Sulfotyrosines of the Kaposi's Sarcoma-Associated Herpesvirus G Protein-Coupled Receptor Promote Tumorigenesis through Autocrine Activation[▽]

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Received 12 September 2009/Accepted 15 January 2010

The Kaposi's sarcoma-associated herpesvirus (KSHV) G protein-coupled receptor (vGPCR) is a bona fide signaling molecule that is implicated in KSHV-associated malignancies. Whereas vGPCR activates specific cellular signaling pathways in a chemokine-independent fashion, vGPCR binds a broad spectrum of CC and CXC chemokines, and the roles of chemokines in vGPCR tumorigenesis remain poorly understood. We report here that vGPCR is posttranslationally modified by sulfate groups at tyrosine residues within its N-terminal extracellular domain. A chemokine-binding assay demonstrated that the tyrosine sulfate moieties were critical for vGPCR association with GRO- α (an agonist) but not with IP-10 (an inverse agonist). A sulfated peptide corresponding to residues 12 through 33 of vGPCR, but not the unsulfated equivalent, partially inhibited vGPCR association with GRO- α . Although the vGPCR variant lacking sulfotyrosines activated downstream signaling pathways, the ability of the unsulfated vGPCR variant to induce tumor growth in nude mice was significantly diminished. Furthermore, the unsulfated vGPCR variant was unable to induce the secretion of proliferative cytokines, some of which serve as vGPCR agonists. This implies that autocrine activation by agonist chemokines is critical for vGPCR tumorigenesis. Indeed, GRO- α increased vGPCR-mediated AKT phosphorylation and vGPCR tumorigenesis in a sulfotyrosine-dependent manner. Our findings support the conclusion that autocrine activation triggered by chemokine agonists via sulfotyrosines is necessary for vGPCR tumorigenesis, thereby providing a rationale for future therapeutic design targeting the tumorigenic vGPCR.

The human Kaposi's sarcoma-associated herpesvirus (KSHV, also known as human herpesvirus 8, or HHV-8) is the etiologic agent for Kaposi's sarcoma (KS), primary effusion lymphoma, and multicentric Castleman's disease (7, 9, 36, 45). These human diseases are angioproliferative malignancies that are largely engendered by excessive inflammation and angiogenic cytokines. Indeed, the KSHV genome encodes an array of polypeptides that are implicated in modulating host inflammatory responses (41), most notably the KSHV G protein-coupled receptor (vGPCR or ORF74). In tissue culture, vGPCR expression activates various signaling pathways and multiple transcription factors that culminate in promoting the production of inflammatory cytokines and angiogenic growth factors, including GRO- α , interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF). These proliferative factors are implicated in KSHV-associated malignancies (3, 6, 12). It was elegantly shown that depletion of VEGF with an antibody significantly diminished vGPCR-induced endothelial cell proliferation, demonstrating the role of growth factors in vGPCR tumorigenesis *ex vivo* (3). Furthermore, vGPCR transgenic mice developed angioproliferative lesions that resemble human KS with similar pathology features (34, 48). These obser-

vations support the hypothesis that vGPCR, along with other viral proinflammatory proteins, drives the angioproliferation of KSHV latently infected endothelial cells, the spindle cells. However, the fact that vGPCR-expressing cells, presumably KSHV-infected cells that undergo lytic replication, account for only a minor fraction of KS lesions casts reasonable doubt on the contribution of vGPCR in KSHV pathogenesis (10, 26). A reconciling hypothesis, supported by accumulating evidence, postulates that vGPCR promotes angiogenesis via both paracrine and autocrine mechanisms, underscoring the significance of proinflammatory cytokines in vGPCR-mediated signaling and tumorigenesis (8, 23).

Because vGPCR is a constitutively active signaling molecule, vGPCR posttranslational regulation may be distinct from that of its cellular counterpart, the human IL-8 receptor (CXCR2). In support of this notion, we have recently demonstrated that the small K7 membrane protein interacts with and retains vGPCR in the endoplasmic reticulum (ER), thereby inducing its rapid degradation by the proteasome (16). As such, K7 coexpression greatly reduced vGPCR signaling in tissue culture and vGPCR tumorigenesis in nude mice (16). These findings define a viral factor that negatively regulates vGPCR tumorigenesis posttranslationally. The significance of K7-induced vGPCR degradation is further supported by the observations that continuous expression of vGPCR is toxic to cells, including the KSHV latently infected PEL cell line (5, 26). Together with the bicistronic translation mechanism and regulation by viral macrophage inflammatory protein (vMIP) che-

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[▽] Published ahead of print on 27 January 2010.

mokines (20, 37), our data imply that KSHV has evolved distinct strategies to delicately regulate vGPCR protein expression and subsequent signal transduction.

Independent of ligand binding, vGPCR is capable of activating downstream signaling pathways including phospholipase C/phosphatidylinositol 3 (PI3)-kinase, mitogen-activated protein kinase/Jun N-terminal kinase (MAPK/JNK), and transcription factors such as NF- κ B and nuclear factor of activated T cells (NFAT) (2, 4, 12, 38). Although chemokines are not necessary for vGPCR constitutive activity, chemokines are shown to modulate vGPCR signaling capacity in cells and are suggested to influence vGPCR tumorigenesis in mice (20–22, 24, 25). For example, IP-10 binding inhibits, while GRO- α association further promotes, signaling downstream of vGPCR. However, mechanistic details of chemokine binding and how vGPCR tumorigenesis is differentially influenced by individual chemokines remain poorly defined. Understanding the molecular details of vGPCR association with chemokines will advance our knowledge of regulatory mechanisms that govern vGPCR signaling and tumorigenesis and will inform efforts to develop therapeutics that target the tumorigenic vGPCR for intervention. Similar to other CXC chemokine receptors, the vGPCR N terminus and extracellular loops collectively contribute to a stable association with its cognate chemokines. Deletion of the N-terminal sequence of vGPCR strongly impairs chemokine binding, but replacement of this domain with corresponding regions from related receptors, including human CXCR3 (receptor for IP-10) and calcitonin receptor type 3, cannot restore chemokine binding and signal modulation (24). Therefore, the N-terminal sequence of vGPCR likely possesses unique amino acids and/or posttranslational modifications that may confer the specificity of vGPCR association with chemokines. In agreement with this notion, one striking feature of vGPCR is its ability to bind a broad spectrum of CC and CXC chemokines. Although multiple negatively charged residues are presumed to interact with positively charged amino acids of CXC chemokines such as the ELR motif, specific molecular determinants within the N terminus that contribute to vGPCR association with individual chemokines have not been well characterized.

