Immunocytochemical Study of Endocytotic Structures Accumulated in HeLa Cells Transformed with a Temperature-sensitive Mutant of Dynamin

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SUMMARY  Dynamin is a 100-kD GTPase, which is required for clathrin-mediated endocytosis. Recent studies have revealed that dynamin is closely involved in clathrin-coated vesicle formation. In this study we investigated the ultrastructure of endocytotic structures accumulated in HeLa cells that were transformed with a temperature-sensitive (ts) mutant of dynamin to clarify which step was blocked in dynats cells. Endocytosis of transferrin receptors was restricted at the level of surface-connected membrane structures. Tubular and vesicular membrane invaginations were accumulated in the cells' peripheral regions, suggesting that the endocytosis was blocked just before the pinching-off steps in coated vesicle formation. The “collared” tubes, which were reported to be localized in nerve terminals in shibirets1 flies and GTP73-treated synaptosomes, were not observed in the dynats cells even at nonpermissive temperature. The distribution pattern of dynamin in deeply invaginated coated pits in dynats cells was similar to that in dynwt cells but not to that in dynK44A cells, which are other endocytosis-defective mutant cells. These morphological data suggest that dynats blocked the pinching-off steps in clathrin-coated vesicle formation, which may be caused by a different mechanism from that of dynK44A cells.

KEY WORDS  dynamin  endocytosis  immunocytochemistry  electron microscopy  coated pit  temperature-sensitive mutant

Dynamin is a 100-kD GTPase, which is required for late stages of clathrin-coated vesicle formation (Baba et al. 1995; De Camilli et al. 1995; Urrutia et al. 1997). Dynamin is a member of the growing family of large GTPases with diverse functions, such as mammalian interferon-induced antiviral MX proteins (Schwemmle et al. 1995), the yeast vesicular transport proteins, VPS1 (Rothman et al. 1990; Vater et al. 1992) and DNM1 (Gammie et al. 1995), and the recently identified mammalian homologue of VPS1, DVLP/DLP1 (Shin et al. 1997; Yoon et al. 1998). Much of our knowledge about dynamin function in vivo derives from the phenotypic analysis of Drosophila bearing the shibire54 mutation (Kosaka and Ikeda 1983a,b). The shibire gene products were later discovered to be 70% identical to mammalian neuronal dynamin (Chen et al. 1991; van der Bliek and M eyerowitz 1991). The most dramatic phenotype of shibire is that the flies become rapidly paralyzed on shifting to the nonpermissive temperature, and the phenotype is reversible by shifting to permissive temperature (Grigliatti et al. 1973). Morphological analyses of neuromuscular junctions from affected flies revealed that the paralysis was due to a depletion of synaptic vesicles caused by a recycling defect (Poodry and Edgar 1979; Kosaka and Ikeda 1983a,b).

Extensive studies on uptake of fluid-phase endocytotic tracers in many tissues established that shibire flies exhibit a pleiotropic and temperature-sensitive defect in endocytosis (Kosaka and Ikeda 1983a,b; Narita et al. 1989; Koenig and Ikeda 1990; Tsuruhara et al. 1990). In some ultrathin sections observed by electron microscopy, a double band of electron density, described as a “collar,” was detected at necks of invaginated pits in synapses (Kosaka and Ikeda 1983a). Although the shibire defect was pleiotropic, such collared pits were not detected on the endocytic profiles accumulating in non-neuronal cells. Under certain
conditions, dynamins were also reported to form collars similar to those in shibireflies. First, incubation of permeabilized synaptosomes with GTPyS led to the accumulation of long invaginations of plasma membranes striated with multiple electron-dense collars, similar to those originally observed in shibire flies (Takei et al. 1995). Immunocytochemical staining with an anti-dynamin monoclonal antibody (Mab) heavily decorated these structures. Second, purified dynamin alone can self-assemble into separate rings and stacks of rings identical in dimensions to the collars (Hinshaw and Schmid 1995). These findings established the functional relationship between shibire and dynamin.

