Identification of a Receptor-Binding Region in the Core Segment of the Human Anaphylatoxin C5a

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Received August 11, 1993®

In order to identify regions of C5a that contribute to receptor binding and functional activity of the anaphylatoxin, a series of peptides was synthesized in which core segments have been attached to C-terminal segments via native peptidic or disulfide bonds. It has been found that residues Arg^{40} and Arg^{46} in the loop-3 region of the core induce a 1000-fold increase in the affinity of the disordered C-terminal segment of C5a. The results obtained from this work lead to the conclusion that the loop-3 region is most likely the core binding site of C5a.

Introduction

Activation of the complement system in response to immunological events results in a cascade of proteolytic cleavages of complement proteins C1–C5 and leads to the assembly of the membrane attack complex capable of cell lysis. A byproduct of this inflammatory response is the release of the anaphylatoxins C3a, C4a, and C5a which interact with cellular components and propagate the inflammatory process.[1] Biological activities of the anaphylatoxins include the contraction of smooth muscle, vasodilation, and increase in vascular permeability. C5a, however, is the principal inflammatory mediator possessing additional biological activities which are mediated through specific receptor–ligand interactions. These activities include an increase in Ca^{2+} mobilization, chemotaxis, a shape change and adhesion to other inflammatory leukocytes,[2] stimulation of the oxidative metabolism of polymorphonuclear leukocytes,[3] and activation of neutrophils and other cells to release tissue-digesting enzymes and other inflammatory substances.[4] C5a has been implicated as a causative or aggravating agent in a variety of inflammatory diseases.[5]

Human C5a is a 74 amino acid peptide that is *N*-glycosylated with a carbohydrate moiety approximately 3 kDa at the aspargine 64 residue.[6] The three-dimensional structure of C5a has been investigated by NMR studies of human[7] and porcine[8] C5a and modeled by comparison to the crystal structure of the homologous C3a using comparative modeling methods.[9] According to these studies, C5a consists of two distinct segments, the disulfide-linked core segment 1–63 and the disordered C-terminal segment 64–74. The core of C5a in turn consists of four helical segments 4–12, 18–26, 32–39, and 46–63 which are folded in an approximately antiparallel bundle and connected by loop segments 13–17, 27–31, and 40–45, respectively. The core is further stabilized by three disulfide bonds formed between Cys^{21}—Cys^{47}, Cys^{22}—Cys^{54}, and Cys^{34}—Cys^{55}. An approximate cartoon representation of the core and the disordered C-terminal segments of C5a is illustrated in Figure 1.

The interaction of C5a with its receptor has been the subject of numerous investigations. Site-directed mutagenesis has identified amino acids in the core and C-terminal segments, such as the loop-1 region, Arg^{40} and Arg^{74}, to play a significant role in receptor binding and biological activity.[10] Neutralizing antibodies to the C5a molecule have implicated the region between Lys^{20} and Arg^{37} to be important for receptor binding.[11] C5a-des-Arg^{74} which is produced from C5a by the action of carboxypeptidase N has markedly reduced biological activity.[12] The truncated C5a (1–69) segment maintains the ability to bind to the C5a receptor on neutrophils but lacks agonist activity.[13] The above literature information clearly indicates the existence of more than one receptor binding region in C5a. It has been suggested[10a] that C5a interacts with its receptor via a "binding" site located at the core and an "activation" site located at the C-terminal region. The mapping, however, of the active sites with synthetic peptides corresponding to various segments of C5a revealed that only the C-terminal peptides were weakly active (k_i = 300 μM) in inhibiting C5a receptor binding.[14] The smallest active segment was found to be a C-terminal octapeptide which induced PMN chemotaxis and lysosomal enzyme release, although it was previously reported to be inactive in vascular permeability.[14,15] In addition, a longer synthetic peptide, Tyr-C5a(55–74), was reported to exhibit spasmolytic activity but lacked chemotactic activity in human neutrophils.[16] Furthermore, modification of the C-terminal octapeptide resulted in the synthesis of analogs with greatly improved receptor binding affinity. The octapeptide analogs exhibited a wide spectrum of C5a agonist effects but with potency weaker than expected by comparison to their binding affinity.[17]

Consideration of the above findings strongly suggests that the design of an effective C5a antagonist should take into consideration both the core "binding" and C-terminal "activation" sites of C5a. The purpose of our investigation was to characterize the pharmacophore of C5a. The information obtained from this study may be useful in the design of an effective C5a receptor antagonist. In order to obtain a better understanding of the nature of the core "binding" site, we have synthesized a series of peptides where segments of the core are appropriately connected to the C-terminal segments of C5a. These peptides are graphically represented in Figure 1, where the shaded areas of the C5a cartoons represent the segments that have been connected via

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® Abstract published in *Advance ACS Abstracts*, July 1, 1994.

