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Characterisation of C5a receptor agonists from phage display libraries

Stuart A. Cain, Adrian Higginbottom, Peter N. Monk*

Academic Neurology Unit, University of Sheffield Medical School, Sheffield S10 2RX, UK

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Abstract

C5a des-Arg^{74} has a 10- to 100-fold lower receptor binding affinity than intact C5a and is only a partial agonist. We have used phage display selection from randomly mutated C5a des-Arg^{74} libraries to isolate variant proteins that can activate C5a receptors with similar potency to C5a. Here we explore the interactions of three variants (V1–3) with C5aR mutated at residues involved in the differential response. The mutant Asp^{282}Arg-C5aR is preferentially activated by C5a des-Arg^{74}, probably due to repulsion between Arg^{74} of C5a and the substituent Arg^{282}. In accordance with this hypothesis, V2 (with a polar C-terminus which has no Arg residue) but not V1 (with a C-terminal Arg residue at position 73) could activate Asp^{282}Arg-C5aR. V3, with a very hydrophobic C-terminus, was the most potent agonist at Asp^{282}Arg-C5aR. Arg^{175} is a potential counterion for the C-terminal carboxylate of C5a. C5aR mutated to either Ala or Asp at this position lost nearly all responsiveness to both C5a and C5a des-Arg^{74}, suggesting that mutation of Arg^{175} caused a non-specific loss of receptor conformation and a loss of signalling capacity. However, V3 could still activate Arg^{175}Asp/Ala-C5aR with the same potency as wild-type C5aR, demonstrating that the mutant receptors retained high signalling capability and showed a specific loss of responsiveness. Thus C5a des-Arg^{74} variants produced by phage display are potentially useful tools for the dissection of ligand–receptor interactions.

Keywords: Complement receptor; Phage display; Random mutagenesis; Pyrimidine analogue; PCR; 8-Oxodeoxyguanosine

1. Introduction

C5a is a potent inflammatory mediator and immunomodulator, which exacerbates tissue damage in *in vivo* models of ischaemia/reperfusion injury [1], immune complex disease [2], the control of infarct size after surgical revascularisation [3], reverse passive Arthus reaction, endotoxic shock [4] and rheumatoid arthritis [5]. The agonist potential of C5a is vested in the C-terminal decapeptide [6–8] and synthetic peptide analogues of this activation domain are full agonists at the C5a receptors (C5aR). The C-terminus of C5a is rapidly desarginated *in vivo* to form C5a des-Arg^{74} [9], a stable form that is only cleared by receptor endocytosis and which has a different spectrum of bioactivity to 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 C5a des-Arg^{74} stimulates only cytokine release [10]. In addition, C5a des-Arg^{74} is only a partial agonist in many cell types.

C5aR is a member of the seven transmembrane receptor superfamily and is usually coupled to G_i-like G proteins in neutrophils and in the rat basophilic leukaemia (RBL-2H3) cell line model for chemoattractant receptor expression [11]. Several C5aR mutations are thought to affect interactions with the C5a C-terminus: (i) the mutation of Glu^{199} to Lys in the second extracellular loop disrupts an interaction with Lys^{68} of C5a [12] and selectively inhibits responses to C5a des-Arg^{74}; (ii) at the extracellular face of transmembrane helix VII, mutation of Asp^{282} [13] inhibits responses to C5a but not to C5a des-Arg^{74}, due to the disruption of an interaction with the basic side-chain of Arg^{74}. Asp^{282}Arg causes a repulsion of the Arg^{74} side-chain of C5a, which does not occur with C5a des-Arg^{74}; (iii) a residue at the extracellular face of transmembrane helix IV,

* Corresponding author. Tel.: +44-114-226-1312; fax: +44-114-226-1201.
E-mail address: p.monk@shef.ac.uk (P.N. Monk).

Abbreviations: C5aR, human complement fragment 5a receptor; WT, wild type; C5a des-Arg^{74}, des arginated C5a; V1–3, variants of C5a des-Arg^{74} selected from randomly mutated phage display libraries; RBL, rat basophilic leukaemia.

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Arg^{175}, is completely conserved in C5aR from different species [14] and is in an analogous position to Arg^{161} of the closely-related C3a receptor [15]. Arg^{175} is proposed to form a docking site for the C-terminal carboxylate group, and so mutation of this residue should disrupt interactions with both intact C5a and C5a des-Arg^{74}.

Recently, we have used a phage display method to select novel ligands from randomly mutated C5a des-Arg^{74} libraries on the basis of affinity for C5aR. These variant proteins, named V1 and V2, have a 5-fold higher affinity for C5aR than wild type (WT) C5a des-Arg^{74}, and are full agonists at C5aR expressed in RBL-2H3 cells. V1 and V2, although they contain a number of different substituted residues, both have a mutation at Cys^{27}, actually found in all functional C5aR ligands from phage display libraries [16,17]. Substitution at Cys^{27} appears to be an essential adaptation to phage display rather than a C5aR-selected mutation, and so the number of possible mutant proteins available for selection from the initial library was limited to those containing Cys^{27} mutants. In this paper we report the selection, from a random mutant library made using a [Cys^{27}Arg]-C5a des-Arg^{74} template, of a new variant, V3. We have analysed receptor activation by the three variants using WT and mutant C5aR. The results show that there are differences in the receptor activation mechanism made by the variants in comparison to C5a des-Arg^{74} and intact C5a and that these may be exploited to further our understanding of C5aR–ligand interactions.

