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Domain structure and intramolecular regulation of dynamin GTPase

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Dynamin is a 100 kDa GTPase required for receptor-mediated endocytosis, functioning as the key regulator of the late stages of clathrin-coated vesicle budding. It is specifically targeted to clathrin-coated pits where it self-assembles into 'collars' required for detachment of coated vesicles from the plasma membrane. Self-assembly stimulates dynamin GTPase activity. Thus, dynamin–dynamin interactions are critical in regulating its cellular function. We show by crosslinking and analytical ultracentrifugation that dynamin is a tetramer. Using limited proteolysis, we have defined structural domains of dynamin and evaluated the domain interactions and requirements for self-assembly and GTP binding and hydrolysis. We show that dynamin's C-terminal proline- and arginine-rich domain (PRD) and dynamin's pleckstrin homology (PH) domain are, respectively, positive and negative regulators of self-assembly and GTP hydrolysis. Importantly, we have discovered that the α-helical domain interposed between the PH domain and the PRD interacts with the N-terminal GTPase domain to stimulate GTP hydrolysis. We term this region the GTPase effector domain (GED) of dynamin.

Keywords: dynamin/endocytosis/GTPase

Introduction

Dynamin is a 100 kDa GTPase that plays a key role in constriction of coated pits and budding of coated vesicles (Damke *et al*., 1994; Hinshaw and Schmid, 1995; Warnock and Schmid, 1996). A model integrating dynamin GTPase activity with guanine nucleotide dependent conformational changes involved in targeting and self-assembly (Hinshaw and Schmid, 1995; Warnock and Schmid, 1996) suggests that dynamin is targeted to coated pits in its GDP-bound form. GTP/GDP exchange then triggers dynamin to self-assemble at the neck of the pit, forming a collar. The assembled dynamin hydrolyzes GTP, undergoing a concerted conformational change in the process, which is somehow required for vesicle budding. The GDP-bound dynamin then disassembles and is recycled. While much work remains to establish the validity of this model and to elucidate mechanisms, it is clear that intramolecular interactions are critical to dynamin's cellular function.

Dynamin differs from most members of the GTPase superfamily in that it is large (100 kDa) and has high endogenous GTPase activity (3–12 min^{-1}) and relatively low affinity for GTP (10–100 μM) (Maeda *et al*., 1992; Shpetner and Vallee, 1992; Tuma *et al*., 1993). Several groups have shown by genetic and biochemical means that dynamin is a homooligomer (Kim and Wu, 1990; Maeda *et al*., 1992; Tuma and Collins, 1995). However, the stoichiometry of dynamin's native oligomeric state has not been definitively established (Hinshaw and Schmid, 1995; Tuma and Collins, 1995).

Dynamin is a complex, multidomain protein. The N-terminal third contains the GTPase domain (see Figure 3B) which is the most highly conserved domain in dynamin family members (Warnock and Schmid, 1996; Urrutia *et al*., 1997). Adjacent to the GTPase domain are two regions of unknown function (designated b and c in Figure 3B) with less homology (20–30%) between dynamin family members. All dynamin isoforms, but not more distantly related family members, contain a pleckstrin homology (PH) domain. PH domains are generally thought to be involved in protein–protein or protein–lipid interactions (Shaw, 1995). Dynamin's PH domain interacts with phosphatidylinositol 4,5-bisphosphate ($PI_{4,5}P_2$)-containing lipid vesicles which stimulate dynamin GTPase activity (Salim *et al*., 1996). C-terminal to the PH domain is another region of unknown function (labeled e in Figure 3B). The computer program PAIRCOILS (Berger *et al*., 1995) weakly predicts a short stretch of coiled-coil in all dynamin isoforms in this region with various probabilities (20–50%), suggesting that this α-helical domain may play a role in intermolecular or intramolecular protein interactions. Additionally, a leucine zipper is predicted in the corresponding region in the more distantly related Mx family members (Melen *et al*., 1992). The C-terminal 100 amino acids of the protein comprise a basic, proline- and arginine-rich domain (PRD).

The PRD promotes dynamin self-assembly *in vitro*. When dialyzed or diluted into low ionic strength buffers, dynamin assembles into sedimentable rings and stacks of rings (Hinshaw and Schmid, 1995). However, after subtilisin treatment to remove the PRD, dynamin no longer self-assembles under these conditions (Hinshaw and Schmid, 1995). Importantly, once stacks are assembled, treatment with subtilisin removes the PRD, but does not cause disassembly. Thus, while the PRD can play a role in initiating assembly, interactions between other sites in dynamin are sufficient to stabilize assembled rings. Dynamin self-assembly in turn potently stimulates GTPase activity (Warnock *et al*., 1996). The GTPase activity of dynamin can also be stimulated by functionally diverse, multivalent effector molecules which interact with dynamin through the PRD (Shpetner and Vallee, 1992; Herskovits *et al*., 1993; Tuma *et al*., 1993; Warnock *et al*., 1995). Given the role of the PRD in assembly, it is likely

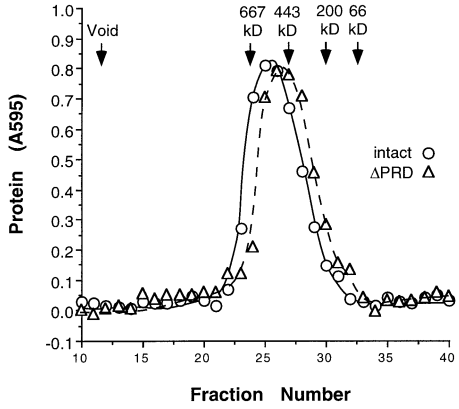

Fig. 1. Dynamin elutes as a multimer following gel filtration chromatography. Superose 6 gel filtration profiles of intact (○) and ΔPRD (△) dynamin in 400 mM potassium phosphate pH 7.0. The elution positions and molecular weights for the void volume, thyroglobulin, ferritin, β-amylase and BSA (arrows, left to right) are indicated.

that these effectors act *in vitro* by triggering or stabilizing dynamin assembly.

