Sulfated tyrosines 27 and 29 in the N-terminus of human CXCR3 participate in binding native IP-10

Original Article

Sulfated tyrosines 27 and 29 in the N-terminus of human CXCR3 participate in binding native IP-10

Jin-ming GAO[1,*], Ruo-lan XIANG[2], Lei JIANG[1], Wen-hui LI[3], Qi-ping FENG[4], Zi-jiang GUO[1], Qi SUN[5], Zheng-pei ZENG[5], Fu-de FANG[4]

[1]Department of Respiratory Disease, [5]Department of Endocrinology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100730, China; [2]Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing 100088, China; [3]Department of Microbiology and Molecular Genetics, Division of AIDS, Harvard Medical School, Boston, Massachusetts 02115, USA; [4]Department of Biochemistry and National Laboratory of Medical Molecular Biology, Basic Institute of Medical Sciences, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 10000S, China

Aim: Human CXCR3, a seven-transmembrane segment (7TMS), is predominantly expressed in Th1-mediated responses. Interferon-γ-inducible protein 10 (IP-10) is an important ligand for CXCR3. Their interaction is pivotal for leukocyte migration and activation. Tyrosine sulfation in 7TMS is a posttranslational modification that contributes substantially to ligand binding. We aimed to study the role of tyrosine sulfation of CXCR3 in the protein's binding to IP-10.

Methods: Plasmids encoding CXCR3 and its mutants were prepared by PCR and site-directed mutagenesis. HEK 293T cells were transfected with plasmids encoding CXCR3 or its variants using calcium phosphate. Transfected cells were labeled with [^{35}S]-cysteine and methionine or [^{35}S]-Na_2SO_3 and then analyzed by immunoprecipitation to measure sulfation. Experiments with ^{125}I-labeled IP-10 were carried out to evaluate the affinity of CXCR3 for its ligand. Calcium influx assays were used to measure intercellular signal transduction.

Results: Our data show that sulfate moieties are added to tyrosines 27 and 29 of CXCR3. Mutation of these two tyrosines to phenylalanines substantially decreases binding of CXCR3 to IP-10 and appears to eliminate the associated signal transduction. Tyrosine sulfation of CXCR3 is enhanced by tyrosyl protein sulfotransferases (TPSTs), and it is weakened by shRNA constructs. The binding ability of CXCR3 to IP-10 is increased by TPSTs and decreased by shRNAs.

Conclusion: This study identifies two sulfated tyrosines in the N-terminus of CXCR3 as part of the binding site for IP-10, and it underscores the fact that tyrosine sulfation in the N-termini of 7TMS receptors is functionally important for ligand interactions. Our study suggests a molecular target for inhibiting this ligand-receptor interaction.

Keywords: tyrosine; sulfation; chemokine receptor; interferon γ-inducible protein 10; CXCR3

Acta Pharmacologica Sinica (2009) 30: 193–201; doi: 10.1038/aps.2008.24; published online 19th January 2009

Introduction

CXCR3, a seven-transmembrane G-protein-coupled chemokine receptor, is expressed on a variety of cells, including activated effector and memory T cells, Th1 lymphocytes, B cells, NK cells and endothelial cells[1–3]. CXCR3 binds to multiple natural ligands, including monokine induced by interferon (IFN)γ (Mig or CXCL9), IFN γ-inducible protein 10 (IP-10 or CXCL10) and IFN-inducible T-cell α chemoattractant (I-TAC or CXCL11). By binding to these ligands, CXCR3 plays a role in many aspects of immune and inflammatory responses, particularly in orchestrating migration and activation of circulating leukocytes[4]. IP-10, which has a molecular mass of 10 kDa, is a member of the CXC superfamily[4]. It is expressed in predominance in Th1-mediated pathological inflammatory processes[5–7]. Blockage of IP-10 inhibits the recruitment of effector T cells to sites of inflammation following injury[8, 9]. On the other hand, IP-10 has been shown to inhibit angiogenesis and to have antitumor properties[10, 11].

Sulfation of tyrosine in chemokine receptors is emerging as a posttranslational modification that contributes substantially to ligand binding. Tyrosine sulfation tends to occur in acidic regions of molecules, usually in regions containing multiple tyrosines[12, 13]. Chemokine receptors, including

* Correspondence to Dr Jin-ming GAO, MD, PhD
E-mail gaojm@pumch.cn
Received 2008-08-16 Accepted 2008-12-15

CCR5, CXCR4, CX3CR1 and CCR2b, are sulfated on their N-terminal tyrosines, and tyrosine sulfation is critical for ligand binding[14–17]. For example, sulfated tyrosines of the amino terminus of CCR5 contribute to the binding of CCR5 to MIP-1α, MIP-1β, and HIV-gp120/CD4 complexes; in addition, they facilitate the entry of HIV-1 into cells expressing CCR5 and CD4[14]. Sulfated tyrosines also contribute substantially to the formation of docking sites for C5a on the human C5aR[18]. Our previous work has shown that single sulfation of tyrosine 174 in the second extracellular loop (ECL2) of human C3aR forms a functional docking site for C3a[19]. Thus, tyrosine sulfation may represent the functional state of many 7-transmembrane segment (7TMS) receptors.

The roles of the extracellular domains of CXCR3 have previously been evaluated by analyzing the binding and function of chimeric CXCR3-CXCR1 receptors[21]. Some studies have shown that the N-terminal extracellular domains of CXCR3 play a role in the binding of IP-10, and that the second extracellular loop is important for receptor activation[20, 21]. Like many other chemokine receptors of the immune system, the N-terminus of CXCR3 contains two tyrosines flanked by an acidic amino acid, suggesting that they are candidates for sulfation[12, 13].