2. Materials and methods

2.1. Materials

RBL cells transfected with WT mutant C5aR [11] were maintained in Dulbecco's Modified Eagle's Medium, supplemented with 10% (v/v) foetal calf serum and 400 μg/mL G418 (Gibco BRL). Mutagenic nucleotide triphosphates (6-(2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-8*H*-pyrimido-[4,5-*C*][1,2]oxazin-7-one; 8-oxo-2′deoxyguanosine) were prepared as described previously [18] or purchased from Amersham-Pharmacia. Receptor mutants were made and expressed in RBL cells as described previously [14]. Homogenous populations of receptor-expressing cells were obtained by fluorescence-activated cell sorting using the anti-human C5a receptor antibody, S5/1 (Serotec).

2.2. Construction of the C5a des-Arg^{74} mutant library

A modified form of the phagemid vector pJ/F2SS was used for expression of the C5a des-Arg^{74} mutant libraries [16]. The mutant libraries were generated by PCR in the presence of mutagenic nucleotide triphosphates as described previously [17], using the template of [Cys^{27}Arg]-C5a des-Arg^{74}. Selection of receptor binding clones from the mutant libraries was performed on RBL cells transfected with human C5aR as previously described [16,17].

2.3. Expression and purification of C5a des-Arg^{74} and mutants

Expression and purification of the recombinant His_6-tagged C5a, C5a des-Arg^{74}, and the C5a des-Arg^{74} variants was performed as described [19]. For cleavage of the His_6 tag from C5a des-Arg^{74} fragments, 200–300 μg of purified recombinant protein was digested with 100 U of rTEV protease in 1 mM DTT and rTEV buffer for 4 hr at 30°. The digested polypeptides were separated from the rTEV protease and cleaved His_6 tag by addition of Ni^{2+}-NTA resin. A short sequence (GGS) remains at the N-terminus of the polypeptides in this production protocol.

2.4. Measurement of receptor activation of RBL cells

Cellular activation was measured as the release of β-hexosaminidase activity from RBL-2H3 cells transfected with human C5aR [16]. The percentage of β-hexosaminidase release was calculated as a percentage of the release in response to a potent stimulus (1 μM C5a, C5a des-Arg^{74} or V3). Data were analysed by GraphPad Prism software and significance assessed by *t*-test.

2.5. [^{125}I]C5a binding assay

Competition 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]. IC_{50} and standard error values were obtained by iterative curve fitting using GraphPad Prism 2.0.

3. Results

3.1. Production of a novel C5a des-Arg^{74} variant by phage display mutagenesis

Following the success of phage display coupled to random mutagenesis for the production of two novel C5a receptor ligands, V1 and V2 [16,17], we attempted to produce a higher affinity ligand by using as template, C5a des-Arg^{74} with the substitution Cys^{27}Arg, a mutation ubiquitously selected from C5a des-Arg^{74} libraries that appears to be an adaptation to phage display or bacterial expression. Three randomly mutated libraries were made, with calculated amino acid mutation rates of 7–10, 5–7 and 3–5%. These libraries were combined, giving a calculated diversity of 6.2×10^5 members, and selected against RBL cells transfected with the human C5aR in four rounds of panning, thus retaining only those phage particles expressing high affinity forms of C5a des-Arg^{74}. The recovery rates (calculated as the number of phage recovered after

