

# Sulfated Tyrosines Contribute to the Formation of the C5a Docking Site of the Human C5a Anaphylatoxin Receptor

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## Abstract

The complement anaphylatoxin C5a and its seven-transmembrane segment (7TMS) receptor play an important role in host defense and in a number of inflammation-associated pathologies. The NH<sub>2</sub>-terminal domain of the C5a receptor (C5aR/CD88) contributes substantially to its ability to bind C5a. Here we show that the tyrosines at positions 11 and 14 of the C5aR are posttranslationally modified by the addition of sulfate groups. The sulfate moieties of each of these tyrosines are critical to the ability of the C5aR to bind C5a and to mobilize calcium. A C5aR variant lacking these sulfate moieties efficiently mobilized calcium in response to a small peptide agonist, but not to C5a, consistent with a two-site model of ligand association in which the tyrosine-sulfated region of the C5aR mediates the initial docking interaction. A peptide based on the NH<sub>2</sub> terminus of the C5aR and sulfated at these two tyrosines, but not its unsulfated analogue or a doubly sulfated control peptide, partially inhibited C5a association with its receptor. These observations clarify structural and mutagenic studies of the C5a/C5aR association and suggest that related 7TMS receptors are also modified by functionally important sulfate groups on their NH<sub>2</sub>-terminal tyrosines.

Key words: C5a • C5aR • tyrosine sulfation • chemotactic receptors • CD88

## Introduction

The anaphylatoxin C5a is a proteolytic fragment of the fifth component of complement generated during activation. C5a and its G protein-coupled, seven-transmembrane segment (7TMS)<sup>1</sup> receptor, C5aR, play central roles in a number of protective and pathological inflammatory processes including rheumatoid arthritis, psoriasis, septic shock, myocardial ischemia injury, acute respiratory distress syndrome, and multiple system organ failure (for reviews, see references 1 and 2).

Like other chemotactic 7TMS receptors of the immune system, including most or all chemokine receptors, the NH<sub>2</sub>-terminal extracellular domain of the C5aR contains several tyrosines adjacent to a number of acidic amino acids

(3). A number of studies with chimeras and point mutations have shown that the NH<sub>2</sub>-terminal domain proximal to these tyrosines plays an important role in the ability of the C5aR to associate with C5a (4–11). Somewhat inconsistently with these studies, a recent nuclear magnetic resonance (NMR) study of the interaction between C5a and an unsulfated peptide based on the C5aR NH<sub>2</sub> terminus (1–30) suggested that the majority of binding energy derived from residues 21–30 of the C5aR NH<sub>2</sub> terminus, distal to the NH<sub>2</sub>-terminal tyrosines at positions 6, 11, and 14 (12).

We have shown previously that a similar acidic and tyrosine-rich motif of the chemokine receptor CCR5 is posttranslationally modified by sulfation of most or all of its four NH<sub>2</sub>-terminal tyrosines, and that tyrosine sulfation contributes to the ability of CCR5 to bind both its natural chemokine ligands and the HIV-1 envelope glycoprotein (3). We have also shown that a tyrosine-sulfated peptide based on the first 22 amino acids of CCR5 blocks HIV-1 entry into CCR5-expressing target cells, and partially inhibits the association of CCR5 with its natural chemokine ligands (13).

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<sup>1</sup>Abbreviations used in this paper: CCR5, CC chemokine receptor 5; Cha, cyclohexylalanine; NMR, nuclear magnetic resonance; 7TMS, seven-transmembrane segment.

Here we demonstrate that two tyrosines of the C5aR NH<sub>2</sub> terminus are sulfated and show that this posttranslational modification is critical for efficient association of intact C5a with the C5aR, but not for the receptor to signal through a small synthetic peptide agonist. We also demonstrate that a tyrosine-sulfated peptide corresponding to residues 7 through 28 of the NH<sub>2</sub> terminus of C5aR, but not its unsulfated equivalent or a doubly tyrosine-sulfated peptide derived from CCR5, partially blocks the association of C5a with its receptor. These data underscore the importance of tyrosine sulfation in many chemotactic and inflammatory processes, and provide new structural insight into the association of C5a with its receptor.

