Biologically Active Conformer of the Effector Region of Human C5a and Modulatory Effects of N-Terminal Receptor Binding Determinants on Activity

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A conformationally biased decapeptide agonist of human C5a ($C5a_{65-74}$Y65,F67,P69,P71,D-Ala73 or YSFKPMPLaR) was used as a functional probe of the C5a receptor (C5aR) in order to understand the conformational features in the C-terminal effector region of C5a that are important for C5aR binding and signal transduction. YSFKPMPLaR was a potent, full agonist of C5a, but at higher concentrations had a superefficacious effect compared to the natural factor. The maximal efficacy of this analogue was 216 ± 56% that of C5a in stimulating the release of β-glucuronidase from human neutrophils. C5aR activation and binding curves both occurred in the same concentration range with YSFKPMPLaR, characteristics not observed with natural C5a or more conformationally flexible C-terminal agonists. YSFKPMPLaR was then used as a C-terminal effector template onto which was synthesized various C5aR binding determinants from the N-terminal core domain of the natural factor. In general, the presence of N-terminal binding determinants had little effect on either potency or binding affinity when the C-terminal effector region was presented to the C5aR in this biologically active conformation. However, one peptide, $C5a_{12-20}$-Ahx-YSFKPMPLaR, expressed a 100-fold increase in affinity for the neutrophil C5aR and a 6-fold increase in potency relative to YSFKPMPLaR. These analyses showed that the peptides used in this study have up to 25% of the potency of C5a in human fetal artery and up to 5% of the activity of C5a in the PMN enzyme release assay.

Introduction

Human C5a anaphylatoxin is a 74-residue glycopolypeptide that is generated as a cleavage product of the fifth component (C5) of the serum complement system. C5a is a multifunctional, proinflammatory molecule that binds to C5a receptors (C5aR) expressed on a variety of cells of myeloid and non-myeloid origin.[1,2] Upon receptor binding, C5a activates an intracellular, G protein-mediated signal transduction pathway that culminates in the expression of smooth muscle contraction,[3] increased vascular permeability,[4] mast cell histamine release,[5] leukocyte recruitment (chemotaxis) and activation,[4,6] and cytokine synthesis and release from monocytes and macrophages.[7−10] The proinflammatory and immunostimulatory actions of C5a have led to the desire to develop specific compounds to modulate these activities. To achieve this goal, further understanding of the C5a structural features important for receptor binding and activation is necessary.

NMR studies of human C5a have shown that the polypeptide consists of two distinct structural domains, the N-terminal core (residues 1−63) and the C-terminal region (residues 64−74).[11] The N-terminal core domain is a highly ordered region made up of a four-helix bundle, interconnected by looped peptidyl segments and stabilized by three disulfide bridges. From a functional perspective, the cationic N-terminal core appears to orchestrate overall C5aR recognition and binding via electrostatic interactions with anionic regions expressed on the surface of the C5aR.[12] In fact, recent site-directed mutagenesis and substitutional studies within the N-terminal core have identified Arg-40 and Arg-46 in peptidyl loop 3 and Lys-12, Lys-19, and Lys-20 in peptidyl loop 1 as residues directly involved in these C5a-C5aR electrostatic interactions.[13,14]

In contrast to the N-terminal core, the C-terminal domain of C5a is a region of considerable flexibility and structural disorder that extends outwardly from the N-terminal core as a finger-like projection. Within the C-terminal domain lies the requisite sequential information necessary for effecting the biological signal(s) characteristic of C5a. This conclusion is based on the observation that peptides synthesized with sequence homology to the C-terminal region ($C5a_{65-74}$) behaved as full agonists relative to the natural factor but at significantly reduced potencies.[10,15,16] Thus, the C-terminal region of C5a is regarded as the biological effector domain of the polypeptide.

Structure−function characterization of the C-terminal effector region of C5a has been difficult because of its inherent conformational flexibility. Consequently, the precise biological role played by topochemical determinants expressed in this region remains poorly understood. We have recently identified the conformational features of C5a agonists derived from the C-terminal effector region of human C5a ($C5a_{65-74}$ or ISHKDMQLGR) that appear responsible for the potent expression of spasmogenic, platelet aggregatory, and neutrophil (PMN) activities.[17,18] These conformational features were identified from a structure−function analysis of a panel of $C5a_{65-74}$ analogues ($C5a_{65-74}$Y65,F67 or YSFKDMQLGR) in which conformational flexibility in the

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Table 1. Spasmogenic Activity of C5a and C5a Analogues on Human Fetal Artery

