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Modification of p115RhoGEF Ser^{330} regulates its RhoGEF activity

Christina R. Chow [a,b], Nobuchika Suzuki [b], Takeshi Kawamura [b], Takao Hamakubo [c], Tohru Kozasa [a,b,*]

[a] Department of Pharmacology, University of Illinois at Chicago, 835 S. Wolcott Avenue Room E403 (m/c 868), Chicago, IL 60612, USA
[b] Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan
[c] Department of Quantitative Biology and Medicine, Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan

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ABSTRACT

p115RhoGEF is a member of a family of Rho-specific guanine nucleotide exchange factors that also contains a regulator of G protein signaling homology domain (RH-RhoGEFs) that serves as a link between Gα13 signaling and RhoA activation. While the mechanism of regulation of p115RhoGEF by Gα13 is becoming well-known, the role of other regulatory mechanisms, such as post-translational modification or autoinhibition, in mediating p115RhoGEF activity is less well-characterized. Here, putative phosphorylation sites on p115RhoGEF are identified and characterized. Mutation of Ser^{330} leads to a decrease in serum response element-mediated transcription as well as decreased activation by Gα13 *in vitro*. Additionally, this study provides the first report of the binding kinetics between full-length p115RhoGEF and RhoA in its various nucleotide states and examines the binding kinetics of phospho-mutant p115RhoGEF to RhoA. These data, together with other recent reports on regulatory mechanisms of p115RhoGEF, suggest that this putative phosphorylation site serves as a means for initiation or relief of autoinhibition of p115RhoGEF, providing further insight into the regulation of its activity.

1. Introduction

The regulator of G protein signaling homology domain containing Rho guanine nucleotide exchange factors (RH-RhoGEFs) is a subfamily of Dbl family RhoGEFs. Approximately 70 Dbl family GEFs have been identified thus far, each containing a tandem Dbl homology (DH)–Pleckstrin homology (PH) domain that catalyzes the exchange of GDP to GTP on Rho family small GTPases [1]. Comprised of three members in mammals: p115RhoGEF, leukemia-associated RhoGEF (LARG), and PDZ-RhoGEF, RH-RhoGEF protein expression is ubiquitous, though expression levels vary in different tissues. For example, p115RhoGEF is highly expressed in hematopoietic tissues [2], LARG is highly widespread, and PDZ-RhoGEF is most highly expressed in neuronal tissues in mice [3]. The RH-RhoGEFs have been shown to specifically activate RhoA, not Rac or Cdc42 [4]. Binding of GTP and subsequent activation of the Rho protein RhoA has been shown to lead to a variety of downstream signaling events, leading to cellular effects such as transformation, proliferation, migration, transcription, and apoptosis [5–7]. The RH-RhoGEFs also contain a regulator of G protein signaling homology (RH) domain at their N-terminus, which serves as a GTPase activating protein (GAP) for the alpha subunit of the heterotrimeric G proteins Gα12 and Gα13 [8]. Gα12/13 are the two members of the G12 family of heterotrimeric G proteins, which are activated upon stimulation of G protein-coupled receptors (GPCRs) such as the lysophosphatidic acid (LPA), protease-activated receptor-1 (PAR-1), angiotensin II, and sphingosine-1-phosphate (S1P) receptors [9–13]. Our lab and others have shown that binding of Gα13 to the RH domain of p115RhoGEF increases its nucleotide exchange activity towards RhoA *in vitro* [14], thus initiating the RhoA signaling cascade. Through their ability to terminate the signal from Gα12/13 and activate RhoA, the RH-RhoGEFs serve as a direct functional link between Gα12/13 signaling and RhoA.

Though the activation of p115RhoGEF by Gα13 is well-known, little else is known about how p115RhoGEF itself is regulated. Expression of p115RhoGEF is upregulated in prostate cancer cells compared to non-cancerous cells [15], and a somatic M165V mutation in its RH domain has been identified in colorectal cancer tumors [16]. There have been reports that p115RhoGEF can bind to proteins such as the hyaluronan receptor CD44v3 in MDA-MB-231 metastatic breast cancer cells [17] and the HIV transmembrane protein gp41 [18,19], modulating signaling pathways within the cell. Additionally, regulatory mechanisms such as homo-oligomerization [20,21], phosphorylation

