The Pleckstrin Homology (PH) Domain of the Arf Exchange Factor Brag2 Is an Allosteric Binding Site*†‡

**Background:** Brag2 is a PH domain-containing Arf guanine nucleotide exchange factor (GEF) that regulates cell adhesion. **Results:** PIP2 association with the PH domain stimulated Brag2 activity. **Conclusion:** PIP2 binding to the PH domain allosterically modifies Brag2 activity. **Significance:** A novel regulatory mechanism for GEFs was identified.

Brag2, a Sec7 domain (sec7d)-containing guanine nucleotide exchange factor, regulates cell adhesion and tumor cell invasion. Brag2 catalyzes nucleotide exchange, converting Arf-GDP to Arf-GTP. Brag2 contains a pleckstrin homology (PH) domain, and its nucleotide exchange activity is stimulated by phosphatidylinositol 4,5-bisphosphate (PIP2). Here we determined kinetic parameters for Brag2 and examined the basis for regulation by phosphoinositides. Using myristoylated Arf1-GDP as a substrate, the $k_{cat}$ was 1.8 ± 0.1 s⁻¹ as determined by single-turnover kinetics, and the $K_m$ was 0.20 ± 0.07 μM as determined by substrate saturation kinetics. PIP2 decreased the $K_m$ and increased the $k_{cat}$ of the reaction. The effect of PIP2 required the PH domain of Brag2 and the N terminus of Arf and was largely independent of Arf myristoylation. Structural analysis indicated that the linker between the sec7d and the PH domain in Brag2 may directly contact Arf. In support, we found that a Brag2 fragment containing the sec7d and the linker was more active than sec7d alone. We conclude that Brag2 is allosterically regulated by PIP2 binding to the PH domain and that activity depends on the interdomain linker. Thus, the PH domain and the interdomain linker of Brag2 may be targets for selectively regulating the activity of Brag2.

Arf-directed guanine nucleotide exchange factors (Arf-GEFs) catalyze the exchange of nucleotide on Arf family GTP-binding proteins (1–3). 15 ArfGEFs have been identified in the human genome and divided into five classes: Big1/2 and Golgi specific Brefeldin A resistant guanine nucleotide exchange factor family, common to fungi, plants, and metazoa, regulate membrane traffic. The other classes of ArfGEFs are found only in metazoans. The Brag family of ArfGEFs, including Brag2, has been implicated in peripheral membrane traffic and in cell adhesion and migration (4, 5). Brag2 has been reported to signal through Arf6 to drive breast cancer invasion (4, 6, 7).

Brag2, also called GEP100 and IQSEC1, is a ~100-kDa protein that contains IQ-like, proline-rich, Sec7, and pleckstrin homology (PH) domains (7) (see Fig. 1B). Brag2 activates Arf6 to regulate cell-substrate and cell-cell adhesion (4–6, 8, 9). Activity has been found to be stimulated by nonphosphorylated peptides from AMPA receptor (10) and phosphopeptides from epidermal growth factor receptor (4). The phosphopeptides from epidermal growth factor receptor are reported to bind to the PH domain of Brag2 to regulate its activity. These observations have been used to explain the effect of epidermal growth factor receptor on cancer cell invasion. Stimulation of Brag2 increases Arf6-GTP levels, which drive the cellular changes responsible for movement of the cancer cells into the normal tissue. Brag2 activity is also regulated by phosphoinositides that presumably bind the PH domain (9). Together, these findings suggest that the PH domain may represent a regulatory motif.

PH domain-mediated regulation of one subtype of ArfGEFs, cytohesin/Grp/ARNO, has been characterized. ARNO GEF activity is autoinhibited by the linker region between the Sec7 and PH domains and a C-terminal amphipathic helix containing a polybasic motif, which physically block the Arf binding site. Binding of Arf6-GTP and phosphoinositides to the PH domain has two functions. One is to recruit ARNO to the membrane surface on which it is active, and the second is to induce a conformational change in the PH domain that relieves autoinhibition. Phosphorylation of serines and threonine within a polybasic motif, which physically block the Arf binding site. Binding of Arf6-GTP and phosphoinositides to the PH domain has two functions. One is to recruit ARNO to the membrane surface on which it is active, and the second is to induce a conformational change in the PH domain that relieves autoinhibition.
ling Brag2 activity with the ultimate goal of using it as a therapeutic target for anticancer cell invasion therapy.

Here we first determined the effect of PIP2 on the fundamental enzymatic parameters \( k_{\text{cat}} \) and \( K_m \) for Brag2. PIP2 increased the ratio \( k_{\text{cat}}/K_m \) from 2.6 \( \times \) 10^3 to 8.8 \( \times \) 10^6 M^{-1} s^{-1}. We then analyzed structural requirements for GEF activity. Unique to Brag2, the linker between the Sec7 and PH domains had a positive effect on activity, and the effect of PIP2 required the N terminus of Arf. These unique features of Brag2 can be used to selectively inhibit Brag2 activity.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**

A mammalian expression vector for Brag2-myc-His and a bacterial expression vector for GST-Brag2-His were kindly provided by Joel Moss (National Heart, Lung, and Blood Institute). Bacterial expression vectors for His-Brag2Sec7-PH (amino acids 499–863), His-Brag2Sec7-linker (amino acids 499–740), and His-Brag2Sec7 (amino acids 499–700) were generated by standard PCR methods using pET19 as an expression vector (EMD Biosciences). Mutants in the PH domain of His-Brag2Sec7-PH (K753S,K756S and R762S) were generated using the QuikChange II site-directed mutagenesis kit (Agilent Technologies).

