Multiple Distinct Coiled-coils Are Involved in Dynamin Self-assembly*

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Dynamin, a 100-kDa GTPase, has been implicated to be involved in synaptic vesicle recycling, receptor-mediated endocytosis, and other membrane sorting processes. Dynamin self-assembles into helical collars around the necks of coated pits and other membrane invaginations and mediates membrane scission. In vitro, dynamin has been reported to exist as dimers, tetramers, ring-shaped oligomers, and helical polymers. In this study we sought to define self-assembly regions in dynamin. Deletion of two closely spaced sequences near the dynamin-1 C terminus abolished self-association as assayed by co-immunoprecipitation and the yeast interaction trap, and reduced the sedimentation coefficient from 7.5 to 4.5 S. Circular dichroism spectroscopy and equilibrium ultracentrifugation of synthetic peptides revealed coiled-coil formation within the C-terminal assembly domain and at a third, centrally located site. Two of the peptides formed tetramers, supporting a role for each in the monomer-tetramer transition and providing novel insight into the organization of the tetramer. Partial deletions of the C-terminal assembly domain reversed the dominant inhibition of endocytosis by dynamin-1 GTPase mutants. Self-association was also observed between different dynamin isoforms. Taken altogether, our results reveal two distinct coiled-coil-containing assembly domains that can recognize other dynamin isoforms and mediate endocytic inhibition. In addition, our data strongly suggests a parallel model for dynamin subunit self-association.

Dynamin is a high molecular mass GTPase (1, 2) that has been implicated in various aspects of endocytosis, including synaptic vesicle recycling, the endocytosis of assorted receptors, internalization of caveolae, and more recently budding from the trans-Golgi network (reviewed in Refs. 3 and 4). Dynamin belongs to a growing family of functionally diverse, large GTPases (4). In mammals, multiple isoforms of dynamin itself have been identified (4, 5), and functional dynamin homologs have been cloned from the lower eucaryotes shibire (Ref. 6) and Caenorhabditis elegans (dyn-1; 8).

Dynamin self-assembly is critical to its function. It has been shown to form helical collars around the necks of coated pits (8) and is postulated to be involved in the subsequent budding of coated vesicles and other early endocytic intermediates. Structures corresponding to the helical collars can be formed in vitro from purified protein (9) as well as in combination with acidic substrata including microtubules (10), phospholipid tubules (11), and native synaptosomal membranes (12). Polymerization is concentration-dependent (13, 14) and inhibited in high ionic strength buffers (9). Self-assembly does not require guanine nucleotides (15, 16), although it is either stimulated or inhibited by them depending upon whether the protein is free or membrane-bound (15, 16). Once polymerized, dynamin can undergo a nucleotide-dependent conformational change that results in membrane fission in vitro (11). In the depolymerized state, dynamin has been reported to exist as either a dimer (16) or a tetramer (17) based on cross-linking studies. Analytical ultracentrifugation has favored tetramer as the predominant species (18).

The regions of dynamin that are important in self-association and in the interaction with lipids and other proteins have been a topic of considerable interest. Dynamin contains a highly conserved, N-terminal GTPase domain of ~300 aa (1) and a centrally located pleckstrin homology (PH) domain that has been reported to bind to phosphatidylinositol 4,5-bisphosphate (19, 20) and G-protein βγ-subunits (21). Downstream of the PH domain are two predicted regions of coiled-coil α-helix, and at the C terminus a 100-aa basic, proline-rich region to which Src homology 3 domains (22–24), acidic phospholipids (13), and microtubules (25) have been shown to bind. This domain has been implicated in targeting dynamin to coated pits through Src homology 3 domains (26).

Dynamin self-association stimulates its GTPase activity. Ligands that bind to the proline-rich domain stimulate both assembly and GTP hydrolysis, and removal of the domain inhibits both activities (13, 22, 25). More recently, proteolytic dynamin fragments missing the proline-rich domain were shown by electron microscopy to be capable of self-assembly when in the presence of GDP and metallofluorides at physiological salt conditions (27) or on lipid bilayer tubes (11).