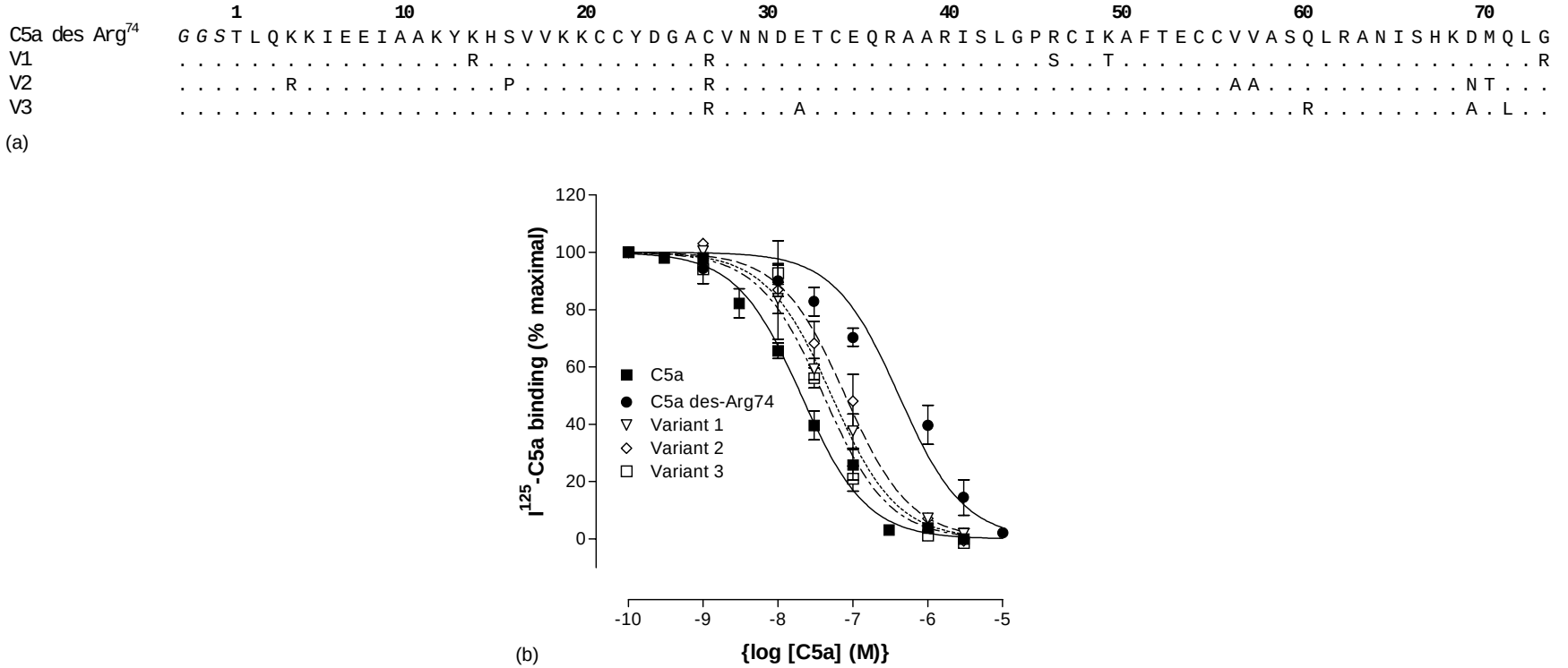

Fig. 1. Sequences and binding affinities of selected variants of C5a des-Arg[74]. (a) The amino acid sequences of the three C5a des-Arg[74] variants selected from randomly mutated phage libraries are shown compared to the sequence of C5a des-Arg[74] (top). The tripeptide extension sequence GGS is shown in italics but residue numbering begins from the first residue of C5a des-Arg[74]. A dot indicates amino acid identity. (b) The binding affinities of the three C5a des-Arg[74] variants are shown. RBL cells transfected with WT C5a receptor were incubated with the indicated concentrations of the variants for 15 min on ice, then [^{125}I]C5a (final concentration 50 pM) was allowed to bind for 15 min. After extensive washing, cell-associated radioactivity was counted.

panning as a percentage of the number of phage applied) were 8.0×10^{-5}, 8.8×10^{-5}, 6.7×10^{-4} and 7.0×10^{-4}% for each panning round. This rise in recovery rates is characteristic of libraries of randomly mutated proteins that are being enriched for phage with high affinity for the target receptor [20,21]. Nineteen clones were selected from the fourth round for sequencing; 11/19 sequences were identical to the previously selected V1 form ($Gln^{3}Pro$, $Cys^{27}Arg$, $Gly^{73}Arg$) but 8/19 were a new sequence, V3 ($Cys^{27}Arg$, $Glu^{32}Ala$, $Gln^{60}Arg$, $Asp^{69}Ala$, $Gln^{71}Leu$) (Fig. 1a), in which two of the polar residues in the C-terminal activation domain are mutated to non-polar amino acids. The binding affinity of V3 for WT-C5aR was measured by competition binding analysis and was found to be intermediate between the affinities for C5a and C5a des-Arg^{74} (Fig. 1b), as found previously for V1 and V2 [22].

3.2. Expression of mutant C5aR in RBL cells

$Glu^{199}Lys$, $Asp^{282}Ala$, $Asp^{282}Arg$ C5aR and two mutants at position 175, $Arg^{175}Ala$ and $Arg^{175}Asp$, were expressed in RBL cells to explore the interactions with C5a, C5a des-Arg^{74} and V1–3. WT and mutant receptors were transfected into RBL cells and subjected to two rounds of fluorescence-activated cell sorting. The expression levels of each receptor, as determined by antibody S5/1 binding, are shown in Fig. 2. The relative degranulation response (see Section 3.3, below) of the transfected RBL cells was determined using a high dose (1 μM) of a potent ligand: C5a (WT, $Glu^{199}Lys$, $Asp^{282}Ala$), C5a des-Arg^{74} ($Asp^{282}Arg$) or V3 ($Arg^{175}Ala$, $Arg^{175}Asp$). Expression levels were high for all receptors and correlated with levels of degranulation, with the exception of $Arg^{175}Asp$-C5aR that, although expressed at high levels at the cell surface, supported a smaller degranulation response than the other receptors. This suggests that a higher percentage of this mutant receptor is not correctly folded and so cannot be activated by ligand. However, there is clearly sufficient active receptor to stimulate measurable degranulation. Using this functional response to measure the activity of different ligands allows only the contribution of active receptors to be taken into account, thus ignoring potential complications due to low affinity binding of ligand to misfolded receptors.

