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The unsulfated extracellular N-terminus of vGPCR reduces the tumorigenicity of hGRO-α in nude mice

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The Kaposi's Sarcoma-associated Herpesvirus (KSHV)-encoded G-protein coupled receptor (vGPCR) is an oncoprotein that is implicated in KSHV-associated malignancies. We previously revealed vGPCR incorporates sulfate groups within its extracellular N-terminal tyrosine residues (Y26 and Y28) and that this tyrosine sulfation is crucial for its tumorigenicity in nude mice. hGRO-α binds vGPCR in a sulfotyrosine-dependent manner and promotes its tumorigenicity through autocrine signaling. Interestingly, an unsulfated vGPCR mutant (yydd-vGPCR) attenuated the tumor growth triggered by hGRO-α. In this study, the extracellular N-terminus of vGPCR (wt-vGN) and an unsulfated vGPCR mutant (yydd-vGN) were individually secreted, expressed and purified. A radioactive labeling assay demonstrated that wt-vGN but not yydd-vGN incorporated [^{35}S]-sulfate. In nude mice, NIH3T3 cells expressing yydd-vGN but not wt-vGN could significantly inhibit the tumor growth triggered by hGRO-α. All our data support the conclusion that the unsulfated extracellular N-terminus of vGPCR reduces the tumorigenicity of hGRO-α.

KSHV, vGPCR, hGRO-α, sulfotyrosine, tumorigenicity

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Kaposi's sarcoma (KS) is an angioproliferative neoplasm consisting of characteristic spindle cells and infiltrating leukocytes, and is a leading cause of morbidity and mortality among the AIDS population. The Kaposi's sarcoma-associated herpesvirus (KSHV, also known as human herpesvirus 8, or HHV-8) is the etiologic agent for Kaposi's sarcoma (KS), as well as primary effusion lymphoma and multicentric Castleman's disease [1–4]. KSHV is a DNA virus and belongs to the gamma-2 herpesvirus family, and is closely related to herpesvirus saimiri (HVS) and rhesus monkey rhadinovirus (RRV). The genome of KSHV is approximately 165–170 kb and contains more than 80 open reading frames. As one of several recently identified human tumor viruses, KSHV has been extensively studied to elucidate the mechanism of its tumorigenicity. Indeed, the KSHV genome encodes an array of proteins that are implicated in modulating host inflammatory responses, angiogenesis and tumor formation, most notably the G protein-coupled receptor (vGPCR or ORF74) [5–7].

vGPCR is the homolog of human interleukin-8 receptor (IL-8R or CXCR2). However, unlike its cellular counterpart, it is constitutively activated even in the absence of ligand association [8]. vGPCR triggers downstream signaling components including the phospholipase C pathway, and PI3 kinase/AKT axis, and has broad signaling effects *in vitro*, activating NF-κB, NFAT and AP-1 [9–14]. Emerging evidence supports that this KHSV lytic gene is an oncogene: vGPCR-transformed cells led to tumorigenesis in nude mice and vGPCR transgenic mice developed the human KS-like

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disease [15,16].

vGPCR constitutively activates downstream signaling even without ligand association. However, chemokines such as GRO-α and IP-10 bind vGPCR and modulate its signaling. Accumulating evidence suggests that the N-terminus of vGPCR is important for its chemokine association and tumorigenicity [17–20]. Understanding the molecular details of vGPCR association with chemokines will advance our knowledge of the regulatory mechanisms that govern vGPCR signaling and tumorigenesis and will inform the efforts to develop therapy targeting vGPCR in the treatment of KS.

Our previous study showed that vGPCR contains sulfated tyrosine residues at the N-terminus (Y26 and Y28), which are necessary for GRO-α but not IP-10 binding. Sulfotyrosines at positions 26 and 28 are not critical for signaling downstream of vGPCR. However, they are crucial for the tumorigenicity of vGPCR in nude mice and human GRO-α (hGRO-α) promotes vGPCR tumorigenesis in a sulfotyrosine-dependent manner. Unsulfated yydd-vGPCR (in which the tyrosines at positions 26 and 28 have been mutated to aspartic acid) was not tumorigenic in nude mice [21]. Interestingly, the tumor forming ability of NIH3T3 cells expressing hGRO-α was consistently reduced by yydd-vGPCR expression [21], which implied yydd-vGPCR (more precisely its unsulfated extracellular N-terminus) decreased the tumorigenicity of hGRO-α.

