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PDZ-RhoGEF Is a Signaling Effector for TROY-Induced Glioblastoma Cell Invasion and Survival

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Abstract

Glioblastoma multiforme (GBM) is the most common type of malignant brain tumors in adults and has a dismal prognosis. The highly aggressive invasion of malignant cells into the normal brain parenchyma renders complete surgical resection of GBM tumors impossible, increases resistance to therapeutic treatment, and leads to near-universal tumor recurrence. We have previously demonstrated that TROY (TNFRSF19) plays an important role in glioblastoma cell invasion and therapeutic resistance. However, the potential downstream effectors of TROY signaling have not been fully characterized. Here, we identified PDZ-RhoGEF as a binding partner for TROY that potentiated TROY-induced nuclear factor kappa B activation which is necessary for both cell invasion and survival. In addition, PDZ-RhoGEF also interacts with Pyk2, indicating that PDZ-RhoGEF is a component of a signalsome that includes TROY and Pyk2. PDZ-RhoGEF is overexpressed in glioblastoma tumors and stimulates glioma cell invasion *via* Rho activation. Increased PDZ-RhoGEF expression enhanced TROY-induced glioma cell migration. Conversely, silencing PDZ-RhoGEF expression inhibited TROY-induced glioma cell migration, increased sensitivity to temozolomide treatment, and extended survival of orthotopic xenograft mice. Furthermore, depletion of RhoC or RhoA inhibited TROY- and PDZ-RhoGEF–induced cell migration. Mechanistically, increased TROY expression stimulated Rho activation, and depletion of PDZ-RhoGEF expression reduced this activation. Taken together, these data suggest that PDZ-RhoGEF plays an important role in TROY signaling and provides insights into a potential node of vulnerability to limit GBM cell invasion and decrease therapeutic resistance.

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Introduction

Glioblastoma multiforme (GBM), a grade IV astrocytoma, is the most common primary central nervous system tumor in human adults and remains largely incurable with a median life expectancy of approximately 15 months [1,2]. Despite advances in therapeutic treatments of GBM, including surgical resection, chemotherapy, and radiation therapy, overall patient survival has not shown significant improvement over the past decade [1]. A hallmark of malignant gliomas is the extensive invasion of tumor cells into the normal brain parenchyma [3], which implies that even extensive resection of the primary tumor mass is not curative. Moreover, the invading

Abbreviations: GBM, glioblastoma multiforme; PRG, PDZ-RhoGEF; NF-κB, nuclear factor kappa B; GEF, guanine nucleotide exchange factor.

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cells are highly resistant to current therapeutic modalities, consequently leading to tumor recurrence [4]. However, the molecular mechanisms underlying glioma cell invasion have remained elusive. Thus, a deeper understanding of the signaling pathways that drive glioma cell invasion as well as the identification and specific targeting of the crucial signaling effectors is needed to ultimately improve the treatments for this disease.

Among the important mediators of glioblastoma cell invasion is TROY, an orphan member of the tumor necrosis factor receptor superfamily, which is widely expressed during embryonic development but whose postnatal expression is tightly regulated [5–8]. Increased expression of TROY has been implicated in several invasive cancers, including melanoma, nasopharyngeal carcinoma, lung cancer, colorectal cancer, and GBM [9–14]. We have previously shown that expression of TROY protein is low in non-neoplastic brain tissue but increases with glial tumor grade and inversely correlates with patient survival [13]. We also noted that TROY mRNA expression was elevated in invasive glioma cells relative to cells in the matched tumor core [14]. Increased expression of TROY stimulated glioma cell invasion *in vitro* and invasion *ex vivo* in brain slices, and induced astrocyte migration *in situ*. TROY-stimulated migration correlated with increased glioma cell resistance to temozolomide (TMZ) or radiation *in vitro via* activation of Akt and the nuclear factor kappa B (NF-κB) [14]. Conversely, knockdown of TROY expression inhibited glioma cell migration and increased sensitivity to TMZ [14]. Furthermore, knockdown of TROY expression alone significantly increased survival in an intracranial xenograft model [14]. Recently, we found that TROY forms a novel complex with epidermal growth factor receptor and that TROY was capable of modulating epidermal growth factor receptor signaling in GBM [15]. However, the signaling pathways and specific downstream effectors involved in TROY-stimulated cell migration and invasion remain largely undefined.

The Rho GTPases, a subgroup of the Ras superfamily, play important roles in a wide spectrum of cellular functions such as actin cytoskeletal reorganization, cell cycle progression, and vesicle trafficking [16]. They act as molecular switches by cycling between an active (GTP-bound) and an inactive (GDP-bound) conformational state. The switch is primarily regulated by guanine nucleotide exchange factors (GEFs), catalyzing the exchange of GDP for GTP, and GTPase-activating proteins, promoting the hydrolysis of GTP bound to Rho GTPases to deactivate the Rho GTPases [17]. Emerging evidence has demonstrated that Rho GEFs link many receptor tyrosine kinases to Rho GTPase activation [18,19]. Given their central role as regulators of the cytoskeleton, cell cycle, cellular polarity, cell adhesion, and cell migration, RhoGEFs have been implicated in cancer cell invasion and tumor progression [20].

