Mapping the Ligand-Binding Site on the C5a Receptor: Arginine^{74} of C5a Contacts Aspartate^{282} of the C5a Receptor†

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ABSTRACT: The interaction between the anaphylatoxin C5a and its receptor involves two distinct sites. One site is formed by acidic residues at the receptor N-terminus and contributes to only ligand binding. The second site, responsible for activation, is less well defined. In this study, we demonstrate that the receptor residue D^{282}, near the extracellular face of transmembrane domain VII, is a component of the second ligand-binding site. Mutation of D^{282} to A decreases the sensitivity of the receptor to activation by intact C5a but not by its less potent metabolite, C5adR^{74}, which lacks the C-terminal arginine^{74}. The mutation of the R^{74} residue of C5a to A causes a 60-fold decrease in wild-type receptor sensitivity, but only a 2-fold decrease for the receptor mutated at D^{282}. In contrast, the mutation of R^{74} to D makes C5a completely inactive on both wild-type and A^{282} C5a receptors. The mutation of D^{282} to R partly restores the response to C5a[D^{74}], which is a more effective ligand than C5a at the mutant receptor. A peptide mimic of the C5a activation domain with a C-terminal R potently activates the wild type but is only a weak agonist at the mutant D^{282}R-C5a receptor. Conversely, a peptide with D at the C-terminus is a more effective activator of D^{282}R than wild-type C5a receptors. These data indicate that the R^{74} side chain of C5a makes an interaction with receptor D^{282} that is responsible for the higher potency of intact C5a versus that of C5adR^{74}.

Complement fragment C5a is a potent inflammatory mediator and immunomodulator, which acts on different organs and cell types to stimulate degranulation, cytokine production, smooth muscle contraction, and increased vascular permeability. C5a plays a role in the pathogenesis of many disorders, including rheumatoid arthritis and adult respiratory distress syndrome, and exacerbates tissue damage in arteriosclerosis and myocardial infarction (*1*). Recently developed C5a receptor antagonists have been effective in in vivo models of ischemia/reperfusion injury, immune complex disease (*2*), the control of infarct size after surgical revascularization (*3*), reverse passive Arthus reaction, and endotoxic shock (*4*).

The agonist potential of C5a is vested entirely in the C-terminal decapeptide (*5*−*7*). The C-terminus of C5a is rapidly desarginated in vivo to form C5adR^{74} [1] (*8*), a stable form that is only cleared by receptor endocytosis and which has a spectrum of bioactivity different from that of intact C5a. For instance, human basophils are stimulated by intact C5a to release lipid mediators (e.g., leukotriene C4) and cytokines (e.g., IL-4 and IL-13), whereas C5adR^{74} stimulates only cytokine release (*9*). The single cell-surface receptor for C5a is a member of the G protein-coupled receptor superfamily (*10*, *11*), which has two distinct regions involved in ligand binding. Ablation or antibody blockade of the N-terminal domain prevents interaction with the core of C5a, but does not inhibit receptor activation by peptide mimics of the C-terminus of C5a (*12*−*15*). Thus, there is a second site that is primarily responsible for receptor activation. Several studies have attempted to locate this region and have identified receptor mutations that interfere with peptide mimic activity. The mutation of R^{206}, near the extracellular face of transmembrane domain V, dramatically affects the agonist activity of peptide mimics but not that of intact C5a (*16*). R^{206} has been proposed to interact with the C-terminal carboxylate group as part of a switching mechanism (*16*). However, another study has suggested that R^{206} is involved in the coupling to intracellular G proteins, which control the affinity state of C5a receptors (*17*). This group has also detected additional receptor residues (R^{175} and D^{282}) that affect ligand binding when mutated, although a direct interaction between these residues and ligand was discounted (*17*). More recently, the use of a powerful genetic technique has enabled the analysis of all transmembrane residues in C5a ligand binding and signaling in yeast (*18*, *19*). On the extracellular side of the receptor, residues R^{206} and D^{282} were identified as important for function but the nature of any interaction with ligand was not investigated. We have previously shown that E^{199}, in the second extracellular loop,

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[1] Abbreviations: C5aR, human complement fragment 5a receptor; D^{282}A, substitution of Ala for Asp^{282} of C5aR; D^{282}R, substitution of Arg for Asp^{282} of C5aR; R^{206}A, substitution of Ala for Arg^{206} of C5aR; E^{199}K, substitution of Lys for Glu^{199} of C5aR; E^{199}K/R^{206}A, double mutant with substitution of Lys for Glu^{199} and Ala for Arg^{206} of C5aR; C5a[A^{74}], substitution of Ala for Arg^{74} of C5a; C5a[D^{74}], substitution of Asp for Arg^{74} of C5a; C5adR^{74}, des arginated C5a; CHA, cyclohexylalanine; CHA-R, PheLysPro$_D$-ChaCha$_D$-Arg; CHA-D, PheLysPro$_D$-ChaCha$_D$-Asp.

