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Role of the Second Extracellular Loop of Human C3a Receptor in Agonist Binding and Receptor Function*

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The C3a anaphylatoxin receptor (C3aR) is a G protein-coupled receptor with an unusually large second extracellular loop (e2 loop, ~172 amino acids). To determine the function of this unique structure, chimeric and deletion mutants were prepared and analyzed in transfected RBL-2H3 cells. Whereas replacement of the C3aR N-terminal segment with that from the human C5a receptor had minimal effect on C3a binding, substitution of the e2 loop with a smaller e2 loop from the C5a receptor (C5aR) abolished binding of ^{125}I-C3a and C3a-stimulated calcium mobilization. However, as much as 65% of the e2 loop sequence (amino acids 198–308) may be removed without affecting C3a binding or calcium responses. The e2 loop sequences adjacent to the transmembrane domains contain multiple aspartate residues and are found to play an important role in C3a binding based on deletion mutagenesis. Replacement of five aspartate residues in the e2 loop with lysyl residues significantly compromised both the binding and functional capabilities of the C3a receptor mediated by intact C3a or by two C3a analog peptides. These data suggest a two-site C3a-C3aR interaction model similar to that established for C5a/C5aR. The anionic residues near the N and C termini of the C3aR e2 loop constitute a non-effector secondary interaction site with cationic residues in the C-terminal helical region of C3a, whereas the C3a C-terminal sequence LGLAR engages the primary effector site in C3aR.

Human C3a is a 77-amino acid protein generated during activation of the complement cascade (1). The anaphylatoxin C3a together with C4a and C5a are involved in mediation of a variety of inflammatory responses (2, 3). C3a is chemotactic for eosinophils and basophils, whereas C5a is chemotactic for eosinophils, basophils, and neutrophils. It was recently reported that C3a activation causes release of effectors from eosinophils, which in turn activate neutrophils (4). Whereas C5a is more potent than C3a in phagocyte chemotaxis and most other functions, C3a can be generated at an approximately 20-fold higher concentration in plasma (5–7). Recent studies suggest that C3a can activate human mast cells (8, 9) and tonsilar lymphocytes (10). The underlying mechanisms for these newly described functions of C3a remain incompletely understood.

Biochemical and pharmacological studies indicate that both C3a and C5a bind and activate G protein-coupled receptors, which then transduce signals through heterotrimeric G proteins (6, 11). To date, our understanding of the anaphylatoxin receptors comes mostly from studies of the C5a receptor. Molecular cloning of C5a receptors from various species has revealed a primary structure containing seven putative transmembrane domains similar to that of other members of the rhodopsin-like G protein-coupled receptor superfamily (12–15). Members of this superfamily include recently cloned receptors for many other chemoattractants and chemokines. Studies using mutagenesis (16, 17) and blocking antibodies (18, 19) indicate that anionic aspartate residues located in the extracellular N-terminal region of the C5a receptor (C5aR)[1] constitute a non-effector interaction site with C5a. High affinity binding and function of the C5a protein requires an additional interaction of its C-terminal region with an effector site in the C5a receptor (20). The C-terminal region of C5a appears to interact with a binding pocket formed by a cluster arrangement of multiple transmembrane helices (20, 21).

The human C3a receptor (C3aR) contain an exceptionally large extracellular loop between the fourth and fifth transmembrane domains (22–24). Subsequent cloning of C3aR from mouse (25, 26), rat (27), and guinea pig (28) confirmed the presence of a large second extracellular loop (e2 loop) in all four species. Extracellular portions of G protein-coupled receptors are believed to provide the sites for interaction with their specific agonists. The extracellular N-terminal regions of G protein-coupled receptors for large glycoprotein hormones are known to be essential for agonist binding (29). Recent studies have also suggested a role for the extracellular loops of G protein-coupled receptors for peptide agonist in both binding and ligand specificity (30). Because the large e2 loop is a unique feature of C3aR, it may play a role in binding the cationic C3a molecule, especially in the absence of anionic clusters in the N-terminal region of the C3aR. In this study, we constructed chimeric and mutant C3a receptors by replacement or progressive deletion of the e2 loop. Analysis of these chimeric and mutant receptors by direct agonist binding and calcium mobilization assays suggested that the large e2 loop is necessary for high affinity C3a binding. Terminal ends on the e2 loop contain anionic residues that may interact with residues in the cationic C3a protein.

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[1] The abbreviations used are: C5aR, C5a receptor; C3aR, C3a receptor; e2 loop, the second extracellular loop; PCR, polymerase chain reaction; HA, hemagglutinin.

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Hu C3aR 162:EIFTTDNHNRCGYKFGLSSSLDYPDF-YG-D-PLENRSLENIVQ 202
Gp C3aR 162:ETFTLENHTICTYNFS-PGSFDYLDYAYARD-AWGYGTPDPIVQ 203
Rt C3aR 162:DLLVVDDYSVCGYNFDSSRAYDYWDYMYNSHLPEINPPDNS--- 202
Mo C3aR 162:DLFIMDNRSICRYNFDSSRSYDYWDYVYKLSLPESNSTDNSTAQ 205
                  * * *     * * *  *

Hu C3aR 203:RPGEMNDRLDP-SSFQTNDHPWTVPTVFQPQTFQRPSADSLPRG 245
Gp C3aR 204:LPGEMEHRSDP-SSFQTQDGPWSVTTTLYSQTSQRPSEDSFHMD 246
Rt C3aR 203:-TGHVDDRTAPSSSVPARD-LWTATTALQSQTFHTSPEDPFSQD 244
Mo C3aR 206:LTGHMNDRSAP-SSVQARDYFWTVTTALQSQPFLTSPEDSFSLD 248
          *       * **    *  *  *    *       *

Hu C3aR 246:SARLTSQNLYSNVFKPADVVSPKIPSGFPIEDHETSPLDNSDAF 289
Gp C3aR 247:SAKLSGQGKYVDV-----VLPTNL-CGLPMEENRTNTLHNA-AF 283
Rt C3aR 245:SA--SQQPHYGG--KPPTVLIATIPGGFPVEDHKSNTL-NTGAF 282
Mo C3aR 249:SA--NQQPHYGG--KPPNVLTAAVPSGFPVEDRKSNTL-NADAF 287
          **    *  *       *       * * *      * *  **