The role of the putative coiled-coil sequences in dynamin self-association is uncertain. Coiled-coil probability is only ~60% (see “Results”), and whether this region actually forms coiled-coil structure, amphipathic α-helix, or neither is uncertain. This region is conserved among the dynamin isoforms and dynamin-related proteins (4), and computer-based analysis (Newcoils; Ref. 28) predicts comparable secondary structure in all of these proteins. The dynamin-related Mx proteins exhibit clear heptad repeats within this region consistent with a leucine zipper structure that, when fused to a reporter gene,

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1 The abbreviations used are: aa, amino acid(s); TFE, trifluoroethanol; PH, pleckstrin homology; HA, hemagglutinin.
was capable of self-association (29).

A potential role for the putative coiled-coil region of dynamin emerged from our previous analysis of the inhibitory endocytic phenotype in mammalian cells transiently transfected with a dominant negative, C-terminal deletion mutant (30, 31). Inhibition was unaffected by removal of the proline-rich domain but was reversed by further deletion into the putative coiled-coil region, suggesting that this part of the molecule might mediate the interaction of mutant dynamin with the endogenous wild-type protein (31). To gain further insight into the mechanism underlying the inhibitory phenotype and to understand the mechanism of dynamin self-association, we have used a series of deletion mutants to define sites of dynamin self-association and identify two distinct regions. Analysis of peptides reveals that at least three sequences in dynamin-1 are capable of self-association. These results suggest a critical role for the coiled-coil region in dynamin self-assembly.

EXPERIMENTAL PROCEDURES

cDNA Constructs—The dynamin-lab isoform (1) was used for most experiments. C-terminal and internal deletion mutants were made via polymerase chain reaction and subcloned into the mammalian expression vector, pSVL (Amersham Pharmacia Biotech). Polymerase chain reaction was also used to fuse the HA (32) or Myc epitope (33) to the N terminus, and all constructs were sequenced to ascertain that the mutations and epitope tags were correct. The dynamin-2b isoform (31) was also Myc-tagged at the N-terminal end using polymerase chain reaction.

Co-Immunoprecipitation Assay for Dynamin Self-association—For the co-immunoprecipitation assays, COS-7 cells were transiently transfected with both HA- and Myc-tagged dynamin-1 constructs using the LipofectAMINE reagent (Life Technologies, Inc.) as directed by the manufacturer. Cytosolic extracts of the co-expressed proteins were prepared by lysing the transfected cells in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and clearing the lysates at 30,000 × g for 20 min to remove any cellular debris. After preclearing the supernatants with protein A beads (Amersham Pharmacia Biotech) for several hours, fresh protein A beads were added along with either anti-HA monoclonal antibody (Babco, Berkeley, CA) or anti-Myc polyclonal antibody (a gift of Dr. Melissa Gee, University of Mass. Medical School, Worcester, MA). Co-immunoprecipitation assays were carried out overnight at 4 °C with end-over-end rotation, after which the beads were washed several times in NET gel buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% gelatin, 0.1% Nonidet P-40) and then resuspended in an equal volume of SDS sample buffer for SDS-polyacrylamide gel electrophoresis. Western analysis was done using either the HA monoclonal antibody (Babco) or the Myc monoclonal antibody (9E10, ATCC, Manassas, VA), and chemiluminescence (Biorad) was used to detect any protein interactions (Kirkgaard and Perry Laboratories).

Yeast Interaction Trap—The LexA-based interaction trap (34) was kindly provided by Dr. Erica Golemis (Fox Chase Cancer Center, Philadelphia, PA) and used to assay for dynamin-dynamin interactions in yeast. Wild-type dynamin-1 was subcloned into the JG202 bait expression vector, which contains a nuclear localization signal. Wild-type and deletion mutants of dynamin were constructed in the JG4-5 prey vector. We tested the suitability of the wild-type dynamin-1 in JG202 as bait and found that it did not activate transcription alone. The lithium acetate method was used to make yeast competent cells and for transformations (35). Dynamin-dynamin interactions were scored positive if transcription of the lacZ and LEU2 reporter genes were activated.

Sucrose Density Gradient Centrifugation—Sucrose density gradients (5–20%) were prepared in 20 mM Tris, pH 7.4, 50 mM KCl buffer. Cytosolic extracts of COS cells transfected with Myc-tagged dynamin constructs were prepared as described above. For each gradient, 500 μl of the extract and 10 μg of each molecular mass marker (Sigma) were loaded. Centrifugation was at 26,500 rpm and 4 °C for 17 h. Fractions were collected at 20-s intervals and analyzed by SDS-polyacrylamide gel electrophoresis. Detection of dynamin self-association using a Myc monoclonal antibody. The positions of the molecular mass markers in the gradients were detected by Coomassie stain. Densitometric measurements were made on a Bio-Rad model GS-700 Imaging Densitometer scanner interfaced to the Bio-Rad Multi-analyst computer program (version 1.1). Protein concentrations were determined by the Bradford assay (Bio-Rad), and the S values for the markers are as follows: bovine serum albumin, 4.5 S; alcohol dehydrogenase, 7.5 S; β-amylase, 9 S.

