The GTPase dynamin is essential for clathrin-mediated endocytosis. Unlike most GTPases, dynamin has a low affinity for nucleotide, a high rate of GTP hydrolysis, and can self-assemble, forming higher order structures such as rings and spirals that exhibit up to 100-fold stimulated GTPase activity. The role(s) of GTP binding and/or hydrolysis in endocytosis remain unclear because mutations in the GTPase domain so far studied impair both. We generated a new series of GTPase domain mutants to probe the mechanism of GTP hydrolysis and to further test the role of GTP binding and/or hydrolysis in endocytosis. Each of the mutations had parallel effects on assembly-stimulated and basal GTPase activities. In contrast to previous reports, we find that mutation of Thr-65 to Ala (or Asp or His) dramatically lowered both the rate of assembly-stimulated GTP hydrolysis and the affinity for GTP. The assembly-stimulated rate of hydrolysis was lowered by the mutation of Ser-61 to Asp and increased by the mutation of Thr-141 to Ala without significantly altering the $K_m$ for GTP. For some mutants and to a lesser extent for WT dynamin, self-assembly dramatically altered the $K_m$ for GTP, suggesting that conformational changes in the active site accompany self-assembly. Analysis of transferrin endocytosis rates in cells overexpressing mutant dynamins revealed a stronger correlation with both the basal and assembly-stimulated rates of GTP hydrolysis than with the calculated ratio of dynamin-GTP/free dynamin, suggesting that GTP binding is not sufficient, and GTP hydrolysis is required for clathrin-mediated endocytosis in vivo.

Dynamin is a large GTPase that is essential for clathrin-mediated endocytosis and synaptic vesicle recycling (1). Dynamin has biochemical properties that distinguish it from typical GTPases (2). Specifically, it has a significantly lower affinity for nucleotide and a higher basal rate of GTP hydrolysis. Most notably, the rate of GTP dissociation from dynamin is $10^4$ times faster than that from, for example, Ras (3, 4). Another distinguishing feature of dynamin is its ability to self-assemble to form higher order structures such as rings and spirals (5). Self-assembly can stimulate dynamin GTPase activity as much as 100-fold (6). Dynamin spirals similar in dimensions to those formed in vitro can be detected as electron dense collars around the elongated necks of endocytic interme-

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The abbreviations used are: GED, GTPase effector domain; GTP$_7$S, guanosine 5’-3-O-(thio)triphosphate; WT, wild type; sw, switch.
ocytosis (14). However, most of the mutants so far studied (K44A, S45N, T65P) are also defective in GTP binding (14–16). One mutation, T65A, was suggested to significantly inhibit GTP hydrolysis without impairing GTP binding (7). However, this assertion is inconsistent with the effects of this highly conserved, switch 1, Thr on GTP binding affinities in other GTPase family members (11) and its role in coordinating Mg\(^{2+}\) for direct interaction with the γ-phosphate of bound GTP. Therefore, it remains uncertain as to whether endocytosis in vivo requires GTP binding and hydrolysis or only GTP binding.

To probe the mechanism of GTP hydrolysis by dynamin and to identify the residues that are critical for nucleotide binding and GTP hydrolysis, we have made a series of new mutations in the switch 1 and 2 regions of the dynamin GTPase domain. Analysis of the kinetic constants for the steady-state GTPase cycle revealed new mutants that differentially affect GTP binding and hydrolysis. The effects of overexpression of these mutants on endocytosis were examined so as to determine whether the rate of endocytosis correlated more closely with the ratio of dynamin-GTP/unoccupied dynamin or with the rate of GTP hydrolysis or both.

EXPERIMENTAL PROCEDURES

Recombinant Baculoviruses and Adenoviruses—Point mutations were generated in human dynamin 1 in a pBluescript vector by the QuiChange method (Stratagene) using the oligonucleotides (Operon) listed in Table I. The BamHI-KpnI fragment containing the entire gene was transferred to pVL1393 vector. The resulting plasmid together with linearized baculovirus DNA (BaculoGold, Pharmingen) was used to generate recombinant baculoviruses by following the manufacturer’s instructions. To generate recombinant adenoviruses, the NdeI-Nhel fragment from pBluescript was subcloned into pADDeT3T7 containing the gene for human dynamin 1 wild type, and the resulting plasmid together with V5 adenovirus DNA was then transfected into HEK293-cre4 cells as described (17).