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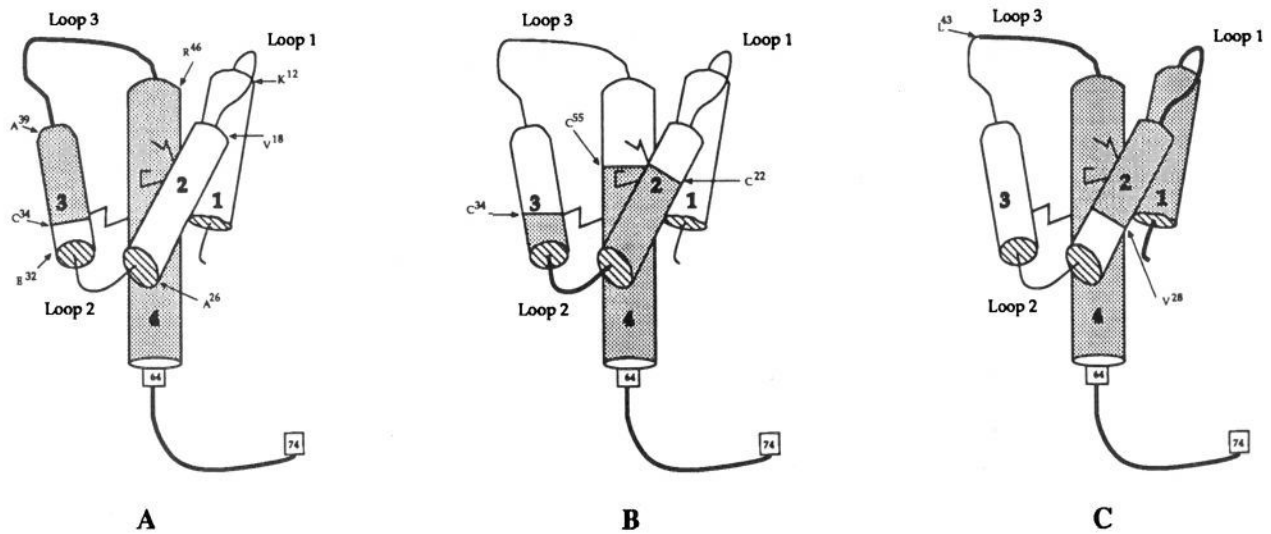

Figure 1. Cartoon representation of a three-dimensional structure of C5a. The core segment of C5a (1−63) is represented by its helical regions (cylinders) connected via loop regions and stabilized by three disulfide bonds in an approximately antiparallel bundle. The orientation of the disordered C-terminal segment 64−74 is arbitrary. Cartoons A, B, and C represent synthetic analogs (shaded areas and heavy lines) in which core segments 34−63, 22−34, and 1−28 are connected to C-terminal segments via native peptidic or disulfide bonds.

C5a Segment 34-74

CEQRAARISLGPRCIKAFTECCVVASQLRANISHKDMQLGR

C5a Segment 22-34

CYDGACVNNDETC

C5a Segment 1-28

TLQKKIEEIAAKYKHSVVKKCCYDGACV

Figure 2. Primary sequence of human C5a segments incorporated into the synthetic C5a analogs.

native peptidic or disulfide bonds. The compounds that incorporated both the "binding" and "activation" sites were expected to show increased affinity to the C5a receptor. In this paper we describe the synthesis and biological evaluation of these compounds.

Synthesis of C5a Partial Structures. The primary sequence of C5a segments depicted in cartoons A, B, and C in Figure 1 is shown in Figure 2. All synthetic peptides described in this work are represented in Table 1. Cartoon A represents a study of the C5a segment 34−74 where the C-terminal segment 64−74 is connected via native peptide bond to segments of the core that include helix 4, loop-3, and part of helix 3 (peptides **1** to **40**). In cartoon B the C5a segment 22−34 (peptide **41**) is connected to the C-terminal segment 54−74 (peptide **3**) via the native Cys^{22}—Cys^{54} and Cys^{34}—Cys^{55} disulfide bonds (peptide **43**). Cartoon C represents the N-terminus segment 1−28 (peptides **44** and **45**) connected to the C-terminal segments 47−74 and 43−74 (peptides **16**, **27**, and **28**) via a single native Cys^{22}—Cys^{54} or Cys^{21}—Cys^{47} disulfide bonds (peptides **46**, **47**, and **48** respectively).

All linear peptides in Table 1 were synthesized by solid-phase peptide synthesis using the OCH_2-PAM-polystyrene resin as polymeric support,[18] with an Applied Biosystems 430A synthesizer. Cleavage of the peptides from the solid support and deprotection was accomplished with HF at 0 °C. The linear peptide **37** was cyclized by air oxidation at high dilution at pH 8 to give compound **39** (cartoon A). The unsymmetrical disulfides **42**, **46**, **47**, and **48** were prepared by air oxidation of equimolar aqueous mixtures of the corresponding linear peptides **3** and **41**, **16** and **44**, **27** and **44**, and **28** and **45**, respectively. In all cases the unsymmetrical disulfides were predominant components in a mixture with the corresponding symmetrical disulfides. The symmetrical disulfides were individually prepared and used for identification purposes with analytical HPLC techniques. The cyclic peptide **43** (cartoon B) was obtained from the corresponding disulfide **42** by oxidation with iodine according to the procedure of Kamber and co-workers.[19] All peptides in Table 1 were purified by RP-HPLC and characterized by FAB-MS and amino acid analysis (Table 2 in the supplementary material).

Biological Evaluation

Receptor Binding. C5a analogs 1−48 were evaluated by their ability to compete for the binding of [^{125}I]-BH-C5a in neutrophil cell membranes as described previously.[20] The IC_{50} values generated by computer analysis of the binding data are shown in Table 1. All compounds with less than 50% inhibition above 100 μM concentrations were considered inactive.

