Phosphatidylinositol (4,5)-Bisphosphate-dependent Activation of Dynamins I and II Lacking the Proline/Arginine-rich Domains*

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Dynamins constitute a family of GTPases that participate in the early stages of endocytosis. The GTPase activity of neuronal specific dynamin I is stimulated by microtubules, negatively charged phospholipid vesicles, and Src homology 2-containing proteins, including Grb2. These activators were previously shown to bind to a proline/arginine-rich domain (PRD) in the carboxyl-terminal region of the enzyme. Dynamin II, which is ubiquitously expressed, has not been purified or characterized previously. In this study, the enzymatic properties of rat dynamin II and of D746, a dynamin II truncation mutant lacking the PRD, have been characterized. Dynamin II has a higher basal activity than dynamin I, but the two types of dynamin are stimulated similarly by microtubules, Grb2, and phospholipids. D746 is not activated by microtubules or Grb2, highlighting the significance of the PRD for these interactions, but it is activated by phospholipid vesicles containing phosphatidylinositol-4,5-bisphosphate. Moreover, in contrast to previous reports, the PRD appears not to be required for phospholipid-stimulated self-assembly of dynamin, which is a key element in the regulation of its activity. Similar results were obtained with bovine brain dynamin I that had been subjected to limited proteolytic digestion to remove the PRD. Our data highlight the potential involvement of dynamin pleckstrin homology domains in the regulation of GTPase activity by phospholipids.

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1 The abbreviations used are: PH, pleckstrin homology domain; PRD proline/arginine-rich domain; PS, phosphatidylinerine; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; MES, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; GST, glutathione S-transferase.

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D746) which lacks this domain was expressed and purified. As expected from previous studies of proteolysed dynamin I lacking the PRD (7, 11), D746 failed to be activated by microtubules or Grb2. Surprisingly, the PRD was not required for expression of phospholipid-stimulated activity or for cooperative GTPase activation because of dynamin self-association. Moreover, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)-stimulated GTPase activity was undiminished at physiological ionic strength. This behavior was also observed using papain-cleaved dynamin I fragments that lack the PRD, indicating that both dynamins can be activated in the absence of that domain.

The PH domain of dynamin I has been expressed and characterized (14, 15). Like many other PH domains, it was found to bind phospholipids, with phosphoinositides having the highest affinity. Moreover, deletion of the PH domain resulted in the loss of PI(4,5)P₂-stimulated GTPase of mutant expressed dynamin I (16). However, the question of whether the PRD is also required for this activation was not addressed. The results presented in the paper suggest that dynamins are subject to multiple modes of regulation, some involving interactions with the PRD, others with the PH domain.

EXPERIMENTAL PROCEDURES

Materials—Phosphocellulose P11 and diethylaminoethylcellulose DE52 were from Whatman. SP-Sepharose and glutathione-Sepharose were from Pharmacia Biotech Inc. PS was from Avanti Polar-Lipids Inc. Phosphatidylincholine (PC) and PI(4,5)P₂ were from Calbiochem. Monoclonal anti-dynamin antibodies against residues 45–358 were from Transduction Laboratories. Anti-peptide polyclonal antibodies against residues 607–624 (PH domain) and residues 840–851 (PRD) were a gift from Dr. Thomas Südhof, University of Texas, Southwestern. Protease inhibitors, papain (EC 3.4.22.3), taxol, and GTP were from Sigma. (γ-³²P)GTP was from Amersham. NIT₂-NTA agarose was from Qiagen.

Construction of Recombinant Dynamins—cDNAs of carboxy-terminal His₆-tagged wild-type dynamin II and D746, a dynamin II mutant truncated after residue 746, were constructed by polymerase chain reaction using the rat dynamin II cDNA as a template (pCMV 96-15) (17). The primers were designed such that a unique 5'-EcoRV site and a 3'-HindIII site were introduced. The amplified DNA was digested with EcoRV and HindIII and ligated to the pGEX-6P-1 expression vector (Pharmacia). The resulting plasmids were transformed into Escherichia coli and expressed as a fusion protein with glutathione S-transferase (GST) and purified on glutathione-Sepharose.

