Identification of ligand effector binding sites in transmembrane regions of the human G protein-coupled C3a receptor

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Abstract

The human C3a anaphylatoxin receptor (C3aR) is a G protein-coupled receptor (GPCR) composed of seven transmembrane α -helices connected by hydrophilic loops. Previous studies of chimeric C3aR/C5aR and loop deletions in C3aR demonstrated that the large extracellular loop2 plays an important role in noneffector ligand binding; however, the effector binding site for C3a has not been identified. In this study, selected charged residues in the transmembrane regions of C3aR were replaced by Ala using site-directed mutagenesis, and mutant receptors were stably expressed in the RBL-2H3 cell line. Ligand binding studies demonstrated that R161A (helix IV), R340A (helix V), and D417A (helix VII) showed no binding activity, although full expression of these receptors was established by flow cytometric analysis. C3a induced very weak intracellular calcium flux in cells expressing these three mutant receptors. H81A (helix II) and K96A (helix III) showed decreased ligand binding activity. The calcium flux induced by C3a in H81A and K96A cells was also consistently reduced. These findings suggest that the charged transmembrane residues Arg161, Arg340, and Asp417 in C3aR are essential for ligand effector binding and/or signal coupling, and that residues His81 and Lys96 may contribute less directly to the overall free energy of ligand binding. These transmembrane residues in C3aR identify specific molecular contacts for ligand interactions that account for C3a-induced receptor activation.

Keywords: G protein-coupled anaphylatoxin C3a receptor; ligand effector binding sites; molecular modeling; site-directed mutagenesis

Anaphylatoxin C3a mediates a wide range of inflammatory responses in a hormone-like fashion. The biologic activities of human C3a include enhanced vascular permeability on small blood vessels, induced smooth muscle contraction, degranulation of mast cells, and chemotaxis of eosinophils and mast cells (Hugli, 1984; Daffern et al., 1995; Hartmann et al., 1997). The complete primary structure of human C3a was determined to comprise 77 amino acid residues (Hugli, 1975), and the three-dimensional structure of C3a has also been solved (Huber et al., 1980; Muto et al., 1985; Chazin et al., 1988).

C3a binds specifically to eosinophils, basophils, neutrophils, and monocytes indicating the presence of C3a receptors on a variety of human leukocytes (Glovsky et al., 1979; Daffern et al., 1995). The human C3a receptor has been cloned (Ames et al., 1996; Crass et al., 1996; Roglic et al., 1996), and the deduced amino acid sequence clearly places this molecule in the superfamily of G protein-coupled receptors (Horn & Vriend, 1998). The C3aR is composed of seven transmembrane segments, but contains an exceptionally large extracellular loop. Further studies indicate that the C3a receptor not only exists on leukocytic cells, but also on a variety of cells and tissues such as adrenal cortex, lung, astrocytes, and microglia (Gasque et al., 1998). Thus, the interactions between C3a and C3aR may involve a number of pathophysiologic events such as vascular, pulmonary (Regal, 1997), and neurodegenerative disorders (Morgan et al., 1997). However, the molecular interactions between C3a and C3aR and the pathophysiologic events that evolved from this interaction remain to be further elucidated.

As more and more GPCRs are cloned and identified, ligand binding and receptor activation studies will certainly help to elu-

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Abbreviations: GPCR, G protein-coupled receptors; C3aR, C3a receptor; C5aR, C5a receptor; FPR, formyl peptide receptor; fMLP, formyl-Met-Leu-Phe; AT1, angiotensin II receptor type 1; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; DMEM, Dulbecco's Modified Eagle Medium; BSA, bovine serum albumin.

cidate the molecular basis of these disorders. Agonists and antagonists of the biogenic amine receptors have been developed and promise to have therapeutic applications. The binding domains for small physiological ligands (e.g., biogenic amines) on GPCRs have been shown to involve residues located in the transmembrane helices (Gether & Kobilka, 1998). Conversely, binding of peptides or glycoprotein hormones to their receptors usually involve interactions with the extracellular regions (Dufau, 1998). These findings have revealed common features for the ligand-binding mechanisms of specific groups of G protein-coupled receptors.

