

Mutations in Human Dynamin Block an Intermediate Stage in Coated Vesicle Formation

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Abstract. The role of human dynamin in receptor-mediated endocytosis was investigated by transient expression of GTP-binding domain mutants in mammalian cells. Using assays which detect intermediates in coated vesicle formation, the dynamin mutants were found to block endocytosis at a stage after the initiation of coat assembly and preceding the sequestration of ligands into deeply invaginated coated pits. Mem-

brane transport from the ER to the Golgi complex was unaffected indicating that dynamin mutants specifically block early events in endocytosis. These results demonstrate that mutations in the GTP-binding domain of dynamin block Tfn-endocytosis in mammalian cells and suggest that a functional dynamin GTPase is required for receptor-mediated endocytosis via clathrin-coated pits.

RECEPTOR-mediated endocytosis occurs via the formation of clathrin-coated vesicles and involves coat assembly, receptor recruitment, coated pit invagination, and finally vesicle budding (Smythe and Warren, 1991; Robinson, 1992; Schmid, 1993). The major structural proteins of the coat, clathrin, and AP complexes have been well characterized (Pearse and Robinson, 1990; Keen, 1990; Brodsky, 1988). While proteins that regulate coated vesicle formation have not been identified (Schmid, 1992), a cell-free assay which reconstitutes receptor-mediated endocytosis has recently revealed a requirement for multiple GTP-binding proteins (Carter et al., 1993).

Dynamin, a GTP-binding protein first isolated from bovine brain tissue (Shpetner and Vallee, 1989; Obar et al., 1990; Scaife and Margolis, 1990; Nakata et al., 1991) is a strong candidate for a GTPase that acts early in endocytosis. Dynamin is a member of a subfamily of structurally related GTP-binding proteins which share considerable sequence homology (43–66%) in the amino-terminal GTPase domain, but more limited homology (<10–30%) in their COOH-terminal regions. Other members of this family (for review see Collins, 1991) include the mammalian Mx-1 related, interferon-inducible proteins which confer viral resistance (Arnheiter and Meier, 1990), MGM1 which plays a role in yeast mitochondrial DNA maintenance (Jones and Fangman, 1992) and VPS1p, a protein involved in vacuolar sort-

ing in yeast (Rothman et al., 1990; Yeh et al., 1991). Whether these proteins have related functions or simply share a common GTPase domain driving divergent functions remains to be determined.

Evidence for the role of dynamin in endocytosis came from the discovery that the *Drosophila* homologue of dynamin is encoded by the *shibire* locus (van der Blik and Meyerowitz, 1991; Chen et al., 1991). Adult flies with a mutation that causes the *shibire* gene product to be temperature sensitive exhibit rapid and reversible paralysis at elevated temperatures (Grigliatti et al., 1973). Morphological analysis of nerve terminals from affected flies revealed a depletion of synaptic vesicles and an accumulation of membrane invaginations and coated pits at the cell surface (Kosaka and Ikeda, 1983).

Other *shibire*-derived tissue and cell lines are similarly affected, demonstrating that the defect is pleiotropic. Results from the fluid-phase uptake of morphological tracers also suggest a specific defect in endocytosis but not secretion (Kosaka and Ikeda, 1983; Tsuruhara et al., 1990). However, a direct role for dynamin in receptor-mediated endocytosis has not been demonstrated in either *Drosophila* or mammalian cells.

The phenotypic analysis of the *shibire* mutation and the identity of the *shibire* gene product as dynamin present a paradox. Dynamin has been characterized in vitro as a microtubule-stimulated GTPase and a putative microtubule-based mechanochemical motor protein (Shpetner and Vallee, 1989; 1992), although the latter activity has not been reproduced with highly purified protein (Maeda et al., 1992). Several studies have shown that polymerized microtubules are not required during the early stages of endocytosis (Oka

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and Weigel, 1983; Morgan and Iacopetta, 1987) but are instead involved in later trafficking events along the endocytic pathway (Matteioni and Kreis, 1987). Thus, if dynamin is a microtubule-dependent GTPase, then the defect in early endocytosis could be an indirect consequence of a block more distal in the endocytic pathway. This paradox can be resolved by identifying the specific stage where dynamin acts.

To provide functional evidence for a direct role of dynamin in receptor-mediated endocytosis, we have used transient expression of mutant dynamin in HeLa cells. Receptor-mediated endocytosis of transferrin was followed using newly developed assays which enable detection of intermediates in coated vesicle formation. We show that transient expression of dynamin mutants containing an altered GTPase domain caused a marked decrease in the rate of endocytosis without affecting protein traffic along the exocytic pathway. Endocytosis was blocked at an intermediate stage in coated vesicle formation. These results provide the first biochemical evidence that dynamin is directly involved in receptor-mediated endocytosis and that the GTP-binding domain is required for its activity.

Materials and Methods

Materials

Anti-Tfn receptor monoclonal antibody, D65 was a kind gift of I. S. Trowbridge and S. White (Salk Institute, San Diego, CA). Anti-clathrin monoclonal antibody, X22, and anti- α -adaptin antibody, AP6, were generous gifts of F. M. Brodsky (University of California, San Francisco). Human diferric transferrin (Tfn)¹ was biotinylated as previously described (Smythe et al., 1992). All other reagents were obtained from Sigma Chem. Co. (St. Louis, MO).

Molecular Analysis and Preparation of Constructs

A human dynamin cDNA fragment had previously been isolated using *Drosophila* cDNA as a heterologous probe (van der Bliek and Meyerowitz, 1991). This fragment was used to screen a cDNA library made from human temporal cortex (Stratagene, La Jolla, CA) after standard protocols (Sambrook et al., 1989). The newly isolated cDNAs were characterized by sequence analysis using oligonucleotides synthesized on a Cyclone Plus DNA synthesizer (Milligen/Bioscience) and Sequenase (Un. States Biochem. Corp., Cleveland, OH). For expression purposes, a linker with the optimal Kozak-consensus and an NcoI site at the AUG initiation codon was inserted into pBluescript (Stratagene). A 1,200-bp NcoI fragment with the 5'-part of human cDNA (starting at the AUG-start codon) was inserted into the NcoI site of the linker followed by selection of a clone with the proper orientation for expression. A 2,550-bp BstXI-HindIII fragment with the 3'-part of the cDNA was added to make the cDNA full-length. The Vaccinia expression construct, pTMI (kindly provided by Dr. B. Moss), was modified to remove the existing AUG. Full-length human dynamin cDNA with the synthetic Kozak-consensus was recloned into the modified pTMI-vector using flanking SpeI and SalI sites. This is the "wild type" construct, called pTMI-Hwt. Mutations were introduced as follows: a complementary pair of oligonucleotides in which the desired mutation had been incorporated was used in two different PCRs with Pfu polymerase (Stratagene).