The intramolecular interactions involved in self-assembly are clearly important for regulating dynamin GTPase. We sought therefore to identify which regions in this multidomain protein are involved in self-assembly interactions. We have used limited proteolysis to generate structural domains of dynamin, which were then isolated and examined for their activities and interactions. We show that dynamin is a tetramer, and that the PH domain and the PRD act as negative and positive regulators of dynamin GTPase, respectively. These domains flank a C-terminal region of dynamin which is associated with the GTPase domain and required for efficient GTP hydrolysis.

Results

Dynamin is a tetramer

Dynamin exists as a homooligomer that elutes from gel filtration on Superose 6 with an apparent mol. wt of >500 kDa (Figure 1). The ΔPRD mutant of dynamin, obtained by deletion of the proline- and arginine-rich C-terminal 100 amino acid residues, eluted only slightly later than intact dynamin. Western blot analysis revealed that native dynamin present in bovine brain cytosolic fractions co-eluted with recombinant dynamin isolated from baculovirus-infected insect cells (unpublished data). Crosslinking studies to determine the oligomerization state of dynamin have yielded conflicting results (Hinshaw and Schmid, 1995; Tuma and Collins, 1995). We therefore subjected both intact and ΔPRD dynamin to analysis by analytical ultracentrifugation to obtain more definitive evidence for the native mol. wt of dynamin oligomers. Under high salt conditions (see Materials and methods), high molecular weight aggregated and/or assembled species were not detected and the major species present when measured at concentrations ranging from 0.1 to 0.6 mg/ml had calculated mol. wts of 414 000 ± 8500 (n = 3) for intact dynamin and 374 000 ± 45 000 (n = 3) for the ΔPRD. Results from representative experiments are shown in Figure 2. The hydrodynamic properties of dynamin are most consistent with dynamin being an elongated tetramer as suggested previously (Hinshaw and Schmid, 1995).

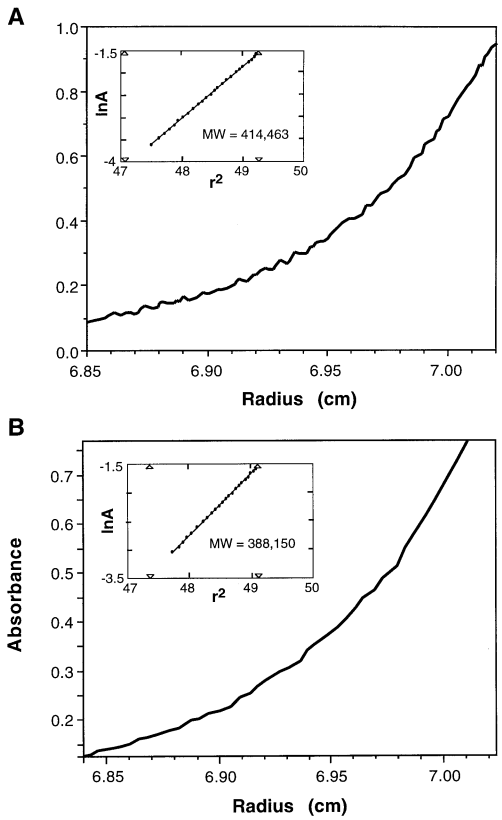

Fig. 2. Analytical ultracentrifugation shows that intact dynamin (**A**) and ΔPRD dynamin (**B**) are tetramers**.** Sedimentation equilibrium profile of dynamins at 0.5 mg/ml in 400 mM potassium phosphate pH 7.0, subjected to centrifugation at 4°C and 7000 r.p.m. The scan was recorded at a wavelength of 228 nm. A graph of ln$A$ versus r^2 is presented in the insets.

Domain structure of dynamin

In order to define the domain structure of dynamin and identify domain interactions involved in oligomerization, we analyzed intact dynamin by limited proteolysis. Figure 3A shows SDS–PAGE of proteolytic products of dynamin after incubation with a variety of proteases for various times. The major products, indicated in Figure 3B, were identified by N-terminal peptide sequencing and/or by Western blot analysis using epitope-mapped antibodies. For fragments where the N- and C-termini have been identified, mol. wts were calculated based on known amino acid composition. Other mol. wts are based on relative mobility on SDS–PAGE. Note that fragments containing region e migrate aberrantly on SDS–PAGE, consistent with their putative α-helical character.

As previously reported (Herskovits *et al*., 1993; Tuma *et al*., 1993; Hinshaw and Schmid, 1995), the PRD was highly protease sensitive: it was digested rapidly by proteinase K (lanes 3 and 4) and trypsin (lanes 5 and 6), and by endoprotease Arg-C and subtilisin (not shown). Digestion with proteinase K, trypsin and endoprotease Lys-C (lanes 7 and 8) each generated a 58 kDa fragment corresponding to the N-terminal half of dynamin and containing the GTPase domain (Figure 3B). The 58 kDa fragment was resistant to further cleavage by Lys-C, but

