Regulation of Dynamin I GTPase Activity by G Protein βγ Subunits and Phosphatidylinositol 4,5-Bisphosphate*

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Hsin Chieh Lin and Alfred G. Gilman‡
From the Department of Pharmacology,
University of Texas Southwestern Medical Center,
Dallas, Texas 75235

Dynamin I is a 100-kDa GTPase that plays an important role in the recycling of synaptic vesicles. Hydrolysis of GTP by dynamin is thought to be a critical step in fission of coated pits to form coated vesicles. We report that the heterotrimeric G protein βγ subunit complex (Gbg) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) are negative and positive regulators of dynamin GTPase activity, respectively. Furthermore, the apparent affinity of dynamin for Gbg is substantially enhanced by PtdIns(4,5)P2. However, the GTPase activity of oligomeric dynamin is unaffected by Gbg. The effects of heterotrimeric G proteins on endocytosis may thus be mediated directly and not involve more remote aspects of their signaling properties.

Originally identified as a microtubule binding protein (1), dynamin, a 100-kDa GTPase, is homologous to the shibire gene product in Drosophila (2–4). Temperature-sensitive mutations in the shibire gene cause paralysis, and morphological examination of synaptic junctions in affected flies demonstrated an apparent failure of fission of vesicles from the presynaptic membrane during endocytosis (5). Furthermore, treatment of nerve terminals with nonhydrolyzable GTP analogs causes tubular invaginations of the plasmalemma, and the walls of these membranous tubules apparently contain rings of dynamin (6). It has thus been hypothesized that dynamin plays a crucial role in the fission reaction during endocytosis and that the regulatory properties of the protein are controlled by cycles of GTP binding and hydrolysis. Unlike classical, low molecular weight GTPases and heterotrimeric G proteins, the affinity of dynamin for GTP and GDP is apparently in the μM (rather than the mM) range (7), and the rate-limiting step in the steady-state hydrolysis of GTP by dynamin is not dissociation of the GDP product. These considerations highlight the importance of knowledge of factors that regulate nucleotide binding and hydrolysis by this protein. To date, it is known that microtubules, a variety of proteins that contain SH3 domains, and phospholipids activate dynamin GTPase activity in vitro (8–11). Interestingly, activation of the dynamin GTPase is apparently the result of positive cooperativity among dynamin molecules in the presence of regulators such as microtubules and phospholipids. Dynamin also belongs to a growing list of proteins that contain a pleckstrin homology (PH) domain (12, 13), and several such proteins are known to interact with both the G protein βγ subunit complex and PtdIns(4,5)P2 (14–18). We have thus examined the effects of these molecules on dynamin GTPase activity.

EXPERIMENTAL PROCEDURES

Affinity Purification of Bovine Brain Dynamin—All procedures were performed at 4 °C. GSTαA was purified as described (19). Bovine brain cytosol (200 mg of protein, 3.6 mg/ml) prepared in buffer A (25 mM Hepes-KOH (pH 7.2), 2 mM MgCl2, 1 mM EDTA, 50 mM KCl, 1 mM DTT), was applied to a 5-ml glutathione-Sepharose column (1-cm diameter) containing 50 μg of bound GSTαA. The column was washed with 100 ml of buffer A, and dynamin was eluted with 25 mM Hepes-KOH (pH 7.2), 2 mM MgCl2, 1 mM EDTA, 500 mM KCl, and 1 mM DTT; 2-ml fractions were collected. Purification of human dynamin I was from SF9 cells.

Purification of Human Dynamin I—A recombinant baculovirus encoding human dynamin I was the generous gift of Dr. S. L. Schmid (20). A 2-liter suspension culture of SF9 cells (1 × 107 cells/ml) was infected with the baculovirus (5 × 108 virions/ml) 64 h prior to harvesting. The following procedures were performed at 4 °C. Cells were suspended in 50 ml of buffer B (50 mM Hepes-KOH (pH 8.0), 50 mM KCl, and 5 mM β-mercaptoethanol) and were disrupted in a Dounce homogenizer. The homogenate was diluted with 50 ml of the same buffer and centrifuged at 100,000 × g for 1 h. The supernatant was applied to a Ni2+-NTA column (15 ml) equilibrated with buffer B. The column was washed with 100 ml of buffer B and 100 ml of buffer C (50 mM Hepes-KOH (pH 8.0), 150 mM KCl, 5 mM β-mercaptoethanol) prior to elution with 30 ml of buffer C containing 100 mM imidazole.

