Clathrin-independent Pinocytosis Is Induced in Cells Overexpressing a Temperature-sensitive Mutant of Dynamin

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Abstract. A stable HeLa cell line expressing a dynamin mutant, dynT6, exhibits a temperature-sensitive defect in endocytic clathrin-coated vesicle formation. DynT6 carries a point mutation, G273D, corresponding to the Drosophila shibireT6 allele. The ts-defect in receptor-mediated endocytosis shows a rapid onset (<5 min) and is readily reversible. At the nonpermissive temperature (38°C) HRP uptake is only partially inhibited. Moreover, when cells are held at the nonpermissive temperature, fluid phase uptake fully recovers to wild-type levels within 30 min, while receptor-mediated endocytosis remains inhibited. The residual HRP uptake early after shift to the nonpermissive temperature and the induced HRP uptake that occurs after recovery are insensitive to cytosol acidification under conditions that potently inhibit receptor-mediated endocytosis of Tf. Together, these results suggest that a dynamin- and clathrin-independent mechanism contributes to the total constitutive pinocytosis in HeLa cells and that dynT6 cells rapidly and completely compensate for the loss of clathrin-dependent endocytosis by inducing an alternate endocytic pathway.

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The monoclonal anti-hemagglutinin antibody, 12CA5, was provided by I. A. Wilson (The Scripps Research Institute, La Jolla, CA). Mouse monoclonal antibodies were purified by affinity chromatography on protein G-Sepharose (Pierce Chemicals, Rockford, IL). Texas Red-conjugated secondary antibody, Texas Red-conjugated transferrin and rhodamine phalloidin were purchased from Molecular Probes (Eugene, OR).

Human diferric transferrin (Tf) was from Boehringer-Mannheim (Indianapolis, IN). Tf was biotinylated as previously described (Smythe et al., 1992). Cytochalasin D, pronase and [4-(2-aminoethyl)-benzenesulfonylfluoride, HCl] (AEBSF) were from Calbiochem-Novabiochem (La Jolla, CA). 5-[N,N-Hexamethylene]-amiloride and reagent-grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Boehringer Mannheim unless otherwise specified.

### Preparation of cDNA Constructs

A temperature-sensitive mutant of human dynamin, designated dyn<sup>ts</sup>, was generated by site-directed mutagenesis of the glycine at position 273 to aspartic acid corresponding to the known point mutation in the shibire<sup>ts</sup> allele (van der Bliek and Meyerowitz, 1991). Dyn<sup>ts</sup> was introduced into the pTｍ1-dyn<sup>ts</sup> constructs using two different PCRs with Phu polymerase (Stratagene Inc., La Jolla, CA) as previously described (van der Bliek et al., 1993). The cDNA was then modified by adding a linker fragment to the 5′ end of the coding sequence for fusion to the sequence, MEYDYFDTYAH, containing an hemagglutinin (HA) epitope, as described in Damke et al. 1994. Subsequently the verified CDNA for the dyn<sup>ts</sup> mutant was subcloned from the modified vaccinia expression constructs pTM1- HA-dyn<sup>ts</sup> into the tetracycline-inducible expression plasmid pUHD10-3 as described (Damke et al., 1994).

### Generation of Stably Transformed Mutant Cell Lines

The stable HeLa cell line tTA-HeLa, expressing the chimeric tetracycline regulatable transcription activator was kindly provided by H. Bujard (ZMBH, Heidelberg, FRG). Cells were maintained in DME supplemented with 10% (vol/vol) FBS, 100 U/ml each of penicillin and streptomycin, 400 μg/ml G418 (Geneticin, Gibco BRL, Gaithersburg, MD). The construct pUHD10-2 encoding HA-tagged dynamin was used for the generation of stably transformed tTA-HeLa cells with tightly regulated expression of dynamin as described in Damke et al. (1995).

### Cell Culture Conditions for tTA HeLa Cells Stably Transformed with the Dynamin cDNA

Cells were cultured at 37°C in DME, 10% FBS, 100 U/ml each of penicillin and streptomycin, 400 μg/ml G418 (selects for transactivator), 1–2 μg/ml tetracycline (suppresses dynamin expression), and 200 ng/ml puromycin to select for positive clones. Puromycin resistant clones were screened for their ability to express dynamin 48 h after induction in the absence of tetracycline at 37°C by SDS-PAGE and Western blot analysis.

For phenotypic analysis, subconfluent cultures were detached with trypsin/EDTA and ~1.2 × 10<sup>6</sup> cells were plated on 100-mm culture dishes in the presence (uninduced) or absence (induced) of tetracycline and incubated at the permissive temperature of 32°C≤72 h before use. At this point the cells were ~65% confluent. As dynamin expression and phenotype were highly dependent on the confinement of the cells it is important to use the cells at subconfluent density for biochemical and morphological analysis. This is also recommended for permanent culture of uninduced cells growing at 37°C. Inducible expression is also dependent on the passage number, and cells were therefore used at low passage number and routinely replaced by freshly thawed cells.

### Western Blot Analysis

Cells were washed twice with PBS and detached with PBS, 5 mM EDTA. Cell pellets were immediately lysed in 1× Laemmli sample buffer and lysates were analyzed by SDS-PAGE on 7.5% gels and electroblotted to BA-S 83 nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) by standard methods (Burnette, 1981). Nonspecific protein binding was blocked with 2% nonfat dry milk powder in Tris-buffered saline containing 0.05% Tween 20. The blots were incubated with the primary anti-dynamin antibody hudy-3 at 0.1 μg/ml, followed by detection with an alkaline phosphatase-conjugated goat anti-mouse antibody (Pierce Chem. Co., Rockford, IL) and developed with an alkaline phosphatase color development kit (Bio-Rad, Hercules, CA).

