Clathrin exchange during clathrin-mediated endocytosis

Xufeng Wu, Xiaohong Zhao, Lauren Baylor, Shivani Kaushal, Evan Eisenberg, and Lois E. Greene

Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

During clathrin-mediated endocytosis, clathrin-coated pits invaginate to form clathrin-coated vesicles (CVs). Since clathrin-coated pits are planar structures, whereas CVs are spherical, there must be a structural rearrangement of clathrin as invagination occurs. This could occur through simple addition of clathrin triskelions to the edges of growing clathrin-coated pits with very little exchange occurring between clathrin in the pits and free clathrin in the cytosol, or it could occur through large scale exchange of free and bound clathrin. In the present study, we investigated this question by studying clathrin exchange both in vitro and in vivo. We found that in vitro clathrin in CVs and clathrin baskets do not exchange with free clathrin even in the presence of Hsc70 and ATP where partial uncoating occurs. However, surprisingly FRAP studies on clathrin-coated pits labeled with green fluorescent protein–clathrin light chains in HeLa cells show that even when endocytosis is blocked by expression of a dynamin mutant or depletion of cholesterol from the membrane, replacement of photobleached clathrin in coated pits on the membrane occurs at almost the same rate and magnitude as when endocytosis is occurring. Furthermore, very little of this replacement is due to dissolution of old pits and reformation of new ones; rather, it is caused by a rapid ATP-dependent exchange of clathrin in the pits with free clathrin in the cytosol. On the other hand, consistent with the in vitro data both potassium depletion and hypertonic sucrose, which have been reported to transform clathrin-coated pits into clathrin cages just below the surface of the plasma membrane, not only block endocytosis but also block exchange of clathrin. Taken together, these data show that ATP-dependent exchange of free and bound clathrin is a fundamental property of clathrin-coated pits, but not clathrin baskets, and may be involved in a structural rearrangement of clathrin as clathrin-coated pits invaginate.

Introduction

Clathrin-mediated endocytosis is one of the major mechanisms employed by cells for the import of receptors from the plasma membrane. In general terms, this process begins with receptors in the plasma membrane binding to the assembly protein (AP)* AP2, one of a family of adaptor proteins that also binds clathrin triskelions and induces them to polymerize to form clathrin-coated pits (Pearse and Robinson, 1990). After its formation, the clathrin-coated pit invaginates and then pinches off to form a clathrin-coated vesicle (CV). Numerous proteins, including amphiphysin, synaptojanin, endophilin, epsin, and dynamin, are involved in this process (Schmid, 1997; Marsh and McMahon, 1999; Brodin et al., 2000). In particular, dynamin is thought to be involved in the pinching-off process, but whether it is acting as a mechanoenzyme (Sweitzer and Hinshaw, 1998; Stowell et al., 1999) or a signaling molecule (Sever et al., 1999) is still not clear. After pinching off, a CV is uncoated rapidly in a process that requires the ATP-binding molecular chaperone of Hsc70 and the J-domain protein auxilin (Ungewickell et al., 1995). This process may also involve hydrolysis of phosphatidylinositol 4,5-biphosphate by synaptojanin (Cremona et al., 1999).

There is strong structural evidence that before they invaginate clathrin-coated pits are planar or slightly curved structures in which the polymerized clathrin triskelions form a hexagonal array. As these planar structures invaginate, they become much more curved, which requires the hexagonal clathrin array to be transformed into a mixture of hexagons and pentagons (Jin and Nossal, 1993). It is not yet clear whether this transformation drives the invagination of the clathrin-coated pits or whether it simply follows the invagination process, which is driven by another mechanism. In either case, the transformation of polymerized clathrin arrays from hexagons to pentagons requires a major structural rearrangement of the polymerized clathrin triskelions. This transformation could occur through addition of clathrin to the edges of a growing clathrin-coated pit with almost no

Address correspondence to Lois Greene, Laboratory of Cell Biology, NHLBI, 50 South Dr., Rm. 2537 MSC 8017, Bethesda, MD 20892-8017. Tel.: (301) 496-1228. Fax: (301) 402-1519.
E-mail: greenel@helix.nih.gov

X. Wu and X. Zhao contributed equally to this work.

*Abbreviations used in this paper: AP, assembly protein; CFP, cyan fluorescent protein; CV, clathrin-coated vesicle; GFP, green fluorescent protein; WT, wild type.

Key words: clathrin; exchange; FRAP; endocytosis; Hsc70

exchange occurring between clathrin in the pit and free clathrin in the cytosol (Kirchhausen, 2000b), a view supported by the observation that invagination can occur apparently in the absence of ATP (Schmid and Smythe, 1991). Alternatively, large scale exchange might accompany invagination and in fact be required for it to occur at a physiological rate.

This latter view is supported by studies on the physiological roles of Hsc70 and auxilin in the cell. Recent studies of yeast (Gall et al., 2000; Pishvaee et al., 2000) and *Caenorhabditis elegans* (Greener et al., 2001) have supported earlier biochemical studies (Ungewickell et al., 1995; Greener et al., 2000; Umeda et al., 2000), suggesting that Hsc70 and auxilin are required for the uncoating of CVs in vivo. In addition, biochemical studies on uncoating of CVs by Hsc70 and auxilin have shown that after a single round of uncoating a stable Hsc70–clathrin–AP complex forms that prevents further uncoating by Hsc70 (Jiang et al., 2000). The occurrence of such a stable complex suggests that Hsc70 may act as a chaperone for the dissociated clathrin–AP complex. These biochemical studies also showed that this complex could transiently rebind to stripped vesicle membranes, implying that Hsc70 is not only required for uncoating but may also be involved in returning clathrin and APs to the plasma membrane to form new clathrin-coated pits. Recent studies on the effect of overexpression of Hsc70 mutants in vivo support the view that Hsc70 and auxilin play multiple roles during clathrin-mediated endocytosis (Newmyer and Schmid, 2001). Therefore, Hsc70 and auxilin may be major players in controlling clathrin dynamics in the cell in general and supporting rapid exchange of clathrin during clathrin-mediated endocytosis in particular.

We have continued to study the role of Hsc70 and auxilin in clathrin dynamics by investigating under what conditions clathrin exchange occurs both in vitro and in vivo. We found that in vitro even in the presence of Hsc70 and ATP under conditions where partial uncoating occurs, we could not detect exchange between free clathrin and clathrin present in CVs or clathrin baskets. However, surprisingly in vivo, under conditions where clathrin-mediated endocytosis was blocked by cholesterol depletion or expression of a dynamin mutant, rapid ATP-dependent exchange of clathrin occurred in the clathrin-coated pits. This suggests that clathrin exchange is a fundamental property of clathrin-mediated endocytosis and may be involved in the structural rearrangement that occurs during invagination of clathrin-coated pits.