Abbreviations: ATRA, All-trans retinoic acid; DH, Dbl homology; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; GAP, GTPase activating protein; HPLC–MS/MS, High pressure/tandem mass spectrometry; LARG, Leukemia-associated RhoGEF; LC/MS/MS, Liquid chromatography/tandem mass spectrometry; LPA, Lysophosphatidic acid; PAR-1, Protease-activated receptor 1; PDZ, Post-synaptic density protein, discs large protein, zonula occludens-1; PH, Pleckstrin homology; PMA, Phorbol 12-myristate 13-acetate; RH-RhoGEF, Regulator of G protein signaling homology domain-containing Rho guanine nucleotide exchange factor; RU, Response units; S1P, Sphingosine-1-phosphate; Sf9, *Spodoptera frugiperda*; SPR, Surface plasmon resonance; TLCK, Nα-tosyl-L-lysine chloromethyl ketone; TPCK, Nα-p-tosyl-L-lysine phenylalanine chloromethyl ketone.

* Corresponding author at: Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan. Tel.: +81 3 5452 5359.
E-mail addresses: crchow@uic.edu (C.R. Chow), suzuki-n@lsbm.org (N. Suzuki), kawamura@lsbm.org (T. Kawamura), hamakubo@lsbm.org (T. Hamakubo), kozasa@lsbm.org (T. Kozasa).

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[22–24], translocation to the plasma membrane [25–28], and auto-inhibition [29] have been shown to regulate the nucleotide exchange activity of p115RhoGEF. In most cases, however, the exact mechanisms by which these phenomena regulate the GEF activity of p115RhoGEF, and thus its role in cells, are not well elucidated.

2. Materials and methods

2.1. Generation of constructs

2.1.1. p115RhoGEF constructs

Single point mutations were introduced into the mammalian expression vector pcDNA3.1 myc containing human p115RhoGEF for use in SRE–luciferase and pulldown assays or the baculovirus transfer vector pFastBac HTc (Invitrogen) harboring human p115RhoGEF using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The S786/S863 double mutants were generated using either pcDNA3.1 myc-p115RhoGEF S786A or pcDNA3.1 myc-p115RhoGEF S786D as the template and the appropriate primers for S863A or S863D. Sense and anti-sense primer pairs used to generate each mutation are as follows:

S14A, 5′-CTCCCCAGGCCCCGCACGGCCTGGCCTGGTTCC-3′; and
5′-GGAACCAGGCCAGGCCGTGCGGGGCCTGGGGAG-3′;
S14D, 5′-CTCCCCAGGCCCCGATCGGCCTGGCCTGGTTCC-3′; and
5′-GGAACCAGGCCAGGCCGATCGGGGCCTGGGGAG-3′;
S330A, 5′-TCTCTGCACCCTCTGGCCCTGGACAGCCCAG-3′; and
5′-CTGGGCTGTCCAGGGCCAGAGGGTGCAGAGA-3′;
S330D, 5′-CTCTGCACCCTCTGGACCTGGACAGCCCAG-3′; and
5′-CTGGGCTGTCCAGGTCCAGAGGGTGCAGAG-3′;
S786A, 5′-CGAGAACCCCTCCTCGCCAGCTCTGAGAACGG-3′; and
5′-CCGTTCTCAGAGCTGGCGAGGAGGGGTTCTCG-3′;
S786D, 5′-CGAGAACCCCTCCTCGACAGCTCTGAGAACGG-3′; and
5′-CCGTTCTCAGAGCTGTGCAGGAGGGGTTCTCG-3′;
S833A, 5′-CCCTTCGGAAAGTGCTGGCCCTGAAGCAGCTTCTG-3′; and
5′-CAGAAGCTGCTTCAGGGCCAGCACTTTCCGAAGGG-3′;
S833D, 5′-CCCTTCGGAAAGTGCTGGACCTGAAGCAGCTTCTG-3′; and
5′-CAGAAGCTGCTTCAGGTCCAGCACTTTCCGAAGGG-3′;
S863A, 5′-GGGGGCGGGCCCCTGGCTCCAGCACGGACCCAGG-3′; and
5′-CCTGGGTCCGTGCTGGAGCCAGGGGCCCGCCCCC-3′;
S863D, 5′-GGGGGCGGGCCCCTGGATCCAGCACGGACCCAGG-3′; and
5′-CCTGGGTCCGTGCTGGATCCAGGGGCCCGCCCCC-3′. Mutated bases are underlined. Introduction of the desired mutation was confirmed by automated dideoxy sequencing of each construct.

2.2. Protein purification

2.2.1. Full-length Gα13

His_6-Gα13 FL was purified from Sf9 cells as described previously [30].

2.2.2. p115RhoGEF (wild-type)

His_6-p115RhoGEF was purified from Sf9 cells as described previously [30].