Proteins, GTPase, and Velocity Sedimentation Assays—Protein purification, dynamin GTPase, and velocity sedimentation assays were performed as described (9). All incubations were at 37 °C unless otherwise noted. Two different dynamin concentrations were used, 1.0 μM for basal and 0.1 μM for lipid tube stimulation GTPase activity. Initial velocities for GTP hydrolysis were measured at varying GTP concentrations and plotted as a function of GTP concentration to obtain the values for $k_{cat}$ and $K_m$ based on the Michaelis-Menten equation. The values from repeated measurements ($n = 3–5$) were summarized in Table II.

Filter Assay for GTP Binding—Dynamin (1.0 μM) was incubated with 100 μM GTP-γ-S (270 μCi/ml, Amersham Biosciences) in the GTPase assay buffer for 20 min at 37 °C or at room temperature in 6-well arrays of PCR tubes. Using a multichannel pipette, samples were applied to nitrocellulose (0.45 μm, BA85, Schleicher and Schuell) in a filter dot-bolt apparatus (Schleicher and Schuell, minifold I) under vacuum. The filter was rapidly washed once with 250 μl of cold buffer. The procedure was repeated, six samples at a time. Each sample was assayed five times. Rapid filtration and washing was necessary for reproducible results given the very high rate of dissociation of GTP from dynamin (3). The dried filter was imaged using an Amersham Biosciences Phosphorimager and quantified using ImageQuant Software. Values for individual mutant dynamins were normalized to wild type dynamin.

Transferin Internalization and Its Correlation with Dynamin GTPase Activity—The kinetics of internalization of biotinylated transferrin into an avidin-inaccessible compartment was determined in tTA-HeLa cells expressing dynamin mutants (n = 6, Fig. 2), exactly as described (18). To determine correlation coefficients, the extent of transferrin internalization at 10 min was plotted as a function of the ratio of dynamin-GTP/dynamin or the relative rate of GTP hydrolysis by dynamin, calculated as indicated below from Michaelis-Menten steady-state kinetics (Equation 1) using 100 μM as the intracellular concentration of GTP (19).

$\text{Rate of GTP hydrolysis} = \frac{k_{cat}}{K_m} \cdot [\text{GTP}]$ (Eq. 5)

RESULTS AND DISCUSSION

We sought to identify critical residues in the dynamin GTPase domain that would exhibit differential effects on GTP binding and/or hydrolysis so as to test for differential requirements for these activities in endocytosis. For other GTPases, the switch (sw) 1 and 2 regions of the GTPase domain undergo major conformational changes upon GTP binding and are proposed to function in sensing the γ-phosphate of bound GTP (Fig. 1). Thus, we chose to mutate three amino acid residues in sw1 and 2 (Ser-61, Thr-65, Thr-141) to amino acids with different biochemical properties that could distinguish their roles in GTP binding and/or catalysis. Ser-61 was mutated to Ala, which would disrupt GTP hydrolysis, and to Asp, which ion required

\begin{table}[h]
\caption{GTPase activity of the dynamin-GTP/dynamin or the relative rate of GTP hydrolysis by dynamin, calculated as indicated below from Michaelis-Menten steady-state kinetics (Equation 1) using 100 μM as the intracellular concentration of GTP (19).}
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Dyn} & \textbf{Dyn-GTP} & \textbf{Dyn-GDP} & \textbf{P_i} \\
\hline
\textbf{K_m} & \textbf{k_{cat}} & \textbf{k_{cat}} & \textbf{k_{cat}} \\
\hline
\textbf{WT} & 102 ± 35 & 2.60 ± 0.98 & 37 ± 18 & 105 ± 47 \\
\textbf{S61A} & 67 ± 10 & 1.94 ± 0.24 & 41 ± 22 & 86 ± 13 \\
\textbf{S61D} & 68 ± 11 & 0.43 ± 0.02 & 56 ± 22 & 26.3 ± 1.5 \\
\textbf{T65A} & 880 ± 330 & 0.075 ± 0.027 & 2115 ± 1413 & 1.22 ± 0.24 \\
\textbf{T65D} & 41 ± 15 & 0.016 ± 0.002 & 513 ± 38 & 0.97 ± 0.20 \\
\textbf{T65H} & 149 ± 21 & 0.018 ± 0.002 & 988 ± 270 & 0.95 ± 0.06 \\
\textbf{T141A} & 174 ± 96 & 0.89 ± 0.79 & 56 ± 20 & 185 ± 55 \\
\textbf{T141D} & 763 ± 120 & 0.15 ± 0.03 & 1940 ± 1200 & 1.50 ± 0.98 \\
\hline
\end{tabular}
\end{table}