Steady-state GTPase Assay—Human dynamin I and other factors were mixed on ice in a final volume of 40 μl of GTPase buffer (20 mM Hepes-NaOH (pH 8.0), 10 mM MgSO4, 1 mM EDTA, 0.001% Lubrol PX, 0.1 mg/ml fatty acid-free bovine serum albumin, and 1 mM DTT). The mixtures were then incubated at 30 °C for 10 min before addition of 10 μl of 1000 μM [γ-32P]GTP (500 cpm/pmol) in GTPase buffer to initiate the reaction. Reactions were terminated by addition of 750 μl of 5% ice cold Norit A, and Pi was quantified as described by Higashijima et al. (21). The length of incubation was adjusted such that no more than 10% of the added GTP was hydrolyzed.

RESULTS AND DISCUSSION

The procedures used to obtain pure dynamin from bovine brain and SF9 cells infected with a recombinant baculovirus encoding human dynamin I (HDYM I) are shown in Fig. 1. Dynamin binds with high affinity to the appendage domain of α-adaptin (19). We took advantage of this interaction to purify dynamin from bovine brain cytosol by affinity chromatography using immobilized glutathione S-transferase-α-adaptin (GSTαA). Although the majority of the dynamin flowed through this column, a significant amount of the protein remained tightly bound after extensive washing and could be eluted with buffer containing 500 mM KCl. Dynamin was highly purified in the later eluting fractions. We also found that recombinant human dynamin expressed in SF9 cells can be affinity-purified by adsorption to Ni2+-NTA columns and eluted with buffer containing 500 mM KCl.

‡To whom correspondence should be addressed: Dept. of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235.

5 The abbreviations used are: PH, pleckstrin homology; GSTαA, glutathione S-transferase-α-adaptin; G protein, heterotrimeric guanine nucleotide-binding protein; PtdIns(4,5)P2, phosphatidylinositol-4,5-bisphosphate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; GTPαS, guanosine 5′-O-(thio)triphosphate.

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tion with imidazole, even though the protein was not polyhistidine-tagged. This material was further purified to homogeneity by phosphocellulose cation exchange chromatography. BIAcore binding assays demonstrated that both bovine brain and recombinant human dynamin I have approximately the same affinity for GSTαA (200 nM) as that reported by Wang et al. (19) (data not shown). The two preparations also gave comparable results in experiments where GTPase activity was measured; the data shown below were all obtained with the recombinant human protein.

Dynamin assembles into stacks of ring-like structures at low ionic strength (and in the absence or presence of nucleotide) (22), and these structures resemble the dynamin oligomers that decorate the neck of deeply invaginated clathrin-coated pits in GTPγS-treated synaptosomes (6). In addition, as mentioned, activation of dynamin GTPase by phospholipids and microtubules is positively cooperative with respect to dynamin concentration. We have discovered that human dynamin I exhibits striking positive cooperativity even in the absence of activators (Fig. 2A). Thus, the specific activity of dynamin GTPase was essentially constant at dynamin concentrations of 10–100 nM but rose severalfold as the protein concentration was elevated beyond that point. The heterotrimeric G protein βγ complex inhibited dynamin GTPase activity profoundly with an IC₅₀ of approximately 400 nM when the dynamin concentration was 5 nM. However, inhibition of GTPase activity by βγ was inapparent when the dynamin concentration was 250 nM or when dynamin GTPase activity was activated by microtubules or the SH3 domain-containing protein GRB2 (added as a GST fusion protein) (Fig. 2B). Identical results were obtained in the presence or absence of GSTαA (Fig. 2C). These results suggest that oligomerization of dynamin prevents inhibition of GTPase activity by βγ, and it is thus possible that the G₃₃₂ binding site in dynamin is masked by oligomerization.