Some immunocytochemical studies of neuronal tissues in shibire flies with an anti-shibire antibody revealed that the shibire protein was mainly located in synaptic vesicles and plasma membrane areas and redistributed to the areas as “hot spots” at nonpermissive temperature (Gass et al. 1995; van de Goor et al. 1995; Estes et al. 1996). Another breakthrough came from studies with cultured cells overexpressing GTPase-defective mutants of dynamin. Biochemical analyses of transiently expressed (Herskovits et al. 1993; van der Bliek et al. 1993) and clonal populations of cells expressing dominant-negative mutants of dynamin (Damke et al. 1994,1995) established that the dynamin activity was specifically required for endocytotic clathrin-coated vesicle formation. Extensive morphological and biochemical examinations of cells expressing the dominant-negative dynK44A mutant, which is defective in GTP binding and hydrolysis, showed that the endocytotic coated vesicle formation was blocked at a stage just after coated pit assembly and invagination but preceding the formation of constricted coated pits (Damke et al. 1994). A previous in vitro analysis established that GTP binding, but not its hydrolysis, was required for formation of the constricted coated pits (Carter et al. 1993). Therefore, these results have established that dynamin is required to form the constricted coated pits and also for the coated vesicle budding (Baba et al. 1995; Damke 1996).

Immunolocalization at light and electron microscopic levels revealed that both the endogenous dynamin (dynamin-2 isoform) and heterologously expressed neuronal dynamin-1 were exclusively localized in clathrin-coated regions on the plasma membrane when they were membrane-associated (Damke et al. 1994). Some immunogold labeling with anti-dynamin MAb revealed that dynamin was uniformly distributed on flat lattices and on shallow coated pits (Damke et al. 1994; Baba et al. 1995; Warnock et al. 1997). In deeply invaginated pits, however, the dynamin could not be detected on the clathrin lattice and instead the immunogold particles appeared to encircle the pits. However, dynamin in dynK44A cells was uniformly distributed throughout the clathrin lattices on the accumulating invaginated coated pits, suggesting that GTP binding might be required for the observed redistribution of dynamin relative to the clathrin lattice. Because the GTP binding, but not GTP hydrolysis, was required to form the constricted coated pits in vitro, it is suggested that the dynamin redistribution might be required for the coated pit formation (Carter et al. 1993). Recently, we have established a stable cell line (dynts cell) overexpressing the temperature-sensitive mutant of dynamin (Damke et al. 1995). Biochemical analyses revealed that endocytosis in dynts cells was blocked at a stage preceding the formation of constricted coated pits after a shift to the nonpermissive temperature. To further characterize endocytosis and dynamin localization in the dynts cells, we have investigated them with various morphological techniques. The endocytosis in dynts cells at nonpermissive temperature was blocked before the pinching-off step in coated vesicle formation, similar to that seen in the dynK44A mutant. However, the redistribution of dynamin to the neck region of invaginated coated pits was not impaired in the dynts cells at nonpermissive temperature.

Materials and Methods

Cell Culture

HeLa cells that had been transformed with wild-type (dynwt) and temperature-sensitive mutant (dynts) of dynamin were maintained in DME supplemented with 10% FCS, 400 μg/ml G418, 2 μg/ml tetracycline, 100U/ml penicillin, and 100 μg/ml streptomycin as previously described (Damke et al. 1994,1995). Before each experiment they were cultured in DME containing 10% FCS in the absence of tetracycline at 30°C for 3 days (Damke et al. 1995).

Antibodies and Other Reagents

Anti-human dynamin MAb, Hedy-1 and anti-human transferrin receptor MAb D65 were kindly provided by Dr. Sandy Schmid (Scripps Research Institute; La Jolla, CA) and Dr. Ian Trowbridge (Salk Institute; La Jolla, CA), respectively. MAb D65 was conjugated to 10-nm colloidal gold particles as previously described (Lamaze et al. 1993).

Morphological Assay for Endocytosis

Dynwt or dynts cells cultured on glass coverslips were incubated with 20 μg/ml BODIPY FL-transferrin (FL-Tfn; Molecular Probes, Eugene, OR) in serum-free medium (DME supplemented with 20 mM Hapes, pH 7.4, and 2% BSA) at 4°C for 30 min. Then they were transferred to 30°C (permissive temperature) or 38°C (nonpermissive temperature) and incubated for 5, 10, and 15 min. The cultured cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), and treated with 0.1% saponin in PBS. The fixed cells were immunostained with anti-HA tag MAb (12CA5;
Boehringer-Mannheim, Mannheim, Germany) and Texas red-conjugated anti-mouse IgG Ab (Molecular Probes). These specimens were observed with a Leica TCS4D confocal laser-scanning microscope (Heidelberg, Germany). To quantify the endocytosis of FL-Tfn, about 200 cells that showed positive dynamin immunostaining were counted in triplicate from each group. The cells showing punctate cytoplasmic staining patterns were scored as endocytosis-positive.

