Improvements in the Minimum Binding Sequence of C5a: Examination of His-67[1]

C5a is a biologically active glycoprotein fragment released during the activation of serum complement proteins. This glycoprotein, representing the N-terminus of the α-chain of complement protein C5, is enzymatically released from the parent molecule by the actions of the complement-derived C5 convertase. C5a shares with C3a and C4a, two other complement-derived anaphylatoxins, the ability to produce a variety of inflammatory responses.[3] However, C5a is believed to play a major role as a potent inflammatory mediator by its additional activities in recruiting and stimulating inflammatory leukocytes. Both C5a and its des Arg-74 derivative exist in vivo as biologically active forms and are both inflammatory mediators. Once it is liberated from its parent molecule, C5a interacts with specific membrane receptors present on white blood cells including polymorphonuclear leukocytes (PMNL), monocytes, basophils, and eosinophils, as well as with tissue resident cells such as macrophages and mast cells. The interaction of C5a with its receptor in target cells stimulates the directed migration (chemotaxis) of leukocytes.[4] C5a induces PMNL shape changes and increased adhesion to other cells and surfaces.[5] C5a stimulates the oxidative metabolism of inflammatory leukocytes as indicated by enhanced superoxide anion generation.[6] The activation of neutrophils and other target cells by C5a also increases the release of granular enzymes,[7] histamine,[8] and other vasoactive inflammatory substances.[9] These substances promote the destruction of tissues, the increase of vascular permeability, the contraction of smooth muscle, and an influx into the tissues of inflammatory cells such as neutrophils, eosinophils, and basophils. C5a may also be important in mediating inflammatory effects of phagocytic mononuclear cells that accumulate at sites of chronic inflammation.[10] Recent studies show that C5a may also exert immunomodulatory effects at sites of inflammation by enhancing antibody production.[11] Thus, the search for a specific C5a receptor antagonist to inhibit the involvement of C5a in inflammation has been an active and ongoing process in recent years because of its therapeutic implications.

Human anaphylatoxin C5a is a single-chain cationic glycoprotein consisting of 74 amino acid residues and an N-linked carbohydrate moiety of approximately 3 kd attached at asparagine 64.[12] Six of the seven cysteines in C5a are linked via intramolecular disulfide bonds to stabilize the folded peptide chain. Based on circular dichroism (CD) spectra, as many as 50% of the residues in C5a are estimated to be in α-helical conformation. The primary structures of human C3a, C4a, and C5a have been elucidated by chemical analysis. The sequence comparison between human C3a and C5a indicates 34% homology, while homology between C4a and C5a is 39%.[3c] The three-dimensional structure of the major portion of C5a has been modeled from the homologous C3a crystal structure by comparative modeling techniques.[13] The "active site", or the minimum active sequence of C3a and C4a, has been identified by synthetic peptide mapping to be the C-terminal penta- and octapeptides.[14,15] Unlike the

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Table I. Inhibition of ^{125}I-rC5a Binding to PMNL Membranes and Biological Activities of Synthetic Peptides[a]

R-AA^{67}-Lys-Asp-Met-Gln-Leu-Gly-Arg-OH

peptide no.	R	AA^{67}	receptor binding: K_i,[c] μM	agonist efficacy[b] CK:[d] % rC5a (EC_{50}, μM)	MPO:[e] % rC5a (EC_{50}, μM)	SVP:[f] % rC5a (EC_{50}, μM)
rC5a			0.00003	100 (0.00014)	100 (0.001)	100 (0.03)
1	H	His	300	129 (110)	–[g]	–
2	H	Ala	inactive[h]	–	–	–
3	H	(1-Me)His	180	88 (110)	–	–
4	H	(3-Me)His	58	90 (17)	–	–
5	H	Thia	20	121 (13)	–	–
6	H	Thi	2.1	142 (0.53)	68 (200)	85 (<1000)
7	H	Cha	13	154 (3)	–	–
8	H	Phe	0.26	132 (0.46)	–	60 (<1000)
9	H	Phg	31	72 (10)	–	–
10	H	hPhe	36	162 (15)	–	–
11	H	DPhe	79	70 (80)	–	–
12		Hca	4.4	84 (0.80)	–	–
13	Ac	Phe	0.20	76 (0.44)	103 (35)	–
14	H-Ile-Ser	Phe	0.39	76 (0.72)	81 (60)	54 (<100)
15	H-Ala-Asn-Ile-Ser	Phe	0.22	85 (0.25)	63 (330)	69 (<100)
16	H-Leu-Arg-Ala-Asn-Ile-Ser	Phe	0.81	133 (1.6)	63 (60)	116 (<300)

