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Synergistic Activation of Dynamin GTPase by Grb2 and Phosphoinositides*

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Hydrolysis of GTP by dynamin is essential for budding clathrin-coated vesicles from the plasma membrane. Two distinct domains of dynamin are implicated in the interactions with dynamin GTPase activators. Microtubules and Grb2 bind to the carboxyl-terminal proline/arginine-rich domain (PRD), whereas phosphoinositides bind to the pleckstrin homology (PH) domain. In this study we tested the effect of different phosphoinositides on dynamin GTPase activity and found that the best activator is phosphatidylinositol 4,5-bisphosphate followed by 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycerol-3-benzyloxyphosphoryl)-D-*myo*-inositol 3,4,5-triphosphate. Phosphatidylinositol 4-phosphate was a weak activator and phosphatidylinositol 3,4-bisphosphate did not activate GTPase at all. We then addressed the question of whether both domains of dynamin, PRD and PH, can be engaged simultaneously, and determined the effects of dual occupancy on dynamin GTPase activity. We found that Grb2 and phosphatidylinositol 4,5-bisphosphate together increased the dynamin GTPase activity up to 4-fold higher than that obtained by these activators tested separately, and also reduced the dynamin concentration required for half-maximal activities by 3-fold. These results indicate that both stimulators can bind to dynamin simultaneously resulting in superactivation of dynamin GTPase activity. We propose that SH3-containing proteins such as Grb2 bind to the dynamin PRD to target it to clathrin-coated pits and prime it for superactivation by phosphoinositides.

Dynamin is a GTPase required for membrane internalization during synaptic vesicle recycling and receptor-mediated endocytosis (for recent reviews, see Refs. 1–5). GTP hydrolysis is necessary for the cellular functioning of dynamin since overexpression of inactive dynamin mutants elicits a dominant inhibitory effect on host cell endocytosis (6, 7). Therefore, the regulation of this enzymatic activity has been under intense investigation. GTPase activity is tightly coupled to dynamin self-assembly, which can occur in the absence of other molecules (8–10), but is facilitated by multivalent surfaces provided by microtubules (11–15) or anionic liposomes (15). Specific activity is also increased by phosphorylation (16) and by interaction with several SH3 domain-containing proteins (17, 18). The mechanisms by which these interactions regulate dynamin GTPase activity are poorly understood.

Until recently it was believed that all dynamin activators interact with a carboxyl-terminal domain of approximately 100 residues designated PRD[1] for its high content of prolines (P) and arginines (R). Negatively charged molecules, such as microtubules and phosphatidylserine-containing liposomes, bind to the PRD via ionic interactions which are disrupted at physiological ionic strength (14). SH3 domain-containing proteins, including Grb2 and amphiphysin, bind tightly to specific proline-rich motifs located in the PRD (18–20).

Another potential site for dynamin interactions is the pleckstrin homology (PH) domain, a module found in numerous signaling and cytoskeletal proteins (21, 22). The dynamin PH domain, consisting of approximately 110 amino acid residues, has been expressed in bacteria and its structure has been solved by x-ray crystallography (23, 24) and NMR (25). Like other PH domains whose structures are known, the dynamin PH domain contains seven amino-terminal β-strands arranged in two antiparallel sheets and a carboxyl-terminal α-helical segment. Harlan *et al.* (21) first showed that the PH domains of numerous proteins can interact with phosphoinositides. Selectivity of distinct PH domains for different phospholipids has been recently reported by Rameh *et al.* (26). Two groups have now shown the binding of phospholipids to recombinant dynamin PH domain (27, 28). Using NMR and fluorescence spectroscopy, Zheng *et al.* (27) determined that the dynamin PH domain binds to PI(4)P, PI(4,5)P_2, and phosphatidylserine with equilibrium dissociation constants of 1.8, 4.4, and 47 μM, respectively. Salim *et al.* (28) found that a mutant form of dynamin lacking only the PH domain was not activable by PI(4,5)P_2, although it retained Grb2-stimulated GTPase activity. We performed the converse experiment, eliminating the carboxyl-terminal PRD (29). As expected, this truncated dynamin was not activable by Grb2 or microtubules, but was stimulated by PI(4,5)P_2, presumably due to an interaction with the PH domain.

This paper addresses two related issues. First, how do specific phosphoinositides affect the GTPase activity of dynamin? The inositol rings of phosphoinositides can be modified at mul-

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[1] The abbreviations used are: PRD, proline/arginine-rich domain; PH, pleckstrin homology domain; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P_2, 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycerol-3-benzyloxyphosphoryl)-D-*myo*-inositol 3,4-bisphosphate; PI(4)P, phosphatidylinositol 4-phosphate; MES, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PI(3,4,5)P_3, 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycerol-3-benzyloxyphosphoryl)-D-*myo*-inositol 3,4,5-triphosphate; GST, glutathione *S*-transferase.

tiple positions by specific kinases and phosphatases, some of which have been implicated in membrane trafficking events (30). If there is specificity in the activation of dynamin by phosphoinositides, then these lipid modifying enzymes are potential regulators of the endocytic process. Second, since dynamin has two interaction sites, the PRD and the PH domain, can these sites be occupied simultaneously and, if so, what is the consequence of simultaneous occupancy for GTPase activation?

EXPERIMENTAL PROCEDURES

Materials—Phosphocellulose (P11) and diethylaminoethylcellulose (DE52) were from Whatman. Methyl sulfonate (SP)-Sepharose and glutathione-Sepharose were from Pharmacia Biotech. PI(4,5)P_2, PI(4)P, and phosphatidylcholine were from Calbiochem. 1-*O*-(1,2-Di-*O*-palmitoyl-*sn*-glycerol-3-benzyloxyphosphoryl)-D-*myo*-inositol 3,4,5-triphosphate ((PI(3,4,5)P_3) and 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-benzyloxyphosphoryl)-D-*myo*-inositol 3,4-bis-phosphate (PI(3,4)P_2) were gifts from Dr. C-S. Chen, University of Kentucky. Protease inhibitors, taxol, papain, thrombin, and GTP were from Sigma. [γ-^{32}P]GTP was from Amersham.

