Biochimica et Biophysica Acta 1853 (2015) 2251–2260

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Biphasic effect of PTK7 on KDR activity in endothelial cells and angiogenesis

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ARTICLE INFO

Article history:
Received 10 February 2015
Received in revised form 27 April 2015
Accepted 5 May 2015
Available online 16 May 2015

Keywords:
Defective receptor protein tyrosine kinase
PTK7
KDR
VEGF
Angiogenesis

ABSTRACT

Protein tyrosine kinase 7 (PTK7) is a member of the defective receptor protein tyrosine kinase family which lacks catalytic activity. Expression of PTK7 is increased in various cancers but its role in carcinogenesis is not well understood. We previously showed that disruption of PTK7 function suppresses VEGF-induced angiogenic phenotypes in HUVECs and mice. Here, we investigated molecular mechanisms for modulating VEGF-induced physiological effects by PTK7. Treatment with a high concentration of extracellular domain of PTK7 (soluble PTK7; sPTK7) or knockdown of PTK7 inhibited VEGF-induced phosphorylation of kinase insert domain receptor (KDR) but did not inhibit phosphorylation of fms-related tyrosine kinase 1 (FLT-1) in HUVECs. PTK7, more specifically sPTK7, interacted with KDR but not with FLT-1 in HUVECs and HEK293 cells. *In vitro* binding assay showed that sPTK7 formed oligomers with the extracellular domain of KDR (sKDR) up to an approximately 1:3 molar ratio, and *vice versa*. sPTK7 at lower molar ratios than sKDR enhanced the binding of VEGF to sKDR. At the same or higher molar ratios, it reduced the binding of VEGF to sKDR. Increasing concentrations of sPTK7 or increasing levels of PTK7 expression first increased and then decreased VEGF-induced KDR phosphorylation, migration, and capillary-like tube formation of HUVECs, as well as *in vivo* angiogenesis. Taken together, our data demonstrates that PTK7 regulates the activity of KDR biphasically by inducing oligomerization of KDR molecules at lower concentrations and by surrounding KDR molecules at higher concentrations.

1. Introduction

A subgroup of receptor protein tyrosine kinases (RPTKs) is defective in tyrosine kinase activity because of alterations in motifs important for catalytic activity [1]. Such defective RPTKs include protein tyrosine kinase 7 (PTK7), HER3 (ErbB3), EphA10, EphB6, and Ryk. Despite their lack of kinase activity, some of these defective RPTKs are known to promote signal transduction by interacting with other proteins. For example, HER3 bound to neuregulin forms heterodimers with other EGFR family members and activates downstream signals, such as PI3-kinase and MAP kinases [2,3]. Ryk interacts with Eph receptors, EphB2 and EphB3, and regulates cell migration during craniofacial and cortical development [4–6]. Ryk also functions as a co-receptor for Wnt signal molecules and modulates Wnt signaling pathways during neural development [7,8].

PTK7, also known as colon carcinoma kinase 4 (CCK-4), is composed of an extracellular domain with seven immunoglobulin-like loops, a transmembrane domain, and a defective tyrosine kinase domain [9–12]. *Drosophila* Dtrk/OTK and chick KLG, which are likely orthologues of human PTK7, contribute to repulsive axon guidance in *Drosophila* development and ventricle segment formation during chick cardiac morphogenesis through interaction with plexins, which are receptors for semaphorins [13,14]. Mammalian PTK7 is known to regulate planar cell polarity (PCP) signaling, based on the finding that mice homozygous for a truncated PTK7 gene were perinatally lethal, with failure of neural closure and misorientation of the stereociliary bundle [15]. Recently it was reported that PTK7 also regulates canonical and non-canonical Wnt signaling pathways [16,17].

PTK7 expression is elevated in various cancers, including esophageal squamous cell carcinoma and colon cancer [18–20]. Conversely, its expression is decreased in other cancers such as metastatic melanoma and ovarian carcinoma [21,22]. These findings suggest that its expression level is not directly related to tumorigenesis. Interestingly, we previously found that PTK7 is shed by ADAM17 and further cleaved by γ-secretase in colon cancer cells [23]. The cleaved PTK7 cytosolic domain translocates from plasma membrane to the nucleus and enhances tumorigenesis.