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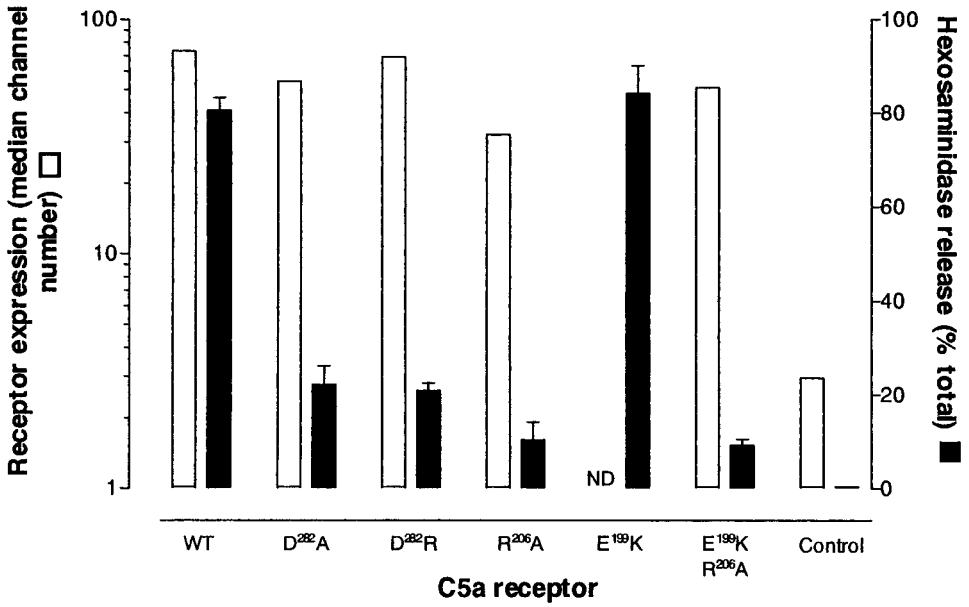

FIGURE 1: Expression of wild-type and mutant C5a receptors in RBL cells. Expression levels of wild-type and mutant C5a receptors in transfected RBL cells were measured by binding of an antibody to the C5aR N-terminus and are shown as median fluorescent channel number. The graph shows the results of a single determination performed contemporaneously with the degranulation assays. The expression level of $E^{199}K$-C5aR has previously been shown to be 3.9-fold lower than that of WT-C5aR (*21*). The degranulation in response to a high dose (1 μM) of C5a or C5adR^{74} ($D^{282}R$ only) was measured as the percentage of hexosaminidase release, as stated in Experimental Procedures. The results are the means of *n* determinations (*n* shown in Table 1 for each receptor type) performed in triplicate ± standard error, expressed as a percentage of the total intracellular hexosaminidase. ND means not determined.

interacts with K^{68} in the C5a C-terminus. This interaction is essential for the activity of peptide mimics and C5adR^{74} but not for intact C5a (*20*−*22*). To date, this is the only well-defined contact between C5a and the activation site of the receptor.

In this study, we have mutated two key charged receptor residues (R^{206} and D^{282}) and examined their potential for interaction with R^{74} of C5a. The results suggest that R^{206} is unlikely to be directly involved in activation by C5a, but that D^{282} interacts with the C-terminal R^{74} residue of C5a.

EXPERIMENTAL PROCEDURES

Construction of Human C5aR Mutants. The mutant C5a receptors were constructed by overlap extension mutagenesis as described previously (*21*). The full-length PCR products were digested with *Eco*RI and *Hin*dIII (Roche Biochemicals) and ligated into the expression vector pEE6 (Celltech Ltd.). The C5aR mutant clones were sequenced using the ABI Big Dye terminator cycle sequencing kit, and the correct constructs were purified using the PC500 Nucleobond Kit (Macherey-Nagel).

Transfection and Cell Culture. RBL-2H3 cells were routinely cultured in Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum, which was supplemented with 400 mg/L G-418 for transfected cells, at 37 °C, in 5% CO_2. RBL-2H3 cells were transfected by electroporation, as previously described (*21*). A monoclonal antibody (S5/1; Serotec), 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.