Purification of Proteins—Dynamin I was purified from bovine brains following a procedure described previously (31). Briefly, fresh bovine brains were homogenized in an Omnimixer with 3 volumes of buffer A, which contains 0.1 M MES, pH 7.0, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM sodium azide, and a mixture of protease inhibitors: 0.2 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of N^{α}-benzoyl-L-arginine methyl ester, N^{α}-*p*-tosyl-L-arginine methyl ester, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, leupeptin, and pepstatin A. The extract following centrifugation was chromatographed on three consecutive ion-exchange columns: DE52-cellulose, P11 phosphocellulose, and SP-Sepharose. Fractions enriched in dynamin were then mixed with microtubules and ultracentrifuged. Dynamin, which co-sediments with microtubules, was released by addition of 10 mM GTP. The supernatant was finally passed through a DE52 column to remove any traces of tubulin. All purification steps were carried out using buffer A and columns were eluted with buffer A containing NaCl.

To remove the carboxyl-terminal PRD, dynamin was proteolyzed with papain at 30 °C for 20 min at an enzyme to dynamin ratio of 1:1000 (w/w). Papain was first activated by incubation on ice for 15 min in a solution containing 0.5 M NaCl, 25 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 5 mM dithiothreitol. Digestion was terminated by adding iodoacetic acid to a final concentration of 5 mM and the digest was mixed with GST-Grb2 coupled to glutathione-Sepharose to remove PRD fragments and any traces of undigested dynamin. This treatment yields fragments of 53 and 32 kDa which are noncovalently associated to each other (29).

GST-Grb2 was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST) in a pGEX-2T vector and purified on glutathione-Sepharose 4B using a standard procedure. Grb2 was obtained by thrombin cleavage of GST-Grb2 in a solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2.5 mM $CaCl_2$ for 4 h at room temperature. Approximately 130 NIH units of thrombin were used to digest 4 mg of GST-Grb2 attached to glutathione-Sepharose 4B beads. The identity of released Grb2 was confirmed by immunoblotting with anti-Grb2 antibodies and anti-GST antibodies. NH_2-terminal sequencing confirmed that GST-Grb2 was cleaved at the expected site, producing full-length Grb2. Tubulin was purified according to the procedure of Williams and Lee (32) but MES rather than PIPES buffer was used. Stable microtubules were obtained by polymerization of tubulin in the presence of taxol at a 2-fold molar excess to tubulin dimer.

Preparation of Phospholipid Vesicles—Phospholipid vesicles were prepared by dissolving phospholipids in chloroform and drying them under a stream of argon. Dried lipids were dissolved in 0.1 M MES, pH 7.0, and sonicated for 10 min at maximum power (Bath sonicator model W185; Heat System Ultrasonics, Inc., Farmingdale, NY). Phosphoinositide vesicles were prepared as mixtures with phosphatidylcholine (PC) at a 1:9 molar ratio (33).

Sedimentation Equilibrium—Sedimentation equilibrium was performed on a Beckman XLA Analytical Ultracentrifuge. The data (absorbances at 280 nm) were collected from cell radii of 6.8–7.2 cm, with a step size of 0.001 cm. Five scans were averaged in the final output. The initial concentrations of GST-Grb2 and Grb2 were 0.2 and 0.5 mg/ml, respectively. Sedimentation analyses of GST-Grb2 and Grb2 were performed in the same buffer (0.1 M MES, pH 7.0, 1.0 mM EDTA, 1.0 mM $MgSO_4$, 1.0 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM NaN_3). GST-Grb2 was centrifuged at 10,000 rpm, and Grb2 at 15,000 rpm in rotor An-60ti using a 12-mm double-sector centerpiece. The background absorbance was estimated by overspeeding at 40,000 rpm until a flat baseline was obtained. The overspeed absorbances were 0.019 and 0.018 for Grb2 and GST-Grb2, respectively. The partial specific volume of Grb2 was calculated to be 0.73 ml/g by the method of Cohn and Edsall (34). The actual molecular masses of Grb2 and GST-Grb2 were calculated to be 26 and 52 kDa, respectively.

GTPase—GTPase activities were measured by the release of $^{32}P_i$ from [γ-^{32}P]GTP (35) after incubation at 37 °C in buffer A containing additionally 1 mM MgGTP. The reaction times varied from 2 to 30 min, depending on dynamin or lipid concentrations, to ensure that less than 15% of GTP was hydrolyzed. Dynamin was preincubated with lipids and/or Grb2 for 10 min at room temperature prior to initiation of the reaction by addition of 5 mM MgGTP. In some experiments (as indicated in the figure legends) buffer A contained additionally 0.1 M NaCl. Although a high concentration of MES was used in these assays, this zwitterionic buffer contributes little to the ionic strength of the solution. Except in the case where the effect of salt was examined (Fig. 3), all GTPase assays were carried out at low ionic strength, allowing a comparison of our results with those of other investigators, who also employ low salt assay conditions (16–18, 28, 36).

Turbidity Measurements—Samples were placed in a 1-cm path length cuvette and absorbance at 340 nm was determine using a Beckman DU 650 spectrophotometer.

Other Methods—Protein concentration was determined as described by Bradford (37) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (38) as modified by Matsudaira and Burgess (39).

RESULTS

Specificity of Dynamin Activation by Phosphoinositides—Inositol phospholipids, which have been implicated in the regulation of dynamin GTPase activity, are subject to phosphorylation and dephosphorylation at multiple positions of the inositol ring. Here we show that the site of inositol phosphorylation is a key determinant for dynamin activation. The order of efficacy is PI(4,5)P_2 > PI(3,4,5)P_3 > PI(4)P (Fig. 1). At concentrations below 1 μM, PI(4)P is somewhat more effective than PI(3,4,5)P_3. However, maximal PI(4)P stimulated activity is only about 50 min^{-1}, less than half the value obtained with PI(3,4,5)P_3 and about one-third of PI(4,5)P_2 stimulated activity. PI(3,4,)P_2, which has the same charge as PI(4,5)P_2, is by far the least effective activator, providing no stimulation even at concentrations as high as 40 μM.

We then asked if PI(3,4)P_2 binds to dynamin despite its inability to stimulate GTPase activity. In a competition assay, PI(4,5)P_2 stimulated activity was measured in the presence of various concentrations of PI(3,4)P_2. If PI(3,4)P_2 binds to dynamin, it should displace PI(4,5)P_2 from its binding site and thus inhibit GTPase activity. However, no inhibition by PI(3,4)P_2 was observed, even at a 10-fold molar excess over PI(4,5)P_2 (Fig. 2). Therefore, PI(3,4)P_2 has a much lower affinity for dynamin than PI(4,5)P_2, or binds at a different site on the molecule.

Dynamin contains two potential sites for phosphoinositide binding, the PH domain and the highly basic carboxyl-terminal PRD. Ionic charges promote the associations of anionic phospholipids with the PRD and interactions are significantly reduced at physiological ionic strength (14). Therefore, by measuring lipid-stimulated GTPase activity in the presence of 100 mM NaCl, as in Fig. 3, it is likely that interactions with the PH domain predominate. In Fig. 3 we show the dependence of GTPase specific activity on dynamin concentration, at fixed concentrations of phosphoinositides. Unlike most enzymes, the specific activity of dynamin is not invariant as a function of dynamin concentration, but increases in a highly cooperative manner (15). This presumably reflects the well established propensity of dynamin to self-associate and the consequent increase in GTPase activity that is coupled to this self-association. We had previously shown that PI(4,5)P_2-containing vesicles provide a multivalent surface that allows cooperative in-

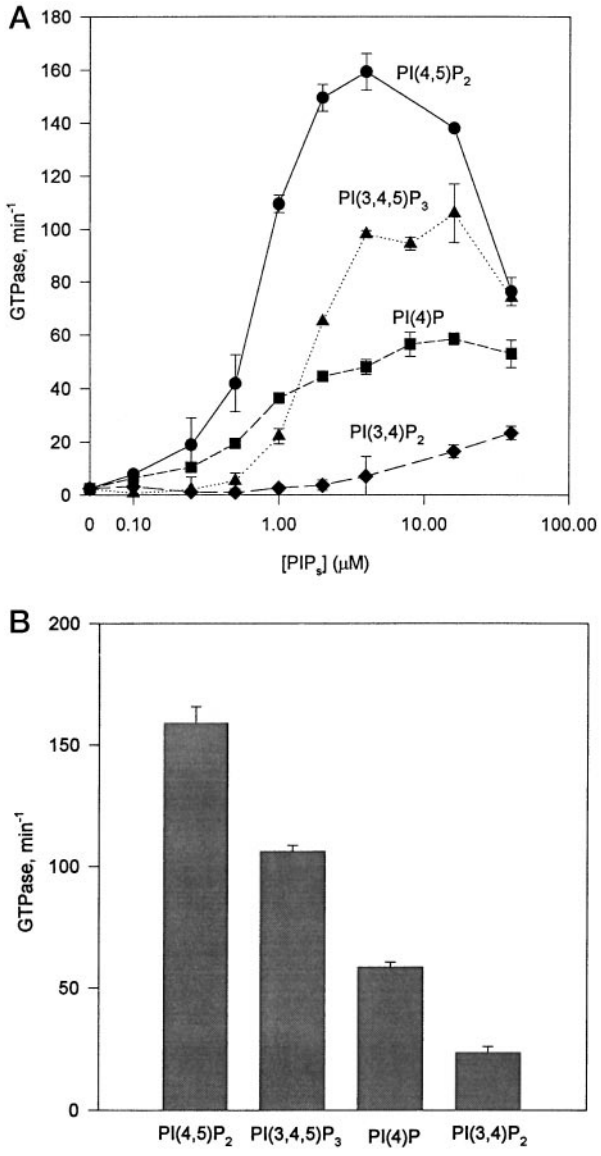

FIG. 1. **Activation of dynamin I GTPase activity by various phosphoinositides.** *A*, dynamin GTPase activity as a function of phosphoinositide concentrations. GTPase of dynamin at 0.1 μM concentration was assayed as described under "Experimental Procedures." GTPase activities decrease at high concentrations of PI(4,5)P_2 and PI(3,4,5)P_3, presumably due to the inhibition of dynamin-dynamin interactions previously shown to occur in the presence of high concentrations of phosphatidylserine or microtubules (14). *B*, data from *panel A* were replotted as a *bar graph* to highlight the differences at maximal activation. Lipid concentrations giving maximal activation were: 4 μM PI(4,5)P_2 and PI(3,4,5)P_3, 16 μM PI(4)P, and 40 μM PI(3,4)P_2. Data represent the mean ± S.E. from three experiments, each done in duplicate for an *n* of 6.

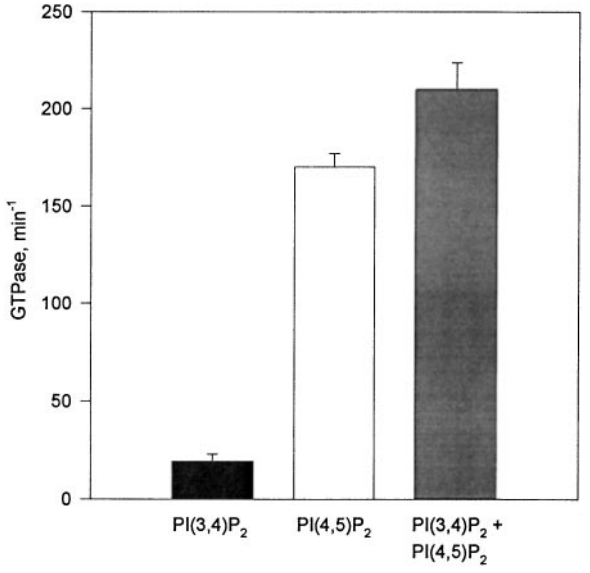

FIG. 2. **PI(4,5)P_2-stimulated dynamin GTPase is not inhibited by PI(3,4)P_2.** The GTPase activity of dynamin (0.1 μM) was assayed in the presence of 4 μM PI(4,5)P_2 and 40 μM PI(3,4)P_2. The *bars* indicate average values of duplicate measurements.