C5a-Induced Ca Rise. C5a analogs were evaluated for functional activity in the calcium rise assay in human neutrophils as described previously.[20] The EC_{50} values in Table 1 indicate the concentration of the compound that produced a calcium rise of 50% of the maximum calcium rise attainable with human recombinant C5a on the same day in human neutrophils. The EC_{50} values were obtained to ascertain the presence of agonist activity and to compare with the receptor binding affinity of the synthetic analogues.

Results and Discussion

Structure−Activity Studies Corresponding to Cartoon A, Figure 1. In this investigation we have studied the effect of the core segment 34−63 on the affinity of peptide **1** which corresponds to the C-terminal

Table 1. Inhibition of [^{125}I]BH-C5a Binding to Neutrophil Membranes and Biological Activity of Synthetic C5a Analogs

	C5a	C5a Analogues[a]	Receptor Binding IC_{50}[b] μM	Ca Rise EC_{50}[c] μM
	C5a		0.000007	0.000037
1	64-74	Ac-NISHKDMQLGR	74	180[d]
2	55-74	CVVASQLRANISHKDMQLGR	10	•
3	54-74	ACM CCVVASQLRANISHKDMQLGR	8	•
4	53-74	ACM ECCVVASQLRANISHKDMQLGR	72	116
5	52-74	ACM TECCVVASQLRANISHKDMQLGR	40	110[d]
6	51-74	ACM FTECCVVASQLRANISHKDMQLGR	35	•
7	50-74	ACM AFTECCVVASQLRANISHKDMQLGR	96	118
8	47-74	ACM ACM CIKAFTE CCVVASQLRANISHKDMQLGR	25	•
9	46-74	ACM ACM RCIKAFTE CCVVASQLRANISHKDMQLGR	0.56	33
10	47-74	AIKAFTEACVVASQLRANISHKDMQLGR	11.4	•
11	46-74	RAIKAFTEACVVASQLRANISHKDMQLGR	0.44	18
12	46-74	AAIKAFTEACVVASQLRANISHKDMQLGR	34.7	•
13	47-74	ACM CIKAFTECCVVASQLRANISHKDMQLGR	92	•
14	46-74	ACM RCIKAFTECCVVASQLRANISHKDMQLGR	0.6	47
15	46-74	ACM ACIKAFTECCVVASQLRANISHKDMQLGR	7.3	•
16	47-74	ACM CIKAFTECAVVASQLRANISHKDMQLGR	25	83
17	46-74	ACM RCIKAFTECAVVASQLRANISHKDMQLGR	1.5	•
18	46-74	ACM ACIKAFTECAVVASQLRANISHKDMQLGR	26.2	•
19	45-74	PRAIKAFTEACVVASQLRANISHKDMQLGR	4.2	•
20	45-74	ACM PRCIKAFTECCVVASQLRANISHKDMQLGR	2.9	•
21	45-74	ACM PRCIKAFTECAVVASQLRANISHKDMQLGR	2.6	•
22	44-74	GPRAIKAFTEACVVASQLRANISHKDMQLGR	2.6	•
23	44-74	ACM GPRCIKAFTECCVVASQLRANISHKDMQLGR	0.44	•
24	44-74	ACM GPRCIKAFTECAVVASQLRANISHKDMQLGR	1.1	•
25	43-74	LGPRAIKAFTEACVVASQLRANISHKDMQLGR	0.34	62
26	43-74	ACM LGPRCIKAFTECCVVASQLRANISHKDMQLGR	0.4	26
27	43-74	ACM LGPRCIKAFTECAVVASQLRANISHKDMQLGR	0.6	< 5[f]
28	43-74	ACM LGPRCIKAFTECAVVASQLRANISHKDMQLGR	0.72	32
29	42-74	SLGPRAIKAFTEACVVASQLRANISHKDMQLGR	1	•
30	42-74	ACM SLGPRCIKAFTECCVVASQLRANISHKDMQLGR	1.3	•
31	41-74	ISLGPRAIKAFTEACVVASQLRANISHKDMQLGR	1	•
32	41-74	ACM ISLGPRCIKAFTECCVVASQLRANISHKDMQLGR	0.9	•
33	40-74	RISLGPRAIKAFTEACVVASQLRANISHKDMQLGR	0.08	37
34	40-74	AISLGPRAIKAFTEACVVASQLRANISHKDMQLGR	1.4	•
35	40-74	ACM RISLGPRCIKAFTECCVVASQLRANISHKDMQLGR	0.24	•
36	40-74	ACM AISLGPRCIKAFTECCVVASQLRANISHKDMQLGR	2.5	•
37	34-74	CEQRAARISLGPRAIKAFTEACVVASQLRANISHKDMQLGR	0.08	24
38	34-74	CEQRAARISLGPRAIKAFTECCVVASQLRANISHKDMQLGR	0.08	•
39	34-74	CEQRAARISLGPRAIKAFTEACVVASQLRANISHKDMQLGP S——S	0.09	25
40	40-54	ACM RISLGPRCIKAFTEC	Inactive[g]	•
41	22-34	ACM CYDGAAVNNDETC	Inactive[g]	•
42	54-74 and 22-34	ACM CCVVASQLRANISHKDMQLGR S S ACM CYDGAAVNNDETC	58	•
43	54-74 and 22-34	CCVVASQLRANISHKDMQLGR S——SS S CYDGAAVNNDETC	Inactive[g]	•
44	1-28	ACM TLQKKIEEIAAKYKHSVVKK CCYDGAAV	Inactive[g]	Inactive
45	1-28	ACM TLQKKIEEIAAKYKHSVVKK CCYDGAAV	Inactive[g]	Inactive at 50
46	47-74 and 1-28	ACM CIKAFTECAVVASQLRANISHKDMQLGR S S TLQKKIEEIAAKYKHSVVKKCCYDGAAV ACM	2.7	50
47	43-74 and 1-28	ACM LGPRCIKAFTECAVVASQLRANISHKDMQLGR S S TLQKKIEEIAAKYKHSVVKKCCYDGAAV ACM	0.7	100[d]
48	43-74 and 1-28	ACM LGPRCIKAFTECAVVASQLRANISHKDMQLGR S——S TLQKKIEEIAAKYKHSVVKKCCYDGAAV ACM	0.41	•