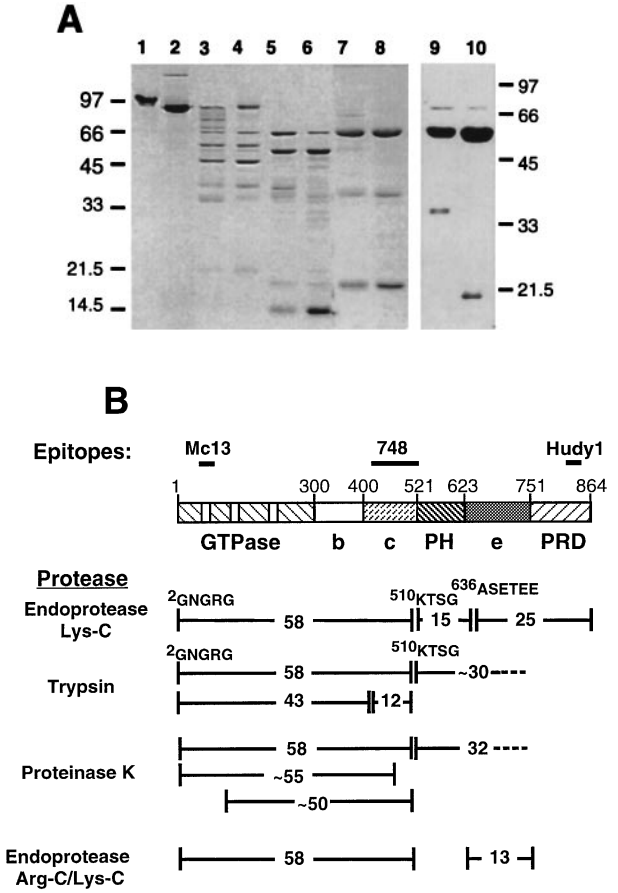

Fig. 3. Domain structure of dynamin dissected by limited proteolysis. (**A**) SDS–PAGE of the proteolytic products of dynamin after incubation with various proteases, as described in Materials and methods. Lane 1, intact dynamin (3 μg); lane 2, ΔPRD dynamin (3 μg); lanes 3 and 4, 1% proteinase K digest, 15 and 60 min, respectively (10 μg); lanes 5 and 6, 1% trypsin digest, 15 and 60 min, respectively (10 μg); lanes 7 and 8, endoprotease Lys-C digest, 1 and 4 h, respectively (10 μg); lane 9, 58 kDa–25 kDa complex isolated following Lys-C digestion (5 μg); lane 10, 58 kDa–13 kDa complex isolated following double digestion with endoproteases Lys-C and Arg-C (7 μg). Lanes 1–8 are from a 13.5% acrylamide gel; lanes 9 and 10 are from a 12% acrylamide gel. The positions of mol. wt markers are indicated. (**B**) Diagram of the domain structure of dynamin and the identity of the major proteolytic fragments as revealed by N-terminal sequencing (as indicated) and Western blot analysis using the antibodies 748, Hudy1 and Mc13, whose epitopes (indicated by the solid bars) have been previously mapped (van der Bliek *et al*., 1993; Warnock *et al*., 1995; Henley and McNiven, 1996). Each of the proteases cleave dynamin at amino acid residue 510, yielding a 58 kDa N-terminal fragment. The mol. wts of the 58, 25, 43 and 15 kDa fragments were calculated based on the known amino acid sequence. For fragments where N- and C-termini have not been identified, the apparent mol. wt as determined by SDS–PAGE is indicated. Note that fragments containing region e, which may encode a coiled-coil, migrate aberrantly on SDS–PAGE.

was cleaved by trypsin to yield a 43 kDa fragment, containing the GTPase domain, and a 12 kDa fragment. Proteinase K (lanes 3 and 4) generated two additional N-terminal fragments of 55 and 50 kDa. Fragments of 30–35 kDa generated by proteinase K and trypsin digestion contain the PH domain and most of the adjacent region e. In addition to the 58 kDa GTPase fragment, Lys-C digestion (lanes 7 and 8) generated a 25 kDa fragment that comprises region e and the PRD, and a 15 kDa peptide corresponding to the PH domain. Thus, the three products of Lys-C digestion of dynamin together make up the entire molecule. A native complex of the 58 and 25 kDa fragments could be isolated from Lys-C-treated dynamin by Q-Sepharose ion-exchange chromatography (lane 9). Digestion of dynamin with a combination of endoproteases Arg-C and Lys-C generated a 58 kDa GTPase fragment and a 13 kDa fragment corresponding to region e which could be subsequently purified by gel filtration chromatography (lane 10).

Domain requirements for dynamin assembly

To examine the domain interactions in both the tetramer and higher-order oligomers of dynamin, we performed crosslinking studies in conjunction with Lys-C treatment. Conditions were chosen (see Materials and methods) which gave efficient crosslinking of intact dynamin into dimers, trimers and tetramers (left panel, Figure 4A). The Lys-C digestion patterns of assembled and unassembled dynamin were the same (right panel, Figure 4A). Thus, dynamin in either the assembled or unassembled state was treated with Lys-C, reacted with the disulfide-containing crosslinker 3,3′-dithiobis[sulfosuccinimidyl propionoate] (DTSSP), and subjected to SDS–PAGE under non-reducing conditions (Figure 4A). The lanes containing the crosslinked sample were then excised, equilibrated with reducing agent and run in the second dimension under reducing conditions. Crosslinked species are found under the diagonal (see arrowheads). The major crosslinks detected in dynamin tetramers under high salt conditions (arrowheads labeled 1 in Figure 4B) occurred between the 58 kDa GTPase fragment and the 25 kDa C-terminal fragment. Since the PRD lacks lysine residues, crosslinking must have occurred through region e. This was confirmed directly by showing that the 58 and 13 kDa products of a Lys-C/Arg-C double digest were crosslinked efficiently to yield a 70 kDa product (not shown). The arrowheads labeled 2 and 3 probably correspond to 25 kDa–25 kDa and 58 kDa–58 kDa crosslinks, respectively. At this resolution, there was no significant difference in crosslinking pattern between unassembled (Figure 4B) and assembled dynamin (Figure 4C), although 58 kDa–58 kDa and 25 kDa–25 kDa crosslinking may have been enhanced. Importantly, no crosslinks were observed to the 15 kDa fragment corresponding to the PH domain (see brackets in Figure 4B and C) in either assembled or unassembled dynamin, suggesting that this domain might not be required for oligomerization.

Further evidence indicating that the PH domain is not required for supramolecular interactions comes from analysis of the self-assembly of Lys-C-treated dynamin. Despite cleavage of the peptide backbone at two sites, Lys-C-treated dynamin assembled upon dilution into low ionic strength conditions (Figure 5, lanes f), as did the intact molecule. Consistent with the crosslinking data, the PH domain remained in the supernatant under assembly conditions, and only the 58 and 25 kDa fragments assembled into sedimentable structures. Structures formed by the isolated 58 kDa–25 kDa complex under low ionic strength were similar to those formed by the intact molecule when examined by negative stain electron microscopy (Figure 6). Thus, the PH domain is not required for dynamin self-assembly.