[a] Thia, 2-thiazoyl-L-alanine; Thi, β-2-thienyl-L-alanine; Cha, L-cyclohexylalanine; Phg, L-phenylglycine; hPhe, L-homophenylalanine; Hca, hydrocinnamoyl. [b] Efficacy compared to C5a and expressed as % rC5a maximum response; potency values are shown in parentheses. [c] K_i: K_i for inhibition of ^{125}I-rC5a binding to human PMNL membranes. [d] CK: PMNL chemokinesis. [e] MPO: PMNL lysosomal enzyme (myeloperoxidase) release. [f] SVP: guinea pig skin vascular permeability increase. [g] –: Peptides not tested. [h] No inhibition of binding at 1 mM of peptide.

studies with C3a and C4a analogues, the synthetic C5a C-terminal octapeptide was originally assessed to be inactive after examination for spasmogenic activity, effects on vascular permeability, and chemotactic activity.[16a] Longer synthetic peptide analogues based on the carboxyl-terminus of human Tyr-C5a (55–74),[16b] rat C5a (58–77), and rat C5a (64–77)[16c] were reported to exhibit spasmogenic activity. However, Tyr-C5a (55–74) did not induce a chemotactic response in human neutrophils.[16b]

Recently, by mapping the active site with synthetic peptides representing various regions of the C5a molecule, the minimum sequence of C5a capable of inhibiting the binding of ^{125}I-C5a to PMNL membranes was reported to be the carboxyl-terminal octapeptide 1 (Table I).[2] In spite of the literature suggesting the existence of more than one receptor binding region of C5a,[17] only peptides containing the C-terminal sequence of C5a were found to inhibit the binding of radiolabeled C5a to its receptor. The carboxyl octapeptide fragment His^{67}-Arg^{74} (1) had a low binding affinity for the C5a receptor of human PMNL membrane fragments, K_i = 300 μM, but was inactive in an intact cell binding assay. This synthetic octapeptide induced both PMNL chemokinesis and lysosomal enzyme release.[2] More recently, an additional binding site near Lys$^{19-20}$ of C5a was indicated by site-directed mutagenesis study.[18] A synthetic peptide based on this region of C5a (residues 19–30) was found to have a low binding affinity for the C5a receptor.[18]

The first steps in our strategy to develop a C5a receptor antagonist was to identify small peptide fragments that have higher binding affinity for the C5a receptor. Although the binding affinity of peptide fragment 1 is low, it represents one of the few small, active peptide fragments of C5a ever identified. With the working model that a key binding region of C5a is the carboxyl-terminal octapeptide, we modified the peptide to increase the binding affinity. This paper reports the synthesis of several His^{67}-modified C5a C-terminal octapeptide analogues with greatly improved binding affinities.

Chemistry

Peptides 2–16 were synthesized by solid-phase methods using a Biosearch 9500 synthesizer. Cleavage and deprotection were accomplished with liquid HF at 0 °C. Purification by reverse-phase high-performance liquid chromatography, followed by lyophilization yielded the desired peptides. The peptides were characterized by FABMS and amino acid analyses.

Biological Evaluation

C5a Receptor Binding Assay. Peptide analogues 2–16 were evaluated as inhibitors for ^{125}I-C5a binding to PMNL membranes. The binding affinities are reported as the apparent dissociation constant (K_i) which was determined from inhibition binding data using the method of Cheng and Prusoff.[19a] The results of these studies are listed in Table I. In general, K_i determinations were not made on

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compounds with less than 50% inhibition at 1 mM and were considered to be inactive.

PMNL Assays. Peptides were tested in a range of concentrations in half-log steps, based on the receptor binding K_i value, to ascertain presence of agonistic activity consistent with C5a receptor interaction. The chemokinesis assay procedure was adapted from the method of Smith et al.[19b] The maximal agonist efficacy of each peptide is shown in Table I and expressed as a percent of the maximal migration obtained with an rC5a control response curve. The EC_{50} concentrations (shown in parentheses) were used as an estimate of potency and were calculated by the method of least-squares regression to obtain the concentration giving a response that was 50% of the maximal response produced by the individual peptides. Myeloperoxidase (MPO) release was measured as described previously.[18] The maximal agonist response of peptides shown in Table I was calculated in comparison to the maximal lysosomal enzyme release produced by 1×10^{-6} M rC5a and the EC_{50} values were calculated as above in reference to the maximal response produced by the respective peptides. For both PMNL assays, efficacy differences of more than 25% from the rC5a control were usually statistically significant; however, differences of more than 50% were found to be reproducible and considered to be of sufficient magnitude to represent meaningful changes in agonist efficacy.

