Published in final edited form as:

J Mol Biol. 2016 December 04; 428(24 Pt B): 4929–4945. doi:10.1016/j.jmb.2016.10.016.

Differences in the Phosphorylation-Dependent Regulation of Prenylation of Rap1A and Rap1B

Jessica M. Wilson¹, Jeremy W. Prokop², Ellen Lorimer¹, Elizabeth Ntantie³, and Carol L. Williams¹

¹Department of Pharmacology and Toxicology, Cancer Center and Cardiovascular Center, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226

²HudsonAlpha Institute for Biotechnology, 601 Genome Way NW, Huntsville, AL 35806

³College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, 1415 South Martin Luther King Jr. Boulevard, Tallahassee, FL 32307

Abstract

Two isoforms of the small GTPase Rap1, Rap1A and Rap1B, participate in cell adhesion; Rap1A promotes steady state adhesion, while Rap1B regulates dynamic changes in cell adhesion. These events depend on the prenylation of Rap1, which promotes its membrane localization. Here, we identify previously unsuspected differences in the regulation of prenylation of Rap1A versus Rap1B, due in part to their different phosphorylation-dependent interactions with the chaperone protein SmgGDS-607. Previous studies indicate that activation of G_{α_s} protein-coupled receptors (GPCRs) phosphorylates S-179 and S-180 in the polybasic region (PBR) of Rap1B, which inhibits Rap1B binding to SmgGDS-607 and diminishes Rap1B prenylation and membrane localization. In this study, we investigate how phosphorylation in the PBR of multiple small GTPases, including K-Ras4B, RhoA, Rap1A, and Rap1B, affects their binding to SmgGDS, with emphasis on differences between Rap1A and Rap1B. We identify amino acids in SmgGDS-607 necessary for binding of Rap1A and Rap1B, and present homology models examining the binding between Rap1A or Rap1B and SmgGDS-607. Unlike Rap1B, phosphorylation in the PBR of Rap1A does not detectably inhibit its prenylation or its binding to SmgGDS-607. Activation of GPCRs suppresses Rap1A prenylation, but unlike this effect on Rap1B, the GPCR-mediated suppression of Rap1A prenylation can occur independently of Rap1A phosphorylation and does not detectably diminish Rap1A membrane localization. These data demonstrate unexpected evolutionarily conserved differences in the ability of GPCRs to regulate the prenylation of Rap1B compared to Rap1A.

Graphical abstract

To whom correspondence should be addressed: Carol Williams, Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI, 53226, USA, Tel.: (414) 955-5640; Fax: (414) 955-6545; williams@mcw.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

protein isoprenylation; G protein-coupled receptor; SmgGDS; Rap1GDS1; polybasic region

INTRODUCTION

The small GTPases Rap1A and Rap1B are encoded by two different genes but have 95% sequence homology¹. Little is known about differences between Rap1A and Rap1B function or regulation, although recent studies suggest that Rap1A maintains steady state cell-cell junctions while Rap1B regulates dynamic changes in cell junctions^{2,3}. Differences in the sequences of Rap1A and Rap1B occur mainly in the C-terminal hypervariable region, which consists of the polybasic region (PBR) and the-CAAX box (Figure 1A). The-CAAX box contains the cysteine that is the site of prenylation, which is necessary for Rap1 membrane localization. Just upstream of the-CAAX box lies the PBR (Figure 1A), which is a string of basic amino acids that assists in binding of Rap1 to the chaperone protein SmgGDS⁴, although the exact binding location on SmgGDS is unknown.

SmgGDS is expressed as two isoforms due to mRNA splicing, named SmgGDS-607 and SmgGDS-558, which regulate prenylation of small GTPases. SmgGDS-607 binds non-prenylated small GTPases and assists in their prenylation, while SmgGDS-558 binds prenylated small GTPases and assists in their membrane localization⁵. Previous examination of SmgGDS-607 binding to another small GTPase, RhoA, identified a highly conserved negative patch on SmgGDS, which the authors labeled as the electronegative patch⁶. The electronegative patch is essential for interactions between the PBR of RhoA and SmgGDS⁶. It is likely that the electronegative patch plays a role in interactions between Rap1 and SmgGDS, but as the previous studies focused on the guanine nucleotide exchange factor (GEF) activity of SmgGDS on RhoA, and SmgGDS is not a GEF for Rap1, the authors did not specifically examine interactions between Rap1 and SmgGDS⁶.

We previously reported that the binding of Rap1B to SmgGDS-607 is diminished when protein kinase A (PKA) phosphorylates S-179 and S-180 in the PBR of Rap1B⁷. We reported that S-179 and S-180 in Rap1B are phosphorylated by treating cells with the general Gα_s activator Cholera toxin (Ctx), or by activating Gα_s protein-coupled receptors (GPCRs) using the adenosine receptor agonist 5'-N-Ethylcarboxamidoadenosine (NECA), or the β-adrenergic receptor agonist Isoproterenol (Iso). This phosphorylation inhibits binding of newly synthesized Rap1B to SmgGDS-607, prevents Rap1B prenylation, and diminishes the membrane localization of Rap1B^{7,8}. In contrast to Rap1B, which has two serines that can be phosphorylated in its PBR, Rap1A contains only one serine in its PBR,

S-180, which is also phosphorylated by PKA^{9, 10}. The effect of S-180 phosphorylation on the binding of Rap1A to SmgGDS-607 or on Rap1A prenylation has not yet been characterized.

SmgGDS binds several additional small GTPases that possess PBRs, including K-Ras4B and RhoA⁴. Both K-Ras4B and RhoA have phosphorylation sites in their PBRs. The phosphorylation of RhoA inhibits interactions with its downstream effectors^{11, 12}. The phosphorylation of K-Ras4B alters the subcellular localization of K-Ras4B as well as its binding to other proteins, such as calmodulin^{13–15}. The effects that PBR phosphorylation might have on the interactions of K-Ras4B and RhoA with SmgGDS have not been previously examined.

Here, we first examine differences in the evolutionary conservation of four small GTPases, Rap1A, Rap1B, K-Ras4B, and RhoA. Next, we examine predicted and *in vivo* differences in binding of Rap1A or Rap1B to SmgGDS as well as differences in binding after phosphorylation of Rap1A or Rap1B. These differences led us to study disparities in the phosphorylation-dependent regulation of the prenylation and localization of Rap1A versus Rap1B. Additionally, we examine whether phosphorylation of other small GTPases alters their binding to SmgGDS. Our findings uncover significant differences in the phosphorylation-dependent binding of Rap1A versus Rap1B to SmgGDS, and differences in their prenylation. Furthermore, we define different phosphorylation-dependent interactions of K-Ras and RhoA with SmgGDS.

RESULTS

Evolutionary Conserved Changes in Rap1A and Rap1B

An in depth evolutionary analysis is one of the best ways to determine conserved versus random differences in sequence, and therefore potentially discover differences in function between similar proteins. Here, we perform the first in depth evolutionary analysis of Rap1A versus Rap1B to elucidate conserved similarities and differences between these two similar Rap1 proteins, as well as examining conserved similarities and differences to two other similar small GTPases that both have a PBR and-CAAX box, K-Ras and RhoA. A sequence alignment of all available NCBI gene sequences for *Rap1A*, *Rap1B*, *RhoA*, and *K-Ras* was created. There were 122 usable species sequences for Rap1A, 131 usable species sequences for Rap1B, 162 usable species sequences for K-Ras, and 154 usable species sequences for RhoA. In the sequence alignment shown in Figure 1A, lime green highlighting indicates conservation between Rap1A and Rap1B but not K-Ras or RhoA, purple highlighting indicates conservation across all four small GTPases, cyan highlighting indicates functional conservation across three of the four small GTPases, and red highlighting indicates that the amino acid is conserved but different in both isoforms of Rap1. All of the conserved but different amino acids (red) are located in the highly variable C-terminus, in the labeled PBR, or the -CAAX box.