In the present study, our data show that two tyrosines, 27 and 29, in the N-terminus of CXCR3 are sulfated, and that these two sulfated tyrosines synergistically play a pivotal role in the efficient association of CXCR3 with intact IP-10, as well as a role in calcium mobilization. CXCR3 variants lacking sulfated tyrosines and ECL2 do not efficiently mobilize calcium in response to IP-10. These data not only underscore the functional importance of sulfated tyrosine in the formation of docking sites for ligand binding but also show that the activation site is not located at the termini of G-protein-coupled receptors (GPCRs).

Materials and methods

Cells, plasmids, antibodies and peptides

HEK 293T and Cf2Th cells were obtained from the American Tissue Type Culture Collection (CRL115544 and CRL1430, respectively). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. An expression plasmid encoding the human CXCR3 fused with an N-terminal FLAG tag and a C-terminal hemagglutinin (HA) tag was generated by PCR from human lung cDNA, and subcloned into the pcDNA 3.1 expression vector (Invitrogen). CXCR3 variants with an N-terminal truncation (ΔNH_2), a deletion of ECL2 (ΔECL2), or one or more tyrosines replaced by phenylalanine were prepared using the PCR-based QuikChange method (Stratagene) using Pfu polymerase. The entire coding sequence of all constructs was checked by sequencing. The anti-FLAG antibody and the anti-HA antibody were purchased from BD Pharmingen (San Diego, CA). IP-10 (PeproTech, Rehovot, Israel) was dissolved in water at 10 μg/μL and diluted into cell suspensions[19]. The expression plasmids for human endothelin receptors A and B were constructed as described above.

Using previously published methods, constructs for the cDNAs of human tyrosyl protein sulfotransferases (TPST) 1 and 2 were amplified by PCR from U87 human astroglioma cell cDNA and subcloned into pcDNA3.1[19, 22]. Small hairpin RNA (shRNA) constructs targeting nucleotides 259-276 of TPST1 and nucleotides 73-94 of TPST2 were generated and subcloned into pBluescript under the control of the murine U6 promoter[19, 23]. All these constructs were kindly provided by Dr Hyeryun CHOE, Harvard Medical School, Boston, MA.

Transfection, metabolic labeling and immunoprecipitation of cxcr3, cxcr3 variants, endothelial receptor (etr) a and b

HEK293T cells were transfected with plasmids encoding CXCR3 or the CXCR3 variants using calcium phosphate[19]. One day later, cells were washed twice with phosphate-buffered saline (PBS), split 1:3, and labeled overnight with [^{35}S]-cysteine and methionine ([^{35}S]-Express, NEN) or [^{35}S]-Na_2SO_3 (NEN). In some cases, cells were treated with 5 μg/mL tunicamycin (Sigma-Aldrich) to inhibit N-glycosylation 5 h prior to and during labeling[14, 19]. For immunoprecipitation, labeled cells were harvested and lysed in 1% N-dodecyl-β-*D*-maltoside (Anatrace) in PBS containing a protease inhibitor cocktail (Sigma-Aldrich and Roche Biochemicals) and 0.2 mmol/L PMSF (Sigma-Aldrich). Cell debris was removed by centrifugation at 18 000×*g* for 5 min at 4 °C, and the supernatants were immunoprecipitated using an anti-HA antibody covalently cross-linked to protein A Sepharose (Amersham Pharmacia Biotech). Immunoprecipitates were washed twice with 1% N-dodecyl-β-*D*-maltoside in PBS containing 0.5% SDS and once with PBS, then eluted with SDS sample buffer under reducing conditions by heating at 55 °C for 10 min. Samples were electrophoresed on 12% SDS-Tris/glycine polyacrylamide gels and analyzed by autoradiography.

O- and N-linked carbohydrates were enzymatically removed from immunoprecipitated CXCR3 under non-denaturing conditions using *O*-glycanase (Glyko) and PNGase F (New England Biolabs), respectively, as recommended by

the manufacturers. The reactions were then mixed with SDS sample buffer and analyzed as above on 12% SDS gels[19].

Flow cytometer analysis

Two days after transfection, HEK 293T cells were harvested in 5 mmol/L EDTA/PBS and 5 mmol/L $MgCl_2$/PBS and washed with PBS. Cells (5×10^5) were incubated with 0.5 μg/mL of the anti-FLAG antibody for 30 min, followed by FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) for 30 min. Finally cells were fixed in 2% paraformaldehyde and analyzed by fluorescence-activated cell sorting (FACS).

Sodium chlorate treatment

Transfected HEK 293T cells were cultured in medium containing 10 mmol/L sodium chlorate for at least 72 h[24].

Binding of IP-10 to cells expressing CXCR3 and CXCR3 variant

Binding experiments were performed using HEK 293T cells transfected with wild-type CXCR3 or CXCR3 variants. Two days after transfection, cells were detached with 5 mmol/L EDTA in PBS, washed with DMEM, counted, and resuspended to a concentration of 2×10^6/mL in binding buffer [20 mmol/L HEPES (pH 7.4), 125 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 5 mmol/L KCl, 0.5 mmol/L glucose, 0.2% BSA, 0.02% sodium azide]. An aliquot of cells was subjected to FACS analysis to verify that the receptors were expressed at comparable levels. For binding assays, an aliquot of 50 μL was incubated with 0.1 nmol/L [^{125}I]-IP10 (NEN Life Science Products) and 0 to 100 nmol/L of unlabeled IP-10 for 30 min at 37 ºC in a final volume of 100 μL. Cells were centrifuged, washed once with binding buffer, and the bound C3a was determined by γ-counting.

For some experiments the degree of sulfation was modified by co-transfecting the CXCR3 with plasmids encoding TPST1 and 2 in order to increase sulfation, or with shRNA constructs directed against TPST1 and 2 in order to inhibit sulfation.

