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Microtubules and Src homology 3 domains stimulate the dynamin GTPase via its C-terminal domain

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ABSTRACT **Dynamin is a 100-kDa GTPase that plays a critical role in the initial stages of endocytosis. Dynamin binds to microtubules, which potently stimulate its GTPase activity. Binding to Src homology 3 (SH3) domains of proteins involved in signal transduction has also recently been reported. In the present study, the protein was digested with a variety of proteases to define its functional domains. Limited digestion with papain split the protein into an ≈7- to 9-kDa microtubule-binding fragment and a 90-kDa nonbinding fragment. Immunoblotting with an antibody to the C-terminal 20 amino acids of rat dynamin showed the small fragment to derive from the C-terminal end of the polypeptide. Microtubule-activated GTPase activity, but not basal GTPase activity, was abolished by papain digestion, identifying the basic, proline-rich C-terminal region of dynamin as an important regulatory site. Bacterially expressed growth factor receptor-bound protein 2 (GRB2) and the SH3 domain of c-Src were also found to stimulate GTPase activity, although to a lesser extent than microtubules. Stimulation of GTPase activity by the recombinant proteins was similarly abolished by papain digestion. These results identify the basic, proline-rich C-terminal region of dynamin as the binding site for both microtubules and SH3 domains and demonstrate an allosteric interaction between this region of the molecule and the N-terminal GTPase domain.**

Dynamin was initially identified as a 100-kDa nucleotide-sensitive microtubule-binding protein (1). In the absence of GTP, dynamin bundled microtubules, forming highly cooperative helical arrays along the microtubule surface (1). The deduced primary sequence of the rat brain protein (2) revealed an N-terminal 300-amino acid domain that contained the three highly conserved consensus-sequence elements characteristic of GTPases. This region of the molecule showed striking sequence similarity to the N-terminal regions of the interferon-inducible antiviral Mx proteins (3) and the product of the yeast vacuolar protein-sorting gene *VPS1* involved in membrane protein sorting (4) and meiosis (5). The dynamin sequence was found to diverge from VPS1 and Mx beyond the GTPase domain and, in addition, to contain a non-conserved 100-amino acid basic, proline-rich extension.

The *Drosophila* gene shibire has been identified as a potential homologue of dynamin, exhibiting 68% amino acid sequence identity with the rat protein and containing a C-terminal extension of comparable length and composition (6, 7). Temperature-sensitive alleles of shibire have a paralytic phenotype (8) that has been attributed to a defect in the reformation of synaptic vesicles at the neuromuscular junction (9, 10) and, more generally, in the budding of coated and noncoated vesicles from the plasma membrane (11, 12). Transfection of mammalian cells with mutant forms of rat dynamin has recently been found to block receptor-mediated endocytosis (13, 14), supporting a similar physiological function for the rat and *Drosophila* proteins.

The role of microtubules in dynamin function is uncertain. Microtubules are not known to be directly involved in the initial stages of endocytosis in which dynamin and shibire have been implicated, nor was evidence for colocalization of dynamin with microtubules observed in transfected (13) or nontransfected (13, 15) cells. Nonetheless, microtubules specifically and potently activate the dynamin GTPase (16). Recently, bacterially expressed SH3 domains and SH3 containing polypeptides were found to bind dynamin in *in vitro* assays (ref. 17; J. Schlessinger, personal communication; K. Seedorf and A. Ullrich, personal communication). This finding is appealing in suggesting a potential link between signal transduction and endocytic pathways. However, the identity of the true physiological effectors for dynamin remains to be fully resolved.

The present study was initiated to gain further insight into the functional organization of the dynamin molecule and to determine whether SH3 domains interact with the same region within the molecule as microtubules. We report here that proteolytic digestion with papain results in a very simple fragmentation pattern, which identifies the C-terminal portion of dynamin as a major interaction site for both microtubules and SH3 domains and is responsible for regulating dynamin GTPase activity.

MATERIALS AND METHODS

Dynamin Purification and Digestion. Dynamin was purified from calf brain as described (18), except 0.2% Triton X-100 (Pierce) was added to the purified protein to prevent aggregation. Papain digestion was done at 21°C with papain, 1:40 (wt/wt) (Boehringer Mannheim) in PG buffer (5 mM sodium phosphate, pH 7.0/50 mM sodium glutamate/1 mM EGTA/1 mM $MgSO_4$/1 mM dithiothreitol), and the digestion was stopped by adding antipain (Boehringer Mannheim) to a final concentration of 50 μg/ml.