Next, we examined differences in nucleotides, instead of amino acids, using a codon selection analysis further gaining an understanding of selective evolutionary pressure between the two proteins. Figure 1B depicts the rate of non-synonymous change of

nucleotides minus the rate of synonymous change of nucleotides at each amino acid (dN-dS) of Rap1A and Rap1B codons combined. This analysis allows us to examine which amino acids stay the same or change due to selective pressure, and which changes are more likely to be random. For example, an amino acid may stay the same throughout all of the species, but if nucleotides encoding that amino acid never change, that amino acid may not be important to the structure and function of the protein but purely conserved based on never having a mutational event that could change the amino acid. On the other hand, if nucleotides encoding a particular amino acid change frequently, but the codon always encodes the same amino acid (codon “wobble”), this indicates that the amino acid is likely important to the structure and function of the protein. Therefore, those amino acids with the lowest normalized dN-dS, due to increased synonymous change relative to the total change, are those amino acids that are most functionally conserved. The most functionally selected amino acids are Q-22, N-47, and T-89, as labeled in Figure 1A and Figure 1B. While Figure 1B shows codon selection for both Rap1A and Rap1B proteins combined, the C-terminus codon selection was divided specifically into Rap1A and Rap1B in Figure 1C. Intriguingly, there is high selection for both S-179 and S-180 of Rap1B, more so than any other C-terminus amino acid in Rap1A and Rap1B. Further evolutionary analysis of all small GTPases is shown in a bootstrap analysis in Supplemental Figure 1. Structural models of all four small GTPases with the GNP bound are shown in Figure 2A. We next used molecular dynamic simulation, which examines the amount of movement or rigidity in each protein in water over 10 nanoseconds (Figure 2B), to determine regions of flexibility of Rap1A, Rap1B, K-Ras, and RhoA. This analysis indicated that in all four small GTPases examined (Rap1A, Rap1B, K-Ras, and RhoA), the PBR-CAAX box (amino acids 170–184 in Rap1, 176–188 in K-Ras, and 182–193 in RhoA) are much more fluid than the rest of the molecule as can be seen by the average movement of each carbon alpha over the simulation (Figure 2B, right). Movement over time was fairly equal for all small GTPases examined (Figure 2B, left). Lastly, the average movement for the amino acids stratified by color in Figure 1A were grouped and compared to each other (Figure 2C). This means that the average movement of amino acids that were conserved among all four small GTPases (purple) was compared to the amino acids conserved among three small GTPases (blue, partial conserved) versus amino acids only conserved in Rap1 (green) versus amino acids that are conserved but different in Rap1A or Rap1B (red). As the amino acids conserved in Rap1A or B, but not both (red) are all found in the PBR-CAAX box, the results in Figure 2B indicate that these amino acids have a large amount of flexibility, as corroborated in Figure 2C. There is also a trend showing that the amino acids conserved in all small GTPases (purple) have the lowest average movement, while those with partial conservation (cyan) have slightly more movement, and those only conserved in Rap1A and B (green) have slightly more movement still. This suggests that the amino acids unique to Rap1A and B (green) are generally more surface exposed, while those conserved among all four small GTPases are often more internal as they are necessary for the general small GTPase structure. This corresponds nicely with the model shown in Figure 1D.

Interaction of SmgGDS with Rap1A and Rap1B

Because SmgGDS-607 participates in the prenylation of Rap1A and Rap1B, and phosphorylation of S-179 and S-180 in Rap1B is known to inhibit binding to SmgGDS-607

and therefore prenylation^{5,7}, we next examined the differences in binding of Rap1A and Rap1B to SmgGDS-607 using computational docking and co-immunoprecipitation in cells. Due to the molecular dynamic simulation showing that the PBR-CAAX box region (amino acid 170–184) of Rap1A/B had significantly more movement than the rest of Rap1A/B, the stable region of both Rap1A and Rap1B (amino acids 1–169) was docked using rigid docking with the PBR-CAAX box allowed to have flexibility in movement. Figure 3A identifies amino acids found previously to be important for binding of RhoA to SmgGDS-607, including those negatively charged amino acids in the electronegative patch⁶. The docking model which is predicted to have the highest binding energy between Rap1A and SmgGDS-607 is shown in Figures 3B and 3C. In Figure 3C, acidic amino acids are red and basic amino acids are blue. The inset in Figure 3C has the basic amino acids in the PBR of Rap1A labeled, and shows clearly how the basic amino acids (blue) in Rap1A likely interact with the acidic amino acids (red) in SmgGDS-607 electronegative patch.

We next examined the immunoprecipitation of Rap1A or Rap1B with wildtype SmgGDS-607 or mutant SmgGDS-607 containing the point mutations (Figure 4) as indicated in Figure 3A. All values were normalized to the amount of Rap1A binding wildtype SmgGDS (Figure 4A) or Rap1B binding wildtype SmgGDS (Figure 4B). Since prenylated Rap1 migrates faster than non-prenylated Rap1 in SDS-PAGE, the prenylated and non-prenylated forms of Rap1B can be detected by a migration shift in immunoblots^{7,16}. As seen in the total Rap1B lysate form, there are doublets indicating that Rap1B continues to get prenylated by endogenous SmgGDS despite the mutation in the transfected SmgGDS and, for some mutations, lack of binding to transfected SmgGDS. This is likely due to the presence of endogenous SmgGDS in cells that continues to assist in prenylation of Rap1B. Similarly, the lysate of Rap1A is entirely in the prenylated form despite the mutations in transfected SmgGDS, some of which affect binding to Rap1A.

Intriguingly, the co-immunoprecipitation analysis suggests that certain amino acids in SmgGDS-607 are required for the binding of Rap1A but not Rap1B, or on the other hand, necessary for the binding of Rap1B but not Rap1A. For example, there was significantly less binding of Rap1B, but not Rap1A, to the M306A mutant of SmgGDS-607 and to the W324A + N328A mutant of SmgGDS-607, demonstrating that these amino acids are more important for binding Rap1B than for binding Rap1A. The D239K + E242K + E246K mutation in SmgGDS-607 significantly reduces binding of Rap1A, but does not significantly influence binding of Rap1B to SmgGDS-607, suggesting these amino acids are important for binding of Rap1A to SmgGDS-607. The importance of several of these amino acids in binding Rap1A and Rap1B is highlighted by our docking modeling between Rap1A or Rap1B to SmgGDS (Figure 5A). Amino acids D239, E242, and E246 are highlighted on SmgGDS, where three amino acid differences are observed between Rap1A and Rap1B (Figure 5), with high selection on K175 of Rap1A, A175 of Rap1B, K179 of Rap1A, and S179 of Rap1B that are predicted to coordinate the SmgGDS-607 amino acids, including amino acids D239, E242, and E246. These findings suggest the importance of the terminal–PBR region of Rap1A and Rap1B in binding to SmgGDS-607.

The Phosphomimetic Form of Rap1A Does not Change Binding to SmgGDS, but the Phosphomimetic Form of Rap1B, RhoA and K-Ras Inhibits Binding to SmgGDS in HEK-293T Cells

Phosphorylation of S-179 and S-180 in Rap1B has previously been shown to inhibit binding to SmgGDS-607⁷; however, the exact involvement of phosphorylation of serine-180 in Rap1A has not been studied. As shown in Figure 5B, the docking studies predict that phosphorylation of S-180 in Rap1A will have minimal alteration to binding of SmgGDS-607, while phosphorylation of Rap1B S-179 decreases interactions between Rap1B and SmgGDS-607. To test these predictions, we performed experiments examining the binding of wildtype and phosphomutant forms of Rap1A and Rap1B to SmgGDS-607 expressed in HEK-293T cells. HEK-293T cells were transfected with myc-small GTPases and HA-SmgGDS-607 or -558. Rap1A-A and Rap1B-AA are both phosphodeficient forms, with the serines in the PBR mutated to alanine, preventing phosphorylation. Rap1A-E and Rap1B-EE are both phosphomimetic forms, with the serines in the PBR mutated to glutamic acid, which has a negative charge mimicking phosphorylation. We found that phosphomimetic Rap1B-EE binds significantly less than wildtype Rap1B to SmgGDS-607 (Figure 6A), consistent with our previous report⁷. Shown here for the first time, phosphomimetic Rap1B-EE also binds significantly less than Rap1B-WT to SmgGDS-558, which is the SmgGDS splice variant that binds only prenylated small GTPases; as Rap1B-EE is not prenylated, it will not bind SmgGDS-558⁵ (Figure 6A). The phosphomutants of Rap1A bind as well as wildtype Rap1A to both SmgGDS-607 and SmgGDS-558 (Figure 6B).

