Chapter 4

The viral G protein-coupled receptor ORF74 unmasks phospholipase C signaling of the receptor tyrosine kinase IGF-1R

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ABSTRACT

Kaposi’s sarcoma-associated herpesvirus (KSHV) encodes the constitutively active G protein-coupled receptor (GPCR) ORF74, which is expressed on the surface of infected host cells and is responsible for the development of the angioproliferative tumor Kaposi’s sarcoma (KS). Furthermore, the insulin-like growth factor (IGF)-1 receptor, a receptor tyrosine kinase (RTK), also plays an essential role in KS growth and survival.

In this study we examined the crosstalk between the structurally and functionally distinct GPCR ORF74 and RTK IGF-1R. Constitutive and CXCL1-induced ORF74 signaling did not transactivate IGF-1R. In contrast, IGF-1 stimulated phospholipase C (PLC) activation in an ORF74-dependent manner without binding to ORF74. Neutralizing IGF-1R with an antibody or silencing IGF-1R expression using siRNA inhibited PLC activation by IGF-1. Transactivation of ORF74 in response to IGF-1 occurred independently of ORF74 ligands, as shown by an N-terminal truncation mutant of ORF74 that is unable to bind chemokines, but is dependent on the constitutive activity of ORF74. Tyrosine residues in the intracellular carboxyl-terminal or intracellular loop 2 of ORF74 are not essential for PLC activation in response to IGF-1. IGF-1-induced PLC activation is specific for ORF74 as IGF-1 was unable to do the same in cells expressing US28, a constitutively active viral GPCR encoded by the human cytomegalovirus (HCMV). Interestingly, IGF does not induce β-arrestin recruitment to ORF74. The proximity ligation assay revealed the close proximity between ORF74 and IGF-1R on the cell surface. Unmasking IGF-1R signaling to PLC in response to IGF-1 is a previously unrecognized action of ORF74.

INTRODUCTION

The insulin-like growth factor 1 (IGF-1) receptor is ubiquitously expressed and is involved in various physiological functions, such as glucose homeostasis and cellular growth. However, IGF-1R and its cognate ligands IGF-1 and IGF-2 have also been recognized for their role in several types of cancer [400], including Kaposi’s sarcoma (KS) [327]. KS is a multifocal vascular neoplasm characterized by proliferating spindle-shaped endothelial cells, infiltrating leukocytes, angiogenesis and lymphangiogenesis. IGF-1R is found in biopsies from KS patients [401]. IGF-1 stimulates KS cell proliferation whereas blocking IGF-1R with a neutralizing antibody or specific small molecule inhibitor reversed this effect and induced apoptosis [327]. Details on the signaling pathways involved in these responses remain to be elucidated.

Kaposi’s sarcoma-associated herpesvirus (KSHV) is the causative agent of KS [53]. In particular the KSHV-encoded G protein-coupled receptor (GPCR) ORF74 plays a key role in KS development, and has consequently been the focus of numerous studies with regard to its signaling and ligand binding properties. ORF74 shows highest sequence identity to human chemokine receptor CXCR2 [75], but acquired additional properties such as promiscuous G protein coupling and constitutive activity. Moreover, ORF74 binds chemokines from the CXC family, including CXCL1, CXCL8 and CXCL10. These chemokines display opposite efficacies: CXCL1 acts as a full agonist, CXCL8 is a low potency (partial) agonist whereas CXCL10 is an
inverse agonist [145]. ORF74 activates several cellular signaling pathways including phospholipase C (PLC), resulting in cell proliferation [147] and transformation [182]. Moreover, ORF74 signaling increases the production and secretion of proinflammatory, angiogenic factors and adhesion molecules [83] that are able to transform adjacent cells through paracrine mechanisms. Transgenic mice expressing ORF74 developed tumors resembling KS lesions in human [77, 78]. In contrast, transgenic mice carrying ORF74 mutants that are deficient in either constitutive activity or chemokine binding fail to develop tumors, indicating that both constitutive and ligand-induced signaling properties of ORF74 are involved in tumorigenesis [155].

Although GPCRs and receptor tyrosine kinases (RTKs) belong to different receptor classes, they can transactivate each other in a bi-directional manner. Several GPCRs have been shown to transactivate IGF-1R, including the neurotensin receptor 1 (NTR1) [402], Angiotensin II type 1 receptor (AT1R) [403], receptors for endothelin-1 (ET-1), bombesin [404] and thrombin [405, 406] and the GABA_A receptor [316]. In contrast, examples of IGF-1R that transactivates GPCRs are less abundant and comprise the chemokine receptors CCR5 [317] and CXCR4 [321] and the pituitary adenyl cyclase activating polypeptide 1 receptor (PAC1R) [322].