Endocytosis Assays—Transferrin uptake was measured in COS cells as described previously (30) with the following modifications. After transient transfection of the cells with Myc-tagged dynamin constructs, the cells were grown in serum-free medium for 24 h. Transferrin endocytosis was then stimulated by adding 20 μg/ml of fluorescein isoioctyanate-labeled transferrin (Molecular Probes) for 10 min at 37 °C, after which uptake was stopped by placing the cells on ice and washing several times with ice-cold phosphate-buffered saline. Cells were then fixed with 4% paraformaldehyde for 15 min at room temperature and subsequently extracted with either ice-cold methanol for 5 min or 0.5% Triton X-100 for 2 min. Cell staining was done as described previously (30), and immunofluorescent imaging was with a Zeiss Axioskop microscope.

Peptide Synthesis and Purification—Synthetic peptides were prepared by solid phase synthesis methodology using a 4-benzylethylamine hydrochloride resin with conventional N-t-butyloxycarbonyl chemistry on an Applied Biosystems model 430A peptide synthesizer as described by Sereda et al. (36). Peptides were cleaved from the resin by reaction with hydrogen fluoride (20 ml/g resin) containing 10% anisole and 2% 1,2-ethanedithiol for 1 h at −5 °C, washed with cold ether several times, extracted from the resin with glacial acetic acid, and then lyophilized. Purification of each peptide was performed by reversed-phase chromatography on a SynChropak semi-preparative C-8 column (inner diameter, 250 × 10 mm; 6.5-μm particle size; 300-A pore size; SynChrom, Lafayette, IN) with a linear AB gradient (ranging from 0.2 to 1.0% B/min) at a flow rate of 2 ml/min, where solvent A is aqueous 0.05% trifluoroacetic acid and solvent B is 0.05% trifluoroacetic acid in acetonitrile. Homogeneity of the purified peptides was verified by analytical reversed-phase chromatography, amino acid analysis, and electrospray quadrupole mass spectrometry.

Circular Dichroism Spectroscopy—CD spectra were recorded on a Jasco J-720 spectropolarimeter (Jasco, Inc.) interfaced to an Epson Equity 380/25 computer running the Jasco DS-500/PS2 system soft-
Dynamin Self-association Is Mediated by Coiled-coil Sequences

were measured from 190 to 255 nm in benign buffer (0.1 M KCl, 0.05 M lacking uracil, histidine, and tryptophan in the presence of 5-bromo-4-deletion mutant preys. Interactions were assayed on galactose plates yeast strain EGY48 using a full-length dynamin-1 bait and dynamin-1 interaction trap.

Constructs.

Is inhibited in the cell overexpressing the K44E mutant construct but is restored in cells expressing the doubly mutant constructs.

RESULTS

Assays for Dynamin Self-association—As a first step in defining a self-association domain in dynamin-1, we tested for interactions between epitope-tagged dynamin-1 constructs co-expressed in COS cells. Full-length Myc- and HA-tagged wild-type dynamin-1 (C851; Fig. 1, A and B; see also Fig. 7) were Myc-tagged, and their expression was detected by the Myc monoclonal antibody. 9E10. Transferrin internalization was measured in COS-7 cells transiently transfected with wild-type dynamin-1 bait and dynamin-1 interaction trap.

Interpretation Program, version 1.01) was employed to calculate the partial specific volume of the protein from the amino acid composition in the NonLin analysis program. The program Sednterp (Sedimentation Equilibrium Analytical Ultracentrifugation—Sedimentation equilibrium ultracentrifugation was performed on a Beckman model XLI Analytical Ultracentrifuge and interference optics as described in the instrument manual (Beckman Instruments). Samples were dialyzed against and run in a 50 mM potassium phosphate, 100 mM KCl, 2 mM dithiothreitol, pH 7 buffer. Six-sector charcoal-filled epon sample cells were used, allowing three concentrations of sample to be run simultaneously. Runs were performed at two or three speeds between 20,000 and 32,000 rpm and were continued until there was no significant difference in scans taken 2 h apart. The data were evaluated using a nonlinear least squares curve-fitting algorithm (37) contained in the Sedimentation Interpretation Program, version 1.01) employed to calculate the partial specific volume of the protein from the amino acid composition as well as the solvent density and viscosity using known values from physical tables.

