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Modification of p115RhoGEF Ser³³⁰ regulates its RhoGEF activity

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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 are less well-characterized. Here, putative phosphorylation sites on p115RhoGEF are identified and characterized. Mutation of Ser³³⁰ 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.

Keywords

p115RhoGEF; Gα13; RhoA; signal transduction

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1. INTRODUCTION

Regulator of G protein signaling homology domain containing Rho guanine nucleotide exchange factors (RH-RhoGEFs) are a sub-family 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 [5,6,7]. 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 G α 12 and G α 13 [8]. G α 12/13 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 [9,10,11,12,13]. Our lab and others have shown that binding of G α 13 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 G α 12/13 and activate RhoA, the RH-RhoGEFs serve as a direct functional link between G α 12/13 signaling and RhoA.

Though the activation of p115RhoGEF by G α 13 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 [22,23,24], translocation to the plasma membrane [25,26,27,28], and autoinhibition [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'-CCTGGGTCCGTGCTGGAGCCAGGGGCCCCGCCCCC-3'; S863D, 5'-GGGGGCGGGCCCCTGGATCCAGCACGGACCCAGG-3'; and 5'-CCTGGGTCCGTGCTGGATCCAGGGGCCCCGCCCCC-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 Ga13—His₆-Ga13 FL was purified from Sf9 cells as described previously [30].

2.2.2. p115RhoGEF (Wild-type) His₆-p115RhoGEF was purified from Sf9 cells as described previously [30]

2.2.3. p115RhoGEF (Mutants)—Sf9 cells expressing mutants of His₆-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 minutes 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 $\mu\text{g/mL}$ PMSF, TPCK, and TLCK; 3.2 $\mu\text{g/mL}$ 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 $\times g$ for 3 minutes 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 $\mu\text{g/mL}$ PMSF, TPCK, and TLCK, 3.2 $\mu\text{g/mL}$ 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 NCBI database. Analysis of the data was carried out with Scaffold software (Proteome Software).

2.5.2. SRE-Luciferase Assays—SRE-Luciferase assays were performed as described [33], with the following modification. HeLa cells were plated in DMEM supplemented with 10% FBS and transfected with 10, 50, or 100 ng of empty pcDNA3.1 myc vector or the indicated p115RhoGEF construct using Lipofectamine 2000 (Invitrogen). After 5 hours, media were changed to DMEM supplemented with 0.5% FBS and incubated for 16 hours before harvest. For immunoblotting of cell lysates, cells were harvested directly into 2X SDS sample buffer and boiled. Cell lysates were separated by SDS-page and myc-p115RhoGEF was detected by Western blotting with α -myc monoclonal antibody (clone 9E10, Covance).

2.5.3. In vitro RhoGEF assays—*In vitro* RhoGEF assays were performed as described [34]. [35 S]-GTP γ S (specific activity = 1,250 Ci/mmol) was obtained from PerkinElmer Life Sciences.

2.5.4. Surface Plasmon Resonance (SPR)—SPR assays were conducted using a Biacore T100. Anti-GST antibody was coupled to a Biacore CM5 Sensor Chip (GE Healthcare) using amine coupling chemistry according to the manufacturer's protocol. The coupling reactions were performed at 10 μ L/min at 25°C using Running Buffer (50 mM HEPES, pH 8.0 at 4°C, 150 mM NaCl, 1 mM EDTA, 10 mM MgCl₂). Flow cell surfaces were activated by a 1:1 mixture of N-hydroxy-succinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide for 5 minutes, followed by injection of α -GST antibody diluted to 30 μ g/ml in Immobilization Buffer (10 mM sodium acetate, pH 5.0) to a density of ~10,000 response units (RU). Residual binding sites on the chip surface were blocked with a 7 minute injection of 1 M ethanolamine. High-affinity GST binding sites on the α -GST antibodies were blocked by 3 sequential injections of recombinant GST (GE Healthcare) diluted to 5 μ g/mL, followed by a 2 minute regeneration using 10 mM glycine-HCl, pH 2.1.

