-specific difference, we measured their effects on glucosaminidase release from a human monocytic cell line, U937, differentiated by treatment with dibutyryl cAMP.

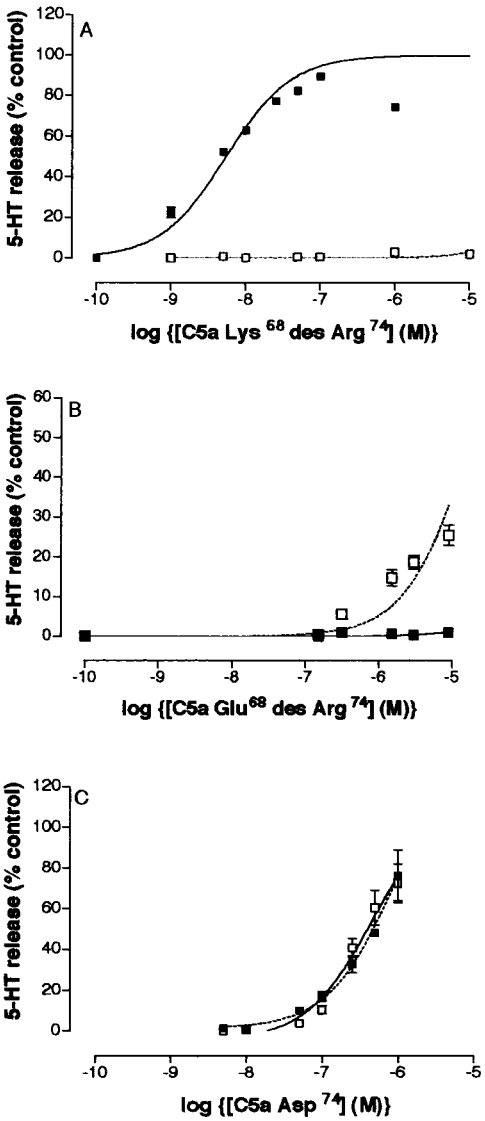

FIGURE 3: The secretory response of RBL cells transfected with the wild-type and mutant human C5a receptors to mutant recombinant C5a des Arg74. RBL cells transfected with either wild-type (Glu^{199}; closed squares) or mutant C5a receptor (Lys^{199}; open squares) were labeled with [^{3}H]5-HT and stimulated with rhC5a [Lys^{68}] des Arg^{74} (A), the substitution mutants rhC5a [Glu^{68}] des Arg^{74} (B) or rhC5a [Lys^{68}] [Asp^{74}] (C). The results are the means ± SE of three separate experiments performed in duplicate and are expressed as a percentage of the response to 250 nM rhC5a. Note the changed *y*-axis scale in panel B.

The responses to rhC5a [Lys^{68}], [Glu^{68}], and [Met^{68}] were similar in this assay (Table 1). As with RBL cells, rhC5a [Lys^{68}] des Arg^{74} had a similar ED_{50} to intact rhC5a [Lys^{68}] but the mutation of Lys^{68} to Glu produced a ligand that was inactive at concentrations of up to 50 μM, and the mutant containing Asp instead of the C-terminal Arg residue was 66-fold less active than wild-type intact C5a. Thus, although the C5aR in U937 cells appears to have a 10-fold higher sensitivity (relative to hC5aR expressed in RBL cells) to rhC5a, the pattern of responsiveness to all forms of rhC5a tested is identical but still does not respond to rhC5a [Glu^{68}] des Arg^{74}.

Inhibition of [^{125}I]rhC5a Binding to C5a Receptor by rhC5a and C5a des Arg^{74} Mutants. The inhibition of [^{125}I]-rhC5a by mutated rhC5a showed similar differences between $wtGlu^{199}R$ and $Lys^{199}R$ to those observed in the [^{3}H]5-HT release assay (Figure 4). Using cells transfected with $wtGlu^{199}R$, rhC5a [Lys^{68}] displaced radiolabeled C5a with an IC_{50} of 10.7nM; $Lys^{199}R$ cells showed an IC_{50} to this ligand of 79.4 nM. The reverse happened with rhC5a [Glu^{68}]; $Lys^{199}R$ gave an IC_{50} of 15.1 nM, and $wtGlu^{199}R$ gave 72.4 nM. However, a different pattern of inhibition was seen when the des Arg^{74} form was used: rhC5a [Lys^{68}] des Arg^{74} gave similar IC_{50} values on $wtGlu^{199}R$ and $Lys^{199}R$ (158 and 110 nM, respectively) whereas rhC5a [Glu^{68}] des Arg^{74} gave values of 1072 and 123 nM for these receptors (Figure 5). Clearly, the ability of the ligands to activate the receptor is not directly related to their ability to displace [^{125}I]rhC5a.

