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Molecular Cloning of Two Isoforms of the Guinea Pig C3a Anaphylatoxin Receptor: Alternative Splicing in the Large Extracellular Loop

Yoshihiro Fukuoka,*† Julia A. Ember,* and Tony E. Hugli3*

The anaphylatoxin C3a is released from C3 during complement activation. C3a is a potent spasmogen and has recently been described as an eosinophil and mast cell chemotactic factor that mediates a number of inflammatory reactions. Previously, we demonstrated the presence of a specific C3a receptor (C3aR) on guinea pig platelets. We report here the isolation of cDNA clones encoding for two isoforms of guinea pig C3aR (gpC3aR). Hydropathy analysis of the deduced amino acid sequence of both gpC3aR clones indicated seven transmembrane domains with a large extracellular (EC) loop between the fourth and fifth transmembrane domains, which is a known characteristic of the human C3aR. Northern blot analysis revealed that the gpC3aR was abundantly expressed on macrophages and in the spleen. A comparison of the deduced amino acid sequence of the larger gpC3aR (gpC3aR-L) with the recently cloned human C3aR indicated a 59.5% identity. The deduced amino acid sequence of the second, smaller cDNA clone was identical with gpC3aR-L, except that it lacked 35 amino acids in the large EC loop. Our evidence indicates that alternative splicing occurred in the large EC loop that accounts for these two isoforms. L cells separately expressing one of these two isoforms of the gpC3aR showed similar high-affinity C3a binding. An RT-PCR analysis documented that both forms of the C3aR were expressed in a variety of guinea pig tissues. The cloning and expression of these two natural forms of gpC3aR cDNA indicated that the deletion of the 35-residue portion of the large EC loop of gpC3aR-L did not alter C3a binding. The Journal of Immunology, 1998, 161: 2977–2984.

Potent bioactive fragments are released during the activation of the complement cascade when they are initiated by immune and inflammatory reactions. One of these fragments, anaphylatoxin C3a, is generated by proteolytic cleavage of the C3 molecule. C3 is a major plasma protein, and as much as 60 μg/ml of C3a can be generated in activated human serum. In addition to smooth muscle contraction and spasmodic activities, C3a stimulates the release of histamine from mast cells (1) and basophils (2), induces cytokine release from macrophages Mφ (3), enhances the generation of reactive oxygen products in eosinophils (4) and neutrophils (5), promotes the chemotaxis of eosinophils (6) and mast cells (7, 8), and activates guinea pig platelet release and aggregation (9). It has also been shown that C3a can alter rat brain function (10) and promote lung injury in vivo (11). C3a reportedly suppresses Ab production in mice (12), and it was reported recently that both C3a and C3aΔεψαβε can regulate the LPS-induced synthesis of TNF-α and IL-1β (13). A number of clinical studies have strongly suggested that C3a participates in the regulation or modulation of various immune and inflammatory responses (14–16).

Human C3a receptor (C3aR) cDNA was recently cloned (17–19) and was shown to belong to the rhodopsin family of G protein-coupled receptors; these receptors have seven transmembrane (TM) domains, which is similar to the receptor for an analogue protein, C5a. However, human C3aR is unique in that it contains a large extracellular (EC) loop of 175 amino acids (aa) between the fourth and fifth TM domains, which is a feature that is rarely found in other receptors of this family. It was suggested that this unusual feature may play a special role in ligand binding.

