Dynamin is a GTP-binding protein that is involved in the release of coated endocytic vesicles from the plasma membrane. We have been characterizing the enzymatic properties of purified rat brain dynamin to better understand how GTP binding and hydrolysis relate to its proposed function. Previously, we have demonstrated that activation of dynamin GTPase results from positive cooperative associations between dynamin molecules as they are bound to a polymeric surface. Our present report has extended these studies and has examined the structural features of dynamin self-association. After treatment with the zero-length protein cross-linking reagent, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, dynamin in solution was found cross-linked into dimers. This homodimer likely reflects the native soluble state of the molecule. After binding to brain vesicles, dynamin was cross-linked into higher order oligomers of greater than 800 kDa. Dynamin, copurified on brain membrane organelles, also formed multimeric complexes when cross-linked suggesting dynamin exists in polymeric form in vivo. No cross-linked species other than homo-oligomers were observed, providing no evidence for close interactions between dynamin and membrane proteins. From experiments examining the effects of GTP, GDP, guanosine 5'-3'-[thio]triphosphate, and 5'-guanylyl-3'-imidodiphosphate on cross-linking, we have determined that both dynamin membrane binding and self-association occur independently from the nucleotide-bound state of the enzyme. An 80-kDa dynamin fragment that is lacking its carboxyl-terminal domain is not cross-linked into higher order oligomers, suggesting that this domain is required for binding of dynamin to membranes and the subsequent enhancement of oligomerization. However, the dynamin fragment was found to form dimers indicating that this domain is not required for dynamin dimerization. Cross-linked dynamin was able to cooperatively bind microtubules, but did not exhibit GTPase activation. We propose that intramolecular cross-links in the dynamin monomer impart structural constraints that prevent the enhancement of GTP hydrolysis. We describe a model of the dynamin activation process to be considered in further investigations of the role for dynamin in endocytic vesicle formation.

Dynam is a GTP-binding protein that functions in the early stages of endocytosis. In particular, it is thought to be required for endocytic coated vesicle formation and budding. Recent reports have suggested that dynamin participates in this process by catalyzing a GTP-dependent fission reaction at the necks of invaginated coated pits that results in the release of coated vesicles from the plasma membrane (1, 2). Mutational analyses of the dynamin GTP-binding site have indicated that GTPase activity is required for in vivo function (2–4). However, it is not clear how dynamin's enzymatic properties are related to its proposed activity in endocytic vesicle formation. We have been investigating the enzymatic properties of purified rat brain dynamin in order to define parameters affecting GTP hydrolysis. Our goal is to identify and characterize the agents which function as physiological regulators of dynamin activity.

Dynam GTP hydrolysis rates are accelerated when in the presence of a diverse set of agents including microtubules (5, 6), acidic phospholipid liposomes (6), rat brain vesicles (6), and certain SH3-domain proteins (7, 8). Dynamin associates with each of these agents via its 100-amino acid carboxyl-terminal domain (6, 8). However, binding of rat brain dynamin to acidic phospholipids or microtubules does not stimulate GTPase activity directly. Rather, binding these agents promotes cooperative associations between dynamin molecules leading to activation of GTP hydrolysis (9, 10). We have determined that at least two dynamin molecules, each of which is a homodimer, must self-associate for optimal GTPase activity to occur (9). Each of the activating agents listed above provide a multivalent surface to which dynamin binds that promotes cooperative associations between dynamin molecules.

It is not clear with which agents (if any) dynamin associates in vivo. Although dynamin is routinely purified from brain preparations by exploiting its affinity for microtubules (6, 11), dynamin has not been shown to colocalize with microtubules in the cell. Instead, biochemical and immunolocalization studies have indicated dynamin plasma membrane associations (2–4, 6, 12, 13). Whether dynamin binds the membrane directly through interactions with acidic phospholipids or binding is mediated by other proteins (SH3-domain or clathrin coat proteins?) is not known.

