

# Activation of p115-RhoGEF Requires Direct Association of $G\alpha_{13}$ and the Dbl Homology Domain<sup>\*[5]</sup>

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**Background:** p115-RhoGEF can be regulated by activated  $G\alpha_{13}$ .

**Results:** Both RGS and DH domains of p115-RhoGEF interact with  $G\alpha_{13}$ .

**Conclusion:** The binding of RGS to  $G\alpha_{13}$  facilitates direct association of  $G\alpha_{13}$  to DH to regulate its exchange activity.

**Significance:** RGS domains can act cooperatively with other domains to mediate effector regulation by G proteins.

RGS-containing RhoGEFs (RGS-RhoGEFs) represent a direct link between the  $G_{12}$  class of heterotrimeric G proteins and the monomeric GTPases. In addition to the canonical Dbl homology (DH) and pleckstrin homology domains that carry out the guanine nucleotide exchange factor (GEF) activity toward RhoA, these RhoGEFs also possess RGS homology (RH) domains that interact with activated  $\alpha$  subunits of  $G_{12}$  and  $G_{13}$ . Although the GEF activity of p115-RhoGEF (p115), an RGS-RhoGEF, can be stimulated by  $G\alpha_{13}$ , the exact mechanism of the stimulation has remained unclear. Using combined studies with small angle x-ray scattering, biochemistry, and mutagenesis, we identify an additional binding site for activated  $G\alpha_{13}$  in the DH domain of p115. Small angle x-ray scattering reveals that the helical domain of  $G\alpha_{13}$  docks onto the DH domain, opposite to the surface of DH that binds RhoA. Mutation of a single tryptophan residue in the  $\alpha 3b$  helix of DH reduces binding to activated  $G\alpha_{13}$  and ablates the stimulation of p115 by  $G\alpha_{13}$ . Complementary mutations at the predicted DH-binding site in the  $\alpha B$ - $\alpha C$  loop of the helical domain of  $G\alpha_{13}$  also affect stimulation of p115 by  $G\alpha_{13}$ . Although the GAP activity of p115 is not required for stimulation by  $G\alpha_{13}$ , two hydrophobic motifs in RH outside of the consensus RGS box are critical for this process. Therefore, the binding of  $G\alpha_{13}$  to the RH domain facilitates direct association of  $G\alpha_{13}$  to the DH domain to regulate its exchange activity. This study provides new insight into the mechanism of regulation of the RGS-RhoGEF and broadens our understanding of G protein signaling.

RGS-RhoGEFs<sup>3</sup> are a homologous subfamily of RhoGEFs (guanine nucleotide exchange factors for Rho proteins) that contain regulator of G protein signaling (RGS) domains. There are three members of this subfamily: p115-RhoGEF (p115), PDZ-RhoGEF (PRG), and leukemia-associated RhoGEF (LARG). They represent potential regulatory links between G protein-coupled receptors that activate the  $G_{12}$  class of heterotrimeric G proteins and RhoA-mediated pathways that lead to cytokinesis and transformation (1, 2). RGS-RhoGEFs catalyze the exchange of GDP for GTP on RhoA, a small GTPase of the Ras superfamily (3). Activated RhoA bound to GTP can then engage downstream effectors and influence cellular functions. Like all members of the large family of RhoGEFs (about 70 in the human genome), the GEF activity of RGS-RhoGEFs resides in their tandemly linked Dbl homology (DH) and pleckstrin homology (PH) domains (4, 5). The RGS homology (RH) domains are situated N-terminal to the DH/PH domains. The RH domains of p115 and LARG function as GTPase-activating proteins (GAPs) for  $G\alpha_{13}$  and  $G\alpha_{12}$  subunits, and binding of  $G\alpha$  subunits to their respective RhoGEFs stimulates their guanine nucleotide exchange activity toward RhoA (6–9). In addition to the RH domain, PRG and LARG also contain an N-terminal PDZ domain that has been shown to mediate interaction of the RGS-RhoGEFs with regulatory proteins (10–12).

The exact mechanism by which  $G\alpha_{13}$  stimulates the exchange activity of p115 remains elusive (7–9). Studies on interactions between RGS-RhoGEFs and activated  $G\alpha_{13}$  have focused primarily on the RH domain, which led to elucidation of crystal structures of the RH domain alone and complexes between RH and  $G\alpha_{13}$  (13–16). The RH domains in RGS-RhoGEFs share low sequence similarity to the canonical RGS domain and require elements outside of the consensus RGS box for proper folding and binding to  $G\alpha_{13}$ . In p115, these include the <sup>23</sup>IIG and <sup>27</sup>EDED motifs located N-terminal to the con-

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<sup>3</sup> The abbreviations used are: GEF, guanine nucleotide exchange factor; RGS, regulators for G protein signaling; RH, RGS homology domain; GAP, GTPase-activating protein; DH, Dbl homology domain; PH, pleckstrin homology domain; PDZ, post synaptic density protein (PSD95); *Drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1); mant-GTP, *N*-methylanthraniloyl-GTP; SAXS, small angle x-ray scattering; p115, p115-RhoGEF; PRG, PDZ-RhoGEF; LARG, leukemia-associated RhoGEF; GTP $\gamma$ S, guanosine 5'-O-(thiotriphosphate).

sensus RGS box and the  $^{163}$ MGM motif C-terminal to the RGS box (see Fig. 1A). When bound to  $G\alpha_{13}$ , RH occupies both the regulator binding and effector binding sites on the GTPase, mainly through direct interaction with its Ras-like domain. The presence of RH is crucial for stimulation of the GEF activity by  $G\alpha_{13}$ , as the activity of a p115 fragment missing the RH domain could not be regulated by  $G\alpha_{13}$  (17). As expected, mutations within the  $^{27}$ EDED motif resulted in the loss of GAP activity and a reduction in binding affinity of p115 toward  $G\alpha_{13}$ . The same mutations, however, had little impact on the stimulation of GEF activity by  $G\alpha_{13}$  (18). This raises the question of whether direct association between RH and  $G\alpha_{13}$  is actually required for the stimulation of GEF activity; it also suggests that regions outside of RH in p115 might interact with  $G\alpha_{13}$  during the activation process. There is evidence that activated  $G\alpha_{13}$  binds weakly to regions outside of RH; however, it has not been shown that the GEF activity of p115 is regulated by such interactions (9, 17).

Here, a combination of molecular cloning, biochemical assays and small angle x-ray scattering (SAXS) was used to examine the interaction of activated  $G\alpha_{13}$  with a C-terminally truncated p115 molecule that includes the tandemly linked DH and PH domains in addition to RH. We show that the  $^{23}$ IIG and the  $^{163}$ MGM hydrophobic motifs, respectively, located N- and C-terminal to the consensus RGS box rather than the  $^{27}$ EDED motif are crucial for the stimulation of the GEF activity of p115 by activated  $G\alpha_{13}$ . Furthermore, activated  $G\alpha_{13}$  interacts with the DH domain via a novel effector binding site located within its helical domain. Mutations of residues in the  $\alpha$ B- $\alpha$ C loop of the helical domain negatively affect stimulation of p115 by  $G\alpha_{13}$ . The additional binding site in p115 for  $G\alpha_{13}$  is located on the side of DH opposite to the binding site for the substrate RhoA. Mutations of residues in the predicted binding site for  $G\alpha_{13}$  on DH abolished stimulation of GEF activity by  $G\alpha_{13}$ . These observations provide a comprehensive model for the molecular mechanism by which the intrinsic exchange activity of RGS-RhoGEFs is regulated by activated G proteins.

## EXPERIMENTAL PROCEDURES

**Protein Expression and Purification**—Coding regions of human p115-RhoGEF were subcloned into a pGEX-KG vector containing the protease recognition site for the tobacco etch virus (pGEX-KG-TEV) for proteolytic cleavage of the expressed domains from glutathione *S*-transferase as described previously (19, 20). His<sub>6</sub> tags were also inserted at the C termini of the p115 coding sequences. The proteins were expressed and purified from *Escherichia coli* strain BL21(DE3) as described (20). The expression and purification of a C-terminally truncated human RhoA (residues 1–181) was carried out as described previously (19). The N-terminally truncated  $G\alpha_{13}$  (41–377) was expressed and purified from insect cells as described (13).