In the current study, we investigated the potential crosstalk between the viral GPCR ORF74 and IGF-1R. We show that ORF74 does not transactivate the IGF-1R. However, constitutive ORF74 activity unmasks IGF-1 and IGF-2 signaling to PLC via IGF-1R. This is the first time that crosstalk of ORF74 with a RTK is reported and contributes to the understanding of the signaling-mechanisms utilized by ORF74.

MATERIALS AND METHODS

MATERIALS
Dulbecco’s modified Eagle’s medium and trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin/streptomycin and Hank’s balanced salt solution (HBSS) were obtained from PAA Laboratories GmbH (Paschen, Austria). Earle’s inositol-free minimal essential medium was from Life Technologies (Carlabad, CA, USA). Na^{125}I, 125I-CXCL10 (2200 Ci/mmol) and myo-[2-3H]inositol (1 mCi/ml) were obtained from Perkin Elmer Life Sciences (Boston, MA, USA). Recombinant human chemokines were obtained from PeproTech (Rocky Hill, NJ, USA) and human recombinant IGF-1 and IGF-2 were from Sigma-Aldrich. The polyclonal antibody recognizing ORF74 was a kind gift of Dr. Hayward (Johns Hopkins University, Baltimore, MD) [337]. Phospho-IGF-1 Receptor β (Tyr^{1135/1136}) antibody (19H7) and total IGF-1 Receptor β (111A9) antibodies were from Cell Signaling Technology (Boston, MA, USA). Anti-β-Actin antibody was purchased from Sigma-Aldrich. Anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated antibodies were obtained from Bio-Rad Laboratories (Veenendaal, the Netherlands). Neutralizing antibodies against IGF-1R (MAB391) and CXCR3 (MAB160) were purchased from R&D systems Inc (Minneapolis, MN, USA). Coelenterazine-h was obtained from Promega (Madison, WI, USA). Linear polyethylenimine (PEI) for transfection was purchased from Polysciences (Warrington, PA,
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USA). For siRNA transfection experiments, Dharmacon On-target plus non-targeting control siRNA (#D-001810-10) and On-target plus human IGF-1R siRNA SmartPool (#L-003012) were purchased from Thermo Scientific (Epsom, UK). Lipofectamine 2000 was purchased from Invitrogen (Paisley, UK). Poly-L-lysine was obtained from Sigma-Aldrich. Duolink PLA probes anti-mouse-PLUS, anti-rabbit-PLUS, anti-rabbit-MINUS, anti-rat-MINUS and Duolink in situ Detection Reagent 563 (red) were purchased from Olink (Uppsala, Sweden).

CELL CULTURE AND TRANSFECTION

HEK293T cells were maintained at 37°C and 5% CO₂ in DMEM supplemented with 10% FBS, 50 IU/ml penicillin and 50 µg/ml streptomycin. HEK293T cells were transfected in a 10 cm culturing dish using linear PEI. Briefly, a total amount of 5 µg DNA (adjusted with empty pcDEF₃) was diluted in a total volume of 250 µl NaCl solution (150 mM). Next, 250 µl NaCl solution containing 30 µg PEI was added to the DNA solution, mixed and incubated for 20 min at 22°C. The mixture was added dropwise to the medium of adherent HEK293T cells. The next day, cells were trypsinized, resuspended and seeded in poly-L-lysine coated assay plates. In IGF-1R knock-down experiments, HEK293T cells (plated in 6-wells plate) were transfected with a total amount of 4 µg DNA (adjusted with empty pcDEF₃) and 100 nM siRNA against IGF-1R using Lipofectamine 2000 according to the manufacturer’s protocol.

DNA CONSTRUCTS

The cDNA of WT-ORF74 (GenBank accession number U71368 with a silent G to T mutation at position 927) was a gift from Dr. Schwartz (University of Copenhagen, Denmark). ORF74 tagged at the N-terminus with the influenza virus hemagglutinin (HA) epitope was described before [235]. The cDNA encoding for US28 (from the VHL/E HCMV strain, GenBank accession number L20501) inserted in pcDNA3 was a gift from Dr. Doms (University of Pennsylvania, Philadelphia, USA). Enhanced yellow fluorescence protein (eYFP) fused in frame to the C-terminus of β-arrestin1 and β-arrestin2 were previously described [31]. An improved variant of Renilla luciferase (Rluc8) was a kind gift from Dr. J. A. Javitch (Columbia University, New York) [339]. Rluc8 was fused to the C-terminus of ORF74 via a SpeI/NotI linker, as previously described [306]. ORF74-Y(326)A was a gift from Dr. Gavard (Institut Cochin CNRS INSERM, Paris, France). The ORF74-Y(149)A, Y(149/326)A and R(3.50(143)A mutants (Ballesteros-Weinstein numbering [340] and UniProt residue numbering between brackets) were constructed by PCR-based site-directed mutagenesis using sense primer 5’-cctggtggcagcctctacgcg-3’ and anti-sense primer 5’-cgctagaggtcttcgctcagcggc-3’ (Y(149)A) or sense primer 5’-cagtctagtgagcgtacctcctg-3 and anti-sense primer 5’-caggaggtacgcaagtctgactg-3’ (R(3.50(143)A). The Δ(1-22)-ORF74 truncation mutant was described before [153]. All constructs were subcloned into pcDEF₃ (a gift from Dr. Langer, Robert Wood Johnson Medical School, Piscataway, NJ) and verified by sequencing.