Hu C3aR 290:LSTHLKLFPSASSNSFYESELPQGFGDYYNLGQFTDDDQVPTP 332
Gp C3aR 284:LSSDLDV-SNATQKCLSTPEPPQDFWD--DLSPFTHEYRTPRL 324
Rt C3aR 283:LSAH-TEPSLT-ASSSPLY-AHDFPDDYFDQLMYGNHAWTP-- 321
Mo C3aR 288:LSAH-TELFPT-ASSGHLY-PYDFQGDYVDQFTYDNHVPTP-- 326
          **                  *              *
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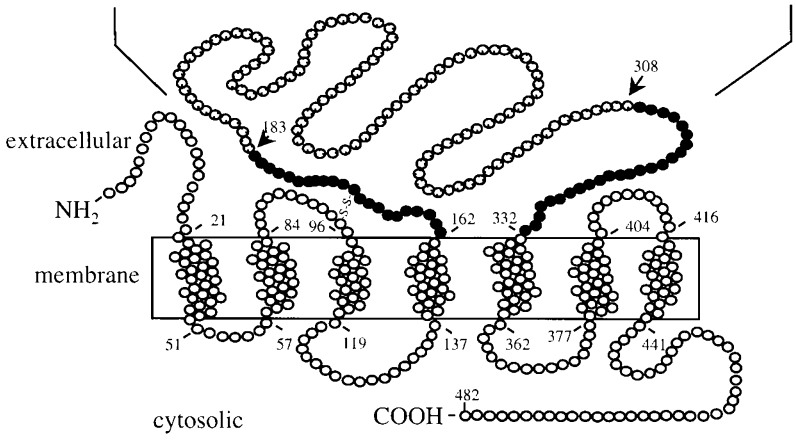
FIG. 1. **Comparison of the e2 loop sequences of C3a receptors from human (*Hu*), guinea pig (*Gp*), rat (*Rt*), and mouse (*Mo*).** The sequences (*top*) are aligned and identical amino acid residues are indicated by *asterisks*. Two regions containing clusters of negatively charged amino acids (Asp and Glu) are *boxed*. Transmembrane and loop structures (*bottom*) are assigned based on hydropathy analysis and comparisons with sequences of other G protein-coupled receptors. *Filled circles* (residues 162–183 and 309–332, corresponding to the sequences set in *bold*) represent sequences of the e2 loop essential for C3aR function as determined by deletion mutagenesis (see text). A disulfide bond between residues 95 and 172 is indicated (*-S-S-*). The sequences were obtained from the GenBank with accession numbers U28488, Z73157, and U62027 (human C3aR), U77460 and U97537 (mouse C3aR), U86379 (rat C3aR), and U86378 (guinea pig C3aR).



EXPERIMENTAL PROCEDURES

Materials—Native C3a was purified from expired human plasma (31). The peptide E7 (WWGKKYRASKLGLAR), the native C3a 18R peptide (ITELRRQHARASHLGLAR), and the octapeptide (AAALGLAR) were synthesized and characterized as described previously (32). Chemicals were purchased from Sigma. Restriction enzymes were obtained from Life Technologies, Inc.

Construction of Chimeric C3aR/C5aR and the e2 Loop Deletion Mutants—The N-terminal C3aR/C5aR chimeric receptor was constructed by substituting amino acids 1–21 of the human C3aR with amino acids 1–37 of the human C5aR. The e2 loop chimeric receptor was generated by replacing amino acids 163–332 of C3aR with amino acids 175–200 of the human C5aR. Briefly, the human C3aR cDNA AZ3B (22) and the human C5aR cDNA (12, 13) were used as templates for amplification by PCR of overlapping cDNA fragments with specific oligonucleotide primers. The fragments were then annealed and amplified again with primers corresponding to the 5′ and 3′ end sequences of the resulting chimeric molecule (33). The full-length PCR products were sequenced to confirm accuracy, digested with *Eco*RI, and cloned into the same site in the pSFFV-neo expression vector (34). To confirm surface expression of the C3aR/C5aR chimera, a hemagglutinin (HA) tag (YPYDVPDYA) was placed after the first methionine in the C3aR/C5aR chimera for detection by the anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim). By using the same PCR strategy, progressive deletions of the e2 loop of C3aR were accomplished, and the resulting cDNAs were subcloned into the same expression vector.

Site-directed Mutagenesis—Using the above PCR-based methodology, synthetic oligonucleotides Lys^{183}-Lys^{186} (5′-tacaaatttggtctctccagctcattaaaatatccaaaaatttttatggag-3′), and Lys^{325}-Lys^{326}-Lys^{327} (5′-ttaggccaattcacaaaaaaaaaacaagtgccaacac-3′), where mutated sequences are underlined, were used to construct the corresponding D183K/D186K, D325K/D326K/D327K, and D183K/D186K/D325K/D326K/D327K mutant C3a receptors. PCR products were then subcloned into the pSFFV-neo expression vector. DNA sequencing was performed to confirm accuracy.

Stable Expression in RBL-2H3 Cells—Ten μg of DNA was linearized with *Xba*I and transfected to RBL-2H3 (ATCC CRL-2256) cells using LipofectAMINE reagent (Life Technologies, Inc.). G418 was added to 300 μg/ml for selection and maintenance of stably transfected cell lines in Dulbecco's modified Eagle's medium supplemented with 10 mM Hepes, 100 mM nonessential amino acids, 2 mM L-glutamine, and 20% heat-inactivated fetal bovine serum (HyClone, Logan, UT).

Intracellular Ca^{2+} Measurement—Transfected RBL-2H3 cells ($\sim 5 \times 10^6$) were harvested using trypsin-free cell dissociation buffer (Life Technologies, Inc.) and loaded with 5 μM Indo-1 AM (Molecular Probes, Eugene, OR) by incubation at 37 °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, IL) detecting at 400 and 490 nm, respectively, with an excitation wavelength of 340 nm. Intracellular free calcium concentration ($[Ca^{2+}]_i$) was calculated as 250 $(F - F_{min})/(F_{max} - F)$, where 250 is the K_d of Indo-1 for calcium (in nM), F is the measured ratio of emission at 400 and 490 nm (A/B), F_{max} is the ratio A/B when Triton X-100 is added to the cells for release of the entire intracellular calcium storage, and F_{min} is the ratio A/B when EGTA is added to cells to chelate the cytoplasmic calcium (35).