**Nucleotide Exchange Assay**—Fluorescence assays measuring the binding of *N*-methylanthraniloyl-GTP (mant-GTP; Invitrogen) were performed on a Fluorolog-3 spectrofluorometer at room temperature ( $\lambda_{\text{ex}} = 356$  nm,  $\lambda_{\text{em}} = 445$  nm, slits = 1/1 nm), as described previously (20). In each assay, 1–2  $\mu$ M RhoA was incubated with 5  $\mu$ M mant-GTP in reaction buffer (25 mM

NaHEPES, pH 8.0, 50 mM NaCl, 1 mM DTT, and 5 mM  $\text{MgCl}_2$ ) in a 200  $\mu$ l cuvette. The exchange reaction was started by the addition of 100 nM p115, in the presence or absence of activated  $G\alpha_{13}$ .

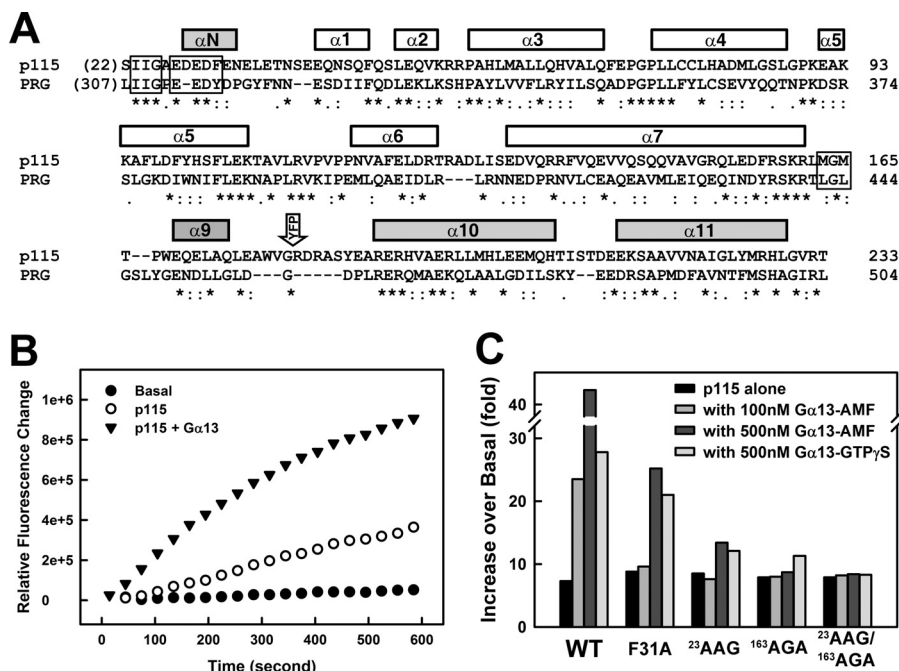
**Binding of GTP $\gamma$ S to  $G\alpha_{13}$** —Purified  $G\alpha_{13}$  was exchanged into binding buffer (20 mM NaHEPES, pH 8, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, and 10  $\mu$ M GDP) and concentrated to 100–250  $\mu$ M. The concentrate was adjusted to 0.5 mM  $\text{MgSO}_4$  and 1 mM GTP $\gamma$ S and incubated at 25 °C for 48–72 h.

**Size Exclusion Chromatography with p115 and  $G\alpha_{13}$ -GTP $\gamma$ S**—Fragments of p115 were mixed with activated  $G\alpha_{13}$  bound to GTP $\gamma$ S, in a buffer containing 25 mM NaHEPES, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5 mM  $\text{MgCl}_2$ . The mixture was concentrated by Amicon-Ultra 4 (10 kDa) concentrators (Millipore) to a final volume of less than 1 ml and then loaded onto Superdex 200/75 columns (Amersham Biosciences) that had been pre-equilibrated with the same buffer.

**Collection of SAXS Data**—The purified RH-L-DH/PH fragment of p115 was dialyzed overnight in 25 mM NaHEPES, pH 8.0, 200 mM NaCl, 5 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 10 mM DTT, and 5% glycerol to ensure a precise buffer match for background subtraction of the scattering arising with the buffer alone from that of the protein sample. The complex of p115 RH-L-DH/PH and activated  $G\alpha_{13}$  bound to  $\text{GDP}\cdot\text{AlF}_4^-\cdot\text{Mg}^{2+}$  was dialyzed overnight in 25 mM NaHEPES, pH 8.0, 200 mM NaCl, 5 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 10 mM DTT, 0.05 mM  $\text{AlCl}_3$ , 5 mM  $\text{MgCl}_2$ , 5 mM NaF, and 5% glycerol. Triton X-100 was added to the sample to a final concentration of 0.5% before the measurement to prevent aggregation. The addition of Triton X-100 had no effect on the stimulation of GEF activity by activated  $G\alpha_{13}$  (supplemental Fig. S1). The samples for SAXS were at a concentration between 1 and 4 mg/ml. Measurements were taken at 10 °C using the SAXS instrument at the BioCAT beamline of the Advanced Photon Source at the Argonne National Laboratory as described previously (20). The samples were analyzed by size exclusion chromatography before and after the SAXS measurements to determine concentration and oligomerization state. Scattering profiles (intensity *I* versus scattering vector *Q*) were reduced, and the SAXS data were merged using IGOR Pro software (WaveMetrics, Inc.) with macros written by the BioCAT staff. Structural parameters and the distance distribution functions, *P*(*r*), were calculated with GNOM (21) using data up to a *Q* of 0.30 and 0.31  $\text{\AA}^{-1}$ , for p115 alone and the p115- $G\alpha_{13}$  complex, respectively.

**Ab Initio Modeling**—Low resolution molecular shape reconstructions from the experimental scattering data were performed with GASBOR (22). GASBOR searches a chain-compatible spatial distribution of an exact number of dummy residues, corresponding to the  $C\alpha$  atoms of protein amino acids. The  $C\alpha$  chain is folded to minimize the discrepancy between the scattering curve calculated from the folded model and the experimental scattering curve. More than 80 GASBOR calculations were performed, and 20 calculations with the smallest standard deviation relative to the experimental data were selected for averaging by DAMAVER (23) to generate the final model; this represents the most probable conformation reconstruction for the protein. The molecular envelopes of RH-L-DH/PH, alone or in a complex with activated  $G\alpha_{13}$ , were calculated based on SAXS data using the program Situs (24),

## DH Domain of p115-RhoGEF Interacts with $G\alpha_{13}$ Helical Domain



**FIGURE 1. Two hydrophobic anchors in the RH domain of p115 are critical for stimulation of GEF activity by  $G\alpha_{13}$ .** *A*, structural based sequence alignment of RH domains from p115 and PRG. The helices are represented with bars on top of the amino acid sequences. The sequence alignment was carried out using the program Clustal W (35). The shaded bars represent additional elements N- and C-terminal to the consensus RGS box (white bars). The  $^{23}$ IIG motif and the  $^{163}$ MGM motif, as well as the  $^{27}$ EDED motif critical for the GAP activity of p115, are boxed. The open arrow labeled YFP indicates the position of the insertion of YFP between helices  $\alpha 9$  and  $\alpha 10$ . *B*, nucleotide exchange assays with p115RH-L-DH/PH and RhoA. For each time course, 2  $\mu$ M RhoA was mixed with 5  $\mu$ M mant-GTP, and the exchange reaction was started at room temperature by the addition of buffer (basal, solid circles), 100 nM p115 (open circles), or 100 nM p115 with 100 nM  $G\alpha_{13}$  bound to GDP·AlF<sub>4</sub><sup>-</sup>·Mg<sup>2+</sup> (GDP·AMF, closed triangles). The subsequent increase in fluorescence ( $\lambda_{\text{ex}} = 356$  nm,  $\lambda_{\text{em}} = 445$  nm) was measured for 10 min. *C*, stimulation of the GEF activity of RH-L-DH/PH by activated  $G\alpha_{13}$  bound to GDP·AMF or GTP $\gamma$ S. Binding of mant-GTP to RhoA was measured as shown in *B*. The initial rates were approximated by linear regression and plotted as the fold increase over basal exchange on RhoA for the indicated conditions: WT, wild-type p115; F31A, Phe-31 in  $^{27}$ EDED motif mutated to alanine;  $^{23}$ AAG,  $^{23}$ IIG mutated to AAG;  $^{163}$ AGA,  $^{163}$ MGM mutated to AGA; and  $^{23}$ AAG/ $^{163}$ AGA, combination of both mutations.

and the crystal structures of DH/PH (20) and the  $G\alpha_{13}$ -RH complex (14) were then fit into the envelope using the program Chimera (25).

