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Residues 21–30 within the Extracellular N-terminal Region of the C5a Receptor Represent a Binding Domain for the C5a Anaphylatoxin*

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The functions of the C5a anaphylatoxin are expressed through its interaction with a cell-surface receptor with seven transmembrane helices. The interaction of C5a with the receptor has been explained by a two-site model whereby recognition and effector sites on C5a bind, respectively, to recognition and effector domains on the receptor, leading to receptor activation (Chenoweth, D. E., and Hugli, T. E. (1980) *Mol. Immunol.* 17, 151–161. In addition, the extracellular N-terminal region of the C5a receptor has been implicated as the recognition domain for C5a, responsible for ~50% of the binding energy of the C5a-receptor complex (Mery, L., and Boulay, F. (1994) *J. Biol. Chem.* 269, 3457–3463; DeMartino, J. A., Van Riper, G., Siciliano, S. J., Molineaux, C. J., Konteatis, Z. D., Rosen, H., and Springer, M. S. (1994) *J. Biol. Chem.* 269, 14446–14450). In this work, the interactions of C5a with the N-terminal domain of the C5a receptor were examined by use of recombinant human C5a molecules and peptide fragments $M_1NSFN_5YTTPD_{10}YGHYD_{15}DKDTL_{20}DLNTP_{25}VDKTS_{30}NTLR$(hC5aRF-1–34), acetyl-$HYD_{15}DKDTL_{20}DLNTP_{25}VDKTS_{30}NTLR$ (hC5aRF-13–34), and acetyl-$TL_{20}DLNTP_{25}VDKTS_{30}$N-amide (hC5aRF-19–31) derived from human C5a receptor. Binding induced resonance perturbations in the NMR spectra of the receptor fragments and the C5a molecules indicated that the isolated N-terminal domain or residues 1–34 of the C5a receptor retain specific binding to C5a and to a C5a analog devoid of the agonistic C-terminal tail in the intact C5a. Residues of C5a perturbed by the binding of the receptor peptides are localized within the helical core of the C5a structure, in agreement with the results from functional studies employing mutated C5a and intact receptor molecules. All three receptor peptides, hC5aRF-1–34, hC5aRF-13–34, and hC5aRF-19–31, responded to the binding of C5a through the 21–30 region containing either hydrophobic, polar, or positively charged residues such as Thr^{24}, Pro^{25}, Val^{26}, Lys^{28}, Thr^{29}, and Ser^{30}. The 21–30 segment of all three receptor fragments was found to have a partially folded conformation in solution, independent of residues 1–18. These results indicate that a short peptide sequence, or residues 21–30, of the C5a receptor N terminus may constitute the binding domain for the recognition site on C5a.

The C5a anaphylatoxin is a 74-residue glycoprotein derived from the fifth component (C5) of the complement system upon proteolytic activation (1). The C5a molecule plays an important role in host defense against invading microorganisms or tumor cells. Inappropriately accumulated C5a, on the other hand, stimulates smooth muscle contraction, causes vasodilation, increases vascular permeability, and can even recruit and stimulate granulocytes leading to the release of inflammatory molecules (2–4). The implication of C5a in various immune and inflammatory diseases prompted extensive structure-and-function studies (5–16). In particular, NMR analysis defined C5a as being composed of a 4-helix core structure (residues 1–64) followed by a 10-residue C terminus with conformational variability (7–9, 16). Determination of the three-dimensional structure of C5a followed by mutagenesis allowed the identification of many C5a residues required for receptor activation and others that may be more important for the structural integrity of the C5a protein (10–15).

There appear to be two major structural elements within C5a required for an effective activation of the C5a receptor (4). The 4-helix region of C5a or residues 1–64 encodes a recognition site for receptor binding (5, 6). The flexible C-terminal residues, on the other hand, comprise the predominant effector site for receptor activation (5, 17–19). Accordingly, peptides derived from the C terminus of C5a were found to be specific agonists of the C5a receptor. These C5a peptides have been useful for mapping the effector sites and led to the development of moderately potent receptor agonists (17–19). A C5a analog truncated at the C terminus, or C5a-1–69, was found to be a C5a receptor antagonist (5). Mutagenesis studies within this portion of C5a identified some residues important for receptor binding (10–15). However, the recognition site has not been defined conclusively because the important residues identified by these studies were distributed almost throughout C5a. Recently, a new C5a antagonist was synthesized, and its solution structure was determined (20). The structural difference between the C5a semi-synthetic antagonist and intact C5a (7–9) provided further insights into the nature of the receptor recognition site on C5a (20).

Since the cloning of the C5a receptor (21, 22), work has been focused on defining the structural features of the receptor molecule required for the C5a-receptor interaction (23–31). The cell-surface receptor for C5a is a member of the G-protein-coupled receptor superfamily, with an extracellular N-terminal region, an integral membrane helical domain, and a C-terminal tail extending into the cytoplasmic space (32). The extracellular and intracellular faces of the C5a receptor have three peptide loops, respectively, each connecting the C and the N termini of the seven individual transmembrane helices. The agonist functions of C5a are thought to be expressed by the binding of its

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TABLE I
The extracellular N-terminal receptor peptides selected for NMR studies

	1 10 20 30
hC5aRF-1–34	MNSFNYTTPDYGHYDDKDTLDLNTPVDKTSNTLR
hC5aRF-13–34	ac-HYDDKDTLDLNTPVDKTSNTLR
hC5aRF-19–31	ac-TLDLNTPVDKTSN-amide

C-terminal tail $M_{70}QLGR_{74}$ (or the effector site) to an activation domain on the receptor formed by the transmembrane helices (21, 22, 29–31). The 4-helix core region or recognition site of C5a may serve to assist receptor activation through interactions with a recognition domain on the extracellular face of the receptor molecule (4, 6, 28, 33). However, there is a lack of detailed structural information about the C5a/C5a-receptor complex (4), or any ligand-receptor complex involving G-protein-coupled receptors (32), for an adequate understanding of the molecular mechanism of receptor activation.

Unfortunately, it is not yet feasible to define in atomic resolution the structural details of the entire C5a/C5a-receptor complex. On the other hand, structural information may be derived through a study of the binding interactions of C5a with soluble fragments of the receptor. Several lines of investigation suggest that the recognition domain for the C5a-receptor interaction is localized in the extracellular N-terminal region of the C5a receptor (4). First, antibodies recognizing the receptor N-terminal fragments interfere with C5a binding (23, 24). Second, truncation of the N terminus of the C5a receptor results in reduced binding of the receptor to intact C5a, but not to the agonist C5a tail peptides (27, 28). Third, partial or complete replacement of the receptor N terminus diminished cell responses to C5a binding (26). Fourth, replacement of some of the N-terminal Asp residues of the receptor with either Ala or Asn results in dramatically decreased affinity for C5a (26, 27). There is therefore a possibility that the receptor N terminus may represent an autonomous binding domain showing specific interactions with C5a. In this paper, we report an NMR characterization of the interactions of the N-terminal fragments of the C5a receptor with C5a and with an antagonist analog, CGS-28805, of C5a. NMR work was facilitated by the finding that the isolated receptor N terminus, residues 1–34 of the human C5a receptor, retained binding to C5a. The NMR results provide additional evidence for specific contacts between C5a and the N-terminal region of the C5a receptor. Receptor residues responsible for these contacts are further localized to residues Asp^{21} to Ser^{30} containing only two out of the five aspartic acids identified by mutagenesis as important for binding to C5a.