SDS-PAGE AND WESTERN BLOT

48 h post-transfection, cells were stimulated with 100 nM CXCL1 and/or 10 nM IGF-1 for indicated time after overnight serum starvation. When neutralizing antibodies were used, cells were pre-incubated for 90 min with 15 ng/ml MAB391 or MAB160 prior to the stimulation with 10 nM IGF-1 for 20 min. Cell lysates were prepared in RIPA-buffer supplemented with α-complete protease inhibitor cocktail (La Roche), 1 mM PMSF, 1mM NaVO₃ and 1 mM NaF, sonificated and protein concentrations were determined using BCA total protein
determination kit (Thermo Fisher Scientific). Equal amounts of protein were resolved by SDS-PAGE analysis using 10% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad) and subsequently blocked for 1 h at 22°C in 5% non-fat milk in 0.1% Tween-20/TBS solution. Then, membranes were incubated overnight at 4°C with indicated primary antibody diluted in 5% BSA in 0.1% Tween-20/TBS solution. The next day, membranes were incubated with HRP-conjugated secondary antibody (diluted in blocking buffer) for 1 h at 22°C. Immunoblots were developed using enhanced chemiluminescence solution (Thermo Fisher Scientific).

**Phospholipase C activation assay**

24 h post-transfection, HEK293T cells transiently transfected with ORF74 (or mutant) were seeded in poly-L-lysine coated 48-wells plates (10⁵ cells/well) (Greiner) and labeled overnight with myo-[2-³H]-inositol (1 μCi/ml) in Earle’s inositol-free minimal essential medium (Gibco) supplemented with 10% FBS, 50 IU/ml penicillin and 50 µg/ml streptomycin. The next day, cells were incubated at 37°C and 5% CO₂ for 90 min in assay buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose, pH 7.4) supplemented with 0.1% BSA and 10 mM LiCl in the absence or presence of indicated concentrations chemokine, IGF-1 or IGF-2. In experiments where neutralizing antibodies were used, the assay buffer was supplemented with 15 µg/ml MAB391 (α-IGF-1R) or MAB160 (α-CXCR3). After incubation, cells were placed on ice and stimulation buffer was aspirated prior to the addition of ice-cold 10 mM formic acid. After incubating the formic acid for 90 min on ice, generated [³H]-inositol phosphates (InsP) were isolated by anion-exchange chromatography (Dowex AG1-X8 columns; Bio-Rad) and counted by liquid scintillation in a Packard liquid scintillation analyzer.

**ß-arrestin recruitment**

HEK293T cells were transiently transfected with a 1:4 ratio of cDNA coding for ORF74-Rluc8 and ß-arrestin1-eYFP or ß-arrestin2-eYFP. 24 h post-transfection, cells were seeded in poly-L-lysine-coated white 96-well culture plates (Greiner) (5·10⁴ cells/well). The next day, cells were washed with HBSS and incubated with fresh HBSS supplemented with 0.1% BSA. Fluorescence was measured on a Victor® multilabel plate reader (PerkinElmer) to monitor expression of eYFP-tagged proteins (excitation 485 nm; emission 535 nm). Cells were incubated with 5 µM coelenterazine-h substrate for 5 min at 37°C. Next, 100 nM CXCL1 or IGF-1 was added and cells were incubated for an additional 10 min at 37°C. For longer stimulation times (1.5 h and 6 h), 100 nM CXCL1 or IGF-1 was added in the growth medium (DMEM + 10% FBS + 1% P/S) and incubated for 1.5 h or 6 h at 37°C and 5% CO₂. Next, stimulation medium was replaced by HBSS supplemented with 0.1% BSA and fluorescence was measured. 5 µM coelenterazine-h substrate was added to the cells and incubated for 15 min at 37°C. When co-stimulated, cells were incubated with 100 nM IGF-1 for 15 min at 37°C, prior to the addition of coelenterazine-h and CXCL1 for 15 min at 37°C. The BRET (emission 535 nm) and Rluc8 signals (emission 460 nm) were measured on a Victor® multilabel plate reader (PerkinElmer). BRET ratios (535/460 emission) were calculated.