To further complicate our understanding of the regulation of the dynamin GTP hydrolytic cycle, it has been demonstrated that activation of dynamin in the presence of acidic phospholipids, rat brain vesicles, microtubules, or Grb2 does not display Michaelis-Menten kinetics (6, 8). Although positive cooperative interactions between dynamin molecules result in optimal activation, it is not known what features of dynamin's interactions with itself or with the binding substrates accounts for the non-classical behavior observed. It is also highly likely that additional regulatory aspects of dynamin function remain to be identified.
In this study we have further defined properties of dynamin GTP binding and hydrolysis. In particular, we have examined dynamin self-association in the presence and absence of activating agents and have determined that dynamin is self-associated when bound to membrane organelles. We have found that dynamin membrane binding and cooperative associations occur independently from nucleotide binding. We have also examined dynamin intra- and intermolecular interactions that are important in the stimulation of dynamin GTPase. We provide a structural model that describes the features of the activation process that must be strongly considered as we continue our investigation of the role dynamin GTP hydrolysis plays in endocytosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Sprague-Dawley rats (Harlan, Indianapolis, IN) between 150 and 250 g were used as the source of brain tissue. GTP (type VII, lithium salt), AMPPNP (lithium salt), GTP·S, GMPPNP, GDP (sodium salt), chymotrypsin, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), and alkaline phosphatase-conjugated secondary antibodies were all purchased from Sigma. Nucleotides were prepared as either 10 mM (GTP·S, GMPPNP, GDP) or 100 mM (GTP, AMPPNP) stocks with equimolar MgSO4. Lipids were purchased from Sigma or Avanti Polar Lipids. Reagents for gel electrophoresis were from ICN (Costa Mesa, CA) and Bio-Rad. Taxol was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Research, National Cancer Institute, and stored as a 10 mM stock in dimethyl sulfoxide at −80°C.

**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 with GTP and AMPPNP, as described (6). Dynamin was further purified by fractionation on a 5-20% linear sucrose gradient (6). The 80-kDa dynamin fragment was produced by a fortuitous proteolytic event that occurs occasionally during routine purification of the intact poly peptide (6).

**Microtubule Preparation**—Tubulin was purified by reversible assembly of microtubules from calf brain cytoskeletal extracts followed by DEAE-Sephadex chromatography (14). Tubulin was polymerized by the addition of equimolar taxol and incubation for 5 min at 37°C (15). The microtubules were diluted in extraction buffer to the desired concentration for use in the assays.

**Brain Vesicle Preparation and Extraction**—A crude coated vesicle preparation was isolated from rat brain as described (6). Briefly, 4–5 g of rat brain were homogenized in an equal volume of extraction buffer and centrifuged at 25,000 × g for 30 min. The supernatant was centrifuged at 138,000 × g for 1 h and the resultant vesicle pellet was either used directly or extracted for 30 min on ice with 1 vol of extraction buffer and centrifuged at 138,000 × g for 1 h. The supernatant was removed and the membrane pellet was washed once in extraction buffer by recentrifugation. All steps were performed at 4°C. The membrane preparations were assayed for protein content and diluted in extraction buffer to the desired concentrations for use in the assays.

Small Unilamellar Vesicle Preparation—Liposomes (25% phosphatidylserine, 75% phosphatidylcholine, w/w) were prepared as described (6). The lipid mixture was dried from chloroform and residual solvent was removed in vacuo. The lipids were resuspended in extraction buffer to the desired concentration and the mixture was sonicated with a probe sonicator until clear.

**GTPase Assays**—Assays were performed in a 100-μl total volume at 37°C for 15 min (9). The concentrations of dynamin and added agents were as indicated in the figure legends. Assay mixtures were incubated for 10 min at room temperature prior to the initiation of the reaction by addition of GTP. GTPase activity was assayed by monitoring [32P]GTP (Amersham Corp.) according to Collins and Vale (16).

**EDC Cross-linking**—Nine volumes of dynamin in the absence or presence of lipids were incubated with 1 volume of EDC at room temperature. Concentrations of the reagents and times of cross-linking are indicated in the figure legends. The cross-linking reactions were stopped by the addition of a 2-fold molar excess of 2-mercaptoethanol over EDC (17). Reaction mixtures were mixed with Laemmli sample buffer and subjected to gel electrophoresis, were used directly in GTPase or microtubule binding assays or were treated with chymotrypsin for protease sensitivity assays. The addition of 2-mercaptoethanol slightly inhibited dynamin GTP hydrolysis rates reflected in lowered maximal activity levels of dynamin when treated with EDC for 0 min.