Results

Biochemical studies on uncoating of CVs by Hsc70 and auxilin have shown that after a single round of uncoating a stable Hsc70–clathrin–AP complex forms that prevents further uncoating by Hsc70 (Jiang et al., 2000). These studies have also shown that this complex rebinds transiently to stripped vesicle membranes, suggesting that under certain conditions clathrin exchange might occur during uncoating in vitro. Therefore, we carried out studies using ^{14}C-labeled clathrin in an attempt to detect this exchange. Interestingly, although we carried out these studies over a wide range of conditions we were unable to detect significant exchange between free

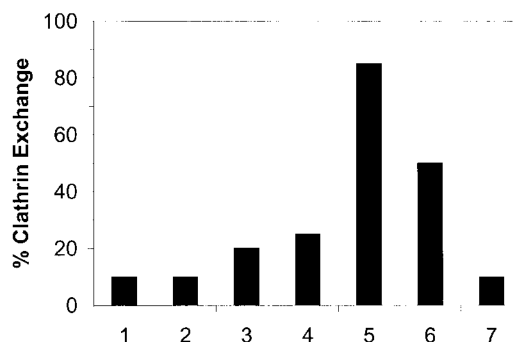

Figure 1. **Exchange of clathrin does not occur to a significant extent in vitro.** (Lane 1) 0.5 μM [^{14}C]clathrin was added to 1.0 μM CVs for 10 min at 25°C at pH 7.0 in buffer A. (Lane 2) Supernatant obtained from the uncoating of 0.5 μM mixed AP-[^{14}C]clathrin baskets was added to 1.0 μM CVs at pH 7.0. (Lane 3) Supernatant from uncoating of 0.5 μM mixed AP-[^{14}C]clathrin baskets was added to 1.0 μM partially uncoated baskets in buffer A at pH 6.5. (Lane 4) Supernatant from the uncoating of 0.5 μM mixed AP-[^{14}C]clathrin baskets was added to 1.0 μM CVs, which were partially uncoated at pH 6.5. (Lane 5) [^{14}C]clathrin was added to crude brain homogenate immediately before the initial homogenization. (Lane 6) [^{14}C]clathrin was added to the crude brain homogenate immediately after homogenization. (Lane 7) [^{14}C]clathrin was added to crude brain homogenate after the first low speed spin.

and bound clathrin in either CVs or artificial clathrin baskets with or without Hsc70 and ATP present. As shown in Fig. 1, using either purified clathrin or clathrin obtained from the uncoating of mixed AP–clathrin baskets in which the clathrin appears to be complexed to Hsc70 and APs (Jiang et al., 2000), there was no significant incorporation of labeled clathrin. This was even the case at pH 6.5, a condition that should favor exchange, since there is only partial uncoating of CVs or baskets at pH 6.5 (Fig. 1, lanes 3 and 4).

Furthermore, when ^{14}C-labeled clathrin was added to crude homogenates of CVs at various stages during their preparation, complete incorporation occurred only when labeled clathrin was added to brain tissue before homogenization (Fig. 1, lane 5). Significantly, less incorporation occurred when labeled clathrin was added immediately after homogenization, and essentially no incorporation occurred when labeled clathrin was added at later steps of the preparation (Fig. 1, lanes 6 and 7). The same results were obtained whether the crude CV preparation was homogenized in homogenization buffer (0.1 M Mes, pH 6.5) or uncoating buffer (buffer A). Taken together, these data suggest that neither CVs nor clathrin baskets show clathrin exchange.

Since clathrin can be dissociated by uncoating in these systems, this lack of exchange is probably due to an inability of free clathrin to bind to the partially uncoated CVs or clathrin baskets. Therefore, we next investigated whether clathrin exchange occurs in clathrin-coated pits in vivo. Of course, clathrin has to be able to bind to the plasma membrane to maintain a pool of clathrin-coated pits. However, it is not clear whether once clathrin binds to the plasma membrane it can dissociate repeatedly and rebind as the clathrin-coated pit invaginates, since it has been reported that Hsc70 does not dissociate clathrin from clathrin-coated pits (Heuser and Steer, 1989).

To investigate clathrin exchange in vivo, we used the technique pioneered by Keen and his associates (Gaidarov et al.,

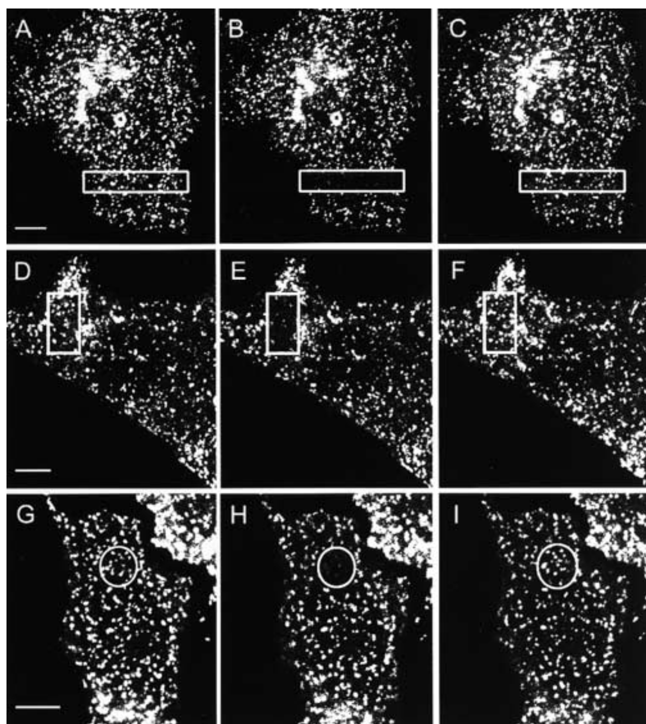

Figure 2. **FRAP GFP-clathrin in HeLa cells at 37°C under different conditions.** Control HeLa cells (A–C); HeLa expressing K44A-dynamin (D–F); and HeLa cells depleted of cholesterol (G–I). Images obtained directly before being photobleached (A, D, and G); images obtained immediately after photobleaching (B, E, and H); images obtained 4 min after photobleaching (C, F, and I). The photobleached area is indicated in each figure. Bars, 7 μm.

1999): expressing green fluorescent protein (GFP)–clathrin by constructing and transfecting HeLa cells with a plasmid encoding human clathrin light chain A with GFP attached at its NH_2 terminus. In agreement with Gaidarov et al. (1999) who used Cos cells in their experiments, confocal microscopy showed numerous GFP-labeled clathrin-coated pits on the plasma membrane of HeLa cells with most of these pits having a diameter of ~0.3 μm or less (Fig. 2 A and Fig. 3 A). After photobleaching a small region of the plasma membrane, there was an immediate 50–80% decrease in the fluorescence intensity of the clathrin-coated pits (Fig. 2 B) followed by an ~80% recovery of fluorescence after 4 min (Fig. 2 C). Quantitative determination of the time course of recovery (Fig. 4, ●, and Table I) showed that the half-life for recovery was about 16 s at 37°C. It seems likely that at least part of this fluorescence recovery is due to clathrin-mediated endocytosis, that is, the simultaneous invagination of bleached clathrin-coated pits and formation of new unbleached clathrin-coated pits. Therefore, to determine whether exchange of clathrin occurs in existing clathrin-coated pits clathrin-mediated endocytosis must be blocked while maintaining the clathrin-coated pits.

