Identification of Receptor-Binding Sites of Monocyte Chemotactic S19 Ribosomal Protein Dimer

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The S19 ribosomal protein (RP S19) cross-linked homo-dimer attracts monocyte migration by binding to C5a receptor on monocytes (H Nishiura, Y Shibuya, T Yamamoto, Laboratory Investigation, 1998, 78:1615–1623). Using site-directed mutants of recombinant RP S19 and synthetic peptides mimicking RP S19 molecular regions, we currently identified the binding sites of the RP S19 dimer to the C5a receptor. The RP S19 dimer activated the receptor by a two-step binding mechanism as in the case of C5a. The first binding site was a basic cluster region containing a -Lys41-His42-Lys43- sequence. The second one was the -Leu131-Asp132-Arg133- moiety, localized 12 residues upstream from the COOH-terminal. The second binding triggered the chemotactic response. The first binding would have a role in achieving a high-binding affinity between the ligand and receptor. The first and second ligand-binding sites of C5a receptor seem to be shared by C5a and the RP S19 dimer, although overall homology between the amino acid sequences of these ligands is only 4%. (Am J Pathol 2001, 159:2293–2301)

We have reported that S19 ribosomal protein (RP S19), a component of the protein-producing machinery (ie, the ribosome), obtains monocyte chemotactic activity when cross-linked intermolecularly between Gln137 and Lys122 by a transglutaminase-catalyzed reaction.^{1–3} The RP S19 dimer with the monocyte chemotactic activity was initially isolated from the extracts of a rheumatoid arthritis synovial lesion¹ and was later revealed to be produced by apoptotic cells.^{3,4}

We also found that the RP S19 dimer exhibits the chemotactic function by means of binding to C5a receptor on monocytes; the apparent chemotactic capacity of the RP S19 dimer as well as of C5a (the complement C5-derived leukocyte chemotactic factor) was strikingly reduced when

the target monocytes were pretreated either with an anti-C5a receptor monoclonal antibody or with a synthetic C5a receptor antagonist, and the RP S19 dimer and C5a competed with each other displaying a similar affinity in binding to leukocytes.⁵ Namely, the RP S19 dimer and C5a share the chemotactic receptor known as C5a receptor, although a calculated homology in the amino acid sequence between RP S19 and C5a is only 4%.

C5a receptor is a member of the G-protein-coupled receptor family and has the structural motif of seven hydrophobic transmembrane α -helices linked by extra- and intracellular hydrophilic loops.^{6,7} Recent advance of the study on the interaction between C5a and C5a receptor has been provided by means of the nuclear magnetic resonance spectroscopic analysis of C5a,⁸ the site-directed mutagenic analyses of C5a and C5a receptor,^{9–13} and the peptide synthesis of C5a receptor agonists and antagonists.^{14–16} By joining the accumulated information, a two-step receptor ligand-binding model has been proposed for the interaction between C5a and C5a receptor.¹⁷ The first binding would occur between the NH₂-terminal acidic portion of the receptor (possibly the second extracellular loop is also involved) and a basic cluster at the core of C5a. The basic cluster is three dimensionally formed by His15, Arg46, and Lys49 residues.⁸ The high-affinity first binding does not activate the receptor, but effectively raises the local concentration of C5a and thereby promotes second binding. The second binding would occur between the COOH-terminal portion of C5a, -Leu72-Gly73-Arg74-COOH,⁹ and transmembranous interhelical regions of the receptor. The second binding triggers the G protein-coupled receptor signaling.

Based on the amino acid sequences of the receptor-binding sites of C5a, we predicted the receptor-binding sites of the RP S19 dimer (Figure 1). The first binding site should be one of the basic clusters on the RP S19 molecule. We have previously reported the presence of two basic clusters such as -Lys23-Lys24-Ser25-Gly26-Lys27-Leu28-Lys29- and -Lys38-Leu39-Ala40-Lys41-His42-Lys43- regions in terms of heparin-binding characteristics.² Using the alanine survey of the basic residues at these sites in the

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MPGVTVKDVN QQEFVRLAA

FLKKSSGKLKV PEWVDTVKLA

KKKELAPYDE NWFYTRAAST

ARHLYLRGGA GVGSMTKIYG

GRQRNGVMPS HFSRGSKSVA

RRVLQALEGL KMVEKDQDGG

RKLTPQGQRD LDRIAGQVAA

ANKKH

Figure 1. S19 ribosomal protein (RP S19) molecular regions as candidates for two C5a receptor-binding sites of the cross-linked RP S19 dimer. Two basic cluster regions as the candidates for the first binding site are indicated with the **white letters**. The candidate for the second binding site is indicated with the **bold letters**. The regions mimicked by the synthetic peptides are **underlined**.

site-directed mutagenesis, and using a competition analysis with synthetic peptides mimicking the basic cluster regions, we have currently determined the first binding site.

Considering the second binding site, the COOH-terminal sequence of RP S19 with -Lys143-Lys144-His145 is totally different from the -Leu72-Gly73-Arg74 of C5a. In our preliminary experiment, a peptide analogue composed of 12 amino acid residues at the COOH-terminal portion of RP S19 did not reproduce the monocyte chemoattraction of the RP S19 dimer at a wide concentration range (see below). Therefore, the COOH-terminal portion of RP S19 is out of the candidate for the second ligand.