Routine Electron Microscopy
The dynwt or dynts cells were labeled with 10-nm gold-conjugated anti-transferrin receptor MAb D65 (D65-gold) at 4C for 60 min (Damke et al. 1994). They were rinsed with PBS three times and incubated in serum-free medium at 30C or 38C for 15 min. After the incubation, some cells were fixed with 2.5% glutaraldehyde in 0.1 M PB for 60 min. To distinguish the surface-connecting membrane invaginations from the enclosed vesicles that had been isolated from the surface membrane, the former structures were externally labeled with ruthenium red (RR), as previously described (Damke et al. 1994). In brief, the cultured cells were fixed with 1.2% glutaraldehyde in 66 mM cacodylate buffer, pH 7.3, containing 0.5 mg/ml RR and then postfixed with 1.6% osmium tetroxide in the same buffer containing 0.5 mg/ml RR (Luft 1971). These specimens were routinely embedded in Quetol 812 as previously described (Damke et al. 1994). Unstained ultrathin sections were observed with an H-600 electron microscope (Hitachi, Japan) at 75 kV.

Pre-embedding Immunocytochemistry
The dynwt or dynts cells were incubated in DME at 38C for 30 min. They were washed five times with KSHM buffer (100 mM potassium acetate, 85 mM sucrose, 20 mM Hepes, 1 mM magnesium acetate, pH 7.4) at 4C. They were then scraped off with a rubber policeman, suspended in KSHM at 4C, and rocked at 4C for 10 min. They were centrifuged at 1000 rpm for 5 min and fixed with 4% paraformaldehyde and 0.05% glutaraldehyde in KSHM for 30 min. The cell pellet was washed in PBS and embedded in thin films of 1.5% agarose for the agarose-embedding method (De Camilli et al. 1983; Takei et al. 1995). The specimens were incubated with 1 mg/ml NaBH4 in PBS for 5 min, washed in PBS, and blocked with 1% BSA/0.1% saponin/PBS for 60 min. They were incubated with the anti-dynamin MAb (Hudy-1) in 1% BSA/0.1% saponin/PBS at 4C for 12 hr, washed extensively with PBS, and incubated with 10-nm colloidal gold-labeled goat anti-mouse IgG antibody (British BioCell; Cardiff, UK) in 1% BSA/PBS at 4C for 12 hr. They were washed with PBS, fixed with 1% osmium tetroxide in PBS for 60 min, routinely dehydrated with ethanol, and embedded in Epon, as described above.

“Ripped-off” Plasma Membrane Preparations for Electron Microscopy
Plasma membrane fragments of the upper cell surface of dynwt or dynts cells were peeled with Formvar-coated nickel grids, which had been treated with poly-l-lysine as previously described (Sanan and Anderson 1991; Damke et al. 1994). Then the specimens on the grids were fixed with 4% glutaraldehyde in KSHM for 30 min and contrasted with serial treatments with 1% osmium tetroxide, 1% tannic acid, and 1% uranyl acetate for 10 min. Some specimens were fixed with a mixture of 4% paraformaldehyde and 1% glutaraldehyde in KSHM for 30 min and then immunostained with the anti-dynamin MAb (Hudy-1) and gold-conjugated

**Figure 1** Confocal laser scanning microscopic images for endocytosis of BODIPY FL-transferrin (FL-Tfn) in dynwt cells (A,B,E,F,I,J) and in dynts cells (C,D,G,H,K,L) at 30C (A,C,E,G,I,K) or 38C (B,D,F,H,L) for 5 min (A-D), 10 min (E-H), and 15 min (I-L). In dynwt cells and dynts cells at 30C, FL-Tfn is rapidly internalized in endocytic vesicles at 10 min. However, in dynts cells at 38C, the uptake of FL-Tfn is severely impaired. Bar = 10 μm.
goat anti-mouse IgG Ab (British BioCell), and finally contrasted as described above. They were observed in an H-600 electron microscope at 75 kV or in an H-8100 electron microscope (Hitachi, Japan) at 100 kV with tilting angles of ±5° for stereo observation. The immunolocalization patterns of dynamin in coated pits were classified into two types, random and circular, as previously reported (Baba et al. 1995; Warnock et al. 1997). Eighty deeply invaginated pits from each condition were randomly selected and scored at a magnification of ×20,000. The data were expressed as percentage of total coated pit number.