Data were (version 1.33a). The temperature-controlled cuvette holder was maintained at 20 °C with a Lauda model RMS circulating water bath. The instrument was calibrated with an aqueous solution of recrystallized d-10-(-)-camphorsulfonic acid at 290.5 nm. Results are expressed as the mean residue molar ellipticity \( \theta \) (degrees cm\(^2\) dmol\(^{-1}\)) calculated from the following equation:

\[
\theta = (\theta_{\text{obs}} - M/10 \times 1 \times c)
\]  

(eq. 1)

where \( \theta_{\text{obs}} \) is the observed ellipticity expressed in millidegrees, \( M \) is the mean residue molecular mass (molecular mass of the peptide divided by the number of amino acids), \( l \) is the optical path length in centimeters, and \( c \) is the final peptide concentration in mg/ml. The CD wave scans were measured from 190 to 255 nm in benign buffer (0.1 M KCl, 0.05 M potassium phosphate, 2 mM dithiothreitol, pH 7) at two peptide concentrations, 1 and 0.1 mg/ml, unless otherwise noted in the text. For samples containing TFE, the above buffer was diluted 1:1 (v/v) with TFE.

Sedimentation Equilibrium Analytical Ultracentrifugation—Sedimentation equilibrium ultracentrifugation was performed on a Beckman model XLI Analytical Ultracentrifuge and interference optics as described in the instrument manual (Beckman Instruments). Samples were dialyzed against and run in a 50 mM potassium phosphate, 100 mM KCl, 2 mM dithiothreitol, pH 7 buffer. Six-sector charcoal-filled epon sample cells were used, allowing three concentrations of sample to be run simultaneously. Runs were performed at two or three speeds between 20,000 and 32,000 rpm and were continued until there was no significant difference in scans taken 2 h apart. The data were evaluated using a nonlinear least squares curve-fitting algorithm (37) contained in the NonLin analysis program. The program Sednterp (Sedimentation Interpretation Program, version 1.01) employed to calculate the partial specific volume of the protein from the amino acid composition as well as the solvent density and viscosity using known values from physical tables.

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Fig. 2. Assay for dynamin-dynamin interaction using the yeast interaction trap. Dynamin-dynamin interactions were assayed in the yeast strain EGY48 using a full-length dynamin-1 bait and dynamin-1 deletion mutant preys. Interactions were assayed on galactose plates lacking uracil, histidine, and tryptophan in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Blue colonies are negative. The nomenclature of the various mutants is shown in Table 1.

Fig. 3. Reversal of endocytic inhibition by deletion of assembly sequences. Fluorescein isothiocyanate-transferrin internalization was measured in COS-7 cells transiently transfected with wild-type dynamin-1 (WT), a dominant inhibitory mutant form of dynamin-1 (K44E), or internal deletions of either of the latter construct (K44E/ΔCC1 or K44E/ΔCC2). All constructs were Myc-tagged, and their expression was detected by the Myc monoclonal antibody 9E10. Transferrin internalization is inhibited in the cell overexpressing the K44E mutant construct but is restored in cells expressing the doubly mutant constructs.

Fig. 4. Heterotypic interaction between the dynamin-1 and dynamin-2 isoforms. A, dynamin-2 (Dyn-2) bait was tested for an interaction with the dynamin-1 (Dyn-1) prey or with the JG4-5 vector as a control. Selection was on galactose lacking uracil, histidine, and tryptophan in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside medium. B, co-immunoprecipitation of dynamin isoforms. Myc-dynamin-2 and HA-dynamin-1 were co-expressed in COS-7 cells and immunoprecipitated using the Myc polyclonal antibody. C, beads alone, control. D, anti-Myc immunoprecipitants blotted with Myc (left) polyclonal or HA (right) monoclonal antibody. The arrow points to the dynamin isoform in each blot. IgG, cross-reacting antibody heavy chain.