EXPERIMENTAL PROCEDURES

Preparation of Peptides and Proteins—Peptide fragments (Table I) derived from residues 1–34 of the human C5a receptor were synthesized using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry on a multiple-channel peptide synthesizer (Symphony Protein Technology Co.). The synthetic peptides were purified by high pressure liquid chromatography using a Vydac C18 reverse-phase column (Vydac, Hesperia, CA) and with a gradient of 10–70% acetonitrile in a flow phase of 99.9% of H_2O and 0.1% of trifluoroacetic acid. The identities of the purified peptides were verified by use of electrospray mass spectrometry, which showed that peptide hC5aRF-1–34 had a molecular mass of 3954 (expected: 3950.79), hC5aRF-13–34 of 2603 (expected: 2602.24), and hC5aRF-19–31 of 1459 (expected: 1458.55). Sequential assignments of the proton NMR resonances also confirmed the expected sequences of all the peptides.

Recombinant C5a (Thr^1-Met) and a C-terminally truncated C5a analog, C5a-1–71 (Thr^1-Met, Cys^{27}-Ser, Gln^{71}-Cys), were obtained from expressing bacterial cell lines as described elsewhere (12). C5a was renatured as the glutathione adduct and CGS-28805 as the cysteine adduct, C5a-1–71 (Thr^1-Met, Cys^{27}-Ser, Gln^{71}-Cys-S-S-Cys), of the C5a-1–71 analog (20). To prepare CGS-28805, cysteine/cystine was used as the redox couple to replace reduced and oxidized glutathione in the renaturation buffer. Electrospray mass spectrometry was used to verify the molecular weight of both proteins. C5a had a molecular weight of 8601 (expected: 8603) and CGS-28805 of 8052 (expected: 8050). The C5a preparation had a binding affinity (IC_{50}) of 0.007 nM whereas that of the CGS-28805 was 0.1 nM based on *in vitro* competitive binding assays against ^{125}I-labeled C5a (12, 14). The CGS-28805 molecule had an antagonist potency (IC_{50}) of better than 400 nM, a value close to those of C5a-1–69 (5) and another C5a antagonist, C5a-1–71 (Thr^1-Met, Cys^{27}-Ser, Gln^{71}-Cys-S-S-Cys-Leu-Gly-D-Arg) (20).

Samples for NMR measurements were prepared by dissolving 1–2 mg of the purified peptides in 450 μl of an aqueous solution containing 20 mM sodium acetate and 0.2 mM EDTA. A volume of 50 μl D_2O was added to the peptide solutions to provide the deuterium lock signal for the NMR spectrometers. The pH values of the peptide samples were adjusted to 5 or 6.8 with dilute NaOH or HCl solutions. The concentrations of the peptides were approximately 0.5 mM for one-dimensional and 1 mM for two-dimensional NMR experiments. Stock solutions of C5a and the C5a antagonist, CGS-28805, were prepared by dissolving desired amounts of the proteins into the same buffers used for the peptide samples. The concentrations of C5a and CGS-28805 were approximately 0.6 mM for two-dimensional NMR measurements.

NMR Measurements—All NMR experiments were carried out on a Bruker AMX2–500 MHz and/or a DRX-500 MHz NMR spectrometer at sample temperatures of 5, 15, and 30 °C. Phase-sensitive detection by time-proportional phase incrementation was employed for both two-dimensional nuclear Overhauser effects, NOESY[1] (34) or ROESY (35, 36), and total correlation, TOCSY (37, 38), experiments. The intense water signal was suppressed either by selective irradiation during the relaxation delay and during the NOE mixing time or by a WATERGATE sequence (39, 40) combined with the flipback of water signals (41, 42). The NOESY spectra were acquired with mixing times of 200 and 350 ms. The ROESY spectra were acquired with a mixing time of 250 ms. TOCSY spectra were obtained with mixing times of 30–60 ms using the TOWNY-16 isotropic mixing sequence (43). All free induction decays were acquired with a size of 2048 complex data points. The free induction decays data matrices were accumulated with sine modulation along the t_1 dimension. The initial t_1 delays were chosen such that the zero-order and first-order phase corrections along the F_1 spectral dimension were 90 and 0, respectively (44). The number of increments along the t_1 dimension was 350–400 for NOESY and ROESY and 300 for TOCSY data sets. The NMR data were processed using an in-house program, nmrDSP, incorporating fast cosine and sine transformation, linear predication, and optimized base-line correction procedures. Prior to spectral transformation, the free induction decays data were multiplied by cosine-squared window functions along both the t_1 and t_2 time dimensions and zero-filled to 1024 real points along the t_1 dimension. Spectral display, analysis, and comparisons were achieved by use of the FELIX software and/or through the graphics interface of the Sybyl NMR module, TRIAD.

Proton Resonance Assignments—Sequence-specific assignments of the proton resonances of the receptor peptides (Tables II–IV) were achieved by use of a combination of two-dimensional TOCSY and NOESY experiments. Intraresidue spin systems were identified based on TOCSY cross-peak patterns. The identified spin systems were assigned to the corresponding residues in the primary sequence through sequential NOE connectivities in the NOESY spectra (45). The δCH protons of Pro^9 and Pro^{25} were used in place of the normal NH for the tracing of the sequential NOE connectivities. The backbone amide proton resonance for Asn^2 was not observed in the TOCSY spectra. The assignment for this residue was made on the basis of sequential NOE connectivities. The two-dimensional NMR spectra allowed the assignment of most of the proton resonances (Tables II–IV), despite that peptide hC5aRF-1–34 contains many repeats of a few residues, for example, 6 Asp and 6 Thr out of its 34 amino acid residues. The assignment of the proton resonances of two shorter peptides, hC5aRF-13–34 and hC5aRF-19–31 (Tables I-IV), was straightforward following similar TOCSY patterns of the common amino acid residues with peptide hC5aRF-1–34.

Assignments of the proton resonances of C5a have been described previously (16, 20). In short, the intraresidue spin systems were identified using through-bond connectivities observed in COSY or TOCSY type experiments. The sequential assignments were achieved by use of

[1] The abbreviations used are: NOESY, two-dimensional nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; ROESY, rotating-frame NOE spectroscopy; TOCSY, total correlation spectroscopy.