Empty-nucleotide RhoA (GST-RhoA*) or GST-RhoA*GTP γ S were generated the day of the assay as described [35]. Basically, GST-RhoA*GDP protein was incubated in Exchange Buffer (50 mM HEPES, pH 8.0 at 4°C, 150 mM NaCl, 10 mM EDTA) in the presence or absence of excess GTP γ S for 30 min at 30°C. Exchange reactions were terminated by the addition of MgCl₂ to 20 mM, and the protein was kept on ice until used.

Kinetic assays were performed at 25°C. GST-RhoA was injected onto the α -GST antibody-coupled chip for 75 seconds at 10 μ L/min (yielding a density of 1000 RU), washed with Running Buffer supplemented with 10 μ M of the appropriate nucleotide for 30 seconds, followed by injection of p115RhoGEF for 90 seconds at 10 μ L/min and 2 minute dissociation using the Kinetics/Affinity Wizard in the Biacore Control Software. Regeneration of the chip surface was performed with a 2 minute injection of 10 mM glycine, pH 2.1 after each binding cycle. All data were corrected for non-specific binding by double subtracting binding responses collected from a blank reference cell as well as buffer injection over a GST-RhoA-immobilized flow cell. Data were fitted to a simultaneous k_a/k_d , 1:1 binding, and global fitting using the BIAevaluation program. Kinetic analyses were not performed when the responses were less than 1/10 of the wild-type p115RhoGEF-RhoA* interaction. The experiments were performed at least three times.

3. RESULTS

3.1. Upregulation of p115RhoGEF during HL-60 cell differentiation

Treatment of HL-60 cells with ATRA or PMA has been shown to cause differentiation to a neutrophil- or macrophage-like phenotype [36]. Upon induction of differentiation with either 1 μ M ATRA or 20 nM PMA, expression of p115RhoGEF was upregulated as early as 24 hours, and sustained through 4 days of treatment (Figure 1a). Though p115RhoGEF expression was upregulated in cells undergoing both treatments, differences can be observed between the two treatments. For example, p115RhoGEF from ATRA-treated cells seems to run slightly slower on an SDS-PAGE gel as compared to p115RhoGEF from untreated or PMA-treated cells. Induction of differentiation of HL-60 cells also resulted in the appearance of a second, higher molecular weight band when the samples were separated by SDS-PAGE. We confirmed that this higher molecular weight band is also p115RhoGEF by Western blotting using an α -p115RhoGEF antibody.

We extracted the bands corresponding to p115RhoGEF from an SDS-PAGE gel and subjected the samples to mass spectrometry using the Orbitrap LC-MS system (Thermo Fisher). After analysis using Scaffold (Proteome Software), we identified five serine residues that were phosphorylated in either ATRA- or PMA-treated cells (Figure 1b). Interestingly, the patterns of phosphorylation were unique to each treatment. Namely, Ser¹⁴, Ser³³⁰, Ser⁷⁸⁶, and Ser⁸³³ were found to be phosphorylated in both treated and untreated HL-60 cells, while Ser⁸⁶³ was phosphorylated after treatment with PMA or ATRA.

3.2. p115RhoGEF S330A and S330D exhibit impaired Rho activity in cells

Overexpression of p115RhoGEF has been previously shown to stimulate RhoA-dependent transcription from the SRE [37]. To explore the role that each of these serine residues plays in the activation of p115RhoGEF, we used QuikChange site-directed mutagenesis to generate the phospho-mimicking (from Ser to Asp) and phospho-defective (from Ser to Ala) mutations of each residue in a mammalian expression vector. Additionally, since these residues were phosphorylated in a differentiation-specific pattern, we made double mutations of Ser⁷⁸⁶/Ser⁸⁶³ to see if a combination of phosphorylation and dephosphorylation at these sites affected the activity of p115RhoGEF. HeLa cells, which endogenously express G α 13, RH-RhoGEFs, and Rho, were transiently co-transfected with plasmids encoding wild-type or mutant p115RhoGEF and a plasmid encoding SRE-directed expression of the luciferase reporter gene. Expression of luciferase was used as an indirect readout of p115RhoGEF-mediated Rho activity in cell lysates. Most of the mutations of p115RhoGEF did not significantly affect activation of Rho in this system (Figure 2a). However, when cells were transfected with S330A, we observed a ~50% decrease in Rho activity, and cells transfected with S330D exhibited a ~75% decrease in Rho activity (Figure 2b).