The N-terminal extracellular domain of vGPCR contains two tyrosines (residues 26 and 28) flanking an aspartic acid, a feature suggestive of tyrosine sulfation. Tyrosine sulfation occurs in the *trans*-Golgi network (TGN) and is catalyzed by two highly conserved tyrosylprotein sulfotransferases (TPST) in mammals, designated TPST1 and TPST2 (35). Sulfated tyrosines have been shown in various experimental settings to greatly enhance receptor-ligand or antigen-antibody interactions (11, 14). Conceivably, tyrosine sulfate moieties may provide a docking site for a surface formed by positively charged residues of a chemokine or other cognate ligands. Indeed, nuclear magnetic resonance (NMR) studies of CXCR4 and its cognate chemokine stromal cell-derived factor-1 α (SDF-1 α) revealed such an interaction (46). Located downstream of a stretch of negatively charged residues, tyrosine sulfate moieties may contribute extra negative charges to the N terminus of vGPCR and likely to a vGPCR association with specific chemokine, offering an opportunity to define the molecular determinants for vGPCR association with chemokines and to uncover roles of chemokines in vGPCR tumorigenesis.

We report here that vGPCR incorporated sulfate groups within its N-terminal tyrosine residues. Tyrosine sulfate moieties were crucial for vGPCR association with an agonist, GRO- α , but not for its association with an inverse agonist, IP-10. Additionally, a sulfated peptide corresponding to residues 12 through 33 of the vGPCR N terminus, but not an unsulfated equivalent, partially blocked vGPCR association with GRO- α . Although the vGPCR variant lacking sulfotyrosines activated signaling pathways in cultured cells, the unsulfated variant demonstrated reduced tumorigenesis in nude mice. In support of the essential roles of sulfotyrosines in vGPCR tumorigenesis, the lack of sulfotyrosines impaired the ability of vGPCR to promote secretion of inflammatory cytokines, some of which function as vGPCR agonists. Moreover, GRO- α triggered vGPCR signaling and potentiated vGPCR tumorigenesis in a sulfotyrosine-dependent manner. These data underscore the importance of sulfated tyrosines in vGPCR association with a chemokine agonist (GRO- α) and reveal the essential roles of chemokines in vGPCR tumorigenesis.

MATERIALS AND METHODS

Cells, plasmids, and peptides. Unless specified, pcDNA5/FRT/TO (Invitrogen) and pCDH-CMV-EF-Puro (System Bioscience) were used for the expression of vGPCR and its variants in which tyrosines at positions 26 and 28 had been altered to aspartic acid individually or simultaneously (Y26D or Y28D for single mutations and YYDD, representing the double mutation) as previously described (16). Briefly, vGPCR and its variants were amplified by PCR and cloned into pcDNA5/FRT/TO between AflII and XhoI or into pCDH-CMV-EF-Puro between EcoRI and BamHI. The CCR5 expression construct is described elsewhere (13).

For protein expression, the hemagglutinin (HA) epitope or the Flag epitope was inserted upstream or downstream of vGPCR coding sequence, respectively. The human GRO- α (hGRO- α) gene was amplified by PCR from the pcDNA3-GRO- α plasmid, a kind gift from Daniel Notterman (Princeton University), and cloned into pCDH-CMV-EF-Puro between XbaI and BamHI. Lentivirus containing wild-type vGPCR, the YYDD variant, or hGRO- α was produced in HEK293T cells as previously described (16). The oligonucleotides encoding the glycogen synthase kinase (GSK) peptide (GSKp; CGPKGPGRRRRTSSF AEG) for AKT/protein kinase B (PKB) kinase assays were synthesized, annealed, and cloned into pGEX-4T-1 between BamHI and XhoI. The GST-GSKp fusion protein was expressed and purified as previously described (17–19).

HEK293T (293T) and NIH 3T3 cells were obtained from the American Type Culture Collection. Immortalized (by simian virus 40 [SV40] large T antigen) murine endothelial cells (SVECs) were a kind gift from Philip Thorpe (UT Southwestern). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 5 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. HEK293T cells were transfected with calcium phosphate as previously described (16, 19). To establish stable cell lines, NIH 3T3 and SVECs were infected with lentivirus containing wild-type vGPCR, the YYDD variant, or human GRO- α and selected with puromycin (1 μ g/ml). Polyclonal cell lines were pooled and used for this study.

Recombinant human GRO- α and IP-10 were purchased from R&D Systems, Inc.; [125 I]GRO- α and [125 I]IP-10 were purchased from PerkinElmer. The tyrosine-sulfated peptide DDDSWNETLNMSGY₂₆DY₂₈SGNFS and the unsulfated equivalent, which correspond to the primary sequence of residues 12 through 33 of vGPCR, were synthesized to 95% purity by Bio-synthesis, Inc. (sulfated tyrosines are numbered and in boldface).