**Radioligand binding experiments**

Human CXCL8 was labeled with ¹²⁵I as previously described [407]. Briefly, 5 µg CXCL8 was incubated with 0.5 mCi ¹²⁵I-Na in 35 µl labeling buffer (125 mM Tris-HCl pH 6.8, 150 mM NaCl)
in Iodo-Gen coated tubes (Pierce Chemical Co., Rockford, IL, USA) for 12 min at 22°C. Iodinated CXCL8 was separated from free iodine using a PD-10 column (GE Healthcare). Incorporation of $^{125}$I and specific activity were subsequently determined using trichloroacetic acid protein precipitation [408]. 24 h post-transfection, HEK293T cells transiently transfected with WT-ORF74 were seeded in poly-L-lysine-coated 48-well plates ($10^5$ cells/well). The next day, binding was performed on whole cells by incubating the cells for 3 h at 4°C with 100 pM $^{125}$I-CXCL8 or $^{125}$I-CXCL10 in binding buffer (50 mM HEPES (pH 7.4), 1 mM CaCl$_2$, 5 mM MgCl$_2$, 0.5% BSA) in the absence or presence of 100 nM unlabeled CXCL1 or IGF-1. After incubation, cells were washed three times with ice-cold binding buffer supplemented with 0.5 M NaCl and subsequently lysed and counted in a Wallac Compugamma counter.

**Cell Surface Receptor Expression ELISA**
HEK293T cells transiently transfected with ORF74 (or mutant) were seeded in a poly-L-lysine-coated 96-well plate ($5 \cdot 10^4$ cells/well) and grown overnight. 48 h post-transfection, cells were fixed for 5 min with 4% formaldehyde in Tris-buffered saline (TBS: 150 mM NaCl, 10 mM Tris-HCl, pH 7.5). Next, cells were incubated with blocking buffer (1% fat-free milk diluted in 0.1 M NaHCO$_3$ (pH 8.6)) for 4 h at 22°C, prior to the overnight incubation at 4°C with rabbit anti-ORF74 antibody diluted in 0.1% BSA/TBS. The next day, cells were washed three times with TBS and subsequently incubated with goat anti-rabbit HRP-conjugated secondary antibody (Bio-Rad) diluted in blocking buffer for 2 h at 22°C. After washing the cells three times with TBS, OPD substrate solution (2 mM o-phenylenediamine (Sigma-Aldrich), 35 mM citric acid, 66 mM Na$_2$HPO$_4$, 0.015% H$_2$O$_2$, pH 5.6) was added to the cells. The enzymatic reaction was terminated by adding 1 M H$_2$SO$_4$ and absorbance was subsequently measured at 490 nm in a PowerWave plate reader (BioTek).

**In situ Proximity Ligation Assay (PLA)**
HEK293T cells were transiently transfected with cDNA coding for WT-ORF74 or ORF74-mVenus and seeded in a 24-well plate ($10^5$ cells/well) containing poly-L-Lysine-coated coverslips. 48 h post-transfection, cells were fixed for 10 min in 4% formaldehyde in PBS and subsequently incubated in blocking solution (5% non-fat milk in PBS) for 30 min at 22°C. Next, cells were incubated with rabbit anti-ORF74 and mouse anti-IGF-1R (MAB391) primary antibodies (diluted in blocking solution) for 1 h at 37°C. After washing with PBS, cells were incubated with anti-mouse-PLUS and anti-rabbit-MINUS secondary antibodies (the PLA probes) and PLA was performed according to the manufacturer’s instructions.

**Data Analysis**
Sigmoidal concentration-response curves or bar graphs were plotted using Graphpad Prism 6 software (GraphPad Software, San Diego). pEC$_{50}$ values were determined by nonlinear regression. Statistical analyses were performed using Graphpad Prism 6 software.

**Results**
ORF74 is unable to induce tyrosine phosphorylation of IGF-1R
Stimulation of HEK293T cells with 10 nM IGF-1 activated endogenous IGF-1R, as revealed by
autophosphorylation of tyrosine residues 1135 and 1136 (Fig. 1A). To determine whether ORF74 transactivates IGF-1R, HEK293T cells were transiently transfected with ORF74. Both constitutive activity of ORF74 and stimulation with 100 nM CXCL1 did not result in phosphorylation of IGF-1R (Fig. 1B), while the IGF-1 induced IGF-1R phosphorylation was unaffected by ORF74 as compared to mock-transfected cells. Similar results were obtained by using 3T3 cells stably expressing ORF74 (data not shown) [182]. These results indicate that ORF74 does not transactivate IGF-1R in the absence or presence of CXCL1.