3.3. p115RhoGEF S330A and S330D fail to induce G α 13-mediated activation of RhoA in vitro

As p115RhoGEF directly activates nucleotide exchange on Rho [14], we investigated whether the Ser³³⁰ mutations rendered p115RhoGEF unable to catalyze nucleotide exchange

on RhoA. We performed an *in vitro* nucleotide exchange assay in which recombinant RhoA was incubated with recombinant p115RhoGEF or the mutants in the presence or absence of recombinant AlF_4^- -activated $\text{G}\alpha_{13}$. At 30°C, RhoA is known to spontaneously exchange GDP for $\text{GTP}\gamma\text{S}$. This exchange was accelerated upon introduction of 5 nM wild-type p115RhoGEF, and further increased by the addition of 100 nM $\text{G}\alpha_{13}$ (Figure 3a). Interestingly, the S330A or S330D mutations did not significantly affect the basal rate of nucleotide exchange compared to wild-type p115RhoGEF *in vitro*, though this decrease is consistent and trends towards significance. However, both the S330A and S330D mutants were significantly impaired in their nucleotide exchange activity in response to $\text{G}\alpha_{13}$ stimulation (Figure 3b–d). In contrast to the results of the SRE-luciferase assay, where S330A caused a ~50% decrease in Rho activation and S330D led to a ~75% decrease, both S330A and S330D exhibited similar exchange activity *in vitro*. However, we attribute this to the fact that the SRE-luciferase assay is an overexpression system which overrides the endogenous regulation mechanisms of p115RhoGEF activity in cells, while the *in vitro* system allows us to compare equivalent amounts of functional protein. Thus, we believe that while the results of the cell-based assays cannot be directly compared to the *in vitro* assays, the two are consistent in exhibiting that mutation at Ser³³⁰ of p115RhoGEF leads to a defect in nucleotide exchange activity.

3.4. The S330A and S330D mutants interact with $\text{G}\alpha_{13}$ in an activation-dependent manner

The best-characterized regulator of p115RhoGEF is $\text{G}\alpha_{13}$. In order to determine whether the decrease in SRE transcription and decreased activity in response to $\text{G}\alpha_{13}$ stimulation was due to a defect in the p115RhoGEF S330A or S330D mutants' ability to properly interact with $\text{G}\alpha_{13}$, we co-transfected HEK 293A cells with myc-p115RhoGEF wild-type or mutants and $\text{G}\alpha_{13}$ and performed a co-immunoprecipitation. Cells were harvested in the absence or presence of AlF_4^- , mimicking the inactive, GDP-bound $\text{G}\alpha_{13}$ or the "active" transition state of $\text{G}\alpha_{13}$. Wild-type p115RhoGEF, as well as the S330A and S330D mutants immunoprecipitated with $\text{G}\alpha_{13}$ in a similar activation-dependent manner, suggesting that the interaction between the mutants and $\text{G}\alpha_{13}$ was not disrupted by these mutations (Figure 4).

3.5. The S330A and S330D mutations alter the kinetics of binding to RhoA

We next performed surface plasmon resonance in order to further characterize the deficiencies in activation exhibited by the S330A and S330D mutants of p115RhoGEF. The binding affinity of p115RhoGEF to RhoA^*GDP , nucleotide-free RhoA (RhoA^*), or $\text{RhoA}^*\text{GTP}\gamma\text{S}$ was measured using a Biacore T100. Anti-GST antibody was coupled to the surface of a Series S CM5 sensor chip (both from GE Healthcare), and GST-RhoA in the appropriate nucleotide state was captured on the chip for 75 seconds at a rate of 10 $\mu\text{L}/\text{min}$. Recombinant wild type or Ser³³⁰ mutants of p115RhoGEF over a range of 10 nM to 1 μM were flowed over the chip at a rate of 10 $\mu\text{L}/\text{min}$ for 90 seconds, then allowed to dissociate for at least 2 minutes. All reactions occurred at 25°C. The kinetic curves were fit globally assuming 1:1 binding and analyzed using BIAevaluation software (GE Healthcare).