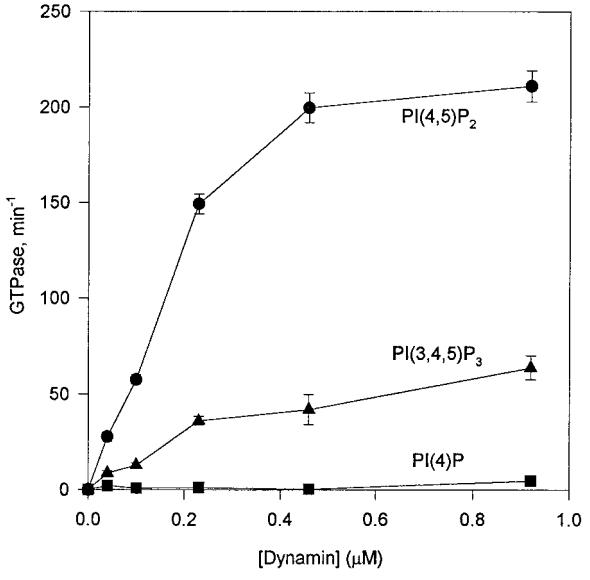

FIG. 3. **Specific GTPase activities in the presence of 4 μM phosphoinositides as a function of dynamin concentration.** Experiments were performed in buffer A containing additionally 0.1 M NaCl. Data represent the mean ± S.E. from two experiments, each done in triplicate for an *n* of 6.

creases in GTPase activity even at relatively low dynamin concentrations (29). Fig. 3 indicates that PI(4,5)P_2 retains its ability to support high specific activity at 100 mM NaCl whereas salt greatly diminishes the efficacies of PI(3,4,5)P_3 and PI(4)P. Therefore, under conditions more closely resembling those in the cell, only PI(4,5)P_2 appears to be a potent stimulator of dynamin activity.

The sensitivity of the PI(3,4,5)P_3-dynamin interaction to ionic strength raised the possibility that this phosphoinositide binds primarily to the PRD. However, this proved not to be the case. To examine this, we deleted the PRD by papain treatment of dynamin, which yields two non-covalently associated fragments of 53 and 32 kDa. The 53-kDa fragment contains the GTP-binding site (thus originating from the NH_2-terminal portion of dynamin), whereas the 32-kDa fragment contains the PH domain and extends to the beginning of the PRD (Fig. 4, see also Ref. 29). As we showed in a prior study, the 53/32-kDa fragments are activated by PI(4,5)P_2, but not by microtubules or Grb2. Activation of the papain fragments by phosphoinositides follows the basic pattern observed with intact dynamin: PI(4,5)P_2 and PI(3,4,5)P_3 are the best activators, PI(4)P is much less effective, and PI(3,4)P_2 fails to activate. Therefore, at low ionic strength, PI(3,4,5)P_3 binds at least as well as PI(4,5)P_2 to a truncated dynamin lacking the PRD. This result agrees with that of Salim *et al.* (28) who demonstrated by surface plasmon resonance that PI(3,4,5)P_3 can bind to expressed dynamin PH domains at low ionic strength but not in the presence of 1 μM Ca^{2+} and 2 mM Mg^{2+}, which more closely reflect physiological conditions. Our results also suggest that a

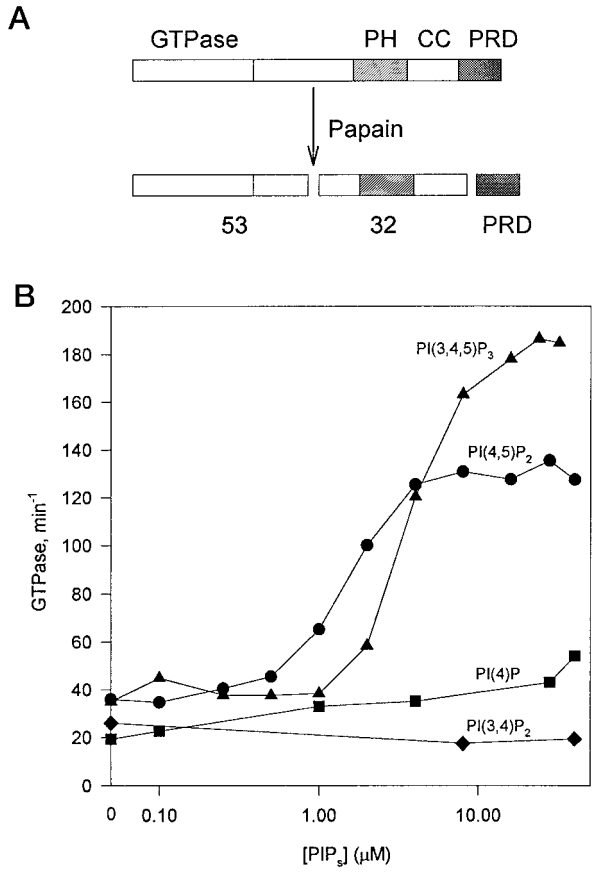

FIG. 4. **Activation of dynamin proteolytic fragments by phosphoinositides.** *A*, scheme of the dynamin digestion pattern. Digestion of dynamin with papain, as described under "Experimental Procedures," yields a 53-kDa fragment which is noncovalently associated with a 32-kDa fragment. *B*, GTPase assays of the 53/32-kDa fragments. Protein concentration in the GTPase assay was 0.12 μM. Each point represents the average of duplicate measurements.

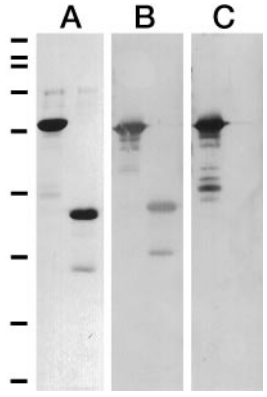

FIG. 5. **Electrophoretic analysis of GST-Grb2 and Grb2.** The *left lane* of each panel shows GST-Grb2, the *right lane* shows Grb2. Molecular mass markers designated on the *left* are 200, 116, 96, 66, 45, 31, 21.5, 14.4, and 6.5 kDa. *Panel A*, Coomassie Blue-stained gel. *Panel B*, immunoblot with anti-Grb2 antibody. *Panel C*, immunoblot with anti-GST antibody.

phosphate at the 5-position of the inositol ring is necessary for high levels of activation, whereas a phosphate at the 3-position reduces the phospholipid activation of dynamin.

Superactivation of Dynamin GTPase Activity by Grb2 and PI(4,5)P$_2$—Most *in vitro* activators of dynamin GTPase activity, including microtubules and SH3-containing proteins, interact solely with the carboxyl-terminal PRD (14, 17, 18). Instead, activation by phosphoinositides involves interactions outside the PRD, presumably with the PH domain (Ref. 29, see also Fig. 4). This raises the question of whether or not phosphoinositides and PRD-binding proteins can interact simultaneously with dynamin and, if so, how simultaneous binding influences GTPase activation. To address this question we set out to determine the effect of Grb2, an SH3-containing protein, on phosphoinositide-stimulated GTPase activity.

First it was necessary to determine the extent to which Grb2 alone could stimulate dynamin activity. All previous studies have utilized expressed fusion proteins of Grb2 and GST. However, since GST has a tendency to dimerize (40), these GST-Grb2 fusion proteins are also predominantly dimeric, as we verified by sedimentation equilibrium (Fig. 6*A*). This multivalency poses a particular problem when considering dynamin GTPase activation, which can even occur upon cross-linking with anti-dynamin antibodies (36). Therefore, we prepared nonfusion Grb2 by thrombin cleavage of GST-Grb2 (Fig. 5), and verified its monomeric nature by sedimentation equilibrium (Fig. 6*B*). Fig. 7 shows that the nonfusion Grb2 stimulates dynamin activity in a dose-dependent manner which plateaus at greater than 2 μM Grb2. In contrast, GST-Grb2 yields a biphasic activation which peaks at 0.5 μM GST-Grb2. These differences may be a consequence of the different complex-forming abilities of Grb2 *versus* GST-Grb2. A low speed co-sedimentation assay revealed that both molecules can cross-link dynamin into large networks (Fig. 8*A*). Under conditions wherein dynamin alone, GST-Grb2 alone, and Grb2 alone remain soluble (13,000 × *g*, 15 min), mixtures of dynamin with either GST-Grb2 or Grb2 are pelletable. The formation of a large complex is also demonstrated by a rapid increase in turbidity when Grb2 or GST-Grb2 are added to dynamin. The traces shown in Fig. 8*B* were obtained using 0.1 μM dynamin and 2 μM Grb2 or GST-Grb2. Clearly, GST-Grb2, which is tetravalent, is a more potent dynamin cross-linker than the divalent Grb2. The nature of these differences is still unclear, although it is possible to draw an analogy with the antigen-antibody precipitin reaction (41) (see "Discussion").

Grb2 activates dynamin GTPase to maximal levels of only 50–60 min^{-1}. Therefore, if Grb2 competes with PI(4,5)P$_2$ for dynamin binding, it should inhibit PI(4,5)P$_2$ stimulated activity. Instead, dynamin specific activity in the presence of both Grb2 and PI(4,5)P$_2$ is considerably higher than the additive activities obtained if the two activators were introduced separately. At 4 μM Grb2, PI(4,5)P$_2$-stimulated GTPase activity was about double that obtained in the absence of Grb2 (Fig. 9). This synergistic effect of Grb2 and phospholipids on GTPase activation provides strong evidence that these two molecules can bind simultaneously to dynamin.

The experiments shown in Fig. 9 were carried out at a fixed concentration of dynamin (0.1 μM). However, as shown above, there is a highly cooperative increase in PI(4,5)P$_2$-stimulated GTPase activity as a function of dynamin concentration. In view of the synergy between Grb2 and PI(4,5)P$_2$, we checked if the cooperative dynamin dependence of activity was also affected by the joint presence of both activators. Fig. 10 shows that Grb2 activates GTPase slightly, compared with the large increase obtained with PI(4,5)P$_2$ alone. However, in the presence of both Grb2 and PI(4,5)P$_2$, there is a marked shift in the dynamin concentration dependence from that obtained in the presence of PI(4,5)P$_2$ alone. At 4 μM PI(4,5)P$_2$, half-maximal activities occur at 0.11 μM dynamin. The effect of adding Grb2, also at 4 μM, reduces these dynamin concentration dependences approximately 3-fold, to 0.032 μM. The synergistic activation by Grb2 and phosphoinositides is particularly prominent at suboptimal levels of dynamin. For example, at 0.05 μM dynamin the GTPase activity is 4-fold higher in the presence of

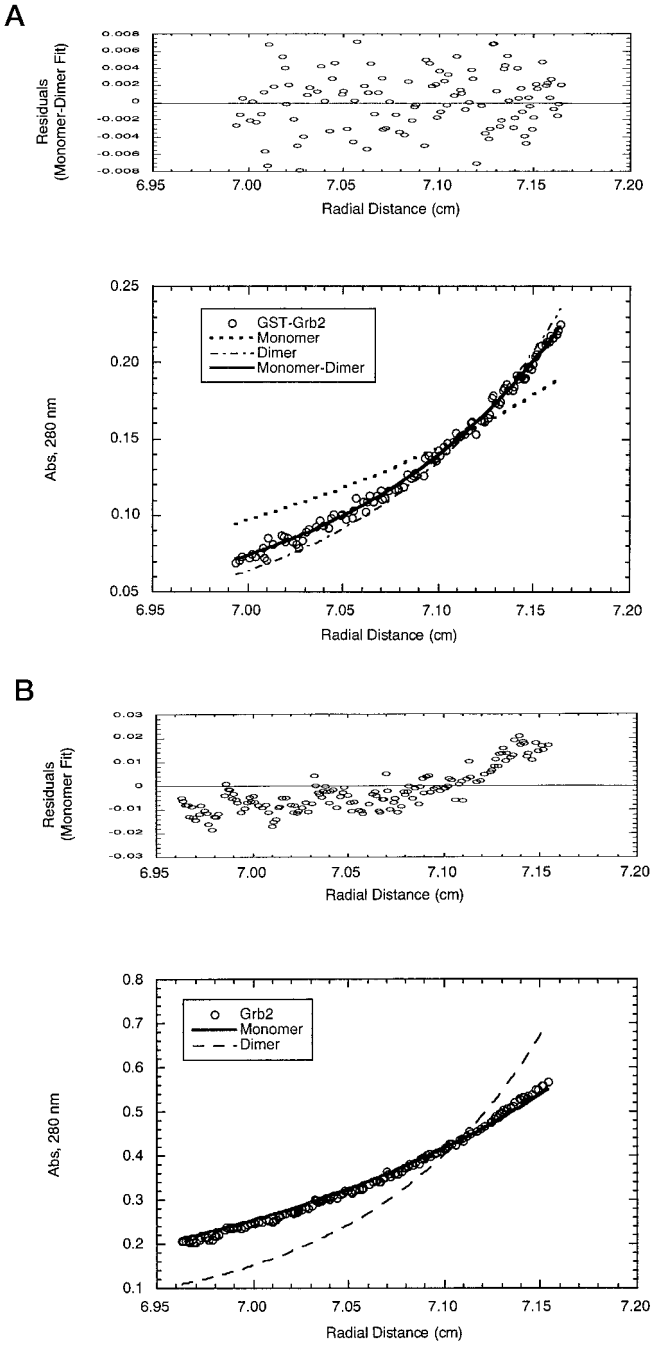

FIG. 6. **Sedimentation equilibrium analysis of GST-Grb2 and Grb2.** *A*, the *lower panel* presents data obtained at 10,000 rpm, 4 °C for GST-Grb2 as well as predicted profiles for monomer (*M*), dimer (*D*), and monomer-dimer equilibrium (*M-D*) model. Initial GST-Grb2 concentration was 0.2 mg/ml. *B*, the *lower panel* presents data obtained at 15,000 rpm, 4 °C for Grb2 and the predicted profiles for monomeric and dimeric species. Initial Grb2 concentration was 0.5 mg/ml. Residual plot for the monomer-dimer fit of GST-Grb2 data and the monomer fit of Grb2 data are shown in the *upper panels*. The analysis indicates that Grb2 alone behaved as a single species of about 28,000 M_r while GST-Grb2 gave a more complex equilibrium profile consistent with a monomer/dimer equilibrium and an association constant (K_a) of 3×10^6 M^{-1}.

both activators than in the presence of PI(4,5)P_2 alone. Since Grb2 has two SH3 domains and dynamin has multiple potential SH3-binding motifs within its PRD, a plausible explanation for the synergistic activation is that Grb2 cross-links dynamin molecules into a cluster by virtue of its two SH3 domains and thus increases its effective concentration on the phospholipid vesicles surface.

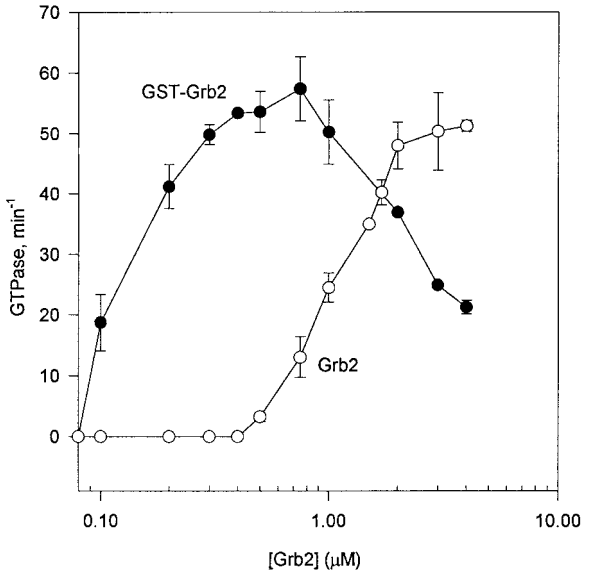

FIG. 7. **Activation of dynamin GTPase by GST-Grb2 and Grb2.** GTPase activities were assayed at 0.1 μM dynamin in the presence of GST-Grb2 or Grb2. Data represent the mean ± S.E. from two experiments, each done in duplicate, for an n of 4.

DISCUSSION

In this paper we present two observations pertinent to the regulation of dynamin: first, at physiological ionic strength dynamin GTPase activity is stimulated by PI(4,5)P_2, less so by PI(3,4,5)P_3, but not at all by PI(4)P or PI(3,4)P_2, and second, another dynamin activator, Grb2, binds to dynamin simultaneously with PI(4,5)P_2, resulting in synergistic rather than additive stimulation of activity.