## Materials and Methods

**Cells, Plasmids, Antibodies, and Peptides.** HEK293T cells were obtained from the American Tissue Type Collection (CRL11554). An expression plasmid encoding the human C5aR with the addition of a nine amino acid extension at its COOH terminus (corresponding to the nine COOH-terminal amino acids of bovine rhodopsin, TETSQVAPA) was generated by PCR amplification of a plasmid encoding human C5aR (14) into a pcDNA3.1 (Invitrogen) expression vector containing a region encoding the bovine rhodopsin COOH terminus (3), facilitating recognition by the antibody 1D4 (provided by the National Cell Culture Center, Minneapolis, MN). Plasmids encoding C5aR variants YFF, YFY, YYF, and L2-FF in which one or more tyrosines were altered to phenylalanine were generated by the QuikChange method (Stratagene), and sequenced in their coding regions. Anti-C5aR antibody S5/1 was provided by Dr. O. Gotze (Georg-August-University, Göttingen, Germany) (15). Tyrosine-sulfated peptides and their unsulfated analogues (TTPDY\*GHY\*DDKDTLNLNTPVDK, asterisks indicate sulfated tyrosines, identified here as “S-peptide”, its unsulfated equivalent identified as “C-peptide”, or a sulfated CCR5-derived peptide, MDYQV-SSPIY\*DINY\*YTSEPSQK, “R5-S-peptide”) were synthesized to 95% purity by American Peptide Company. Small peptide C5aR agonist (F-K-A-dCha-Cha-dR, where Cha indicates cyclohexylalanine; reference 6) was synthesized to 95% purity by New England Peptide.

**Labeling and Immunoprecipitation of C5aR and C5aR Variants.** HEK293T cells were transfected with plasmid encoding wild-type C5aR or C5aR variants by the calcium phosphate method. Approximately 36 h after transfection, cells were washed twice in PBS, divided, and labeled with [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine or with [<sup>35</sup>S]sulfate overnight. In some experiments, 5 μg/ml tunicamycin (Sigma-Aldrich) was added to the cell culture media 5 h before radiolabeling, and during the labeling to inhibit N-glycosylation of the receptor. Labeled cells were lysed in 1% Cymal-6 (Anatrace) in PBS containing a protease inhibitor cocktail (Sigma-Aldrich) and 0.2 mM PMSF. Labeled receptor was immunoprecipitated in the presence of 1% SDS with 1D4 antibody covalently linked to Sepharose beads (Amersham Pharmacia Biotech). Immunoprecipitates were washed in PBS containing 1% Cymal-6 and 1% SDS, incubated at 55°C for 10 min in reducing sample buffer, and analyzed by 10% SDS-PAGE.

**Binding of C5a to Cells Expressing C5aR Variants or in the Presence of Sulfated and Unsulfated Peptides.** Binding experiments were performed with HEK293T cells transfected as described above. 2 × 10<sup>5</sup> cells were incubated with 0.2 nM [<sup>125</sup>I]C5a (Du-

pont/NEN Life Science Products) and 0 to 300 nM unlabeled C5a (Sigma-Aldrich) for 30 min at 37°C in binding buffer (50 mM Hepes, pH 7.4, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.2% BSA, and 0.02% NaN<sub>3</sub>). In parallel, FACS<sup>®</sup> analysis was performed on 5 × 10<sup>5</sup> of the same cells stained with 0.5 μg/ml of the anti-C5aR antibody S1/5 (15) and then with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich).

Inhibition of C5a binding by a sulfated C5aR-derived peptide (S-peptide, corresponding to residues 7–29 of the C5aR), its unsulfated equivalent (C-peptide), or sulfated peptide corresponding to the 22 NH<sub>2</sub>-terminal residues of CCR5 (R5-S-peptide) was determined by incubating cells with 0.2 nM [<sup>125</sup>I]C5a in the presence or absence of peptides at the concentrations indicated.