Calcium influx mediated by the cxcr3 and cxcr3 variants

Cf2Th cells were transfected with CXCR3 or CXCR3 variants using the Lipofectamine method (Invitrogen). One day later, the transfected Cf2Th cells were split into two parts. One aliquot of cells was subjected to flow cytometric analysis to quantify receptor expression as previously described[19]. The other aliquot was transferred to flasks and grown in DMEM for at least 24 h. Cells were harvested, counted, and incubated with the indicator dye Fura-2-AM (Molecular Probes) for 1 h at 37 ºC in 20 mmol/L HEPES (pH 7.4), 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 125 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L glucose, and 0.2% BSA. Cells were washed twice and resuspended in the same buffer to a concentration of 1×10^6/mL. Changes in intracellular calcium concentration in response to native IP-10 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[19].

Statistical analysis

To calculate relative K_d values, binding data were analyzed using Prism software (GraphPad) running an algorithm for nonlinear regression of homologous one-site competition binding with ligand depletion[25]. Data are expressed as mean±SEM. Comparison between multiple groups was performed by ANOVA. A value of $P<0.05$ was considered a significant difference.

Results

CXCR3 is posttranslationally modified by N-glycosylation and tyrosine sulfation.

Sulfate can be incorporated into proteins at sites of N- or O-linked glycosylation or on tyrosines[13]. As shown in Figure 1A, immunoprecipitated CXCR3 is present in predominance as a form migrating with an apparent molecular weight of about 45 kDa. Because CXCR3 has two predicted sites of N-linked glycosylation that are also substrates for sulfation[12], treating immunoprecipitated CXCR3 with PNGase F produces a mature form of CXCR3 of about 35 kDa. The predicted amino acid sequence includes a number of potential O-linked glycosylation sites in the N-terminal extracellular domain and in ECL2, and when these glycans are removed by treating immunoprecipitated CXCR3 with O-glycanase, the CXCR3 band does not shift on SDS-PAGE (Figure 1B). We conclude that wt CXCR3 is modified by N-linked, but not O-linked, glycosylation.

Two tyrosines in the N-terminus of CXCR3 are adjacent to an acidic amino acid, which is a motif similar to that predicted for tyrosine sulfation. To test whether these tyrosines of CXCR3 are sulfated, we added tunicamycin to the cell medium before and during labeling to remove N-linked glycosylation. As shown in Figure 1C, cells expressing wild-type (wt) CXCR3 labeled with [^{35}S]cysteine and methionine

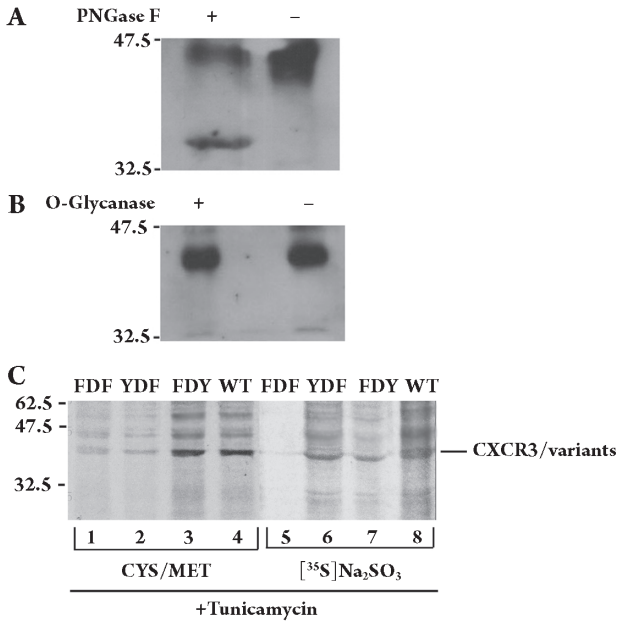

Figure 1. Tyrosines 27 and 29 in the N-terminus of the CXCR3 are sulfated. (A, B) HEK 293T cells were transfected with plasmids encoding wild-type CXCR3 with N- and C-terminal epitope tags. CXCR3 immunoprecipitated from [^{35}S]-cysteine- and methionine-labeled cells was treated with O-glycanase or PNGase F as indicated. (C) HEK 293T cells were transfected with plasmids encoding wild-type CXCR3 or the CXCR3 mutants YDF, FDY, and FDF. After 24 h the transfected cells were divided up and labeled with [^{35}S]-cysteine and methionine or with [^{35}S]-Na_2SO_3. Both sets of cells were treated with the N-glycosylation inhibitor tunicamycin 5 h before and during labeling. Twenty-four hours after labeling, cells were harvested, lysed and immunoprecipitated with an antibody against the C-terminal HA tag. Immunoprecipitates were analyzed under reducing conditions by electrophoresis on 12% SDS polyacrylamide gels. Gels were dried and exposed to X-ray film. Numbers at the left indicate the positions of molecular weight markers.

show a single major form of CXCR3 migrating at about 35 kDa (lane 4). As expected, only the mature band of CXCR3 is sulfated (Figure 1C, Lane 8). Figure 1C also shows that a variant of CXCR3 in which tyrosines 27 and 29 in the N-terminus were changed to phenylalanine did not incorporate detectable levels of [^{35}S]-Na_2SO_3, even after extended exposure (Lane 5). Thus, we infer that tyrosines in the N-terminus of CXCR3 are sulfated and that tyrosine sulfation is independent of N-glycosylation elsewhere in CXCR3.

Sulfated tyrosines 27 and 29 at the N-terminus of CXCR3 are required for the protein to bind IP-10

Posttranslational modifications are known to affect receptor function, so we sought to identify the role of sulfation in the interactions between CXCR3 and IP-10. Sodium chlorate ($NaClO_3$) is a selective nontoxic inhibitor of adenosine triphosphate (ATP) sulfurylase, which is required for formation of phosphoadenosine phosphosulfate (PAPS), the donor for sulfation reactions[26]. We used $NaClO_3$ to treat cells expressing wt CXCR3.