Purification of Dynamin I—Bovine brains were homogenized with 10% MES buffer and sonicated for 10 min at maximum power (Bath sonicator model W185; Heat System Ultrasorins, Inc., Farmingdale, NY). PS and PS/PC vesicles gave similar level of GTPase activation, although at different total lipid concentrations. In this study, only vesicles containing 100% PS were used. PI(4,5)P₂ was prepared as a mixture with PC in a 1:9 molar ratio.

Other Methods—Protein concentration was determined as described by Bradford (20) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (21) as modified by Matsudaira and Burgess (22). Immunoblot analysis was carried out by the method of Towbin et al. (23) as described previously (24).

RESULTS

Characterization of the GTPase Activities of Dynamin II—Because dynamin II has never been purified, there has been no previous characterization of its properties. To study dynamin II, rat dynamin II with a COOH-terminal His₆ tag was expressed from recombinant baculovirus in Sf9 cells and purified by chromatography on nickel resin. The expressed protein migrates with an apparent molecular weight of 101,000 on SDS-polyacrylamide gel electrophoresis (Fig. 1), consistent with the monomer molecular weight of 98,169 calculated from the amino acid composition. Differences in the two types of dynamin suggested the possibility of differences in their regulation. Therefore, the GTPase activity of the purified enzyme was assayed in the presence or absence of four known activators of dynamin I: phospholipid vesicles consisting of 100% PS or 10% PI(4,5)P₂ and 90% PC, GST-Grb2, and microtubules. Dynamin II has a 10-fold higher basal activity than bovine brain dynamin I (~20 min⁻¹ versus ~2 min⁻¹ for dynamin I). Nevertheless, the enzymatic properties of the two dynamins are similar (Fig. 2). GTPase activation profiles of both dynamins are biphasic, and maximal activities are reached at similar dynamin:activator ratios. Microtubules and anionic liposomes are the most effective activators, accelerating the GTPase of each to more than 200 min⁻¹. Grb2, expressed as a fusion protein with GST, stimulated the GTPase of each to a maximal level of ~55 min⁻¹. Although specific activities of dynamins were slightly variable from preparation to preparation (see also Ref. 7), we observed dramatic variations of the enzyme activation depending on the nature of the phospholipids, with up to 5-fold differences in activation using different lipid batches but the same dynamin preparation. This may account in part for the lower PI(4,5)P₂-stimulated GTPase ac-
Activities of human dynamin I reported earlier\(^2\) (9). Nevertheless, both forms of dynamin showed nearly identical activation patterns, regardless of the maximal specific activities achieved.

**Activation of the GTPase Activity of Truncated Dynamin II, D746**—The PRD contains binding sites for all the currently known stimulators of dynamin GTPase activity, including microtubules, Src homology 3-containing proteins, and negatively charged phospholipids. Hence, it has been proposed that this domain is required for dynamin activation (1), and data supporting this view have been presented (7, 11). It has been shown recently, however, that expressed dynamin I PH domains interact with certain negatively charged phospholipids, e.g. PI(4,5)P\(_2\) (\(K_D = 4.4 \mu M\)) and PS (\(K_D = 47.2 \mu M\)) (15). In light of these observations we reexamined the question of whether or not the PRD is indeed essential for phospholipid-stimulated GTPase activity. A recombinant protein containing residues 1–746 of rat dynamin II was expressed in Sf9 cells from a recombinant baculovirus and purified on nickel resin (Fig. 3, inset). Like full-length dynamin II, D746 has a 10-fold higher basal GTPase activity than dynamin I, but, as expected, its ability to be activated by microtubules or GST-Grb2 is nearly abolished (Fig. 3). In contrast, the GTPase activity of D746 is stimulated potently by negatively charged phospholipid vesicles. Vesicles containing PI(4,5)P\(_2\) were especially efficacious. Thus, despite deletion of the PRD, two of the activators retained the ability to activate the truncated protein. Therefore, we conclude that the PRD is not required for all modes of dynamin activation.

