Analysis of Post-translational CCR8 Modifications and Their Influence on Receptor Activity

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Post-translational modifications of the extracellular portions of receptors located in the cell membrane can contribute to modulating their biological activity. Using a mutagenesis approach in which single or multiple Tyrr-to-Phe, Thr-to-Ala, Ser-to-Ala, and Asn-to-Gln substitutions were made at the appropriate positions, we analyzed the sulfation and glycosylation state of the murine CCR8 chemokine receptor, and the way in which these post-translational modifications affect CCR8 activity. A Y14F15-to-F14F15 CCR8 mutant was less sulfated than the wild-type receptor. An N8-to-Q8 mutant was less glycosylated than wild-type, and a double T10T12-to-A10A12 mutant showed even less glycosylation. We established a flow cytometric analysis with an Fc-fused form of mouse CCL1 to determine precisely the ligand-binding activity of these mutants. Single mutants at amino acid positions 8, 10 or 12 bound CCL1-Fc similarly to wild-type CCR8, whereas the F14F15 double mutant was essentially inactive and the A10A12 double mutant showed about 65% of wild-type ligand-binding activity. Calcium flux activity assays were performed with these mutants, yielding results consistent with those from the ligand binding assays. These data indicate that sulfation at specific positions of the N-terminal domain of mouse CCR8 is critical for its biological activity, whereas glycosylation has a minor influence.

Post-translational modifications of amino acid residues located in the extracellular or intracytoplasmic domains of cell membrane receptors can modify their signaling activity of these proteins. In the case of chemokine receptors, a family of seven-transmembrane domain proteins with critical roles in the control of basal and pathological leukocyte trafficking (1), alteration of their activity may modulate the migratory routes of immune cells. Chemokine receptors are known to undergo a variety of post-translational modifications. For instance, phosphorylation of specific intracellular serine residues in the C-terminal region of chemokine receptors is essential for their signal transduction function (2). In some chemokine receptors, extracellular regions are also known to be post-translationally modified. Human chemokine receptors CCR2b, CCR5, CXCR1, and CXCR4 are reported to be sulfated and/or glycosylated at their N-terminal extracellular domains. In vitro, these modifications have diverse consequences for receptor ligand-binding activities, as well as for their function as human immunodeficiency virus coreceptors (3–6).

CCR8, the receptor for the β-chemokine CCL1 (7–9), is expressed in Th2-polarized T cells (10, 11) and has been implicated in allergic inflammation (12), although whether CCR8 has a critical role in asthma remains controversial (13–15). CCR8 also has a potential role in atherogenesis (16), can act as a human immunodeficiency virus coreceptor (17), and is a target for certain virally encoded chemokines (18). We characterized the mouse version of CCR8, which has only one murine ligand, CCL1 (8). We developed mouse CCR8-specific antibodies and used them to study the tissue expression of this molecule, which was highest in thymocytes committed to the CD4+ T cell lineage (19).

The results of that study suggested that modifications in extracellular regions of CCR8 could mask or form part of epitopes recognized by the antibodies. Using a mutagenesis approach and metabolic labeling studies, we have now performed a detailed study of potential post-translational modifications of murine CCR8. Our results indicate that tyrosines at positions 14 and 15 in mouse CCR8 are sulfated amino acid residues, whereas asparagine 8 and threonines 10 and 12 are glycosylated. CCR8 mutants in which those residues are replaced by amino acids subject neither to sulfation nor to glycosylation are affected differently in their ability to bind CCL1, as well as in their signaling. All together, the results show that tyrosine sulfation in the CCR8 N-terminal domain is important for full activity of this chemokine receptor, whereas the influence of asparagine and threonine glycosylation on the receptor activity is less critical.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle medium (BioWhittaker, Verviers, Belgium) containing 10% fetal calf serum and supplemented with glutamine, penicillin, and streptomycin. Methionine- and cysteine-free medium consisted of RPMI 1640 (BioWhittaker); sulfatate-free medium consisted of Eagle's modified minimal essential medium (ICN Biomedicals, Aurora, OH), supplemented with 1 mM CaCl2. In some cases tunicamycin (5 μg/ml) or monensin (5 μM; both from Sigma, St. Louis, MO) was added to the culture. When necessary, G418 (1 mg/ml; Promega, Madison, WI) was used for selection.

