Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1214–1218, February 1994
Biochemistry

Two-site binding of C5a by its receptor: An alternative binding paradigm for G protein-coupled receptors

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Communicated by Edward M. Scolnick, October 20, 1993

ABSTRACT **The guanine nucleotide-binding protein-coupled receptor superfamily binds a vast array of biological messengers including lipids, odorants, catecholamines, peptides, and proteins. While some small molecules bind to these receptors at a single interhelical site, we find that the binding domain on the receptor for the inflammatory protein C5a is more complex and consists of two distinct subsites. This more elaborate motif appears to be an evolutionary adaptation of the simpler paradigm to which a second interaction site has been added in the receptor N terminus. Surprisingly, occupation of only one of the subsites is required for receptor activation. The two-site motif is not unique to the C5a receptor but appears to be widely used by the superfamily to accommodate macromolecular ligands.**

The 74-aa glycoprotein C5a evokes a variety of responses *in vivo* and *in vitro*, implying that it is a principal mediator of inflammatory responses (1, 2). C5a is a potent chemotaxin and secretagogue for granulocytes and macrophages; it activates the respiratory burst in these cells and modulates their adhesive properties. The effects of C5a are amplified by its ability to stimulate the release of other mediators including histamine, prostaglandins, leukotrienes, interleukin (IL) 1, and IL-6 (1–3).

All of the effects of C5a are initiated when it binds to its cell surface receptor, a member of the guanine nucleotide-binding protein (G protein)-coupled receptor superfamily (4, 5). The superfamily consists of over 100 members and binds a variety of ligands ranging in complexity from small molecules to moderately sized proteins. Despite this biologic diversity, a general model for the structure of these receptors has emerged: an extracellular N terminus, seven membrane-spanning helices connected by alternating intracellular and extracellular loops, and an intracellular C terminus (6, 7). The amino acid sequence of the C5a receptor is consistent with this model and like most members of the family has a short N terminus of about 30 residues in length (4, 5).

Family members such as rhodopsin and the β-adrenergic receptor bind their ligands at a single domain, which lies in the receptor's hydrophobic core, between the helices and below the upper plane of the cellular membrane (6, 8). However, it is unclear whether this binding motif is also used by other members of the superfamily, especially those that interact with more complex ligands like C5a, or whether the motif is altered to accommodate the larger agonists. The little information that exists comes largely from studies with the glycopeptide hormone receptors, a branch of the superfamily characterized by a greatly extended extracellular N terminus (9, 10). These receptors, in contrast to rhodopsin and the β-adrenergic receptor, appear to bind ligands by means of this enlarged N terminus (11, 12). We now report that the binding site of the C5a receptor is more complex and consists of two physically separable domains. The first domain is composed of the N terminus and possibly the external loop between helices 2 and 3. The second domain interacts with the C terminus of C5a and probably, as in the other superfamily members, lies between the transmembrane helices.

EXPERIMENTAL PROCEDURES

Materials. Human C5a was purified from activated plasma as described (13). Human recombinant monocyte IL-8 was obtained from Peprotech, ^{3}H-labeled fMet-Leu-Phe (fMLP) was from New England Nuclear, and the *Plectruerys tristes* venom was purchased from The Spider Farm (Black Canyon, AZ). All peptides were synthesized on a MilliGen/Biosearch 9500 and analyzed by mass spectrometry. Membranes from human neutrophils and transfected 293 cells were prepared as described (13).

Iodinations. ^{125}I-labeled C5a (^{125}I-C5a) was prepared as described (13), and the ^{125}I-labeled Tyr-Phe-Lys-Ala-Cha-Cha-Leu-DPhe-Arg (C-009; Cha = cyclohexylalanine) was made using the same procedures. The monoiodo derivative of ^{125}I-labeled C-009 (^{125}I-C-009) was isolated by reverse-phase HPLC.

Binding and Degranulation Assays. Assays with ^{125}I-C5a or ^{3}H-labeled fMLP (^{3}H-fMLP) and human neutrophil membranes were carried out as described for C5a (13). Assays using ^{125}I-C-009 were performed with similar procedures except that the second wash buffer was 50 mM ([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)-1-propanesulfonic acid (Taps) at pH 8.5 containing 0.5 M NaCl. Binding assays with membranes prepared from transfected 293 cells used the same procedures except that 50 μM *o*-phenanthroline was added to the assay buffers. Degranulation was assessed by monitoring the release of myeloperoxidase as described (14).