**Protein Preparations**

Brag2-myc-His expressed in HEK293T cells was purified using nickel-nitriilotriacetic acid-agarose column. GST-Brag2-His expressed in bacteria was purified using glutathione-Sepharose 4B (GE Healthcare). His-Brag2Sec7-PH, His-(K753S,K756S)Brag2Sec7-PH, His-(R762S)Brag2Sec7-PH, His-Brag2Sec7-linker, and His-Brag2Sec7 were expressed in bacteria and purified using a His-Trap HP column followed by a HiLoad16/60 Superdex 75 column (GE Healthcare). The expression and purification of myristoylated Arf1 (myrArf1), (L8K)Arf1, and (Δ17)Arf1 have been described previously (13–17).

**Lipid Binding Assay**

Large unilamellar vesicles (LUVs) were prepared by extrusion with lipids purchased from Avanti Polar Lipids as described previously (18, 19). They contained molar ratios of 40% phosphatidylcholine, 25% phosphatidylethanolamine, 15% phosphatidylserine, 10% cholesterol, 9% phosphatidylinositol, and 1% phosphatidylinositol 4,5-bisphosphate. For the PIP2 titration experiments, phosphatidylinositol 4,5-bisphosphate was varied from 0, 0.1, 0.25, 0.5, to 1%. The amount of phosphatidylinositol was changed accordingly from 10, 9.9, 9.75, 9.5, to 9%. The purified recombinant protein His-Brag2Sec7-PH (800 nm) alone or with \( 1 \mu M \) myrArf1, (Δ17)Arf1, or (L8K)Arf1 was incubated with sucrose-loaded LUVs containing 500 \( \mu M \) total phospholipids at 30 °C for 5 min. The LUVs were precipitated by ultracentrifugation at 100,000 \( \times \) g for 15 min at 4 °C, and the proteins precipitated with LUVs were separated by SDS-PAGE and visualized by Coomassie Blue staining. The signal was quantified from densitometric traces using ImageJ software.

**Brag2 GEF Activity**

The conversion of Arf-GDP to Arf-GTP was followed in one of three ways.

**Fixed Time Point Assay for Determination of \( C_{50} \)—**Brag2-catalyzed GTP\( \gamma \)S binding to Arf-GDP was measured using nucleotide exchange buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 2 mM MgCl2, 1 mM EDTA, 1 mM ATP, 5 \( \mu M \) GTP\( \gamma \)S, and [35S]GTP\( \gamma \)S (for specific activity of \( \sim 10,000 \) cpn/ml) as described (9, 18, 20, 21). High [MgCl2] was used in this reaction to slow down the spontaneous nucleotide exchange. The reactions also contained 0.5 mM LUVs and 0.5 \( \mu M \) Arf-GDP with different concentrations of Brag2. The reactions were incubated at 30 °C for 3 min and terminated with 2 ml of ice-cold 20 mM Tris, pH 8.0, 100 mM NaCl, 10 mM MgCl2, and 1 mM dithiothreitol. Protein-bound nucleotide was trapped on nitrocellulose, and the bound radioactivity was quantified by liquid scintillation counting.

**Substrate Saturation Experiments**—Brag2 GEF activity was determined under conditions satisfying the steady state assumption using a FluorMax3 spectrophotometer (Jobin Yvon Horiba, Edison, NJ). The conversion of Arf1-GDP to Arf1-GTP was monitored by fluorescence (excitation, 297 nm; emission, 340 nm). Arf1-GTP has a greater emission than Arf1-GDP; therefore, the conversion results in an increase in fluorescent signal. The reaction contained 25 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 2 mM MgCl2, 1 mM EDTA, and 0.5 mM LUV with or without 1% PIP2. When GTP\( \gamma \)S was the substrate being varied, 0.1 nM His-Brag2Sec7-PH, 5 \( \mu M \) myrArf1 (saturating concentration), and 0.5–100 \( \mu M \) GTP\( \gamma \)S were included. When Arf1-GDP or Arf6-GDP was the substrate being varied, 0.1 (for LUVs containing 1% PIP2) or 0.5 nM (for LUVs lacking PIP2) His-Brag2Sec7-PH, 100 \( \mu M \) GTP\( \gamma \)S, and different concentrations of myrArf1 or myrArf6 were included.