where $K_m = (k_{cat} + k_{cat})k_{cat}$.
for nucleotide binding. On the other hand, mutation to Asp or His might increase nucleotide affinity if their greater ability to pull protons would correlate with their ability to coordinate Mg\(^{2+}\) ion. Finally, GTP hydrolysis requires the positioning and/or activation of a nucleophilic water molecule at the backside of the \(\gamma\)-phosphate of the bound GTP. In Ras-like GTPases, this is accomplished by a highly conserved glutamine residue within G3. This glutamine residue is not conserved in dynamin family members and corresponds to Met-140 in dynamin-1. Thus, to test the hypothesis that the neighboring Thr-141 in sw2 could instead play a role in positioning and/or activating the water molecule, it was mutated to Ala and Asp with the expectation that these mutations might reduce or increase the rate of GTP hydrolysis, respectively.

Each of these mutations was generated in human dynamin-1, and the proteins were expressed and purified from insect cells infected with recombinant baculoviruses. To assess the role of each residue in GTP binding and hydrolysis, kinetic analyses were performed to measure both basal and assembly-stimulated GTPase activity of the individual mutant dynamins. Basal GTPase activity was measured at low concentrations of dynamin and at physiological salt concentrations to prevent dynamin self-assembly into higher order structures. Assembly-stimulated GTPase activity was determined using phosphatidylinositol 4,5-diphosphate-containing lipid nanotubes as a template for dynamin self-assembly. Under these conditions, the basal GTPase activity of wild type dynamin was stimulated 40–50-fold when measured in the presence of lipid nanotubes, consistent with previous reports (6, 9).

**Fig. 1.** Dynamin domain structure and sequences of four conserved GTP binding elements in the dynamin GTPase domain. Sw1 and Sw2 regions associated with the \(\gamma\)-phosphate-sensing elements G2 and G3 are indicated. Point mutations were made in Sw1 and Sw2 residues at the indicated positions (arrow).}

As previously reported (7, 16), we found that Thr-65 mutations (T65A, T65D, T65H) greatly reduced both basal and assembly-stimulated GTPase activity (36–100-fold), demonstrating that Thr-65 has an important role in catalysis (Table II). However, these mutations exhibited differential effects on the \(K_m\) for GTP. Contrary to previous observations (7), but as predicted for other GTPases (11), the T65A mutation lowered the apparent affinity for GTP by 8-fold for basal and by \(\sim\)50-fold for assembly-stimulated GTPase activity (Table II). Mutation of Thr-65 to a carboxylate residue, Asp, slightly increased the apparent GTP affinity of unassembled dynamin (2-fold) but significantly lowered that of assembled dynamin (14-fold). Similarly, although the T65H mutation showed a minimal effect on the \(K_m\) for GTP of unassembled dynamin (less than 2-fold), the \(K_m\) for assembled dynamin increased by 27-fold. The dramatic changes in the \(K_m\) for GTP observed in the presence of lipid tubules suggest that the GTP binding pocket can undergo significant conformational changes upon dynamin self-assembly. Consistent with this, the \(K_m\) of wild type dynamin-1 for GTP decreases \(>\)2-fold upon self-assembly.