In this study, chimeras comprising the extracellular N-terminus of vGPCR or yydd-vGPCR and mouse Fc (fragment, crystallizable) region were constructed (wt-vGN-mFc and yydd-vGN-mFc, respectively). These fusion proteins were individually secreted, expressed and purified by affinity chromatography from whole cell lysates or the media supernatant. Radioactive labeling demonstrated that wt-vGN-mFc but not yydd-vGN-mFc recruited [^{35}S]-sulfate. In nude mice experiments, the tumor forming ability of NIH3T3 cells expressing hGRO-α was consistently reduced by the expression of yydd-vGN-mFc but not by wt-vGN-mFc expression. Also, the tumorigenicity of vGPCR *in vivo* was reduced by yydd-vGN-mFc. All these data support the conclusion that the unsulfated extracellular N-terminus of vGPCR reduces the tumorigenicity of hGRO-α *in vivo*.

1 Materials and methods

1.1 Cells and plasmids

HEK293T (293T) cells and NIH3T3 cells were obtained from the American Type Culture Collection (ATCC, Manassas/USA). NIH3T3 cells stably expressing vGPCR or hGRO-α were kept in the lab. All cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin.

DNA fragments encoding the extracellular N-terminal region (amino acid 1–49 of vGPCR (wt-vGN) or yydd-vGPCR (yydd-vGN) were PCR-amplified and cloned into pSecTag2A (Invitrogen) between the *Asc* I and *Xho* I sites. Mouse Fc fragment (mFc) was PCR-amplified using a plasmid encoding LSF-mouse Fc hinge as the template (a kind gift from Dr. Feng PingHui, USC) and cloned into the above constructs between the *Xho* I and *Apa* I sites (pSecTag2A-wt-vGN-mFc and pSecTag2A-yydd-vGN-mFc accordingly).

For lentiviral vector constructs, wt-vGN-mFc or yydd-vGN-mFc were excised and cloned into pCDH-CMV-EF-Puro (System Bioscience) between the *Nhe* I and *Pme* I sites (CDH-wt-vGN-mFc and CDH-yydd-vGN-mFc accordingly). Lentivirus containing wt-vGN-mFc or yydd-vGN-mFc was made as described previously and NIH3T3 cells stably expressing wt-vGN-mFc or yydd-vGN-mFc were established as previously [22].

1.2 Expression and purification of wt-vGN and yydd-vGN

293T cells were transfected with either pSecTag-wt-vGN-mFc or pSecTag-yydd-vGN-mFc (10 dishes (100 mm×20 mm) of cells for per construct). Cells were harvested at 48 h post-transfection and lysed in Lysis Buffer-Tween (50 mmol L^{-1} Tris-Hcl, pH 7.4, 150 mmol L^{-1} NaCl, 1% NP-40, 5 mmol L^{-1} EDTA, 0.05% Tween-20) containing protease inhibitor cocktail (Roche, Shanghai, China). Both the cell lysates and the media supernatant were incubated with 5% Ni-NTA Magnetic Agarose Bead suspension (QIAGEN, Shanghai, China) on ice for 1 h. The beads were washed and eluted according to the manufacturer's protocol. A total of 100 μL eluate of each sample was obtained, of which 10 μL was used for Coomassie brilliant blue (CBB) staining.

For the secreted expression of wt-vGN-mFc or yydd-vGN-mFc, 2 mL of media from CDH-wt-vGN-mFc or CDH-yydd-vGN-mFc transfected 293T cells seeded in 6-well-plates was used in affinity chromatography, while 20 μL eluate of each sample was obtained and used for immunoblotting.

1.3 Immunoblotting

Immunoblot assays were performed as previously described [21]. Immunoblot detection of the wt-vGN-mFc and yydd-vGN-mFc was performed either with anti-Penta.His antibody (1∶2500; Sigma) or with secondary antibody (goat anti-mouse IgG peroxidase conjugate) alone.

