Sulfation of Tyrosine 174 in the Human C3a Receptor Is Essential for Binding of C3a Anaphylatoxin

Jinming Gao‡, Hyeryun Choe‡, Dalena Bota‡, Paulette L. Wright§, Craig Gerard‡, and Norma P. Gerard‡¶

From the ‡Perlmutter Laboratory and Department of Pediatrics, Children’s Hospital and the §Brigham and Women’s Hospital, Department of Medicine, Division of AIDS, Harvard Medical School, Boston, Massachusetts 02115

The complement anaphylatoxin C3a and its cellular seven-transmembrane segment receptor, C3aR, are implicated in a variety of pathological inflammatory processes. C3aR is a G-protein-coupled receptor with an exceptionally large second extracellular loop of 172 amino acids. Previously reported deletion studies have shown that at least part of this region plays a critical role in binding C3a. Our data now demonstrate that five tyrosines in the second extracellular loop of the C3aR are posttranslationally modified by the addition of sulfate. Blocking sulfation by mutation of tyrosine to phenylalanine at positions 184, 188, 317, and/or 318 does not affect ligand binding or signal transduction. However, when tyrosine 174 is mutated to phenylalanine, binding of native C3a is completely blocked. This variant efficiently mobilizes calcium in response to synthetic C3a agonist peptides, but not to native C3a. Our data now demonstrate that tyrosine sulfation in other extracellular domains can function for ligand interactions as well.

The human C3a anaphylatoxin is a 77-amino acid protein generated by proteolysis of C3 during activation of the complement cascade (1, 2). C3a is a potent inflammatory mediator, inducing smooth muscle contraction, increased vascular permeability, arachidonic acid metabolism, cytokine release, cellular degranulation, and chemotaxis (3–9). The C3a receptor, C3aR, is a G-protein-coupled receptor with a highly acidic amino-terminal extracellular domain (10–12). Despite significant sequence homology with the receptor for the C5a anaphylatoxin, C5aR, the C3aR is devoid of the highly acidic and tyrosine-rich amino terminus that participates in its association with C5a (12–14). The unusual structural feature represented by the large ECL2 in the C3aR suggested that it might be involved in this ligand-receptor interaction. Indeed, several studies with chimeras and point mutants have shown that ECL2 is at least part of the binding site for C3a (12, 15, 16).

Tyrosine sulfation is a posttranslational modification occurring on a number of secreted proteins, including complement factor VIII, cholecystokinin, and gastrin (17–20). This modification tends to occur in acidic regions of proteins, usually those containing multiple tyrosines (21, 22). Several chemokine receptors, including CCR5, CXCR4, C3CR1, and CCR2b, have been shown to be sulfated on tyrosines in their amino-terminal domains, and at least some of this sulfation is critical for ligand binding (23–26). For example, sulfotyrosine in the amino-terminal sequence of CCR5 is required for binding of the natural ligands MIP-1α, MIP-1β, and RANTES (regulated on activation normal T cell expressed and secreted) and certain HIV-1 GP120/CD4 complexes as well as the binding and entry of the CCR5-using strains of HIV-1 (23). Amino-terminal sulfation of tyrosines in the C5a receptor also contributes to formation of the docking (but not the signaling) site for the C5a anaphylatoxin (14). Thus, tyrosine sulfation is a critical modification for conferring the natural function of a number of 7TMS receptors.

To date, all the reported functional sulfotyrosines identified in 7TMS receptors have occurred in the amino-terminal extracellular domains. In contrast to the C5aR, which has an acidic and tyrosine-rich motif at its amino terminus, the C3aR does not (11, 12). There are nine predicted tyrosines in the full-length human C3aR, seven of which are located at positions 174, 184, 188, 255, 306, 317, and 318, all in ECL2, and some of these are flanked by acidic amino acids. The two remaining tyrosines are in the sixth and seventh transmembrane sequences. The multiplicity of tyrosines, coupled with the unusually large ECL2, raised the possibility that tyrosine sulfation may occur in this domain and that this modification may contribute to the binding of C3a.