no.	peptide	*n*	$pD_2 \pm SE$	EC_{50} (μM)	Δ^a	% potency[b]	selectivity[c]
	C5a	37	7.87 ± 0.07*	0.02	0	100	2.6
2	YSFKDMQLGR	35	4.96 ± 0.08	9.6	3.00 ± 0.1	0.1	8.3
10	YSFKDMPLaR	28	6.32 ± 0.1	0.5	1.66 ± 0.1	2.2	151
54	YSFKPMPLaR	34	6.75 ± 0.09	0.2	1.11 ± 0.09	7.8	24
64	Ac-YSFKPMPLaR	8	6.98 ± 0.15	0.1	0.74 ± 0.21	9.6	89
83	Ahx-YSFKPMPLaR	10	7.16 ± 0.07*	0.07	0.79 ± 0.1	16	45
68	DW-Ahx-YSFKPMPLaR	7	6.96 ± 0.26	0.2	0.91 ± 0.25	8.5	66
70	KW-Ahx-YSFKPMPLaR	11	7.11 ± 0.16	0.1	0.86 ± 0.16	14	32
73	K-Ahx-YSFKPMPLaR	12	7.14 ± 0.18*	0.09	0.84 ± 0.17	14	59
76	KGK-Ahx-YSFKPMPLaR	10	6.98 ± 0.15	0.1	0.97 ± 0.17	11	47
78	KGKGK-Ahx-YSFKPMPLaR	12	6.98 ± 0.20	0.1	0.99 ± 0.22	10	63
86	KGKGKGGG-YSFKPMPLaR	11	7.14 ± 0.12*	0.07	0.83 ± 0.18	15	27
79	KGKGK-YSFKPMPLaR	10	6.81 ± 0.24	0.2	1.2 ± 0.21	6.2	26
82	$C5a_{37-46}$	3	3.56 ± 0.03	272	4.32 ± 0.18	0.004	
84	$C5a_{12-20}$	3		*d*			
66	$C5a_{40-64}$(A47,A54)-YSFKPMPLaR	8	5.84 ± 0.19	1.5	1.82 ± 0.18	1.5	1.1
85	$C5a_{37-64}$(S47,S54,S55)-YSFKPMPLaR	9	6.56 ± 0.18	0.3	1.47 ± 0.17	3.4	23
74	$C5a_{37-46}$-Ahx-YSFKPMPLaR	10	7.32 ± 0.16*	0.06	0.63 ± 0.15*	24	126
87	$C5a_{12-20}$-Ahx-YSFKPMPLaR	11	7.11 ± 0.20	0.08	0.87 ± 0.22	13	8.5

[a] Mean log concentration ratio ± SEM [pD_2(C5a) − pD_2(peptide)]. [b] Relative potency of peptide as percent C5a [antilog (−Δ) × 100]. [c] Antilog[pD_2(fetal artery) − pD_2(PMN enzyme release)]. [d] No response at concentrations up to 1 mM. *Significant increase in potency above **54** ($p < 0.05$).

Table 2. Pharmacological Activities of C5a and C5a Analogues in Human PMN Enzyme Release

no.	peptide	*n*	$pD_2 \pm SE$	EC_{50} (μM)	Δ^a	% potency[b]	efficacy % C5a max[c]
	C5a	20	7.46 ± 0.08	0.03	0	100	100
2	YSFKDMQLGR	5	4.04 ± 0.14	92	3.42 ± 0.37	0.04	87 ± 10
10	YSFKDMPLaR	6	4.14 ± 0.11	72	3.35 ± 0.26	0.04	174 ± 36
54	YSFKPMPLaR	8	5.37 ± 0.10	4.1	2.06 ± 0.16	0.9	216 ± 56**
64	Ac-YSFKPMPLaR	4	5.03 ± 0.41	9.3	2.44 ±0.77	0.4	148 ± 27
83	Ahx-YSFKPMPLaR	4	5.51 ± 0.21	3.1	1.90 ± 0.33	1.2	180 ± 41
68	DW-Ahx-YSFKPMPLaR	4	5.14 ± 0.45	7.2	2.30 ± 0.63	0.5	173 ± 53
70	KW-Ahx-YSFKPMPLaR	5	5.60 ± 0.26	2.5	1.72 ± 0.24	1.9	134 ± 15
73	K-Ahx-YSFKPMPLaR	3	5.37 ± 0.19	4.3	1.93 ± 0.11	1.2	145 ± 19
76	KGK-Ahx-YSFKPMPLaR	4	5.31 ± 0.22	4.9	1.99 ± 0.22	1.0	156 ± 20
78	KGKGK-Ahx-YSFKPMPLaR	4	5.18 ± 0.17	6.7	2.20 ± 0.24	0.6	173 ± 7**
86	KGKGKGGG-YSFKPMPLaR	4	5.71 ± 0.18	2.0	1.74 ± 0.21	1.8	177 ± 37
79	KGKGK-YSFKPMPLaR	4	5.40 ± 0.13	3.9	2.05 ± 0.19	0.9	198 ± 25**
82	$C5a_{37-46}$	2		*d*			
84	$C5a_{12-20}$	2		*d*			
66	$C5a_{40-64}$(A47,A54)-YSFKPMPLaR	3	5.79 ± 0.29	1.6	2.08 ± 0.49	0.8	218 ± 19
85	$C5a_{37-64}$(S47,S54,S55)-YSFKPMPLaR	3	5.20 ± 0.22	6.3	2.34 ± 0.23	0.5	180 ± 46
74	$C5a_{37-46}$-Ahx-YSFKPMPLaR	4	5.22 ± 0.28	5.9	2.15 ± 0.33	0.7	176 ± 43
87	$C5a_{12-20}$-Ahx-YSFKPMPLaR	5	6.18 ± 0.15*	0.7	1.29 ± 0.19*	5.2	215 ± 37**

[a] Mean log concentration ratio ± SEM [pD_2(C5a) − pD_2(peptide)]. [b] Relative potency of peptide as percent C5a [antilog (−Δ) × 100]. [c] Maximum efficacy relative to C5a (100%). [d] No response at concentrations up to 1 mM. *Significant increase in potency above **54** ($p < 0.05$). **Significant increase above C5a efficacy ($p < 0.05$).