Results

Endocytosis of Transferrin in dyn⁵ Cells at Nonpermissive Temperature

The endocytosis of BODIPY FL-conjugated transferrin (FL-Tfn) was observed under the confocal laser scanning microscope. After incubation at 30°C for 5 min, FL-Tfn was readily internalized as bright dots in both dyn⁵/wt cells (Figure 1A) and dyn⁵ cells (Figure 1C). After 10 min at 30°C, the FL-Tfn was then accumulated in perinuclear regions (Figures 1E and 1G), which are known to be late endosomes or trans-Golgi network compartments. After incubation at 38°C, similar dot-like fluorescence staining with FL-Tfn was observed within 5 min in dyn⁵/wt cells (Figure 1B). In dyn⁵ cells, however, both internalization and intracellular accumulation of FL-Tfn were rarely observed even after incubation for 15 min (Figure 1L). For quantitation of endocytosis at 30°C and 38°C for 15 min, only the cells with intense punctate fluorescence staining were scored as endocytosis-positive. As shown in Figure 2, more than 95% of the dyn⁵ cells endocytosed the FL-Tfn at both 30°C and 38°C. In addition, the dyn⁵ cells also endocytosed FL-Tfn at a similar level to the dyn⁵/wt cells at 30°C. On the contrary, only 15% of the dyn⁵ cells at 38°C were positive for FL-Tfn endocytosis.

Ultrastructure of Dyn⁵ Cells at Nonpermissive Temperature

Dyn⁵ or dyn⁵ cells were observed after the incubation at 30°C or 38°C for 30 min. At 30°C, the ultrastructure of dyn⁵ cells was indistinguishable from that of dyn⁵/wt cells (not shown), as previously reported (Damke et al. 1995). At 38°C, however, large electron-dense aggregates were accumulated in the cytoplasm of dyn⁵ cells (Figures 3B and 3C, arrows). These aggregates were observed neither in dyn⁵/wt cells (Figure 3A) nor in dyn⁵ cells at 30°C (not shown). The aggregates were found to contain dynamin by pre-embedding immunogold labeling with the anti-dynamin MAb (Figures 3C–3E). As shown in Figures 3D and 3E, only the surfaces of tight aggregates were densely labeled with colloidal gold. At higher magnification, they appeared as tubular stacks of rings with diameters of about 45.0 ± 7.1 nm (n = 50) (Figure 3E, arrowheads). Although they were mainly located in perinuclear regions, no specific organelles were involved in the formation of the tubular stack of rings. No collared tube-like structures, which had been reported in both nerve terminals of shibire⁶ flies (Kosaka and Ikeda 1983a,b) and perforated synaptosomes treated with GTPyS (Takei et al. 1995), were detected in the present study.

Endocytosis of Transferrin in Dyn⁵ Cells at the Electron Microscopic Level

Transferrin receptors on the plasma membrane were labeled at 4°C as described in Materials and Methods and their endocytosis at 38°C was examined by electron microscopy. The immunogold particles were localized only on the cell surface in both dyn⁵/wt cells and dyn⁵ cells at 4°C (not shown). After incubation at 38°C for 10 min, they were internalized into endosomes in the deep cytoplasm of dyn⁵/wt cells (Figure 4A, arrows).
Localization of Dynamin in Coated Pits
At 30°C, dyn^{ts} cells endocytosed gold particles into vesicles near the cell surface (Figure 4B, small arrows) and in the deep cytoplasm (large arrow). As shown at higher magnification (inset), those gold-containing vesicles were ruthenium red-negative. On the contrary, most D65-gold particles were retained on the cell surface or in membrane invaginations near the cell surface at 38°C in the dyn^{ts} cells (Figure 4C, arrowheads). Gold particles were observed in ruthenium red-positive invagination (Figure 3C, inset).