It is well known that guinea pig tissue and guinea pigs in general are highly responsive and sensitive to C3a; consequently, guinea pigs are frequently used for the investigation of anaphylatoxin-induced inflammatory diseases. Injecting human C3a along with a carboxypeptidase B-type inhibitor causes shock and sudden death of the guinea pig (20). Guinea pig ileal contraction and platelet aggregation are responses that are commonly used to measure C3a activity (1). We previously demonstrated a C3a-C3aR complex in guinea pig platelets using chemical cross-linking (9). Furthermore, the phosphorylation of a guinea pig platelet protein was induced by C3a stimulation (21). The presence of the C3aR on guinea pig Mφ was reported previously using a functional assay (22, 23). We now report the molecular cloning of two forms of guinea pig C3aR (gpC3aR) and provide evidence for alternative splicing as a mechanism that results in the deletion of a part of the large EC loop. The functional characterization of the two expressed forms of gpC3aR indicates that this deletion in the large EC loop has virtually no effect on C3a-binding affinity. The cloning of these gpC3aR genes provides us with new opportunities for characterizing the role of C3aR in inflammation.
Materials and Methods

Genomic DNA analysis and screening of guinea pig spleen cDNA library

Genomic DNA was prepared from guinea pig liver, and 3 μg of DNA was digested by restriction enzymes at 37°C for 24 h. The samples were electrophoresed on 0.8% agarose gel in Tris-acetate EDTA buffer and blotted to Hybond N* nylon membrane (Amersham, Arlington Heights, IL). Human C3αR cDNA was generated by RT-PCR using human C3αR-specific primers that were based on the reported sequence (17–19) and cDNA that was prepared from PMA-differentiated U937 cells. The sequence of the sense primer was 5′-GTAAGGCCTTTTCTCTGCGACCAAT-3′ (base pairs (bp) 1–27), and the sequence of the antisense primer was 5′-GGCT GCTCCACATTTTCACAGTGACTC-3′ (bp 1465–1435). After the PCR product had been confirmed as C3αR cDNA by sequence analysis, it was labeled with [α-32P]deoxyCTP using a prime-It II random primer labeling kit (Stratagene, La Jolla, CA). Hybridization was performed with 32P-labeled human C3αR cDNA in QuikHyb solution (Stratagene) for 1 h at 65°C. The membranes were washed once in 0.1% SSC at room temperature for 30 min and twice in 0.5% SSC at 60°C for 15 min; they were then exposed to x-ray film (X-OMAT, Kodak, Rochester, NY) using an image intensifying screen at −70°C for 24 h. The cDNA library that was constructed by the ZAP II vector (Stratagene) with poly(A)+ RNA that had been isolated from guinea pig (Hartley) spleen was kindly provided by Dr. M. Nonaka (Nagoya City University, Nagoya, Japan). Approximately 5 × 108 plaques were transferred to a Colony/Plaque Screen membrane (New England Nuclear, Boston, MA) and probed with 32P-labeled human C3αR cDNA. Hybridization was performed in 5× SSC, 0.02% SDS, 0.1% Sarkosyl (Fluka, Ronkonkoma, NY), 1% blocking reagent (Boehringer Mannheim, Indianapolis, IN), and 0.1 mg/ml salmon sperm DNA (Stratagene) at 60°C for 16 h as described previously (24). The membranes were washed twice in 2× SSC and 0.5% SDS at room temperature for 15 min and then twice in 1× SSC and 0.5% SDS at 60°C for 20 min. The positive plaques were purified and transformed into plasmids by the automatic excision process of ZAP II arms using R408 helper phage (Stratagene) according to the manufacturer’s instructions. The cloned fragments were sequenced using an automated DNA sequencer (373A, Applied Biosystems, Foster City, CA). The sequence results were assembled and aligned using GeneSect-max software, (GeneSect-max Software Development, Tokyo, Japan).

Expression of cloned cDNA in mammalian cells

The coding region of the gpC3αR cDNA was amplified using a 5′-specific primer containing a HindIII restriction site and a 3′-specific primer containing an XhoI site. The PCR product was subcloned into the HindIII and XhoI restriction sites of a mammalian expression vector, pcNYB, that was kindly provided by Dr. J. Miyazaki (Osaka University, Osaka, Japan) and was transfected into pCV108-neo into mouse L cell fibroblasts using Lipofectin (Life Technologies, Gaithersburg, MD). Cells were grown in DMEM containing 800 μg/ml of G418 (Life Technologies) for 2 wk to select the resistant clones and were subsequently maintained in DMEM containing G418 at 300 μg/ml.