**Effect of Proteolytic Digestion on the GTPase Activity of Bovine Brain Dynamin I**—Previous studies demonstrating the importance of the PRD for phospholipid-stimulated GTPase activity examined proteolytic fragments of dynamin I lacking the COOH-terminal domain (7, 11). Our results with D746 prompted us to reevaluate these earlier findings. Bovine brain dynamin I was subjected to limited papain digestion, yielding known stimulators of dynamin GTPase activity, including microtubules, Src homology 3-containing proteins, and negatively charged phospholipids. Hence, it has been proposed that this domain is required for dynamin activation (1), and data supporting this view have been presented (7, 11). It has been shown recently, however, that expressed dynamin I PH domains interact with certain negatively charged phospholipids, e.g. PI(4,5)P\(_2\) (\(K_D = 4.4 \mu M\)) and PS (\(K_D = 47.2 \mu M\)) (15). In light of these observations we reexamined the question of whether or not the PRD is indeed essential for phospholipid-stimulated GTPase activity. A recombinant protein containing residues 1–746 of rat dynamin II was expressed in Sf9 cells from a recombinant baculovirus and purified on nickel resin (Fig. 3, inset). Like full-length dynamin II, D746 has a 10-fold higher basal GTPase activity than dynamin I, but, as expected, its ability to be activated by microtubules or GST-Grb2 is nearly abolished (Fig. 3). In contrast, the GTPase activity of D746 is stimulated potently by negatively charged phospholipid vesicles. Vesicles containing PI(4,5)P\(_2\) were especially efficacious. Thus, despite deletion of the PRD, two of the activators retained the ability to activate the truncated protein. Therefore, we conclude that the PRD is not required for all modes of dynamin activation.

\(^2\) In Ref. 9, PI(4,5)P\(_2\)-stimulated GTPase activities of up to 25 min\(^{-1}\) were reported for dynamin I. However, those assays were performed at exceedingly low dynamin concentrations (5 nm) and far from the optimal dynamin/lipid molar ratios of about 1:10–1:20 (see Fig. 8). This, together with the potential variation caused by differences among lipid batches discussed under "Results," may explain the 10-fold differences in specific activities between this paper and the earlier report.
an 88-kDa fragment that lacks the PRD, as determined by its failure to be recognized by an antibody against residues 837–851 (Fig. 4B) and its inability to bind to GST-Grb2 (25). The 88-kDa fragment is gradually digested to an 85-kDa polypeptide, which is cleaved further to 53- and 32-kDa fragments. Recognition by antibodies raised against the GTP binding domain (residues 45–358) (Fig. 4C) confirms that the 53-kDa fragment contains the catalytic site. The 32-kDa fragment contains the PH domain and is thus recognized by antibodies raised against a peptide corresponding to residues 607–624 (Fig. 4D). The latter two fragments appear to exist in a nonco-

valent complex because they comigrate on a Superose 12 gel filtration column (data not shown). Not only the 85-kDa but also the 53-kDa/32-kDa fragments retain GTPase activity that is approximately 10-fold higher than the basal activity of undigested dynamin I (Fig. 5B). In agreement with previous reports (7, 11), dynamin I fragments lacking the PRD are not stimulated by microtubules or by GST-Grb2 (Fig. 5A). However, as predicted from analysis of D746, activation by PS or P(4,5)P₂ vesicles is undiminished.