***P. tristes*.** Venom and Protease Treatments. To test effects on binding and degranulation, neutrophil membranes or intact neutrophils were preincubated with crude venom or purified protease for 20 min at 22°C, the binding was initiated by the addition of labeled ligand, and then the assays were carried out as described above. The ability to cleave the purified C5a receptor (15) was examined by incubating 50 ng of pure protease or buffer with receptor from 1.5×10^9 human neutrophils for 90 min at 4°C in a total volume of 440 μl. The reactions were stopped by adding 5× Laemmli sample buffer; the mixtures were boiled, concentrated, and subjected to SDS/PAGE. To examine the ability of the enzyme to cleave the C5a receptor in intact human neutrophils, 1×10^9 cells in 3 ml of Hanks' balanced salts buffered to pH 7.2 with 25 mM Hepes were incubated with or without 20 ng of protease for 2

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Abbreviations: IL, interleukin; G protein, guanine nucleotide-binding protein; fMLP, fMet-Leu-Phe; C-009, Tyr-Phe-Lys-Ala-Cha-Cha-Leu-DPhe-Arg (Cha = cyclohexylalanine).

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hr at 22°C. The cells were washed twice by centrifugation to remove the protease, and membranes were prepared as described (13). One-sixth of each sample was subjected to SDS/PAGE on a 14% gel, and Western blotting was performed. The blots were probed with a rabbit polyclonal antibody raised to the receptor C-terminal peptide RESKSFTRSTVDT and then developed with the Amersham ECL kit.

Receptor Cloning and Mutagenesis. The human C5a receptor was cloned from human bone marrow RNA by reverse transcriptase–PCR using primers designed according to the published sequence for the receptor (4, 5). The expression cassette containing the C5a receptor was constructed by generating two individual PCR products, the sequences of which overlapped at a unique *Sca* I restriction site within the receptor coding sequence. These products were simultaneously ligated into the mammalian expression vector pHIV-LTRHygl. A cDNA representing the N-terminal truncation of the C5a receptor was created using PCR (16, 17). The initial codon for the truncated receptor was built into a primer designed to anneal to the wild-type receptor cDNA beginning at nucleotide 76 (Asn-23). The sequence for the primer is 5′-TTAAGCTTGCTAGCCCACCATGAACACCCCTGTGGAT-3′. In all other respects the construction of the expression vector for the truncated receptor is identical to that of the wild-type (J.D., unpublished results). The accuracy of the receptor sequences were confirmed by sequencing.

Transfection. For transient expression, 2×10^6 293 cells (ATCC CRL 1573; ref. 18) in 100-mm tissue culture plates were transfected with 20 μg of plasmid DNA by calcium phosphate precipitation (19, 20). Cells were incubated with the precipitate for 16 hr at 37°C, refed, and harvested 40 hr posttransfection.

RESULTS

Antagonists of Intact C5a Fail to Antagonize C-Terminal Peptides. The current three-dimensional model for C5a is that of a compact, disulfide-bonded core (residues 1–60) and an unstructured C terminus (residues 63–74) (21). The C-terminal eight amino acids contain at least part of the binding site on the molecule, because a synthetic peptide consisting of these residues [C5a-(67–74)] inhibited C5a binding with a K_i of 300 μM and was found to be a functional agonist (22, 23). Subsequent studies succeeded in producing peptides with much higher affinities (23, 24). For example, substitution of phenylalanine for histidine at residue 67 [C5a(67F–74)] increased the potency of the peptide 500-fold (see also Fig. 1*A*). We have extended these studies to design the peptide C-009, which has a K_i for the C5a receptor of 8 nM (Fig. 1*A*) and is a functional agonist (data not shown). The presence of the tyrosine allows for iodination and establishment of a peptide binding assay. As shown in Fig. 1*B* both C5a (IC_{50} = 0.1 nM) and unlabeled C-009 (IC_{50} = 10 nM) inhibit the binding of ^{125}I-C-009 with concentration dependencies similar to those obtained from competition experiments against ^{125}I-C5a. Moreover the competition is specific since neither the chemotactic peptide fMLP nor the chemokine IL-8 inhibits the binding of C-009 (data not shown).

