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The role of the ShcD and RET interaction in neuroblastoma survival and migration

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ABSTRACT

Preliminary screening data showed that the ShcD adaptor protein associates with the proto-oncogene RET receptor tyrosine kinase. In the present study, we aimed to investigate the molecular interaction between ShcD and RET in human neuroblastoma cells and study the functional impact of this interaction. We were able to show that ShcD immunoprecipitated with RET from SK-N-AS neuroblastoma cell lysates upon GDNF treatment. This result was validated by ShcD-RET co-localization, which was visualized using a fluorescence microscope. ShcD-RET coexpression promoted ShcD and RET endosomal localization, resulting in unexpected inhibition of the downstream ERK and AKT pathways. Interestingly, ShcD-RET association reduced the viability and migration of SK-N-AS cells. Although ShcD was previously shown to trigger melanoma cell migration and tumorigenesis, our data showed an opposite role for ShcD in neuroblastoma SK-N-AS cells via its association with RET in GDNF-treated cells. In conclusion, ShcD acts as a switch molecule that promotes contrasting biological responses depending on the stimulus ad cell type.

1. Introduction

Adaptor proteins are key factors in maintaining intracellular homeostasis. These proteins form a dynamic link between the receptor and effector motifs of signalling pathways, enabling cellular signals to be transduced and appropriate physiological responses to be generated [1]. Any disorder in the expression of adaptor proteins may lead to a disease condition or, in some cases, may trigger cancer formation [2]. The Src homology and collagen (Shc) family of adaptor proteins modulates the dynamics of various intracellular cascades downstream of different receptor tyrosine kinases (RTKs) through the family's various Shc isoforms, which have multiple domains [3,4]. The recently identified melanoma-associated adaptor ShcD belongs to the Shc family of adaptor proteins [5]. ShcD expression was found to be upregulated in invasive melanoma [5,6], which led researchers to propose ShcD as a therapeutic target for melanoma treatment [7].

Previous studies showed that ShcD associates with various tyrosine kinase receptors, such as muscle specific kinase receptor (MUSK), epidermal growth factor receptor (EGFR), neurotrophic receptors (TrkA/B/C), RET, anaplastic lymphoma kinase (ALK), Met, insulin growth factor-1 receptor (IGF-1 R), ErbB2/4 and vascular endothelial growth factor receptor-3 (VEGR-3 [6,8,9], albeit little has been reported regarding the consequences of ShcD-receptor interactions.

The ShcD and MUSK association was shown to interfere with acetylcholine receptor phosphorylation at the neuromuscular junction [9], while the ShcD and TrkB interaction was suggested to have a role in brain cell differentiation and development in the presence of brain-derived growth factor (BDNF) [10]. A study by Wills and co-workers also presented an interesting and unique role for ShcD in promoting EGFR phosphorylation in a ligand-independent manner [6]. Notably,

Abbreviation: Akt,, Protein kinase B;; ALK,, Anaplastic Lymphoma Kinase; CMV,, Cytomegalovirus; DMEM,, Dulbecoo Modified Eagle's Medium;; DNA,, Deoxyribonucleic Acid; ECL,, Enhanced Chemiluminescence;; EGF,, Epidermal Growth Factor;; EGFR,, Epidermal Growth Factor Receptor;; ERK,, Extracellular Signal–Regulated Kinases;; FBS,, Fetal Bovine Serum; FGFR,, fibroblast growth factor receptors; GDNF,, Glial Cell Line-Derived Neurotropic Factor;; GFLs,, GDNF Family Ligands;; GFP,, Green Fluorescent Protein; GPCR,, G-Protein Coupled Receptor; GRB2,, Growth Factor Receptor-Bound Protein 2;; HGFR,, hepatocyte growth factor receptor;; HRP,, Horseradish Peroxidase; hrs,, Hours; IGF,, Insulin Growth Factor;; LB,, Luria-Bertani; mAb,, Monoclonal Antibody; MAP,, Mitogen-Activated Protein;; MAPK,, Mitogen-Activated Protein Kinases; min,, Minute; MuSK,, Muscle Specific Kinase; NFDM,, Non-Fat Dry Milk; PBS,, Phosphate-Buffered Saline; PBST,, Phosphate-Buffered Saline Tween; PDGF,, Platelet-Derived Growth Factor;; PI3K,, Phosphoinositide 3-Kinase; PMSF,, Phenylmethylsulfonyl Fluoride; pAb,, Polyclonal Antibody; pTyr,, Phospho-Tyrosine; PVDF,, Polyvinylidene Fluoride; RET,, Rearranged During Transfection; rpm,, revolution per minute;; RT,, Room Temperature;; RTKs,, Receptor Tyrosine Kinase; SDS-PAGE,, Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis; ShcD,, Src Homology And Collagen D; Src,, Proto-Oncogene Tyrosine-Protein Kinase Src; TKRs,, Tyrosine Kinase Receptor;; TrkA/B/C,, Tropomyosin-Related Kinase Receptor A/B/C