1.4 Labeling of wt-vGN and yydd-vGN

293T cells were transfected with pSecTag2A-wt-vGN-mFc or pSecTag2A-yydd-vGN-mFc. Cells were passeged at 10 h post-transfection. Approximately 36 h after transfection, the cells were radiolabeled with [^{35}S]-methionine/cysteine or

[^{35}S]-sulfate for 10 h. Labeled cells were lysed and used in affinity chromatography. The eluate was used for autoradiography as previously described [22].

1.5 Tumor formation assays in nude mice

All animal experiments were carried out according to the institutional ethical guidelines for animal experiments at the Guangzhou Institute of Medicine and Health, Chinese Academy of Sciences. NIH3T3 cells stably expressing the empty vector (CDH-CMV-EF-Puro) and NIH3T3 cells stably expressing vGPCR, hGRO-α, wt-vGN-mFc or yydd-vGN-mFc were injected subcutaneously into the flanks of 3- to 5-week-old mice (athymic, nude/nude). Nude mice were sacrificed four weeks after inoculation, and the tumor weights were determined as described before [22].

Total RNA was isolated from the tumors and cDNA was prepared using the kit according to the manufacturer's instructions (Invitrogen, Shanghai, China). The expression of hGRO-α, vGPCR, wt-vGN-mFc and yydd-vGN-mFc mRNA in the tumors was detected by RT-PCR, as previously reported [21,22].

2 Results

2.1 wt-vGN but not yydd-vGN is modified by sulfation

pSecTag2A-wt-vGN-mFc and pSecTag2A-yydd-vGN-mFc contained the vGN-mFc chimera, in which the Igκ leader sequence was upstream of the DNA fragment (vGN) encoding 1–49 amino acids of vGPCR or yydd-vGPCR, and the mFc fragment with 6×His.tag tail was fused to the 3′ end of vGN (Figure 1). The vGN-mFc chimeras of CDH-wt-vGN-mFc or CDH-yydd-vGN-mFc were identical to their pSecTag2A counterparts apart from the restriction sites.

293T cells were transfected with pSecTag-wt-vGN-mFc or pSecTag-yydd-vGN-mFc. wt-vGN-mFc and yydd-vGN-mFc were purified and used for CBB staining or immunoblotting. One exclusive band of approximately 40–45 kD was identifid by CBB staining for each sample, matching the predicted size of wt-vGN-mFc or yydd-vGN-mFc (Figure 2). The wt-vGN-mFc band was of a weaker intensity than that of yydd-vGN-mFc, implying lower expression levels. Immunoblotting with anti-Penta His antibody verified that the purified proteins were wt-vGN-mFc and yydd-vGN-mFc. Immunoblotting without primary antibody demonstrated that both wt-vGN-mFc and yydd-vGN-mFc were secreted into the media. Additionally, the band for yydd-vGN-mFc was wider than that of wt-vGN-mFc, which correlated with the CCB staining result (Figure 2).

The results of [^{35}S]-methionine/cysteine labeling demonstrated that both the lanes for wt-vGN-mFc and yydd-vGN-mFc had bands of 40–45 kD, confirming the CBB staining results. In the [^{35}S]-sulfate labeling assay, a band of 40–45 kD was found exclusively in the wt-vGN-mFc lane, clearly demonstrating that only wt-vGN-mFc incorporated [^{35}S]-sulfate (Figure 2). All the data elucidated that both wt-vGN-mFc and yydd-vGN-mFc were secreted, and wt-vGN but not yydd-vGN possessed tyrosine sulfation

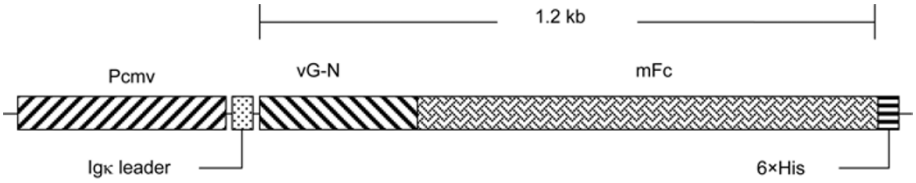

Figure 1 Diagram of wt-vGN-mFc and yydd-vGN-mFc. Pcmv, cmv promoter; Igκ leader, Igκ-chain leader sequence; vGN, wt-vGPCR or yydd-vGPCR extracellular N-terminus (1–49 amino acids); mFc, mouse Fc hinge; 6×His, 6×His tag.