Either of the two mutagenic oligos was combined with an appropriate upstream or downstream primer yielding two PCR products that were then fused by PCR in a second step with the upstream and downstream primers, but without the mutagenic primers. Products of the fusogenic PCR reactions were cleaved with SacII to yield 1,350 bp fragments, which were then recloned into SacII-cut and dephosphorylated pTMI-wild-type construct. The recloned constructs were sequenced to identify mutants and to ensure that they contained only the intended mutations. Supercoiled plasmid DNA, necessary for transient expression, was isolated with alkaline lysis followed by CsCl-gradient purification (Sambrook et al., 1989). The cDNA that was

chosen for the pTMI-Hwt construct corresponds to the most prevalent COOH-terminal splice variant, i.e., without an insert (see Fig. 1). The internal splice variants were represented equally among the cDNAs; pTMI-Hwt has the second of the two in Fig. 1. To confirm that the variants were products of alternative splicing, cross-hybridizing phage was isolated from a library of human lymphocyte genomic DNA inserted in lambda DASH (Stratagene). Genomic EcoRI fragments were then subcloned into pBlue-script and the splice sites were sequenced using oligonucleotides generated while sequencing the cDNAs. RNA expression was analyzed by hybridizing a human multiple tissue Northern blot (Clontech laboratories, Palo Alto, CA) with a radiolabeled 1,400-bp fragment of human dynamin cDNA corresponding to the 5'-half of the mRNA. The blot was washed with a final wash at high stringency ($0.2 \times$ SSPE at 65°C). The blot contained 2 μ g poly(A) + RNA per lane with size markers of 9.5, 7.5, 4.4, 2.4, and 1.35 kb.

Transfection Procedure

HeLa cells were maintained in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS and 100 U/ml each of penicillin and streptomycin in a 5% humidified CO₂ incubator. HeLa cells (1.5×10^6) were plated in 60-mm dishes containing a coverslip the day before transfection. On occasion, cells were used 2.5 h after plating 1.5×10^6 cells/60-mm dish. Before infection, the cells were washed twice with DMEM, and then infected with vaccinia T7 RNA polymerase recombinant virus (vTF7-3, Fuerst et al., 1986), at a multiplicity of 10 PFU/cell for 30 min with intermittent rocking in a 37°C incubator. The inoculum was removed and the cells transfected with 7 μ g pTMI plasmid DNA containing dynamin constructs prepared as described above that had been previously mixed for 15 min at room temperature in 1 ml of DMEM and 20 μ l of transfection reagent (TransfectACE) prepared as described (Rose et al., 1991). 2 h after infection, rifampicin (100 μ g/ml final concentration) was added to the cells and expression was allowed to proceed for the indicated times.

Anti-Dynamin Antibodies and Western Analysis

E. coli carrying the expression vector pET11D containing a *Drosophila* dynamin fragment (residues 359–615) produced an abundant 30-kD protein upon induction by IPTG. The protein was gel-purified, electroeluted, and used as antigen to generate polyclonal antibodies (748) from rabbits by standard procedures. Serum from this rabbit contained an antibody immunoreactive against the 30-kD protein and a 100-kD protein in HeLa cells and bovine brain extracts (data not shown). The antibody also recognized recombinant wild-type and mutant molecules. For Western blot analysis, samples from the resuspended cells used in transport assays were lysed by the addition of 1% Triton X-100 in PBS, containing 5 mM MgCl₂ and 1 mM AEBSF (Calbiochem, La Jolla, CA). The samples were pelleted to clear the nuclei, precipitated with 10% TCA, washed with ice cold acetone, resuspended in Laemmli sample buffer, and then analyzed by SDS-PAGE. The gels were electroblotted onto nitrocellulose, blocked with 5% nonfat dry milk powder in TBS containing 0.1% Tween 20 incubated with a 1:1,500 dilution of the rabbit anti-dynamin antibody (748) followed by detection with an HRP-conjugated secondary antibody (Pierce, Rockford, IL), and then developed with ECL detection system (Amersham Corp., Arlington Heights, IL) according to the manufacturer's directions. Signals from various exposure times were quantitated using a scanning densitometer (Model GS300, Hoefer Sci. Instrs., San Francisco, CA) connected to an IBM-XT with the integrating software (Model GS350, Hoefer Sci. Instrs.). Linearity was established using bovine brain cytosolic fractions to generate a standard curve.

Subcellular Fractionation

Cells from one 60-mm dish were harvested at room temperature in PBS containing 5 mM EDTA and washed at 4°C by centrifugation, and resuspended in 0.5 ml 0.25 M sucrose, 1 mM MgCl₂, 2 mM EGTA, 25 mM Hepes, pH 7.4. Cells were then lysed by rapid freezing in liquid nitrogen, followed by slow thawing in a room temperature water bath. Soluble and particulate fractions were obtained after centrifugation for 30 min at 50 K rpm (110 k g) in a TLA100.2 (Beckman Instrs. Inc., Fullerton, CA) rotor. Pellets were resuspended in 0.5 ml and equal amounts of pellet and supernatant fractions were solubilized in 0.5% TX-100 and precipitated with 10% TCA. SDS-PAGE and Western blot analysis were performed as described above.

Assay of Tfn Endocytosis

After the appropriate period of protein expression (usually 5 h), the cells

1. Abbreviations used in this paper: endo H, endoglycosidase H; Tfn, transferrin; VSV-G, Vesicular Stomatitis virus G.

were washed 1 × in dPBS, harvested at room temperature in dPBS containing 5 mM EDTA, pelleted in a Beckman J6-B centrifuge at 1,000 rpm, and resuspended in dPBS containing 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 0.2% BSA. For endocytosis assays, aliquots of cells were incubated at 37°C for the indicated times in the presence of BSST (2 μg/ml final concentration). The cells were then returned to ice, pelleted, and the supernatant aspirated. Latency to MesNa and avidin was determined as previously described (Smythe et al., 1992; Carter et al., 1993). Internalized Tfn was expressed as the percent of total surface-bound Tfn. The level of Tfn internalization was normalized to the amount of internalization for mock-infected cells to account for day to day variation; however, the rate and extent of internalization after 5 h of vaccinia infection in the presence of rifampicin was typically between 60–90% of that observed for control cells which had not been infected.

Assay of ER-to-Golgi Transport

HeLa cells were infected and transfected as described above using 5 μg of dynamin containing plasmid and 5 μg of a plasmid encoding Vesicular Stomatitis virus G (VSV-G) protein (pAR-G, Tisdale et al., 1992). After the transfection period, the cells were pulse-labeled for 10 min in methionine-free media containing 20 μCi TRANS ³⁵S-label, and then chased for 1 h in DMEM/10% FCS. The chase was terminated by the addition of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF) and VSV-G protein was immunoprecipitated using a monoclonal antibody to VSV-G (clone 8G5). Immune precipitates were washed, boiled, and eluted VSV-G protein was digested with endoglycosidase H (endo H) (Boehringer Mannheim, Indianapolis, IN), and analyzed by SDS-PAGE and fluorography (Tisdale, 1992).

Indirect Immunofluorescence

Coverslips containing transfected cells were incubated with 4 μg/ml anti-Tfn receptor monoclonal antibody (D65) for 30 min at 37°C in dPBS ++ (with 1 mM CaCl₂, 1 mM MgCl₂) containing 5 mM glucose and 0.2% BSA. Excess antibody was removed by washing at 4°C and the cells were incubated with 50 μg/ml avidin in dPBS/BSA. Excess avidin was removed by washing and the cells were fixed for 20 min at room temperature with 2.5% formaldehyde. Internalized biotinylated-HTR-D65 was detected by permeabilizing the fixed cells with 0.05% saponin and probing with Texas red-conjugated ultraavidin (Leinco Technologies, 2 μg/ml) in permeabilization buffer (PBS⁺⁺/0.05% saponin and 4% goat serum). The cells were then washed extensively and mounted in Aqua-Polymount (Polysciences, Warrington, PA) and viewed under a BioRad confocal scanning microscope.

For indirect immunofluorescence of coat proteins, cells were fixed for 30 min in 2.5% formaldehyde, permeabilized as described above, and incubated with primary antibody (5 μg/ml AP.6, 20 μg/ml X22) in permeabilization buffer for 30–60 min at room temperature. Primary antibodies were visualized with Texas red goat anti-mouse IgG secondary reagents (Molecular Probes, Eugene, OR).