As shown in Figure 2A, the binding affinity of CXCR3 for IP-10 was substantially lower in HEK 293T cells treated with 10 mmol/L $NaClO_3$ than in the same cells without $NaClO_3$ treatment. The K_d values were 1.4±0.2 nmol/L for untreated cells and 72.3±0.2 nmol/L for $NaClO_3$-treated cells.

We next explored the binding site for IP-10 on CXCR3 in greater detail. As shown in Figure 2B, cells expressing wt CXCR3 showed affinity for IP-10 similar to that of cells expressing the ΔECL2 variant. In contrast, cells expressing ΔNH2 did not show detectable binding to IP-10. These data show that the N-terminus of CXCR3 is necessary for binding IP-10.

To further examine the importance of tyrosines 27 and 29 at the N-terminus for ligand binding, we studied the ability of IP-10 to associate with wt CXCR3 or with variants in which at least one of the two tyrosines in the N-terminus was mutated to phenylalanine. Transfected HEK 293T cells expressed similar levels of wt CXCR3 or variants, as indicated by the fluorescence intensity of anti-FLAG antibody staining. These cells were tested in competition binding experiments with [^{125}I]IP-10 and increasing concentrations of unlabeled IP-10 as indicated. As shown in Figure 2C, the cells expressing CXCR3 mutants, FDY and YDF, showed lower binding affinity to [^{125}I]IP-10 than did cells expressing wt CXCR3. However, only HEK 293T cells expressing the FDF variant failed to bind detectably to [^{125}I]IP-10. These data indicate that sulfated tyrosines 27 and 29 of CXCR3 are important for the association of CXCR3 with IP-10.

Sulfated tyrosines 27 and 29 of CXCR3 are functionally important for calcium mobilization induced by IP-10

The model for ligand-receptor association has been well established in GPCRs, with the docking site at the N-terminus and activation site in the other parts of the receptor, including ECL2 and transmembrane domains[18]. As shown in Figure 2C, the binding site of IP-10 is at the N-terminus of CXCR3. It is speculated that binding of CXCR3 to IP-10 triggers intracellular signaling events, such as calcium mobilization.

Therefore, we investigated whether the sulfated tyrosines in CXCR3 affect calcium influx using the calcium mobilization assay. Since for most G-protein-coupled receptors, Cf2Th cells appear to express an intact Ca^{2+} signaling pathway[19], we examined the ability of Cf2Th cells trans-

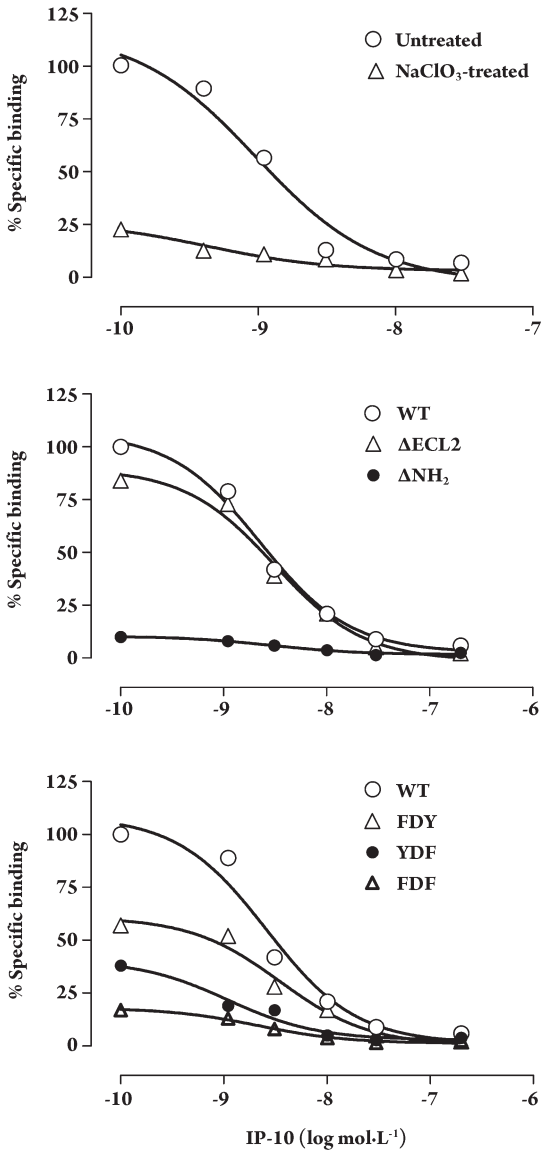

Figure 2. Sulfated tyrosines 27 and 29 in the N-terminus of CXCR3 are necessary for IP-10 binding. (A) HEK 293T cells were transfected with the plasmid encoding wild-type CXCR3. One day later, cells were divided into two parts. One aliquot was treated with 10 mmol/L $NaClO_3$ for 72 h, while the other aliquot was grown without $NaClO_3$ treatment for 3 days. (B) HEK 293T cells were transfected with plasmids encoding wild-type CXCR3 or the CXCR3 variants ΔNH2 and ΔECL2. (C) HEK 293T cells were transfected with plasmids encoding wild-type CXCR3 or the CXCR3 variants YDF, FDY or FDF. Transfected cells were incubated with 0.1 nmol/L [^{125}I]IP-10 and the indicated concentrations of unlabeled IP-10 as described in Materials and Methods. Cells were washed, and bound [^{125}I]IP-10 was determined by γ-counting. Aliquots of the same transfections were analyzed for receptor expression levels by flow cytometry using the anti-FLAG tag antibody to verify that receptors were expressed at comparable levels. Data are expressed as % maximal binding.

fected with wt CXCR3 or the variants FDY, YDF, and FDF to mobilize calcium in response to IP-10. IP-10 at 200 nmol/L effectively induced effective calcium mobilization in cells expressing wt CXCR3, YDF, or FDY (Figure 3A and B), whereas no such induction was observed in cells expressing the FDF variant (Figure 3C). In a parallel experiment, cells expressing ΔECL2 failed to respond robustly to IP-10 stimulation (Figure 3D). Taken together, these data show that ECL2 domain contains the site of activation that allows CXCR3 to induce signal transduction.