Previously, we had shown that the GTPase activity of dynamin is stimulated by liposomes containing PI(4,5)P_2, even in the absence of its COOH-terminal proline and arginine-rich domain (29). This domain was earlier thought to participate in all interactions leading to GTPase activation. It is likely that PI(4,5)P_2 stimulates GTPase activity by interacting with the dynamin PH domain, because recombinant PH domains bind to phosphoinositides and dynamin mutants lacking the PH domain are not activated by these phospholipids (27, 28). In the present study we found that PI(3,4,5)P_3 is also a potent stimulator of dynamin, but only at low ionic strength. In contrast, PI(4)P and PI(3,4)P_2 are poor activators under all conditions examined. These results prompt us to speculate that in cells, dynamin activity can be reduced or terminated by the action of a phosphatidylinositol-5-phosphatase, such as synaptojanin, which converts PI(4,5)P_2 or PI(3,4,5)P_3 to PI(4)P and PI(3,4)P_2, respectively (42, 43). Synaptojanin is enriched at clathrin-coated pits and interacts with amphiphysin, a dynamin-binding protein also enriched at the coated pit (42, 44).

Grb2, an adaptor protein that consists of two SH3 domains flanking a central SH2 domain, binds exclusively to the dynamin PRD (17, 18). Presumably, Grb2 stimulates dynamin GTPase activity as a consequence of cross-linking the enzyme by virtue of its two SH3 domains. We found that dynamin and Grb2 form large complexes, as monitored by light scattering and low-speed centrifugation. Although the role of Grb2 in endocytosis is uncertain, it has been speculated to serve as a linker between activated tyrosine kinase receptors and dynamin (45). Until now, all *in vitro* studies of Grb2-dynamin interactions have employed bacterially-expressed fusion proteins with GST. We treated GST-Grb2 with thrombin to obtain nonfusion Grb2 and confirmed that it is monomeric, in contrast to GST-Grb2 which is predominantly dimeric. Thus, GST-Grb2

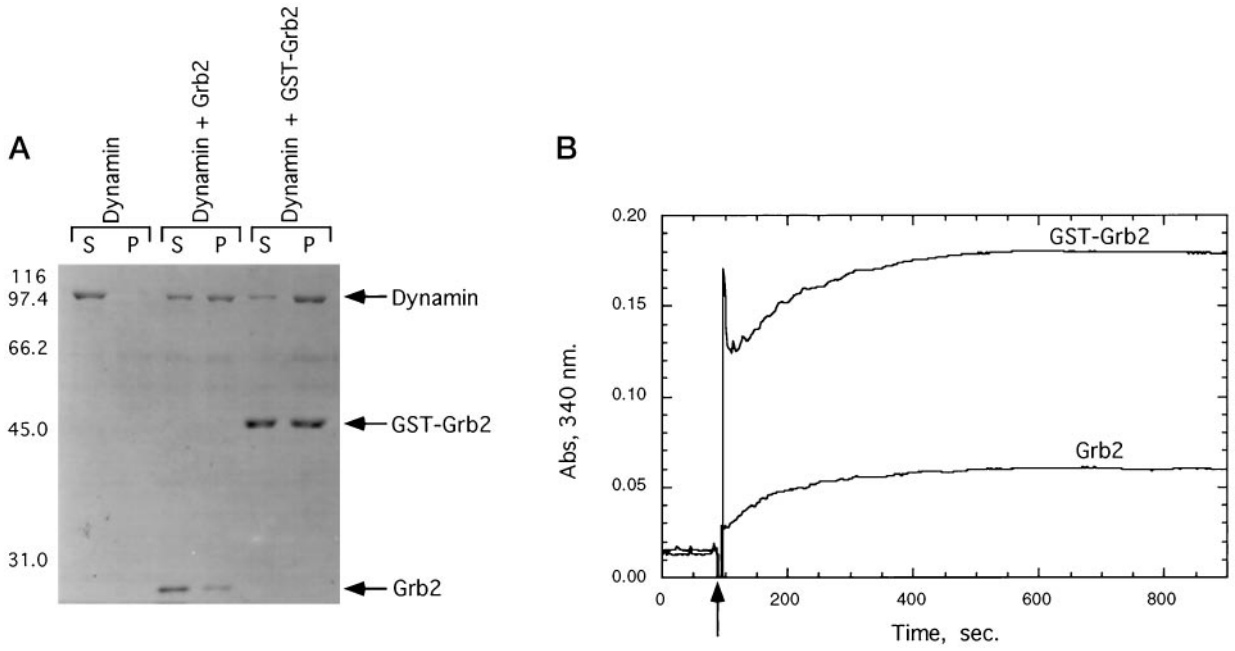

FIG. 8. **Dynamin cross-linking by Grb2 and GST-Grb2.** *A*, co-sedimentation assay. Dynamin (0.3 μM) was mixed with 1 μM of either Grb2 or GST-Grb2, incubated for 10 min at room temperature, and centrifuged at 13,000 $\times$ *g* for 15 min. Pellets were resuspended to equal volumes as supernatants and aliquots of each were electrophoresed. The gel was stained with Coomassie Brilliant Blue. Molecular weights of markers are shown on the *left*. *B*, turbidity was measured as absorbance at 340 nm. Cuvettes contained 0.1 μM dynamin I. At a time designated by the *arrow*, 2 μM Grb2 or GST-Grb2 were added. In the absence of dynamin, neither Grb2 nor GST-Grb2 gave any increase in absorbance.