**Figure 4**  Endocytosis of anti-transferrin receptor MAb D65 conjugated to colloidal gold (D65-gold). The plasma membranes and surface-channeled membranes are labeled with ruthenium red (RR) after incubation with D65-gold for 10 min. In dyn^{ts} cells incubated at 38°C, D65-gold particles are internalized in RR-negative endosomes (A, arrows) in the deep cytoplasm. In dyn^{ts} cells incubated at 30°C (B), they are internalized to RR-negative vesicles near the cell surface (small arrows) and in the deep cytoplasm (large arrow). (Inset) Higher magnification of white rectangular area. Gold particles are observed in RR-negative vesicles. On the contrary, in the dyn^{ts} cells incubated at 38°C (C), most D65-gold particles remain in RR-positive membrane invaginations (arrowheads). (Inset) Higher magnification of white rectangular area. Bars = 0.5 μm; insets = 100 nm.

Tubular Membrane Invaginations in dyn^{ts} Cells
The vesicular profiles and shallow membrane invaginations were labeled near the surface membranes in dyn^{wt} cells at 30°C (Figure 5A) and 38°C (Figure 5B) and in dyn^{ts} cells at 30°C (Figure 5C). In dyn^{ts} cells incubated at 38°C (Figures 5D–5H), irregular tubular membrane structures (arrows) were labeled with ruthenium red. In addition, coated pit-like structures were often observed at tips of these tubular membrane invaginations (Figure 5F, arrowheads).

**Figure 5**  Electron micrographs of dyn^{wt} cells and dyn^{ts} cells after labeling with ruthenium red (RR). The dyn^{wt} cells were incubated at 30°C (A) or at 38°C (B). The dyn^{ts} cells were also incubated at 30°C (C) or at 38°C (D–H). Only in the dyn^{ts} cells incubated at 38°C are RR-positive long tubular structures (D–H, arrows) observed. The tips of those tubular structures are often clathrin-coated (F, arrowheads). Bar = 100 nm.

Deeply Invaginated Coated Pits in Dyn^{ts} Cells
To identify clathrin-coated structures in large areas of upper plasma membranes, fragments of the plasma membranes were isolated using "ripped-off" membrane preparations and processed for transmission electron microscopy (Damke et al. 1994). The dyn^{wt} and dyn^{ts} cells were examined after incubation at 30°C or 38°C for 10 min. The clathrin-coated structures were classified into three types; flat lattices on the surface membrane, shallow coated pits, and deeply invaginated coated pits with high electron density around their necks. As shown in Figure 6A, the flat lattice...
large arrows) and shallow coated pits (small arrows) were observed in the dynwt cells. In the dyn15 cells, however, deeply invaginated coated pits (arrowheads) were markedly increased in number (Figure 6B). For quantitative analyses, the isolated membranes were randomly photographed at a magnification of 20,000. The numbers of clathrin-coated structures were counted and expressed as mean numbers per \( \mu m^2 \) of membrane area. As shown in Figure 7, in the dyn15 cells at 38°C the numbers of deeply invaginated pits were markedly increased.

Redistribution of Dynamin in Coated Pits of Dyn15 Cells

The immunogold localization of dynamin was examined on the ripped-off membranes obtained from the dynwt and dyn15 cells at 38°C, as revealed in stereo pictures (Figure 8). The immunogold particles were observed on the flat clathrin lattice (Figures 8A and 8B, large arrows), shallow coated pits (Figure 8A, small arrow), and deeply invaginated pits (Figures 8B, arrowheads). Two predominant patterns were detected relative to deeply invaginated pits, as previously reported (Warnock et al. 1997). One was the random distribution of immunogold particles over the coated pits (Figure 9A). The other pattern was a circular distribution of immunogold particles around the pits (Figure 9E). The deeply invaginated pits were randomly selected and scored for dynamin distribution.

Figure 6 Electron micrographs of “ripped-off” plasma membrane preparations from dynwt cells (A) or dyn15 cells (B) incubated at 38°C. Flat clathrin lattice (large arrow), shallow coated pits (small arrows), and deeply invaginated pits (arrowheads) are observed on the cytoplasmic side of plasma membranes. In the dyn15 cells, clusters of deeply invaginated-coated pits (B, arrowheads) are often observed. Bars = 100 nm.