C-terminal end was restricted. These studies showed that the potent expression of biological activity required ligand presentation of a twisted, helix-like conformation in the region spanning residues 65−69, extended backbone conformation for residues 70−71, and, for residues (71)72−74, a β-turn of either type II or type V for spasmogenic activities and a β-turn of type V for PMN activities. We hypothesized that these conformational features may also represent important topochemical determinants within the C-terminal effector region of the natural factor that are involved in C5aR binding and activation.

In this paper, we first present the biological results of a conformationally biased decapeptide agonist of C5a, YSFKPMPLaR. These results support a new, refined view of the conformational features in the C-terminal effector region of C5a that appear important in C5aR binding and activation. In the second part of our investigation, various N-terminal core binding determinants were synthesized onto the N-terminal end of YSFKPMPLaR. These peptides were used to investigate the modulatory effects that N-terminal core binding determinants have on the activity of the C-terminal effector region when it is presented to the C5aR in a well-defined, biologically active conformation.

Results and Discussion

The C5a agonist peptides used in this investigation are listed in Tables 1−3 along with the biological parameters that characterize their ability to induce smooth muscle contraction of human fetal artery (Table 1), to stimulate β-glucuronidase release from human PMNs (Table 2), and to compete with [^{125}I]C5a for binding to the C5aR expressed on whole PMNs (Table 3). For each peptide listed in Tables 1 and 2, a full dose−response curve was generated together with C5a in the same tissue or cell preparation. EC_{50} values (effective concentration of peptide at 50% of the maximal response) were determined from these dose−response curves and pD_2 values [−log EC_{50} (M)] calculated from individual curves relative to the C5a maximum response. The potency of each peptide relative to C5a was reported in each experiment as the log potency ratio at the C5a EC_{50} level. Using the latter approach, indi-

Table 3. Receptor Binding Affinity of C5a and C5a Analogues in Human PMNs

no.	peptide	*n*	IC_{50}[a] (μM)	Δ[b]	% C5a affinity[c]	EC_{50}/I_{C50}[d]
	C5a	14	0.0006	0	100	53
2	YSFKDMQLGR	3	1.3	3.67 ± 0.07	0.022	72
10	YSFKDMPLaR	3	3.7	4.13 ± 0.3	0.007	19
54	YSFKPMPLaR	10	6.0	3.84 ± 0.22	0.014	0.7
83	Ahx-YSFKPMPLaR	3	5.5	4.33 ± 0.44	0.005	0.6
66	$C5a_{40-64}$(A47,A54)-YSFKPMPLaR	2	1.2	3.44 ± 0.34	0.04	1.4
82	$C5a_{37-46}$	2	397	5.97 ± 0.33	0.0001	
84	$C5a_{12-20}$	3	133	5.63 ± 0.20	0.0002	
85	$C5a_{37-64}$(S47,S54,S55)-YSFKPMPLaR	3	1.4	3.72 ± 0.06	0.019	4.6
74	$C5a_{37-46}$-Ahx-YSFKPMPLaR	4	0.7	3.12 ± 0.39	0.075	8.3
87	$C5a_{12-20}$-Ahx-YSFKPMPLaR	3	0.07	1.98 ± 0.31	1.04	9.6

[a] IC_{50}, concentration of peptide causing a 50% inhibition of the binding of [^{125}I]C5a to intact PMNs. [b] Mean log concentration ratio ± SEM [log IC_{50}(C5a) − log IC_{50}(peptide)]. [c] Relative affinity of peptide for PMN receptor as percent C5a affinity [antilog (−Δ) × 100]. [d] Ratio of concentration agonist potency (EC_{50}) to concentration inhibiting [^{125}I]C5a binding.

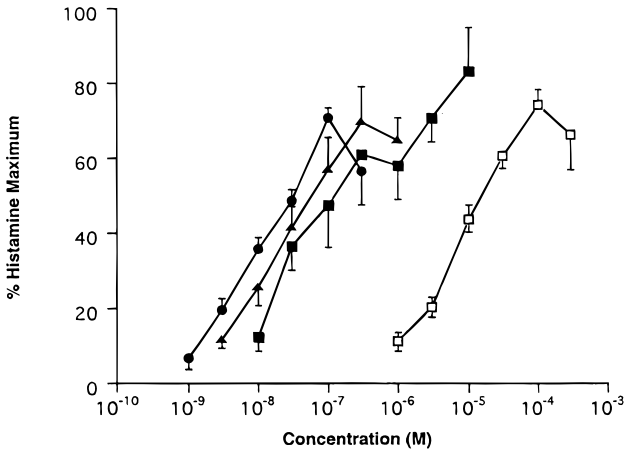

Figure 1. Spasmogenic effects of C5a and C5a analogues on human fetal artery. Tissue strips were exposed to C5a or peptides and, following contraction, to a supramaximal concentration of histamine (10 μM): C5a (●) (n = 37); C-terminal conformational restriction results in an increase in potency with YSFKPMPLaR (■) (n = 34) over YSFKDMQLGR (□) (n = 35); a further increase in potency is seen with the addition of the N-terminal extension, $C5a_{37-46}$-Ahx-YSFKPMPLaR (▲) (n = 10). Note the biphasic response seen with YSFKPMPLaR. Points represent the mean response and vertical bars the SEM.