3.3. Analysis of the activation of mutant C5aR by C5a des-Arg^{74} variants

Receptor activation was measured as the secretion of β-hexosaminidase during RBL cell degranulation. As found previously, C5a des-Arg^{74} was only a partial agonist at WT-C5aR (stimulating only 79% of maximal release at 1 μM) whereas all of the variants were full agonists (stimulating 92–110% of maximal release; Table 1) but with EC_{50} values closer to those of C5a des-Arg^{74} than intact C5a. The response patterns were very different for the mutant receptors. As found previously, $Glu^{199}Lys$-C5aR is potently activated by intact C5a ($EC_{50} = 6$ nM) but not by C5a des-Arg^{74} ($EC_{50} > 10$ μM) [23]. This receptor mutant is also not activated by V1 at concentrations up to 3 μM, suggesting that this variant interacts with C5aR like C5a des-Arg^{74}. V2 caused weak receptor activation, suggesting that it has properties intermediate between those of C5a and C5a des-Arg^{74}, but V3 caused full, potent activation of $Glu^{199}Lys$ receptors suggesting that it interacts with receptor more like intact C5a (Fig. 3, Table 1). $Asp^{282}Arg$ and $Asp^{282}Ala$-C5aR showed

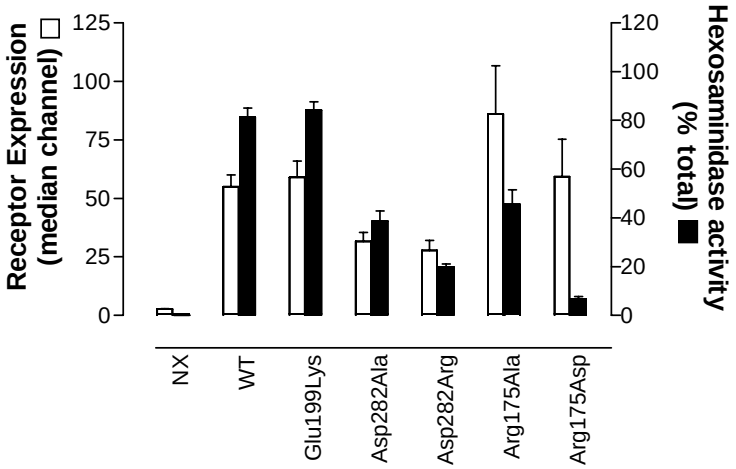

Fig. 2. Cell surface expression levels and relative ligand-stimulated degranulation of RBL cells transfected with WT and mutant C5aR. RBL cells transfected with WT or the indicated mutant C5a receptor were subjected to two rounds of fluorescence-activated cell sorting using an anti-C5a receptor antibody, selecting the top 10% each time to give homogenous populations of high expressing cells. Receptor expression levels (open bars) were determined by flow cytometry and are shown as median fluorescent channel numbers, mean ± SE from three separate determinations. Untransfected control cells (NX) are also shown. Degranulation was stimulated with 1 μM of a potent ligand for each receptor: WT, $Glu^{199}Lys$, $Asp^{282}Ala$—C5a; $Asp^{282}Arg$—C5a des-Arg^{74}; $Arg^{175}Ala$, $Arg^{175}Asp$—V3, and measured as the release of β-hexosaminidase from intracellular granules as a percentage of total cellular β-hexosaminidase activity. The values shown are means ± SE from 4 to 16 separate experiments.

Table 1
Summary of the effects of C5aR ligands on the degranulation of transfected RBL cells

