GTP hydrolysis by dynamin is required to drive coated vesicle budding at the plasma membrane. A diverse set of molecules including microtubules, grb2, and acidic phospholipids stimulate dynamin GTPase activity in vitro, although the physiological relevance of these effectors remains to be determined. Dynamin has been shown to assemble around microtubules, the most potent stimulatory molecule, into structures indistinguishable by electron microscopy from collars captured in vivo at the necks of endocytic coated pits. Under low ionic strength conditions purified dynamin self-assembles into rings and helical stacks of rings. Here we show that dynamin self-assembly stimulates its GTPase activity as much as 10-fold. Thus, we identify dynamin, itself, as the first effector of dynamin GTPase activity known to be physiologically relevant. Assembled dynamin's stimulated GTPase activity is not dependent on the direct interaction of high affinity GTP binding sites since a mutant defective in GTP binding and hydrolysis can coassemble with and stimulate GTP hydrolysis by wild-type dynamin. Finally, we find that GTP destabilizes assembled dynamin structures, suggesting that the activated rates of GTP hydrolysis reflect a continuing cycle of assembly, GTP hydrolysis, and disassembly.

Dynamins, a 100-kDa GTPase, is specifically required for endocytic clathrin coated vesicle formation (1, 2). Mutations in shibire, the Drosophila homologue of dynamin, cause a pleiotropic defect in endocytosis (3, 4). Similarly, transient overexpression of GTPase-defective dynamin mutants blocks receptor-mediated endocytosis in mammalian cells (5, 6). More detailed phenotypic analysis of stable transfectants overexpressing the K44A dynamin mutant defective in GTP binding and hydrolysis revealed that clathrin-coated vesicle budding was blocked at a stage following coated pit assembly and invagination, but preceding the formation of constricted coated pits (7). Receptor-bound ligands that accumulate in these late intermediates in coated vesicle budding were sequestered from the necks of endocytic profiles accumulating at nerve terminals in the Drosophila shibire mutant (10). The suggestion that these collars might correspond to dynamin was confirmed by electron microscopy-immunolocalization of dynamin to electron dense helical bands that assembled on membrane invaginations in permeabilized synaptosome preparations incubated with GTPγS, a nonhydrolyzable analogue of GTP (11). These results, together with the immunolocalization of endogenous and overexpressed dynamin molecules specifically to clathrin coated pits (1, 7) and the GTP-requirements for coated vesicle formation in vitro (12), suggested an early working model (1, 9) for dynamin function. We have proposed that dynamin is targeted to and evenly distributed on clathrin lattices in its unoccupied or GDP-bound form. GTP-GDP exchange then triggers dynamin to redistribute from the clathrin-lattice and to assemble into collars at the necks of now constricted coated pits. Finally, GTP hydrolysis by dynamin is required, perhaps to trigger a concerted conformational change by the assembled dynamin, for vesicle detachment and also for recycling of dynamin for reutilization.

Thus both the self-assembly and GTPase properties of dynamins are integral to its function. Compared to other members of the GTPase superfamily, dynamin has a relatively high intrinsic rate of GTP hydrolysis (13–16). Dynamin's GTPase activity can be stimulated in vitro by a diverse group of effectors which include microtubules (13), glutathione S-transferase fusions of SH3 domain-containing proteins (17, 18), and acidic phospholipids (15). Each of these effectors binds to dynamin through its ~100-amino acid C-terminal proline-arginine-rich domain (PRD), and each is multivalent. The importance of multivalent interactions in stimulating dynamin GTPase activity was established using monoclonal antibodies directed against dynamin's PRD. In these experiments, the stimulatory activity of intact IgGs was enhanced by further cross-linking, while monovalent Fab fragments could not stimulate dynamin GTPase activity unless they were cross-linked (16). The finding that both microtubule- and phospholipid-stimulated dynamin GTPase activity shows strong cooperativity also suggested that dynamin-dynamin interactions were required for stimulated GTPase activity (19).