One method of blocking endocytosis is to express the dynamin mutant K44A (Damke et al., 1994). EM has shown (Damke et al., 1994) that the diameter of the coated pits on the membrane was unaffected by overexpression of mutant dynamin, but there was a change in distribution with a decrease in flat lattices and an increase in curved lattices, which often appeared clustered. Consistent with that study, we

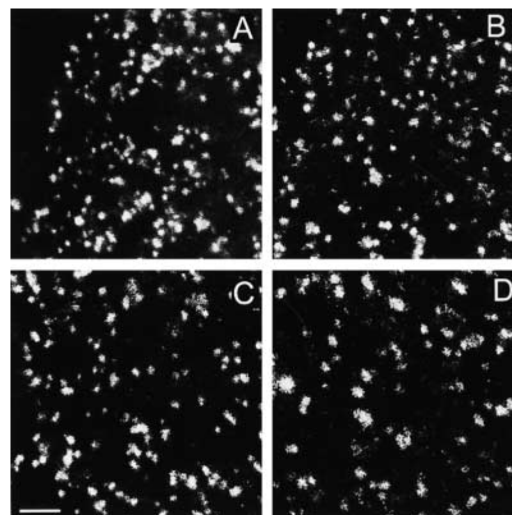

Figure 3. **Determination of the size of the clathrin-coated pits.** CFP-clathrin in HeLa cells were imaged using the 413-nm laser as described in Materials and methods to maximize the resolution of the pit size. Images were acquired with a zoom such that each pixel repents 30 nm in length thereby enabling individual pits to be displayed clearly on the screen. HeLa cells (A); HeLa cells expressing WT dynamin (B); HeLa cells expressing K44A dynamin (C); HeLa cells depleted of cholesterol (D). Bar, 2 μm.

found that in cells expressing K44A dynamin the GFP-clathrin pits are much more clustered, especially around the periphery of the cell (Fig. 2 D). This clustering caused about two-thirds of the fluorescent spots to appear >0.3 μm. Despite the clustering of the pits in these cells, we were able to find areas away from the periphery in which most of the pits were normal size. Therefore, we could easily photobleach fields containing both clustered and normal-sized pits. As a control, we also examined fluorescence recovery of clathrin-coated pits in cells expressing wild-type (WT) dynamin.

To our surprise, we found that the fluorescence of clathrin-coated pits in HeLa cells expressing mutant dynamin recovered after photobleaching (Fig. 2, D–F). The rate of this recovery, which had a half-life of about 16 s, was essentially the same in cells expressing mutant dynamin as in cells expressing WT dynamin (Fig. 4, △ and ▲, and Table I). As expected, the presence of WT dynamin did not affect the rate or magnitude of the recovery (Fig. 4 and Table I). However, as shown in Table I in cells expressing mutant dynamin there was a small reduction in the magnitude of recovery compared with control cells or cells expressing WT dynamin, especially at 37°C. This may be due to the cells expressing mutant dynamin being more sensitive to photobleaching, particularly at higher temperature. In the cells expressing mutant dynamin, we observed no difference in the recovery of fluorescence in normal-sized and clustered clathrin-coated pits.

Another method of blocking clathrin-mediated endocytosis is to deplete cholesterol from the plasma membrane (Rodal et al., 1999; Subtil et al., 1999); EM of these membranes showed that cholesterol depletion caused an accumulation of flat coated pits and a corresponding decrease in deep coated pits (Rodal et al., 1999; Subtil et al., 1999). In agreement, we observed by fluorescence of the GFP-clathrin that about half of the clathrin-coated pits appeared to be

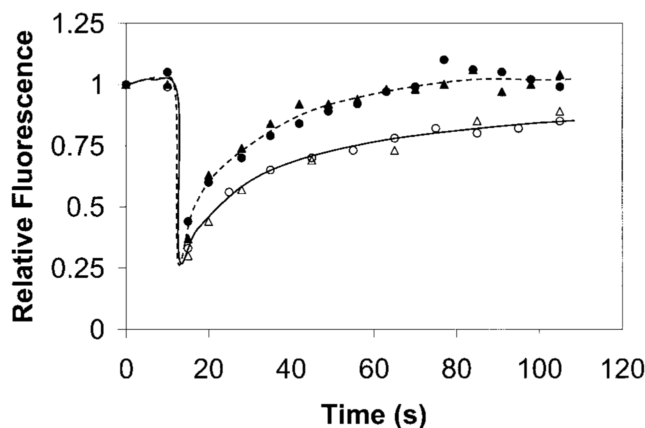

Figure 4. **Kinetics of GFP-clathrin recovery after photobleaching at 37°C.** HeLa cells (●), HeLa cells expressing WT dynamin (▲), HeLa cells expressing K44A-dynamin (△), and HeLa cells depleted of cholesterol (○) were photobleached at 15 s and then scanned at low laser power.

~50% larger than the clathrin-coated pits observed in control cells (Fig. 2 G and Fig. 3 D). As observed in cells expressing K44A dynamin, GFP-clathrin was able to recover after photobleaching in cholesterol-depleted cells (Fig. 2, G–I). Furthermore, the rate of fluorescence recovery was almost the same as that obtained with control HeLa cells (Fig. 4 and Table I). As observed with K44A dynamin-expressing cells, in cholesterol depleted cells the magnitude of recovery was again reduced slightly, suggesting the pits were more sensitive to photobleaching. In summary, by using two different methods to block clathrin-mediated endocytosis without disrupting clathrin-coated pits we have shown that the bleached clathrin in clathrin-coated pits is replaced rapidly by unbleached clathrin from the cytosol.

In these experiments, we have assumed that most of the fluorescent spots on the plasma membrane are individual clathrin-coated pits and not some larger structure. To confirm this assumption, we used cyan fluorescent protein (CFP)–clathrin rather than GFP-clathrin because its lower emission wavelength allows a more accurate sizing of small structures. In addition, we optimized the resolution by using a smaller pinhole and higher magnification (see Materials and methods). Measurement of the size of the CFP-clathrin–coated pits in control HeLa cells (Fig. 3 A) suggested that almost 58% of the pits were ≤0.2 μm in size, 38% were between 0.2 and 0.3 μm, and less than 4% were >0.3 μm, in good agreement with the sizes observed for individual clathrin-coated pits by EM. Similar results were observed for cells expressing WT (Fig. 3 B) and K44A dynamin (Fig. 3 C). We also carried out similar studies in cholesterol-depleted cells (Fig. 3 D), and interestingly the fluorescent spots appeared to be somewhat larger in size: ~40% of the spots were ≤0.2 μm, ~50% were between 0.2 and 0.3 μm, and almost 10% were >0.3 μm. This again is in good agreement with EM studies, showing that clathrin-coated pits are enlarged in cells depleted of cholesterol. Finally, we found that the CFP-clathrin fluorescent spots colocalize almost completely with YFP-AP2 fluorescent spots (unpublished data) and are identical in size and structure to clathrin-coated pits visualized in fixed cells by immunofluorescence using various anticlathrin and anti-AP2 antibodies (unpublished data). Therefore, we conclude that for the most part the fluorescent spots we are observing in vivo are individual clathrin-coated pits.

To confirm that clathrin-mediated endocytosis was markedly inhibited in HeLa cells that either were expressing K44A dynamin or were cholesterol depleted, we measured the rate of ^{125}I-transferrin internalization. In both cases, we found that the rate of transferrin uptake was reduced significantly with cholesterol depletion causing an ~75% reduction and mutant dynamin causing a 90% reduction (unpublished data). However, interestingly we also found that even in WT cells, the rate constant for fluorescence recovery (4.3×10^{-2} s^{-1}) (Table I) was ~14 times the rate constant we measured for transferrin uptake ($3.1 \pm 0.8 \times 10^{-3}$ s^{-1}; $n = 4$). This could occur because most of the replacement of bleached clathrin is not due to endocytosis but rather to a process such as clathrin exchange. On the other hand, it could occur because only ~7% of the transferrin receptors on the plasma membrane are located in clathrin-coated pits at any one time. In support of the latter possibility, Sandvig and his colleagues (Hansen et al., 1992; Rodal et al., 1999) using immunogold studies found that only ~10% of the transferrin receptors on the plasma membrane were localized in clathrin-coated pits. On the other hand, using essentially the same technique Damke et al. (1994) found that 20–30% of the transferrin receptors were localized in clathrin-coated pits.

