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DHHC protein-dependent palmitoylation protects regulator of G-protein signaling 4 from proteasome degradation

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ABSTRACT

Regulator of G-protein signaling 4 (RGS4), an intracellular modulator of G-protein coupled receptor (GPCR)-mediated signaling, is regulated by multiple processes including palmitoylation and proteasome degradation. We found that co-expression of DHHC acyltransferases (DHHC3 or DHHC7), but not their acyltransferase-inactive mutants, increased expression levels of RGS4 but not its Cys2 to Ser mutant (RGS4C2S). DHHC3 interacts with and palmitoylates RGS4 but not RGS4C2S *in vivo*. Palmitoylation prolongs the half-life of RGS4 by over 8-fold and palmitoylated RGS4 blocked α_{1A}-adrenergic receptor-stimulated intracellular Ca^{2+} mobilization. Together, our findings revealed that DHHC proteins could regulate GPCR-mediated signaling by increasing RGS4 stability.

Structured summary:
MINT-8049215: *Rgs4* (uniprotkb:P49799) *physically interacts* (MI:0915) with *DHHC3* (uniprotkb:Q8R173) by *anti-tag coimmunoprecipitation* (MI:0007)

1. Introduction

The basic unit of a G-protein coupled receptor (GPCR) signaling system contains four major components: receptor, G-protein, effector and regulator of G-protein signaling (RGS) protein [1]. G-proteins, classified into G_s, G_i, G_q and G_{12} subfamilies, stimulate intracellular signal proteins (effectors) when GTP binds to the G-protein in response to ligand-activation of GPCRs. Signaling ends when the G-protein hydrolyzes the bound GTP. RGS proteins increase GTP hydrolysis rates of G-proteins up to 1000-fold, thus profoundly inhibiting downstream consequences of GPCR activation.

Regulator of G-protein signaling 4 (RGS4) regulates signaling mediated by GPCR coupled to G_i and G_q, and plays an important role in regulation of the cardiovascular [2] and central nervous systems [3] as well as tumorigenesis [4]. We reported that RGS4 down-regulation in breast cancer cells is due to proteasome degradation, and that proteasome blockade increased RGS4 protein to levels that markedly inhibit breast cancer metastatic abilities [5]. Interestingly, RGS4 is not intrinsically unstable, but is made unstable by *N*-arginylation [6,7] at the oxidized N-terminal Cys (Cys2) [8] and then ubiquitinated and degraded by proteasomes [6,9]. Thus, identification of mechanisms underlying the regulation of oxidation-sensitive proteasome degradation of RGS4 is important.

RGS4 is also known to be palmitoylated, a reversible thioester attachment of palmitate to Cys in a protein. Previous studies demonstrated that the primary palmitoylation site of RGS4 was Cys2 [10,11]. Since oxidation of Cys2 is critical for RGS4 degradation [8], it would be important to determine whether palmitoylation regulates RGS4 protein stability.

Palmitoyl acyltransferases are integral membrane proteins with a conserved DHHC (Asp-His-His-Cys) motif, and are largely localized to Golgi [12]. The DHHC motif is crucial for enzyme activity since mutation of Cys to Ser eliminates palmitoyltransferase activity [13]. A recent study demonstrated that DHHC3 and DHHC7 can palmitoylate Gα to control Gα localization to the plasma membrane, thus regulating GPCR signaling [13]. In the present study, we sought to determine if DHHC proteins palmitoylate RGS4 and how this impacts RGS4 stability given the convergence of these processes on the Cys2 residue. Our data are the first to show that DHHC3 and DHHC7 are capable of palmitoylating RGS4 *in vivo*, which prolongs RGS4 half-life by interfering with its proteasome degradation, thus attenuating α_{1A}-adrenergic receptor (α_{1A}-AR)-stimulated intracellular Ca^{2+} mobilization.

Abbreviations: GPCR, G-protein coupled receptor; RGS4, regulator of G-protein signaling 4; RGS4C2S, RGS4 Cys2 to Ser mutant

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2. Materials and methods

2.1. Cell lines and reagents

HEK293 cells and MDA-MB-231 breast cancer cells were from American Type Culture Collection, and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS, Invitrogen). Primary antibodies include mouse anti-HA (Covance), rabbit anti-Myc (ABM), and goat anti-β-actin (Santa Cruz Biotechnology). Hydroxylamine, 2-bromopalmitate (2-BP), dithiothreitol (DTT), cycloheximide, and MG132 were from Sigma–Aldrich. 17-Octadecynoic acid (17-ODYA) was from Cayman Chemical Company.

2.2. Western blot analysis

Proteins were extracted from cells using 1× radioimmunoprecipitation assay lysis buffer (RIPA; Santa Cruz), subjected to 12% SDS–PAGE and transferred to Immobilon-FL membranes (Millipore). RGS4 was detected by its HA-tag while DHHC3 and DHHC7 were detected by their Myc-tags. IRDye700- or IRDye800-labeled secondary antibodies were used for protein band detection by a LI-COR Odyssey imaging system (LI-COR Biosciences).

