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 Gs, Gi, Gq and G12 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 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 α1A-adrenergic receptor-stimulated intracellular Ca²⁺ mobilization. Together, our findings revealed that DHHC proteins could regulate GPCR-mediated signaling by increasing RGS4 stability.

Keywords:
- G-protein coupled receptor
- Regulator of G-protein signaling 4
- Proteasome degradation
- Palmitoylation
- DHHC acyltransferases
- α1A-Adrenergic receptor
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). Hydroxyamine, 2-bromopropylate (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 hydroxyamine treatment, cell lysate (30 μg) was incubated with DTT (200 μM), 0.5 M hydroxyamine (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% CO₂ and 95% N₂. 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 normoxia 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, pH 7.4, 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).

Octadecenoylated 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 Ca²⁺ in HEK293 cells expressing α₁A-AR

HEK293 cells were stably transfected with mouse α₁A-AR and α₁A-AR expression was verified with [³H]prazosin, as described [16]. Intracellular Ca²⁺ was measured using the fluorescent Ca²⁺ indicator Fluo-4. Fluorescence of cell suspensions (1 × 10⁶ 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 Ca²⁺ 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...
proteasome blockade. Fig. 1B shows that this effect is not confined to MDA-MB-231 cells since similar results are seen in HEK293 cells. Thus, we confirmed that the Cys2 residue is critical for oxidation-sensitive proteasome degradation of RGS4 in cells [8].

3.2. Co-expression of active DHHC proteins increases expression levels of RGS4 but not RGS4C2S mutant

The Cys2 residue of RGS4 also is the primary target for palmitoylation [10,11]. We investigated whether palmitoylation affects RGS4 degradation. Palmitoylation of RGS4 in vivo has been shown in mammalian cells, but the acyltransferase for RGS4 palmitoylation is unknown. Recently, a family of acyltransferases characterized by a DHHC domain has been identified as enzymes for protein palmitoylation [17]. Fig. 2A shows the effects of Myc-tagged DHHC3 or DHHC7 expression on co-expressed HA-tagged RGS4 or RGS4C2S mutant in HEK293 cells. For WT RGS4 (left), expression of DHHC3 or DHHC7 led to large increases in RGS4 protein. In contrast, RGS4C2S protein is readily detected, and there was no effect of DHHC3 or DHHC7 expression on its levels (right).

Fig. 2. Co-expression of active DHHC proteins increases expression levels of RGS4 but not RGS4C2S mutant. HEK293 cells were transfected with HA-tagged WT RGS4 or RGS4C2S mutant in the absence or presence of Myc-tagged DHHC3, DHHC7 or their inactive C157S or C160S mutants. Proteins in cell lysate were analyzed by Western blot using anti-HA, anti-Myc and anti-β-actin antibodies. Images shown are representatives of three separate experiments. (A). Co-expression of DHHC3 or DHHC7 only caused WT RGS4 to accumulate in cells and migrate more rapidly during electrophoresis (B). Acyltransferase activity is critical for DHHC3 and DHHC7 to modulate RGS4.

Fig. 3. DHHC3 palmitoylates RGS4 in HEK293 cells. Cell lysates were analyzed by Western blot. Images are representatives of 3–6 separate experiments. (A). DHHC3 increased RGS4 protein in a dose-dependent manner, which was blocked by 2-BP (100 μM). (B). DTT or hydroxylamine reversed DHHC3-induced increase in the mobility of RGS4. (C). HEK293 cells co-transfected with GFP-tagged DHHC3 and RGS4 or RGS4C2S were metabolically labeled with 17-ODYA. RGS4 and RGS4C2S were immunoprecipitated and biotin labeled. Samples were treated with Tris–HCl (lanes 1–3) or hydroxylamine (lanes 4–6) for 1 h prior to Western blot analysis using anti-HA antibody and streptavidin-IRDye800. (D). DHHC3 forms complex with RGS4 but not RGS4C2S in HEK293 cells. Expression of DHHC3-Myc was analyzed by Western blot (upper panel), HA-tagged RGS4, RGS4C2S, and associated DHHC3-Myc were immunoprecipitated using anti-HA affinity agarose beads and detected by Western blot using anti-HA and anti-Myc antibodies, respectively (lower panel).
Interestingly, a comparison of RGS4 mobility on the SDS–PAGE gel reveals that co-expression of DHHC3 or DHHC7 causes RGS4 but not RGS4C2S mutant protein to migrate more rapidly during electrophoresis (lanes 2 and 3 vs. lanes 5 and 6).

The C157S mutation of DHHC3 or the C160S mutation of DHHC7 renders these proteins unable to palmitoylate [13]. As shown in Fig. 2B, WT Myc-tagged DHHC3 or DHHC7 maintained RGS4 protein levels and the increased rate of RGS4 band migration in comparison to the acyltransferase-inactive DHHC mutants. This suggests that the effects of DHHC proteins on RGS4 were probably caused by palmitoylation.