A number of weak antagonists of C5a have been described. The diaminoquinoline, L-584,020, is a competitive inhibitor, which blocks C5a binding to neutrophil membranes with an IC_{50} of 3 μM (ref. 25; Fig. 2) but which has no effect on the binding of either fMLP (Fig. 3*A*) or leukotriene B_4 (data not shown) to their respective G-protein-coupled receptors. This compound is a functional antagonist inhibiting C5a but not fMLP-induced degranulation from human neutrophils with an IC_{50} of 8 μM (Fig. 2*B*). In contrast, L-584,020 has little

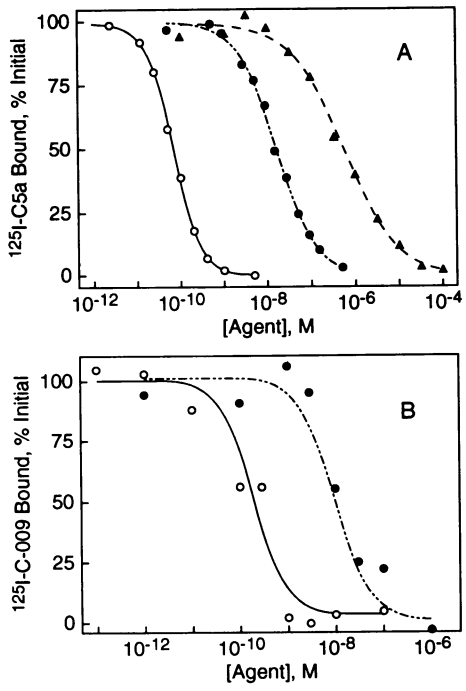

FIG. 1. Equilibrium binding properties of the C-terminal peptides on human neutrophil membranes. (*A*) C5a (○), C-009 (●), or C5a-(67F–74) (▲) was used to compete against ^{125}I-C5a. (*B*) C5a (○) or C-009 (●) was used to compete against ^{125}I-C-009. Each value is the average of triplicate determinations.

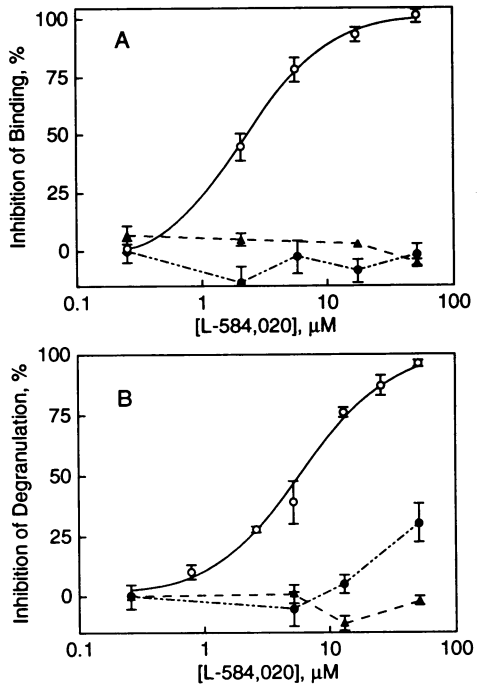

FIG. 2. L-584,020 is a C5a but not a C-terminal peptide antagonist. (*A*) Effect of L-584,020 on the binding of ^{125}I-C5a (○), ^{125}I-C-009 (●), or ^{3}H-fMLP (▲) to human neutrophil membranes. The values shown are the averages of duplicate determinations except those for ^{125}I-C-009, which are the averages of four measurements. (*B*) Ability of L-584,020 to inhibit C5a (○), C5a-(67F–74) (●), or fMLP (▲) stimulated degranulation. The procedure used was the same as that described previously (14) except that each sample contained L-584,020 at the appropriate concentration or an aliquot of dimethyl sulfoxide (final dimethyl sulfoxide concentration was 0.1%). The agonist concentrations used were 0.3 nM (C5a), 8 μM [C5a-(67F–74)], and 10 nM (fMLP). The data represent the averages of triplicate determinations.