C3a Binding Assays—Iodination of C3a with ^{125}I was performed using the IODO-BEAD iodination reagent (Pierce). The average specific activity of ^{125}I-labeled C3a was 520 Ci/mmol. The saturation binding curve was generated using increasing concentrations of labeled C3a with 100-fold excess concentration of unlabeled C3a. For each duplicate measurement, $\sim 5 \times 10^5$ cells resuspended in Earl's balanced salt buffer (10 mM Hepes, 0.5% bovine serum albumin, 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

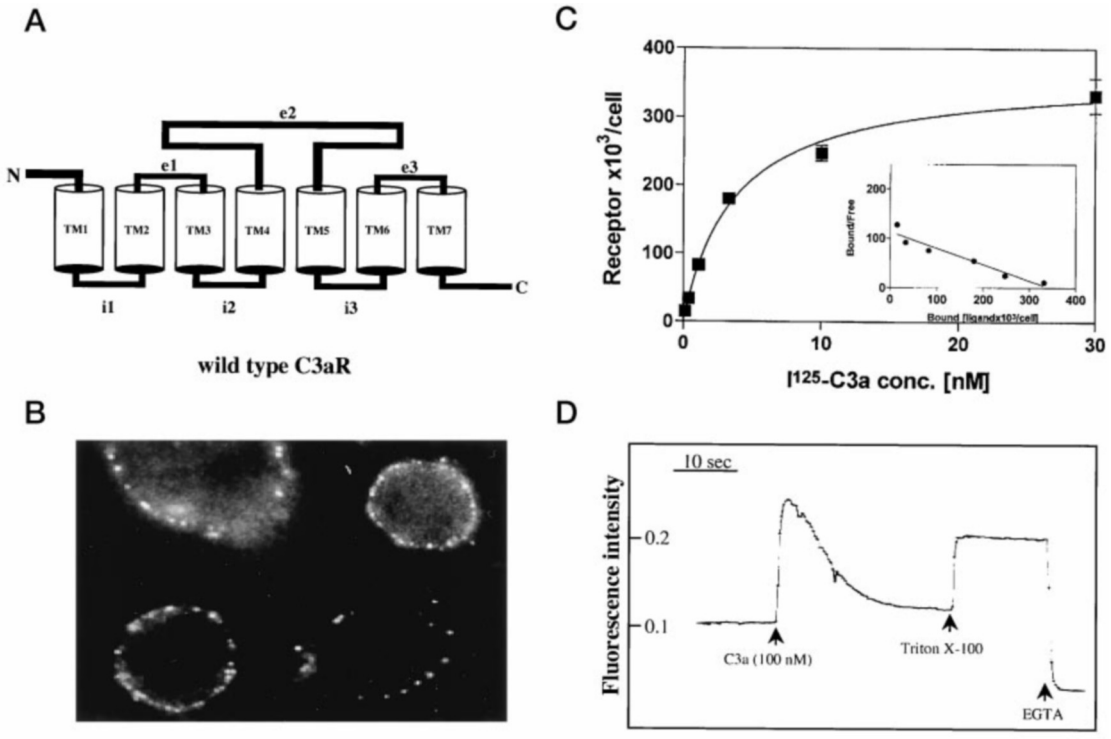


FIG. 2. **Exogenous expression and functional analysis of the human C3aR in RBL-2H3 cells.** *A*, schematic representation of the C3aR with the extracellular (*e*) and intracellular (*i*) loops marked and the transmembrane domains (*TM*) numbered. *B*, cell surface expression of the C3aR was detected by immunofluorescent staining using an anti-C3aR antibody and a rhodamine-conjugated second antibody as described under "Experimental Procedures." *C*, binding of ^{125}I-C3a by the C3aR in transfected cells. Data shown are the means of specific binding collected from three separate measurements, each performed in duplicate. *Inset*, Scatchard plot of the specific binding data. *D*, a representative result of calcium mobilization in the transfected cells in response to C3a. Relative fluorescent intensity was measured in cells loaded with Indo-1 AM. The experiment was conducted as described under "Experimental Procedures." *Arrows* mark the time of addition of the indicated ligand and reagents.

TABLE I
Characterization of C3aR/C5aR chimeras

Construct	K_d[a]	Receptor no./cell[a]	$[Ca^{2+}]_i$[b]
	nM		
Wild-type C3aR	3.85 ± 0.15	~360,000	Yes
HA-tagged wild-type C3aR	3.25 ± 0.05	~174,000	Yes
C3aR/C5aR N-terminal chimera	5.51 ± 0.60	~153,000	Yes
HA-tagged C3aR/C5aR e2 loop chimera	ND[c]	NA[d]	No

[a] Mean and S.E. values are based on specific binding of ^{125}I-C3a (n = 3).
[b] $[Ca^{2+}]_i$, intracellular calcium mobilization, was measured at 100 nM C3a.
[c] No detectable binding.
[d] Not applicable.

room temperature for 60 min. Unbound ^{125}I-C3a was separated by spinning through a 100-μl phthalate oil cushion. Curve fitting and statistical analysis were conducted as follows. The saturation binding curve was determined by nonlinear regression analysis using the GraphPad Prism program (GraphPad, San Diego, CA). The program uses the least sum-of-squares method. Saturation binding curves best fitted the one-site binding model (hyperbola, $Y = B_{max} \times X/K_d + X$, where X is the concentration of the ligand) with curve fitting values ranging between 0.95 and 0.99.

Immunofluorescent Staining—Transfected RBL-2H3 cells, grown on 18-mm glass coverslips in six-well culture dishes, were washed with 1× phosphate-buffered saline and fixed in 1% paraformaldehyde for 3 min. After blocking with 2% goat serum in phosphate-buffered saline for 20 min, the anti-HA tag, monoclonal antibody 12CA5, or the anti-C5aR-(9–29) polyclonal antibody against the human C5aR N terminus (18) was added to 2 μg/ml. The cells were incubated at room temperature for 1 h, washed briefly with phosphate-buffered saline, and incubated with rhodamine-conjugated secondary antibody at 7 μg/ml for an additional hour. Specimens were mounted and observed under a Nikon epifluorescent microscope.

Flow Cytometry—Transfected RBL-2H3 cells were harvested in trypsin-free cell dissociation buffer, blocked for 30 min on ice with 1% goat serum in phosphate-buffered saline, and incubated with a rabbit polyclonal antibody against C3aR for 1 h on ice. The antibody was generated against the entire e2 loop, which was expressed in *Escherichia coli* and used as immunogen. Fluorescein isothiocyanate-labeled goat anti-rabbit secondary antibody was then added at 1:250 dilution. The cells were further incubated on ice for an additional hour and assayed on a FACScan flow cytometer (Beckton Dickinson, Mountain View, CA).

