The Pleckstrin Homology Domains of Dynamin Isoforms Require Oligomerization for High Affinity Phosphoinositide Binding

Daryl E. Klein, Anthony Lee, David W. Frank‡, Michael S. Marks‡, and Mark A. Lemmon§

From the Department of Biochemistry and Biophysics and Johnson Research Foundation and the Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6089

The dynamins are 100-kDa GTPases involved in the scission event required for formation of endocytic vesicles. The two main described mammalian dynamins (dynamin-1 and dynamin-2) both contain a pleckstrin homology (PH) domain, which has been implicated in dynamin binding to (and activation by) acidic phospholipids, most notably phosphoinositides. We demonstrate that the PH domains of both dynamin isoforms require oligomerization for high affinity phosphoinositide binding. Strong phosphoinositide binding was detected only when the PH domains were dimerized by fusion to glutathione S-transferase, or via a single engineered intermolecular disulﬁde bond. Phosphoinositide binding speciﬁcities agreed reasonably with reported effects of different phospholipids on dynamin GTPase activity. Although they differ in their ability to inhibit rapid endocytosis in adren al chromaffin cells, the dynamin-1 and dynamin-2 PH domains showed identical phosphoinositide binding speciﬁcities. Since oligomerization is required for binding of the dynamin PH domain to phosphoinositides, it follows that PH domain-mediated phosphoinositide binding will favor oligomerization of intact dynam in (which has an inherent tendency to self-associate). We propose that the dynamin PH domain thus mediates the observed cooperative binding of dynamin to membranes containing acidic phospholipids and promotes the self-assembly that is critical for both stimulation of its GTPase activity and its ability to achieve membrane scission.

The dynamins are GTPases of 100 kDa that play a key role in the scission event leading to endocytic vesicle formation (1–3). In cells expressing mutated dynamin-1 defective in GTP binding and hydrolysis (4–6), receptor-mediated endocytosis is inhibited, and invaginated coated pits accumulate, on which dynamin appears uniformly distributed (6). If GTP hydrolysis (but not binding) by dynamin is inhibited in synaptosomes with GTPγS,1 constricted coated pits accumulate, in which dynamin forms a “collar” around the constriction (7). A similar collar occurs in shibire Drosophila, which have a mutated dynamin homolog (8). A model has thus emerged for the role of dynamin in receptor-mediated endocytosis (1, 2) in which it is ﬁrst targeted to clathrin-coated pits in a GDP-bound (or nucleotide-free) form. Upon GTP binding, dynamin is proposed to self-assemble at the necks of invaginated coated pits to form collars, and GTP hydrolysis by dynamin in the collars is ﬁnally thought to pinch off the endocytic vesicle.

Three mammalian dynamin isoforms are known (1, 3, 9). Dynamin-1 is expressed only in neurons (10, 11), dynamin-2 is ubiquitously expressed (11, 12), while dynamin-3 is restricted primarily to the testes (13). Each isoform has multiple domains. The N-terminal ~300 amino acids comprise the GTPase domain, which is followed by two ~100-amino acid regions of unknown function. A pleckstrin homology (PH) domain extends from residues 521 to 623 (in human dynamin-1), followed by a 130-amino acid domain that interacts with the GTPase domain and acts as a GTPase effector (14). Finally, the C-terminal 100 amino acids form a proline/arginine-rich domain (PRD), which binds in vitro to several SH3 domains (15), and is important in targeting dynamin to coated pits (16). Recent studies have demonstrated a specific role for PRD binding to the amphipathic SH3 domain in recruiting dynamin to coated pits (17, 18).

In the absence of membranes, puriﬁed dynamin forms a tetramer (14), which further self-assembles into rings or spirals when subjected to low ionic strength conditions (19) or (at physiological ionic strength) to GDP plus metallo ﬂuorides (20). The assemblies are morphologically similar to the collars seen in constricted coated vesicles in vivo, and their formation requires the PRD, but not the PH domain (14, 19). The function of dynamin in endocytosis requires both its self-assembly and GTPase activity. Self-assembly enhances the GTPase activity of dynamin; an effect that can be mimicked in vitro by several multivalent dynamin-binding molecules including microtubules (21), glutathione S-transferase (GST)/SH3 domain fusion proteins (e.g. from Grb2) (15), and bivalent antibodies (22). The GTPase activity of puriﬁed dynamin also shows a cooperative dependence on its concentration (23), which correlates with self-assembly (23, 24). Many activators of dynamin in vitro thus appear to exert their effect simply by enhancing dynamin self-assembly. This is also likely to occur in vivo, although the mechanism of dynamin self-association on coated vesicles is not clearly understood.
Phosphoinositide Binding by Oligomeric Dynamin PH Domains