Here we show that dynamin self-assembly in vitro stimulates GTPase activity in the absence of any effector molecules. GTP binding and hydrolysis, in turn, destabilize the dynamin assemblies, suggesting a cycle of GTP hydrolysis, disassembly, and reassembly. The ability of dynamin to stimulate its own

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Dynamin’s endogenous GTPase activity is not dependent on a high affinity GTP binding site, since a mutant defective in GTP binding and hydrolysis can coassemble with and stimulate GTP hydrolysis by wild-type dynamin.

EXPERIMENTAL PROCEDURES

Materials—Protease inhibitor mixture tablets were from Boehringer Mannheim and calpain inhibitor I was obtained from Calbiochem. Serum-free medium, EX-CELL 401, was purchased from JRH Biosciences (Lenexa, KS). Fetal calf serum and Fungizone solution were from Irvine Scientific (Santa Ana, CA). The supplier of Macro-Prep High Q strong anion exchange support and Macro-Prep Ceramic Hydroxyapatite was Bio-Rad. [α-32P]GTP was from Amersham Corp. GTTP and GDP were acquired from Sigma and GTTP-s was from Boehringer Mannheim. Unless otherwise indicated, all other chemicals were reagent grade.

Expression of Recombinant Dynamin in Sf9 Cells and Purification of Dynamin Protein—Human neuronal dynamin (dynamin-1) and K44A dynamin-1 containing a lysine to alanine conversion at amino acid 44 (7) were expressed in recombinant baculovirus-infected Sf9 cells exactly as described previously (16). However, a new purification procedure was used. Cells were infected at 3–5 plaque-forming units/cell with high titer virus stocks and then harvested 65 h later by centrifugation at 500 × g for 10 min, washed once with phosphate-buffered saline and repelleted. The cell pellet obtained from a 0.5-liter culture was resuspended in 25 ml of Hepes column buffer (HCB, 20 mM Hepes, pH 7.2; 2 mM EGTA, 1 mM MgCl2, 1 mM DTT) containing 100 mM NaCl (referred to as HCB100), a protease inhibitor mixture tablet, and 1 mM PMSF. All subsequent steps were performed at 4 °C. Cells were homogenized by N2-cavitation at 500 p.s.i. for 25 min prior to slow release. The homogenate was diluted 2-fold in HCB0 (no NaCl) and then centrifuged at 50,000 rpm for 60 min in a Beckman Ti60 rotor. The supernatant was collected.

The supernatant was brought to 30% ammonium sulfate by slow addition of salt. After 10 min of slow stirring, the solution was centrifuged for 10 min in a Beckman JA-20 rotor at 10,000 × g. The pellet was gently resuspended using a loose fitting Dounce in 10 ml of HCB50 (50 mM NaCl) with protease inhibitors. The resuspended fraction was re-centrifuged for 10 min at 10,000 × g to remove aggregated protein. The solubilized 30% NH4SO4 cut was then applied to a High-Q strong anion exchange (Bio-Rad) column (10 × 1.5 cm) pre-equilibrated in HCB50. The column was washed with 50 ml of HCB50 and then with 50 ml of HCB100. Dynamin was finally step eluted in a volume of about 10 ml of HCB50. Protease inhibitor mixture, 40 μM calpain inhibitor 1, and 1 mM PMSF were added to the pooled High Q eluate which was then brought to 5 mM CaCl2. The dynamin fraction was loaded onto a 10-ml Macro-Prep Ceramic Hydroxyapatite column (Bio-Rad) pre-equilibrated with HCB250, 5 mM CaCl2. The column was washed with 30 ml of 200 mM KPO4, pH 7.2 and then eluted in 400 ml KPO4 (pH 7.2). K44A and wild-type dynamin-1 behaved identically during this procedure and yielded up to 15 mg of dynamin from 1 × 109 infected Sf9 cells. Purity was greater than 95% as judged by Coomassie Blue staining following SDS-PAGE. Aliquots were stored at −80 °C in 400 mM KPO4 containing 1 mM DTT, 40 μM calpain inhibitor 1, and 1 mM PMSF.