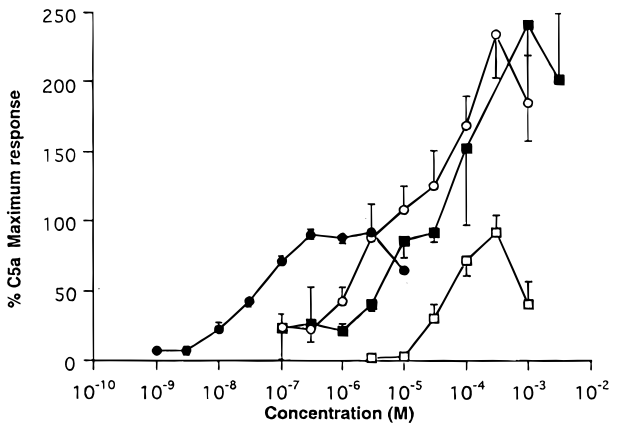

Figure 2. Release of β-glucuronidase from PMNs by C5a and C5a analogues: superefficacious activity of conformationally constrained analogues. Data are shown for C5a (●) (n = 20), $C5a_{12-20}$-Ahx-YSFKPMPLaR (○) (n = 5), YSFKPMPLaR (■) (n = 8), and YSFKDMQLGR (□) (n = 5). Results are expressed as percentage of C5a maximal response in each experiment. Points represent the mean and vertical bars the SEM.

vidual differences in tissue and cell sensitivity to C5a were minimized.[19] Thus, two measures of receptor sensitivity were obtained for the purpose of quantitative comparisons of the potencies of the peptides. In the PMN enzyme release assay, dose−response curves were biphasic for peptides containing the conformationally constrained agonist YSFKPMPLaR (discussed in the next section). Moreover, these peptides consistently expressed greater efficacy than C5a in the higher concentration ranges. In these cases, peptide potency was determined relative to the EC_{50} of C5a.

Conformational Characterization of the C-Terminal Agonist YSFKPMPLaR. In previous studies we showed that peptide **10** (YSFKDMPLaR), an analogue of our standard peptide **2** (YSFKDMQLGR) in which Gln-71 was replaced with Pro and Gly-73 with D-Ala, was about 30-fold more potent than peptide **2** in spasmogenic and platelet aggregatory responses[17] but equipotent to peptide **2** in PMN responses.[18] Furthermore, it was shown that peptide **10** adopted a helix-like conformation for residues 65−69, elongated backbone conformation for residues 70−71, and a β-turn of either type II or type V for residues (71)72−74 and that these conformational features were correlated with the increase in potency exhibited by peptide **10**.[17,18,20]

Peptide **54**, $C5a_{65-74}$Y65,F67,P69,P71,D-Ala73 or YSFKPMPLaR, like peptide **10**, possessed the biologically important, C-terminal β-turn but had a Pro-for-Asp substitution at position 69. This substitution afforded a significant ($P < 0.05$) 3−4-fold increase in spasmogenic potency (Table 1, Figure 1) and a 20-fold increase ($P < 0.05$) in PMN response relative to peptide **10** (Table 2, Figure 2). Peptide **54** expressed nearly 8% of C5a potency in contracting smooth muscle of human fetal artery and 0.9% of C5a potency in stimulating enzyme release from PMNs.

In peptide **54**, the presence of Pro at position 69 influences topochemistry in two important ways. First, it eliminates the contribution made to C5aR binding and activation by the carboxyl side chain of Asp-69. Second, Pro-69 forces the pre-proline residue, Lys-68, into a region of sterically allowed (ϕ,ψ) angular space characteristic of an elongated backbone conformation.[21] This conformational effect is shown in the Ramachandran plot of Figure 3. The presence of Pro-69 in peptide **54** changes the sterically allowed assignment of residues 68 and 69 in peptide **10** from the region straddling quadrants B and R into the boxed region of quadrant B only. Thus, the presence of extended backbone conformation well into the region that was previously viewed as being dominated by a twisted, helix-like conformation appears to be biologically preferred in these assays.