Consistent with previously published data [2,38], wild-type p115RhoGEF has relatively low affinity for RhoA^*GDP (Figure 5a) but binds with high affinity to RhoA^* (Figure 5d). The

affinity of this interaction is so high that the Biacore could not detect dissociation of p115RhoGEF from RhoA*, thus prohibiting the calculation of the kinetic constant, K_D . The S330A and S330D mutants bind RhoA* with an association constant very similar to that of wildtype ($4.39 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for wild-type, $3.59 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for S330A, and $4.34 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for S330D), though the interactions of S330A and S330D are of lower affinity, as evidenced by the lower total amplitude of the curves as compared to wild-type as well as measurable dissociation from RhoA*, which yields affinities of $2.84 \times 10^{-9} \text{ M}$ and $3.14 \times 10^{-9} \text{ M}$, respectively (Figure 5e,f, Table 1). Additionally, we were able to measure the binding affinity of p115RhoGEF S330A and S330D for RhoA*GTP γ S (Figure 5g-i). The curves generated for the interaction between wild-type p115RhoGEF and RhoA*GTP γ S did not appropriately fit the data curves, so we instead calculated the steady-state affinity for that interaction. Wild-type p115RhoGEF binds with an apparent K_d of $1.40 \times 10^{-6} \text{ M}$, while S330A and S330D bind with similar affinities of $4.92 \times 10^{-8} \text{ M}$ and $6.42 \times 10^{-8} \text{ M}$, respectively (Table 1).

4. DISCUSSION

The linker between the RH and DH-PH domains of p115RhoGEF is a string of approximately 169 amino acids, which is predicted to be flexible and have little secondary structure (Figure 6). Other Dbl family GEFs, such as proto-Dbl, Vav1, and p63RhoGEF have been shown to be autoinhibited by a variety of mechanisms, involving phosphorylation or binding of an activating protein to relieve this autoinhibition [39,40,41]. Among the RH-RhoGEFs, PDZ-RhoGEF has been shown to be autoinhibited by interaction of the RH domain with an acidic patch at the C-terminus of its linker region [42]. Recently, it has been reported that the linker region of p115RhoGEF also regulates its activity, however, the mechanism by which this autoinhibition occurs or is relieved is not well-defined [29]. Based on the data that we present in this study, we propose that a region of the linker around Ser³³⁰ plays a role in the autoinhibition of p115RhoGEF by coordinating the structure of the linker to bring the RH and DH-PH domains into an optimal orientation for nucleotide exchange on RhoA. Mutation of Ser³³⁰ constrains p115RhoGEF in a conformation that renders it less sensitive to activation by Gα13, thus maintaining a more inactive state. It is possible that the hydroxyl group of the serine residue forms contacts with other parts of the linker or another part of p115RhoGEF necessary for complete GEF activity, and mutating this residue disrupts these interactions. However, in the absence of a structure of full-length p115RhoGEF, either by itself or in complex with RhoA, it is difficult to accurately predict the exact role of Ser³³⁰ in coordinating the conformation of this protein.