Dialysis of Dynamin and Assay of GTPase Activity—Dynamin was transferred to HCB150 or GTTPase assay buffer referred to as PH buffer (20 mM Pipes, 20 mM Hepes, 2 mM MgCl2, 1 mM EGTA, 1 mM DTT, pH 7.0), by overnight dialysis with two buffer changes. Spectra/Por 2 dialysis membrane tubing (Spectrum Medical Industries, Houston, TX) with a molecular weight cut-off of 12,000–14,000 or a framed dialysis membrane for a microdialyzer system 100 apparatus (Pierce) with a molecular weight cut-off of 8,000 were used with equivalent results.

GTTPase assays were performed in PH buffer with 1 mM DTT and 0.1% bovine serum albumin in a final volume of 20 μl, essentially as described elsewhere (7, 16). The final ionic strength of the GTTPase assay buffer was always adjusted to that of PH buffer and control experiments confirmed that addition of small amounts of HCB150 to GTTPase assays, while maintaining final ionic strength, had no effect on GTTPase activity. Reactions were initiated by the addition of GTTP (0.1 μCi of [α-32P]GTTP (Amersham). 1.5-μl aliquots were removed at each time point and spotted onto cellulose polyethyleneimine thin layer chromatography plates with fluorescent indicator (J. T. Baker, Inc.). Nucleotides were resolved by TLC in 1 M LiCl2, 2 M formic acid (1:1). Quantitation of GTTP and GDP at each time point was performed on a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA). Rates of GTTP hydrolysis were calculated from a minimum of five time points and expressed as the percent of GDP per GTTP plus GDP.

Dynamin Assembly Assay and SDS-PAGE Analysis—Dynamin self-assembly into oligomeric structures was assayed as described previ-
RESULTS

We showed previously that purified dynamin self-assembles into sedimentable structures composed of rings and small stacks of rings upon dilution into low ionic strength buffers (9). Interestingly, the GTPase activity of dynamin is routinely measured under low ionic strength conditions (13–15). We therefore used a sedimentation assay to determine whether dynamin self-assembly occurs under GTPase assay conditions. Thus, dynamin was diluted into GTPase assay buffer (referred to here as PH buffer, see “Experimental Procedures”) and subjected to sedimentation analysis as described previously (9). The SDS-polyacrylamide gel in Fig. 1 (lanes a) shows that >95% of dynamin is found in the supernatant in the starting buffer, HCB150 which contains 150 mM NaCl. However, upon 8-fold dilution into PH buffer, >75% becomes sedimentable (Fig. 1b). The structures assembled by dynamin in PH buffer were indistinguishable from those described previously (9) (data not shown). As reported previously (9) self-assembly at low ionic strength does not require either the presence of guanine nucleotides or dynamin’s high affinity GTP binding activity since the K44A mutant of dynamin defective in GTP binding and hydrolysis (7) shows comparable sediment activity (Fig. 1, c and d).

It had previously been shown that stimulated dynamin GTPase activity was highly cooperative (19). Given dynamin’s ability to self-assemble under GTPase assay conditions, we examined the concentration dependence of dynamin’s intrinsic GTPase activity. For this experiment stock solutions of dynamin were prepared in HCB150 and then diluted 8-fold into PH buffer at 4 °C so that the final concentration of NaCl in the assay remained constant. GTPase assays were transferred to 37 °C and initiated by addition of [α-32P]GTP. The kinetics of GTP hydrolysis were determined for each concentration of dynamin as described under “Experimental Procedures.” The rate of GTP hydrolysis increased in a nonlinear fashion with increasing dynamin concentration (Fig. 2A). A plot of the specific activity of dynamin’s intrinsic GTPase (Fig. 2B) gave a sigmoidal curve showing a sharp concentration dependence and reaching maximal specific activity at 2 μM dynamin. These data suggested that dynamin’s intrinsic GTPase activity could be stimulated by self-assembly.