In the sequence of second ligand moiety of C5a, the side chains of Leu72 and Arg74 are important. A Leu72Asp mutant of C5a reduced the neutrophil chemotactic capacity to <1% compared to that of the native C5a.⁹ An Arg74Ala mutant of C5a exhibited a 1000-fold less activity than the wild type in the intracellular calcium mobilization assay using neutrophils.¹⁰ The importance of the α -carboxyl group of Arg74 of C5a was also demonstrated. A C5a mutant bearing one Gly residue elongated at position 75 possessed only 12% of the neutrophil chemotactic potency of the wild form.⁹ According to the information, we found a sequence of -Leu131-Asp132-Arg133- in RP S19. In this sequence, the side chains of Leu and Arg are present with the same orientation as the COOH-terminal of C5a. If the β -carboxyl group of Asp132 of RP S19 functions equivalently to the α -carboxyl group of Arg74 of C5a, the -Leu131-Asp132-

Arg133- sequence of RP S19 would satisfy the conditions required for the second ligand for the induction of the receptor activation as the -Leu72-Gly73-Arg74 of C5a does. Using the site-directed mutagenesis of recombinant RP S19, and using the peptide synthesis, we currently examined our working hypothesis.

Materials and Methods

Reagents and Others

RPMI 1640 medium was purchased from Nissui Pharm. Co. (Tokyo, Japan). Fetal bovine serum was a product of Gibco BRC (Paisley, Scotland). Mono-Poly Resolving Medium was a product of Flow Laboratory (Herts, UK). Bovine serum albumin (BSA), formyl-Met-Leu-Phe (f-MLF), and zymosan A were purchased from Sigma Chemical (St. Louis, MO). A multiwell chamber for chemotaxis assay was obtained from Neuro Probe (Bethesda, MD). Nuclepore filters were purchased from Nuclepore (Pleasant, CA). Restriction endonucleases (*Nde*I, *Bam*HI), Plasmid Pure Prep, TaKaRa RNA PCR kit, DNA ligation kit, Microcon & Micropure, and *Escherichia coli* strain JM109-competent cells were purchased from TaKaRa Biomedicals (Otsu, Japan). Native Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA). Jetsorb gel extraction kit was purchased from Genomed GmbH (Bad Oeynhausen, Germany). SeaKem GTG agarose and NuSieve GTG agarose were purchased from FMC (Rockland, ME). *E. coli* strain BL21(DE3)-competent cells and plasmid pET11a were obtained from Novagen Inc. (Madison, WI). Type II transglutaminase purified from the guinea pig liver was purchased from Oriental Yeast Co. (Osaka, Japan). Factor XIIIa was supplied by the Institute of the Chemo Sero Therapy (Kumamoto, Japan). NMePhe-Lys-Pro-dCha-dCha-dArg, which had been synthesized as described previously,¹⁸ was a kind gift from Dr. M. Mizuno of the Third Department of Internal Medicine, Nagoya University, Nagoya, Japan. All other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) or from Wako Pure Chemicals (Osaka, Japan) unless otherwise specified. Zymosan-activated plasma was prepared according to the method of Fernandez and colleagues¹⁹ with a modification as described previously.²⁰

Oligonucleotides

Primer nucleotides were synthesized based on the cDNA sequence of RP S19 previously reported.²¹ A list of the synthesized primers named P1 to P12 is shown in Table 1. The primers with odd and even numbers are sense and antisense primers, respectively. P1 and P2 primers were used for the preparation of a wild-type rRP S19, and primers from P3 to P12 were used for the mutants of RP S19. P1 primer consisting of nucleotides from number 13 to number 45, in which 20A, 21C, and 22G were changed to C, A, and T, respectively, to construct the *Nde*I restriction site, and P2 primer consisting of nucleotides from number 472 to number 443, in which 466T, 464T, and 463G were changed to A, C, and C, respectively, to

Table 1. List of the Synthesized Primer Nucleotides

No.*	Nucleotide sequence
P1	5'AGGCCGCCATATGCCTGGAGTTACTGTAAAGA3'
P2	5'GCATGGATCCTTCTAATGCTTCTTGTGGC3'
P3	5'TCCTCGCAGCGTCCGGGGCGCTGGCAGTCCCCGAAT3'
P4	5'GGACTGCCAGCGCCCCGGACGCTGCGAGGAAGGCTGCC3'
P5	5'CCGTCGCGCTGGCCAGCGGCCGACAGCTTGCTCCCTA3'
P6	5'GCTCTGCGGCCGCGCCAGCGACGCGTATCCACCCATT3'
P7	5'GAGATGACGACAGAATCGCCGGAC3'
P8	5'CTGTCGTCTCTTTGTCCCTGA3'
P9	5'ATCTGGGCAGATCGCCGGACAGG3'
P10	5'ATTCTGCCAGATCTCTTTGTCC3'
P11	5'TGGACGCAATCGCCGGACAGGTGGCAGCTGC3'
P12	5'GCGATTGCGTCCAGATCTCTTTGTC3'

*The odd and even numbers are sense and antisense primers, respectively.

P1 and P2, wild-type RP S19; P3 and P4, Lys23Ala, Lys24Ala, Lys27Ala, and Lys29Ala RP S19; P5 and P6, Lys38Ala, Lys41Ala, His42Ala, and Lys43Ala RP S19; P7 and P8, Leu131Asp RP S19; P9 and P10, Asp132Gly RP S19; P11 and P12, Arg133Ala RP S19.

construct the *Bam*HI restriction site were synthesized by a DNA synthesizer model 381A (Perkin Elmer-Applied Biosystems, Foster City, CA). P3 to P12 primers that contain mutations were made to order by TaKaRa Biomedicals. Vector pET11a primers (T7 promoter primer and T7 terminator primer) were also obtained from TaKaRa Biomedicals.