2.3. 2-BP, DTT, and hydroxylamine treatment

HEK293 cells were transfected with RGS4 and DHHC3 for 30 h and then were treated without or with 2-BP (100 μM) for 4 h before extracting protein with RIPA buffer. For DTT or hydroxylamine treatment, cell lysate (30 μg) was incubated with DTT (200 μM), 0.5 M hydroxylamine (pH 7.0) or 0.5 M Tris–HCl (pH 7.0) at room temperature for 1 h, respectively. The mixtures were then analyzed by Western blot.

2.4. Hypoxia treatment and RGS4 degradation analysis [14]

Hypoxic conditions were established by evacuating oxygen from a sealed chamber with 5% CO_2 and 95% N_2. HEK293 cells transfected with RGS4 or RGS4 Cys2 to Ser mutant (RGS4C2S) with or without DHHC3 were cultured under hypoxia for 24 h to accumulate RGS4 protein. Then, the cells were cultured under nomoxia in the presence of cycloheximide (100 μM). The disappearance of the accumulated RGS4 protein pool was followed over time by Western blot.

2.5. Detection of palmitoylated RGS4 using Cu(I)-catalyzed azide-alkyne cycloaddition reaction (click chemistry)

HEK293 cells co-transfected with GFP-tagged DHHC3 and HA-tagged RGS4 were cultured in media containing 5% charcoal-stripped FBS for 24 h. The media was changed to serum-free DMEM for 1 h and then labeled with the lipid 17-ODYA (100 μM in DMEM and 1% fatty-acid free BSA) for 8 h. Cells were lysed with buffer (50 mM Tris–HCl, pH7.4, 150 mM NaCl, 2% Triton X-100, protease inhibitors). RGS4 was immunoprecipitated using anti-HA affinity agarose beads and eluted with 200 μg/ml HA peptide (Sigma–Aldrich).

Octadecynoylated RGS4 protein in the immunoprecipitates was linked to biotin-azide reporter groups via click chemistry [15]. Samples were analyzed by Western blot using anti-HA antibody to detect HA-tagged RGS4 and streptavidin-IRDye800 (LI-COR) to detect 17-ODYA labeled proteins via the attached biotin.

2.6. Co-immunoprecipitation assay

Myc-tagged DHHC3 was co-transfected with control vector, HA-tagged RGS4 or RGS4C2S mutant into HEK293 cells. After 30 h, cell lysates were immunoprecipitated with anti-HA affinity agarose beads. The immunoprecipitate was subjected to Western blot analysis for RGS4 and DHHC3.

2.7. Measurement of intracellular Ca^{2+} in HEK293 cells expressing α_{1A}-AR

HEK293 cells were stably transfected with mouse α_{1A}-AR and α_{1A}-AR expression was verified with [^{3}H]prazosin, as described [16]. Intracellular Ca^{2+} was measured using the fluorescent Ca^{2+} indicator Fluo-4. Fluorescence of cell suspensions (1×10^6 cells) were measured on a Varian Cary Eclipse fluorescence spectrometer at 30 °C using 485 nm excitation/515 nm emission. Maximum fluorescence was obtained with the calcium ionophore A23187 (2 μM). Changes in intracellular Ca^{2+} were quantified by the ratio of phenylephrine-induced peak fluorescence vs. the maximum fluorescence.

3. Results and discussion

3.1. Cys2 is critical for oxidation-sensitive proteasome degradation of RGS4 in cells

Breast cancer MDA-MB-231 cells have very low levels of endogenous RGS4 because of rapid proteasome degradation [5]. As shown in Fig. 1A, MDA-MB-231 cells transfected with C-terminal HA-tagged wild-type (WT) RGS4 also have very low levels of recombinant RGS4 unless grown under hypoxic conditions, when RGS4 levels approximate those seen in the presence of the proteasome inhibitor MG132. Mutating the Cys2 residue to Ser in RGS4 (RGS4C2S) eliminates susceptibility to oxidation such that RGS4 levels are high and similar during normoxia, hypoxia or

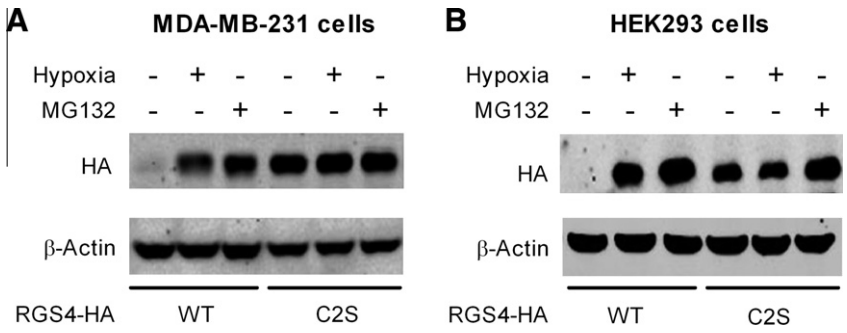

Fig. 1. Cys2 is critical for oxidation-sensitive proteasome degradation of RGS4 protein in MDA-MB-231 cells (A) and HEK293 cells (B). Cells were transfected with 1 μg plasmids encoding HA-tagged WT RGS4 or RGS4C2S mutant. Cells under hypoxia (+) or nomoxia (−) for 24 h were treated without or with 20 μM MG132 for 4 h. Proteins in cell lysate were subjected to Western blot analysis with anti-HA and anti-β-actin antibodies. Images shown are representatives of four separate experiments.