**Microtubule Binding Assays**—EDC-cross-linked dynamin (100 mM) was incubated in the presence or absence of 0.3 mg/ml microtubules for 10 min at room temperature. Assay volumes were corrected to 100 μl with extraction buffer. The mixtures were centrifuged at 25,000 × g for 30 min at 37°C. The resultant microtubule pellet was resuspended to volume with extraction buffer and both the supernatant and pellet samples were mixed with Laemmli sample buffer in preparation for gel electrophoresis.

**Chymotrypsin Digestion**—Dynamin (40 nm) in the presence or absence of lipid vesicles, or EDC-cross-linked dynamin, was incubated with 0.25 μg/ml chymotrypsin at room temperature for 60 min. Digests were stopped by the addition of 5 mM phenylmethylsulfonyl fluoride and further diluted in Laemmli sample buffer for gel electrophoresis.

**Negative Stain Electron Microscopy**—EDC-cross-linked dynamin (45 nm) was incubated with 0.05 mg/ml microtubules for 10 min at room temperature. The mixtures were adsorbed onto carbon-coated Formvar grids for 2 min followed by fixation with 0.5% glutaraldehyde and staining. Grids were washed with 1% uranyl acetate and stained with 2% j EOL (Peabody, MA) JEM 100CX transmission electron microscope.

**RESULTS**

**Dynamin Forms Higher Order Oligomers When Bound to Endogenous Vesicles**—Our previous enzymatic analysis of purified dynamin revealed cooperativity in the activation of GTP hydrolysis rates (9). We chose to examine the self-association of dynamin by more structural means to extend these results in order to better understand how these properties relate to GTP binding and hydrolysis. For this purpose, we developed a protein cross-linking strategy to examine dynamin self-association properties in vitro. We chose the zero-length cross-linking reagent, EDC, as it will covalently cross-link peptides that are immediately adjacent, i.e. those dynamin molecules that are self-associated. Rat brain vesicles, salt extracted to remove peripheral proteins, were used as the dynamin binding surface, as we have characterized them as stimulators of dynamin GTPase, and they may well represent the in vivo binding site (6). Dynamin binding to these vesicles likely occurs through interactions with acidic phospholipids (6). Purified rat brain dynamin was treated with 10 mM EDC for time periods of up to 1 h in the presence or absence of brain vesicles (10 μg of protein/ml), conditions that provide optimal activation of dynamin GTPase activity (6), and the reaction was stopped by the addition of 2-mercaptoethanol. The extent of cross-linking was monitored on Western blots by the appearance of dynamin immunoreactive species with observed electrophoretic mobilities greater than 100 kDa, the molecular weight of the dynamin monomer.

The extent of dynamin self-association in solution, the absence of lipid vesicles, was limited (Fig. 1). Within 5 min of EDC treatment, dynamin dimers (200-kDa immunoreactive species) were formed and, after 60 min, were the prominent oligomers detected. Higher order cross-linked species were also observed, but only after 30–60 min of EDC treatment and in much lower amounts (Fig. 1). In the presence of brain vesicles, the extent of dynamin oligomerization was enhanced (Fig. 1).

As was observed for dynamin in the absence of brain vesicles,
Dynamin self-association is enhanced in the presence of brain vesicles. Dynamin (60 nM) was treated with 10 mM EDC for 0, 5, 15, 30, or 60 min in the absence or presence of lipid vesicles and the samples were processed for Western blot analysis using anti-dynamin antibodies. The relative levels of dynamin monomer remaining, as determined by densitometric analysis, are indicated below each lane of the blot. The molecular masses of the dynamin immunoreactive species are indicated at the right of the figure in kilodaltons. The corresponding Coomassie Blue-stained gel revealed only minor cross-linking of other vesicle proteins (data not shown).

The prevalence of dynamin dimer formation, both in the absence and presence of vesicles, indicates that this is the minimal dynamin multimer and represents the native form of dynamin. This result agrees with size estimations obtained from sucrose density gradient sedimentation values (22), gel permeation chromatography (23), and electron microscopic observations (11, 23). However, recent reports have suggested that dynamin exists in its native form as a tetramer (21). Data for these reports were obtained using significantly higher dynamin concentrations which may have promoted some self-association.