Preparation of Wild-Type and Mutant Types of Recombinant RP S19 Molecules

Six types of recombinant RP S19, the wild type and five mutants (Lys23Ala, Lys24Ala, Lys27Ala, and Lys29Ala RP S19, Lys38Ala, Lys41Ala, His42Ala, and Lys43Ala RP S19, Leu131Asp RP S19, Asp132Gly RP S19, and Arg133Ala RP S19) were prepared. The cDNA for the wild-type RP S19 was prepared as described previously.⁵ The mutant RP S19 were prepared by the polymerase chain reaction-mediated site-directed mutagenesis according to the method of Brendan²² as described previously.² pET11a bearing an insert RP S19 cDNA (recombinant plasmid) was purified from a positive colony culture, and analyzed for the DNA sequence using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit for performing fluorescence-based dideoxysequencing reactions according to the method of Prober and colleagues²³ to confirm the site-directed mutagenesis. These constructs of the wild-type and mutant RP S19 recombinant plasmids were transformed to the expression host *E. coli* BL21(DE3) competent cells.

The wild-type and mutant RP S19 molecules extracted from the periplasmic fraction of the *E. coli* were purified by high-performance liquid chromatography (HPLC) using a SP-5PW column (Tosoh, Tokyo, Japan) and a HiTrap heparin column (Pharmacia), in this order, as described previously.⁴ The preparations of the wild-type and mutant recombinant RP S19 thus obtained demonstrated single bands with an apparent molecular size of 15.5 kd in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), respectively (data

not shown). Judging from the SDS-PAGE patterns, their purity was always >95%.

Preparation of Dimers of Recombinant RP S19

Factor XIIIa and type II transglutaminase catalyze the production of the chemotactically active RP S19 dimer, and the catalysis with the former enzyme requires heparin as a cofactor.^{2,3} Some types of the recombinant RP S19 were treated with factor XIIIa (final concentration, 1 U/ml) in the presence of heparin (1 U/ml), 1 mmol/L dithiothreitol, and 5 mmol/L CaCl₂ for 60 minutes at 37°C as described previously.² The remaining types of the recombinant RP S19 were treated with type II transglutaminase (final concentration, 0.5 U/ml) in the same conditions except for the absence of heparin as described previously.³ The cross-linked dimers of the recombinant proteins were then purified by immunoaffinity column chromatography with an anti-isopeptide bond monoclonal antibody column (Coval Ab, Lyon, France), and by HPLC with a Hitachi 5C4-300 column (Nacalai Tesque, Kyoto, Japan) as described previously.² Each cross-linked dimer enriched in a HPLC fraction was evaporated with a vacuum centrifuge concentrator (Savant). When the quality of the preparations was examined by SDS-PAGE, it was always >90% (the minor contaminant in each preparation was the monomer, data not shown).

The protein concentration of these dimers thus prepared was determined by the absorbance at 280 nm under the assumption that absorbance unit 1.0 was equivalent to 1 mg/ml. The lyophilized samples were dissolved in sterilized phosphate-buffered saline (PBS, pH7.4) containing 0.1 mg/ml BSA, and used in the chemotaxis assay.

Preparation of Analogue Peptides

The peptides were synthesized by the solid-phase fluorenylmethoxycarbonyl (Fmoc) method on alkoxybenzyl alcohol resin. The synthesized peptides were separated from the resin with reagent K (TFA/phenol/H₂O/thioanisole/ethanedithiol = 82.5/5/5/5/2.5), purified by HPLC with a YMC C18 preparation column (Yamamura, Kyoto, Japan) and freeze-dried. Their structures were confirmed by fast atom bombardment mass spectrometry. The lyophilized peptides were dissolved into PBS containing 0.1 to 2.0 mg/ml BSA and used in the chemotaxis assay.

Monocyte Chemotaxis Assay

Monocytes were isolated from heparinized human venous blood of healthy donors according to the method of Fernandez and colleagues¹⁹ as described previously.¹ The monocytes were suspended at a density of 1×10^6 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum for the multiwell chamber assay. In some experiments, the indicator monocytes were pretreated with analogue peptides for 10 minutes at 37°C. The multiwell chamber was handled according to the method of Falk and colleagues²⁴ using a nucleopore filter with a pore

size of 5 μm . When the synthetic peptides were used as the chemoattractant, we added BSA in the solvent at a concentration between 0.1 mg/ml and 2 mg/ml to prevent an apparent loss of the peptides. After incubation for 90 minutes, each membrane was separated, fixed with methanol, and stained with Giemsa solution. The total number of monocytes that had migrated beyond the lower surface of the membrane was counted in five high-power fields. The results were expressed by the number of migrated monocytes.

Results

Identification of First Binding Site of RP S19 Dimer to C5a Receptor

In terms of heparin-binding capacity, RP S19 has two apparent basic clusters, which are -Lys23-Lys24-Ser25-Gly26-Lys27-Leu28-Lys29- and -Lys38-Leu39-Ala40-Lys41-His42-Lys43- regions.² We hypothesized that one of these clusters would be the first binding site.

Monocyte Chemotactic Activity of Dimers of RP S19 Mutated at One of Basic Cluster Regions

We prepared two recombinant RP S19 mutants in which all of the basic amino acid residues in each basic cluster were substituted by Ala residues. The Lys23Ala, Lys24Ala, Lys27Ala, and Lys29Ala RP S19 and the Lys38Ala, Lys41Ala, His42Ala, and Lys43Ala RP S19 thus prepared, and the wild-type recombinant RP S19 were, respectively, treated with type II transglutaminase. The reason why type II transglutaminase was used instead of factor XIIIa (plasma transglutaminase) is that the former cross-links RP S19 independently to heparin³ different from the latter enzyme. The Lys23Ala, Lys24Ala, Lys27Ala, and Lys29Ala RP S19 is not effectively dimerized by factor XIIIa even in the presence of heparin, because of the loss of the heparin-binding capacity of the mutant RP S19.² Judging from the SDS-PAGE patterns, there was no significant difference among the dimerization ratios of these RP S19 molecules when catalyzed by type II transglutaminase (data not shown).

