Cellular Signalling 25 (2013) 2646–2651

Contents lists available at ScienceDirect

Cellular Signalling

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

Transglutaminase 2 as a novel activator of LRP6/β-catenin signaling

S. Deasey, D. Nurminsky, S. Shanmugasundaram, F. Lima, M. Nurminskaya *

Dept. of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201, United States

ARTICLE INFO

Article history:
Received 25 July 2013
Accepted 24 August 2013
Available online 29 August 2013

Keywords:
Transglutaminsase 2
LRP5/6
ß-catenin

ABSTRACT

The β-catenin signaling axis is critical for normal embryonic development and tissue homeostasis in adults. We have previously shown that extracellular enzyme transglutaminase 2 (TG2) activates β-catenin signaling in vascular smooth muscle cells (VSMCs). In this study, we provide several lines of evidence that TG2 functions as an activating ligand of the LRP5/6 receptors. Specifically, we show that TG2 synergizes with LRP6 in the activation of β-catenin-dependent gene expression in Cos-7 cells. Interfering with the LRP5/6 receptors attenuates TG2-induced activation of β-catenin in Cos-7 cells. Further, we show that TG2 binds directly to the extracellular domain of LRP6, which is also able to act as a substrate for TG2-mediated protein cross-linking. Furthermore, inhibitors of TG2 protein cross-linking quench the observed TG2-induced β-catenin activation, implicating protein cross-linking as a novel regulatory mechanism for this pathway. Together, our findings identify and characterize a new activating ligand of the LRP5/6 receptors and uncover a novel activity of TG2 as an agonist of β-catenin signaling, contributing to the understanding of diverse developmental events and pathological conditions in which transglutaminase and β-catenin signaling are implicated.

1. Introduction

Wnt/β-catenin signaling is a key signal transduction pathway in normal development and disease [1]. The "canonical" pathway is activated by the binding of a secreted lipid-modified glycoprotein from the Wnt protein family [2] to a cell surface receptor complex, consisting of a serpentine receptor from the Frizzled (Fzd) family and a low-density-lipoprotein-related protein co-receptor LRP5 or LRP6 (LRP5/6). Binding of a Wnt ligand leads to LRP5/6 oligomerization and phosphorylation of its cytoplasmic tail [3–5]. This results in recruitment of Dishevelled, thereby disrupting the destruction complex containing Axin, Adenomatosis Polyposis Coli (APC) and Glycogen Synthase Kinase 3 (GSK3), which targets β-catenin for proteasomal degradation. Stabilized β-catenin can then translocate to the nucleus, where it forms a complex with transcription factors of the TCF/LEF family and activates expression of target genes [6].

Mutations in LRP5/6 signaling are associated with bone disorders, abnormal ocular vascularization, early onset cardiovascular disease and metabolic syndromes [5,7,8]. However, not all of these phenotypes may be attributable to altered activation by ligands of the Wnt family. For instance, during ocular vascularization activation of canonical β-catenin signaling is mediated by a Norrin–LRP5–Fzd interaction [9]. Activation by Norrin utilizes both the LRP5/6 and Fzd-4 co-receptors, similar to Wnt ligands; however, Fzd-independent mechanisms of β-catenin activation have also been reported. For instance, PTH activates β-catenin through the LRP6/PTHR1 complex [10], while cysteine knot proteins SOST/Sclerostin and WISE bind directly to LRP5/6 to either inhibit or activate signaling in a context-dependent manner [11–13]. Similarly, Dkk1, a protein of the Dickkopf family, binds to LRP6 and antagonizes signaling by preventing ligand binding [14]. The multiplicity of LRP5/6-regulated biological processes suggests that identification of novel interacting ligands will advance understanding of diverse and significant aspects of normal development and its pathological disruptions.

Previously, we have shown that extracellular enzyme tissue transglutaminase (TG2) activates β-catenin signaling in primary mouse vascular smooth muscle cells (VSMCs) and interacts with the LRP5 receptor on the cell surface [15]. In mammals, eight Ca^{2+}-dependent transglutaminases (TGases) have been identified, comprising a family of enzymes that catalyze formation of intra- or intermolecular glutamyl-lysine-protein cross-links. TG2 is unique owing to its ability to catalyze more than one enzymatic reaction and interact with a number of cell surface receptors upon externalization into the extracellular matrix (ECM) [16]. Inside the cell, TG2 is able to act as a deaminase, GTPase, protein kinase and protein disulphide isomerase [17]. While in the ECM TG2 can interact with β1 and β3 integrins [18], the atypical G-protein-coupled receptor GPR56 [19], VEGF receptor 2 [20] and LRP5 [15]. We have recently shown that the protein cross-linking catalytic activity of TG2 is critical for warfarin-induced activation of β-catenin signaling in VSMCs [21]. Taking into consideration that TG2 is often externalized and activated in response to tissue insult [22] and that this often correlates with activation of β-catenin signaling in disease,