RNA analysis

Total RNA was separated from different guinea pig tissues, and peritoneal MDC were elicited by an i.p. injection of paraffin oil using an RNAeasy kit (Qiagen, Chatsworth, CA). Total RNA was separated as described above; sscDNA was synthesized using the Superscript preamplification system for first strand cDNA synthesis (Life Technologies) according to the manufacturer’s instructions. The sense primer 5′-CGTTCACCTA GAAAACCCAAG-3′ (bp 488–510) and the antisense primer 5′-TGCCGCTCAATTTCCACAGGACCTA-3′ (bp 943–919) were synthesized to amplify the cDNA of the large EC loop of gpC3αR. The PCR was performed with 2 μl of cDNA, 0.25 μg of each primer, 0.2 mM deoxynucleotide triphosphates, reaction buffer (Stratagene), and 1.5 U of Taq DNA polymerase (Stratagene) for 30 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min. The PCR products were visualized on a 1% agarose gel with ethidium bromide fluorescence. As a control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified using human primers (Stratagene).

Ab production and flow cytometry

Antiserum was raised against a synthetic peptide based on the large EC loop region of gpC3αR, AWYGTPDVPLQPG (residues 192–202). Lysine and alanine were added to permit the conjugation of the peptide to keyhole limpet hemocyanin with glutaraldehyde. A total of 800 μg of the conjugate that had been emulsified in CFA or IFA were injected s.c. into New Zealand white rabbits. The reactivity of the Abs produced was determined using a peptide-specific ELISA as described previously (25).

Cells were suspended in FACS buffer (HBSS containing 10% FCS, 1 mg/ml goat IgG, and 0.1% NaN3) and incubated with anti-gpC3αR serum and guinea pig immune rabbit serum at 1:100 dilution for 1 h. After washing with PBS, cells were incubated with the F(ab′)2, fragments of FITC-conjugated goat anti-rabbit IgG (Jackson Immunosresearch Laboratories, West Grove, PA) on ice for 1 h. Cells were analyzed on a FACScan cytometer (Becton Dickinson, San Jose, CA) using the CellQuest program.

Binding assay

C3α and C3αdesArg were isolated from activated human serum as described previously (26). The iodination of C3α and C3αdesArg with 125I was performed using the Iodo-beads Iodination Reagent (Pierce, Rockford, IL). The average specific activity of the labeled material was 420 Ci/mmol. Competitive binding was determined by incubating transfected L cells (5 × 105) for 60 min at room temperature in 200 μl of Earl’s balanced salt solution containing 0.5% BSA with 1 nM 125I-labeled human C3α and in the presence of increasing concentrations of unlabeled human C3α. The carboxyl terminus of human C3α, WWGKKYRASKLGGLAR (27). Unbound, labeled C3α was separated by sedimentation through a mixture of a dibutyryl- and dioctyl-phthalate oil layer for 1 min. The cell-bound 125I-labeled human C3α were counted in a Cobra Autogamma counter (Packard Instrument Company, Meriden, CT). Under these conditions, the 50% inhibitory dose (IC50) was determined based on a nonlinear regression analysis using the Prism program (GraphPad Software, San Diego, CA). Saturation binding was determined by incubating increasing quantities of 125I-labeled human C3α ± 100-fold excess of unlabeled human C3α as described above. The saturation-binding curve was used to determine Kd and receptor density based on nonlinear regression and Scatchard analysis using the GraphPad Prism program. In separate experiments, stably transfected L cells were preincubated with protein A-purified anti-gpC3αR Ab (0.5–2.3 μg/ml) or control IgG for 20 min at room temperature before the 125I-labeled C3α-binding assay was performed.