Results

Molecular Analysis of Human Dynamin

Human dynamin cDNA was cloned for use in the functional studies in HeLa cells described below, to avoid problems that might have arisen with a heterologous expression system. A human brain cDNA library was probed with radiolabeled *Drosophila shibire* cDNA, followed by reprobating with the human fragments that were initially obtained. A cDNA spanning the entire coding region of the human *shibire* homologue was constructed from two overlapping fragments, and then sequenced. The deduced protein is 864 amino acids of which 99% are identical to rat dynamin (Fig. 1). Both human and rat sequences are similar to the *Drosophila shibire* sequence (69%) and this similarity extends throughout the molecule, strongly suggesting that function is conserved. Similar to rat and *Drosophila*, human dynamin has an NH₂-terminal GTPase domain that is highly conserved in VPS1p, MX-proteins, and MGM1p. The remainder of the molecule is divergent compared to these other family members and includes a proline/arginine-rich COOH-terminal domain specific to the dynamin homologues.

Analysis of a large collection of cDNAs revealed alternative splicing at two positions in the coding region (shown in Fig. 1). This has not been reported for rat dynamin, but is comparable to the alternative splicing found in *Drosophila* (van der Blik and Meyerowitz, 1991; Chen et al., 1991). Each human variant cDNA was found in multiple copies and the points of divergence correspond to exon-intron boundaries that include splice-site consensus sequences in the corresponding genomic DNA (data not shown). Thus it is unlikely that these represent cloning artifacts or incomplete processing. The first variation is the replacement of a 139-nt exon in the middle domain by an alternative exon of equal length. These two exons were equally represented in the cloned cDNA collection. Both exons encode 47-amino acid segments which differ by 15 amino acids (Fig. 1).

The second splice variation occurs at a position close to the COOH-terminus and involves the insertion of mini-exons. Surprisingly, *Drosophila* RNA is also alternatively

	element I	
MGNRGMEDLIPLVNLRLQDAFSAIGQNADLDLPQIAVVGGSAGKSSVLENFVGRDLPFRSGSIVTRRPLVLQLVNATTEYAEFLHCKGKK		90
	element II	
FTDFEEVRLIEAETDRVTGTNGKISPVPIINLRVYSPHVLNLTLDLPGMTKVPVQDQPPDIEFQIRDMLMQFVTKENCLILAVSPANSD		180
	element III	
LANSALKVAKEVDPQQRGTIGVITKLDLMDDEGTDARDVLENKLLPLRRGYIGVVNRSQKIDGKDKITAAALAEKRFPLSHPSYRHLAD		270
	internal variants	
RMGTPYLQKVLNQLTNHIRDTPGLRNKLSQQLSIEKEVEEYKNFRPDDPARKTKALLQMVQQFAVDFEKRIEGSGDQIDTYELSGGA		360
	internal variants	
RINRIFHERFPFELVKMEFDEKELRREISYAIAKNIHGI	RTGLFTPDLAFEATVKKQVQKLKEPSIKCVDMVVELTATIRKCKSEK	450
	RTGLFTPDMAFETIVKQVKKIREFPCIKCVDMVISELSTVRQCTTK	
	internal variants	
RLREEMERIVTTHIREREGRTKEQVMLLIDIELAYMNTNHEDFIFGANAQQRSNQMNKKTSGNQDEILVIRKQWLTINNIGIMKGGSK		540
YWFVLTAEENLSWKDDEEKEKMYLSDVNLKLRDVEKGFMSKXIFALFNTEQRNVYKDYRQLELACETQEEVDSWKASFVLRAGVYPERV		630
GDKEKASETEENGSDSFMHSDPQLERQVETIRNLVDSYMAIVNKTVRDLMPKTIHMLMINNTEKFEIFSELLANLYSCGDQNTLMEESAE		720
QAQRREMLRMYHALKEALSIIIGNINTTIVSTPMPFPVDDSWLQVQVSPVAGRRSPTSSPTPQRRAVPPARPGRGPAFPPPPAGSALG		810
	C-terminal variants	
GAPPVPSRPGASPDPPFGPPFQVPSRENRAPPGVP	SRSQQASPSRPEsprppfdl	864
	SQPIGSGKSIPS	856
	RITISDP	851
	mRNA has	
	no insert	
	4 bp insert	
	37 bp insert	

Figure 1. Deduced amino acid sequence of human dynamin. Sequences that are conserved within the GTPase superfamily are indicated as element I, II, and III. The five amino acids that are different in rat dynamin are indicated above or below the main sequence in bold type. The two points of alternative splicing are shown as alternate translation. In the internal variants a segment of 47 amino acids is replaced. At the COOH-terminus, the prevalent variant has no insert, the second variant has a 4-bp insert that changes the reading frame, and the third variant has a short insert with its own stop codon.

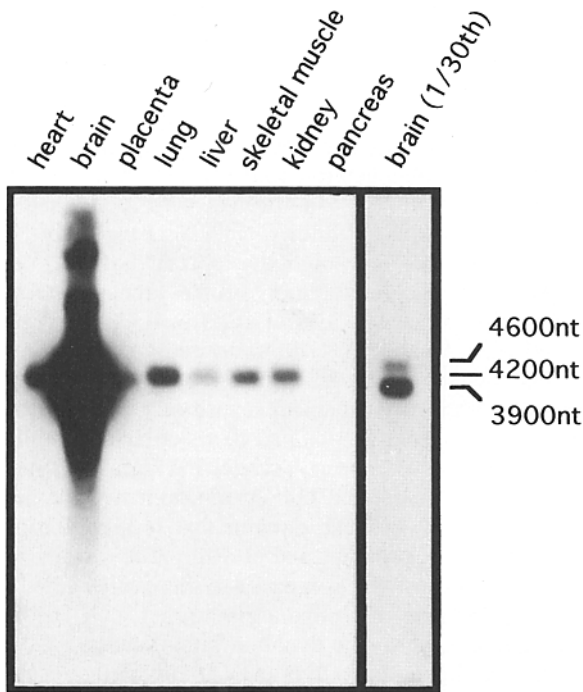


Figure 2. RNA blot analysis of human dynamin expression. An RNA blot containing poly(A)⁺ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas was hybridized with radiolabeled cDNA corresponding to the 5'-half of the dynamin sequence. The size of the dynamin RNAs was determined with an RNA ladder. A comparison between the high level of expression in brain and lower levels in other tissues was made using different exposure time (30 min vs 14 h).

spliced at this position, although it is through replacement of alternative 3'-exons rather than through the insertion of mini-exons (van der Blik and Meyerowitz, 1991). In a collection of 16 human cDNAs we found four variations at this position: (1) without a mini-exon (in 10 cDNAs), (2) with a 4-bp insertion which results in a frameshift and thus a different COOH-terminus (in 4 cDNAs), (3) a 37-bp insertion which contains a stop codon (in 1 cDNA), and (4) the same insertion encoding an identical COOH-terminus but with additional noncoding sequence (in 1 cDNA). The 37-bp insertion is present in the published sequence of rat dynamin (Obar et al., 1990). It seems likely that the other splice variants will also be found in rat. The 3'-untranslated sequence is common to all cDNAs. The cDNA that we have used for the experiments described below was the most abundant in the collection. It has the second of the two internal variants shown in Fig. 1 and lacks the COOH-terminal mini-exon.

Human dynamin is expressed in all tissues that were examined by RNA blot analysis (Fig. 2). The RNA levels are similar in most tissues, except in the human brain where it is at least 30-fold higher. High levels of dynamin expression were also found in rat brain (Scaife and Margolis, 1990; Nakata et al., 1991) and in the *Drosophila* nervous system (Chen et al., 1992; Ding, D., and A. M. v. d. Blik, unpublished results). Human dynamin RNAs are 4,200 nt, except in the brain which has a 3,900-nt and a 4,600-nt transcript. These differences in length are not accounted for by the known patterns of alternative splicing and possibly reflect the use of alternative transcriptional initiation sites. The elevated levels

of expression in the brain may reflect a high demand for membrane retrieval at the synapse. Dynamin expression in all tissues, at various levels, is consistent with the pleiotropic phenotype found in *Drosophila* mutants (Poodry et al., 1973).

Transient Expression of Dynamin in HeLa Cells

To determine if dynamin is required for receptor-mediated endocytosis in HeLa cells, we expressed wild-type and mutant human dynamin constructs using a transient expression system (Fuerst et al., 1986, 1989). The generation and functional analysis of *trans*-dominant mutations in several GTPase family members has established the validity of this approach (Der et al., 1988; Bucci et al., 1992; van der Sluijs et al., 1992; Tisdale et al., 1992). Site-directed mutagenesis was performed to introduce two mutations which significantly reduce nucleotide-binding affinities in all members of the GTPase superfamily so far studied (Fig. 3 *a* and references therein). The two mutations are designated element I (K⁴⁴→A) and element III (K²⁰⁶→D) and involve replacing critical lysine residues required for nucleotide binding. Identical results were obtained with both mutants (data not shown) and so studies presented here focus on the element I mutation. This nucleotide-binding element is also conserved in ATPases (Walker et al., 1982; Fry et al., 1986). While GTP binding has not been directly measured for the transiently expressed wild-type and mutant dynamin molecules, the conserved lysine residue directly interacts with the bound nucleotide and its mutagenesis reduces both ATP (Meluh and Rose, 1990) and GTP (Bourne et al., 1990, 1991) binding.

For expression studies, HeLa cells were first infected with vaccinia T7 RNA polymerase recombinant virus (vTF7-3, Fuerst et al., 1986), and then transfected with the expression vector pTMI containing a cDNA fragment encoding wild-type or mutant dynamin. Rifampicin was added to the culture medium to block maturation of the vaccinia capsid and to reduce vaccinia-associated cytotoxic events (Moss et al., 1969). Transfection efficiencies ranged from 25 to 60%. Since an antibody capable of recognizing dynamin by immunofluorescence was not available, transfection efficiencies were assessed by immunofluorescence of cells transfected with VSV-G protein in parallel (data not shown, see Tisdale et al., 1992).

Expression of mutant and wild-type dynamin was monitored by Western blot analysis using a rabbit polyclonal antibody (748), which is monospecific for dynamin.

A 100-kD protein, corresponding to dynamin, was detected in nontransfected HeLa cells and was substantially increased in cells transfected with either the wild-type or the mutant dynamin constructs (Fig. 3 *b*). This band was not detected by preimmune serum (data not shown). Detection of a minor band at ~45 kD was variable and levels of this protein were unaffected by infection or transfection. Dynamin expression was observed 3 h after transfection and continued to accumulate over 12 h. By 5 h after transfection, both wild-type and mutant dynamin accumulated in the cell population to 3.5–14-fold higher than endogenous levels (average ~7.5-fold).

Subcellular fractionation of HeLa cells in isotonic sucrose indicated that dynamin was approximately equally distrib-

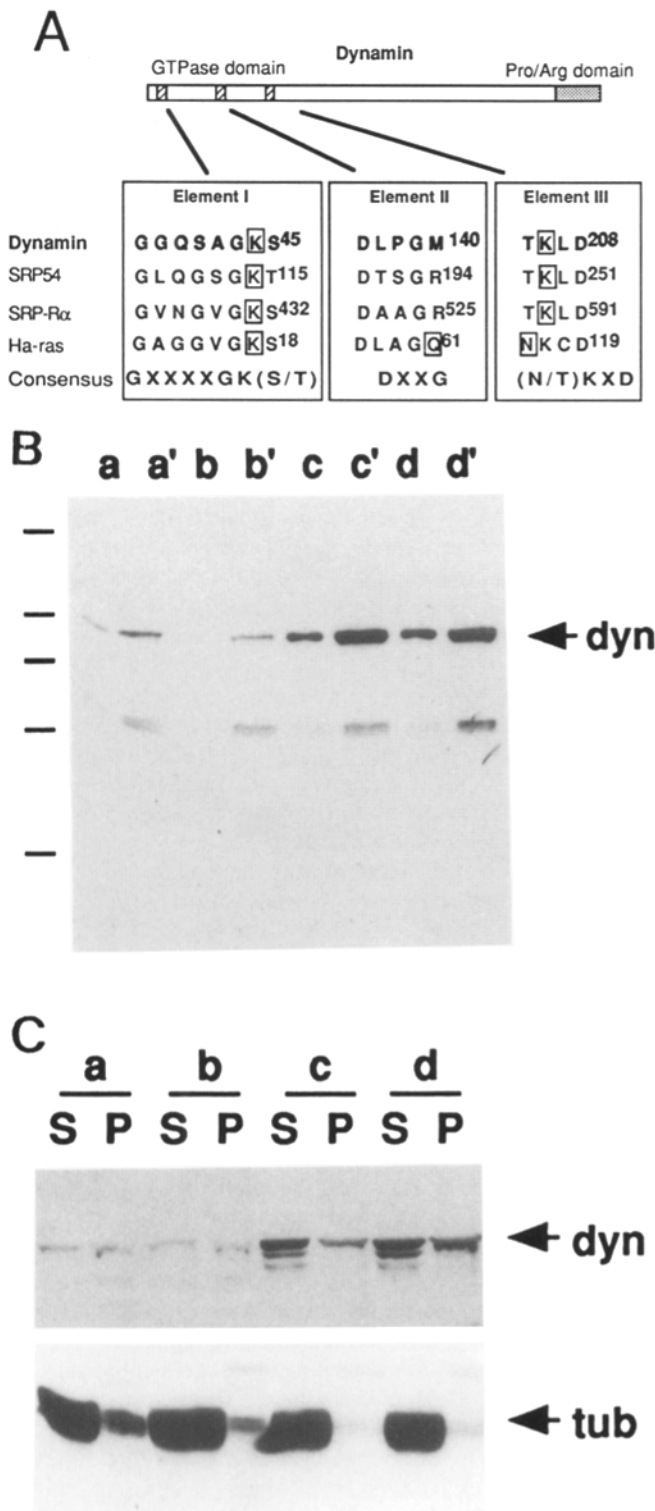


Figure 3. Transient expression of wild-type and mutant dynamamin constructs in HeLa cells. (A) Point mutations in the nucleotide-binding region of dynamamin family members which interfere with function. Shown are the domain structure of dynamamin and the three GTP-binding elements conserved among all members of the GTPase superfamily. The invariant lysines in element I and element III interact directly with bound nucleotides (Pai et al., 1990). Two dynamamin mutations, K⁴⁴→A in element I and K²⁰⁶→D, predicted to have impaired guanine-nucleotide-binding affinities were generated by site-directed mutagenesis. Analogous point mutations shown to result in a severely impaired guanine-nucleotide binding

uted between the particulate and the soluble fraction (Fig. 3 c, lanes a) as determined by densitometric analysis (see Materials and Methods). This distribution was similar to that reported for brain tissue and PC12 cells (Scaife and Margolis, 1990). Tubulin served as a control for the almost quantitative release of soluble proteins. The distribution of dynamamin was unaltered in virally infected and mock-transfected cells (Fig. 3 c, lanes b). At high levels of expression (~13-fold over endogenous), the amount of sedimentable wild-type dynamamin increased ~4-fold (Fig. 3 c, lanes c), while most of the excess dynamamin was present in the soluble pool. Similar results were obtained with mutant dynamamin except that the amount of sedimentable dynamamin was approximately eightfold over endogenous (Fig. 3 c, lanes d). Lysis under higher salt conditions results in complete redistribution of dynamamin into the soluble pool (data not shown). These results suggest that dynamamin interacts peripherally with a saturable component on membranes and that this interaction might be modulated by GTP binding.