**Single Turnover Assay**—Single turnover analyses were performed using an SF-2004 stopped flow instrument (KinTek Corp., Austin, TX). MyrArf1 or myrArf6 preloaded with mant-GDP was rapidly mixed with an equal volume of His-Brag2Sec7-PH. To load myrArf1 (or myrArf6) with mant-GDP, 0.2 \( \mu M \) mant-GDP was incubated at 30 °C for 1–2 h in 25 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 0.5 mM MgCl2, 1 mM EDTA, and 0.5 mM LUV with or without 1% (5 \( \mu M \)) PIP2. At the end of the incubation, MgCl2 was added to reach a final concentration of 2 mM. His-Brag2Sec7-PH was in the same buffer as for loading except 2 mM Mant-GDP was present and mant-GDP was not. The conversion of Arf-mantGDP to Arf-GTP was monitored by a FRET signal resulting from resonance energy transfer from tryptophan in Arf to the methylantranoyl group on GDP. The excitation wavelength was set at 297 nm, and the photomultiplier tube filter cutoff was 400 nm. Arf-mantGDP has a FRET signal, whereas Arf-GTP does not; therefore, the conversion results in a decrease in fluorescent signal.

**Modeling**

Secondary structure prediction was done using a consensus of different prediction programs (22). A docking calculation of the putative linker helix to the complex of Brag2 Sec7 domain
with myrArf was performed using the EMAP program of CHARMM (23, 24). The myrArf was created by superposing the NMR structure of myrArf (Protein Data Bank code 2K5U (25)) onto the structure of (Δ17)Arf1 bound to ARNO1 Sec7 domain (Protein Data Bank code 1RE0 (26)). Homology modeling was done using Prime (Schrödinger Inc., New York, NY) to replace the ARNO1 sequence with that of Brag2. The random coil portion of the Brag2 linker was docked interactively using MacroModel (Schrödinger Inc.), and the Sec7 domain, putative linker helix, and random coil region were linked using Prime.

Miscellaneous Methods

Protein concentration was estimated using the Bio-Rad dye binding assay. Arf concentration was determined by GTPγS binding as described (27). Graphs were prepared and parameter estimates were obtained using GraphPad Prism®. Alignments based on crystal structures were performed using the Protein Structure Alignment tool in Maestro (Schrödinger Inc.). Experiments with myrArf6 are described in the supplemental Methods.

RESULTS AND DISCUSSION

General Experimental Considerations for the Kinetics and Biochemistry of Arf and Arf-directed Guanine Nucleotide Exchange Factors—Arfs are myristoylated proteins (referred to as myrArf here) that bind to GDP with pm affinities (21, 29). Nucleotide exchange on myrArf requires that nucleotide evacuates the binding site on myrArf followed by nucleotide binding to the empty site. MyrArf is not stable without nucleotide and precipitates from solution. The function of the exchange factor is to accelerate nucleotide dissociation and stabilize the empty form of Arf. We consider the reaction a simplified Ping Pong Bi Bi (30) (Fig. 1A) based on available biochemical and crystallographic data (31–33). Bi Bi refers to two substrates and two products. Ping Pong refers to a mechanism in which the enzyme cannot bind two substrates simultaneously. One substrate binds to the enzyme; the enzyme releases the first product and is then able to bind to the second substrate. In this scheme, the first substrate, Arf-GDP, binds to the GEF, releasing the product, GDP, and leaving the complex of GEF-empty Arf. We consider this a second form of the enzyme to which the second substrate, GTP, binds. The product, Arf-GTP, is released, generating the initial form of the enzyme.

The role of a membrane surface was considered in examining Brag2 activity. Neither of the Brag2 substrates, myrArf-GDP and GTP, is membrane-restricted. Therefore, surface dilution does not have to be considered when examining initial rates. However, the enzyme-substrate complex (i.e. Brag2-myrArf) and the product myrArf-GTP are tightly associated with membranes, and myrArf-GTP cannot accumulate without a hydrophobic surface. We provided LUVs as the surface.

The experiments presented here used myrArf1-GDP and mutants of Arf1 as substrates for Brag2. MyrArf6 was also used for substrate saturation and single turnover experiments with results that were very similar to those obtained with myrArf1. Because Brag2 has been reported to be an Arf6 exchange factor, we provide the data for myrArf6 in supplemental Results and Discussion, Figs. S1–S3, and Tables S1–S3 to provide a documented comparison with Arf1. The data do not add significantly to the test of the hypothesis that PIP2 binding to the PH domain regulates GEF activity and, therefore, are not included in the main text.

Brag2 Fragment Comprising Sec7 and PH Domains (His-Brag2sec7-PH) as a Model for Studying Regulation of Brag2—We sought a recombinant form of Brag2 to examine phosphoinositide dependence. Although we have prepared full-length recombinant Brag2 suitable for some biochemical analyses, at
this time, we are not able to obtain a sufficiently pure, homogeneous, stable full-length Brag2 of adequate concentration for the planned kinetic experiments. We were able to prepare a protein comprising the Sec7 and PH domains with a His10 tag fused to the N terminus (His-Brag2<sub>sec7-PH</sub>). We first determined whether His-Brag2<sub>sec7-PH</sub> was activated by PIP2 to a similar extent as full-length Brag2. Two preparations of full-length Brag2 were used for the comparison: (i) Brag2 expressed in bacteria as a GST fusion protein that also contained a His6 tag (GST-Brag2-His) and (ii) Brag2 with myc and His6 tags on the C terminus (Brag2-myc-His) expressed in and purified from mammalian cells (see Fig. 1B for schematic of recombinant Brag2 proteins used in these experiments). The concentration of the full-length Brag2 was estimated by comparing the intensity of staining with standards run on the same polyacrylamide gel.

To determine the relative effect of PIP2 on exchange factor activity, the Brag2 recombinant proteins were titrated into reactions containing myrArf1-GDP (0.5 μM), GTPγS (5 μM), and LUVs with or without PIP2 (Fig. 1C and Table 1). The concentration of Brag2 that resulted in 50% exchange of nucleotide on Arf (we call this concentration the C<sub>50</sub>), which is roughly proportional to the inverse of enzymatic power (28), was determined. All preparations of Brag2, including His-Brag2<sub>sec7-PH</sub>, had 15–20-fold more specific activity in the presence of PIP2 than in its absence. His-Brag2<sub>sec7-PH</sub> was 6–10-fold more active than either full-length Brag2. Given the similar effects of PIP2 on the activity of full-length Brag2 and His-Brag2<sub>sec7-PH</sub>, His-Brag2<sub>sec7-PH</sub> was used for our subsequent work aimed at understanding the mechanisms by which PIP2 binding to Brag2 stimulates GEF activity.

Effect of PIP2 on Kinetic Parameters of His-Brag2<sub>sec7-PH</sub>—We determined the effect of PIP2 on kinetic parameters for His-Brag2<sub>sec7-PH</sub> using myrArf1-GDP as the substrate. The analysis for myrArf1-GDP is simplified if analyzed with saturating concentrations of the second substrate, GTP (we used GTPγS, an analog of GTP that is slowly hydrolyzed) (see supplemental Appendix for equations used for analysis). To establish the necessary concentration of GTPγS, we determined the enzymatic parameters with GTPγS as the varied substrate (Fig. 2). Arf1-GDP was fixed at a saturating concentration (>20 K<sub>m</sub>, see next paragraph). The reaction was followed continuously by measuring tryptophan fluorescence, which increases when Arf switches from the GDP- to GTP-bound forms (27). Initial rates were estimated and plotted against the concentration of GTPγS, and the data were fit to a Michaelis-Menten equation. The K<sub>m,GTPγS</sub> was 1 μM. The k<sub>cat</sub> calculated from the V<sub>max</sub> was 8/s (Table 2).

The effect of PIP2 on the kinetic parameters using myrArf1-GDP as the varied substrate was determined (Fig. 3 and Table 2). In these experiments, the dependence of the initial velocity of the exchange reaction on myrArf1-GDP concentration was determined, and the results were analyzed using the Michaelis-Menten equation. In the presence of PIP2, the K<sub>m,Arf1-GDP</sub> was 0.2 μM, and k<sub>cat</sub> was 8/s (calculated from the V<sub>max</sub>) (Fig. 3 and Table 2). In the absence of PIP2, the K<sub>m</sub> was 2.2 μM, 11-fold greater than in the presence of PIP2. More His-Brag2<sub>sec7-PH</sub> was used for experiments in the absence of PIP2 than in the presence, so the V<sub>max</sub> was greater than the V<sub>max</sub> determined in the presence of PIP2. The calculated k<sub>cat</sub> for which V<sub>max</sub>/([His-Brag2<sub>sec7-PH</sub>]) was 6.4/s, which is similar to that determined in the presence of PIP2.

We also determined the enzymatic parameters using single turnover experiments. In these experiments, the complex Arf1-mantGDP was used as a substrate, which was detected as resonance energy transfer from the tryptophans in Arf1 to the methylanthronoyl group on GDP. Nucleotide dissociation was detected as the loss of resonance energy transfer. The rate of nucleotide dissociation with increasing concentrations of His-Brag2<sub>sec7-PH</sub> was measured using a stopped flow instrument. At saturating concentrations of His-Brag2<sub>sec7-PH</sub> the observed rate (k<sub>obs</sub>) is equal to the k<sub>cat</sub>; thus, the single turnover approach has the advantage that k<sub>cat</sub> is determined directly. The concentration of His-Brag2<sub>sec7-PH</sub> at which the observed rate is 1/2 of the k<sub>cat</sub> (we call this the Brag2<sub>50</sub>) is between 1/2 K<sub>m</sub> and K<sub>m</sub>. We...
found that Brag2_{50} for Arf1 was 15-fold greater in the absence than in the presence of PIP2. The value of $k_{cat}$ in the absence of PIP2 was $\frac{1}{2}$ the $k_{cat}$ determined in the presence of PIP2 (Fig. 4 and Table 3), consistent with the notion that PIP2 acts as an allosteric modifier of Brag2.

The efficiency of an enzyme (also called enzymatic power) is expressed as the ratio $k_{cat}/K_m$. To obtain estimates of $k_{cat}$ and $K_m$, we used both substrate saturation and single turnover experiments. We are most confident in the $K_m$ determined by substrate saturation because the Arf concentration was determined by titrating GTP binding sites. The concentration of Brag2 was estimated using a dye binding assay and, therefore, may not accurately represent molar mass. We are most confident in the $k_{cat}$ determined by titrating GTP binding sites. The concentration of Brag2 was estimated using a dye binding assay and, therefore, may not accurately represent molar mass. We are most confident in the $k_{cat}$ determined from single turnover studies because this is a direct measurement. In substrate saturation studies, the $K_m$ is calculated from $V_{max} = k_{cat}[Brag2]$. An error in estimating Brag2 concentration would be propagated to the calculation for $k_{cat}$. Using the values in which we are most confident, we calculate a $k_{cat}/K_m$ of $2.6 \pm 0.9 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ in the absence of PIP2 and $8.8 \pm 3.1 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ in the presence of PIP2. There is a 34-fold increase in activity due to PIP2.

The value of $k_{cat}/K_m$ in the presence of PIP2 is 20–30% the value of the most efficient exchange factors described, EFs ($4.25 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) (34) and RCC1 ($3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) (35), and 5–20% that of Arf GTPase-activating proteins, which are enzymes that inactivate Arf (ASAP1, $5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) (19). An ideal enzyme, limited by the rate of molecular collision, would have a $k_{cat}/K_m$ of $10^8 \text{ M}^{-1} \text{s}^{-1}$ (36, 37). Although Brag2 is efficient, it is theoretically possible that another stimulatory factor could increase activity 10–20-fold beyond that achieved with PIP2.

**TABLE 3** Kinetic parameters determined from single turnover experiments

| Brag2_{so} | $k_{cat}$ |
|------------|-----------|
| + PIP2     | 0.067 ± 0.009 | 1.77 ± 0.06 |
| − PIP2     | 0.99 ± 0.06   | 0.58 ± 0.01  |

The PH Domain of His-Brag2_{sec7-PH} Is Necessary for Robust GEF Activity—The activities of recombinant proteins comprising the Sec7 (His-Brag2_{sec7}), Sec7-linker (His-Brag2_{sec7-linker}), and Sec7-PH (His-Brag2_{sec7-PH}) domains (schematically represented in Fig. 5A) were compared to examine the role of the PH domain in Brag2 GEF activity. MyrArf1-GDP was used as the substrate, and LUVs containing PIP2 were present to stabilize the product of the reaction, myrArf1-GTPγS. In the experiment presented in Fig. 5B, more than 30% of the myrArf1 exchanged nucleotide when incubated with 0.14 nm His-Brag2_{sec7-PH}, whereas 100 nm Sec7-linker (His-Brag2_{sec7-linker}) induced 20% exchange, and 100 nm Sec7 alone (His-Brag2_{sec7}) induced 10% exchange. Based on these rates, His-Brag2_{sec7-PH} had more than 700-fold greater activity than either His-Brag2_{sec7-linker} or His-Brag2_{sec7}. In the absence of PIP2, His-Brag2_{sec7-PH} had more than 50-fold the activity of either His-Brag2_{sec7-linker} or His-Brag2_{sec7} (not shown). These results indicate that the PH domain is a critical regulator of Brag2 activity.

![FIGURE 3. MyrArf and PIP2 dependence of Brag2-catalyzed nucleotide exchange: substrate saturation experiments.](image)

![FIGURE 4. Effect of PIP2 on Brag2-catalyzed exchange of nucleotide on Arf: single turnover experiments.](image)
To test the idea that PIP$_2$ binding to the PH domain regulates GEF activity, recombinant proteins with changes in residues predicted to bind PIP$_2$ were examined. The Brag2 PH domain does not align well with typical PIP$_2$ or phosphatidylinositol 1,4,5-trisphosphate binding PH domains, such as from ARNO (see alignment in Fig. 5C). However, a crystal structure of the PH domain of Brag2 is available (MMDB accession number 89889). Assuming the loop between strands $\beta_9$ and $\beta_10$ (in Fig. 5C, loop residues are indicated by a “C” and $\beta$ strand residues are indicated by an “E” above the sequence) contains the PIP$_2$ binding site (double underlined region in sequence), mutations were introduced into the PH domain of His-Brag2Sec7-PH (Fig. 5C, highlighted in blue). Two constructs were generated, one with serine substitutions of lysines at positions 753 and 756 (K753S,K756S) and another with a serine substitution of arginine at position 762 (R762S). Both mutants had GEF activity when assayed in the presence of 5 $\mu$M PIP$_2$. His-(R762S)Brag2Sec7-PH had less activity than wild type His-Brag2Sec7-PH (Fig. 5D and Table 4).