SmgGDS is known to bind the PBR of several other small GTPases, including K-Ras4B and RhoA. Both of these small GTPases are phosphorylated in the PBR; RhoA is phosphorylated at S-188¹¹ and K-Ras4B is phosphorylated at S-181¹³ (Figure 5C), both sites of which are under high selection (RhoA S-188) or high conservation (K-Ras4B S/T-181). Similar to Rap1, we created phosphomutants with the indicated serine in the PBR mutated to alanine for a phosphodeficient mutant (RhoA-A and K-Ras-A), or the serine in the PBR mutated to glutamic acid for a phosphomimetic mutant (RhoA-E, K-Ras-E). Examination of SmgGDS binding to RhoA revealed significantly less binding of SmgGDS-607 to phosphomimetic RhoA-E, while no change was detected in binding of SmgGDS-558 to the phosphomutants of RhoA compared to wildtype RhoA (Figure 6C). Very intriguingly, examination of SmgGDS binding to K-Ras4B revealed significantly less binding of phosphomimetic K-Ras4B-E and less drastically, phosphodeficient K-Ras4B-A, to SmgGDS-607 but not to SmgGDS-558 (Figure 6D).

Prenylation Differences Between Rap1A and Rap1B

We found that SmgGDS-607 binds equally well to wildtype Rap1A and phosphomimetic Rap1A-E in HEK293T cells (Figure 6B). Since SmgGDS-607 assists in prenylation of Rap1⁵, we next examined if the phosphomimetic form of Rap1A suppresses prenylation of Rap1A, similar to the reduced prenylation of the phosphomimetic form of Rap1B⁷. HEK-293T cells were transfected with either myc-tagged Rap1A or Rap1B in the wildtype forms, or in the mutant forms that are prenylation deficient (SAAX mutant), phosphomimetic (Rap1A-E or Rap1B-EE), or phosphodeficient (Rap1A-A or Rap1B-AA).

Since prenylated Rap1 migrates faster than non-prenylated Rap1 in SDS-PAGE, the prenylated and non-prenylated forms of Rap1 can be detected by a migration shift in immunoblots^{7, 16}. In agreement with our previous results^{7, 8}, myc-Rap1B is approximately 75% prenylated (Figure 7A, lane 5), while phosphodeficient myc-Rap1B-AA is almost fully prenylated (Figure 7A, lane 6). Phosphomimetic myc-Rap1B-EE stays in a non-prenylated form (Figure 7A, lane 7) that migrates with the prenylation deficient Rap1B-SAAX mutant (Figure 7A, lane 8). In contrast to myc-Rap1B, we found that myc-Rap1A is fully prenylated (Figure 7A, lane 1). Phosphodeficient myc-Rap1A-A is similarly fully prenylated (Figure 7A, lane 2). We observed that both myc-Rap1A-A and myc-Rap1B-AA migrate slightly faster than the wildtype forms of Rap1A and Rap1B, respectively (compare lanes 1 versus 2, and lanes 5 versus 6, in Figure 7A). This faster migration of the phosphodeficient mutants probably results from the loss of the hydroxyl group on the serine in the PBR that is mutated to alanine in these phosphodeficient mutants. Interestingly, phosphomimetic myc-Rap1A-E migrates at the same rate as wildtype myc-Rap1A (Figure 7A, lanes 1 and 3), which is faster than the migration of non-prenylated myc-Rap1A-SAAX (Figure 7A, lane 4). These results suggest that the phosphorylation status of S-180 in Rap1A does not affect its prenylation, consistent with our observation that the phosphomimetic status of S-180 in Rap1A does not affect its binding to SmgGDS-607 (Figure 6B).

To examine the localization of Rap1A and Rap1B in cells, we transfected HEK-293T cells with GFP-Rap1A or GFP-Rap1B. As shown in Figure 7C, there is more membrane and junctional localization of GFP-Rap1A than GFP-Rap1B, which correlates with the increased steady state prenylation of Rap1A compared to Rap1B, as shown in Figure 7A. GFP-Rap1B has a perinuclear localization, as well as a cytoplasmic localization. This distribution is similar to the previously reported difference in the localization of Rap1A and Rap1B in HUVEC cells³.

Our docking predictions suggest the location of S-179 and not S-180 to be found at the interaction interface with SmgGDS-607 (Green, Figure 5A), with S-179 of Rap1B still having potential to hydrogen bond with SmgGDS-607. Computational modeling of the phosphorylation of S-179 and not S-180 can be seen to potentially alter this interaction with SmgGDS-607 (Figure 5B). Mutations of Rap1B with only S-179 or only S-180 were performed to examine the effect of phosphorylation of each individual serine, with Rap1B-AS representing the phosphodeficient form at only S-179, Rap1B-ES representing the phosphomimetic form of only S-179, Rap1B-SA representing the phosphodeficient form of only S-180, and Rap1B-SE representing the phosphomimetic form of only S-180. Figure 7C shows the results of both the prenylation of these single mutations of Rap1B, as well as their binding to SmgGDS-607. As expected, the mutation of S179 (Rap1B-ES) to the phosphomimetic form inhibits the prenylation of Rap1B, as it runs with the higher non-prenylated band of myc-Rap1B-WT and the single non-prenylated band for myc-Rap1B-EE, which is always non-prenylated. Intriguingly, the mutation of S180 (Rap1B-SE) to the phosphomimetic form also inhibits prenylation of Rap1B, supporting our previous studies showing the double mutation Rap1B-EE inhibits prenylation of Rap1B^{7, 8} but conflicting with our model in Figure 5B showing that only S-179, and not S-180, is involved in binding to SmgGDS-607. As further support that phosphorylation of both amino acids are important for binding to SmgGDS-607, only the double mutant Rap1B-EE trends towards a significant

decrease in binding to SmgGDS-607, while neither individual phosphomimetic mutant has any significant change in binding to SmgGDS-607 (Figure 7C).

G α_s Activation Induced by Cholera Toxin or the Adenosine Receptor Agonist NECA Phosphorylates S-180 of Rap1A

It was previously reported that Rap1B is phosphorylated in HEK293T cells by activating G α_s with Ctx, by activating adenosine receptors with NECA, or by activating β -adrenergic receptors with Iso^{7,8}. To determine whether these agonists also induce phosphorylation of Rap1A, we examined the effects of Ctx, NECA, and Iso on Rap1A phosphorylation. Myc-Rap1A is phosphorylated by treating HEK293T cells with Ctx (Figure 8A, lane 2) and NECA (Figure 8B, lane 2). This phosphorylation occurs on S-180, because neither Ctx nor NECA induces phosphorylation of the Rap1A phosphomutants that have S-180 mutated to alanine (Rap1A-A) or glutamate (Rap1A-E) (Figure 8A and B). Unlike the Iso-induced phosphorylation of Rap1B⁸, we did not detect Iso-induced phosphorylation of Rap1A (Figure 8A, lane 3). Examination of the prenylation-resistant myc-Rap1A-SAAX mutant indicates that it is phosphorylated in cells treated with Ctx (Figure 8A, lane 11), indicating that phosphorylation can occur before Rap1A is prenylated.

The immunoblotted proteins reveal some unexpected changes in their prenylation. Iso decreases steady-state Rap1A prenylation, as indicated by the Iso-induced migration shift of Rap1A in the immunoblot (Fig. 8A, lane 3). Since Iso does not induce detectable phosphorylation of Rap1A, we hypothesize that Iso inhibits Rap1A prenylation by some other G α_s -dependent event that does not involve Rap1A phosphorylation. In support of this hypothesis, treatment of the cells with NECA, Ctx, and Iso decreases the prenylation of both Rap1A-A and Rap1A-E, even though these Rap1A mutants are not detectably phosphorylated by the agonists. As expected, the Rap1A-SAAX mutant migrates with the upper band of all the other forms (Fig. 8A, lanes 10–12, and Fig. 8B, lanes 7 and 8), indicating that the top band of the other forms is the non-prenylated form of Rap1A.

G α_s Activation Can Inhibit Prenylation of Rap1A Independently of S-180 Phosphorylation

The studies described above examined steady state Rap1A prenylation, possibly resulting in underestimation of the effects of the agonists on prenylation. Therefore, we next examined the rate of prenylation instead of steady-state prenylation. A mevastatin-block-and-release assay was used to examine the rate of Rap1A prenylation. In this assay, cells transfected with the wildtype or mutant forms of Rap1A were treated with mevastatin for 18–22 hours, washed, and then treated with the agonists for the indicated times before the cells were lysed and examined for Rap1A prenylation. Mevastatin inhibits isoprenoid synthesis, but prenylation rapidly resumes when the cells are washed to remove the mevastatin. Previous studies using this assay indicated that treatment with Ctx, Iso, and NECA slows the rate of Rap1B prenylation in a phosphorylation-dependent manner, with phosphomimetic Rap1B-EE exhibiting minimal or undetectable prenylation in both the absence and the presence of the agonists^{7,8}. Intriguingly, Rap1A behaves differently than Rap1B in this assay, as shown in Figure 9. In striking contrast to phosphomimetic Rap1B-EE, we found that phosphomimetic Rap1A-E undergoes robust prenylation, resulting in similar rates of prenylation for wildtype Rap1A, phosphodeficient Rap1A-A, and phosphomimetic Rap1A-E

(Figure 9). Treatment with Ctx, Iso, or NECA slows the rate of prenylation of wildtype Rap1A (Figure 9), consistent with the results shown in Figure 8 indicating that these agonists inhibit steady-state prenylation of wildtype Rap1A. Most interestingly, we found that NECA significantly inhibits prenylation of wildtype Rap1A, phosphodeficient Rap1A-A, and phosphomimetic Rap1A-E, indicating that NECA suppresses prenylation regardless of the phosphorylation status of Rap1A (Figure 9). This finding provides strong evidence for the phosphorylation-independent suppression of Rap1A prenylation, consistent with the results shown in Figure 8. Taken together, these data indicate that $G\alpha_s$ activation inhibits prenylation of Rap1A in a manner mostly independent of Rap1A phosphorylation.

Localization of Rap1A Is Not Detectably Changed by S-180 Phosphorylation

We next examined the localization of the GFP-tagged wildtype and mutant forms of Rap1A in HEK293T cells (Figure 10). Visual inspection of the images indicated that wildtype Rap1A is localized at the plasma membrane and cell junctions (Figure 10), similar to the results shown in Figure 7B. Both phosphodeficient and phosphomimetic Rap1A are also localized at the plasma membrane and cell junctions (Figure 10), consistent with the robust prenylation of these Rap1A mutants (Figure 8 and 9). Ctx slightly diminishes the membrane and junctional localization of the wildtype and mutant forms of Rap1A, but the specific Adenosine 2_B Receptor agonist Bay 60–6582 and the β -adrenergic agonist Iso do not detectably alter Rap1A localization (Figure 10). These results agree with the prenylation data shown in Figures 8 and 9 which indicate that treatment with Ctx suppresses Rap1A prenylation more effectively than any other $G\alpha_s$ activator examined.

DISCUSSION

This study identifies intriguing differences in the interaction of Rap1A and Rap1B with SmgGDS-607, and the regulation of Rap1A and Rap1B prenylation. We previously reported that phosphorylation of Rap1B at S-179 and S180 inhibits the interaction of Rap1B with SmgGDS-607 and suppresses Rap1B prenylation ⁷. In contrast to Rap1B, our current studies indicate that phosphorylation of Rap1A at S-180 does not detectably alter the interaction of Rap1A with SmgGDS-607 nor does it alter Rap1A prenylation. Furthermore, we found that activation of GPCRs coupled to $G\alpha_s$ more effectively suppress the prenylation and membrane localization of Rap1B ^{7, 8} than Rap1A (Fig. 8–10). These differences in the mechanisms that regulate the prenylation and membrane localization of Rap1A and Rap1B help define why Rap1B participates in dynamic changes in cell adhesion induced by extracellular signals, while Rap1A is relatively insensitive to these signals and maintains steady-state adhesion ^{2, 3}.

These studies indicate that phosphorylation of both S-179 and S-180, as well as each individually, of Rap1B form a prenylation switch that is evolutionary conserved and different from Rap1A, whose prenylation is unaffected by phosphorylation of S-180. Our docking models (Figure 5) indicate that amino acid 179 of both Rap1A and Rap1B potentially binds SmgGDS-607, while amino acid 180 may not, initially suggesting that phosphorylation of S179 and not S180 would likely alter Rap1 interaction with SmgGDS. Co-immunoprecipitations in HEK-293T cells support part of this model, indicating that the

single phosphomimetic form of S-180 (Rap1B-SE) does not influence binding to SmgGDS-607, but surprisingly, the phosphomimetic form of only S-179 (Rap1B-ES) does not inhibit binding to SmgGDS-607 either. This suggests that phosphorylation of both amino acids S-179 and S-180 may be necessary to inhibit binding to SmgGDS-607. On the other hand, the presence of only one serine, S-180, in Rap1A prevents this mechanism for regulation of prenylation. This deviation in Rap1A and Rap1B regulation is found throughout the majority of analyzed vertebrates. Amino acid 179 of Rap1A is found as a K in 112 out of 122 vertebrate species analyzed, while Rap1B has an S/T at this location in 130 out of 131 species analyzed. The selection rate (dN-dS) of amino acid S179 on Rap1B (Figure 1C) suggests that throughout evolution a critical function is found for this amino acid. 32 (of 65 analyzed) additional invertebrate species have an S/T at amino acid 179, suggesting that the phosphorylation control at amino acid 179 is likely a deeply conserved Rap1B prenylation control switch that predates the invertebrate-vertebrate evolutionary divergence. Although the docking model (Figure 5B, right) predicts that S-180 may be less involved in regulation of binding to SmgGDS-607, it has a similar dN-dS value as S-179 (Figure 1C), suggesting that both amino acids may be evolutionarily conserved as a prenylation switch. This hypothesis is supported by our cellular data showing that the phosphomimetic form of both S-179 and S-180 (Rap1B-EE, Figure 6A), but not the phosphomimetic form of either individual serine (Rap1B-SE or Rap1B-ES, Figure 7C), inhibits binding to SmgGDS-607. On the other hand, K-179 and S-180 of Rap1A have a dN-dS value less indicative of a strong evolutionary conservation of function (Figure 1C). This is also supported by our observation that wildtype Rap1A and phosphomimetic Rap1A-E form equally stable complexes with SmgGDS-607 in HEK293T cells (Figure 6B) with no alteration of prenylation of phosphomimetic Rap1A-E (Figure 7A, Figure 8), further suggesting that amino acid S-180 is not involved in a phosphorylation-dependent prenylation control of Rap1A. However, as the dN-dS value of S-180 of Rap1A does suggest it may serve a conserved function, phosphorylation of Rap1A has already been shown to have an alternate function, with S-180 phosphorylation leading to decreased binding of Raf-1, allowing Raf-1 activation¹⁷.