Labeling and immunoprecipitation of vGPCR and vGPCR variants. 293T cells were transfected with plasmids encoding either wild-type vGPCR, the YYDD variant, or CCR5 (as a positive control). Cells were split at 10 h posttransfection. Approximately 36 h after transfection, cells were radiolabeled with [35 S]methionine-cysteine or [35 S]sulfate for 10 h. Labeled cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA) containing a protease inhibitor cocktail (Roche). Centrifuged cell lysates were cleared with protein A/G agarose and incubated with anti-Flag or anti-CCR5 (C9) antibody, respectively. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

FIG. 1. The KSHV vGPCR efficiently incorporates radiolabeled sulfate. (A) The KSHV vGPCR, human cytomegalovirus (HCMV) US28, and murine gamma herpesvirus 68 (γ HV68) vGPCRs contain a putative tyrosine sulfation motif within their N-terminal extracellular domains. These putative tyrosine sulfation motifs are underlined. (B) Wild-type (WT) vGPCR efficiently incorporates radiolabeled sulfate. HEK293T cells were transfected with plasmids encoding wild-type vGPCR, a variant in which tyrosines 26 and 28 had been altered to aspartic acid (designated YYDD), or a plasmid encoding CCR5 that serves as a positive control. Cells were split, labeled with [35 S]methionine-cysteine (Met/Cys) or [35 S]sulfate (SO_4), and lysed. Cell lysates were incubated with anti-Flag or C9 anti-CCR5 antibody. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. (C) Experiment similar to panel B except that cells were cotransfected with plasmids encoding wild-type vGPCR or the YYDD variant and TPST2 as indicated. (D) Experiment similar to panel B except that cells were transfected with plasmids encoding wild-type vGPCR, YYDD, and Y26D variants as indicated. Numbers at the bottom indicate relative ratios of sulfated vGPCR normalized against [35 S]Met-Cys-labeled vGPCR.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblot analyses were performed as previously described (18, 19). Immunoblot detection was performed with anti-AKT antibody (1:500; Cell Signaling), anti-Flag M2 antibody (1:5,000; Sigma), anti-HA (1:2000; Covance), anti-actin (1:30,000; Abcam), and antibody specific for phospho-AKT (1:1,000; Cell Signaling). Proteins were visualized with chemical luminescent detection reagent (Pierce) and detected by Fuji Phosphor Imager LAS-3000.

Chemokine-binding assays. Chemokine-binding experiments were carried out as previously described (15). Briefly, NIH 3T3 cells stably expressing wild-type vGPCR (NIH 3T3/vGPCR) or the YYDD variant (NIH 3T3/YYDD) were treated with 5 mM EDTA and resuspended in DMEM. Then, 2×10^5 cells were incubated with 50 pM [125 I]GRO- α or [125 I]IP-10 and 0 to 250 nM unlabeled human GRO- α or IP-10 as a competitor for 45 min at 37°C in 100 μ l of binding buffer (50 mM HEPES, pH 7.5, 1 mM CaCl_2 , 150 mM NaCl, 5 mM MgCl_2 , 5% dialyzed FBS). Cells were washed with binding buffer three times and bound [125 I]GRO- α was quantitated by scintillation counting.

For vGPCR cellular surface expression, NIH 3T3 cells stably expressing wild-type vGPCR or the YYDD variant were used. The vGPCR on the cell surface was detected by immunofluorescence without permeabilization, and intracellular vGPCR was similarly detected after permeabilization with 0.2% Triton X-100 in phosphate-buffered saline (PBS). Alternatively, NIH 3T3 stable cells were fixed and incubated with 1 μ g/ml anti-HA antibody. Cellular surface expression of vGPCR was quantitated by flow cytometry assay.

For peptide competition, 2×10^5 NIH 3T3 cells stably expressing vGPCR were incubated with 50 pM [125 I]GRO- α and the indicated amount (see Fig. 3) of a peptide corresponding to residues 12 through 33 of vGPCR with either the sulfated tyrosines at positions 26 and 28 or unmodified tyrosines at these positions, or cells were incubated with only 50 pM [125 I]GRO- α (control), and bound [125 I]GRO- α was quantitated as above.

Determination of AKT activation. For chemokine stimulation, exponentially proliferating SVEC stable cells were starved overnight in serum-free medium

and stimulated with 25 nM GRO- α or IP-10 for 15 min or left untreated. Whole-cell lysates were prepared and used to determine the levels of phospho-AKT or total AKT by immunoblot analysis.

To assess AKT kinase activity, NIH 3T3 stable cells were harvested and lysed with kinase lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 0.5 mM dithiothreitol [DTT]), and lysates were precipitated with 0.5 μ g of anti-AKT antibody (Santa Cruz). After extensive washing with lysis buffer and then one wash with kinase reaction buffer (1 mM DTT, 5 mM KCl, 2 mM MgCl_2 , 2 mM MnCl_2 , 10 mM NaF, 25 mM HEPES, pH 7.5), precipitated AKT was used to phosphorylate the glutathione *S*-transferase (GST) fusion protein containing a GSK peptide (GSKp) *in vitro*. Reaction mixtures containing 0.5 μ g of GST-GSKp and 10 μ Ci of [γ - 32 P]ATP in 30 μ l were carried out for 45 min at 25°C. Samples were analyzed by SDS-PAGE and autoradiography.

Luciferase reporter assay. HEK293T cells in 12-well plates were transiently transfected with a reporter cocktail and 150 ng of plasmids expressing wild-type vGPCR or the YYDD variant as previously described (16). The reporter cocktail contained 50 ng of plasmids expressing firefly luciferase under the control of response elements of NFAT or NF- κ B transcription factor and 50 ng of plasmid expressing β -galactosidase. At 36 h after transfection, cells were harvested and lysed on ice. The centrifuged supernatant was used to measure luciferase and β -galactosidase activity according to the manufacturer's protocol (Promega).

Tumor formation *in vivo*. All animal experiments were carried out according to the National Institutes of Health principles of laboratory animal care and approved by the University of Texas Southwestern Medical Center. As previously reported (16, 30), NIH 3T3 cells and NIH 3T3 cells stably expressing wild-type vGPCR, the YYDD variant, or hGRO- α were injected subcutaneously into the flanks of 3- to 5-week-old mice (athymic, nude/nude; Jackson Laboratory). Nude mice were sacrificed 3 to 4 weeks after inoculation, and the tumor weight was determined.

