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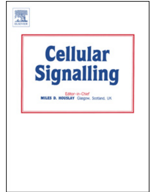

Regulator of G protein signaling 20 enhances cancer cell aggregation, migration, invasion and adhesion☆

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ABSTRACT

Several RGS (regulator of G protein signaling) proteins are known to be upregulated in a variety of tumors but their roles in modulating tumorigenesis remain undefined. Since the expression of RGS20 is elevated in metastatic melanoma and breast tumors, we examined the effects of RGS20 overexpression and knockdown on the cell mobility and adhesive properties of different human cancer cell lines, including cervical cancer HeLa, breast adenocarcinoma MDA-MB-231, and non-small cell lung carcinoma H1299 and A549 cells. Expression of RGS20 enhanced cell aggregation, migration, invasion and adhesion as determined by hanging drop aggregation, wound healing, transwell chamber migration and invasion assays. Conversely, shRNA-mediated knockdown of endogenous RGS20 impaired these responses. In addition, RGS20 elevated the expression of vimentin (a mesenchymal cell marker) but down-regulated the expression of E-cadherin, two indicators commonly associated with metastasis. These results suggest that the expression of RGS20 may promote metastasis of tumor cells.

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1. Introduction

Increasing evidence suggests that altered expression and/or activation of G protein-coupled receptors (GPCRs) are associated with numerous types of tumors [1]. Modulation of tumor progression by GPCRs is often achieved through direct or indirect regulation of signals that control cell cycle progression and cell proliferation [2,3]. Although not classified as receptors for growth factors, many GPCRs possess the ability to regulate mitogenic pathways that are typically stimulated by receptor tyrosine kinases (RTKs) that respond to ligands such as epidermal growth factor and nerve growth factor. A notable example is the ability of GPCRs to activate the family of mitogen-activated protein kinases including extracellular signal-regulated kinases and c-Jun N-terminal kinases [4]. By comparison, the mechanisms by which GPCRs affect tumor progression through the modulation of angiogenesis or metastasis are poorly understood. Nevertheless, it has been demonstrated that stimulation of GPCRs by lysophosphatidic acid and chemokines [5,6] can induce tumor cell migration and the production of angiogenic factors.

Transactivation of RTKs has long been known to provide an avenue for GPCRs to modulate cellular responses and it often engages signaling molecules beside the typical G proteins [7]. In this regard, numerous studies have shown that the family of RGS (regulator of G protein signaling) proteins participate in the regulation of tumorigenesis and cancer development, and these effects appear to be independent of their canonical function as GTPase activating proteins for the Gα subunits [8]. At least six different RGS proteins (RGS2, 4, 10, 16, 17, 19) are capable of regulating cell proliferation [9–14]. Evidence for potential involvement in tumorigenesis is perhaps strongest for RGS17 among these RGS proteins. Analysis of human familial lung cancer tumors has identified RGS17 as a commonly induced gene [15], which is also up-regulated in prostate [12], breast [13] and liver [16] cancers. RGS17 apparently induces tumor cell proliferation in these tissues through the cyclic AMP-PKA-CREB pathway [12]. Interestingly, we have similarly demonstrated that RGS19 (also known as Gα-interacting protein or GAIP), which belongs to the same RZ/A subfamily of RGS proteins as RGS17, can stimulate cell proliferation in multiple cell types by enhancing Akt signaling and deregulating cell cycle control [14,17]. Up-regulation in ovarian cancer cells [18] and its ability to tap into RTK signaling [19] tend to implicate RGS19 in tumorigenesis. Whether other RGS proteins of the RZ/A subfamily can similarly affect cell proliferation remains largely unknown.

As a RZ/A family member, RGS20 (also known as RGSZ1) shares about 62% similarity with RGS17 and they have the same cysteine-rich motif in the N-terminal domain [20]. RGS20 is highly expressed in brain especially in the caudate nucleus and the temporal lobe [21], and its transcript is detectable in the eye and female sex organs [22].

Abbreviations: EMT, epithelial-mesenchymal transition; GPCR, G protein-coupled receptor; HINT, histidine triad nucleotide binding protein; RTK, receptor tyrosine kinase; RGS, regulator of G protein signaling; TLR, Toll-like receptor.

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The transcript level of RGS20 is significantly elevated in melanoma and metastatic breast cancer cells [23,24]. RGS20 is usually regarded as a $G\alpha_z$-selective GAP; however, it can also interact with other $G\alpha_i$ subunits to attenuate $G\alpha_i$-mediated cell signaling [25]. Unlike RGS19, overexpression of RGS20 in human embryonic kidney 293 (HEK293) cells did not stimulate cell proliferation [14] and it also failed to induce neoplastic transformation in NIH3T3 fibroblasts [26]. Although RGS20 does not appear to be oncogenic itself, we observed that its coexpression in NIH3T3 fibroblasts augmented oncogenic Ras^{GV}-induced foci formation [26]. Moreover, tumors arising from 3T3/Ras^{GV}/RGS20 cells appeared earlier than 3T3/Ras^{GV} cells in nude mice assays [26]. Because changes in cell survival and adhesion properties can modulate both *in vitro* and *in vivo* colony formations, we thus examined the effects of RGS20 expression on cell viability, aggregation, adhesion, migration, and invasion in a variety of cell types.