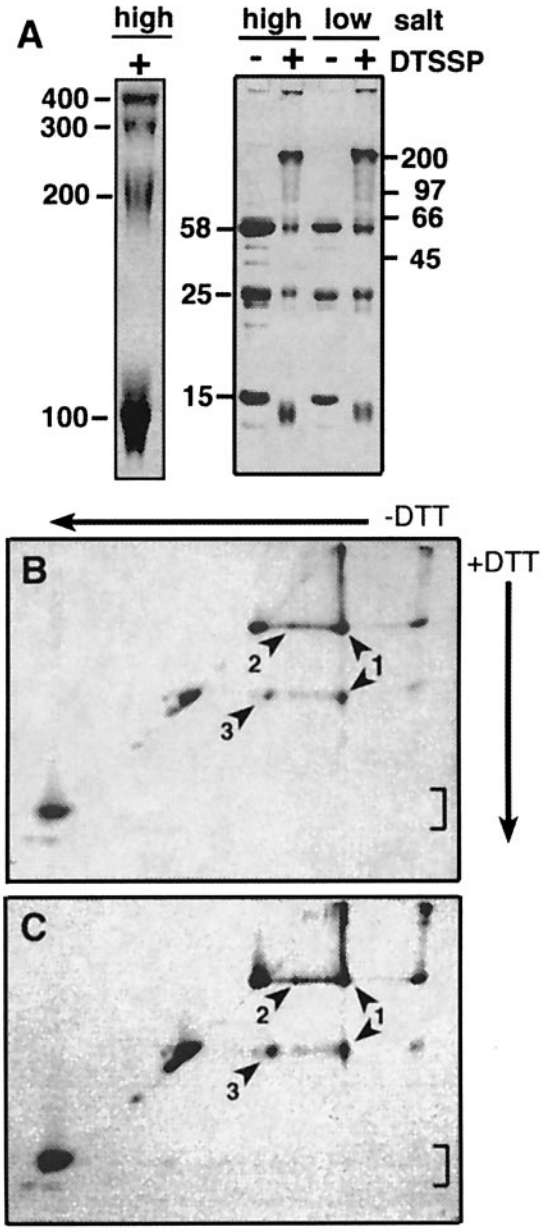

Fig. 4. Interactions between N- and C-terminal domains identified by limited proteolysis and crosslinking. Peak dynamin fractions eluting from Superose 6 gel filtration were crosslinked with Bis(sulfosuccinimidyl suberate) (BS^3) and electrophoresed as described in Materials and methods. Monomers, dimers, trimers and tetramers can be seen in the left-hand panel in (**A**). Dynamin tetramers in either high (unassembled) or low (assembled) ionic strength buffers were treated with Lys-C and interacting fragments crosslinked using the cleavable reagent DTSSP. The right-hand panel in (A) shows the digestion and crosslinking products analyzed by SDS–PAGE under non-reducing conditions. Lanes from gels containing crosslinked samples were excised, incubated with DTT and subjected to a second dimension of SDS–PAGE under reducing conditions. Panel (**B**) shows crosslinked species derived from unassembled dynamin; panel (**C**) shows assembled dynamin. Spots below the diagonal that are aligned vertically indicate species that crosslinked to each other. Arrowheads labeled 1 indicate the major 58 kDa–25 kDa crosslinked species in dynamin tetramers and higher order oligomers. Arrowhead 2 probably corresponds to 58 kDa–58 kDa crosslinks; arrowhead 3 probably corresponds to 25 kDa–25 kDa crosslinks. Brackets indicate the position of the 15 kDa fragment.

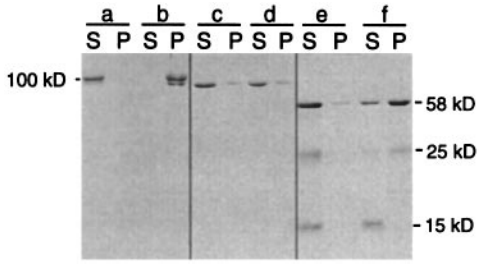

Fig. 5. The PRD, but not the PH domain, is necessary for assembly. A velocity sedimentation assay (Hinshaw and Schmid, 1995) was used to analyze the self-assembly of intact (lanes a and b), ΔPRD (lanes c and d), and Lys-C-treated dynamin (lanes e and f). Dynamins were diluted 6-fold into either assembly (HCB0; b, d and f) or non-assembly (HCB150; a, c and e) conditions. S indicates soluble fraction, and P the fraction that pelleted during centrifugation at 100 000×*g* for 10 min. The soluble and pelleted fractions were separated and analyzed by SDS–PAGE.

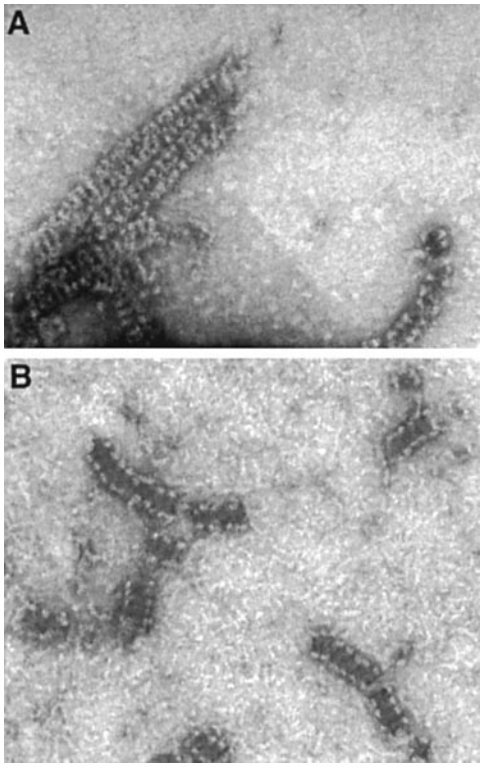

Fig. 6. The 58 kDa–25 kDa complex forms structures similar to those formed by intact dynamin by negative stain electron microscopy. Intact dynamin (**A**) or 58 kDa–25 kDa complex (**B**) was dialyzed against HCB20, then diluted to 0.1 mg/ml and examined as in Materials and methods. Magnification is 67 000×.