Here we demonstrate that five of the seven tyrosines in ECL2 of the C3aR are sulfated, but only one, tyrosine 174, is essential for binding and signaling with native C3a. The C3aR variant in which tyrosine 174 was mutated to phenylalanine mobilized calcium in response to 8- and 15-residue synthetic peptides containing the carboxyl terminus of C3a, consistent with a two-site model of ligand association. These data not only define the precise structural requirement for the C3a docking site but also demonstrate that tyrosine sulfation can occur at sites distinct from the amino-terminal regions of 7TMS receptors.

Received for publication, June 9, 2003, and in revised form, July 15, 2003
Published, JBC Papers in Press, July 18, 2003, DOI 10.1074/jbc.M306061200

* This work was supported by National Institutes of Health Grants HL36162 (to N. P. G.), AI41851 (to C. G.), and AI43891 (to H. C.).
† To whom correspondence should be addressed: Perlmutter Laboratory, Children’s Hospital, 320 Longwood Ave., Boston, MA 02115. Tel.: 617-355-6737; E-mail: Norma.Gerard@FCH.harvard.edu.
‡ The abbreviations used are: C3aR, C3a receptor; C5aR, C5a receptor; CCR5, CC chemokine receptor 5; MIP, macrophage-inflammatory protein; 7TMS, seven-transmembrane segment; ECL, extracellular loop; HEK, human embryonic kidney; TPST, tyrosyl protein sulfotransferase; shRNA, small hairpin RNA; PBS, phosphate-buffered saline.

37902 This paper is available on line at http://www.jbc.org
**EXPERIMENTAL PROCEDURES**

**Cells, Plasmids, Antibodies, and Peptides—HEK 293T and C2Th cells were obtained from the American Type Culture Collection (ATCC; CRL-1554 and CRL-1430, respectively). 293 GP packaging cells were purchased from BD Biosciences. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin, and streptomycin. An expression plasmid encoding the human C3aR with the addition of a ten-amino acid tag at its amino terminus (Myg tag) and a nine-amino acid extension at its carboxyl terminus (C9 tag) was obtained by PCR amplification of human genomic DNA and subcloned into the pcDNA 3.1 expression vector (Invitrogen). All the C3aR variants in which one or more tyrosines were mutated to phenylalanine (see Table I) were made by the PCR-based QuickChange method (Stratagene) and confirmed by sequencing the entire reading frame. The antibody 1D4, which recognizes the C9 tag, and 9E10, which binds the Myg tag, were provided by the National Cell Culture Center (Minneapolis, MN). The C3aR agonist peptides, containing 8 and 15 residues (AAAGLGLAR and WWGKKYRASKLGLAR, respectively) (27), were synthesized to 95% purity by New England Peptide. Lyophilized peptides were dissolved in water at 10 μg/mL and diluted into cell suspensions. The wild type C3aR coding sequence containing both amino- and carboxyl-terminal transmembrane domains and the variants C3aR YFFFF and FYYYY were cloned into the retrovector pQCXIX (BD Biosciences) for transduction into C2 cells. The cDNAs for human tyrosyl protein sulfotransferases (TPSTs) 1 and 2 were amplified by PCR from U87 human astrogliaoma cell cDNA and subcloned into pcDNA 3.1 (28). Small hairpin RNA (shRNA) constructs targeting nucleotides 259–276 of TPST1 and nucleotides 73–94 of TPST2 were generated and subcloned into pBabePuro under the control of the murine U6 promoter (29).

**Labeling and Immunoprecipitation of C3aR and C3aR Variants—** HEK 293T cells were transfected with plasmids encoding C3aR or the C3aR variants using calcium phosphate (14). One day later, cells were washed twice with phosphate-buffered saline (PBS) and subcultured into three separate aliquots. One of each of the flasks was labeled with [35S]sulfate (PerkinElmer Life Sciences) overnight, and the third was retained for binding and flow cytometry. Cells were treated with 3 mg/mL tunicamycin (Sigma-Aldrich) to inhibit N-glycosylation (Sigma-Aldrich) to inhibit N-glycosylation 5 h prior to and throughout the labeling period (14). For immunoprecipitation, labeled cells were harvested and lysed in 1% N-dodecyl-β-D-maltoside (Anatrace) in PBS containing a protease inhibitor mixture (Sigma-Aldrich and Roche Applied Science) and 0.2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Cell debris was removed by centrifugation at 18,000×g for 5 min at 4 °C, and the supernatants were immunoprecipitated in the presence of the anti-C9-tag antibody, ID4, covalently cross-linked to protein A-Sepharose (Amersham Biosciences). Immunoprecipitates were washed twice with 1% N-dodecyl-β-D-maltoside in PBS containing 0.5% SDS and once with PBS and then eluted with SDS sample buffer under reducing conditions by heating at 55 °C for 10 min and analyzed by autoradiography following electrophoresis on 12% SDS Tris-glycine polyacrylamide gels (Invitrogen).