One class of molecules that enhance both the GTPase activity of dynamin and its self-assembly in vitro is acidic phospholipids, including the phosphoinositides (25, 26). Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$) appears to be the most potent (27), although PtdIns(3,4,5)P$_3$ also has a strong stimulatory effect (28), and significant effects are seen with other acidic phospholipids (25–29). Deletion of the PH domain from dynamin-1 abolishes the ability of PtdIns(4,5)P$_2$ to stimulate GTPase activity (27), while deletion of the PRD has no effect (29). Thus, as for other PH domains (30, 31), there is evidence that phosphoinositide binding to the dynamin PH domain (DynPH) plays a role in its activation. Nonetheless, we have not been able to detect binding of isolated DynPH to any phosphoinositide or inositol phosphate in a variety of assays (32, 33), although others have (27, 34) (see below). Furthermore, while the isolated dynamin-1 PH domain (Dyn1PH) inhibits rapid endocytosis (RE) following stimulated catecholamine secretion from adrenal chromaffin cells (35), other PH domains that bind strongly to PtdIns(4,5)P$_2$ have no effect. The PH domain from dynamin-2, which shares 81% identity with Dyn1PH, does not affect RE (35). Motivated by these observations, we reinvestigated phosphoinositide binding by dynamin PH domains in an effort to understand discrepancies in the literature and to determine whether functional differences (in RE) between Dyn1PH and Dyn2PH can be explained by the phosphoinositide binding specificities or affinities.

Dyn1PH has been shown to bind weakly to the PtdIns(4,5)P$_2$ head group, inositol-1,4,5-trisphosphate (Ins(1,4,5)P$_3$), in NMR studies, with reported $K_{d}$ values of 4.3 mM (34) and 1.2 mM (27). Zheng et al. (34) also found that PtdIns(4,5)P$_2$ and PtdIns-4-P can bind Dyn1PH, but only when detergent-solubilized phospholipids were used, and the final detergent concentration was below critical micelle concentration. In contrast, Salim et al. (27) found that Dyn1PH binds to PtdIns(4,5)P$_2$-containing vesicles, using a GST fusion protein of Dyn1PH immobilized on a biosensor chip. In our own studies with isolated Dyn1PH, we have been unable to detect significant binding to any phosphoinositide (32, 33).

In this report, we demonstrate that the PH domains from dynamin-1 and dynamin-2 bind with much higher affinity to phosphoinositides when they are oligomeric; PH domain dimerization was required for detection of significant phosphoinositide binding. Since intact dynamin forms tetramers and higher order assemblies, and this behavior is critical for its physiological function, we suggest that PH domain-mediated binding of dynamin to the membrane surface in vivo requires oligomerization. Furthermore, since Salim et al. (27) used a (dimeric) GST fusion protein and were able to detect Dyn1PH binding to PtdIns(4,5)P$_2$, our findings provide an explanation for the disagreement between previous studies. Phosphoinositide binding by dimeric dynamin PH domains shows specificity similar to that seen for stimulation of dynamin GTPase activity in vitro (28). Dyn1PH and Dyn2PH gave identical results, despite their different abilities to inhibit rapid endocytosis in adrenal chromaffin cells (35).

We suggest that PH domain-mediated binding of dynamin to phosphoinositide-containing membranes can occur only coincident with, or following, its self-assembly. This is likely to explain the observed cooperativity in dynamin binding to membranes, the stabilization of dynamin oligomers by vesicles containing acidic phospholipids (26), the influence of phosphoinositides on dynamin GTPase activity, and it is likely to have important functional implications.

EXPERIMENTAL PROCEDURES

Phospholipids and Inositol Phosphates—PtdIns-4-P, PtdSer, PtdIns-(4,5)P$_2$, and Ins(1,4,5)P$_3$ were from Sigma. Dipalmitoyl PtdIns-5-P, PtdIns(3,4)P$_2$, and PtdIns(3,4,5)P$_3$ were from Matreya (Pleasant Gap, PA). PtdCho and di(dibromostearoyl) PtdCho were from Avanti (Birmingham, AL).

Production of Dynamin PH Domains—Monomeric Dyn1PH and Dyn2PH were produced exactly as described previously (33, 35). For GST fusion proteins, fragments with the same amino acid boundaries were subcloned into pGEX-2T and pGEX-2TK (Amersham Pharmacia Biotech), for centrifugation experiments and dot-blot experiments, respectively. For GST fusion protein purification, cells were lysed by sonication in 50 mM Tris, pH 8.0, 150 mM NaCl, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM DTT. Protein concentrations were determined by the Bradford method (Sigma) for 15 min at 4 °C. Beads were washed four times in lysis buffer and once in lysis buffer containing 1 mM NaCl, and protein was eluted with 15 mM reduced glutathione. Glutathione was removed by size-exclusion chromatography.

Dot-blot Assay—Purified GST fusion proteins (pGEX-2TK) were labeled with [$^{32}$P]ATP plus 10–20 units of protein kinase A (Sigma) for 30 min at room temperature in 50 mM potassium phosphate, pH 7.15, 10 mM MgCl$_2$, 5 mM NaF, 4.5 mM DTT. After washing extensively with phosphate-buffered saline, containing 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride, [$^{32}$P]-labeled protein was eluted from glutathione-agarose using 15 mM reduced glutathione in phosphate-buffered saline and dialyzed (0.2 µM) prior to use for dot-blot assays.

Phospholipids at 2 mg/ml in 1:1 chloroform:methanol solution (containing 0.1% HCl) were spotted (2 µl) onto nitrocellulose sheets in the pattern shown in Fig. 1. After drying, nitrocellulose was blocked overnight at 4 °C in Tris-buffered saline plus 3% bovine serum albumin (without detergent). [$^{32}$P]-labeled GST-PH fusion protein at 0.5 µg/ml in Tris-buffered saline, 3% bovine serum albumin was then used to probe the phosphoinositide-containing nitrocellulose for 30 min at room temperature. Filters were washed five times with Tris-buffered saline (without detergent) and dried, and bound radioactivity was visualized using a PhosphorImager (Molecular Dynamics).

Production of Disulfide-linked PH Domain Dimers—The single cysteine in Dyn1PH (Cys$^{80}$ of human dynamin-1) was mutated to serine using polymerase chain reaction mutagenesis as described previously (35). A unique N-terminal cysteine was then introduced by polymerase chain reaction, and the mutated product (CysDyn1PH) was expressed from pET11a in Escherichia coli BL-21 as described previously (33). The N terminus of CysDyn1PH has the sequence MCKTSG. DTT was maintained at 5 mM during initial purification steps. Following ion-exchange and ammonium sulfate precipitation, protein was dialyzed overnight into 50 mM sodium phosphate, pH 7.4, containing no DTT (buffer, pH 7.4, containing no DTT). Cu(II) 1,10-phenanthroline was then used to catalyze oxidation for disulfide-mediated dimerization. In gel filtration, the oxidized protein gave two incompletely resolved peaks corresponding to the dimer and monomer, respectively. Fractions were taken from the beginning of the dimer peak and determined by nonreducing SDS-polyacrylamide gel electrophoresis, analytical ultracentrifugation, and light-scattering measurements to contain ~90% PH domain dimer. A similar procedure was followed for Dyn2PH.

Analytical Ultracentrifugation—Sedimentation equilibrium experiments employed the XL-A analytical ultracentrifuge (Beckman). Samples were loaded into six-channel epon charcoal-filled centerpieces, using quartz windows. Experiments were performed at 25 °C using two different speeds (10,000 and 15,000 rpm), detecting at 280 nm, with identical results. Solvent density was taken as 1.033 g/ml, and the partial specific volume of Dyn1PH was estimated from its amino acid composition as 0.734 ml g$^{-1}$. Experiments were performed with 6 and 15 µM protein, using oxidized CysDyn1PH from the dimer peak obtained in gel filtration chromatography. In each case, experiments were performed both with (1 mM) and without the reducing agent tris(2-carboxyethylphosphine) hydrochloride (TCEP). Data were fit using the Origin program, a data analysis software (Beckman/MicroCal). Randomly distributed residuals were obtained using fits to a single ideal species, examples of which are shown in Fig. 2.

Centrifugation Assays for PH Domain Binding to Lipid Vesicles—Small unilamellar vesicles (SUVs) were generated by co-dissolving di(dibromostearoyl) phosphatidylcholine (PtdCho) with a single phosphoinositide or phosphatidylserine (PtdSer) at 3% (molar) in 1:1 chloroform:
Our primary motivation was the observation that Dyn1PH, but not Dyn2PH, inhibits RE in adrenal chromaffin cells when introduced as the monomeric protein (35). To test whether this functional distinction could be explained by differences in phosphoinositide-binding affinities, we measured the ability of unlabeled Dyn1PH and Dyn2PH (expressed from a pET vector) to compete with $^{32}$P-labeled GST-Dyn1PH for binding to PtdIns(3,4,5)P$_3$ immobilized on small nitrocellulose discs. Surprisingly, no competition by the unlabeled PH domains was seen until they were added at concentrations $>$100 $\mu$m (not shown). Since GST-Dyn1PH is present in these assays at 0.5 $\mu$g/ml ($\sim$0.01 $\mu$m), this result argues that fusion of Dyn1PH to GST enhances its affinity for the immobilized phosphoinositide by more than 10$^4$-fold. Using a gel filtration assay (30) and the PEG-precipitation assay of Fukuda et al. (41) (not shown), we found that fusion to GST does not significantly increase binding affinity of the dynamin PH domains for soluble inositol phosphate head groups. $K_D$′s for binding of dynamin PH domains to [H]Ins(1,4,5)P$_3$ or [H]Ins(1,3,4)P$_3$ were estimated to be in the several hundred micromolar to millimolar range, as reported previously (27, 34), regardless of fusion to GST (not shown). The difference between GST fusion proteins and proteins expressed as free PH domains is therefore not likely to be a simple artifact of PH domain misfolding that leads to loss of a high affinity binding site.