The Dynamin Carboxyl-terminal Domain Is Required for Enhanced Self-association—Dynamin associates with each of its “activating” agents through its carboxyl-terminal domain (6, 8). We investigated whether this domain was also involved in dynamin self-association. For these experiments, we examined the oligomerization properties of an 80-kDa dynamin proteolytic fragment that we have previously characterized as lacking its carboxyl-terminal domain (6). Purified 80-kDa dynamin was incubated with EDC in the presence or absence of salt-stripped brain vesicles (10 µg of protein/ml) and the extent of oligomerization was determined (Table I). In the absence or presence of vesicles, only 32 and 29% of the truncated dynamin was cross-linked, respectively. Unlike intact dynamin, oligomerization of the fragment was not enhanced in the presence of vesicles, but rather closely approximated values obtained for intact dynamin in the absence of vesicles (37%, see Table I). In fact, only dimer formation was observed for experiments involving the 80-kDa fragment. These results support the conclusion that the dynamin carboxyl-terminal domain is not involved in dynamin dimer formation (6), but indicates that higher order oligomers of truncated dynamin do not form in solution. In the presence of vesicles, the carboxyl-terminal domain appears to be re-
pressed Mx1 protein has been shown to self-associate when bound to membranous organelles. These results indicate that dynamin is self-associated and this self-association is enhanced in the presence of 1 mM GTP. As shown in Fig. 4, the rate of dynamin self-association is not enhanced in the presence of GTP. In the absence or presence of lipid vesicles, the relative levels of dynamin oligomeric species (>200 kDa) were determined and are expressed as % dynamin oligomerized. The addition of 1 mM GTP had no effect on the rates of dynamin oligomerization (●, □).

**FIG. 3.** Dynamin is self-associated when isolated from rat brain. A high speed pellet preparation (HSP) enriched in clathrin-coated vesicles (1.9 mg of protein/ml) was treated with 10 mM EDC for 0, 15, 30, or 60 min and the samples processed for Western blot analysis with anti-dynamin antibodies. For comparison, purified dynamin (53 nM) that was cross-linked in the presence of a salt-stripped HSP preparation (vesicles) is also shown. The dashed to the right of the figure correspond to the molecular mass of the dynamin immunoreactive monomer (100 kDa), dimer (200 kDa), and other higher order cross-linked species (>200 kDa). The corresponding Coomassie Blue-stained gel revealed only minor cross-linking of other vesicle proteins (data not shown).

**Table I**

| Time of EDC treatment (min) | 80 kDa Cross-linked | 80 kDa Dimerized | 80 kDa + vesicles Cross-linked | 80 kDa + vesicles Dimerized | 100 kDa Cross-linked | 100 kDa Dimerized |
|---------------------------|---------------------|------------------|-------------------------------|----------------------------|---------------------|-------------------|
|                           | %                   |                  |                               |                            | %                   |                  |
| 0                         | 0                   | 0                | 0                             | 0                          | 0                   | 0                |
| 15                        | 4                   | 4                | 4                             | 4                          | 11                  | 11               |
| 30                        | 10                  | 10               | 29                            | 29                         | 34                  | 22               |
| 60                        | 32                  | 32               | 29                            | 29                         | 37                  | 27               |

Dynamin Is Self-associated When Isolated from Rat Brain—We wished to determine whether dynamin exists in a multimeric state in vivo. We have previously shown that dynamin is not self-associate. To examine the effects of GTP on dynamin oligomerization, dynamin was treated with 10 mM EDC in the presence of 1 mM Mg²⁺-GTP. As shown in Fig. 4, the rate of dynamin oligomerization was not altered by the addition of GTP. In the presence and absence of GTP, 25 and 26% dynamin were cross-linked into higher order complexes after 1 h, respectively. These results indicate that, unlike Mx1, dynamin self-association is not enhanced in the presence of GTP.
the addition of GDP did not disrupt dynamin self-association. This indicates that dynamin binding to membranes is not regulated by its nucleotide-bound state such that it does not cycle on and off as it hydrolyzes GTP.