RESULTS

The vGPCR N-terminal extracellular domain is modified by sulfate. Visual inspection of the vGPCR extracellular amino acid sequence reveals a motif, two tyrosine residues (at positions 26 and 28) flanking an aspartic acid, a feature suggestive of tyrosine sulfation (Fig. 1A). Interestingly, putative tyrosine sulfation sequences are also found within the N termini of human cytomegalovirus US28 (GPCR) and murine gamma herpesvirus 68 vGPCR (ORF74) (Fig. 1A) (32, 47). This observation suggests that tyrosine sulfate moieties may play important roles for vGPCR functions. To examine whether vGPCR incorporates [³⁵S]sulfate, a variant containing aspartic acid residues at positions 26 and 28, designated YYDD, was created. Plasmids containing wild-type vGPCR and the YYDD variant were transfected into 293T cells. Cells were split and labeled with either [³⁵S]methionine-cysteine or [³⁵S]sulfate. Precipitated vGPCR and CCR5, a positive control, were analyzed by autoradiography. It was found that wild-type vGPCR incorporated significant amounts of sulfate at a comparable level to that of CCR5, while the YYDD variant incorporated a minimal level of sulfate (Fig. 1B). This supports the idea that vGPCR is modified by sulfate at tyrosine residues within the N terminus of vGPCR. There are two major species of approximately 43 and 45 kDa that are likely due to differential glycosylation as analyzed by immunoblotting (data not shown). A protein band corresponding to the size of a vGPCR dimer was also observed. The dimer form was also observed for CCR5, and we found that these seven-transmembrane proteins often dimerize/oligomerize in solution. Notably, vGPCR aggregates after heat denaturation and was observed predominantly in wells after electrophoresis.

The tyrosine sulfation of vGPCR was further examined under conditions where TPST2, a cellular tyrosylprotein sulfotransferase, was overexpressed. As shown in Fig. 1C, exogenous TPST2 greatly increased the sulfate incorporation of vGPCR. To examine whether both tyrosine residues are modified by sulfate, vGPCR variants containing a single aspartic acid at position 26 or 28 were expressed for sulfate incorporation analysis. Surprisingly, the Y28D variant was not detectable by immunoblot analysis and was expressed at a very low level, as determined by immunofluorescence microscopy (data not shown). Nevertheless, the Y26D variant consistently incorporated [³⁵S]sulfate at a reduced level compared to that of wild-type vGPCR, suggesting that both tyrosine residues are utilized for sulfate addition (Fig. 1D). Collectively, these data support the conclusion that the N terminus of vGPCR is modified by posttranslational sulfate addition.

Sulfotyrosines of vGPCR are crucial for vGPCR association with GRO- α but not for its association with IP-10. Tyrosine sulfate moieties were previously shown to mediate protein-protein interactions such as receptor association with cognate chemokines (14, 15, 46). To examine whether sulfated tyrosine residues are important for vGPCR association with chemokines, we performed chemokine-binding assays with human chemokines (GRO- α and IP-10) against murine NIH 3T3 cells to minimize chemokine-binding to endogenous receptors. To do this, NIH 3T3 cells stably expressing HA-tagged wild-type vGPCR or the YYDD variant were established. The surface expression of wild-type vGPCR and the YYDD variant was

examined by immunofluorescence. It was found that wild-type vGPCR and the YYDD variant were expressed well on the cell surface (Fig. 2A). Flow cytometry analyses further support the conclusion that the YYDD variant was expressed on the cell surface in NIH 3T3 cells at a similar, albeit slightly reduced, level as the wild-type vGPCR (Fig. 2B). Given that vGPCR displays promiscuous association with a number of chemokines, we initially focused on vGPCR association with GRO- α and IP-10. While GRO- α is an agonist that stimulates vGPCR-dependent signaling, IP-10 is an inverse agonist of vGPCR (27, 40). We examined the ability of GRO- α to bind vGPCR with a chemokine competition assay. NIH 3T3 stable cells as described above were used to bind [¹²⁵I]GRO- α with increasing amounts of unlabeled GRO- α as indicated. Figure 2C demonstrates that cells expressing wild-type vGPCR bound [¹²⁵I]GRO- α with a 50% inhibition concentration (IC₅₀) of 12 nM $\pm$ 3 nM. In contrast, the YYDD variant could not detectably associate with [¹²⁵I]GRO- α when measured without the lowest concentration (0.1 nM) of unlabeled GRO- α (Fig. 2C). These results indicated that tyrosine sulfate moieties are critical for vGPCR association with GRO- α . Similarly, the chemokine-binding assay was carried out to assess the ability of vGPCR to associate with IP-10. Surprisingly, wild-type vGPCR and the YYDD variant bound [¹²⁵I]IP-10 with similar affinities (measured IC₅₀s of 18 $\pm$ 2 nM and 35 $\pm$ 5 nM, respectively) (Fig. 2D). Interestingly, the maximal binding ability of the YYDD variant was approximately 65% of that of wild-type vGPCR. This may be due to a slightly reduced expression of the YYDD variant as observed in NIH 3T3 stable cells (Fig. 2B). Taken together, these data demonstrated that the tyrosine sulfate moieties are critical for vGPCR association with GRO- α but not for vGPCR association with IP-10.

A sulfated peptide corresponding to residues 12 through 33 of the vGPCR N terminus, but not an unsulfated equivalent, partially inhibits vGPCR association with GRO- α . To further test the role of the sulfate group in GRO- α -binding, peptides corresponding to residues 12 through 33 of vGPCR with or without sulfate addition at positions 26 and 28 were synthesized (Fig. 3A). To assess the effect of these two peptides on vGPCR association with GRO- α , a chemokine-binding assay was carried out using NIH 3T3 cells stably expressing wild-type vGPCR. As shown in Fig. 3B, the sulfated peptide partially inhibited vGPCR association with GRO- α in a dose-dependent manner. The reduction of bound [¹²⁵I]GRO- α was approximately 30% and 45% at peptide concentrations of 50 μ M and 100 μ M, respectively. However, the unsulfated equivalent had no detectable effect on vGPCR association with GRO- α under the same conditions, indicating a specific effect of the sulfate moieties. These data suggest that the sulfotyrosines may provide a docking site on the N terminus of vGPCR for GRO- α binding. Together with the fact that the YYDD variant failed to bind GRO- α , these findings indicate that the tyrosine sulfate moieties are responsible for the specificity of the vGPCR interaction with GRO- α .