Expression and Purification of C5a Mutants and Peptide Synthesis. Expression and purification of the recombinant His_6-tagged C5a, C5adR^{74}, C5a[A^{74}], and C5a[D^{74}] was performed as described previously (*23*, *24*). For cleavage of the His_6 tag, 200−300 μg of purified recombinant protein was digested with 100 units of rTEV protease in 1 mM DTT and rTEV buffer for 4 h at 30 °C. The digested polypeptides were separated from the rTEV protease and cleaved His_6 tag by addition of Ni^{2+}−NTA resin. The peptide mimics of the C5a C-terminus (CHA-R, FKPD-ChaChaD-R; CHA-D, FKPD-ChaChaD-D) were synthesized as described previously (*20*).

Assessment of Receptor Activation of RBL Cells. Receptor activation was assessed as the release of β-hexosaminidase from intracellular granules, as described previously (*24*). The percentage of β-hexosaminidase release was calculated as a percentage of the maximal release (1 μM C5a or 1 μM C5adR^{74} for D^{282}R-C5aR). The total hexosaminidase content was determined following cell lysis with 0.1% NP-40. The EC_{50} and standard error values were obtained by iterative curve fitting using GraphPad Prism 2.0.

[^{125}I]C5a Binding Assay. Binding assays using 50 pM [^{125}I]-rhC5a were performed on adherent C5aR-transfected RBL cells in 96-well microtiter plates as described previously (*21*). The IC_{50} and standard error values were obtained by iterative curve fitting using GraphPad Prism 2.0.

RESULTS

Production of Transfected RBL Cells. A number of mutations with the potential to affect the interaction with ligand were introduced into C5aR. Wild-type (WT) and mutant receptors expressed in RBL cells were subject to two rounds of FAC sorting using a monoclonal antibody that recognizes the N-terminus of C5aR. Expression levels after sorting are shown in Figure 1. WT-C5aR was expressed at the highest levels, but all of the mutants were expressed at approximately equivalent levels. Using a very high dose of either C5a or C5adR^{74} (1 μM), the maximal degranulation response of these cell lines was measured (Figure 1). WT-