**Effect of Intra- and Intermolecular Cross-link Formation on Dynamin GTPase Activity**—Since optimal dynamin GTPase activity requires that at least two dynamin molecules (dimers) must be self-associated (9), we reasoned that enhanced activity may be observed under conditions that stabilize oligomer formation. To test this hypothesis, we assayed the GTPase activity of dynamin that was cross-linked in the presence of 25% phosphatidylserine liposomes (0.1 mg/ml) or 0.15 mg/ml microtubules. For these experiments, increasing concentrations of EDC were added to dynamin that was prebound to either agent. The cross-linking reactions were stopped after 20 min by the addition of 30 mM 2-mercaptoethanol and the mixture was assayed for GTPase activity. Surprisingly, the addition of only 1 mM EDC drastically inhibited stimulated rates of dynamin GTP hydrolysis in the presence of either microtubules or liposomes (Fig. 5A). To ensure that the cross-linking reaction was not interfering with the dynamin active site, we cross-linked dynamin in the absence of liposomes or microtubules, and assayed for basal GTP hydrolysis rates. As shown in Fig. 5B, these rates were not altered by treatment with EDC, suggesting that intramolecular cross-links or modifications did not involve the GTPase active site.

The inhibition of dynamin GTPase observed in Fig. 5A occurred at low EDC concentrations that do not induce intermolecular cross-links as assayed by monomer content on Coomassie Blue-stained gels (data not shown). Rather, intramolecular cross-links or modifications were likely being formed that disrupted the activation of dynamin GTP hydrolysis through self-association. To examine the effects of this modification, dynamin was treated with EDC under conditions that did not favor intermolecular cross-link formation (Table III). The treated dynamin was incubated with liposomes or microtubules for 10 min to allow binding, and GTPase activity was measured. As shown in Table III, treatment with EDC drastically inhibited the stimulated rates of dynamin GTP hydrolysis. Upon treatment of dynamin with 15 mM EDC, GTPase activity levels were inhibited by 83% (in the presence of liposomes) and 88% (in the presence of microtubules). Even at this high EDC concentration, intermolecular cross-links were not being formed; only a small proportion (4 or 18%, in the presence of liposomes or microtubules, respectively) of dynamin was cross-linked into dimers.

These results indicate that EDC treatment was inducing either intramolecular cross-links or modifications that were...
disrupting the dynamin GTPase activation process. One possible explanation is that dynamin was being modified such that activator-dynamin interactions were being prevented. To test this possibility, we assayed for binding of EDC-cross-linked dynamin to microtubules. Dynamin was cross-linked for 20 min with 15 mM EDC. The reaction was stopped with 2-mercaptoethanol and the mixture was incubated in the presence of 0.3 mg/ml microtubules for 10 min. The microtubules and associated dynamin were recovered by centrifugation. In Fig. 6, the results of this experiment are shown. In the absence of microtubules, the EDC-cross-linked dynamin remained in the supernatant, indicating that these complexes are not forming large aggregates that could sediment under these conditions. However, in the presence of microtubules, virtually all of the dynamin (both monomer and cross-linked species) was recovered in the pellet fraction indicating that treatment with EDC was not disrupting dynamin-microtubule interactions.

Another possible explanation for the inhibition of GTPase activity observed is that EDC was modifying dynamin such that dynamin-dynamin interactions were being prevented. To test this possibility, we examined dynamin cooperative binding to microtubules. After treatment with 10 mM EDC for 5 min (a combination sufficient to inhibit stimulated GTP hydrolysis rates), dynamin was incubated with microtubules to allow binding and the samples were processed for negative stain electron microscopy. As shown in Fig. 7A, dynamin bound microtubules with uniform spacing (13 nm) and appeared in clusters. This pattern of dynamin binding to microtubules has been previously observed and represents cooperative associations between dynamin molecules (11). Even after 30 min of cross-linking, dynamin was still able to bind microtubules in a cooperative manner (Fig. 7B). This indicates that neither dynamin-microtubule nor dynamin-dynamin interactions were perturbed by EDC treatment.

To better understand how EDC treatment was inhibiting dynamin-activated GTP hydrolysis, it was necessary to determine whether intramolecular cross-links were being formed and identify the regions of the dynamin molecule that were involved. Dynamin was treated with 10 mM EDC for the indicated times and subsequently digested with 0.25 μg/ml chymotrypsin at room temperature for 60 min (Fig. 8). In Fig. 8A, a silver-stained gel of the peptide maps obtained is shown (A). The corresponding Western blot was probed with anti-dynamin antibodies specific for the carboxyl-terminal domain (B). The position of the dynamin monomer and a cluster of dynamin carboxyl-terminal fragments are indicated in kilodaltons.