Expression of Mutant Dynamamin Inhibits Receptor-mediated Endocytosis of Transferrin

To follow receptor-mediated endocytosis in transfected HeLa cells, Tfn was biotinylated via a cleavable disulphide bond (the complex is referred to as BSST) for use as a ligand. Internalization was measured by following the latency of transferrin to the low molecular weight reducing agent, MesNa (β -mercaptoethane sulfonic acid) as previously described (Smythe et al., 1992; Carter et al., 1993). The experiment presented in Fig. 4 demonstrates that both the rate and extent of endocytosis of transferrin were inhibited by >40% in cells overexpressing mutant dynamamin as compared to mock-transfected cells (i.e., vaccinia-infected cells treated with TransfectAce). HeLa cells overexpressing wild-type dynamamin showed neither inhibition nor stimulation of endocytosis over mock-infected cells, indicating that the level of dynamamin expression was not rate-limiting. In general, mock transfection or transfection with wild-type dynamamin resulted

in dynamamin family members are boxed. References to the mutations are as follows: *SRP54*, Bernstein, H., and Walter, P., personal communication; *SRP-R α* , Rapiejko and Gilmore, 1992; *Ha-ras*, Der et al., 1988; Sigal et al., 1986. (B) Western blot analysis of wild-type and mutant dynamamin overexpression. Vaccinia-infected cells transfected with a plasmid encoding wt or mutant constructs of dynamamin were allowed to express protein for 5 h, harvested and processed for Western blot analysis as described in Materials and Methods. Lane a, a' untreated cells; lane b, b', mock-transfected cells (which were infected with vaccinia virus and transfected without plasmid); lane c, c', cells transfected with wild-type dynamamin; and lanes d, d' cells transfected with the element I mutation. a-d, 10 μ g of cell lysate protein. a'-d', 50 μ g of cell lysate protein. (C) Subcellular distribution of endogenous and overexpressed dynamamin. Control, mock-transfected, and transfected cells were lysed by a freeze-thaw cycle and lysates were resolved into particulate and soluble fractions as described in Materials and Methods. Equal amounts of soluble (S) and particulate (P) fractions were analyzed by SDS-PAGE and Western blotting. Tubulin, detected with a polyclonal anti-tubulin antibody (ICN, Irvine, CA) was used as a control to monitor release of soluble contents. Lanes a, untreated cells; lanes b, mock-transfected cells; lanes c, cells transfected with wild-type dynamamin; and lanes d, cells transfected with the element I mutation.

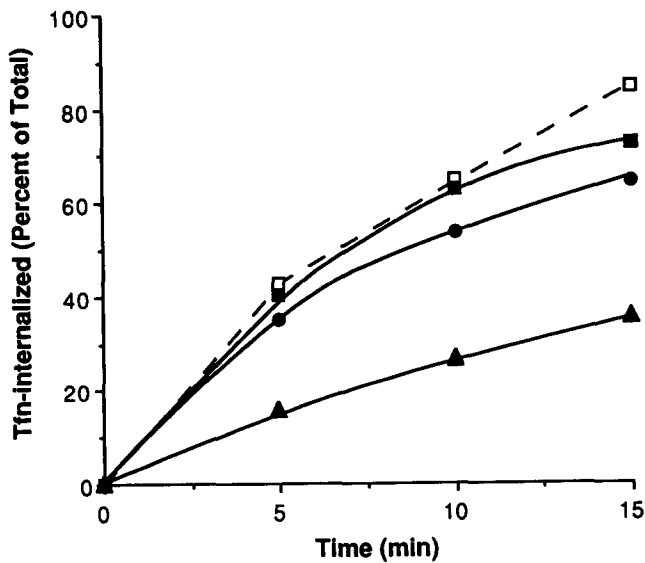


Figure 4. Inhibition of Tfn endocytosis caused by expression of mutant dynamin. HeLa cells were harvested after 5 h of protein expression and assayed for Tfn internalization as described in Materials and Methods. (□) untreated cells; (■) mock-transfected cells; (●) cells transfected with wild-type dynamin; and (▲) cells transfected with mutant dynamin. In this experiment, the level of overexpression of transfected dynamin was six-fold over endogenous.

in partial reduction of endocytosis as compared to untreated cells. While significant inhibition of receptor-mediated endocytosis was always observed in cells overexpressing mutant dynamin molecules, the extent of inhibition varied. In all experiments ($n = 12$), the extent of inhibition as compared to cells expressing equivalent levels of wild-type dynamin was $38 \pm 13\%$ (range 19–59%).

A correlation between the extent of inhibition of receptor-mediated endocytosis and the levels of expression of mutant dynamin molecules in the transfected population is shown in Fig. 5. Overexpression from the transfected dynamin constructs could be detected at 3 h after transfection and dynamin continued to accumulate in the transfected cell population over the 5-h time course shown (Fig. 5 B). At the indicated times, cells expressing either wild-type or mutant dynamin were harvested and incubated for 15 min with BSST. As can be seen in Fig. 5 A (solid bars), the inhibition of Tfn internalization relative to mock-transfected controls increased with increasing levels of expression of mutant dynamin. The degree of inhibition of receptor-mediated endocytosis in cells overexpressing mutant dynamin was 31, 59, and 65% at 3, 4, and 5 h after transfection, respectively. At 5 h after transfection, cells expressing wt dynamin exhibited partially reduced endocytosis, possibly due to prolonged viral infection. Inhibition due to expression of mutant dynamin is therefore measured relative to cells expressing wt dynamin. These results demonstrate that expression of mutant dynamin greatly reduces receptor-mediated endocytosis in mammalian cells.

Immunofluorescence Assay Reveals a Subpopulation of Cells with Severely Inhibited Endocytosis

The most likely explanation for the partial block in endocy-

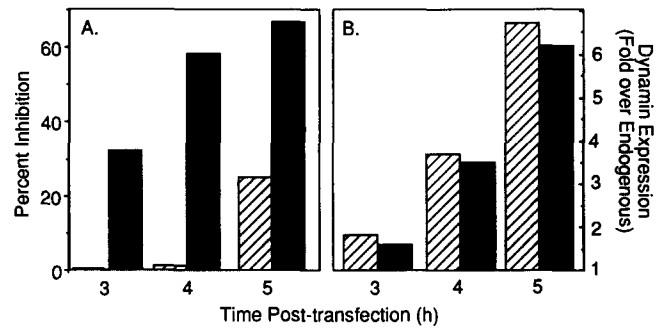


Figure 5. Time course of dynamitin expression and inhibition of endocytosis. Vaccinia-virus infected HeLa cells were transfected with either wild-type dynamitin (striped bars) or ele 1 GTP-binding domain mutant dynamitin (solid bars), and incubated for the indicated times before harvesting. (A) Inhibition of Tfn endocytosis expressed relative to the level of endocytosis in mock-transfected cells at 3 h was determined as described in Materials and Methods. (B) Dynamitin expression relative to endogenous levels was determined by Western blotting analysis as described in Materials and Methods.