Because of the presence of Pro residues at positions 69 and 71, this region in peptide **54** likely adopts a

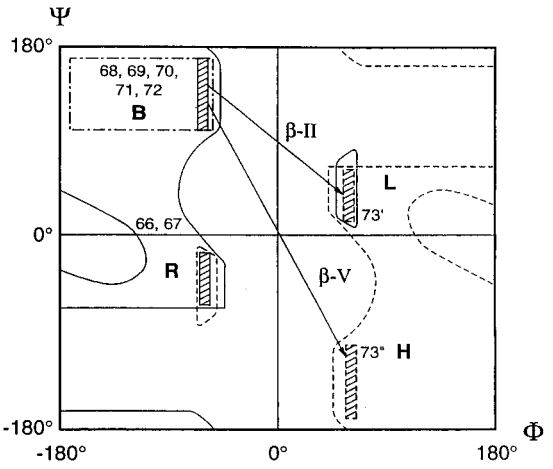

Figure 3. Ramachandran plot for YSFKPMPLaR. Ramachandran plot showing the sterically allowed conformational space occupied by the residues in the decapeptide agonist YSFKPMPLaR (peptide **54**). The terminal residues Tyr-65 and Arg-74 are omitted. Sterically allowed space for L- and D-residues is contained within the solid and dashed lines, respectively. The narrow, vertical regions depict sterically allowed space for L-Pro (left-hand quadrants outlined with solid lines) and D-Pro (right-hand quadrants outlined with dashed lines). The boxed region in quadrant B is the sterically allowed conformational space occupied by the pre-proline residues. B, R, L, and H refer to the (ϕ,ψ) regions that correspond to β-conformations, right-handed helices, left-handed helices, and high-energy conformations, respectively.

polyproline type II (P_{II}) conformation. It is noteworthy that P_{II} types of extended conformations are being shown to play an increasingly important role in directing the molecular recognition of peptides and proteins.[22] It is also possible that the extended backbone conformation imposed on Lys-68 by the presence of Pro-69 may allow the positively charged amino side chain of Lys-68 to more effectively interact with a negatively charged residue within the C5aR. Such a possibility is supported by a recent study that showed that Lys-68 in the natural factor interacts with the negatively charged carboxyl side chain of Glu-199 in the C5aR and that this electrostatic interaction is correlated with C5aR binding and potency.[23]

Addition of N-Terminal Core Binding Determinants. Using peptide **54** as a biologically active template, different C5aR binding determinants from the N-terminal core of C5a were added to the N-terminus of peptide **54**. These peptides were designed to evaluate the modulatory effects N-terminal core binding determinants have on C5a activity when the C-terminal effector region is presented to the C5aR in a specific, biologically active conformation. The C5aR binding determinants chosen for this study were peptidyl stretches containing basic residues in order to mimic the electrostatic interactions known to occur between the cationic N-terminal core and the anionic, surface-exposed regions of the C5aR.

The first panel of peptides was synthesized with different combinations of positively charged (Lys) residues added to the N-terminal end of peptide **54** (peptides **70**, **73**, **76**, **78**, **79**, and **86**). Ahx and/or Gly were used as spacers between the effector template and the Lys extensions. In peptide **70**, Trp (W) was used as a hydrophobic determinant in a manner similar to its use in generating a C3a superagonist.[24] Peptide **68**, which contained an N-terminal Asp residue, was generated as a negative control to peptide **70**. Peptides **64** and **83** were synthesized as controls to assess the effects of removing the N-terminal positive charge and adding the hydrophobic Ahx spacer group to the N-terminus of peptide **54**.

In a second panel, peptidyl stretches corresponding to the C5aR binding determinants found in the loop 1 ($C5a_{12-20}$ or KYKHSVVKK) or loop 3 ($C5a_{37-46}$ or RAARISLGPR) regions of the C5a N-terminal core were added to the N-terminus of peptide **54**. These two loop regions of C5a contain residues that have been directly implicated in ligand−receptor interactions: specifically, Lys-12, Lys-19, and Lys-20 in loop 1 and Arg-40 and Arg-46 in loop 3.[13,14] Consequently, peptides **87** and **74** were synthesized with the loop 1 and loop 3 regions, respectively, separated by an Ahx linker. Peptides **66** and **85**, analogues of $C5a_{40-74}$ and $C5a_{37-74}$, respectively, incorporated the important loop 3 C5aR binding region from the N-terminal core of C5a with the biologically active effector conformation presented by peptide **54**. These two functional regions of C5a were brought together by the intervening sequence of the natural factor rather than by the Ahx spacer. Peptides **82** and **84** correspond, respectively, to the loop 3 and loop 1 regions of the N-terminal core and were synthesized as controls to peptides **74** and **87**, respectively.

Activity in Fetal Artery. All peptides tested in this study were spasmogens in the fetal artery assay and full agonists relative to C5a (Table 1, Figure 1). The presence of the different N-terminal extensions resulted in significant potency increases in several, but not all, of the analogues in the fetal artery assay when analyzed as pD_2 or log potency ratios (Table 1). The largest increase in potency was seen with peptide **74**, which was 3-fold more potent than peptide **54**. Peptide **74** expressed about 25% of the molar potency of C5a in this assay when analyzed as the log potency ratio.

The dose−response curve for peptide **54** was biphasic with higher concentrations (3−10 μM) increasing the contraction of the fetal artery tissue following a plateau that occurred at 0.3−1.0 μM (Figure 1). Since peptide **54** was routinely included as a standard peptide in this assay to gauge the sensitivity of each preparation, a wider range of concentrations was included for this peptide than for C5a and the other analogues. Consequently, experiments using supramaximal concentrations of other C5a analogues were not routinely performed, and such data were not collected for other peptides used in this assay.