WT

K44E

K44E/ΔCC1

K44E/ΔCC2

α-myc

Transferrin
precipitation experiments, interactions persisted in prey devoid of either the GTP-binding domain (N272; Fig. 2; see also Fig. 7) or the PH domain (ΔPH; Fig. 2; see also Fig. 7). C-terminal deletions missing some or all of the potential coiled-coil forming sequences resulted in no interaction with the full-length bait (C695, C648, and C514; Fig. 2; see also Fig. 7) as did partial or complete removal of the coiled-coil region between aa 654–741 (ΔCC1, ΔCC2, and ΔCC12; Fig. 2; see also Fig. 7), consistent with our co-immunoprecipitation data.

These data were consistent with our hypothesis that dominant inhibitory dynamin polypeptides act through interaction with endogenous wild-type dynamin protein (30). To test this possibility directly, we assayed for transferrin uptake in cells overexpressing dominant negative dynamin-1 K44E mutants that also had deletions in the self-assembly region. Fig. 3 shows that removal of either of the two self-association sequences identified in our co-immunoprecipitation and yeast interaction trap assays resulted in reversal of the dominant negative endocytic phenotype, i.e. the resumption of transferrin uptake. Because dynamin-1-K44E exhibits its inhibitory effects in cells tagged dynamin-1 constructs were analyzed in 5–20% sucrose density gradients and immunoblotted using anti-Myc antibody. The blots were quantified by densitometry. The densitometric scans were normalized for expressed protein concentration for each construct. S values for markers used are shown at the top. A, data for full-length dynamin-1 (C851) and C-terminal deletions C750 and C695. B, data for full-length dynamin-1 (C851) and the internal deletions ΔPH and ΔCC12.

![Sucrose density gradient centrifugation of various dynamin-1 C-terminal deletion mutants. COS-7-expressed Myc-tagged dynamin-1 constructs were analyzed in 5–20% sucrose density gradients and immunoblotted using anti-Myc antibody. The blots were quantified by densitometry. The densitometric scans were normalized for expressed protein concentration for each construct. S values for markers used are shown at the top. A, data for full-length dynamin-1 (C851) and C-terminal deletions C750 and C695. B, data for full-length dynamin-1 (C851) and the internal deletions ΔPH and ΔCC12.](image-url)

![Buffer-dependent dissociation of C733. The C-terminal dynamin-1 truncation construct C733 was analyzed in 5–20% sucrose density gradients containing either the standard gradient buffer (20 mM Tris, pH 7.4, 50 mM KCl) (A) or 20 mM Hepes, pH 7, 50 mM KCl (B). Other conditions are as described in the legend to Fig. 5.](image-url)
containing primarily the dynamin-2 and dynamin-3 isoforms (30), we also tested for an interaction between isoforms. Interaction between dynamin-1 and dynamin-2 could be readily detected both by the yeast two-hybrid assay (Fig. 4A) and by co-immunoprecipitation (Fig. 4B).

To learn more about the identity of the dynamin self-association products, lysates of COS-7 cells expressing either epitope-tagged wild-type or mutant dynamin-1 were analyzed by sucrose density gradient centrifugation. We established that the COS-expressed Myc-tagged dynamin-1 isoform behaved similarly to rat brain dynamin-1 in the sucrose density gradients (data not shown). Both ran as a 7.5 S peak, indicating that they were not in a highly assembled state under these conditions. The S value was unaffected by the removal of either the proline-rich (Fig. 5A; see also Fig. 7) or PH domains (Fig. 5B; see also Fig. 7). However, deletions involving the self-assembly region identified by our co-immunoprecipitation and yeast interaction trap experiments resulted in a shift of the 7.5 S peak to 4.5 S (C695; Fig. 5A; see also Fig. 7; and ΔCC12; Fig. 5B; see also Fig. 7), consistent with dissociation to a smaller species. No such shift was produced by deletion to aa 733 (C733; Figs. 6 and 7), which reverses the dominant inhibitory endocytic effect (31), despite removal of several amino acids from the C terminus of the self-assembly domain. However, partial dissociation was observed in a different gradient buffer (20 mM Hepes, pH 7, 50 mM KCl; Figs. 6 and 7). These results indicated a decrease in the affinity between polypeptides, although the ability to self-associate was not abolished completely.