Sulfotyrosines are not critical for signaling downstream of vGPCR. Previous studies have defined the signaling events downstream of vGPCR, typically including the PI3 kinase/AKT pathway and transcription factors of NF- κ B and NFAT family (6, 12, 31, 33, 38, 42, 44). We have examined the effects of sulfotyrosines on selected vGPCR-dependent signaling

FIG. 2. Sulfotyrosines of vGPCR at residue 26 and 28 are necessary for vGPCR association with GRO- α but not for vGPCR association with IP-10. (A) Control NIH 3T3 (Vector) and NIH 3T3 cells stably expressing HA-tagged wild-type vGPCR (WT) or the YYDD variant were fixed, permeabilized (Perm) or not permeabilized (Non-Perm), incubated with anti-HA (green) and anti-calreticulin (red) (as a permeability control) antibodies, and analyzed by immunofluorescence microscopy. (B) NIH 3T3 cells stably expressing the HA-tagged wild-type vGPCR or the YYDD variant were incubated with anti-HA antibody, fixed, and analyzed by flow cytometry. (C) NIH 3T3 cells stably expressing wild-type vGPCR or the YYDD variant were incubated with 20 pM [¹²⁵I]GRO- α and the indicated amounts of unlabeled human GRO- α competitor. Cells were washed, and bound [¹²⁵I]GRO- α was quantitated by scintillation counting. (D) Experiment similar to panel C except that cells were incubated with 20 pM [¹²⁵I]IP-10 and the indicated amounts of unlabeled human IP-10. For both panels C and D, data represent the mean of at least three independent experiments, and error bars denote standard deviations. The binding of [¹²⁵I]GRO- α and [¹²⁵I]IP-10 to NIH 3T3/Vector cells (endogenous receptors) is approximately 5% $\pm$ 2% of that of NIH 3T3/vGPCR cells.

events including the activation of NF- κ B, NFAT, and AKT kinase. First, the ability of wild-type vGPCR and the YYDD variant to activate NFAT and NF- κ B transcription factors was assessed by luciferase-based reporter assays. In transiently transfected 293T cells, wild-type vGPCR and the YYDD variant potently activated NFAT transcription factor to similar levels in a dose-dependent manner (Fig. 4A). Moreover, similar levels of NF- κ B activation were also observed for wild-type vGPCR and the YYDD variant (Fig. 4B). These findings support the idea that sulfotyrosines are dispensable for vGPCR-mediated signal transduction. Thus, the activation of AKT kinase by vGPCR was assessed by an *in vitro* kinase assay where AKT was precipitated, and its kinase activity to phosphorylate a GST fusion protein containing the GSK peptide was examined by autoradiography. As shown in Fig. 4C, the AKT precipitated from NIH 3T3 cells expressing wild-type vGPCR and the YYDD variant have significantly higher levels of kinase activity than AKT precipitated from control NIH 3T3 cells. However, the kinase activity of AKT in NIH 3T3 cells expressing the YYDD variant was lower than that in NIH 3T3 cells expressing wild-type vGPCR. Nevertheless, these experiments support the conclusion that the tyrosine sulfate moieties

are not critical for vGPCR to activate downstream signaling events.

Diminished tumor formation and altered cytokine secretion induced by the unsulfated vGPCR variant. To test whether sulfotyrosines are important for vGPCR tumorigenesis, NIH 3T3 cells stably expressing wild-type vGPCR or the YYDD variant were examined for their relative expression levels. The HA-tagged vGPCR was precipitated with and analyzed by immunoblotting with anti-HA antibody. This showed that the YYDD variant was expressed at a higher level than the wild-type vGPCR in NIH 3T3 stable cell lines (Fig. 5A). Given that vGPCR is toxic to cells (5, 26) and that, as we have shown, vGPCR expression reduces cell proliferation (16), we examined the effects of the YYDD variant on cell proliferation. Surprisingly, the expression of the YYDD variant did not affect cell proliferation, whereas wild-type vGPCR significantly increased the doubling time of NIH 3T3 cells (Fig. 5B). This observation indicates that the YYDD variant is not toxic to NIH 3T3 cells and suggests that the loss of function of the YYDD variant permits higher levels of expression. Next, the roles of sulfotyrosines in vGPCR tumorigenesis were examined by tumor formation in nude mice. vGPCR is believed to induce

FIG. 3. The sulfated peptide but not the unsulfated equivalent partially inhibits vGPCR association with GRO- α . (A) The sulfated peptide corresponding to residues 11 to 33 of the vGPCR N terminus is shown. The peptide carrying sulfate groups at positions 26 and 28 (denoted by *) and its unsulfated equivalent were synthesized and purified. (B) NIH 3T3 cells stably expressing wild-type vGPCR were incubated with 20 pM [125 I]GRO- α and the indicated amount of a peptide corresponding to residues 12 through 33 of the vGPCR with the sulfated tyrosines at position 26 and 28 (vG-N-S) or with unmodified tyrosines at these positions (vG-N), or cells were incubated with buffer only; cells were washed, and bound [125 I]GRO- α was quantitated by scintillation counting. Data represent the mean of four independent experiments, and error bars denote standard deviations. *, $P < 0.01$ relative to vG-N as calculated by a Student's t test.

tumor formation via both autocrine and paracrine mechanisms. Thus, NIH 3T3 cells stably expressing wild-type vGPCR or the YYDD variant (1×10^6 cells) were mixed with regular NIH 3T3 cells (1×10^6), the cells were injected into nude mice, and tumor formation was monitored. Three weeks postinoculation, mice inoculated with NIH 3T3/vGPCR developed sizable tumors, whereas no tumor was observed in nude mice inoculated with NIH 3T3/vector or NIH 3T3/YYDD cells (Fig. 5C). Tumors derived from NIH 3T3/vGPCR weighed

between 500 mg and 950 mg (Fig. 5D). Moreover, vGPCR expression in tumor tissues was confirmed by reverse transcriptase PCR, suggesting that vGPCR is necessary for tumor formation *in vivo* (Fig. 5E). The striking loss of the ability of the YYDD variant to cause tumors in nude mice indicates that the sulfotyrosines are critical for vGPCR tumorigenesis *in vivo*.