Intracellular (IC) Ca2+ measurement

L cells that had been stably transfected with gpC3αR cDNA were harvested and resuspended at 3 × 105 cell/ml in HBSS and then loaded with 5 μM of Indo-1/AM (Molecular Probes, Eugene, Oregon) for 30 min at 37°C. Cells were washed twice and resuspended in HBSS at 1.5 × 106 cell/ml. Cells were diluted 20 times with HBSS, placed in a continuously stirred cuvette at 37°C, and monitored in an SLM-8000 spectrophotometer (SLM-Aminco, Urbana, IL) at 400 nm and 490 nm with an excitation wavelength of 340 nm as described previously (28). Cells were stimulated with a bolus dose of C3α (1–100 nM final concentration). Triton X-100 (0.2% final concentration) was added to fully release the IC calcium, and EGTA (10 mM) was added to chelate the cytoplasmic calcium. The results are expressed as the ratio of fluorescence measured at 400 nm and 490 nm.

Results

Cloning of two forms of gpC3αR and RT-PCR analysis

When the large EC loop cDNA of human C3αR was used to probe guinea pig genomic DNA, no band was detected by Southern hybridization (data not shown). However, as shown in Figure 1, a 32P-labeled open reading frame of human C3αR cDNA cross-hybridized with guinea pig genomic DNA. These results suggest that the cDNA sequence for the large EC loop in C3αR may not be well conserved between species. The pattern of hybridization in Figure 1 also gave evidence for a single copy gene of gpC3αR. The fragments of genomic DNA that hybridized with human C3αR cDNA were ~3.5 kb (BamHI), ~3 kb (EcoRI), ~4–5 kb ( HindIII), and ~7 kb (PstI).

Northern blotting experiments by Ames et al. (17) have shown that human C3αR was abundantly expressed in the spleen and lung. Therefore, we screened a guinea pig spleen cDNA library using the

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entire coding region of human C3aR cDNA. Seven clones were isolated, and one of the clones contained a probable initiation site (i.e., Met) that was followed by an open reading frame encoding 475 aa and containing seven putative TM domains (Fig. 2). Hydrophy analysis of this clone showed the presence of a large EC loop between TM4 and TM5, which is a known characteristic of human C3aR (17–19) and mouse C3aR (29). This clone had a 59.5% overall identity with human C3aR. Therefore, we concluded that this clone represented gpC3aR. There were five predicted N-linked glycosylation sites; one site was near the N terminus, and four were in the large EC loop region. A putative polyadenylation signal (AATAAA) was located at 676 bp downstream from the TAA stop codon.

An analysis of the remaining clones revealed a unique clone that had N-terminal and C-terminal sequences that were identical with the first clone but was smaller in size. Based on its homology, which was determined by sequence analysis, this clone was identified as a second C3aR clone. The deduced amino acid sequence of the second clone was identical with that of the first clone from the N-terminal end to residue 253 in the large EC loop and from residue 289 of the large EC loop to the C terminus. In other words, the second gpC3aR clone was characterized as having a 35-aa deletion when compared with the first clone. This deletion involved nucleotides 761 to 865, which encode aa residues 254 to 288 in the large EC loop (see Fig. 3, A and B). The first “long” clone and the second “short” clone are designated as gpC3aR-L and gpC3aR-S, respectively. Of the seven clones that were isolated from the spleen cDNA library, five clones were identical with gpC3aR-L, and two clones were identical with gpC3aR-S.

Guinea pig RNA was isolated from the heart, kidney, lung, spleen, liver, and brain; RNA was also isolated from peritoneal Mφ that had been elicited by an i.p. injection of paraffin oil. An RT-PCR analysis using primers that amplify the large EC loop showed two clear bands of 450 bp and 350 bp in every tissue (Fig. 4). Both fragments were sequenced, and we confirmed that the ~350-bp fragment represented a deleted form of the ~450-bp fragment. It was concluded that the ~450-bp form was derived from the large EC loop of gpC3aR-L, while the ~350-bp form represented that of gpC3aR-S. Similarly, two bands were detected in cDNA that had been isolated from guinea pig megakaryocytes (data not shown).