The mutants were used to correlate PIP$_2$ binding to Brag2 activation. We first determined binding to LUVs containing variable concentrations of PIP$_2$, to determine relative affinities. The mutant His-(K753S,K756S)Brag2Sec7-PH bound PIP$_2$ in LUVs less tightly than the wild type protein (Fig. 5E). We did not detect binding of His-(R762S)Brag2Sec7-PH to PIP$_2$-containing LUVs. The effect of the mutations on binding to LUVs correlated with the effect of PIP$_2$ on Brag2 activity. PIP$_2$-stimulated activity was detected for His-(K753S,K756S)Brag2Sec7-PH, but the PIP$_2$ dependence was shifted to the right. At 5 $\mu$M PIP$_2$ (the condition used to determine whether the proteins had activity in Fig. 5D), His-(K753S,K756S)Brag2Sec7-PH had ~80% the
activity of wild type protein. PIP₂ had no effect on the activity of His-(R762S)Brag₂Sec7-PH (Fig. 5F). These findings indicate that the PH domain and PIP₂ binding to the PH domain are required for maximal Brag₂ activity.

**PH Domain and Interdomain Linker Promote Activity of Sec7 Domain in His-Brag₂Sec7-PH**—We next tested the idea that PIP₂ binding to the PH domain relieved autoinhibition. The function of the PH domain in controlling autoinhibitory motifs within ARNO was uncovered using recombinant Arf lacking the N terminus ((Δ17)Arf₁) (11), which does not require a hydrophobic surface for nucleotide exchange (21). We used a similar approach to examine the possibility of autoinhibition in Brag₂.

The predictions for the autoinhibition model are that His-Brag₂Sec7 should be more active than His-Brag₂Sec7-PH using (Δ17)Arf₁ as a substrate in the absence of phosphoinositides and that the activity of His-Brag₂Sec7-PH would be increased by PIP₂ and possibly by a soluble PIP₂ analog. We compared activities of His-Brag₂Sec7, His-Brag₂Sec7-linker, and His-Brag₂Sec7-PH (Fig. 5A) using (Δ17)Arf₁ as a substrate. In contrast to the predictions of the autoinhibition model, we found that His-Brag₂Sec7-PH and His-Brag₂Sec7-linker were more active than His-Brag₂Sec7 (Fig. 6C and Table 5) and that LUVs with PIP₂ had no effect on the activity of any of these recombinant Brag₂ proteins when using (Δ17)Arf₁ as a substrate (Fig. 6C). Also in contrast to the prediction of the autoinhibition mechanism, a soluble analog of PIP₂ did not affect activity (Fig. 6D). We conclude that PIP₂-mediated regulation of Brag₂ activity does not involve rearrangements that relieve inhibition. Instead, the linker contributes to Brag₂ activity.

The result that Brag₂ is not regulated in the same manner as ARNO is consistent with the lack of homology in the regions responsible for autoinhibition in ARNO (Figs. 5C and 6A). In the sequence shown in Fig. 5C, the polybasic motif in ARNO that is necessary for autoinhibition (11) is underlined. There is little similarity to Brag₂. In Fig. 6A, the linker following the Sec7 of ARNO is underlined. This motif, which contributes to autoinhibition in ARNO (11), also has little similarity to Brag₂.

The greater activity of the Brag₂ recombinant proteins with the linker could be due to interaction of the linker with Arf₁. The Sec7 domain comprises 10 α helices, A through J, with a prominent hydrophobic groove in which Arf binds. Motifs immediately C-terminal of helix J are near the hydrophobic groove and have been previously reported to interact with Arf₁ (38). A consensus of secondary structure prediction programs predicted that the first part of the Brag₂ linker region, residues 704–716, should form a helix (Fig. 6B). This putative helix is amphipathic. A docking calculation of the putative helix to the Brag₂-myrArf complex was performed, testing all possible locations of the helix placed immediately C-terminal of Brag₂ helix J. Many possible docking sites were predicted, including one that also interacted with switch 1 of myrArf (Fig. 6B). Such an interaction could explain why the Brag₂ constructs containing the linker are more active; however, further experiments would be needed to prove this linker/myrArf interaction.

The Amino Terminus of Arf Is Necessary for Regulated Activity—The lack of effect of PIP₂ on the activity of His-Brag₂Sec7-PH when using (Δ17)Arf₁ as a substrate was evidence that regulation was not through autoinhibition. Alternative mechanisms include PIP₂ concentrating Brag₂ together with myrArf₁-GDP on a membrane or PIP₂ stimulating interaction of the N terminus of Arf₁ directly with Brag₂. In initial experiments to distinguish between these possibilities, we studied the Arf mutant (L8K)Arf₁ as a substrate. This mutant is not myristoylated, eliminating the possibility that the myristate could account for the observed effects. In addition, (L8K)Arf₁ binds GTP independently of lipids, and neither (L8K)Arf₁-GDP nor (L8K)Arf₁-GTP binds to lipids (13, 14). Therefore, effects of the membrane on activity could be separated from effects of the membrane on product accumulation.