We used a different approach to determine the fraction of transferrin receptor associated with clathrin-coated pits, carrying out single turnover experiments in which transferrin receptors on the cell surface were first loaded with transferrin, excess transferrin washed away, and then uptake of bound transferrin measured. If transferrin external to the clathrin-coated pits is transferred rapidly into the pits, the rate of uptake of this external transferrin will be linear with a rate constant equal to the rate of endocytosis times the fraction of receptor that is localized in the pits. Then, after this external transferrin is depleted a first-order process will be-

Table I. **Flourescence recovery after photobleaching**

Treatment	37°C		28°C	
	Recovery	**$t_{1/2}$**	**Recovery**	**$t_{1/2}$**
	%	s(n)	%	s(n)
Control	80 ± 9	16.2 ± 4.8 (11)	76 ± 11	29.9 ± 11.4 (24)
WT-dynamin	86 ± 9	16.5 ± 3.8 (6)	76 ± 12	23.2 ± 5.2 (12)
K44A-dynamin	72 ± 20	15.8 ± 6.5 (13)	77 ± 15	35.1 ± 16.2 (16)
Cholesterol depleted	63 ± 15	16.4 ± 6.8 (10)	68 ± 12	34.5 ± 11.6 (39)

Values given are mean ± average deviation.

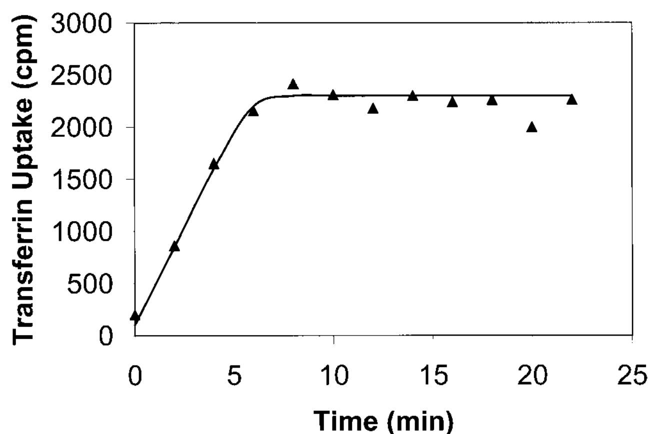

Figure 5. **Single turnover of transferrin uptake at 28°C.** ^{125}I-labeled transferrin was preloaded onto HeLa cells at 4°C. The cells were then washed in PBS and transferred to 28°C. At the indicated times, the surface-bound transferrin was removed, cells were washed in PBS, and the internalized transferrin measured after solubilization of the cells.

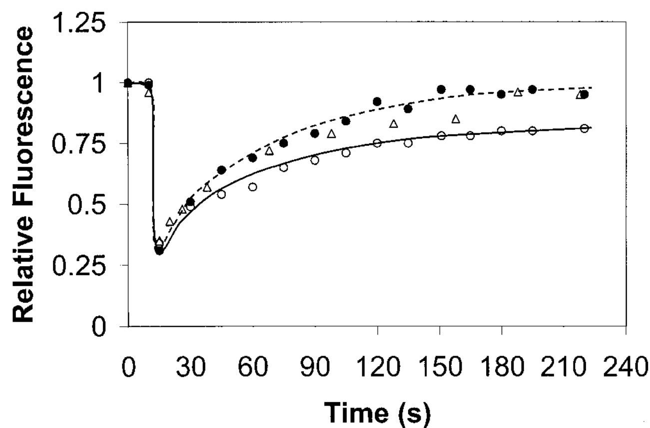

Figure 6. **Kinetics of GFP-clathrin recovery after photobleaching at 28°C.** HeLa cells (●), HeLa cells expressing K44A-dynamin (△), and HeLa cells depleted of cholesterol (○) were photobleached at 15 s. After photobleaching, a time series of scanning were performed at low laser power.

gin as the transferrin left in the pits is internalized. For example, if half of the bound transferrin was located in clathrin-coated pits, 50% of the transferrin would be taken up linearly, and then 50% would be taken up in a first-order process with a rate constant equal to the rate that clathrin-coated pits are internalized. We carried out the single turnover experiments at 28°C because at 37°C the rate of uptake was too rapid to get an accurate time course. The results in Fig. 5 demonstrate that the rate of transferrin uptake is essentially linear until ≥90% of the transferrin bound to receptors is taken up by the cell. These data suggest strongly that most of the transferrin on the plasma membrane is not located in clathrin-coated pits. In agreement with this finding, we found that at both 37 and 28°C when we measured the rate constant for disappearance of fluorescent pits it was approximately one order of magnitude faster than the rate of transferrin uptake, confirming that at both temperatures only ∼10% of the transferrin is located in clathrin-coated pits. Therefore, at least part of the explanation for why the rate of fluorescence recovery is 14 times faster than the rate of transferrin uptake at 37°C is because most of the transferrin is not located in clathrin-coated pits.

However, interestingly when we measured the rate of fluorescence recovery at 28°C (2.3×10^{-2} s^{-1}) (Table I) we found that it was ∼30 times faster than the rate obtained for transferrin uptake ($8.1 \pm 1.4 \times 10^{-4}$ s^{-1}; $n = 8$) at the same temperature (and about threefold faster than the rate of disappearance of fluorescence pits) because as the temperature is decreased from 37 to 28°C the rate of transferrin uptake decreased by a factor of four, whereas the rate of clathrin replacement decreased only by a factor of two. Since it is unlikely that only 3% of the transferrin on the membrane is located in clathrin-coated pits at 28°C, these data suggest that at this temperature a considerable part of the clathrin replacement is indeed due to clathrin exchange rather than endocytosis. In confirmation, as we observed at 37°C, at 28°C both the rate and magnitude of clathrin replacement after photobleaching is nearly the same in cells when endocytosis is blocked by either cholesterol depletion or expression of the K44A dynamin mutant as in control or WT dynamin-expressing cells (Fig. 6 and Table I). Therefore, as the temperature decreases replacement of bleached clathrin becomes dominated by clathrin exchange rather than endocytosis, whereas at 37°C the rates of endocytosis and clathrin exchange appear to be similar. Of course, if the rates of endocytosis and clathrin exchange were identical at 37°C the rate of fluorescence recovery in control cells should be about twice the rate in cells where endocytosis is blocked, since both endocytosis and clathrin exchange occur in WT cells. In fact, we found that the rates of fluorescence recovery are about equal in control and blocked cells at 37°C, but this could easily be due to variability in the measurement or to a twofold faster rate of clathrin exchange occurring in blocked cells compared with control cells.

These data strongly suggest that, overall, replacement of bleached clathrin occurs in cells where endocytosis is blocked. However, to make certain that this actually occurs in the specific cells subjected to photobleaching we simultaneously monitored transferrin internalization and clathrin replacement after photobleaching in these cells. As shown previously (Gaidarov et al., 1999), we found that expression of GFP-clathrin light chain had no effect on endocytosis of transferrin, analyzed by examining the uptake of Cy5 transferrin both in transfected and nontransfected cells over a wide range of times. To follow the photobleaching simultaneously with transferrin uptake, we first incubated the cells with Cy5-transferrin for 5 min at 37°C, conditions in which there is significant uptake of transferrin if endocytosis is not blocked. Fig. 7, A, B, D, and H, shows that during this period of time no transferrin was taken up by cells depleted of cholesterol or expressing the K44A dynamin, nor was transferrin taken up by these cells during the 5 min incubation at 28°C after photobleaching. Nevertheless, the bleached clathrin in the clathrin-coated pits in these cells was replaced almost completely by unbleached clathrin during this same period of time (Fig. 7, E–G and I–K). This clearly establishes that exchange is occurring when endocytosis is blocked.