Abbreviations: Fzd, Frizzled; LRP5/6, LRP5 or LRP6; APC, Adenomatosis Polyposis Coli; GSK3, Glycogen Synthase Kinase 3; TG2, Tissue transglutaminase; VSMCs, Vascular smooth muscle cells; TGases, Transglutaminases; ECM, Extracellular matrix; MEFs, Mouse embryonic fibroblasts; gpTG2, Guinea pig liver TG2; E. coli, Escherichia coli; hTG2, Human TG2.

* Corresponding author at: Dept. of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 N Greene St, Baltimore, MD 21201, United States. Tel.: +1 410 706 7469.
E-mail address: mnurminskaya@som.umaryland.edu (M. Nurminskaya).

0898-6568/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cellsig.2013.08.016

further identification and characterization of biologically relevant TG2-receptor interactions are important for a better understanding of cardiovascular disease and other conditions.

The previously reported interaction of TG2 with LRP5 on the cell surface of osteogenic cells [15] supports the hypothesis that induction of the β-catenin signaling pathway by TG2 is mediated by the LRP5/6 receptors. However, recent findings implied that TG2 activates β-catenin signaling through a c-Src-dependent mechanism [23], thus putting the role of LRP5/6 into question. In this study we found that TG2 binds directly to and cross-links the LRP5/6 receptor. Moreover, we found that LRP5/6 is required for TG2-induced activation of β-catenin. These results may help us to understand the intertwined diversity of the biological functions of LRP5/6 and TG2 and suggest oligomerization of the LRP receptor by cross-linking as a mechanism of activation.

2. Experimental procedures

2.1. Cell culture, plasmid constructs, DNA transfection and luciferase assay

Cos-7 cells [24] obtained from ATCC and primary mouse embryonic fibroblasts (MEFs) from LRP5 null mice and their wild type siblings were maintained in Dulbecco's modified Eagle's medium supplemented with 100 U/mL of penicillin G, 100 μg/mL of streptomycin, and 10% fetal bovine serum (complete DMEM) in 5% CO_2 at 37 °C. Cells stably carrying TCF/LEF firefly luciferase reporter and constitutively active renilla luciferase reporter were established using lentivirus-based constructs, according to the manufacturer's instructions (SABiosciences). Puromycin-resistant colonies were expanded and maintained in complete DMEM supplemented with 1 μg/mL puromycin. For assays cells were seeded at 5×10^3 cells/well in 96-well plates and transiently transfected with plasmids for expression of LRP5, LRP6, TG2, Dkk1 and Wnt3a, using FuGene 6 (Roche). Where indicated in the text, MEFs were also transfected with dsRNA targeting LRP5 or LRP6 using HiPerfect reagent (Qiagen). Dual luciferase assay was performed two days post-transfection to measure the luciferase activity. Renilla activity was used as the internal normalization control. In separate experiments, Cos-7 cells were transiently transfected with TCF/LEF luciferase reporter construct TOP-FLASH and a β-galactosidase expression plasmid (Upstate) together with other expression constructs. In this case the β-galactosidase activity, measured using specific substrate (Promega), was used as the reference to normalize for transfection efficiency. When the medium was supplemented with TG2 protein, the medium was changed to 1% serum 24 h post-transfection, and supplemented with purified guinea pig liver TG2 (gpTG2) (Sigma) (0.01 U/mL, unless indicated otherwise) or purified recombinant human TG2 (N-zyme, 300 ng/mL) (N-Zyme), and cells cultured for 48 h. The plasmid for transient expression of secreted human TG2 was previously described [25]. Expression constructs for GST-TG2 fusion proteins were kindly provided by Dr. A Belkin (University of Maryland). Plasmids for transient expression of human LRP5, LRP6 and Wnt3a were kindly provided by Dr. X He (Harvard Medical School). Expression construct for Dkk1 was a gift from Dr. F Long (University of St. Louis).