Mutagenesis and Transfection—The construction of a mammalian expression plasmid containing the coding sequence of mouse CCR8 cDNA has been described previously (8). A synthetic DNA fragment encoding a c-Myc protein epitope was introduced at the 5′ end of the
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Mouse CCR8 was analyzed for ligand binding and function. CCR8 is an N-terminal sulfated glycoprotein.

**RESULTS**

**Myc-tagged Mouse CCR8 Is Expressed Functionally at the Cell Surface**—The study of potential extracellular post-translational modifications of mouse CCR8 required a tool to measure precisely cell surface expression levels of the protein independently of such modifications. We therefore produced a plasmid encoding a version of CCR8 with a c-Myc epitope tagged at its N-terminal end. This CCR8 variant was tested for expression and function in transfected HEK 293 cells. Tagged CCR8 was recognized by an anti-myc mAb (Fig. 1A), yielding a FACS staining pattern that closely matched that of the CCR8-specific mAb 8F4 (19). HEK 293 stable transfectant cells expressing myc-tagged or untagged CCR8 were obtained and tested for ligand-induced Ca\(^{2+}\) flux. The ligand sensitivity of CCR8 was assessed.

**FIG. 1.** Myc-tagged CCR8 and human Fe-fused CCL1 are biologically active. A, HEK 293 cells transfected with a myc-tagged wild-type CCR8-expressing plasmid were stained with the anti-CCR8 8F4 (gray line) or the anti-myc 9E10 (black line) mAb. Control stainings are shown for non-transfected HEK 293 cells with the same mAb (dotted line). B, HEK 293 cells expressing myc-tagged or untagged wild-type CCR8 were tested in Ca\(^{2+}\) flux assays. Buffers, 0.1, 1, or 10 nM mCCL1 were added at 50, 100, 200, and 300 s, respectively; sharp, high spikes along the fluorescence trace mark the exact time of additions. Indo 1 fluorescence reflects intracellular Ca\(^{2+}\) concentration. C and D, wild-type CCR8-expressing cells were analyzed in the FACS ligand binding assay; in C, the binding profile is shown at decreasing concentrations of CCL1-Fc-conditioned medium; in D, competition is shown for binding of CCL1-Fc-conditioned medium (10% v/v) by purified CCL1.

37°C, excited with light at 335 nm, and stimulated with various concentrations of purified mouse CCL1; intracellular Ca\(^{2+}\) levels were assessed as Indo 1 fluorescence at 390 nm and recorded as a time trace in an F2500 fluorometer (Hitachi, Tokyo, Japan).

Confocal Microscopy Analysis—Transfected HEK 293 cells were cultured in poly-L-lys-coated glass Lab-Tek II chamber slides (Nalge Nunc, Naperville, IL). Culture medium was withdrawn, and the cells were washed, fixed in 2% paraformaldehyde (20 min, room temperature), and permeabilized with 0.1% saponin in PBS for 15 min. After thorough washing, slides were incubated with a culture supernatant from the anti-CCR8 mAb-producing hybridoma 3E19, which recognizes a fixation-resistant epitope at the C-terminal end of CCR8. Cells were further washed and incubated with an Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes). Saponin was maintained throughout the process to this step. After two final washes, slides were mounted and visualized on a Leica DM IRB fluorescence microscope. Serial confocal images along the z-axis were acquired using an Ar-Kr laser scanning TCS-NT Leica system; images were selected corresponding to thin optical cell sections at the nuclear level to assess CCR8 distribution. Samples were in duplicate, and at least two independent transfections were performed for each CCR8 variant.