**Peptide Analysis**—The domain implicated in dynamin-1 self-assembly contains two short, closely spaced sequences (aa 654–681 and aa 712–740) predicted by the Newcoils program (28) to have a propensity for coiled-coil formation based on analysis of a database of known coiled-coil sequences (Fig. 8A). However, the probability is only ~60%, with no more than four heptad repeats predicted for each region. Newcoils also predicts at even lower probability three short sequences downstream of the GTPase domain between aa 295–470 (Fig. 8A). Paircoils, which is based on pairwise residue correlations (38) yielded a very different set of predictions, including a short sequence in near the center of the dynamin-1 polypeptide (Fig. 8B). Because of the disparity in the two predictive approaches, we also examined dynamin-1 using a recently developed nonstatistically based algorithm termed Stablecoils, which is based on the direct analysis of coiled-coil formation by synthetic peptides of

![Fig. 7. Summary of the mapping results](image_url)

**Fig. 7. Summary of the mapping results.** Schematic representation summarizing the cDNA constructs used to map the C-terminal self-assembly region by co-immunoprecipitation (Co-IP), yeast two-hybrid (2-Hybrid), and sucrose density gradients (S-value). ++, very good interaction; +, good interaction; +/-, detectable interaction; −, no interaction; Pro-rich, proline-rich domain.

![Fig. 8. Secondary structure analysis](image_url)

**Fig. 8. Secondary structure analysis.** Newcoils (A), Paircoils (B), and Stablecoils (C) plots of dynamin-1 are shown, along with sequences selected for the design of synthetic peptides (bottom).
systematically varied composition (39). The aa 651–675 region was judged to be the most hydrophobic with the highest propensity for amphipathic helix formation (Fig. 8C). A lower, but significant, propensity for coiled-coil formation was observed for aa 715–738, and additional sequences were identified in the central portion of the polypeptide that differed from those identified by Newcoils and Paircoils (Fig. 8).

To determine which of these short sequences in fact had properties consistent with coiled-coil formation, we analyzed synthetic peptides corresponding to five distinct regions by circular dichroism and equilibrium ultracentrifugation (Table I). The CD spectrum of the D1 peptide, which was derived near the GTPase domain of the dynamin-1 polypeptide, was predominantly random coil. We note, however, that in 50% TFE, a helix-inducing hydrophobic solvent, it could be forced into an \( \alpha \)-helical conformation, indicating that this region of dynamin has the intrinsic ability to adopt a helical secondary structure (data not shown). Similarly, the centrally derived D5 peptide exhibited a mixture of random coil and \( \beta \)-sheet, but not \( \alpha \)-helical, structure in benign solution (Fig. 9A). In contrast, peptides D2, D3, and D4 exhibited \( \alpha \)-helical CD spectra, with the characteristic minima at 208 and 222 nm (Fig. 9B). An increase in \( \alpha \)-helicity (10–20%) was observed for the D2 and D4 peptides at higher concentrations, whereas limited solubility of D3 precluded comparable analysis. CD spectra for a mixture of D2 and D4 were additive (data not shown). Curiously, D3/D4 or D2/D3 mixtures were less than additive (data not shown). This may be due to nonspecific interactions by D3, which also caused a similar, but smaller, decrease in the mean residue molar ellipticity at 222 nm when mixed with a known coiled-coil forming peptide from an unrelated protein, MMKif (Ref. 39 and data not shown). Peptides D2, D3, and D4 all exhibited low stability and were completely unfolded at low concentrations of guanidine HCl (0.75M; Fig. 10). Table II summarizes the CD data.

Equilibrium ultracentrifugation of peptides D2, D3, and D4 revealed that each is capable of oligomerization. The sedimentation data for D2 and D4 each fit to a homogenous species with mass average molecular masses of 14,051 and 15,210 Da, respectively, in both cases consistent with the formation of a tetramer (Fig. 11, A and B). D3 was observed to have a mass average molecular mass of 23,873 Da, which approaches a hexameric species (Fig. 11C). This suggests that D3 may not have a strictly defined oligomeric state but does have the ability to form helical bundles.

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### Table I

| Peptide | Sequence |
|---------|----------|
| D1 (295–318) | Ac-CGGGLENKQSOLLTRKPEEKDEYKHF-amide |
| D2 (323–352) | Ac-CGGARKTAKLQMQQGAFEDFKEKIESGDQGD-amide |
| D3 (652–681) | Ac-CGGDPQERQVETIRNLDSIAGNAKVTVDLM-amide |
| D4 (712–740) | Ac-CGGNTLEDSAESDCGREDHLMWARKEALS-amide |
| D5 (372–404) | Ac-CGGRFFPFLVELFREDKKEKRESYMAIVGHGTRFT-amide |

* Peptide sequence name followed by residues (indicated in parentheses) substituted from that of native rat dynamin-1.