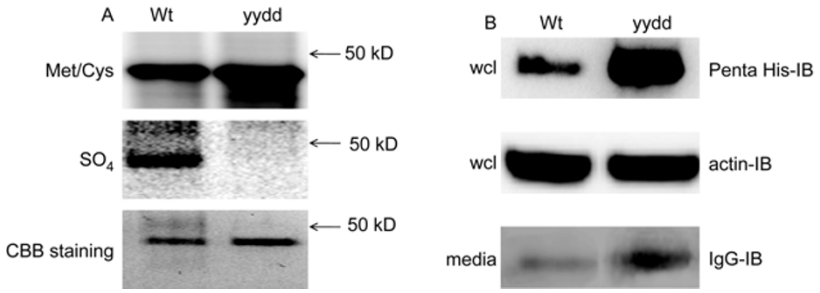

Figure 2 wt-vGN but not yydd-vGN efficiently incorporates radiolabeled sulfate. wt-vGN-mFc and yydd-vGN-mFc were purified from both cell lysates and media supernatant. The purified peptides were isolated by 10% SDS-PAGE and used for experiments as described in Materials and methods. A, [^{35}S]-methionine/cysteine (Met/Cys) labeling (upper); [^{35}S]-sulfate (SO_4) labeling (middle); coomassie brilliant blue staining (bottom). B, 5% input for Penta-his IB (upper); 5% input for actin immunoblotting (middle); eluate from media supernatant for immunoblotting, in which only the goat anti-mouse IgG peroxidase secondary conjugate was used (bottom). CBB staining: coomassie brilliant blue staining. Wt, wt-vGN-mFc; yydd, yydd-vGN-mFc; wcl, whole cell lysate; media, media supernatant.

modifications.

2.2 Unsulfated vGN reduces the tumorigenicity of hGRO-α in nude mice

In our previous study, the average tumor weight of the nude mice injected with NIH3T3/hGRO-α cells mixed with NIH3T3/yydd-vGPCR cells was only 38% of that of nude mice injected with NIH3T3/hGRO-α cells alone [21]. This suggested that yydd-vGPCR (precisely its unsulfated extracellular N-terminus) could affect the tumorigenicity of hGRO-α. To investigate if the unsulfated N-terminus of yydd-vGPCR reduces the tumorigenicity of hGRO-α, nude mice were subcutaneously injected with NIH3T3 cells stably expressing hGRO-α, wt-vGN-mFc, or yydd-vGN-mFc (Table 1). The average tumor weight of the hGRO-α group was 1571.625 mg, 1994.875 mg for the hGRO-α/wt-vGN group and 602.4 mg for the hGRO-α/yydd-vGN group (Figure 3A), demonstrating that the tumorigenicity of hGRO-α was greatly reduced by yydd-vGN but not wt-vGN. RT-PCR showed that hGRO-α, wt-vGN and yydd-vGN were well expressed in the nude mouse tumors (Figure 3B). The average tumor weight of hGRO-α/yydd-vGN group was only 38% of that of the hGRO-α group and the tumor weight of the hGRO-α/wt-vGN group was 125% of that of the hGRO-α group. These data correlated with our previous data, in which the average tumor weight of the hGRO-α/yydd-vGPCR group was only 38% of that of the hGRO-α group [21].

Table 1 NIH3T3 cells to test the effect of vGN on the tumorigenicity of hGRO-α *in vivo*[a)]

	Cell number ($\times 10^6$)			
Mouse group	Puro	hGRO-α	wt-vGN	yydd-vGN
Control	6	0	0	0
hGRO-α	5	1	0	0
hGRO-α/wt-vGN	3	1	2	0
hGRO-α/yydd-vGN	3	1	0	2

a) Puro, NIH3T3 stably expressing CDH-CMV-EF-Puro empty vector; hGRO-α, NIH3T3 expressing hGRO-α; wt-vGN, NIH3T3 expressing wt-vGN-mFc; yydd-vGN, NIH3T3 expressing wt-vGN-mFc.