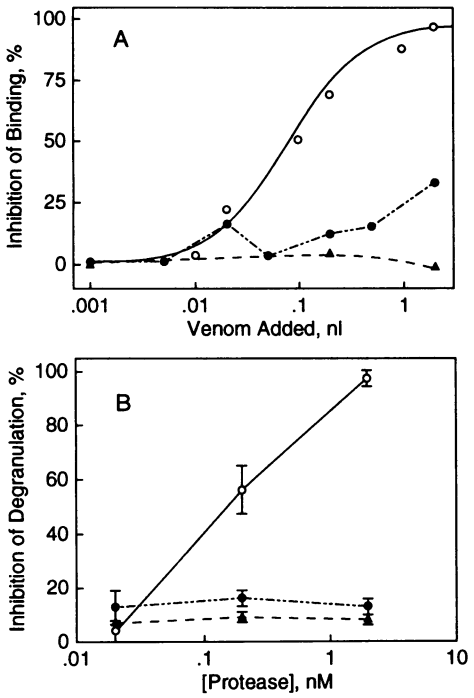

FIG. 3. The protease-treated C5a receptor does not bind or respond to C5a but does bind and respond to C-terminal peptides. (*A*) Effects of the venom on the binding of ^{125}I-C5a (○), ^{125}I-C-009 (●), or ^{3}H-fMLP (▲) to human neutrophil membranes. The data shown are averages of duplicate determinations except those for ^{125}I-C-009, which are the averages of quadruplicate measurements. (*B*) The C5a inhibitory component in the venom, a protease, was purified, and its effect on the ability of C5a and C5a-(67F–74) to stimulate degranulation is shown. Freshly isolated human neutrophils were preincubated for 20 min with the indicated concentrations of protease and then tested for their ability to release myeloperoxidase in response to 0.3 nM C5a (○), 8 μM C5a-67F–74 (●), and 10 nM fMLP (▲). All values represent the averages of triplicate determinations.

effect on either the binding or functional activity of the C-terminal peptides of C5a. As shown in Fig. 2*A*, the compound does not inhibit the binding of C-009 at concentrations as high as 50 μM. Similarly, the compound has little effect on the degranulation induced by C5a-(67F–74) (Fig. 2*B*). Two other antagonists of C5a, poly(L-arginine) (26) and 1-(2-ethyl-4-amino-5-pyrimidylmethyl)-4-vinylpyridinium bromide hydrobromide, exhibit similar behavior to L-584,020 as neither has any substantial effect on the activity of the C-terminal peptides (data not shown). Thus all three molecules show differential activity against intact C5a as compared to the C-terminal peptides.

C-Terminal Peptides but not C5a Bind to and Activate Proteolytically Cleaved Receptor. The venom of the spider *P. tristes* is an extremely potent inhibitor of C5a binding (Fig. 3). Moreover, this inhibition is specific, because the venom had no effect on the binding of either fMLP (Fig. 3*A*) or leukotriene B_4 (data not shown) to their respective G protein-coupled receptors. The active component of the venom was isolated and found to be a metalloproteinase (T.E.R., unpublished observations) with a molecular mass of 20 kDa. Since the protease inhibited C5a binding to intact cells, to membrane preparations, and to purified receptor, the actions of the enzyme must reflect its ability to cleave either C5a or the receptor. C5a is not the target since (*i*) the inhibitory potency of the protease increased when it was preincubated with whole cells or membranes, and washing the membranes or cells prior to addition of C5a did not substantially diminish activity, and (*ii*) extensive incubation with the enzyme failed to change either the mobility of C5a on SDS/PAGE (Fig. 4*C*) or its elution pattern on reverse-phase HPLC (data not shown).

To examine the enzyme's effect on the receptor, the protease was incubated with detergent-solubilized and affinity-purified C5a receptor. As reported (15), the purified receptor exhibits three bands on SDS/PAGE—a band at 42 kDa, which represents the binding subunit of the receptor, and bands at 41 and 36 kDa, which are the α and β subunits of the receptor-associated G proteins, respectively (Fig. 4*A*, lane 2). Incubation with the venom results in the loss of the 42-kDa band and the appearance of a new band at 31 kDa (Fig. 4*A*, lane 3). Some loss of 41-kDa band is also apparent. Since detergent extraction exposes the cytoplasmic as well as the extracellular regions of the receptor, the cleavage pattern seen in this experiment may not be entirely representative of what occurs in intact cells. In particular, the G proteins are inaccessible to the enzyme as are the intracellular loops of the receptor-binding subunit. To assess the effects of the venom protease on the receptor in intact cells, neutrophils were incubated with the protease and washed extensively to remove the enzyme, and membranes were prepared. The membranes were subjected to SDS/PAGE, and Western blotting was performed. The blot was probed with a rabbit polyclonal antibody raised against a peptide from the receptor C terminus. As shown in the untreated control (Fig. 4*B*, lane 4), the antibody identifies a single band with a molecular

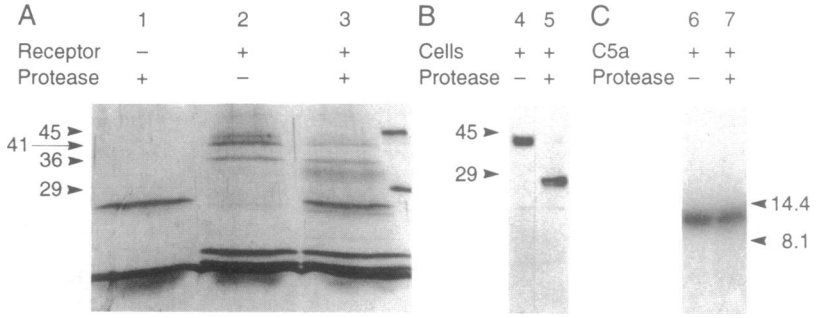

FIG. 4. The *P. tristes* protease cleaves the C5a receptor. (*A*) Affinity-purified receptor was incubated with protease, and the results were analyzed on an SDS/PAGE (4–20%) gel. The patterns were visualized by silver staining. Lane 1, protease plus buffer; lane 2, purified receptor plus buffer; lane 3, receptor plus protease. (*B*) Intact neutrophils were incubated with protease, and membranes were prepared. The Western blot from a SDS/14% PAGE gel was probed with a rabbit polyclonal antibody raised against a receptor C-terminal peptide. Lane 4, neutrophils plus buffer; lane 5, neutrophils plus protease. Similar results were also obtained in experiments in which boiling SDS was added directly to the washed cells (data not shown). The antibody had no reactivity with the protease (data not shown). Binding assays demonstrated that the protease treatments in both *A* and *B* totally abrogated C5a binding. (*C*) Lack of effect of the protease on C5a. Aliquots of ^{125}I-C5a were incubated for 2 hr at 22°C with or without 15 ng of protease in 250 μl of Hanks' balanced salts containing 25 mM Hepes (pH 7.2). Twenty-five microliters of each sample was then subjected to SDS/PAGE on an 18% gel, and autoradiography was performed. Lane 6, C5a plus buffer; lane 7, C5a plus protease. Molecular size markers (in kDa) are indicated.

mass of 42 kDa. Incubation of the cells with the protease caused a loss of the 42-kDa receptor and the concomitant appearance of a 31-kDa fragment (Fig. 4*B*, lane 5), a result similar to that obtained with the purified receptor. Since only the extracellular portions of the receptor are exposed to the protease in this experiment, the 11-kDa reduction in mass localizes the cleavage site to the external loop between transmembrane helices 2 and 3. While the receptor C-terminal antibody does not allow visualization of any N-terminal fragment produced on cleavage, we have failed to find this fragment in experiments employing the purified receptor and silver-stained gels, suggesting that the protease also destroys the N terminus of the receptor.

Thus, the protease abrogates C5a binding because it cleaves the receptor. As expected, the loss of binding results in the failure of C5a to stimulate degranulation (Fig. 3*B*). However, protease treatment has little effect on the ability of the receptor to bind the C-terminal peptides (Fig. 3*A*) or to activate degranulation in response to the peptides (Fig. 3*B*). Thus cleavage of the receptor produces a truncated molecule that has greatly diminished ability to bind and respond to intact C5a but has normal avidity and response to C-terminal peptides of C5a.

N-Terminal Truncation of the C5a Receptor Causes a Loss of C5a but Not C-Terminal Peptide Binding. To more precisely localize the external region of the receptor that interacts with C5a but not the C-terminal peptides, a recombinant receptor lacking residues 1–22 was prepared and expressed in 293 cells. Since initial experiments with the mutated receptor indicated greatly diminished C5a binding, all further experiments were carried by competition against ^{125}I-C-009. As shown in Fig. 5*A*, the N-terminal deletion has little effect on the binding of C-009 as the IC_{50} values measured on the wild-type and truncated receptors are indistinguishable. In contrast, the truncation has a dramatic effect on the binding of C5a, producing a 1000-fold decrease in affinity (Fig. 5*B*). Attempts to examine the effects of the deletion on receptor activation were not successful because, in our hands, the wild-type receptor was not functionally coupled in 293 cells.

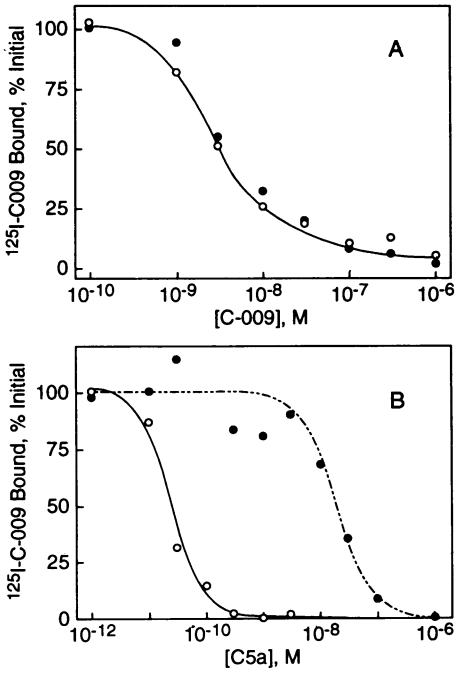

FIG. 5. N-terminal truncation of the receptor decreases C5a but not C-terminal peptide binding. Wild-type C5a receptor (○) and a truncated receptor lacking residues 1–22 (●) were examined for their ability to bind the C-terminal peptide C-009 (*A*) or C5a (*B*) by competition against ^{125}I-C-009. All results are the averages of quadruplicate determinations.

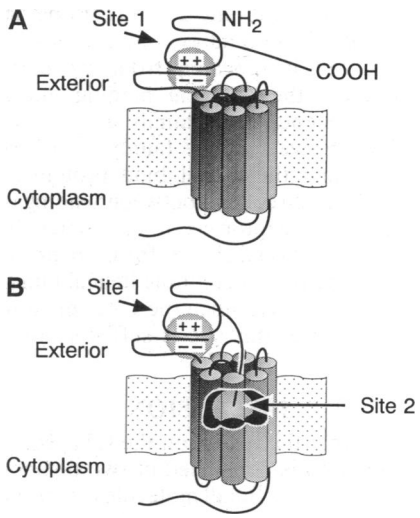

FIG. 6. Model for the binding of C5a to its receptor. As shown in *B* the interaction takes place at distinct sites. The first, designated site 1, is between the N terminus, and possibly the second extracellular loop of the receptor, and the core of C5a. Specifically, we believe the primary interaction is between several of the aspartic acid residues in the N terminus and Arg-40 and possibly Arg-37 and His-15 of C5a. The second interaction site, designated site 2, is between the C terminus of C5a and the interhelical region of the receptor. The primary interaction involves Arg-74 and Lys-68 of C5a (Z.K. and S.J.S., unpublished observations). We believe that the initial site of productive contact takes place at site 1 as depicted in *A*. The contact at site 1 effectively raises the local concentration of C5a and thereby promotes the more difficult interaction at site 2. In this regard, preliminary studies indicate that the binding rate of C5a is significantly greater than that of C-009 (S.J.S. and M.S.S., unpublished observations).

Two-Site Model. The properties of the proteolyzed and truncated receptors, taken together with the differential effects of the C5a antagonists, argue strongly that the interaction domain between C5a and its receptor is composed of two physically distinct and separable loci (Fig. 6). The first is between the core of C5a and a portion of the receptor that includes the N terminus and possibly the external loop between helices 2 and 3 or both (site 1 in Fig. 6). It is this interaction that is disrupted by the antagonists. Structure–activity studies of L-584,020 and analogs (25) demonstrate that the two amino groups of the molecule are essential for activity, as is the distance between them, implying that ionic interactions with appropriately spaced negatively charged groups of the receptor provide the binding energy. While it is not necessarily true that the energetically important contacts between C5a and the receptor are the same, site-directed mutagenesis implicates Arg-40 and possibly His-15 as being necessary for binding (27). Thus, it seems likely that, as for the antagonists, the binding energy is due to contact between negatively charged residues on the receptor with positively charged residues on the ligand. In this regard the N terminus of the receptor contains seven aspartic acid residues, whereas the loop between helices 2 and 3 does not contain a single negatively charged residue (4, 5), suggesting that the N terminus is the more likely candidate for the extracellular (site 1) binding domain. The results with the truncated receptor, in which six of the seven aspartic acid residues have been deleted, clearly demonstrates the role of the N terminus in the site 1 binding domain.

