Activation of Dynamin GTPase Is a Result of Positive Cooperativity*

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Dynamin is a GTP-binding protein thought to be involved in the early stages of endocytosis. Presently, it is not known how dynamin GTP binding and hydrolysis are related to its role in vivo. We previously characterized the ability of acidic phospholipid vesicles and microtubules to strongly stimulate the GTPase activity of purified brain dynamin. In a further analysis of dynamin enzymatic properties, we have found that the increase of dynamin GTP hydrolysis in the presence of activating agent depends on enzyme concentration. At low enzyme concentration, little or no activation is observed. Plots of dynamin GTPase activity with increasing enzyme concentration in the presence of either activating agent are strongly sigmoidal, indicating that positive cooperativity is responsible for the increased activity observed. A Hill coefficient of 2.3 was determined, implying that at least two dynamin molecules associate for maximal GTPase activity. No cooperative effects in GTP binding were observed. Linear transformation of reaction velocity versus enzyme concentration data indicate an apparent $K_m$ for dynamin-dynamin interactions of 37 nm, which is significantly lower than the physiological concentration of dynamin in brain. These results suggest that cooperative interactions between dynamin molecules are responsible for the apparent activation of GTPase observed and are likely involved in dynamin function in vivo.

Dynamin is a member of a family of novel GTP-binding proteins (1) (reviewed in Ref. 2), whose members include the interferon-inducible vertebrate Mx proteins involved in conferring viral resistance (reviewed in Ref. 3), the Vps1/Spo15 gene product that is required for proper sorting of vacuolar proteins and spindle pole separation in yeast (4, 5) and the Mgm1 gene product that is thought to be important in yeast mitochondrial genome maintenance (6, 7). Based on its sequence similarity with the Drosophila melanogaster shibire gene product, dynamin is thought to function in the early stages of endocytosis (8, 9). Despite the apparent divergent functions among this family of GTP-binding proteins, all members share considerable sequence identity in the amino-terminal two-thirds of the molecule, particularly in the region around the tripartite GTP-binding domain. The carboxyl termini differ substantially in length and sequence, and they are likely responsible for conferring the functional differences observed.

Although mutational analyses of the GTP-binding sites of dynamin and Vps1/Spo15 have demonstrated that GTPase activity is required for in vivo function (10-12), very little is known about the enzymatic properties of these proteins. Dynamin, Vps1/Spo15, and Mxl exhibit intrinsic GTPase activities of $-15$, $-0.04$, and $-6$ mol of GTP hydrolyzed/min/mmol of protein, respectively (10, 13, 14). These basal turnover rates are significantly higher than that reported for the low molecular weight GTP-binding proteins, such as ras (0.008/min), and are comparable to those of heterotrimeric G-proteins (2-4/min) (see Ref. 15). Most GTP-binding proteins hydrolyze GTP in a conditional manner, i.e. a hormone stimulus or binding of a regulatory protein is required for GTP hydrolysis. This possibility has not been well explored for the dynamin family of proteins. Only dynamin has been shown to exhibit GTPase activity that is strongly stimulated in the presence of other agents (up to $-200$ mol of GTP hydrolyzed/min/mmol of protein).

Dynamin GTPase was initially characterized as being potently activated by microtubules (16). We have shown that endogenous brain vesicles and acidic phospholipids activate rat brain dynamin GTPase in a similar manner to that provided by microtubules, in which stimulation of dynamin GTPase occurs at low activator concentrations, followed by a decrease back to near basal GTPase with further increases in "activator" concentration (13). Recent reports have also demonstrated that some SH3 domain proteins are capable of stimulating dynamin GTPase as well (17, 18). It is not presently known what structural or enzymatic properties of dynamin or of its interactions with each of these agents are contributing to the biphasic activation profiles observed. However, we and others have shown that the dynamin carboxyl-terminal domain mediates the association of dynamin with acidic phospholipids, microtubules, and certain SH3 domains leading to its activation (13, 18).

The present study was aimed at identifying the features of dynamin in its interaction with activating agents that are important in regulating GTPase activity. The marked similarity of dynamin behavior (binding and enzymatic activities) in the presence of each of the activating agents (microtubules, acidic phospholipids, endogenous rat brain vesicles, and SH3 domain-containing proteins), suggests that the mechanism of activation is conserved among these agents. Further analysis of dynamin GTPase activity revealed positive cooperativity with regard to enzyme concentration, suggesting that direct interactions between dynamin molecules, in the presence of either microtubules or acidic phospholipids, are responsible for the activation of GTP hydrolysis observed.