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1. Abbreviations used in this paper: BSST, biotinylated-transferrin; wt, wild-type; tet, tetracycline; Tfn, transferrin; Tfn-R, transferrin-receptor; HA, hemagglutinin; ts, temperature-sensitive.
Internalization Assays

Internalization assays using biotinylated-transferrin (BSST) were performed as previously described (van der Bieke et al., 1993). Cells induced at the permissive temperature were detached with PBS, 5 mM EDTA at RT for 5 min, briefly rinsed, and resuspended in PBS containing 1 mM MgCl₂, 1 mM CaCl₂, and 0.2% BSA, pH 7.4. The cell suspensions were then preincubated for various times at 30 or 38°C in waterbaths in which temperature was carefully adjusted using a digital thermometer. After the preincubation, cells were rapidly cooled on ice and the ligand was added as a concentrated solution to a final concentration of 8 μg/ml BSST. The cell suspensions were then split into 50-μl aliquots (corresponding to 2 × 10⁶ cells) for continuous internalization of BSST at 30 or 38°C for various times. Endocytosis was stopped by returning the samples to ice. Internalization of ligand was quantified after processing the samples for measuring avidin inaccessibility or MesNa resistance according to Carter et al. (1993).

Fluid Phase Uptake

Cells were grown on 35-mm plates for 72 h at 32°C (dyeK446 cells for 48 h at 37°C) in the absence of tetracycline, washed twice with PBS and incubated with 4 mg/ml HRP in DME/20 mM Heps, 0.2% BSA, pH 7.4, for various times. The uptake was terminated by aspirating the medium and by six subsequent washes with PBS, 1 mM MgCl₂, 1 mM CaCl₂, 0.2% BSA, pH 7.4, for 5 min at 4°C (Marsh et al., 1987). After two additional rinses with PBS at 4°C the cells were detached with 0.1% pronase in PBS, pH 7.4, which also removed any HRP unspecifically bound to the cell surface. The cell suspensions were pelleted through a sucrose cushion (0.5 M phosphate buffer, pH 7.4, which also removed any HRP unspecifically bound to the cell surface. The cell suspensions were then split into 50-μl aliquots (corresponding to 2 × 10⁶ cells) for continuous internalization of BSST at 30 or 38°C for various times. Endocytosis was stopped by returning the samples to ice. Internalization of ligand was quantified after processing the samples for measuring avidin inaccessibility or MesNa resistance according to Carter et al. (1993).

Cytosol Acidification

For acidiﬁcation of the cytosol, wild-type or mutant cells grown at the permissive temperature were rinsed two times with DME-H (DME containing 2 mM L-glutamine and 20 mM Heps, without bicarbonate), incubated for 30 min in DME-H, 0.2% BSA, rinsed twice in DME-H, 0.2% BSA, pH 5.0 and then incubated in 10 mM acetic acid in DME-H, 0.2% BSA, pH 5.0. To prepare DME-H, 1 M acetic acid, pH 5.0 (adjusted with NaOH) was diluted 1:100 into DME-H (adjusted to pH 5.0 by HCl) as described (Hansen et al., 1993). Controls were incubated in DME-H, 0.2% BSA, pH 5.0 or pH 7.4. Depending on the time of preincubation at the nonpermissive temperature all solutions were prewarmed to 30 or 38°C. Then the medium was aspirated and the cells were incubated for up to 60 min in DME-H, 0.2% BSA containing HRP (4 mg/ml) and supplemented with 5 mM glucose at 30 or 38°C. Subsequently cells were processed as described above. For transferrin internalization cells in suspension were incubated under identical conditions in the presence of 8 μg/ml BSST as ligand.

Indirect Immunofluorescence

Dynamin wild-type and dyn- mutant TATAHeLa cells were grown in the absence of tetracycline on glass coverslips for 72 h at 32°C to ~60% confluency. The cells were incubated with TexasRed-conjugated transferrin (50 μg/ml) in serum free medium (DME, 20 mM Heps, 0.2% BSA) at 30 or 38°C for 10 min. Then the cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min at room temperature, permeabilized with 0.05% saponin in PBS for 10 min, and then blocked in 1% BSA, 5% normal goat serum, 0.01% saponin, 0.02% sodium azide in PBS for 60 min. The cells were incubated with anti-HA mAb 12CA5 (2 μg/ml) against the COOH-terminal end of the ~300-amino acid G273D mutation, designated dyn-8, was made by site-directed mutagenesis. The cDNA encoding dyn-8 with an NH2-terminal HA-epitope tag was inserted into a tetracycline (tet)-responsive expression vector (pUHD10-3) and used to transform TATATATAHeLa cells expressing a tet-regulatable chimeric transcription activator (Gossen and Bujard, 1992; Damke et al., 1995). Clonal cell lines showing tight regulation of dynamin expression by tetracycline were selected and amplified, as previously described (Damke et al., 1994, 1995). Western blot analysis (Fig. 1) shows the tet-regulatable expression of dynamin constructs in these stably transformed TATATATA-HeLa cells. In the presence of tetracycline the expression of neuronal dynamin is not detectable with the dynamin-1 specific monoclonal antibody, hudy-3. In the absence of tetracycline, dynamin expression was induced to >20-fold over endogenous dynamin-2 (Damke et al., 1994). Equal expression levels were obtained in cells expressing either wt or mutant dynamin and when cells were incubated for either 48 h at 37°C or for 72 h at 32°C.

HeLa Cells Expressing Dynamin-8

Show a Temperature-sensitive Defect in Receptor-mediated Endocytosis

Initial attempts to detect an endocytosis defect in transiently transfected cells expressing dyn-8 using the vaccinia

![Figure 1. Western blot analysis with the anti-dynamin-1 mAb, hudy-3, demonstrates the tight control of tetracycline-regulatable expression of wild-type and ts-dynamin. The levels of protein accumulation were equivalent when cells were induced for 72 h at 32°C or for 48 h at 37°C. Lysates of equal numbers of cells were processed as described in Materials and Methods.](image-url)
virus transfection system (van der Bliek et al., 1993) were unsuccessful (A. M. van der Blick, T. E. Redelmeier, and S. L. Schmid, unpublished data). Similarly, when tetracycline was removed to induce dyn expression at 37°C, endocytosis was unaffected (data not shown) even though expression levels of recombinant dynamin were as high as for other mutants (Fig. 1). We reasoned that, since the mutation was predicted to be temperature-sensitive for function, it might be necessary to synthesize the mutant protein at the permissive temperature to generate a correctly folded and otherwise functional protein. Several temperatures were tested and we found that the optimum temperature for cell growth and induction of functional dyn was 32°C. As can be seen in Fig. 1, 72 h after induction at 32°C dyn accumulated in HeLa cells to high levels. When assayed at 30°C (the experimentally determined permissive temperature), endocytosis of transferrin (Tfn) in these cells was found to occur with the same rates and efficiencies as in cells expressing wt dynamin (Fig. 2 A). In contrast, when measured 15 min after shifting the cells to 38°C (the experimentally determined nonpermissive temperature) Tfn-internalization was severely inhibited in dyn cells as compared to dyn cells (Fig. 2 B). The extent of inhibition of the initial rate of Tfn uptake at the nonpermissive temperature in dyn cells (81 ± 11%, n = 5) was similar to that previously reported (Damke et al., 1994) in cells expressing the dominant-interfering mutant, dynK44A (82 ± 8%, n = 10). Previous studies established that overexpression of wt dynamin had no effect on either the rate or extent of endocytosis (Damke et al., 1994).

Consistent with the shibire phenotype, cells expressing dyn showed a rapid onset of the temperature-sensitive defect. The data in Fig. 3 A show that even without a preshift to the nonpermissive temperature, the amount of Tfn internalized after 10 min at 38°C was inhibited by ~45%. In fact, inhibition was apparent even at the earliest time (2.5 min) of Tfn internalization at 38°C (not shown). Maximal inhibition was obtained by 10–15 min after shift to the
nonpermissive temperature and inhibition persisted even when cells were maintained for 12 h at 38°C (Fig. 3 A). These results suggest a direct role for mammalian dynamin in endocytic coated vesicle formation.

The temperature-sensitive inhibition of Tfn-internalization was reversible. Tfn internalization (Fig. 3 B) began to recover immediately after return to the permissive temperature and by 20 min, Tfn-internalization in dyn ts mutants recovered to ~75% of wt cells assayed under identical conditions. The complementary immunofluorescence experiment in Fig. 4 shows that Texas Red-conjugated Tfn was efficiently internalized into cells expressing dyn wt during a 10-min incubation and accumulated in the recycling endosomal compartment at both permissive and nonpermissive temperatures (Fig. 4, a' and c', respectively). Similarly, internalization of Tfn at 30°C was normal in dyn wt cells (compare b' with a'). In contrast, intracellular accumulation of Tfn was severely reduced when the dyn ts cells were incubated at 38°C (compare Fig. 4, d' to c'). However, the ts-defect in endocytosis was rapidly reversed by returning the cells to 30°C for 10 min before addition of Tfn and further incubation at 30°C (Fig. 4 e').

The immunofluorescence pattern for dyn wt and dyn ts at the permissive and nonpermissive temperatures is also shown in Fig. 4 (a–e). As previously described (van der Bliek et al., 1993; Damke et al., 1994), most of the overexpressed dynamin was cytosolic, suggesting that the membrane association of dynamin is saturable. Strikingly, at the nonpermissive temperature the dyn wt mutant formed large aggregates in the cytoplasm (Fig. 4 d). These aggregates were reminiscent of those previously seen in cells expressing a 272-amino acid NH2-terminal deletion mutant of dynamin lacking the GTPase domain (Herskovits et al., 1993). Aggregation was apparently reversible as the dynamin aggregates dispersed within 30 min after returning cells to the permissive temperature (Fig. 4 e).

The most striking morphological phenotype of dyn K44A cells examined at longer times after induction was that cells became flat and spread radially (Damke et al., 1994). This was associated with rearrangements of the actin cytoskeleton from radial stress fibers to the cell periphery. Our earlier arguments that this was an indirect consequence of the block in endocytosis were confirmed using the dyn ts cells since no such alterations were detected even after several hours at the nonpermissive temperature (not shown). Ultrastructural analysis of dyn wt cells was performed using thin section electron microscopy. The amorphous structures of the dynamin aggregates formed after 15 min at the nonpermissive temperature are visible in Fig. 5 A. Preembedding and immunogold labeling confirmed that these large aggregates were heavily stained with the dynamin specific antibody, hudy-1 (not shown, but see Fig. 4 d). Curved coated pits accumulated at the cell surface of dyn wt cells (see for example, Fig. 5 B). Quantitation of the number of cell surface coated pits was performed as described in Materials and Methods and revealed an approximately fourfold increase in the number of endocytic coated pits in dyn wt cells held at the 38°C for 15 min (89 coated pits/mm cell surface; 982 µm counted) as compared to dyn wt cells at 38°C (21 coated pits/mm cell surface; 516 µm counted). This accumulation was even greater than that observed at the surface of dyn K44A mutant cells (Damke et al., 1994) and probably reflects the finding that the long membrane invaginations, often with coated ends, seen in dyn K44A cells did not accumulate at early times in the dyn ts cells (Fig. 5 A).

**The Formation of Constricted Coated Pits Is Blocked in dyn wt Cells**

We have previously shown that expression of the dyn K44A mutant defective in GTP binding and hydrolysis blocked endocytic coated vesicle formation at a stage after coated pit assembly and invagination but preceding the formation of constricted coated pits (Damke et al., 1994). Coated vesicle formation was blocked at a similar stage in dyn ts cells. The sequestration of receptor-bound ligands, biotinylated via cleavable disulfide bonds, into constricted coated pits can be distinguished biochemically from their subsequent internalization into sealed coated vesicles (Schmid and Smythe, 1991; Carter et al., 1993). Ligands sequestered into constricted coated pits become inaccessible to avidin, but remain accessible to the small membrane impermeant reducing agent, MesNa (β-mercaptoethanol sulfonic acid). The data in Fig. 6 show that sequestration and internalization are equally inhibited in dyn ts cells indicating that the block in endocytosis precedes the formation of constricted coated pits. These results are consistent with the phenotype of shibire flies in which coated pits accumulating at the cell surface of nonneuronal cells remain accessible to large probes such as wheat germ agglutinin-conjugated HRP (Kosaka and Ikeda, 1983a).