A comparison between the molecular models shown here and the models shown in the studies conducted by Sondek and colleagues⁶ highlights some important considerations. Sondek's group identified a conserved electronegative patch in SmgGDS that they hypothesized was important for binding the PBR of RhoA, as well as a second highly conserved binding surface⁶. Both sites were important for the GEF activity of SmgGDS on RhoA⁶. The authors hypothesized that the conserved binding surfaces (including D240, E243, E247, E254, D256, M307, N343, H380, S384, N388, N395, K396) binds to the body of RhoA⁶, but they were unable to perform docking studies examining the full proteins. Our examination of the full protein sequences of Rap1A binding to SmgGDS-607 in Figure 3 identifies the same electronegative patch on SmgGDS as binding the PBR of Rap1, supporting that this region of acidic amino acids is conserved to allow functional connections between various small GTPases and SmgGDS.

The fact that a single phosphomimetic mutant can inhibit the prenylation of Rap1B without detectably inhibiting binding to SmgGDS-607 was somewhat surprising. These results suggest there may be another mechanism for regulation of prenylation that is still

phosphorylation-dependent but not reliant on binding to SmgGDS-607. These data agree with our previously suggested model that prenylation can occur, in some cases, without interactions involving SmgGDS-607⁵. Furthermore, prenylation of Rap1A is also modulated in a mechanism without apparently involving SmgGDS-607, as evidenced by the fact that activation of $G\alpha_s$ signaling through Ctx or NECA can inhibit prenylation in a non-phosphorylation-dependent manner.

Unexpectedly, we found that GPCRs can regulate the prenylation of Rap1A independently of the phosphorylation status of Rap1A. This response differs from Rap1B, whose prenylation is regulated almost exclusively by GPCR-dependent changes in Rap1B phosphorylation^{7,8}. GPCR activation may slow the prenylation of Rap1A independently of its phosphorylation status due to additional $G\alpha_s$ -mediated signals that inhibit the activities of other proteins, such as the prenyltransferase. It is intriguing to speculate that these additional $G\alpha_s$ -mediated signals might also slow the prenylation of Rap1B, but the effects of these phosphorylation-independent signals on Rap1B prenylation would not be detected easily due to the much stronger inhibitory effects that phosphorylation of Rap1B has on Rap1B prenylation.

We also examined changes in binding of SmgGDS to other small GTPases with phosphomimetic and phosphodeficient mutations. SmgGDS is known to act as a GEF for RhoA as well as regulating RhoA prenylation^{5,6}. RhoA is phosphorylated by both PKA and PKG^{11,12}. As we show here that the phosphomimetic form of RhoA inhibits binding to SmgGDS-607, but not SmgGDS-558, it is possible that phosphorylation of RhoA decreases activation of RhoA by inhibiting the GEF activity of SmgGDS on RhoA through preventing their interaction with each other. As support for this idea, several studies have shown that activation of the cGMP-dependent protein kinase, which phosphorylates S-188 on RhoA, can decrease RhoA activation, decrease stress fiber formation, and decrease the activation of downstream RhoA effectors^{11,18}. Furthermore, phosphorylation through PKA has also been shown to decrease RhoA membrane translocation^{11,12,19}. Future studies will examine whether this decrease in binding of SmgGDS-607 to phosphorylated RhoA alters the activation, prenylation, or other characteristics of RhoA. Of note, quantification of co-immunoprecipitations should be interpreted with caution due to the inherent multiple levels of normalization²⁰, but are best used for examining large differences in binding between mutations of the same molecule, such as the Rap1B-EE and K-Ras-E mutations in Figure 6. Therefore, when interpreting the results in Figure 4, it is likely most of the mutations of SmgGDS-607 which had a very large decrease in binding (which was most of the mutations) have a real effect on binding to Rap1B, while some like H379A+S383A which had a very small but significant effect may not actually influence binding. In the same way, many previous studies have consistently seen decreased binding of Rap1B-EE to SmgGDS-607^{7,8}, so the lack of statistical significance in Figure 7C is more likely a function of the inherent problem with quantification than an indication that the Rap1B-EE mutant does not lose binding to SmgGDS-607.

The function of SmgGDS interactions with K-Ras is still unknown. Studies indicate that SmgGDS may regulate K-Ras prenylation only under special circumstances⁵. There are several studies showing that phosphorylation of K-Ras can regulate its localization and

binding to other proteins, such as calmodulin^{13, 14}. Future studies will be needed to characterize how decreased binding of phosphorylated K-Ras to SmgGDS affects K-Ras or SmgGDS functions or localization.

Taken together, these results identify phosphorylation-dependent and-independent differences in the binding of Rap1A, Rap1B, and other small GTPases to SmgGDS. Our data indicate that the ability of SmgGDS-607 to bind these GTPases is inhibited more by the double phosphorylation of Rap1B than by the single phosphorylation of Rap1B or of Rap1A. These differences likely contribute to our finding that GPCRs that activate Gα_s very effectively suppress the prenylation and membrane localization of Rap1B in a phosphorylation-dependent manner^{7, 8}, whereas the same GPCRs less effectively regulate membrane localization of Rap1A.

METHODS AND MATERIALS

Cell Culture and Materials

Human embryonic kidney 293T (HEK-293T) cells were grown in Dulbecco's modified eagle medium (DMEM) with 1% penicillin/streptomycin, 1% sodium pyruvate and 10% FBS. Ctx (Calbiochem, Cat. No. 227035) was used at a concentration of 0.1 µg/ml, Iso was used at a concentration of 0.1 µM, NECA was used at a concentration of 10 µM, and Bay 60-6583 (Bay) (Tocris, Cat. No. 4472) was used at concentration of 0.1 µM unless otherwise stated. The antibodies used were monoclonal rabbit anti-Rap1B (Cell signaling, 36E1), mouse anti-c-myc (Santa Cruz Biotechnology, 9E10, sc-40), polyclonal rabbit anti-c-myc (Covance, PRB-150P-200), rabbit anti-HA (Covance, PRB-101P-500) and mouse anti-HA (Covance, MMS-101P-1000, 16B12). Mevastatin was used at a concentration of 10 µM.

Examination of Prenylation Differences

HEK-293T cells were plated in 12 well plates (2.0×10^5 cells/well). The next day, they were transfected with the indicated myc-Rap1A or myc-Rap1B complementary DNA (cDNA) using Lipofectamine 2000 (Invitrogen). They were lysed the next day and subjected to SDS-PAGE, then transferred to PVDF membranes, and then western blotting was performed. As the prenylated Rap1A or Rap1B migrates faster than the non-prenylated Rap1A or Rap1B during SDS-PAGE, as previously described⁷, the migration shift detected in immunoblots could be used to examine changes in Rap1A prenylation. Densitometry was conducted using ImageJ software and used to calculate the ratio of the lower prenylated band to the total doublet bands.

Analysis of Rap1A phosphorylation by in vivo ³²P labeling

The ³²P labeling assay was performed as described previously⁷. Briefly, HEK-293T cells were transfected with the indicated cDNA encoding myc-tagged Rap1A or myc-Rap1A mutant and incubated overnight. The next day, the cells were washed and phosphate-free DMEM containing 200 µCi/ml of ³²P-orthophosphate with or without 10 µM Iso, 10 µM NECA, or 0.1 µg/ml Ctx was added to the cells and incubated for 3 hours. The cells were then washed twice in PBS, lysed in NP-40 lysis buffer with phosphatase and protease inhibitors, and immunoprecipitated with rabbit-anti-Myc antibody (Sigma No.C3956) and

Protein A agarose beads (Invitrogen No. 15918-014). Samples were subjected to SDS-PAGE and transferred to PVDF membranes. After immunoblotting using a mouse-anti-Myc antibody (Abcam No. 18185), phosphoimages of the immunoblots were obtained using a Storm 820 Phosphoimager.

Fluorescence Microscopy

HEK-293T cells were plated on 35 mm MatTek glass bottom plates. Two days later, cells were transfected with cDNA encoding green fluorescent protein (GFP)-tagged Rap1A or Rap1B and treated with the indicated drug 90 minutes later. The next day they were imaged using a Nikon A1-R confocal microscope.