EXPERIMENTAL PROCEDURES

Materials —Male Sprague-Dawley rats (Harlan, Indianapolis, IN) between 150 and 250 g were used as the source of brain tissue. Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). GTP (type VII, lithium salt) and AMPPNP (lithium salt) were purchased from Sigma. Taxol was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Research, National Cancer Institute, and stored as a 10$\mu$ stock solution in dimethyl sulfoxide at $80^\circ C$.

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1 The abbreviations used are: AMPPNP, 5'-adenylyl-β,γ-imidodiphosphate; Pepes, 1,4-piperazinediethanesulfonic acid.
Dynamin Purification—Dynamin was purified from rat brain by coassembly with microtubules in extraction buffer (50 mM Hepes, 50 mM Pipes, 2 mM MgCl₂, 1 mM EDTA, pH 7.0) followed by elution from the microtubules with GTP and AMPPNP (13, 19). The eluted dynamin was further purified by fractionation on a 5–20% linear sucrose gradient as previously described (13).

Liposome Preparation—Liposomes (small unilamellar vesicles) were prepared from mixtures of phosphatidylinerine and phosphatidyldiglycerol by first drying from the chloroform in which they were stored. Residual solvent was removed in vacuo for 30 min to 1 h. The dried lipids were reconstituted to the desired concentration in extraction buffer and sonicated with a probe sonicator until the solution clarified. For each experiment, the phosphatidylinerine content of the liposomes is given as a weight percent of total phospholipid.

Microtubule Preparation—Tubulin was isolated from calf brain by reversible assembly of microtubules following DEAE-Sephadex chromatography (20). Tubulin was polymerized for use in assays by adding equimolar taxol (21) and incubating for 5 min at 37 °C. The microtubules were diluted in extraction buffer to the desired concentration for use in assays.

GTPase Assays—Assays were performed in 100 μl of 0.5 x extraction buffer at 37 °C for 15 min. The concentrations of dynamin and added agents were as indicated in the figure legends. Assay mixtures were incubated for 5–10 min at room temperature prior to initiation of the reaction by addition of GTP. Activity was monitored by measuring 32P release from 0.5–0.75 mM [γ-32P]GTP (Amersham Corp.) according to Collins and Vallee (22).

Protein Determination—Protein content was determined either using BCA reagent (Pierce) or by laser scanning densitometry of Coomassie Blue-stained gels using bovine serum albumin as a standard.

RESULTS

Enzyme and Activator Concentration Effects on Dynamin GTPase—We previously reported that dynamin activation by microtubules, acidic phospholipids, and endogenous rat brain vesicles occurred in a biphasic manner (13) (Fig. 1). As the concentration of activator increased, dynamin GTPase activity increased until an optimum was reached, beyond which dynamin activity decreased. Our data suggest that microtubules and phospholipids do not act as Michaelis-Menten activators, with reversible associations between the enzyme and activating agent, but rather dynamin remains stably bound to both of these polymers. Concentrations of GTP less than 5 mM do not elute dynamin from microtubules, and additions of NaCl to an ionic strength identical to 10 mM GTP completely elute dynamin, suggesting that the elution behavior observed in previous studies is due to ionic strength effects.2 If the microtubules and acidic phospholipids acted only as stable binding sites, the concentrations required for optimal activation might be considered a reflection of both binding affinity and number of binding sites available relative to enzyme concentration. To examine whether dynamin GTPase depends on activator concentration, such that an optimal ratio of dynamin molecules to number of binding sites is required for maximal activation, the experiments shown in Figs. 1 and 2 were performed.

In Fig. 1 the concentration of microtubules or acidic phospholipids required for maximal activation of different dynamin concentrations was determined. At the two dynamin concentrations assayed (62 and 124 nM), optimal activation occurred at identical microtubule concentrations (Fig. 1A), but the maximal specific GTPase activity was different at the two enzyme concentrations. A similar result was obtained when acidic phospholipids were used as the activating agent (Fig. 1B). At 11 nM dynamin, minimal activation by phospholipid was observed (1.2-fold increase over basal levels). However, at both 33 and 66 nM dynamin, optimal activation levels were observed at 5 μg/ml phospholipid. In this experiment as well, lower dynamin concentrations were activated to lesser extents than the higher dynamin levels, with increases of 1.2-, 3.2-, and 6.4-fold over basal GTPase observed for 11, 33, and 66 nM dynamin, respectively. Although the extent of dynamin activation differed between the two agents tested, the similarity in dynamin enzymatic behavior was striking. GTPase activation was dependent on enzyme concentration, but the concentration of activator (either microtubules or phospholipid) required for maximal effect did not vary with dynamin content in the assay.