Together, these results establish that the human dyn wt mutant is functionally homologous to *Drosophila shibire* mutant. Using this mutation, we have generated the first mammalian cell line with a rapid and reversible temperature-sensitive defect in receptor-mediated endocytosis.

**Fluid Phase Endocytosis Is Only Partially Inhibited in dyn wt Cells**

We previously showed that nonselective pinocytosis of the fluid phase marker, HRP, continued in dyn K44A cells at the same rates as in dyn wt cells (Damke et al., 1994). Since clathrin-dependent endocytosis was severely inhibited we argued that a clathrin-independent pathway of pinocytosis may have been induced to compensate for the loss of the clathrin-dependent contribution to bulk pinocytosis. The rapid and selective temperature-sensitive inhibition of clathrin-dependent endocytosis in the dyn ts cells has allowed us to confirm this hypothesis. The data in Fig. 7 A show that at the permissive temperature fluid phase endocytosis of HRP occurs with equal efficiency in dyn wt and dyn ts cells. In contrast to results obtained with the dyn K44A cells, fluid phase endocytosis of HRP at the nonpermissive temperature was reduced by ~50% in dyn ts cells compared to dyn wt cells (Fig. 7 B). However, while Tfn-internalization in dyn wt cells was potently inhibited at the nonpermissive temperature, substantial fluid phase uptake continued.

Importantly, at later times of incubation at the nonpermissive temperature, although clathrin-dependent Tfn endocytosis remained blocked, fluid phase endocytosis of HRP fully recovers. The data in Fig. 8 show that after a brief lag but within 30 min, fluid phase uptake recovers to wt levels. In fact, at 60 and 120 min after shift to the non-
Figure 4. The immunofluorescence pattern for overexpressed dyn wt (a and c) and dyn as (b, d, and e) detected with anti-HA antibody 12CA5 followed by a BODIPY FL-conjugated secondary antibody and for internalized Texas Red-conjugated transferrin (a'–e'). Cells were incubated at the permissive temperature (a and b; 30°C) or the nonpermissive temperature (c and d; 38°C) for 10 min with Texas Red-Tfn. Alternately, cells were preincubated at 38°C for 10 min, returned to 30°C for 30 min and then incubated with Texas Red-Tfn for 10 min at 30°C (e). At the nonpermissive temperature dynamin forms large aggregates in the cytosol (d) and cells do not internalize Tfn (d'). These aggregates were dispersed within 30 min when the cells are returned to the permissive temperature (e) and the ability of cells to internalize transferrin was restored (e').
permissive temperature we observed a small but reproducible overcompensation to as much as 20% higher than wt levels. After 12 h of incubation at the nonpermissive temperature the rate of fluid phase endocytosis has been restored to wt levels. At this time point, Tfn internalization was still potently inhibited (see Fig. 3 A). This result was consistent with dyn<sup>K46A</sup> cells that showed wt levels of fluid phase endocytosis 48 h after induction of dynamin expression (Damke et al., 1994). These results suggest that a constitutive clathrin-independent pathway for pinocytosis occurs in tTA-HeLa cells and confirm our earlier suggestion that this pathway is up-regulated in compensation for the loss of clathrin-dependent endocytosis.

A Clathrin-independent Mechanism for Pinocytosis in dyn<sup>b</sup> Cells

Several possible mechanisms of clathrin-independent pinocytosis have been described (reviewed by van Deurs et al., 1989; Watts and Marsh, 1992). These can be pharmacologically distinguished from clathrin-dependent endocytosis, which is selectively inhibited by K<sup>+</sup>-depletion, cytosol acidification or hypertonicity (Larkin et al., 1983; Daukas and Zigmond, 1985; Sandvig et al., 1987). Cytosol acidification, one of the best characterized of these treatments, causes the accumulation of adherant coated pits at the plasma membrane (Heuser, 1989; Hansen et al., 1993) and effectively inhibits receptor-mediated endocytosis while bulk endocytic processes continue (Sandvig et al., 1987). Likewise, with dyn<sup>wt</sup> cells, cytosol acidification severely (>85%) reduced receptor-mediated endocytosis of Tfn (Fig. 9 A) while fluid phase endocytosis of HRP was only partially (~50%) inhibited (Fig. 9 B). The acid-resistant component of HRP endocytosis corresponded quantitatively to the residual HRP uptake that occurred in dyn<sup>b</sup> cells at early times after shift to the nonpermissive temperature (Fig. 9 B, compare dyn<sup>wt</sup> [open bars] with dyn<sup>b</sup> 15 min [striped bars]). Furthermore, the residual HRP uptake in dyn<sup>b</sup> cells was insensitive to cytosol acidification, consistent with the notion that it occurred via a clathrin-independent mechanism. Importantly, the residual Tfn uptake in dyn<sup>b</sup> cells was also resistant to cytosol acidification (Fig. 9 A), suggesting that it occurred through bulk pinocytic mechanisms and further strengthening our argument for a potent block in clathrin-dependent endocytosis in dyn<sup>b</sup> cells.
Figure 6. Expression of the dyn ts mutant blocks BSST internalization preceding its sequestration into constricted coated pits. Cells expressing either wild-type (●, ○) or the dyn ts mutant (▲, △) were harvested 72 h after induction at 32°C, preshifted for 10 min at 38°C, and incubated with BSST for the indicated times at 38°C. The extent of Tf internalization was assessed by avidin inaccessibility (●, ▲) or MesNa-resistance (○, △) in four independent experiments as described in Materials and Methods.