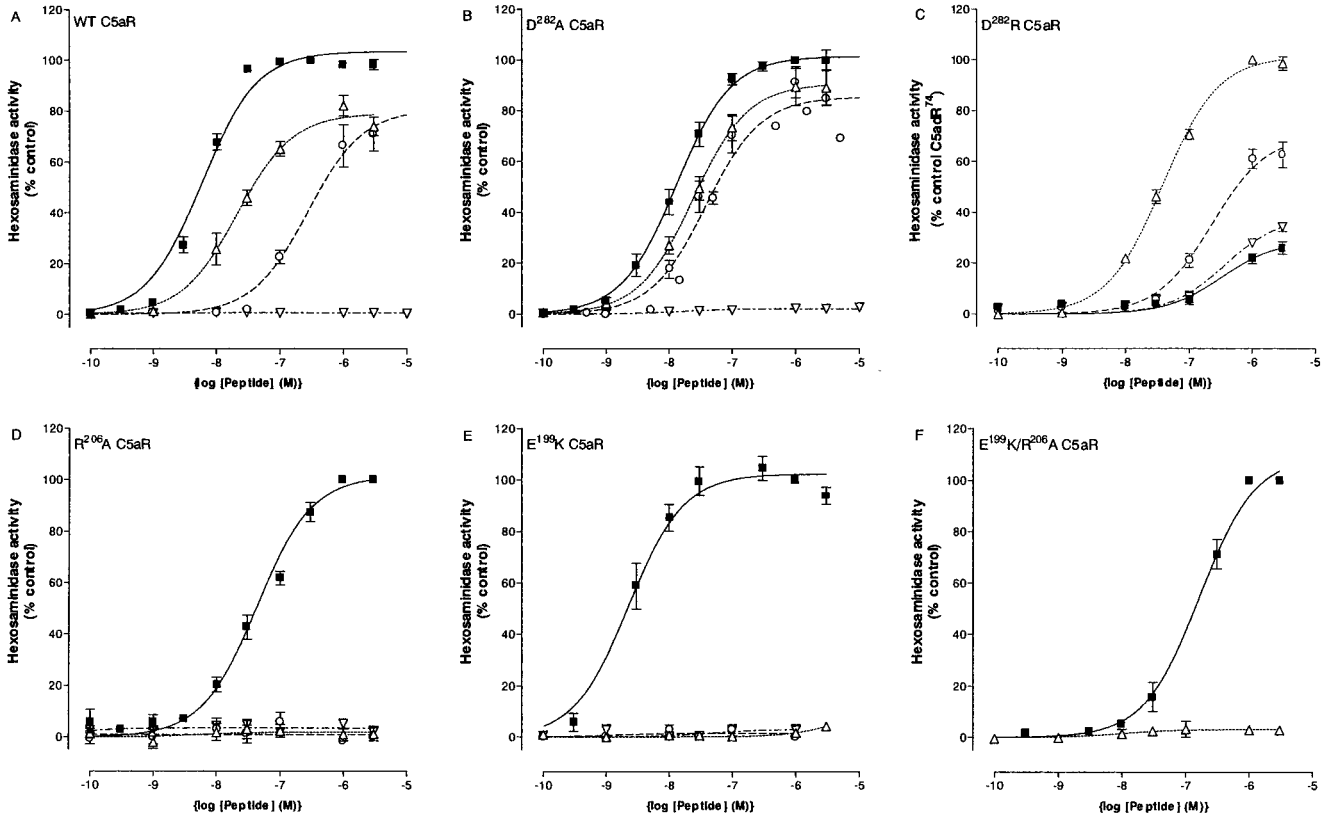

FIGURE 2: Degranulation of wild-type and mutant C5a receptor-transfected RBL cells in response to C5a, C5a[A^{74}], C5a[D^{74}], and C5adR^{74}. RBL cells transfected with the stated wild-type (WT) or mutant C5a receptor were treated with the stated concentrations of C5a (■), C5adR^{74} (△), or C5a with the C-terminal arginine substituted with alanine, C5a[A^{74}] (○), or aspartate, C5a[D^{74}] (▽). Degranulation was assessed as hexosaminidase release, as described in Experimental Procedures. The results are the means of *n* separate experiments (*n* shown in Table 1) performed in triplicate ± standard error, expressed as a percentage of the response to 1 μM C5a or C5adR^{74}.

Table 1: Summary of the Effects of C5aR Ligands on the Degranulation of Transfected RBL Cells[a]

	WT-C5aR			D^{282}A-C5aR			D^{282}R-D282R−C5aRC5aR			R^{206}A-C5aR		
ligand	pD_{2E} ± SE	EC_{50}	*n*	pD_{2E} ± SE	EC_{50}	*n*	pD_{2E} ± SE	EC_{50}	*n*	pD_{2E} ± SE	EC_{50}	*n*
C5a	8.24 ± 0.03	5.82 (100)	16	7.83 ± 0.05[b]	14.6 (100)	9	6.49 ± 0.16[b]	325 (29)	4	7.40 ± 0.04[b]	40.0 (100)	10
C5adR^{74}	7.67 ± 0.07	21.2 (79)	16	7.62 ± 0.07[c]	24.5 (91)	9	7.42 ± 0.03[d]	38.2 (100)	7	<5		3
C5a[A^{74}]	6.57 ± 0.12	272 (66)	16	7.50 ± 0.07[b]	29.0 (88)	9	6.65 ± 0.09[c]	227 (70)	4	<5		3
C5a[D^{74}]	<5		16	<5		9	6.44 ± 0.12	365 (38)	4	<5		3
CHA-R	6.65 ± 0.09	223 (88)	4	5.43 ± 0.08[b]	3715 (199)	3	4.28 ± 0.05[b]	52890 (181)	3	<2		3
CHA-D	2.82 ± 0.40	1.5×10^6 (88)	4	4.56 ± 0.19[d]	27350 (56)	3	5.09 ± 0.09[e]	819 (102)	3	<2		3

[a] $pD_{2E} = -\log EC_{50}$, where EC_{50} is the concentration (nanomolar) resulting in 50% of maximal degranulation. *n* is the number of separate experiments performed in triplicate. The values in brackets show release as a percentage of maximal C5a (or C5adR^{74}) stimulation. [b] Significantly different from that of WT-C5aR ($p < 0.05\%$; *t* test). [c] Not significantly different from that of WT-C5aR ($p > 5\%$). [d] Significantly different from that of WT-C5aR ($p < 5\%$). [e] Significantly different from that of WT-C5aR ($p < 0.5\%$).

and E^{199}K-C5aR RBL cells secreted 80% of the total intracellular hexosaminidase; D^{282}A- and D^{282}R-C5aR (in response to C5adR^{74}) secreted 20−30%, but R^{206}A and E^{199}K/R^{206}A secreted only 10%, despite the nearly equivalent receptor expression levels of these mutants. This shows that some inactive receptor is present at the cell surface but that all cell lines carry functional receptor populations.