O-linked carbohydrates were enzymatically removed from immunoprecipitated C3a receptors using O-glycanase (Glyko) and neuraminidase. N-linked carbohydrates were removed with PNGase F (New England Biolabs), as described by the manufacturer under non-denaturing conditions, eluted with SDS sample buffer, and analyzed as above on 12% SDS gels.

**Binding of C3a to Cells Expressing C3aR and C3aR Variants—** Binding experiments were performed using HEK 293T cells transfected with wild type C3aR or C3aR variants. Two days after transfection, cells were detached with 5 mM EDTA in PBS, washed with Dulbecco’s modified Eagle’s medium, counted, and resuspended in binding buffer (20 mM HEPES, pH 7.4, 125 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 5 mM KCl, 0.5 mM glucose, 0.2% bovine serum albumin, and 0.02% sodium azide) at 2 × 105/mL. An aliquot of cells was subjected to flow cytometry analysis to control for the relative expression levels of the receptors. Briefly, 5 × 105 cells were incubated with 0.5 μg/ml anti-Myc antibody 9E10 followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology), fixed in 2% paraformaldehyde, and analyzed using a FACS-Star cytometer and CellQuest software (BD Biosciences). For binding, duplicate aliquots of 50 μl were incubated with 0.1 nM 125I-C3a (PerkinElmer Life Sciences) and 0–200 nM unlabeled C3a (Advanced Research Technologies) for 30 min at 37 °C in a final volume of 100 μl. Cells were centrifuged, washed once with binding buffer, and bound C3a was determined by γ-counting. Binding data were subjected to nonlinear regression analysis using Prism software (GraphPad).

For some experiments, the degree of sulfation was modified by cotransfecting the C3aR variant YFFFF with plasmids encoding TPST1 and 2 to increase sulfation or with shRNA constructs directed against TPST1 and 2 to inhibit sulfation. Controls included co-transfection of the C3aR variant with pcDNA3.1 without insert.

**Calcium Mobilization Mediated by the C3aR and C3aR Variants—** The transduction vector pQCXIX (BD Biosciences) encoding the wild type C3aR or C3aR variants FYYYY and YFFFF (see Table I) were co-transfected into 293 GP packaging cells (BD Biosciences) with plasmids encoding the vesicular stomatitus virus G protein (VSV-G) envelope and Gag/Pol using calcium phosphate. Two days later, the culture supernatants were collected, and virus particles were concentrated by centrifugation at 15,000 × g for 75 min. This concentrated virus was adsorbed in 0.5-mL cell culture medium and used to infect C2 cells, which were plated at 3 × 105 cells per well in 12-well plates 1 day before. One day later, the infected C2 cells were transferred to flasks and grown in Dulbecco’s modified Eagle’s medium for at least 24 h. Receptor expression was quantified by flow cytometric analysis as described above. Cells were harvested, counted, and incubated with the indicator dye Fura-2-AM (Molecular Probes) for 1 h at 37 °C in 20 mM HEPES, pH 7.4, 1 mM CaCl2, 1 mM MgCl2, 125 mM NaCl, 5 mM KCl, 0.5 mM glucose, and 0.2% bovine serum albumin. Cells were washed twice and resuspended in the same buffer at 1 × 106/mL. Changes in intracellular calcium concentration in response to C3a or C3a synthetic peptide agonists were determined fluorometrically at 37 °C by monitoring the emission at 510 nm and the excitation at 340 and 380 nm as a function of a time. Responses were quantified as the peak of the fluorescence ratio of 340/380 nm. Measurements were made using cells from at least three independent experiments.