Activity in PMNs. In the PMN enzyme release assay, only peptide **87** was significantly more potent ($P < 0.05$) than peptide **54** when analyzed by pD_2 or log potency ratios (Table 2, Figure 2). Peptide **74**, which was the most potent analogue in the fetal artery, did not exhibit an increase in potency over peptide **54** in the PMN assay. These results demonstrate that the potency changes effected by the addition of the N-terminal core binding determinants to peptide **54** were not correlated in the two assays.

During the course of the PMN studies, it was noted that the ability of most peptides to induce the release of β-glucuronidase was consistently greater than the maximum effect expressed by C5a (Table 2, Figure 2). The increased efficacy of these peptides amounted to about 150−200% that of the C5a maximum effect.

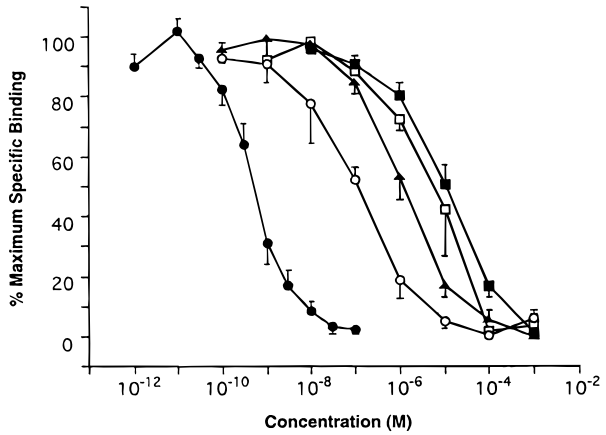

Figure 4. Binding curves of C5a and C5a analogues on intact PMNs. Binding assays were performed by the addition of increasing concentrations of unlabeled C5a or peptide in the presence of a constant amount of [^{125}I]C5a (see the Experimental Section). Data are plotted as the percentage of specific binding of [^{125}I]C5a. Data shown are for C5a (●) (n = 14), $C5a_{12-20}$-Ahx-YSFKPMPLaR (○) (n = 3), YSFKPMPLaR (■) (n = 10), $C5a_{37-46}$-Ahx-YSFKPMPLaR (▲) (n = 10), and YSFKDMQLGR (□) (n = 3). Points represent the mean value and vertical bars the SEM.

Because of this finding, the pD_2 values for the peptides were calculated by reference to the C5a EC_{50} values in order to standardize procedures used with the fetal artery analyses. It is not yet known if the secondary phase of the enzyme release induced by the analogues is mediated exclusively by C5aRs. The flexible, unconstrained peptide **2** was the only peptide in this study that did not exhibit increased efficacy relative to C5a (Table 2, Figure 2).

Binding to C5aRs on Whole PMNs. The functional data presented above demonstrated a number of phenomena relating to the pharmacological activity of the C-terminal agonist peptides. To determine if the differences observed in agonist potency were related to corresponding changes in the affinity for the C5aR, the ability of selected peptides to compete with [^{125}I]C5a for binding to C5aRs expressed on intact PMNs was evaluated (Table 3, Figure 4). Binding assays were not attempted in fetal artery because of tissue complexity and cellular heterogeneity.

Our binding studies were performed on whole PMNs rather than PMN membranes, which are most commonly used in binding studies of the C5aR.[13,25,26] The preference of PMN membranes may have emanated, in part, from a report by the Abbott group who showed that the affinity of other C5a agonist analogues for competing with [^{125}I]C5a for the C5aR in intact PMNs was very low, whereas the affinity of both C5a and analogue peptides in PMN membranes was considerably higher.[26] These investigators suggested that the differences in affinity may be due to the uncoupling of the C5aR−G protein complex during the preparation of the membranes resulting in an increase in the observed C5aR affinity. For the present investigation, however, we chose to use whole PMNs for two reasons. First, we required viable PMNs in order to make direct comparisons between the phenomenon of ligand binding to the C5aR and its relationship to signal transduction. Second, we have shown that our conformationally biased agonists of C5a express potencies and affinities amenable to the undertaking of such a study on whole PMNs. Although binding affinities of C5a peptide analogues have been reported in the nanomolar range in PMN membranes,[13,25,26] it should be borne in mind that these affinities are meaningful only when expressed relative to that of C5a under the same experimental conditions. Consequently, we have expressed our data, both functional and binding, relative to the activity of C5a in the same experiment.

A comparison of the C5a dose−response curve with the C5a binding curve demonstrated that the ability to bind the C5aR occurred at a lower concentration of C5a than did the ability to activate the C5aR (EC_{50}/IC_{50} = 53) (Table 3). In contrast, both C5aR binding and activation curves for the conformationally biased peptide **54** were essentially coincidental (EC_{50}/IC_{50} = 0.7). Increased affinity for the C5aR over peptide **54** was observed when certain N-terminal core binding determinants were attached to peptide **54** (i.e., peptides **74** and **87**). The N-terminal core binding determinants alone, $C5a_{12-20}$ (peptide **84**) and $C5a_{37-46}$ (peptide **82**), competed weakly with [^{125}I]C5a for binding to the C5aR and displayed IC_{50}s around 100−400 μM yet were inactive in the PMN assays (Table 2). For peptide **87**, which was significantly more potent than peptide **54** in the PMN assay, the binding curve was shifted 2 log units to the left of the binding curve for peptide **54**. Interestingly, the shift in the activation curve relative to peptide **54** was smaller (0.8 log unit). These results show that increased affinity to the C5aR can be achieved by the addition of certain N-terminal core binding determinants to the conformationally biased effector region, i.e., peptide **54**. However, these results also demonstrate that an increase in C5aR binding affinity alone does not necessarily result in a corresponding increase in agonist potency.