The second interaction locus is between the C terminus of C5a and some portion of the receptor that is not affected by the N-terminal truncation or by the protease. While at present this locus has not been identified, we believe that it

is likely to be interhelical (site 2 in Fig. 6) by analogy with models proposed for the β-adrenergic receptor and rhodopsin. Such a location is appealing from two points of view. First, it conserves the classical binding motif of the G protein-coupled receptor superfamily and provides an evolutionary bridge between those family members that bind small molecules and those that bind proteins. Second, it provides a relatively direct link between binding of the ligand and triggering the receptor as it eliminates the need to propagate a conformational change from a region on the cell's exterior through the transmembrane helical bundle to a loop in the cytoplasm. Clearly, since the C-terminal peptides are agonists, interaction at this site is sufficient to activate the receptor.

DISCUSSION

We have shown that the region on the C5a receptor responsible for binding C5a is composed of two distinct and physically separable subsites. Small molecules, such as retinal and catecholamines, are known to bind to their G protein-coupled receptors at a single interhelical site. The two-site motif appears to be an adaptation of this simpler paradigm in which a second extracellular binding site has been added to accommodate larger molecules such as C5a. This motif may be widely used by the G protein-coupled receptor superfamily to bind peptides and proteins. There is a body of data implicating the extended N terminus of the much larger glycoprotein hormone receptors as the primary site of interaction with their ligands (11, 12, 28), as well as some evidence for interhelical contacts (29, 30). Similarly, experiments with chimeric receptors imply that the different ligand-binding specificities of the two IL-8 receptors are dictated by the extracellular N termini (31), and site-directed mutagenesis studies of IL-8 suggest that two regions of the ligand are involved in the interaction (32, 33). In addition we have found that all site 1 C5a antagonists also inhibit the binding of IL-8, a result that implies the presence of a site 1-like domain on the IL-8 receptors (S.J.S., unpublished observations). The alternative explanation, that the activity of compounds is due to some generic nonspecific effect, such as membrane disruption, is unlikely since none of the molecules blocked the binding of either fMLP or leukotriene B_4 to their respective G protein-coupled receptors. Finally, recent studies have shown that the extracellular loops play a role in determining the ligand specificity of another G protein-coupled receptor, neurokinin 1 (34).

The identification of the two-site binding motif provides a starting point for more detailed studies of the structure–function relationships in the C5a receptor and for a molecular understanding of how ligand binding initiates the variety of distinct cellular processes produced by C5a. It also poses questions as to the functional relationships between sites 1 and 2. First, is interaction at site 2 alone as efficient at activating the receptor as the simultaneous occupation of both sites? Second, do the various biological responses evoked by C5a behave in a concordant manner on subsite activation? Third, can the receptor be activated by interaction at site 1 alone? Answers to these questions as well as the precise identification of the residues involved at the two binding sites await additional experiments.

Finally, the two-site binding paradigm provides a rational strategy for developing agonists and antagonists to receptors with macromolecular ligands. While targeting of receptors has been an area of success for mechanism-based disease treatments, the approach has been largely restricted to receptors for simple, small molecules. Attempts to develop therapeutics for receptors that bind proteins have been almost entirely unsuccessful, presumably because of the difficulties involved in emulating, or inhibiting, the interactions between a receptor and a large ligand. The potential for subsite interaction simplifies this problem and may greatly extend the set of receptors amenable to pharmacological intervention.

The authors are grateful to Dr. Seth Lederman (Columbia University) for his generous gift of human bone marrow and Bruce Dougherty for the pHIVLTRHygl vector. We also thank L. Peterson and L. Wicker for comments on the manuscript and N. Sigal for discussion and support.

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