**Calcium Mobilization Mediated by the C5aR and a C5aR Variant.** Approximately 3 × 10<sup>6</sup> HEK293T cells were transfected with 15 μg of plasmid encoding wild-type C5aR or the YFF variant together with 15 μg of plasmid encoding the G protein component G<sub>α16</sub>, which has been shown necessary for C5a-mediated signal transduction (16). Cells were harvested at 48 h, washed, 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 CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 125 mM NaCl, 5 mM KCl, 0.5 mM glucose, and 0.2% BSA (17). Cells were washed twice and resuspended in the same buffer at 2 × 10<sup>6</sup>/ml. Changes in intracellular calcium concentrations in response to C5a or a small synthetic peptide C5aR agonist (F-K-A-dCha-Cha-dR; reference 6) were determined by monitoring the emission at 510 nm of the excitation at 340 and 380 nm as a function of time. Responses were quantified as the peak of the ratio of 340/380 nm wavelengths.

## Results

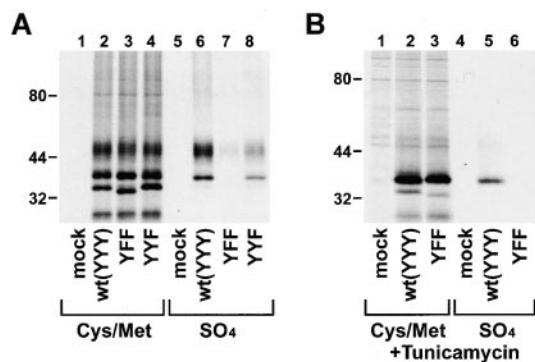
**Tyrosines at Positions 11 and 14 of the C5aR Are Sulfated.** Human C5aR has five tyrosines in its extracellular domains: at positions 6, 11, and 14 at its NH<sub>2</sub> terminus (see Fig. 4 B) and at positions 181 and 192 in its second extracellular loop (14). Among these, all but tyrosine 6 are immediately adjacent to one or more acidic residues, a feature suggestive of tyrosine sulfation (18–20). Tyrosine 6 is also adjacent to the NH<sub>2</sub>-terminal glycosylation site of the C5aR at asparagine 5 and as such is less likely to be sulfated than the remaining tyrosines (18). We also speculated that the relatively accessible NH<sub>2</sub>-terminal domain of the receptor would be more likely to be sulfated than the second extracellular loop of the C5aR, consistent with our observations in the case of the chemokine receptor CCR5 (3). We therefore focused our initial investigations on the NH<sub>2</sub>-terminal tyrosines 11 and 14.

We first examined whether the C5aR could efficiently incorporate [<sup>35</sup>S]sulfate. An expression plasmid encoding the human C5aR with a nine amino acid COOH-terminal tag derived from bovine rhodopsin was transfected into HEK293T cells. Cells were divided, radiolabeled with either [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine or [<sup>35</sup>S]sulfate, lysed, and lysates were immunoprecipitated and analyzed by SDS-PAGE. Fig. 1 A demonstrates that when tagged wild-type C5aR was expressed in 293T cells and labeled with [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine, four discrete bands could be observed (Fig. 1 A, lane 2). The top two bands appear to represent mature glycosylated and unglycosylated forms of

the receptor (compare lane 2 of Figs. 1, A and B), whereas the bottom two bands migrate faster than unmodified full-length C5aR and may represent proteolytic products of the receptor, as suggested by pulse chase analysis (data not shown). Lane 6 of Fig. 1 A demonstrates that two larger forms of wild-type C5aR specifically incorporated [<sup>35</sup>S]sulfate, implying that these forms transit through the trans-Golgi network where sulfation occurs (18, 21, 22), whereas the smaller bands observed in lysates labeled with [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine did not incorporate [<sup>35</sup>S]sulfate (compare lanes 2 and 6 of Fig. 1 A). We conclude that the full-length C5aR is modified by the addition of sulfate and that at least some of these sulfate moieties are independent of glycosylation present on the molecule.