ORF74 unmasks PLC activation in response to IGF-1

Stimulation of HEK293T cells with 100 nM IGF-1 did not increase inositol phosphate (InsP) production in HEK293T cells, indicating that PLC is not directly activated by IGF-1R (Fig. 2A). ORF74 constitutively increased PLC activity by 5-fold as compared to mock-transfected cells (Fig. 2A), which is in line with previous findings [145, 147, 148]. CXCL1 had no effect on PLC activity in mock-transfected cells, but increased constitutive signaling of ORF74 by 2-fold in a dose-dependent manner (pEC50 = 8.7 ± 0.1) (Fig. 2B), as previously reported [145]. Interestingly, stimulation of ORF74-expressing HEK293T cells with IGF-1 also increased PLC activity in a dose-dependent manner with a potency (pEC50 = 8.8 ± 0.2) and Emax (2.1-fold over basal) comparable to CXCL1 (Fig. 2B). IGF-2, another ligand of IGF-1R, is also able to increase PLC activation in ORF74 expressing cells (Fig. 2B). Co-stimulation of increasing concentrations of CXCL1 in combination with 10 nM IGF-1 resulted in an additive PLC activation (Fig. 2C) without affecting the potency of CXCL1 (pEC50 = 8.5 ± 0.2). The inverse agonist CXCL10 inhibited both constitutive and IGF-1-induced PLC activation in ORF74-expressing cells in a dose-dependent manner (Fig. 2D), indicating that constitutive ORF74 signaling is required for PLC responsiveness to IGF-1. The potency of CXCL10 to inhibit ORF74 signaling was not affected by co-stimulating with IGF-1 (pEC50 CXCL10 = 8.1 ± 0.1; pEC50 CXCL10 and IGF-1 = 8.1 ± 0.1), suggesting that there is no competition between ORF74 chemokines and IGF-1 or allosteric cooperativity.

The mutant ORF74-R3.50A is expressed at the cell surface at levels comparable to WT-ORF74 (Fig. 2E), but is unable to constitutively activate PLC (Fig. 2F), as previously reported [196]. Moreover, PLC activity is not stimulated by IGF-1 in cells expressing this ORF74-R3.50A mutant, confirming that ORF74-mediated G protein activation is essential for IGF-1-induced PLC activation.

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**Figure 1. ORF74 does not transactivate IGF-1R in HEK293T cells.** HEK293T cells were transiently transfected with empty vector (mock) (A) or HA-ORF74 (B) and stimulated with 100 nM CXCL1 or 10 nM IGF-1 for indicated time. IGF-1R activation was determined by immunoblotting with an antibody recognizing the phosphorylated IGF-1R on Tyr1135/1136. Total IGF-1R levels were determined as loading controls. Representative blots of three experiments are shown.
Upon activation, GPCRs are phosphorylated and consequently recruit β-arrestins to prevent further G protein-mediated signaling and induce internalization [409, 410]. IGF-1 increases PLC activity in ORF74-Rluc8-expressing cells to the same extent as compared to WT-ORF74-expressing cells (Fig. 3A). To test whether IGF-1 also induced β-arrestin recruitment to ORF74, cells were transfected with ORF74-Rluc8 in combination with β-arrestin1- or β-arrestin2-eYFP and stimulated with CXCL1 or IGF-1 for various time periods. CXCL1 (100 nM) increased bioluminescence resonance energy transfer (BRET) between Rluc8 and eYFP as a result of β-arrestin1 (Fig. 3B) or β-arrestin2 (Fig. 3C) recruitment to ORF74. In contrast, IGF-1 (100 nM) was unable to recruit β-arrestin1 or β-arrestin2 to ORF74 for all tested time-points. When co-stimulated, IGF-1 increased CXCL1-induced β-arrestin1 recruitment by 1.2-fold (Fig. 3D), whereas it had no effect on CXCL1-induced β-arrestin2 recruitment to ORF74 (Fig. 3E).

**IGF-1 activates PLC via IGF-1R**

To investigate whether IGF-1 binds to ORF74, whole cell binding experiments were performed. Both $^{125}$I-CXCL8 and $^{125}$I-CXCL10 were used as they bind to different populations of ORF74 [367, 411]. As expected, CXCL1 was able to displace both $^{125}$I-CXCL8 (Fig. 4A) and $^{125}$I-
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CXCL10 (Fig. 4B) from ORF74-expressing cells. However, 100 nM IGF-1 did not affect binding of 125I-CXCL8 nor 125I-CXCL10 to ORF74, indicating that IGF-1-induced PLC activation did not result from IGF-1 binding to the chemokine-binding site of ORF74.

To confirm that IGF-1-induced PLC activation is mediated via IGF-1R, cells were pre-treated with the antibody MAB391 that specifically recognizes the extracellular α-subunit of IGF-1R and consequently inhibits the binding of IGF-1 to IGF-1R [411, 412]. Indeed, MAB391 reduced IGF-1-induced IGF-1R phosphorylation, while control antibody MAB160 (recognizing chemokine receptor CXCR3) was ineffective (Fig. 5A). MAB391 fully blocked IGF-1-induced PLC activation, but had no significant effect on CXCL1-induced signaling (Fig. 5B).

Moreover, downregulation of endogenous IGF-1R expression levels by siRNA (Fig. 5C) completely inhibited IGF-1-induced PLC activation in ORF74-expressing cells (Fig. 5D), whereas
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Non-targeting siRNA had no effect. IGF-1R siRNA treatment did not affect ORF74-induced constitutive PLC activation (data not shown) or PLC activation in response to CXCL1 (Fig. 5D). These data demonstrated that IGF-1R is required for IGF-1 induced PLC activation.