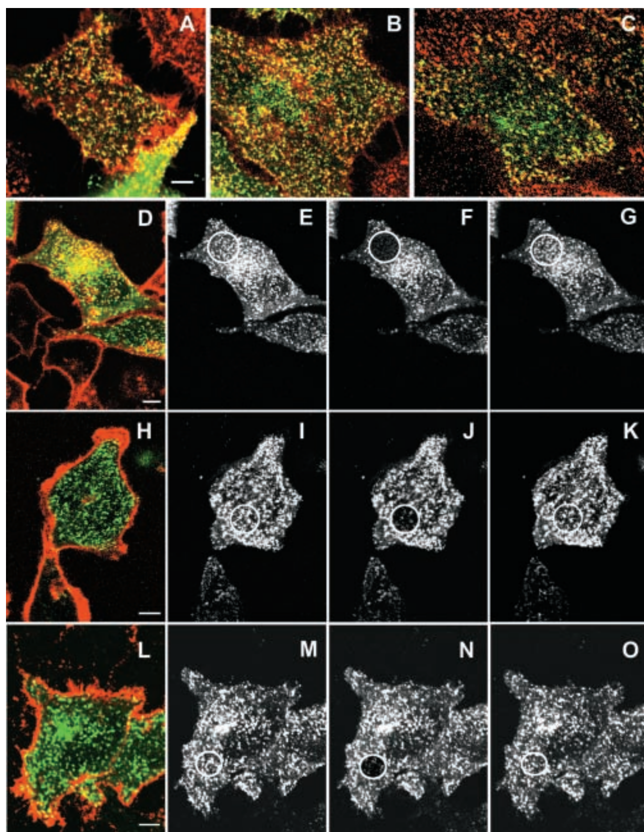

Figure 7. **GFP-clathrin exchanges in cells in which transferrin uptake is blocked.** Cy5-conjugated transferrin (4 μg/ml) was added to cholesterol-depleted HeLa cells (A and D–G), HeLa cells expressing K44A dynamin (B and H–K), or HeLa cells incubated and imaged at 15°C (C and L–O). Cholesterol-depleted cells or cells expressing K44A dynamin were incubated for 5 min at 37°C before being imaged at 28°C. (A–C) Basal surface of cells showing that transferrin is on the membrane and concentrates in most of the clathrin-coated pits; absence of transferrin internalization (D, H, and L); cells immediately before photobleaching (E, I, and M); cells immediately after photobleaching (F, J, and N); cells 5 min after photobleaching (G,K, and O). The photobleached area is indicated in each figure Bars: (A–C) 5 μm; (D–K) 6 μm; (L–O) 5 μm.

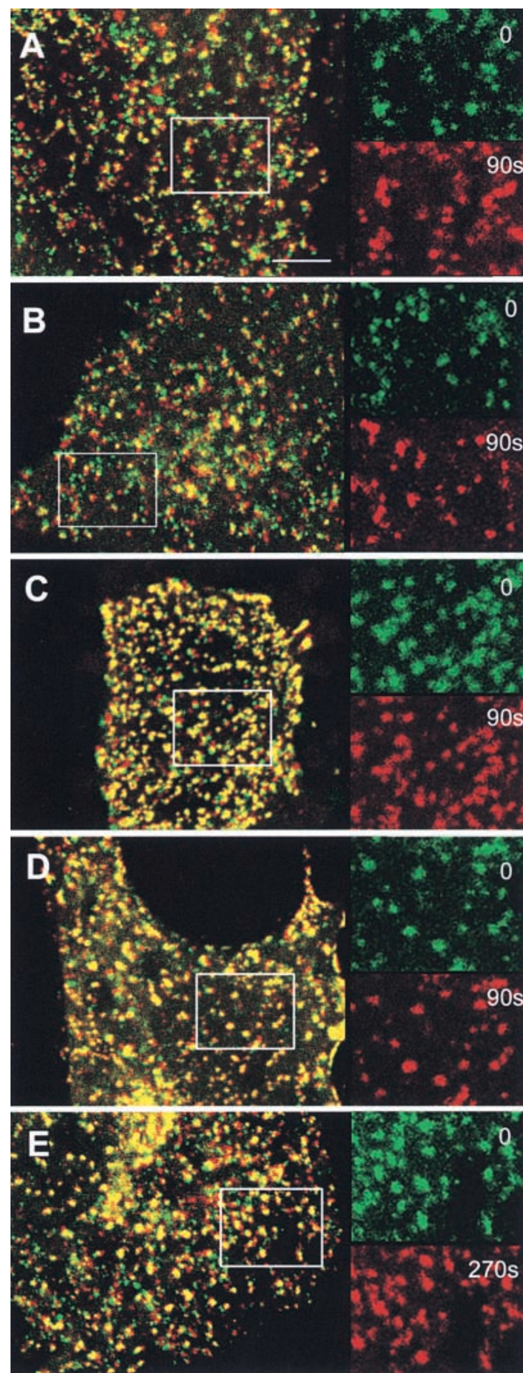

Figure 8. **CFP-clathrin–coated pit pattern is not changed significantly in cells undergoing exchange when endocytosis is blocked.** Control HeLa (A); HeLa expressing WT dynamin (B); HeLa expressing K44A dynamin (C); cholesterol depleted HeLa (D); HeLa cells incubated at 15°C (E). Cells were imaged with a 63×,1.4 NA objective as described in Materials and methods, and images collected were overlayed at 0 and 90 s in A–D and 0 and 270 s in E. The inset magnifies the boxed area (length of box is 8 μm) to show the distribution of clathrin-coated pits at the two different times. Bar, 5 μm.

Using this same technique, we next tested our observation that as the temperature is decreased the rate of clathrin exchange does not decrease as rapidly as the rate of endocytosis. We carried out this experiment at 15°C where previous studies have shown that transferrin uptake by clathrin-coated pits continues to occur but at a much decreased rate compared with 37°C (Cao et al., 1998). We found that over a period of 30 min, transferrin uptake did indeed occur at 15°C (unpublished data), but over a period of 5 min almost no transferrin was taken up by the cells at 15°C (Fig. 7, C and L). On the other hand, in these same cells at 15°C after photobleaching of the pits almost complete replacement of the bleached clathrin was observed over a period of 5 min (Fig. 7, M–O) and in fact was nearly complete in 3 min (unpublished data). Therefore, at 15°C where the rate of endocytosis is reduced greatly, replacement of clathrin by clathrin exchange continues to occur at a rapid rate. These data demonstrate clearly that replacement of bleached clathrin by endocytosis and by exchange of clathrin are two separate processes. They also show that clathrin exchange does not occur just when endocytosis is blocked by artificial treatment of the cells but also when the rate of endocytosis is reduced by decreasing the temperature.