Secretory Response of Transfected RBL Cells to C5a and C5adR^{74}. To determine whether the selected receptor residues are involved in an interaction with R^{74} of C5a, we compared the secretory responses of wild-type and mutant C5aR-transfected RBL cells to intact C5a and C5adR^{74} (Figure 2A−F). The EC_{50} for C5a was increased by R^{206}A (7-fold), E^{199}K/R^{206}A (27-fold), and D^{282}A (2-fold) and slightly decreased by E^{199}K. For WT-C5aR, C5adR^{74} was only a partial agonist, stimulating 79% of the response to intact C5a. For D^{282}A-C5aR, however, C5adR^{74} was a nearly full agonist (91%; Figure 2B and Table 1) but with an EC_{50} value similar to that of WT-C5aR. D^{282}R-C5aR exhibited a complete reversal of the responsiveness of WT-C5aR to these ligands. C5a was only a weak agonist (29%) with an EC_{50} that was 56-fold higher than that of WT, whereas C5adR^{74} caused a much larger secretory response with an EC_{50} that was only 2-fold lower than that of WT (Figure 2C and Table 1). In contrast, cells bearing E^{199}K-, R^{206}A-, or E^{199}K/R^{206}A-C5aR did not respond at all to C5adR^{74} (Figure 2D−F).

Secretory Response of Transfected RBL Cells to C5a[A^{74}] and C5a[D^{74}]. The pattern of responses to C5adR^{74} suggested the possibility that R^{74} could interact with D^{282} of C5aR. This was further investigated by making C5a mutants containing either A or D instead of R at position 74, substituting nonpolar or acidic side chains, respectively, but

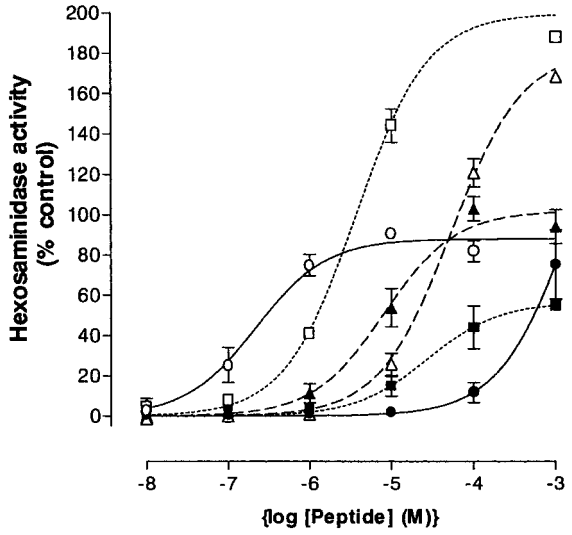

FIGURE 3: Degranulation of wild-type and mutant C5a receptor-transfected RBL cells in response to peptide mimics of the C5a C-terminus. RBL cells transfected with the wild-type (WT) (○ and ●, solid lines) $D^{282}A$ (□ and ■, dashed lines), or $D^{282}R$ (△ and ▲, dotted lines) C5a receptor were treated with the stated concentrations of the peptide mimics of the C5a C-terminus, CHA-R (white symbols), or CHA-D (black symbols). The results are the means of *n* separate experiments (*n* shown in Table 1) performed in triplicate ± standard error, expressed as a percentage of the response to 1 μM C5a or $C5adR^{74}$.

positioning the C-terminal carboxylate approximately where it might be in wild-type C5a. $C5a[A^{74}]$ was a partial agonist for WT-C5aR, stimulating only 66% of the maximal response to intact C5a with an EC_{50} that was 47-fold higher (Figure 2A and Table 1) but was nearly a full agonist on $D^{282}A$-C5aR cells (88% of the response to C5a) with an EC_{50} similar to those of both $C5adR^{74}$ and intact C5a (Figure 2B and Table 1). $C5a[D^{74}]$ had no activity at all on cell lines transfected with either receptor. In contrast, $D^{282}R$-C5aR cells responded to $C5a[D^{74}]$ more strongly than to intact C5a, with 38 and 29% of the maximal response to $C5adR^{74}$, respectively (Figure 2C and Table 1). However, $C5a[A^{74}]$ was a more potent agonist than either C5a or $C5a[D^{74}]$, with a lower EC_{50} value and a higher percentage release (70% of the response to $C5adR^{74}$). $R^{206}A$- and $E^{199}K$-C5aR did not respond to either $C5a[A^{74}]$ or $C5a[D^{74}]$ at concentrations up to 10 μM (Figure 2D,E).