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the data have not revealed the exact downstream consequence of the ShcD-receptor association with known interacting receptors.

In an unpublished study, Fagiani demonstrated that ShcD is expressed by neural crest-derived cancers, such as gliomas and neuroblastomas [5]. Additionally, RET tyrosine kinase receptor is essential for neuronal development [11,12]; it has also been shown to promote neuroblastoma prosurvival signalling and migration [13,14]. Another study by Smith and colleagues showed that RET and ShcD associate at the exogenous level in human embryonic kidney cells [8], though no further experiments were performed to elucidate the functional impact of this interaction.

The RET (rearranged during transfection) receptor is one of the tyrosine kinase receptor which is critical for normal development and wellbeing of different cell types [15]. RET is a single-pass transmembrane and similar to the other TKRs, RET possess an extracellular domain, an intracellular domain and a transmembrane membrane region. The RET signalling is triggered through the binding of glial cell line-derived neurotropic factor (GDNF) family ligands (GFLs), which includes GDNF, artemin (ARTN), neurturin (NRTN) and persephin (PSPN) [16].

Based on these observations, further study is needed to decipher the role of the molecular interaction between ShcD and RET in neuroblastoma cells since the role of RET in neuronal cell migration and survival is well determined [16]. In addition, we aimed to explore the effect of this association on downstream signalling and its effects on cellular biological responses, particularly survival and migration.

2. Materials and methods

2.1. Plasmids, antibodies, and growth factors

A full-length clone DNA of the *Homo Sapiens* RET proto-oncogene transcript variant 2 with a C-terminal MYC tag, GFP construct and MYC-tag negative construct were obtained from Sino Biological, Inc, China. GFP-ShcD and empty vector constructs were provided by Dr. Sally A. Prigent, University of Leicester, UK and described previously by Ahmed and Prigent [17].

The glial derived neurotrophic factor (GDNF) from Sigma-Aldrich, UK was prepared in sterile, molecular-grade water to a concentration of 20 μg/ml.

The following primary antibodies were used for immunoblotting, immunoprecipitation and immunofluorescence: anti-RET (sc-9996; Santa Cruz, USA), anti-MYC (ab9106; Abcam, UK), anti-MYC tag (ab18185; Abcam), anti-phospho-tyrosine (ab179530; Abcam), anti-ShcD (sc-165482; Santa Cruz, USA), anti-AKT1/2/3 (ab179463; Abcam), anti-phospho-AKT1/2/3 (sc-7985; Santa Cruz), anti-PKC (ab179522; Abcam), anti-GFP (sc-9996; Santa Cruz), anti-phospho-RET (sc-20252; Santa Cruz), anti-ERK1/2 (9102S; Cell Signalling), anti-phospho-ERK1/2 (4370S; Cell Signalling), anti-β actin (4970S; Cell Signalling), anti-GAPDH (ab37168; Abcam), anti-RET (ab134100; Abcam) and anti-RAB7 (ab198337). Horseradish peroxidase-conjugated anti-goat (ab97023, Abcam), anti-mouse (7076S; Cell Signalling) and anti-rabbit (7074S; Cell Signalling) secondary antibodies were used for immunoblotting. Donkey anti-rabbit IgG Alexa Fluor 647 and goat anti-mouse IgG Alexa Fluor 405 from Abcam were used for immunostaining.