To date, our understanding of the anaphylatoxin receptors comes mostly from studies of the C5a receptor (C5aR). In the C5a and C5aR interaction, two distinct binding sites were described between the ligand and receptor (for reviews, see Ember & Hugli, 1997; Ember et al., 1998). The N-terminus of C5aR constitutes a secondary binding site and is completely distinct from the primary effector site that is essential for receptor activation (Siciliano et al., 1994; Monk et al., 1995). In the C5a anaphylatoxin, His15 in the N-terminal region, Arg40 in the disulfide-linked core region, and Lys68, Leu72 and Arg74 in the C-terminal region were all shown to participate in C5aR binding (Mollison et al., 1989). However, binding site interactions proposed for the C3a/C3aR appear to differ from the C5a/C5aR model. The C3aR has a shorter N-terminal region (21 amino acids) and an exceptionally large extracellular loop compared with C5aR. Previous studies have demonstrated that replacement of the N-terminal segment of human C3aR with that of the human C5aR had a minimal effect on C3aR receptor function. However, the Asp residues (Asp183 and Asp186; Asp325, Asp326, and Asp327) near either end of the large extracellular loop2 in C3aR did affect C3aR activity and may be involved in secondary (noneffector) interactions with C3a (Chao et al., 1999). Thus, the primary and secondary binding sites in C3aR appear to be contiguous. Therefore, elucidation of the primary effector binding site in human C3aR was the particular focus of this study.

Since the C-terminal sequence LGLAR in C3a, including the guanidino and carboxyl groups of the Arg, was shown to be essential for effector function (Caporale et al., 1980), our hypothesis was that charged residues in the transmembrane regions of C3aR may provide electrostatic interactions for the ligand. We selected the charged residues in the transmembrane regions of C3aR for our study. These residues were substituted with Ala using site-directed mutagenesis, and analysis of these mutant receptors was carried out by direct agonist binding and calcium mobilization assays. The study identifies at least three residues (Arg161, Arg340, and Asp417) that appear to be essential for the primary or effector ligand binding, and several sites that may provide secondary binding interactions or structural stability.

Results

Candidate residues for mutational analyses in the human C3aR were selected by the following criteria: (1) The guanidino and carboxyl groups of the C-terminal Arg77 residue in C3a are essential and thought to bind within a transmembrane binding pocket. Therefore, charge-charge interactions between Arg77 in C3a and residues in C3aR should occur at both acidic (Asp or Glu), and basic (Lys, Arg, or His) side chains in the receptor. Consequently, we focused our efforts on residues in the transmembrane region that have charged side chains. (2) Subsequent cloning of C3aR from mouse (Hsu et al., 1997; Tornetta et al., 1997), rat (Fukuoka et al., 1998a), and guinea pig (Fukuoka et al., 1998b) demonstrated

that sequences of the C3a receptors from these species were highly conserved, especially in the transmembrane domains. Human C3a cross-reacts with the C3aR in mouse, rat, and guinea pig tissue; consequently, the binding residues must have remained conserved for this family of receptors from different species. Therefore, we selected only the conserved charged residues in the transmembrane regions for mutation.

Based on these criteria, we selected Asp68 and His81 (helix II), Lys96 (helix III), Arg161 and Glu162 (helix IV), Arg340 (helix V), His394 (helix VI), and Glu406 (loop VI/VII), Asp417 and His418 (helix VII) as candidates for Ala replacement (see Fig. 1 for locations). Replacement by Ala was used to ensure removal of existing side-chain charges without introducing novel or alternative functional groups. PCR-mediated site-directed mutagenesis was performed, and the mutant receptors were cloned into the eukaryotic expression vector pSFFV-neo. The clones were completely sequenced to confirm that the correct amino acid substitution had been made.