To examine this phenomenon over a wider range of dynamin concentrations, we assayed dynamin GTPase activity in the presence of microtubules at a constant molar ratio of 1 dynamin monomer to 20 tubulin dimers. Total activity is plotted.

2 P. L. Tuma and C. A. Collins, unpublished results.
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In the presence of microtubules deviated significantly from the namin to microtubule concentration was necessary and sufficient at the optimal concentration of each activating agent content.

Modulate GTPase activity.

were assayed in the presence of 0.15 mg/ml microtubules

dynamin activity in the presence of 0.15 mg/ml microtubules was measured.

Cooperativity Effects on Dynamin GTPase Activity—The sigmoidal curves observed in Figs. 2 and 3 are indicative of positive cooperativity between dynamin molecules (23). Analysis of the rate of GTP hydrolysis by dynamin at constant activator concentration revealed no sigmoidal character. The positive cooperativity, therefore, is the result of interactions between dynamin molecules and activator, not dynamin and GTP. This was demonstrated by generating Lineweaver-Burk plots of GTP hydrolysis in the presence of microtubules (Fig. 5A) or acidic phospholipids (Fig. 5B).

Straight lines were obtained, indicating no positive cooperativity in GTP binding. However, when plots of reciprocal activity were plotted against $1/\text{[dynamin]}$ in the presence of microtubules (Fig. 5C) or acidic phospholipids (Fig. 5D), profiles were generated that are expected of systems that display positive cooperativity (23).

We generated modified Hill plots to examine the relationship of the log ($V/V_{max} - V$) to the log[dynamin] (Fig. 6). When dynamin was assayed in the presence of a constant concentration of microtubules (0.15 mg/ml), the resulting velocity versus enzyme concentration plot had a positive slope, with $n_H = 2.1$ (Fig. 6A). Similar results were obtained for assays carried out in the presence of liposomes (Fig. 6B), with $n_H = 2.2$. The average Hill coefficient was found to be $2.3 \pm 0.2$ ($n = 7$); implying that at least two dynamin molecules interact in order to achieve maximal GTPase activity in the presence of either microtubules or phospholipids.

To calculate constants for the interactions between dynamin molecules, secondary double reciprocal plots were generated (23). The linear transformation shown in Fig. 7 was based on data presented in Fig. 3A. The $y$-intercept reflects the reciprocal of the apparent $V_{max}$ for the reaction (used to generate the Hill plots shown in Fig. 6), while the $x$-intercept indicates the reciprocal of the $K_m$ for dynamin interactions with itself. The average value for $K_m$-dynamin was $37 \pm 8 \text{ nm}$ ($n = 7$), considerably below the endogenous concentration of dynamin in brain (see below).

**FIG. 3.** Cooperative interactions between dynamin molecules modulate GTPase activity. Increasing concentrations of dynamin were assayed in the presence of 0.15 mg/ml microtubules (A) or 0.1 mg/ml 50% phosphatidylserine liposomes (B). Data are presented as means of duplicate specific activity measurements. The basal GTPase activity for the experiment shown in A was 46 nmol/min/mg, and for B was 176 nmol/min/mg.

Increasing dynamin concentration is shown. If the ratio of dynamin to microtubule concentration was necessary and sufficient to achieve optimal activation, a straight line with an intercept at the origin should result. Although the basal activity was proportional to enzyme concentration, dynamin activity in the presence of microtubules deviated significantly from the straight line expected, with minimal activation at low dynamin content.

Since all concentrations of dynamin tested were optimally stimulated by nearly identical amounts of activating agents, and dynamin exhibited higher specific activity levels as enzyme concentration increased, it appeared that activation was largely dependent on enzyme concentration. In the experiment shown in Fig. 3, varying concentrations of dynamin were assayed at the optimal concentration of each activating agent (determined as for Fig. 1). As the concentration of dynamin was increased in the presence of constant microtubule concentration, the specific activity profile again approximated a sigmoidal curve rather than a straight horizontal line (Fig. 3A).

Similar results were obtained using acidic phospholipid-containing liposomes (Fig. 3B).