Mevastatin-block-and-release

The mevastatin-block-and-release assay was performed as described previously ⁷. HEK-293T cells were transfected with myc-tagged Rap1A and treated with 10 μ M mevastatin 90 minutes later. Mevastatin inhibits isoprenoid synthesis, therefore inhibiting prenylation. Cells were washed with phosphate buffered saline (PBS) 18–22 hours after the addition of mevastatin, re-plated with or without the indicated drug, and then lysed at the indicated time points after removal of mevastatin. Cell lysates were then subjected to SDS-PAGE, transferred to a PVDF membrane, and then western blotting was performed. Densitometry using ImageJ software was used to determine the ratio of prenylated Rap1A to total Rap1A.

Co-Immunoprecipitations

HEK-293T cells were transfected using Lipofectamine 2000 with cDNAs encoding HA-tagged SmgGDS and myc-tagged small GTPases. The next day, 90–100% confluent cells were lysed in an NP-40 lysis buffer and a portion of the total lysate fraction was taken. The remaining lysate was rocked with HA-agarose beads (Sigma) for at least 1.5 hours. Beads were washed four times and then boiled with 2x SDS-lysis buffer. Both the total fraction and the immunoprecipitates were subjected to SDS-PAGE, transferred to a PVDF membrane, and western blotting was performed using the rabbit anti-myc and rabbit anti-HA antibody. Densitometry was performed, and normalized as follows:

$$(\text{Optical Density (OD)})_{\text{Small GTPase in IP}} / \text{OD}_{\text{SmgGDS in IP}}$$

Sequence Analysis of Rap1A, Rap1B, RhoA, and K-Ras4B

Sequences for *Rap1A*, *Rap1B*, *RhoA*, and *K-Ras4B* were obtained from the NCBI gene database such that only a single open reading frame (ORF) DNA sequence per species was used. We used 122 sequences for Rap1A, 131 sequences for Rap1B, 162 sequences for K-Ras4B, and 154 sequences for RhoA. All 569 sequences were aligned with ClustalW (Codons) ²¹ in MEGA 5.10 ²². Codon selection analysis was performed on all sequences for the four proteins using the joint Maximum Likelihood reconstructions of ancestral states under a Muse-Gaut model ²³ of codon substitution and Tamura-Nei model ²⁴ of nucleotide substitution.

Protein Models for RAP1A, RAP1B, and SmgGDS

Homology modeling was performed using YASARA²⁵ for the full-length human Rap1A (UniProt accession P62834), Rap1B proteins (P61224), K-Ras4B (P01116), and RhoA (P61586) using YASARA2 knowledge-based force field minimizations of 5 merged models. Molecular dynamic simulations were then performed independently on all four models by placing the protein in a simulation box extended 5 Å from all atoms, pKa predictions for side chains at pH 7.4, water at 0.997g/mL, and NaCl at 0.9% mass fraction, using the AMBER03 force field calculations²⁶. Simulations were performed for 10 nanoseconds capturing atom positions every 25 picoseconds resulting in a total of 400 simulation snapshots. Analysis of these 400 simulation snapshots was performed, calculating global movement of carbon alphas at each time point and the average movement of each carbon alpha over the entire simulation. Identified regions from our sequence alignments were then grouped for average movement over all four proteins, calculating the standard error of the mean for each grouping.

Docking of Rap1A and Rap1B to SmgGDS-607

Docking was done using the full models for either Rap1A or Rap1B docked to the Sondek model of SmgGDS-607⁶. To dock the Rap1A model, the Rap1A model was trimmed to amino acids 1–171 removing the variable C-terminal amino acids 172–184 of the PBR and CAAX. Docking was then performed using AutoDock LGA²⁷ with 100 rigid ligands (Rap1A) docked to 5 ensembles of the receptor (Sondek model of SmgGDS-607). Clustered docking sites with alignments less than 1 Å were merged and AutoDock ranking performed. To dock the variable C-terminal region of Rap1A and Rap1B, amino acids of 172–184 for each were docked using AutoDock Vina²⁸ with 100 flexible ligands (either Rap1A 172–184 or Rap1B 172–184) docked to 5 ensembles of the receptor (Sondek SmgGDS-607). Docking was repeated by adding phosphorylation to S-179 of Rap1B (p1Rap1B), S-180 of Rap1A/B (p2Rap1A/B), or S-179 and S-180 of Rap1B (ppRap1B) and the docking analysis repeated for each modified peptide. In all, 3000 ligand predictions were calculated for SmgGDS-607 binding. The compiled docking of the rigid and flexible regions of Rap1A were merged, taking the highest ranked flexible PBR region located near the top rigid body docking, followed by 20 rounds of energy minimizations of the model in the AMBER03 force field.

Statistics

A two-way ANOVA was used to examine differences in prenylation rate in the mevastatin-block-and-release assay. To compare the binding of various small GTPase with mutations to the–WT form, the co-immunoprecipitations were first normalized as described in the co-immunoprecipitation section, then normalized to–WT, and then an one-way ANOVA with Dunnett's Multiple Comparison test was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported, in whole or in part, by funding from the Nancy Laning Sobczak, Ph.D., Breast Cancer Research Award, Medical College of Wisconsin (C.L.W.) and NIH grants R01CA188871 (C.L.W.) and K01ES025435 (J.W.P.). Jessica Wilson is a member of the Medical Scientist Training Program at the Medical College of Wisconsin, which is partially supported by training grant T32-GM080202 from the NIGMS. This work is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

The abbreviations used are

A₂BR	Adenosine A2B Receptor
βAR	β-adrenergic receptor
Bay	Bay 60-6583
Ctx	Cholera toxin
T_{1/2}	half time
Iso	Isoproterenol
GPCR	G protein-coupled receptors
PBS	Phosphate buffered saline
PBR	Polybasic region
PKA	Protein kinase A
Prop	Propranolol

References

1. Altschuler D, Lapetina EG. Mutational analysis of the cAMP-dependent protein kinase-mediated phosphorylation site of Rap1b. *J Biol Chem.* 1993; 268:7527–7531. [PubMed: 8463283]
2. Wittchen ES, Nishimura E, McCloskey M, Wang H, Quilliam LA, Chrzanowska-Wodnicka M, Hartnett ME. Rap1 GTPase activation and barrier enhancement in RPE inhibits choroidal neovascularization in vivo. *PloS One.* 2013; 8:e73070.doi: 10.1371/journal.pone.0073070 [PubMed: 24039860]
3. Wittchen ES, Aghajanian A, BurrIDGE K. Isoform-specific differences between Rap1A and Rap1B GTPases in the formation of endothelial cell junctions. *Small Gtpases.* 2011; 2:65–76. DOI: 10.4161/sgtp.2.2.15735 [PubMed: 21776404]
4. Williams C. The polybasic region of Ras and Rho family small GTPases: a regulator of protein interactions and membrane association and a site of nuclear localization signal sequences. *Cell Signal.* 2003; 15:1071–1080. DOI: 10.1016/S0898-6568(03)00098-6 [PubMed: 14575862]
5. Berg TJ, Gastonguay AJ, Lorimer EL, Kuhnmuench JR, Li R, Fields AP, Williams CL. Splice Variants of SmgGDS Control Small GTPase Prenylation and Membrane Localization. *J Biol Chem.* 2010; 285:35255–35266. DOI: 10.1074/jbc.M110.129916 [PubMed: 20709748]
6. Hamel B, Monaghan-Benson E, Rojas RJ, Temple BRS, Marston DJ, BurrIDGE K, Sondek J. SmgGDS Is a Guanine Nucleotide Exchange Factor That Specifically Activates RhoA and RhoC. *J Biol Chem.* 2011; 286:12141–12148. DOI: 10.1074/jbc.M110.191122 [PubMed: 21242305]
7. Ntantie E, Gonyo P, Lorimer EL, Hauser AD, Schuld N, McAllister D, Kalyanaraman B, Dwinell MB, Auchampach JA, Williams CL. An adenosine-mediated signaling pathway suppresses prenylation of the GTPase Rap1B and promotes cell scattering. *Sci Signal.* 2013; 6:ra39.doi: 10.1126/scisignal.2003374 [PubMed: 23716716]