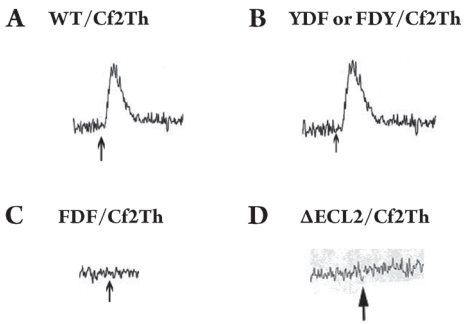

Figure 3. The CXCR3 variant FDF does not mobilize calcium in response to IP-10. Cf2Th cells were transfected with wild-type CXCR3 or with the CXCR3 variants YDF, FDF, or ΔECL2, as described in Materials and Methods. Cells were loaded with the calcium indicator dye Fura-2 and assessed for their ability to mobilize calcium in response to IP-10, which was added at the time points indicated by the arrows. Calcium flux was displayed as the fluorescence ratio of 340 nm to 380 nm. Receptor expression was determined by staining with the anti-FLAG-tag antibody. Mean fluorescence levels were 59.3 for wild-type CXCR3, 48.0 for YDF, 58.9 for FDF and 56.2 for ΔECL2; for mock-transfected cells, the value was 7.7. The data shown are representative of three independent experiments.

Enzymatic regulation of the sulfation of tyrosines 27 and 29 of CXCR3

One possible explanation for a lack of IP-10 binding to the CXCR3 variant FDF is the absence of sulfate on tyrosines 27 and 29. An alternative explanation is that the expression of phenylalanine at this site alters receptor conformation due to the increase in hydrophobicity resulting from the absence of the hydroxyl group[19]. We addressed these possibilities by altering the extent of CXCR3 sulfation. In order to increase CXCR3 sulfation, HEK 293T cells were transfected with the plasmid encoding wt CXCR3 alone or in combination with the plasmids encoding TPST1 and 2. As shown in Figure 4A, cells co-transfected with the plasmids encoding wt CXCR3 and TPST 1 and 2 showed higher levels of sulfation (lane 4). In order to decrease CXCR3 sulfation, HEK 293T cells were transfected with the plasmid encoding wt CXCR3 or they

were co-transfected with wt CXCR3 and shRNA constructs. Cells co-transfected with the plasmids encoding wt CXCR3 and shRNAs showed lower sulfation signal compared with cells transfected with wt CXCR3 (lane 5). Furthermore, we assayed whether TPST and shRNA affect the ability of cells expressing wt CXCR3 to bind to IP-10. As shown in Figure 4B, while CXCR3 expression levels were quite similar, TPST 1 and 2 increased mean maximal binding by approximately 30%. In contrast, the shRNA constructs decreased mean maximal binding by approximately 30%. Therefore, sulfated tyrosines at the N-terminus of CXCR3, not conformational change in the protein, are required for the protein to associate with IP-10.

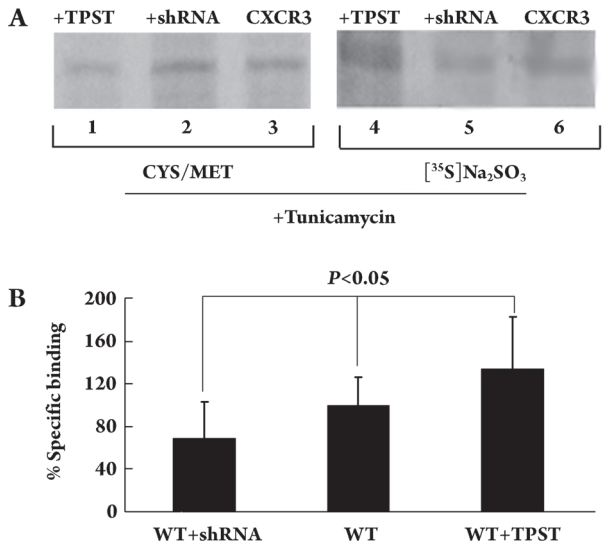

Figure 4. Tyrosine sulfation of CXCR3 is critical for binding affinity to IP-10. (A) HEK 293T cells were co-transfected with the CXCR3 plasmid alone or with plasmids encoding TPSTs or with shRNAs targeting TPSTs as indicated. Transfections were carried out in the presence of tunicamycin and labeled with [^{35}S]cysteine and methionine or [^{35}S]-Na_2SO_3. Receptors were immunoprecipitated and analyzed as described for Figure 1. (B) Cells from the same transfection were tested for maximal binding of [^{125}I]IP-10 after verifying by flow cytometric analysis that expression levels of the receptors were comparable. Only transfected cultures showing similar levels of expression were used in the analysis. Results are expressed as mean±SEM of four independent experiments.