**PH Domain Dimerization Is Required for High Affinity Phosphoinositide Binding**—Since GST fusion proteins are known to form tight dimers (42, 43), we reasoned that the enhanced affinity of GST-PH proteins for immobilized phosphoinositides might reflect an avidity effect resulting from their association in a dimer. Indeed, several examples have been reported in which GST can functionally replace a native oligomerization domain (44–46) or enhance apparent binding affinities through avidity effects (47, 48). Since purified dynamin is tetrameric (14) and self-assembles to form still higher order oligomers in fulfilling its endocytotic functions (1–3), Dyn1PH will also be oligomeric in its native context.

To investigate directly the effect of oligomerization on phosphoinositide binding by dynamin PH domains, we developed a system in which self-association could easily be controlled. Dyn1PH was mutated to contain a single cysteine at its amino terminus (N-terminal sequence MCKTSGN...), with the only native cysteine (Cys$^{607}$ of human dynamin–1) replaced by serine. Mutated Dyn1PH (CysDyn1PH) was expressed without fusion to GST (see "Experimental Procedures"). By oxidizing purified protein in the presence of copper(II) 1,10-phenanthroline, disulfide-mediated dimerization of CysDyn1PH was induced with yields greater than 90%. The dimer was purified by gel filtration and could be reduced to its monomeric form using DTT or TCEP. Sedimentation equilibrium analytical ultracentrifugation (Fig. 2) showed that oxidized CysDyn1PH sediments as a single ideal species with a molecular mass of 33.2 ± 5.2 kDa (from six independent experiments at three different concentrations; error is quoted as the S.D. for fits to the six different data sets). In the presence of 1 mM TCEP, the same protein sedimented as a single species of 15.7 ± 0.8 kDa. The predicted monomeric molecular mass of CysDyn1PH is 14.8 kDa, arguing that oxidation induces only dimerization, which can be reversed completely by addition of reducing agent. Fits of sedimentation equilibrium data to a model of two independent species (monomer and dimer) also gave randomly distributed residuals (indicative of a good fit), with 94 ± 9% dimer in the oxidized samples and 5.5 ± 4% dimer in the reduced samples.