While the trends shown in Fig. 2 were consistently obtained, we noted several experimental factors affecting dynamin’s GTPase activity. For example, when dialysis was performed in the absence of DTT, higher specific activities were obtained at all concentrations of dynamin (not shown). When dynamin was dialyzed into buffer containing 100 mM NaCl (as opposed to 150 mM) higher specific activities were obtained at higher concentrations of dynamin. Finally, preincubation of dynamin (for 30–90 min) after dilution into GTPase buffer resulted in higher specific activities at lower concentrations of dynamin. Preincubation of dynamin for 30–90 min after dilution into GTPase buffer resulted in higher specific activities at lower concentrations of dynamin. This result probably reflects the slower kinetics of self-assembly at lower concentrations of dynamin. It should be noted that the high variation of intrinsic GTPase rates for dynamin reported in the literature, from as low as <1 min⁻¹ (14, 18) to as high as 23 min⁻¹ (15), probably reflect differences in protein handling and assay conditions.

To further test the relationship between dynamin GTPase activity and self-assembly, dynamin was preassembled by dialysis into PH buffer (9) and then assayed for intrinsic GTPase activity upon dilution into PH buffer containing increasing concentrations of NaCl. Other samples were diluted in parallel and subjected to sedimentation analysis. As can be seen, dynamin’s intrinsic GTPase activity (Fig. 3A) decreased with increasing salt concentrations in parallel with the disassembly of sedimentable structures (Fig. 3B).

These results suggest that dynamin-dynamin interactions are important for stimulated GTPase activity. Does the stimulation of dynamin GTPase activity in assembled structures require that the neighboring dynamin molecules are themselves able to bind and hydrolyze GTP? To address this question, wild-type dynamin was coassembled with increasing amounts of a mutant dynamin (designated K44A) that is defective in GTP binding and hydrolysis by virtue of a point mutation in the first conserved GTP binding element (7). As seen in Fig. 1 (lanes 7 and 8), K44A dynamin was able to self-assemble into structures indistinguishable from wild-type dynamin (see also Hinshaw and Schmid (9)). However, as shown in Fig. 4, A and B (open circles), the K44A mutant on its own had little or no intrinsic GTPase activity, nor could its
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GT Prepare activity by stimulated mixtures of microtubules (not shown, but see Damke et al. (7)). Nonetheless, when K44A dynamin was allowed to coassemble with a fixed and small amount of wild-type dynamin during dialysis into PH buffer, these mixtures showed greatly stimulated GTPase activity dependent on the concentration of K44A dynamin (Fig. 4, closed triangles). In fact, the K44A mutant appeared to be even more effective than wild-type dynamin at stimulating specific GTPase activity. The maximum specific activity of wild-type dynamin alone under these assay conditions was 9.4 min⁻¹ (Fig. 2B), while when coassembled with K44A mutant, wild-type dynamin hydrolyzed GTP at a rate of 20 min⁻¹ (Fig. 4B).

In an effort to determine why wild-type dynamin showed greater GTPase activity when coassembled with the K44A mutant dynamin than with itself, we examined the effects of GTP on the stability of the assembled dynamin structures. The experiment in Fig. 5 shows that incubation of preassembled wild-type dynamin (lanes a) with either GTP (lanes b–d) or GTPγS (lanes e and f) destabilized the sedimentable structures, releasing dynamin into the supernatant. In fact, GTPγS was more potent than GTP. A 58% release of sedimentable dynamin occurred in the presence of 10 μM GTPγS (lanes e), while only a 42% release occurred following incubation in 250 μM GTP (lanes c). GDP, however, was considerably less effective at destabilizing assembled dynamin with only 32% released at 1 mM GDP (lanes i).