tos in the cell population, given that transfection efficiencies are 25–60%, is cell-to-cell variability in the levels of dynamitin expression. For this reason, an immunofluorescence assay was developed to demonstrate that receptor-mediated endocytosis is strongly inhibited in a subpopulation of cells. In this single cell assay, transfected HeLa cells expressing mutant and wild-type dynamitin were incubated for 30 min at 37°C with a biotinylated-monoclonal antibody (HTR-D65) which recognizes the ectodomain of the Tfn receptor (White et al., 1990). Unlike BSST, this antibody ligand will not be recycled and will accumulate in cells capable of efficient endocytosis (Weissman et al., 1986). Subsequently, the cells were washed and incubated for 30 min at 4°C with avidin to mask accessible biotin residues. The cells were washed, fixed, permeabilized, and incubated with Texas-red-conjugated-avidin to detect the internalized, biotinylated-anti-Tfn antibody. These results illustrated in Fig. 6, demonstrate that mock-transfected cells (Fig. 6; A) or transfected cells overexpressing wild-type dynamitin (Fig. 6; B) showed efficient and uniform internalization of receptor-bound antibody. In marked contrast, a subpopulation of transfected cells expressing the mutant dynamitin failed to internalize any detectable anti-Tfn mAbs (Fig. 6, C and D, arrowheads). The extent of inhibition determined biochemically corresponded to the fraction of cells unable to internalize HTR-D65 as detected by immunofluorescence. For example, in two experiments in which endocytosis was inhibited in cells expressing mutant dynamitin by 29 and 37% as compared to cells expressing wild-type dynamitin, 23 and 32% of ~400 cells counted for each condition showed inhibited endocytosis by immunofluorescence. Taken together, these assays provide strong evidence that functional dynamitin is required for endocytosis of the Tfn receptor.

Mutant Dynamitin Blocks an Intermediate Stage in Coated Vesicle Formation

We have previously shown that early and late stages in coated vesicle formation can be distinguished by determining the inaccessibility of bound ligand either to bulky probes such as

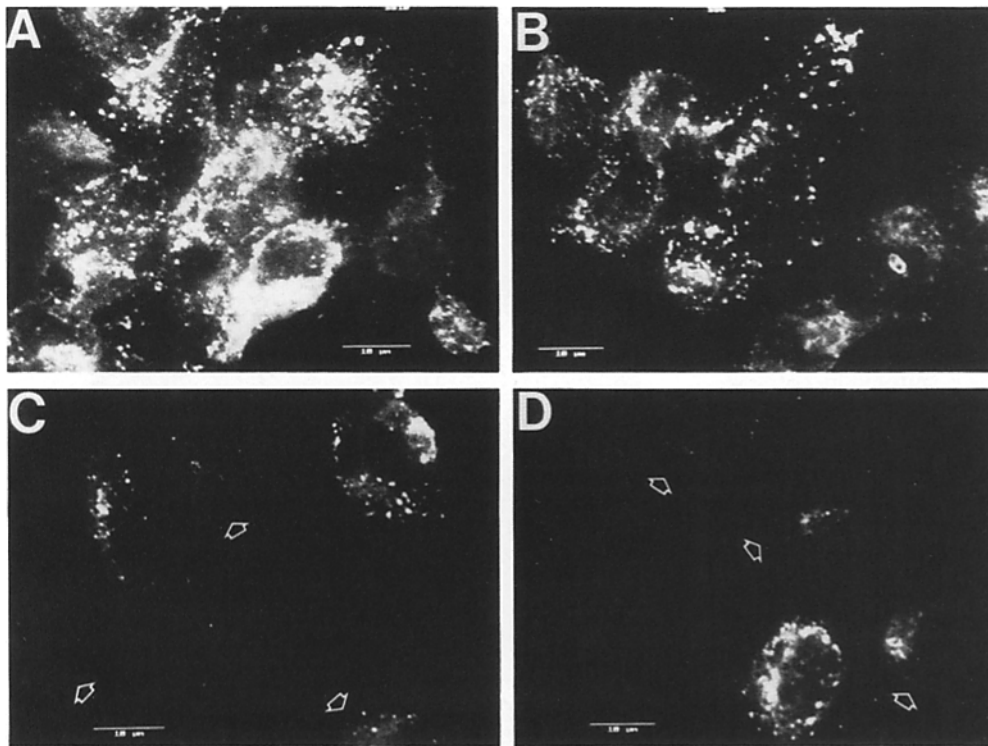


Figure 6. Complete inhibition of Tfn-receptor internalization in a subpopulation of cells expressing mutant dynamin. Cells grown, infected, and transfected on coverslips as described in Materials and Methods were allowed to internalize surface bound biotinylated anti-Tfn-R antibody, HTR-D65, for 30 min at 37°C. Residual surface bound biotinylated antibody was masked with avidin, cells were fixed, permeabilized, and internalized HTR-D65 was subsequently detected with a Texas-red-conjugated ultra-avidin as described in Materials and Methods. Mock-transfected cells (A) and cells expressing wild-type dynamin (B) showed uniformly high levels of intracellular HTR-D65. In contrast, a subpopulation of cells transfected with ele I GTP-binding domain mutant dynamin (C and D) were incapable of internalizing detectable HTR-D65 (open arrowheads). The level of inhibition determined biochemically in this experiment was 38% over wild-type.

avidin or antibodies, or to small probes such as MesNa. Internalization of biotinylated ligands into coated vesicles renders them inaccessible to both large and small probes; however, ligands sequestered into deeply invaginated coated pits become inaccessible to avidin while remaining accessible to MesNa (Schmid and Carter, 1990; Schmid and Smythe, 1991). Under normal conditions, the rate and extent of endocytosis as measured using the two probes is identical. However, in cells depleted of ATP, membrane fission is selectively blocked resulting in fivefold greater sequestration from avidin as compared to MesNa (Schmid and Carter, 1990). These two probes were used to determine the stage at which coated vesicle formation was blocked in cells expressing mutant dynamin. The results, illustrated in Fig. 7, demonstrate that the sequestration of Tfn into deeply invaginated coated pits was as effectively inhibited as Tfn internalization in HeLa cells expressing mutant dynamin. These experiments suggest that dynamin activity is required for a relatively early stage of endocytosis, preceding both coated pit invagination and the membrane fission event leading to coated vesicle formation.