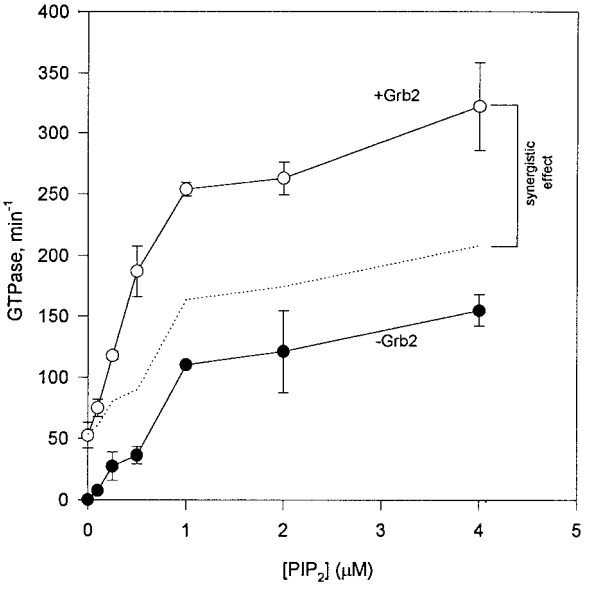

FIG. 9. **Grb2 potentiates GTPase activity of dynamin stimulated by PI(4,5)P$_2$.** The GTPase activity of dynamin (0.1 μM) was assayed as a function of PI(4,5)P$_2$ concentration in the absence or presence of 4 μM Grb2. *Dotted line*, theoretical curve showing expected activities if stimulation by PI(4,5)P$_2$ and Grb2 were simply additive. Data represent the mean $\pm$ S.E. from two experiments, each done in duplicate, for an *n* of 4.

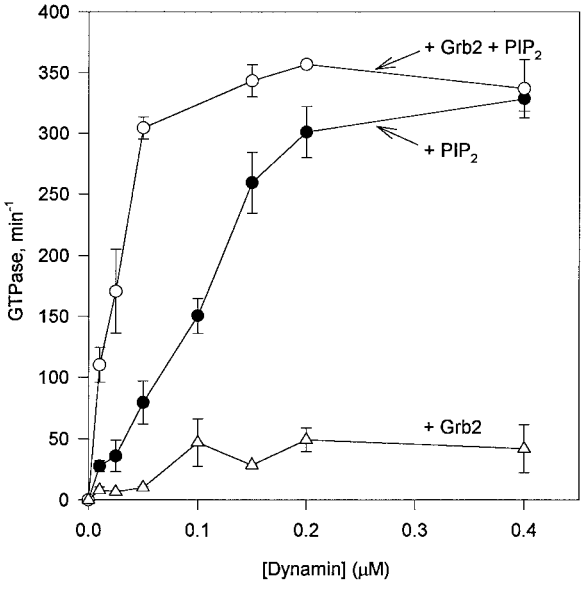

FIG. 10. **Stimulation of dynamin GTPase activity by Grb2 and PI(4,5)P$_2$.** GTPase activity was assayed as a function of dynamin concentration at fixed concentrations (4 μM) of Grb2 and phosphoinositide. The values obtained with Grb2 alone were subtracted. In the absence of PI(4,5)P$_2$ or Grb2, increases in activity as a function of dynamin concentration were negligible throughout the concentration range tested. Data represent the mean $\pm$ S.E. from two experiments, each done in duplicate, for an *n* of 4.

contains four dynamin-binding SH3 domains whereas Grb2 itself has only two, presumably accounting for the greater efficacy of GTPase stimulation by GST-Grb2 which we observed. The biphasic nature of GTPase activation by GST-Grb2 is analogous to the precipitin reaction between antigens and antibodies (41). In the presence of excess GST-Grb2, single dynamin molecules are coated by multiple molecules of GST-Grb2, thus preventing the formation of a cross-linked dynamin lattice needed for GTPase activation. Monomeric Grb2, which is bivalent, would be expected to yield a similar drop in GTPase activity but at higher concentrations than those obtained with the tetravalent GST-Grb2. Because dynamin GTPase activity is stimulated by cross-linking, even with anti-dynamin antibodies (36), our results suggest that previous reports of dynamin activation by GST-SH3 fusion proteins should be re-evaluated and that only monomeric SH3-containing species should be used in the future to avoid artifactual cross-linking caused by GST dimerization.

Compared with PI(4,5)P$_2$, Grb2 by itself is a relatively weak

stimulator of dynamin GTPase activity. Its major effect on activity is the synergistic stimulation with PI(4,5)P_2 which is most pronounced at low dynamin concentrations. Because there is no evidence for an interaction between Grb2 and PI(4,5)P_2, the simplest explanation for this synergy involves simultaneous binding of the two activators to dynamin. For example, Grb2 may cluster dynamin molecules by cross-linking their SH3 binding domains, thus increasing the effective concentration of dynamin bound to PI(4,5)P_2-containing liposomes. In the absence of Grb2, dynamin molecules would be dispersed on the liposome surface until high enough concentrations were reached to promote the dynamin-dynamin interactions required for high enzymatic activity.

The ability of dynamin to interact simultaneously with phospholipids and Grb2 raises the possibility of simultaneous interaction with other SH3-containing proteins such as amphiphysin (20). The importance of amphiphysin for dynamin targeting was highlighted by the recent study of Shupliakov *et al.* (46) who introduced expressed amphiphysin SH3 domains into lamprey neurons, displacing dynamin from the coated pit and blocking synaptic membrane recycling.

There is strong evidence that SH3-binding motifs in the PRD are required to target dynamin to coated pit regions of the plasma membrane (19). Likewise, it appears that phosphoinositides bind to the dynamin PH domain and potently stimulate GTPase activity by this interaction. Based on these results and our current observations, we propose the following model for dynamin regulation: dynamin is first recruited to the coated pit by SH3-containing protein, *e.g.* Grb2 or amphiphysin. Once there, dynamin's interaction with the membrane is strengthened by the binding of its PH domain with PI(4,5)P_2 and, perhaps, PI(3,4,5)P_3 which may also orient dynamin to favor its polymerization. In a similar manner, binding of β-adrenergic receptor kinase to $G_{\beta\gamma}$ is favored by the presence of both inositol phospholipids and $G_{\beta\gamma}$ on the membrane (47). In the case of dynamin, these phospholipids also promote self-association and GTPase stimulation, which could be reversed by the enzymatic action of a phosphoinositide 5-phosphatase, *e.g.* synaptojanin. This model is highly speculative, but it incorporates the key observation presented in this paper, *i.e.* that phospholipids and SH3-containing proteins bind simultaneously to distinct domains of the dynamin molecule and synergistically activate dynamin GTPase.

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