Microtubule Binding and GTPase Assays. Calf brain tubulin was prepared by the reversible assembly method followed by DEAE-Sephadex chromatography (19). Microtubules were assembled by using taxol and resuspended in PG buffer (18). For microtubule-rebinding assays, microtubules were added to the papain digests to a final concentration of 0.125 mg/ml, incubated at room temperature for 5 min, and then centrifuged at 30,000 × *g* for 30 min at 37°C. GTP hydrolysis was measured in PG or PEM/G buffer (5 mM Pipes, pH 7.0/50 mM sodium glutamate/1 mM EGTA/1 mM $MgSO_4$/1 mM dithiothreitol) containing 1 mM GTP, using a malachite green colorimetric (20) or [γ-^{32}P]GTP isotopic assay (16). Glu-

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Abbreviations: SH3, Src homology 3; GRB2, growth factor receptor-bound protein 2.

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tathione S-transferase fusion proteins containing GRB2, GRB2 SH3 (N terminal), GRB2 SH3 (C terminal), and c-Src SH3 were gifts of Michael Waterfield (Ludwig Institute for Cancer Research) (17).

Other Methods. Protein was quantitated by using the Bradford method (Bio-Rad). Electrophoresis was done with 7% polyacrylamide gels (21) or 10% Tris/tricine polyacrylamide gels to resolve low-molecular-mass fragments (22). Antibody against the dynamin C terminus was raised by immunization with a synthetic peptide corresponding to the 20 C-terminal amino acids of the rat brain sequence (VPSRPNRAPPGVPRITISDP) (13). Immunoblotting was done as described (23) at a 1:500 dilution of an IgG fraction of the antipeptide serum, followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody, as recommended by the manufacturer (Promega).

RESULTS

Papain Digestion of Dynamin. To define the site of interaction of dynamin with microtubules and obtain insight into the structural organization of the dynamin molecule, we exposed it to a number of proteases. Trypsin, chymotrypsin, subtilisin, thermolysin, V8 protease, and papain produced prominent fragments at 90, 60, and 40 kDa, whereas elastase was relatively ineffective in cleaving the protein. Papain digestion resulted in almost complete conversion of the 100-kDa dynamin polypeptide to a 90-kDa fragment (Fig. 1*a*). In some experiments a trace level of an ≈95-kDa fragment could be detected at early digestion times. Neither dynamin nor the 90-kDa papain fragment could be sequenced by Edman degradation, suggesting that the fragment might derive from the N terminus of the protein.

To resolve this issue, we raised a polyclonal antibody to a synthetic 20-amino acid peptide corresponding to the C terminus of rat brain dynamin and used it to immunoblot the papain digest (Fig. 1*b*). The immunoreactivity of the 100-kDa dynamin electrophoretic species decreased with digestion time. No corresponding immunoreactivity appeared at 90 kDa or 95 kDa. Instead, a prominent 7- to 9-kDa immunoreactive fragment was observed, which reached a maximum level when conversion from the 100-kDa to the 90-kDa species was complete. A minor 10-kDa immunoreactive fragment could sometimes be detected at early time points, and a minor 4-kDa immunoreactive fragment was seen with more prolonged digestion. Together, the data are most readily explained by the existence of a primary papain cleavage site at 7–9 kDa from the C terminus of dynamin. Secondary cleavage sites may also exist to account for the minor high- and low-molecular-mass fragments.