vGPCR is a constitutively active signaling molecule that activates multiple proinflammatory pathways, culminating in promoting cytokine production. Accumulating evidence supports the conclusion that vGPCR promotes tumor formation by stimulating proinflammatory cytokine production. The sulfotyrosines are not critical for vGPCR to constitutively activate downstream signaling events (Fig. 4); therefore, we examined cytokine secretion induced by vGPCR. Cytokines of particular interest to this study include those that are implicated in KSHV-associated malignancies and that are capable of modulating vGPCR-dependent signal transduction (39, 40). Murine endothelial SVEC cell lines stably expressing wild-type vGPCR and the YYDD variant were established with lentiviral infection. A multiplex array analysis revealed that wild-type vGPCR potentially increased IL-6 and MIP-2 secretion in SVECs (Fig. 5F). However, the levels of IL-6 and MIP-2 secreted from SVECs expressing the YYDD variant were approximately 45% of those of SVECs expressing wild-type vGPCR. Surprisingly, the YYDD variant, but not wild-type vGPCR, significantly increased tumor necrosis factor alpha (TNF- α) secretion in SVECs. Notably, TNF- α is a proapoptotic cytokine that possesses antitumor and antiviral activity. IL-6 and KC secretion in NIH 3T3 cells expressing wild-type vGPCR and the YYDD variant showed a similar pattern although MIP-2 and TNF- α were not detectable in NIH 3T3 cells (data not shown). Thus, these results suggest that sulfotyrosines are critical for vGPCR to induce proliferative cytokines and to inhibit proapoptotic cytokine secretion. Collectively, these data support the critical roles of tyrosine sulfate moieties in vGPCR-dependent cytokine production and tumorigenesis *in vivo*, implying that vGPCR association with agonistic proinflammatory cytokines

FIG. 4. Sulfotyrosines of vGPCR are not critical for its constitutive activity. (A) HEK293T cells were cotransfected with NFAT-luciferase reporter construct (50 ng), β -galactosidase reporter construct (50 ng), and plasmids (150 ng) containing wild-type vGPCR or the YYDD variant. Cells were harvested at 36 h posttransfection, and luciferase and β -galactosidase activities were determined. Data represent the mean of at least three independent experiments, and error bars denote standard deviation. (B) Experiment similar to panel A except that cells were transfected with an NF- κ B-luciferase reporter construct. (C) NIH 3T3 cells stably expressing wild-type vGPCR or the YYDD variant were lysed with buffer containing 1% NP-40. Precipitated AKT was incubated with purified GST-GSK and [γ - 32 P]ATP, and radiolabeled GST-GSK was analyzed by SDS-PAGE and autoradiography. Precipitated AKT was also analyzed by immunoblotting with anti-AKT antibody (bottom panel). Numbers indicate the relative intensity of phosphorylated GST-GSKp normalized to total AKT. Data represent three independent experiments.

FIG. 5. Sulfotyrosines are essential for vGPCR tumorigenesis. (A) Relative expression of wild-type vGPCR and the YYDD variant in stable NIH 3T3 (1×10^7) cells was analyzed by immunoprecipitation and immunoblotting with anti-HA antibody. For loading controls, whole-cell lysates (WCL) were analyzed by immunoblotting with anti-actin antibody (bottom). (B) Exponentially proliferating NIH 3T3/vector (Vec), NIH 3T3/vGPCR (WT), and NIH 3T3/YYDD cells were counted every 48 h to calculate their doubling times. *P* values as indicated were calculated with Student's *t* test relative to NIH 3T3/Vector cells. (C and D) NIH 3T3 cells stably expressing wild-type vGPCR or the YYDD variant, together with NIH 3T3 cells (1:1), were inoculated into the flanks of 3- to 5-week-old nude mice. Nude mice were sacrificed at 3 weeks after inoculation and photographed (C), and the tumor weight was determined (D). (E) Total RNA extracted from tumors derived from NIH 3T3/vGPCR cells was used for reverse transcription-PCR (RT-PCR) analyses with primers specific for vGPCR and β -actin. N, negative control (total RNA of tumor tissue derived from NIH 3T3/hGRO- α); P, positive control with a plasmid containing vGPCR. (F) Amounts of secreted IL-6, MIP-2, and TNF- α in the supernatant of SVEC/vector (lane 1), SVEC/vGPCR (lane 2), or SEVC/YYDD (lane 3) cells were determined by a multiplex cytokine array. Data represent mean values, and error bars denote standard deviations, with *P* values relative to SVEC/vector cells as calculated by Student's *t* test. *, *P* < 0.04.

mediated by sulfotyrosines contributes to vGPCR tumorigenesis.

GRO- α promotes vGPCR tumorigenesis in a sulfotyrosine-dependent manner. vGPCR promotes the expression of proinflammatory cytokines in endothelial cells (Fig. 5F and data not shown). Notably, KC and MIP-2 are mouse equivalents of the human GRO- α and potentiate vGPCR-dependent signaling. The observation that the tyrosine sulfate moieties are necessary for vGPCR association with GRO- α provides an opportunity to elucidate the roles of GRO- α in vGPCR signaling and tumorigenesis. To examine signaling events downstream of vGPCR in response to GRO- α stimulation, SVEC stable cells were cultured in conditioned medium without serum for 12 h, and GRO- α was added for 15 min before harvest for immunoblot analyses with antibodies to phospho-AKT and total AKT. As expected, GRO- α potently increased AKT phosphorylation in SVECs expressing wild-type vGPCR. However, the ability of GRO- α to stimulate AKT phosphorylation was greatly diminished in SVECs expressing the YYDD variant

(Fig. 6A). In contrast, IP-10 treatment increased the phosphorylation of AKT to similar levels in SVECs expressing wild-type vGPCR and the YYDD variant (Fig. 6B), consistent with the observation that sulfotyrosines are dispensable for vGPCR association with IP-10 (Fig. 2D). These findings demonstrate the functional significance of sulfotyrosines in vGPCR association with GRO- α .