### RESULTS

*Two Conserved Hydrophobic Motifs outside of the Canonical RGS Box in p115-RhoGEF Are Critical for Stimulation of GEF Activity by  $G\alpha_{13}$* —The GEF activity of RGS-RhoGEFs can be stimulated by activated  $G_{12}$  class  $G\alpha$  subunits *in vivo*, and some of the RGS-RhoGEFs can be stimulated *in vitro* by activated  $G\alpha_{13}$  (1). In the case of p115,  $G\alpha_{13}$  bound to GDP·AlF<sub>4</sub><sup>-</sup>·Mg<sup>2+</sup> (GDP·AMF), which mimics the transition state of GTP hydrolysis, is more efficient at stimulating the GEF activity of p115 than  $G\alpha_{13}$  bound to GTP $\gamma$ S, a nonhydrolyzable analog of GTP (Fig. 1C). This is anticipated because p115 is a GAP for  $G\alpha_{13}$ , and it has a much higher affinity toward  $G\alpha_{13}$  bound to GDP·AMF than toward  $G\alpha_{13}$  bound to GTP. The GAP activity of RGS-RhoGEFs is conveyed by the RH domain (14), particularly by a series of acidic residues followed by a phenylalanine located N-terminal to the RGS box (the  $^{27}$ EDED motif; Fig. 1A). A recent study of the interfaces observed in various complexes of RH- $G\alpha_{13}$  revealed two conserved hydrophobic anchors in RH (13): the  $^{23}$ IIG motif at the very N terminus of RH and the  $^{163}$ MGM motif just C-terminal to the RGS box (Fig. 1A). Mutation of either one of the two hydrophobic anchors greatly diminished binding between RH and activated  $G\alpha_{13}$  (data not shown) and greatly reduced stimulation of p115 by

$G\alpha_{13}$  (Fig. 1C). However, the reduction can be at least partially recovered by using higher concentrations of activated  $G\alpha_{13}$  (Fig. 1C).  $G\alpha_{13}$  could not activate p115 containing mutations of both isoleucines of  $^{23}$ IIG and the two methionines of  $^{163}$ MGM, even in the presence of higher concentrations of activated  $G\alpha_{13}$  (Fig. 1C). In contrast, the F31A mutation had much less impact on  $G\alpha_{13}$ -stimulated GEF activity of p115 (Fig. 1C). This is consistent with an earlier study demonstrating that mutations within the  $^{27}$ EDED motif led to a loss of GAP activity and a reduction in binding affinity between RH and  $G\alpha_{13}$ , but the same mutations did not diminish  $G\alpha_{13}$ -stimulated GEF activity in the background of full-length p115 (18). In fact, the GAP activity is not required at all for the stimulation of GEF activity by  $G\alpha_{13}$ . The replacement of RH in p115 with that from PRG, which is not a GAP, had little effect on  $G\alpha_{13}$ -stimulated GEF activity (supplemental Fig. S2) (17). Taken together, these data show that the physical association of RH and  $G\alpha_{13}$ , mediated by the two conserved hydrophobic anchors outside of the RGS box, is critical for stimulation of GEF activity by activated  $G\alpha_{13}$ .

*A Binding Site for Activated  $G\alpha_{13}$  outside of the RH Domain*—The interaction between RH domains and activated  $G\alpha_{13}$  has been well characterized (13, 14). The binding affinities between RH and  $G\alpha_{13}$  range between 10 nM to 1  $\mu$ M, depending on the specific RH domain and the state of the GTPase.<sup>4</sup> Binding of

<sup>4</sup> Z. Chen, L. Guo, J. Hadas, S. Gutowski, S. R. Sprang, and P. C. Sternweis, unpublished data.

$G\alpha_{13}$  to regions outside of the RH domain has been suggested (9, 17), but the evidence is limited. The affinity between truncated p115 missing RH and activated  $G\alpha_{13}$  is low, such that no physical association between the two could be observed by size exclusion chromatography (Fig. 2). In contrast, activated  $G\alpha_{13}$  and RH form a stable complex that is dependent on two interfaces (13, 14). The N-terminal 41 amino acids in RH include one of these interfaces, the <sup>23</sup>IIG motif and the <sup>27</sup>EDEF motif, which are located N-terminal to the consensus RGS box. Truncation of this N-terminal segment ( $\Delta^N$ RH; Fig. 2A) results in a sharp decrease in the affinity of the RH domain itself toward activated  $G\alpha_{13}$ . Although the intact RH domain readily forms a stable complex with activated  $G\alpha_{13}$ ,  $\Delta^N$ RH failed to form a complex with  $G\alpha_{13}$ -GTP $\gamma$ S that can be detected by size exclusion chromatography (Fig. 2B). A fragment of p115 consisting of L-DH/PH also failed to bind  $G\alpha_{13}$ -GTP $\gamma$ S (Fig. 2B). However, the fragment consisting of both  $\Delta^N$ RH and L-DH/PH can form a weak but readily observable complex with the activated  $\alpha$  subunit (Fig. 2B). This strongly suggests the existence of a weak binding site for activated  $G\alpha_{13}$  within the L-DH/PH region in addition to the known site in  $\Delta^N$ RH.

**Solution Structure of the C-terminally Truncated p115—**Dimerization of p115 in solution is dependent on the domain C-terminal to PH (1). Thus, the C-terminally truncated proteins used in Figs. 1 and 2 are monomeric. We determined the solution structure of the C-terminally truncated p115 (RH-L-DH/PH) at 20 Å resolution using SAXS. The molecular envelope of RH-L-DH/PH was obtained by *ab initio* shape reconstruction from experimental SAXS data (22). The experimental scattering profile is shown in Fig. 3A. Low angle scattering intensity calibration with cytochrome *c* indicated that RH-L-DH/PH exists as a monomer in solution, with a distance distribution function,  $P(r)$ , that is characteristic of an elongated molecule (Fig. 3A, inset). The calculated molecular envelope of RH-L-DH/PH confirms this, with the longest dimension of the molecule at ~260 Å. The crystal structure of p115 DH/PH (20) fits well into the calculated molecular envelope (Fig. 3B), occupying half of the envelope. The shape and volume of this region closely resembles those calculated for solution envelopes of the DH/PH domains in the absence of RH (20) or without the linker (data not shown). The linker region and RH putatively occupy the volume of the molecular envelope below the DH domain.

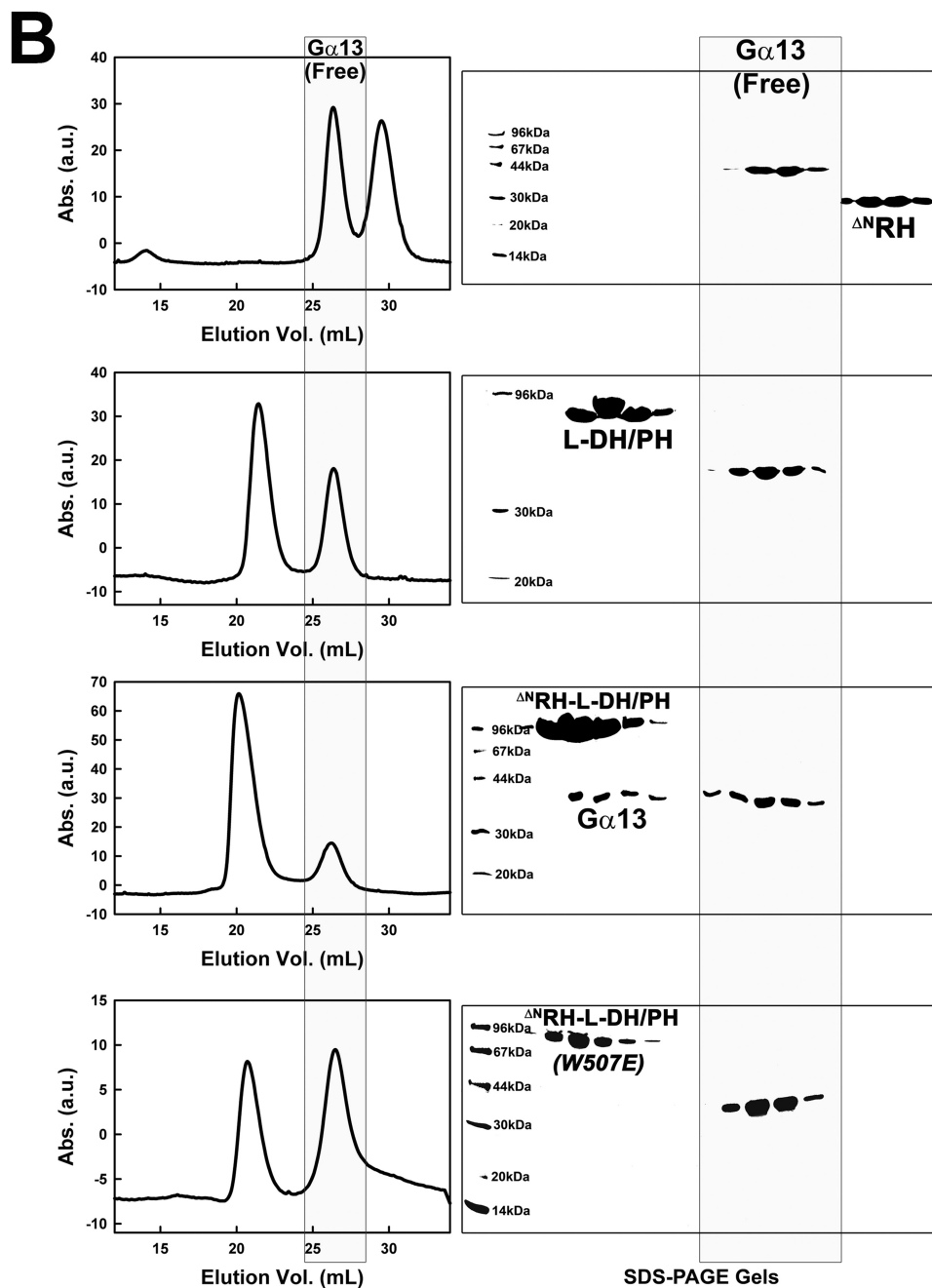
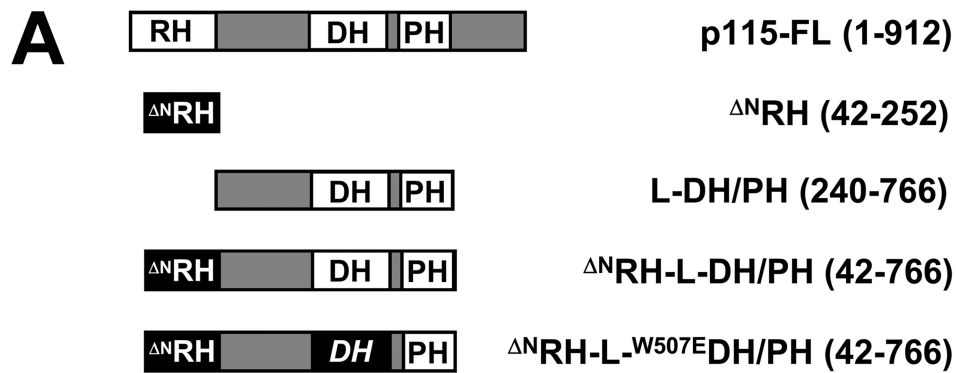
The spatial relationship between DH/PH and RH is not evident. The linker region is disordered in the crystal structure of L-DH/PH and partially disordered in solution (20). Crystal structures of RH domains are available; however, the exact location of RH within the volume below DH (Fig. 3B) could not be unambiguously determined. To identify the location of RH, we inserted YFP between helices  $\alpha 9$  and  $\alpha 10$  of RH (Fig. 1A) and determined the molecular envelope of the <sup>YFP</sup>RH-L-DH/PH fusion protein by SAXS (supplemental Fig. S3). The YFP fusion protein is elongated, with the longest dimension similar to that of the native p115. The portion of the <sup>YFP</sup>RH-L-DH/PH envelope containing the DH/PH domains can be overlapped relatively well with that of the native protein. There is almost no change in shape or volume at the distal end (supplemental Fig. S3) of the envelope, suggesting that YFP, and hence the RH domain, is not located in this area. The major difference

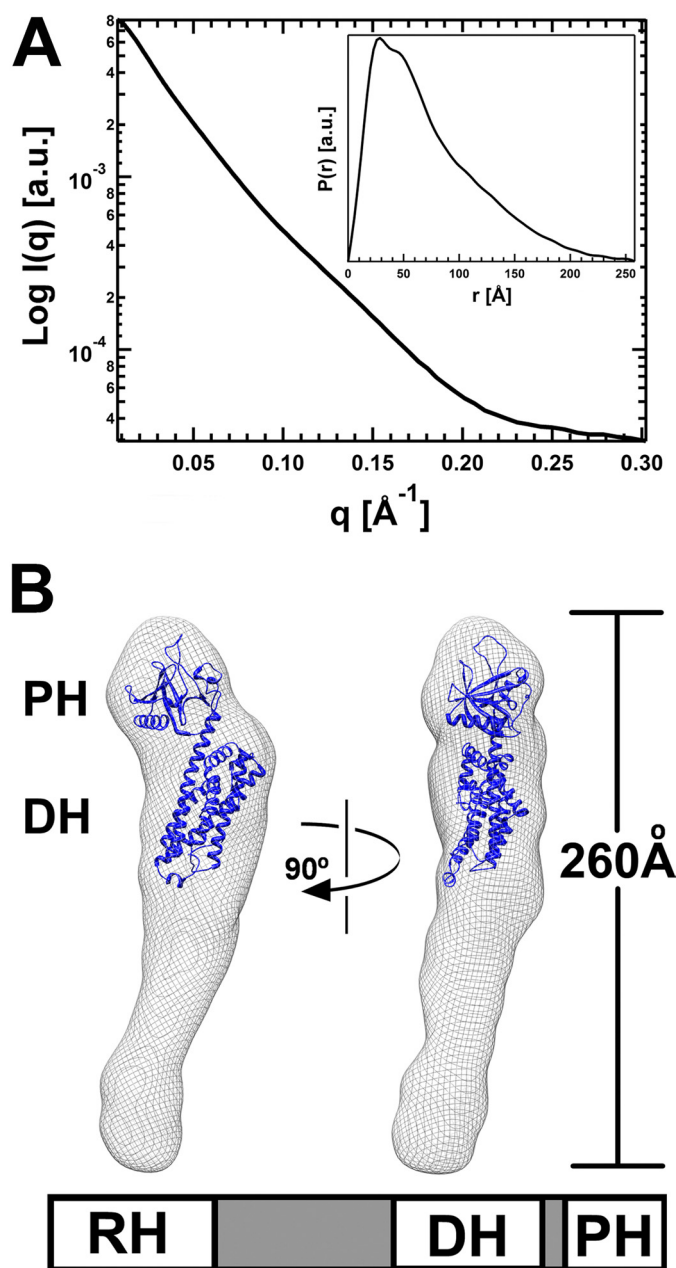
between the two envelopes is evident in the center region underneath the DH domain. Insertion of YFP in RH caused a 20° bend of the molecule in the middle, a strong indication that RH (and YFP) is located in this region, near the bottom of the DH domain. The close proximity of RH to DH is also supported by the calculated molecular envelope of RH-L-DH/PH bound to activated  $G\alpha_{13}$ .

**Solution Structure of a p115- $G\alpha_{13}$  Complex—**The solution structure of RH-L-DH/PH from p115 (Fig. 3B) in a complex with activated  $G\alpha_{13}$  bound to GDP·AMF was determined by SAXS at a resolution of 20 Å. The experimental scattering profile is shown in Fig. 4A. Low angle scattering intensity calibration with cytochrome *c* indicated that the complex is monomeric in solution. Comparison of the  $P(r)$  function of p115 alone and that of the complex indicates that the overall shapes of the two are similar, with the longest dimension being 260 Å in both cases (Fig. 4B). The molecular envelope of the RH-L-DH/PH- $G\alpha_{13}$  complex was obtained by *ab initio* shape reconstruction from experimental SAXS data. Although the overall shape of the complex resembles that of the RH-L-DH/PH alone, a large increase in volume (~65,000 Å<sup>3</sup>) was observed in the midsection of the molecular envelope, near the bottom of the modeled DH domain. This additional volume can accommodate a  $G\alpha_{13}$  molecule (calculated at ~50,000 Å<sup>3</sup>). As discussed above, binding of the RH domain to activated  $G\alpha_{13}$  is critical for both the activation of p115 and the formation of a stable complex between p115 and  $G\alpha_{13}$  in solution (Fig. 2B). It is therefore reasonable to assume that the RH domain remains bound to  $G\alpha_{13}$  in this complex. Hence, the RH domain is located near the DH domain where the increase in mass is observed, as concluded from inspection of the molecular envelope of the YFP fusion protein (supplemental Fig. S3). The crystal structure of the p115-RH- $G\alpha_{13}$  complex (14) can be fit into the calculated solution envelope together with the DH/PH domains (Fig. 4C). In this model, activated  $G\alpha_{13}$  contacts the DH domain, opposite to the site at which RhoA binds (Fig. 4D). Importantly, the binding site for  $G\alpha_{13}$  on the DH domain does not overlap with the binding site for RhoA. The DH domain appears to form an interface with the helical domain of  $G\alpha_{13}$  rather than the Ras-like domain, which is engaged with the RH domain as observed in the structures of RH- $G\alpha_{13}$  complexes (13, 14). Thus,  $G\alpha_{13}$  utilizes both the helical and the Ras-like domains for interaction with p115. We do note that the positioning of RhoA in this model (Fig. 4D) is based exclusively on crystal structures of DH/PH-RhoA complexes (26, 27). A solution structure of the ternary complex may provide further insight into the orientation of RhoA in the presence of  $G\alpha_{13}$ .