Together, these data suggest that residues in switch 1 are involved in both GTP binding and hydrolysis and that they undergo assembly-dependent conformational changes. Interestingly, Ser-61 and Thr-65 are both located within the sequence \(57\text{LPRGSGIVTR}\)66, which is highly conserved across the entire dynamin family and corresponds to a region previously identified in Mx1 as a self-assembly motif (20). Although the effect of self-assembly on GTP binding may in part be mediated through conformational changes in sw1, these sw1 mutations appear not to be defective in self-assembly or in assembly-stimulated GTP hydrolysis per se. Indeed, the degree of lipid tubule-dependent GTPase stimulation for most of these mutants, including S61A, S61D, T65D, T65H, was similar to or better than that observed for wild type dynamin (44–61-fold compared with 40-fold). Moreover, velocity sedimentation demonstrated that these mutations are competent to self-assembly on lipid nanotubes (Fig. 2). The T65A mutation also showed significant (\(\sim\)16-fold) assembly-dependent stimulation of GTPase activity and was unimpaired in its ability to self-assemble (Fig. 2) as reported by others (7).

**A Mutation in Switch 2 Increases Dynamin Basal GTPase Activity**—We next examined the effects of mutation of the switch 2 residue, Thr-141. Contrary to our hypothesis that Thr-141 could be involved in positioning and/or activating the nucleophile water molecule, its change to a small hydrophobic residue (Ala) increased the \(k_{cat}\) by 2-fold for both basal and assembled GTPase activity with only small affects on \(K_m\). Also unexpectedly, mutation to Asp, a potentially stronger activator of water, inhibited GTP binding and hydrolysis. Together, these results suggest that a hydrophobic environment is preferred in this region of the nucleotide binding pocket at the transition state and indicate that Thr-141 has roles in both GTP binding and hydrolysis, although the exact mechanism remains to be established. Although velocity sedimentation analysis established the ability of these mutants to self-assem-
Effect of Dynamin GTPase Domain Mutants on Endocytosis

Fig. 3. **Filtration assay for GTPγS binding to wild type and mutant dynamins.** Duplicate samples of wild type and mutant dynamins (1.0 μM) as indicated were incubated with 100 μM GTPγS for 20 min at room temperature and then subjected to rapid filtration and washing under vacuum, as described under "Experimental Procedures." Filter-bound dynamin-GTPγS was quantified by PhosphorImager analysis, and the resulting values were normalized to the amount bound to WT dynamin. The data shown are the averages ± S.E. for five such independent experiments. Indistinguishable results were obtained for incubations at 37 °C.

Table III

| Temperature | WT dynamin | T65A dynamin |
|-------------|------------|--------------|
| °C | K_m (μM) | k_cat (min⁻¹) | K_m (μM) | k_cat (min⁻¹) |
| ___ | ___ | ___ | ___ | ___ |
| 22 | 3.4 ± 1.7 | 0.19 ± 0.03 | 97 ± 28 | 0.017 ± 0.002 |
| 37 | 102 ± 35 | 2.60 ± 0.98 | 880 ± 330 | 0.075 ± 0.027 |
| 47 | 141 ± 17 | 7.6 ± 0.2 | 44 ± 8.2 | 0.062 ± 0.004 |

Lipid tubule.

**Temperature-dependent Effects on GTP Binding and Hydrolysis**—Contrary to previous reports (7), our data establish that in addition to its reported effects on hydrolysis, mutation of Thr-65 to Ala substantially decreases GTP binding based on both K_m values and direct measurements. One possible explanation for this discrepancy is that we measure GTPase activity at the physiologic temperature of 37 °C, whereas the previous findings were based on measurements performed at room temperature (7). Indeed, we find that both kinetic parameters for dynamin GTPase activity, K_m and k_cat, are significantly affected by incubation at lower temperatures (Table III). Thus, both the basal and assembly-stimulated rates of GTP hydrolysis for wild type dynamin are ~10-fold lower when assayed at 22 °C as compared with 37 °C, which is significantly greater than observed for many enzymes (22). Similarly, when measured at 22 °C, the K_m for GTP is ~30-fold reduced for wild type dynamin measured under basal GTPase assay conditions and ~6-fold reduced when measured in the presence of lipid tubules. The T65A mutant shows a pronounced defect in GTP hydrolysis at both temperatures. Strikingly, there is an ~9-fold differential in K_m for GTP measured under basal conditions at the two temperatures and an ~50-fold differential when measured in the presence of lipid tubules (Table III). Indeed, when measured at 22 °C, our findings correspond to those reported by Marks et al. (7). The relatively strong temperature dependence of these parameters of dynamin GTPase activity suggests that upon GTP binding and hydrolysis significant conformational changes may occur in the non-reacting parts of dynamin (23) in addition to the active site and explains previous discrepancies in reported k_cat and K_m values for both wild type and T65A mutant dynamins (6, 7, 12).

A **Search for Catalytic Residues in the GTPase Domain**—Dynamin has a relatively high intrinsic rate of GTP hydrolysis, at least when measured at physiologically relevant temperatures, suggesting that residues essential for catalysis are intrinsic to the GTPase domain in the unassembled state. Thus, we propose that dynamin assembly-stimulated GTPase activity, mediated by the GTPase activating protein activity of GED, functions to better position intrinsic catalytic residues for more efficient hydrolysis of GTP. This situation is more akin to that of Ga subunits, whose intrinsic rate of GTP hydrolysis (1–2 min⁻¹) is stimulated ~50–100-fold by RGS-type GTPase activating proteins, which alter the conformation of active site residues in the switch regions of the Ga GTPase domain for more effective catalysis (11, 24). Among these is a highly conserved arginine residue in Ga subunits that is located in sw1 near the invariant G2 Thr, which functions to neutralize negative charges that develop in the transition state during catalysis (24). Although we have established that mutation of the sw1 and sw2 residues, Ser-61 and Thr-141 alter catalysis, neither is essential for GTP hydrolysis. Similarly, although mutation of Thr-65 severely impairs GTP hydrolysis, it also severely compromises GTP binding, and thus, a direct role in catalysis cannot be determined. In an attempt to identify essential catalytic residues within the dynamin GTPase domain that are specifically required for GTP hydrolysis, we mutated

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**Footnote:** M. Leonard, unpublished data.
three conserved Arg residues within the sw1 region, Arg-54, Arg-59, and Arg-67; however, neither of these mutations significantly perturbed basal GTPase activity. Others have established that the R66A mutation equally impairs both GTP binding and hydrolysis (7). Thus, the catalytic Arg within the dynamin GTPase domain, if it exists, remains elusive.

The Dependence of Endocytosis on GTP Binding or Hydrolysis—We have generated a new series of mutant dynamins that exhibit differential and graded effects on $K_m$ and $k_{cat}$ for both basal and assembly-stimulated GTPase activities (Fig. 4A). With these mutants in hand we sought to determine whether endocytosis rates would correlate more directly with the ratio of GTP bound:unoccupied dynamin, the rate of GTP hydrolysis, or both. To this end, recombinant adenoviruses expressing wild type and mutant dynamins unilaterally infected tTA-treated HeLa cells overexpressing mutant dynamins (Fig. 4, B and C). Consistent with previous results (7, 16, 25), overexpression of either dyn1-T65F (open squares) or dyn1-T65D (black inverted triangles), which were severely defective in both GTP binding and hydrolysis, potently inhibited transferrin internalization. Also as expected, dyn1-S61A (black circles), which exhibited near wild type GTP binding and hydrolysis activities, had no effect on endocytosis as compared with dyn1-WT (open circles). In contrast, endocytosis was significantly inhibited by overexpression of dyn1-S61D (diamonds), which was defective in GTP hydrolysis, but exhibited normal GTP binding. Finally, endocytosis was, if anything, slightly stimulated by overexpression of dyn1-T141A (black triangles).