The observation that nonhydrolyzable GTP analogues were at least as effective as GTP in destabilizing dynamin assemblies allowed us to compare the relative stability of preassembled K44A dynamin to wild-type dynamin in the presence of GTPγS. In this way, the differential hydrolysis of GTP by wild-type and K44A dynamin would not affect the results. The data in Fig. 6 shows the effect of increasing concentrations of GTPγS on the stability of preassembled wild-type dynamin (open squares), K44A mutant dynamin (open circles) or a 4:1 (K44A: wild-type) mixture of the two (closed triangles) as assessed by sedimentation analysis. As expected, assembled K44A dynamin required 20-fold more GTPγS to trigger its disassembly than did wild-type dynamin, reflecting its greatly reduced affinity for GTP (7). Moreover, when wild-type dynamin was coassembled with the K44A mutant at the ratio that gave near-maximum stimulation of GTPase activity (see Fig. 4B), these coassembled structures were as resistant to GTPγS-induced disassembly as the K44A mutant itself.

**FIG. 5.** Guanine nucleotides destabilize self-assembled structures of dynamin. The stability of self-assembled dynamin structures (a) to increasing concentrations of GTP (b–d), GTPγS (e and f), and GDP (g–i) were determined. Dynamin was preassembled by dialysis overnight into PH buffer. Eightfold dilutions of dynamin were made into PH buffer containing the varying concentrations of nucleotide. The assays were kept at 4°C for 10 min before separation into soluble (S) and pelletable (P) fractions by ultracentrifugation and analysis as described in Fig. 1.

**DISCUSSION**

We have shown that dynamin’s intrinsic GTPase activity is significantly enhanced under conditions that favor self-assembly of dynamin into rings and helical stacks of rings. This directly confirms previous suggestions based on the cooperative behavior of dynamin’s GTPase activity when stimulated by acidic phospholipids and microtubules (19), that dynamin-dynamin interactions were key to regulating its GTPase activity. Moreover, we have shown that stimulation of dynamin GTPase does not depend on the high affinity binding or hydrolysis of GTP by neighboring dynamin molecules. While the nature of the dynamin-dynamin interactions required for stimulated GTPase activity remain to be determined, we have identified dynamin as the first effector of its GTPase activity known to be physiologically relevant. In this regard it is of interest to note that all of the suppressors of shibire so far identified through genetic analysis are intra-allelic (20), consistent with the suggestion the dynamin-dynamin interactions are critical to its in vivo function.

Interestingly, we also show that GTP binding destabilizes...
GTPase rate is independent of protein concentration. We assume that conformational changes triggered by GTP hand hydrolysis is required, perhaps to trigger a conformation change in dynamin, as it does for other members of the GTPase superfamily. The GTP-induced conformational change may be necessary in vivo to alter dynamin interactions with partners on the clathrin lattice releasing it for assembly. Assembled dynamin is destabilized by GTP or GDP/GTPγS in vitro, yet GDP/GTPγS stabilizes helical dynamin stacks triggering their exaggerated assembly on synaptosomal membrane fractions (11). These differences suggest that other factors may be required in vivo to regulate both dynamin assembly and disassembly and that these factors might be membrane associated. It will be essential to identify components that interact with dynamin in vivo in order to determine their role in regulating dynamin targeting, GTPase activity, and assembly. The assays developed here will be important in testing the function and determining the mechanism of action of both dynamin and its partners.

Fig. 6. Coassembly of wild-type dynamin with K44A dynamin stabilizes sedimentable structures to disassembly by guanine nucleotides. Wild-type (●) and K44A (▲) dynamin and a 1:4 mix of wild type:K44A (▲) were preassembled by dialysis into PH buffer overnight. Eightfold dilutions of the dynamins were made into PH buffer containing varying concentrations of GDP/GTPγS. The assays were performed exactly as described in Fig. 5.

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