ETRA and ETRB are not sulfated on tyrosines

We extended our study to examine whether tyrosine sulfation is a universal posttranslational modification that happens in other 7TMS. Unlike chemokine receptors, receptors for endothelin A and B have tyrosines adjacent to basic amino acids at ECL2. As demonstrated in Figure 5, sulfate

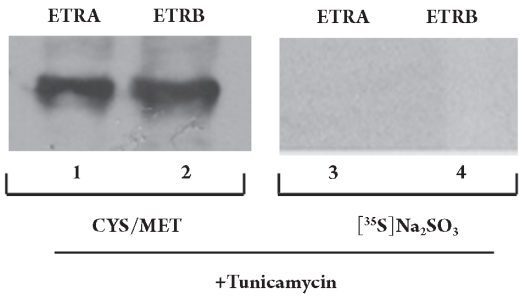

Figure 5. ETRA and ETRB are not tyrosine-sulfated. HEK 293T cells were transfected with plasmids encoding endothelin receptors A and B carrying an HA tag at the C-terminus. One day later, transfected cells were labeled in the presence of tunicamycin as described in Figure 1.

moiety was not detectably incorporated in either ETRA or ETRB, indicating that ETRA and ETRB are not sulfated posttranslationally (Figure 5).

Discussion

We have shown here that tyrosines 27 and 29 in the N-terminus of CXCR3 are sulfated, and that these two sulfated tyrosines play an important role in binding IP-10. In this way, the sulfated tyrosines play a role in signal transduction and calcium mobilization in response to IP-10. This observation confirms the two-site model for ligand-receptor binding and activation of CXCR3.

Sulfation of tyrosines is a posttranslational modification occurring late in the trans-Golgi network and found on a wide spectrum of secreted proteins, as well as on extracellular regions of many membrane proteins of multicellular eukaryotes[27]. It has been estimated that up to 1% of all tyrosines in eukaryotic proteins may be sulfated[28]. Sulfated tyrosines at the N-termini of chemokine receptors play a critical role in helping these receptors bind their natural ligands. These include CCR5, CXCR4, CCR2b, and CX3CR1 as well as Duffy antigen receptor for binding chemokines[14–17, 29]. Previous work has indicated that sulfated tyrosines in the N-terminus of chemotactic C5aR help to form the docking site for C5a, but not the activation site[18]. In addition, our work on the structure-function of human C3aR has shown evidence for a two-step model for C3aR function, in which the binding site is at tyrosine 174 in ECL2 and the activation site lies at some distance away from the binding site[19].

The molecular cloning of CXCR3 places this molecule in the superfamily of G-protein-coupled receptors[30–32]. A splice variant of CXCR3 called CXCR3-B has recently been cloned. CXCR3-B has a longer N-terminal extracellular domain containing 52 amino acids, and it lacks a sulfat-

able tyrosine[33]. In contrast, CXCR3 has a highly anionic tyrosine-rich N-terminal segment. Tyrosines 27 and 29 lie adjacent to an acidic amino acid, which is essential for tyrosine sulfation, and the present study shows that both of these tyrosines are sulfated (Figure 2C). However, the two tyrosines are sulfated to the different extent. Single tyrosine-to-phenylalanine mutations at position 27 or 29 of CXCR3 cause the loss of sulfation signal seen in wt CXCR3, and these mutations greatly reduce the protein's binding affinity for IP-10. Only a mutant FDF, in which both tyrosines 27 and 29 are changed to phenylalanine, fails to detectably incorporate sulfate. Cells expressing FDF do not efficiently bind IP-10.

Our data support the notion that tyrosine sulfation sites are often clustered and that sulfation of one site may depend on the negatively charged sulfates already present at other sites[34]. For example, the tyrosines of CCR5 at positions 3–15 are sulfated in a stepwise manner; tyrosines 14 and 15 are sulfated first, followed by tyrosines 10 and 3[35]. In the present study, to verify that the reduced binding of IP-10 by cells expressing FDF reflects the loss of sulfate and not a change in receptor conformation, we attempted to increase or decrease the extent of sulfation by co-transfecting cells with the expression plasmids for CXCR3 and TPSTs, or with CXCR3 and shRNAs targeting TPSTs. If sulfate were important for ligand binding, an increase or decrease in sulfation without a change in the level of receptor expression would lead to increased or decreased ligand binding[19]. Our data suggest that TPSTs increased tyrosine sulfation and the affinity of the receptor for IP-10. Conversely, when co-transfections were performed with shRNA constructs targeting TPSTs, both sulfation and binding were inhibited, without a significant change in receptor expression. The observation that CXCR3 binding was reduced only partially reflects enzymatic activity of TPSTs synthesized prior to transfection with shRNAs.

Tyrosine sulfation has been identified as a key mediator of protein-protein interactions involved in leukocyte adhesion, hemostasis and chemokine signaling[26]. Sulfate is a charged and highly polarizable moiety that is likely to contribute free energy to binding. Here we have shown not only that tyrosines 27 and 29 are sulfated but also that these sulfated moieties are critically important for binding native IP-10. Our data that double sulfated tyrosines contribute to ligand binding are inconsistent with the previous report showing that single mutation of tyrosine 27 or 29 of CXCR3 decreases its ability to bind its ligands, including IP-10[36]. There are several possible explanations for these inconsistencies. First of all, the previous authors report that the molecular weight of human CXCR3 is about 70 kDa, whereas our study shows that the molecular weight of human mature CXCR3 is about 45 kDa, consistent with the predicted amino acid composition. Secondly, tyrosines 27 and 29 are separated by one acidic amino acid, suggesting that both of these two tyrosines should be sulfated[12, 35, 37]. Thirdly, the previous authors were unable to exclude other factors that may have affected the binding of CXCR3 to IP-10, such as N-linked glycosylation. More importantly, those authors did not determine that the decreased binding affinity was due to a loss of sulfate or a change in receptor conformation. In contrast, we have used TPSTs and shRNAs to determine that sulfated tyrosines, not a conformational change in the receptor, affect the binding of receptor and ligand. Our observations in the present study clearly demonstrate that sulfated tyrosines 27 and 29 are functionally more important than other extracellular domains in binding IP-10.