IGF-1 ACTIVATES PLC IN A CHEMOKINE-INDEPENDENT MANNER

To investigate the possible involvement of de novo released ORF74-stimulating chemokines (e.g. CXCL1) in response to IGF-1 stimulation, PLC activation mediated by the N-terminal truncation mutant ∆(1-22)-ORF74 was determined. Deletion of the first 22 amino acids of ORF74 did not affect basal signaling levels compared to WT-ORF74, but disabled CXCL1-induced signaling (Fig. 6) as previously reported [153]. In contrast, IGF-1 was still able to activate PLC in ∆(1-22)-ORF74-expressing cells (Fig. 6), indicating that IGF-1-induced PLC activation is independent of endogenous ORF74 agonists.

IGF-1 ACTIVATES PLC INDEPENDENTLY OF ORF74 TYROSINE PHOSPHORYLATION

To investigate the possibility that PLC activation in response to IGF-1 is regulated by tyrosine phosphorylation of ORF74, tyrosine residues in ICL2 (Y149) and the C-tail (Y326) of ORF74 were Ala-substituted. ORF74-Y(149)A and ORF74-Y(326)A are expressed at the cell surface at levels similar to WT-ORF74, whereas cell surface expression of the double mutant ORF74-Y(149/326)A was 1.2-fold higher as compared to WT-ORF74 (Fig. 7A). All three ORF74 mutants significantly activated PLC in a constitutively active manner as compared to mock-transfected cells (dotted line Fig 7B), but PLC activation by ORF74-Y(149)A, ORF74-Y(326)A and ORF74-Y(149/326)A was 2.5-, 1.6- and 3.2-fold lower, respectively, as compared to WT-ORF74 (Fig. 7B). This indicates that the tyrosine residues Y(149) and Y(326) play a role in constitutive ORF74 signaling. CXCL1 and IGF-1 significantly increased constitutive PLC activation of all mutants (Fig. 7B), showing

Figure 4. IGF-1 does not bind to ORF74. HEK293T cells transiently transfected with WT-ORF74 or empty vector DNA (mock) were incubated with 0.1 nM 125I-CXCL8 (A) or 125I-CXCL10 (B) in the absence or presence of 100 nM CXCL1 or IGF-1. Data are presented as fold over mock and represent mean ± SEM of pooled data from three independent experiments each performed in triplicate. Statistical differences between vehicle- and CXCL1- or IGF-1-treated ORF74-expressing cells were determined by one-way ANOVA and Bonferroni test. **** p ≤ 0.0001, 'NS' means not significant.
that tyrosine phosphorylation of Y(149) and Y(326) of ORF74 is not essential for IGF-1-induced PLC activation. However, IGF-1 (and CXCL1)-induced signaling did not reach the signaling levels as obtained for WT-ORF74. This indicates that a certain level of constitutive activity is required for IGF-1-induced PLC activation and is in line with the results obtained with the signaling-deficient mutant ORF74-R3.50(143)A (Fig. 2F).

**IGF-1-induced PLC activation is specific for ORF74**

To examine whether IGF-1-induced PLC activation is specific for ORF74, cells were transfected with US28 DNA. US28 is a GPCR encoded by the human cytomegalovirus (HCMV) and also activates PLC in a constitutively active manner [203, 204]. Constitutive PLC activation mediated by US28 is significantly higher compared to that of ORF74 (Fig. 8A). The human chemokine CCL5 increased the basal signaling of US28 to PLC, whereas IGF-1 had no effect (Fig. 8B).

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**Figure 5. IGF-1 activates PLC via IGF-1R.** (A) HEK293T cells were pre-incubated with MAB391 (anti-IGF-1R) or MAB160 (anti-CXCR3) prior to the stimulation with IGF-1. IGF-1R activation in response to vehicle or 10 nM IGF was determined by immunoblotting with an antibody recognizing the phosphorylated IGF-1R on Tyr1135/1136. Total IGF-1R expression levels were determined using an antibody recognizing IGF-1R independently of its phosphorylation state. (B) HEK293T cells were transiently transfected with WT-ORF74 and vehicle-stimulated (white bars) or stimulated with 10 nM CXCL1 (black bars) or 10 nM IGF-1 (striped bars) in combination with MAB391 or MAB160 and PLC activation was determined by measuring inositol phosphate accumulation. (C, D) HEK293T cells were transfected with WT-ORF74 or empty vector DNA (mock) in combination with non-targeting control siRNA or siRNA targeting IGF-1R. Total IGF-1R and β-actin levels were determined by immunoblotting. Data are presented as fold over vehicle-treated cells and are mean ± SEM of pooled data from three independent experiments each performed in triplicate or representative blots of three experiments are shown. Significant differences in PLC activation between vehicle and corresponding IGF-1- or CXCL1-treated cells (B, D) or between CXCL1-stimulated cells transfected with control siRNA or siRNA targeting IGF-1R (D) were determined by one-way ANOVA and Bonferroni test or a Student t-test, respectively. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, 'NS' means not significant.
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ORF74 and IGF-1R are in close proximity