**RESULTS**

**Myc-tagged Mouse CCR8 Is Expressed Functionally at the Cell Surface**—The study of potential extracellular post-translational modifications of mouse CCR8 required a tool to measure precisely cell surface expression levels of the protein independently of such modifications. We therefore produced a plasmid encoding a version of CCR8 with a c-Myc epitope tagged at its N-terminal end. This CCR8 variant was tested for expression and function in transfected HEK 293 cells. Tagged CCR8 was recognized by an anti-myc mAb (Fig. 1A), yielding a FACS staining pattern that closely matched that of the CCR8-specific mAb 8F4 (19). HEK 293 stable transfectant cells expressing myc-tagged or untagged CCR8 were obtained and tested for ligand-induced Ca\(^{2+}\) flux. The ligand sensitivity of CCR8 was assessed.

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cells transfected with the tagged version was similar to that of cells expressing the untagged version; they responded readily to 0.1 and 1 nM CCL1 and showed a typical desensitization pattern following addition of 10 nM CCL1 (Fig. 1B). The myc-tagged CCR8 was thus expressed efficiently and functioned similarly to the untagged version.

CCL1-Fc and CCL1 Have Similar CCR8-binding Activity—To analyze the ligand-binding activity of CCR8 and its variants, we produced a human Fc-fused version of CCL1. Conditioned medium (CM) containing CCL1-Fc and secondary FITC-conjugated reagents recognizing the Fc portion were used to stain cells transfected with a plasmid encoding CCR8 or the void vector. At high CCL1-Fc concentrations, CCR8-expressing cells stained intensely; staining faded with dilution and was no longer detectable at CCL1-Fc CM concentrations below 0.01% (v/v) (Fig. 1C). The vector-transfected cells did not stain above background level at any CCL1-Fc CM concentration; in addition, mock CM was produced and tested similarly on CCR8-expressing cells; as predicted, no staining was detected above background levels (not shown). To verify that Fc-linked cell staining was a reliable measure of CCL1 binding to its receptor, CCR8-expressing cells were incubated with 10% CCL1-Fc CM in the presence of purified mouse CCL1 at various concentrations. At 0.25 nM, purified CCL1 competed slightly for CCL1-Fc staining; competition increased gradually by augmenting CCL1 concentration up to 200 nM, above which CCL1-Fc staining was negligible (Fig. 1D). The CCL1 concentration yielding half-maximal competition was ~2 nM, which matches the dissociation constant reported for the CCL1-CCR8 interaction (8). CCL1-Fc CM is thus a reliable tool for measuring ligand-binding activity of CCR8-bearing cells by flow cytometry.

Flow Cytometric Analysis of CCL1 Binding to CCR8—Simultaneous measurement of cell surface CCR8 expression and CCL1 binding in a single flow cytometry assay would facilitate testing the activity of receptor variants. This was analyzed by incubating myc-tagged CCR8-transfected cells with CCL1-Fc CM, with anti-myc 9E10 mAb, or with both simultaneously. Secondary FITC- or PE-conjugated reagents were used to develop bound Fc or myc epitopes, respectively. Cells stained with CCL1-Fc alone were recorded as typical FITC fluorescent events (Fig. 2A), and cells stained with the anti-myc 9E10 mAb were essentially PE events (Fig. 2B); cells stained with both reagents displayed a linear distribution along the FL1/FL2 diagonal (Fig. 2C). Anti-myc 9E10 mAb binding was inhibited weakly by CCL1-Fc binding (74.2% PE+ cells in plot B versus 60.7% PE+ cells in plot C), although strong overall correlation was observed between CCR8 levels and CCL1-Fc binding. In addition, CCL1-Fc binding to myc-CCR8 was estimated to have a $K_d$ of 1–2 nM, concurring with previously published results for the untagged CCL1:CCR8 pair (8). These results allowed specific gating for myc-associated fluorescence, i.e. CCR8 levels, and measuring the amount of Fc-associated fluorescence, i.e. CCL1 binding, only for the specifically gated cell population. By gating on myc, it was thus possible to compare the ligand-binding activity of CCR8 variants with different cell surface expression efficiency.