Table 1: Glucosaminidase Release-stimulatory Activity of Recombinant Human C5A on Differentiated U937 Human Monocytic Cells[a]

ligand	ED_{50} (nM)
rhC5a [Lys^{68}]	0.85 ± 0.22 (n = 7)
rhC5a [Lys^{68}] des Arg^{74}	1.00 ± 0.09 (n = 3)
rhC5a [Glu^{68}]	2.20 ± 1.20 (n = 6)
rhC5a [Glu^{68}] des Arg^{74}	ND
rhC5a [Met^{68}]	5.80 ± 3.70 (n = 3)
rhC5a [Asp^{74}]	56.0 ± 5.60 (n = 5)

[a] U937 cells were differentiated using dibutyryl cAMP and treated with ligand for 3 min. Glucosaminidase activity was measured as described in Experimental Procedures. The number of individual experiments performed in duplicate is given in brackets. ND = no activity detected at up to 50 μM ligand.

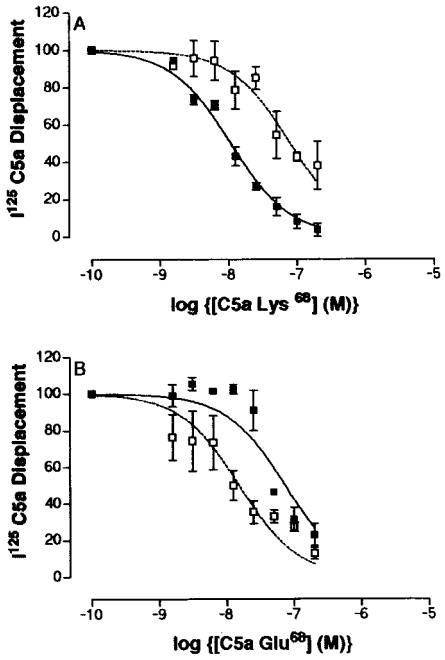

FIGURE 4: Displacement of [^{125}I]rhC5a by rhC5a. RBL cells transfected with either wild-type (Glu^{199}; closed squares) or mutant C5a receptor (Lys^{199}; open squares) were incubated with 50 pM [^{125}I]rhC5a and the stated concentration of wild-type rhC5a (Lys^{68}, panel A) or the substitution mutant rhC5a [Glu^{68}] (B). Results are the means of three separate experiments performed in duplicate ± SE.

DISCUSSION

The wild-type human C5a receptor ($wtGlu^{199}R$) transfected into the rat basophilic leukemia cell line responds equally effectively to both rhC5a and rhC5a des Arg^{74}. Purified human C5a des Arg^{74}, which has extensive glycosylation on

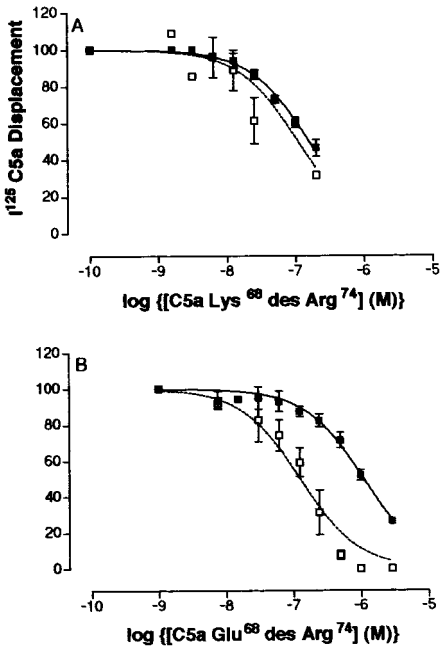

FIGURE 5: Displacement of [^{125}I]rhC5a by rhC5a des Arg^{74}. RBL cells transfected with either wild-type (Glu^{199}; closed squares) or mutant C5a receptor (Lys^{199}; open squares) were incubated with 50 pM [^{125}I]rhC5a and the stated concentration of wild-type rhC5a [Lys^{68}] des Arg^{74} (A) or the substitution mutant rhC5a [Glu^{68}] des Arg^{74} (B). Results are the means of three separate experiments performed in duplicate ± SE.