The physical association of IGF-1R with CXCR4 [147], the PAC1R [322] or GABA_B [413] was suggested to underlie the crosstalk between IGF-1R and these GPCRs. The in situ proximity ligation assay (PLA) is an antibody-based method that allows visualization of the interaction between two proteins. Close proximity between WT-ORF74 and endogenous IGF-1R was detected on the cell surface of HEK293T cells (Fig. 9A). Similar results were obtained with ORF74 C-terminally fused to mVenus (Fig. 9B). Only cells expressing ORF74-mVenus showed the typically red fluorescent PLA clusters, demonstrating the specificity of the PLA signal. No close proximity between US28 and IGF-1R was detected on intact HEK293T cells (data not shown).
**DISCUSSION**

The viral GPCR ORF74 plays a key role in the initiation [77, 78, 383] and progression [84] of KS. The RTK IGF-1R and its cognate ligands IGF-1 and IGF-2 are essential for KS growth and survival [327], but the mechanism is unknown. Crosstalk between GPCR and RTK signaling is critical for multiple physiological functions, but is also involved in cancer [313, 414, 415]. Although most GPCRs that transactivate IGF-1R use mechanisms independent of the re-

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**Figure 8. IGF-1-induced PLC activation is specific for ORF74.** (A) PLC activation was determined by measuring inositol phosphate accumulation in HEK293T cells transiently transfected with WT-ORF74 (A) or WT-US28 (A, B) that were vehicle-treated (A, B) or stimulated with 100 nM IGF-1 or CCL5 (B). Data are presented as fold over mock-transfected cells (A) or as fold over basal US28 activity (B) and are mean ± SEM of three independent experiments each performed in triplicate. Significant differences between the constitutive activity of ORF74 and US28 (A) or between vehicle- and corresponding CCL5- or IGF-1-treated cells (B) were determined by one-way ANOVA and Bonferroni test, **p ≤ 0.01, ****p ≤ 0.0001, ‘NS’ means not significant.

**Figure 9. Close proximity between ORF74 and IGF-1R.** HEK293T were cells transiently transfected with WT-ORF74 (A) or ORF74-mVenus (B) and in situ proximity ligation assay was performed to identify the close proximity between ORF74 and endogenous IGF-1R, as visualized by red fluorescent dots. ORF74-mVenus expression is shown in green and the nuclei are in blue. Representative images of at least three independent experiments are shown.
lease of IGF-1 or IGF-2 [316, 402-404], the V2 vasopressin receptor (V2R) has recently been shown to induce metalloproteinase-mediated shedding of a factor that activates IGF-1R in an autocrine/paracrine manner in HEK293 cells [315]. ORF74 is expressed by only a minority of cells in KS lesions [337], but has been shown to activate neighboring cells through the release of paracrine growth factors [81, 83]. In fact, KSHV infection of human microvascular endothelial cells (HMVEC-d) induces the secretion of growth factors including IGF-1 [330]. However, in this study we were unable to detect increased phosphorylation of endogenous IGF-1R in HEK293T cells or 3T3 cells expressing the constitutive active ORF74 in response to vehicle or CXCL1. Yet, ORF74-induced IGF-1R transactivation cannot be ruled-out and might be cell type-dependent [314, 416].

Our studies did however show IGF-1R-mediated transactivation of ORF74. IGF-1 does not directly activate PLC in HEK293T cells endogenously expressing IGF-1R. However, co-expression of ORF74 renders HEK293T cells sensitive to PLC signaling in response to IGF-1. The lack of IGF-1 signaling to PLC in mock- and ORF74-R3.50(143)A-transfected cells in combination with the inhibitory effect of the inverse agonist CXCL10 on IGF-1-induced PLC activation shows the dependency of constitutive ORF74 activity.

IGF-1 has been shown to transactivate GPCRs by transcriptional upregulation or enzymatic activation of GPCR agonists. Stimulation of MCF-7 breast carcinoma cells with IGF-1 results in de novo synthesis and secretion of CCL5. Subsequent autocrine and/or paracrine activation of CCR5 by CCL5 leads to IGF-1-induced cell chemotaxis [317]. In addition, IGF-1 activates sphingosine kinase, which catalyzes the formation of sphingosine-1 phosphate (S1P), resulting in increased secretion of S1P and subsequent activation of the S1P1 receptor [320]. On the other hand, also mechanisms independent of the release of GPCR ligands have been described to contribute to the IGF-1-induced transactivation of GPCRs [321, 322]. For example, competitive PAC1R antagonists were unable to inhibit IGF-1-induced transactivation of the PAC1R [322]. Furthermore, IGF-1 transactivates CXCR4 without inducing the synthesis and secretion of the CXCR4 ligand CXCL12 [321]. To identify the mode of action involved in the IGF-1/ORF74 crosstalk, IGF-1-induced secretion of ORF74 agonists (e.g. CXCL1) was investigated. IGF-1 has been reported to enhance CXCL1 expression in mouse embryonic fibroblasts [417]. In addition, endothelial cells have been shown to release CXCL1 from small granules in response to histamine [418, 419]. However, although Δ(1-22)-ORF74 is unable to bind chemokines [153], this mutant is still responsive to IGF-1, showing that IGF-1-induced PLC in ORF74-expressing cells is not the result of the upregulation and/or release of chemokines. This is also in line with our observation that IGF-1, in contrast to CXCL1, fails to induce β-arrestin recruitment to ORF74.