2. Materials and methods

2.1. cDNA constructs, cell culture and transfection

The cDNAs encoding H-Ras^{GV}, and HA-tagged RGS19 and RGS20 were purchased from Missouri S&T cDNA Resource Center (Rolla, MO, USA). HEK293 cells (ATCC®CRL-1573™), mouse fibroblast NIH3T3 cells (ATCC®CRL-1658™), human non-small cell lung carcinoma H1299 (ATCC®CRL-5803™) and A549 cells (ATCC®CCL-185™), human breast adenocarcinoma MDA-MB-231 cells (ATCC®HTB-26™) and human cervical cancer HeLa cells (ATCC®CCL-2™) were purchased from American Type Culture Collection (ATCC; Rockville, MD, U.S.A.). NIH3T3 stable cells were established and maintained as previously described [14,26]. HEK293 cells were transiently transfected with 15 μg of cDNA encoding RGS20 using LipofectAMINE PLUS™ reagents from Invitrogen (Carlsbad, CA, U.S.A). For stable transfection in MDA-MB-231 cells, cells were transfected with 5 μg of cDNA encoding RGS20 using Lipofect AMINE® 2000 from Invitrogen and selected by 0.6 mg/ml of G418 for 2–3 weeks. Expression of the RGS20 construct was confirmed by immunoblots.

2.2. Reverse-transcription PCR

Total RNA of cells was isolated with TRIzol® reagent obtained from Invitrogen. cDNA samples were prepared using SuperScript® III First-Strand Kit (Invitrogen). Reverse-transcription PCR was performed using AccuPrime™ Pfx SuperMix (Invitrogen). The primers used were as follows: 5′–GCT CCT ACT CTG GAA GAA GT–3′ and 5′–GAA GTT GAG CAT CAT CGA AT–3′ for RGS20; 5′–TGA TGA CAT CAA GAA GGT GGT GAA G–3′ and 5′–TCC TTG GAG GCC ATG TGG GCC AT–3′ for GAPDH (Human glyceraldehyde 3-phosphate dehydrogenase).

2.3. Stable knockdown and rescue of RGS20 by shRNA

Stable knockdown of RGS20 in HeLa and H1299 cells and the subsequent characterization of HeLa/shRGS20 and H1299/shRGS20 cells have been described previously [26]. The same approach employing the pKAR vector system containing specific shRNA constructs was used to generate stable knockdown of RGS20 in A549 cells. The specific shRNA construct against RGS20 was created by annealing the following pair of primers into the pKAR vector: 5′–TTT GGA GAA GTG ATC AAC AGA AAC GAA TTT CTG TTG ATC ACT TCT CTT TTT–3′ and 5′–CTA GAA AAA GAG AAG TGA TCA ACA GAA ATT CGT TTC TGT TGA TCA CTT CTC–3′. A549 cells were transfected with 5 μg of shRNA against RGS20 using LipofectAMINE® 2000 and maintained in 0.5 mg/ml of puromycin for 3 weeks. For the rescue experiment in H1299 and A549 cells, the shRNA-resistant cDNA construct of HA-tagged RGS20 (RGS20') was created by silencing mutation of RGS20 at the shRNA corresponding sequence. The primers for RGS20' were 5′–TCC CCC GGG TGA GGG AGG TAA TAA AC–3′ and 5′–CCG CTC GAG CTA TGC TTC AAT AGA TTT CT–3′. 5 μg of RGS20' cDNA was transiently transfected into RGS20-knockdown stable cells using LipofectAMINE® 2000. The knockdown and rescue efficiency of RGS20 was confirmed by RT-PCR.

2.4. MTT cell proliferation assay

A549 stable cells were seeded in 96-well plates at 2000 cells per well and growth medium was replenished every 2 days. At various time points, cell proliferation was measured by Cell Proliferation Kit I (MTT) from Roche (Basel, Switzerland) and expressed as the absorbance at 570 nm as previously described [14].

2.5. Focus formation assay

Five thousand stably transfected NIH3T3 cells were mixed with 1×10^6 untransfected NIH3T3 cells and seeded on a 10 cm plate. In the control group, the transfected cells were replaced by untransfected cells. Cells were cultured for 14 days with the media changed every 3 days. Foci were visualized by Giemsa staining as described previously [26].

2.6. Hanging drop aggregation assay

The hanging drop aggregation assay was based on the method described previously [27]. Cells were suspended in culture medium and 5000 cells were placed in 20 μl droplets on the inner surface of the lid covering a Petri dish. 10 ml PBS was added to the dish to prevent evaporation of the hanging drops. Cells were cultured for 18 h and the number of cells was counted from micrographs recorded at the beginning and the end of the incubation. Cell aggregation was expressed as the aggregation index according to the formula $(N_0 - N_t)/N_0$ where N_0 is the number of total cells and N_t represents the number of non-aggregated cells at the end of the incubation period. To examine the adhesive strength of cellular aggregates, parallel cultures were triturated 10 times through a yellow micropipette tip. Relative particle size of cell aggregates after trituration was estimated by determining the mean area of particles (~200) in the micrographs using Image J analysis. Mean area of particles was expressed in units of 1000 $pixel^2$.

2.7. Wound healing assay

Confluent monolayers of cells were serum starved overnight. After washing with PBS, cells were scratched with yellow micropipette tips and cultured for 12 or 24 h. Images were captured at regular intervals during cell migration. The migration rate was quantified by the ratio of open space, which was not covered by migrated cells, using Image J software.

2.8. Transwell chamber migration and invasion assay

For the migration assay, 2×10^4 cells were seeded in the polycarbonate membrane insert of permeable supports with 8.0 μm pore size from Corning Inc. (New York, USA) and serum starved overnight before the assay. For the invasion assay, the inserts were coated with 50 μl Matrigel (BD Biosciences; New Jersey, USA) before seeding cells, and 600 μl culture medium with 10% FBS was added to the lower chamber of the transwell plate. After incubating for 24 h, the insert was washed gently with PBS and cells were removed from the top layer with cotton strips. Cells that have passed through the membrane were fixed by 4% PFA for 10 min and stained with 0.5% crystal violet for 10 min. Photos were taken randomly under phase contrast microscope and cell number per field was counted.

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