Targeting of Potential Post-translational Modification Sites in the CCR8 Amino Acid Sequence—Post-translational modifications relevant to chemokine receptor function have been described mainly at the N-terminal domain. In particular, protein sulfation at tyrosine residues, as well as both N- and O-glycosylation have been reported for other CC chemokine receptors. There is no strict consensus site for tyrosine sulfation, but sulfated tyrosines in CCR5 and CCR2b are adjacent to acidic amino acids (3, 4). We detected four such potential sulfation sites in two segments of the mouse CCR8 sequence predicted to be extracellular, three at the N-terminal domain and an additional site in the second extracellular loop (Fig. 3A). For N-linked glycosylation, the asparagine residue at CCR8 position 8
conforms to requirements of the well defined consensus site for this type of modification (asparagine-X-serine/threonine) (8). Finally, O-linked glycosylation is the least characterized modification in terms of consensus sites, but this modification often occurs within threonine and/or serine residue clusters. We identified two such sites, an N-terminal domain threonine pair that partially overlaps the N-glycosylation consensus site, and a threonine/serine pair at the third extracellular loop (Fig. 3A).

To study potential post-translational modifications of mouse CCR8, we generated a set of myc-tagged CCR8 mutants at these nine positions. Conservative changes were designed, consisting of tyrosine-to-phenylalanine, which is not subject to sulfation, asparagine-to-glutamine, not subject to N-glycosylation, and threonine- or serine-to-alanine, not subject to O-glycosylation (Fig. 3B).

**CCR8 Is Modified by Sulfate—Myc-tagged CCR8-expressing cells were incubated with either [35S]methionine/cysteine or sodium [35S]sulfate, followed by myc immunoprecipitation, electrophoresis, and autoradiography.** Three bands were observed in autoradiographs corresponding to wild-type CCR8-expressing cells cultured with [35S]methionine/cysteine or sodium [35S]sulfate, followed by myc immunoprecipitation, electrophoresis, and autoradiography. The upper band migrated as host c-Myc and was present in all immunoprecipitates analyzed, including a mock-transfected control (Fig. 4A, lane 1). Two bands were seen corresponding to CCR8; the upper band was broad and migrated at 41 kDa; the lower, sharper band migrated as 35 kDa (Fig. 4A, lane 1).

Similar results were obtained in Western blot analysis of unlabeled cells (see below). The autoradiograph of wild-type CCR8-expressing cell cultures treated with [35S]sulfate showed one band corresponding to the 41-kDa form (Fig. 4B, lane 1). This showed that CCR8 is indeed a sulfated protein and that sulfate is incorporated into the low mobility protein form.

To analyze whether the targeted tyrosine residues were involved in CCR8 sulfation, cells transfected with plasmids encoding the appropriate mutants were labeled, immunoprecipitated, and autoradiographed. For correct comparison of the sulfation level of CCR8 variants, autoradiographs were densitometered, and the amount of [35S]methionine/cysteine label was used to normalize the amount of [35S]sulfate label for protein mass. All the single mutants incorporated [35S]sulfate label in amounts similar to wild-type (Fig. 4A, lane 2–5); double or quadruple mutants involving tyrosines 14 and 15 significantly reduced the [35S]sulfate label (Fig. 4B, lanes 6 and 7), whereas all mutants were similarly labeled by [35S]methionine and [35S]cysteine (Fig. 4A, lanes 2–7). These results suggest that tyrosines 14 and 15 are indeed subject to sulfation and that there is additional sulfate incorporation throughout the molecule not involving the targeted tyrosines. A slight decrease in CCR8 apparent molecular weight was seen in the [35S]methionine/cysteine autoradiograph in samples from single mutants at positions 14 or 15, particularly at the lower band; this mobility shift was even more marked in the double or quadruple mutants involving these positions (Fig. 4A, lanes 4–7). Similar small electrophoretic mobility shifts were also reported for sulfatable tyrosine mutants of C5a receptor (21).