All mutant receptors were stably expressed in RBL-2H3 cells and analyzed for their binding affinity toward C3a and for C3a-induced calcium mobilization (see Table 1). The cells were further examined by flow cytometry to confirm surface expression of the mutant receptors (Fig. 2). Most of the mutant receptors were expressed at levels comparable to the wild-type C3aR, except for E162A and H394A, which expressed at lower levels (Column 3, Table 1).

Substitution of Ala for charged residues did not always affect receptor affinity for the ligand. As shown in Figure 3, competitive ligand binding assays demonstrated that five of the mutants exhibited IC_{50} values ranging from 0.4 to 1.0 nM, which are comparable to that of the wild-type. These mutants were D68A (helix II), E162A (helix III), H394A (helix VI), E406A (loop VI/VII), and H418A (helix VII). However, E162A and H394A

Table 1. Characterization of site specific mutants of human C3aR

C3aR mutants	K_d^a (nM)	Receptors/cell ^b	EC_{50}^c [calcium flux] (nM)	Δ MFI ^d
Wild-type	2.33	184,000	2.50 ± 0.20	32.8
D68A	3.65	176,000	1.76	49.5
H81A	80.6	256,000	70.2	60.6
K96A	12.0	157,000	51.5	37.7
R161A	N.D. ^e	N.D.	$>5,000^f$	50.5
E162A	1.39	96,000	2.07	24.5
R340A	N.D.	N.D.	$>1,000^f$	71.7
H394A	1.16	48,000	12.3	19.7
E406A	2.18	192,000	2.22	58.6
D417A	N.D.	N.D.	$>5,000^f$	42.0
H418A	3.32	144,000	4.70	43.3

^a K_d are based on specific binding of ^{125}I -C3a as determined by nonlinear regression.

^bReceptors/cell rounded to nearest thousand based on B_{max} of the saturation curve.

^c EC_{50} values of calcium flux are based on intracellular calcium mobilization assays ($n = 2-3$).

^d Δ MFI, mean fluorescent intensity minus the negative control MFI value.

^eN.D., not detectable.

^fLess than 50% response up to this level.

Fig. 1. A primary and secondary structure model of the human G protein-coupled C3a receptor. Transmembrane helices were assigned based on hydropathy analysis and comparisons with known sequences of other GPCRs, and the boundary residues are numbered. A disulfide bond exists between residues Cys95 and Cys172 (-S-S-). The residues that were selected for mutagenesis are highlighted in bold type and numbered.

mutants showed apparent reductions in B_{max} values, indicating either a reduced number of high-affinity binding sites on the cell surface or efficacy of transporting the mutant receptors to the membrane surface was reduced (Fig. 3). The K_d values for these mutants as measured by saturation ligand binding assay ranged from 1.16 to 3.65 nM, which were comparable to the wild-type K_d of 2.33 nM (see column 2, Table 1). The receptor number per cell calculated for these five mutants corresponded to the competitive ligand binding analysis, showing that E162A and H394A express fewer receptors (96,000 and 48,000 receptors/cell, respectively), compared with wild-type C3aR (184,000 receptors/cell) (Table 1). These results were also consistent with the flow cytometry analysis of the mutants (see column 5, Table 1). These findings argue against major involvement of these residues in ligand binding and indicate that the structural change of C3aR after removal of the charged side chains were minimal.

To investigate whether the mutations in C3aR affect the ligand effector binding, calcium mobilization assays were performed. We used native C3a, C3a analog peptide E7, which contains hydrophobic residues and the LGLAR effector sequence, as well as the octapeptide AAALGLAR. Addition of the ligand to transfected RBL-2H3 cells leads to a calcium flux that can be detected by Indo-1 fluorescence intensity changes. The five mutants D68A, E162A, H394A, E406A, and H418A retained normal calcium flux responsiveness to C3a, as well as to C3a analog peptide E7 and the octapeptide AAALGLAR. Two hundred nanomolars E7 peptide and 20 μ M AAAL GLAR peptide induced the same size signal as 100 nM of native C3a (Fig. 4, see WT C3aR). The C3a concentrations (1 to 10 nM) needed to induce 50% calcium mobilization

(EC_{50} values) in these five mutants were comparable to levels that stimulated the wild-type C3aR (column 4, Table 1).