Structural similarities between guinea pig, human, and mouse C3aR

A comparison of the deduced amino acid sequence of gpC3aR with human C3aR showed 59.5% overall identity, while 57.0% overall identity existed between gpC3aR and mouse C3aR. The alignment of these three receptors is shown in Figure 5. The TM regions of C3aR from these three species were the most highly conserved (50–90%), much like the comparison of C5aR from several species (30–33). The IC region of C3aR from these three species appeared less conserved (31–84%), with IC2 being the most and IC3 being the least conserved. A putative substrate motif (XKSXXK) for protein kinase C (34) was found in the IC3 (residues 360–366) region, and this motif was conserved in all three species. Five seryl residues and two threonyl residues were conserved in the C-terminal region of guinea pig, mouse, and human.
C3aR; these residues may represent phosphorylation sites that become modified as a result of C3a stimulation. A similar abundance of Ser/Thr phosphorylation sites (up to six) occurred in the C-terminal region of the C5aR (35). The N-terminal region of the C3aR (EC1) from these three species was moderately conserved (47%). All known C3aRs have a relatively shorter N-terminal region than C5aR, and only one Asp residue was found in this region of guinea pig and human C3aR compared with six in human C5aR (36). Surprisingly, the large EC loop (EC3) exhibited the lowest level of identity (26%) along with a number of gaps that appeared when interspecies comparisons were made, especially between residues 253 and 261 (numbering is based on the human C3aR sequence). An N-linked glycosylation site at Asn-9 in human C3aR was conserved. However, glycosylation sites in the large EC loop (one site for human C3aR, three or four sites for gpC3aR-S and gpC3aR-L, respectively, and three sites for mouse C3aR) were not conserved.

**Northern blot analysis of gpC3aR in various tissues**

Several tissues and peritoneal Mφ were analyzed by Northern blotting for their expression of mRNA (Fig. 6). The cDNA for gpC3aR-L was used as the hybridization probe. In Mφ for their expression of mRNA (Fig. 6). The cDNA for gpC3aR-L, respectively, and three sites for mouse C3aR) were not conserved.

**Flow cytometry analysis of the expression of gpC3aR on L cells stably transfected with two forms of gpC3aR cDNA and platelets**

The expression of gpC3aR protein on stably transfected L cells was confirmed using a polyclonal Ab that had been raised against a gpC3aR peptide and by FACS analysis. The histogram of gpC3aR-L- and gpC3aR-S-transfected L cells indicated a high degree of expression of both forms of C3aR protein on stably transfected L cells (Fig. 7, A and B). Untransfected L cells showed no specific Ab binding (data not shown). Isolated guinea pig platelets also showed specific staining by anti-gpC3aR peptide (Fig. 7C), which confirms our previous report of C3aR expression on guinea pig platelets using chemical cross-linking techniques (9).

**Binding of C3a to L cells stably transfected with two forms of gpC3aR cDNA**

Saturation-binding experiments showed that C3a bound to both forms of gpC3aR in transfected L cells in a specific and saturable manner (Fig. 8A). Scatchard analysis of the binding curve revealed a dissociation constant (Kd) of 10.1 nM for gpC3aR-L and 6.2 nM for gpC3aR-S, respectively. The average number of sites per cell was 3.3 × 10^5 and 2.3 × 10^5, respectively. 125I-labeled C3a binding to both forms of gpC3aR was competed by either the C3a or the synthetic C3a analogue peptides (Fig. 8B). An IC50 of 19.0 nM for gpC3aR-L and 15.3 nM for gpC3aR-S, respectively, was determined when 1 nM 125I-labeled human C3a was competed with human C3a. The IC50 for competition by the synthetic C3a analogue peptide was 5.95 μM for gpC3aR-L and 3.88 μM for gpC3aR-S, respectively. Protein A-purified anti-C3aR polyclonal Ab that had been raised against gpC3aR peptides based on the sequence of the large EC loop (residues 192–206) failed to interfere with 125I-labeled C3a binding even at the highest concentration tested (data not shown). Recently, it was demonstrated that both C3a and C3aDesArg can regulate the synthesis of TNF-α and IL-1β (13). Therefore, we examined the possibility that C3aDesArg may bind differently to the two isoforms of gpC3aR. However, radiolabeled C3aDesArg demonstrated no specific binding to either form of gpC3aR (data not shown); consequently, the C3aDesArg-induced functions that have been observed in lymphocytes (37) appear to be mediated through different receptors or mechanisms.