3.3. DHHC3 palmitoylates RGS4 in HEK293 cells

Fig. 3A shows DHHC3 dose effects on HA-tagged RGS4 band intensity and mobility on SDS–PAGE, effects that can be antagonized by treatment of cells for 4 h with 2-BP, a competitive inhibitor of palmitoylation. We also treated protein from DHHC3-transfected cells with DTT or hydroxylamine to cleave the thioester bond of palmitoylated proteins. As shown in Fig. 3B, DTT or hydroxylamine reduces mobility of the prominent faster migrating RGS4 band (lane 3 vs. lanes 1 and 2) to the same position as the RGS4C2S mutant (lane 4). This suggests that the mobility shift of RGS4 was probably due to palmitoylation.

Protein palmitoylation has historically been detected by incorporation of radiolabeled palmitate, but acetylene-fatty acids such as 17-ODYA have recently been shown to function as a metabolically incorporated probe for profiling protein palmitoylation in mammalian cells [15]. As shown in Fig. 3C, click chemistry-detected 17-ODYA incorporation into RGS4 was entirely dependent on Cys2 since this band was eliminated by the C2S mutation (lane 3 vs. lane 2), and by hydroxylamine (lane 5 vs. lane 2).

Co-immunoprecipitation of Myc-tagged DHHC3 with HA-tagged RGS4 was performed using anti-HA antibody-agarose beads. Data in Fig. 3D indicates that DHHC3 interacts with RGS4, but not RGS4C2S, again showing the importance of the Cys2 residue for DHHC3-dependent palmitoylation of RGS4.
3.4. DHHC3-dependent palmitoylation protects RGS4 from proteasome degradation

We next determined if increased RGS4 protein levels seen with palmitoylation were due to prolongation of RGS4 half-life. HEK293 cells with hypoxia-induced RGS4 accumulation were transferred to normoxia in the presence of cycloheximide to block new protein synthesis. The disappearance of pre-accumulated RGS4 was then followed over time by Western blot. Fig. 4A shows a representative Western blot indicating that RGS4 was degraded rapidly and that MG132 blocked degradation. In contrast, degradation of oxidation-resistant RGS4C2S mutant is much slower. Interestingly, co-expression of DHHC3 slowed the degradation rate of RGS4. RGS4 had a half-life of approximately 30 min while the half-lives of RGS4C2S mutant and RGS4 that was palmitoylated by DHHC3 exceeded 4 h (Fig. 4B). Thus, DHHC3-dependent palmitoylation protects RGS4 from proteasome degradation and extends its half-life by over 8-fold.

3.5. Palmitoylated RGS4 attenuates \( \alpha_{1A}-\text{AR} \)-dependent intracellular Ca\(^{2+} \) mobilization

\( \alpha_{1A}-\text{AR} \) predominantly couple to \( G_{o} \)-mediated phosphoinositide hydrolysis pathways which mobilize intracellular Ca\(^{2+} \) [18]. As shown in Fig. 5A, in HEK293 cells stably expressing \( \alpha_{1A}-\text{AR} \)-RGS4, the \( \alpha_{1A}-\text{AR} \) agonist phenylephrine caused a transient increase in the Ca\(^{2+} \) indicator Fluo-4 fluorescence that lasted approximately 1 min. Phenylephrine increased intracellular Ca\(^{2+} \) in a concentration-dependent manner (Fig. 5A, inset) with an EC\(_{50} \) of 77 ± 15 nM. The \( \alpha_{1A}-\text{AR} \) antagonist prazosin blocked phenylephrine-stimulated Ca\(^{2+} \) mobilization. Expression of RGS4 alone had little effect, presumably due to rapid degradation of expressed RGS4 protein. In contrast, co-transfection of RGS4 with DHHC3 resulted in 60% reduction of phenylephrine-stimulated intracellular Ca\(^{2+} \) as compared to cells expressing DHHC3 alone (Fig. 5B). Thus, palmitoylated RGS4 protein can attenuate \( \alpha_{1A}-\text{AR} \)-dependent Ca\(^{2+} \) mobilization in vivo.

4. Conclusion

We confirmed that RGS4 protein levels are regulated by the N-end rule for proteasomal degradation via a Cys2-dependent oxidation of RGS4 [6–9]. In addition, our study provides the first evidence that RGS4 is palmitoylated by DHHC3 and DHHC7 acyltransferases in vivo and that Cys2 of RGS4 is essential for this palmitoylation. Palmitoylation usually increases protein affinity for cell membranes as a possible regulatory mechanism. However, palmitoylation of RGS4 is not required for its attachment to the plasma membrane [19]. We demonstrated that the palmitoylation of RGS4 in vivo prolongs RGS4 half-life by interfering with its oxidation-dependent degradation, and that this palmitoylated RGS4 can attenuate functions of GPCRs such as \( \alpha_{1A}-\text{AR} \) in cells. Thus, the present findings revealed a novel mechanism by which DHHC acyltransferase-mediated palmitoylation. Moreover, our data raise the interesting possibility that in addition to controlling G-protein localization [13], DHHCs may also modulate GPCR signaling events through regulation of RGS4 stability.

Acknowledgements

Supported by National Institute of Health (R011CA125661), Nebraska State LB595, National Basic Research Program of China (2004CB720000) and the National Natural Science Foundation of China (30900246).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fleb.2010.10.052.

References