In this study, we sought to identify downstream effectors involved in TROY-induced glioma cell migration and invasion. We identified PDZ-RhoGEF (ARHGEF11) as a component of a signalsome that includes TROY and the non–receptor tyrosine kinase Pyk2 [13]. PDZ-RhoGEF expression is significantly increased in GBM tumors and stimulates the migration of TROY-expressing GBM cells. PDZ-RhoGEF can exchange for both RhoA and RhoC linking TROY signaling to Rho activation. The current results substantiate a role for PDZ-RhoGEF as an effector of TROY signaling and suggest that PDZ-RhoGEF may represent a novel target to inhibit GBM cell invasion.

Materials and Methods

Cell Culture

Authenticated human astrocytoma cell lines U87MG and T98G (American Type Culture Collection), human kidney epithelial cell line 293 cells, and T98G cells transduced with a shRNA targeting TROY [14] as well as the 293/NF-κB-luc reporter cell line [15] were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen), 1% nonessential amino acids, 2 mmol/l glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2. When indicated, cells were serum starved by replacing the culture media with DMEM supplemented with 0.1% bovine serum albumin (BSA). GBM43 and GBM10 are primary GBM patient-derived xenografts (PDX) obtained from the Mayo Clinic Brain SPORE [21]. These PDX were established directly from patient surgical samples and maintained as subcutaneous flank xenografts through serial passaging in immune-deficient mice. Extensive phenotypic and genotypic characterizations of these models as well as their growth properties in flank and brain and the response of orthotopic tumors to various therapies are available at https://www.mayo.edu/research/labs/translational-neuro-oncology/mayo-clinic-brain-tumor-patient-derived-xenograft-national-resource. Fresh flank tumors were resected, processed to single cell suspension by mechanical dissociation, and maintained in neurosphere media (DMEM/F12 containing 2% B-27 supplement, 20 ng/ml bFGF, and 20 ng/ml EGF).

Antibodies, Expression Constructs, and Reagents

A polyclonal PDZ-RhoGEF antibody was purchased from Novus Biologicals (Littleton, CO). Antibodies to HA-epitope tag, α-tubulin, β-tubulin, and RhoC were purchased from Cell Signaling Technologies (Beverly, MA). A rabbit polyclonal antibody to TROY was produced by Cocalico Biologicals (Reamstown, PA) using a peptide mapping to the TROY amino terminus conjugated to KLH. The anti-RhoA antibody and the anti–PDZ-RhoGEF monoclonal antibody were obtained from Santa Cruz biotechnology (Dallas, TX). The anti-Myc monoclonal antibody (9E10), the anti-Rac1 monoclonal antibody, and the anti-Pyk2 polyclonal antibody were obtained from Millipore (Bedford, MA). The anti-FLAG antibody was obtained from Sigma (St. Louis, MO). The polyclonal anti-AU1 epitope antibody and the polyclonal anti-Myc epitope antibody were obtained from Bethyl Laboratories (Montgomery, TX). The anti-phosphotyrosine mAb pY20 was from BD Biosciences (San Jose, CA). The β-actin monoclonal antibody was obtained from ThermoFisher Scientific (San Jose, CA). Alexa Fluor 546–labeled goat anti-rabbit antibody and Alexa Fluor 488–labeled goat anti-mouse antibody were purchased from Invitrogen (Carlsbad, CA). The 3X HA epitope-tagged wild-type (WT) TROY construct was constructed as previously described [13]. The AU1 epitope-tagged TROY was generated by replacing the 3X HA epitope with the AU1 epitope (DTYRYI) by PCR. Generation of the FLAG-epitope tagged wild-type Pyk2 and kinase-deficient Pyk2 K457A variant has been previously described [22]. Plasmids encoding rat PDZ-RhoGEF and leukemia-associated Rho guanine nucleotide exchange factor (LARG) [23] were generously provided by Zhekang Ying (University of Maryland School of Medicine). To generate FLAG epitope-tagged wild-type PDZ-RhoGEF, the coding sequence of PDZ-RhoGEF was cloned in-frame downstream of a 3X FLAG epitope in pcDNA3. Recombinant E1-deleted adenovirus for this construct was prepared using the Ad-Easy system as previously described [24]. Collagen was obtained from Advanced Biomatrix (San Diego, CA). EGF was obtained from Invitrogen (Carlsbad, CA).

Generation of NF-κB Response Element–Driven Firefly Luciferase Reporter Stable Cell Lines Overexpressing HA Tagged TROY

A cDNA fragment encoding WT TROY with a C-terminal 3X HA epitope-tag was subcloned into the lentiviral transfer vector pCDH GFP

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