[a] Underlined amino acids indicate substitution in native sequence. [b] IC_{50} values in human neutrophil membranes. [c] Concentration required to induce 50% of the maximal calcium rise obtainable with human recombinant C5a in human neutrophils. [d] Estimated by extrapolation. Highest concentration tested gave less than 50% increase in calcium. [e] Not determined. [f] Lower concentrations not run. [g] No inhibition of binding at 100 μM of analog.

segment 64–74 (peptides **2**–**40**, Table 1 and Figure 3). All residues of helix 4 from 47 to 63 (peptides **2** through **8**) make essentially no significant contribution to the receptor binding affinity of peptide **1**. This finding is in good agreement with the results reported earlier by Kawai and co-workers.[14] It should be noted at this point that due to the relatively low affinity of peptides **1**–**8**, the observed differences in their IC_{50}'s were not considered to be significant. The introduction of Arg^{46} on the other hand (peptide **9**, IC_{50} = 0.56 μM) produced a 45-fold increase in the affinity of peptide **8** (IC_{50} = 25 μM). Moreover, peptide **9** exhibited 150 times greater binding

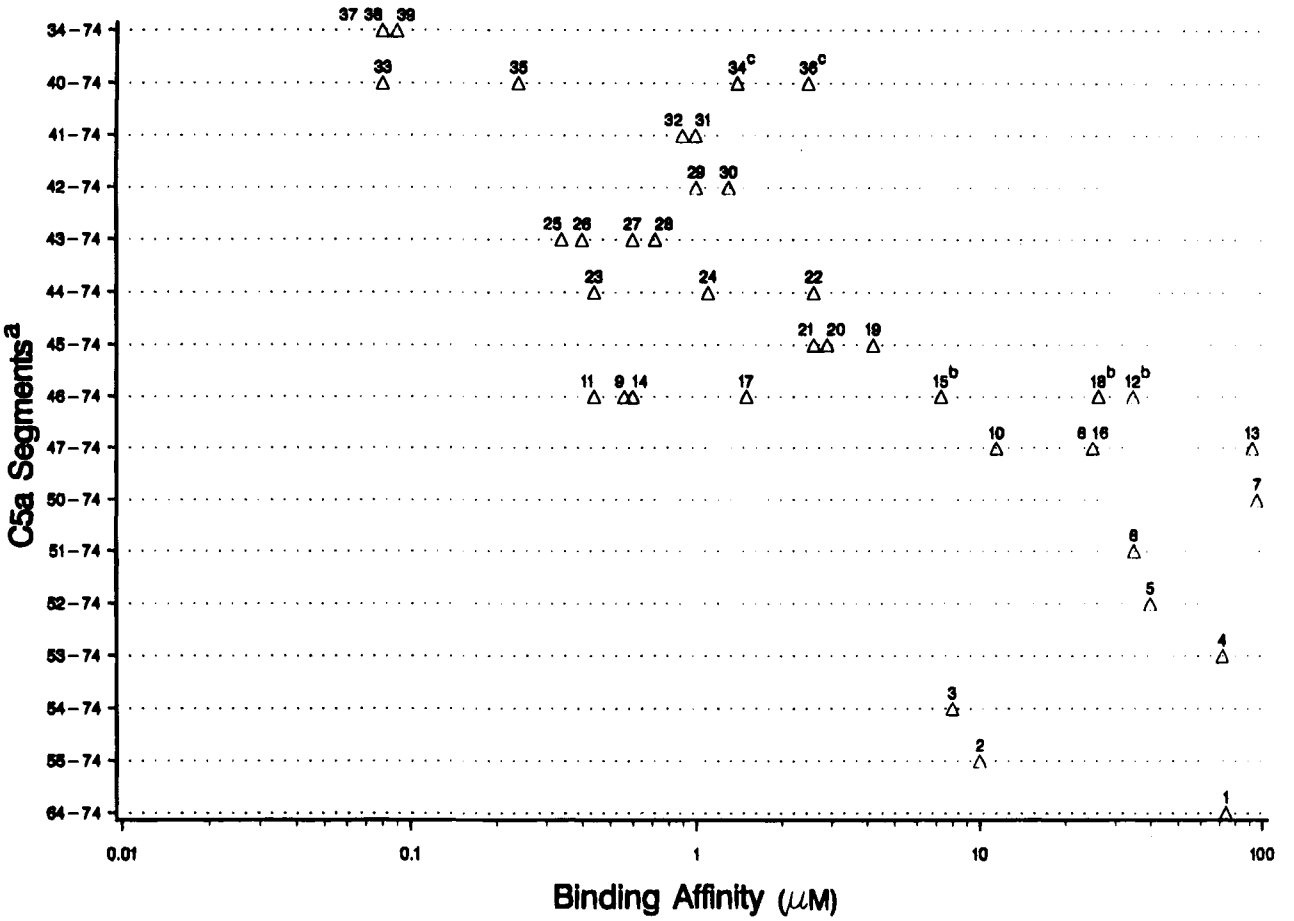

Figure 3. Schematic representation of the effects of Arg^{40} and Arg^{46} on the receptor binding affinity of C-terminal C5a segments. (**a**) Ordinate axis represents C5a segments for which peptide analogs were synthesized. The numbered peptides (see Table 1) were positioned on the chart as triangles according to their IC_{50} values and their relationships to the corresponding C5a segment. (**b**) In this analog Arg^{46} has been substituted by Ala. (**c**) In this analog Arg^{40} has been substituted by Ala.