Asn^{64}, is 10-fold less active on these cells (*23*), suggesting that the presence of glycosylation at the C-terminal of C5a can interfere with receptor activation and cause a substantial loss of activity. However, the mutation of the receptor residue Glu^{199} to Lys (Lys^{199}R) completely abolishes the response of transfected cells to either form of C5a des Arg^{74} but has only a small effect on the response to intact rhC5a. This change of activity is also seen with peptides which mimic the C-terminus of C5a. We tested whether this was also true for rhC5a des Arg^{74}, by constructing rhC5a [Glu^{68}] and rhC5a [Glu^{68}] des Arg^{74}. rhC5a [Glu^{68}] showed slightly increased effectiveness on Lys^{199}R relative to wtGlu^{199}R whereas rhC5a [Glu^{68}] des Arg^{74} was completely ineffective on wtGlu^{199}R, but retained some activity on the mutant C5a receptor. This shows that an interaction between Lys or Glu^{68} of C5a and Glu or Lys^{199} of the receptor is critical for receptor activation by C5a des Arg^{74} and peptide analogues but not for C5a. The 3.9-fold lower receptor expression level on Lys^{199}R (relative to wtGlu^{199}R) cells does not appear to have a significant affect on the response to ligand. Maximal 5-HT release in response to a supra-optimal concentration (250 nM) of wild-type rhC5a [Lys^{68}] is actually higher for Lys^{199}R compared to wtGlu^{199}R cells and the sensitivity of Lys^{199}R to rhC5a [Glu^{68}] is increased, indicating that sufficient Lys^{199} receptors are present to fully activate the cells. Thus, the lack of a response of Lys^{199}R cells to rhC5a [Lys^{68}] des Arg^{74} is due to the loss of the interaction between Glu^{199} and Lys^{68} and is not dependent on receptor expression levels. This is confirmed by the absence of a response in U937 cells to rhC5a [Glu^{68}] des Arg^{74}, despite the 10-fold lower ED_{50} values of the responses to other ligands. Lys^{199}R cells also clearly responded to rhC5a [Glu^{68}] des Arg^{74}, demonstrating the effect of partially restoring the interaction with receptor residue 199.

Taken together, these data suggest that both the peptide analogues and C5a des Arg^{74} might have a different binding mode to full-length C5a. We examined this possibility by attempting to inhibit [^{125}I]rhC5a binding using wild-type and mutant ligands: rhC5a [Lys^{68}] and rhC5a [Glu^{68}] had reciprocal effects on wtGlu^{199}R and Lys^{199}R, with a 7-fold shift in IC_{50} values. rhC5a [Lys^{68}] des Arg^{74} had nearly identical effects on wtGlu^{199}R and Lys^{199}R in contrast to the results obtained in activation assays, whereas rhC5a [Glu^{68}] des Arg^{74} had a 7-fold higher affinity for Lys^{199}R over the wild-type receptor. This reinforces the evidence which shows that binding is only partly dependent on interactions at the C-terminus and that a considerable proportion of the binding energy results from interactions elsewhere with the C5a molecule. There is also a poor correlation between binding and receptor activation; rhC5a [Lys^{68}] des Arg^{74} is able to fully activate wtGlu^{199}R cells at very low receptor occupancy rates whereas near maximal occupancy of the Lys^{199}R receptor does not lead to cell activation. This is also seen with rhC5a [Glu^{68}] des Arg^{74}, where concentrations of ligand which cause 50% inhibition of [^{125}I]rhC5a binding to wtGlu^{199}R binding cause no detectable receptor activation. It appears that C5a and C5a des Arg^{74} bind to the C5aR in a similar way, but activate the receptor by different mechanisms. A disparity between binding and activation has also been observed in the chemotactic responses of human monocytes to C5a des Arg^{74} (*8*, *9*).