The exchange on (L8K)Arf₁ catalyzed by His-Brag₂Sec7-PH, His-Brag₂Sec7-linker, and His-Brag₂Sec7 in the absence of LUVs with PIP₂ was examined. There was little exchange using any of these forms of Brag₂ compared with the rate observed with (Δ17)Arf₁ (Fig. 6E). Activity of His-Brag₂Sec7-PH but not of His-Brag₂Sec7-linker or His-Brag₂Sec7 increased at least 40-fold by including LUVs with PIP₂ and was about 3-fold more efficient with (L8K)Arf₁ than with (Δ17)Arf₁ (Fig. 6E and Table 6). Soluble PIP₂ had no effect on activity (Fig. 6F). In short, the N terminus of Arf is required for activation of Brag₂ by PIP₂.

**PIP₂-dependent Recruitment of Brag₂ and Arf to Membranes Does Not Completely Account for Regulation**—Both Brag₂ and Arf₁ have been reported to bind PIP₂. To further test the idea that concentrating the two proteins on a surface may account for at least part of the effect of PIP₂ on Brag₂ activity, we directly measured binding of both proteins to LUVs. In these experiments, LUVs containing increasing concentrations of PIP₂ were incubated with either His-Brag₂Sec7-PH, myrArf₁, or both. The LUVs were separated from bulk solution, and the bound proteins were measured. His-Brag₂Sec7-PH bound to the LUVs with a Kᵦ of 2 μM for PIP₂ (Fig. 7, A and B). In contrast, PIP₂ had little effect on myrArf₁ binding to LUVs (Fig. 7, C and D). When His-Brag₂Sec7-PH and myrArf₁ were incubated together, both proteins bound efficiently to LUVs in the absence of PIP₂. PIP₂ had a small effect on binding when the two proteins were incubated together, but the increase was not sufficient to account for the 30–40-fold change in activity (Fig. 7, A–D). (Δ17)Arf₁ and (L8K)Arf₁ were also examined. Neither bound efficiently to LUVs, and neither PIP₂ nor Brag₂ increased their association with LUVs. We conclude that PIP₂ does not increase activity by concentrating Brag₂ with myrArf₁ on a membrane surface.

**Potential Regulatory Mechanisms of PIP₂**—The regulatory effect of PIP₂ required the N terminus of Arf and a membrane surface. These requirements and the computer modeling have led us to consider three roles of PIP₂. First, PIP₂ may bind to Arf through lysines 10, 15, and 16 in the N terminus to stabilize nucleotide-free Arf associated with Brag₂. Previously, PIP₂ was found to stabilize nucleotide-free Arf when Mg²⁺ was buffered to ~1 μM (39). The second role of PIP₂ may be to bind to the PH domain to control the conformation and orientation of the linker domain. The N terminus of Arf may be important to see this effect; modeling supports the idea that the linker between the PH domain and Sec7 domain may interact with switch 1 of Arf. The linker may also bind to the N terminus of Arf. Past the helix that may interact with switch 1, the residues in the linker
are predicted to be in a random coil structure. In the structural model in Fig. 6B, the possibility of contact between the linker and the N-terminal region of Arf is shown. This structural model is hypothetical as the current data only imply the possibility of physical interaction between the linker and Arf. We are currently devising tests of this idea. The third effect of PIP2 could be to anchor the enzyme/substrate complex to the membrane where the transition state (i.e. nucleotide-free Arf-Brag2) may be stabilized.

A Unique Aspect of the Arf Nucleotide Exchange Reaction Can Explain the Effect of Membrane on Reaction Rate—A unique aspect of nucleotide exchange on Arf is that, although both
substrates (myrArf1-GDP and GTP) are soluble, the enzyme-substrate complex and one product (myrArf1-GTP) are membrane-associated. Therefore, when using myrArf1 as a substrate, a hydrophobic surface is required for product accumulation. MyrArf1 also has the property that in the presence of lipids its affinity for GTP is higher than its affinity for GDP, whereas the affinity of (Δ17)Arf1 for GTP and GDP is similar (21). We found that catalysis on Arf mutants that do not associate with the membrane is 1–2% that of myrArf1, which has high affinity for membranes. Two factors may explain the difference in reaction rates. First, the equilibrium of myrArf1-GDP and myrArf1-GTP lies more strongly in the direction of myrArf1-GTP in the presence of membranes than do the equilibria between (Δ17)Arf1-GDP and (Δ17)Arf1-GTP or between (L8K)Arf1-GDP and (L8K)Arf1-GTP. Based on the Haldane relationship \( K_{eq} = V_{\text{forward}} K_{\text{reverse,forward}}/K_{\text{reverse}} K_{\text{forward}} \), at the very least, the relative forward reaction is more efficient than the reverse when using myrArf1, and it is plausible that the absolute rates may be different. The second explanation for the difference in rates is that the myristoylated N terminus of Arf may anchor the enzyme-substrate complex in the membrane with the consequence of accelerating step 1 or step 2 in the reaction scheme shown in Fig. 1A.