After a prolonged (120 min) incubation at the nonpermissive temperature fluid phase uptake in the dyn ts cells had returned to near normal levels. This induced pinocytic pathway was now resistant to the effects of cytosol acidification (Fig. 9, compare dyn ts 120 min, [open and striped bars]). Again, Tf uptake at this time point remained severely inhibited. Cells expressing the constitutively defective dyn K44A mutant provide an additional control for long-term incubations in the absence of functional dynamin. As for the dyn ts cells, both the residual Tf uptake and the induced HRP uptake in these cells were resistant to the inhibitory effects of cytosol acidification (Fig. 9, A and B). To rule out effects on endosomal trafficking that might lead to reduced accumulation of HRP (Cupers et al., 1994), the data in Fig. 9 were derived from an early time point after HRP uptake (15 min) before the inflection in the initial rate of uptake occurred (see Fig. 7). In addition, to ensure that cytosol acidification effects were related to internalization and not recycling, the rates of efflux (regurgitation) of HRP in the dyn ts mutant cells were measured directly and found to be unaffected (not shown). This is consistent with our previous finding that Tf recycling was unaffected in dyn K44A cells. These results confirm that the compensation in bulk endocytosis was mediated by an acid-resistant pinocytic process mechanistically distinct from clathrin-mediated endocytosis.

The effect of other pharmacological treatments on the clathrin-independent pathway induced in dyn ts cells was examined to further characterize this pathway. Specifically we examined the effects of cytochalasin D, which has been shown to selectively inhibit endocytosis at the apical membrane of polarized epithelial cells (Gottlieb et al., 1993) and to inhibit the internalization of caveolae-associated proteins in some cells (Parton et al., 1994) and hexameth-ylamiloride, which has been shown to selectively inhibit macropinocytosis (West et al., 1989; Hewlett et al., 1994). Neither the residual HRP uptake at early times after shift to the nonpermissive temperature nor the induced uptake occurring after prolonged incubations were affected by these treatments (data not shown).

Since there was significant residual fluid phase uptake in dyn ts cells, HRP was used as a probe to compare the ultrastructure of the early endocytic pathway at permissive and nonpermissive temperatures. 10 min after incubating wild-type cells at 38°C with HRP, most of the electron-dense DAB reaction product could be detected in large heterogeneously shaped endosomal vesicles (100-600-nm diam) and thin tubules (Fig. 5 C). A similar distribution of internalized HRP was seen in dyn ts cells when incubated at the permissive temperature of 30°C (Fig. 5 D). In contrast, when the dyn ts cells were incubated at 38°C for 10 min, HRP labeling was predominantly detected in a small (<70-nm diam) and more homogeneous population of vesicles.
Figure 8. Recovery of fluid phase endocytosis in dynwt cells after shift to the nonpermissive temperature. Dynwt and dynts cells were grown on 35-mm dishes for 72 h at 32°C in the absence of tetracycline. After incubation at the nonpermissive temperature for the indicated times, fluid phase uptake of HRP (4 mg/ml) was followed for 15 min at 38°C. Cells were rapidly cooled by repeated washes on ice and samples were processed as described in Materials and Methods (n = 3).

Together with the acid-resistant properties of HRP uptake in the dynts cells, these results were most consistent with a role for the noncoated vesicles described by Sandvig, van Deurs, and colleagues (Hansen et al., 1993; Sandvig and van Deurs, 1994) in compensating for the loss of clathrin-dependent endocytosis.

Interestingly, larger endosomal structures were not readily detectable after HRP uptake in dynts cells at early times after shift to the nonpermissive temperature (Fig. 5 C). However, after prolonged incubation (120 min) at the nonpermissive temperature, when the bulk rate of endocytosis had recovered to wt levels, large endosomal compartments could again be labeled after a 10-min incubation with HRP (Fig. 5 F). These data strongly suggest that the early endosomal compartment rapidly contracts (within 10 min) and later expands in response to the magnitude of incoming endocytic membrane. Although quantitative morphometric analyses will be necessary to confirm this hypothesis, similar observations have been made in studies on shibire (Kessell et al., 1989; Koenig and Ikeda, 1990).

Discussion

We have generated a mammalian cell line with a specific and molecularly defined temperature-sensitive defect in receptor-mediated endocytosis. These cells overexpress a human dynamin mutant (G273D) that is homologous to the shibirest allele in Drosophila. In many ways, the phenotype of these cells resembles that of shibire flies. Receptor-mediated endocytosis occurs at wild-type rates at the permissive temperature of 30°C but is severely inhibited at the nonpermissive temperature of 38°C. The ts phenotype shows rapid onset and is reversible upon return to the permissive temperature. Invaginated, but not constricted, coated pits accumulate on the cytoplasmic surface of the plasma membranes. Like the previously characterized HeLa cell line expressing the dynK44A mutant defective in GTP binding and hydrolysis, the dynts cells exhibit a specific defect in endocytic clathrin-coated vesicle formation.

Dynamin Functions as an Oligomer in Coated Vesicle Budding

Previous genetic analysis of shibire heterozygotes had suggested that the shibire gene product acts as a multimeric protein (Kim and Wu, 1990). Our finding, that purified dynamin exists as a tetramer that spontaneously self-assembles in vitro into rings and helical stacks of rings (Hinshaw and Schmid, 1995), confirms this hypothesis. The dynamin rings formed in vitro are identical in dimension to “collars” originally observed around the necks of the membrane invaginations that accumulated at nerve terminals in shibire flies incubated at the nonpermissive temperature (Kosaka and Ikeda, 1983b; Koenig and Ikeda, 1989). More recently, when permeabilized synaptic terminals isolated from rat brain were incubated with GTPγS, the nonhydrolyzable analogue of GTP, bands of dynamin were ob-
served encircling the elongated necks of coated pits that accumulated (Takei et al., 1995). Thus, in both shibire and GTPγS-treated nerve termini, dynamin apparently accumulates in ring structures at the necks of invaginated coated pits clearly segregated from the clathrin lattice at their base. This distribution contrasts with the immunolocalization of the dynK44A mutant that is uniformly distributed throughout the clathrin lattices of flat and curved coated pits (Damke et al., 1994). These results suggested a model (Hinshaw and Schmid, 1995) in which dynamin, in its unoccupied or GDP-bound form, is first recruited to and uniformly distributed on flat or curved clathrin lattices. Subsequent GTP-GDP exchange would result in a conformational change that alters dynamin-dynamin interactions or interactions between dynamin and some, as yet unidentified, targeting and/or assembly molecule. GTP-bound dynamin is redistributed to the necks of invaginated pits and assembles into collars, resulting in the formation of constricted coated pits. Since the dynK44A has reduced affinity for GTP, this step is blocked (Damke et al., 1994). Conversely, in the presence of a nonhydrolyzable analogue of GTP, these structures become exaggerated (Takei et al., 1995). We have proposed that GTP hydrolysis would result in a second concerted conformation change of the dynamin collar to drive vesicle budding (Hinshaw and Schmid, 1995).