The absence of Arg^{74} appears to determine the response to ligands with an improper interaction with the receptor at Glu^{199}. However, the substitution of Asp for Arg^{74} produces a ligand which is equally active on wtGlu^{199}R and Lys^{199}R, suggesting that it is the position of the C-terminal carboxylate group which switches the receptor from a state in which an interaction between Glu^{199}/Lys^{68} (or Lys^{199}/Glu^{68}) is essential for activation to one in which this interaction is of secondary importance. However, we cannot eliminate the possibility that the side chain of the C-terminal amino acid (in this case carboxylate) may also influence receptor responsiveness. A potential switching mechanism involving Arg^{74} has been demonstrated which suggests that a transient interaction may occur between the C-terminal carboxylate group and receptor residue, Arg^{206} (*25*). This interaction is proposed to deflect the guanidino group of the receptor Arg^{206} residue, allowing the Arg^{74} guanidino group to dock with an unspecified receptor residue providing most of the ligand-binding energy. However, this effect appears to be restricted to C-terminal peptide analogues because intact C5a was found to be equally active on both wild-type (Arg^{206}) and mutated (Ala^{206}) C5aR although C5a des Arg^{74} was not tested in this system.

An additional role for Arg^{74} has recently been proposed following the detailed structural definition of the C-terminus of C5a by NMR (*3*). Residues Asp^{69}−Arg^{74} have been shown to form a helix, folded back into the core of the molecule stabilized by an interaction between the side chains of Arg^{74} and Arg^{62}. In this model, Lys^{68} is positioned in the interhelical loop, with the side chain accessible for possible interaction with receptor residues. In the absence of Arg^{74}, it is possible that the C-terminal helical domain is not stabilized (and so is invisible during determination of solution structure by NMR) and that Lys^{68} of C5a des Arg^{74} is presented to the receptor in a different, or less rigid, conformation. The concomitant inability of the side chain to penetrate a receptor-

binding pocket containing Glu^{199} may partly explain the lower binding affinity of C5a des Arg^{74} relative to intact C5a. When Lys is substituted for Glu^{199}, the more rigid C-terminal of C5a may still be able to force the side chain of Lys^{68} into this pocket despite the charge repulsion, thus allowing suboptimal activation of the receptor; C5a des Arg^{74} cannot force this interaction due to the lack of structure at the C-terminus and so the ability to activate the receptor is lost. A similar mechanism may also explain why the peptide analogues are also sensitive to Glu/Lys substitutions in both ligand and receptor in that the lack of an interaction with the receptor N-terminal domain (which has been shown to contribute 50% of the binding energy) may prevent the peptide Lys residue penetrating a receptor-binding pocket when Glu^{199} has been substituted by Lys^{199}. Hydrophobic interactions may also be involved in the interaction between Lys^{68} of C5a and the binding pocket around Glu^{199} (*28*). The substitution of Glu for Lys^{68} in C5a des Arg^{74} only partly restores mutant receptor activation, suggesting that perhaps the short side chain of Glu relative to Lys causes the loss of these additional hydrophobic interactions that are necessary for full activity.

In summary, the human C5a receptor can display a degree of ligand selectivity giving different responses to C5a and C5a des Arg^{74} in different cellular backgrounds. In this paper, we have shown that this selectivity may involve an interaction between Lys^{68} of C5a and Glu^{199} of the receptor. In the absence of this interaction, C5a des Arg^{74} is not able to activate the receptor whereas the response to intact C5a is largely unaffected.

ACKNOWLEDGMENT

The authors wish to acknowledge the Yorkshire Cancer Research Campaign for the use of the FACS facilities.