At higher dynamin concentrations, it appeared that activity was reaching saturating levels. To ensure that the apparent saturation was not due to depletion of nucleotide in the assay mixture or due to product inhibition of the reaction, we assayed the concentration of inorganic phosphate released during the course of the assay (Fig. 4). In the presence of optimal microtubule concentrations (0.15 mg/ml for this experiment), phosphate release was linear with time at both high dynamin concentrations (156 nm) and at the concentration of dynamin typically assayed (56 nm). This indicates that dynamin was not being inhibited by the products of the reaction during this time course. After 15 min, the standard assay reaction time, 9.5 nmol of GTP, representing only 14% of added GTP, were hydrolyzed by 156 nm dynamin. The remaining concentration of GTP in this experiment was 580 µM, significantly above the $K_m$ of dynamin for GTP in the presence of microtubules or acidic phospholipids (159 µM (Fig. 5A) and 29 µM (Fig. 5B), respectively, and 12 µM for basal GTP hydrolysis (data not shown)).

**DISCUSSION**

**FIG. 4.** The rate of GTP hydrolysis is linear during the course of the assay. The levels of GTP hydrolyzed by 56 (○) and 156 nm (C) dynamin in the presence of 0.15 mg/ml microtubules were measured. Basal activity was not subtracted from total.

Previous work (19, 24) has shown that dynamin associates with microtubules in clusters, thought to be a reflection of cooperativity in binding (16, 19). At least two interchangeable activator binding sites must be present on each dynamin molecule, since dynamin is capable of mediating cross-links between individual microtubules (19), as well as between micro-
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Fig. 5. Lineweaver-Burk analysis of dynamin GTPase activity with respect to GTP and dynamin concentration. Dynamin GTPase was assayed in the presence of 0.15 mg/ml microtubules (A and C) or 0.1 mg/ml of 25% (B) or 50% (D) phosphatidylserine liposomes. Reciprocal plots are shown with respect to GTP concentration in A and B and with respect to dynamin concentration in C and D. Units for 1/[GTP] are expressed as μM⁻¹ (A and B) and for 1/[dynamin], μM⁻¹ (C and D). The values for Kₘ,GTP based on linear regression analysis of the data shown are 159 μM for A, and 34 μM for B, and are representative of five determinations.

The major finding from our study is that the activation of dynamin GTPase appears to be largely due to interactions between dynamin molecules once bound to microtubules or liposomes, rather than a direct activation of dynamin by these agents. The degree to which dynamin could be activated was a function of enzyme concentration, with only slight stimulation by microtubules or lipids occurring at low (10 nM) dynamin levels. This was not a reflection of decreased affinity between dynamin and activating agent, since significant binding was observed at these concentrations (data not shown). We have not yet been able to determine experimentally whether the remaining stimulation of GTPase activity observed at low enzyme levels can be attributed to direct activation by microtubules or lipids, or reflects interactions between dynamin molecules even when they are diluted among a large excess of available binding sites.

The carboxyl-terminal domain of dynamin responsible for binding microtubules and acidic phospholipids is extremely basic (pI = 12.5) and proline-rich. Such regions generally form extended structures that have been described as “sticky arms” (for review, see Ref. 27) that are involved in multiple weak binding interactions that together promote stable associations. We suggest that dynamin binding along a polymeric substrate (microtubules or liposomes in this case) allows for the appropriate orientation and proximity for dynamin interactions to occur. This result contrasts with studies describing microtubule activation of cytoplasmic dynein (28, 29) and kinesin (30, 31) where microtubules act as a Michaelis-Menten activator, and saturation curves reflect binding affinities for the transient interactions which occur between these motor molecules and their cytoskeletal substrate during the enzymatic cycle.

The sigmoidal nature of the enzyme concentration profiles in the present study are indicative of positive cooperative interactions between dynamin molecules (23). A Hill coefficient of 2.3 for dynamin in the presence of microtubules or acidic phospholipids was determined, indicating that at least two dynamin molecules (with each molecule at least a dimer) must associate for maximal GTPase activity. Based on these data, the Michaelis constant for dynamin interactions was determined to be 37 nM. We have estimated the dynamin concentration in rat brain to be ~2.5 μM (data not shown). This value is in agreement with previous calculations that dynamin comprises 1.5% of total extractable protein in brain (32). This concentration is significantly higher than the apparent Kₘ, suggesting that dynamin interactions are likely in a physiological context. The dynamin content of non-neuronal tissues is significantly less than that of...
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at their site of action could lead to cooperative behavior even

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