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VANGL2 interacts with integrin αv to regulate matrix metalloproteinase activity and cell adhesion to the extracellular matrix

Tammy N. Jessen, Jason R. Jessen*

Department of Biology, Middle Tennessee State University, 1301 East Main Street, Murfreesboro, TN 37132, USA

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ABSTRACT

Planar cell polarity (PCP) proteins are implicated in a variety of morphogenetic processes including embryonic cell migration and potentially cancer progression. During zebrafish gastrulation, the transmembrane protein Vang-like 2 (VANGL2) is required for PCP and directed cell migration. These cell behaviors occur in the context of a fibrillar extracellular matrix (ECM). While it is thought that interactions with the ECM regulate cell migration, it is unclear how PCP proteins such as VANGL2 influence these events. Using an in vitro cell culture model system, we previously showed that human VANGL2 negatively regulates membrane type-1 matrix metalloproteinase (MMP14) and activation of secreted matrix metalloproteinase 2 (MMP2). Here, we investigated the functional relationship between VANGL2, integrin αvβ3, and MMP2 activation. We provide evidence that VANGL2 regulates cell surface integrin αvβ3 expression and adhesion to fibronectin, laminin, and vitronectin. Inhibition of MMP14/MMP2 activity suppressed the cell adhesion defect in VANGL2 knockdown cells. Furthermore, our data show that MMP14 and integrin αv are required for increased proteolysis by VANGL2 knockdown cells. Lastly, we have identified integrin αvβ3 as a novel VANGL2 binding partner. Together, these findings begin to dissect the molecular underpinnings of how VANGL2 regulates MMP activity and cell adhesion to the ECM.

1. Introduction

Planar cell polarity (PCP) refers to the polarized organization of cellular structures, such as actin, in relation to the plane of a tissue [1]. This concept was originally used to describe the polarization of cuticular structures of the insect epidermis [2]. In the fly, a highly conserved core module of six PCP proteins was identified and shown to regulate PCP in multiple epithelial tissues including the wing and eye [1]. The core PCP genes are *van gogh*, *prickle*, *dishevelled*, *frizzled*, *flamingo*, and *diego* [3–5]. The vertebrate *van gogh* ortholog, *vang-like 2* (*vangl2*), was first identified in 2001 when it was found to be the genetic defect in *Loop-tail* mouse mutants [6,7]. Here, recessive mutations in *vangl2* result in severe neural tube defects associated with abnormal morphogenesis of the floor plate neuroectoderm [6,7]. Humans with mutations in VANGL1 or VANGL2 also develop neural tube closure defects [8]. Accumulating data also suggest a role for VANGL proteins during tumor progression and invasion [9,10]. In zebrafish, *vangl2* was identified as the defective gene in *trilobite* mutant embryos noted for having a strong convergence and extension gastrulation phenotype [11,12]. Underlying convergence and extension of embryonic tissues are a variety of cell behaviors including directed migration and mediolateral intercalation [13–15]. Loss of Vangl2 protein function in *trilobite* mutant embryos produces rounder cells that migrate dorsally along indirect or meandering trajectories [11]. Zebrafish gastrulation cell movements occur in the context of a fibrillar extracellular matrix (ECM) network [16]. Knockdown of zebrafish fibronectin using antisense morpholino oligonucleotides produces a convergence and extension phenotype similar to, but weaker than, the *vangl2/trilobite* mutant phenotype [16]. Data from the frog gastrula have demonstrated that an intact fibronectin ECM is necessary for the formation of polarized membrane protrusions that drive mediolateral cell intercalation [17]. However, despite the long recognized role for fibronectin during vertebrate gastrulation [18–20], the functional relationship between the ECM and PCP proteins such as VANGL2 is unclear.