Secretory Responses of Transfected Cells to a Peptide Mimic of the C5a C-Terminus. The peptide mimic CHA-R was a potent stimulator of secretion from WT-C5aR-transfected RBL cells and had full agonist activity, with an EC_{50} of 223 nM (Figure 3 and Table 1). On $D^{282}A$- and $D^{282}R$-C5aR, CHA-R had higher EC_{50} values (3715 and 52 890 nM, respectively) but with substantial increases in the size of the maximal response relative to that of WT-C5aR (Figure 3 and Table 1). A modified form of this peptide, with D instead of R at the C-terminus (CHA-D), was only a weak agonist on WT-C5aR (EC_{50} = 1.5 mM), but significantly more potent at $D^{282}A$- and $D^{282}R$-C5aR (Figure 3 and Table 1), with EC_{50} values of 27 350 and 819 nM, respectively. Unlike CHA-R, CHA-D did not increase the magnitude of the maximal response for the mutant receptors. In contrast, $R^{206}A$-C5aR did not respond at all to CHA-R or CHA-D even at 10 mM (Table 1).

Binding of [^{125}I]C5a to Wild-Type and Mutant C5a Receptors. For WT-C5aR, [^{125}I]C5a binding was readily detectable and accurate inhibition curves could be constructed (Table 2). However for $D^{282}A$-, $D^{282}R$-, and $R^{206}A$-C5aR, the level of [^{125}I]C5a binding was reduced nearly 10-fold (data not shown) relative to that of WT-C5aR, despite the apparently high levels of receptor expression demonstrated by flow cytometry (Figure 1). Binding to $E^{199}K$- and $E^{199}K/R^{206}A$-C5aR was not performed. WT-C5aR bound $C5adR^{74}$, $C5a[A^{74}]$, and $C5a[D^{74}]$ with 32-, 215-, and 271-fold lower affinities than intact C5a, respectively (Table 2). Similarly, $D^{282}A$- and $D^{282}R$-C5aR bound $C5adR^{74}$ with 40−50-fold lower affinity than intact C5a (Table 2). WT- and $D^{282}A$-C5aR bound C5a with a identical affinity that was 4-fold higher than that of $D^{282}R$-C5aR (Table 2). Unfortunately, accurate data for the C5a mutants could not be obtained due to the low levels of [^{125}I]C5a binding observed with these cells. $R^{206}A$-C5aR bound C5a with a 7-fold lower affinity than WT-C5aR but had an affinity similar to that for $C5adR^{74}$ (Table 2).

DISCUSSION

The mechanism by which C5a activates its receptor is not understood in detail, but C5a and its more stable metabolite, $C5adR^{74}$, are known to interact differently with the C5a receptor. In this paper, we have mutated two charged residues predicted to be involved in the ligand-binding site of the C5aR, to establish their roles in the interaction with the side chain and carboxylate group of the C-terminal R^{74} residue of C5a.

An important criterion for the identification of a receptor residue that can interact with R^{74} would be that a mutation would have a significantly stronger effect on activation by intact C5a than by $C5adR^{74}$, which would already be lacking this interaction. R^{206} of C5aR has been proposed to act as a molecular switch, leading to receptor activation when displaced by electrostatic interaction with the carboxylate group of R^{74}. However, this effect has only been demonstrated with short peptide mimics of the C5a C-terminus (*16*). Our data indicate that R^{206} is unlikely to be directly involved in interactions with C5a, despite the 7-fold decrease in receptor responsiveness caused by mutating R^{206} to A and a similar decrease in the binding affinity for C5a. If R^{206} interacted with the R^{74} carboxylate group of C5a, then $C5adR^{74}$ and the intact ligand would be expected to have a similar potential to activate the mutant receptor, due to the lack of an interaction at residue 74. However, although $R^{206}A$-C5aR binds $C5adR^{74}$ with an affinity similar to that of WT-C5aR, no receptor activation occurs with this ligand. $C5a[A^{74}]$ is also inactive on $R^{206}A$-C5aR. $C5adR^{74}$ but not C5a requires an interaction between K^{68} and E^{199} of the receptor for activation to occur, due to the loss of the interaction with the C-terminal carboxylate of R^{74} (results presented here and in refs *20* and *21*). If the $R^{206}A$ mutation ablated a critical interaction with R^{74}, we would expect that the double mutant receptor, $E^{199}K/R^{206}A$, would not respond to intact C5a; however, the double mutant has an EC_{50} for C5a that is only 4-fold higher than that of $R^{206}A$-C5aR.