The monocyte chemotactic capacities of these mutant and wild-type dimers in the chemotaxis chamber assay are shown in Figure 2. As the positive control, the wild-type RP S19 dimerized with factor XIIIa in the presence of heparin² was used. Whereas, the dimers of the Lys23Ala, Lys24Ala, Lys27Ala, and Lys29Ala RP S19 and the wild-type RP S19 exhibited comparable chemotactic activities to the positive control, the dimer of the Lys38Ala, Lys41Ala, His42Ala, and Lys43Ala RP S19 demonstrated a significantly reduced activity.

These results strongly suggested that the first ligand site of the RP S19 dimer would be the basic cluster region from Lys38 to Lys43.

Figure 2. Monocyte chemotactic activity of dimers of recombinant RP S19 mutated at one of the basic cluster regions. The cross-linked dimers of wild-type or mutated forms of recombinant RP S19 were subjected to the monocyte chemotaxis assay at the final concentrations of 10^{-9} mol/L using the multiwell chamber and a Nuclepore filter with a pore size of 5 μm . After a 90-minute incubation, the membrane was stained with Giemsa solution and the total number of monocytes migrated beyond the membrane was counted in five high-power fields of light microscopy. Values are expressed as mean $\pm$ SD. Three experiments were performed for each examination. The **dotted column**, the **hatched column with thin slash**, and the **hatched column with thick slash** denote the monocyte chemotactic activity of the dimers of the wild-type RP S19 (Wild), of the Lys23Ala, Lys24Ala, Lys27Ala, and Lys29Ala RP S19 (M 13), and of the Lys38Ala, Lys41Ala, His42Ala, and Lys43Ala RP S19 (M 14), respectively. These dimers were prepared with type II transglutaminase (type II TG). The positive controls (**open columns**) are 10^{-9} mol/L, 10^{-10} mol/L, and 10^{-11} mol/L of the RP S19 dimerized with factor XIIIa (plasma transglutaminase) in the presence of heparin, and the negative control (**open column**) is PBS containing 0.1 mg/ml BSA.

Inhibition of Chemotactic Activity of RP S19 Dimer by Basic Cluster Analogue Peptide

Basic cluster analogue peptides with 14 and 15 amino acids corresponding to Leu22 to Asp35 and to Val37 to Asn51 were, respectively, examined for the inhibitory capacities against the monocyte chemotaxis attracted by the RP S19 dimer. In the chamber assay, the indicator monocytes were pretreated with one of the peptides at various concentrations for 10 minutes at 37°C and used. At concentrations up to 10^{-3} mol/L, these peptides did not influence the viability of the monocytes. A representative experiment using these peptides at a concentration of 10^{-4} mol/L is shown in Figure 3. The pretreatment with the peptide (Ac-VKLAKHKELAPYDE) mimicking the sequence from Val37 to Asn51 prevented the chemotactic response to the RP S19 dimer. In contrast to this, the peptide (Ac-LKKSGKLKVPWVD), mimicking the sequence from Leu22 to Asp35, did not prevent the response. Similar results were obtained at the concentration of 10^{-3} mol/L. The prevention by Ac-VKLAKHKELAPYDE was not observed when the monocytes were attracted with the bacterial chemotactic peptide, f-MLF, instead of the RP S19 dimer. This indicated the specific interaction of Ac-VKLAKHKELAPYDE to the C5a receptor on the monocytes, because monocytes recognize f-MLF with the formyl-peptide receptors but not with the C5a receptor.

To further restrict the essential site as the first ligand in the Val37 to Asn57 region, we synthesized the former

Figure 3. Inhibition of chemotactic activity of RP S19 dimer by basic cluster analogue peptide, Ac-VKLAKHKELAPYDE. The indicator monocytes in the chemotaxis assay were pretreated with either of the basic cluster analogue peptides or a derivative at a concentration of 10^{-4} mol/L, or with the vehicle buffer (the medium for the cell suspension, **open columns**) for 10 minutes at 37°C , and used for the chemotaxis assay attracted by the RP S19 dimer (10^{-9} mol/L), formyl-Met-Leu-Phe (f-MLF, 10^{-9} mol/L) or PBS containing 0.1 mg/ml of BSA (PBS). No. 1 peptide (**hatched columns with thin slash**) denotes Ac-LKKSGKLKVPWEVD mimicking the sequence from Leu22 to Asp35 of RP S19, no. 2 peptide (**hatched columns with thick slash**) denotes Ac-VKLAKHKELAPYDE mimicking the sequence from Val37 to Asn51, and no. 3 peptide (**dotted columns**) denotes Ac-VKLAAAELAPYDE, a derivative of no. 2 peptide substituted the three basic residues in tandem (Lys-His-Lys) by Ala residues. Values are mean $\pm$ SD. Three experiments were performed for each examination. The numbers with parentheses on the vertical axis are the values for the f-MLF-induced chemotaxis.

peptide but substituted the tandem basic residues of the Lys-His-Lys moiety by Ala-Ala-Ala sequence, such as Ac-VKLAAAELAPYDE. This peptide did not reproduce the inhibition of the RP S19 dimer-induced chemotaxis when pre-exposed to the indicator monocytes at the concentration of 10^{-4} mol/L (Figure 3). Although the experiment at the concentration of 10^{-3} mol/L could not be done because of a low solubility of this peptide in PBS containing BSA, the experimental results at concentrations up to 10^{-4} mol/L indicate that the Lys41-His42-Lys43 moiety is essential for the first ligand function.