A Model for the Defect in dyna Cells

How might the dyna phenotype be explained in the context of this working model for dynamin function? First, our model predicts that dynamin functions as a large homo-oligomer involving the concerted action of multiple dynamin polypeptides (Hinshaw and Schmid, 1995). This would explain how expression of the dyna mutant in the presence of endogenous wild-type dynamin gives a ts-phenotype. At the levels of overexpression achieved (>20-fold) each functional oligomer would contain very few wt dynamin polypeptides, thus the dyna phenotype would be dominant.

Interestingly, when synthesized at 37°C the dyna phenotype is silent and there is no apparent interference with the function of wt-dynamin. This suggests that dyna is misfolded under these conditions so that it cannot interact with endogenous dynamin. An important consequence of this observation is that during long-term incubation at 38°C, newly synthesized dyna would be nonfunctional and the mutant hetero-oligomers would be gradually replaced with wt dynamin homo-oligomers. This could explain why phenotypes seen at longer incubation times with the dynK44A mutant, such as alterations in the actin cytoskeleton, are not observed in dyna cells.

The mutation that generates the ts phenotype (G273D) exchanges a flexible glycine residue for a less flexible aspartic acid residue at the boundary between the GTPase domain and the COOH-terminal two-thirds of dynamin. When overexpressed in HeLa cells, the dyna mutant, like the dynK44A mutant, blocks the formation of constricted coated pits, suggesting that it is also defective in GTP binding. In contrast, collared and, according to our model, constricted coated pits accumulate at the nerve termini of Drosophila expressing the analogous mutant (Kosaka and Ikeda, 1983b), suggesting that the shibire gene product might be defective in GTP hydrolysis. However, the conserved GTP binding elements in dynamin are unchanged by this mutation so that neither GTP binding nor hydrolysis are likely to be affected by the dyna mutation. Instead, we speculate that the COOH-terminal two-thirds of dynamin is uncoupled from the NH2-terminal GTPase domain by this mutation. Interestingly, the aggregate formed by dyna at the nonpermissive temperature are similar to those formed by a mutant dynamin lacking the entire GTPase domain (Herskovits et al., 1993). Large-scale expression of recombinant dyna mutant is being performed to directly test this hypothesis.

The suggestion that the dyna mutant is defective in transmitting conformational information from its NH2-terminal GTPase domain to its COOH-terminal domains would reconcile the apparent phenotypic differences between dyna HeLa cells and shibire neurons. Such a defect would block both the conversion of dynamin-GTP to dynamin-GDP (preventing the formation of constricted coated pits, as in the dynK44A mutant), and the conversion of dynamin-GTP to dynamin-GDP (leading to the accumulation of collars, as seen in GTPγS treated neurons). The phenotype observed would depend on the stage in its GTP cycle predominantly occupied by dynamin before the temperature shift. Interestingly, as in HeLa cells, collared pits were not observed in non-neuronal tissues of shibire flies (Kosaka and Ikeda, 1983a). Therefore it is possible that in neurons, dynamin might be specifically held at a late stage, in its GTP-bound form, to facilitate rapid endocytosis after depolarization. The selective regulation of neuronal dynamin-1 by phosphorylation/dephosphorylation (Robinson et al., 1993) might be involved in triggering dynamin function for rapid synaptic vesicle recycling (Morris and Schmid, 1995).

Dynamin and Clathrin-independent Pinocytosis

Apparently unlike the shibire phenotype, fluid phase endocytosis continues at the nonpermissive temperature in HeLa cells expressing the dyna mutant. At early times after shift to the nonpermissive temperature, fluid phase endocytosis is partially inhibited reflecting the contribution of clathrin-mediated endocytosis to overall pinocytosis in HeLa cells. Strikingly, after prolonged incubation at the nonpermissive temperature fluid phase uptake increases to fully compensate for the loss of clathrin-dependent endocytosis. Studies on fluid phase uptake in shibire flies have been limited to early times after shift to the nonpermissive temperature. Therefore it is possible that a compensatory pathway would also be induced in shibire cells after prolonged incubation. It is also possible that the relative contributions of clathrin-dependent and clathrin-independent pinocytosis varies between cell types as it does in different organisms. Clathrin deficient yeast, for example, show only a partial reduction in endocytosis (Payne et al., 1988) whereas fluid phase uptake is inhibited by ~80% in clathrin-deficient Dictyostelium (Russetti et al., 1994).

The rapid block in clathrin-mediated endocytosis in dyna cells has revealed the existence of a constitutive clathrin-independent mechanism for endocytosis in HeLa cells. A number of studies have established that fluid
phase pinocytosis can continue under conditions where receptor-mediated uptake is severely inhibited (Daukas and Zigmond, 1985; Moya et al., 1985; Sandvig et al., 1987). Morphological studies have identified smooth endocytic vesicles as mediators of these clathrin-independent endocytic events (Huet et al., 1980; Hansen et al., 1991). However, these studies have largely relied on the use of treatments with poorly understood mechanisms of inhibition of clathrin-dependent endocytosis, namely K⁺-depletion, cytosol acidification and hypertonicity. These treatments have variable effects on different cell types and are not always selective (reviewed by van Deurs et al., 1989). Others have argued that the rate of clathrin-dependent endocytosis can account for total fluid phase uptake (Marsh and Helenius, 1980; Griffiths et al., 1989; Pelchen-Matthews et al., 1991) and that clathrin-independent pathways might only be induced in response to the nonphysiological conditions required to inhibit clathrin-dependent endocytosis (Watts and Marsh, 1992; Cupers et al., 1994). As a result the significance and even the existence of a constitutive clathrin-independent pathway for endocytosis has been questioned (Watts and Marsh, 1992).