The $D^{282}A$-C5aR mutant expressed in RBL cells was able to respond robustly to C5a, stimulating quite high levels of degranulation despite showing low levels of [^{125}I]C5a bind-

Table 2: Binding Affinities of C5aR Ligands[a]

	WT-C5aR			D^{282}A-C5aR			D^{282}R-C5aR			R^{206}A-C5aR		
ligand	$pD_{2I} \pm SE$	IC_{50}	*n*	$pD_{2I} \pm SE$	IC_{50}	*n*	$pD_{2I} \pm SE$	IC_{50}	*n*	$pD_{2I} \pm SE$	IC_{50}	*n*
C5a	7.86 ± 0.03	14.0	9	7.85 ± 0.12[b]	14.0	3	7.24 ± 0.18[c]	57.8	2	7.02 ± 0.13[c]	94.8	3
C5adR^{74}	6.34 ± 0.08	450	9	6.19 ± 0.28[b]	649	3	5.64 ± 0.20[d]	2300	2	6.36 ± 0.12[b]	436	3
C5a[A^{74}]	5.55 ± 0.08	3010	8	–			–			–		
C5a[D^{74}]	5.42 ± 0.06	3790	8	–			–			–		

[a] $pD_{2I} = -\log IC_{50}$, where IC_{50} is the concentration (nanomolar) resulting in 50% inhibition of the maximum level of binding of [^{125}I]rhC5a. *n* is the number of separate experiments performed in triplicate. [b] Not significantly different from that of WT-C5aR ($p > 5\%$). [c] Significantly different from that of WT-C5aR ($p < 0.05\%$; *t* test). [d] Significantly different from that of WT-C5aR ($p < 0.5\%$; *t* test).

ing. Thus, a significant proportion of the surface-expressed protein is correctly folded in the presence of this mutation. The EC_{50} for C5a was increased 2-fold by this mutation, but the EC_{50} for C5adR^{74} was unchanged. The ratio of the EC_{50} values for C5adR^{74}/C5a was >3 for WT-C5aR and <2 for D^{282}A-C5aR. Importantly, the C5a[A^{74}]/C5a ratio was also <2 for the mutant receptor but >46 for WT-C5aR. These data suggested that the presence of a guanidino group at position 74 of C5a was less important for the activation of D^{282}A-C5aR than for that of WT. The further mutation of D^{282} to R drastically reduced the responsiveness to C5a, with a 62-fold increase in EC_{50} relative to that of WT-C5aR, despite the small (4-fold) change in binding affinity. However, the responsiveness of D^{282}R-C5aR to C5adR^{74} was only slightly affected, with a <2-fold increase in EC_{50}, although the binding affinity was increased by >5-fold relative to that of WT-C5aR. C5a could stimulate only 29% of the maximal response to C5adR^{74}. C5a[A^{74}] had a similar EC_{50} and maximal response in both WT- and D^{282}R-C5aR whereas C5a[D^{74}], completely ineffective on WT- and D^{282}A-C5aR, was actually a more potent agonist than C5a at the D^{282}R receptor, presumably because of the partial reconstitution of the interaction at D^{282}. The relative inactivity of C5a[D^{74}] on D^{282}R-C5aR compared to either C5a[A^{74}] or C5adR^{74} may be explained by the insufficient steric resemblance of the reconstituted interaction produced by simply switching the residues between the ligand and receptor, or possibly by the adoption of a less favorable conformation of the C-terminus of C5a[D^{74}] relative to the A^{74} form. Although the data show that both C5a[A^{74}] and C5a[D^{74}] can bind to the WT-C5aR equally well, the separation of binding and activation sites on C5aR means that an unfavorable conformation of the C5a C-terminus would not necessarily be detected in a binding assay.