PI(4,5)P₂-stimulated GTPase Activity Occurs at Physiological Ionic Strength—Activation of dynamin I GTPase activity in vitro by microtubules and PS vesicles is apparent only under conditions of low ionic strength (7) (Fig. 6A). The same is true for expressed rat dynamin II (data not shown). This salt sensitivity has raised a question about the physiological relevance of these activators (2). In contrast, Grb2-stimulated activity was not reduced by salt (Fig. 6A), and PI(4,5)P₂-stimulated activities of both intact and truncated dynamins were only slightly reduced at NaCl concentrations higher than 150 mM (Fig. 6B).

Cooperative Activation of D746 and Dynamin I Fragments—In the presence of microtubules or PS vesicles, the specific GTPase activity of dynamin I increases cooperatively as a function of dynamin concentration (8) (Fig. 7). This behavior is characteristic of self-associating enzymes having activities that depend on their state of oligomerization (26). Tuma and Collins (8) proposed that dynamin GTPase activation involves dynamin self-association of dynamin molecules which is facilitated by their alignment on multivalent surfaces such as microtubules or PS vesicles. As discussed above, the interactions between dynamin I and these anionic surfaces are believed to be mediated by the positively charged PRDs. However, our results demonstrate that the PRD is not required for cooperative activation by PI(4,5)P₂ because it occurs not only with intact dynamins (Fig. 8, A and C) but also with D746 (Fig. 8D) and with the 53-kDa/32-kDa proteolytic fragments (Fig. 8B), which lack the PRD. Specific GTPase activities rise with increasing dynamin concentrations until they reach a maximum and thereafter decline. These maxima occur at different dynamin concentrations but at similar dynamin:phospholipid ratios. A plausible explanation for this behavior is that after maximal activities are achieved at a given dynamin:phospholipid ratio, steric blocking prevents further dynamin binding and activation. Because maxima occur at dynamin:phospholipid ratios of about 1:200, each dynamin molecule apparently

Fig. 5. GTPase activity of the proteolytic fragments of bovine brain dynamin I. Dynamin I was digested for various times as described in Fig. 4. Panel A, GTPase activities of undigested dynamin I or dynamin I digest (each at a final concentration of 0.01 mg/ml) were measured in the presence of 1 μM microtubules (□), 1 μM GST-Grb2 (■), 10 μM PS (○), and 0.5 μM PI(4,5)P₂ (△). Phospholipid vesicles were composed of either 100% PS or 10% PI(4,5)P₂ and 90% PC. The specific activities of undigested dynamins (0 time) were 120 min⁻¹ (microtubules, MT), 32 min⁻¹ (GST-Grb2), 128 min⁻¹ (PS), and 63 min⁻¹ (PI(4,5)P₂). The lower activities of intact dynamin are caused by iodoacetic acid (4 mM), used to terminate the digestions. Panel B, basal GTPase activity of dynamin I digest. Data represent the mean ± S.E. from three separate experiments, each done in duplicate for an n of 6.

Fig. 4. Identification of the proteolytic fragments of bovine brain dynamin I. Dynamin I was digested with papain (1:1,000 w/w) at 30 °C for the times indicated. Proteolysis was terminated by the addition of 4 mM iodoacetic acid. Panel A, Coomassie Blue-stained gel showing the digestion patterns. Panels B–D, immunoblots of dynamin fragments using antibodies raised against different domains: panel B, recognition of the PRD (antibody against residues 840–851); panel C, recognition of the GTP binding domain (antibody against residues 45–358); and panel D, recognition of the PH domain (antibody against residues 607–624). Panel E shows a scheme of the digestion products.
occupies an area of the liposome surface containing 100 lipid molecules (approximately 6 nm × 6 nm), if only the outer monolayer is considered.

Our results indicate that the COOH-terminal PRDs of dynamin I and II are not required for the activation of dynamin GTPase activity by phospholipids. In view of the well-documented interactions between specific phospholipids and dynamin PH domains (15, 16), it seems likely that activation occurs as a consequence of phosphoinositide binding to this domain. Significantly, this binding can take place even at physiological ionic strength, unlike the interaction between dynamin and microtubules, another potent stimulator of GTPase activity. Finally, if GTPase activation is contingent upon dynamin self-association on a multivalent matrix, then our data suggest that dynamin molecules align themselves on membranes via phospholipid-PH domain interactions.