An Additional Activator or Regulator May Affect Brag2 Activity—We examined truncated Brag2 constructs to identify the critical role of the PH domain in catalytic regulation. However, other domains of Brag2 may also regulate its activity. The preparations of full-length Brag2 that we examined had less activity than His-Brag2Sec7-PH. Although we cannot exclude that a fraction of the full-length proteins was not active due to improper folding, the result is also consistent with the idea that a motif outside of the Sec7-PH domains has an autoinhibitory function. A number of ligands could relieve such inhibition. The activity of Brag2 has been reported to be increased by binding to peptides from AMPA receptor (10) and phosphorylated peptides from epidermal growth factor receptor (4). Although these bind to the PH or Sec7-PH domains, they could affect activity by a mechanism distinct from the effect of PIP2 and could involve other domains of Brag2. Other binding partners might also contribute to Arf specificity by restricting Brag2 localization or by changing the qualitative interaction with the substrate.

Explanation for the \( k_{\text{cat}} \) Effect of PIP2 Observed in Single Turnover but Not Substrate Saturation Experiments—In single turnover experiments, PIP2 was found to affect the \( K_m \) and \( k_{\text{cat}} \) of the reaction, whereas in substrate saturation experiments, only the \( K_m \) was affected. The trivial explanation for the differ-

### TABLE 5
Effect of interdomain linker on activity using (Δ17)Arf1

| C_{50} | His-Brag2Sec7-PH | His-Brag2Sec7-linker | His-Brag2Sec7 |
|-------|-----------------|---------------------|---------------|
| 55 ± 10 | 84 ± 10         | 138 ± 17p|     |

*p < 0.001 comparing His-Brag2Sec7-PH with His-Brag2Sec7-linker.

### TABLE 6
Effect of PIP2 on His-Brag2Sec7-PH activity for myrArf1, (L8K)Arf1, and (Δ17)Arf1

|        | MyrArf1 | (L8K)Arf1 | (Δ17)Arf1 |
|--------|---------|-----------|-----------|
| +PIP_2 | 0.44 ± 0.03 | 24.2 ± 2.1 | 68.6 ± 13.2 |
| −PIP_2 | 6.45 ± 0.74 | >800       | 61.7 ± 13.1 |

### FIGURE 7
Brag2, myrArf1-GDP, (Δ17)Arf1-GDP, and (L8K)Arf1-GDP binding to LUVs. His-Brag2Sec7-PH (0.8 μM) with or without 1 μM myrArf1-GDP, (Δ17)Arf1, or (L8K)Arf1 was incubated with 500 μM sucrose-loaded LUVs containing the indicated concentrations of PIP2. Vesicles were recovered by centrifugation, and associated proteins were separated by SDS-PAGE. The amount of lipid-bound protein was determined by densitometry of the Coomassie Blue-stained gels with standards on each gel. Primary data for His-Brag2Sec7-PH binding for representative experiments are shown in A, and primary data for Arf binding are shown in C. Standards for the representative experiments are shown in A and C. The summary of experiments for His-Brag2Sec7-PH binding is shown in B, and the summary of experiments for Arf binding is shown in D. Averages and S.E. for two to four experiments are presented.
Allosteric Regulation of Brag2 Sec7 Domain

![Kinetic Scheme Diagram](image)

FIGURE 8. Modified kinetic scheme. In this kinetic scheme, which is a modification of the scheme in Fig. 1A, E represents a ground state of Brag2, and E* represents a transition state intermediate. AD, Arf1-GDP; AT, Arf1-GTP; A, Arf1 (nucleotide-free); T, GTP; D, GDP. Steps measured in single turnover experiments are highlighted, and steps measured in substrate saturation experiments are circled.

ence is that the relatively large error associated with parameters determined from substrate saturation experiments disguised the 3-fold difference that was apparent in the more precise and accurate determination by single turnover experiments. Excluding this explanation, the difference could be related to the particular steps of the reaction being measured. In single turnover studies, a single round of GDP release was measured. An effect on the first or second step of the reaction (see Figs. 1A and 8) would account for a change in both $K_m$ and $k_{cat}$. In the case of substrate saturation, GTP binding and ArfGTP release also determine reaction rate. If one of these steps were rate-limiting, a change in step 1 or step 2 might affect the $K_m$ for the first substrate without affecting the $V_{max}$ and calculated $k_{cat}$. Other explanations include hysteretic effects on Brag2 during the catalytic cycle. For example, the reaction scheme in Fig. 8 shows the transition state toward release of product ($E^*$) that relaxes slowly toward the ground state. Single turnover studies measure steps 1–3 in this scheme. Substrate saturation may primarily measure the cycle of steps 7, 3, 4, and 5. Future work will focus on determining specific reaction steps affected by ligand binding to the PH domain.

Summary—Our results support a model in which the PH domain of Brag2 is an allosteric binding site regulating catalysis, whereas the linker between the Sec7 and PH domains contributes to activity. Together, these represent a regulatory mechanism unique to Brag2.

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