TABLE II
Proton chemical shifts of peptides hC5aRF-1–34 at 15 °C and pH 5

Residue	H^N	H^α	H^β	Others
Met^1		4.12	[a]	2.15, 2.58
Asn^2	8.30	4.72	2.68, 2.63	
Ser^3	8.45	4.36	3.77, 3.77	
Phe^4	8.29	4.48	2.98, 2.92	7.26, 7.12
Asn^5	8.36	4.64	2.72, 2.61	
Tyr^6	8.08	4.54	3.04, 2.94	(H^δ) 7.09; (H^ϵ) 6.79
Thr^7	8.09	4.34	4.13	(H^γ) 1.13
Thr^8	8.15	4.57	4.19	(H^γ) 1.26
Pro^9		4.30	[a]	2.15, 1.63, 1.96; (H^δ) 3.83, 3.68
Asp^{10}	8.33	4.53	2.66, 2.55	
Tyr^{11}	8.13	4.47	3.10, 2.92	(H^δ) 7.09; (H^ϵ) 6.79
Gly^{12}	8.35	3.77, 3.77		
His^{13}	8.12	4.63	3.20, 3.07	($H^{\delta 2}$) 7.15; ($H^{\epsilon 1}$) 8.54
Tyr^{14}	8.34	4.54	3.05, 2.88	(H^δ) 7.09; (H^ϵ) 6.79
Asp^{15}	8.43	4.59	2.71, 2.59	
Asp^{16}	8.27	4.53	2.72, 2.72	
Lys^{17}	8.32	4.27	[a]	1.82, 1.66, 1.43
Asp^{18}	8.36	4.64	2.78, 2.68	
Thr^{19}	8.02	4.27	4.27	(H^γ) 1.24
Leu^{20}	8.21	4.29	[a]	1.65, 1.57, 0.91, 0.85
Asp^{21}	8.37	4.59	2.78, 2.61	
Leu^{22}	8.28	4.30	[a]	1.66, 1.63, 0.93, 0.87
Asn^{23}	8.48	4.73	2.83, 2.77	
Thr^{24}	8.02	4.53	4.11	(H^γ) 1.24
Pro^{25}		4.45	[a]	2.30, 2.04, 1.92; (H^δ) 3.87, 3.69
Val^{26}	8.32	4.05	2.04	(H^γ) 0.94, 0.94
Asp^{27}	8.48	4.62	2.74, 2.64	
Lys^{28}	8.50	4.38	[a]	3.00, 1.93, 1.76, 1.68, 1.45
Thr^{29}	8.34	4.33	4.33	(H^γ) 1.22
Ser^{30}	8.31	4.45	3.88, 3.88	
Asn^{31}	8.52	4.78	2.86, 2.80	
Thr^{32}	8.14	4.30	4.21	(H^γ) 1.21
Leu^{33}	8.28	4.36	[a]	1.66, 1.63, 0.93, 0.87
Arg^{34}	7.94	4.17	[a]	1.85, 1.71, 1.58; (H^δ) 3.18

[a] The proton chemical shifts of the β-protons of these residues were not distinguished from other side chain protons.

TABLE III
Proton chemical shifts of hC5aRF-13–34 at 15 °C and pH 5

Residue	H^N	H^α	H^β	Others
His^{13}	8.34	4.59	3.12, 3.02	($H^{\delta 2}$) 7.18; ($H^{\epsilon 1}$) 8.54
Tyr^{14}	8.34	4.57	3.03, 2.85	(H^δ) 7.06; (H^ϵ) 6.78
Asp^{15}	8.41	4.58	2.68, 2.58	
Asp^{16}	8.28	4.51	2.70, 2.70	
Lys^{17}	8.33	4.25	[a]	2.97, 1.90, 1.80, 1.75, 1.64, 1.40
Asp^{18}	8.34	4.63	2.83, 2.67	
Thr^{19}	8.04	4.24	4.24	(H^γ) 1.17
Leu^{20}	8.21	4.27	[a]	1.63, 1.54, 0.85
Asp^{21}	8.35	4.57	2.77, 2.61	
Leu^{22}	8.27	4.28	[a]	1.59, 1.63, 0.85
Asn^{23}	8.46	4.69	2.80, 2.73	
Thr^{24}	8.02	4.51	4.08	(H^γ) 1.22
Pro^{25}		4.43	[a]	2.28, 2.01, 1.96, 1.88 (H^δ) 3.66, 3.84
Val^{26}	8.31	4.03	2.02	(H^γ) 0.92, 0.92
Asp^{27}	8.47	4.60	2.74, 2.64	
Lys^{28}	8.50	4.35	[a]	2.97, 1.90, 1.80, 1.75, 1.64, 1.40
Thr^{29}	8.32	4.30	4.30	(H^γ) 1.20
Ser^{30}	8.30	4.43	3.86, 3.86	
Asn^{31}	8.51	4.75	2.83, 2.87	
Thr^{32}	8.13	4.27	4.18	(H^γ) 1.18
Leu^{33}	8.27	4.34	[a]	1.59, 1.63, 0.85
Arg^{34}	7.94	4.16	[a]	1.82, 1.68, 1.55, (H^δ) 3.15

[a] The proton chemical shifts of the β-protons of these residues were not distinguished from other side chain protons.

the sequential NOE contacts observed in the NOESY spectra of C5a (45). Similar approaches were applied to the assignments of the proton resonances for the C5a antagonist, CGS-28805.

RESULTS AND DISCUSSION

The Isolated N-terminal Domain of the C5a Receptor Retains Binding to C5a—Interactions between the receptor peptide hC5aRF-1–34 and C5a were followed by NMR spectroscopy through perturbations of the proton resonances of the receptor

TABLE IV
Proton chemical shifts of hC5aRF-19–31 at 15 °C and pH 5

Residue	H^N	H^α	H^β	Others
Thr^{19}	8.28	4.18	4.08	(H^γ) 1.12
Leu^{20}	8.42	4.28	[a]	1.55, 1.49, 0.84, 0.80
Asp^{21}	8.45	4.51	2.70, 2.53	
Leu^{22}	8.41	4.23	[a]	1.58, 1.50, 0.86, 0.79
Asn^{23}	8.52	4.64	2.74, 2.69	
Thr^{24}	8.04	4.44	4.02	(H^γ) 1.17
Pro^{25}		4.38	[a]	2.28, 2.02, 1.89, (H^δ) 3.90, 3.72
Val^{26}	8.38	3.95	1.96	(H^γ) 0.87, 0.87
Asp^{27}	8.52	4.54	2.68, 2.58	
Lys^{28}	8.56	4.30	[a]	1.86, 1.69, 1.61, 1.38
Thr^{29}	8.36	4.25	4.18	(H^γ) 1.14
Ser^{30}	8.36	4.48	3.80, 3.80	
Asn^{31}	8.47	4.61	2.75, 2.69	

[a] The proton chemical shifts of the β-protons of these residues were not distinguished from other side chain protons.