PTK7 mRNA levels are modulated during vascular endothelial growth factor (VEGF)-induced capillary-like tube formation by human umbilical vein endothelial cells (HUVECs) [24]. Functional disruption of PTK7 by treatment with the PTK7 extracellular domain (soluble PTK7; sPTK7) as a decoy receptor or knockdown of PTK7 decreased

Abbreviations: FLT-1, fms-related tyrosine kinase 1; HUVEC, human umbilical vein endothelial cell; KDR, kinase insert domain receptor; PTK7, protein tyrosine kinase 7; RPTK, receptor protein tyrosine kinase; VEGF, vascular endothelial growth factor.

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http://dx.doi.org/10.1016/j.bbamcr.2015.05.015
0167-4889/

VEGF-induced migration, invasion, and tube-formation of HUVECs, and angiogenesis *in vivo* [24]. Therefore, PTK7 at the plasma membrane probably plays an important role in VEGF-mediated signaling. To further understand the effect of PTK7 on signaling pathways induced by VEGF, we identified molecules interacting with PTK7. We then analyzed the changes in the activity of PTK7-interacting proteins upon exposure to different PTK7 levels, as well as the effects of different PTK7 levels on migration and tube-formation by HUVECs, and on angiogenesis *in vivo*. Based on the results, we propose a model for the action of PTK7.

2. Materials and methods

2.1. Antibodies

The following antibodies were used: anti-FLT-1, anti-phospho-Erk, anti-Erk, anti-p38, anti-VEGF, and anti-β-actin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho-KDR (Y951), anti-KDR, anti-phospho-p38, anti-phospho-Akt (S473), anti-Akt, anti-phospho-eNOS (S1177), anti-eNOS, anti-phospho-JNK, and anti-JNK antibodies from Cell Signaling Technology (Beverly, MA, USA); anti-phospho-tyrosine (clone 4G10) antibody from Upstate (Lake Placid, NY, USA); anti-FLAG-M2 antibody from Sigma-Aldrich (St. Louis, MO, USA); anti-penta-His antibody from Qiagen (Cambridge, MA, USA); anti-phospho-FLT-1 (Y1213) antibody from R&D Systems (Minneapolis, MN, USA), and horseradish peroxidase-conjugated goat anti-mouse-IgG, anti-rabbit-IgG or anti-human-Fc antibodies from KOMA Biotech (Seoul, Korea). Rabbit anti-PTK7 anti-serum was described previously [24].

2.2. Cell culture

HUVECs were grown in M199 medium supplemented with 20% FBS (Hyclone, South Logan, UT, USA), 5 U/ml heparin, and 3 ng/ml bFGF (Upstate) and were used in passages 5–8. Human embryonic kidney (HEK) 293 cell lines were grown in DMEM supplemented with 10% bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were grown at 37 °C in 5% CO_2.

2.3. Expression and knockdown vectors

pRSET-A-KDR harboring human full-length kinase insert domain receptor (KDR; VEGF receptor 2) cDNA and pCI-Neo-FLT-1 encoding human fms-related tyrosine kinase 1 (FLT-1; VEGF receptor 1) were a kind gift from Dr. Yong Song Gho (POSTECH, Korea). pcDNA3-sKDR-Fc and pcDNA3-sFLT-1-Fc encoding the extracellular domain of KDR (amino acids 1–764) and FLT-1 (amino acids 1–687) with a C-terminal Fc fragment, respectively, were generous gifts from Dr. Gou Young Koh (KAIST, Korea). Construction of the following expression vectors is described in the Supplementary materials: pcDNA3-hPTK7-FLAG encoding human PTK7 with a C-terminal Flag tag, pcDNA3-PTK7-TM-Cyt-FLAG encoding transmembrane and cytosolic domains of human PTK7 with a C-terminal Flag tag, and pcDNA3.1-Kozak-KDR encoding human KDR. Constructs pLKO.1-shRNA-PTK7-6433 and -6434 for human PTK7 knockdown vectors and pLKO.1-control (Sigma-Aldrich) were described previously [20].