For the carboxyl-terminal domain, striking differences in the peptide maps were noticed (Fig. 8B). At time 0 (no EDC cross-linking), a characteristic chymotrypsin peptide map of dynamin carboxyl-terminal derived fragments was generated with immunoreactive peptides at approximately 50, 44, 42, 36, 21, 20.5, and 18.5 kDa. The fragments ranging from 18.5 to 21 kDa have been previously described as the regions deleted from the 80-kDa dynamin (6). Within 5 min of EDC treatment, the levels of 18.5–21-kDa fragments detected were significantly reduced and by 15 min, were barely detectable. The 36-44-kDa fragments were also present in reduced amounts as the extent of dynamin cross-linking was increased. Similar alterations in peptide maps were obtained from digests of dynamin when
Dynamin binds a multivalent surface (step 1) and adopts an orientation that promotes cooperative associations (step 2). Upon binding and hydrolysis of GTP, the dynamin molecules undergo a concerted conformational change (step 3). The molecules either return to a starting conformation (step 3) or are released from the membrane (step 4). Dynamin monomers are represented by ovals with the carboxyl-terminal domains extending away from the intact molecule.

Cross-linked in the presence of lipid vesicles (data not shown). The altered sensitivity of the treated dynamin to digestion by chymotrypsin indicates that EDC was inducing intramolecular cross-links that involved the dynamin carboxyl-terminal domain. This rigid domain likely extends away from the rest of the molecule (26), and upon treatment with EDC, may be covalently attached back to the intact molecule or adjacent molecule thus altering the availability of these sequences to digestion by chymotrypsin. The cross-links not only decrease the conformational freedom of this domain, but also likely confer structural rigidity to the whole dynamin molecule. This, in turn, may prevent conformational changes required for activated GTP hydrolysis. Recent reports have postulated that dynamin conformational flexibility is important for pinching off coated vesicles from the plasma membrane (1). These experiments provide evidence that such conformational changes are directly related to dynamin GTP binding and hydrolysis.

**DISCUSSION**

Our previous analysis of dynamin enzymatic properties revealed that cooperative associations between dynamin molecules, as they are bound to a polymeric surface, are responsible for activation of GTP hydrolysis. In this study, we extended our analysis and have provided structural evidence that dynamin self-associates in vitro in the presence of brain vesicles. We have also determined that membrane-bound dynamin, copurified with a clathrin-coated vesicle preparation, is organized into high order polymers. Both dynamin-membrane and dynamin-dynamin interactions occur independently of its nucleotide-bound state. We found that structural constraints, imposed by the formation of intramolecular cross-links involving the dynamin carboxyl-terminal domain, inhibit dynamin-activated GTP hydrolysis.

From these results and our biochemical analysis of dynamin, we have developed a structural model that describes the dynamin activation process (Fig. 9). In step 1, a dynamin dimer (its native state) binds a multivalent surface via its carboxyl-terminal domain that extends from the intact molecule. Upon binding, dynamin adopts an orientation that promotes cooperative interactions between dynamin molecules resulting in polymer formation (step 2). GTP binding, hydrolysis, and release of products (GDP and Pi) are associated with a concerted conformational change in the polymer that likely promotes the release of endocytic vesicles from the plasma membrane in vivo (step 3). Upon completion of the hydrolytic cycle, the dynamin molecules either reset to a starting conformation or are released from the binding surface (step 4). We and others have shown that both membrane binding and self-association are not dependent on dynamin nucleotide binding (steps 1, 2, and 4). Other signaling events are likely required for regulation of membrane binding, initiation of the GTP hydrolytic cycle and the resetting process (see below). Treatment of dynamin with EDC inhibits step 3 of the activation process by conferring structural rigidity to the molecule, preventing the necessary conformational changes associated with stimulated rates of GTP hydrolysis.