This study also provides the first report of the binding kinetics of full-length p115RhoGEF and the various nucleotide states of RhoA. As predicted from previous reports [2,38], the affinity between p115RhoGEF and RhoA* is very high, such that it cannot be measured using our system. S330A and S330D also bind RhoA* with high affinity, though the kinetic parameters can be measured on the Biacore T100 and, therefore, we conclude that these interactions are of lower affinity than the wild-type. We also determined that the affinity between p115RhoGEF or the Ser³³⁰ mutants and RhoA*GDP is much lower than that of the p115RhoGEF-RhoA* interaction. Consistent with other reports of interactions between GTP γ S-loaded small GTPases and RhoGEFs [43,44], we demonstrate that wild-type

p115RhoGEF has some affinity for RhoA*GTP γ S. The S330A and S330D mutants have higher affinity and bind to RhoA*GTP γ S with a much different kinetic profile than the wild-type. The measured K_D for binding of the S330A and S330D mutants to RhoA*GTP γ S are 4.92×10^{-8} M and 6.42×10^{-8} M, respectively, while the calculated K_D for binding of wild-type p115RhoGEF to RhoA*GTP γ S is 1.40×10^{-6} M (Table 1). This observed change in kinetics may reflect a preference of wild-type, active p115RhoGEF for RhoA*, as would be expected, and of the mutated, inactive p115RhoGEF for RhoA*GTP γ S.

One explanation for this phenomenon is that the binding of p115RhoGEF to RhoA* may occur through a separate interface than the one that is required for the p115RhoGEF-RhoA*GTP γ S interaction, thus regulating its activity. This mechanism can be observed in crystal structures of the interaction between PDZ-RhoGEF and RhoA. In this case, RhoA*GTP γ S interacts exclusively with the PH domain [43], whereas RhoA* primarily interacts through the DH domain and makes minimal contact with the PH domain [43,45]. While no physiological consequence of the difference in these interactions has been observed, it is tempting to speculate that the differences in binding contribute to regulation of the GEF activity of PDZ-RhoGEF. It is possible that in p115RhoGEF, this interface is more solvent-exposed in the p115RhoGEF S330A or S330D mutants, thereby increasing its affinity for RhoA*GTP γ S. Our data suggest that the preferential binding of the G α 13-insensitive p115RhoGEF Ser³³⁰ mutants to RhoA*GTP γ S may act as a feedback mechanism to maintain an inactive GEF in the presence of adequate concentrations of activated RhoA within the cell, however, more work needs to be done to further explore this hypothesis.

Although much is known about the mechanism by which p115RhoGEF is activated by G α 13, little is known about other means that regulate p115RhoGEF activity. This study has identified new potential regulatory phosphorylation sites on p115RhoGEF, as well as characterized the putative phosphorylation site Ser³³⁰. We propose models for novel regulatory mechanisms of p115RhoGEF, which are supported by previous reports of mechanisms such as autoinhibition [29] and post-translational modification [22,23,24]. Additionally, we provide the first reports of the kinetic binding parameters of the p115RhoGEF-RhoA* and RhoA*GTP γ S interaction. However, given the wide range of these studies and the various cellular effects of p115RhoGEF, these mechanisms are likely to be complex. Ongoing studies to obtain a crystal structure of the complex of full-length p115RhoGEF with RhoA as well as to identify the kinases and/or phosphatases involved in its post-translational modification will be critical in further understanding the role of these mechanisms in regulating the activity of p115RhoGEF.

CONCLUSIONS

p115RhoGEF is expressed throughout the body and is implicated in several disease processes, including cancer, hypertension, and cardiac hypertrophy [15,17,22]. In this study, we have demonstrated that p115RhoGEF protein is upregulated and phosphorylated during the differentiation of HL-60 cells. Additionally, we showed that mutation of p115RhoGEF Ser³³⁰ inhibits its SRE transcriptional activity and the ability to activate RhoA in response to stimulation by G α 13. This report presents the first report of measured binding kinetics between full-length p115RhoGEF and RhoA* and RhoA*GTP γ S, as well as suggests that

mutating this serine residue shifts the binding preference of p115RhoGEF from RhoA* to RhoA*GTP γ S.