RESULTS

A prominent feature of the human C3aR is a large extracellular loop between the fourth and fifth transmembrane domains (the e2 loop). Subsequent cloning of C3aR from mouse, guinea pig, and rat confirmed the presence of a large e2 loop in all four species (Fig. 1). A comparison between human C3aR and C5aR indicated that C3aR, unlike C5aR, does not have a highly anionic N-terminal segment, known to participate in the C5a-C5aR interaction. Therefore, the unique structural features represented by the large e2 loop in c3aR suggests that it may play a significant role in receptor-ligand interactions.

To examine this possibility, we utilized stable transfectants of RBL-2H3 cells that express the human C3aR for measuring agonist binding and calcium mobilization. Cell surface expression of the recombinant C3aR was confirmed by immunofluorescence staining with a rabbit antibody against the e2 loop of the C3aR. A typical periplasmic membrane staining pattern was observed in the transfected cells (Fig. 2*B*) but not in untransfected RBL-2H3 (not shown). Direct binding of the transfected cells with ^{125}I-labeled C3a revealed a high binding affinity (K_d = 3.85 ± 0.15 nM; Fig. 2*C* and Table I). C3a stimulation efficiently mobilized calcium with an EC_{50} of 2.52 ± 0.10 nM (Fig. 2*D* and Table II). These results indicated that the human C3aR was functionally expressed in the RBL-

TABLE II
Characterization of e2 loop deletion mutants of C3aR

Construct	Deletion region	K_d[a]	Receptor no./cell[a]	EC_{50}[b]
		nM		*nM*
Wild type C3aR	None	3.85 ± 0.1	~360,000	2.52 ± 0.10
Δ284–308	N-terminal	2.91 ± 0.1	~670,000	1.76 ± 0.15
Δ240–308	N-terminal	3.05 ± 0.1	~790,000	22.51 ± 1.04
Δ212–308	N-terminal	2.85 ± 0.1	~800,000	10.45 ± 0.86
Δ198–308	N-terminal	5.01 ± 0.2	~900,000	5.85 ± 0.70
Δ183–308	N-terminal	13.87 ± 2.7	~280,000	90.15 ± 2.22
Δ174–308	N-terminal	ND[c]	ND	NA[d]
Δ212–308	C-terminal	2.85 ± 0.1	~800,000	10.45 ± 0.86
Δ212–323	C-terminal	8.89 ± 0.6	~230,000	184.12 ± 11.50
Δ212–327	C-terminal	12.75 ± 0.4	~332,000	120.40 ± 10.85
Δ212–332	C-terminal	57.33 ± 9.9	~329,000	65.81 ± 2.01

[a] Mean and S.E. values are based on specific binding of ^{125}I-C3a ($n = 3$).
[b] Mean and S.E. values are based on intracellular calcium mobilization assay ($n = 3$).
[c] No detectable binding.
[d] Not applicable.

2H3 cells.

To investigate the role of the N-terminal segment and the e2 loop of C3aR in agonist binding, chimeric receptors were prepared by interchanging portions of the human C3aR and C5aR as illustrated in Fig. 3. Replacement of the N-terminal segment of C3aR (21 amino acids) with the equivalent segment from C5aR (37 amino acids) resulted in a very small effect to the binding affinity for C3a (Fig. 3*A, top panel*, and Table I). This structural change did not confer C5a binding capability on the chimera either (data not shown). Similarly, N-terminal tagging with HA had little influence on C3a binding (Fig. 3, *A* and *B*, *middle panels*, and Table I). Taken together, these results suggest that the C3aR differs from C5aR in its utilization of the N-terminal segment for agonist binding.

The role of the e2 loop in agonist binding was next examined in a chimera of C3aR in which the entire 172-residue e2 loop was replaced with the corresponding 25-residue loop from the human C5aR (Fig. 3*A, bottom panel*). Cell surface expression of the stable transfectants was confirmed by immunofluorescent staining with an anti-HA tag antibody (Fig. 3*B, bottom panel*). The transfected cells, however, displayed no specific binding for C3a, nor did the cells respond to C3a with calcium mobilization (Table I). Thus, replacement of the C3aR e2 loop with the e2 loop from human C5aR led to an apparent loss of C3aR functions.

The human C3aR shares approximately 40% overall sequence homology with human C5aR. Published data indicate that these two receptors specifically respond to their respective agonists on both peripheral blood leukocytes and transfected mammalian cells. To examine whether exchanging the e2 loops between these two receptors produced altered binding specificity, we evaluated binding and calcium mobilization using human C5a as the agonist. No binding or calcium response to C5a was detected in cells expressing the C5a e2 loop replacement in C3aR mutant (data not shown). These results suggested that replacement of the entire e2 loop in C3aR fails to change binding specificity of this receptor from C3a to C5a.

Results from the above experiments indicated that the large e2 loop in C3aR was required for the binding of C3a. However, although the large e2 loop was present in C3a receptors from the four species cloned to date, a comparison of the e2 loop sequences from human, mouse, guinea pig, and rat revealed less sequence homology than exists in other regions of the receptor. For example, the human and mouse C3a receptors share 65% identity overall, but the e2 loop regions have only a 45% sequence identity (25, 26). This finding raised the question of whether only a limited portion of the e2 loop is important for C3a binding. To address this issue, mutants created by sequential deletion of portions of the e2 loop sequence from the human C3aR were designed (Fig. 4). Six deletion mutants were created by systematic removal of N-terminal segments beginning at residue 308 near the C terminus of the e2 loop (Fig. 4*A*). Analysis of these modified receptors in transfected RBL-2H3 cells showed that four of the six mutant receptors were almost fully functional in agonist binding and calcium mobilization assays, compared with the wild-type C3aR (Table II). Deletion of sequences N-terminal to residue 197 resulted in a receptor with much reduced binding affinity and calcium mobilization capability as reflected by marked increases in the K_d and EC_{50} values for Δ183–308 (Table II). Removal of the additional nine residues between 174 and 183 led to a receptor (Δ174–308) essentially devoid of all functions. Deletion of this stretch of the C3aR sequence (amino acids 174–182) likely disrupted the global conformation of C3aR because of its proximity to a conserved cysteine residue at position 172. This deletion also resulted in poor expression of the C3aR mutant in transfected cells as determined by immunofluorescent microscopy using an anti-HA tag (data not shown). Thus, deletion mutagenesis suggested that the N terminus of the e2 loop contains a segment between residues 182–197 that is necessary for high affinity C3a binding.