2.2. Cell culture, transfection and GDNF treatment optimization

The neuroblastoma cell line SK-N-AS was obtained from ECACC (Sigma-Aldrich, UK). The cells were maintained at 5% CO_2 and 37 °C in DMEM supplemented with 10% foetal bovine serum (FBS), 1 mM MEM non-essential amino acids, 5 mM L-glutamine and 1% penicillin/streptoMYCin (P/S). For co-immunoprecipitation, cells were seeded in 100-mm culture dishes with 10 ml of media. For the immunofluorescence analysis and wound healing assay, cells were seeded on sterile glass coverslips in 6-well plates with 2 ml of media. For the MTT and caspase 3/7 assays, cells were seeded in 96-well plates with 200 μl of complete media. The cells were transfected with 2 μg of control vector (FLAG-HIS empty vector) as mock transfection, GFP, MYC tag negative vector, GFP-ShcD, MYC-RET or co-transfected with GFP-ShcD and MYC-RET plasmid DNA following the TurboFect manufacturer's guide (Thermo Fisher Scientific; R0531). The same amount of DNA was used for transfection in the case of the individual transfection of GFP, MYC, GFP-ShcD or MYC-RET; the control vector was used to equalize the amount. After transfection, the cells were starved with DMEM containing 0.1% FBS for 4 h and treated with 200 ng/ml for 40 min for the downstream signalling dissection experiment. While for the wound migration the cells were untreated or treated with 200 ng/ml GDNF for 24 h in 10% FBS containing medium. In the assessment of cell viability experiments, the cells were either kept in 1% FBS containing medium or in % FBS containing medium with 200 ng/ml GDNF for 48 h.

2.3. Cell lysate preparation and immunoprecipitation

Following GDNF treatment, cells were washed twice with ice-cold PBS and lysed using pre-chilled Triton lysis buffer containing 1% Triton lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100), 50 mM NaF, 1 mM Na3VO4, 1 mM PMSF and 2% protease inhibitors. The cell lysates were centrifuged at 14,000 rpm for 10 min at 4 °C to remove the cell debris. The protein analysis was performed using a Thermo Scientific Pierce BCA Protein Assay Kit. The sample buffer (3× SB, 100 mM DTT) was then added to the cell lysate of each sample. The samples were stored at (−20 °C). The next day, the samples were heated at 95 °C for 5 min and resolved on an SDS-PAGE gel.

For co-immunoprecipitation, a 25-μl slurry of protein G-sepharose beads (Sigma-Aldrich, UK; P3296) was conjugated with 2–5 μg of the primary antibody and/or control antibody. After immobilizing the antibodies with beads, ~ 500 μl of the cell lysate was added to the beads and kept for incubation at 4 °C for 2 h with gentle rocking. After the incubation, the beads were washed 4 times with 500 μl of washing buffer (1% Triton lysis buffer, 1 mM PMSF, 50 mM NaF and 1 mM Na_3VO_4); 50 μl of the sample buffer (3× SB, 70 mM DTT) was added to the bead pellet and incubated at 95 °C for 5 min immediately before gel loading.

2.4. Immunoblotting

Whole cell lysates (WCLs) or immunoprecipitates were separated on 8–10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Immobilon-P) using a semidry Turbo Transfer system (Bio-Rad). Membranes were blocked in 5% BSA or NFDM in Tris-buffered saline/Tween-20 (TBST) for 1 h. Then, the membrane was incubated with the primary antibody overnight at 4 °C. After incubation, the membranes were washed 4 times with TBST for 10 min each with agitation. The secondary antibody coupled to HRP was diluted in the same blocking buffer for 1 h at room temperature. The membrane was washed 4 times with TBST, and the bands were visualized using ECL chemiluminescence detection reagent (Thermo Scientific Pierce; PL209759A).

2.5. Immunofluorescence staining, confocal microscopy, and colocalization analysis

After GDNF treatment, the culture medium was aspirated from each glass coverslip, rinsed twice with cold 1× PBS and fixed by incubation with 3.7% (v/v) formaldehyde in PBS for 20 min at RT. The cells were then washed three times with PBS for 1–2 min. Thereafter, the cells were permeabilised with 0.1% Triton X-100 in PBS for 10 min at RT, blocked with 3% BSA in PBS for 30 min-1 h at RT and incubated with the primary antibody at 4 °C overnight. Following incubation, the cells were rinsed with PBS, incubated in a sealed, light-impermeable