These results were surprising because charged side chains in the transmembrane regions of the C3aR family were conserved, suggesting a major influence on either receptor structure or function. Asp68 in helix II of C3aR is highly conserved in all GPCRs, indicating a crucial structural or functional role for this residue. The corresponding Asp residue was shown to be involved in protein folding or ligand binding in a number of GPCRs. Mutation of Asp82 in human C5aR, corresponding to Asp68 in C3aR, affected receptor expression in which the D82A mutant receptor was retained intracellularly (Raffetseder et al., 1996). Replacement of Asp71 with Ala in the formyl peptide receptor (FPR) resulted in decreased fMLP binding and a significant loss in calcium mobilization (Prossnitz et al., 1995). However, mutation of charged residues such as Asp68 to Ala in C3aR did not appear to affect either structure or function, which was unexpected.

Substitution of Ala at positions Arg161, Arg340, and Asp417 completely abolished C3a binding activity (Fig. 3), while full expression of these mutant receptors on the cell surface was confirmed by flow cytometry (see Table 1). As shown in Figure 4, the R161A, R340A, and D417A mutants exhibited no calcium flux signal when exposed to 100 nM C3a ligand. However, R340A did respond weakly to ligand concentrations of 1.0 μ M, while R161A and D417A responded weakly to 5.0 μ M C3a. This indicates that mutants R161A, R340A, and D417A were at least 2,000, 400, and 2,000 times less active, respectively, than the wild-type C3aR. No calcium flux was observed in these mutants with either 200 nM E7 peptide, or 100 μ M octapeptide AAALGLAR. This behavior is in

Fig. 2. Flow cytometric analysis of RBL-2H3 cells transfected with human C3a receptors. The cells were incubated with rabbit polyclonal antibody directed against the large extracellular loop2 (thick lines) or normal rabbit serum as control (thin lines), followed by staining with a FITC-labeled goat antirabbit IgG secondary antibody. Mutants D68A, H81A, K96A, R161A, E162A, R340A, H394A, E406A, D417A, and H418A all exhibited receptor expression. Wild-type C3aR expression is comparable to that of mutant D68A expression as determined in a separate experiment. Note that RBL-2H3 cells do not express endogenous C3aR (not shown).

contrast to the mutants D68A, E162A, H394A, E406A, H418A, and the wild-type C3aR, which showed normal receptor binding and responded to low nanomolar concentrations of C3a.

These results are compatible with the hypothesis that Arg161, Arg340, and Asp417 represent C3aR residues essential for C3a effector binding. Each of these C3aR mutants binds polyclonal antibody specific for the large extracellular loop, which may play an important role in receptor folding and activation. This antibody was used to obtain the flow cytometry data confirming that the mutant receptors were all expressed on cell surface. Therefore, we conclude that the conformational change of C3aR induced by these point mutations was minimal.

Mutants H81A and K96A showed reduced C3a binding capacity, having IC_{50} of 18.5 and 5.5 nM, respectively (Fig. 3). The K_d measured by saturation ligand binding assays was 35-fold and 5-fold higher than that of the wild-type, while the receptor num-

Fig. 3. Competitive ligand binding curves of RBL-2H3 cells expressing human C3aR. The IC_{50} values of D68A, E162A, H394A, E406A, and H418A deduced from these experiments ranged from 0.4 to 1.0 nM, comparable to that of wild-type. K96A and H81A have IC_{50} values of 5.5 and 18.5 nM, respectively. IC_{50} values for mutants R161A, R340A, and D417A could not be determined (N/A: not available). Note that R161A, R340A, and D417A showed no binding and H81A showed little binding.