**Mutations in a Predicted Binding Site for  $G\alpha_{13}$  on DH Abolish  $G\alpha_{13}$ -stimulated GEF Activity—**The model of the RH-L-DH/PH- $G\alpha_{13}$  complex derived from interpretation of SAXS data features an interface between the DH domain of p115 and the helical domain of  $G\alpha_{13}$  (Fig. 4C). Structures of DH domains from RGS-RhoGEFs (20, 26, 27) consist of six major  $\alpha$ -helices (Fig. 5A). Unlike p115, the GEF activity of PRG, a member of the RGS-RhoGEF family, cannot be stimulated by activated  $G\alpha_{13}$  *in vitro*, even though its RH domain binds tightly to the  $\alpha$  subunit (13). Thus, differences in the amino acid sequences and tertiary structures of the DH domains of p115 and PRG provide clues to

DH Domain of p115-RhoGEF Interacts with  $G\alpha_{13}$  Helical Domain





**FIGURE 3. Solution structures of p115 RH-L-DH/PH.** A, solution x-ray scattering profile for p115 RH-L-DH/PH. The distance distribution function (*inset*),  $P(r)$ , of RH-L-DH/PH was computed from the x-ray scattering using the program GNOM (21). B, solution structure of RH-L-DH/PH of p115. The solution structure (molecular envelope) is depicted as a mesh and superimposed onto the crystal structure of the p115 DH/PH domains (*blue ribbon*). The large unoccupied region in the molecular envelope beneath the DH domain presumably contains the linker region and RH. The schematic representation of RH-L-DH/PH of p115 is depicted underneath the molecular envelope.

the location of the binding site at which  $G\alpha_{13}$  exerts GEF stimulatory activity toward p115. Two DH segments that are poorly conserved between p115 and PRG were identified at the puta-

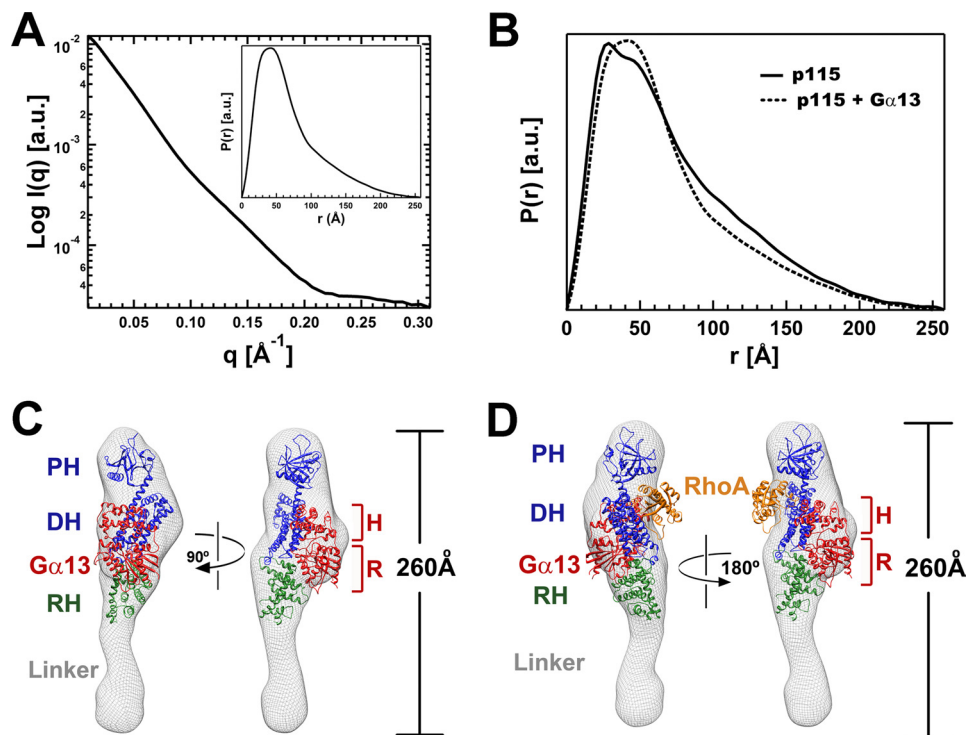
tive interface between DH and  $G\alpha_{13}$  in the SAXS model of the RH-L-DH/PH- $G\alpha_{13}$  complex (Fig. 5, A and B). The first segment is located in the  $\alpha 2b$  helix, consisting of amino acids 475–483 ( $\alpha 2b$ ). The second segment is located within the  $\alpha 3b$  helix, including residues 503–507 ( $\alpha 3b$ ). None of these residues in  $\alpha 2b$  or  $\alpha 3b$  are involved in binding to RhoA (Fig. 5A). Replacement of residues in  $\alpha 2b$  or  $\alpha 3b$  in p115 with those from PRG had little effect on the basal exchange activity of p115 toward RhoA, indicating that these substitutions do not affect the structure of the DH recognition site for RhoA (Fig. 5C and supplemental Fig. S4). Although changes in  $\alpha 2b$  had no effect on GEF activity stimulated by  $G\alpha_{13}$ , swapping of residues from PRG into  $\alpha 3b$  in p115 abolished stimulation by  $G\alpha_{13}$  *in vitro* (Fig. 5C).

Three of the five amino acids in  $\alpha 3b$  are exposed to solvent in the crystal structures of p115 and PRG DH domains and are not conserved in the amino acid sequences of the domains (Fig. 5B). These include Glu<sup>504</sup> (Ala<sup>821</sup> in PRG), Ser<sup>506</sup> (Glu<sup>823</sup> in PRG), and Trp<sup>507</sup> (Glu<sup>824</sup> in PRG). Single point mutations at any of these three positions in p115 had varied effects on stimulation of GEF activity by  $G\alpha_{13}$  (Fig. 5D). Mutation of Ser<sup>506</sup> to glutamate in p115 had no effect on this regulation. The change of glutamate 504 to alanine decreased the response of p115 to activated  $G\alpha_{13}$ . Mutation of the exposed tryptophan 507 to glutamate, the corresponding residue in PRG, completely abolished  $G\alpha_{13}$ -stimulated GEF activity of p115. None of these point mutations affected the basal exchange activity of p115 in the absence of  $G\alpha_{13}$ . The p115 fragment,  $\Delta^N$ RH-L-DH/PH, bearing the W507E mutation also failed to bind activated  $G\alpha_{13}$  as assayed by size exclusion chromatography (Fig. 2). Mutations of Trp<sup>507</sup> to either a tyrosine or an alanine also greatly reduced  $G\alpha_{13}$ -stimulated GEF activity of p115 (Fig. 5E).