The effect of overexpression of dynamin mutants on coat assembly, an earlier stage of endocytosis, was examined by indirect immunofluorescence of the AP2 complex, a major plasma membrane coat protein. The results are shown in Fig. 8. The punctate staining pattern characteristic of cell surface-coated pits was uniformly observed in untreated cells (Fig. 8; A) and was unaltered in transfected cells expressing

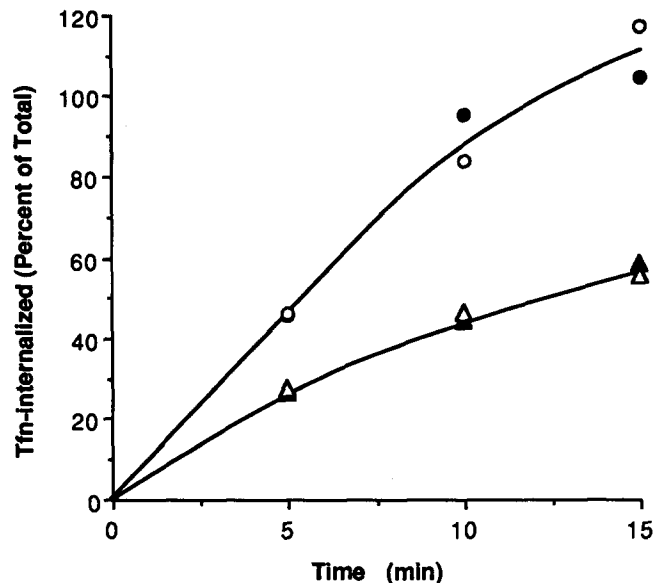


Figure 7. Expression of mutant dynamin blocks Tfn internalization at an intermediate stage in coated vesicle formation. Cells expressing either (○, ●) wild-type dynamin or ele I GTP-binding domain mutant dynamin (△, ▲) were harvested after an 8-h expression and incubated with BSST for the indicated times. The extent of Tfn internalization was assessed either by MesNa-resistance (●, ▲) or avidin accessibility (○, △) as described in Materials and Methods.

either wild-type (Fig. 8, *B*) or mutant dynamin (Fig. 8, *C*). This distribution was uniformly observed throughout the population even though endocytosis of Tfn in these cells was 40% inhibited. The distribution of clathrin, as detected by indirect immunofluorescence was similarly unaffected (data not shown). These results are consistent with the accumulation of coated pits seen by EM of *Drosophila shibire* mutants (Kosaka and Ikeda, 1983) and suggest that the initiation of coat assembly is unaltered by mutant dynamin.

Mutant Dynamin Does Not Inhibit Transport from ER to the Golgi Complex

Proteins involved in vesicular transport could be specific to a single step or could participate in multiple membrane trafficking events (Rothman and Orci, 1992). To determine whether mutant dynamin had a specific effect on the endocytic pathway, we investigated the consequence of its overexpression on the secretory pathway. Transport along the early part of the secretory pathway was monitored by cotransfecting vaccinia-infected HeLa cells with T7-expression constructs encoding VSV-G and constructs encoding wild-type or mutant dynamin. VSV-G which serves as a marker for transport, acquires two endoglycosidase H(endoH)-sensitive N-linked oligosaccharide chains in the ER that are processed to endoH-resistant forms upon transport to the *cis*/medial Golgi compartments. The results presented in Fig. 9 demonstrate that pulse-labeled VSV-G was quantitatively (>90%) converted to the endoH resistant form after a 60-min chase. The extent of transport to the Golgi was unaffected in cells cotransfected with either wild-type or mutant dynamin constructs. In parallel experiments, endocytosis was inhibited by 30–40% (data not shown). Preliminary immunofluorescence showed an unaltered distribution of VSV-G protein in cells expressing mutant and wild-type dynamin suggesting that export from the Golgi and delivery to the cell surface was also unaffected (data not shown). More thorough examination of the effects of dynamin on post-Golgi trafficking to the cell surface or to lysosomes awaits the generation of stable transformants. These results rule out the possibility of nonspecific cytotoxic effects and indicate that mutant dynamin expression causes a specific reduction in the early stages of receptor-mediated endocytosis.

Discussion

Dynamin Is Required for Receptor-mediated Endocytosis

We have shown that receptor-mediated endocytosis is inhibited in mammalian cells that overexpress mutant dynamin. Using assays which enable detection of intermediates in coated vesicle formation, the dynamin mutants were found to block endocytosis at a stage preceding the sequestration of ligands into deeply invaginated coated pits. The distribution of clathrin and APs, the major coat constituents, was unaffected in cells overexpressing these dynamin mutants, suggesting that the block follows coat assembly. These results demonstrate that mutations in the GTP-binding domain of dynamin block Tfn-endocytosis in mammalian cells, suggesting that a functional dynamin GTPase is required for receptor-mediated endocytosis via clathrin-coated pits.

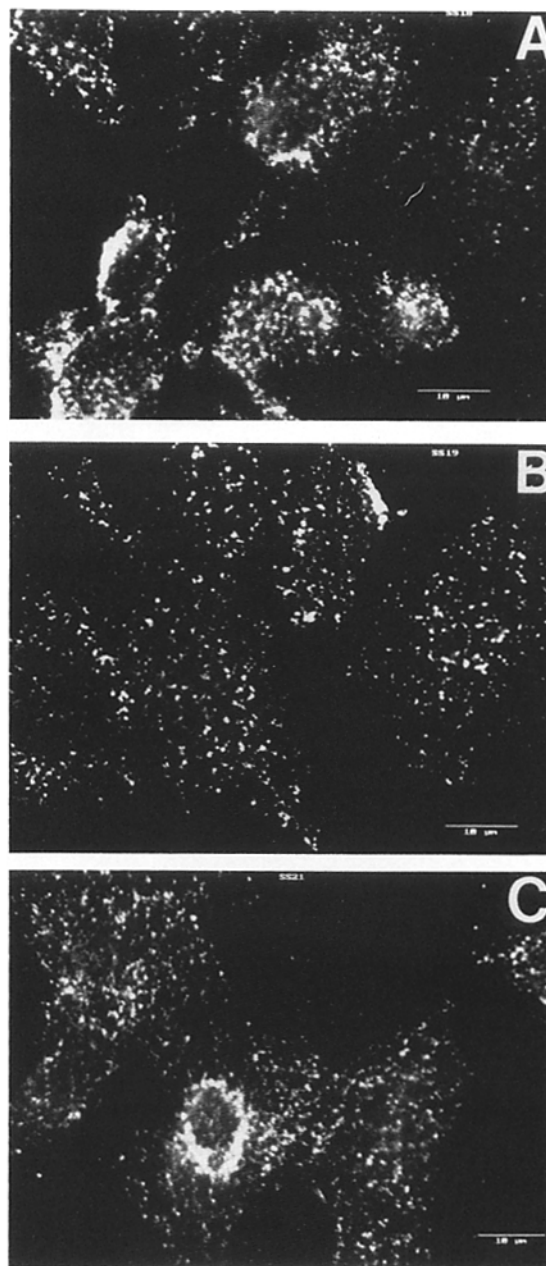


Figure 8. The distribution of α -adaptins is unaffected by overexpression of mutant dynamin. The distribution of α -adaptins was determined by indirect immunofluorescence using the AP.6 mAb as described in Materials and Methods. (*A*) mock-transfected cells; (*B*) cells expressing wild-type dynamin; (*C*) cells expressing mutant dynamin. The level of inhibition determined biochemically in this experiment was 40% over wild-type.

The mutations constructed in elements I and III of the dynamin GTP-binding domain were predicted, by analogy to *ras* (Sigal et al., 1986; Der et al., 1988), SRP-R α -subunit (Rapiejko and Gilmore, 1992), and SRP54 (Bernstein, H., and P. Walter, personal communication) to reduce its affinity for both GTP and GDP. Here we report that point mutations in dynamin predicted to reduce GTP-binding have a dominant negative effect on endocytosis when overexpressed in mammalian cells. Interestingly, a number of mutations in the

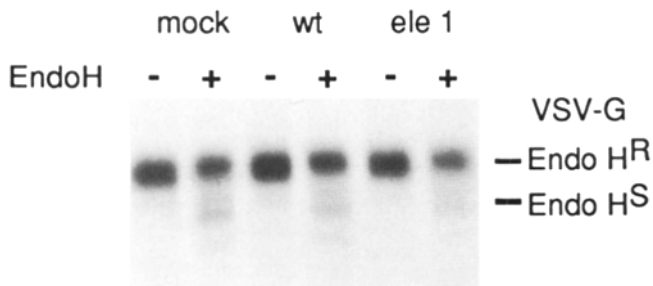


Figure 9. Dynamins do not inhibit VSV-G transport between the ER and the Golgi stack. Vaccinia-infected cells were transfected with a plasmid containing the indicated dynamin construct and a plasmid encoding VSV-G protein (pAR-ts). Conversion of pulse-labeled VSV-G protein from an EndoH sensitive form found in the ER to an EndoH resistant form associated with the Golgi complex was determined as described in Materials and Methods.