To further examine the roles of GRO- α in modulating vGPCR tumorigenesis, NIH 3T3 cells stably expressing human GRO- α were established. NIH 3T3 cells stably expressing wild-type vGPCR or the YYDD variant, with or without NIH 3T3 cells expressing GRO- α , were inoculated into nude mice. After 3 weeks, mice were sacrificed, and tumor weight was measured. In agreement with previous data (Fig. 5D), wild-type vGPCR, but not the YYDD variant, potently induced tumor formation in nude mice. Not surprisingly, NIH 3T3 cells expressing human GRO- α also induced sizable tumors in nude mice (Fig. 6C). We found that tumorigenesis of wild-type vGPCR was greatly increased by NIH 3T3 cells expressing hGRO- α (Fig.

FIG. 6. GRO- α promotes vGPCR signaling and tumorigenesis in a sulfotyrosine-dependent manner. (A and B) SVECs stably expressing wild-type vGPCR (WT) or the YYDD variant were starved in serum-free conditioned medium and left untreated or were treated with GRO- α for 15 min (A) or IP-10 for 30 min (B). Whole-cell lysates were analyzed by immunoblotting with antibodies to phosphorylated AKT (p-AKT), total AKT (AKT), or β -actin (as a loading control). Numbers indicate the relative intensity of p-AKT by densitometry. Data represent three independent experiments. (C) NIH 3T3 cells stably expressing wild-type vGPCR or the YYDD variant were mixed with NIH 3T3 cells stably expressing human GRO- α or control NIH 3T3 stable cells and inoculated into nude mice. Mice were sacrificed at 3 weeks postinoculation, and tumor size was determined. *P* values between any two groups were calculated by Student's *t* test. *, *P* < 0.05. (D) vGPCR mRNA in tumors derived from NIH 3T3/hGRO- α mixed with NIH 3T3/vGPCR cells (lanes 1 to 4) or NIH 3T3/YYDD cells (lanes 5 to 8) was determined by RT-PCR with total RNA and primers specific for vGPCR, hGRO- α , and β -actin. (E) A hypothetical model of sulfotyrosines and chemokine agonists in vGPCR signaling and tumorigenesis. Chemokine agonists (GRO- α) bind vGPCR in a sulfotyrosine-dependent manner. Chemokine association promotes vGPCR signaling, leading to further production of proinflammatory cytokines including vGPCR chemokine agonists. Secreted cytokines and growth factors stimulate the proliferation of vGPCR-expressing cells in an autocrine manner and bystander cells in a paracrine manner. The autocrine feedback by chemokine agonists is critical for vGPCR tumorigenesis *in vivo*.

6C). The average weight of tumors derived from mixed NIH 3T3 cells that express vGPCR and GRO- α was approximately 2-fold of the sum of tumor weight of NIH 3T3 cells that expressed vGPCR or GRO- α individually. Interestingly, tumors derived from NIH 3T3 cells expressing the human GRO- α were consistently reduced by NIH 3T3 cells expressing the YYDD variant (Fig. 6C). Finally, vGPCR mRNA was detected in tumors derived from NIH 3T3 cells expressing wild-type vGPCR and GRO- α but was absent in tumors derived from NIH 3T3 cells expressing the YYDD variant and GRO- α (Fig. 6D). These results support the conclusion that wild-type vGPCR, but not the YYDD variant, is necessary to promote tumor formation in nude mice. Taken together, these findings identify a potent effect of GRO- α in promoting vGPCR signaling and tumorigenesis in a manner that is dependent on sulfotyrosines.

DISCUSSION

We have demonstrated here that the KSHV vGPCR is post-translationally modified by sulfate groups at tyrosine residues within the N-terminal extracellular domain. The tyrosine sulfate moieties are crucial for vGPCR association with an agonist, GRO- α . However, the lack of these sulfotyrosines had a marginal effect on vGPCR association with IP-10, an inverse agonist (Fig. 2C and D). The latter observation confirms that

the YYDD variant preserves conformational and functional integrity. In support of the essential roles of sulfate moieties in vGPCR's interaction with chemokines, the sulfated peptide containing residues 12 through 33 of vGPCR partially inhibited GRO- α binding to vGPCR (Fig. 3). An unsulfated equivalent failed to do so, indicating that the loss of sulfate, not change of amino acid, affects the vGPCR interaction with GRO- α . These experiments define the tyrosine sulfate moieties as a molecular determinant for vGPCR association with GRO- α , an important proinflammatory cytokine implicated in vGPCR tumorigenesis and KSHV-associated malignancies (28, 29, 39). Our findings are consistent with a previous report that the N-terminal deletion of residues 2 through 11 reduces vGPCR association with IP-10 while having no effect on vGPCR association with GRO- α (24). Located at residues 26 and 28 and downstream of the IP-10 binding determinant, the tyrosine sulfate groups identified here are responsible for vGPCR association with GRO- α . Our attempt to evaluate the contribution of individual tyrosine residues to vGPCR sulfate incorporation was not successful because the Y28D variant was expressed at a very low level (data not shown). However, the Y26D variant was well expressed and incorporated sulfate groups at a reduced level compared to incorporation by the wild-type vGPCR (Fig. 1D), implying that both tyrosine residues are utilized for sulfate incorporation. We note that the unsulfated YYDD mutant is expressed at a slightly reduced level on the

cell surface (Fig. 2B) although the total protein amount of the YYDD mutant is higher than that of wild-type vGPCR (Fig. 5A). Nevertheless, the YYDD variant was expressed and displayed on the cell surface at a similar level to that of wild-type vGPCR, permitting the investigation of the roles of tyrosine sulfate moieties in vGPCR chemokine-binding, signaling, and tumorigenesis.