**RESULTS**

**Tyrosines in the ECL2 of the C3aR Are Sulfated—** Previous work has shown that the sulfation of tyrosines on the extracellular amino-terminal domain of the C5a anaphylatoxin receptor is a critically important posttranslational modification for ligand recognition (14). Given the similarities of the C3a and C5a anaphylatoxins and their receptors, we hypothesized that a similar posttranslational modification might be important for C3aR function as well. The C3aR has no sulfatable tyrosines in its extracellular amino-terminal sequence; however, seven are evident in the unusually large ECL2 consisting of 172 amino acids (12). In addition, the tyrosines at positions 184, 188, 317, and 318 are flanked by acidic residues characteristic of preferred sites for sulfation (21, 22). Studies with chimeric C3a/C5a receptors and several CLE2 deletion mutants have shown that ECL2, particularly its amino acids adjacent to the fourth and fifth transmembrane sequences, is essential for C3a binding (15, 16). We therefore tested the hypothesis that one or more of the tyrosines in this region is sulfated and plays a role in C3a binding and signal transduction. Tyrosines at positions 174, 184, 188, 317, and 318 in ECL2 were individually and multiply mutated to phenylalanine, as indicated in Table I. Constructs were transfected into mammalian cells and tested for expression, binding, and signal transduction.

To facilitate the determination of relative levels of cell surface expression, expression plasmids were engineered to encode the wild type or mutant C3aRs fused in-frame with an amino-terminal Myc tag, which is recognized by the antibody 9E10. Immunoprecipitations were accomplished using the antibody 1D4, which is specific for the in-frame C9-tag at the carboxyl terminus, corresponding to the nine carboxyl-terminal amino acids of rhodopsin. Control experiments demonstrated that these modifications had no detectable effect on binding or signal transduction compared with native C3aR lacking these tags (not shown). Transfected HEK 293T cells were assessed for surface expression of wild type C3aR or C3aR variants by flow cytometry using the anti-Myc tag antibody and mean fluorescence compared for each experiment. Simultaneously, cells from the same transfection were labeled with [35S]cysteine and [35S]methionine or [35S]sulfate and lysed, and C3aRs were immunoprecipitated with the anti-C9 antibody, 1D4,
cross-linked to Protein A-Sepharose beads. To ensure selection of sulfation exclusively on tyrosines, tunicamycin was also included in the culture medium (10–12).

As shown in Fig. 1, cells expressing the wild type C3a receptor and grown in the absence of tunicamycin reveal a predominant form migrating with an apparent molecular mass of ~90 kDa that is labeled with both [35S]Cys/Met and [35S]sulfate. A minor form migrates at ~50 kDa that labels only with [35S]Cys/Met and is presumably an immature intracellular receptor. The addition of tunicamycin to the culture medium prior to and during labeling results in a mature C3aR of 75–80 kDa in addition to the non-sulfated form at ~50 kDa. The predicted amino acid sequence includes a number of potential O-linked glycosylation sites in the amino-terminal extracellular domain and ECL2, and when these are removed by O-glycanase treatment of immunoprecipitated C3aR, the receptor migrates with an apparent molecular mass of ~60 kDa.

Because a previous report indicated an important role for sequences adjacent to transmembrane sequences 4 and 5 (15), we focused first on the tyrosines in these regions and generated the mutant receptors outlined in Table I. As shown in Fig. 2, only when all five tyrosines at positions 174, 184, 188, 317, and 318 in the ECL2 were changed to phenylalanine was the ability to incorporate [35S]sulfate lost, suggesting that tyrosines at 255 and 306 may not be sulfated. As we have observed previously with other 7TMS receptors (14, 23), when multiple tyrosines are mutated to phenylalanine in the C3aR, particularly the YYYYY and FFFFF variants (see Table I), their electrophoretic mobility is increased, likely due to alterations in SDS binding.

C3a Binding to Wild Type C3aR and C3aR Variants—To examine the functional importance of the sulfated tyrosines in the ECL2, we next compared the ability of125I-C3a to associate with the wild type C3aR and C3aR variants, as indicated by their fluorescence intensity of anti-Myc-tagged antibody staining, levels of wild type C3aR or C3aR variants, as indicated by their tyrosines in ECL2 was replaced with phenylalanine. Transfected HEK 293T cells expressing similar levels of wild type C3aR or C3aR variants, as indicated by their fluorescence intensity of anti-Myc-tagged antibody staining, were tested in competition binding experiments with 125I-C3a and increasing concentrations of unlabeled C3a as indicated.