Inhibition of Chemotactic Activity of C5a by First Ligand Analogue Peptide of RP S19 Dimer

The first ligand portion of C5a is also a basic cluster, which is three dimensionally formed by His15, Arg46, and Lys49 residues.⁸ The next question was whether the tandem basic sequence of the RP S19 dimer, the Lys41-His42-Lys43 moiety, was equivalent to the basic cluster of C5a in the C5a receptor binding. To answer this question, we examined the inhibitory capacity of the first ligand analogue peptide of the RP S19 dimer to the monocyte chemotaxis induced by C5a in the experimental system described above. In this experiment, ZAP was used as the source of C5a.

As shown in Figure 4, the first ligand analogue peptide of the RP S19 dimer (Ac-VKLAKHKELAPYDE), but not the other basic cluster analogue peptide (Ac-LKKSGKLKVPWEVD), inhibited the C5a-induced monocyte chemotaxis in a dose-dependent manner. Furthermore,

Figure 4. Inhibition of chemotactic activity of C5a by first ligand analogue peptide of RP S19 dimer, Ac-VKLAKHKELAPYDE. The indicator monocytes in the chemotaxis assay were pretreated with one of the three peptides described in Figure 3, or with the vehicle buffer (**open columns**) for 10 minutes at 37°C , and used for the chemotaxis assay attracted by zymosan-activated plasma (ZAP, 10%) containing C5a or by PBS containing 0.1 mg/ml of BSA (PBS). The **hatched columns with thin slash** denote Ac-LKKSGKLKVPWEVD mimicking the sequence from Leu22 to Asp35 of RP S19 (no. 1 peptide), the **hatched columns with thick slash** denote Ac-VKLAKHKELAPYDE mimicking the sequence from Val37 to Asn51 (no. 2 peptide), and the **dotted columns** denote Ac-VKLAAAELAPYDE, a derivative of no. 2 peptide substituted the three basic residues in tandem (Lys-His-Lys) by Ala residues (no. 3 peptide). Values are mean $\pm$ SD. Three experiments were performed for each examination.

the first ligand analogue peptide where the tandem basic residues were substituted by Ala residues (Ac-VKLAAAELAPYDE) did not possess the inhibitory capacity even at a concentration of 10^{-4} mol/L.

Lack of Chemoattracting Capacity of Basic Cluster Analogue Peptides

C5a receptor activation capacities of these peptides were also examined using monocytes without the pretreatment. None of these peptides themselves attracted the monocytes at a wide range concentration from 10^{-7} mol/L to 10^{-3} mol/L. A representative experiment at a concentration of 10^{-4} mol/L is shown in Figure 5.

These results indicate that the basic cluster at the region from Val37 to Asn51 is the first ligand site of the RP S19 dimer to C5a receptor. The first binding itself does not trigger the intracellular signaling of C5a receptor as in the case of C5a.

Identification of Second Binding Site of RP S19 Dimer to C5a Receptor

Our working hypothesis for the second binding site with the activating capacity to C5a receptor is the moiety with -Leu131-Asp132-Arg133- of the RP S19 dimer. This hypothesis bases on an assumption that the β -carboxyl group of Asp132 of RP S19 is equivalent to the α -carboxyl group of Arg74 of C5a as schematically shown in Figure 6.

Figure 5. Lack of chemoattracting capacity of first ligand analogue peptide. The basic cluster analogue peptides were subjected to the monocyte chemotaxis assay at the final concentrations of 10^{-4} mol/L. No. 1 peptide (hatched columns with thin slash) denotes Ac-LKKSGKLVPEWVD mimicking the sequence from Leu22 to Asp35 of RP S19, and no. 2 peptide (hatched columns with thick slash) denotes Ac-VKLAKHKELAPYDE mimicking the sequence from Val37 to Asn51. The positive control is the RP S19 dimer (10^{-9} mol/L), and the negative control is PBS containing 0.1 mg/ml of BSA. Values are mean $\pm$ SD. Three experiments were performed for each examination.

Monocyte Chemotactic Activity of Dimers of RP S19 Mutants

To examine the working hypothesis three mutants with either Leu131Asp, or Asp132Gly, or Arg133Ala of recombinant RP S19 were synthesized, and their cross-linked homodimers were prepared. In terms of the efficacy of the factor XIIIa-catalyzed dimer formation, no significant

Figure 6. Schematic diagrams of second receptor-binding site of C5a and of candidate for it of RP S19 dimer. In the second binding with the C5a receptor activation, the side chains of Leu72 and Arg74, and the COOH-terminal α -carboxyl group of Arg74 of C5a are essential. The moiety with Leu131-Asp132-Arg133 of RP S19 possesses the same two side chains and β -carboxyl group of Asp132. It is assumed that the β -carboxyl group of Asp132 functions equivalently as the α -carboxyl group of Arg74.

Figure 7. Monocyte chemotactic activity of dimers of recombinant RP S19 mutated at Leu131-Asp132-Arg133 moiety. The dimers of the wild-type RP S19 (Wild), of the Leu131Asp RP S19 (L131D), of the Asp132Gly RP S19 (D132G), and of Arg133Ala RP S19 (R133A) were subjected to the monocyte chemotaxis assay at the final concentrations of 10^{-9} mol/L. The negative control is PBS containing 0.1 mg/ml BSA. Values are mean $\pm$ SD. Three experiments were performed for each examination.

difference was present among the mutants and the wild-type (data not shown).