peptide accompanying complex formation. Fig. 1 shows the amide NH region of the proton NMR spectra of peptide hC5aRF-1–34 (Fig. 1*a*) and the same peptide in the presence of a less than stoichiometric amount of C5a (Fig. 1*b*). Comparison of the two spectra shows that only a subset of the proton resonances from the receptor peptide (Fig. 1*a*) were selectively perturbed upon the addition of C5a (Fig. 1*b*). The perturbed resonances exhibited changes in chemical shifts and some broadening in line widths, indicating a fast association and dissociation of the C5a-peptide complex (46). Most of the resonance perturbations are relatively small, presumably as a result of low affinity of the isolated receptor N terminus for C5a and/or a minimal change of the conformations of the receptor peptide between the free and the C5a-bound states (46). The binding experiment was also carried out (Fig. 1*c*) with an antagonist analog of C5a, CGS-28805, a molecule lacking 3 residues from the C-terminal tail of native C5a (see the "Experimental Procedures"). Addition of the C5a antagonist had larger effects on the proton NMR spectra of the receptor peptide as more resonance signals appear to be shifted or broadened (Fig. 1*c*). The pattern of resonance perturbations remained the same when the experiments were repeated at a higher temperature of 30 °C or at pH 6.8 (spectra not shown) for both complexes. The isolated N-terminal domain, hC5aRF-1–34, of the C5a receptor therefore retains binding to C5a even in the absence of the C-terminal agonist tail of intact C5a (as in CGS-28805).

The perturbed proton resonances were assigned to the specific residues of the receptor peptide based on a comparison of the two-dimensional TOCSY spectra of the receptor peptide in the presence and absence of C5a. Fig. 2*A* shows the superposition of the NH side chain connectivities of peptide hC5aRF-1–34 before (in *blue*) and after (in *pink*) the addition of C5a at ~0.13 molar ratio to the peptide. Clearly, most of the peptide peaks are not affected by C5a binding although slight perturbations can be seen along the NH resonance frequencies of residues Thr^{24}, Val^{26}, Asp^{27}, Lys^{28}, and Thr^{29}. These same residues of the receptor peptide are perturbed in the presence of ~0.15 molar ratio of the C5a antagonist, CGS-28805 (Fig. 2*B*), but with more dramatic change of resonances than by C5a (Fig. 2*A*). Binding of the C5a antagonist also perturbed the NH signals of residues Asp^{21} and Ser^{30}. Residues Val^{26}, Asp^{27}, and Lys^{28} displayed significant broadenings in their NH resonances in addition to shifts of the resonance frequencies (Fig. 2*B*). The amide proton resonances of Val^{26} and Asp^{27} were shifted downfield, whereas the NH signals of Thr^{24}, Lys^{28}, and Thr^{29} moved upfield in the presence of both C5a and CGS-28805. Resonance perturbations were also observed for the βCH and γCH_3 protons of Val^{26} and for the αCH and one of δCH protons of Pro^{25}, despite the lack of significant effects on the side chain proton

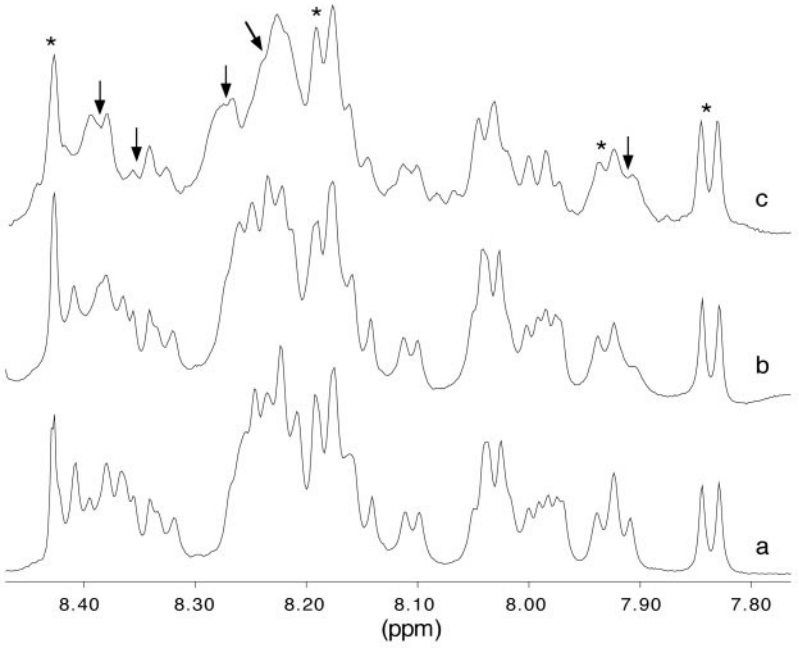

FIG. 1. **Amide region of the proton NMR spectra of peptide hCRF-1–34 at pH 5 and 15 °C.** *a*, free hCRF-1–34; *b*, in the presence of human C5a; and *c*, in the presence of CGS-28805, an antagonist of C5a. The concentration of the receptor peptide is 0.6 mM and those of the C5a ligands are 0.075 and 0.09 mM, respectively. Proton resonances were perturbed upon the addition of the C5a proteins to the peptide solution. The most affected resonances are indicated by *arrows*, and the unperturbed resonances are labeled with *asterisks*.

resonances of other residues in the same peptide. Differential broadening of the amide proton resonances of Val^{26}, Asp^{27}, and Lys^{28} of the receptor peptide indicates that these protons must have significantly different resonance frequencies and chemical environment upon binding to C5a, given that the C5a and the antagonist were present in less than 15% of the molar concentration of the receptor peptide (Figs. 1 and 2).

Receptor binding assays showed that the C5a receptor has high affinity interactions with intact C5a with a K_d (or IC_{50}) in the subnanomolar range, between 4 pM and 5 nM depending on the types of assays used (4, 6, 9, 12, 26, 33). Mutagenesis experiments indicated that the receptor N terminus contributed to almost half of the binding energy between the C5a ligand and the surface-expressed intact receptor molecules (27). A two-site binding mode between C5a and the receptor (4, 6, 28) predicts that the isolated receptor N terminus can have a binding affinity for C5a with a K_d value as low as ~1 μM. Indeed, NMR results here indicate that there are interactions between C5a and the isolated N-terminal receptor domain, hC5aRF-1–34, involving specific residues (Figs. 1 and 2). These interactions appear to be enhanced when C5a was replaced by a C5a antagonist, CGS-28805, with three residues removed from the C terminus of C5a. Consistent patterns of resonance perturbations on the receptor peptide hC5aRF-1–34 by both C5a and the C5a antagonist suggest that interactions detected by NMR (Figs. 1 and 2) are very likely consequences of specific binding between the isolated receptor N terminus and the C5a molecules.

The N-terminal Receptor Fragment Binds to the Recognition Site within C5a—Interactions of the isolated receptor N terminus with C5a were also examined by following the changes in the proton NMR spectra of C5a induced by the binding of the receptor peptide. Again, there were localized changes in the one-dimensional proton spectra of C5a upon the addition of peptide hC5aRF-1–34. It was difficult to analyze the binding effects using these one-dimensional NMR data since C5a with 74 residues had a significantly more complex proton spectrum (not shown) than that for the 34-residue receptor peptide (Fig. 1*A*). On the other hand, the structured C5a produces well resolved two-dimensional NOESY spectra (7–9) with which perturbed resonances can be analyzed and assigned. In the presence of the receptor peptide, C5a and CGS-28805 exhibited a large number of the NOE connectivities characteristic of the three-dimensional structure of C5a (16, 20). Except for shifts in the positions of a small number of cross-peaks, the NOESY spectra remain essentially unchanged (spectra not shown) without the intensity distortions or grossly broadened peaks seen with the spectrum of the receptor peptide (Fig. 2).