To compare phosphoinositide binding by CysDyn1PH dimers and monomers, we used an ultracentrifugation assay to measure their binding (with and without 1 mM TCEP) to SUVs...
containing different single phosphoinositides at 3 mol% in PtdCho. Brominated PtdCho was used to increase SUV density for efficient pelleting by ultracentrifugation (39). As shown in Fig. 3A, dimeric CysDyn1PH bound significantly to vesicles containing PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, or PtdIns(4,5)P_2. In contrast, TCEP-reduced CysDyn1PH monomers bound only weakly to the same vesicles (Fig. 3B). Partition coefficients (K_p) for vesicle binding by dimeric Dyn1PH (Table I) are only 6–8 times lower than those reported for binding of the PLC-δ1 PH domain to vesicles containing 3% PtdIns(4,5)P_2 in a background of 2:1 PtdCho/PtdSer (49). If it is assumed that Dyn1PH binds to phosphoinositides with a stoichiometry of 1:1 (i.e. two per dimer), K_p values for binding to PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, and PtdIns(4,5)P_2 can be estimated as approximately 11, 14, and 9 μM, respectively; all within a factor of 10 of the highest affinities reported for PH domain binding to phosphoinositides in lipid vesicles (30, 32, 49). Dimeric CysDyn1PH bound much more weakly to vesicles containing PtdIns-4-P, PtdIns-3-P, or PtdSer (Fig. 3A; Table I), and did not bind significantly to vesicles containing no acidic phospholipid (not shown). This experiment shows that disulfide-mediated dimerization of CysDyn1PH leads to a significant increase in its affinity for specific phosphoinositides in SUVs. The effect is seen only for PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, and PtdIns(4,5)P_2. For PtdIns-3-P, PtdIns-4-P, or PtdSer (at 3 or 20%), binding affinity is enhanced by only a factor of 2 (or less) upon dimerization. Residual binding seen for the reduced protein in Fig. 3B is likely to reflect both the small amount of dimer that remains (≤5%), and the intrinsic (very low) affinity of monomeric Dyn1PH for these vesicles. In contrast with the findings of Salim et al. (27), addition of divalent cations (1 μM Ca^{2+}, 1 mM Mg^{2+}) had no effect on either the specificity or affinity of phosphoinositide binding by dimeric CysDyn1PH. We suggest that the effect of divalents seen previously (27) may be specific either to the biosensor assay or to the nature of the large unilamellar vesicles employed. Indeed, although Salim et al. (27) also reported a divalent cation requirement for specificity in binding of the Btk PH domain to phosphoinositides, Rameh et al. (50) did not. It should also be noted that, while PtdIns(4,5)P_2 binds quite strongly to dimeric CysDyn1PH in our centrifugation assay, it does not give a significant signal in the dot-blot assay (Fig. 1). This discrepancy is quite reproducible, and we do not currently have a satisfactory explanation. Since the 3-phosphorylated phosphoinositides used here are synthetic dipalmitoylated lipids, and PtdIns(4,5)P_2 is derived from bovine brain, the discrepancy could reflect differences in acyl chain composition. This could affect the mode of their association with nitrocellulose or could (in vesicle assays) allow interactions of CysDyn1PH with PtdIns(4,5)P_2 acyl chains that are not possible with the 3-phosphoinositides. Using a mutated form of Dyn2PH with a cysteine introduced at the N terminus of the wild-type PH domain (CysDyn2PH), oxidized dimers could also be obtained, although at a lower yield than with CysDyn1PH. As with CysDyn1PH, CysDyn2PH dimers could be reduced to their monomeric form, and showed the same enhanced binding to phosphoinositide-containing vesicles (data not shown). We also tested binding of the monomeric PLC-δ1 PH domain to the same vesicles both with and without TCEP, to control for possible indirect effects of this reducing agent. As expected (32, 49), monomeric PLC-δ1 PH binding to PtdIns(4,5)P_2-containing vesicles was 5–10-fold stronger than binding of dimeric CysDyn1PH or CysDyn2PH, but was completely unaffected by the addition of TCEP (data not shown). Comparison of Phosphoinositide Binding Specificity for Dimeric Dyn1PH and Dyn2PH—Having established that disulfide-mediated dimerization is sufficient for significant phosphoinositide binding by both Dyn1PH and Dyn2PH, we next compared their binding affinities directly. Yields of disulfide-linked dimer differed for CysDyn1PH and CysDyn2PH, which would complicate a direct comparison of vesicle binding by the two proteins. We therefore used purified GST fusion proteins rather than the disulfide-mediated dimers. Since GST dimerizes with K_p < 1 μM (43), purified GST-PH fusions will be more than 80% dimeric under the conditions of our experiments (where [GST-DynPH] is 10 μM). Since GST-Dyn1PH and GST-Dyn2PH are dimerized by the same mechanism, and studied at the same concentrations, a comparison of the two isoforms is straightforward. As shown in Fig. 4 and Table II, GST-Dyn1PH and GST-Dyn2PH bind with essentially the same affinity to any given phosphoinositide, consistent with their 81% sequence identity. Addition of TCEP had no effect on binding of either GST fusion protein to phosphoinositide-containing vesicles (not shown), confirming that the effects seen in Fig. 3 reflect reduction of the...
intermolecular disulfide bond and not phosphoinositide binding itself. Molar partition coefficients ($K$) for binding of the GST fusion proteins to SUVs (Table II) were lower than those for dimeric CysDyn1PH (Table I), but followed the same trends with different phosphoinositides. The reduction in $K$ values for GST fusion proteins may reflect the presence of up to 20% monomeric GST-DynPH ($K_a$ for dimerization $\leq 1 \mu M$) in the experiment, which will not bind significantly to the vesicles, so reducing apparent affinity. This difference does not affect our ability to compare the selectivities of GST-Dyn1PH and GST-Dyn2PH.

Fig. 4 shows that both PH domains have the same selectivity, with phosphoinositide binding affinity following the order $\text{PtdIns(3,4,5)P}_3 \approx \text{PtdIns(4,5)P}_2 \approx \text{PtdIns(3,4)P}_2 > \text{PtdIns}-3-P > \text{PtdIns}-4-P > \text{PtdSer}$ (not shown) $> \text{PtdSer}$. As discussed below, this specificity agrees reasonably well with the reported specificity of dynamin GTPase activation by phosphoinositides (28).

Phosphoinositide Binding by a Dyn1PH Dimer Is Reduced by a Specific Mutation in a Variable Loop—We were concerned that the increased phosphoinositide binding affinity of dimeric DynPH might simply reflect nonspecific electrostatic attraction of the positively charged face of DynPH (33) for the negatively charged membrane surface. To test more thoroughly for binding specificity, we analyzed the effects on binding of mutations in the variable loops of Dyn1PH. Each PH domain of known structure has three loops that are most variable in both length and sequence, and these coincide with the positively charged face of the electrostatically polarized PH domain (51). The x-ray crystal structure of the PLC-8, PH domain in complex with Ins(1,4,5)P_3 identified these variable loops as providing the majority of interactions between Ins(1,4,5)P_3 and the PH domain (52). We previously generated a series of Dyn1PH mutations in which the three variable loops were replaced individually with those from the PLC-8, PH domain (35). The mutations are detailed in Fig. 5A, and none affects our ability

![Graph A: Oxidized (Dimer)](image)

![Graph B: Reduced (Monomer)](image)