Ligand	Receptor																	
	WT-C5aR			$Glu^{199}Lys$-C5aR			$Asp^{282}Ala$-C5aR			$Asp^{282}Arg$-C5aR			$Arg^{175}Ala$-C5aR			$Arg^{175}Asp$-C5aR		
	pD_2[a] ± SE	EC_{50}[b]	N[c]	pD_2 ± SE	EC_{50}	N	pD_2 ± SE	EC_{50}	N	pD_2 ± SE	EC_{50}	N	pD_2 ± SE	EC_{50}	N	pD_2 ± SE	EC_{50}	N
C5a	8.24 ± 0.03	5.82 (100)	16	8.21 ± 0.08 (ns)	6.13 (100)	4	7.83 ± 0.05***	14.6 (100)	9	6.49 ± 0.19***	325 (29)	4	6.30 ± 0.17***	502 (55)	3	6.65 ± 0.33***	223 (20)	3
C5a des-Arg^{74}	7.67 ± 0.07	21.2 (79)	16	<5		4	7.62 ± 0.07 (ns)	24.5 (91)	9	7.34 ± 0.03***	46.0 (100)	11	<5		3	5.36 ± 0.87***	4392 (12)	3
V1	7.72 ± 0.08	19.0 (108)	3	<5		4	7.46 ± 0.31 (ns)	34.7 (14)	3	7.15 ± 0.29 (ns)	71.2 (25)	2	<5		2	7.15 ± 0.16*	71.0 (50)	3
V2	7.80 ± 0.10	15.9 (92)	3	6.43 ± 0.37*	369 (23)	4	8.22 ± 0.20 (ns)	6.00 (59)	3	7.64 ± 0.08 (ns)	23.0 (114)	4	<5		3	<5		2
V3	7.53 ± 0.11	29.3 (110)	3	8.27 ± 0.10**	5.40 (103)	3	7.82 ± 0.06 (ns)	15.2 (149)	3	7.68 ± 0.08 (ns)	21.0 (161)	3	7.53 ± 0.04 (ns)	29.7 (100)	7	7.28 ± 0.07 (ns)	52.8 (100)	9

[a] $pD_2 = -\log EC_{50}$.
[b] EC_{50} = concentration (nM) resulting in 50% of maximal degranulation measured response to 1 μM of a potent ligand for each receptor: WT, $Glu^{199}Lys$, $Asp^{282}Ala$—C5a; $Asp^{282}Arg$—C5a des-Arg^{74}; $Arg^{175}Ala$, $Arg^{175}Asp$—V3.
[c] N = number of separate experiments performed in triplicate; significantly different from WT-C5aR: ns >5%.
* <5% (*t*-test); ** <0.5% (*t*-test); *** <0.005% (*t*-test).