**Functional response to C3a by L cells stably transfected with two forms of gpC3aR cDNA**

To compare the functional response of gpC3aR-L and gpC3aR-S with C3a, the stably transfected L cells were loaded with Indo-1, and C3a-induced Ca^{2+} mobilization was analyzed. As shown in Figure 9, 100 nM of human C3a induced similar changes in the fluorescence ratio for L cells that had been transfected with either form of gpC3aR cDNA. The responses of both forms of transfected L cells to C3a were concentration-dependent in the 1 nM to 100 nM range (data not shown). The repeated addition of C3a to each of the transfected L cell lines led to a loss of the Ca^{2+} mobilization response, suggesting a homologous desensitization of
C3aR (data not shown). Untransfected L cells failed to respond to C3a (data not shown).

Discussion

The gpC3aR gene was isolated because guinea pigs are known for their high sensitivity to C3a and are frequently used as an experimental animal model in anaphylatoxin-induced inflammatory diseases, including systemic anaphylaxis, asthma, and various models of pulmonary disease (1). The cloning of gpC3aR together with our recently cloned gpC5aR (33) provides us with valuable molecular tools for monitoring the expression and regulation of anaphylatoxin receptors in in vivo models of inflammatory diseases. We isolated the gpC3aR-L from a guinea pig spleen cDNA library and found it to be 59.5% identical with the amino acid sequence of human C3aR (17–19) and 57% identical with that of mouse C3aR (29). Like the human and mouse receptors, gpC3aR-L also contains a unique and unusually large EC loop between TM4 and TM5. gpC3aR-S was isolated from the same guinea pig spleen cDNA library. From our sequence analysis, it was determined that gpC3aR-S cDNA was an altered form of gpC3aR-L cDNA with alternative splicing occurring in the large EC loop. We detected the expression of both forms of gpC3aR by RT-PCR analysis of RNA from several tissues using primers that were specific for the large EC loop. Both fragments (∼450 bp and ∼350 bp) were sequenced, and we confirmed that the ∼350-bp fragment was a spliced form of the ∼450-bp fragment. The potential alternative splice sequence, AG/GT, was found at the 5’ donor junction in the large EC loop of gpC3aR-L (Fig. 3A). A 105-bp deletion of gpC3aR could arise from a deletion in the C3aR gene itself that would generate two allelic forms; this possibility was reported for a defective CC chemokine receptor (CCR)5 allele that contained an internal 32-bp deletion as found in HIV exposed-uninfected individuals (38, 39). The other possibility is that the two forms could be derived from two tandem genes, as was reported in the mouse decay-accelerating factor (DAF) gene (40). When we amplified the large EC region by PCR using genomic DNA from guinea pig liver, only the

**FIGURE 5.** Deduced amino acid sequence of gpC3aR-L compared with human and mouse C3aR. The putative TM domains (I–VII) are overlined. Asterisks denote the identities in all sequences, and the gaps are denoted by dashes. Potential glycosylation sites are indicated in bold. The peptide sequence including residues 192–206 was used to make Ab. The alternatively spliced portion (residues 254–288) is boxed.

**FIGURE 6.** Northern blot analysis of gpC3aR expression on tissues and cells. A total of 6 μg of total RNA was loaded on formaldehyde agarose gel. The blotted nylon membrane was probed with 32P-labeled gpC3aR-L cDNA. Lane 1, heart; lane 2, kidney; lane 3, lung; lane 4, spleen; lane 5, liver; lane 6, brain; lane 7, peritoneal Mφ. A photograph of the ethidium bromide-stained agarose gel is shown on the left.
A ~450-bp band was detected (data not shown). This finding indicated that the ~350-bp band was neither derived from a defective C3aR allele nor derived from tandem genes, and that the large EC loop was derived from a single exon.