Agonist Selectivity. Although the EC_{50} values for C5a were similar in fetal artery and PMNs (21 and 34 nM, respectively), there were marked differences in the potencies of the agonist peptides in the two tissues used in this study. This difference has been expressed as a function of the relative selectivity of the peptides in the two assays: i.e., selectivity = antilog[pD_2(fetal artery) − pD_2(PMN enzyme release)] (Table 1). This measure of selectivity showed that all peptides used in this study were more potent in smooth muscle contraction of human fetal artery than in enzyme release from PMNs.

Pharmacological Analysis. It is interesting to note that when compared to the conformationally flexible peptides **2** and **10**, the potency expressed by the more conformationally restricted peptide **54** was less dependent upon its affinity to the C5aR. This was demonstrated in PMNs where peptide **54** was significantly more potent than peptides **2** and **10**, despite the fact that all three peptides expressed similar C5aR binding affinities. These results suggest that the expression of potency by agonists of the C-terminal effector region of C5a is conferred by topochemical features other than those exclusively involved in C5aR binding affinity. This lack of correlation between binding affinity and agonist potency lends additional support to our hypothesis that the presentation of an optimized conformation in the C-terminal effector region of C5a to the activation domain of the C5aR is a critical factor that dictates the magnitude of the potency expressed by C-terminal agonists of C5a. In agreement with this hypothesis, the

ratio of EC_{50}/IC_{50} for peptides **2**, **10**, and **54** (Table 3) decreased as the degree of conformational flexibility increased; i.e., there was a positive correlation between the extent of conformational restriction (and resulting agonist topography) and the potency of C5aR activation.

The dose−response relationships for C5aR binding and enzyme release in PMNs were almost coincidental for peptide **54**. These results suggest that for a potent, conformationally biased C-terminal agonist of C5a, C5aR binding and activation are tightly coupled ($EC_{50}/IC_{50} = 0.7$). C5aR binding and activation with natural C5a, however, is different. The natural factor is thought to bind first to the extracellular loops expressed on the surface of the C5aR.[27] This initial interaction provides the bulk of the ligand−receptor binding energy and may also serve to orient the signal transducing, C-terminal region for proper interaction within the activating region of the C5aR. Since the C-terminal effector region of C5a is conformationally flexible, many energy-minimized conformers are presented to the C5aR. Thus, C5aR activation is delayed relative to the overall rate of C5aR binding. This is reflected by our observation that the binding curve for C5a was positioned well to the left of the activation curve by a factor of about 1.7 log units.

On the basis of this observation, we hypothesized that the binding and activation curves of peptide **54** when coupled to the N-terminal core binding determinants would resemble those of the natural factor. In fact, this was shown to be the case with peptide **87**; i.e., the binding curve was positioned well to the left of the activation curve. However, where the concentration ratio of binding to activation for C5a was 1/50, that for peptide **87** was 1/10, indicating that C5aR activation and binding were more tightly coupled for peptide **87** than for the natural factor. Thus, the 100-fold increase in affinity for the C5aR expressed by peptide **87** only resulted in a 6-fold increase in potency, suggesting that the presentation of the C-terminal effector region of the peptide to the receptor was not optimized.

With the noteworthy exception of peptide **87** in PMN responses, the addition of N-terminal core C5aR binding determinants to peptide **54** did not result in significant increases in agonist potency. Potency increases over peptide **54** were a maximum of 2−3-fold and reached statistical significance only in the fetal artery assay where the greatest potency, observed with peptide **74**, amounted to 25% of C5a. This increase in potency was not observed in enzyme release from PMNs. In fact, peptide **74** was equipotent to peptide **54**, again illustrating the lack of concordance of agonist potencies in the different assays. However, the binding affinity of peptide **74** for the C5aR expressed on PMNs was 5-fold higher than peptide **54**, suggesting that although binding to the C5aR occurred at lower concentrations, the coupling of the conformationally biased effector region to the activation domain of the C5aR was not efficient. This may be explained by the Ahx spacer group not being of optimal length to allow for the effective presentation of the effector moiety to the activation site of the C5aR.

As mentioned, the conformationally biased peptides consistently caused significantly higher amounts of β-glucuronidase release from PMNs than either C5a or the conformationally flexible, C-terminal agonist peptide **2**. This increased efficacy was also observed in fetal artery for peptide **54** as shown by the biphasic nature of the dose−response curve. It is not yet known if the superefficacy exhibited by the conformationally biased agonist analogues is mediated exclusively by the C5aR. It should be noted, however, that others have reported similar results with C5a agonist analogues showing increased efficacy relative to C5a in other cellular systems.[10,16,28] This novel activity of C5a agonist analogues may also reflect an intrinsic property of the transmembrane site within the C5aR where these agonists are thought to interact. It may be that the conformationally biased agonists interact with this site in such a way that they do not interact with potential regulatory sites expressed in other regions of the C5aR. This may result in continued signal transduction and/or activation of other C5aR-linked intracellular signaling pathways. Studies are underway to investigate the mechanism of this superefficacious activity.