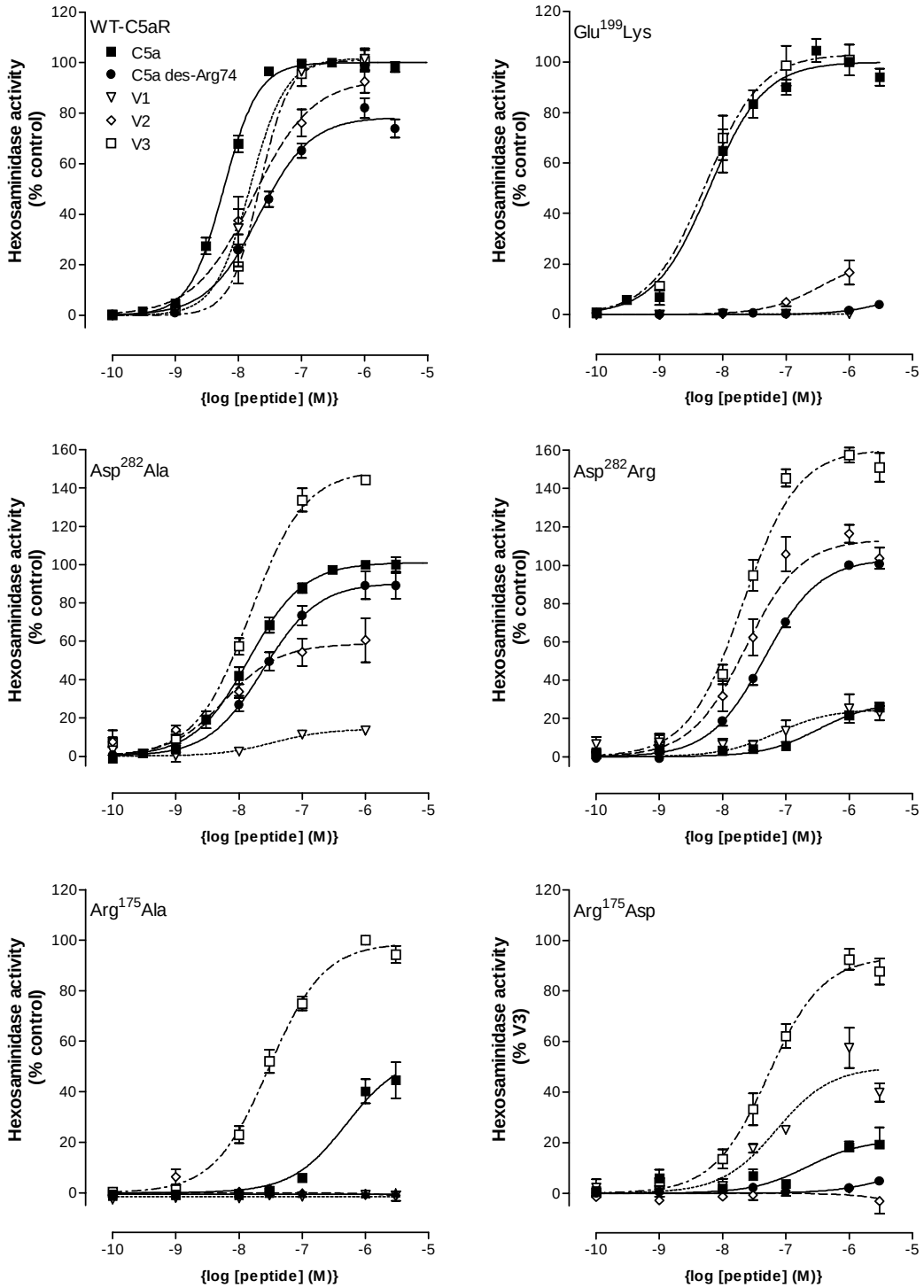

Fig. 3. Receptor activation assays in RBL cells transfected with WT and mutant C5aR. Sorted populations of RBL cells transfected with WT or the indicated mutant C5a receptor were incubated with the stated concentrations of C5a, C5a des-Arg^{74} or the three selected C5a des-Arg^{74} variants (V1–3). Degranulation was assessed as the release of β-hexosaminidase, as described in Section 2, and is shown as a percentage of degranulation in response to 1 μM of a potent ligand for each receptor: WT, Glu^{199}Lys, Asp^{282}Ala—C5a; Asp^{282}Arg—C5a des-Arg^{74}; Arg^{175}Ala, Arg^{175}Asp—V3. Results are the means of 2–16 separate experiments (see Table 1) performed in triplicate ±SE.

increased responses to C5a des-Arg^{74}, relative to intact C5a (Fig. 3, Table 1), as found previously [13]. Only V2 and V3 were full agonists at these mutant receptors; V1 was as poor an agonist as intact C5a at Asp^{282}Arg and considerably less active than C5a at Asp^{282}Ala-C5aR (Fig. 3, Table 1) indicating that the presence of a C-terminal Arg residue, even at position 73, can inhibit activation of these mutants. The mutation of Arg^{175} to Ala completely inhibited the degranulation response to C5a des-Arg^{74} and substantially reduced the response to C5a suggesting that

mutation of this residue may prevent receptor activation. The mutation of Arg^{175} to Asp had an even more dramatic effect on activation by C5a, although some activation by C5a des-Arg^{74} is detectable (Fig. 3, Table 1). However, the Arg^{175} mutant receptors were clearly still able to bind ligand and signal, as both V3 (Arg^{175}Ala and Arg^{175}Asp) and V1 (Arg^{175}Asp) could stimulate high levels of degranulation with EC_{50} values <100 nM. This indicates that significant numbers of both Arg^{175} mutants are functional and so the lack of responsiveness to C5a and C5a des-Arg^{74} is likely to be due to the loss of a specific receptor–ligand interaction.

4. Discussion

A novel high affinity ligand was produced from a randomly mutated [Cys^{27}Arg]-C5a des-Arg^{74} library by selection for binding to C5aR expressed on RBL cells. The [Cys^{27}Arg]-C5a des-Arg^{74} mutant was used as the template for the library because this mutation was always selected from previous C5a or C5a des-Arg^{74} libraries [16,17], and appeared to be an essential mutation for either phage display or bacterial expression. The selection procedure resulted in a modest 10-fold increase in phage recovery (the ratio between input and output phage number) typical of phage display of polypeptides such as C5a or of antibodies [21,24,25]. However, only two sequences, V1 and V3, were isolated from an initial library containing $>6 \times 10^5$ different sequences indicating that a high degree of selection had occurred. The affinity of the newly selected ligand, V3, for WT-C5aR was more than 10-fold higher than WT C5a des-Arg^{74}, although still not as high as intact C5a and was similar to the two previously selected C5a des-Arg^{74} variants, V1 and V2. V3 has only one residue substituted in the alpha-helical core (Glu^{32}Ala), but this is at a site not previously described as having a role in receptor binding. It seems likely that the mutations with most effect on binding are Asp^{69}Ala and Gln^{71}Leu because increased hydrophobicity at the C-terminus has previously been shown to confer higher affinity for C5aR in mutants of C5a des-Arg^{74} [2]. As the three variants appear to have properties intermediate between those of C5a des-Arg^{74} and intact C5a, we investigated the effects of the variants on a number of C5aR mutants that are known to differentially affect the responses to C5a des-Arg^{74} and intact C5a.

The mutation of Glu^{199} to Lys has been shown to completely inhibit receptor activation by C5a des-Arg^{74} and also the mutant Arg^{74}Ala-C5a [12], suggesting that the interaction between Glu^{199} and Lys^{68} of C5a is essential only when a further interaction, between the side-chain of Arg^{74} and the receptor, is missing. Surprisingly, V1, with Gly^{73}Arg, also could not activate Glu^{199}Lys-C5aR, indicating that the interaction between receptor and Arg^{73} of V1 was not identical to that with Arg^{74} of intact C5a. In contrast, the more hydrophobic variants, V2 and V3, could activate the receptor, with V3 being as potent as C5a. This suggests that Arg^{74} may be required in C5a to maintain the correct structure of the C5a C-terminus, as proposed by Zhang *et al*. [26], in addition to requirements for direct Arg^{74} side-chain and carboxylate interactions with receptor. Due to the mutated residues, the C-termini of V2 and V3 may assume this correct structure in the absence of Arg^{74}, whereas V1, although it can make some interactions with receptor through Arg^{73}, does not form the correct C-terminal structure for Glu^{199}Lys receptor activation.