bers expressed on the cell surface were comparable or even somewhat higher than that of wild-type C3aR (Table 1). Cells expressing mutants H81A and K96A did exhibit a calcium signal when exposed to C3a (Fig. 4), but the EC_{50} was 70.2 and 51.5 nM, respectively, for H81A and K96A. That is 25- to 35-fold higher than for the wild-type. However, when 200 nM E7 peptide or 20 μ M octapeptide was used, we observed little if any calcium flux for H81A and K96A. At least 100 μ M octapeptide was needed to induce even low level calcium flux from the H81A mutant. These calcium flux data indicated that the ligand effector binding to H81A and K96A mutants was also consistently reduced.

Discussion

The receptor binding sites in human C3a have been well defined. The three-dimensional structure of human C3a consists of a large globular core of four closely packed α -helices covalently linked by three disulfide bonds with a C-terminal flexible irregular structure (Huber et al., 1980). The C-terminal region of C3a is folded in a pseudo- β -turn and is stabilized by an adjacent α -helical segment according to NMR studies (Chazin et al., 1988). The C-terminal 21 residues fragment of C3a (i.e., C3a 57–77) has been shown to retain all of the biologic activities of the natural molecule (Lu et al., 1984; Ember et al., 1991). Synthetic peptide analogs of C3a demonstrated that the primary effector binding site in C3a exists in the irregular C-terminal region (LGLAR sequence). Removal of Arg77 from the C-terminal end of C3a abolishes C3aR affinity and most biologic activities, and substitution with Lys77 gave at least 300 times less activity. Blockage with amide on carboxyl group of Arg77 in C3a was at least 1,000 times less active (Caporale et al., 1980; Erickson et al., 1981; Unson et al., 1984). Therefore, we selected charged residues in C3aR as candidates for site-directed mutagenesis as a means to define the primary determinants involved in C3a binding.

We identified five transmembrane amino acid residues in C3aR that may participate in binding C3a. These residues are located in the 2nd (His81), 3rd (Lys96), 4th (Arg161), 5th (Arg340), and 7th (Asp417) transmembrane segments. Mutations that affect ligand

Fig. 4. Calcium mobilization in RBL-2H3 cells transfected with wild-type or mutant C3aR. The cells were stimulated with the intact C3a, peptide E7, or the octapeptide AAALGLAR (octa), at concentrations of 100, 200, and 20 μM, respectively, unless otherwise noted. Relative fluorescence intensity is shown. C3aR mutants D68A, E162A, H394A, E406A, and H418A gave responses similar to the wild-type C3aR (A) at the concentrations mentioned above. Mutants (B) H81A and (C) K96A exhibited higher EC_{50} values to all three ligands tested (C3a, E7, and octa). Mutants (D) R161A, (E) R340A, and (F) D417A responded weakly to higher concentration of C3a, but did not respond to higher concentration of octapeptide (e.g., 100 μM). Agonist-specific stimulation was followed by addition of 0.2% Triton X-100 to release the intracellular calcium (the largest increase of signals) and 10 mM EGTA to chelate the calcium (loss of signals)

binding in C3aR were compared with mutagenesis data reported for other seven-transmembrane domain receptors.

The human C5aR transmembrane segments showed the highest homology with C3aR. As demonstrated by sequence alignment, C5aR contains an Arg206 in a position corresponding to Arg340 in human C3aR. Strong evidence exists for a direct interaction between Arg206 in the C5aR (helix V) and a carboxyl group in the ligand (DeMartino et al., 1995; Raffetseder et al., 1996). C5a peptide analogs and receptor mutagenesis revealed that the guanidino group of Arg74 in C5a makes the energy-yielding contact with the receptor, while the free carboxylate of Arg74 in C5a negates "electrostatic" interference with Arg206 of the receptor (DeMartino et al., 1995). Substitution of Arg206 by Ala or Gln in C5aR resulted in partial or full expression of the mutant receptor but caused reduction in binding capacity, indicating this charged residue is needed for ligand interactions (Raffetseder et al., 1996).

Mutation of Arg341 in guinea pig C3aR (corresponding to Arg340 in human C3aR) by Ala or Gln completely inactivated the molecule (Fukuoka et al., 1999). Furthermore, mutagenesis of the cationic residue corresponding to Arg340 in human C3aR clearly affected ligand binding in a number of other GPCRs, including the angiotensin II receptor type 1 (AT1) (Hunyady et al., 1996) and type 2 (AT2) (Pulakat et al., 1998).

In the angiotensin/receptor AT1 interactions, the guanidino group of Arg2 and the carboxyl terminus of the ligand, together with aromatic side groups of Tyr4 and His6, were essential for receptor binding. Detailed mutagenesis studies demonstrated that the terminal carboxyl group of angiotensin II binds to Lys199 in helix V of AT1 (corresponding to Arg340 in C3aR) (Hunyady et al., 1996; Inoue et al., 1997). Meanwhile, the ligand binding affinity was greatly reduced in AT1 mutants such as K102A in helix III (corresponding to K96A in C3aR) and R167A in helix IV (corresponding to R161A in C3aR), indicating the importance of these regions in formation of a transmembrane binding pocket. These results were surprisingly similar to the pattern obtained from the C3aR mutagenesis data, suggesting that the Arg340 (and/or Arg161) in C3aR interacts with the carboxyl group of the C-terminal Arg77 in C3a.

Asp281 in helix VII of AT1 (corresponding to Asp417 in C3aR) was identified as a major docking point for a charge-charge interaction with Arg2 of the ligand angiotensin (Hunyady et al., 1996; Inoue et al., 1997). In the bradykinin receptor (B2 type), a salt bridge occurs between the guanidino group of the C-terminal Arg9 in bradykinin and Asp286 in the B2 receptor (corresponding to Asp417 in C3aR) (Jarnagin et al., 1996). In contrast, the corresponding Asp284 in FPR does not appear to be involved in ligand binding to fMLP (Miettinen et al., 1997), presumably due to the absence of a cationic group in this ligand. Instead, Arg84 (corresponding to His81 in C3aR) and Lys85 in helix II of FPR were suggested to play a role in fMLP binding (Quehenberger et al., 1994; Mills et al., 1998). These results further suggest that the guanidino group of C-terminal Arg77 in C3a may interact with Asp417 in C3aR. Therefore, the C-terminal end of C3a occupies a primary ligand binding site located in a pocket formed by transmembrane helices II, III, IV, V, and VII in C3aR. The ligand-specific transmembrane binding pocket is a common feature of these GPCRs, while the extracellular regions of the receptor provides additional energy to stabilize binding of the larger ligands.

Meanwhile, molecular modeling of GPCRs provide a valuable tool that is complementary to, but independent from, the site-directed mutagenesis studies. Initially, a human C3aR model was constructed by substituting the C5aR model (Grötzinger et al., 1991) provided by the GPCR database (Horn et al., 1998). The helix bundle appears to be consistent with the Baldwin model (Baldwin, 1993). However, Arg340 in helix V of C3aR points away from the bundle, which is inconsistent with the mutagenesis data. Using the Swiss-Model program, a model of C3aR was constructed for the seven-transmembrane domains, in which the neuropeptide Y1 receptor template was used. This model is consistent with our mutagenesis data. Each helix is positioned next to its neighbor in the sequence, and helices I, IV, and V are the most exposed to the lipid surrounding the receptor while helix III the least exposed (Fig. 5A). Using this C3aR model and the C3a crystal structure, accessibility of the primary C-terminal binding site in C3a was examined for interactions with human C3aR.