The results presented here provide strong evidence for a constitutive clathrin and dynamin-independent pathway for pinocytosis in HeLa cells and further suggest that this pathway can be up-regulated to compensate for the loss of clathrin-dependent endocytosis. In dyn²⁸⁹ cells, Tf uptake is severely inhibited by cytosol acidification while HRP uptake is partially inhibited. Importantly, the residual uptake of both Tf and HRP that occurs in dyn²⁸⁹ cells early after shift to the nonpermissive temperature and the induced uptake of HRP that occurs at later times after shift to the nonpermissive temperature are resistant to cytosol acidification. Thus the pinocytosis that occurs in dyn²⁸⁹ cells can be mechanistically distinguished from clathrin-dependent endocytosis. Induction of bulk-phase endocytosis to fully compensate for inhibition of clathrin-mediated endocytosis was recently observed in rat fetal fibroblasts after K⁺-depletion or incubation in hypertonic media (Cupers et al., 1994). Based on our results, the 30-min incubations required for these inhibitory effects would suffice for induction of an alternate pinocytic pathway.

Interestingly, yeast cells expressing a temperature-sensitive defect in a clathrin-mediated pathway also induce a compensatory clathrin-independent pathway when maintained at the nonpermissive temperature. At early times after shift to the nonpermissive temperature, chc-ts1 yeast cells carrying a temperature-sensitive clathrin mutant show a strong defect in sorting of vacuolar enzymes. However, the chc-ts1 cells regain the ability to efficiently sort vacuolar enzymes when maintained at the nonpermissive temperature for 3 h (Seeger and Payne, 1992). The nature of this compensatory pathway for efficient vacuolar enzyme sorting in yeast is also unknown.

After prolonged incubation at the nonpermissive temperature HRP uptake increases approximately twofold recovering to wt levels. In contrast, the residual Tf uptake is not apparently increased. Some of this residual Tf uptake may occur due to an incomplete block in coated vesicle formation. Alternatively, it is possible that Tf-R are not freely mobile in the membrane, perhaps trapped in coated pits, and are therefore not a reliable bulk mem-

References
Burnette, W. N. 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiiodinated protein A. Anal. Biochem. 112:195–203.
Carter, L. L., T. E. Redelmeier, L. A. Woollenweber, and S. L. Schmid. 1993. Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis. J. Cell Biol. 120:37–45.
Cupers, P., A. Veithen, A. Kiss, P. Bauduin, and P. J. Courtoy. 1994. Clathrin polymerization is not required for bulk-phase endocytosis in rat fetal fibroblasts. J. Cell Biol. 127:725–735.
Damske, H., T. Baba, D. E. Warnock, and S. L. Schmid. 1994. Inhibition of mutant dynamin specifically blocks endocytic vesicle formation. J. Cell Biol. 124:915–934.
Damske, H., M. Gossen, S. Freundlieb, H. Bujard, and S. L. Schmid. 1995. Tightly regulated and inducible expression of a dominant interfering dynamin mutant in stably transformed HeLa cells. Methods Enzymol. 257:209–220.

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References
Burnette, W. N. 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiiodinated protein A. Anal. Biochem. 112:195–203.
Carter, L. L., T. E. Redelmeier, L. A. Woollenweber, and S. L. Schmid. 1993. Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis. J. Cell Biol. 120:37–45.
Cupers, P., A. Veithen, A. Kiss, P. Bauduin, and P. J. Courtoy. 1994. Clathrin polymerization is not required for bulk-phase endocytosis in rat fetal fibroblasts. J. Cell Biol. 127:725–735.
Damske, H., T. Baba, D. E. Warnock, and S. L. Schmid. 1994. Inhibition of mutant dynamin specifically blocks endocytic vesicle formation. J. Cell Biol. 124:915–934.
Damske, H., M. Gossen, S. Freundlieb, H. Bujard, and S. L. Schmid. 1995. Tightly regulated and inducible expression of a dominant interfering dynamin mutant in stably transformed HeLa cells. Methods Enzymol. 257:209–220.
Daukas, G., and S. H. Zigmond. 1985. Inhibition of receptor mediated but not fluid phase endocytosis in polymorphonuclear leukocytes. J. Cell Biol. 101:1673–1679.
Gossen, M., and H. Bujard. 1992. Tight control of gene expression in mamalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA. 89:5547–5551.
Gottlieb, T. A., I. E. Ivanov, M. Adesnik, and D. D. Sabatini. 1993. Actin microfilaments play a critical role in endocytosis at the apical not the basolateral surface of polarized epithelial cells. J. Cell Biol. 120:695–710.
Griffiths, G., R. Back, and M. Marsch. 1989. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. J. Cell Biol. 109:2703–2720.
Hansen, S. H., K. Sandvig, and B. van Deurs. 1991. The pseudosomal compart-
Herskovits, J. S., C. C. Burgess, R. A. Obar, and R. B. Vallee. 1993. Effects of potassium depletion, hypertonic medium, and cytosol acidification. J. Cell Biol. 121:61–72.

Herskovits, J. S., C. C. Burgess, R. A. Obar, and R. B. Vallee. 1993. Effects of mutant rat dynamin on endocytosis. J. Cell Biol. 122:565–578.

Heuser, J. 1989. Effects of cytoplasmic acidification on clathrin lattice morphology. J. Cell Biol. 108:401–411.

Hewlett, L. J., A. R. Prescott, and C. Watts. 1994. The coated pit and macropinocytic pathways serve distinct endosome populations. J. Cell Biol. 124:689–701.

Hinshaw, J. E., and S. L. Schmid. 1995. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. Nature (Lond.) 374:190–192.

Huet, C., J. F. Ash, and S. J. Singer. 1980. The antibody-induced clustering and endocytosis of HLA antigens on cultured human fibroblasts. Cell 21:429–438.

Kesseli, I., B. D. Holst, and T. F. Roth. 1989. Membrane intermediates in endocytosis are labile, as shown in a temperature-sensitive mutant. Proc. Natl. Acad. Sci. USA. 86:4998–4972.