Four additional mutant receptors were constructed by progressive deletion of residues 212–332 near the C terminus of the e2 loop in C3aR (Fig. 4*B*). When analyzed in transfected RBL-2H3 cells, all but one mutant receptor (*i.e.* Δ212–308) displayed altered binding and calcium mobilization capabilities (Table II). There was a gradual decrease in C3a binding because residues 309–331 were deleted from this region of the e2 loop. These deletions also reduced the calcium response (Table II). These results suggested that deletions at the C terminus of the e2 loop of C3aR also altered C3a binding.

Because deletion of a large portion of the e2 loop (from residues 198–308, 111 residues or 65% of the e2 loop) did not significantly affect C3a binding, we concluded that only the N and C termini of the e2 loop contained elements necessary for C3a-C3aR interaction. Charged residue side chains play important functions in receptor-ligand interaction as reported in other studies of G protein-coupled receptors including C5aR (21). An examination of the e2 loop sequence identified several negatively charged residues in the same N- and C-terminal regions that were shown to affect function when deleted (Fig. 1, *boxed areas*). This includes an aspartic acid triplet located a few residues from the boundary of the fifth transmembrane domain (Fig. 1, *second boxed area*). To test the function of these residues, the triplet was replaced with three lysyl residues that carry opposite charges on the side chain (Fig. 5). Functional

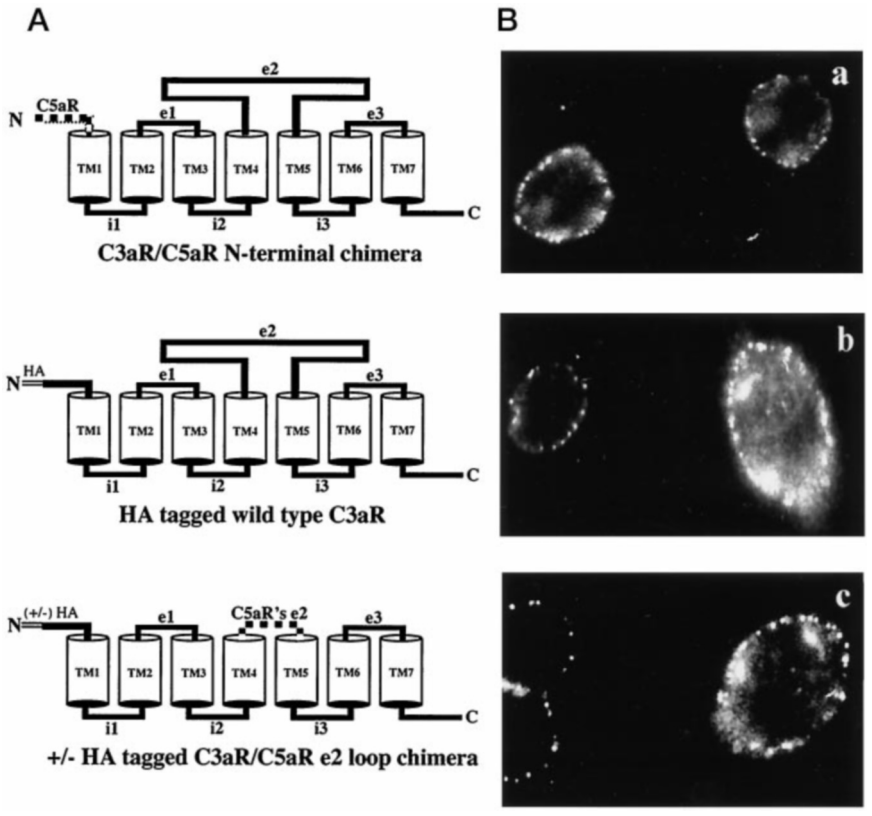


FIG. 3. **Construction and expression of C3aR/C5aR chimeric receptors.** *A*, schematic representation of the C3aR constructs with or without HA tagging. The C3aR/C5aR N-terminal chimera (*top panel*) was made by substituting the N-terminal sequence of C3aR (amino acids 1–21) with the corresponding sequence from C5aR (amino acids 1–37). The *middle panel* depicts an N-terminal HA-tagged (YPYDVPDYA) wild-type C3aR. A C3aR/C5aR e2 loop chimera (*bottom panel*) was prepared by replacing the entire e2 loop of C3aR (amino acids 162–332) with its corresponding loop from C5aR (amino acids 175–200). A similar construct was made by adding the HA tag to the N terminus, and this construct was used for immunofluorescent staining. *B*, confirmation of cell surface expression of these receptors by immunofluorescent staining. Shown in each *panel* are representative cells transfected with the corresponding constructs on the *left* side. An anti-C5aR polyclonal antibody was used for detecting expression of the C3aR/C5aR N-terminal chimera. The anti-HA monoclonal antibody 12CA5 was used for the other two receptors. Rhodamine-conjugated secondary antibodies were used for detection of fluorescence.