2.2.3. p115RhoGEF (mutants)

Sf9 cells expressing mutants of His_6-p115RhoGEF were resuspended in lysis buffer (20 mM HEPES, pH 8.0; 100 mM NaCl; 10 mM imidazole; 10 mM β-mercaptoethanol, 16 μg/mL PMSF, TPCK, and TLCK; and 3.2 μg/mL leupeptin and lima bean trypsin inhibitor) and lysed with a Potter-Elvehjem grinder. The lysate was clarified by centrifugation at 100,000 ×*g* for 30 min at 4 °C. The soluble portion of the lysate was diluted 2-fold with lysis buffer and applied to a nickel-NTA column pre-equilibrated with lysis buffer. The column was washed with 20 bed volumes of wash buffer (20 mM HEPES, pH 8.0; 400 mM NaCl; 20 mM imidazole; 10 mM β-mercaptoethanol; 16 μg/mL PMSF, TPCK, and TLCK; 3.2 μg/mL leupeptin and lima bean trypsin inhibitor), and bound protein was eluted with elution buffer (20 mM HEPES, pH 8.0; 400 mM NaCl; 300 mM imidazole; 10 mM β-mercaptoethanol; 16 μg/mL PMSF, TPCK, and TLCK; 3.2 μg/mL leupeptin and lima bean trypsin inhibitor). Peak fractions containing p115RhoGEF were pooled, concentrated and exchanged to exchange buffer (20 mM HEPES, pH 8.0; 150 mM NaCl; 10 mM β-mercaptoethanol; 10% glycerol; 16 μg/mL PMSF, TPCK, and TLCK; 3.2 μg/mL leupeptin and lima bean trypsin inhibitor) using a Spin-X UF 30k concentrator (Corning). The protein was aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C until use.

2.2.4. RhoA

GST-tagged RhoA was purified from *Escherichia coli* as described previously [30].

2.3. Generation of antibodies

Mouse monoclonal antibody Y0920, directed against the C-terminus of p115RhoGEF, was generated at the University of Tokyo by immunizing mice with peptides representing residues 812–912 of human p115RhoGEF isoform 2 as previously described [31].

2.4. Cell culture

2.4.1. Routine cell culture

HEK 293 and HeLa cells were cultured in DMEM + 10% FBS in 10 cm dishes.

2.4.2. Differentiation of HL-60 cells

HL-60 cells were cultured in RPMI + Glutamax (Gibco) in the presence of 10% FBS. Cells were treated with ATRA (Sigma-Aldrich) to a final concentration of 1 μM or PMA (Sigma-Aldrich) to a final concentration of 20 nM for the indicated time, harvested, and subjected to immunoprecipitation.

2.5. Biochemical experiments

2.5.1. Shotgun proteomics for native p115RhoGEF phosphorylation

p115RhoGEF mAb-crosslinked Protein G-conjugated magnetic beads were prepared as described [32] using α-p115RhoGEF mAb Y0920. Native p115RhoGEF was immunopurified from HL-60 cells treated with or without ATRA (1 mM) or PMA (20 nM) at the indicated timepoint, as previously described [32]. Basically, after the indicated amount of time, cells were harvested by centrifugation at 1000 ×*g* for 3 min at 4 °C and resuspended in 0.5% NP-40 lysis buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 0.2 mM EDTA, 0.1 mM DTT, 0.5% IGEPAL CA-630 [Sigma-Aldrich], 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 10 mM sodium orthovanadate, 16 μg/mL PMSF, TPCK, and TLCK, 3.2 μg/mL leupeptin and lima bean trypsin inhibitor), then subjected to immunoprecipitation with α-p115RhoGEF mAb. Immunoprecipitated proteins were eluted from the beads using RapiGest SF Surfactant (Waters Corporation) and precipitated using trichloroacetic acid. The resulting pellet was resuspended in SDS sample buffer and the proteins were analyzed by SDS-PAGE followed by Western blotting or staining with Sypro Ruby.

To analyze the p115RhoGEF phosphorylation sites, liquid chromatography–tandem mass spectrometry (LC/MS/MS) and a capillary reverse phase HPLC–MS/MS system (ZAPLOUS System; AMR Inc.), were performed as previously described [32]. For the identification of p115RhoGEF-specific phosphorylation sites, MS/MS data were processed with Mascot® software (version 2.1.04, Matrix Science) against the NCBInr database. Analysis of the data was carried out with Scaffold software (Proteome Software).