Cells transfected with vector only were treated similarly and yielded only the band corresponding to host c-Myc (Fig. 4, A and B, lanes 8).

**Inhibition of Sulfation Impairs CCR8 Ligand Binding—** Cells transfected with myc-tagged CCR8 were depleted of sulfate and incubated with 10 mM sodium chloride, a concentration reported to inhibit sulfation (22). Control cells were incubated in the presence of sulfate. Cells were harvested and analyzed in the CCL1-Fc binding FACS assay. The results indicated that even cells with low surface CCR8 expression, cultured in the presence of sulfate, bound CCL1 as predicted.
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(FIG. 4C). To the contrary, sulfate-depleted cells cultured in sodium chlorate bound small amounts of CCL1 only when expressing high CCR8 levels (Fig. 4D). This result indicated that sulfation is necessary for the full ligand-binding activity of CCR8.

Mutations at Tyrosines 14 or 15 Decrease CCR8 Ligand-binding Activity—Cells transfected with wild-type myc-tagged CCR8 or selected tyrosine-to-phenylalanine mutants were analyzed in the CCL1-Fc binding FACS assay. A gate was established for similar medium to high levels of CCR8 expression, and the mean Fc-associated fluorescence intensity was determined for gated cells on each sample. The results indicated that a single mutation at tyrosine 14 (YFYF) significantly decreased CCR8 ligand-binding activity; a mutation at tyrosine 15 (YFYY) provoked a greater decrease in binding, and a double mutation at both positions (YFFY) virtually abolished CCR8 ligand binding. At the CCL1-Fc concentration tested, impairment of binding activity was not greater after quadruple mutation (FFFF) than after double mutation of tyrosines 14 and 15 (YYFY), the quadruple mutant was also inactive in this assay (Fig. 5C). The Ca<sup>2+</sup> flux results were thus fully consistent with those of the ligand binding assay, supporting a central role for tyrosines 14 and 15 in CCR8 biological activity.

CCR8 Is Modified by Oligosaccharide: Sialic Acids Are Important for Ligand Binding—G protein-coupled receptors can be O- and/or N-glycosylated. To analyze the presence of sialic acid moieties linked to CCR8 amino acid residues, myc-tagged CCR8-expressing cells were incubated with neuraminidase, immunoprecipitated, and analyzed in Western blot. Blots developed with anti-myc rabbit antibody showed that, compared with untreated samples (Fig. 6, lane 1), enzyme treatment produced a slight increase in the electrophoretic mobility of the upper CCR8 band (Fig. 6, lane 2), indicating that this receptor is modified by sialic acid. To analyze the potential N-glycosylation of CCR8, similar cell samples were cultured with the N-glycosylation inhibitor tunicamycin, harvested, and processed as above. This treatment produced an even larger downward shift in the migration of the upper band, as well as a shift in the lower band (Fig. 6A, lane 3), suggesting that both CCR8 protein forms are modified by N-linked oligosaccharides. Untreated or neuraminidase- or tunicamycin-treated cells were also analyzed in the CCL1-Fc binding assay, to determine the mean Fc-associated fluorescence intensity of cells gated for
similar medium to high CCR8 expression levels. The results indicated that a lack of sialic acids significantly impairs CCR8 interaction with CCL1 (Fig. 6B); on the contrary, N-glycosylation does not appear to be essential for this interaction.

To analyze whether the targeted asparagine, threonine, and serine residues were involved in CCR8 glycosylation, cells transfected with plasmids encoding the corresponding mutants were similarly immunoprecipitated and tested using Western blot analysis. Single mutants at asparagine 8 (N8Q) or threonine 10 (ATTs) yielded a CCR8 banding pattern identical to that of tunicamycin-treated cells (Fig. 6C, lanes 2 and 3). The single mutant at threonine 12 (TATS) and the double mutant at threonine 259 and serine 260 (TTAA) behaved similarly to wild-type CCR8 (Fig. 6C, lanes 1, 4, and 6). Finally, the double mutant at threonines 10 and 12 (AATS) showed a shift in the lower band similar to that observed for tunicamycin-treated cells, and an even larger shift in the upper band, with mobility near that of the lower band (Fig. 6C, lane 5).