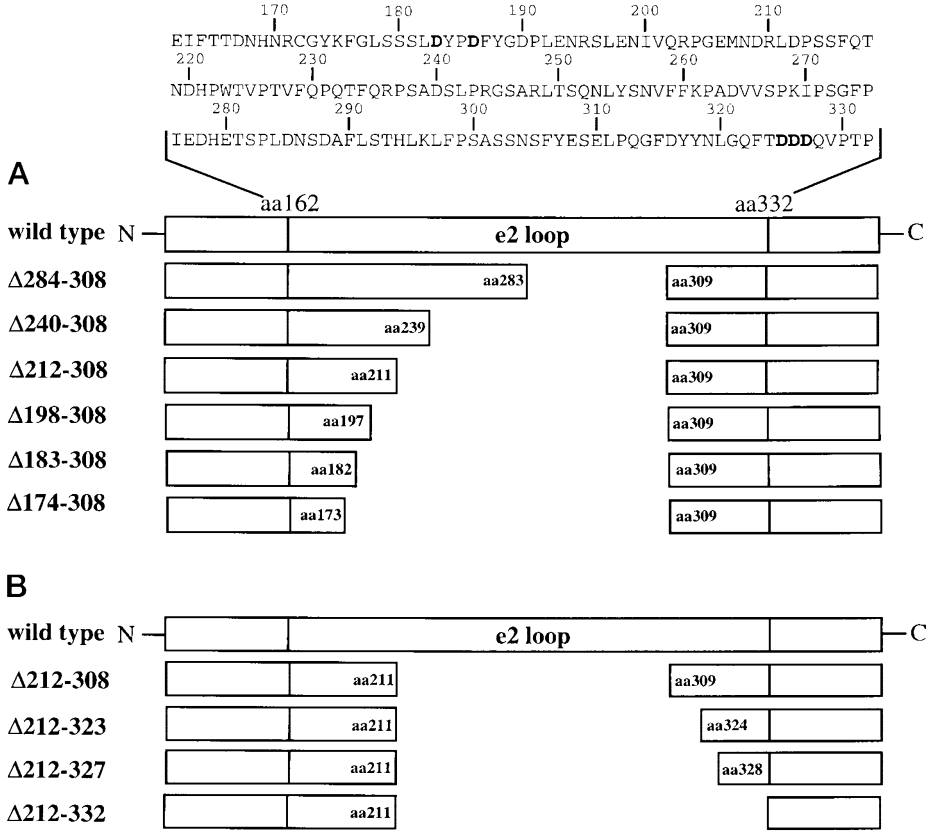


FIG. 4. **Schematic representation of the N-terminal and C-terminal deletions of the e2 loop.** The e2 loop sequence is shown at the *top*. *A*, the N-terminal e2 loop deletion mutants were constructed by progressive deletion of the e2 loop from residue 308 to 173. *B*, the C-terminal e2 loop deletion mutants were constructed by progressive deletion from residue 212 to 332. Both groups of deletion mutants were constructed by alteration of the C3aR cDNA and subsequent expression in RBL-2H3 cells, as described under "Experimental Procedures."

analysis of this mutant receptor (D325K/D326K/D327K) revealed a 6-fold decrease in C3a binding affinity (K_d increased from 3.85 to 22.12 nM) and a 14-fold drop in calcium mobilization capability (EC_{50} values changed from 2.52 to 34.56 nM), suggesting that these negatively charged residues on C3aR contribute to C3a binding (Table III). The effect of D325K/D326K/D327K changes on C3a binding results from either important alterations in charge-charge interactions within

C3aR or between C3a and its receptor. Because the N and C termini of the e2 loop should be proximal to one another at the membrane surface, we also examined the function of anionic residues located near the amino end of the e2 loop. Aspartic acids at positions 183 and 186 of the e2 loop have been conserved in C3aR from four different species (Fig. 1), and these residues are also located in a segment that affects C3a binding when deleted (Table II). Replacement of these two residues by cationic lysines resulted in a receptor (D183K/D186K) having a nearly 4-fold decrease in binding affinity and a 40-fold decrease in calcium response to C3a (Table III). The resultant receptor was also expressed at a lower density on the cell surface, which may be partially responsible for the reduced calcium mobilization. These results suggest a critical role for aspartic acid residues at the N terminus of the e2 loop, in either C3a binding or maintenance of overall receptor structure.

Finally, the combined effect of Asp^{183}-Asp^{186} and Asp^{325}-Asp^{326}-Asp^{327} replacement by lysines in a mutant C3aR (D183K/D186K/D325K/D326K/D327K) was measured. Whereas the resultant receptor was expressed on the cell surface, as determined by flow cytometry with an anti-C3aR antibody (Fig. 5*B*) and evidenced by low level calcium mobilization in response to C3a (Fig. 5*C*), the binding affinity for C3a was elevated to micromolar range (Table III). These results suggested interaction between some or all of the negatively charged residues in the e2 loop of C3aR and positively charged residues on the C3a molecule. To examine this possibility, short C3a analog peptides, that have previously been shown to activate C3aR (32), were used in the calcium mobilization assays to evaluate wild-type and the D183K/D186K/D325K/D326K/D327K mutant C3a receptors. The first two analog peptides employed here are E7 (WWGKKYRASKLGLAR) and C3a-derived 18R (ITELRRQHARASHLGLAR), both having positively charged residues N-terminal to the functionally essential sequence LGLAR. Like native C3a, these two peptides were much less effective in stimulating calcium mobilization from cells expressing the D183K/D186K/D325K/D326K/D327K mutant receptor than from cells expressing the wild-type C3aR (Fig. 5*C*, compare *right panels* with *left panels*). A semiquantitative analysis of the extent of calcium mobilization indicated that the intact C3a was 35–40% active on the D183K/D186K/D325K/D326K/D327K mutant receptor as compared with the wild-type C3aR, whereas the two peptides retained only ~2% activity on the mutant receptor. This difference may be a result of additional receptor contact sites in native C3a that are missing from the much shorter analog peptides.

Having established that substitution of five aspartic acid residues in the e2 loop by lysyl residues reduced the activity of both C3a and two C3a analog peptides containing cationic residues, we next examined whether peptides without the cationic resides were affected by the Asp to Lys replacement. The octapeptide AAALGLAR can activate C3aR when used at micromolar concentrations (Fig. 5*C*, *bottom row*). Approximately 65–70% of this activity was retained when the D183K/D186K/D325K/D326K/D327K mutant C3aR was evaluated by the calcium mobilization assay. These results indicated that a C3a effector peptide lacking additional cationic residues was minimally impacted by the Asp to Lys replacement in C3aR. These results support our hypothesis that charge-charge interactions between C3aR and C3a involve one or more of the aspartic acid side chains at positions 183, 186, 325, 326, and 327 of the e2 loop in C3aR.