Previously, we provided evidence that fibronectin is a substrate for membrane type-1 matrix metalloproteinase (Mmp14) in the zebrafish gastrula [16,21]. Matrix metalloproteinases (MMPs) cleave ECM substrates to control a variety of processes including cell adhesion, migration, and invasion [22]. Our work identified Mmp14 as a regulator of PCP during gastrulation [23] and we showed that similar to *vangl2*,

Abbreviations: ECM, extracellular matrix; Fn, fibronectin, ITGAV, integrin αv; ITGB3, integrin β3; Ln, laminin; MMP2, matrix metalloproteinase 2; MMP14, membrane type-1 matrix metalloproteinase; PCP, planar cell polarity; VANGL2, Vang-like 2; Vn, vitronectin

* Corresponding author.
E-mail address: jason.jessen@mtsu.edu (J.R. Jessen).

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mmp14 also exhibits a strong genetic interaction with *glypican4* [23,24]. Glypican4 is a cell-surface proteoglycan thought to function as the co-receptor for Wnt11/Wnt5b-dependent non-canonical PCP signaling [25–27]. These developmental studies did not provide evidence for a mechanistic relationship between zebrafish Vangl2 and MMP-dependent proteolysis of ECM proteins. Using human HT-1080 fibrosarcoma cells (a fibroblast-like cell type that expresses PCP proteins and exhibits a high level of MMP14/MMP2-dependent invasiveness [28]), we showed that human VANGL2 regulates MMP14 and activation of the secreted MMP2 zymogen [29,30]. MMP2 activation is initiated at the cell surface by MMP14 cleavage of the 68 kDa pro-enzyme to produce a 64 kDa intermediate form of MMP2 followed by autocatalysis and production of the 62 kDa mature enzyme [31–33]. Our results demonstrated that siRNA-mediated knockdown of human VANGL2 increases MMP2 activation and cleavage of ECM substrates [29,34] suggesting that the normal function of VANGL2 is to inhibit or restrict MMP2 activity. Significantly, these data were relevant to zebrafish embryonic development. We found that *vangl2/trilobite* mutant embryos have decreased levels of fibronectin protein due to increased MMP proteolytic activity [30]. Moreover, injection of *mmp14* antisense morpholinos into *vangl2/trilobite* mutant embryos partially suppresses the convergence and extension phenotype [30]. Notably, our data from HT-1080 cells also showed that loss of VANGL2 function both increases the number of invadopodia membrane protrusions and decreases the number of paxillin positive focal adhesions [30,34]. The ability of VANGL2 to impact these events suggests that one function of this transmembrane PCP protein might be to regulate cell-ECM interactions underlying processes such as adhesion and membrane protrusive activity. Yet, it remains unknown molecularly how VANGL2 function influences MMP2 activity and whether this actually affects cell adhesion to ECM proteins.

It is thought that proteolytic cleavage of proteins such as fibronectin by MMP14 or MMP2 alters cellular interactions with the ECM and subsequently behaviors like migration [35,36]. Integrins are key regulators of cell adhesion to the ECM and play important roles during assembly of fibrillar ECM proteins [37]. Integrins themselves can also influence the activation of secreted MMP2 [38,39]. The integrin αvβ3 heterodimer in particular was shown to physically interact with MMP14 and MMP2 [40–42]. The concept that integrins and MMP14 can cooperate to increase MMP2 activation provides an attractive mechanism to regulate cell-ECM interactions underlying cell polarity and migration. Therefore, we hypothesized that VANGL2's ability to regulate MMP2 activation and ECM protein cleavage might somehow involve or require integrin αvβ3. In this report, we used HT-1080 cells to examine the functional relationship between human VANGL2, integrin αvβ3, and MMP2 activation. Our data show that VANGL2-dependent regulation of MMP14/MMP2 activity and cell surface integrin αvβ3 expression influence cell adhesion to the ECM. The ability of VANGL2 to regulate MMP2 activation requires both integrin αv and MMP14. We further show that integrin αvβ3 is a novel VANGL2 binding partner. Together, our results support a model whereby VANGL2 acts to inhibit or limit MMP2 proteolytic activity while promoting integrin mediated cell-ECM adhesion.