Figure 7 Quantitative analyses of clathrin-coated structures on the cytoplasmic side of plasma membranes. Although the numbers of flat and shallow coated pits are variable among the various conditions, the numbers of deeply invaginated coated pits were sharply increased in the dyn15 cells incubated at 38°C (ts38). Values are the mean ± SD.
Localization of Dynamin in Coated Pits

(Figure 10). The data show that the ratio of random distribution to circular distribution was unchanged between dyn\textsuperscript{wt} cells and dyn\textsuperscript{ts} cells at 30\textdegree C or 38\textdegree C, indicating that the redistribution of dynamin in coated pits was not impaired in the dyn\textsuperscript{ts} cells even at nonpermissive temperature.

**Discussion**

In the present study, the dyn\textsuperscript{ts} cells were further examined by extensive morphological approaches. At 5 min after a temperature shift to 38\textdegree C, the endocytosis of transferrin in the dyn\textsuperscript{ts} cells was severely blocked at the fluorescence microscopic level (Figure 1), as reported previously (Damke et al. 1995). The dyn\textsuperscript{ts} mutant cells formed large dynamin aggregates in the cytoplasm at nonpermissive temperature. The intensity of dynamin immunostaining was instead decreased throughout the cytoplasm when the aggregates were formed as spotty areas. It is known that most overexpressed dynamin is cytosolic, suggesting that membrane-associated sites were saturable (Damke et al. 1994, 1995). These data suggest that cytosolic mutant dynamin may be self-assembled to form large aggregates at nonpermissive temperature. However, such aggregates were found in ~50\% of endocytosis-blocked cells, and they were not necessarily responsible for the impaired endocytosis.

The dynamin aggregates, as observed at higher magnification by electron microscopy, appeared similar to self-assembled stacked rings of dynamin in vitro, as previously described (Hinshaw and Schmid 1995). They appeared to be made up of tubular structures without any membranous components. Immunogold labeling with an anti-dynamin MAb indicated that they contained dynamin. At the light microscopic level, similar cytoplasmic aggregates were reported to exist...
in COS-7 cells expressing a 272-amino-acid N-terminal deletion mutant of dynamin lacking the GTPase domain (Herskovits et al. 1993). These dynamin aggregates were not described in the case of shibire⁵⁵ flies, which were extensively studied by light and electron microscopy. The shibire⁵⁵ and dyn⁵⁵ mutations, which occur at the C-terminal end of the GTPase domain, may prevent the transmission of GTP-dependent conformational changes between the otherwise active GTPase and effector domains of dynamin (van der Bliek and Meyerowitz 1991; Damke et al. 1995). The aggregation of dynamin in the dyn⁵⁵ cells may be due to the discommunication between the GTPase domain and the effector domains, which may result in uncontrolled self-assembly in their cytoplasm.

In the present study, we have confirmed that the endocytosis in dyn⁵⁵ cells is blocked at the pinching-off step at nonpermissive temperature, as revealed by various morphological techniques. The previous morphological and biochemical studies on HeLa cells expressing the GTPase-defective mutant of dynamin revealed that the endocytotic coated vesicle formation is blocked at a stage after coated pit assembly and invagination but preceding the formation of constricted coated pits (Damke et al. 1994). The constricted coated pits were usually detected as deep invaginations with channels connecting to the surface plasma membranes. Very few surface-channeled membrane invaginations were found in the dynwt cells, which suggested that such intermediate structures were short-lived. In contrast, many RR-positive membrane profiles, especially long-necked tubular invaginations, were observed near the plasma membrane in dyn⁵⁵ cells at 38C (Figures 5D–5H). They were similar to those in dynK⁴⁴⁴A cells, as previously described (Damke et al. 1994). The collar structure, originally described in nerve terminals of shibire⁵⁵ flies (Kosaka and Ikeda 1983a,b), was never found around necks of those invaginations in the present study. Recently, Hinshaw and Schmid (1995) showed that the dynamin was spontaneously self-assembled into separate rings and stacks of interconnected rings, comparable in dimension to the collar structure localized at synaptic terminals of shibire flies. A similar accumulation of dynamin was also found in GTPγS-treated synaptosomes (Takei et al. 1995). Why are these collars not found in the dyn⁵⁵ cells, which are assumed to be a mammalian homologue of the shibire⁵⁵ mutant? One possible explana-
tion is that neuron-specific factors are required for dynamin to form the collar structure. In fact, even in shibire6 flies, collared pits were found only in neuronal cells and not in nephrocytes or oocytes (Kessell et al. 1989; Tsuruhara et al. 1990). Furthermore, we have examined ultrastructures of perforated A431 cells, which were incubated with both GTPyS and K562 cell cytosol (Baba et al. 1995; Warnock et al. 1997). Both A431 and K562 cells are non-neuronal and contain only ubiquitous dynamin-2 (Warnock et al. 1997). We could not find any collar structures in the A431 cells, which were extensively studied by electron microscopy (Warnock et al. 1997). These results indicate that some factors in neuronal cells may be required for collared pit formation. These neuron-specific factors, however, are not necessarily required for endocytosis of transferrin because the dyns cells, which were transformed HeLa cells, endocytosed transferrin at 30°C as effectively as dynwt cells.