8. Wilson JM, Lorimer E, Tyburski MD, Williams CL. β -Adrenergic receptors suppress Rap1B prenylation and promote the metastatic phenotype in breast cancer cells. *Cancer Biology & Therapy*. 2015; 16:1364–1374. DOI: 10.1080/15384047.2015.1070988 [PubMed: 26209110]
9. Hata Y, Kaibuchi K, Kawamura S, Hiroyoshi M, Shirataki H, Takai Y. Enhancement of the actions of smg p21 GDP/GTP exchange protein by the protein kinase A-catalyzed phosphorylation of smg p21. *J Biol Chem*. 1991; 266:6571–6577. [PubMed: 1901063]
10. Quilliam LA, Mueller H, Bohl BP, Prossnitz V, Sklar LA, Der CJ, Bokoch GM. Rap1A is a substrate for cyclic AMP-dependent protein kinase in human neutrophils. *J Immunol*. 1991; 147:1628–1635. [PubMed: 1908879]
11. Sawada N, Itoh H, Yamashita J, Doi K, Inoue M, Masatsugu K, Fukunaga Y, Sakaguchi S, Sone M, Yamahara K. cGMP-dependent protein kinase phosphorylates and inactivates RhoA. *Biochem Biophys Res Commun*. 2001; 280:798–805. DOI: 10.1006/bbrc.2000.4194 [PubMed: 11162591]
12. Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *EMBO J*. 1996; 15:510–519. [PubMed: 8599934]
13. Alvarez-Moya B, Lopez-Alcala C, Drosten M, Bachs O, Agell N. K-Ras4B phosphorylation at Ser181 is inhibited by calmodulin and modulates K-Ras activity and function. *Oncogene*. 2010; 29:5911–5922. DOI: 10.1038/onc.2010.298 [PubMed: 20802526]
14. Alvarez-Moya B, Barcelo C, Tebar F, Jaumot M, Agell N. CaM interaction and Ser181 phosphorylation as new K-Ras signaling modulators. *Small GTPases*. 2011; 2:99–103. DOI: 10.4161/sgtp.2.2.15555 [PubMed: 21776410]
15. Bivona TG, Quatela SE, Bodemann BO, Ahearn IM, Soskis MJ, Mor A, Miura J, Wiener HH, Wright L, Saba SG, Yim D, Fein A, Perez de Castro I, Li C, Thompson CB, Cox AD, Philips MR. PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-XL on mitochondria and induces apoptosis. *Mol Cell*. 2006; 21:481–493. DOI: 10.1016/j.molcel.2006.01.012 [PubMed: 16483930]
16. Gutierrez L, Magee AI, Marshall CJ, Hancock JF. Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *EMBO J*. 1989; 8:1093–1098. [PubMed: 2663468]
17. Hu CD, Kariya K, Okada T, Qi X, Song C, Kataoka T. Effect of phosphorylation on activities of Rap1A to interact with Raf-1 and to suppress Ras-dependent Raf-1 activation. *J Biol Chem*. 1999; 274:48–51. [PubMed: 9867809]
18. Gudi T, Chen JC, Casteel DE, Seasholtz TM, Boss GR, Pilz RB. cGMP-dependent protein kinase inhibits serum-response element-dependent transcription by inhibiting rho activation and functions. *J Biol Chem*. 2002; 277:37382–37393. DOI: 10.1074/jbc.M204491200 [PubMed: 12119292]
19. Aburima A, Wraith KS, Raslan Z, Law R, Magwenzi S, Naseem KM. cAMP signaling regulates platelet myosin light chain (MLC) phosphorylation and shape change through targeting the RhoA-Rho kinase-MLC phosphatase signaling pathway. *Blood*. 2013; 122:3533–3545. DOI: 10.1182/blood-2013-03-487850 [PubMed: 24100445]
20. Janes KA. An analysis of critical factors for quantitative immunoblotting. *Sci Signal*. 2015; 8:rs2.doi: 10.1126/scisignal.2005966 [PubMed: 25852189]
21. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007; 23:2947–2948. btm404 [pii]. [PubMed: 17846036]
22. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. 2011; 28:2731–2739. DOI: 10.1093/molbev/msr121 [PubMed: 21546353]
23. Muse SV, Gaut BS. A likelihood approach for comparing synonymous and nonsynonymous nucleotide substitution rates, with application to the chloroplast genome. *Mol Biol Evol*. 1994; 11:715–724. [PubMed: 7968485]

24. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol.* 1993; 10:512–526. [PubMed: 8336541]
25. Krieger E, Joo K, Lee J, Lee J, Raman S, Thompson J, Tyka M, Baker D, Karplus K. Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: four approaches that performed well in CASP8. *Proteins: Structure, Function, and Bioinformatics.* 2009; 77:114–122. DOI: 10.1002/prot.22570
26. Duan Y, Wu C, Chowdhury S, Lee MC, Xiong G, Zhang W, Yang R, Cieplak P, Luo R, Lee T. A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations. *Journal of Computational Chemistry.* 2003; 24:1999–2012. DOI: 10.1002/jcc.10349 [PubMed: 14531054]
27. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry.* 2009; 30:2785–2791. DOI: 10.1002/jcc.21256 [PubMed: 19399780]
28. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry.* 2010; 31:455–461. DOI: 10.1002/jcc.21334 [PubMed: 19499576]

Research Highlights

- Structural evolutionary conserved differences in Rap1A and Rap1B are examined.
- C-terminal polybasic region differences regulate binding to SmgGDS and prenylation.
- Phosphorylation of serine 180 does not inhibit prenylation of Rap1A, unlike Rap1B.
- GPCR activation inhibits Rap1A prenylation independently of S-180 phosphorylation.

FIGURE 1. Evolutionary conservation analysis of Rap1A and Rap1B reveals that the functionally conserved differences between Rap1A and Rap1B are located in the C-terminal PBR and CAAX box

(A) Sequence alignments of 122 Rap1A sequences, 131 Rap1B sequences, 154 RhoA sequences, and 162 K-Ras sequences. A black asterisk (*) indicates conservation within the isoform, and a red asterisk (*) indicates conservation between sequences for both Rap1A and Rap1B isoforms. A period (.) indicates loose conservation. A lime green highlight indicates conservation between Rap1A and Rap1B but not K-Ras or RhoA. A purple highlight indicates conservation across all four small GTPases, A red highlight indicates that the amino acid is conserved but different between the two Rap1 isoforms. The three amino acids with labels above the sequences are functionally conserved in Rap1. **(B)** Codon selection analysis of all amino acids in the 253 Rap1 sequences that were examined. The graph depicts the functional conservation of each codon, as indicated by negative values of normalized dN–dS. dN–dS is the rate of non-synonymous change of nucleotides minus the rate of synonymous change of nucleotides at each amino acid. The three functionally conserved amino acids that are labeled in the graph are the same as those labeled in A. **(C)** Codon selection analysis of the C-terminal amino acids for Rap1A and Rap1B. The graph depicts the functional conservation of each codon, as indicated by negative values of normalized dN–dS. The functionally conserved amino acids in Rap1B that are labeled in the graph (S179 and S180) are the same as those labeled p1 and p2 in A. **(D)** Model of Rap1A. The color scheme is identical to that of the labels in part A. A gray GTP is added.

FIGURE 2. Molecular dynamic simulation of Rap1A and Rap1B reveals that the C-terminal PBR/CAAX box is flexible, while amino acids 1-171 are rigid

(A) Model of each small GTPase with GNP bound. (B) The rigidity of Rap1A, Rap1B, K-Ras, and RhoA was examined using a molecular dynamic simulation. Total movement of the entire protein over time (left) and movement of each amino acid over a fixed amount of time (right). (C) Average side chain RMSD movement of each group of amino acids as labeled in Figure 1A. Bars indicate standard error of the mean for all sites in color groupings from Figure 1 over all four proteins.