Domain requirements for GTPase activity

We next analyzed the domain requirements for GTP hydrolysis. Dynamin exhibits an intrinsic rate of GTP hydrolysis, which is independent of self-assembly, and corresponds to that observed for the ΔPRD dynamin (Warnock,D.E. *et al*., 1997). In addition, dynamin GTPase can be stimulated by self-assembly when assayed at higher protein concentrations (Warnock *et al*., 1996). When assayed at low concentrations (1 μM), intact and ΔPRD dynamin (filled circles and filled triangles, respectively, in Figure 7A) exhibit similar GTPase activity (2 ± 0.6 min^{-1} and 1.6 ± 0.5 min^{-1}, respectively; $n = 4$ for all). Interestingly, the GTPase activity of the 58 kDa–25 kDa

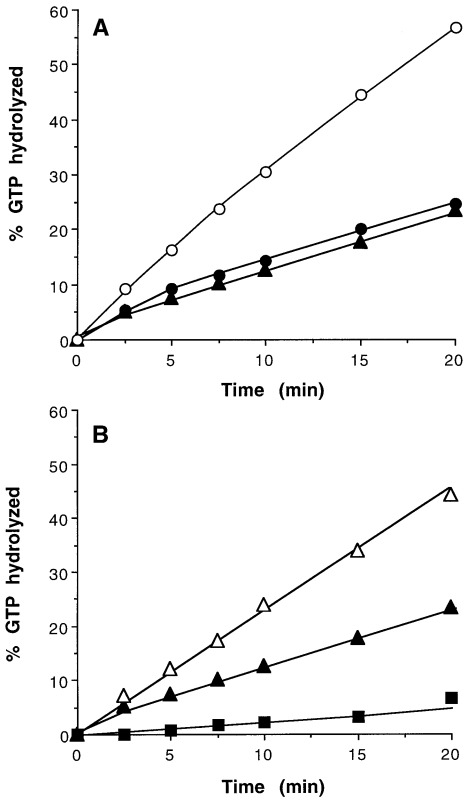

Fig. 7. GTPase activity of intact, ΔPRD, 58 kDa–25 kDa, 58 kDa–13 kDa and trypsin-treated dynamin. GTPase activity of dynamin and proteolytic domains was assayed as described in Materials and methods. Protein concentrations used were 1 μM. (**A**) Intact dynamin (●), ΔPRD dynamin (▲), or 58 kDa–25 kDa (○) complex. (**B**) Replotted ΔPRD dynamin (▲), 58 kDa–13 kDa (△) complex and the 60 min trypsin digest of dynamin (■), which comprises 58 kDa and 43 kDa N-terminal fragments. Data shown are representative of three experiments.

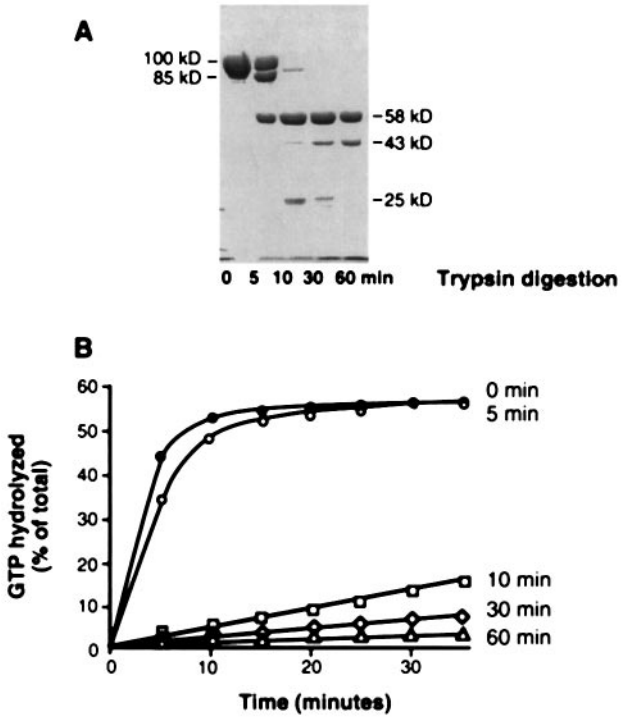

Fig. 8. Loss of the 25 kDa fragment correlates with a decrease in GTPase activity. (**A**) SDS–PAGE of time course of 1% tryptic digest of dynamin, as in Materials and methods for Figure 3A. Each lanes contained 5 μg. (**B**) GTPase assay of 0.5 μM dynamin from each time point shown in (A). Data shown are representative of three experiments, and were collected as indicated in Materials and methods.

complex (open circles; 3.5 ± 0.2 min^{-1}) was consistently nearly twice that of the intact molecule. Similarly, the 58 kDa–13 kDa complex derived from Lys-C/Arg-C double digestion (Figure 7B; open triangles) showed higher GTPase activity (2.5 ± 0.3 min^{-1}) than ΔPRD dynamin (1.6 ± 0.5 min^{-1}; filled triangles). These data indicate that the PH domain is not required for GTP hydrolysis, and that it may act as a negative regulator of GTPase activity in the context of the intact molecule.

To test whether the GTPase domain alone was sufficient for hydrolysis, or whether the 13 kDa domain was necessary, we attempted to functionally isolate the GTPase domain from the remainder of the molecule. Since the 13 kDa domain is tightly bound to, and purifies with, the 58 kDa domain even in high salt, this was accomplished by analysis of the endpoint products of trypsin digestion (see Figure 3B). As can be seen in Figure 7B, this mixture of 58 and 43 kDa GTPase domain-containing fragments (filled squares) hydrolysed GTP much more slowly (0.32 ± 0.2 min^{-1}; n = 3) than ΔPRD dynamin. These data suggested that the C-terminal regions of dynamin might be required for efficient GTP hydrolysis. This was confirmed by analysis of GTPase activity throughout a time course of trypsin digestion (Figure 8A and B). The loss of GTPase activity correlated with loss of intact dynamin and proteolysis within the C-terminal 30 kDa fragment. Since cleavage of the PRD does not affect intrinsic GTPase activity, these data suggest that the 13 kDa domain has a role in GTP hydrolysis. At late time points, when the C-terminal domain was no longer detected, the rate of GTP hydrolysis was reduced ~5-fold relative to ΔPRD dynamin, and ~8-fold relative to the 58 kDa–13 kDa complex. These data indicate that the 58 kDa fragment alone inefficiently hydrolyzes GTP and that an intact 13 kDa domain is necessary for full intrinsic GTPase activity.