In the present study, clathrin-coated structures on the plasma membrane were easily observed with the ripped-off membrane technique (Sanan and Anderson 1991; Baba et al. 1995). The ultrastructural findings indicated that the accumulation of coated pits in dyns cells at 38°C, as shown in Figure 6B, was comparable to that previously reported in dynK44A cells (Damke et al. 1994). The accumulated coated pits were individually demarcated in the dyns cells. In contrast, grape-shaped aggregates of coated pits were predominantly observed in dynK44A cells. Although both dynK44A and dyns cells are GTPase-defective and endocytosis-impaired mutants, their guanine nucleotide binding state was different (Warnock and Schmid 1996). The dynK44A is defective in both GTP binding and hydrolysis, whereas the dyns can bind to GTP but has impaired intramolecular interaction between the GTPase domain and the putative GTPase effector domain. This difference in dysfunction of dynamin GTPase may account for the variously shaped accumulations of coated pits.

As previously reported with dynK44A cells (Damke et al. 1994), overexpressed dynamin in dyns cells was also exclusively localized on clathrin-coated regions. The present findings again showed that dynamin-binding sites were saturable on the plasma membrane (Baba et al. 1995; Damke 1996). However, the distribution pattern of dynamin was different in the deeply invaginated pits. As previously reported (Damke et al. 1994; Baba et al. 1995), most dynamin in dynK44A cells was randomly distributed on the deeply invaginated coated pits, suggesting that dynamin’s ring formation around the neck of coated pits was inhibited. On the contrary, in dyns cells, the ratio of random distribution of dynamin to its circular distribution was similar to that in dynwt cells even at 38°C. These results suggest that dynamin’s ring may not properly work for pinching-off of coated pits in dyns cells. We have recently reported the distribution of endogenous dynamin-2 on deeply invaginated pits in intact and perforated A431 cells (Baba et al. 1995; Warnock et al. 1997). In the intact A431 cells, the dynamin was preferentially localized at the neck of deeply invaginated coated pits. Moreover, in the perforated A431 cells incubated with GTP or GTPyS, dynamin showed a circular distribution that was similar to that in intact cells. However, when the perforated cells were incubated with GDPyS, the dynamin was evenly distributed over the deeply invaginated pits (Warnock et al. 1997). It was suggested that GTP binding, but not GTP hydrolysis, was required for dynamin’s redistribution to the neck of the coated pits.

The present study, because dynamin was located in the neck of coated pits in dyns cells at 38°C, they were probably in the GTP-bound state. Alternatively, the localization of dyns protein may not be regulated by its guanine nucleotide binding state, because the dyns protein is presumed to be defective in transmitting signals between the GTP binding domain and the C-terminal effector domains.

The present morphological findings on dyns cells are consistent with our current working model for a role of dynamin in coated vesicle formation (Baba et al. 1995; Warnock and Schmid 1996), except that the dynamin in dyns cells was normally redistributed to the neck of coated pits. Rapid onset and reversible blockade of endocytosis in the dyns cells may be quite useful for dynamic molecular research in cell biology. For example, the dyns cell system is suited for studying a regulatory role of endocytosis in other cellular processes, such as signal transduction, cell adhesion and cell locomotion, because a simple temperature shift may have a greater advantage than conventional endocytosis-blocking procedures, such as cytoplasmic acidification (Sandvig et al. 1987), hypertonic treatment (Daukas and Zigmond 1985), or potassium depletion (Sandvig et al. 1985).

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