As shown in Fig. 3, HEK 293T cells expressing the C3aR variants YFFFF, YYYY, and YFFFF bound C3a with affinities similar to the wild type receptor. Uniquely, binding to C3aR YYYY was not detected. Cell surface expression of this mutant receptor was essentially the same as the wild type receptor.

Calcium Mobilization through C3aR and Its Mutants—In the case of the C5a receptor, intact C5a was unable to induce a calcium flux when two of the three amino-terminal tyrosines were mutated to phenylalanine, although a synthetic peptide agonist could still activate the same cells (13). These data were interpreted as further supporting the two-site model for ligand-receptor interactions (30). Synthetic peptide agonists corresponding to the carboxyl-terminal 8 or 15 amino acids of C3a have also been described (27), and we sought to study them in parallel experiments.

In 293T cells the C3a receptor, like the C5a receptor, requires co-transfection with Go16 to elicit a calcium flux (31). Because we have no convenient method to monitor expression

---

**Table I**

**Deduced amino acid sequences flanking the tyrosine residues in ECL2 of the C3a receptor**

| Position of Tyr in C3aR mutants | Amino acid sequence flanking tyrosines in ECL2 of the C3a receptor | Tyrosine sulfate | \( K_d \) |
|---------------------------------|---------------------------------------------------------------|---------------|----------|
| WT (YYYYY)                      | RCGYEKFEG SLDYPDFYG FGDYNYNLG ...                            | +             | 6.1 ± 1.0 (n = 7) |
| FYYYYY                          | RCGYKFG SLDYPDFYG FGDYNNLG ...                               | +             | ND (n = 4) |
| YYYYF                           | RCGYKFG SLDYPDFYG FGDYNNLG ...                               | +             | 3.5 ± 0.1 (n = 3) |
| FFYYFF                          | RCGYKFG SLDYPDFYG FGDYNNLG ...                               | +             | 7.3 ± 1.6 (n = 3) |
| FFFFFF                          | RCGYKFG SLDYPDFYG FGDYNNLG ...                               | +             | ND (n = 3) |
| FFFFFF                          | RCGYKFG SLDYPDFYG FGDYNNLG ...                               | +             | 6.7 ± 1.4 (n = 3) |

---

**Fig. 1. Tyrosines in ECL2 of the C3a receptor are sulfated.**

A. HEK293T cells were transfected with plasmids encoding the wild type C3a receptor with amino and carboxyl-terminal epitope tags. After 24 h they were divided and labeled with [35S]cysteine and [35S]methionine or [35S]methionine or [35S]sulfate in the presence or absence of the N-glycosylation inhibitor tunicamycin (Tmn) before and during labeling as indicated. After an additional 24 h, cells were harvested and lysed, and C3a receptors were immunoprecipitated with the antibody 1D4, which recognizes the carboxyl-terminal C9 tag. Immunoprecipitates were analyzed under reducing conditions by electrophoresis on 12% SDS-polyacrylamide gels. Gels were dried and exposed to x-ray film.

B. In 293T cells the C3a receptor, like the C5a receptor, requires co-transfection with Go16 to elicit a calcium flux (31). Because we have no convenient method to monitor expression
of Ga16, we used Cf2 cells, which appear to endogenously express an intact Ca\(^{2+}\) signaling pathway for most G-protein-coupled receptors. These cells are relatively inefficiently transfected, so we packaged the wild type C3aR or C3aR variant expressing transduction vectors in murine leukemia viral particles pseudo-typed with the vesicular stomatitis virus G protein and used them to transduce Cf2 cells. Cells expressing wild type C3aR or its variants, C3aR YFFFF, which binds native C3a normally, and C3aR FYYYY, which does not, were tested for the ability to mobilize calcium in response to native C3a or the two synthetic C3a agonist peptides. As shown in Fig. 4, cells expressing wild type C3aR or C3aR YFFFF induced effective calcium signals in response to 100 nM native C3a, whereas the FYYYY variant failed to do so. In contrast, all three receptors, wild type C3aR, C3aR FYYYY, and C3aR YFFFF, responded almost equally well to both of the synthetic C3a agonist peptides. As shown in Fig. 4, cells expressing wild type C3aR or C3aR YFFFF induced effective calcium signals in response to 100 nM native C3a, whereas the FYYYY variant failed to do so. In contrast, all three receptors, wild type C3aR, C3aR FYYYY, and C3aR YFFFF, responded almost equally well to both of the synthetic C3a agonist peptides. Thus, the lack of calcium mobilization by the FYYYY variant is not due to a global alteration of receptor structure. The data further suggest that the C3a receptor, like the C5a receptor (14, 30), presents distinct docking and activation sites for a ligand.