The monocyte chemotactic capacities of the dimers of the wild-type and the mutants at the final concentration of 10^{-9} mol/L are comparatively shown in Figure 7. All of the mutant protein dimers demonstrated significantly lower chemotactic activities when compared to that of the wild-type RP S19 dimer. These reduced activities of the dimerized mutants were observed in a wide concentration range from 10^{-13} mol/L to 10^{-7} mol/L (data not shown). Therefore, the side chains of Leu131, Asp132, and Arg133 are all equally important for the chemoattractant capacity of the RP S19 dimer.

These results strongly support our working hypothesis that the moiety with -Leu131-Asp132-Arg133- of the RP S19 dimer would function as the moiety with -Leu72-Gly73-Arg74-COOH of C5a in the second binding with the receptor activation, in which the β -carboxyl group of Asp132 of RP S19 plays the same role as the α -carboxyl group of Arg74 of C5a.

Monocyte Chemotactic Activity of Analogue Peptides

To confirm the above data, we prepared an analogue peptide of RP S19 that possessed 18 amino acid residues from Gly127 to Lys144 (Ac-GQRDLDRAGQVAAANKK). The COOH-terminal His145 was omitted in this peptide to clear-cut that the COOH-terminal did not participate in the monocyte chemoattraction. This peptide possessed the monocyte chemoattractant activity. The dose-activity relationship of this peptide is shown in Figure 8. The pattern is bell-shaped, which is typical for the leukocyte chemotactic factors. The apparent peak concentration of this peptide is 10^{-7} mol/L, that is, 100-fold higher than that of the RP S19 dimer.

To further confirm the evidence, we prepared two additional analogue peptides of RP S19. One had the same sequence as the peptide mentioned above, except for

Figure 8. Monocyte chemotactic activity of peptide analogue of possible second ligand site of RP S19 dimer. An analogue peptide of RP S19 composed of 18 amino acid residues from Gly127 to Lys144 including the Leu-Asp-Arg sequence such as Ac-QQRDLRIAGQVAAANKK (no. 4 peptide, **open circles**), and its derivative in which an Asp residue corresponding to Asp132 of RP S19 had been substituted by Gly residue (Ac-QQRDLGRIAGQVAAANKK, no. 5 peptide, **filled circles**) were subjected for the monocyte chemotaxis assay at various concentrations from 10^{-10} mol/L to 10^{-3} mol/L in PBS containing 2 mg/ml BSA. A peptide mimicking the COOH-terminal portion of RP S19 (IAGQVAAANKKH, no. 6 peptide, **hatched circles**) was also subjected to the chemotaxis assay. The negative control was PBS containing 2 mg/ml BSA. Values are mean $\pm$ SD. Three experiments were performed for each examination.

the substitution of the Asp residue that corresponded to Asp132 of RP S19 by Gly residue such as Ac-QQRDLGRIAGQVAAANKK. The other possessed only the COOH-terminal 12 amino acids residues behind the Leu131-Asp132-Arg133 portion of RP S19 such as IAGQVAAANKKH. The 18 amino-acid peptide with the Asp to Gly substitution exhibited a greatly reduced monocyte chemotactic activity, and the peptide-mimicking COOH-terminal 12 amino-acid residues did not exhibit the activity at all (Figure 8). These peptides, as well as the chemotactic peptide, did not exhibit any apparent toxic effect to monocytes at concentrations of up to 10^{-3} mol/L.

Competition between Chemotactic Analogue Peptide and RP S19 Dimer

The monocytes were pretreated with either the functional analogue peptide (10^{-4} mol/L) or the Asp-Gly-substituted peptide (10^{-4} mol/L) for 10 minutes at 37°C , and used for the chemotaxis assay attracted by the RP S19 dimer (10^{-9} mol/L). As shown in Figure 9, the pretreatment with the functional peptide, but not with the Asp-Gly-substituted peptide, prevented the chemotactic response to the RP S19 dimer. The specificity of this competition was further confirmed with f-MLF as the che-

Figure 9. Competition between RP S19 dimer and analogue peptides of its second ligand site in monocyte chemotaxis. The indicator monocytes in the chemotaxis assay were pretreated with either the chemotactic analogue peptide (Ac-QQRDLRIAGQVAAANKK, 10^{-4} mol/L, **hatched columns with thick slash**), or the modified one with the Asp-Gly substitution (Ac-QQRDLGRIAGQVAAANKK, 10^{-4} mol/L, **hatched columns with thin slash**), or the vehicle buffer (**open columns**) for 10 minutes at 37°C , and used for the chemotaxis assay attracted by either the RP S19 dimer (10^{-9} mol/L), f-MLF (10^{-9} mol/L), or PBS containing 0.1 mg/ml of BSA (PBS). Values are mean $\pm$ SD. Three experiments were performed for each examination.

moattractant. The pretreatment with the functional analogue peptide (Ac-QQRDLRIAGQVAAANKK) did not affect the f-MLF-induced monocyte chemotaxis.

These results indicate again that the second ligand site of the RP S19 dimer, which induces C5a receptor activation, is the -Leu131-Asp132-Arg133- moiety, but not the COOH-terminal portion of RP S19.

Inhibition with Authentic C5a Receptor Antagonist of Receptor Activation by Second Ligand Analogue Peptide of RP S19 Dimer

The antagonist effect of NMePhe-Lys-Pro-dCha-dCha-dArg peptide to C5a receptor has been established demonstrating the inhibition of C5a¹⁸ and of the RP S19 dimer.⁵ The effect of the C5a receptor antagonist on the monocyte chemoattraction induced by the second ligand analogue peptide of the RP S19 dimer was examined. As shown in Figure 10, the C5a receptor antagonist inhibited the monocyte chemotactic response to the RP S19 analogue peptide in a dose-dependent manner. By this result, it was confirmed that the RP S19 analogue peptide attracts monocytes via the C5a receptor. This result also indicates that the second ligand interaction site of the C5a receptor is common in the C5a and the RP S19 dimer bindings.