* Amino acid sequence of the peptides. Residues proposed to form the 3–4 hydrophobic repeat (positions “a” and “d” of the heptad repeat “abcdefg,” characteristic of coiled coils) are underlined. “Ac” denotes N-acetyl, and “amide” denotes C-amide. A Cys-Gly-Gly linker has been added to the native sequences.
DISCUSSION

Our results implicate two portions of dynamin in self-association, a complex region located between the PH and proline-rich domains and a region near the center of the polypeptide (Fig. 12A). We will refer to these regions as the C-terminal and central self-assembly domain, respectively. Our peptide analysis further identified coiled-coil forming sequences within both regions (Fig. 12A) and implicates coiled-coils as a primary mode of dynamin-dynamin interaction. Our results confirm our hypothesis that the C-terminal region mediates the interaction between dominant inhibitory dynamin mutants and endogenous wild-type protein. Furthermore, the identification of two distinct coiled-coil domains has important implications for dynamin structural organization, as discussed below.

Nature of Self-assembly Species—A significant issue in this study is the stage in self-assembly in which these regions participate. Dynamin has been reported to exist in the unassembled state primarily as a tetramer (14, 16), although whether this species represents a stable, minimal assembly unit is unclear. Dynamin and the Mx proteins also assemble to partial rings, complete rings, and helical stacks of rings. Our analysis has dealt primarily, if not exclusively, with dynamin in the disassembled state. Our immunoprecipitation studies were performed at a sufficiently high ionic strength range to prevent assembly (14). The sucrose gradient analysis, although performed at lower ionic strength, revealed directly that the protein was in the disassembled state. The S value of 7.5 is consistent with a tetramer, albeit a highly assymetric structure. This species is clearly oligomeric in view of the sharp decrease in S value caused by small changes in C-terminal sequence. The state of self-association of dynamin-1 expressed in yeast is uncertain. The N-terminal fusion sequences could inhibit assembly, although N-terminal GFP-dynamin appears to function normally in cells (5).2 Because our mapping studies are so closely consistent with the results of our co-immunoprecipitation and centrifugation analysis, it is likely that the two-hybrid analysis primarily reflects monomer-dimer or monomer-tetramer formation, with one intriguing exception.

Previous studies of dynamin pointed to a role for the proline-rich domain in dynamin self-assembly (9, 14, 16). However, in

2 R. J. Vasquez and R. B. Vallee, unpublished observations.
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Fig. 12. Models for the structural organization of dynamin tetramers. A, summary of peptide analysis. Dark bars, location of peptides that were α-helical by CD analysis; dotted bars, location of peptides that were random coil or a mixture of random coil and β-sheet by CD; Assembly, self-assembly regions identified in this paper; R, shortest C-terminal deletion (aa 733) at which receptor-mediated endocytosis is observed in a dominant negative dynamin-1 mutant (see Ref. 31). B, dynamin-1 polypeptides are shown either in a parallel (model I) or anti-parallel (model II) orientation with coiled-coil sequences corresponding to peptides D2, D3, and D4 linking the individual polypeptides together.

these cases effects on polymer formation were assayed. Our data indicate that the proline-rich region is not required for self-association to the dimer or tetramer level; hence, it must be required for higher order assembly steps. We did observe diminution in the intensity of the yeast two-hybrid reaction using the C750 construct in the prey vector (Fig. 2), although the extent of co-immunoprecipitation was not affected by a comparable deletion, nor was the mutant protein observed to dissociate to the 4.5 S species by sucrose density gradient centrifugation. How the yeast result is to be interpreted is unclear. It may indicate greater sensitivity in detecting changes in affinity between dynamin subunits, or it may indicate that some extent of co-polymerization of bait and prey dynamin does actually occur in this system. We do note that although we tested for interactions between identical truncation mutant constructs in our other assays, full-length bait was used in the two-hybrid analysis. Thus, it is also possible that interaction between wild-type and the C750 mutant dynamin could be sterically affected by the proline-rich region.