This study also demonstrates that although there is a good overall correlation between the potencies of C5a agonist analogues in the spasmogenic and PMN assays, there are also clear exceptions. For example, peptide **10** was about 20-fold more potent than peptide **2** in the fetal artery assay, but both peptides were equipotent in the PMN assay. Analysis of the selectivity of the peptides for C5aRs associated with fetal artery or PMNs demonstrated that the conformationally constrained peptides were more potent in fetal artery. The reasons for this agonist selectivity are not yet known but may be related to as yet undefined differences in the C5aRs in the two tissue and cell types. Pharmacological analyses with C5aR antagonists[25] will clarify the situation. Whether a series of C5a analogues possessing different topographical features that exhibit selectivity for the C5aR expressed on PMNs can be developed is yet to be determined. The ability to generate highly potent, selective compounds may be useful in the augmentation of certain C5a-mediated activities.

Summary and Conclusions

A constrained carboxy terminal decapeptide analogue of C5a (peptide **54**) has been developed with increased agonist potency. This increase in potency appears to be due to the introduction of a polyproline type II conformation that allows for more favorable interactions between the peptide and the C5aR. In the second part of our investigation, N-terminal binding determinants were attached to the highly potent agonist peptide **54**. The results show that decapeptide agonist affinity for the C5aR can be increased by 2 orders of magnitude using this approach. Furthermore, the results indicate that the C5aR binding and signal transduction events are more tightly coupled with conformationally restricted agonists than with the natural factor or C-terminal agonists with a higher degree of conformational flexibility. These findings suggest that when presented to the C5aR in a biologically active conformation, the C-terminal effector region of C5a plays a prominent role in C5aR recognition and binding.

Experimental Section

Abbreviations used: EC_{50}, effective concentration at half-maximal response; HPLC, high-performance liquid chromatography; pD_2, $-\log EC_{50}$; PMNs, polymorphonuclear leukocytes (neutrophils); Ahx, ϵ-aminocaproic acid; A, alanine; C,

cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; W, tryptophan; Y, tyrosine. Uppercase letters designate the L-stereoisomeric form of the amino acid and lowercase letters the D-stereoisomeric form.

Peptide Synthesis, Purification, and Characterization. All peptides were synthesized by standard solid-phase methods on an Applied Biosystems (Foster City, CA) Model 430A synthesizer. Syntheses were performed on a 0.25 mmol scale on a *p*-(hydroxymethyl)phenoxymethyl polystyrene resin using the *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) synthetic scheme. Peptides were purified by analytical and preparative HPLC on columns packed with C_{18}-bonded silica gel and were characterized by amino acid compositional analysis. The details of these methods have been previously reported.[17]

Human Fetal Artery Assay. Spasmogenic activity was measured by the ability of peptides to induce smooth muscle in human fetal artery. The methods of tissue preparation and assay performance were identical with those published previously.[17,29] Full dose−response curves for C5a (human recombinant C5a; Sigma) or individual peptides were generated in each experiment, and the EC_{50} values (concentration of peptide producing 50% of the maximal response to each peptide) were calculated. pD_2 transforms [−log EC_{50} (M)] were calculated for each dose−response curve and means ± SE obtained for each peptide. The log potency ratio [pD_2(C5a) − pD_2(peptide)] was calculated in each experiment to minimize the effects of biological variation in tissue sensitivity to C5a or peptides on the calculations of relative potencies.[19]

Enzyme Release from PMNs. β-Glucuronidase release from human PMNs was determined as described by Schroder.[30] Briefly, human PMNs were pretreated with cytochalasin B (5 μg/mL, 10 min, 37 °C). The cells (10^6) were incubated in the presence of stimulants for 60 min at 37 °C in a final volume of 200 μL and then centrifuged. The supernatant was collected, and 50 μL was incubated with 50 μL of 0.01 M *p*-nitrophenyl β-D-glucuronide (in 0.1 M sodium acetate at pH 4 for 18 h) as triplicate determinations in 96-well microtiter plates. The reaction was stopped by the addition of 100 μL of 0.4 M glycine buffer (pH 10). The reaction mixture developed color that was read at 415 nm in a microtiter plate reader.

C5a Receptor Binding Assays. All assays were performed using freshly prepared human PMNs isolated as previously described.[18] The assay buffer consisted of 50 mM HEPES, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% bovine serum albumin. In assays performed at 4 °C, buffer, Hunter/Bolton labeled [^{125}I]C5a (20 pM) (New England Nuclear, MA), unlabeled C5a or peptide, and PMNs (0.25 × 106) were added sequentially to a final volume of 200 μL. After incubation for 60 min, the samples were filtered through Millipore GVWP 0.22 μm membranes. The concentration of each peptide was determined in triplicate, and tubes were washed twice with 2 mL of ice-cold buffer. Filters were dried and counted in an LKB gamma counter. Nonspecific binding was assessed by including 1 mM peptide or 100 nM C5a, which typically resulted in 10−15% of total binding.

Statistical Analysis of Results. Statistical analyses were performed by ANOVAR followed by post-hoc Dunnett tests.

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