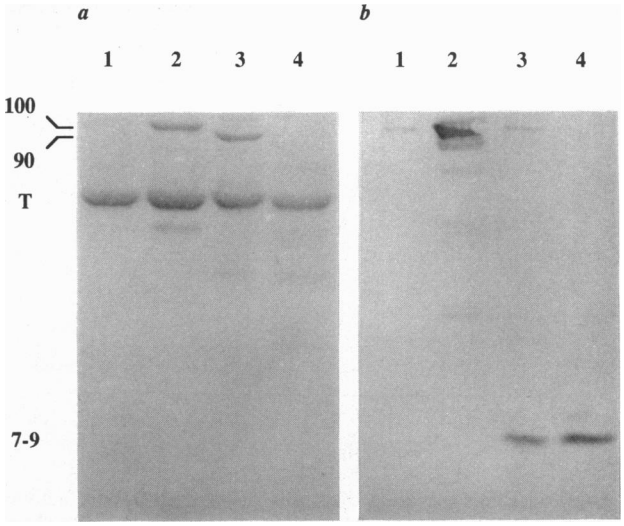

FIG. 2. Microtubule-binding by dynamin fragments. Dynamin was exposed to papain for a series of times, and the resulting fragments were tested for cosedimentation with microtubules. Samples were subjected to SDS/PAGE and stained with Coomassie brilliant blue (*a*) or immunoblotted using the antibody to the dynamin C terminus (*b*). Lanes: 1 and 2, supernatant and pellet, respectively, for intact dynamin; 3 and 4, supernatant and pellet, respectively, from digested dynamin. 100, Intact dynamin; 90, 90-kDa fragment; T, tubulin; 7-9, low-molecular-mass C-terminal dynamin fragment. Intact dynamin cosedimented with microtubules, as did a substantial fraction of the 7- to 9-kDa fragment, while the 90-kDa fragment remained in the supernatant.

Effect of Digestion on Microtubule Binding and GTPase Activity. The fragments were evaluated for their ability to bind to microtubules (Fig. 2*a*). No binding was observed with the 90-kDa fragment alone, indicating that the C-terminal end of the molecule was essential for this interaction. Significantly, the 7- to 9-kDa fragment could bind to and cosediment with microtubules (Fig. 2*b*). This result indicated a direct role for this region in microtubule binding. The binding efficiency was reduced relative to the intact protein, suggesting a role for other parts of the molecule, either in microtubule binding or in maintaining the proper conformation of the C-terminal domain.

In view of the effect of microtubules on the dynamin GTPase (16), the effect of papain digestion on GTPase activity was evaluated (Fig. 3). Papain digestion completely abolished microtubule stimulation of the GTPase activity with a time-course similar to that of the disappearance of the

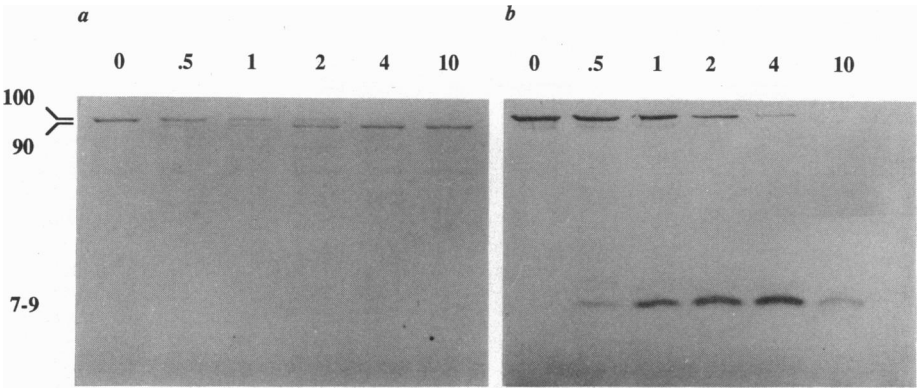

FIG. 1. Time-course of papain digestion. Dynamin fragments were separated by SDS/PAGE and stained with Coomassie brilliant blue (*a*) or immunoblotted with an antibody to the dynamin C terminus (*b*). A 7- to 9-kDa immunoreactive fragment and a 90-kDa nonreactive fragment were produced, indicating a primary papain cleavage site near the dynamin C terminus. Times of digestion (in minutes) are at top; polypeptide sizes in kDa are at left.

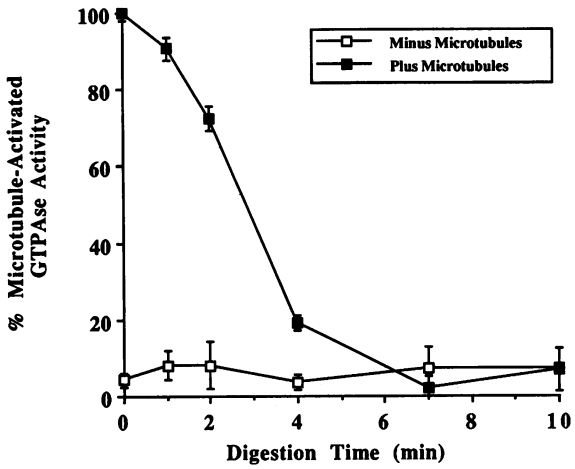

FIG. 3. Effect of papain digestion on dynamin GTPase activity. Microtubule-activated (■) and basal GTPase (□) activity are shown as function of papain digestion time. The microtubule-activated GTPase activity was abolished by papain digestion, which had no apparent effect on basal GTPase activity. Vertical lines indicate the range of duplicate data points.

intact dynamin polypeptide (compare with Fig. 1). In contrast to this result, no significant effect on the basal GTPase activity was seen. This result is consistent with the apparent insensitivity of the N-terminal GTP-binding domain to degradation by papain (Fig. 1). We also note that the lack of any detectable stimulation of the basal GTPase activity by removal of the C-terminal domain argues against a possible role for this region as an endogenous inhibitor of dynamin activity.

Effect of SH3-Containing Proteins on Dynamin GTPase Activity. Despite the potent activation of the dynamin GTPase by microtubules (16), the biological relevance of this interaction has been called into question by evidence indicating a role for dynamin in the initial stages of endocytosis (6, 7). Recently, a number of bacterially expressed recombinant glutathione *S*-transferase fusion proteins containing SH3 domains were found to bind dynamin *in vitro* (17). SH3 domains are present in many proteins associated with receptor tyrosine kinase complexes thought to be internalized via the endocytic pathway; thus, an interaction between SH3 domains and dynamin would provide a potential mechanism to target receptors for internalization.

To gain further insight into the likely physiological significance of these interactions, a number of glutathione *S*-transferase fusion proteins containing different SH3 domains were assayed for their effect on dynamin steady-state GTPase activity (Table 1; Fig. 4). Both the SH3 domain of c-Src and

Table 1. Effect of SH3 domain-containing fusion proteins on dynamin GTPase activity

Addition	GTPase activity, nmol/min per mg
None	9.9 ± 2.0
GST	17.0 ± 0.1
c-Src SH3	85.0 ± 12.4
GRB2	131.9 ± 11.9
GRB2 SH3 (N)	16.4 ± 0.9
GRB2 SH3 (C)	12.0 ± 1.4
Microtubules	658.2 ± 8.3

Dynamin GTPase activity was assayed in the presence of either the indicated glutathione *S*-transferase (GST) fusion proteins (0.08 mg/ml) or microtubules (0.12 mg/ml). Errors indicate the range of duplicate data points. (N), N-terminal SH3 domain; (C), C-terminal SH3 domain.

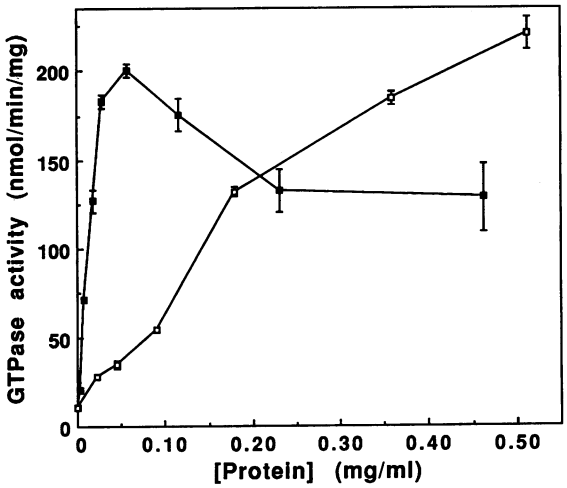

FIG. 4. Dependence of dynamin GTPase activity on GRB2 (■) or c-Src SH3 (□) concentration. In this experiment, activity in the presence of microtubules (0.11 mg/ml) was 802 ± 51 nmol/min per mg. Vertical lines indicate the range of duplicate data points.

the full-length GRB2 protein potently stimulated GTPase activity, by as much as 20.7- and 18.6-fold, respectively. Stimulation was not observed with the individual GRB2 N-terminal or C-terminal SH3 domains. Maximal stimulation by full-length GRB2 was seen at 0.06 mg/ml (Fig. 4), but activity declined at higher GRB2 concentrations, behavior very similar to that seen with microtubules (16). However, the maximal activity seen with GRB2 was considerably lower than that observed with microtubules. c-Src SH3 stimulated dynamin GTPase activity to levels above those observed with GRB2 or the individual GRB2 SH3 domains. Stimulation by c-Src SH3 showed no evidence of saturating even at the highest concentration examined (0.51 mg/ml).