**DISCUSSION**

The molecular cloning of the C3a receptor places this molecule in the superfamily of G-protein coupled 7TMS receptors (10–12). Despite a number of similarities, the C3a receptor does not have a highly acidic tyrosine-rich amino-terminal

---

**An Essential Role for Tyrosine Sulfation in the C3a Receptor**

37905

---

**FIG. 2.** Five tyrosines in ECL2 of the C3a receptor are modified by sulfation. A, HEK 293T cells were transfected with the plasmids encoding wild-type C3aR (wt) or its variants, YYYYY and YYYYF, as indicated (see Table I for identification of the variant receptors). Cells were split and labeled with \(^{35}\)S-cysteine and \(^{35}\)S-methionine or \(^{35}\)S-sulfate in the presence of tunicamycin, and C3a receptors were purified and analyzed as described for Fig. 1. B, the C3aR variants, YYYYY, YYYYF, and YYYYF, were transfected and analyzed as described for panel A. Only when all five tyrosines were mutated to phenylalanine was labeling with \(^{35}\)S-sulfate substantially reduced.

**FIG. 3.** Sulfotyrosine 174 in the ECL2 of the C3a receptor forms the binding site for C3a. A, HEK 293T cells were transfected with plasmids encoding wild type C3aR (YYYYY) or the C3aR variants YYYYY or YYYYF. Cells were incubated with 0.1 nM \(^{125}\)I-C3a and the indicated concentrations of unlabeled C3a as described under “Experimental Procedures.” Cells were washed, and bound \(^{125}\)I-C3a was determined by \(^{125}\)I-counting. Aliquots of the same transfections were analyzed for C3aR expression levels by flow cytometry using the anti-Myc-tagged antibody 9E10. Each point was determined in duplicate, and data are expressed as the percentage of specific binding determined from at least three independent experiments. B, cells were treated in a manner identical to that described for panel A, except that ligand binding to the C3aR variants YYYYF and YYYYF are compared with wild type C3aR.
sequence like the C5a receptor, which we have recently shown to be involved in its interaction with C5a (14). The predicted extracellular amino-terminal sequence of the C3a receptor is relatively short (~21 amino acids) and has been shown not to participate in C3a binding (15, 16). Instead, the C3aR has a large ECL2 that contains seven potentially sulfatable tyrosines. Here, we have shown that five of the seven tyrosines in this region of the C3a receptor are sulfated and that sulfotyrosine 174 alone plays a critical role in the binding and signaling of native C3a. The absence of sulfotyrosine 174 does not alter transduction in response to either of the two synthetic C3a peptide agonists tested, suggesting first, that this tyrosine-mutated C3aR variant is not completely inactivated. Second, the data lend support to a two-site model for ligand-receptor interactions similar to C5a and C5aR interactions as well as interactions between a number of other peptide ligand receptor pairs (30, 32). The observation that native C3a does not signal in C3aR FYYYY-expressing cells while agonist peptide responses are not altered suggests that the “docking” step induces a conformational change in the ligand, exposing a structure that can subsequently activate the cell.

Sulfation of tyrosines is a posttranslational modification occurring late in the trans-Golgi network (33), and estimates indicate that up to 1% of all tyrosines in eukaryotic proteins are sulfated (34). This modification is most appreciated on a large number of secreted proteins and in the extracellular domains of membrane-bound proteins (21, 33). More recently, we and others have demonstrated the existence of sulfated tyrosines in amino-terminal sequences of several chemoattractant receptors that play a critical role in receptor function (23–26). In the case of the chemokine receptors CCR5, CXCR4, CCR2b, and CX3CR1, it is not clear whether distinct docking and activation