FIGURE 3. Homology modeling predicts that the binding energy between Rap1A and SmgGDS-607 is higher than the binding energy between Rap1B and SmgGDS-607
(A) A model of SmgGDS-607, with red highlighting identifying the amino acids previously reported to participate in binding of RhoA. **(B)** The model depicting the highest energy binding between Rap1A (cyan) and SmgGDS-607 (grey with the same amino acids highlighted in red as in A). The inset shows the PBR-CAAX box of Rap1A (cyan) with the same SmgGDS-607 amino acids highlighted in red as in A. **(C)** Similar model as depicted in B, with acidic amino acids in SmgGDS-607 highlighted in red, and basic amino acids in SmgGDS-607 highlighted in blue. The armadillo repeat that is present in SmgGDS-607 but absent in SmgGDS-558 is highlighted in green. The inset depicts the labeled basic amino acids in the PBR of Rap1A interacting with the red acidic amino acids in SmgGDS-607.

FIGURE 4. Identification of amino acids in SmgGDS-607 that are required for binding to Rap1A and Rap1B

HEK-293T cells were transfected with a cDNA encoding myc-Rap1A (**A**) or myc-Rap1B (**B**), plus a cDNA encoding HA-tagged SmgGDS-607 possessing the wildtype (WT) sequence or the indicated mutation. Cell lysates were immunoprecipitated using HA antibody, and the immunoprecipitates were immunoblotted using myc antibody (to detect myc-tagged Rap1 proteins) and HA antibody (to detect HA-tagged wildtype or mutant SmgGDS-607). The graphs depict the relative amount of myc-Rap1A (**A**) or myc-Rap1B (**B**) that co-precipitated with wildtype or mutant SmgGDS-607-HA, normalized to the amount of myc-Rap1A (**A**) or myc-Rap1B (**B**) that co-precipitated with wildtype SmgGDS-607-HA, as described in *Methods*. Each graph presents data from three independent experiments. Representative immunoblots are shown beneath the graphs. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

Figure 5. Modeling of PBR-CAAX Box of Rap1A and Rap1B and Sequence Alignment of PBR-CAAX Box of Rap1A, Rap1B, RhoA, and K-Ras4B

(A, B) A model of the PBR-CAAX box of Rap1A and Rap1B binding to SmgGDS with acidic amino acids shown in red, basic amino acids shown in dark blue, and serines that can be phosphorylated shown in lime green. Line between S179 of Rap1B and D239 of SmgGDS in (A) represents possible hydrogen bond. In (B), the serines are phosphorylated, changing the configuration of S179 and preventing the possible hydrogen bond between S179 on Rap1B and D239 on SmgGDS that was previously seen in part (A). (C) Sequence alignment of indicated number of species of the PBR-CAAX box of Rap1A, Rap1B, RhoA, and K-Ras4B. Normalized-(dN-dS) is shown below each amino acid, with larger values indicating greater conservation. Cyan indicates basic amino acid, lime green represents serine phosphorylation site.

FIGURE 6. Phosphomimetic form of Rap1B, RhoA, and K-Ras, but not Rap1A, diminishes binding to SmgGDS-607 in HEK-293T Cells

HEK-293T cells were transfected with a cDNA encoding HA-tagged SmgGDS-607 or SmgGDS-558, plus a cDNA encoding the indicated myc-tagged GTPase with a wildtype sequence (WT) or with a phosphodeficient mutation (AA or A) or phosphomimetic mutation (EE or E) in the PBR. Cell lysates were immunoprecipitated using HA antibody, and the immunoprecipitates were immunoblotted using myc antibody (to detect the myc-tagged GTPase) and HA antibody (to detect HA-tagged SmgGDS). The graphs show the relative amount of each myc-tagged GTPase that co-precipitated with HA-tagged SmgGDS, normalized to bound HA-tagged SmgGDS and then the amount of the wildtype myc-tagged GTPase in the immunoprecipitates, as described in *Methods*. Each graph presents data from three to nine independent experiments.

FIGURE 7. Rap1A exhibits more prenylation and membrane localization than Rap1B at steady state in HEK-293T cells

(A) HEK-293T cells were transfected with the indicated wildtype or mutant form of myc-tagged Rap1A or Rap1B, and cell lysates were immunoblotted using myc antibody. Different exposures of the chemiluminescence reaction during immunoblotting are indicated by the times listed at the left of the immunoblots. The prenylated (pren) and non-prenylated (np) forms of the GTPases were identified by their different migration rates. The immunoblots are representative of 3 independent experiments. (B) HEK-293T cells expressing GFP-tagged Rap1A or Rap1B were imaged by fluorescence microscopy. The region inside the box in the top image is enlarged in the bottom image. The results are representative of at least 3 experiments. (C) HEK-293T cells were transfected with a cDNA encoding HA-tagged SmgGDS-607, plus a cDNA encoding the indicated myc-tagged Rap1B with a wildtype sequence (WT) or with a phosphodeficient mutation (AA, AS, or SA) or phosphomimetic mutation (EE, ES, or SE) in the PBR. The first letter indicates the mutation at S-179 and the second indicates the mutation at S-180. Cell lysates were immunoprecipitated using HA antibody, and the immunoprecipitates were immunoblotted using myc antibody (to detect the myc-tagged Rap1B) and HA antibody (to detect HA-tagged SmgGDS). The graphs show the relative amount of each myc-tagged GTPase that co-precipitated with HA-tagged SmgGDS, normalized to bound HA-tagged SmgGDS and then the amount of the wildtype myc-tagged GTPase in the immunoprecipitates, as described in *Methods*. The western blot shows the total Rap1B lysates and the prenylated (pren) and non-prenylated (np) forms of the GTPases were identified by their different migration rates. The immunoblot is representative of 3 independent experiments.

FIGURE 8. Treatment with Ctx and NECA, but not Iso, promotes phosphorylation of Rap1A at S-180
HEK-293T cells expressing the indicated myc-tagged wildtype or mutant Rap1A proteins were pulsed with ³²P orthophosphate for 3 hours in the presence or absence of Ctx (0.1 μg/ml) or Iso (10 μM) (**A**) or in the presence or absence of NECA (10 μM) (**B**). The myc-tagged GTPase was immunoprecipitated and immunoblotted using myc antibody. The immunoblot was analyzed by phosphoimaging to detect the ³²P-labeled proteins in the immunoblot. The results are representative of two independent experiments.

FIGURE 9. G_{aq} Activation by Ctx, Iso, or NECA can suppress prenylation of Rap1A independently of the phosphorylation status of S-180 in Rap1A

HEK-293T cells expressing the indicated myc-tagged wildtype or mutant Rap1A proteins were incubated with mevastatin (10 μ M, 20–24 hr), washed, and then incubated in the presence or absence of Ctx (0.1 μ g/ml) (A), Iso (10 μ M) (B), or NECA (10 μ M) (C). Cell lysates were collected at the indicated times and immunoblotted using myc antibody. The faster migrating form of Rap1A detected in the immunoblots is prenylated (p), whereas the slower migrating form of Rap1A is non-prenylated (np). Densitometric analysis of the immunoblots provided the values used to calculate the ratio of prenylated Rap1A to total Rap1A, which are graphed to the right of the immunoblots. Data are the mean $\pm$ SEM, with $n=3$. A two-way ANOVA with Bonferroni Multiple Comparisons, examining time and drug, was performed on the ratios of prenylated to total Rap1A. Significance shown on the graphs is the Bonferroni Multiple Comparison p-value due to the effect of Ctx, NECA, or Iso as indicated, with ** indicating $p<0.01$ and **** indicating $p<0.0001$. Time effects were significant by two-way ANOVA for all three drug treatments. * on Western Blot indicates molecular weight marker of 26 kDa.

FIGURE 10. The membrane localization of Rap1A is not detectably altered by mutation of S-180 or by treatment with Iso or Bay, but is slightly diminished by treatment with Ctx
HEK-293T cells were transfected with the indicated forms of GFP-tagged wildtype or mutant Rap1A, and cultured overnight in the absence or presence of Ctx (0.1 $\mu\text{g/ml}$), Iso (10 μM), or Bay (0.1 μM). The cells were imaged by fluorescence microscopy. All images are the same magnification and representative of three independent experiments.