To determine whether SH3 domains stimulated the dynamin GTPase activity via the C-terminal domain of dynamin, the effect of papain digestion was evaluated. Fig. 5 shows that GRB2 activation was completely abolished by

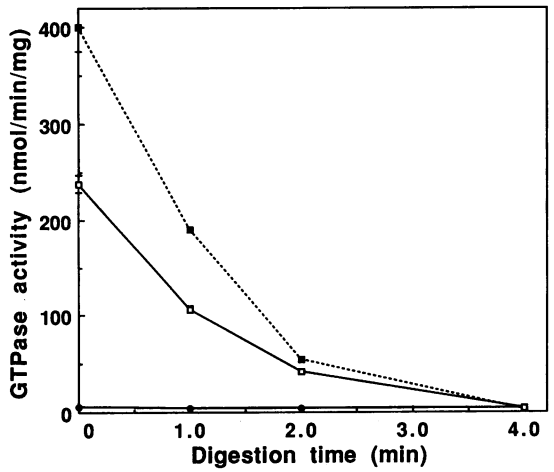

FIG. 5. Effect of papain digestion on GRB2 stimulation of dynamin GTPase activity. GRB2-activated (□), microtubule-activated (■), and basal GTPase activities (●) are shown as a function of papain digestion time. Zero time-point represents activity without papain. GRB2-stimulated GTPase activity was abolished by papain digestion with a time course similar to that seen for microtubule-stimulated activity. Vertical lines indicate the range of duplicate data points, which generally lay within size of symbols. Levels of activation by microtubules and SH3 domains varied somewhat between dynamin preparations (compare with Table 1).

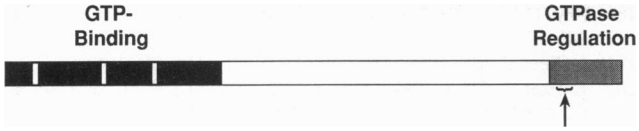

FIG. 6. Primary structural organization of dynamin polypeptide. Conserved region with consensus GTPase sequence elements indicated by open bars is shown at left; C-terminal 100-amino acid basic, proline-rich region is shown at right. Arrow, primary papain cleavage site.

papain digestion, and the decline in GRB2 activation closely paralleled the decline in microtubule activation.

DISCUSSION

We have shown that papain removes a small fragment from dynamin responsible for both microtubule binding and stimulation of its GTPase activity. We find that c-Src SH3 and full-length GRB2 stimulate the dynamin GTPase and that this effect, like microtubule activation, is abolished by removing the C-terminal fragment. Together these results establish the C-terminal region of rat dynamin as a probable regulatory site.

Based on its size, this fragment must contain a substantial portion of the compositionally distinctive basic (pI = 12.5) and proline-rich (32%) C-terminal region identified in the dynamin primary sequence (2). As noted earlier, the known dynamin-related proteins Mx (3), the *VPS1* gene product VPS1p (4, 5), and the more recently described yeast mitochondrial genome maintenance protein MGM-1p (24) all lack a comparable basic and proline-rich C-terminal domain. Our current results suggest that these proteins are not regulated by the same factor(s) that modulate dynamin activity. We note, however, that reconciling a report of microtubule binding by the *VPS1* gene product (5) with our present analysis is difficult.

Our results provide a number of insights into dynamin structure. The effect of removing the C-terminal papain fragment on GTPase activity seems surprising in view of the substantial distance between this fragment and the N-terminal GTPase domain. However, our results could indicate that the two functional domains are juxtaposed in the folded molecule. Such an interaction could occur within the individual dynamin polypeptide or between polypeptides within a multimeric complex.