![Fig. 4. The C3aR variant FYYYY does not mobilize calcium in response to native C3a but responds robustly to C3a synthetic peptide agonists.](image-url)
Tyrosine sulfation has been identified as a key mediator of protein-protein interactions involved in leukocyte adhesion, hemostasis, and chemokine signaling (36). Sulfate is a charged and highly polarizable moiety that is likely to contribute significant free energy to the binding. Here we have shown that tyrosine at 174 is not only sulfated but that this sulfated moiety is critically important for C3a binding. This result is consistent with the previous report using deletion mutants of the C3aR, wherein deletion of amino acids from 198 to 308 in ECL2 (as much as 65% of this region) did not affect C3a binding or calcium mobilization (15). The removal of residues 174 to 183 resulted in a total loss of receptor function. Our findings demonstrate the importance of sulfotyrosine at 174 for C3a receptor function. It is curious that additional tyrosines in ECL2 are sulfated but have no apparent role in C3a-C3aR interactions. That finding, coupled with the unusual size of this receptor domain, suggests the possibility of additional ligands. Potential candidates might include other products of C3 that result from complement activation. Alternatively, this region may associate with another cell surface protein possibly modulating signal transduction.

Taken together, our data support a two-site model in which the binding/docking site for the native ligand is separable from the activation/effect site. In other characterized receptors with peptide ligands, the docking/binding site resides in the acidic amino acid and tyrosine-rich amino termini, and the activation site includes ECL2 and transmembrane helices. In the case of the C3aR, the docking site also resides in ECL2 but is clearly separable from the activation site. Even though five tyrosines in this region of the C3aR are sulfated, only the tyrosine at 174 is involved in C3a binding.

REFERENCES
1. Cochrane, C. G., and Muller-Eberhard, H. J. (1968) J. Exp. Med. 127, 371–386
2. Hugli, T. E. (1975) J. Biol. Chem. 250, 8293–8301

An Essential Role for Tyrosine Sulfation in the C3a Receptor

Fig. 5. The C3a receptor variant YFFFF is maximally sulfated in 293T cells. A, HEK293T cells were co-transfected with the C3aR variant YFFFF, with or without plasmids, encoding TPST1 and 2 as indicated in the presence of tunicamycin and labeled with [35S]cysteine and [35S]methionine or [35S]sulfate. Receptors were immunoprecipitated and analyzed as described for Fig. 1. (The Cys/Met labeled band at ~45,000 kDa, indicated as TPST, co-purifies with the C3a receptor because it also contains a C9 tag.) B, cells from the same transfection were tested for binding of [125I]-C3a as described for Fig. 3 following flow cytometric analyses of receptor expression levels. Only transfections with similar expression levels were used. Overexpression of TPST does not increase the extent of receptor sulfation or ligand binding.