Biochemical subfractionation of rat brain has shown dynamin peripheral association with membranous organelles (6, 12–13). Immunolocalization studies at both the light and electron microscopic levels have shown that dynamin is found at the plasma membrane (2–4). However, it is not known whether dynamin binds membranes directly or through other membrane-associated proteins. It is compelling to speculate that dynamin is binding acidic phospholipid domains of the membrane. Acidic phospholipid liposomes and salt-stripped brain vesicles have both been shown to support dynamin-activated GTP hydrolysis in vitro (6). Furthermore, cross-linking experiments of isolated brain vesicles with which dynamin associates provided no evidence for dynamin-receptor interactions (Fig. 3). However, it is possible that dynamin-receptor binding is substoichiometric, as one dynamin molecule binds a receptor, it adopts an orientation that favors cooperative associations such that binding proceeds without additional receptor-mediated interactions.

Whether dynamin binds membrane lipids or proteins, the interactions are likely GTP-insensitive (Fig. 4B). This conclusion is supported by the observation that mutant dynamin molecules that bind GTP with greatly reduced affinity maintain their plasma membrane localization when overexpressed in HeLa cells (2). Although dynamin binding to microtubules has been regarded as GTP-sensitive, the ionic strength of the added nucleotide is sufficient to disrupt ionic interactions between dynamin and microtubules that results in an apparent GDP-dependent elution (9). Furthermore, we found that dynamin was not eluted from membranes by the addition of GDP (Table I). Although biochemical subfractionation studies have shown that dynamin partitions approximately equally between soluble and particulate fractions (6, 12–13), our results suggest that dynamin membrane binding is not regulated by GTP hydrolysis and occurs independently from the nucleotide-bound state of the enzyme. Since self-association stimulates dynamin GTPase, there must be other signals to regulate membrane binding, initiate the GTP hydrolytic cycle, and/or reset the process. The dynamin carboxyl-terminal 20-kDa fragment is required for vesicle binding and subsequent enhancement of oligomerization in vitro (this report) and targeting to membranes in vivo (4). The pleckstrin homology domain of the dynamin molecules used in these studies remained intact and recently it has been shown that this domain can bind membrane lipids (27). It is possible that lipid binding by this domain may be involved in the regulation of these processes. Also, dynamin carboxyl-terminal phosphorylation, as regulated by synaptic terminal hyperpolarization, remains a possible mechanism for the regulation of dynamin membrane binding (13, 28).

Once bound to membranes in vivo, dynamin likely adopts an orientation that favors positive cooperative associations, resulting in dynamin polymer formation. In the absence of brain vesicles, the extent of dynamin oligomerization is limited primarily to dimer formation. However, upon the addition of ves-
icles to the mixture, dynamin rapidly forms multimeric complexes. That dynamin polymer formation occurs is further supported by studies performed in isolated synaptosomes that were treated with GTPγS. These synaptosomes were characterized by large tubular plasma membrane invaginations which were positive for high density dynamin immunoreactivity along their lengths (1). Although high concentrations of dynamin have been shown to self-associate in the absence of polymeric binding sites in a GTP-independent manner (21), we suggest that in vivo, dynamin-dynamin interactions occur following binding to membranes.

The finding that a dynamin-related protein, Mx1, also self-associates in vitro suggests that this property may be conserved among the dynamin family members. In fact, a “self-assembly domain” has been identified in Mx1 that is shared among all the related proteins (25). However, our present analysis suggests that dynamin self-association is regulated differently. Soluble Mx1 self-assembles in vitro and this is enhanced in the presence of GTP (25). This protein is found in rat liver nuclei in what appears to be Mx1 aggregates (25). Our analysis has provided direct evidence that both dynamin membrane binding and self-association occur independently from the GTP hydrolytic cycle. Mutational analyses of dynamin molecules have shown that GTPase activity is required for its role in endocytosis (2–4) indicating that other properties of dynamin must be regulated by GTP binding and hydrolysis. Our experiments indicate that dynamin structural rigidity, resulting from EDC-induced intramolecular cross-links, inhibit association-stimulated GTPase activity. This suggests that conformational flexibility, in combination with membrane binding and self-association, is required for dynamin function. We propose that dynamin polymers, as they are bound to the membrane, are able to bind GTP and upon hydrolysis, undergo a concerted conformational change of the polymer relative to the surface. We suggest that this GTP hydrolysis-dependent conformational change brings the opposing membranes of the neck of the invaginated coated pit into close proximity, allowing membrane fusion and subsequent release of the endocytic vesicle.

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