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The abbreviations used are

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	N α -tosyl-L-lysine chloromethyl ketone
TPCK	N α -p-tosyl-L-lysine phenylalanine chloromethyl ketone

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Highlights

- p115RhoGEF is upregulated during HL-60 differentiation with ATRA or PMA.
- Differentiation of HL-60 cells leads to specific phosphorylation of p115RhoGEF.
- Mutation of p115RhoGEF Ser³³⁰ decreases its activity in cells and *in vitro*.
- We report kinetic parameters of the p115RhoGEF-RhoA binding interaction.

Figure 1. p115RhoGEF is upregulated and specifically phosphorylated during HL-60 cell differentiation with ATRA or PMA

a) HL-60 cells were treated with 1 μ M ATRA or 20 nM PMA to induce differentiation to neutrophil- or macrophage-like cells, respectively. At the indicated timepoint, cells were harvested and lysates were subjected to immunoprecipitation for p115RhoGEF using anti-p115RhoGEF monoclonal antibody Y0920. Upregulation of p115RhoGEF was observed 24 hours after treatment and sustained through 4 days of treatment. Full phenotypic differentiation of HL-60 cells was obtained 4 days after induction of treatment. **b)** Serine residues 14, 330, 786, and 833 were constitutively phosphorylated in both treated and untreated HL-60 cells (black diamonds), while serine 863 was phosphorylated after PMA or ATRA treatment (white oval).

Figure 2. Mutation of p115RhoGEF S330 to alanine or aspartic acid decreases SRE-mediated transcription

HeLa cells were transfected with luciferase under the control of the SRE promoter along with empty vector or the indicated p115RhoGEF construct and grown in the presence of 0.5% FBS for 16 hours. Lysates were analyzed for luciferase activity and data were normalized to β -gal to account for differences in transfection efficiency. a) Representative panel of Ser to Ala or Ser to Asp mutations. S786D, 863D, and the S786/S863 double mutants have no effect on luciferase activity as compared to wild-type, while S330A significantly reduces luciferase activity. b) Dose-dependent increase in luciferase activity after transfection with 10, 50, or 100 ng of p115RhoGEF. Total cell lysate was immunoblotted for myc-p115RhoGEF to show equal expression of transfected constructs. Data are presented as the mean $\pm$ S.E.M. of triplicate determinations from one representative experiment after analysis using one-way ANOVA followed by Dunnett's post-test to determine significance between cells transfected with wild-type p115RhoGEF as compared to mutant transfected cells. Three independent experiments were performed, with similar results. *, $p < 0.05$; ***, $p < 0.001$.

Figure 3. p115RhoGEF S330 mutants have decreased RhoGEF activity *in vitro* in response to Ga13

Recombinant wild-type p115RhoGEF or S330 mutants were incubated with recombinant RhoA in the absence (gray dashed line) or presence (solid black line) of recombinant Ga13, and the amount of [³⁵S]-GTPγS bound to RhoA was determined by filter binding. Basal binding of [³⁵S]-GTPγS to RhoA is shown by the solid gray line. Results are shown as **a-c**) a timecourse of 20 minutes or **d**) a percentage of total [³⁵S]-GTPγS bound at 20 minutes. Data were analyzed using one-way ANOVA followed by Dunnett's post-test. **: $p < 0.01$ over wild-type p115RhoGEF; ***: $p < 0.005$ over wild-type p115RhoGEF.

Figure 4. p115RhoGEF S330A and S330D co-immunoprecipitate with Ga13

a) HEK 293T cells were transfected with Ga13 and the indicated myc-p115RhoGEF construct. Cell lysates were immunoprecipitated with anti-myc antibody 9E10 in the absence or presence of AlF₄⁻ to activate Ga13. Immunoprecipitates were separated by SDS-PAGE and analyzed by western blotting (anti-myc 9E10, anti-Ga13 B860). **b)** Quantitation of Ga13 co-immunoprecipitated with myc-p115RhoGEF wild-type or mutants. Data shown are representative of 4 independent experiments. Data were analyzed using one-way ANOVA followed by Dunnett's post-test. ^: not statistically significant compared to Ga13-GDP – wild-type p115RhoGEF; ns: not statistically significant compared to Ga13-GDP-AlF₄⁻ wild-type p115RhoGEF.