potency than peptide 1. In order to verify the apparent significant contribution of Arg^{46} on the receptor binding affinity of peptide **9**, three additional pairs of peptides, **10** and **11**, **13** and **14**, **16** and **17**, which corresponded to segments 47–74 and 46–74, respectively, were prepared. These peptides differ only in the substitution at positions 47, 54, and 55, which are the sites of disulfide linkages in the core of C5a. As shown in Table 1 and Figure 3, peptides **11**, **14**, and **17** displayed 26, 150, and 16 times greater binding affinities than the peptides **10**, **13**, and **16**, respectively. To rule out the possibility that the peptides **9**, **11**, **14**, and **17** are artificially good due to other factors such as a positive N-terminus, peptides **12**, **15**, and **18** in which Arg^{46} was replaced by Ala were prepared. These peptides exhibited 79, 12, and 17 times weaker affinities than the corresponding Arg^{46}-bearing peptides **11**, **14**, and **17**, respectively. The above results are consistent with the conclusion that Arg^{46} is essential for the increased receptor binding affinity exhibited by peptides analogs corresponding to the C5a segment 46–74.

The effect of the loop-3 region on the affinity of peptide **1** was subsequently examined. Thus, peptides **19–36** were prepared. Figure 3 illustrates clearly that, with the exception of peptides **19–21** produced by the introduction of Pro^{45} and Gly^{44}, the IC_{50}'s of peptides **23** and **24** (segment 44–74), **25**, **26**, **27** and **28** (segment 43–74), **29** and **30** (segment 42–74), **31** and **32** (segment 41–74) fall essentially within the 0.44–1.5 μM range of IC_{50}'s exhibited by the peptides corresponding to segment 46–74. The slightly higher IC_{50}'s exhibited by peptides **19–21** are still well bellow the IC_{50}'s of the low-affinity peptides corresponding to segments 47–74 through 64–74. The introduction of the last amino acid of the loop-3 region, Arg^{40}, produced a 12-fold increase in the binding affinity of peptide **31** (peptide **33**, IC_{50} = 0.08 μM) and a 4-fold increase in the binding affinity of peptide **32** (peptide **35**, IC_{50} = 0.24 μM). Furthermore, peptides **33** and **35** exhibit receptor binding affinities that are 900 and 300 times higher, respectively, than the affinity of the peptide **1**. Considering the large size of peptides **31** and **32** as well as the relatively high binding potencies of peptides **33** and **35**, the observed increases in the binding affinities produced by the introduction of Arg^{40} can be considered to be significant. As in the case of Arg^{46}, replacement of Arg^{40} with Ala in peptides **33** and **35** produced a nearly proportional 17- and 10-fold reduction in their binding affinity (peptides **34** and **36**, IC_{50} = 1.4 and 2.5 μM, respectively). It is interesting to note that Arg^{40} was found to contribute to the C5a receptor binding by site-directed mutagenesis.[10] Further lengthening of the peptide chain to Cys^{34} (peptides **37** and **38**, IC_{50} = 0.08 μM) did not alter the effects produced by residues Arg^{40} and Arg^{46} even when conformational rigidity was introduced by the formation of the native Cys^{34}–Cys^{55} disulfide bond (peptide **39**, IC_{50} = 0.09 μM). The latter result can be attributed to the β-turn conformational effects of Gly^{44}-Pro^{45} dipeptide that preserves the conformational integrity of the loop-3 region.[21]