Discussion

The present study revealed that the RP S19 dimer interacts with the C5a receptor by the two-step binding mechanism as in the case of C5a. All of the experimental results using the mutant RP S19 dimers and the

Figure 10. Inhibition with synthetic C5a receptor antagonist of monocyte chemotaxis induced by second ligand analogue peptide of RP S19 dimer. Monocytes were pretreated with various concentrations of a synthetic receptor antagonist (NMePhe-Lys-Pro-dCha-dCha-dArg) for 10 minutes at 37°C, then C5a (10^{-9} mol/L, **open circles**) or the second ligand analogue peptide of the RP S19 dimer (Ac-GQRDLRIAGQVAAANKK, 10^{-7} mol/L, **closed circles**) was given to induce the chemotaxis. Values are mean $\pm$ SD. Three experiments were performed for each examination.

synthetic analogue peptides indicate that the first binding site of RP S19 dimer is the basic cluster from Lys38 to Lys43 containing Lys41-His42-Lys43 sequence, and the second one is the moiety with Leu131-Asp132-Arg133. Although the first binding does not trigger the G-protein-coupled signaling, it would play a role in the high-affinity interaction between the RP S19 dimer and C5a receptor. This is indicated by a concentration difference between the RP S19 dimer (10^{-9} mol/L)^{3,5} and the second ligand analogue peptide (10^{-7} mol/L) (Figure 8) in inducing the maximum chemotactic response of monocytes.

The pretreatment of monocytes with the first ligand analogue peptide of the RP S19 dimer also prevented the C5a-induced monocyte chemotaxis (Figure 4). As the first ligand function, the Lys41-His42-Lys43 sequence of the RP S19 dimer seems equivalent to the basic cluster of C5a formed by His15, Arg46, and Lys48 residues. This would indicate that C5a and the RP S19 dimer share the first ligand acceptor site of C5a receptor molecule. The first ligand acceptor site of the receptor to C5a was indicated to be the NH₂-terminal acidic portion of the receptor.²⁵ However, as for the role of Asp residues forming the acidic portion, it has not yet been determined whether they directly interact with the basic residues of the first ligand of C5a via a salt bridge or they involve in folding a high-affinity-binding pocket to the first ligand.²⁵ The present results, showing that the first ligand moiety of the RP S19 dimer is also composed of the basic residues, seem to support the former possibility.

The present study also indicated that the moiety with -Leu131-Asp132-Arg133- of the RP S19 dimer functions in the same way as the moiety with -Leu72-Gly73-Arg74-COOH of C5a in the C5a receptor activation. A good way to confirm this from the opposite side might be to perform an experiment using a double mutant of RP S19 in which Asp132 is changed to Gly and the COOH-terminal portion after Arg133 is truncated. However, this experiment cannot be done theoretically, because the RP S19 mutant having the truncated COOH-terminal sequence of -Leu131-Gly132-Arg133-COOH lacks the Gln137 residue that is essential to form the cross-linked dimer. Even in the absence of this experiment, the present data strongly suggest that an identical region of C5a receptor interacts with these moieties of the RP S19 dimer and C5a, respectively, and that the receptor activation mechanism is, therefore, the same between the RP S19 dimer and C5a. A mechanism model in the C5a receptor activation by C5a was proposed.¹² In this model, the side chain guanidino group of Arg206 of C5a receptor is initially recognized by the α -carboxyl group of C5a by a positive electrostatic interaction, but subsequently, the side chain of Arg206 relocates by a negative electrostatic interaction with the guanidino group side chain of Arg74 of C5a. The conformational change with the relocation of Arg206 of the C5a receptor is a positive signal for triggering the G-protein-coupled transduction mechanism.¹² In this molecular mechanism, the requirement of the orientation between the guanidino group and carboxyl group of Arg74 of C5a does not seem very restricted, because the Arg74 of C5a could be substituted by D-Arg, preserving the agonistic capacity, at least when C5a mimicking peptides were used.¹² In the case of the RP S19 dimer, the β -carboxyl group of Asp132 and the guanidino group of Arg133 would drive the same molecular machinery. The molecular mechanism without the requirement of a restricted orientation between the guanidino and carboxyl groups would provide the same functional capacity between the RP S19 dimer with the -Leu131-Asp132-Arg133- moiety and C5a with the -Leu72-Gly73-Arg74-COOH moiety.

The RP S19 analogue peptides mimicking the first binding site and the second binding site, respectively, function in their monomer forms. In contrast to this, the cross-linked dimerization at Gln137 is essential for RP S19 to gain the chemotactic function in either case, catalyzed by factor XIIIa²¹ or by type II transglutaminase.³ The monomer form of RP S19 did not compete with the radiolabeled C5a in the C5a receptor binding.⁵ It seems reasonable to speculate that both of the moieties with the functions in the first binding and the second binding are hidden in the conformation of the monomer of RP S19.

As described previously, the RP S19 dimer functions as a receptor antagonist to the C5a receptor of polymorphonuclear leukocytes.⁵ How the RP S19 dimer behaves oppositely between monocytes and polymorphonuclear leukocytes in terms of the C5a receptor interaction is a very interesting issue to be solved in the near future.