GTP binding versus hydrolysis

The reduced activity of the GTPase domain could be due to impaired nucleotide binding or catalysis or both. Therefore, we tested whether the dynamin fragments could bind GTP as effectively as the intact molecule. Dynamin was incubated with [α-^{32}P]GTP, subjected to a UV photocrosslinking procedure (Yue and Schimmel, 1977) to form covalent bonds between the nucleotide and the protein, and then analyzed by SDS–PAGE. The Coomassie-blue-stained gel of these species and the corresponding autoradiograph are shown in Figure 9 panels A and B, respectively. All fragments containing the GTPase domain were able to bind GTP. These included intact as well as ΔPRD dynamin, the 58 kDa fragment of both the 58 kDa–25 kDa and 58 kDa–13 kDa complexes and the 58 and 43 kDa fragments from trypsin digestion. GTP binding was specific, as labeling was prevented in the presence of excess unlabeled GTP. Figure 9C shows a titration of unlabeled GTP for intact and trypsinized dynamin. The affinity for GTP did not seem to vary between these molecules, and the half-maximal disappearance of labeling

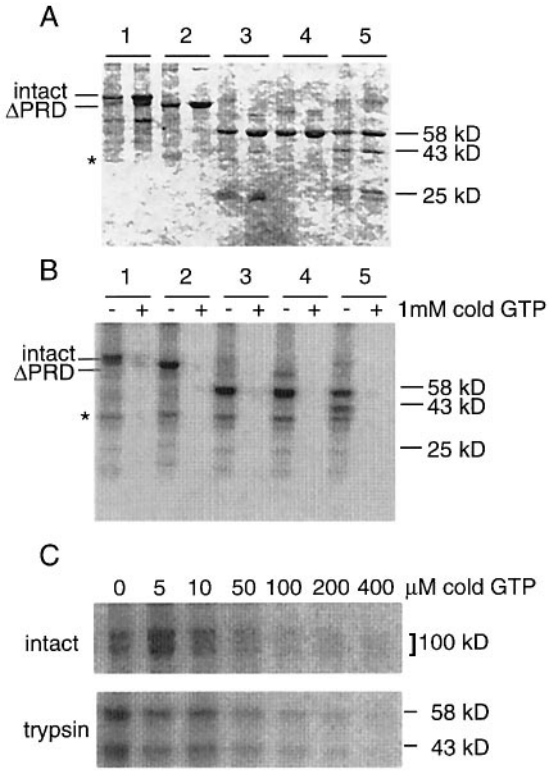

Fig. 9. Isolated N-terminal GTPase domains retain their ability to bind GTP. Dynamin and its proteolytic products were incubated with 5 μM [α-^{32}P]GTP in the presence or absence of 1 mM unlabeled GTP as indicated, and irradiated with UV light as described in Materials and methods. The reactions were analyzed by SDS–PAGE and stained for protein (**A**) before autoradiography (**B**). Lane 1, intact dynamin; lane 2, ΔPRD dynamin; lane 3, 58 kDa–25 kDa complex; lane 4, 58 kDa–13 kDa complex; lane 5, trypsin-treated dynamin. Molecular weights are indicated for dynamin and major fragments. The band at ~35 kDa (indicated by the star) that binds GTP appears to be a minor contaminant, because it is seen in intact dynamin as well as the digested forms, and is only very weakly detected by Coomassie blue staining. (**C**) The indicated amounts of excess unlabeled GTP were added to compete with GTP binding of intact and trypsinized dynamin as described in Materials and methods.

occurred between 10 and 50 μM, corresponding well with previously obtained values for K_m (Maeda *et al*., 1992; Shpetner and Vallee, 1992; Tuma *et al*., 1993). Thus, the compromised GTP hydrolysis of trypsin-treated dynamin was not due to a defect in nucleotide binding. These data are consistent with the hypothesis that association of the 13 kDa fragment derived from the α-helical domain with the GTPase domain is required for efficient GTP hydrolysis.

Discussion

We have established that dynamin is a tetramer by a combination of crosslinking and analytical ultracentrifugation. A previous study on dynamin in dilute solution used crosslinkers with a zero-length spacer arm, and although dimers were the prevalent crosslinked species, larger species were also detected (Tuma and Collins, 1995). Given that crosslinking under these conditions is inefficient, any crosslinking products observed should be authentic. Therefore, these previous findings are also consistent with dynamin being a homotetramer. The tetrameric nature of dynamin has interesting implications for analysis of dominant-negative mutants *in vivo*, where the formation of heterotetramers between endogenous and exogenous dynamin can affect the interpretation of results.

Limited proteolysis was used in combination with further crosslinking studies to identify domain interactions involved in dynamin tetramerization. The major domain contact in dynamin tetramers is between the 58 kDa N-terminal GTPase fragment and region e within the 25 kDa Lys-C C-terminal fragment. Homotypic interactions between 58 kDa fragments and 25 kDa fragments were also detected, although to a lesser extent. Region e contains sequences with a weak coiled-coil character, consistent with its involvement in protein–protein interactions. Leucine repeats near the C-terminus of a number of Mx family proteins have also been implicated in oligomerization (Melen *et al*., 1992). A second motif required for higher order assembly has been identified within the GTPase domain of Mx1 (Nakayama *et al*., 1993). Thus Mx family members and dynamin appear to require both C- and N-terminal sequences for oligomerization.