For quantitative analysis, the extent of endocytosis in vivo at 10 min was plotted as a function of either the calculated ratio of dynamin-GTP/dynamin (Fig. 5, A and C) or as a function of their relative rates of GTP hydrolysis (Fig. 5, B and D), as determined in vitro based on Michaelis-Menten steady-state kinetics (see “Experimental Procedures”). We observed no apparent correlation between the rates of endocytosis and the degree of dynamin-GTP loading, calculated based on the basal GTPase properties of dynamin (Fig. 5A) and only a weak correlation when calculated based on assembly-stimulated GTPase activity (Fig. 5C). In particular, dyn1-S61D (Fig. 5, black diamonds), which exhibited near wild type GTP binding as determined either by $K_m$ measurements or direct filter binding assays, was a significant dominant-negative inhibitor of endocytosis. The dyn1-T65D mutant is also a significant outlier in this analysis, at least when based on basal GTPase properties (Fig. 5A); however, the disparity in apparent affinity for GTP as assessed by $K_m$ values compared with direct GTP binding assays renders results from this mutant difficult to interpret. Nonetheless, data represented in Fig. 5, A and C, argue that GTP binding is not sufficient for dynamin function in clathrin-mediated endocytosis but suggest that conformational changes induced by both GTP binding and self-assembly may be important for dynamin function in vivo.

In contrast, we observed a strong correlation between the rate of endocytosis and the calculated rates for both basal and assembly-stimulated GTP hydrolysis (Fig. 5, B and D). Transferrin internalization was reduced in cells overexpressing mutant dynamins with lower rates of GTP hydrolysis. We (16) and others (7, 25) report that GTPase-defective T65F and T65A mutants strongly inhibited transferrin internalization; however, because these mutations also severely impair GTP binding, the reasons for their inhibitory effects on endocytosis are

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3 S. P. Sholly, M. Leonard, and S. Schmid, unpublished data.
Effect of Dynamin GTPase Domain Mutants on Endocytosis

difficult to interpret. Our observation that overexpression of dyn1-S61D (black diamonds), which has a 4–5-fold lower rate of GTP hydrolysis with little change in affinity for GTP, reduces the rate of transferrin internalization by ~60% and is, by contrast, more informative.

We have previously shown that overexpression of dynamin mutants specifically defective in self-assembly and thereby assembly-stimulated GTPase activity can accelerate a rate-limiting step in clathrin-mediated endocytosis (12, 26). These mutations, located in GED, did not significantly affect the basal rate of GTP hydrolysis (12). In contrast, each of the mutations studied here have equal effects on both basal and assembly-stimulated rates of GTPase activity. Thus, the tight correlation we see here between basal GTPase activity and endocytosis rates may reflect a specific role for dynamin basal rate of GTP hydrolysis in clathrin-mediated endocytosis. Recent studies at the synapse (27) confirm our earlier observations in nonneuronal cells (14) that unassembled dynamin is present on coated pits from their earliest stages of assembly and maturation. Dynamin interacts with multiple SH3 domain-containing effector molecules, and these interactions are likely to occur from the outset of dynamin association with the emerging coated pit. Thus, basal GTPase activity may play a role in regulating dynamin interactions with itself and with partner proteins during earlier stages of coated pit formation and maturation. Further studies will be needed to assess whether these effector interactions are regulated by and/or regulate the dynamin cycle of GTP binding and hydrolysis.

There are also several not mutually exclusive possibilities for the role of dynamin assembly-stimulated GTPase activity in clathrin-mediated endocytosis. Assembly-stimulated GTP hydrolysis may be directly involved in driving conformational changes in assembled dynamin that mediate membrane fission (5, 6). Alternatively, GTP hydrolysis by assembled dynamin may function to dismantle the fission machinery for recycling in vivo. Indeed, GTP hydrolysis in vitro has been shown to trigger the rapid disassembly of dynamin (21, 28), and this interpretation would be consistent with recent observations that GTP hydrolysis by dynamin may not be required for a single round of vesicle formation in vitro (29). There is precedence for this model in that GTP hydrolysis is required for the in vivo function and recycling of other GTPases such as Sar, Arf, SRP54, and SRP receptor, although GTPase defective mutants of each of these proteins are able to support single rounds of activity in vitro (30–34). Analysis of the effects of acute inhibition of dynamin GTPase activity in vivo, as opposed to long term overexpression experiments, will be needed to resolve these issues. In addition, the generation and analysis of new classes of mutants that selectively impair either assembly-stimulated or basal GTPase activities would be informative.

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