Fig. 1 A also shows that a variant of the C5aR in which NH<sub>2</sub>-terminal tyrosines 11 and 14 are modified to phenylalanine (denoted YFF to indicate amino acid present at positions 6, 11, and 14, respectively, of the variant receptor) only weakly incorporated radiolabeled sulfate in its glycosylated form, and could not incorporate sulfate in its mature unglycosylated form (Fig. 1 A, lanes 3 and 7). Another receptor variant, YFF, in which tyrosine 14 alone was altered to phenylalanine, could incorporate radiolabeled sulfate in both its glycosylated and unglycosylated forms, but substantially less efficiently than wild-type C5aR (Fig. 1 A, lane 8). A receptor variant (YFY) in which tyrosine 10 alone was altered to phenylalanine also incorporated substantially lower levels of radiolabeled sulfate than wild-type C5aR receptor (data not shown). Taken together, these data show that tyrosines at positions 11 and 14 are modified by sulfate.

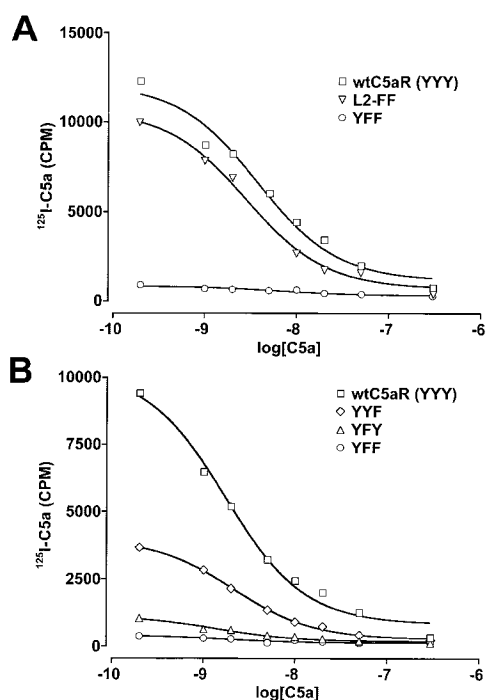
We further investigated the source of the residual sulfation observed with the YFF variant. Constructs expressing wild-type C5aR or the YFF variants were transfected into



**Figure 1.** Wild-type C5aR, but not a C5aR variant lacking tyrosines 11 and 14, efficiently incorporates radiolabeled sulfate. (A) HEK293T cells were transfected with vector alone or plasmids encoding wild-type (wt) C5aR (YYY, denoting NH<sub>2</sub>-terminal tyrosines at positions 6, 10, and 14), or variant receptors in which tyrosines 11 and 14 (YFF) or tyrosine 14 alone (YFY) had been altered to phenylalanine. Cells were split, radiolabeled with [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine or [<sup>35</sup>S]sulfate, as indicated, and lysed. Cell lysates were incubated with the antibody 1D4 that recognizes a tag COOH-terminal to C5aR and C5aR variants, and immunoprecipitates were analyzed by SDS-PAGE. (B) Experiment similar to panel A except that cells were treated with the N-glycosylation inhibitor tunicamycin before and during radiolabeling.

HEK293T cells and treated with the N-glycosylation inhibitor tunicamycin. Cells were divided, radiolabeled with [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine or [<sup>35</sup>S]sulfate in the presence of tunicamycin, and analyzed as above. Fig. 1 B shows that the majority of immunoprecipitated wild-type and YFF C5aR was found in an unglycosylated form (Fig. 1 B, lanes 2 and 3). Wild-type C5aR could efficiently incorporate [<sup>35</sup>S]sulfate (Fig. 1 B, lane 5), but the YFF C5aR variant could not incorporate detectable [<sup>35</sup>S]sulfate (Fig. 1 B, lane 6), even after extended exposure (data not shown). We conclude that tyrosines 11 and 14 are the only tyrosines present on the C5aR posttranslationally modified by sulfate and that the N-linked glycosylation of the C5aR may also incorporate some radiolabeled sulfate.