The possibility of self-association is also raised in considering the implications of the present results for the organization of dynamin within microtubule bundles. In our initial characterization of dynamin, we observed that it formed extensive, highly cooperative helical arrays of cross-bridges between microtubules (1). However, while cross-bridging requires at least two microtubule-binding sites, our current analysis reveals only a single site within the dynamin polypeptide (Fig. 6). Together these observations can be explained by the formation of dynamin dimers or higher-order multimers. Alternatively, dynamin may contain a low-affinity microtubule-binding site outside the C-terminal region, the activity of which might only be evident within cooperative arrays of the protein. An additional possibility is that removal of the C-terminal domain by papain digestion abolishes the activity of a second microtubule-binding site, but we consider this latter explanation unlikely.

Physiological Interactions with the Dynamin C Terminus. The identity of the physiological ligand or ligands for the C-terminal portion of dynamin remains an important, but incompletely resolved, issue. We report here that at least two SH3 domain-containing polypeptides not only interact with dynamin, as previously observed, but substantially stimulate its GTPase activity. This observation adds strong support to the potential physiological relevance of these interactions.

Our data indicate that microtubules and SH3 domains interact with the C-terminal domain of dynamin, which has characteristics consistent with binding to both structures. The microtubule-binding domains of microtubule-associated proteins are basic and somewhat high in proline content (see, for example, refs. 25 and 26), whereas sequences capable of binding Abl-SH3 have been found to contain clusters of prolines (27). In fact, several sequences comparable to the consensus derived for the interaction between Abl-SH3 and its binding partner 3BP-1 are present in the dynamin C terminus, and a direct interaction between the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (p85α) and a synthetic peptide corresponding to amino acids 786–806 in rat dynamin has been reported (17). Whether other regions within the C-terminal region can also interact with SH3 domains and how rigorously the consensus sequence defined for Abl-SH3 applies to other SH3-containing proteins remain to be seen.

Which of the interactions observed *in vitro* is significant in the cell remains a critical question. There appears to be considerable variability in the ability of different SH3 domains to stimulate the dynamin GTPase, and the highest levels of GTPase stimulation were always seen with microtubules (Table 1; Fig. 4). However, we note that in preliminary experiments, mixtures of GRB2 and microtubules containing equimolar concentrations of GRB2 and tubulin dimers produced levels of GTPase stimulation only as high as those seen with GRB2 alone, suggesting that GRB2 competes favorably with microtubules for an overlapping site (H.S.S., unpublished work). GRB2 has recently been found by both genetic and biochemical means to interact specifically with the GTP exchange protein SOS (28–35). Whether alternative physiological roles exist for GRB2, such as one involving dynamin, remains to be answered.

Interestingly, the individual GRB2 domains had much lower GTPase stimulatory activity than did intact GRB2 (Table 1). This observation may reflect an increased tendency to fold incorrectly. Alternatively, the observation may indicate a cooperative interaction of multiple SH3 domains with dynamin. In this context, we speculate that the microtubule surface represents an ideal substrate for interacting with dynamin, in view of the extensive, extremely regular array of tubulin subunits on the microtubule surface. Conceivably, microtubules may serve as an extremely effective mimic of a cellular condition in which SH3-containing proteins become clustered beneath the plasma membrane, as might be imagined to occur during the earliest stages of endocytosis.

Our analysis of the role of rat dynamin in receptor-mediated endocytosis has also pointed to the importance of the C-terminal domain (13). Overexpression of dynamin containing point mutations in or deletions of the GTPase domain had a dominant inhibitory effect on transferrin uptake. Removal of the C-terminal 188-amino acids completely reversed this effect. A smaller C-terminal deletion had no effect, defining amino acids 663–794 as an important functional region. Based on the present data, this region may be the same as that involved in the interaction of dynamin with other proteins and in regulating GTPase activity. However, further high-resolution analysis of this domain will now be necessary to address this issue.

Note Added in Proof. We note the presence of the sequence AGSAL within the basic, proline-rich domain of dynamin (AQSLL in shibire), which is related to the G5 guanine ring-binding consensus sequence element in the small GTPases and EfTu. This observation raises the possibility of direct participation of the dynamin C-terminal domain in GTP and GDP binding.

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