Overall, the results indicate that the upper band in [35S]methionine/cysteine autoradiography and Western blot of wild-type CCR8 samples corresponds to the full glycosylated and sulfated protein; the lower band is a subglycosylated, nonsulfated form. The electrophoretic mobilities of these two bands were somewhat surprising, because their apparent molecular weights were inconsistent with that calculated from the amino acid sequence of the nonglycosylated myc-tagged mouse CCR8 protein (41,616 Da). CCR8 samples cannot be boiled for SDS-PAGE in a standard way due to protein aggregation; instead, they are heated to 50 °C. This low temperature processing may result in incomplete denaturation, causing the migration anomaly. The results also suggest that CCR8 is N-glycosylated at asparagine 8, that threonine 10 is required for N-glycosylation, as predicted from its position in the consensus motif, and that threonines 10 and/or 12 are O-glycosylated.

A Double Mutation at Threonines 10 and 12 Affects CCR8 Ligand-binding Activity, Whereas Mutations at Threonine 10 Alter Receptor Trafficking to the Cell Membrane—Cells transfected with wild-type myc-tagged CCR8 or selected mutants were tested in the CCL1-Fc binding FACS assay. A gate was established for similar medium to high CCR8 expression levels, and mean Fc-associated fluorescence intensity was determined for the gated cells on each sample. The results indicated that the double mutation at threonines 10 and 12 clearly impaired binding, reducing it to ~65% of the wild-type value (Fig. 6D). Other CCL1-Fc concentrations yielded similar results (not shown) and allowed estimation of a $K_D$ of 4 nM for the interaction with the AATS mutant, approximately double than that estimated for wild-type CCR8. Conversely, neither single mutations at threonines 10/12 nor the double mutation at threonine 259 and serine 260 significantly decreased CCR8 ligand-binding activity.

Calcium flux assays using cells transfected with these mutants showed that, with minor variations, most CCR8 mutants showed chemokine sensitivity similar to that of the wild-type. All responded to 0.1 nM CCL1 except those involving threonine 10, which required a 100-fold greater CCL1 concentration (10 nM) to elicit Ca$^{2+}$ mobilization; even at this concentration the flux was low (Fig. 6E). Overall, the results support an important role for glycosylatable threonines 10 and 12 in CCR8 biological activity.

Mutations at amino acid residues subject to glycosylation can affect the trafficking of membrane proteins within the cell (23). We performed confocal microscopy analysis using permeabilized, CCR8-stained transfected cells to study the intracellular trafficking of CCR8 variants. Fig. 7 shows thin confocal sections selected at middle z-axis of representative cell clusters for each preparation. As transient transfecants were used, weak and bright staining were observed on different cells. Distinct degrees of intracellular staining were observed in each cell, mainly in perinuclear granules. In addition, bright membrane-associated staining was clearly detected in cells transfected with wild-type, TATS, or TTAA CCR8 variants; transfectants with the N8Q mutant showed weaker, but clear cell surface-associated staining. Finally, cells transfected with the single ATTs or the double AATS mutants showed very weak membrane staining, similar to that of wild-type CCR8-transfected cells incubated with monensin, a glycoprotein secretion inhibitor. Cells expressing CCR8 tyrosine-to-phenylalanine mutants were analyzed, with results similar to those of the wild-type receptor (not shown). These data suggest that a lack of glycosylatable threonine 10, but not threonine 12, partially impairs CCR8 transport to the cell membrane.

These CCL1-Fc binding studies used cells gated by flow cytometry for strictly comparable membrane CCR8 levels, regardless of intracellular CCR8 content. Calcium flux assays were performed using transient transfecants in which surface CCR8 levels were matched after pilot tests with different DNA doses for each CCR8 variant. For mutants involving threonine 10, higher DNA doses were needed to obtain cell surface expression similar to that of wild-type CCR8.