The GTPase and PH domains were not required for dynamin self-association in our assays. The former result contrasts with those for the antiviral Mx proteins. Among the dynamin-related family members, the structure of these proteins has been the most extensively characterized. Three self-assembly regions have been mapped within the Mx1 protein. The monomer-to-polymer transition was assayed by gel filtration chromatography of full-length and truncation mutant Mx constructs. This analysis identified one self-assembly site within the GTPase domain (40) and a second near the C terminus (29). No role was detected for a putative leucine zipper domain further downstream at the extreme C terminus. In contrast, the latter region was implicated in dimerization in a separate study in which it was fused to a reporter enzyme. The basis for the difference in these results is unclear. The latter results, however, seem to be more nearly consistent with our findings for dynamin. Although we find no obvious sequence homology between the leucine zipper region of Mx and the C-terminal assembly domain of dynamin, their secondary structures seem to be related. Thus, it is likely that C-terminal coiled-coils may play a common role throughout the greater dynamin protein family, a conclusion supported by secondary structure predictions for other family members (41).

Role of Coiled-coils—Secondary structure predictions for dynamin-1 by the Newcoils, Stablecoils, or Paircoils algorithms suggested several possible coiled-coil regions in dynamin-1, although the predictions differed substantially. We tested the ability of five of these regions to form interacting α-helical structure using synthetic peptides. Intriguingly, the predictive success of each algorithm was variable. It is not yet clear whether this outcome reflects the choice of peptide boundaries or features of the predictive schemes. Three of the five peptides exhibited clear evidence for α-helix formation by CD spectroscopy: D3 and D4 within the C-terminal self-assembly domain, identified in our analysis of recombinant dynamin polypeptides, and D2, derived from the center of the polypeptide. D3 was predicted to have a 60% probability of coiled-coil formation by Newcoils, only 11% probability by Paircoils, and the strongest propensity for coiled-coil formation using Stablecoils. D4 also exhibited 60% probability using Newcoils and approximately half the propensity of D3 using Stablecoils. Results were most surprising for the other peptides. D2 showed insignificant probability using Newcoils, only slightly positive probability using Stablecoils, and 11% using Paircoils. We considered D1 worth examining because, despite moderate predictive scores from Newcoils and Stablecoils, the homologous region within dynamin-2 exhibited 95% probability using Newcoils. Nonetheless, the D1 peptide was observed as a random coil by CD. It is possible that the coiled-coil forming sequence within the complete dynamin-1 polypeptide is more extensive, encompassing the D1 region and other regions as well.

In the case of the C-terminal assembly domain our data support the existence of two short stretches of α-helix (D3 and D4) separated by a 31-aa intervening sequence of undetermined structure (pI = ~5.3). Whether this region also participates in coil formation is uncertain; however, the D3 coil cannot be much longer than predicted because proline residues flank the sequence on either side. Although the sequence of this C-terminal assembly domain is conserved among the dynamin
isomers (79%), only the D3 region is consistently predicted by the Newcoils program to form a potential coiled-coil, albeit with varying probability (42–60% between rat dynamin isomers). It is of interest that the D4 region has very low coiled-coil probability in dynamin-2. Inspection of the latter sequence suggests that a single amino acid gap accounts for this difference. That dynamin-2 co-immunoprecipitated with dynamin-1 is significant; this result implies that isomers are interchangeable in the cell. It helps to explain the dominant inhibition of endocytosis by heterologous isomers. In addition, it raises questions about models that implicate dynamin isomers in distinct cellular functions (5). Conceivably, targeting signals exist that override the tendency toward miscibility and segregate isomers to different membranous compartments. However, dynamins appear to be predominantly cytoplasmic (26), where our results suggest they would be free to intermix. Finally, deletion of either of these regions in the dynamin-1-K44E mutant reversed the dominant negative endocytic effect and resulted in transferrin uptake, indicating that these regions are indeed functionally important.

CD spectra of mixtures revealed no obvious evidence for heterodimerization. However, despite their relatively short lengths, peptides D2, D3, and D4 all clearly self-associated. D2 and D4 were observed to be tetrameric, with no evidence for higher order aggregates. That self-association involved coiled-coil formation is supported by the increase in higher order aggregates. Although this is an attractive possibility, it remains to be seen. However, it now seems clear that the coiled-coil segments of dynamin represent a new, functionally important element. Further analysis of these regions should be of considerable value in understanding how assembly of this remarkable protein is controlled.

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