Guinea Pig Skin Vascular Permeability. The in vivo agonist activities of peptides were determined by comparing their ability to cause Evans blue dye leakage as a marker of increased vascular permeability when injected intradermally, a predominantly mass cell-dependent response in the guinea pig.[3,19c] Responses of peptides, shown in Table I, were compared to the maximal response produced by a control rC5a injection included with each test group.

Biological Activities and Discussion

To evaluate the binding affinities and to study the structure–activity relationships of the analogues, the PMNL membrane binding assay was used. The minimum binding sequence was recently reported to be the carboxyl octapeptide 1, K_i = 300 μM.[2]

The importance of His^{67} to receptor binding is suggested in the report[2] because His^{67} deletion in 1 resulted in reduced inhibition of binding. The potential contribution of His^{67} in receptor binding was therefore reassessed. In the present study, the role of the imidazole ring in the His side chain was examined by replacing the imidazole ring with a hydrogen to give peptide **2**. The Ala for His^{67} change preserves the overall peptide backbone and more carefully assesses the importance of this His side chain. This modification caused a decrease in binding affinity and reaffirmed the importance of the His side chain.

Histidine is an interesting residue since it can bind to the receptor protein either by specific hydrogen-bonding interactions or by other interactions characteristic of the aromatic ring.[20] Modifications of the imidazole side chain of histidine residue in peptides can often produce drastic changes in biologic activity.[20,21] At physiological pH, it may exist in two possible tautomers.[22a] The N^{τ}–H and N^{π}–H tautomers of histidine have been observed by ^{13}C NMR spectroscopy in bacitracin,[22b] angiotensin,[22c] and thyliberin (TRH).[22d] Both of the imidazole nitrogens are able to donate or accept a hydrogen bond while interacting with the receptor protein. Peptides **3–5** explore the effect of His side chain basicity upon interactions of the ligand to its receptor. Peptides **3** and **4**, with the imidazole nitrogens methylated at positions 1 and 3 respectively, are slightly better in binding than the parent compound. The extra steric bulk introduced by the methylation seems not to interfere with receptor binding. Both methylated histidines are more basic (1-MeHis, pK_a = 6.48 and 3-MeHis, pK_a = 6.56) than histidine (pK_a = 6.0) itself.[23] Because of the relatively small changes in binding, it is difficult to draw correlations between basicity and affinity observed with these analogues. However, replacing histidine with 3-(4-thiazolyl)-L-alanine in **5**[24] resulted in substantial improvements in affinity (K_i = 20 μM). Thiazolylalanine is an unnatural amino acid that results from the replacement of the N^{τ}–H of histidine with a sulfur atom. In **5**, the planarity and the five-membered ring geometry of the imidazole group is preserved without drastically changing the steric environment of the His residue. On the other hand, thiazolylalanine side chain is substantially less basic (pK_a of thiazole = 2.44)[25] and is not expected to be protonated at physiological pH. These results indicate that the imidazole ring probably interacts with the receptor in its uncharged form, and a neutral aromatic ring system might increase the binding affinity. Another interesting

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His surrogate is 3-(2-thienyl)-L-alanine (Thi), in which the imidazole of His is replaced by thiophene. This heterocycle, which lacks the polar nitrogens, approximates the planar, five-membered ring geometry of the imidazole moiety. Thus peptide **6** shows an impressive 120-fold increase in binding (K_i = 2 μM). The study with peptides **2–6** suggests that His^{67} is not interacting with the receptor protein through hydrogen bonding. It is interesting that the progressive increase in binding affinity parallels the gradual change in hydrophobicity of the peptide while the steric environment stays relatively constant. But it is still unclear whether the hydrophobicity or the aromaticity is more important for high affinity.

Substituting His^{67} with cyclohexylalanine (Cha), an unnatural amino acid residue known to exert strong hydrophobic interactions,[26] gives peptide **7** with 20-fold improved affinity. The increase in affinity is relatively small compared to that of Thi replacement. This difference indicates either the planarity of the thiophene ring or its aromaticity is required for effective interactions with the receptor. The next logical His replacement is phenylalanine, a natural amino acid which is an important residue for many ligand–receptor interactions because of the aromaticity and hydrophobicity of its side chain.[27] The synthetic peptide **8**, with Phe for His^{67} (K_i = 0.26 μM), is a 1000-fold increase in binding affinity compared to the native octapeptide **1**.