*Sulfate Moieties at Tyrosines 11 and 14 of C5aR Contribute to C5a Association.* We next investigated the ability of C5a to bind wild-type C5aR and C5aR variants in which one or both of the sulfated tyrosines had been altered to



**Figure 2.** Sulfate moieties at C5aR tyrosines 11 and 14 contribute to C5a association. (A) HEK293T cells were transfected with plasmids encoding wild-type (wt) C5aR (YYY; □), a C5aR variant in which tyrosines 11 and 14 were altered to phenylalanine (YFF; ○), or a construct in which two tyrosines in the C5aR second loop were altered to phenylalanine (L2-FF; ▽). Cells were incubated with the 0.2 nM [<sup>125</sup>I]C5a and the indicated amounts of unradiolabeled C5a competitor. Cells were washed, and bound [<sup>125</sup>I]C5a was quantitated by scintillation counting. FACS<sup>®</sup> measurements were performed in parallel on the same cells with the anti-C5aR antibody S5/1 (reference 15). Mean fluorescence for cells expressing wild-type C5a was measured at 374; for L2-FF, 352; and for YFF, 532. Mean fluorescence for mock transfected cells was 7.3. (B) An experiment similar to that in panel A using HEK293T cells expressing the wild-type C5aR (YYY; □), or the variants YFF (◇), YFY (△), or YFF (○). In this experiment, mean fluorescence values of cells expressing wild-type C5aR (YYY) was 383; YFF, 400; YFY, 383; YFF, 430; and mock-transfected, 6.6. Experiments are representative of two (A) or three (B) experiments performed with similar results.

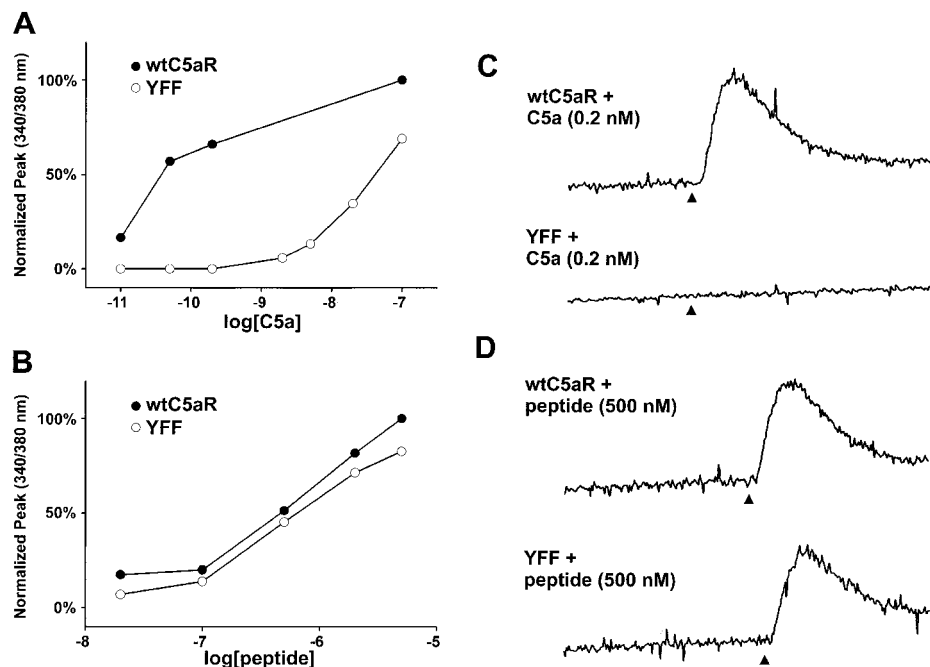
phenylalanine. HEK293T cells were transfected with wild-type C5aR, the YFF variant, or a C5aR variant in which the two tyrosines of the second extracellular loop of C5aR (tyrosines 181 and 192) had been altered to phenylalanine (denoted L2-FF). The ability of cells transfected with each of these constructs to bind [<sup>125</sup>I]C5a was assayed in the presence of increasing concentration of unlabeled C5a competitor. In parallel, an aliquot of these same cells was assayed by FACS<sup>®</sup> analysis of their surface expression of the receptor using the S5/1 anti-C5a antibody (15). Fig. 2 A demonstrates that cells transfected with the wild-type C5aR or the L2-FF variant expressed comparable levels of receptor (Fig. 2 legend) and bound [<sup>125</sup>I]C5a with similar affinities (measured dissociation constants of  $2.7 \pm 0.7$  nM and  $2.6 \pm 0.8$  nM for wild-type C5aR and the L2-FF variant, respectively). In contrast, the YFF C5aR variant expressed comparably or more efficiently than wild-type C5aR, but could not detectably associate with [<sup>125</sup>I]C5a (Fig. 2 A and Fig. 2 legend).

We also examined the specific contribution of each of the two tyrosine sulfate moieties of the C5aR to C5a binding by assaying receptor variants (YFY and YFF) in which tyrosines 11 and 14 had been altered individually to phenylalanine. Fig. 2 B shows that cells transfected with either the YFY variant or the YFF variant bound [<sup>125</sup>I]C5a substantially less efficiently than wild-type C5aR, but more efficiently than the YFF variant, despite the fact that all receptors expressed at comparable levels (Fig. 2 legend). The C5aR variant lacking a sulfate moiety at position 11 (YFY) bound [<sup>125</sup>I]C5a less efficiently than the variant lacking a sulfate at position 14 (YFF), suggesting a greater role for

the sulfate moiety at position 11. These data show that both tyrosine sulfate moieties of the C5aR NH<sub>2</sub> terminus contribute to the formation of a C5a binding site.

*The Efficiency of Calcium Mobilization by the C5aR Is Not Dependent on Sulfated Tyrosines.* Like many 7TMS superfamily receptors with protein ligands, the C5aR binds its ligand at two conformationally distinct receptor sites (9, 10, 23, 24). The first or docking site, which includes the NH<sub>2</sub>-terminus of the receptor, is known to contribute substantially to the binding energy of the receptor–ligand association, whereas the second or activation site is thought to include a pocket formed by the transmembrane helices. This second site is likely the sole site involved in the association of 7TMS receptors with small nonprotein ligands such as dopamine or acetylcholine, and is sufficient for receptor signaling with a small peptide in the case of the C5aR (6, 23). We therefore sought to determine if sulfated tyrosines of the NH<sub>2</sub> terminus of C5aR contributed to the formation of the second receptor site as well as the first.

Dose–response curves for changes in intracellular calcium induced in cells expressing wild-type C5aR or the YFF variant incubated with either C5a or a small peptide agonist (F-K-A-dCha-Cha-dR) are shown in Fig. 3. This latter agonist has been previously demonstrated to signal through the C5aR independently of the receptor NH<sub>2</sub> terminus (6). Expectedly, as shown in Fig. 3 A, a 200- to 1,000-fold higher concentration of C5a was necessary to induce a similar level of calcium mobilization in cells expressing the YFF variant compared with cells expressing wild-type C5aR, reflecting the substantially reduced ability of C5a to bind the YFF receptor observed in Fig. 2. In



**Figure 3.** A C5aR variant lacking tyrosines 11 and 14 mobilizes calcium in response to a small peptide agonist, but not to C5a. 293T cells were transfected with plasmid encoding wild-type (wt) C5aR (●) or the YFF C5aR variant (○) and a plasmid encoding the G protein component G<sub>α16</sub>. Cells were then labeled with calcium indicator dye Fura-2, and assayed for their ability to mobilize calcium in response to the indicated concentrations of C5a (A) or a small peptide C5aR agonist (reference 6) (B). Calcium mobilization is shown as the difference between the flux peak and the background levels of the ratios between the emission observed at 510 nm after excitation at 340 and 380 nm wavelengths. (C) Representative peaks of the type used to generate the dose–response curves in panel A observed with cells expressing wild-type C5aR or the YFF variant, as indicated, and treated with 0.2 nM C5a. (D) Representative peaks of the type used to generate the curves in B, treated with 500 nM agonist peptide. Triangles indicate time of introduction of C5a (C) or peptide agonist (D). A representative experiment of two experiments performed is shown in A–D.

contrast, the same cells expressing wild-type C5aR or the YFF variant responded nearly equivalently to a small peptide agonist that requires only the second site of association to mobilize calcium (Fig. 3, B and D). These data demonstrate that tyrosine sulfation contributes to the formation of the first site of C5a association, but not significantly to the second site or to the ability of the C5aR to signal.

*A Tyrosine-sulfated Peptide Partially Blocks Association of C5a with C5aR.* Tyrosine-sulfated peptides based on the NH<sub>2</sub> terminus of the chemokine receptor CCR5 have been shown to specifically block chemokine and HIV-1 gp120 association with CCR5 at high concentrations (13, 25). Fig. 4 A demonstrates that an analogous peptide, corresponding to residues 7 through 28 of the C5aR NH<sub>2</sub> terminus and sulfated at tyrosines 11 and 14 (identified as “S-peptide”; Fig. 4 B), blocked approximately half of the specific association of 0.2 nM [<sup>125</sup>I]C5a with C5aR at 200 μM concentration. The same concentration of an unsulfated peptide of the same sequence (“C-peptide” in Fig. 4 A), or a doubly sulfated control peptide based on the NH<sub>2</sub> terminus of CCR5 (“R5-S-peptide” in Fig. 4 A) had no significant effect on the ability of [<sup>125</sup>I]C5a to bind the C5aR. Assuming that C5a bound to peptide lacks the ability to bind C5aR, the affinity of the S-peptide for C5a can be placed at ~200 μM, a value similar to an affinity of the chemokine macrophage inflammatory protein (MIP)-1α for the R5-S-peptide (13). These relatively low affinities may reflect the participation of other components of the receptor in ligand binding, and the high entropic barriers that a relatively disordered peptide must overcome to adopt

its binding conformation. These data suggest a direct physical interaction between C5a and the tyrosine-sulfated NH<sub>2</sub> terminus of the C5aR, rather than an indirect role for this region of C5aR as has been suggested (2, 12).

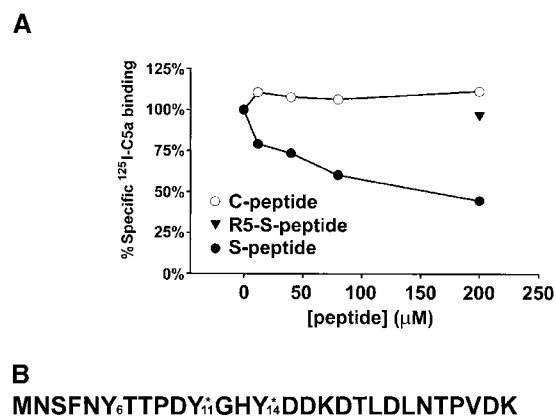
## Discussion

We have shown here that the C5aR contains two tyrosine sulfate moieties located at positions 11 and 14 in the NH<sub>2</sub> terminus of the receptor. These sulfates appear to play a critical role in the association of C5aR with C5a, but no significant role in the ability of the receptor to transduce a signal and mobilize calcium in response to a small peptide agonist. This latter observation confirms the conformational and functional integrity of the C5aR variants used in this study. We have also shown that at high concentration, a tyrosine-sulfated peptide based on the NH<sub>2</sub> terminus of the C5aR specifically inhibits the association of the C5aR with C5a. These observations are consistent with a physical interaction between the tyrosine-sulfated region of C5aR and C5a.

The presence of two sulfate moieties on the C5aR that contribute to its association with C5a may resolve apparently contradictory observations of other studies. A C5aR variant lacking residues 1–22 bound C5a significantly less efficiently than wild-type C5aR (4, 6, 11). Also, alteration of aspartic acids 10, 15, and 16 of C5aR had a similarly pronounced effect on C5a binding (4). However, Chen et al. (12) reported that in an NMR study of C5a bound to an unsulfated NH<sub>2</sub>-terminal C5aR peptide, only residues 21–30 of the C5aR NH<sub>2</sub> terminus bound C5a. Our data suggest that the discrepancy between this study and the previous mutagenic studies of C5aR is a consequence of the use of unsulfated peptide in the former NMR study. Our data also raise the possibility that perturbation of the C5aR NH<sub>2</sub>-terminal aspartic acids interferes with C5a binding (4) in part by interfering with acidic motif necessary for tyrosine sulfation (19, 20).

Chen et al. also investigated proton resonance perturbations of C5a residues in the presence of the same NH<sub>2</sub>-terminal C5aR peptide (12). They observed a number of shifts at residues in the first loop and second helix of C5a, but did not detect resonance shifts for a set of mostly positively charged residues in the third and fourth helix of C5a implicated by mutagenesis in binding the C5aR (9, 12, 26, 27). These C5a residues include Arg 37, Arg 40, Leu 43, Arg 46, Lys 49, Glu 53, and Arg 62. Most of these residues are distal from the COOH terminus of C5a which interacts with the second or activation site of the receptor, and which is implicated in signaling. Thus, given the charge complementarity of these residues and the NH<sub>2</sub> terminus of C5aR, it is likely that some of these residues interact with the region of the C5aR NH<sub>2</sub> terminus that includes sulfated tyrosines 11 and 14. It will be interesting to determine if a sulfated peptide induces chemical shifts in C5a in addition to those observed by Chen et al. (12).

The prevalence of and functional role for sulfated tyrosines at the NH<sub>2</sub> terminus of at least two 7TMS recep-



**Figure 4.** A sulfated peptide derived from the C5a NH<sub>2</sub> terminus partially inhibits C5a association with the C5aR. (A) 293T cells transfected with wild-type C5aR were incubated with 0.2 nM [<sup>125</sup>I]C5a and the indicated amount of a peptide corresponding to residues 7 through 28 of the C5aR, with sulfated tyrosines at positions 11 and 14 (S-peptide; ●), or with unmodified tyrosines at these positions (C-peptide; ○), or with a doubly sulfated peptide based on the 22 NH<sub>2</sub>-terminal residues of the chemokine receptor CCR5 (R5-S-peptide; ▼). Cells were washed, and bound [<sup>125</sup>I]C5a was measured by gamma counting. (B) The primary sequence of the NH<sub>2</sub>-terminal 28 residues of the C5aR with positions of tyrosines indicated. Sulfated tyrosines 11 and 14 are indicated with asterisks. The sequence of the C5aR corresponding to the sulfated peptide (S-peptide) used in panel A is underlined. A representative experiment of two similar experiments is shown.

tors, CCR5 and the C5aR, that mediate chemotaxis suggest that unique properties of the sulfate moiety contribute to the function or regulation of these receptors. For example, the sulfate moiety is labile at acidic pH, suggesting the possibility that internalization of the ligand-bound receptor into a low pH compartment and consequent loss of the sulfate moiety facilitates the release and degradation of ligand and recycling of the receptor, possibly after resulfation in the trans-Golgi network (18, 20, 21). Rapid dissociation of ligand and receptor recycling may be especially useful in the case of chemotactic receptors that must respond quickly to minute concentration gradients of ligand. Also of note is that many 7TMS receptors with relatively large protein ligands have NH<sub>2</sub> termini that suggest the presence of sulfated tyrosines (3), whereas most 7TMS receptors with small molecule ligands lack a similar tyrosine sulfation motif. Thus, in many cases sulfated tyrosines may provide the primary binding energy for ligand association at the docking site of the receptor, positioning the ligand appropriately for interaction with the second, activation site. The presence and functional role of sulfate moieties on the chemotactic receptors CCR5 (3), CCR2 (28), and C5aR suggest that most of the many chemotactic 7TMS receptors containing acidic and tyrosine-rich regions in their extracellular domains are indeed tyrosine sulfated, and that sulfate moieties contribute to the function of these receptors as well.

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