2.4. Transfection of expression and knockdown vectors

For stable expression in HEK293 cells, subconfluent cells were transfected by the calcium phosphate method [25] and were grown in the presence of 1.2 mg/ml G418 for two weeks. G418-resistant colonies were isolated as individual clones or cultured as mixed populations. For transient expression in HEK293 cells, transfection was performed by the same procedures as for stable expression. For transient transfection into HUVECs, subconfluent cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfected cells were used two days after transfection.

2.5. Transfection of siRNA

siRNA mixture against human PTK7 was transfected into HUVECs as described previously [24].

2.6. Purification of sPTK7, sKDR, and sFLT-1

Purification of human and mouse sPTK7 with a C-terminal His tag (sPTK7-His) was previously described [24]. For purification of human sKDR and human sFLT-1 fused to a C-terminal Fc (sKDR-Fc and sFLT-1-Fc), stable clones expressing sKDR-Fc or sFLT-1-Fc were cultured in serum-free medium for seven days. The conditioned medium was subjected to ammonium sulfate precipitation with 70% saturation. The pellets were dissolved in 0.1 M Tris–HCl (pH 8.0) containing 1 mM PMSF and 1 mM EDTA. Protein-A Sepharose (Sigma-Aldrich) was added to the supernatant, washed with 0.1 M Tris–HCl (pH 8.0), and eluted with 0.1 M glycine–HCl (pH 3.0). The eluent containing sKDR-Fc was neutralized with 0.1 volume of 1 M Tris–HCl (pH 8.0) immediately and dialyzed against PBS [137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 2 mM KH_2PO_4 (pH 7.4)] containing 1 mM PMSF.

2.7. Growth factor stimulation of HUVECs

Subconfluent HUVECs were serum-depleted in M199 medium supplemented with 1% FBS for 6 h. If necessary, the depleted cells were pre-incubated with the indicated proteins for 30 min. The cells were stimulated with 0.5 nM VEGF (KOMA Biotech), for 5 min for analysis of receptors, or for 10 min for analysis of other signaling molecules.

2.8. Immunoprecipitation, pull-down assays and immunoblotting

Cells were lysed with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40) containing 5 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail III (Calbiochem, La Jolla, CA, USA). For immunoprecipitation, lysates were incubated with the indicated antibodies and protein-A/G agarose (Upstate) or mouse anti-FLAG M2-agarose. For pull-down assays, cell lysates and *in vitro* binding mixtures containing sPTK7-His, sKDR-Fc, or sFLT-1-Fc were incubated with Ni^{2+}-NTA agarose and protein-A Sepharose, respectively. The protein-bound resins were washed with lysis buffer or, in the case of the His-tag pull-down assay, with PBS containing 20 mM imidazole. For immunoblotting, cell lysates or immunoprecipitated or pulled-down proteins were resuspended in SDS-sample buffer, subjected to SDS-PAGE, and transferred to PVDF membranes. Immunoblotting was performed using the indicated antibodies, and immunoreactivity was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA, USA) and a LAS-3000 imaging system (Fuji, Tokyo, Japan).

2.9. Chemotactic migration assay and capillary-like tube formation

These assays were performed as described previously [24] except that migration and tube formation assays were incubated for 4 h and 24 h, respectively.

2.10. Ethics statement

The protocol for animal studies was approved by the Institutional Animal Care and Use Committee of Yonsei University (Permit Number: 20130100). Animal studies were performed in accordance with the guidelines of the Committee.

2.11. In vivo Matrigel plug assay

Ice-cold Matrigel™ (BD Biosciences, Bedford, MA, USA) (0.25 ml) was mixed with 16 units of heparin, 2.5 nM mouse VEGF (KOMA