The shifted proton resonances of C5a in the presence of the hC5aRF-1–34 peptide were assigned to the corresponding residues in C5a. Fig. 3 shows the chemical shift differences for the C5a resonances in the absence and presence of the receptor peptide at 0.3 molar ratio to C5a. Clear resonance perturbations (>0.01 ppm) by peptide binding were observed for the NH protons of Val^{17p},[2] Lys^{19p}, and Ser^{42p}. In addition, detectable peak shifts (0.005–0.01 ppm) were found for the NH proton resonances of Val^{18p}, Lys^{20p}, Cys^{21p}, Tyr^{23p}, Ala^{38p}, and Gly^{44p}. Compared with free C5a, the backbone amide proton resonances of Val^{17p}, Lys^{20p}, Tyr^{23p}, and Ser^{42p} were shifted downfield, whereas those of Val^{18p}, Lys^{19p}, Cys^{21p}, Ala^{38p}, and Gly^{44p} were moved to upfield (Fig. 3). Furthermore, resonance shifts were also detected for the βCH, ϵCH, and δCH protons of His^{15p} with the result for the ϵCH proton shown in Fig. 3. More pronounced effects were observed when the experiments were carried out with the C5a antagonist, CGS-28805, as shown in Fig. 3. It is also seen that binding of the receptor peptide perturbed the same set of residues in both the native C5a and the C5a antagonist.

Residues of C5a contributing to receptor binding have been identified through chemical modification and mutagenesis studies. Table V is a list of the C5a residues implicated in receptor binding by mutagenesis and those whose proton resonances were perturbed significantly by the receptor peptide hC5aRF-1–34. Residues with resonance perturbations are localized within the 15–44 region of C5a, whereas the isolated receptor N terminus did not perturb any residues from the agonist C-terminal tail of C5a. The binding interactions between C5a and the isolated receptor peptide detected by NMR are specific effects since the NMR signals of C5a perturbed by the receptor peptide correspond closely to those C5a residues identified by mutagenesis as important for binding to intact

[2] The residue number for C5a is followed by a lowercase letter p to distinguish from that for the C5a receptor fragments.

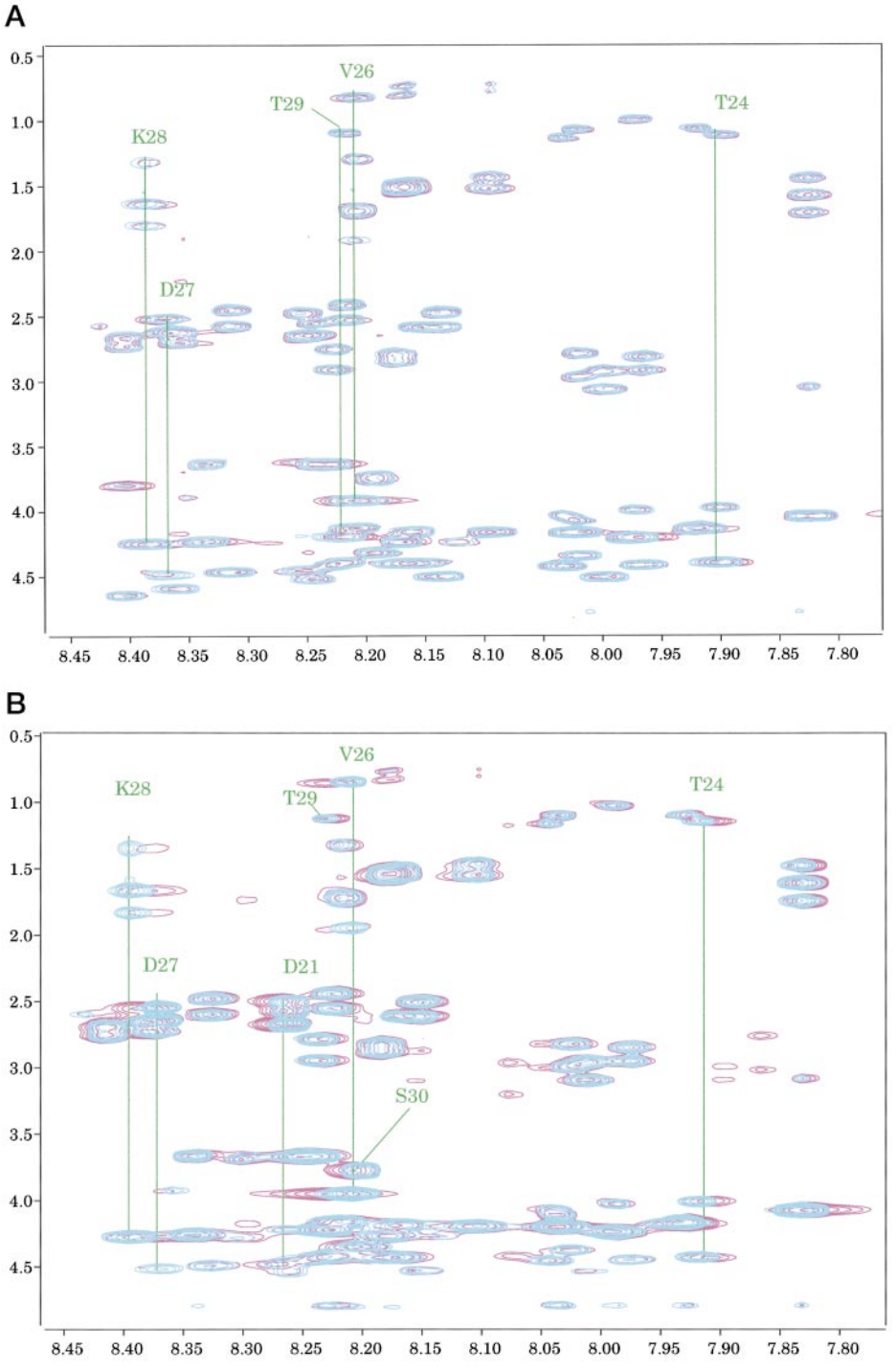

FIG. 2. **The NH region of the TOCSY spectra for hC5aRF-1–34 in the presence and absence of C5a and the C5a antagonist.** The TOCSY spectra of the free peptide hC5aRF-1–34 are shown in *blue* and in *pink* are the spectra in the presence of C5a (*A*) or the C5a antagonist (*B*). The concentration of the receptor peptide is 1 mM, and those of the proteins are 0.13 mM for C5a and 0.15 mM for the C5a antagonist, CGS-28805. Only a few resonances were perturbed by C5a binding (see also Fig. 1), whereas most amide protons remained at their original positions. The perturbed resonances are labeled by their corresponding residues (see also Tables II–IV). Both frequency shifts and line broadening were observed for the perturbed resonances. Note that resonance perturbations are for the same residues of hC5aRF-1–34 in the presence of either C5a (*A*) or the C5a antagonist, CGS-28805 (*B*).

receptor molecules (Table V). The NMR results therefore provided additional evidence for the interaction between C5a and its receptor involving a contact between a recognition site on C5a and the N-terminal region of the C5a receptor (6, 28). The receptor recognition site on C5a is most likely organized by residues 15–44 within the helical core structure of C5a. In addition, the C5a antagonist, CGS-28805, binds to the isolated receptor N terminus peptide, hC5aRF-1–34, using the same contact surface as in the agonist C5a. Interestingly, the antagonist CGS-28805 appears to have enhanced interactions with the receptor peptide despite that it had a somewhat decreased binding affinity (~0.1 nM) compared with the native C5a molecule (~0.007 nM) in receptor binding assays (see "Experimental Procedures").