We have previously shown that the Arg^{74} side-chain of intact C5a interacts with a residue located at the extracellular face of transmembrane helix 7, Asp^{282}. Peptide analogues of the C5a C-terminus have indicated that this may be due to the loss of an electrostatic interaction between Asp^{282} and Arg^{74} side-chains [13]. Thus, V1 with a C-terminal Arg^{73} residue may be expected to resemble C5a (an ineffective ligand due to the repulsion between receptor Arg^{282} and the C-terminal ligand Arg^{74} residue) rather than C5a des-Arg^{74} in its effect on C5aR mutated at Asp^{282}. This is, in fact, the case: V1 is a much weaker agonist at D^{282} mutants than V2 and V3, at least in terms of the maximal response. This effect is clearest on Asp^{282}Arg-C5aR: V1 has similar activity to intact C5a, whereas V2 and V3 are super-agonists (relative to C5a des-Arg^{74}), confirming the role of the Arg^{74} side-chain in C5aR activation. It should be noted that V1 is also a very weak agonist at Asp^{282}Ala, where no side-chain repulsion should occur, suggesting that the lack of the correct C-terminal conformation is also affecting interactions with both Glu^{199} and Asp^{282} mutant C5aR.

We hypothesised that Arg^{175} might be involved in interactions with the C-terminus of C5a because of the loss of ligand binding by C3a receptors mutated at the analogous residue, Arg^{161} [15], suggesting an interaction with the C-terminal carboxylate of C3a. In contrast, a previous study of C5a receptor mutants has shown that mutation of Arg^{175} to Ala does not appear to affect C5a binding affinity [27] but reduces the number of binding sites through an unknown mechanism. However, these authors did not look at the effects of Arg^{175}Ala on receptor activation. We show here that receptor activation by both C5a and C5a des-Arg^{74} is profoundly affected by mutation of Arg^{175} to Ala or Asp but that this is not simply due to a global misfolding of the receptor that would prevent cellular activation through receptor interaction with any ligand. The activity of V3 on Arg^{175}Ala/Asp-C5aR clearly shows that a significant fraction of the expressed mutant receptor is in a sufficiently normal conformation for signalling. Interestingly, V1 can stimulate 50% of the maximal response on Arg^{175}Asp, despite being inactive on Arg^{175}Ala-C5aR. This may be due to an interaction between the basic side-chain of the Arg^{73} residue of V1 and the Asp at position 175 of the receptor, suggesting that Arg^{175} is normally proximal to the C-terminus of bound

C5a. It is apparent from these data that Arg^{175} is involved in interactions with C5a, and that mutations of this residue do not simply result in loss of receptor conformation although further work is required to elucidate the role of Arg^{175} in receptor activation by C5a.

In summary, we have shown that variants of C5a des-Arg^{74} isolated from randomly mutated phage display libraries may be valuable tools for the dissection of receptor–ligand interactions. In particular, C5aR mutated at residue Arg^{175} are potently activated by V3 but not by the natural C5aR ligands, C5a and C5a des-Arg^{74}, suggesting that this residue is a previously unrecognised component of the ligand binding/receptor activation mechanism.

Acknowledgments

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References

[1] Arumugam TV, Shiels IA, Strachan AJ, Abbenante G, Fairlie DP, Taylor SM. A small molecule C5a receptor antagonist protects kidneys from ischemia/reperfusion injury in rats. Kidney Int 2003; 63:134–42.

[2] Heller T, Hennecke M, Baumann U, Gessner JE, zu Vilsendorf AM, Baensch M, Boulay F, Kola A, Klos A, Bautsch W, Kohl J. Selection of a C5a receptor antagonist from phage libraries attenuating the inflammatory response in immune complex disease and ischemia/reperfusion injury. J Immunol 1999;163:985–94.

[3] Riley RD, Sato H, Zhao ZQ, Thourani VH, Jordan JE, Fernandez AX, Ma XL, Hite DR, Rigel DF, Pellas TC, Peppard J, Bill KA, Lappe RW, Vinten-Johansen J. Recombinant human complement C5a receptor antagonist reduces infarct size after surgical revascularization. J Thorac Cardiovasc Surg 2000;120:350–8.

[4] Strachan AJ, Woodruff TM, Haaima G, Fairlie DP, Taylor SM. A new small molecule C5a receptor antagonist inhibits the reverse-passive Arthus reaction and endotoxic shock in rats. J Immunol 2000;164: 6560–5.

[5] Woodruff TM, Strachan AJ, Dryburgh N, Shiels IA, Reid RC, Fairlie DP, Taylor SM. Antiarthritic activity of an orally active C5a receptor antagonist against antigen-induced monoarticular arthritis in the rat. Arthritis Rheum 2002;46:2476–85.