DISCUSSION

Little was known about the function of the large extracellular loop of the C3aR, a unique feature of this G protein-coupled receptor. In this study we examined the role of the e2 loop of C3aR in agonist binding and receptor activation. Construction and analysis of chimeric receptors between C3aR and C5aR led

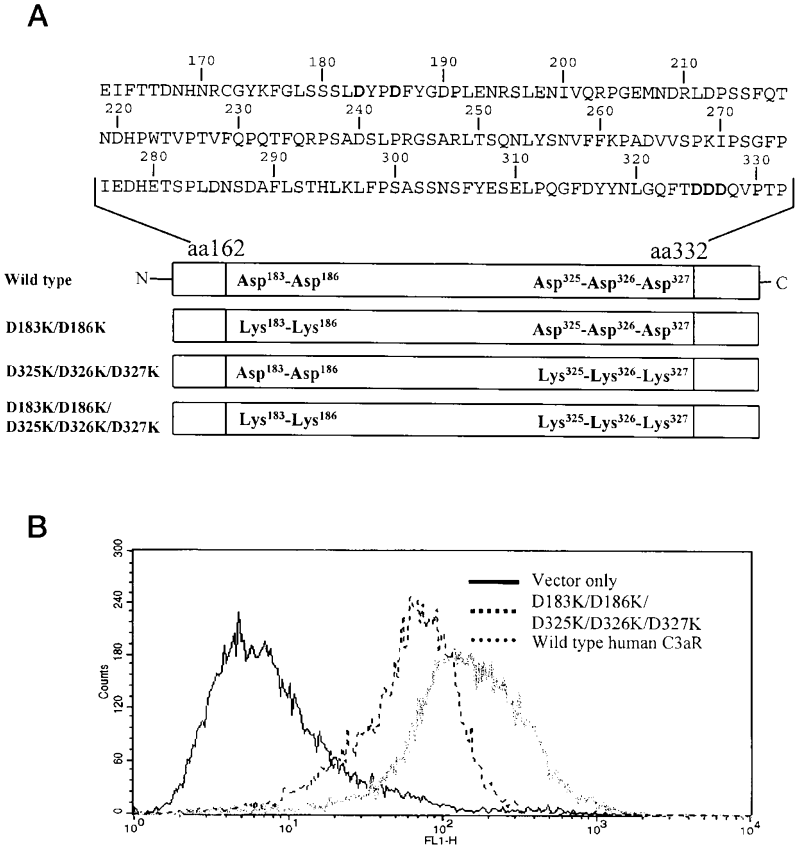


FIG. 5. **Construction and analysis of C3aR with Asp → Lys mutations.** *A*, schematic representation of the three Asp → Lys mutant C3a receptors. The Asp and Lys residues are marked in their relative positions within the e2 loop. The e2 loop sequence is shown on the *top*, with the candidate Asp residues in *bold*. *B*, flow cytometric analysis of RBL-2H3 cells stably transfected with the D183K/D186K/D325K/D326K/D327K mutant C3aR (*dashed line*), wild-type C3aR (*dotted line*), and the expression vector only (*solid line*). The cells were incubated with a rabbit polyclonal antibody directed to the e2 loop and stained with a fluorescein isothiocyanate-labeled goat anti-rabbit IgG secondary antibody, as described under "Experimental Procedures." *C*, calcium mobilization in RBL-2H3 cells transfected with the D183K/D186K/D325K/D326K/D327K mutant C3aR (*right panels*) and the wild-type C3aR (*left panels*) (on next page). Representative traces are shown based on three independent measurements. The intact C3a (100 nM, *top row*), E7 peptide (200 nM, *second row* from *top*), 18R peptide (200 nM, *third row* from *top*), and the octapeptide (10 μM, *bottom row*) were added at the times indicated. Relative fluorescence intensity is shown.

to the conclusion that the e2 loop but not the N-terminal region of the C3aR is indispensable for C3a binding. Because sequences of the e2 loop are the least conserved across species, progressive deletion was adopted to localize the specific regions of the loop important for C3a binding. It was surprising to find that nearly two-thirds of the loop sequence could be removed without affecting C3a binding. This finding parallels a recent study reporting the presence of an isoform of the guinea pig C3aR, which lacks 35 amino acids (residues 254–288) from the large extracellular loop and maintains activity (36). The observation that this shorter C3aR isoform also binds C3a with high affinity was in agreement with our own result, further indicating that part of the C3aR loop structure is not essential for agonist binding. Only when portions of the e2 loop sequences near the transmembrane domains were deleted did the resulting receptors display reduced binding affinity for C3a. Data from our subsequent studies suggested that anionic residues at the terminal ends of the e2 loop likely form contact sites for C3a. Our findings differ from a recent report claiming that residues 185–193 of the e2 loop represent an immunodominant domain but not a ligand-binding region because antibodies interacting with this region failed to block C3a-induced calcium mobilization (37). In contrast, our data showed that when sequences within this region (residues 174–197) were deleted or when the aspartate residues were replaced by lysyl residues, both calcium mobilization and agonist binding decreased. Consequently, we conclude that both the N- and C-terminal ends of the e2 loop are involved in agonist binding.

Previous studies of the C5a receptor resulted in the identification of charged amino acids in the second extracellular loop, the fifth transmembrane domain, and the N-terminal regions that serve as contact sites for C5a binding (17, 21, 38). These results support a two-site binding model, which was proposed earlier based on both biochemical and functional assays (39). The model predicts the presence of a non-effector or internal recognition site and an effector or activation site within the C5a protein, which interact with corresponding binding sites on C5aR. With the cloning of the C5aR cDNA, it has been