Residues 21–30 of the C5a Receptor Constitute a Binding Domain for C5a—Fig. 2 showed that only 7 out of a total of 34 residues of the receptor peptide have specific resonance shifts and/or line broadening in the presence of C5a. Both C5a and CGS-28805 perturbed the receptor N-terminal fragment in the region of residues 21–30, whereas no significant changes were found for residues 1–18. These results indicate that C5a may contact the C-terminal portion of the receptor peptide hC5aRF-1–34 independent of the N-terminal residues 1–18 of the same peptide. Two shorter receptor peptides (hC5aRF-13–34 and hC5aRF-19–31 in Table I) were synthesized, and their interactions with CGS-28805 were examined. These two subfragments were found to not only bind to the C5a antagonist but also the perturbed resonances by the antagonist in the shorter

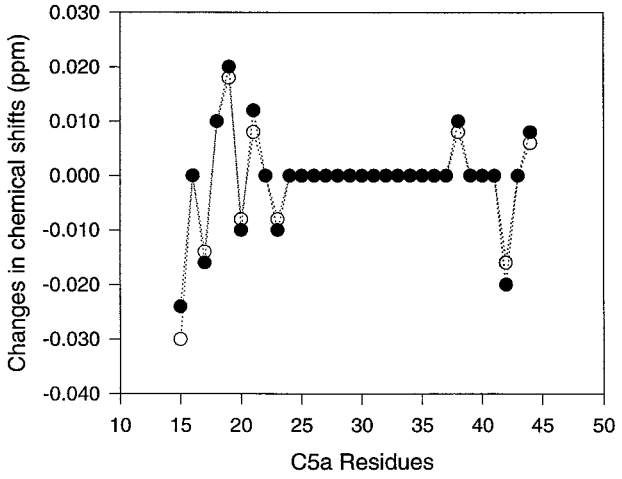

FIG. 3. **Resonance perturbations in C5a (*open circles*) and in CGS-28805 (*filled circles*) induced by the binding of the hC5aRF-1–34 peptide.** The values of the changes were the chemical shift differences between the resonances of the proteins before and after the addition of the peptide. All illustrated changes were for backbone amide protons except for residue 15 (His^{15p}) for which the side chain ϵCH proton was used. Only residues 15–44 were shown since no detectable resonance shifts were observed for the residues beyond this region of the C5a sequence. The concentrations of C5a and CGS-28805 were approximately 0.6 mM in acetate buffer at pH 5 and 30 °C in the absence or presence of ~0.2 mM of the hC5aRF-1–34 peptide.

peptides belong to the same set of residues as observed for hC5aRF-1–34 (Fig. 4). Frequency shifts were observed for the amide proton resonances of Thr^{24}, Val^{26}, Asp^{27}, Lys^{28}, and Ser^{30} of hC5aRF-13–34. Obvious perturbations were found for the amide protons of residues Thr^{24}, Val^{26}, Asp^{27}, and Lys^{28} of hC5aRF-19–31.

Binding induced perturbations of proton resonances identified with the three receptor peptides, hC5aRF-1–34, hC5aRF-13–34, and hC5aRF-19–31 (Fig. 4), suggest that the contact region for C5a may be localized within the segment of residues 21–30 in the N terminus of the C5a receptor. For the shorter receptor fragments, there appear to be fewer residues with resonance perturbations that are mostly centered around the hydrophobic residue Val^{26} (Fig. 4). This result is not surprising in light of the fact that residues Val^{26}, Asp^{27}, and Lys^{28} have the most severely perturbed proton resonances with both resonance shifts and line broadening effects in the full-length peptide hC5aRF-1–34 (Fig. 2*C*). It is also possible that the truncated receptor fragments may have a binding mode slightly different from the longer peptide hC5aRF-1–34. The surface area on C5a contacted by the shortest receptor fragment, hC5aRF-19–31, was then examined by following the resonance perturbations in CGS-28805 induced by the binding of this shorter receptor fragment. Clear peak shifts were observed for residues His^{15p}, Val^{17p}, Val^{18p}, Lys^{19p}, Lys^{20p}, Ser^{42p}, and Gly^{44p} of CGS-28805 as seen for the binding of peptide hC5aRF-1–34 (Fig. 3). However, the shorter receptor peptide did not perturb the resonances of residues Cys^{21p}, Tyr^{23p}, and Ala^{38p}. On the other hand, residues Ala^{50p} and Phe^{51p} were slightly perturbed (spectra not shown) which were not affected by the binding of the longer peptide hC5aRF-1–34. Regardless, all the C5a residues with resonance perturbations are still localized in the same structural region of C5a molecules (16, 20). The shortest receptor peptide hC5aRF-19–31 therefore retains specific binding to the C5a antagonist, suggesting that residues in this region of the receptor represent a binding site for C5a.

Mutagenesis studies showed that residues throughout the extracellular N-terminal domain of the C5a receptor, especially

TABLE V
C5a residues contributing to receptor binding and with perturbations of the proton resonances by the receptor fragment, hC5aRF-1–34

C5a residues	Biochemical/ mutagenesis[a]	Proton resonance perturbations[c]
Loop 1-helix 2 region		
His^{15p}	++	++
Val^{17p}	−	++
Val^{18p}	+++[b]	+
Lys^{19p}	+	++
Lys^{20p}	+	+
Cys^{21p}	++	+
Tyr^{23p}	++[b]	+
Helix 3-loop 3 region		
Arg^{37p}	++	−
Ala^{38p}	−	+
Arg^{40p}	+	−
Ser^{42p}	−	++
Leu^{43p}	+	−
Gly^{44p}	−	+
Helix 4 region		
Arg^{46p}	++	−
Lys^{49p}	+	−
Glu^{53p}	+	−
Arg^{62p}	+	−
C-terminal tail region		
Leu^{72p}	+	−
Gly^{73p}	++	−
Arg^{74p}	++++	−

[a] Residues were identified by mutagenesis or chemical modification of C5a, as in Johnson and Chenoweth (51); Mollison *et al.* (10, 11); Bubeck *et al.* (15); Toth *et al.* (12). Activities affected by mutations are indicated by ++++, more than 100-fold decrease in binding; +++, ~100-fold decrease; ++, ~10-fold decrease; +, effects less than 5-fold; −, no effects on binding. The C5a residues were replaced by Ala in the mutagenesis experiments, and the binding assays were carried out with intact receptor molecules.