References

1. Nishiura H, Shibuya Y, Matsubara S, Tanase S, Kambara T, Yamamoto T: Monocyte chemotactic factor in rheumatoid arthritis synovial tissue: probably a cross-linked derivative of S19 ribosomal protein. *J Biol Chem* 1996, 271:878–882
2. Nishiura H, Tanase S, Shibuya Y, Nishimura T, Yamamoto T: Determination of the cross-linked residues in homo-dimerization of S19 ribosomal protein concomitant with exhibition of monocyte chemotactic activity. *Lab Invest* 1999, 79:915–923
3. Nishimura T, Horino K, Nishiura H, Shibuya Y, Hiraoka T, Tanase S, Yamamoto T: Apoptotic cells of an epithelial cell line, AsPC-1, release monocyte chemotactic S19 ribosomal protein dimer. *J Biochem* 2001, 129:445–454
4. Horino K, Nishiura H, Ohsako T, Shibuya Y, Hiraoka T, Kitamura N, Yamamoto T: A monocyte chemotactic factor, S19 ribosomal protein dimer, in phagocytic clearance of apoptotic cells. *Lab Invest* 1998, 78:603–617
5. Nishiura H, Shibuya Y, Yamamoto T: S19 ribosomal protein cross-linked dimer causes monocyte-predominant infiltration by means of molecular mimicry to complement C5a. *Lab Invest* 1998, 78:1615–1623
6. Gerard NP, Gerard C: The chemotactic receptor for human C5a anaphylatoxin. *Nature* 1991, 349:614–617
7. Boulay F, Mery L, Tardif M, Bouchon L, Vignais P: Expression cloning of a receptor for C5a anaphylatoxin on differentiated HL-60 cells. *Biochemistry* 1991, 30:2993–2999
8. Zhang X, Boyer W, Toth MJ, Wennogle L, Gonnella NC: Structural definition of the C5a C terminus by two-dimensional nuclear magnetic resonance spectroscopy. *Proteins* 1997, 28:261–267
9. Mollison KW, Mandecki W, Zuiderweg ERP, Fayer L, Fey TA, Krause RA, Conway RG, Miller L, Edalji RP, Shallcross MA, Lane B, Fox JL, Greer J, Carter GW: Identification of receptor-binding residues in the inflammatory complement protein C5a by site-directed mutagenesis. *Proc Natl Acad Sci USA* 1989, 86:292–296
10. Toth MJ, Huwyer L, Boyer WC, Braunwalder AF, Yarwood D, Hadala J, Haston WO, Sills MA, Seligmann B, Galakatos N: The pharmacophore of the human C5a anaphylatoxin. *Protein Sci* 1994, 3:1159–1168
11. Kolakowski LF, Lu B, Gerard C, Gerard NP: Probing the "Message: address" sites for chemoattractant binding to the C5a receptor. Mutagenesis of hydrophilic and proline residues within the transmembrane segments. *J Biol Chem* 1995, 270:18077–18082
12. DeMartino JA, Konteatis ZD, Siciliano SJ, Van Riper G, Underwood DJ, Fisher PA, Springer MS: Arginine 206 of the C5a receptor is critical for ligand recognition and receptor activation by C-terminal hexapeptide analogs. *J Biol Chem* 1995, 270:15966–15969
14. Ember JA, Sanderson SD, Taylor SM, Kawahara M, Hugli TE: Biological activity of synthetic analogues of C5a anaphylatoxin. *J Immunol* 1992, 148:3165–3173
15. Finchi AM, Vogan SM, Sherman SA, Kirnarsky L, Taylor SM, Sanderson SD: Biologically active conformer of the effector region of human C5a and modulator effects of N-terminal receptor binding determinants on activity. *J Med Chem* 1997, 40:877–884
16. Zhang X, Boyer W, Galakatos N, Gonnella NC: Solution structure of a unique C5a semi-synthetic antagonist: implications in receptor binding. *Protein Sci* 1997, 6:65–72
17. Siciliano SJ, Rollins TE, DeMartino J, Konteatis Z, Malkowitz L, Van Riper G, Bondy S, Rosen H, Springer MS: Two-site binding of C5a by its receptor: an alternative binding paradigm for G protein-coupled receptors. *Proc Natl Acad Sci USA* 1994, 91:1214–1218
18. Konteatis ZD, Siciliano SJ, Van Riper G, Molineaux CJ, Pandya S, Fischer P, Rosen H, Mumford RA, Springer MS: Development of C5a receptor antagonists: differential loss of functional responses. *J Immunol* 1994, 153:4200–4205
19. Fernandez HN, Henson PM, Otani A, Hugli TE: Primary structural analysis of the polypeptide portion of human C5a anaphylatoxin: polypeptide sequence determination and assignment of the oligosaccharide attachment site in C5a. *J Immunol* 1978, 120:109–117
20. Matsubara S, Yamamoto T, Tsuruta T, Takagi K, Kambara T: Complement C4 derived monocyte-directed chemotaxis-inhibitory factor. *Am J Pathol* 1991, 138:1279–1291
21. Kondoh N, Schweinfest CW, Henderson KW, Papas TS: Differential expression of S19 ribosomal protein, laminin-binding protein, and human lymphocyte antigen class 1 messenger RNAs associated with colon carcinoma progression and differentiation. *Cancer Res* 1992, 52:791–796
22. Brendan C: Mutagenesis by the polymerase chain reaction. *Current Protocols in Molecular Biology*. Edited by MA Frederick, B Roger, EK Robert, DM David, JG Seidman, AS John, S Kevin. New York, John Wiley & Sons, Inc, 1997, pp 8.5.1–8.5.10
23. Prober JM, Trainor GL, Dam RJ, Hobbs FW, Robertson CW, Zangursky RJ, Cocuzza AJ, Jensen MA, Baumeister K: A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* 1987, 238:336–341
24. Falk W, Goldwin RH, Leonard EJ: A 48 well micro-chemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J Immunol Methods* 1980, 33:239–247
25. Mery L, Bouly F: The NH₂-terminal region of C5aR but not that of FPR is critical for both protein transport and ligand binding. *J Biol Chem* 1994, 269:3457–3463