The peptide mimic of the C5a C-terminus, CHA-R, was an agonist at WT-, D^{282}A-, and D^{282}R-C5aR, but the EC_{50} values for the mutants were 17- and 237-fold higher than that for WT. These data suggest that a repulsive interaction between the C-teminal residue of the peptide and the receptor residue at position 282 can inhibit peptide activity. A modified form of the peptide with a C-terminal D residue, CHA-D, was synthesized to confirm this observation. This peptide had a pattern of activity that was the opposite of that of CHA-R, having EC_{50} values at D^{282}A- and D^{282}R-C5aR 55- and 1831-fold lower than at WT-C5aR, respectively. The rank order of EC_{50} values to CHA-R is as follows: $D^{282}R \gg D^{282}A >$ WT; however, to CHA-D it is as follows: WT > $D^{282}A \gg D^{282}R$. The presence of an attractive rather than a repulsive interaction with residue 282 partly restores agonist activity, but the absence of an interaction (i.e., in D^{282}A-C5aR) has only a mildly deleterious effect on peptide activity. This is similar to the only partial reconstitution of activity on D^{282}R-C5aR using C5a[D^{74}], perhaps indicating that the substitution causes a significant change in the conformation of both the peptide and the full-length protein. This conformational sensitivity may also explain the difference between the data presented here and the data from an earlier publication (*21*) in which C5a[D^{74}] is an agonist at WT-C5aR, albeit with a 164-fold higher EC_{50} than C5a. The mutant C5a used in this case was not produced with an N-terminal His_6 tag extension and was affinity purified using a monoclonal antibody. It is likely that the His_6 tag, which is cleaved after Ni^{2+} affinity purification to leave a tripeptide N-terminal extension on C5a, can interfere with the correct refolding of the C5a C-terminus. We have previously shown that the presence of the tripeptide extension changes C5adR^{74} from a full to a partial agonist (*25*). An additional explanation of the only partial restoration of agonist activity by the reciprocal exchange of residues at D^{282} and R^{74} is that features of the R^{74} side chain in addition to charge are important. In support of this, a C5a mutant with K substituted for R^{74} was found to be less active than C5adR^{74} on human neutrophils (*5*). As previously reported, CHA-R has no activity on R^{206}A-C5aR, although it has been shown to bind to this C5aR mutant with high affinity (*16*). Substitution at R^{206} has also been shown to inhibit a constitutively activated C5aR mutant (*29*). Taken together, this evidence suggests that mutation at R^{206} simply makes the receptor unable to respond to suboptimal stimuli.

All C5aRs from other species sequenced to date have a D at the position equivalent to D^{282} except mouse and rat C5aR [which both bind human C5a with equally high affinity (*26*)] which have N at this position. It has been noted, however, that both have an E residue one turn lower in helix VII (*17*) that may fulfill a function similar to that of D^{282} in the human receptor. The mutation of C5aR D^{282} to A has been previously shown to completely inhibit ligand binding (*17*) in transfected COS cells, whereas the mutation of D^{282} to N fully restored binding activity (*17*). However, these authors examined only ligand binding, not receptor activation. In the RBL system, we found that although the number of binding sites appeared to be reduced by the D^{282}A mutation, the binding affinity of active receptors was identical to that of WT-C5aR. It is possible that substituting N instead of A for D^{282} simply produces a greater number of active receptors, at least in COS cells, allowing binding affinity to be measured. Clearly, the size of the subset of active D^{282}R-C5aR and D^{282}A-C5aR on RBL cells is sufficient to allow a substantial secretory response. A 14-fold increase in the numbers of active WT-C5aR expressed on RBL cells has previously been shown not to affect the EC_{50} for C5a (*28*). It is therefore unlikely that the variations in expression levels

of mutant C5aRs determine the responsiveness of receptors to different ligands. In support of this, neither WT- nor D^{282}A-C5aR responds to C5a[D^{74}] despite the difference in the expression levels of these receptors. Similarly, the sensitivity of both D^{282}A- and D^{282}R-C5aR to activation by C5adR^{74} and the reversal of the rank order of responses to peptide mimics when the C-terminal residue is substituted are strong evidence for a specific effect of the receptor mutations, rather than global conformational changes that nonspecifically inactivate the receptor.

In this report, we have presented data that suggest an interaction between D^{282} of C5aR and R^{74} of C5a. The lack of this interaction probably explains much of the reduced potency of C5adR^{74} relative to intact C5a in the RBL system, confirming the previous observation that the partial loss of activity of C5adR^{74} is mainly due to the loss of the guanidinium function (*5*). We have previously shown that purified human C5adR^{74} (N-glycosylated at the C-terminus) has a 10-fold higher EC_{50} for transfected RBL cells than the bacterially expressed (i.e., aglycosylated) C5adR^{74} used in our report (*20*). The enzymatic deglycosylation of C5adR^{74}, but not intact C5a, has been shown to increase activity on human neutrophils 10-fold (*29*). Thus, the $D^{282}-R^{74}$ interaction may confer the 10^1-10^4-fold higher potency of C5a relative to C5adR^{74} that is observed in several cell types, e.g., neutrophils and mast cells (*30*−*34*).

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