*The  $\alpha B$ - $\alpha C$  Loop in the Helical Domain of  $G\alpha_{13}$  Is a Novel Effector Binding Motif*—The  $\alpha 3b$  helix in the DH domain of p115, which includes Trp<sup>507</sup>, forms a protruding bulge on the surface of DH that is predicted to interact with the helical domain of  $G\alpha_{13}$  in our model (Fig. 6A). Q-SiteFinder, a ligand-binding site prediction program (28), identifies a hydrophobic pocket between the  $\alpha B$  and  $\alpha C$  helices of  $G\alpha_{13}$  in the helical domain (Fig. 6, B–D) as a possible site for protein-protein interaction. The shape of this pocket appears roughly complementary to the protruding bulge formed by Trp<sup>507</sup> from DH. These surfaces are in close proximity in the low resolution model of the p115- $G\alpha_{13}$  complex derived from SAXS (Fig. 4C). Part of the  $\alpha 3b$  helix from DH, including residues 502–510, can be docked into this hydrophobic pocket in the  $\alpha B$ - $\alpha C$  loop on  $G\alpha_{13}$  using the program PatchDock (29). In this model, Trp<sup>507</sup> from DH docks into the hydrophobic pocket on  $G\alpha_{13}$  formed by the side chains of Phe<sup>125</sup>, Ala<sup>129</sup>, Pro<sup>130</sup>, Met<sup>131</sup>, Ala<sup>132</sup>, Val<sup>137</sup>, Val<sup>141</sup>, and Tyr<sup>145</sup> (Fig. 6D). Glu<sup>504</sup> from DH also makes

**FIGURE 2. An additional binding site for activated  $G\alpha_{13}$  outside of RH.** A, schematic representation of truncated forms of p115 used; the included amino acids are listed in parentheses. B, *left panels*, chromatograms from size exclusion chromatography with p115 and  $G\alpha_{13}$ -GTP $\gamma$ S are aligned based on elution volumes; peaks corresponding to free (unbound)  $G\alpha_{13}$  with elution volumes around 26 ml are outlined in a gray box. *Right panels*, SDS-PAGE gels showing components of eluted peaks of protein. Lanes on gels are aligned based on volumes of elution. Molecular masses of protein standard makers (*first lanes on the left*) are labeled. Fractions corresponding to free (unbound)  $G\alpha_{13}$  are outlined in a gray box. The same amount of  $G\alpha_{13}$ , which was premixed with equal molar of the p115 fragment, was used in all four experiments. The scale of absorption (*Abs.*) in the last panel (at *bottom*) was changed because of variations in detector efficiency.

## DH Domain of p115-RhoGEF Interacts with $G\alpha_{13}$ Helical Domain



**FIGURE 4. Solution structures of the complex of p115 RH-L-DH/PH bound to  $G\alpha_{13}$ -GDP-AMF.** *A*, solution x-ray scattering profile for the complex, with the distance distribution function (*inset*),  $P(r)$ . *B*, comparison of the distance distribution functions of p115 alone and its complex with  $G\alpha_{13}$ . The overall shapes of the two are similar, with the longest dimensions being identical at  $\sim 260$  Å. *C*, solution structure of RH-L-DH/PH of p115 bound to  $G\alpha_{13}$ -GDP-AMF. The solution structure (molecular envelope) is depicted as a *mesh* and superimposed onto the crystal structures of the p115 DH/PH domains (*blue ribbon*) and the complex of RH (*green ribbon*) bound to  $G\alpha_{13}$ -GDP-AMF (*red ribbon*). The helical domain and the Ras-like domain of  $G\alpha_{13}$  are outlined with *brackets* and labeled with H and R, respectively. The unoccupied region in the molecular envelope beneath the RH domain is likely to be the location for most of the linker region. *D*, a model for the ternary complex of RH-L-DH/PH bound to activated  $G\alpha_{13}$  and nucleotide-free RhoA. RhoA (*orange ribbon*) was modeled into the binding site on DH/PH of p115 based on crystal structures of PRG-DH/PH or LARG-DH/PH bound to nucleotide-free RhoA (26, 27). The location of RhoA was not experimentally determined by SAXS in this study.

van der Waals contacts with residues from this region. Single point mutations of these hydrophobic residues in the  $\alpha B$ - $\alpha C$  region had varied effects on the stimulation of GEF activity of p115 by  $G\alpha_{13}$  *in vitro* (Fig. 6E). Mutation of Ala<sup>129</sup> to lysine or Ala<sup>132</sup> to leucine resulted in a 40% reduction in stimulation of GEF activity of p115 by  $G\alpha_{13}$ . Similar reduction of stimulation of p115 was observed with a double mutation of Phe<sup>125</sup> and Tyr<sup>145</sup> to alanines. Such a reduction in stimulation of p115 correlated with a lowered efficacy of the mutant when compared with the wild-type  $G\alpha_{13}$  (Fig. 6F). These mutations had no effect on the intrinsic GTPase activity of  $G\alpha_{13}$  or its ability to bind guanine nucleotide (supplemental Fig. S5).

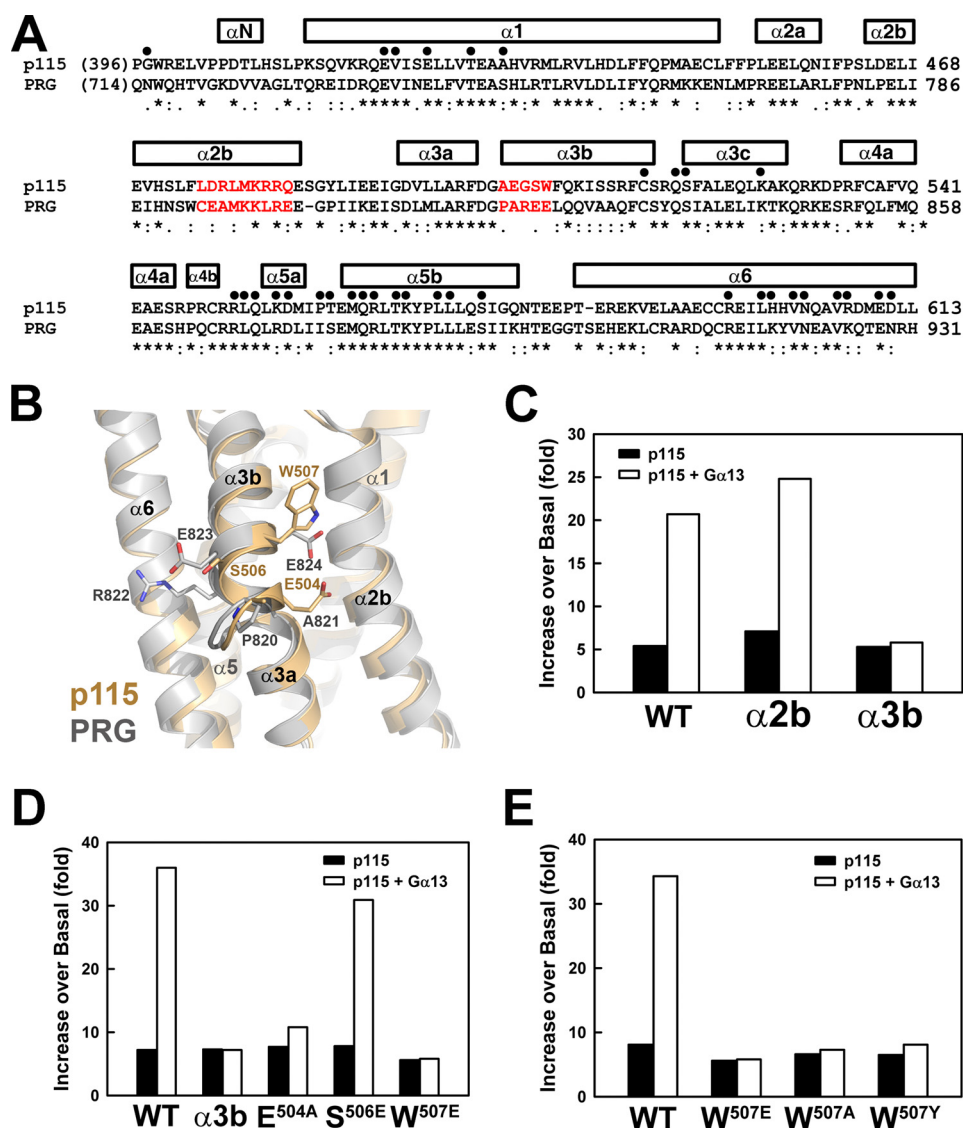
### DISCUSSION

Earlier studies on interactions between RGS-RhoGEFs and activated  $G\alpha_{13}$  have been primarily focused on RH and its interaction with  $G\alpha_{13}$ . The residues in the  $\alpha 3b$  helix of DH described here are the first outside of the RH domain to be implicated as direct mediators for activation by G proteins. The binding affinity between DH and activated  $G\alpha_{13}$  is low; hence DH alone may not be sufficient to associate with the  $\alpha$  subunit. An important function of the RH domain, as suggested by data presented in this report, might be to hold activated  $G\alpha_{13}$  in a position for optimized interaction with the  $\alpha 3b$  helix in the DH domain of p115.