2.2. Western blot analysis and immunoprecipitation

For Western blotting, antibodies against LRP6 (clone C-10, Santa Cruz), and phospho-LRP6 (Ser1490) (Antibody #2568, Cell Signaling) were used at a 1:1000 dilution. Secondary anti-mouse or anti-rabbit HRP-conjugated antibodies (Pierce) were used at a 1:10,000 dilution. The presence of His-tagged proteins was detected using the HRP-conjugated His probe (Pierce) at a 1:10,000 dilution. HRP-conjugated proteins were detected by the chemiluminescence method (Pierce). For immunoprecipitation, antibodies against GST (clone GST-2, Sigma) or TG2 (Abcam) were utilized and protein A/G-Sepharose (Pierce) beads were used to purify the protein complexes.

2.3. TG2 over-expression in zebrafish larvae

Expression construct encoding externalized human TG2 in RCASBP(A) vector was used [25]. The TG2 open reading frame was excised from the RCAS-TG2 vector with ClaI restriction digest and inserted into the ClaI site of the pCS2+ vector. For RNA synthetic reaction pCS2+-TG2 was linearized with NSiI and mMessage mMachine (Ambion) was used to synthesize RNA as directed by the manufacturer. RNA was cleaned with MEGAclear kit (Ambion). RNA was dissolved in water to a concentration of 60 μg/mL and a final concentration of 0.1% phenol red was added to allow for visualization during injections. Approximately, 1 μL of RNA was injected into zebrafish embryos at the 2–8-cell stages. Embryos were fixed in 4% PFA 24 h after injection and analyzed by microscopy with a Nikon T-80 microscope equipped with SPOT RT slider real-time CCD camera. 83 embryos were analyzed.

2.4. TG activity assay

TG cross-linking activity was assayed as previously described [26]. 96-well microtiter plates (Maxisorp NUNC) were incubated overnight with 250 μL of 1 mg/mL *N,N′*-Dimethylcasein (Sigma-Aldrich) in 5 mM Sodium Carbonate (pH 9.8), and blocked with 200 μL of 0.1% bovine serum albumin (BSA) (HyClone) in 5 mM Sodium Carbonate (pH 9.8) for 1 h at 37 °C. LRPΔC (R & D Systems), purified guinea pig liver TG2 (80 ng) (Sigma-Aldrich) and Ez-link Pentylamine-Biotin (2.5 mM) (Pierce) were incubated at 37 °C for 1 h in reaction buffer (100 mM Tris–HCl pH 8.5, 6.7 mM $CaCl_2$, 13.3 mM DTT) with increasing molar concentrations of LRPΔC. Incorporated Ez-link Pentylamine-Biotin was detected with 1:5000 ExtrAvidin-Peroxidase (Sigma) and Super AquaBlue ELISA Substrate (eBioscience) followed by reading the absorbance at 405 nm on a Polarstar Optima plate reader.

2.5. Statistical tests

For all figures, error bars represent Standard Errors and P values were calculated by a one-tailed student's *T* test (*$p < 0.05$, **$p < 0.01$). At least three independent replicates were performed for each experiment.

3 . Results

3.1. Extracellular TG2 activates β-catenin signaling in Cos-7 cells

Previously, we have shown that exogenously purified TG2 activates β-catenin signaling in mouse VSMCs [15]. In this study, we sought to identify the target position for TG2 activity in the β-catenin signaling pathway. For this, we monitored activation of this pathway using a luciferase reporter driven by TCF/LEF-binding promoter (TOP-FLASH) in Cos-7 cells, which are conventionally used for the analysis of β-catenin signaling. Similar to its effect on mouse VSMCs, extracellular TG2 purified from guinea pig liver tissue (gpTG2) induced activation of β-catenin in Cos-7 cells in a dose-dependent manner (Fig. 1A). We examined levels of gpTG2 ranging from 1.5×10^{-4} to 1×10^{-2} U/mL as this is within the range of endogenous TG2 levels in smooth muscle cells [27]. To confirm that this effect was specific to TG2 and not due to possible contaminants from the liver tissue, we showed a similar induction of the TOP-FLASH reporter with recombinant human TG2 purified from *Escherichia coli* (*E. coli*) (Fig. 1B, hTG2). In addition, overexpression of human TG2, fused to a secretory peptide to assure its externalization, also activated the TOP-FLASH reporter in Cos-7 cells (Fig. 1B, sTG2). In aggregate, these observations strongly indicate that extracellular TG2 can serve as an activator of β-catenin signaling.

Activation of canonical β-catenin signaling by Wnt ligands results in phosphorylation of the serine residue in the NPPPSPATE motif of the intracellular LRP6 domain [5]. This activation can be inhibited by extracellular DKK proteins [28] and by the small molecule inhibitor FJ9, which specifically disrupts intracellular interactions of the Dishevelled