**DISCUSSION**

In addition to classic gene expression regulation, biological functions of chemokine receptors may also be subject to regulation at post-translational stages. In particular, tyrosine sulfation proved important for ligand-binding activity of several human chemokine receptors (3–6). Nonetheless, no mouse G protein-coupled receptor has to date been reported to be tyrosine-sulfated. Here we present data demonstrating that this is indeed the case for mouse CCR8.

Our analysis maps potentially sulfated tyrosines to the N-terminal domain. Specifically, tyrosines at positions 14 and 15 appear to be sulfation substrates, although their degree of sulfation is probably not homogeneous. Single tyrosine-to-phenylalanine mutations at mouse CCR8 position 14 or 15 did not cause a significant loss of the protein sulfate load compared with the wild-type protein, whereas a double mutation at both amino acid positions clearly decreased receptor sulfate content.
This indicates that both sites are subject to sulfation, although they are not modified to the same extent in all CCR8 molecules. Our data suggest that, in the absence of one of these tyrosines, the other is sulfated more extensively. One explanation would be the existence of steric hindrance in the use of adjacent tyrosines by the sulfation machinery. Alternatively, tyrosines 14 and 15 may be important for the sulfation of a non-tyrosine CCR8 substrate. Indeed, there is non-tyrosine-linked sulfate in mouse CCR8. Tyrosines 3 and 185 appear not to be sulfated, because even a double mutant at both positions incorporates \[^{35}S\]sulfate in amounts similar to that of wild-type protein (not shown); in addition, the quadruple \(\text{FFFF}^{m}\) mutant incorporates similar amounts of \[^{35}S\]sulfate as the double \(\text{YYFF}^{m}\) mutant lacking tyrosines 14 and 15. Tyrosine 170, which is at the limit between the predicted fourth CCR8 transmembrane domain and the second extracellular loop, could potentially be another CCR8 sulfation site. This tyrosine is nonetheless distant from acidic amino acid residues and adjacent to a basic amino acid, an environment that appears to exclude tyrosine 170 as sulfation substrate. A truncated form of mouse CCR8 lacking the 28 N-terminal amino acids is not sulfated (not shown). For all these reasons, we believe that the extra sulfate detected is probably linked to an oligosaccharide moiety located in the CCR8 N-terminal domain. Sulfate-linked oligosaccharide is reported to modify the N-terminal domains of CXCR4 and C5aR (5, 21). Remarkably, all sulfate in mouse CCR8 was in the fully glycosylated, unsulfated CCR8 proteins in the glycosylated protein form. Sulfate is known to be linked to core carbohydrate chains. Although the amount of oligosaccharide incorporated to the neighboring carbohydrate, the AATS mutant lacks an additional small oligosaccharide moiety. In wild-type CCR8, this oligosaccharide would be O-linked to threonine 10, to threonine 12, or to both in a non-homogeneous way. This situation resembles that of tyrosine 14 and 15 sulfation, because the extent of post-translational modification at the threonine 10 and 12 residues seems to be interdependent. Apparently, if glycosylation at position 10 is not possible, position 12 can accept some oligosaccharide; if there is no glycosylation at residue 12, threonine 10 incorporates it all. The fact that threonine 10 forms part of the N-glycosylation motif complicates this issue further, because both N- and O-glycosylation could exert mutual influence. Glycosyltransferase access to sites otherwise masked by neighboring carbohydrate has been reported for other mutant G protein-coupled receptors (25). Tyrosine-to-phenylalanine mutations at positions 14 and/or 15 caused slightly higher CCR8 electrophoretic mobility in SDS-PAGE, which was particularly evident for the subglycosylated protein form. This may reflect the potential influence of those two amino acid residues on the amount of oligosaccharide incorporated to the neighboring N-glycosylation site.