Although vGPCR possesses a constitutive signaling capacity that does not require chemokine association, specific chemokines can further enhance and others can inhibit the constitutive activity (2, 21, 27). Taking advantage of the posttranslational sulfation, we have examined the roles of tyrosine sulfate moieties in vGPCR signaling and tumorigenesis. It has been shown that vGPCR activates multiple proliferative signaling pathways, leading to the activation of NF- κ B and NFAT transcription factors (3, 12, 38). We examined the effects of tyrosine sulfate moieties on vGPCR constitutive activity and induced signaling in response to GRO- α . The YYDD variant, compared to wild-type vGPCR, appears equally capable of activating NF- κ B and NFAT transcription factors, as measured by reporter assays (Fig. 4A and B). This agrees with previous findings that chemokine association is not critical for the constitutive activity of vGPCR to activate various downstream signaling pathways (24, 40). Indeed, the YYDD variant, although less efficiently than wild-type vGPCR, increased the steady level of AKT kinase activity, which is indicative of PI3 kinase activation (Fig. 4C). The reduced activity of the YYDD variant to engage downstream signaling pathways may reflect the contribution of a positive feedback by agonist chemokines such as GRO- α , KC, and MIP-2. It is conceivable that these chemokines enhance signaling events, collectively known as vGPCR "constitutive activity," downstream of vGPCR at steady state.

Although the YYDD variant activated NF- κ B and NFAT transcription factors as potently as wild-type vGPCR, as determined by reporter assays (Fig. 4A and B), SVECs expressing wild-type vGPCR secreted significantly more IL-6 and MIP-2 than those expressing the YYDD variant. It is likely that vGPCR promotes cytokine production through both transcriptional and translational regulations. In fact, Sodi et al. reported that vGPCR drives the endothelial cell transformation by enhancing translation through the PI3-kinase-AKT-mTOR pathway (43). The reduced cytokine production in NIH 3T3 cells expressing the YYDD variant agrees with the lower levels of phosphorylated AKT in those cells than in NIH 3T3 cells expressing wild-type vGPCR. These findings suggest that vGPCR engages distinct signaling pathways to promote transcription (driven by NF- κ B and NFAT) and translation (downstream of AKT and/or other unknown pathways). Perhaps, sulfotyrosines are distinctively required for vGPCR to promote translation. Thus, future experiments are necessary to address this hypothesis.

In contrast to the minor difference of constitutive activity between wild-type vGPCR and the YYDD variant, GRO- α addition potently promoted AKT phosphorylation in SVECs expressing wild-type vGPCR while its ability to increase AKT phosphorylation was greatly reduced in SVECs expressing the YYDD variant (Fig. 6A). In contrast, IP-10 treatment increased AKT phosphorylation to similar levels in NIH 3T3 cells expressing wild-type vGPCR and the YYDD variant (Fig. 6B), supporting the observation that sulfotyrosines are dis-

pensable for vGPCR association with IP-10 (Fig. 2D). Interestingly, GRO- α increased AKT phosphorylation in SVECs expressing the YYDD variant (Fig. 6A) although the YYDD variant no longer associated with GRO- α (Fig. 2D). This is likely due to the binding of GRO- α to other receptors endogenous to SVECs, while the YYDD variant is able to "prime" signaling molecules downstream of vGPCR. One possible priming mechanism may be physical association or recruitment of signaling molecules such as PI3 kinase and/or AKT to the proximity of the plasma membrane. Future experiments are necessary to investigate the underlying molecular mechanisms. Furthermore, forced expression of GRO- α significantly increased tumor growth in the presence of NIH 3T3 cells expressing wild-type vGPCR but failed to do so in the presence of NIH 3T3 cells expressing the YYDD variant (Fig. 6C). Surprisingly, the expression of the YYDD variant reduced tumor formation induced by GRO- α expression, suggesting a potential dominant negative effect. One possible scenario is that specific chemokine agonists bind the YYDD variant independent of tyrosine sulfate moieties but fail to activate signaling events downstream of vGPCR, thereby sequestering chemokines and inhibiting GRO- α -induced tumor formation. Nevertheless, these experiments demonstrated that GRO- α stimulated vGPCR signaling and tumorigenesis in a sulfotyrosine-dependent manner. Our findings identified an essential role of GRO- α in promoting vGPCR signaling in tissue culture and vGPCR tumorigenesis in nude mice.

Our findings presented here support a model by which vGPCR induces tumor formation, with an emphasis on the autocrine stimulation by chemokine agonists. Within this model, chemokine agonists (such as GRO- α) bind vGPCR in a sulfotyrosine-dependent manner and promote vGPCR signaling, thereby boosting the production of cytokines and growth factors in vGPCR-expressing cells (Fig. 6E). Secreted chemokine agonists further amplify vGPCR-mediated signaling events and cytokine production through binding to the sulfated N terminus of vGPCR. Such an amplification feedback loop consisting of vGPCR and its cognate chemokines secreted by vGPCR-expressing cells is necessary for vGPCR tumorigenesis. Our experiments demonstrating the positive effect of GRO- α in modulating vGPCR tumorigenesis agree with the hypothesis that vGPCR induces angiogenesis via a combination of paracrine and autocrine mechanisms (8). Conceivably, disrupting the chemokine association mediated by tyrosine sulfate moieties of vGPCR would abrogate the autocrine feedback loop and reduce vGPCR tumorigenesis. It would be interesting to examine whether the YYDD variant possesses residual tumorigenic activity. Therefore, we may identify additional sequences and cognate chemokines important for vGPCR signaling and tumorigenesis. These data collectively support the essential roles of chemokines in promoting vGPCR tumorigenesis, pointing to the importance of elucidating the intracellular signaling cascades that lead to chemokine production. The importance of these tyrosine sulfate moieties reflects the critical roles of positive feedback by chemokines in vGPCR signaling and tumorigenesis, suggesting that interfering with the regulatory circuit may effectively inhibit vGPCR tumorigenesis. Overall, our study defined a molecular determinant that contributes to vGPCR association with a cognate agonist chemokine and identified an essential role of GRO- α in

vGPCR tumorigenesis. These findings also provide proof-of-principle for future therapeutics targeting the tumorigenic vGPCR.

ACKNOWLEDGMENTS

We thank Nicholas Conrad for critical reading of the manuscript.

This work was supported by NIH grants CA117809 (R21) and CA134241 (R01) and the UT Southwestern Endowed Scholar program. H. Feng was partly sponsored by the Chinese Ministry of Education (grant number 207077) and Hunan Education Department (06B054).

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