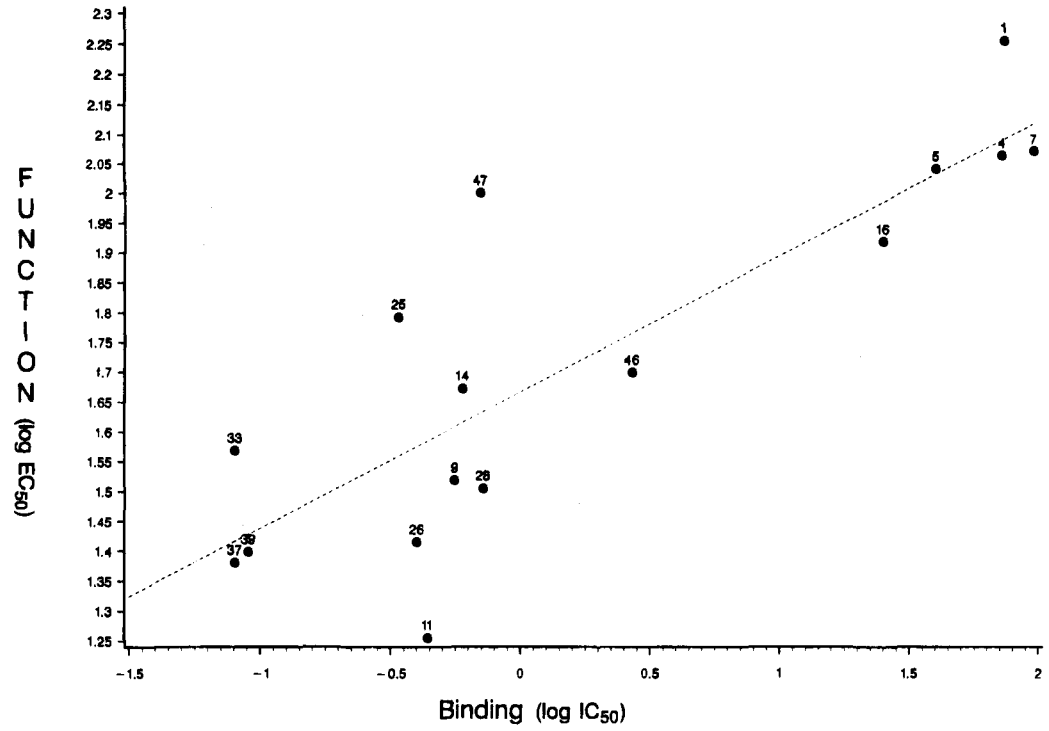

Figure 4. The log of the IC_{50} values (μM) from the binding experiments are plotted as a function of the log of the EC_{50} values (μM) from the Ca rise assay. A significant correlation was obtained ($r = 0.84$, $p < 0.001$) between these two sets of values. The best fit line is shown, along with the respective compound number.

It should be emphasized at this point that the contribution of both loop-3 and C-terminal segments are required to achieve maximum effect in the receptor binding affinity of these compounds. Core segments that included the loop-3 region, in the absence of C-terminal segments, were found to be inactive in inhibiting C5a receptor binding. Peptide **40**, corresponding to segment 40–54, as well as a previously reported segment $33-43^{14}$ were found to be inactive in inhibiting C5a receptor binding.

Structure–Activity Studies Corresponding to Cartoon B, Figure 1. The contribution of segment 22–34, which is centered by the loop-2 region to the receptor binding affinity of C5a, was subsequently examined. Segment 22–34 with Cys^{27} substituted by Ala (peptide **41**) was found to be inactive in the receptor binding assay. Peptide **41**, when attached to C-terminal peptide 54–74 via the native Cys^{34}—Cys^{55} disulfide bond (peptide **42**) produced a 10-fold decrease in the affinity of the parent C-terminal peptide **3**. The formation of the second native disulfide bond led to the inactive peptide **43**. These results indicate that segment 22–34 does not directly contribute to the C5a receptor binding. This conclusion is also in agreement with results obtained from site-directed mutagenesis.[10]

Structure–Activity Studies Corresponding to Cartoon C, Figure 1. The effect of the remaining portion of the core was examined with segment 1–28. As with segment 22–34, Cys^{27} was substituted with Ala and Cys^{21}, Cys^{22} residues were selectively protected with ACM groups (peptides **44** and **45**) to allow attachment to appropriate C-terminal segments of C5a. Peptides **44** and **45** were inactive in the receptor binding assay. Peptide **44**, when attached to the C-terminal peptide **16** via the native Cys^{22}—Cys^{54} disulfide bond produced a 10-fold increase in the affinity of the latter (peptide **46**, $IC_{50} = 2.7$ μM). In view of the weak binding potency of **16** ($IC_{50} = 25$ μM), the observed 10-fold increase in the affinity of **46** is not considered to be significant. In a similar operation, peptide **44** did not affect the affinity of the intrinsically more potent peptide **27** (peptide **47**, $IC_{50} = 0.7$ μM). No change in affinity was also observed with peptide **48** ($IC_{50} = 0.41$ μM) which resulted from the connection of peptides **28** and **45** via the Cys^{21}—Cys^{47} disulfide bond. The formation of the second native disulfide bond between ACM protected Cys residues in **46**, **47**, and **48** that would have assured a proper orientation of the two C5a segments was not accomplished. The above findings suggest that segment 1–28 does not significantly contribute to the C5a receptor binding. Site-directed mutagenesis studies on the other hand, have implicated loop-1 as a region contributing to the C5a receptor binding.[10]

C5a-Induced Ca Rise. The calcium rise assay values presented in Table 1 were used to estimate the functional activity of the C5a analogues. Maximum activity has been attained with all analogues tested in this assay. There were no cases where partial agonist activity was apparent. From Table 1, both binding and functional data were obtained for 16 analogs of C5a. When the IC_{50} values from the binding experiments were compared with EC_{50} values obtained from the Ca rise assay for these compounds, a significant correlation (Figure 4) was found (Pearson Product-Moment, $r = 0.84$, $p < 0.001$). Although certain compounds, such as **47**, appear to be more effective inhibitors in the binding assay as compared to their potency in stimulating a rise in calcium, a significant correlation was obtained. C5a itself was not included in the correlation because it is more than 4 log units away from the next most potent compound.