There are two mechanisms which could account for replacement of bleached clathrin with unbleached clathrin of cells where clathrin-mediated endocytosis is blocked. First,

even though endocytosis is not occurring there may be dissolution and replacement of whole clathrin-coated pits. Alternatively, individual clathrin molecules in the pits may exchange. To investigate this question, we followed the fate of individual pits for 90 s to determine if whole pits were replaced with new pits at a different location or whether the clathrin in preexisting pits gradually exchanged. We found that in control HeLa cells a considerable fraction of clathrin replacement occurred because photobleached pits were replaced by new pits (Fig. 8 A). The same result was obtained in cells expressing WT dynamin (Fig. 8 B) as expected since endocytosis is occurring in these cells.

In contrast, in cells where endocytosis was blocked either by expression of the K44A mutant (Fig. 8 C) or by cholesterol depletion (Fig. 8 D) more than two-thirds of the clathrin replacement was caused by exchange of clathrin in existing pits, whereas less than one-third of the replacement was due to dissolution and replacement of whole clathrin-coated pits. These experiments were carried out in areas of the cells where the clathrin-coated pits were not clustered, since with clustered pits it is difficult to distinguish dissolution and replacement of whole pits from clathrin exchange. We did note that in cholesterol-depleted cells the clathrin-coated pits appeared to be considerably more mobile than in control cells. Nevertheless, when we followed the fate of individual clathrin-coated pits it was clear that most of the clathrin replacement was due to exchange of clathrin rather than dissolution and replacement of pits. Furthermore, this same effect occurred at 15°C (Fig. 8 E). Over a period of 4.5 min, during which time we found that endocytosis is blocked but complete replacement of bleached clathrin occurred, no new pits formed but rather the bleached clathrin in the pits exchanged with unbleached clathrin. Therefore, our data strongly suggest that when endocytosis is blocked by agents that have relatively little effect on the structure of clathrin-coated pits the bound clathrin rapidly exchanges with free clathrin in the cytosol.

Since we were unable to detect clathrin exchange on CVs in vitro, we were next interested in determining whether or not clathrin exchange occurs on CVs in vivo before irreversible uncoating occurs. This is a difficult question to answer because even when CVs are visible briefly in vivo they rapidly uncoat. However, it has been reported that when cells are exposed to either hypertonic sucrose or potassium depletion, both the number and size of the clathrin-coated pits on the plasma membrane are reduced dramatically, and clathrin microcages form under the plasma membrane where the clathrin-coated pits were located previously (Heuser and Anderson, 1989). These microcages appeared to be very similar in structure to the subset of relatively small clathrin baskets formed by polymerization of clathrin and APs in vitro. Therefore, we were interested in determining if these microclathrin baskets would show clathrin exchange in vivo.

We found that when cells were treated with either hypertonic sucrose or were potassium depleted, there were still GFP fluorescent spots on the membrane. However, there were somewhat fewer spots and with a broader size distribution, which became more pronounced as we observed the cells over longer time periods. Since it is quite possible that confocal microscopy would not distinguish between GFP-clathrin–coated pits and GFP-clathrin microcages occurring directly under the plasma membrane, we carried out photobleaching experiments on the fluorescent spots to determine if they show the same exchange properties as clathrin-coated pits. We found that in contrast to clathrin-coated pits fluorescent spots in cells treated by either hypertonic sucrose (Fig. 9, A–C) or potassium depletion (Fig. 9, D–F) showed no recovery from photobleaching. Therefore, these treatments dramatically change the properties of the clathrin structures at the plasma membrane, perhaps by converting clathrin-coated pits into clathrin microcages that no longer show clathrin exchange.

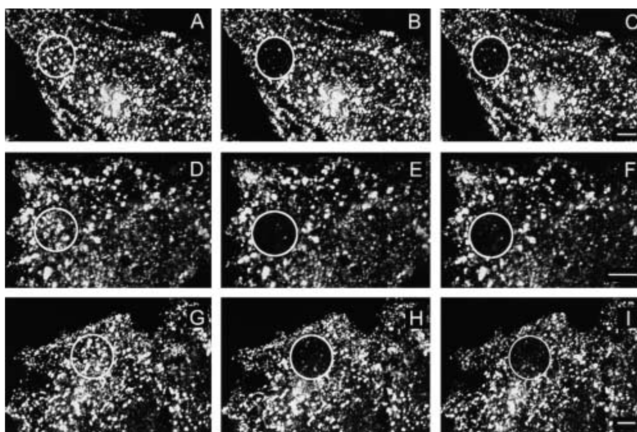

Figure 9. **Absence of clathrin exchange in cells treated with hypertonic sucrose, depleted of K^+, or depleted of ATP.** HeLa cells were treated as described in Materials and methods. Cells treated with hypertonic sucrose (A and C); cells depleted of K^+ (D–F); cells depleted of ATP (G–I). Cells were imaged at 28°C before photobleaching (A, D, and G), immediately after photobleaching (B, E, and H), and 5 min after photobleaching (C,F,I). The photobleached area is indicated in each figure. Bars, 5 μm.

Finally, we tested whether ATP is required for exchange of clathrin in clathrin-coated pits by depleting ATP from the cells. Initially, the appearance of the coated pits was only affected slightly by ATP depletion, but with time the pits appeared to become clustered. However, in both cases the GFP-clathrin fluorescence showed no recovery after photobleaching either at 37 or 28°C even after 10 min after photobleaching (Fig. 9, G–I). Shorter times of ATP depletion had proportionately less effect, presumably because residual ATP remained in the cell. Therefore, ATP is required for clathrin exchange in clathrin-coated pits at the plasma membrane.

Discussion

In this study, we continued our investigation of the role of Hsc70 and auxilin in clathrin dynamics by determining under what conditions clathrin exchange occurs both in vitro and in vivo. The in vivo experiments were carried out with clathrin containing GFP clathrin light chain A, raising the possibility that we are observing light chain rather than clathrin exchange. However, since the light chains bind extremely tightly to the clathrin heavy chains and show very little exchange with free light chains even over a period of a week in vitro (Winkler and Stanley, 1983) it is very unlikely

that any of the exchange we observe in vivo is due to light chain exchange.

In vitro, we did not observe clathrin exchange with CVs or baskets even in the presence of Hsc70 and ATP at pH 6.5 where partial uncoating occurred. In agreement with this result, we found that hypertonic sucrose or potassium depletion, which have been reported to block endocytosis in vivo by transforming clathrin-coated pits into clathrin microcages just below the surface of the plasma membrane (Heuser and Anderson, 1989), not only block endocytosis but also block clathrin exchange. However, our data also suggest that the structures that are induced to form by hypertonic sucrose or potassium depletion in vivo are not identical to clathrin baskets formed in vitro since they are stable for long periods of time and therefore apparently cannot be uncoated by Hsc70 and auxilin present in the cytosol.

It is not possible to determine whether clathrin exchanges under conditions where clathrin-mediated endocytosis occurs because formation of new clathrin-coated pits as old ones bud off would mask any exchange on preexisting pits. However, in contrast to hypertonic sucrose and potassium depletion cholesterol depletion and expression of the dynamin mutant K44A block endocytosis without removing the clathrin-coated pits from the plasma membrane. Therefore, we tested the effect of these agents on clathrin exchange and remarkably found that exchange of clathrin occurred at nearly the same rapid rate and to the same extent as in control HeLa cells. Likewise, when the rate of endocytosis was markedly reduced by decreasing the temperature of the cells clathrin exchange continued to occur at a rapid rate.