Of the charged residues selected for mutagenesis, Asp68 is located most deeply in the membrane, being on the intracellular side

Fig. 5. The proposed three-dimensional orientation of human C3aR transmembrane helices was modeled after the known structure of the rhodopsin receptor. **A:** Stereo view of the transmembrane helices as viewed from the extracellular space. **B:** Side view of C3aR model showing transmembrane helices I-IV in the foreground, and C3a ligand on the top. Helices in C3aR are colored: I (residues 22–46), pink; II (residues 60–84), red; III (residues 94–118), magenta; IV (residues 139–163), blue; V (residues 333–357), cyan; VI (residues 377–401), green; VII (residues 414–438), yellow. The critical residues (Arg161, Arg340, and Asp417) are indicated. The model was generated using the automated rule-based method by submitting the C3aR helical segments I-VII to neuro peptide Y1 receptor template. C3a ligand is colored orange. Side chains of the C-terminal sequence LGLAR in the C3a ligand are indicated. Possible contact interactions occur between the essential carboxyl group of Arg77 in C3a and Arg340 (or Arg161) in C3aR, and between the essential guanidino group of Arg77 and Asp417 in C3aR.

of helix II, which we conclude makes it inaccessible to the ligand. Most of the other residues are located near the end of the respective helix on the extracellular side. Arg340 in helix V and His394 in helix VI are located two helical turns below the membrane boundary. There is open space above the side chain of Arg 340, which permits the side chain to be freely exposed and flexible. Arg161 (helix IV) and Asp417 (helix VII) are located on the boundary between helix and the extracellular loop, and point to the transmembrane pocket. The distances between Arg161 or Arg340 guanidino groups in C3aR and the carboxyl group of Asp417 in this model are 7.5 and 8.0 Å, respectively. It is possible that an Arg group (e.g., Arg77 in C3a; both guanidino and carboxyl ends are separated by up to 9.0 Å in distance) can reach across both side chains of the critical residues in C3aR. Based on the experimental data and molecular modeling, we propose that the C3a C-terminal peptide interacts with C3aR in a ligand-binding pocket located at the extracellular transmembrane interface (Fig. 5B). The essential carboxyl group of Arg77 in C3a may interact with the guanidino side chain of Arg340 (or Arg161) in C3aR and the essential guanidino group of Arg77 may interact with the carboxyl side chain of Asp417 in C3aR.

In summary, the results from our studies support the hypothesis that the C3a ligand interacts with C3aR at multiple sites and that the effector site is in a ligand-binding pocket formed by a cluster of transmembrane helices. Our mutagenesis studies and molecular modeling approaches have helped to further refine the C3a binding sites on C3aR.

Materials and methods

Materials

Human C3a was purified from expired plasma (Hugli et al., 1981). The C3a analog peptides E7 (WWGKKYRASKLGLAR) and octapeptide (AAALGLAR) were synthesized and characterized as described previously (Ember et al., 1991). All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, Massachusetts). DNA polymerase (native Pfu) were purchased from Stratagene (La Jolla, California). Rabbit polyclonal antiserum against the entire large extracellular loop of C3aR was prepared against the purified sequence expressed in *Escherichia coli* strain (Chao et al., 1999). All other materials were reagent grade and were obtained from commercial sources.

Site-directed mutagenesis

Single substitution mutants were constructed in plasmid pSFFV-neo by using two-stage polymerase chain reaction (PCR) (Higuchi et al., 1988). Briefly, the human C3aR cDNA AZ3M (Roglic et al., 1996) were used as templates for amplification by PCR of overlapping cDNA fragments with specific oligonucleotide primers. Synthetic oligonucleotides were designed in which the corresponding site is changed to Ala. The first stage PCR fragments were annealed and amplified again with primers corresponding to the 5' and 3' end sequences of the resulting molecule. The 5' end primer

(5'-GAAGAATTCAGCTACTGTCTC-3', EcoRI site underlined) and 3' end primer (5'-ACGAATTCATCACACAGTTGTACTAT TTC-3') were used. The full-length PCR products were then digested with EcoRI and cloned into the same site in the pSFFV-neo expression vector (Fuhlbrigge et al., 1988). DNA sequencing of the entire C3aR cDNA was performed on ABI Model 373A DNA Sequencer to confirm accuracy.