**DISCUSSION**

Many characteristics of the regulation of dynamin activity have been described. The general features of this regulation, as they are currently understood, include a low basal GTPase of the unassembled enzyme, the ability to self-associate on an appropriate matrix, and cooperative autoactivation as a consequence of suitable alignment on the matrix. The PRD has been thought to account for interactions with GTPase regulators and for mediating self-assembly and autoactivation through these interactions. Instead, our findings indicate that the PRD is not required for all of the regulatory properties listed above: (i) Removal of the dynamin I PRD by limited proteolysis results in a 10-fold increase in its basal GTPase activity; (ii) dynamin lacking the PRD is activated by phospholipids; and (iii) dynamin lacking the PRD is activated by phospholipids; and (iii) the PRD is not required for the cooperative increase in GTPase activity.
activity when activated by PI(4,5)P₂. If increases in specific activity reflect dynamin self-association, then it appears that the PRD is not required for PIP₂-driven polymerization. In the presence of PI(4,5)P₂, stimulation of GTPase activities of intact dynamin and dynamin lacking the PRD is not affected by physiological ionic strength. In contrast, two of the PRD-mediated interactions, i.e. those with microtubules and PS vesicles, occur only at low ionic strength. The interaction between the PRD and Grb2 is resistant to physiological salt concentration, but Grb2-stimulated GTPase activity is relatively low compared with maximal microtubule- or lipid-stimulated activities. Salim et al. (16) were the first to demonstrate that activation of dynamin I GTPase by phosphoinositides involves regions of dynamin outside the PRD. They showed that dynamin I mutants with deleted PH domains expressed Grb2-stimulated GTPase activity but were not activable by PI(4,5)P₂. Our observation that D746 and papain digests of dynamin I can be activated by phospholipids despite their lack of PRDs suggests that interactions with the PH domains are both necessary and sufficient for enzyme activation. Although the mechanism of GTPase activation remains to be established, it is likely that dynamin self-association, promoted by phospholipid binding to the PH domains, is involved. However, a more direct effect of phospholipids on catalysis cannot be ruled out.

Rat dynamin I and II have an overall sequence identity of 79%, but their PRDs are only 46% identical (2). However, the PRDs of the two forms of dynamin have nearly the same isoelectric point (∼12.5) and percentage of prolines (∼30%). This similarity of composition most likely accounts for the almost identical interactions of dynamins I and II with their PRD-binding activators, Grb2 and microtubules. The PH domains of the two dynamins have 84% sequence identity, explaining the similar activation properties observed in the presence of phospholipid vesicles, which may interact with both the PH domains and the PRDs. Aside from the approximately 10-fold higher basal activity of dynamin II, the only other physical difference we detected between dynamins I and II is the greater tendency of dynamin II to aggregate in the absence of activators (not shown). However, it should be noted that dynamin I was purified from bovine brain, whereas dynamin II is a recombinant protein with a His₆ tag at the carboxyl terminus. Native dynamin II has not yet been isolated from cells or tissues.

Removal of the dynamin I PRD by papain cleavage results in a 10-fold increase in basal GTPase activity. This finding, which disagrees with an earlier observation (11), suggests that the PRD is inhibitory in native dynamin, perhaps being displaced by phospholipids alone probably cannot account for specific targeting of dynamin to the coated pits, a function more suitable for suitable binding partners such as Grb2 (29), amphiphysin (30), or AP2 (31). Therefore, we propose that dynamin is recruited to the coated pit by these proteins, many of which interact with the PRD, and that GTPase activity at the coated pit is stimulated by phosphoinositides, primarily via interactions with the PH domain. The validity of this model is currently being investigated in our laboratory.

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