The AATS mutant showed a doublet band in Western blot, suggesting that some oligosaccharide is present on the molecule at sites other than asparagine 8, threonine 10, or threonine 12. Our data show that neither threonine 259 nor serine 260 is glycosylated, as judged by the electrophoretic mobility of the double \(\text{TTAA}^{m}\) mutant, although other non-clustered threonine and serine residues in the CCR8 extracellular domains may be subject to glycosylation. It is also possible that the AATS doublet band does not reflect differences in CCR8 glycosylation, but other post-translational modifications or partial proteolytic degradation.

Neuraminidase treatment resulted in a small loss of CCR8 mass, as deduced from electrophoretic mobility changes. Neuraminidase hydrolyzes sialic acids that are terminally linked to core carbohydrate chains. Although the amount of these acids in CCR8 seems to be small, their chemical nature could be important for receptor function. Sialic acids would increase the extra negative charge at the CCR8 N-terminal region, in addition to that provided by sulfate. Indeed, we found that neuraminidase-treated cells expressing wild-type CCR8 bound CCL1 less efficiently than untreated cells. A similar result was reported for CCR5 (26). This again supports a relevant role for electrostatic forces in the interaction between basic chemokines and the N-terminal domains of chemokine receptors.

The \(\text{Ca}^{2+}\) flux activity of glycosylation-targeted mutants differs from that of the tyrosine mutants in that there is no direct match to the ligand binding data. Both the single and double mutants involving threonine 10 showed decreased sensitivity to CCL1 in \(\text{Ca}^{2+}\) flux, whereas only the double AATS mutant showed reduced ligand binding. The ATTS mutant bound the chemokine similarly to the wild-type receptor at all concentrations tested (not shown), suggesting that the mutation affects post-binding steps specific for CCR8 signal transduction. Confocal microscopy analysis showed that both mutants affected in threonine 10 had a dramatically reduced ratio of membrane-associated to cytoplasmic protein, probably caused by impaired trafficking of the nascent protein to the plasma membrane.

Glycosylation is an important step in the intracellular traffic of certain G protein-coupled receptors (27). Indeed, confocal
images of monensin-treated cells expressing wild-type CCR8 were similar to those of untreated threonine 10 mutants. Transfection conditions for our functional analysis were adjusted for similar variant CCR8 membrane expression levels; threonine 10 mutants were thus probably overloaded with intracellular protein compared with other CCR8 variants. This intracellular receptor overload may sequester cytoplasmic molecules required for signal transduction, reducing that available for signaling from the membrane-docked receptor. This would explain why the ATTS mutant is able to bind CCL1 efficiently for signaling from the membrane-docked receptor. This would also account for the distinct behavior of the ATTS mutant, because a protein conformation potentially active for ligand binding at low temperature might be unstable at 37 °C.

The mouse CCR8 shows 71% overall amino acid sequence identity with its human counterpart, which is reduced to 50% at the N-terminal domain (8). The threonine 10/12 and tyrosine 14/15 pairs are conserved between human and murine CCR8, but the human protein is two amino acid residues longer than the murine form, with a threonine at position 9 and a tyrosine at position 13 that are putative substrates for modification. This probably adds complexity to the potential post-translational modifications in human CCR8 and may explain the discrepancy in reports on mouse CCL1 activity with human CCR8 (8, 10, 28, 29).

Our results on post-translational modifications of mouse CCR8 are based on transfections of a human cell line. HEK 293 cells were used, because they expressed mouse CCR8 more efficiently than the murine lymphoid cell lines tested. Although mouse cell modification of CCR8 may differ quantitatively from that of the human host cells, it is unlikely that these modification patterns would differ qualitatively. Study of the mouse CCR8 in vivo post-translational modification pattern will require development of modification-specific antibodies, for which the CCR8 variants we have generated will be valuable tools.

In conclusion, these results present mouse CCR8 as the first murine G protein-coupled receptor shown to be tyrosine-sulfated. We also show a major role for tyrosine sulfation in chemokine binding and describe a complex pattern of CCR8 N- and O-glycosylation, supporting the concept that these post-translational modifications may contribute to physiological regulation of chemokine function.

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