Kim, Y.-T., and C.-F. Wu. 1990. Allelic interactions at the shibire locus of Drosophila: effects on behaviour. J. Neurogenet. 7:1–14.

Koenig, J. H., and K. Ikeda. 1990. Transformational process of the endosomal compartment in nephrocytes of Drosophila melanogaster. Cell Tissue Res. 262:233–244.

Kosaka, T., and K. Ikeda. 1983a. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of Drosophila melanogaster, shibire. J. Cell Biol. 97:499–507.

Kosaka, T., and K. Ikeda. 1983b. Possible temperature-dependent blockage of membrane retrieval and endocytosis of HLA antigens on cultured human fibroblasts. J. Cell Biol. 103:1–8.

Larkins, D. M., M. S. Brown, J. L. Goldstein, and R. G. W. Anderson. 1983. De novo synthesis of the high-density lipoprotein receptor in human fibroblasts. J. Cell Biol. 97:926–936.

Larkin, J. M., M. S. Brown, J. L. Goldstein, and R. G. W. Anderson. 1983. Multiple domains of the high-density lipoprotein receptor are required for receptor function. J. Cell Biol. 97:926–936.

Larkins, D. M., M. S. Brown, J. L. Goldstein, and R. G. W. Anderson. 1983. Mutations in the human apolipoprotein B receptor lead to the type III hyperlipoproteinemia phenotype. J. Biol. Chem. 258:299–304.

Morris, S. A., and S. L. Schmid. 1995. Synaptic vesicle recycling: the Ferrari of endocytosis? Curr. Biol. 5:113–115.

Moys, A., Dautry-Varsat, B. Goud, D. Louvard, and P. Bougnet. 1985. Inhibition of coated pit formation in HepG2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. J. Cell Biol. 101:548–559.

Nakata, T., A. Iwamoto, Y. Noda, R. Takemura, H. Yoshikura, and N. Hirokawa. 1991. Predominant and developmentally regulated expression of dynamin in neurons. Neuron. 7:461–469.

Nakata, T., R. Takemura, and N. Hirokawa. 1993. A novel member of the dynamin family of GTP-binding proteins is expressed specifically in the testis. J. Cell Sci. 105:1–5.

Parton, R. G., B. Joggerst, and K. Simons. 1994. Regulated internalization of caveolae. J. Cell Biol. 127:1199–1215.

Payne, G. S., D. Baker, E. van Tuinen, and R. Schekman. 1988. Protein transport to the vacuole and receptor-mediated endocytosis by clathrin heavy chain-deficient yeast. J. Cell Biol. 106:1453–1461.

Polchen-Matthews, A., J. E. Armes, G. Griffiths, and M. Marsh. 1991. Differential endocytosis of CD4 in lymphoid and non-lymphocytic cells. J. Exp. Med. 173:575–587.

Pooomy, C. A., and L. Edgar. 1979. Reversible alterations in the neuromuscular junction of Drosophila melanogaster bearing a temperature-sensitive mutation, shibire. J. Cell Biol. 81:520–527.

Robinson, P. J., J. M., Sontag, J.-P. Liu, E. M. Fykse, C. Slaughter, H. McManus, and T. C. Südhof. 1993. Dynamin GTPase regulated by protein kinase C phosphorylation in nerve terminals. Nature (Lond.) 365:163–166.

Ruscetti, T. J. A. Cardelli, M. N. Swanson, and T. J. O'Halloran. 1994. Clathrin heavy chain functions in sorting and secretion of lysosomal enzymes in Dictyostelium. J. Cell Biol. 126:343–352.

Sandvig, K., and B. van Deurs. 1994. Endocytosis and intracellular sorting of ricin and Shiga toxins. FEBS Lett. 346:99–102.

Sandvig, K., S. Olins, O. W. Petersen, and B. van Deurs. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. J. Cell Biol. 105:679–689.

Schmid, S. L., and E. Smythe. 1991. Stage-specific assays for coated pit formation and coated vesicle budding in vitro. J. Cell Biol. 114:869–880.

Seeger, M., and G. S. Payne. 1992. A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast. EMBO J. 11:2811–2818.

Shpetner, H. S., and R. B. Vallee. 1989. Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. Cell. 59:421–432.

Smythe, E. T. E. Redelmeier, and S. L. Schmid. 1992. Receptor-mediated endocytosis in semi-intact cells. Method Enzymol. 219:223–234.

Sontag, J. M., E. M. Fykse, Y. Ushkaryov, J.-P. Liu, P. J. Robinson, and T. C. Südhof. 1994. Differential expression and regulation of multiple dynamins. J. Biol. Chem. 269:4547–4554.

Takei, K., P. McPherson, S. L. Schmid, and P. De Camilli. 1995. Tubular membrane invaginations coated by dynamin rings are induced by GTPyS in nerve terminals. Nature (Lond.). 374:185–190.

Tsuru hara, T., J. H. Koenig, and K. Ikeda. 1990. Synchronized endocytosis studied in the oocyte of a temperature-sensitive mutant of Drosophila melanogaster. Cell Tissue Res. 258:209–207.

van der Bliek, A. M., and E. M. Meyerowitz. 1991. Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. Nature (Lond.). 353:411–414.

van de Breek, A. M., T. E. Redelmeier, H. Damke, E. J. Tisdale, E. M. Meycroowitz, and S. L. Schmid. 1993. Mutations in human dynamin block an intermediate stage in coated vesicle formation. J. Cell Biol. 122:553–563.

van Deurs, B., O. W. Petersen, S. Olins, and K. Sandvig. 1989. The ways of endocytosis. Int. Rev. Cytol. 117:131–177.

Warrack, D. E., L. J. Terlecky, and S. L. Schmid. 1995. Dynamin GTPase is stimulated by crosslinking through the C-terminal proline-rich domain. EMBO J. 14:1322–1328.

Watts, C., and M. Marsh. 1992. Endocytosis: what goes in and how? J. Cell Sci. 103:1–8.

West, M. A., M. S. Bretscher, and C. Watts. 1989. Distinct endocytotic pathways in EGF stimulated human carcinoma A431 cells. J. Cell Biol. 109:2731–2739.