Fig. 6. Sulfation of tyrosine 174 is responsible for C3a binding. A, HEK293T cells were co-transfected with the C3aR variant YFFFF with or without shRNAs targeting TPST1 and 2, as indicated, in the presence of tunicamycin and labeled with [35S]cysteine and [35S]methionine or [35S]sulfate. Receptors were immunoprecipitated and analyzed as described for Fig. 1. B, cells from the same transfection were tested for binding of [125I]-C3a as described for Fig. 3 following flow cytometric analyses of receptor expression levels. Only transfections with similar expression levels were used. Neither sulfation nor receptor binding is completely inhibited, as shRNAs cannot inactivate TPSTs already synthesized.
3. Gerard, C., and Gerard, N. P. (1994) *Annu. Rev. Immunol.* 12, 775–808
4. Vegt, W. (1996) *Complement* 3, 177–188
5. Stimler, N. P., Hugli, T. E., and Bloor, C. M. (1980) *Am. J. Pathol.* 100, 327–348
6. Stimler-Gerard, N. P., and Galli, S. J. (1987) *J. Immunol.* 138, 2299–2304
7. Daffern, P. J., Pfeifer, P. H., Ember, J. A., and Hugli, T. E. (1995) *J. Exp. Med.* 181, 2119–2127
8. Nilsson, G., Johnell, M., Hammer, C. H., Tiffany, H. L., Nilsson, K., Metcalfe, D. D., Siegbahn, A., and Murphy, P. M. (1996) *J. Immunol.* 157, 1693–1698
9. Humbles, A. A., Lu B., Nilsson, C.A., Lilly, C., Israel, E., Fujiwara, Y., Gerard, N. P., and Gerard, C. (2000) *Nature* 406, 998–1001
10. Roglic, A., Prossnitz, E. R., Cavanagh, S. L., Pan, Z., Zou, A., and Ye, R. D. (1996) *Biochim. Biophys. Acta* 1295, 39–43
11. Crass, T., Raffetseder, U., Martin, U., Grove, M., Klos, A., Kohl, J., and Bautsch, W. (1996) *Eur. J. Immunol.* 26, 1944–1950
12. Ames, R. S., Li, Y., Sarau, H. M., Nuthulaganti, P., Foley, J. J., Ellis, C., Zeng, Z., Su, K., Jurewicz, A. J., Hertzberg, R. P., Bergsma, D. J., and Kumar, C. (1996) *J. Biol. Chem.* 271, 20231–20234
13. Gerard, N. P., and Gerard, C. (1991) *Nature* 349, 614–617
14. Farzan, M., Schnitzler, C. E., Vasilieva, N., Leung, D., Kuhn, J., Gerard, C., and Choe, H. (2001) *J. Exp. Med.* 193, 1059–1066
15. Chao, T. H., Ember, J. A., Wang, M., Bayon, Y., Hugli, T. E., and Ye, R. D. (1999) *J. Biol. Chem.* 274, 9721–9728
16. Crass, T., Ames, R. S., Sarau, H. M., Nuthulaganti, P., Foley, J. J., Kohl, J., Klos, A., and Bautsch, W. (1999) *J. Biol. Chem.* 274, 8367–8370
17. Huttner, W. B. (1988) *Annu. Rev. Physiol.* 50, 363–376
18. Baeuerle, P. A., and Huttner, W. B. (1985) *J. Biol. Chem.* 260, 6434–6439
19. Bundgaard, J. R., Dyhr, J., and Rehfeld, J. F. (1997) *J. Biol. Chem.* 272, 21760–21765
20. Humes, C., Huttner, W. B., Carvallo, D., and Degryse, E. (1990) *J. Biol. Chem.* 265, 32974–32978
21. Rosenquist, G. L., and Nicholas, H. B., Jr. (1993) *Protein Sci.* 2, 215–222
22. Farzan, M., Mirzabekov, T., Kolchinsky, P., Wyatt, R., Cayabyab, M., Gerard, N. P., Gerard, C., Sodroski, J., and Choe, H. (1995) *Cell* 96, 667–676
23. Farzan, M., Babcock, G. J., Vasilieva, N., Wright, P. L., Kiprilov, E., Mirzabekov, T., and Choe, H. (2002) *J. Biol. Chem.* 277, 29484–29489
24. Prehusanovsky, A. A., Dragan, S., Kawano, T., Gavrilin, M. A., Gulina, I. V., Chakravarty, L., and Kolattukudy, P. E. (2000) *J. Immunol.* 165, 5295–5303
25. Fong, A. M., Alam, S. M., Imai, T., Haribabu, B., and Patel, D. D. (2002) *J. Biol. Chem.* 277, 19418–19423
26. Ember, J. A., Johansen, N. L., and Hugli, T. E. (1991) *Biochemistry* 30, 3603–3612
27. Choe, H., Li, W., Wright, P. L., Vasilieva, N., Venturi, M., Huang, C.-C., Grondner, C., Darman, T., Zwick, M. B., Wang, L., Rosenberg, E. S., Kwog, P. D., Burton, D. R., Robinson, J. E., Sodroski, J. G., and Farzan, M. (2003) *Cell* 114, 161–170
28. Sui, G., Soohoo, C., Affar, E. B., Gay, F., Shi, Y., and Forrester, W. C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 5515–5520
29. Kolakowski, L. F., Jr., Lu, B., Gerard, C., and Gerard, N. P. (1995) *J. Biol. Chem.* 270, 18077–18082
30. Amatruda, T. T., III, Gerard, N. P., Gerard, C., and Simon, M. I. (1993) *J. Biol. Chem.* 268, 10139–10144
31. Gether, U., and Kohlika, B. K. (1998) *J. Biol. Chem.* 273, 17979–17982
32. Huttner, W. B. (1988) *Annu. Rev. Physiol.* 50, 363–376
33. Kaese, M., and Huttner, W. B. (1985) *J. Biol. Chem.* 260, 6434–6439
34. Bundgaard, J. R., Vuetz, J., and Rehfeld, J. F. (1997) *J. Biol. Chem.* 272, 21760–21765
35. Kehoe, J. W., and Bertozzi, C. R. (2000) *Chem. Biol.* 7, R57–R61