Figure 5. p115RhoGEF S330 mutants have altered binding kinetics to RhoA*GTP γ S compared to wild-type

Anti-GST antibody was coupled to a Series S CM5 sensor chip using the GST capture kit (all from GE Healthcare). Recombinant GST-RhoA was stripped of GDP and trapped in the nucleotide-free state or loaded with GTP γ S by incubating with 10 mM EDTA in the absence or presence of excess GTP γ S for 30 minutes at 30°C, then quenched by addition of MgCl₂ to 20 mM. For each injection, similar amounts (as determined by RU) of **a-c**, GST-RhoA*GDP; **d-f**, GST-RhoA*; or **h-j**, GST-RhoA*GTP γ S were captured on the chip, washed, then recombinant wildtype p115RhoGEF (**a, d, h**), S330A (**b, e, i**), or S330D (**c, f, j**) were injected onto the chip over a range of 10 nM - 1 μ M. The association time between GST-RhoA and p115RhoGEF or mutants was 90 seconds, followed by a dissociation time of 2 minutes. Binding and kinetics were determined using the Biacore T100 surface plasmon resonance system. *Red lines* indicate experimental data. *Black lines* indicate fitting data analyzed as simultaneous k_a/k_d , 1:1 binding, and local fitting using the BIAevaluation software (GE Healthcare). Data shown are representative of at least 3 experiments.

Figure 6. Prediction of disorder in the linker region of p115RhoGEF

The 169 residue linker region was submitted to analysis using the disorder prediction programs Regional Order Neural Network (RONN, available at <http://www.strubi.ox.ac.uk/RONN>), Prediction of Intrinsically Unstructured Proteins (IUPred, available at <http://iupred.enzim.hu/>), and DisEmbl (available at <http://dis.embl.de/>) or the secondary structure predicting algorithm PHD, available at <http://www.predictprotein.org/>). H indicates the predicted presence of a helical structure, d indicates disorder, and the numbers 1–9 indicate the probability of disorder where 1 is low and 9 is high. Ser³³⁰ is indicated in pink.

KINETIC PARAMETERS OF p115RhoGEF Ser³³⁰ MUTANTS BINDING TO RhoA

Table 1

ka, association constant; *kd*, dissociation constant; *KD* = *kd/ka*; equilibrium constant. Reported numbers are representative of rawdata generated from at least three unique runs and evaluated using BIAevaluation software, as indicated in Figure 5.

Protein	RhoA*GDP			RhoA*			RhoA*GTPγS		
	<i>ka</i>	<i>kd</i>	<i>KD</i>	<i>ka</i>	<i>kd</i>	<i>KD</i>	<i>ka</i>	<i>kd</i>	<i>KD</i>
	<i>M</i> ⁻¹ <i>s</i> ⁻¹	<i>s</i> ⁻¹	<i>M</i>	<i>M</i> ⁻¹ <i>s</i> ⁻¹	<i>s</i> ⁻¹	<i>M</i>	<i>M</i> ⁻¹ <i>s</i> ⁻¹	<i>s</i> ⁻¹	<i>M</i>
Wildtype	ND ^a	ND	ND	4.39 × 10 ⁴	§ ^b	§	4.26 × 10 ⁴	7.65 × 10 ⁻⁴	1.40 × 10 ⁻⁶
S330A	ND	ND	ND	3.59 × 10 ⁴	1.02 × 10 ⁻⁴	2.84 × 10 ⁻⁹	5.33 × 10 ⁴	2.62 × 10 ⁻³	4.92 × 10 ⁻⁸
S330D	ND	ND	ND	4.34 × 10 ⁴	1.36 × 10 ⁻⁴	3.14 × 10 ⁻⁹	4.36 × 10 ⁴	2.80 × 10 ⁻³	6.42 × 10 ⁻⁸

^a ND; kinetic analysis was not done because the response was less than 1/10 compared to the wild-type p115RhoGEF-RhoA* interaction.

^b §; unique dissociation constants could not be determined by the BIAevaluation software.