**Fig. 3.** Significant binding of an oxidized Dyn1PH dimer, but not a reduced monomer, to phosphoinositides in small unilamellar vesicles. Binding to SUVs containing 3% (mol) of each phosphoinositide noted was analyzed using the centrifugation assay described under “Experimental Procedures.” Experiments were performed in the absence of reducing agent (oxidized), where CysDyn1PH is dimeric (see Fig. 2), and in the presence of 1 mM TCEP, which reduces the intermolecular disulfide bond to yield monomeric CysDyn1PH. Data points are “Experimental Procedures.” Experiments were performed in the absence of reducing agent (oxidized), where CysDyn1PH is dimeric (see Fig. 2), and in the presence of 1 mM TCEP, which reduces the intermolecular disulfide bond to yield monomeric CysDyn1PH. Data points are

**Table I**

| Phospholipid          | $K \pm \text{S.D.}^{a}$ (dimer) | $K \pm \text{S.D.}^{a}$ (monomer) |
|-----------------------|-------------------------------|----------------------------------|
|                       | $K \pm \text{S.D.}^{a}$ (dimer) | $K \pm \text{S.D.}^{a}$ (monomer) |
| $\mu M$               | $\mu M$                        | $\mu M$                         |
| PtdIns(3,4,5)P$_3$     | 2753 ± 434                     | 11                               |
| PtdIns(4,5)P$_2$       | 3333 ± 377                     | 9                               |
| PtdIns(4,5)P$_2^2$     | 2179 ± 191                     | 14                              |
| PtdIns-3-P             | 294 ± 38                       | 106                             |
| PtdIns-4-P             | 353 ± 16                       | 85                              |
| PtdSer                 | 191 ± 44                       | 157                             |

$^{a}$ $K$ represents the molar partition coefficient for vesicle binding (see “Experimental Procedures”), and makes no assumption of lipid-binding stoichiometry (40). Total lipid concentrations were divided by two to approximate the available lipid present on the outer leaflet of the vesicles (40).

$^{b}$ $K_p$ values have been approximated by assuming that the PH domain binds only to the phosphoinositide, and does so with a stoichiometry of one available phosphoinositide per Dyn1PH monomer. $K_p = 0.03K$.
to generate pure, soluble protein. The VL-1 and VL-3 mutants are both direct loop swaps, which do not alter the net charge of the PH domain. A similar swap of VL-2 reduced protein solubility significantly, so we have analyzed instead only a VL-2 mutant that increases the net positive charge of Dyn1PH by substituting 4 acidic residues with their amides. PtdIns(4,5)P2 binding by each of the mutated PH domains (as GST fusion proteins) is compared with that of wild-type GST-Dyn1PH in Fig. 5B. Analysis of PtdIns(3,4,5)P3 binding gave identical results (not shown). The VL-1 and VL-2 mutants behaved indistinguishably from wild-type GST-Dyn1PH, whereas the VL-3 loop swap reduced PtdIns(4,5)P2 binding to the level seen for PtdSer binding by wild-type GST-Dyn1PH. Thus, of two mutations that conserve net charge, one abolishes PtdIns(4,5)P2 binding, and one has no effect. The mutation that increases net charge (VL-2) did not enhance PtdIns(4,5)P2 binding. Together, these results argue strongly against a nonspecific electrostatic interaction being responsible for the observations reported here.

Previous analyses of NMR chemical shift changes (27, 34) indicated likely binding sites on Dyn1PH for the PtdIns(4,5)P2 head group (which binds with a millimolar range $K_d$). The precise details of the proposed binding site differ between the reports, but both agree that it occurs on the positively charged face of the PH domain containing the variable loops. We were unable to test the role of the basic side chains in VL-2, since mutations in this region adversely affected protein stability. However, deletion of Lys90 in the VL-3 loop swap abolished PtdIns(4,5)P2 binding, consistent with implication of this residue in Ins(1,4,5)P3 binding by NMR, and the reduced binding of a K90M mutant to PtdIns(4,5)P2 reported by Salim et al. (27). Taken together, analysis of the three mutants described here is in general agreement with the studies of Zheng et al. (34) and Salim et al. (27), and argues that the phosphoinositide binding observed for dimeric Dyn1PH involves a specific binding site.

It is of particular interest that the effects on phosphoinositide binding of the mutations listed in Fig. 5A do not correlate with their abilities to inhibit RE in adrenal chromaffin cells (35) (Table III). Only one mutant (VL-1) was impaired in its ability to inhibit RE (35), yet this mutant resembles wild-type Dyn1PH in its phosphoinositide binding (Fig. 5A; Table III). Similarly, the only mutation that impaired phosphoinositide binding by Dyn1PH (VL-3) was previously found to have no effect on the ability of Dyn1PH to inhibit RE (35). If it is assumed that RE inhibition by Dyn1PH reflects sequestration of some critical component, the lack of correlation in Table III argues that this component is not a phosphoinositide. This finding adds substantial strength to the suggestion that an additional (or alternative) Dyn1PH-specific ligand is involved in this endocytosis process (35).
DISCUSSION