Alternative splicing within a single exon appears to be an uncommon event; however, there are a few examples of this phenomenon. The Ser/Thr-rich a, b, and c domains of the guinea pig DAF gene were encoded by a single exon. It was concluded that the deletion of the b domain or the c domain resulted from alternative splicing (41). The human monocyte chemoattractant protein-1 receptor occurred within exon 2 at a potential splicing site, AG/GT, to produce the CCR2A isoform.

The expression of gpC3aR mRNA was analyzed by RT-PCR using primers that were specific for the large EC loop; this analysis revealed that both gpC3aR-L and gpC3aR-S were expressed in tissues such as the heart, kidney, lung, spleen, liver, and peritoneal Mϕ. Although it was not quantitatively measured, it was evident that the alternatively spliced form of C3aR was also distributed in all of the tissues examined. A high expression of gpC3aR mRNA was detected in the spleen and Mϕ by Northern blot analysis. In the case of human and mouse C3aR, the detection of a second large minor transcript was also reported (18, 19, 29). It is possible that the broad C3aR signals in the guinea pig spleen and Mϕ originate from the two isoforms of C3aR that have a 105-bp difference in size. An additional possibility is that another polyadenylation site exists downstream of the presented 3′ untranslated region (Fig. 2) of the gpC3aR gene. It has been reported that four polyadenylation
that this feature might form a ligand-binding site for C3a that would act in a manner similar to the N-terminal-binding domain of the C5aR. It has been well established that the N-terminal region of the C5aR contains a binding site for C5a, and presumably there are negatively charged Asp residues in this region of the C5aR that interact with the positively charged side chains on C5a (36). The C3aR has an N-terminal region that is relatively short compared with C5aR and contains fewer negatively charged side chains near the N terminus. On the other hand, the large EC loop contains a number of Asp residues, suggesting that this region might provide a binding pocket for the many positively charged groups on the cationic C3a molecule. Naturally existing gpC3aR-S, with its shorter EC3 loop region, serves as a model for testing this hypothesis. Both gpC3aR-S and gpC3aR-L exhibited similar functional behavior as determined by the binding assays and Ca$^{2+}$ influx measurements when stably expressed on L cells. These results indicated that the 35 aa (residues 254–288) that were deleted from the large EC loop of gpC3aR-L had no role in ligand binding. Consequently, the deletion of these residues appeared to cause no significant conformational changes in C3aR. In addition, since Ab generated against an adjacent 15-residue region (residues 192–206) of the large EC loop did not compete with ligand binding (data not shown), we can exclude almost 30% (35/124) of the large EC loop as contributing to a C3a-binding site. The gpC3aR-S actually showed higher (less than twofold) affinity to C3a than did gpC3aR-L as evidenced by both the $K_d$ and IC$_{50}$ measurements in the binding assay; however, we do not know whether this difference has biologic significance. Based on our results, we concluded that these two isoforms of gpC3aR are not analogous to the high and low affinity C3aR that were detected on the human mast cell line HMC-1 (51).

In summary, two isoforms of gpC3aR were cloned and identified. To our knowledge, this is the first report that describes the isoforms of anaphylatoxin receptors (i.e., either C3aR or C5aR). We have concluded that gpC3aR-S was derived from gpC3aR-L by alternative splicing in the large EC loop region. Both forms displayed similar C3a-binding affinity and Ca$^{2+}$ influx activity, and both C3aR mRNAs were expressed in various tissues and Mφ. With the cloning of gpC3aR, we can monitor the expression and regulation of C3aR in experimental diseases using the guinea pig model. This new molecular approach promises to greatly advance our understanding of the cellular targets and functional roles for C3a in inflammatory disease. The expression of two isoforms of C3aR also provides a naturally occurring model for exploring the contributions of the large EC loop region of C3aR to C3a binding.

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