In addition to its structural role, the interaction between the GTPase domain and the 13 kDa domain is required for efficient GTPase activity. The GTPase activity of the 58 and 43 kDa tryptic digest fragments is strongly impaired, even though they encode the entire GTPase domain. In contrast, the 58 kDa–13 kDa complex isolated following Lys-C/Arg-C double digestion efficiently hydrolyzes GTP. These results establish that interactions between the 13 kDa domain and the GTPase domain are required for efficient GTP hydrolysis. Therefore, we propose the name GTPase effector domain, or GED, for this region. As yet, it is unclear which step of the GTPase cycle is catalyzed by the GED. However, it is likely that the GED serves either as a GTPase activating protein (GAP) (to accelerate GTP hydrolysis) or as a guanine nucleotide exchange factor (GEF) (to accelerate GDP dissociation or exchange) (reviewed in Bourne *et al*., 1991), because GTP binding was unaffected in its absence.

The existence of an intramolecular regulatory region is not without precedent in the GTPase superfamily. Ef-Tu encodes an effector domain (Nock *et al*., 1995) that increases the rate of GTP hydrolysis. Similarly, the α subunit of heterotrimeric GTPases consists of two domains—a small Ras-like domain that hydrolyzes GTP, and an activating domain that stimulates the hydrolysis of GTP through interaction with the GTPase domain (Markby *et al*., 1993). Given that dynamin is a tetramer, two models consistent with the crosslinking results are possible (Figure 10). In the first, interactions occur between the N- and C-terminal regions of the same polypeptide (model 1). In the second, these interactions are proposed to occur between adjacent, antiparallel polypeptides (model 2). We favor this latter possibility because it is more consistent with self-assembly proceeding by reiteration of tetramer contacts. Through domain exchange between subunits, conformational changes due to GTP hydrolysis in one tetramer could be propagated throughout an assembled dynamin ring to effect concerted activity of the collar, as we have proposed occurs at later stages of endocytosis. Regulation of the GTPase domain of one polypeptide by the GED of another in the tetramer could allow all the

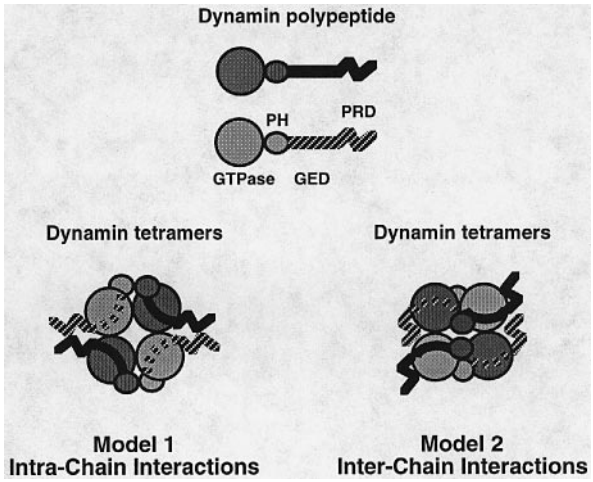

Fig. 10. Two models for the structure and function of the GTPase effector domain (GED). The GED could catalyze GTP hydrolysis through interactions with the GTPase domain on the same polypeptide (model 1), or on an adjacent polypeptide (model 2) in the dynamin dimers/tetramers.

molecules in a collar to 'talk' to each other, making such a concerted activity possible. Experiments are under way to distinguish between the two models.

Studies on the antiviral Mx proteins, which have low homology to dynamin, have suggested that a 10 kDa C-terminal domain of the protein folds back on to the GTPase domain and regulates its activity. Strikingly, while large (250 amino acid) internal deletions of MxA reduce GTP hydrolysis by ~10-fold, C-terminal deletions of as few as seven amino acids completely inactivate the protein (Schwemmle *et al.*, 1995). Mx proteins are oligomeric (Melen *et al.*, 1992; Nakayama *et al.*, 1993; Richter *et al.*, 1995) and it has not been established whether these interdomain contacts are within or between chains. In contrast to our results with dynamin, C-terminal deletion mutants of MxA are unable to bind GTP. Thus, it remains to be seen whether the mechanism of GTP hydrolysis is the same for both groups of GTPases.

The PH domain of dynamin is not required for oligomerization, self-assembly or GTPase activity. In fact, the 58 kDa–25 kDa and 58 kDa–13 kDa complexes, which constitute proteolytically-derived PH domain deletions of intact and ΔPRD dynamin, respectively, have higher intrinsic GTPase activities than their PH domain-containing counterparts. These results suggest that, *in vitro*, the unliganded PH domain may be a negative regulator of GTPase activity. Interestingly, two possible ligands of the PH domain have opposite effects on dynamin GTPase activity. $PI_{4,5}P_2$-containing lipid vesicles stimulate dynamin GTPase (Zheng *et al.*, 1995; Salim *et al.*, 1996), while βγ subunits of heterotrimeric G-proteins are inhibitory (Lin and Gilman, 1996). Together these data are consistent with a role for the PH domain in intramolecular regulation of dynamin GTPase activity. Other dynamin family members, such as the yeast Vps1p, that lack a PH domain appear, nevertheless, to function in intracellular trafficking.

The PRD, in contrast, appears to act as a positive regulator of dynamin GTPase activity (Herskovits *et al.*, 1993; Warnock *et al.*, 1995, 1996). The effects of the ΔPRD are largely mediated through regulation of dynamin self-assembly (Warnock,D.E. *et al.*, 1997). The mechanism by which the PH domain regulates GTPase activity remains to be determined. Interestingly, the GED is located between the PH domain and the PRD. Because both these domains influence dynamin's GTPase rate, the GED may integrate signals from these two regulators to control dynamin function.

Materials and methods

Materials

Trypsin and Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) were from Sigma. Lys-C and proteinase K were from Boehringer Mannheim. Crosslinkers BS^3 and DTSSP were from Pierce. [α-^{32}P]GTP at 400 mCi/mmol was from Amersham. Mouse monoclonal antibody Hudy-1 (Warnock *et al.*, 1995) and rabbit polyclonal antibody 748 (van der Bliek *et al.*, 1993) were as previously described. Antipeptide antibody MC13 (Henley and McNiven, 1996) was generously provided by Mark McNiven (Mayo Clinic, Rochester, MN).