The DH and RH domains of RGS-RhoGEFs are separated by long linker regions. Interpretation of the size exclusion chromatography data (Fig. 2) and the low resolution molecular envelopes of  $G\alpha_{13}$  with p115 (Fig. 4) is most consistent with a model in which  $G\alpha_{13}$  interacts simultaneously with the RH and the DH domains of p115. Because the 150-residue linker region in p115 intervenes between RH and DH, it must therefore adopt a hairpin conformation, in which the N and C termini of the linker are nearly juxtaposed. The linker region from p115 would occupy the portion of the molecular envelope distal to the PH domain and beneath the RH domain (Fig. 4). The linker region in p115 appears to be rigid, because molecular envelopes of p115 alone and its complex with  $G\alpha_{13}$  are of the same length. Our results differ from a recent study of PRG by SAXS (30). Like that of p115, the RH-L-DH/PH fragment of PRG is also an elongated structure. However, the RH and DH modules of PRG are well separated in the SAXS model by the linker region. The linker connecting RH and DH in PRG appears to be very flexible and changes both in length and in overall shape in the presence of the N-terminal PDZ domain (not present in p115) or when bound to RhoA. It is not clear how  $G\alpha_{13}$  would affect the overall shape of PRG in solution, because PRG cannot be stimulated by  $G\alpha_{13}$  *in vitro*.

The hairpin conformation of the p115 linker in our model allows the C terminus of the linker to contact the RH domain as

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**FIGURE 5. Mutations at the predicted  $G\alpha_{13}$ -binding site on DH abolish the  $G\alpha_{13}$ -stimulated GEF activity of p115.** *A*, sequence alignment of DH domains from p115 and PRG. Residues involved in contacts with nucleotide-free RhoA are marked with *dots* over the alignment. Helices are represented with *bars* on top of the amino acid sequences. The sequence alignment was carried out using the program Clustal W (35). The two potential binding sites for  $G\alpha_{13}$  targeted for mutagenesis are colored *red*. *B*, ribbon diagrams with superimposed structures of DH domains from p115 (*wheat*) and PRG (*gray*). Side chains in  $\alpha$ 3b are depicted as sticks. Oxygen and nitrogen atoms are colored *red* and *blue*, respectively. *C*, stimulation of the GEF activity of RH-L-DH/PH by activated  $G\alpha_{13}$ . Binding of mant-GTP to RhoA was measured as shown in Fig. 1*B*. Initial rates were approximated by linear regression, and the increases of exchange rate over basal RhoA were plotted for the following proteins: *WT*, wild-type p115;  $\alpha$ 2*b*, mutant p115 where residues 475–483 were substituted with corresponding residues from PRG; and  $\alpha$ 3*b*, mutant p115 where residues 503–507 were substituted with corresponding residues from PRG. *D*, effects of single point mutations in the  $\alpha$ 3*b* region on stimulation by  $G\alpha_{13}$ . *E*, effects of single point mutations of Trp<sup>507</sup> on stimulation by  $G\alpha_{13}$ .

the polypeptide chain returns from its excursion to the hairpin back toward the DH domain. This is supported by the observation that C-terminally extended RH constructs (RH-L) are not stably expressed unless the full-length linker is included in the construct (data not shown). RH-L constructs missing the C-terminal 40 residues in the linker region did not express well as soluble protein. Furthermore, removal of the N-terminal ~100 residues of the linker region (RH- $\Delta$ N<sub>2</sub>-L-DH/PH; Fig. 7*A*) did not affect stimulation by  $G\alpha_{13}$  (Fig. 7*B*) or the apparent interaction between DH and  $G\alpha_{13}$  (Fig. 7*C*). Complete removal of the linker region, however, led to a significant increase in the basal exchange activity of p115 and diminished stimulation by  $G\alpha_{13}$  (Fig. 7*B*). This is consistent with recent reports in which the linker region in RGS-RhoGEFs has been shown to play an auto-

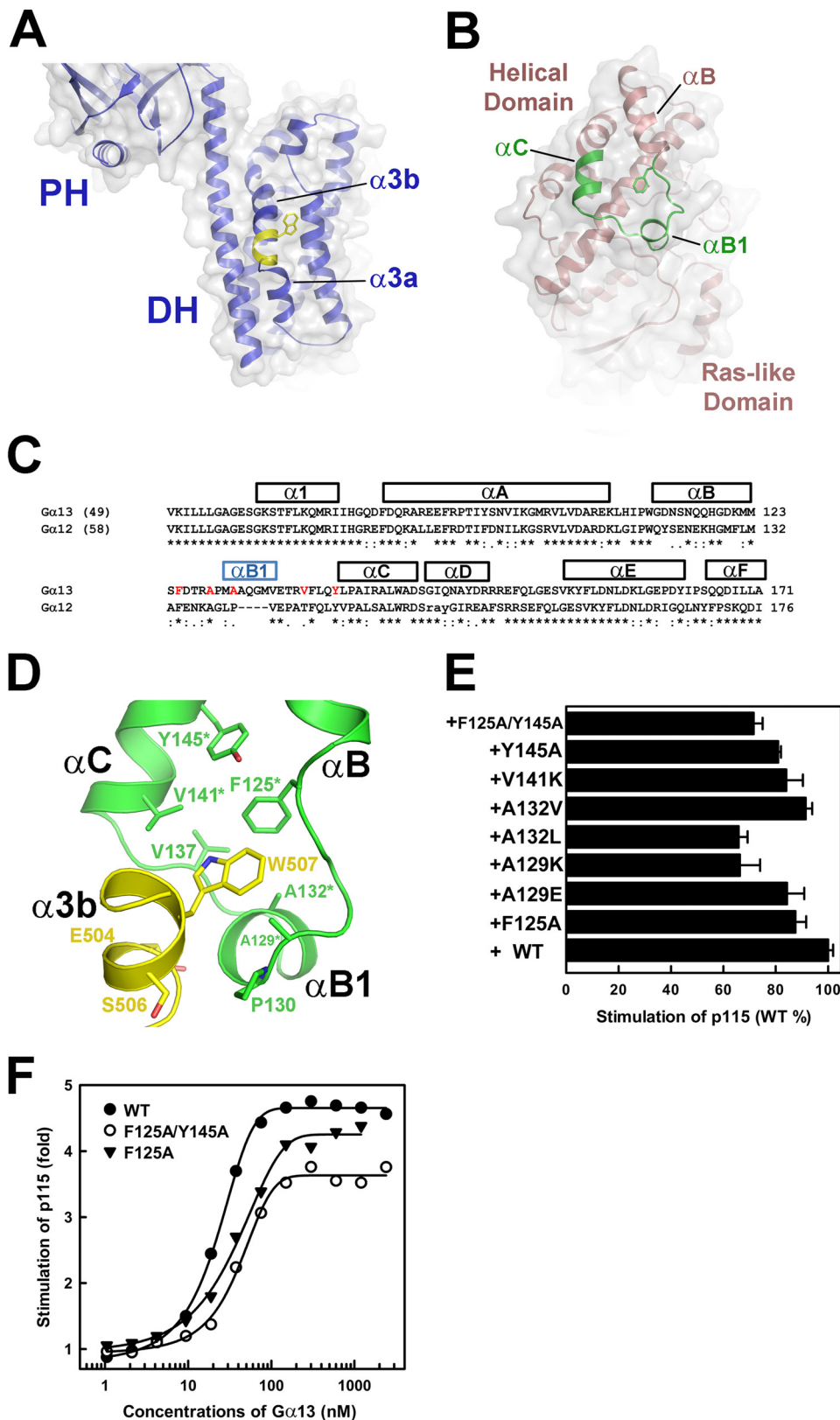
inhibitory role (20, 31). The presence of the 40 amino acids in the linker immediately preceding the start of DH lowers the basal exchange activity of these RhoGEFs. A recent study suggests that this segment from the linker perturbs the folding of the GEF switch at the N terminus of DH in p115 (20). The same segment in the linker would interact with RH in our model. A possible scenario for  $G\alpha_{13}$ -stimulated GEF activity is that the binding of  $G\alpha_{13}$  to RH and DH leads to conformational changes at the RH-L interface, which leads to formation of a productive interface between the GEF switch and RhoA that is required for enhanced GEF activity. Thus, binding of activated  $G\alpha_{13}$  to RH, and to a less extent to DH, relieves the autoinhibition imposed by the linker region of p115.