Cell culture

Two micrograms of DNA was linearized with Xba I and transfected to RBL-2H3 cells (ATCC CRL-2256) using Fugene-6 reagent (Boehringer Mannheim, Indianapolis, Indiana). G418 was added to 0.5 mg/mL for selection and maintenance of stably transfected cell lines in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum.

Flow cytometric analysis

Transfected RBL-2H3 cells were harvested in 0.526 mM EDTA buffer (Irvine Scientific, Santa Ana, California), resuspended in PBS with 10% fetal calf serum and 1 mg/mL goat IgG. The 10^6 cells were incubated with 1:200 diluted rabbit polyclonal antibody serum against the C3aR loop2 for 1 h on ice or normal rabbit serum as a negative control, washed with PBS for three times. Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) was then added at 1:250 dilution. The cells were further incubated on ice for 1 h, washed and assayed on a FACscan flow cytometer using CellQuest program (Beckton Dickinson, San Jose, California). Viable transfected cells were electronically gated based on forward (FSC) and side (SSC) light scatter.

Receptor binding

Iodination of C3a with ^{125}I was performed using the Iodo-beads iodination reagent (Pierce, Rockford, Illinois). For each duplicate measurement, $\sim 5 \times 10^5$ cells resuspended in Earl's balanced salt buffer (0.5% BSA, 10 mM Hepes, pH = 7.4) were mixed with various concentrations of ^{125}I -C3a and unlabeled C3a in a final volume of 100 μL . The mixture was incubated at room temperature for 60 min. Unbound ^{125}I -C3a was separated by sedimenting through a 100 μL phthalate oil cushion and the cells were counted in a COBRA Auto-Gamma counter (Packard Instrument, Co., Meriden, Connecticut). Curve fitting and statistical analyses for K_d determinations were conducted as follows: saturation binding curve was determined by nonlinear regression analysis using the GraphPad Prism program (GraphPad Software, Inc., San Diego, California). The program uses the least sum-of-squares method. Saturation binding curves best fitted the one-site binding model (hyperbola, $Y = B_{\text{max}} \times X/K_d + X$, where X is the concentration of the ligand) with curve fitting values ranging between 0.95 and 0.99. The K_d values are reported as the average of duplicate determinations. The receptors/cell values were calculated using the B_{max} from the curve fitting results and the specific activity of the radio-labeled ligand.

Calcium mobilization assay

Transfected RBL-2H3 cells ($\sim 5 \times 10^6$) were harvested in 0.526 mM EDTA buffer and loaded with 5 μM Indo-1 AM (Molecular Probes, Eugene, Oregon) by incubation at 37 $^\circ\text{C}$ for 30 min.

Intracellular free calcium was measured with $\sim 2 \times 10^5$ cells in a 0.25 mL final volume. Continuous fluorescent measurements of calcium-bound and free Indo-1 were made using an SLM 8000 photon counting spectrofluorometer (SLM-Aminco, Urbana, Illinois), as described previously (Chao et al., 1999). EC_{50} values were determined based on relative fluorescence values obtained in the range of 0.1 to 1,000 nM C3a using nonlinear regression analysis by the Graph Pad Prism program.

Molecular modeling

Human C3aR model was generated by using SWISS-MODEL program (Peitsch et al., 1996). The coordinates of each helix are generated by the protein modeling tool ProMod. A model of neuropeptide Y1 receptor, derived from human rhodopsin structure and supported by sufficient mutagenesis data (Du et al., 1997), was used as a template. A final energy minimization was performed to improve the stereochemistry and to remove the unfavorable contacts. Coordinates for the crystal structure of the C3a ligand (residues 13–77) were determined by Huber (Huber et al., 1980). All other computer graphic work was performed on MidasPlus program (UCSF, California) linked to an SGI work station.

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