Posttranslational modification occurs in chemokine and chemotactic receptors, including C3aR, C5aR, CCR5, CX3CR1, and CC2b[15–19]. We hypothesized that tyrosine sulfation might be a universal phenomenon in other 7TMS GPCRs, such as ETRA and ETRB[38]. However, we did not observe the sulfation of tyrosines in ETRA and ETRB. Thus tyrosine sulfation in 7TMS GPCRs should be further examined in order to determine its functional importance for this case of receptor-ligand interaction.

Activation of the G-protein-coupled receptor CXCR3 by IP-10 has been proposed to occur by a "two-step/two-site" mechanism[20, 21]. Traditionally, chemokine receptors are considered to possess two regions of interaction: a docking/binding domain rich in the acidic amino acid and tyrosine-rich N-terminus confer binding affinity; while an activation site, which includes the ECL2 and transmembrane helices, mediates the biological activity of the ligand[18, 19, 39, 40]. Our data imply that sulfated tyrosines 27 and 29 in the N-terminus of CXCR3 constitute the binding site of the receptor, which positions IP-10 appropriately for interaction with the activation site.

Many chemokine receptors have been suggested as therapeutic targets for inflammation[41]. Because the pathogenesis of inflammation involves the interaction of many receptors on various cells[4], targeting an individual receptor may not generate a desirable outcome. Many chemokine receptors use sulfated tyrosine(s) to bind their ligands[37]. Thus, inhibition of tyrosine sulfation on these receptors may serve as a therapeutic target for inflammatory diseases.

Abbreviations

CXCR3, CXC receptor 3; C3aR, C3a receptor; C5aR, C5a receptor; CCR5, CC chemokine receptor 5; 7TMS, 7-transmembrane segment; GPCR, G-protein-coupled receptor; ECL, extracellular loop; HIV, human immunodeficiency virus.

Acknowledgements

This work was supported in part by grants from the National Natural Science Foundation of China (No 30470767), the Beijing Natural Science Foundation (No 7072063), and the Ministry of Education of China (No NCET 06-0156).

We are grateful to Dr Hyeryun CHOE for helpful encouragement and plasmids, Dr Paulette L WRIGHT for critical reading and editing of this manuscript, Drs Wei CUI and Ding-hua LIU for assistance with FACS analysis and Dr Zhiying ZHAO for help in performing binding experiments.

Author contribution

Jin-ming GAO designed and performed the experiment and wrote the manuscript. Ruo-lan XIANG, Lei JIANG and Qi-ping FENG performed the experiments. Wen-hui LI designed the primers. Zi-jiang GUO and Qi SUN carried out immunoprecipitation and binding experiments. Zheng-pei ZENG and Fu-de FANG were actively involved throughout the entire study.

References

1 Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, *et al*. Chemokine receptor specific for IP-10 and mig: structure, function, and expression in activated T-lymphocytes. J Exp Med 1996; 184: 963–9.

2 Thomas SY, Hou R, Boyson JE, Means TK, Hess C, Olson DP, *et al*. CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. J Immunol 2003; 171: 2571–80.

3 Romagnani P, Annunziato F, Lasagni L, Lazzeri E, Beltrame C, Francalanci M, *et al*. Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. J Clin Invest 2001; 107: 53–63.

4 Luster AD. Chemokines-chemotactic cytokines that mediate inflammation. N Engl J Med 1998; 338: 436–45.

5 Sørensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, *et al*. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. J Clin Invest 1999; 103: 807–15.

6 Zhao DX, Hu Y, Miller GG, Luster AD, Mitchell RN, Libby P. Differential expression of the IFN-gamma-inducible CXCR3-binding chemokines, IFN-inducible protein 10, monokine induced by IFN, and IFN-inducible T cells alpha chemoattractant in human cardiac allografts: association with cardiac allograft vasulopathy and acute rejection. J Immunol 2002; 169: 1556–60.

7 Medoff BD, Wain JC, Seung E, Jackobek R, Means TK, Ginns LC, *et al*. CXCR3 and its ligands in a murine model of obliterative bronchiolitis: regulation and function. J Immunol 2006; 176: 7087–95.

8 Hancock WW, Gao W, Csizmadia V, Faia K, Shemmeri N, Luster AD. Donor-derived IP-10 initiates development of acute allograft rejection. J Exp Med 2001; 193: 975–80.

9 Luster AD, Greenberg SM, Leder P. The IP-10 chemokine binds to a specific cell surface heparin sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. J Exp Med 1995; 182: 219–31.

10 Arenberg DA, Kunkel SL, Polverini PJ, Morris SB, Burdick MD, Glass MC, *et al*. Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. J Exp Med 1996; 184: 981–92.

11 Luster AD, Leder P. IP-10, a CXC chemokine, elicits a potent thymus-dependent antitumor response *in vivo*. J Exp Med 1993; 178: 1057–65.

12 Niehrs C, Huttner WB, Carvallo D, Degryse E. Conversion of recombinant hirudin to the natural form by *in vitro* tyrosine. Differential substrate specifities of leech and bovine tyrosylprotein sulfotransferases. J Biol Chem 1990; 265: 9314–8.

13 Rosenquist GL, Nicholas HB Jr. Analysis of sequence requirements for protein tyrosine sulfation. Protein Sci 1993; 2: 215–22.

14 Farzan M, Mirzabekov T, Kolchinsky P, Wyatt R, Cayabyab M, Gerard NP, *et al*. Tyrosine sulfation of the amino-terminus of CCR5 facilitates HIV-1 entry. Cell 1999; 96: 667–76.