REFERENCES

1. Gallin, J. I., Goldstein, I. M., and Snyderman, R. (1992) *Inflammation: Basic Principles and Clinical Correlates*, 1st ed.; Raven Press, New York.
2. Zuiderweg, E. R., and Fesik, S. W. (1989) *Biochemistry 28*, 2387−2391.
3. Zhang, X. L., Boyar, W., Toth, M. J., Wennogle, L., and Gonnella, N. C. (1997) *Proteins: Struct., Funct., Genet. 28*, 261−267.
4. Mollison, K. W., Mandecki, W., Zuiderweg, E. P., Fayer, L., Fey, T. A., Krause, R. A., Conway, R. G., Miller, L., Edalji, R. P., Shalcross, M. A., Lane, B., Fox, J. L., Greer, J., and Carter, G. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 292−296.
5. Bubeck, P., Grotzinger, J., Winkler, M., Kohl, J., Wollmer, A., Klos, A., and Bautsch, W. (1994) *Eur. J. Biochem. 219*, 897−904.
6. Toth, M. J., Huwyler, L., Boyar, W. C., Braunwalder, A. F., Yarwood, D., Hadala, J., Haston, W. O., Sills, M. A., Seligmann, B., Galakatos, N. (1994) *Protein Sci. 3*, 1159−1168.
7. Bokisch, V. A., and Muller-Eberhardt, H. J. (1970) *J. Clin. Invest. 49*, 2427−2434.
8. Yancey, K. B., Lawley, T. J., Dersookian, M., and Harvath, L. (1989) *J. Invest. Dermatol. 92*, 184−189.
9. Marder, S. R., Chenoweth, D. E., Goldstein, I. M., and Perez, H. D. (1985) *J. Immunol. 134*, 3325−3331.
10. Burgi, B., Brunner, T., and Dahinden, C. A. (1994) *Eur. J. Immunol. 24*, 1583−1589.
11. Fureder, W., Agis, H., Willhein, M., Bankl, H. C., Maier, U., Kishi, K., Muller, M. R., Czerwenka, K., Radaszkiewicz, T., Butterfield, J. H., Klappacher, G. W., Sperr, W. R., Oppermann, M., Lechner, K., and Valent, P. (1995) *J. Immunol. 155*, 3152−3160.
12. Werfel, T., Oppermann, M., Butterfield, J. H., Begemann, G., Elsner, J., Gotze, O., and Zwirner, J. (1996) *Scand. J. Immunol. 44*, 30−36.
13. El-Lati, S. G., Dahinden, C. A., and Church, M. K. (1994) *J. Invest. Dermatol. 103*, 803−806.
14. Senior, R. M., Griffin, G. L., Perez, H. D., and Webster, R. O. (1988) *J. Immunol. 141*, 3570−3574.
15. Gerard, C., Chenoweth, D. E., and Hugli, T. (1981) *J. Immunol. 127*, 1978−1982.
16. Gerard, C., and Gerard, N. P. (1991) *Nature 349*, 614−617.
17. Boulay, F., Tardif, M., Brouchon, L., and Vignais, P. (1991) *Biochemistry 30*, 2993−2999.
18. Pease, J. E., Burton, D. R., and Barker, M. D. (1994) *Eur. J. Immunol. 24*, 211−215.
19. De Martino, J. A., Van Riper, G., Siciliano, S. J., Molineaux, C. J., Knoteatis, Z. D., Rosen, H., and Springer, M. (1994) *J. Biol. Chem. 269*, 14446−14450.
20. Mery, L., and Boulay, F. (1994) *J. Biol. Chem. 269*, 3457−3463.
21. Opperman, M., Raedt, U., Hebell, T., Schmidt, B., Zimmerman, B., and Gotze, O. (1993) *J. Immunol. 151*, 3785−3794.
22. Morgan, E. L., Ember, J. A., Sanderson, S. D., Scholz, W., Buchner, R., Ye, R. D., and Hugli, T. E. (1993) *J. Immunol. 151*, 377−388.
23. Monk, P. N., Barker, M. D., Partridge, L. J., and Pease, J. E. (1995). *J. Biol. Chem. 270*, 16625−16629.
24. Raffetseder, U., Roper, F., Mery, L., Gietz, C., Klos, A., Grotzinger, J., Wollmer, A., Boulay, F., Kohl, J., and Bautsch, W. (1996) *Eur. J. Biochem. 235*, 82−90.
25. DeMartino, J. A., Konteatis, Z. D., Siciliano, S. J., Van Riper. G., Underwood, D. J., Fischer, P. A., and Springer, M. S. (1995) *J. Biol. Chem. 270*, 15966−15969.
26. Siciliano, S. J., Rollins, T. E., DeMartino, J., Konteatis, Z., Malkowitz, L., VanRiper, G., Bondy, S., Rosen, H., and Springer, M. S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1241−1218.
27. Monk, P. N., Pease, J. E., Marland, G., and Barker, M. D. (1994) *Eur. J. Immunol. 24*, 2922−2925.
28. Vogen, S. M., Finch, A. M., Wadi, S. K., Thatcher, J., Monk, P. N., Sanderson, S. D., and Taylor, S. M. (1999) *J. Pept. Res. 53*, 8-17.

BI990139Q