[6] Mollison KW, Mandecki W, Zuiderweg ER, 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.

[7] 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: identification of receptor-binding residues in the inflammatory complement protein C5a by site-directed mutagenesis. Eur J Biochem 1994;219:897–904.

[8] Toth MJ, Huwyler L, Boyar WC, Braunwalder AF, Yarwood D, Hadala J, Haston WO, Sills MA, Seligmann B, Galakatos N, Bubeck P, Grotzinger J, Winkler M, Kohl J, Wollmer A, Klos A, Bautsch W. The pharmacophore of the human C5a anaphylatoxin. Protein Sci 1994;3:1159–68.

[9] Bokisch VA, Muller-Eberhard HJ. Anaphylatoxin inactivator of human plasma: its isolation and characterization as a carboxypeptidase. J Clin Invest 1970;49:2427–36.

[10] Eglite S, Pluss K, Dahinden CA. Requirements for C5a receptor-mediated IL-4 and IL-13 production and leukotriene C4 generation in human basophils. J Immunol 2000;165:2183–9.

[11] Monk PN, Pease JE, Barker MD. C5a stimulus-secretion coupling in rat basophilic leukaemia (RBL-2H3) cells transfected with the human C5a receptor is mediated by pertussis and cholera toxin-sensitive G proteins. Biochem Mol Biol Int 1994;32:13–20.

[12] 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.

[13] Cain SA, Coughlan T, Monk PN. Mapping the ligand-binding site on the C5a receptor: arginine74 of C5a contacts aspartate282 of the C5a receptor. Biochemistry 2001;40:14047–52.

[14] Cain SA, Woodruff TM, Taylor SM, Fairlie DP, Sanderson SD, Monk PN. Modulation of ligand selectivity by mutation of the first extracellular loop of the human C5a receptor. Biochem Pharmacol 2001;61: 1571–9.

[15] Sun J, Ember JA, Chao TH, Fukuoka Y, Ye RD, Hugli TE. Identification of ligand effector binding sites in transmembrane regions of the human G protein-coupled C3a receptor. Protein Sci 1999;8:2304–11.

[16] 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.

[17] Cain SA, Williams DM, Harris V, Monk PN. Selection of novel ligands from a whole-molecule randomly mutated C5a library. Protein Eng 2001;14:189–93.

[18] Zaccolo M, Williams DM, Brown DM, Gherardi E. An approach to random mutagenesis of DNA using mixtures of triphosphate derivatives of nucleoside analogues. J Mol Biol 1996;255:589–603.

[19] Paczkowski NJ, Finch AM, Whitmore JB, Short AJ, Wong AK, Monk PN, Cain SA, Fairlie DP, Taylor SM. Pharmacological characterization of antagonists of the C5a receptor. Br J Pharmacol 1999;128:1461–6.

[20] Hennecke M, Otto A, Baensch M, Kola A, Bautsch W, Klos A, Kohl J. A detailed analysis of the C5a anaphylatoxin effector domain: selection of C5a phage libraries on differentiated U937 cells. Eur J Biochem 1998;252:36–44.

[21] Kola A, Baensch M, Bautsch W, Klos A, Kohl J. Analysis of the C5a anaphylatoxin core domain using a C5a phage library selected on differentiated U937 cells. Mol Immunol 1999;36:145–52.

[22] Cain SA, Williams DM, Harris V, Monk PN. Selection of novel ligands from a whole-molecule randomly mutated C5a library. Protein Eng 2001;14:189–93.

[23] Monk PN, Barker MD, Partridge LJ, Pease JE. Mutation of glutamate 199 of the human C5a receptor defines a binding site for ligand distinct from the receptor N terminus. J Biol Chem 1995;270:16625–9.

[24] Hennecke M, Kola A, Baensch M, Wrede A, Klos A, Bautsch W, Kohl J. A selection system to study C5a–C5a-receptor interactions: phage display of a novel C5a anaphylatoxin, Fos-C5aAla27. Gene 1997;184:263–72.

[25] Bugli F, Bastidas R, Burton DR, Williamson RA, Clementi M, Burioni R. Molecular profile of a human monoclonal antibody Fab fragment specific for Epstein-Barr virus gp350/220 antigen. Hum Immunol 2001;62:362–7.

[26] Zhang X, Boyar W, Toth MJ, Wennogle L, Gonnella NC. Structural definition of the C5a C terminus by two-dimensional nuclear magnetic resonance spectroscopy. Proteins 1997;28:261–7.

[27] Raffetseder U, Roper D, Mery L, Gietz C, Klos A, Grotzinger J, Wollmer A, Boulay F, Kohl J, Bautsch W. Site-directed mutagenesis of conserved charged residues in the helical region of the human C5a receptor. Arg206 determines high-affinity binding sites of C5a receptor. Eur J Biochem 1996;235:82–90.