2. Materials and methods

2.1. Cell culture and MMP inhibition

Human fibrosarcoma HT-1080 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and used throughout this study (cells were only used below passage 25). Cells were cultured in DMEM media (4.5 g/L glucose, L-glutamine, and sodium pyruvate) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and antibiotics (100 units/mL penicillin G sodium and 100 μg/mL streptomycin). Cells were incubated at 37 °C in the presence of 5% CO_2. MMP inhibition was achieved by incubating cells 48 h in 10 μM of the broad-spectrum MMP inhibitor GM6001 (EMD Millipore, Billerica, MA).

2.2. siRNA transfection and knockdown

Cells were seeded overnight in 6- or 12-well plates and transfected at 40–50% confluence DharmaFECT 4 lipid reagent (GE Dharmacon, Lafayette, CO). The siRNA pools were used at 100 nM while the single siRNAs were used at 25 nM. The following siRNAs were used in this study: Dharmacon human VANGL2 (L-010581) and integrin αv (L-004565) ON-TARGETplus SMARTpool siRNA, Dharmacon human integrin αv single siRNAs (J-004565-08 and J-004565-10), Ambion (Thermo Fisher Scientific, Waltham, MA) human MMP14 Silencer (ID#104074) and Silencer Select (ID#s8877) siRNA, and Dharmacon Non-Targeting #2 ON-TARGETplus SMARTpool Control siRNA (D-001810). For all siRNA experiments, cells were transfected for 4 days and protein knockdown was confirmed using western blot (see below for procedure and antibodies). The MMP14 Silencer siRNA (see Supplementary Fig. 1) and the integrin αv siRNA pool (see Supplementary Fig. 2) were used throughout this study.

2.3. Plasmid DNA transfection and overexpression

Expression plasmids containing full length human VANGL2 and the control plasmids were used in this study. For the adhesions assays, VANGL2/pcDNA3 was used and for co-immunoprecipitation experiments, GFP-VANGL2/pCS2 was used. The control plasmids were pcDNA3 and pEGFPN1. Cells were seeded in 6- or 12-well plates and transfected at 80–90% confluence using Lipofectamine 3000 reagent (Thermo Fisher Scientific) following the manufacturer's instructions. For all plasmid overexpression experiments, cells were transfected for 48 h and protein expression was verified using western blot (see below for procedure and antibodies).

2.4. Co-immunoprecipitation and antibodies

Cells used for co-immunoprecipitation experiments had their proteins extracted using a Tris lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 0.1% Triton-X, pH 7.5) and protein concentrations were determined using the BCA assay. Lysates at a concentration of 200–300 μg were treated or untreated with 3–10 μg of antibodies [integrin αvβ3 (MAB1976), EMD Millipore; VANGL2 (21492-1-AP), Proteintech, Rosemont, IL. Lysates were incubated with antibodies for 2 h at 4 °C then added to prepared protein G magnetic beads (Thermo Fisher Scientific) or anti-GFP mAb-magnetic beads (Medical & Biological Laboratories CO., LTD, Nagoya, Japan). Beads and lysates were incubated overnight at 4 °C with rotation. Beads were washed with Tris lysis buffer and then denatured using Laemmli sample buffer containing β-mercaptoethanol at 95 °C for 6 min.

2.5. Western blot and antibodies

Protein obtained from co-immunoprecipitation experiments and whole-cell lysates were separated by 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose or PVDF membranes using a Trans-Blot Turbo (Bio-Rad, Hercules, CA). Non-specific binding to membranes was blocked with TBS-Tween (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 0–5% non-fat milk depending on the antibody used and membranes were incubated overnight at 4 °C with primary antibody in block solution. Membranes were then incubated with peroxidase-conjugated affinity-purified donkey anti-rabbit, anti-mouse, or anti-rat secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). TrueBlot anti-rabbit or anti-mouse IgG HRP secondary antibodies (Rockland Antibodies & Assays, Limerick, PA) were used for certain western blots. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo