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Auxilin-Dynamin Interactions Link the Uncoating ATPase Chaperone Machinery with Vesicle Formation

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Summary

The large GTPase dynamin is required for budding of clathrin-coated vesicles from the plasma membrane, after which the clathrin coat is removed by the chaperone Hsc70 and its cochaperone auxilin. Recent evidence suggests that the GTP-bound form of dynamin may recruit factors that execute the fission reaction. Here, we show that dynamin:GTP binds to Hsc70 and auxilin. We mapped two domains within auxilin that interact with dynamin, and these domains inhibit endocytosis when overexpressed in HeLa cells or when added in a permeable cell assay. The inhibition is not due to impairment of clathrin uncoating or to altered clathrin distribution in cells. Thus, in addition to its requirement for clathrin uncoating, our results show that auxilin also acts during the early steps of clathrin-coated vesicle formation. The data suggest that dynamin regulates the action of molecular chaperones in vesicle budding during endocytosis.

Introduction

Clathrin-coated vesicle (CCV) formation at the plasma membrane underlies the process of receptor-mediated endocytosis and proceeds through morphologically and biochemically distinct steps (Kirchhausen, 2000; Schmid, 1997). Coat assembly is initiated with the recruitment of adaptor proteins (APs) to the membrane, where they recruit clathrin and interact with cargo proteins. Clathrin assembly drives the early stages of membrane curvature, leading to formation of the invaginated pit. Subsequently, the invaginated pit becomes constricted, and finally, membrane fission releases the coated vesicle from the membrane. After vesicle release, the coat is disassembled by Hsc70 (Schlossman et al., 1984) and its cochaperones, auxilin-1 (neuronal isoform) (Ungewickell et al., 1995) or auxilin-2 (ubiquitous form) (Greener et al., 2000; Scheele et al., 2001). Whereas Hsc70 is widely known for its CCV uncoating role, it has recently also been implicated in the early growth of coated pits (Newmyer and Schmid, 2001).

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The large GTPase, dynamin, plays an essential role in clathrin-mediated endocytosis (Schmid et al., 1998; Sever, 2002). Dynamin self-assembles into supramolecular coiled structures in vitro (Hinshaw and Schmid, 1995). Assembly stimulates its basal GTPase activity 10- to 100-fold in vitro (Tuma, 1994; Warnock et al., 1996), and GTP hydrolysis drives dynamin disassembly (Maeda et al., 1992; Warnock et al., 1996). These and other observations led to the model that dynamin is a "mechanochemical" enzyme whose stimulated rate of GTP hydrolysis is required to pinch off vesicles from the plasma membrane (Danino and Hinshaw, 2001; Hinshaw and Schmid, 1995; McNiven, 1998; Warnock and Schmid, 1996).

Dynamin's GTPase activity is controlled by an intramolecular domain termed GED (*G*TPase *e*ffector *d*omain, amino acids 618–752 of dynamin) that becomes activated upon dynamin self-assembly (Muhlberg et al., 1997; Sever et al., 1999). Two novel mutations in this domain impair dynamin's assembly-stimulated GTPase activity (Sever et al., 1999) and thus prolong the lifetime of dynamin in the GTP-bound state (dynamin:GTP). Thus, GED appears to be an intramolecular GTPase activating protein (GAP). Unexpectedly, overexpression of dynamin bearing these mutations accelerated the rates of endocytosis (Sever et al., 1999). Subsequent morphological and biochemical analyses revealed that both mutants increased the rate at which constricted coated pits were formed, identifying this step as the rate-limiting step for endocytosis (Sever et al., 2000). Importantly, while the GED mutation that impaired GTP hydrolysis via a defect in self-assembly increased the overall rate of endocytosis, a catalytic mutation in GED also inhibited the subsequent budding step. Together, these results suggested that dynamin acts as a classical regulatory GTPase whose GTP-bound form recruits downstream effectors that execute the constriction step and that impairment of dynamin disassembly as a result of impaired stimulated rate of GTP hydrolysis inhibits the subsequent budding step. Although the idea that dynamin is a classical regulatory GTPase has been challenged (Marks et al., 2001; but see Damke et al., 2001a), more recent data support it. Thus, analogous GED mutations within Dnm1, another dynamin family member, gave identical in vivo phenotypes, including stimulation of mitochondrial fission (Fukushima et al., 2001). In the case of MxA, a dynamin family member with antiviral activity, mutations within GED that impair self-assembly did not abolish antiviral activity (Janzen et al., 2000). These data suggest that all dynamin family members function as regulatory GTPases.

If dynamin acts as a classical regulatory GTPase (Sever et al., 1999), it should be possible to identify effectors that interact with the GTP-bound form of dynamin. Using nucleotide-specific dynamin affinity columns, we have identified two proteins, Hsc70 and auxilin, that specifically interact with dynamin:GTP. The interaction of both proteins with dynamin is direct. We identified two regions in auxilin that interact with dy-

namin, and either their overexpression in HeLa cells or their addition to a permeable cell assay potently inhibits endocytosis. Since the inhibitory phenotype was not due to impairment of vesicle recycling or clathrin dynamics, and could be rescued by addition of dynamin, our data establish a role for auxilin in endocytosis at the formation of constricted coated pits. We propose that dynamin regulates the chaperone machinery to induce controlled conformational changes within the clathrin coat that in turn drive vesicle constriction and fission.

Results

Biochemical Identification of Dynamin:GTP Binding Partners

We used nucleotide-specific affinity chromatography to identify proteins that interact with dynamin in the GTP-bound state. To develop appropriate biochemical conditions for affinity chromatography, we first used the GTPase domain of dynamin as bait (Figure 1A). The GTPase domain fused to GST was immobilized on a column and incubated with rat brain cytosol (RBC) that was supplemented with GED, dynamin's putative GAP domain, and either GDP or GTPγS. GAP domains are known to bind to their cognate GTPases in a GTP-specific fashion (Bourne et al., 1991; Mittal et al., 1996; Scheffzek et al., 1998). After binding, the resin was washed extensively with 250 mM NaCl to generate the first wash (Figure 1B, lanes 1–3), and proteins were subsequently eluted by switching the nucleotide in the column without changing the ionic strength ("second wash"; Figure 1B, lanes 4–6). As shown in Figure 1B (lane 6), GED bound to dynamin's GTPase domain in the presence of GTPγS was released from the column only when GTPγS was replaced by GDP. These observations, together with our previous demonstration that GED potently stimulates dynamin's GTPase activity in a fashion that is sensitive to aluminum fluoride (Sever et al., 1999), strongly argue that GED is a GAP, and we will hereafter refer to it as such. In addition to dynamin's GAP, full-length dynamin from the RBC was also enriched on the GTPγS column (Figure 1B, lane 6).

To identify novel proteins that bind to the GTP-bound form of dynamin, full-length dynamin-1 (the neuronal isoform) was immobilized on the column. A discrete set of $\sim$30 proteins from RBC that was not present on the GST-column (Figure 1C, lane 3) was eluted from the dynamin column in the second nucleotide wash (Figure 1C, lanes 4 and 5). The majority of the proteins bound to dynamin independently of the nucleotide present. This was expected given that a number of SH3 domain-containing proteins have been shown to interact with dynamin's C-terminal proline-arginine rich domain (PRD) independently of nucleotide (Schmid et al., 1998; Slepnev et al., 1998). Thus, amphiphysin-1 bound to the column independently of nucleotide (Figure 1D-I), as did its interacting proteins clathrin, and the α-adaptin subunit of the AP-2 adaptor complex (Slepnev et al., 1998) (Figures 1D-II and 1D-III). In addition, there was also nucleotide-independent enrichment of α/β-tubulin, as expected given that dynamin was originally isolated as a microtubule binding protein (Shpetner and Vallee, 1989) (Figure 1D-IV). Importantly, the γ-adaptin subunit of the AP-1 complex, which functions in conjunction with the ubiquitously expressed dynamin-2 isoform at the trans-Golgi network (Cao et al., 1998; Jones et al., 1998), did not interact with dynamin-1 on the column, even though it was abundant in the extract (Figure 1D–V). Similarly, the early endosomal marker EEA1 (Mu et al., 1995) was not recruited (Figure 1D-VI). Interestingly, endophilin, an SH3 domain protein proposed to be a dynamin effector (Schmidt et al., 1999), was not bound to the column (Figure 1D-VII). Dynamin derived from RBC (as confirmed by mass spectrometry) bound to the GST-dynamin column independently of the nucleotide present (Figure 1D-VIII), consistent with the finding that dynamin self-assembles in a nucleotide-independent manner under some circumstances (Hinshaw and Schmid, 1995; Maeda et al., 1992; Sweitzer and Hinshaw, 1998; Takei et al., 1998). Together, these observations provide compelling evidence that proteins from the extract bound the immobilized dynamin in a highly specific manner.

Dynamin:GTP Binds Directly to Hsc70 and Auxilin

At least three polypeptides were eluted from the GTPγS column but not from the GDP column (Figure 1C, compare lanes 4 and 5; nucleotide specific polypeptides are marked by asterisks). Mass spectrometry and Western blotting identified the largest of these as Hsc70 (Figure 1D-IX; and data not shown). The fact that Hsc70 was released only upon addition of GDP demonstrates that its interaction with dynamin is nucleotide-dependent and argues that it is not simply binding unfolded dynamin on the column. The specificity of Hsc70 action depends on its interactions with cochaperones (Kelley, 1998). A known partner of Hsc70 in vesicle uncoating during endocytosis is the DnaJ type cochaperone, auxilin (Ungewickell et al., 1995). We therefore tested whether dynamin also recruits auxilin. As shown in Figure 1D-X, auxilin was recruited on the dynamin:GTPγS column.

We next asked whether Hsc70 and auxilin bind dynamin directly. We used Hsc70 purified from bovine brains and recombinant auxilin expressed in bacteria. As shown in Figures 2A and 2B (lane 7), both Hsc70 and auxilin interacted efficiently with dynamin, and the interaction was GTPγS dependent (compare lanes 6, 7, and 8). The nucleotide specificity of auxilin binding is particularly apparent if one considers that binding to dynamin:GDP is largely nonspecific (Figure 2B, compare lanes 5 and 6). Due to the presence of a number of proteolytic fragments of auxilin (Figure 2B, lower bands), we next tested whether dynamin can bind a recombinant His-tagged auxilin fragment (aux$^{405\text{-}910}$) (Greener et al., 2000). As shown in Figure 2C, aux$^{405\text{-}910}$ also exhibited GTP-specific binding to dynamin, and we used this fragment in subsequent assays. Binding of dynamin's GAP to dynamin:GTPγS (Figure 2D, lane 7) was used as a control for nucleotide-dependent assay conditions. Together, these data indicate that Hsc70 and auxilin bind to dynamin:GTP directly and independently of one another. This is consistent with a model in which dynamin is not the substrate of Hsc70, but rather serves to recruit Hsc70 and auxilin to a specific location within coated pits.

Auxilin, but Not Hsc70, Inhibits the GTPase Activity of Dynamin

Until now, dynamin itself was the only known protein to interact with dynamin in a GTP-specific fashion (Carr

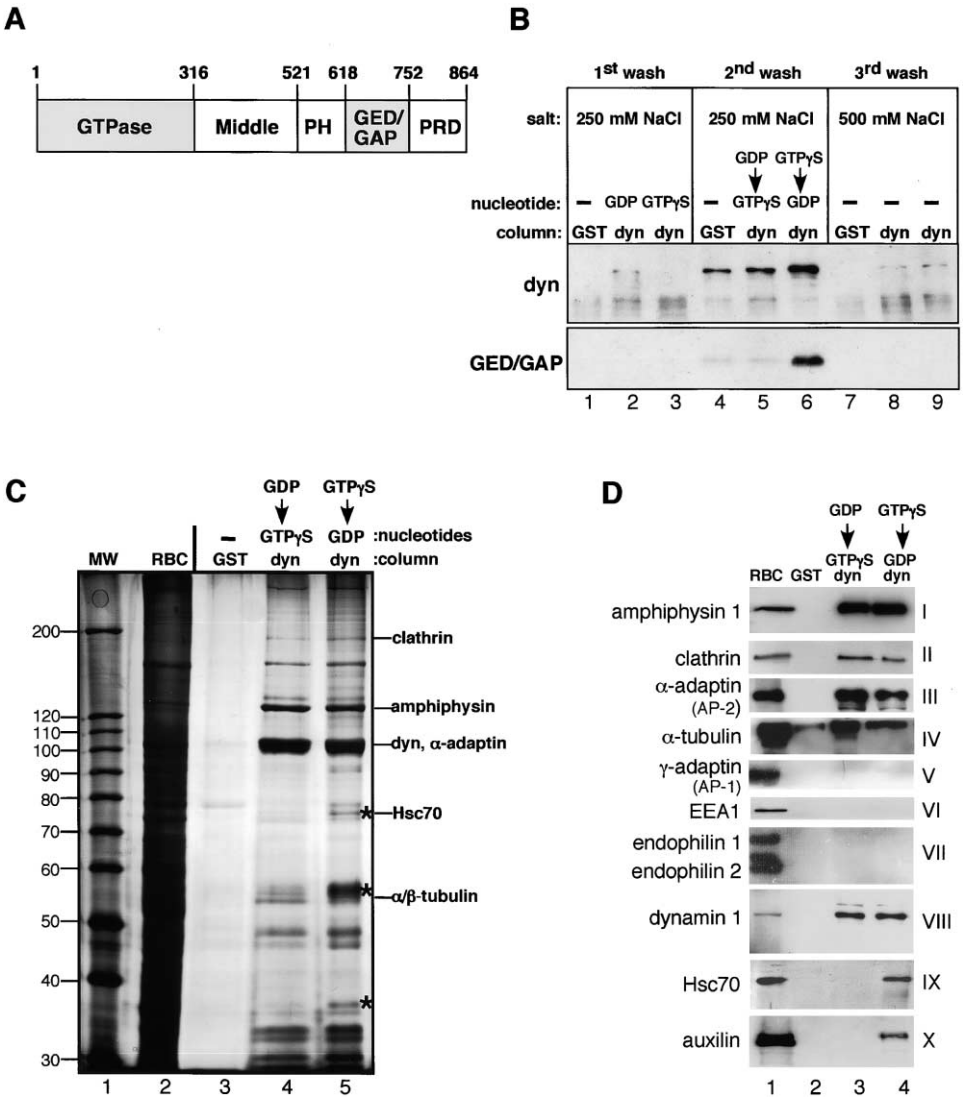

Figure 1. Identification of Proteins that Interact Specifically with Dynamin

(A) Domain structure of dynamin showing the N-terminal GTPase domain, the PH (pleckstrin-homology) domain, the GAP domain (previously referred to as a GED; Muhlberg et al., 1997; Sever et al., 1999), and the C-terminal PRD (proline/arginine-rich) domain.

(B) GST or GST-GTPase was incubated initially with rat brain cytosol (RBC) in the presence of 0.5 mM GDP (lane 2), GTPγS (lane 3), or no nucleotide (lane 1). Proteins eluted with the indicated washes were analyzed by Western blotting with anti-GAP antibody: first wash, buffer containing 250 mM NaCl and the nucleotide used during binding (lanes 1–3); second wash, 250 mM NaCl containing no nucleotide (lane 4), GTPγS (lane 5), or GDP (lane 6); third wash, buffer containing 500 mM NaCl lacking nucleotides (lanes 7–9).

(C) Silver staining of the input RBC (lane 2), the material eluted from immobilized GST (lane 3), material eluted from immobilized GDP-dynamin with GTPγS (lane 4), and material eluted from immobilized GTPγS-dynamin with GDP (lane 5). Proteins (lanes 3–5) were eluted in buffer containing 250 mM NaCl as described for (B). Eluted proteins specific to GST-dynamin:GTPγS (lane 5) are labeled with an asterisk (*).

(D) Immunoblot analysis of the material affinity-purified in (C), lanes 3–5. Experiments were performed with 10 μg RBC and 30% of total eluted proteins shown in (C).

and Hinshaw, 1997; and Figures 1B and 2D), and this interaction leads to potent stimulation of dynamin's GTPase activity ("assembled rate") (Tuma, 1994; Warnock et al., 1996). We therefore tested whether Hsc70 and auxilin could influence the GTPase activity of dynamin and found that they did not stimulate dynamin's basal GTPase rate (data not shown). We next asked whether auxilin or Hsc70 affected the GTPase rate of assembled dynamin. To this end, auxilin or Hsc70 was added to reactions containing dynamin and its GAP domain, whose interaction with dynamin stimulates GTPase activity of dynamin in a manner that resembles assembly (Sever et al., 1999). In this assay, auxilin$^{405-910}$, but not Hsc70, almost completely inhibited GAP-mediated stimulation (Figure 3A). Potent inhibition of dynamin's GTPase activity was also achieved with full-length auxilin (data not shown). In addition, auxilin could also inhibit the stimulated GTPase activity of the ubiquitously expressed dynamin-2 isoform (data not shown). Auxilin's inhibition of dynamin exhibited highly cooperative behavior (Figure 3B). Dynamin-dynamin interactions (Tuma, 1994; Warnock et al., 1996) as well as GAP-dynamin

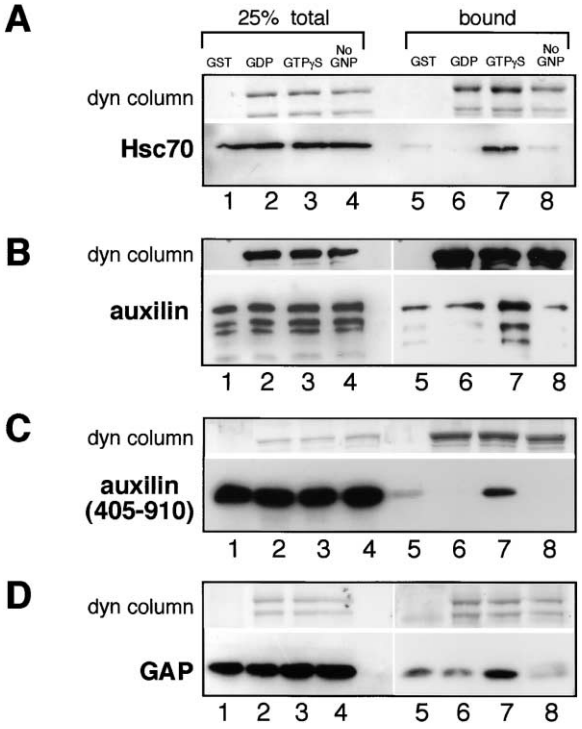

Figure 2. Dynamin:GTP Binds Directly to Hsc70 and Auxilin

GST-dynamin attached to Glutathione-Sepharose was used as bait and incubated with 2 μM of native brain Hsc70 (A), recombinant auxilin (B), recombinant auxilin$^{405-910}$ (C), and GAP (D). All incubations were performed in buffer HCB100 with the indicated nucleotide for 60 min at 4°C. 25% of the incubation mixture was loaded as a total protein standard (lanes 1–4), and remaining beads were extensively washed and bound protein eluted with boiling (lanes 5–8). 0.5 mM guanine nucleotides, as indicated in the figure, were present at all times. The samples were analyzed by SDS-PAGE and immunoblotting.

interactions (Sever et al., 1999) exhibit the same high degree of cooperativity. These data suggest that auxilin may induce the same allosteric conformational changes within dynamin as the interactions taking place during dynamin assembly. Importantly, an increased concentration of added GAP could overcome inhibition by auxilin (Figure 3C), demonstrating that GAP competes for auxilin binding to dynamin. We previously showed that the GAP domain, on its own, stimulates GTP hydrolysis by interacting with the GAP domain within unassembled dynamin (Sever et al., 1999). Indeed, we could not detect binding between auxilin and the isolated GAP or GTPase domains (data not shown). Therefore, auxilin's ability to abrogate GAP stimulation suggests that auxilin is likely binding both the GAP and the GTPase domains *within* dynamin.

To demonstrate directly that auxilin interacts with the GAP domain within full-length dynamin, we performed sedimentation assays that monitor dynamin assembly, previously shown to be mediated by GAP-GAP interactions (Muhlberg et al., 1997; Sever et al., 1999; Warnock et al., 1996). In this assay, assembly of dynamin upon dilution into low ionic strength buffer is measured by the absence of dynamin remaining in the supernatant after centrifugation. As shown in Figure 3D, the addition of purified GAP inhibits dynamin assembly as previously reported (Sever et al., 1999). In a similar manner, the addition of auxilin$^{405-910}$ significantly inhibited dynamin self-assembly, whereas GST had no effect. Together, these results identify auxilin as a novel dynamin:GTP binding protein that interacts with dynamin's GAP domain and possibly its GTP-bound GTPase domain. These interactions appear to induce the same allosteric conformational changes within dynamin tetramers that occur during dynamin self-assembly.

Auxilin Contains Two Dynamin Binding Domains

To localize dynamin's binding domain within auxilin, we purified different segments of auxilin from bacteria (Figures 4A and 4B), and their inhibitory activity was examined in the GAP/dynamin GTPase assay. Two nonoverlapping domains, aux$^{405-591}$ and aux$^{591-814}$, inhibited the assembled GTPase rate of dynamin (Figures 4A and 4C). Aux$^{405-591}$ bound slightly better than aux$^{405-910}$ but did not completely abolish the stimulated rate of GTP hydrolysis (compare black circles in Figure 3B and Figure 4C), whereas aux$^{591-814}$ bound 4-fold less avidly than aux$^{405-910}$ yet inhibited the stimulated rate completely. Amino acids within aux$^{405-591}$ have not been implicated in any specific function, whereas amino acids within aux$^{591-814}$ interact with clathrin and AP-2 (Holstein et al., 1996; Scheele et al., 2001). These results suggest that there might be a temporal and/or spatial order by which auxilin engages in interactions with clathrin, AP-2, and dynamin. Thus, we examined whether auxilin fragments harboring a dynamin binding site could interfere with auxilin's known role in uncoating CCVs. To this end, auxilin fragments were titrated into an assay in which CCV uncoating is mediated by auxilin already present on coated vesicles isolated from bovine brain, and added native Hsc70. As expected given previous results (Holstein et al., 1996), aux$^{405-910}$, which contains both the DnaJ and clathrin binding domains, did not inhibit uncoating, whereas aux$^{405-814}$ and aux$^{591-814}$, which both lack the J domain, did (Figure 4D). Importantly, aux$^{405-591}$, which contains dynamin but not clathrin binding domains, did not inhibit CCV uncoating. Together, these results show that auxilin contains at least two dynamin binding sites: one exclusive for dynamin and one involved in multiple protein interactions. Interestingly, auxilin also contains more than one binding site for clathrin, and these sites are also tandemly arranged (Scheele et al., 2001). Therefore, it seems that auxilin is capable of engaging in numerous interactions with proteins involved in endocytosis.

Auxilin-Dynamin Interactions Are Essential for CCV Formation

We next examined whether interactions between dynamin and auxilin are required for endocytosis. HeLa cells stably expressing a chimeric tetracycline-regulatable transcription activator (tTA-HeLa) were infected with adenoviruses recombined with different auxilin cDNA fragments. Within infected cells, a single round of biotinylated transferrin (BXX-Tfn) sequestration into constricted coated pits and coated vesicles was measured by its acquired inaccessibility to exogenously added avidin (Carter et al., 1993). Cells infected with the

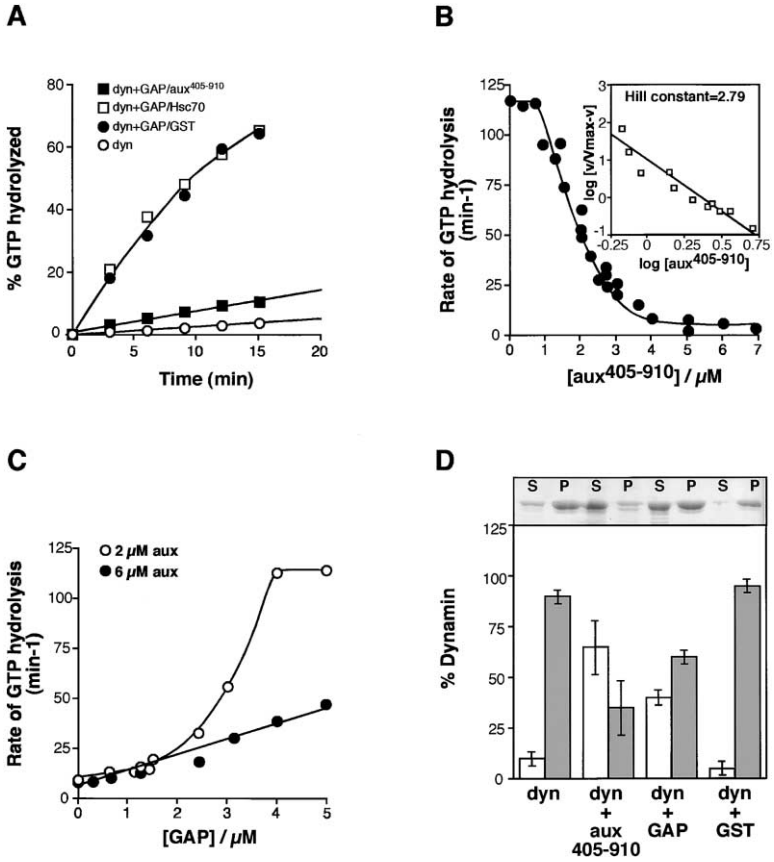

Figure 3. Auxilin Inhibits GAP-Stimulated GTP Hydrolysis by Dynamin

(A) Rate of GTP hydrolysis in reactions containing 0.15 μM dynamin (open circle); dynamin and 4 μM GAP (black circle); dynamin, 4 μM GAP, and 6 μM Hsc70 (open square); or dynamin, 4 μM GAP, and 6 μM aux$^{405-910}$ (black square).

(B) The GTPase activity of 0.15 μM dynamin in the presence of 4 μM GAP was measured in the presence of increasing concentrations of aux$^{405-910}$. Inset: the data were replotted to derive the Hill constant.

(C) The GTPase activity of 0.15 μM dynamin in the presence of 2 μM (open circle) or 6 μM aux$^{405-910}$ (black circle) was measured in the presence of increasing concentrations of GAP.

(D) Dynamin was incubated with 6 μM aux$^{405-910}$, GAP, or GST for 30 min on ice in HCB52 and then diluted 10-fold into HCB0 to induce self-assembly. Self-assembly reactions were kept at room temperature for 10 min before separation into supernatant (white bars) and pellet (gray bars) fractions by ultracentrifugation. The error bars reflect the ±SD from five independent experiments.

aux^{WT} virus were also grown in the presence of tetracycline, which inhibits protein expression, as a control for viral infection (Figure 5, open square). The kinetics of BXX-Tfn uptake and recycling in uninfected cells were indistinguishable from those measured in virally infected cells grown in the presence of tetracycline, demonstrating that the infection did not affect Tfn trafficking (data not shown). If interactions between auxilin and dynamin are important for CCV formation, then aux$^{405-591}$ and aux$^{591-814}$ are expected to act as dominant-negatives for endocytosis by competing with the endogenous auxilin-2 for dynamin. In agreement with this hypothesis, overexpression of aux$^{405-591}$ decreased the rate and extent of receptor-mediated endocytosis approximately 2-fold when compared to uninduced cells (Figure 5, compare black circles with open squares). More potent inhibition of endocytosis, approximately 4-fold, was observed in cells overexpressing aux$^{591-814}$ (Figure 5, open circles). Given that both adenoviruses gave a similar level of infection efficiency (>90%), with similar levels of protein expression (Figure 5, inset), aux$^{591-814}$ acts as the more potent inhibitor. This difference might be due to the clathrin binding domain within aux$^{591-814}$ either having an additional inhibitory effect on uncoating CCVs (but see below) or better targeting of this fragment to the active site at the plasma membrane where it can encounter dynamin.

In agreement with published data (Zhao et al., 2001), overexpression of aux^{WT} and aux$^{405-910}$ resulted in significant impairment of Tfn endocytosis (Figure 5, black triangles and black diamonds). Inhibition of endocytosis in this case has been explained as an indirect consequence of auxilin's ability to sequester clathrin into nonfunctional cytosolic granules (Zhao et al., 2001; and see below). Since auxilin contains a putative lipid binding domain (Figure 4A, amino acids 1–356), which may play a role in auxilin recruitment to the plasma membrane, we asked whether overexpression of this domain could inhibit endocytosis. As shown in Figure 5 (gray squares), overexpression of aux$^{1-405}$ had no effect on endocytosis, suggesting that this domain likely plays a postbudding role.

Importantly, none of the fragments affected recycling of Tfn to the cell surface, as seen by the fact that after the initial uptake at 5 min, the internalized BXX-Tfn was efficiently recycled ($t_{1/2}$ ~15 min) out to the cell surface (Figure 5, later time points). Since defective clathrin disassembly would limit the cellular sorting of Tfn and prevent recycling, our results suggest that the observed inhibition of Tfn internalization by the auxilin fragments is not principally due to impaired CCV uncoating. Lack of a CCV uncoating defect in vivo by aux$^{591-814}$ is most likely due to the relatively low levels of protein expression that occur with adenoviral expression compared to in vitro inhibitory concentrations (Figure 4D). The absence of an uncoating defect suggests that the potent inhibition of endocytosis by aux$^{591-814}$ occurred prior to or included CCV formation.

To further test the hypothesis that aux$^{405-591}$ and aux$^{591-814}$ specifically impair early stages of endocytosis regulated by dynamin, we examined steady-state clathrin distribution using immunofluorescence, since

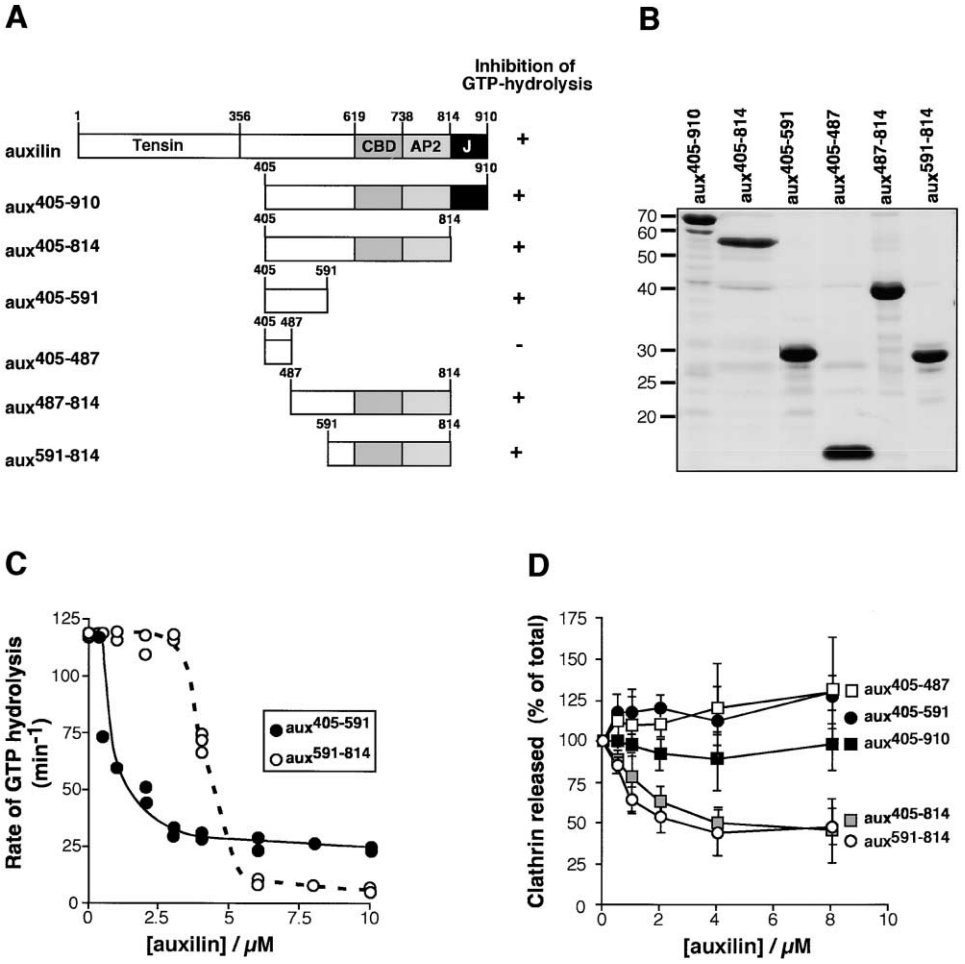

Figure 4. Mapping the Dynamin Binding Site on Auxilin

(A) Domain structure of auxilin and His-tagged auxilin fragments showing tensin, clathrin binding (CBD), adaptor binding (AP2), and DnaJ (J) domains. The inhibitory effect of the indicated fragments on dynamin GTP hydrolysis is shown on the right.

(B) Coomassie blue staining of the purified proteins.

(C) Concentration dependence of inhibition of GAP-stimulated GTP hydrolysis by aux$^{405-591}$ (black circle) and aux$^{591-814}$ (open circle) in the presence of 4 μM GAP and 0.15 μM dynamin.

(D) Effects of auxilin fragments on Hsc70-mediated clathrin uncoating. CCVs were incubated in the presence of native Hsc70 (0.4 μM) and increasing concentrations of auxilin fragments. The error bars reflect the ±SD from four independent experiments.

auxilin has been shown to inhibit endocytosis by sequestering clathrin into nonfunctional cytosolic granules (Zhao et al., 2001). As previously published (Zhao et al., 2001), cytosolic granules that contain auxilin and clathrin were found in cells overexpressing either aux^{WT} or the PTEN domain-lacking aux$^{405-910}$ fragment (Figures 6A and 6B). In contrast, cells overexpressing aux$^{405-591}$ or aux$^{591-814}$ did not show formation of clathrin/auxilin granules (Figures 6C and 6D), and the distribution of auxilin, clathrin, and dynamin in these cells was the same as in cells expressing aux$^{1-405}$ (data not shown), a fragment that did not inhibit endocytosis (Figure 5), as well as in uninfected cells (Figures 6A–6D, see arrows). Together, our data strongly argue that there are two different mechanisms by which auxilin can inhibit endocytosis: indirectly, by aberrant polymerization of cytosolic clathrin (as in the case of aux^{WT} and aux$^{405-910}$), and directly, by acting as dominant-negatives for endocytosis by competing with endogenous auxilin-2 for dynamin (as in the case of aux$^{405-591}$and aux$^{591-814}$).

To further test this hypothesis, we used a perforated-cell assay that reconstitutes a single round of CCV formation that is dependent on dynamin (Hill et al., 2001; Simpson et al., 1999; Smythe et al., 1989) and independent of clathrin recycling (Smythe et al., 1989, 1992). As shown in Figure 7A, aux$^{405-591}$ and aux$^{591-814}$ inhibited sequestration of BXX-Tfn in a concentration-dependent manner, with aux$^{591-814}$ causing more potent inhibition, as observed in intact cells (Figure 5A). The effective concentration and the extent of inhibition were similar to previously reported effects of different SH3-domains in this assay (Hill et al., 2001; Simpson et al., 1999). Addition of aux$^{405-910}$ also inhibited endocytosis, in agreement with its ability to sequester cytosolic clathrin into nonfunctional aggregates. Importantly, aux$^{405-487}$ and GST, neither of which binds to dynamin, had no effect.

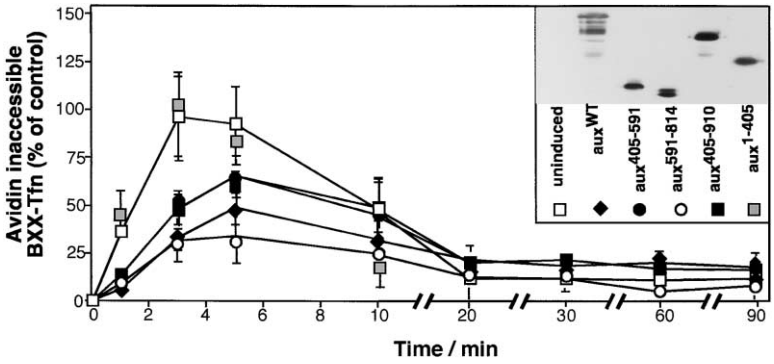

Figure 5. Overexpression of Auxilin Fragments Dominantly Interferes with Tfn Receptor Endocytosis but Not with Recycling

tTA-HeLa cells overexpressing aux^{WT} (black diamonds), aux$^{405-910}$ (black squares), aux$^{591-814}$ (open circles), aux$^{405-591}$ (black circles), and aux$^{1-405}$ (gray squares) were harvested 18 hr after adenoviral infection. Single-round kinetics of Tfn internalization and recycling were determined by first incubating cells with an excess of BXX-Tfn for 30 min on ice, washing away unbound ligand, and internalizing cell surface-bound BXX-Tfn at 37°C. Control uninduced cells (open squares) were infected with aux^{WT}-encoding adenoviruses cultured in the presence of tetracycline to suppress expression. Inset shows the expression levels for different auxilin proteins from a single representative assay analyzed by Western blotting. The error bars reflect the ±SD from five independent experiments.

To confirm that the auxilin-mediated inhibition in the perforated-cell assay involved direct interactions with dynamin, auxilin domains were preincubated with purified dynamin before addition into the assay. As expected, dynamin completely abrogated the inhibitory effect of aux$^{405-591}$ and aux$^{591-814}$ (Figure 7B, columns 5–10). Interestingly, addition of dynamin rescued the inhibitory effect of aux$^{405-910}$, although with greater variability (see error bar in Figure 7B, lane 6), suggesting that increasing the dynamin concentration may promote dynamin-auxilin interactions that either compete with clathrin-auxilin interactions that promote aberrant coat assembly or redirect inhibitory levels of auxilin to modulate clathrin dynamics normally at the coated pit. Alternatively, aux$^{405-910}$ might, in addition to polymerizing clathrin, act as a weak dominant-negative for auxilin's function at early stages of endocytosis.

Immunodepletion of dynamin from the cytosol reduced transferrin uptake to a similar extent as addition of the auxilin domains (Figure 7B, compare columns 3, 5, 7, and 9; and Hill et al., 2001; Simpson et al., 1999). Aux$^{591-814}$ reduced transferrin uptake even further than dynamin depletion, most likely due to its ability to efficiently target dynamin already present on the membranes. Thus, the tested auxilin fragments effectively sequestered dynamin levels found in the cytosol and even appeared to neutralize dynamin primed at the membrane for endocytosis, suggesting that dynamin-auxilin interactions are important for CCV formation.

Discussion

Here we report novel interactions between dynamin and the chaperones Hsc70 and auxilin. The interactions are direct and nucleotide-dependent, identifying Hsc70 and auxilin as proteins that specifically interact with dynamin in its GTP-bound form. Auxilin binds to dynamin's intramolecular GAP domain, whereas Hsc70 likely binds to

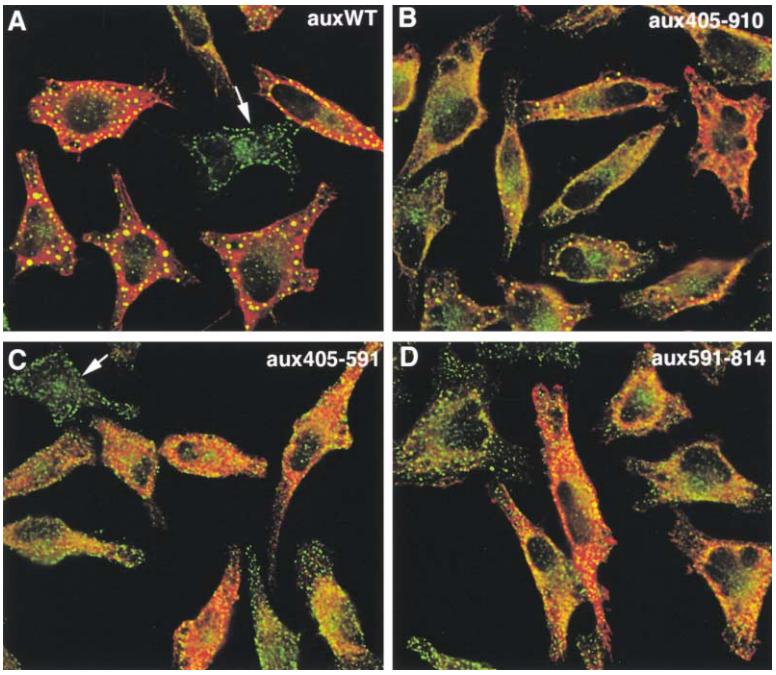

Figure 6. Steady-State Distribution of Clathrin and Adenovirus-Expressed Auxilin Fragments

tTA HeLa cells expressing HA-tagged aux^{WT} (A), aux$^{405-910}$ (B), aux$^{405-591}$ (C), and aux$^{591-814}$ (D) were analyzed 18 hr post-infection. Fixed and permeabilized cells were incubated with anti-HA tag monoclonal antibody HA.11 (red) and anti-clathrin light chain antibody (green). Untransfected cells are marked with arrows.

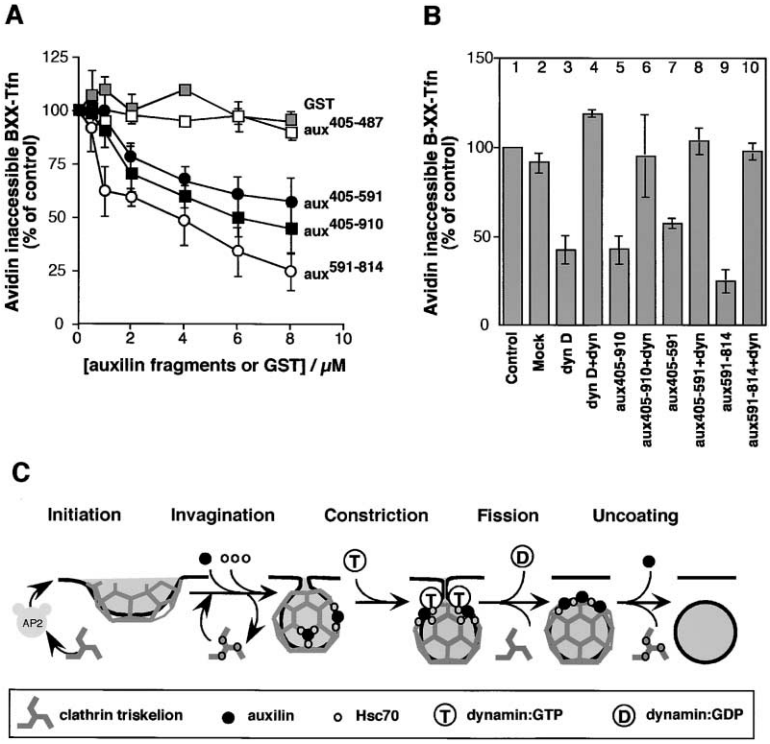

Figure 7. Interactions between Dynamin and Auxilin Are Important for Endocytosis

(A) Endocytosis of biotinylated transferrin (BXX-Tfn) in perforated A431 cells is inhibited by addition of auxilin fragments. Membranes were incubated with increasing concentrations of GST (gray squares), aux$^{405-487}$ (open squares), aux$^{405-910}$ (black squares), aux$^{405-591}$ (black circles), or aux$^{591-814}$ (open circles) for 20 min on ice before addition of cytosol. The amount of internalized BXX-Tfn after 10 min at 37°C was determined by avidin inaccessibility, as previously described (Smythe et al., 1992), and plotted as % of uptake in the absence of auxilin fragments. Results are the average of three independent experiments ± SD.

(B) Inhibition of endocytosis by auxilin fragments can be overcome by addition of dynamin. The experiments were performed essentially as under (A), using 6 μM of different auxilin fragments, with the exception that 1.25 μM of purified dynamin (dyn) was added as indicated. Lane 2, mock-depleted cytosol. Lanes 3 and 4, dynamin-depleted cytosol (dyn D). Results are the average of five independent experiments ± SD.

(C) New model for the role of dynamin in formation of CCVs. Coat assembly starts with recruitment of adaptors (AP2) followed by recruitment of clathrin. Oligomerized clathrin recruits auxilin, which in turn recruits Hsc70. Exchange of cytosolic clathrin with assembled clathrin due to activity of Hsc70/auxilin is believed to drive invagination of coated pits. Dynamin:GTP is recruited through an as yet unknown mechanism to the neck of the invaginating pit, and it redistributes Hsc70/auxilin to this location. Hsc70/auxilin chaperone activity in turn induces conformational changes within the clathrin coat that drive formation of the constricted coated pit. GTP hydrolysis returns dynamin to the cytosol, disengages its interactions with Hsc70/auxilin, and allows unhindered coat rearrangement that executes the budding of free CCVs. Thus, dynamin GTP hydrolysis may temporally couple collared neck disassembly with localized clathrin uncoating to yield fission of the coated pit. In summary, we propose that the major endocytic role of dynamin is to concentrate the uncoating chaperone machinery at the neck of the coated pit to mediate constriction and fission of the emerging coated vesicle.

another region of dynamin. Auxilin contains two dynamin bindings sites, and their overexpression inhibits endocytosis. We propose that dynamin-auxilin interactions are essential for CCV formation at the plasma membrane.

A Role for Hsc70/Auxilin in the Early Stages of Endocytosis

Mounting evidence directly indicates that Hsc70 is required for the early stages of endocytosis that also require dynamin, and various lines of circumstantial evidence have also implicated auxilin (Chang et al., 2002; Höning et al., 1994; Morgan et al., 2001; Newmyer and Schmid, 2001). A role for auxilin in vesicle formation was suggested by its interactions with the appendage domain of AP-2 (Owen et al., 1999) and by studies showing that during early stages of invagination in HeLa cells, membrane-bound clathrin exchanges with cytosolic clathrin (Wu et al., 2001). This exchange was ATP dependent, suggesting involvement of auxilin/Hsc70. These results notwithstanding, studies on the exact role(s) of auxilin in endocytosis have been hampered by its sequestration of clathrin into nonfunctional aggregates (Zhao et al., 2001). Here, we show that cellular expression of aux$^{405-591}$, which binds to dynamin, inhibits endocytosis without leading to sequestration of clathrin (Figure 6). The inhibition occurred in single-round endocytosis assays in vivo and in vitro (Figures 5 and 7A), and the fragment had no effect on vesicle uncoating or recycling (Figures 4D and 6). We showed that aux$^{405-910}$ inhibited dynamin assembly (Figure 3D), and it is expected that the minimal dynamin binding fragment, aux$^{405-591}$, would have the same effect given its ability to impair assembly-stimulated GTP hydrolysis by dynamin (Figure 4C). Therefore, there is a formal possibility that aux$^{405-591}$ inhibits early steps of endocytosis by inhibiting dynamin self-assembly. This interpretation assumes that dynamin self-assembly is essential for fission of CCVs, an assumption most strongly supported by the observation that GTP hydrolysis of assembled dynamin can lead to fragmentation of synthetic lipid tubes in vitro (Sweitzer and Hinshaw, 1998). However, the significance of this assay for vesicle budding in vivo has not been documented. Importantly, in the one instance where the cellular role of dynamin self-assembly was directly tested (expression of dyn^{K694A}, an assembly-impaired mutant of dynamin in HeLa cells), endocytosis was *stimulated*. Therefore, the inhibitory effects of aux$^{405-591}$ on endocytosis strongly support the model that auxilin functions at early stages of endocytosis.

Is Dynamin a Classical Regulatory GTPase?

Dynamin has been viewed as a pinchase that executes the fission reaction (Danino and Hinshaw, 2001; Hinshaw and Schmid, 1995; Marks et al., 2001; McNiven, 1998;

Warnock and Schmid, 1996) and a classical regulatory GTPase that recruits effectors of fission (Fish et al., 2000; Sever et al., 1999, 2000). In support of the latter, mutation in the GAP domain of dynamin family member Dnm1 activates this protein's function in mitochondrial fission (Fukushima et al., 2001). Recently, the regulatory model gained further support from the crystal structure of the GTPase domain of *Dictyostelium discoideum* dynamin, which has extensive structural homology to Ras-like regulatory GTPases (Neimann et al., 2001). In this paper, we provide further evidence for the regulatory model. First, we show that the putative GAP domain of dynamin binds to dynamin in a GTP-dependent fashion, as seen for other GAP-GTPase interactions (Bourne et al., 1991; Mittal et al., 1996; Scheffzek et al., 1998). Second, we have identified two putative dynamin downstream effectors, Hsc70 and auxilin. Interestingly, while auxilin interacts only with dynamin in the GTP-bound conformation, as predicted for bona fide downstream effectors, neither the GTPase domain nor the GAP domains are by themselves sufficient for auxilin binding (data not shown). However, in the context of full-length dynamin, the GAP is required for auxilin binding (Figure 3), whereas the requirement for the GTPase domain remains unknown. Importantly, the *Dictyostelium discoideum* dynamin structure suggests that the GAP interacts directly with the catalytic loops (switch 1 and 2) of the GTPase. This observation suggests that the nucleotide state of dynamin might be transmitted to its GAP. Consistent with this, dynamin self-assembly involves GTP-dependent GAP-GAP interactions (Carr and Hinshaw, 1997; Sever et al., 1999). Therefore, auxilin-GAP interactions could be sufficient for auxilin to sense the nucleotide state of dynamin. While it is unprecedented for an effector to bind via the GTPase's GAP, it should be noted that dynamin's intramolecular GAP remains *inactive* until it is stimulated during dynamin self-assembly. In summary, the stimulatory in vivo phenotypes of mutations in the GAP domains of dynamin family members (Fukushima et al., 2001; Sever et al., 1999, 2000) and the identification of proteins that bind dynamin:GTP (present paper) support the model that dynamin functions as a regulator of endocytosis.

A Clathrin-Centric Model for Endocytosis

In light of our findings, we propose new roles for dynamin, Hsc70, and auxilin in endocytosis (Figure 7C). The affinity of auxilin for clathrin should be sufficient for its recruitment to coated pits, where it in turn recruits Hsc70. Given that dynamin:GTP is concentrated at the necks of coated pits that are undergoing constriction (Baba et al., 1995; Damke et al., 1994; De Camilli and Takei, 1996; Warnock and Schmid, 1996), the role of dynamin:GTP might be to specifically enrich Hsc70/auxilin at the interface between the neck and coated pit, thereby accelerating clathrin exchange and thus facilitating constriction and fission of the invaginated pit. Regardless of the exact mechanism by which dynamin modulates the activity of the chaperone machinery, it seems most likely that the constriction and fission reactions are driven by several rounds of assembly and disassembly of a dynamin/Hsc70/auxilin complex, given that Hsc70 acts stoichiometrically rather then catalytically (a single Hsc70 monomer per clathrin heavy chain). In this view, the role of dynamin is to provide spatial and temporal regulation of sequential reactions required for coat reorganization during constriction and budding. In this context, it is also important to note that since auxilin competes for dynamin self-assembly through binding to dynamin's GAP and thus inhibits assembly-stimulated GTP hydrolysis, it effectively prolongs dynamin in the GTP-bound state. This property of the system should allow auxilin to persist at the site of action, and auxilin release may in fact result from dynamin's basal GTPase activity. Clearly, future work will be needed to elucidate how dynamin affects the chaperone's ATPase cycle and how these interactions influence the functions of Hsc70 and auxilin.

Chaperone-mediated clathrin dynamics now appear to be involved in pit invagination (Greener et al., 2001; Wu et al., 2001), constriction and subsequent fission (Newmyer and Schmid, 2001; and this study), and uncoating (Chang et al., 2002; Greener et al., 2000; Schlossman et al., 1984; Ungewickell et al., 1995). In this view, all stages of endocytosis can be understood in terms of differentially regulated, chaperone-driven clathrin coat rearrangements. Therefore, formation of CCVs at the plasma membrane might not differ conceptually from the formation of other coated vesicles, COPI and COPII, where coat assembly alone is the major driving force behind vesicle formation. Given that dynamin is the prototype of a large family of GTPases that all function in processes that are most likely driven by rearrangement of multiprotein complexes (fusion and fission), this work also raises the intriguing possibility that all these GTPases function as regulators of different chaperone machineries.

Experimental Procedures

Preparation of cDNA Constructs and Protein Expression

The GST domain from a pGEX vector was PCR amplified and cloned in-frame within the original pFASTBac-dynamin^{wt} to generate a vector coding for N-terminally tagged GST-dynamin. Expression and purification of GST-GTPase and GED was as previously described (Sever et al., 1999). pGEX-4T1-auxilin was a generous gift from E. Ungewickell (Hanover Medical School, Hanover, Germany). Vectors coding for 6×His fusion proteins of aux$^{405\text{-}910}$ (pQE30-Aux54) and aux$^{405\text{-}814}$ (pQE30-ΔJ) were generous gifts of L.E. Greene (NIH, Bethesda, MD) (Greener et al., 2000). His-tagged fusion proteins were expressed and purified following the standard batch method described by QIAGEN. For adenovirus expression of the proteins in tTA-HeLa cells, fragments of auxilin were PCR amplified using an N-terminal primer containing an NdeI restriction site, and a C-terminal primer containing a PstI restriction site. Fragments were cloned into NdeI-PstI sites of the pADtet7 encoding HA-tag vector (Altschuler et al., 1998; Hardy et al., 1997).

Purification of Dynamin Effectors

One liter of insect Tn5 cells was infected with viruses expressing GST-dynamin and dynamin at a 2:1 ratio as previously described (Damke et al., 2001b). The protein was purified using glutathione beads according to manufacturer's instructions (Amersham Biosciences) with the following minor modifications. Cells were broken in HCB100 buffer (20 mM HEPES, 1 mM $MgCl_2$, 1 mM EGTA [pH 7.0], 100 mM NaCl) by sonication (Damke et al., 2001b). This procedure resulted in 5–10 mg of GST-dynamin bound to 1 ml of glutathione Sepharose 4B beads. This material was then incubated in modified HCB100 (5 mM $MgCl_2$, 0.1% Triton X-100, and 0.5 mM GDP or GTPγS) for 10 min at room temperature to allow binding of the nucleotides to dynamin. Subsequently, beads were incubated for 90 min at 4°C with 0.5 ml of RBC (10 mg/ml) obtained as follows:

12 rat brains were homogenized in 25 ml of buffer containing 20 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT, 10 μM pepstatin, 5 μM aprotinin, 1 μg/ml TLCK, 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and calpain inhibitor at 0.5 μg/ml (Calbiochem), using a Dounce homogenizer and ten passes of each of the A and B pestles. The homogenate was centrifuged at 7000 × g for 15 min. After addition of 1% Triton X-100, the supernatant was rocked at 4°C for 1 hr, followed by a second centrifugation in a Ti45 rotor (Beckman Instruments) at 100,000 × g for 1 hr. Nucleotides were removed by passing cytosol through PD-10 columns (Amersham Biosciences), GDP or GTPγS was added (0.5 mM), and the extract was batch-bound to the affinity resin in HCB buffer with 150 mM NaCl. After 60 min incubation at 4°C, the mixture was poured into a column and washed with ten volumes of modified HCB (100 mM NaCl), followed by ten volumes of HCB (250mM NaCl), with all washes containing the original nucleotide. Bound proteins were eluted with 1.5 column volumes of the same buffer incubated with beads for 10 min at 4°C, with the nucleotides present in the buffer being swapped. In case of GST affinity chromatography, there was no nucleotide in the buffers.

Antibodies

The following antibodies were generous gifts: anti-α adaptin, AP.6, and anti-clathrin TD1 from S.L. Schmid (The Scripps Research Institute, La Jolla, CA) with permission from F.M. Brodsky (The University of California at San Francisco, San Francisco, CA), anti-endophilins from P. DeCamilli (Yale University, New Haven, CT). Anti-GED antibodies were already described (Sever et al., 2000). Anti-Hsc70, anti-amphiphysin, anti-γ-adaptin, and anti-EEA1 antibodies were obtained from StressGen Biotechnologies, and anti-α-tubulin was from Sigma-Aldrich. Polyclonal anti-auxilin antibodies were prepared against bovine $aux^{405\text{-}814}$ using Pocono Rabbit Farm's standard protocol.

Immunofluorescence

18 hr post-infection, adenovirus-treated cells grown on coverslips were washed briefly in PBS, fixed with 3% formaldehyde in PBS containing 1 mM $CaCl_2$ and 1mM $MgCl_2$, and permeabilized with PBS, 1 mM $CaCl_2$, 1mM $MgCl_2$, 0.1% saponin, and 5% sheep serum. The cells were incubated with a monoclonal anti-HA tag antibody HA.11 (Covance) and polyclonal antibodies to either the clathrin light chain from F.M. Brodsky or anti-GED. The primary antibodies were detected with the following secondary antibodies: Rhodamine red-conjugated donkey anti-mouse or FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories). Immunofluorescence data (0.15 μM sections) were collected on an Olympus Inverted System Microscope IX70 and were deconvolved with a Deltavision restoration microscopy system (Applied Precision).

Biochemical Assays

Assembly assays and GAP-stimulated GTPase assays were performed essentially as described (Damke et al., 2001b), with the exception that dynamin was preincubated for 20 min on ice with different concentrations of both GAP and auxilin domains. In pull-down assays, GST-dynamin (3 μg) was bound to the glutathione beads and incubated with purified proteins as indicated in the figure legend. The concentration of the proteins was adjusted to be 2 μM in 40 μl of HCB100 buffer with addition of 5 mM $MgCl_2$, 0.1% Triton X-100, and 0.5 mM GNPs. Beads were collected by centrifugation, washed two times with 100 μl of the same buffer, then resuspended in 20 μl sample buffer and analyzed by SDS-PAGE. Proteins were detected by Western blotting. Bovine Hsc70 (StressGen Biotechnologies) did not contain any bound nucleotide based on the product analysis by manufacturer.

Clathrin Release Assay

Hsc70-mediated clathrin release from isolated bovine brain CCVs was performed for 8 min at 25°C in 20 mM HEPES (pH 7.0), 25 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM Mg^{2+}/ATP as previously described (Hannan et al., 1998), except 0.4 μM Hsc70 and 8 μg CCV protein were used.

Adenoviral Expression of Auxilin Fragments in tTA HeLa Cells

Adenoviruses carrying the auxilin genes were prepared from the pADtet7 auxilin constructs as described (Altschuler et al., 1998; Hardy et al., 1997). Within this expression system, the gene is introduced under the control of a tetracycline-repressible promoter. For all of the assays described below, 2×10^6 cells were plated on 10 cm dishes. Following adherence, the cells were infected for 2 hr with the respective viruses and were subsequently incubated in fresh DME/10% FBS for 16 hr. To control for viral effects, 1 μg/ml tetracycline was included with adenovirally infected aux^{WT} cells to give an uninduced sample.

Single Round of Endocytosis and Recycling from the Endosomal Compartment

Biotinylated transferrin (BXX-Tfn) was generated as described (Smythe et al., 1992). The internalization of prebound BXX-Tfn and recycling was performed as described (Sever et al., 2000). Briefly, adenovirally infected cells were grown in the presence or absence of tetracycline. Cells were detached with PBS/5 mM EDTA at room temperature for 5 min, briefly rinsed, and resuspended in ice-cold PBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% BSA, and 5 mM glucose at 2×10^6 cells/ml. Cells (50 μl) were incubated with 2 μg/ml of BXX-Tfn for 30 min on ice. After the unbound ligand was removed through washing, internalization of prebound BXX-Tfn was determined by incubating cells at 37°C and using the avidin assay to mask unsequestered biotin.

Perforated Cell Assay for Formation of Endocytic Coated Vesicles

The semi in vitro internalization assay using A431 cells was performed essentially as described (Smythe et al., 1992) with minor modifications. For auxilin domain-inhibition experiments, permeabilized cells were preincubated with auxilin domains for 20 min on ice before addition of cytosol. In rescue experiments, auxilin domains were preincubated with 5 μg of purified dynamin (1.25 μM final concentration in the assay) for 10 min on ice, before addition of membrane. 30 μl of the assay was then further submerged on ice for 15 min, and after addition of cytosol and nucleotides, cell membranes were incubated at 37°C for 10 min to allow for endocytosis. For dynamin depletion and rescue experiments, the GST-SH3 domain of amphiphysin, a generous gift of S. L. Schmid (the Scripps Research Institute, La Jolla, CA), was bound to glutathione beads. Cytosolic fractions derived from K562 cells (0.2 ml containing ~3 mg total proteins) were incubated with ~50 μl of affinity matrix for 1 hr at 4°C, with rotation. As a control, mock-treated cytosol was incubated with the same amount of beads lacking GST-SH3 domain.

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