Two questions arise in regard to the clathrin exchange that occurs when endocytosis is blocked. First, are the fluorescent structures that we observe actually clathrin-coated pits, and second, is clathrin exchange occurring in these structures, or are we simply observing their dissolution and reformation. In regard to the first question, it has been suggested that the GFP-clathrin structures observed by Gaidarov et al. (1999) in Cos cells are actually clathrin reservoirs rather than clathrin-coated pits (Kirchhausen, 2000b). However, since our data suggest that many if not most of the structures that we observe are close to the resolution of the confocal microscope, that is, they are ~0.2–0.3 μm in diameter, about the size of a clathrin-coated pit (Kirchhausen, 2000a), it seems likely that they are either clathrin-coated pits or perhaps in some cases small clusters of clathrin-coated pits. Furthermore, these structures appear to be about the same size as the clusters of microcages induced by the action of sucrose or potassium depletion, again suggesting that they are not large clathrin reservoirs.

As for whether these clathrin-coated pits are undergoing clathrin exchange or dissolution and reformation, our data suggest that in the large majority of cases clathrin exchange is occurring, and only a minority of the clathrin-coated pits are undergoing dissolution and reformation during the time period in which the photobleached clathrin is replaced. This is in contrast to the situation when clathrin-mediated endocytosis is active; in this case, much of the clathrin replacement is due to replacement of CVs that have budded off from the membrane. Therefore, our data suggest that the structures we are observing are clathrin-coated pits, and these pits are undergoing clathrin exchange even when they are blocked from completing the process of invagination and pinching off. Furthermore, our data show that this exchange is ATP dependent. Therefore, it seems very unlikely that this clathrin exchange only occurs when endocytosis is blocked. Rather, the ATP-dependent exchange of free and bound clathrin in vivo is most likely a fundamental property of clathrin-coated pits, independent of the process of endocytosis.

The question then arises as to whether this clathrin exchange is involved in the transition that occurs when flat clathrin-coated pits invaginate and become spherical CVs. There are two views on how this transition might occur. First, it has been suggested on theoretical grounds that if, when the clathrin lattice opens, two adjacent clathrin triskelions exchange before the lattice closes preexisting hexagons present in the flat pits can be transformed into the pentagons required for invagination to occur (Jin and Nossal, 1993). On the other hand, it has been suggested that invagination might occur by adding new clathrin to the edges of already existing clathrin-coated pits so that pentagons form de novo (Kirchhausen, 2000b). Although the data presented in this paper do not distinguish between these two models, they do show that clathrin exchange occurs in clathrin-coated pits in vivo, and furthermore, although it is possible that the very core of the pit does not show complete exchange, exchange is not just occurring on the edges of the pits, since we see almost complete recovery of fluorescence, that is, almost complete replacement of bleached clathrin in cells where endocytosis is blocked. Therefore, clathrin exchange could be involved in the transformation of hexagons to pentagons as invagination occurs. Alternatively, even if the curved lattice grows out from existing flat pits, clathrin exchange throughout most of the pit may be necessary for error correction to ensure that hexagons and pentagons form in the correct position on the curved lattice of the invaginating coated pit; similar error correction has been suggested to occur during formation of viral coats (Steven et al., 1992). If clathrin exchange is indeed involved in the transition from flat pits to curvilinear vesicles, it might be expected to occur at about the rate as endocytosis itself, and this is indeed what we observe at 37°C, although as the temperature is decreased clathrin exchange becomes much more rapid than endocytosis.

Our data also demonstrate that ATP is required for clathrin exchange to occur. This raises the possibility that the ATP-binding chaperone Hsc70 is not only involved in the uncoating of CVs but is involved in the clathrin exchange that occurs during the earlier stages of clathrin-mediated endocytosis. There is now strong evidence that Hsc70 and the J-domain protein auxilin are required for the uncoating of CVs in vivo. Experiments with yeast and *C. elegans* demonstrate that when auxilin is deleted (Gall et al., 2000; Pishvaee et al., 2000) or prevented from forming by RNA_i (Greener et al., 2001) clathrin-mediated endocytosis is blocked, an effect that is lethal in the case of *C. elegans*. However, there has always been a problem as to how the uncoating of CVs by Hsc70 is regulated so that no uncoating occurs until the CVs bud off from the plasma membrane at which point very rapid uncoating is triggered. The observation that clathrin not only detaches from CVs after the vesicles pinch off but is actually detaching and reattaching to the plasma membrane

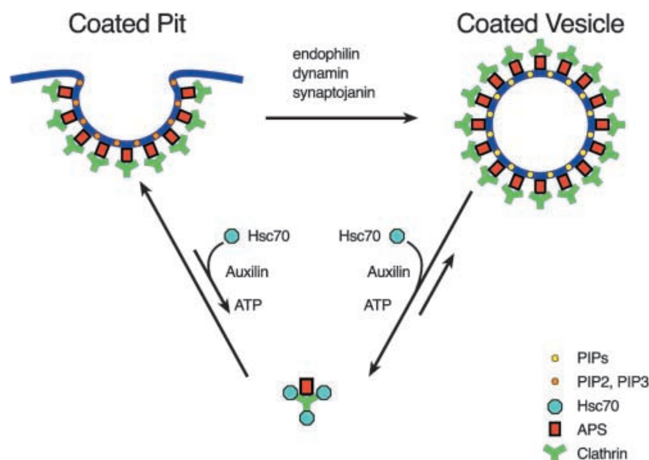

Figure 10. **Model of clathrin exchange in vivo.** See text for details.

throughout the endocytic process allows us to examine this problem from a new perspective.

Since exchange is constantly occurring, the actual uncoating step may only involve a small change in the rates of clathrin attachment to and detachment from the membrane, particularly if either attachment or detachment is a cooperative process. Thus, as shown in the model in Fig. 10 Hsc70 may not only be involved in the uncoating of CVs after they pinch off from the membrane but may also be involved in the detachment of clathrin from clathrin-coated pits during earlier steps in endocytosis and perhaps in the rebinding of clathrin as well. In support of this view, our biochemical experiments suggest that Hsc70 is involved in both chaperoning dissociated clathrin and in the rebinding of clathrin to the membrane (Jiang et al., 2000). In addition, when auxilin RNA_i was used to block formation of auxilin in *C. elegans* not only was clathrin-mediated endocytosis blocked, but in addition FRAP studies showed that in contrast to what occurs in WT *C. elegans* replacement of clathrin after photobleaching no longer occurred in the coelomocytes of RNA_i worms (Greener et al., 2001). Although this could be due to clathrin-mediated endocytosis being blocked in the RNA_i worms, it might also be due to clathrin exchange in clathrin-coated pits being blocked in the absence of auxilin, that is, in the absence of Hsc70 action.

If small changes in the rate constants for attachment or detachment of clathrin after formation of CVs indeed trigger complete uncoating of the vesicles, a possible candidate that might cooperatively affect these rates is synaptojanin as shown in Fig. 10. Synaptojanin is a polyphosphoinositide phosphatase that dephosphorylates phosphoinositides at 3, 4, and 5 of the inositol ring (Brodin et al., 2000), and there is evidence already that synaptojanin and Hsc70 are involved in uncoating, since CVs accumulate in the synapses of synaptojanin knock-out mice (Cremona et al., 1999) and also in lamprey synapses when synaptojanin activity at the clathrin-coated pit is blocked by microinjection of an inhibitory peptide that prevents synaptojanin from binding to endophilin (Gad et al., 2000). In this regard, preliminary experiments in vitro suggest that cytosol from synaptojanin knock-out mice is less effective in uncoating CVs than cytosol from WT mice (Cremona et al., 1999), an effect that does not occur with clathrin baskets, which lack membranes. Therefore, the presence of phosphatidylinositol 4,5-biphosphate or perhaps phosphatidylinositol 3,4,5-trisphosphate (Gaidarov et al., 2001) in the plasma membrane may cooperatively increase the binding of AP2 and clathrin to the plasma membrane.