GTP-binding domain of VPS1_p, a dynamin-related protein in yeast, including some which do not affect GTP-binding, have dominant negative effects on vacuolar sorting (Vater et al., 1992). This result supports the importance of the GTPase domain for dynamin function. In contrast to the stimulatory effect of overexpression of the small GTP-binding proteins rab5 (Bucci et al., 1992) and rab4 (van der Sluijs et al., 1992) on endocytosis in mammalian cells, overexpression of wild-type dynamin had no detectable effect. This result suggests that the level of dynamin is not normally "rate-limiting" for endocytosis in mammalian cells.

Dynamins were originally identified as a microtubule-stimulated GTPase (Shpetner and Vallee, 1989, 1992; Maeda et al., 1992), however the significance of this association is unclear (Vallee, 1992). Drugs which influence the polymerization or depolymerization of microtubules do not alter the rate or extent of receptor-mediated endocytosis (Oka and Weigel, 1983; Morgan and Iacopetta, 1987). Our finding that dynamins are required for receptor-mediated endocytosis in mammalian cells is therefore inconsistent with its previously suggested role as a microtubule-based mechanochemical motor (Shpetner and Vallee, 1989). Recent results have shown that while highly purified preparations of dynamins retain their microtubule-stimulated GTPase activity, they lack microtubule-based motor activity (Maeda et al., 1992). Given our results, it remains likely that other, as yet unidentified proteins or protein complexes regulate dynamin GTPase activity in vivo.

Overexpression of mutant dynamins appeared to specifically inhibit receptor-mediated endocytosis: transport of VSV-G protein from the ER to the Golgi was unaffected and preliminary results suggested that delivery to the cell surface was also unaffected. Given the incomplete inhibition in these transient transfection assays, more thorough analysis of the effects of overexpression of these dynamin mutants on other vesicular trafficking pathways, including fluid-phase endocytosis, receptor-recycling, post-Golgi trafficking, and lysosomal targeting will await the generation of inducible stably transformed cell lines.

The *Shibire* Phenotype and the Role of Dynamins in Endocytosis

Dynamins were first linked to endocytosis through the discov-

ery that the *Drosophila* homologue of dynamin is encoded by the *shibire* gene (van der Bliek and Meyerowitz, 1991; Chen et al., 1991). *Shibire* was identified earlier as a mutation that causes temperature sensitive paralysis in adult flies (Grigliatti et al., 1973) and was shown to affect development if the temperature was shifted at a critical time (Poody et al., 1973). Evidence suggesting that *shibire* affects endocytosis has come largely through morphological studies which have shown that conditional paralysis correlates with a depletion of synaptic vesicles, and a concomitant increase in coated pits at nerve endings (Koenig and Ikeda, 1989). In other cell types, for example, nephrocytes (Kosaka and Ikeda, 1983; Koenig and Ikeda, 1990) or oocytes (Kessel et al., 1989), shift to the nonpermissive temperature also leads to the apparent disappearance of the endosomal compartment and an increase in cell surface area, reflected by the formation of large membrane infoldings. Coated pits, which appear normal, are often seen at the bottom of these membrane invaginations.

These electron microscopic observations, coupled to the rapid kinetics of paralysis and its reversal in *shibire* mutants (<1 min) are compatible with a defect in the early stages of endocytosis. For lack of a suitable receptor in *Drosophila*, functional studies have been limited to morphological studies after the uptake of electron dense fluid phase markers. These studies support the hypothesis that the *shibire* mutation is defective in endocytosis (Kosaka and Ikeda, 1983; Tsurahara et al., 1990). By testing the effect of mutant dynamins on transferrin uptake in HeLa cells, we have provided the first functional evidence that the mutant protein blocks receptor-mediated endocytosis at an intermediate stage in coated vesicle formation.

Human Dynamins Expression and Alternative Splicing

The human cDNA is the third dynamin homologue to be cloned. What distinguishes dynamins from related proteins (for review see Collins, 1991) is a proline rich (30%) and highly basic COOH-terminus that extends ~100 amino acids. This domain is similar in composition to the COOH-terminal domain of synapsin I, which has been implicated in the binding of synapsin I to a synaptic vesicle protein (Benfenati et al., 1992). The extreme COOH-termini of both dynamins and synapsin I are alternatively spliced (Sudhof et al., 1989). Whether this reflects a common function in membrane binding remains to be tested. Multiple electrophoretically distinct isoforms of dynamins have been detected in both rat and *Drosophila* protein extracts (Scaife and Margolis, 1990; Chen et al., 1991; Faure et al., 1992). These may correspond to the splice variants detected in *Drosophila* (van der Bliek and Meyerowitz, 1991; Chen et al., 1991) and in humans.

Given that mutations in dynamins disrupt endocytosis, it is of no surprise that the gene is expressed in all tissues examined thus far. However, it is striking that expression is at least 30-fold higher in the human brain. The coat proteins clathrin and APs, by comparison, are expressed at 5–10-fold higher levels in brain (Kirschhausen et al., 1987; Robinson, 1989). Whole-mount in situ of *Drosophila* embryos show very high dynamin expression throughout the nervous system (Chen et al., 1992; Ding, D., and A. M. v. d. Bliek, unpublished results). Dynamins are highly expressed in the rat cerebellar

cortex, the hippocampus, and the cerebral cortex (Nakata et al., 1991; Faire et al., 1992) similar to the distribution of AP subunit transcripts (Robinson, 1989). Recent studies have demonstrated that coated vesicles isolated from synaptic termini are almost exclusively involved in recycling the constituents of synaptic vesicles (Maycox et al., 1992). Thus, high levels of dynamin expression in nerve cells are consistent with its role in expediting synaptic vesicle recycling.

Conclusion

What role might dynamin play in receptor-mediated endocytosis and coated vesicle formation? Our results show that mutant dynamin blocks endocytosis after the initiation of coat assembly but before coated pit invagination. This specifically assigns a role for dynamin in the intermediate stages on the temporal sequence of events that can be distinguished biochemically: coat assembly, receptor recruitment, coated pit invagination and vesicle budding (Schmid, 1993). Consistent with this assignment and with the known affinity of dynamin for GTP, coated pit invagination *in vitro* requires $\sim 10 \mu\text{M}$ GTP (Carter et al., 1993). Members of the GTPase superfamily, in general, act as monitors and molecular switches which either trigger an event or ensure the fidelity of vectorial reactions (Bourne et al., 1990, 1991). During coated pit invagination dynamin might monitor receptor recruitment, necessary coat protein rearrangements, or the assembly of a molecular complex required for invagination or budding. In response to completion of these requisites to vesicle formation, dynamin might trigger invagination. While we cannot rule out that dynamin plays an active, mechanochemical role in the invagination and/or pinching process, this seems less likely given its membership in the GTPase superfamily. Our observation that dynamin is equally distributed in particulate and soluble fractions suggests cycling on and off the membrane as part of its function. Further characterization of the role of dynamin will follow from identification of interacting proteins.

Genbank Accession Numbers

The human dynamin cDNA sequences are accessible under GenBank accession numbers L07807-L07810.

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