We show in this report that oligomerization is required for significant binding of the dynamin-1 and dynamin-2 PH domains to phosphoinositides in a lipid bilayer or immobilized on nitrocellulose. This finding resolves a disagreement between earlier reports. Previous studies that did not detect significant binding of Dyn1PH to phosphoinositides used either monomeric PH domain (32, 33) or monomeric phosphoinositides (50). The study that did demonstrate Dyn1PH binding to phosphoinositide-containing vesicles (27) used dimeric GST fusion protein that was further oligomerized by immobilization on a biosensor surface.
Phosphoinositide Binding by Oligomeric Dynamin PH Domains

The soluble head group of PtdIns(4,5)P$_2$ binds to Dyn1PH with a $K_D$ between 1.2 mM (27) and 4.3 mM (34), corresponding to a binding energy ($\Delta G$) of $-3.2$ to $-4$ kcal/mol. Dimerization of Dyn1PH does not appear to enhance the affinity of this interaction significantly, indicating that the binding sites themselves are not altered. Rather, the observations here seem to reflect a simple avidity effect, with the energies of phosphoinositide binding by the two PH domains in a dimer being additive. For dimeric CysDyn1PH, the partition coefficient for binding to PtdCho vesicles containing 3% PtdIns(4,5)P$_2$ suggests a $K_D$ of 9 $\mu M$ for PtdIns(4,5)P$_2$ binding (assuming 1:1 stoichiometry). This $K_D$ would correspond to a $\Delta G$ of $-6.9$ kcal/mol for binding of the Dyn1PH dimer to PtdIns(4,5)P$_2$-containing vesicles, or approximately twice the $\Delta G$ for binding of the monomer to free lipid head group. Thus, if the assumption concerning stoichiometry is correct, the energies of the monomeric interactions would appear to be additive in binding of the dimer to PtdIns(4,5)P$_2$-containing vesicles.

Although isolated Dyn1PH crystallized as a dimer (33, 53), x-ray scattering (33), and analytical ultracentrifugation (54) studies showed that it is monomeric at concentrations up to 10 $\text{mg/ml}$ (680 $\mu M$), and tends to oligomerize only above 1 $\text{mM}$ (54). Oligomerization of Dyn1PH therefore requires that it is present in a molecule that self-associates through other interactions. Purified dynamin--1 is a tetramer in solution at a concentration of 5 $\mu M$ (14), and although the weak tendency of Dyn1PH to self-associate could contribute to this self-association, it is not required (14). If the avidity effect that we have described for Dyn1PH dimers is also permitted by the arrangement of PH domains in a dynamin tetramer, the apparent $K_D$ for binding of the tetramer to PtdIns(4,5)P$_2$ in vesicles would be predicted to be less than 0.1 $\text{nm}$.

**Implications for Dynamin Function**—Since the PH domain of dynamin binds more strongly to phosphoinositides when oligomeric, it follows that binding to phosphoinositides in a membrane will stabilize the formation of dynamin oligomers. Although the PH domain of dynamin is not required for in vitro formation of tetramers and higher order assemblies in the absence of phospholipids (14), our findings predict that it is required for the ability of acidic phospholipids to stabilize dynamin oligomers. At relatively low concentrations, under conditions where dynamin self-association is not complete, the PH domain avidity effect reported here will lead to cooperativity in binding of dynamin to phosphoinositide-containing membranes, and will enhance its self-association. Indeed, Tuma and Collart (26) observed this experimentally, showing by chemical cross-linking that dynamin oligomerization is enhanced significantly by the presence of brain-derived vesicles when studied at approximately physiological protein concentrations. This effect was accompanied by enhanced dynamin GTPase activity (25), which several studies have shown requires its self-assembly (24), and also showed strong positive cooperativity with respect to the dynamin concentration (23). These studies argue that self-assembly through cooperative binding to phosphoinositide-containing membranes, likely mediated by the PH domain, can activate dynamin in vitro. Stimulation of dynamin GTPase activity has been reported for vesicles containing PtdIns(4,5)P$_2$ (27–29), PtdIns(3,4,5)P$_3$ (28), PtdIns-4-P (28), PtdSer (25, 29), or other acidic phospholipids (25). The stimulatory effect of PtdIns(4,5)P$_2$ is abolished upon deletion of the PH domain from dynamin--1 (27). Where efficacies have been compared, relative abilities of different phospholipids to activate dynamin agree well with the phosphoinositide binding specificity of dimeric Dyn1PH reported here. One exception is that PtdIns(3,4)P$_2$ was not a strong activator of dynamin GTPase (28), despite binding being indistinguishable from PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$ in our studies.

Swetzer and Hinshaw (55) recently showed that, when added to PtdSer vesicles, purified dynamin--1 induces the formation of membrane tubes around which dynamin is assembled in a helical array. Remarkably, addition of GTP stimulated vesiculation of these membrane tubes (55), showing that no additional components are required for vesicle scission by dynamin. Analysis of proteolytic fragments indicated that the PH domain of dynamin--1 is required for its assembly on membrane tubes, while the PRD was necessary for neither assembly nor vesiculation. The helical tubes observed were similar in dimensions to the collars formed around the necks of clathrin-coated pits in shibire Drosophila (8), and formed under conditions that do not favor assembly in the absence of lipids (19, 20). Thus, Swetzer and Hinshaw (55) argue that the lipid vesicles provide a surface on which dynamin self-assembly is favored, as previously suggested by chemical cross-linking studies (26), and further that the PH domain participates in this process. Our studies argue that binding of the PH domain to acidic phospholipids could explain these observations.

The coupling of membrane association and self-assembly of dynamin appears to be critical for its physiological function. We propose that the properties of the dynamin PH domain are uniquely suited to this requirement. A PH domain that binds very weakly to membranes as a monomer, but strongly as an oligomer (through avidity effects), will drive cooperative assembly on the membrane surface when placed in the context of a molecule (such as dynamin) that is capable of self-association. This could be true for all PH domains, although those that bind with high affinity to phosphoinositides in their monomeric form (e.g. PH domains from PLC-δ1, Btk, and Grp1) will be capable of membrane binding without oligomerization. Coupling of oligomerization to membrane binding would only be required when the monomeric PH domain binds weakly, and is possible only if its host molecule has an inherent tendency to self-associate.

The specificity of lipid binding by the oligomeric Dyn1PH could define the particular regions on the membrane surface at which dynamin self-assembly is promoted. De Camilli et al. (56) have suggested that molecules such as the inositol 5-phosphatase synaptojanin (which dephosphorylates PtdIns(4,5)P$_2$) play a role in generating membrane patches of unique phosphoinositide composition. Synaptojanin colocalizes with amphiphysin (57) and dynamin (58) in nerve endings. Since PtdIns(4,5)P$_2$ is the preferred ligand for the oligomeric dynamin PH domain (among the more abundant phospholipids), it might be speculated that dynamin self-assembly into collar-like structures will be most efficient in regions of a pre-endocytotic invagination at which synaptojanin has not dephosphorylated PtdIns(4,5)P$_2$.

**Absence of Correlation between Phosphoinositide Binding and Inhibition of Rapid Endocytosis**—As reported by Artalejo et al. (35), isolated Dyn1PH inhibits RE when introduced into adrenal chromaffin cells. The PLC-δ1 PH domain has no effect,
Phosphoinositide Binding by Oligomeric Dynamin PH Domains

...despite binding with high affinity to PtdIns(4,5)P_2 (32). This finding argues that Dyn1PH does not inhibit RE by competition with endogenous dynamin for PtdIns(4,5)P_2 binding (35). We show here that the phosphoinositide binding specificities of Dyn1PH and Dyn2PH are identical, yet only Dyn1PH can inhibit RE (35). Furthermore, the effects of Dyn1PH mutations on RE inhibition and phosphoinositide binding are not related (Table III). A Dyn1PH mutant that does not inhibit RE (VL-1) still binds phosphoinositides, while another mutant that does not bind phosphoinositides (VL-3) can inhibit RE. These findings lend weight to the suggestion (35) that binding to something other than a phosphoinositide is responsible for the effects of Dyn1PH on RE. Similar suggestions have been made for the PH domains from IRS-1 (59) and cytohesin-1 (60).

In contrast with the case for RE, we have been unable to detect inhibition of receptor-mediated endocytosis upon over-expression of Dyn1PH or Dyn2PH in HeLa cells, as assessed by studies of fluorescently labeled epidermal growth factor or transferrin uptake. Both PH domains were overexpressed as fusions with green fluorescent protein, either alone or with a leucine zipper to drive dimerization. From the model suggested above for the role of the dynamin PH domain, it is expected that the isolated PH domain will not be able to inhibit endogenous dynamin by competing for phospholipid binding sites on the membrane. Cooperative membrane binding of intact dynamin will be much stronger than that for the isolated PH domain (monomeric or dimeric), as a result of dynamin-dynamin interactions mediated by regions outside the PH domain. Studies of PH domain mutations in intact dynamin will be required for a direct test of the importance of the phosphoinositide binding reported here.

Conclusions—It is clear that the PH domain of dynamin is required for stimulation of its GTPase activity by phosphoinositides (27). It also appears that the PH domain is required for self-assembly of dynamin into helical tubes on membrane surfaces (55). We have shown that, while phosphoinositide binding by dynamin PH domain monomer is barely detectable, dimers of the PH domain bind strongly. We propose that this characteristic of the dynamin PH domain allows it to mediate cooperative binding of intact dynamin to membranes that contain phosphoinositides or other acidic phospholipids. The cooperativity would arise from well documented dynamin-dynamin interactions that do not involve the PH domain (14). This mechanism could allow dynamin self-assembly to occur at membrane sites enriched in certain phosphoinositides, which may be important for the apparent redistribution of dynamin to the necks of membrane invaginations prior to vesicle fission. Finally, we would suggest that some other PH domains that bind only weakly to membranes when monomeric may also play a role in self-assembly of their host molecules at the membrane surface.

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