The role of G protein-coupled receptor kinases (GRKs) and other serine/threonine kinases in GPCR phosphorylation and regulation has been known for some decades. However, the discovery that some GPCRs are tyrosine phosphorylated has slowly emerged and might contribute to the transactivation of GPCRs in response to RTK activation [335]. For example, PAC1R is tyrosine phosphorylated by Src in response to IGF-1 and blocking Src results in the complete inhibition of PAC1R-mediated cAMP production in response to IGF-1 [322]. However, it remains to be elucidated how tyrosine phosphorylation of PAC1R contributes to the production of cAMP. Ala-substitution of tyrosine residues in the C-tail and ICL2 of ORF74 did
not inhibit PLC activation in response to IGF-1, suggesting that ORF74 employs a different transactivation mechanism as compared to PAC1R.

Recently it has been shown that GRKs phosphorylate IGF-1R in response to IGF-1, resulting in β-arrestin recruitment and subsequent regulation of IGF-1R-dependent MAPK and Akt activation [420]. Hypothetically, GRK and subsequent β-arrestin recruitment to IGF-1R in response to IGF-1 stimulation might diminish their availability to desensitize ORF74, which could explain the observed increase in PLC activity. Indeed, GRKs are known to inhibit ORF74-induced signaling [182, 278], putatively by facilitating the recruitment of β-arrestins. However, β-arrestin1/2 recruitment to ORF74 in response to CXCL1 is unaffected (or even increased) when co-stimulated with 100 nM IGF-1 (Fig. 3C and 3D). This indicates that ORF74 could still be desensitized in the presence of IGF-1 and excludes GRK scavenging by IGF-1R as a possible mechanism for the crosstalk between IGF-1R and ORF74. The increased β-arrestin1 recruitment upon co-stimulation with CXCL1 and IGF-1 as compared to the stimulation with CXCL1 alone, might be explained by conformational changes in ORF74 as a result of the transactivation by IGF-1R. This increased signal is not observed for β-arrestin2 and might be due to differential phosphorylation requirements.

The HCMV-encoded GPCR US28 constitutively activates similar signal transduction pathways as ORF74, including PLC [203]. Surprisingly, IGF-1 is unable to increase constitutive US28 signaling to PLC. This not only shows that the observed crosstalk with IGF-1/IGF-1R is specific for ORF74 but also suggests that the point of signal convergence occurs at the level of ORF74 instead of activated Goq or PLC. However, the mechanism utilized by ORF74 to activate PLC remains to be elucidated. Although ORF74 activates PLC in a PTX-insensitive manner [177], a role for Goq has not been proven. Hence, US28 and ORF74 might activate PLC via different mechanisms and it can not be excluded that IGF-1 stimulation results in the activation of proteins downstream of ORF74 (e.g. G proteins) rather than ORF74 itself. The inability of IGF-1 to induce β-arrestin1/2 recruitment to ORF74 further supports this hypothesis. On the other hand, IGF-1R might transactivate ORF74 in a biased manner, leading to PLC activation but not β-arrestin recruitment.

ORF74 and IGF-1R form heteromeric complexes at the cell surface, but no close proximity could be observed between US28 and IGF-1R. This might be due to the differential localization of US28 as compared to ORF74 [288]. IGF-1R was also found to co-immunoprecipitate with CXCR4 [321] and PAC1R [322], but strong evidence that these interactions are critical for the observed crosstalk is lacking. Disrupting GPCR/RTK interactions would provide direct proof, but is also challenging as it should not directly interfere with downstream signaling and requires information about the interface of the two receptors.

Taken together, we describe for the first time crosstalk between vGPCR ORF74 and the human RTK IGF-1R. ORF74 renders PLC activation in HEK293T cells sensitive to IGF-1 stimulation. Unmasking IGF-1-induced PLC signaling by ORF74 might contribute to the complex signaling network of ORF74 and hijacking of IGF-1R and its ligands might be a strategy of KSHV to increase PLC-mediated oncogenic signaling. Future studies are required to demonstrate whether ORF74 also induces crosstalk with other RTKs that are involved in KS (e.g. the vascular endothelial growth factor receptor [325] and the platelet-derived growth factor receptor [328]).