2.3 Unsulfated vGN reduces the tumorigenicity of vGPCR in nude mice

Our previous study demonstrated that hGRO-α binds vGPCR in a sulfotyrosine-dependent manner, and suggested that hGRO-α promotes vGPCR tumorigenicity through autocrine signaling. To further test if the unsulfated N-terminus of yydd-vGPCR affects hGRO-α autocrine signaling in vGPCR tumorigenicity, nude mice were subcutaneously injected with NIH3T3 cells stably expressing vGPCR, wt-vGN-mFc, or yydd-vGN-mFc (Table 2). The average tumor weight of the vGPCR group was 659.5 mg, 685 mg for the vGPCR/wt-vGN group and 417.5 mg for the vGPCR/yydd-vGN group (Figure 4A). RT-PCR confirmed good expression of vGPCR, wt-vGN and yydd-vGN in the nude mouse tumors (Figure 4B). The average tumor weight of the vGPCR/yydd-vGN group was 60% of that of the vGPCR group and the average tumor weight of the vGPCR/wt-vGN group was almost identical (105%) to that of the vGPCR group. These data support the conclusion that unsulfated yydd-vGN inhibits vGPCR tumorigenicity in nude mice.

3 Discussion

vGPCR is a bona fide signaling molecule that is implicated in KSHV-associated malignancies. This seven membrane-spanning protein is constitutively activated; however, ligand association upregulates (GRO-α) or downregulates (IP-10) its signaling. Our previous study revealed that tyrosine sulfation on Y26 and Y28 of the N-terminus of vGPCR is crucial for GRO-α but not IP-10 association. An unsulfated YYDD mutant (yydd-vGPCR) demonstrated similar signaling as that of wild-type vGPCR, however, the tumorigenicity of yydd-vGPCR in nude mice completely disappeared. A mode of action was proposed based on our previous study, whereby hGRO-α promotes signaling and tumorigenicity in a sulfotyrosine-dependent manner [21]. Interestingly, in nude mice experiments, when NIH3T3 cells expressing yydd-vGPCR were mixed with hGRO-α expressing NIH3T3 cells, the tumorigenicity of hGRO-α was reduced

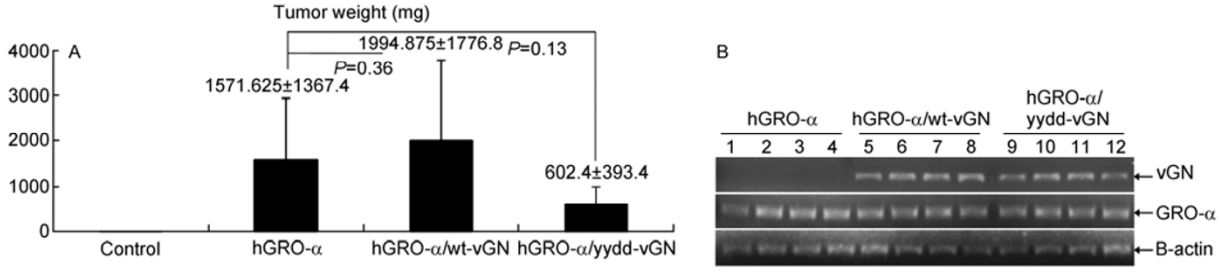

Figure 3 Unsulfated yydd-vGN affects the tumor formation triggered by hGRO-α *in vivo*. A, NIH3T3 cells stably expressing hGRO-α, wt-vGN-mFc or yydd-vGN-mFc were inoculated into the flanks of 3- to 5-week-old nude mice. Nude mice were sacrificed four weeks after inoculation and the tumor weight was determined. Data represent mean values and error bars denote standard deviations (average tumor weight±SD), with *P*-values relative to hGRO-α group as calculated by Student's *t*-tests. B, The expression of hGRO-α, wt-vGN-mFc and yydd-vGN-mFc in the tumors was detected by RT-PCR.