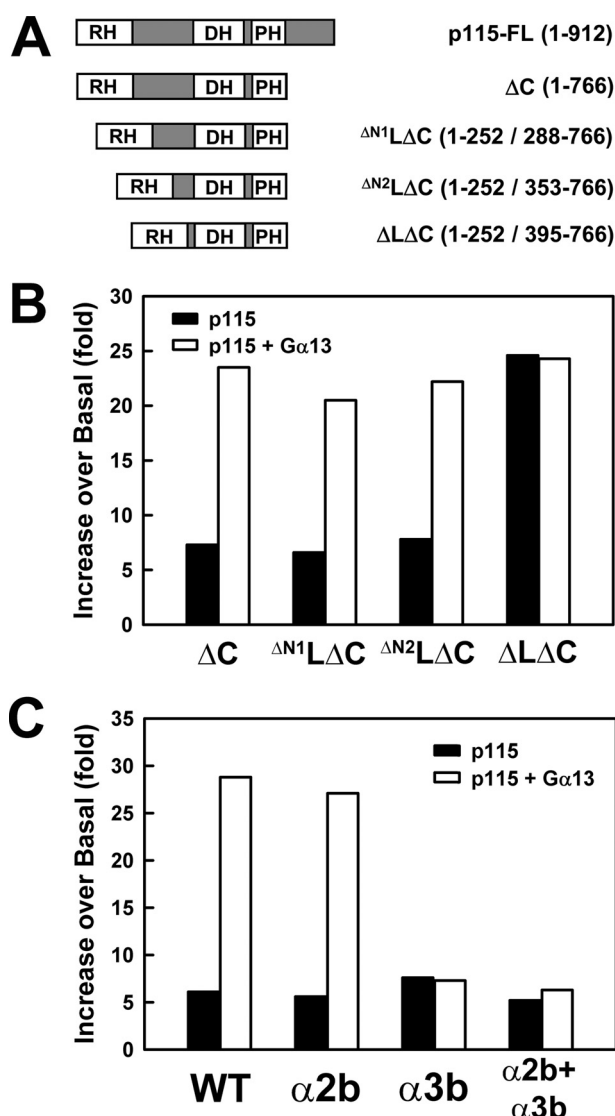


## DH Domain of p115-RhoGEF Interacts with $G\alpha_{13}$ Helical Domain

Interaction between the DH domain and the  $\alpha$  subunit of a G protein has been reported before. p63-RhoGEF, a GEF for the small GTPase Rho, can be activated by  $G\alpha_q$ . In the crystal structure of the  $G\alpha_q$ -p63-RhoGEF complex, parts of the  $\alpha 2$  and  $\alpha 3$

helices from the DH domain also make contact with the  $G\alpha_q$  subunit (32), but in this case, the segments in  $G\alpha_q$  that interact with DH come from the Ras-like domain of  $G\alpha_q$ , rather than its helical domain. Nevertheless, interactions between the DH





**FIGURE 7. Removal of part of the linker region does not affect stimulation of p115 by  $G\alpha_{13}$ .** *A*, schematic representation of the truncated forms of p115 used; the included amino acids are listed in parentheses. *B*, stimulation of the GEF activity of RH-ΔL-DH/PH by activated  $G\alpha_{13}$ . Binding of mant-GTP to RhoA was measured as shown in Fig. 1. Removal of the first 100 amino acids in the linker region had no effect on stimulation by  $G\alpha_{13}$ . The last 40 residues within the linker region immediately preceding DH is important for activation by  $G\alpha_{13}$ . *C*, stimulation of the GEF activity of mutated RH-ΔN2L-DH/PH by activated  $G\alpha_{13}$ . The constructs are: *WT*, wild-type p115-ΔN2LΔC; *α2b*, mutant p115-ΔN2LΔC where residues 475–483 were substituted with corresponding residues from PRG; *α3b*, mutant p115-ΔN2LΔC where residues 503–507 were substituted with corresponding residues from PRG; and *α2b+α3b*, mutant p115-ΔN2LΔC where both α2b and α3b regions were substituted with corresponding regions from PRG.

domains of RhoGEFs and their regulatory proteins might be a common mechanism for regulation.

The proposed binding of the p115 DH domain to the helical domain of  $G\alpha_{13}$  defines a new effector interface for heterotrimeric G proteins and the potential to define the contribution of direct activation of the GEF activity of p115 in physiological function. All heterotrimeric G protein α subunits possess two domains: a Ras-like domain that makes direct contacts with regulators and effectors and a less well functionally characterized helical domain. Thus far, the only protein-protein interactions that have been ascribed to the helical domain are between  $G\alpha_i$  and the GoLoco motif of RGS14 (33) and between  $G\alpha_{13}$  and the RH domain of RGS-RhoGEFs (13, 14). The α3b helix in the DH domain of p115, which includes Trp<sup>507</sup>, forms a protruding bulge on the surface of DH that is predicted to interact with the helical domain of  $G\alpha_{13}$  in our models (Fig. 4). One of the potential binding sites for the DH domain of p115 within the helical domain of  $G\alpha_{13}$  includes the αB-αC helices, because these surfaces are in close proximity in the low resolution model of the p115- $G\alpha_{13}$  complex derived from SAXS (Figs. 4C and 6). The hydrophobic pocket in the helical domain is unique to  $G\alpha_{13}$ . As described in previous studies (14), a notable feature of the helical domain of  $G\alpha_{13}$  that is not present in other known  $G\alpha$  structures is the “helical insert” (αB1) between the αB and αC helices, which forms part of this hydrophobic pocket (Fig. 6, B–D). In  $G\alpha_{12}$ , the other G protein that interacts with the RGS-RhoGEFs, the loop connecting the αB and αC helices is shortened by four amino acids (Fig. 6C). Comparison of the structures of  $G\alpha_{13}$  and  $G\alpha_{12}$  (34) reveals that αB1 is not present in  $G\alpha_{12}$ . The GEF activity of p115 can be stimulated by activated  $G\alpha_{13}$ , but not  $G\alpha_{12}$ , *in vitro* (7). The lack of the αB1 helix or an intact hydrophobic pocket in the helical domain of  $G\alpha_{12}$  might explain this observation. The GEF activity of p115 can be stimulated by activation of  $G\alpha_{12}$  *in vivo*. However, this activation is likely due to the localization of p115 to the plasma membrane where free RhoA is located. The conformation of the helical domain is not significantly different between the GDP- and the GTP-bound forms of G protein α subunits. Therefore it is anticipated that any specific regulation of effectors by this region would require coincident interactions of effector molecules with the conformationally flexible switch regions within the Ras-like domain as observed here. This could represent a common mechanism by which RGS domains in effector proteins could both regulate turnover of the G proteins and provide assistance to other sites that regulate effector activities.

**FIGURE 6. A binding pocket for DH within the helical domain of  $G\alpha_{13}$ .** *A*, ribbon diagram of the DH domain of p115. The molecule is positioned such that the proposed  $G\alpha_{13}$ -binding site on DH (yellow) is facing the reader. The side chain of Trp<sup>507</sup> is shown as sticks. The surface of DH is shown and colored gray. *B*, ribbon diagram of  $G\alpha_{13}$ . The molecule is positioned such that the proposed DH binding site (green) is facing the reader. The side chain of Phe<sup>125</sup> is shown as sticks. The surface of  $G\alpha_{13}$  is shown and colored gray. *C*, structural based sequence alignment of  $G\alpha_{13}$  and  $G\alpha_{12}$  helical domains. Helices are represented with bars on top of the amino acid sequences. The sequence alignment is carried out by the program Clustal W (35). Residues targeted for mutagenesis from the hydrophobic pocket are colored red. *D*, ribbon diagram depicting a modeled interface between α3b and the hydrophobic pocket in the helical domain of  $G\alpha_{13}$  using the same color scheme as in A and B. Side chains of residues involved are depicted as stick models. Residues from the helical domain of  $G\alpha_{13}$  that impact the stimulation of p115 when mutated are marked with asterisks. *E*, stimulation of the GEF activity of full-length p115 by activated  $G\alpha_{13}$ . Reactions contained 2 μM RhoA, 30 nM p115, and 300 nM  $G\alpha_{13}$  (wild type or mutant). The effect of mutations in  $G\alpha_{13}$  on the initial rate of the GEF activity of p115 is compared against wild-type  $G\alpha_{13}$  (100%). Each experiment was repeated at least twice. *F*, stimulation of p115 with different concentrations of  $G\alpha_{13}$ . The initial rate of the GEF activity of p115 (approximated by linear regression with the first 4 min of a 6-min reaction) was assessed in the presence of increasing concentrations of activated  $G\alpha_{13}$  (wild type or mutant). The reactions contained 2 μM RhoA and 30 nM p115: WT (closed circles), F125A (closed triangles), F125A/Y145A (open circles).

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