[b] The replacement of residue Val^{18p} may result in the misfolding of the mutant protein (Toth *et al.* (12)); Tyr^{23p} was identified through chemical modification (Johnson and Chenoweth (51)).

[c] The degrees of resonance perturbations are indicated as (see Fig. 3): ++, more than 0.015 ppm of changes in chemical shift; +, perturbations in the range of >0.005 and <0.015 ppm; −, no perturbations detected.

aspartic acid residues, are important for the interaction of the intact receptor molecule with C5a (25–27). Furthermore, a mutant receptor missing the first 22 residues from the N terminus had reduced binding to C5a by 600-fold, and a receptor without residues 1–30 had the affinity for C5a reduced by almost 50,000 (27, 28). The C5a receptor lost the binding for C5a after Asp^{10}, Asp^{15}, and Asp^{16} or all the 5 Asp residues, Asp^{10}, Asp^{15}, Asp^{16}, Asp^{21}, and Asp^{27}, were replaced by Asn residues (26). Interestingly, little effects on affinity were found when either Asp^{10}, Asp^{27}, or both Asp^{21} and Asp^{27} were replaced by Asn (26). On the other hand, the receptor has a decreased binding affinity of more than 40-fold after Asp^{10}, Asp^{15}, Asp^{16}, Asp^{18}, and Asp^{21} were substituted with Ala residues (27). Another 15-fold reduction of binding affinity was found when Asp^{27} was replaced with Ala within a receptor missing the first 22 residues (27). Taken together, the mutagenesis results demonstrate that all the Asp residues play some role in determining the binding of the intact receptor to C5a. Only two (Asp^{21} and Asp^{27}) out of the six Asp residues in the receptor peptide are perturbed by C5a binding based on the analysis of the proton NMR signals in the isolated receptor peptides. The other residues in the 21–30 region of the receptor are either positively charged or with hydrophobic side chains, such as Leu^{21}, Pro^{25}, Val^{26}, and Lys^{27} (Fig. 4). Residues 21–30 of the receptor N terminus may therefore be involved in direct contacts with C5a, whereas other important residues within the region of 1–18 of the receptor may contribute to the C5a binding through indirect interactions.

The 21–30 Region of the Receptor N Terminus Has a Partially

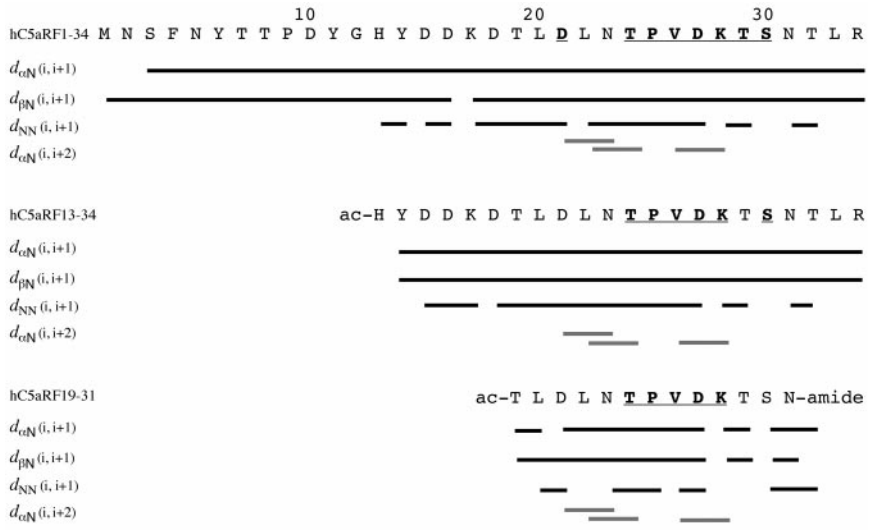

FIG. 4. **Summary of the NOE (or ROESY) connectivities observed for the three receptor peptides, hC5aRF-1–34, hC5aRF-13–34, and hC5aRF-19–31.** Most of the NOEs were those observed at a temperature of 15 °C with NOE mixing times of 350 ms for NOESY and 250 ms for ROESY (for peptide hC5aRF-19–31). Three $d_{\alpha N}(i,i+2)$ NOE contacts between Asp^{21} and Asn^{23}, between Leu^{22} and Thr^{24}, and between Val^{26} and Lys^{28} were found at 5 °C and are shown by *hatched lines*. The residues with resonance perturbations upon the binding of CGS-28805 (Fig. 2) are shown by the *underlined* and *bold letters*.