Table 2 NIH3T3 cells to test the effect of vGN on the tumorigenicity of vGPCR *in vivo*[a)]

	Cell number ($\times 10^6$)			
Mouse group	Puro	vGPCR	wt-vGN	yydd-vGN
Control	2.5	0	0	0
vGPCR	2.4	0.1	0	0
wt-vGN/vGPCR	1.9	0.1	0.5	0
yydd-vGN/vGPCR	1.9	0.1	0	0.5

a) Puro, NIH3T3 stably expressing CDH-CMV-EF-Puro empty vector; vGPCR, NIH3T3 expressing vGPCR; wt-vGN, NIH3T3 expressing wt-vGN-mFc; yydd-vGN, NIH3T3 expressing wt-vGN-mFc.

by 60% compared with the hGRO-α only group. Thus, we hypothesized that these important residues of the extracellular N-terminus of vGPCR might facilitate hGRO-α to associate with its other cell surface-expressing receptors (named GR here) and regulate their signaling. hGRO-α could not associate with yydd-vGPCR and the unsulfated N-terminus of this molecule might block hGRO-α association with GR through some unknown mechanism. This could explain why the average tumor weight of nude mice injected with NIH3T3 cells expressing yydd-vGPCR mixed with NIH3T3 cells expressing hGRO-α was much reduced compared with those injected with NIH3T3 hGRO-α cells alone.

yydd-vGN-mFc and wt-vGN-mFc were expressed and purified in this study to investigate if the unsulfated extracellular N-terminus of vGPCR negatively regulates the tumorigenicity of hGRO-α. mFc was introduced to facilitate the expression and characterization of the secreted peptides. The [^{35}S]-sulfate labeling assay verified that wt-vGN-mFc but not yydd-vGN-mFc has the tyrosine sulfation modification, which implies these two peptides are suitable for *in vivo* studies mimicking the N-terminus of wt-vGPCR and yydd-vGPCR accordingly.

Injection of nude mice with NIH3T3 cells expressing hGRO-α mixed with NIH3T3/yydd-vGN-mFc cells resulted in far smaller tumors than injection with NIH3T3/hGRO-α cells. Furthermore, the average tumor weight of the hGRO-α/wt-vGN group was higher (125%) than that of the hGRO-α only group. This correlated with our previous data. This may be because wt-vGN-mFc enhances the association of hGRO-α with GR. However, it cannot function as a receptor and promote tumor formation through an autocrine pathway as vGPCR does, which may explain why the average tumor weight of the hGRO-α/vGPCR group is almost two fold that of the hGRO-α group plus the vGPCR group in our previous study.

hGRO-α promotes vGPCR tumorigenicity through autocrine signaling and yydd-vGN could attenuate the tumorigenicity of hGRO-α, implying that the tumorigenicity of vGPCR expressing NIH3T3 cells could be affected by blocking hGRO-α association with its cellular receptor. Nude mice data verified this possibility, in which the average tumor weight of vGPCR/yydd-vGN group was 60% of that of the vGPCR group, and the average tumor weight of the vGPCR/wt-vGN group was almost identical (104%) to that of vGPCR group (Figure 4).

Based on the data generated in this study and our previous study, we present a model in which the tumorigenicity of hGRO-α is diminished by the unsulfated extracellular N-terminal region of vGPCR (Figure 5). In this model, the extracellular N-terminus of vGPCR associates with hGRO-α to promote hGRO-α association with its cellular receptor. Its unsulfated counterpart, the N-terminus of yydd-vGPCR, cannot bind hGRO-α and can block hGRO-α association with its cellular receptor. We hypothesize that vGPCR has two docking sites in its intracellular N-terminus, which can associate with hGRO-α and the cell surface hGRO-α receptor (GR) separately. The sulfation modification at the N-terminus of vGPCR works like a "swivel clamp" in the scaffold to "fasten" hGRO-α to GR, enabling binding between hGRO-α and GR. In contrast, the N-terminus of yydd-vGPCR could not bind hGRO-α because of the loss of the sulfotyrosines. However, it could still occupy the docking site in GR and inhibit hGRO-α association with GR, explaining why yydd-vGPCR could reduce the tumorigenicity of hGRO-α *in vivo*. Secreted yydd-vGN, which has the same sequence and a similar structure to the unsulfated N-terminus of yydd-vGPCR, could reduce the tumorigenicity