Buffers

HCB is 20 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.0. Numbers after HCB refer to the concentration (mM) of NaCl; for example, HCB150 contains 150 mM NaCl.

Dynamin expression and purification

Wild-type dynamin was expressed in Tn*5* cells and purified as described previously (Warnock *et al.*, 1996), except that no $CaCl_2$ was added to buffers during hydroxyapatite chromatography. The ΔPRD dynamin construct was provided by Hanna Damke, and was generated by the introduction of a point mutation encoding a stop codon at amino acid residue 751 into the hemagglutinin-tagged wild-type dynamin sequence, and transferred into the baculovirus expression system. The protein product has a mol. wt of 85 kDa, and was purified by the same procedure as the intact molecule.

Analytical ultracentrifugation

Sedimentation velocity (SV) and sedimentation equilibrium (SE) centrifugation of dynamin at a concentration of 0.1–0.6 mg/ml (determined by A_{280} where the A_{280} of 1 mg/ml dynamin was 0.464) in 400 mM potassium phosphate pH 7.0 were performed using a Beckman analytical ultracentrifuge model XLA, equipped with absorption optics. SV runs were carried out at 56 000 r.p.m., 8°C, in a 12 mm double-sector cell (Epon). Scan records were taken in the range of 220–235 nm. SE runs were performed at 4°C, 7000 r.p.m., in 4 mm cell. The molecular masses (M_r) were calculated from the SE runs, using a floating baseline computer program that adjusts the baseline absorbance to obtain the best linear fit of ln*A* versus r^2 (where *A* is absorbance and *r* radial distance). The partial specific volume of 0.725 cm^3/g based on the amino acid composition of dynamin was used for all calculations. The density and viscosity of the buffer, for correction to 20°C water, were taken from the *CRC Handbook of Chemistry and Physics*, 70th edn.

Limited proteolysis and N-terminal sequence analysis of dynamin

Purified dynamin at 1 mg/ml in HCB150 was digested at 1% (w/w) with proteinase K and trypsin. Proteinase K was resuspended in 100 mM Ca^{2+} to yield a final Ca^{2+} concentration of 10 mM in the reaction. Endoprotease Lys-C solution (30 U/ml) in 100 mM ammonium bicarbonate, pH 8.0, was used as a 10× stock for digestion. All digests were performed on ice, and reactions were terminated by boiling in sample buffer. Lys-C-treated dynamin was bound to Q-Sepharose resin in HCB150 and eluted in HCB500 to isolate the 58 kDa–25 kDa complex. The protein was then dialyzed against HCB150. To generate the 58 kDa–13 kDa complex, dynamin in HCB250 was digested with 0.25% (w/w) endoprotease Arg-C and 0.5× endoprotease Lys-C for 16 h, precipitated with 35% ammonium sulfate, and purified by gel filtration on Superose 6. Peak fractions were pooled and concentrated using a Centricon 10 (Amicon). N-terminal peptide sequence analysis was performed by the Scripps Research Institute Protein Chemistry Core Facility on fragments after transfer to polyvinylidene difluoride (PVDF) filters.

Crosslinking

Dynamin or ΔPRD dynamin (100 μg) was chromatographed on a Superose 6 gel filtration column in 400 mM potassium phosphate,

pH 7.0. Peak protein fractions (0.5 mL) were crosslinked with 5 mM BS^3 on ice for 1 h and quenched by addition of glycine to 100 mM. Protein was then precipitated with 10% trichloroacetic acid (TCA), washed with cold acetone, and resuspended in Laemmli sample buffer containing 4 M urea for electrophoresis on a 6% acrylamide gel. For two-dimensional analysis, 100 μg of dynamin at 1 mg/ml after overnight dialysis into HCB0 (low salt; assembled) or HCB150 (high salt; unassembled) was digested for 4 h with Lys-C, as described above. The Lys-C digest was stopped with the addition of TLCK to 1 mM and the mixture incubated with 5 mM DTSSP for 1 h on ice before quenching with 100 mM glycine. The sample was then precipitated with 10% TCA, washed with cold acetone, resuspended in 30 μl of urea sample buffer and analyzed by SDS–PAGE on a 13.5% acrylamide gel under non-reducing conditions. Lanes containing crosslinked species were excised, soaked in running buffer with 10 mM dithiothreitol (DTT) for 15 min at room temperature and placed on the stacking gel of a second 13.5% gel for SDS–PAGE under reducing conditions in the second dimension.

Assembly and GTPase assays

Dynamin self-assembly and GTP hydrolysis assays were performed as described elsewhere (Warnock *et al*., 1996), with the exception that HCB45 was used as assay buffer.

GTP binding assays

The GTP binding assay was a modification of the method of Yue and Schimmel (1977). Incubations were on ice for 10 min and contained 3 μg protein in 20 μl HCB45 buffer, 0.3 μCi of [α-^{32}P]GTP and indicated amounts of unlabeled GTP. UV irradiation was carried out at room temperature for 10 min at 1500 μW/cm^2 in a Fisher Biotech UVXL-1000 UV crosslinker. The volumes of samples were made up to 50 μL with 0.15% Triton X-100 in HCB45, then precipitated for SDS–PAGE analysis with 10% TCA. The pellets were washed in cold acetone, resuspended in urea sample buffer and analyzed by SDS–PAGE on either a 12% or 10% acrylamide gel. Gels were stained for protein, dried and exposed on to a Phosphorimager cassette; they were then scanned and the images were viewed with ImageQuant software.

Negative stain electron microscopy

Intact dynamin and the 58 kDa–25 kDa complex were dialyzed against HCB20, then diluted to 0.1 mg/ml. Samples were incubated on electron microscopy grids for 5 min, then washed extensively in HCB20. Grids were stained with 2% uranyl acetate, dried and observed on a Philips CM-10 electron microscope at 50 000× magnification.

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