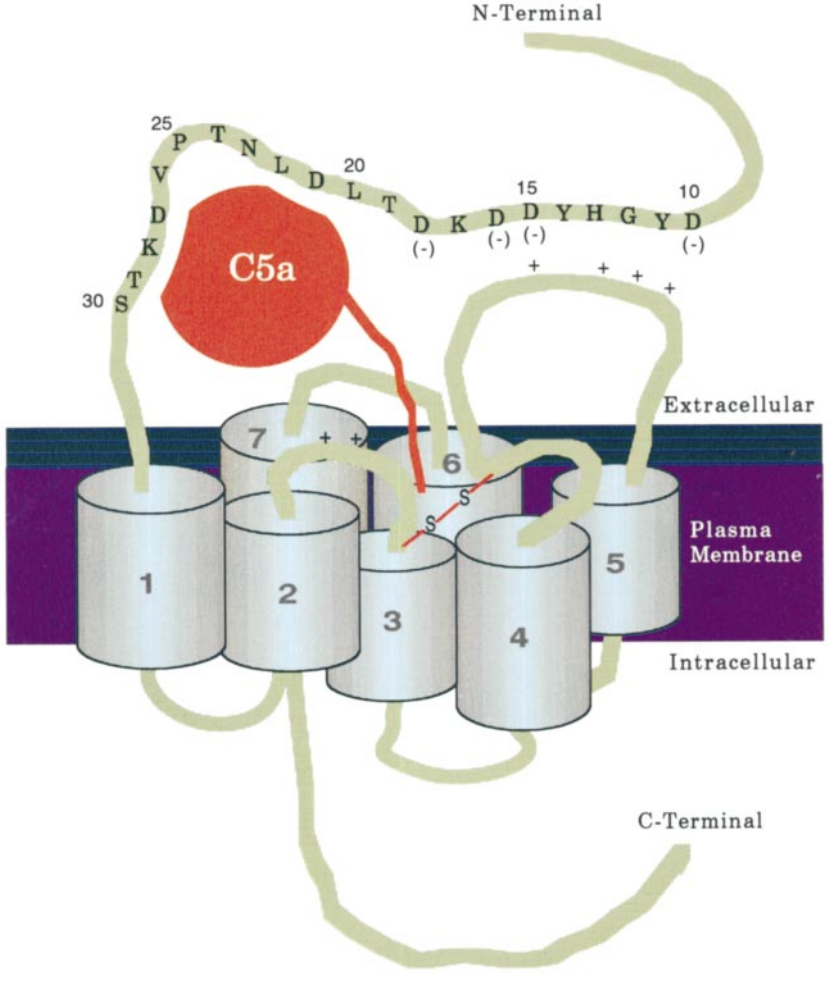

FIG. 5. **A model for the interaction of C5a with its receptor.** Seven transmembrane helices are shown by *cylinders*. Their arrangement is based on a model of the C5a receptor given by Grotzinger *et al.* (49). A disulfide bond between Cys^{109} in the first extracellular and Cys^{189} in the second extracellular loops is shown by a *red line*. The C5a protein core only contacts residues 21–30 of the receptor N terminus. Residues 1–18 (in particular residues 10–18) of the receptor N terminus may interact with the positively charged and hydrophobic stretch of residues within the extracellular loops (the second extracellular loop shown here) of the C5a receptor. The C5a protein molecule is held in a proper position by the N terminus of the receptor, allowing its C-terminal tail to bind to and activate the receptor.

Folded Conformation in Solution—Potential interactions within the receptor N terminus were examined through an analysis of nuclear Overhauser effects (NOEs) between all the protons of the receptor peptide hC5aRF-1–34. Two-dimensional NOE (NOESY) experiments were performed at temperatures of 5 and 15 °C. At 15 °C, many NOE connectivities were observed between residues next to each other in the peptide sequence (spectra not shown). In particular, sequential NOEs were observed between the amide protons of the stretch of residues from His^{13} to Thr^{32} but not for residues Met^{1} to Gly^{12}. In addition, existence of NOE contacts between the αCH proton of the preceding residue to the δCH protons of Pro^{9} and Pro^{25} indicates both proline residues assume the *trans* conformation in peptide hC5aRF-1–34. On the other hand, there were no medium and long range NOE contacts, indicating that the free hC5aRF-1–34 peptide is in a state of dynamic averaging of many conformations with no particularly well defined secondary structures. Upon a lowering of the temperature to 5 °C,

long range NOE contacts were still absent. Only three medium range $d_{\alpha N}(i,i + 2)$ NOEs between residues Asp^{21} and Asn^{23}, Leu^{22} and Thr^{24}, and between Val^{26} and Lys^{28} were observed in the NOESY spectra of hC5aRF-1–34 acquired at this temperature (Fig. 4). Therefore, there appear to be no contacts between the N-terminal residues 1–18 and residues 21–34 in the receptor peptide hC5aRF-1–34.

The existence of non-sequential NOEs, along with many sequential NH-NH NOE contacts, indicates that the free hC5aRF-1–34 peptide may have locally folded conformations within residues 20–30 or the C-terminal region of the peptide. The consecutive NH-NH NOE connectivities suggest the existence of some population of α or 3_{10} helices. However, the absence of characteristic $(i,i + 3)$ NOE contacts and presence of three medium range NOEs suggest that some turn-like local structure may exist in the free hC5aRF-1–34 peptide. One possible conformation within residues 20–30 of the hC5aRF-1–34 peptide could be a nascent helix involving residues from Leu^{20} to Thr^{24} followed by a β turn starting from Pro^{25} to Lys^{28}. It should be noted that this folded conformation is located within the same region where most residues have perturbed proton resonances upon the binding of C5a (Fig. 4).

Similar conformations in peptide hC5aRF-1–34 may also exist in the two shorter receptor peptides hC5aRF-13–34 and hC5aRF-19–31. In the case of hC5aRF-13–34, sequential NOE connectivities were observed throughout the peptide sequence, including many NOEs between the sequential amide protons (Fig. 4). The three medium range NOEs observed with peptide hC5aRF-1–34 were also present in this truncated peptide. Almost identical chemical shifts were found for the proton resonances of residues 14–34 with the exception of His^{13} which became the N terminus in the peptide (Table III). The further truncated peptide hC5aRF-19–31 exhibited very few NOEs in the NOESY spectra, apparently as a result of its small size. Rotating-frame NOE spectra (ROESY) were instead acquired which helped the identification of these missing NOEs. Several sequential NH-NH ROESY contacts were observed in addition to the sequential αCH-NH and βCH-NH ROESY connectivities. Most importantly, the three non-sequential $d_{\alpha N}(i,i + 2)$ NOE contacts present in two longer peptides were also observed in the shortest peptide (Fig. 4). In addition, the peptide hC5aRF-19–31 still has proton chemical shifts similar to those of the corresponding residues in hC5aRF-1–34 (Table IV). These results indicate that the folding of residues 21–30 in the isolated C5a receptor N terminus is independent of the presence of residues 1–18 in hC5aRF-1–34. The folded conformations in the receptor peptides may be important for the specific interaction with C5a since the same residues are strongly affected by C5a binding.

A Model for the C5a-Receptor Complex—Identification of residues 21–30 of the isolated receptor N terminus as a contact site for C5a provides an explanation for the apparent interspecies variations within the amino acid sequence of the receptor N-terminal domain (47). In fact, human, dog, and mouse C5a receptors share a stronger sequence similarity in the region of residues Thr^{19} to Ser^{30} of the human sequence, as noted previously (25). Human C5a binds to both dog and mouse C5a receptors (21, 47), presumably because a similar conformation of the C5a binding region, or residues 21–30 of the human receptor, is stabilized through interactions of the different receptor N terminus (residues 1–18) with the corresponding extracellular regions of the receptor variants. The N-terminal region for intramolecular interactions within the C5a receptor may be localized within residues $D_{10}YGHYD_{15}DKD$ of the human receptor, since a chimeric C5a receptor with the first 8 residues replaced with those from a formylpeptide receptor functioned normally, whereas replacement of the first 13 residues resulted in loss of binding affinity for C5a (26). The target binding site for residues Asp^{10} to Asp^{18} in the receptor N terminus may be the second and/or the third extracellular loops, since additional loop-swap experiments suggested that, besides the N terminus, both the second and the third extracellular loops of the C5a receptor are important for C5a binding (48).

The results discussed so far can be represented by a model describing potential interactions within the C5a-receptor complex (Fig. 5). The spatial arrangement of the seven transmembrane helices is based on a computer-generated model for the C5a receptor (49, 50). In this model, residues 10–18 of the receptor N terminus make intramolecular contacts with the extracellular loops (most likely the second extracellular loop) of the C5a receptor, thereby stabilizing a conformation of residues 21–30 of the receptor N terminus required for the "recognition site" binding to C5a. Upon binding, C5a is positioned by the N terminus of the receptor to permit a proper interaction of its agonistic C terminus with the cell-surface expressed receptor, leading to receptor activation. Further work is in progress to elucidate the detailed intermolecular interactions within the C5a-receptor peptide complex and the potential conformational changes in C5a upon binding of the N-terminal domain of the C5a receptor.

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