Even if synaptojanin and Hsc70 are involved in clathrin uncoating in vivo, it is still difficult to explain why the clathrin baskets induced to form under the plasma membrane by hypertonic sucrose or why potassium depletions are not uncoated by Hsc70 in the same way that they are uncoated in vitro. Perhaps these clathrin cages act as clathrin reservoirs and their state of polymerization is regulated by their level of phosphorylation. In any event, much more work will be required to understand fully the regulation of clathrin polymerization and depolymerization that occurs during clathrin-mediated endocytosis. Further studies on coated pit dynamics both in vivo and in permeabilized systems may help reveal the factors that are involved in this regulation.

Materials and methods

Materials

ATP, MES, Hepes, sucrose, deoxyglucose, imidazole, tetracycline, methyl-β-cyclodextrin, oubain, and ATP agarose were from Sigma-Aldrich. ^{125}I-transferrin and [^{14}C]formaldehyde were from New England Nuclear. All media and supplements were obtained from Biofluids, Inc. Fugene6 was from Roche Molecular Biochemicals, and Cy5-conjugated transferrin was from Jackson ImmunoResearch Laboratories, Inc.

Preparation of proteins

CVs, clathrin, mixed APs, mixed AP–clathrin baskets, and Hsc70 were prepared from fresh calf brains as described previously (Jiang et al., 2000). Purified clathrin was trace labeled with [^{14}C]formaldehyde as described previously (Jiang et al., 2000). Hsc70 and clathrin baskets were suspended in buffer A (20 mM imidazole, 25 mM KCl, 10 mM ammonium sulfate, 2 mM magnesium acetate, pH 7.0) unless otherwise noted.

Clathrin exchange

The exchange of clathrin was performed by adding ^{14}C-labeled clathrin, using either free clathrin or clathrin generated from the uncoating of labeled mixed AP baskets, to different nonradioactive substrates including CVs, mixed AP baskets, and partially uncoated coated vesicles and baskets. In experiments using brain homogenates, ^{14}C-labeled clathrin was added to the brains before homogenization in the Waring blender, after homogenization, and after the first low speed spin that removes most of the heavy cellular debris. Uncoating of CVs or mixed AP baskets by Hsc70 was carried out as described previously (Jiang et al., 2000) in either buffer A (pH 7.0), or when uncoating was at pH 6.5 buffer A contained 20 mM MES instead of imidazole. The clathrin released after uncoating and the clathrin incorporated into the nonradioactive substrate during the exchange were determined by centrifugation of the samples in the TL100 at 380,000 *g* for 6 min. Samples were then counted in a Beckman Coulter LS 3801 liquid scintillation counter.

Cloning of the GFP-clathrin light chain

Human Placenta Quick Clone cDNA (CLONTECH Laboratories, Inc.) was screened for clathrin light chain A by PCR using primers 5′-GATCTCGAGATGGCTGAG-3′ and 5′-CAGATGTAG TGTTTCCACAG-3′. An XhoI site was introduced at the beginning of the clathrin light chain A during PCR. The 700 bp PCR product was cloned into pCR II TOP10 (Invitrogen) followed by subcloning the XhoI to EcoRI fragment into pEGFP-C3 vector (CLONTECH Laboratories, Inc.). The full 700 bp clathrin light chain A fragment was sequenced to confirm the accuracy of the PCR product.

Cell culture and transfection

HeLa cells were maintained in DME supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 U/ml) in a humidified incubator with 5% CO_2 at 37°C. HeLa cells stably expressing WT and mutant (K44A) dynamin were grown under control of a tetracycline pro-

moter and induced by removal of tetracycline (Damke et al., 1995). Cells were transfected with the GFP-clathrin light chain with Fugene6. Hypertonic treatment was performed by incubating the cells in 0.2 M sucrose for 45 min at 37°C. Potassium depletion was performed by hypotonic swelling of the cells in 50% diluted DME containing 1 mM oubain and then incubating them in ice-cold K^1-free medium (100 mM NaCl, 50 mM Hepes, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM glucose at pH 7.3) for 20 min. Cellular ATP was depleted by incubating the cells for 40 min at 37°C using 50 mM 2-deoxyglucose and 0.02% sodium azide in glucose-free medium. Cholesterol was depleted by incubating the cells in 10 mM methyl-β-cyclodextrin for 30 min at 37°C (Rodal et al., 1999; Subtil et al., 1999).

Transferrin internalization

Cells were grown overnight in 6-well cell culture dishes. They were then washed once in PBS, incubated 30 min at 37°C in DME containing 0.5% BSA before incubating the cells at 4°C for 15 min in the presence of ^{125}I-labeled transferrin. In routine experiments, the cells were either transferred directly to 28 or 37°C to determine the amount of transferrin internalized and surface bound transferrin at six different times. The data were then graphed as a ratio of internalized transferrin per surface transferrin versus time. The reciprocal of the slope gave the first order rate constants (Subtil et al., 1999). Values given in the text are shown ± SD. For a single round of internalization, the same protocol was followed except after incubating with transferrin on ice the cells were washed two times with PBS, incubated with DME and at the various times surface, and internalized transferrin were measured. Surface-bound transferrin was dissociated by incubating cells with 0.5% acetic acid and 0.5 M NaCl, whereas internalized transferrin was determined after removing the surface-bound transferrin, washing with PBS, and then lysing the cells for 5 min at 37°C with 1% Triton X-100 and 0.5% SDS.

Confocal microscopy

Cells grown on 25 mm^2 coverslips were typically transferred to DME phenol-free medium containing 20 mM Hepes, pH 7.4, 30 min before imaging on a ZEISS LSM 510 confocal microscope. When indicated, Cy5-conjugated human transferrin, which was imaged using a 633-nm laser, was added to follow transferrin internalization. CFP-clathrin was imaged using a 413-nm laser line with a 63×, 1.4 NA objective and a pinhole size of 1 airy unit. Under these conditions, minimum optically resolvable object is 120 nm. GFP-clathrin was imaged and photobleached using 488-nm laser with a 40×, 1.4 NA objective. A defined region was photobleached at a high laser power to result in a 50–80% reduction in the fluorescence intensity. The recovery of fluorescence was monitored by scanning the whole cell at low laser power. Temperature was regulated at 37°C either by using a Bioptechs chamber or else using a heating fan and a YSI precision thermometer to check the temperature and at 15°C using a Micro Incubator by Micro Devices. To analyze the rate of recovery, we compared the fluorescence of the photobleached area to that of an adjacent unbleached area with similar GFP-clathrin pit density. For each time point, we then normalized the relative fluorescence of the bleached to the nonbleached area to correct for any change due to either drift in focal plane or photobleaching during the low light scanning. Only samples in which this correction did not significantly affect the data (<20% correction compared with the uncorrected data) were used in the analysis.

We would like to thank Dr. Sandra Schmid for the stable HeLa cell lines expressing WT and K44A dynamin.

Submitted: 20 April 2001
Revised: 7 September 2001
Accepted: 7 September 2001

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