Vesicles containing acidic phospholipids or salt-stripped clathrin-coated vesicles activate dynamin GTPase activity (11). However, it remains unclear which specific lipids activate the GTPase in vivo. PH domains from several proteins bind phospholipids and display particularly high affinity for PtdIns(4,5)P₂ (14, 15). Although a PH domain derived from dynamin did not bind to vesicles containing different inositol phosphates, including PtdIns(4,5)P₁ (15), the question of interaction of intact dynamin with PtdIns(4,5)P₂ has not been addressed. We prepared vesicles using mixtures of several individual phospholipids (10% w/w) with phosphatidylcholine, which does not activate dynamin GTPase activity (11). Vesicles containing PtdIns(4,5)P₂ stimulated dynamin GTPase activity to a much greater extent than did vesicles containing the other phospholipids tested (Table I). Vesicles prepared from total brain lipids also activated dynamin GTPase activity in a concentration-dependent manner (Fig. 3A), and this effect was blocked by neomycin, which interacts with PtdIns(4,5)P₂ (Fig. 3B). The basal GTPase activity of dynamin was not altered by neomycin, suggesting specificity in its inhibitory action. Furthermore, vesicles containing 10% PtdIns(4,5)P₂, 90% phosphatidylcholine were potent activators of dynamin GTPase (EC₅₀ ~ 10 μM PtdIns(4,5)P₂), and this effect was blocked by stoichiometric amounts of neomycin (Fig. 3C). These results strongly suggest that PtdIns(4,5)P₂ is a specific activator of the GTPase activity of dynamin I.

We next tested the capacity of G₃₃₂ to inhibit dynamin I GTPase stimulated by either brain lipid or PtdIns(4,5)P₂-containing liposomes. The IC₅₀ for βγ was approximately 40 nM in
each case (Fig. 3D), representing a 10-fold increase in potency over the effects of Gαi, observed in the absence of activators. These concentrations of Gαi, are comparable to those necessary for other well-characterized effects of this subunit complex (23). The capacity of Gαi to inhibit PtdIns(4,5)P2-stimulated GTPase activity suggests that the mechanism of stimulation of this reaction by phospholipids differs from that utilized by microtubules or GRB2. The inhibitory effect of Gαi was prevented by stoichiometric concentrations of a GDP-bound G protein α subunit (Fig. 3E). These experiments were performed with a GTPase-deficient mutant of Gαi (Arg178→Cys) to eliminate the confounding effect of the GTPase activity of the wild type protein.

Models describing the role of dynamin self-assembly in budding of clathrin-coated vesicles have been presented (6, 22). With the knowledge that clathrin-coated vesicles have been found to contain G proteins (24, 25), we propose the following model. In the first step, it has been assumed that GDP-bound or nucleotide-free dynamin is recruited from the cytosol to clathrin-coated pits. However, in the absence of an unknown regulator of cytosolic dynamin GTPase activity, the protein would presumably be saturated with substrate and hydrolyzing nucleotide slowly but futilely. Association of dynamin with coated vesicles, presumably mediated by adaptins, would facilitate interaction of dynamin with both Gαi and PtdIns(4,5)P2. Inhibition of GTPase activity by Gαi could promote accumulation of dynamin in the GTP-bound form, although this remains to be demonstrated. Alternatively, Gαi may directly prevent oligomerization of dynamin. Sequestration of Gαi, possibly by a G protein α subunit or some other interacting protein, would permit assembly of dynamin at the neck of the budding coated pit (step 2). With an elevated GTPase activity caused by oligomerization, PtdIns(4,5)P2, and/or other factors, the synchronous hydrolysis of GTP by the assembled dynamin would trigger fission, with resultant formation of a coated vesicle (step 3). This process may well require factors that serve as effectors for GTP-dynamin. Finally, dissociation of oligomeric dynamin could favor its return to the cytoplasm. Phosphorylation and dephosphorylation of dynamin I, controlled by the state of polarization of the synapse, may also play important roles in regulating binding and release of the protein from coated vesicles (26). A recent report suggested that dynamin is linked to the internalization of a prototypical G protein-coupled receptor (27). Although much remains to be learned about this complex regulatory cycle, we suggest that the subunits of heterotrimeric G proteins are involved directly and not as more remote initiators of regulatory signals.

### Table I

| Phospholipids | GTPase | pmol Pi·pmol dynamin·min⁻¹ |
|--------------|--------|---------------------------|
| None         | 2.7    |
| PC alone     | 2.7    |
| PS          | 4.1    |
| PE          | 2.6    |
| PtdIns      | 3.0    |
| PtdIns(4)P  | 3.0    |
| PtdIns(4,5)P₂| 14.6   |

*PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine.*

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Regulation of Dynamin I GTPase Activity

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