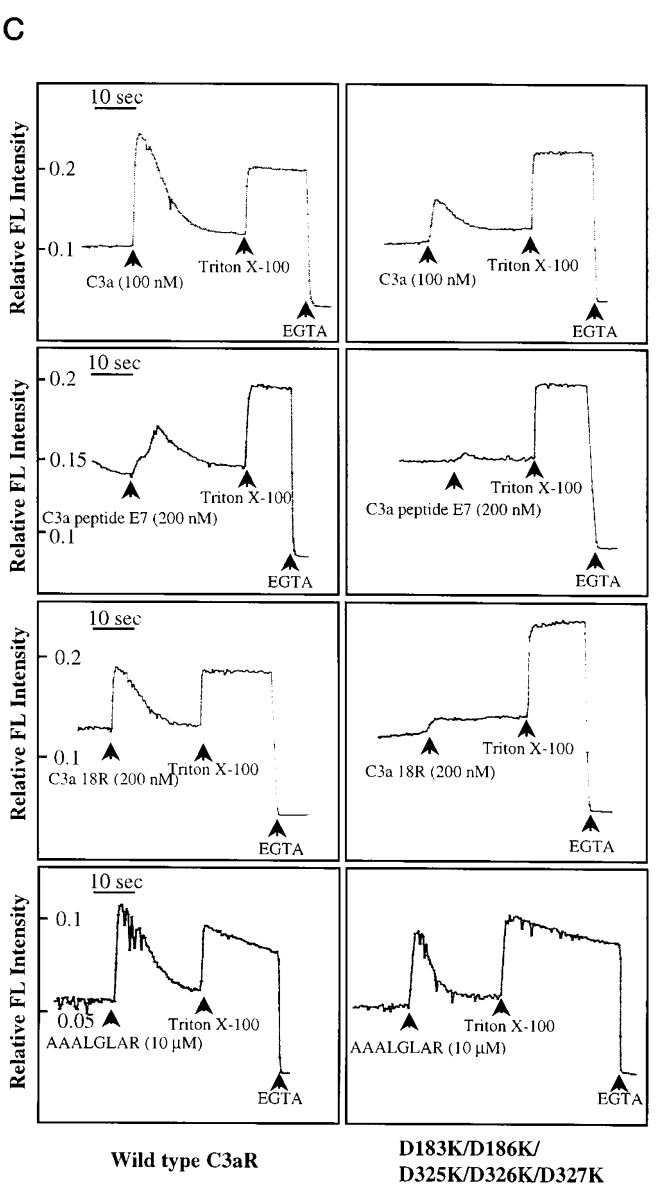


FIG. 5—*continued*

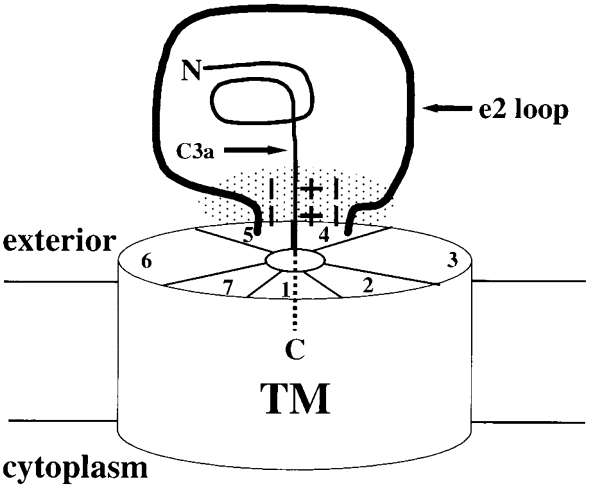


FIG. 6. **A model proposed for the interaction of C3a with its receptor.** The receptor is schematically shown as a cylinder divided into seven sections corresponding to the seven transmembrane domains. The two negatively charged regions of the receptor's e2 loop are marked by − signs and highlighted in the *shaded area*. Positively charged residues in the C3a molecule near the C-terminal effector region are indicated by + signs. The model depicts possible charge-charge interactions between C3a and its receptor. This schematic has been adapted from Ember and Hugli (7).

TABLE III
Characterization of D183K/D186K, D325K/D326K/D327K, and D183K/D186K/D325K/D326K/D327K mutants of C3aR

Construct	K_d[a]	Receptor no./cell[a]	EC_{50}[b]
	nM		*nM*
Wild-type C3aR	3.85 ± 0.15	~360,000	2.52 ± 0.10
D325K/D326K/D327K	22.12 ± 2.38	~216,000	34.56 ± 1.45
D183K/D186K	14.21 ± 1.04	~55,000	101.03 ± 5.62
D183K/D186K/D325K/D326K/D327K	>1 μM	ND[c]	210.03 ± 15.42

[a] Mean and S.E. values are based on specific binding of ^{125}I-C3a (n = 3).
[b] Mean and S.E. values are based on intracellular calcium mobilization assay (n = 3).
[c] Not determined.

shown that negatively charged residues in the e2 loop, as well as in the N-terminal region of C5aR, interact with positively charged residues in C5a at positions 12, 37, and 40 and in the effector region near the C terminus. Interactions between C5aR and the non-effector site residues 12, 37, and 40 in C5a likely are followed by insertion of the C-terminal effector domain of C5a into a binding pocket formed by the C5aR transmembrane domains, thereby leading to receptor-mediated cellular activation (20, 21). These findings provide important clues for our understanding of C3a-C3aR interactions. There are similarities between the modes of action for C3a and C5a, *e.g.* each having an effector site at the C terminus and cationic residues in other parts of the molecules that participate in binding. However, there are major structural differences as well between C3aR and C5aR. The human C3aR has a relatively short (21 amino acids) N-terminal region containing only one aspartate residue, which does not align with aspartate residues at the N-terminal end of C5aR. In our studies, replacement of the C3aR N-terminal region with the N-terminal region of C5aR resulted in a chimeric C3aR that displays wild-type levels of binding affinity for C3a. This finding suggested that the N-terminal region of the human C3aR does not play as important a role in agonist recognition as was the case for C5aR. Likewise, C3aR and C5aR interact only with their respective agonists and retain high specificity even in chimeric form. Replacement of either the N-terminal region or the e2 loop of the C3aR with the equivalent structure from the human C5a did not confer C5a binding capability. These results differ from a previous report indicating a promiscuous response for anaphylatoxins in *Xenopus* oocytes (40).

Our combined results support a model that depicts a non-effector C3a binding/recognition site in the extracellular loop of the receptor, as proposed in a recent review article (7). This recognition site contains negatively charged aspartate residues in the N and C termini of the e2 loop. These anionic residues may interact with positively charged amino acid side chains near the C terminus of the C3a molecule, thus facilitating agonist binding (Fig. 6). Our results indicate that the aspartate residues in both terminal segments of the e2 loop play a concerted role in C3a binding. Significant C3a binding was maintained when only one of the two aspartic residue clusters in the e2 loop was replaced by lysyl residues, but not when both were replaced. However, complete replacement of the five anionic residues (D183K/D186K/D325K/D326K/D327K mutant) caused a very significant reduction in C3a binding. Results obtained from calcium mobilization studies using synthetic C3a analog peptides also supported the presence of charge-charge interactions between the wild-type C3aR and these peptide agonists.

Similar to the intact C3a molecule, the peptides E7 and 18R contain the effector sequence LGLAR at their C-terminal end and two to three cationic residues near the N terminus. In comparison with C3a, the peptides were much less effective in mobilizing intracellular calcium in the transfected cells expressing the D183K/D186K/D325K/D326K/D327K mutant receptor than in the wild-type C3aR. It is possible that there are multiple, cooperating recognition sites for C3a-C3aR interaction. The localization of one of these sites in the e2 loop of the human C3aR should facilitate the identification of other agonist recognition and receptor activation sites within the C3aR.

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