The optimal steric requirements for the Phe residue in peptide **8** was studied next. The side-chain length of phenylalanine was found to be sensitive to modification. Analogues with a side chain either one methylene shorter or longer, L-phenylglycine (Phg^{67}) and L-homophenylalanine (hPhe^{67}) in analogues **9** and **10**, respectively, are 100-fold less avid in binding compared to **8**. In order to study the charge and chirality requirements of the Phe^{67}-receptor interaction with **8**, the contribution of the N-terminal basic amino group has to be assessed first. Acetylated peptide **13** is equal to **8** in binding affinity, suggesting that the unprotected N-terminal amino group is not an important feature required for binding. Deletion of the N-terminal amino group to give the hydrocinnamoyl (Hca^{67}) peptide **12** resulted in a 20-fold loss of affinity. This could be explained by the loss of either chirality or conformational stability. Replacing the Phe residue with D-Phe results in 200-fold decrease in affinity (peptide **11**). It is concluded that Phe^{67} in the modified peptide interacts with the receptor in a very stereospecific manner.

A single substitution of His^{67} in the minimum binding sequence of C5a with Phe has boosted the binding affinity by 1000-fold. At present, it is not clear whether the Phe substitution may have some profound effect on the bioactive conformation of the peptide or if we have improved the interactions of residue 67 with the receptor. It may also be possible that Phe^{67} is interacting with some auxiliary binding site normally not available for His^{67} on the receptor. A point C5a mutant, with Ala^{67} for His^{67}, did not change the activity of C5a.[17] However, another mutant substituting Phe for His^{67} was made based on the present synthetic peptide work and was found to boost the potency of native rC5a, suggesting an auxiliary binding site at C5a 67.[18] One other interesting possibility is that we might have unknowingly synthesized a peptide fragment that represents another region of the C5a molecule which is the true minimum binding sequence of the protein. Examination of the known primary sequences of the human anaphylatoxins C3a, C4a, and C5a and those of other species revealed the presence of only one phenylalanine at residue 53. It is highly conserved not only among anaphylatoxins but also among different species. Is it possible for **8** to span some discontinuous binding sites near the Phe^{53} binding region? In the reported "active site" mapping with synthetic peptides, the specific activity of peptides based on the C-terminal sequence of C3a increases steadily as the length of the synthetic peptides is extended gradually.[3c] If Phe is simply re-enforcing the favorable His^{67} interaction of the native sequence with the receptor, a similar improvement in affinity with extended C5a carboxyl-terminal Phe^{67} peptides should be observed. However, peptides **14–16** which are extended Phe^{67} peptides, do not show any improved binding affinity as compared to **8**.

The finding that all of the peptides tested display full agonist activity in triggering two major functional responses of human PMNL is surprising in view of their small size relative to that of C5a itself, and the evidence that regions of the C5a molecule in addition to the C-terminus contribute to its receptor binding and biological activity.[17,18] Indeed, two peptides with enhanced chemokinetic efficacy compared to rC5a (>150% response) could be classified as superagonists. The role of the particular position 67 substitutions, Cha and hPhe, respectively, in conferring this property is not clear. The PMNL responses appear to be attributable to interaction of the peptides with the C5a receptor because potencies of the functional responses agree well with the binding affinities. The chemokinetic responses reached EC_{50} levels at concentrations within severalfold of the binding K_i values. Inducing MPO release required consistently higher concentrations of peptides than for stimulating locomotion, which is a well-known property of chemoattractants such as C5a.[3] The further ability of members of this series of peptides to trigger biologic responses in vivo is established by their activity in the vascular permeability assay. Because C5a has a broad dose–response curve for inducing vascular leakage (not shown) and requires much higher concentrations to reach a maximum, the peptides were tested at a single high concentration to determine whether or not they retained this undesirable agonist response. Vascular leakage involves additional targets, e.g., mast cells,[3,19c] indicating that the C5a receptor interaction and agonist properties of the C-terminal peptide analogs are not confined to PMNL.

Conclusions

A 1000-fold boost of binding affinity of the C5a C-terminal octapeptide has been realized by a single Phe for His^{67} substitution. However, extension of the Phe^{67} C5a C-terminal peptides up to 14-mers does not further improve the affinity as compared to the Phe^{67} C-terminal octapeptide. These peptides, although representing a small portion of the C5a molecule, are agonists as determined by PMNL chemokinesis, PMNL myeloperoxidase release and guinea pig skin vascular permeability assays. As such, they are interesting small molecule mimics of C5a and serve as potent leads in the development of C5a antagonists. We have recently demonstrated the applicability of this Phe for His^{67} modification in other series of ana-

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Supplementary Material Available: Experimental and characterization data for the compounds discussed in this work (5 pages). Ordering information is given on any current masthead page.

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