15 Farzan M, Babcock GJ, Vasilieva N, Wright PL, Kiprilov E, Mirzabekov T, *et al*. The role of post-translational modifications of the CXCR4 amino terminus in stromal-derived factor 1 alpha association and HIV-1 entry. J Biol Chem 2002; 277: 29484–9

16 Preobrazhensky AA, Dragan S, Kawano T, Gavrilin MA, Gulina IV, Chakravarty L, *et al*. Monocyte chemotactic protein-1 receptor CCR2B is a glycoprotein that has tyrosine sulfation in a conserved extracellular N-terminal region. J Immunol 2000; 165: 5295–303.

17 Fong AM, Alam SM, Imai T, Haribabu B, Patel DD. CX3CR1 tyrosine sulfation enhances fractalkine-induced cell adhesion. J Biol Chem 2002; 277: 19418–23.

18 Farzan M, Schnitzler CE, Vasilieva N, Leung D, Kuhn J, Gerard C, *et al*. Sulfated tyrosines form the docking site of the human C5a anaphylatoxin receptor. J Exp Med 2001; 193: 1059–66.

19 Gao J, Choe H, Bota D, Wright PL, Gerard C, Gerard NP. Sulfation of Tyrosine 174 in the human C3aR is essential for binding of C3a anaphylatoxin. J Biol Chem 2003; 278: 37902–8.

20 Xanthou G, Williams TJ, Pease JE. Molecular characterization of the chemokine receptor CXCR3: evidence for the involvement of distinct extracellular domains in a multi-step model of ligand binding and receptor activation. Eur J Immunol 2003; 33: 2927–36.

21 Clark-Lewis I, Mattioli I, Gong JH, Loetscher P. Structure-function relationship between the human chemokine receptor CXCR3 and its ligands. J Biol Chem 2003; 278: 289–95.

22 Choe H, Li W, Wright PL, Vasilieva N, Venturi M, Huang CC, *et al*. Tyrosine sulfation of human antibodies contributes to recognition of the CCR5 binding region of HIV-1 gp120. Cell 2003; 114: 161–70.
23 Sui G, Soohoo C, Affarel B, Gay F, Shi Y, Forrester WC. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc Natl Acad Sci USA 2002; 99: 5515–20.
24 Xia L, Ramachandran V, McDaniel JM, Nguyen KN, Cummings RD, McEver R. N-terminal residues in murine P-selectin glycoprotein ligand-1 required for binding to murine P-selectin. Blood 2003; 101: 552–9.
25 Swillens S. Interpretation of binding curves obtained with high receptor concentrations: practical aid for computer analysis. Mol Pharmacol 1995; 47: 1197–203.
26 Kehoe JW, Bertozzi CR. Tyrosine sulfation: a modulator of extracellular protein-protein interactions. Chem Biol 2000; 7: R57–61.
27 Huttner WB. Tyrosine sulfation and the secretory pathway. Annu Rev Physiol 1988; 50: 363–76.
28 Baeuerle, PA, Huttner, WB. Tyrosine sulfation of yolk proteins 1, 2, and 3 in *Drosophila melanogaster.* J Biol Chem 1985; 260: 6434–39.
29 Choe H, Moore MJ, Owens CM, Wright PL, Vasilieva N, Li W, *et al*. Sulphated tyrosines mediate association of chemokines and *Plasmodium vivax* Duffy binding protein with the Duffy antigen/receptor for chemokines (DARC). Mol Microbiol 2005; 55:1413–22
30 Gerard C, Gerard NP. The pro-inflammatory seven transmembrane segment receptors of the leukocyte. Curr Opin Immunol 1994; 6: 140–5.
31 Lu B, Humbles A, Bota D, Gerard C, Moser B, Soler D, *et al*. Structure and function of the murine chemokine receptor CXCR3. Eur J Immunol 1999; 29: 3804–12.
32 Colvin RA, Campanella GS, Sun J, Luster AD. Intracellular domains of CXCR3 that mediate CXCL9, CXCL10, and CXCL11 function. J Biol Chem 2004; 279: 30219–27.
33 Lasagni L, Francalanci M, Annunziato F, Lazzeri E, Giannini S, Cosmi L, *et al*. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor-4. J Exp Med 2003; 197: 1537–49.
34 Bundgaard JR, Vuust J, Rehfeld JF. New consensus features for tyrosine O-sulfation determined by mutational analysis. J Biol Chem 1997; 272: 21700–5.
35 Seibert C, Cadene M, Sanfiz A, Chait BT, Sakmar TP. Tyrosine sulfation of CCR5 N-terminal peptide by tyrosylprotein sulfotransferases 1 and 2 follows a discrete pattern and temporal sequence. Proc Natl Acad Sci USA 2002; 99: 11031–6.
36 Colvin RA, Campanella GS, Manice LA, Luster AD. CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. Mol Cell Biol 2006; 26: 5838–49.
37 Liu J, Louie S, Hsu W, Yu KM, Nicholas HB Jr, Rosenquist GL. Tyrosine sulfation is prevalent in human chemokine receptors important in lung disease. Am J Respir Cell Mol Biol 2008; 38: 738–43.
38 Elshourbagy NA, Korman DR, Wu HL, Sylvester DR, Lee JA, Nuthalaganti P, *et al*. Molecular characterization and regulation of the human endothelin receptors. J Biol Chem 1993; 268: 3873–9.
39 Crump MP, Gong JH, Loetscher P, Rajarathnam K, Amara A, Arenzana-Seisdedos F, *et al*. Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. EMBO J 1997; 16: 6996–7007.
40 Gether U, Kobilka BK. G protein-coupled receptors. II. Mechanism of agonist activation. J Biol Chem 1998; 273: 17979–82.
41 Barnes PJ, Shapiro SD, Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. Eur Respir J 2003; 22: 672–88.