In synopsis, core segments of C5a have been studied for their ability to contribute to the receptor binding and biological activity of the anaphylatoxin. Our results

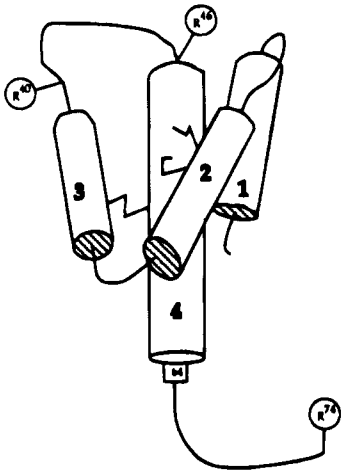

Figure 5. Highlighted amino acid residues found to contribute to binding and receptor activation of C5a.

indicate that the loop-3 region of the core and in particular the amino acid residues Arg^{40} and Arg^{46} contribute to a near 1000-fold increase in the affinity of the C-terminal segment of C5a. The contribution of both amino acid residues Arg^{40} and Arg^{46} as well as the contribution of both loop-3 and C-terminal regions are required in order to achieve maximum effect. Our results further indicate that the region 22-34, which is centered by loop-2, and the region 1–28, which is centered by loop-1, do not appear to contribute to the C5a receptor binding. It is reasonable, therefore, to conclude that the core binding site of C5a is located at loop-3 segment 40–46. In Figure 4, the C5a cartoon highlights the amino acid residues found by structure–activity relationship studies to contribute to receptor binding and functional activity of C5a. Studies are presently in progress to establish the spatial relationship between these important residues in the active conformation of C5a and further assess the structure–activity relationship of C5a analogs.

Experimental Section

Synthesis. All linear peptides were synthesized on an Applied Biosystems Synthesizer 430A using O-CH_2-PAM polystyrene resin[18] as solid support. All Boc-amino acid side chains were protected with TFA-stable protecting groups such as Acm(Cys), benzyl (Ser, Thr, Glu), 2-bromobenzyloxycarbonyl (Tyr), 2-chlorobenzyloxycarbonyl (Lys), cyclohexyl (Asp), *p*-methylbenzyl (Cys), 2,4-dinitrophenyl (His), and tosyl (Arg). Amino acid coupling reactions were performed with symmetrical anhydrides preformed with DCC as dehydrating reagent with the exception of Asn, Gln, and Arg, where DCC and HOBT were used directly as the coupling reagents. Amino acid couplings that proceeded with difficulty were encounterd with His^{67}, Val^{57}, Val^{56}, and Arg^{46}. In these cases, the reactions were monitored using the ninhydrin color test,[22] and double coupling reactions were performed when necessary. The cleavage of the peptides from the solid support was performed with liquid hydrogen fluoride at 0 °C for 60 min in the presence of 10% anisole with or without 2% of *p*-thiocresol. The crude peptide was first washed with dry ethyl ether and extracted with 10% aqueous acetic acid. The extracts were lyophilized, and the residual crude peptide was purified by RP-HPLC on a Waters Delta Prep 3000 using a YMC, C18, S-15 μm 50 × 500 mm column. The peptides were eluted with a gradient of A and B solution system (solution A, water with 0.1% TFA, and solution B, acetonitrile/water/TFA, 60:40:0.1) usually varying from 20 to 60% of solution B. Peptides with unprotected cysteines were stored at 0 °C under inert atmosphere and an Ellman's test[23] was performed prior to their use in biological assays.

All peptides were characterized by amino acid analysis performed with a Water Picotag System, and the FAB-MS values were obtained on a TSQ 70 Triple Stage Quadrapole Mass Spectrometer (Finnigan MAT, San Jose, CA) employing xenon gas and FABIINF source (VCR Group, Middlesex, UK). The analytical results are summarized in Table 2 in the supplementary material.

Synthesis of the Cyclic Peptide 39. After HPLC purification, the linear peptide **37** was dissolved in 0.1 M aqueous solution of ammonium acetate at a concentration of 0.1 mg/mL. The pH of the solution was adjusted to 8 with concentrated ammonium hydroxide, and the mixture was stirred in an air atmosphere at room temperature. The reaction was monitored by analytical HPLC to completion. The pH of the solution was adjusted to 5 with glacial acetic acid, and the mixture was passed through a YMC C18, 40–63-μm column. The column was washed with water and the product was eluted with solvent B described above. The peptide eluents were condensed at aspirator pressure to a small volume and lyophilized. The cyclic peptide was further purified by RP-HPLC as described above.

Synthesis of the Unsymmetrical Disulfide 42. Peptide **41** (34.5 mg, 22 × 10^{-3} mmol) and peptide **3** (63.2 mg, 22 × 10^{-3} mmol) were dissolved in 0.3 M aqueous solution of mercaptoethanol (58 mL) adjusted to pH 7.1 with ammonium hydroxide. The mixture was allowed to stand at room temperature overnight and lyophilized for 48 h. The residue was dissolved in water (15 mL), the pH was adjusted to 8, and the mixture was stirred in the air and monitored by analytical HPLC until peptides **41** and **3** were consumed and three new components of the desired peptide **42** (major) and the two symmetrical disulfides (minor) were formed. The mixture was lyophilized and the product was purified by RP-HPLC as described above. In a similar manner the unsymmetrical disulfides **46**, **47**, and **48** were synthesized from the corresponding purified monomers **16** and **44**, **27** and **44**, **28** and **45**, respectively.

Synthesis of Peptide 43. The unsymmetrical disulfide **42** (25 mg, 5.66 × 10^{-3} mmol) was dissolved in water/methanol/acetic acid (39:57:4, 115 mL). A methanolic solution of iodine (0.1%, 5 mL) was added, and the mixture was stirred at room temperature for 16 h. The excess iodine was destroyed with the addition of zinc dust. The solution was filtered, and the filtrates were lyophilized. The peptide was purified by RP-HPLC as described above.