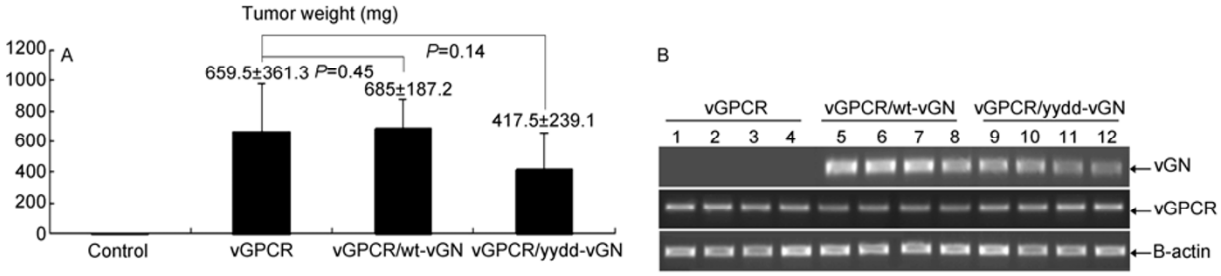

Figure 4 Unsulfated yydd-vGN affectes the tumor formation triggered by vGPCR *in vivo*. A, NIH 3T3 cells stably expressing vGPCR, wt-vGN-mFc or yydd-vGN-mFc were inoculated into the flanks of 3- to 5-week-old nude mice. Nude mice were sacrificed four weeks after inoculation, and the tumor weight was determined. Data represent mean values and error bars denote standard deviations (average tumor weight±SD), with *P*-values relative to vGPCR group as calculated by Student's *t*-tests. B, The expression of vGPCR,wt-vGN-mFc and yydd-vGN-mFc in the tumors was detected by RT-PCR.

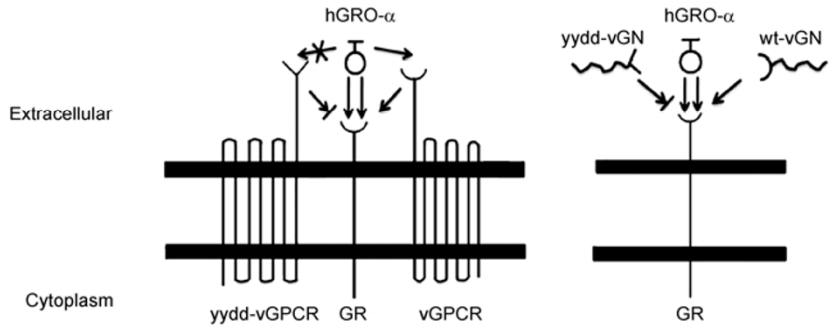

Figure 5 A hypothetical model of the extracellular N-terminus of vGPCR function in hGRO-α signaling. vGPCR acts as both a receptor and a ligand on the cellular surface. Left panel, hGRO-α binds to vGPCR in a sulfotyrosine-dependent manner, and the sulfate modified extracellular N-termini of vGPCR enables hGRO-α association with its cellular receptor (GR). The sulfation-free vGPCR mutant (yydd-vGPCR) cannot associate with hGRO-α, and its unsulfated extracellular N-terminus blocks hGRO-α association with GR. Right panel, wt-vGN and yydd-vGN have the same amino acid sequence and similar structure to the extracellular N-terminus of vGPCR or yydd-vGPCR accordingly. Sulfated wt-vGN promotes hGRO-α association with GR. However, unsulfated yydd-vGN blocks hGRO-α association with GR and negatively regulates downstream signaling.

of hGRO-α and reduce the tumorigenicity of vGPCR in nude mice by blocking the hGRO-α association with its cellular receptor (GR). This provides a good opportunity for the development of therapeutic agents targeting hGRO-α that could be applied to the treatment of cancers caused by KSHV vGPCR or abnormal hGRO-α expression.

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