Receptor Binding Assay. Binding of [^{125}I]Bolton–Hunter labeled C5a to PMN membranes was measured as previously described[24] with modifications. PMNs were resuspended in Hank's balanced salt solution (without Ca^{2+} and Mg^{2+} and containing 10 mM Hepes (pH 7.3), 2.5 mM $MgCl_2$, 100 units/mL of DNAse 1, 0.1 mM PMSF, 10 μg/mL aprotining, and 10 μg/mL leupeptin) and were equilibrated at 400 psi for 20 min at 4 °C in a nitrogen cavitation bomb. After evacuation into 3 volumes 0.5 M $KHCO_3$ containing 25 mM EDTA and the protease inhibitors listed above, the gelatinous material was removed with forceps, and the mixture was centrifuged at 400*g* for 10 min at 4 °C. The supernatant was centrifuged at 10000*g* for 20 min at 4 °C; afterwards, the resulting supernatant was centrifuged at 50000*g* for 60 min at 4 °C. The pellets from aliquots representing 200 × 10^6 cells were stored at −70 °C. For binding studies, these membranes were resuspended at an equivalent of 20 × 10^6 cells/mL (10 mL/tube) in 50 mM Hepes, pH 7.3 containing 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.1% bacitracin, and 0.5% BSA. After further 1:75 dilution with the same buffer, 400 μL of this suspension were added to duplicate tubes containing 50 μL of [^{125}I]BH-C5a (specific activity 2200 Ci/mmol, DuPont/NEN, Boston, final concentration 4.0 pM) and 50 μL of buffer or peptide. Nonspecific binding was determined in the presence of 10 nM unlabeled C5a. The binding reaction was initiated by the addition of tissue and continued for 120 min at 4 °C. Bound and free radioactivity was separated by vacuum filtration through Whatman GF/C glass fiber filters (pretreated for 90 min with 0.05% PEI) using Brandel Cell Harvester (Gaithersburg, MD). Filters were washed with 3 × 5 mL of ice-cold 5 mM Tris buffer, pH 7.4, and counted in a Genesys multiwell

Gamma counter. Data were analyzed using the nonlinear regression analysis program, RS/1 (Bolt, Beranek and Newman, Boston).

C5a-Induced Ca Rise Assay. Recombinant human C5a or peptide was dissolved in Hank's buffer containing 0.01% Tween-20, and all stock dilutions of C5a, peptide, and [^{125}I]-BH-C5a were made in this buffer. The acetoxymethyl ester or fura-2 (fura 2AM, Molecular Probes) was dissolved in DMSO. Neutrophils were purified from human peripheral blood by sedimentation in 6% hetastarch (HESPAN, DuPont, Waukegan, IL) followed by counter-flow elutriation as described previously.[25] Purified cells (2×10^6/mL) were mixed with 0.2 μM fura 2AM and incubated for 30 min at 37 °C in HEPES-buffered Hank's solution containing calcium and magnesium. The loaded cells were washed once and stored on ice until use at a concentration of 2×10^6 cells/mL in 0.01 M Hepes buffered Hank's medium without calcium and magnesium. Fifteen minutes before assay, the cell suspension was transferred to a cuvette containing a stir bar and repleted with calcium and magnesium. The cell suspension was incubated with stirring at 37 °C. Assays were terminated within 4 h of cell purification, and a standard control response was obtained periodically to assure that the cell responses were not changing over the time of the experiment. The calcium rise was determined fluorometrically using an SLM 8000 spectrofluorometer (SLM-Aminco Instruments, Urbana, IL) as described previously.[26]

Acknowledgment. We thank Dr. Louis Martin for the FAB-MS determinations, Dr. Matthew Toth, Dr. Lawrence Wennogle, and Dr. Jan von Östrom for their helpful suggestions, and Aida Navarrete and Yueh-Ling Hsieh for their assistance in the preparation of the manuscript.

Supplementary Material Available: Table 2 of FAB-MS and amino acid analyses on the synthetic peptides used in the study (3 pages). Ordering information is given on any current masthead page.

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(1) (a) Symbols and abbreviations are in accordance with recommendation of the IUPAC–IUP commission on biochemical nomenclature. Other abbreviations: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; BH-C5a, Bolton–Hunter C5a; C5a, complement 5a; DCC, *N,N'*-dicyclohexylcarbodiimide; FAB-MS, fast-atom-bombardment mass spectroscopy; HOBT, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PAM, phenylacetamidomethyl; PMN, polymorphonuclear leukocytes, TFA, 1,1,1-trifluoroacetic acid. (b) Frank, M. M. The Complement System in Host Defense and Inflammation. *Rev. Infect. Dis.* **1979**, *1*, 483–501. (c) Hugli, T. E. The Structural Basis for Anaphylatoxin and Chemotactic Functions of C3a, C4a and C5a. *CRC Crit. Rev. Immunol.* **1981**, *1*, 321-366. (d) Hugli, T. E. Structure and Function of the Anaphylatoxins. *Springer Semin. Immunopathol.* **1984**, *7*, 193–219. (e) Hugli, T. E.; Muller-Eberhard, H. J. Anaphylatoxins: C3a and C5a. *Adv. Immunol.* **1978**, *26*, 1–53.

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