Exchange of clathrin, AP2 and epsin on clathrin-coated pits in permeabilized tissue culture cells

Yang-In Yim, Sarah Scarselletta, Fang Zang, Xufeng Wu, Dong-won Lee, Young-shin Kang, Evan Eisenberg and Lois E. Greene*

Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, MD 20892-0301, USA
*Author for correspondence (e-mail: greenel@helix.nih.gov)

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Summary

Clathrin and clathrin adaptors on clathrin-coated pits exchange with cytosolic clathrin and clathrin adaptors in vivo. This exchange might require the molecular chaperone Hsc70 and J-domain-protein auxilin, which, with ATP, uncoat clathrin-coated vesicles both in vivo and in vitro. We find that, although Hsc70 and ATP alone could not uncoat clathrin-coated pits, further addition of auxilin caused rapid uncoating of clathrin but not AP2 and epsin. By contrast, cytosol uncoats clathrin, AP2 and epsin from pits in permeabilized cells, and, concomitantly, these proteins in the cytosol rebind to the same pits, establishing that, like in vivo, these proteins exchange in permeabilized cells.

Dissociation and exchange of clathrin in permeabilized cells can be prevented by inhibiting Hsc70 activity. The presence of clathrin-exchange in the permeabilized system substantiates our in vivo observations, and is consistent with the view that Hsc70 and auxilin are involved in the clathrin-exchange that occurs as clathrin-coated pits invaginate in vivo.

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Introduction

Clathrin-mediated endocytosis is one of the major mechanisms employed by the cell for the import of receptors from the plasma membrane. In general terms, this process involves receptors in the plasma membrane binding to a clathrin adaptor protein, such as AP2, which induces clathrin polymerization and the formation of clathrin-coated pits (Pearse and Robinson, 1990; Ehrlich et al., 2004). Clathrin-coated pits are dynamic structures; both clathrin and AP2 exchange with their cytosolic proteins, not only during endocytosis, but also when endocytosis is blocked (Wu et al., 2001; Moskowizt et al., 2003). Following their formation, clathrin-coated pits invaginate. This process involves many proteins, including endophilin and epsin, both of which have been reported to be directly involved in inducing membrane curvature as the pits invaginate (Ford et al., 2002; Schmidt et al., 1999). In addition, the membrane protein amphiphysin has recently been found to function in sensing, and perhaps even generating, the membrane-bending events required for vesicle formation (Peter et al., 2004). The coated pits then pinch off in a process that involves the GTP-binding protein dynamin (Danino and Hinshaw, 2001), and perhaps also actin and actin-related proteins in some cell types (Qualmann et al., 2000; Fujimoto et al., 2000). Following pinching off, clathrin-coated vesicles (CVs) are then uncoated in a process that requires the ATP-binding molecular chaperone Hsc70 and a specific clathrin-binding J-domain protein, either auxilin in neuronal tissues (Ungewickell et al., 1995) or GAK (auxilin2) in non-neuronal tissues (Greener et al., 2000; Umeda et al., 2000). Apparently, uncoating also involves the hydrolysis of phosphoinositide-4,5-phosphate by synaptojanin (Stenmark, 2000), which might reduce the binding strength of AP2, and indirectly clathrin, to the plasma membrane (Gaidarov et al., 1996). Although a different set of proteins is involved in the budding of CVs from the trans-Golgi network (TGN), here too, the clathrin-coated pits appear to be dynamic structures with clathrin and assembly proteins exchanging during the budding process and also when this process is blocked (Wu et al., 2001; Wu et al., 2003; Puertollano et al., 2003).

The observation that clathrin-exchange is a fundamental property of clathrin-coated pits both at the plasma membrane and the TGN, suggests that this exchange might be related to the structural changes that occur as clathrin-coated pits invaginate. Before they invaginate, clathrin-coated pits are planar or slightly curved structures, in which the polymerized clathrin triskelions form a hexagonal array (Crowther and Pearse, 1981). By contrast, the invaginated pits appear to be considerably more curved (Pearse et al., 2000). Invagination may occur partially through the addition of clathrin to the edges of a growing pit, but there also appears to be an increase in curvature that would require transformation of the polymerized clathrin arrays from hexagons to pentagons (Guichet et al., 2002). This, in turn, would require clathrin-exchange to take place as invagination occurs. Alternatively, Ehrlich et al. have suggested a mechanism, whereby clathrin coats might change their curvature without any exchange taking place (Ehrlich et al., 2004).

We have previously observed that clathrin exchange requires ATP and that, in Caenorhabditis elegans, clathrin exchange was prevented when RNA interference was used to block the
formation of auxilin (Greener et al., 2001). On this basis we proposed that, rather than clathrin passively dissociating from pits, Hsc70, auxilin and ATP are required to actively unravel individual clathrin molecules from the intricate clathrin lattice present in the pits (Wu et al., 2001). However, previous studies found that, whereas Hsc70 was able to uncoat CVs in the presence of ATP, it was not able to uncoat clathrin-coated pits that were present on plasma membrane fragments prepared by brief sonication of tissue-culture cells (Heuser and Steer, 1989). At the time this was done, it was not yet known that auxilin in addition to Hsc70 is required for the uncoating of CVs. In this present study, we therefore investigate, whether Hsc70 in combination with auxilin can uncoat clathrin-coated pits on the plasma membrane of permeabilized cells. We found that a combination of Hsc70 and auxilin uncoats clathrin-coated pits in an ATP-dependent reaction in permeabilized cells. In the presence of cytosol, concomitant with clathrin-uncoating, clathrin, AP2 and epsin exchange on the pre-existing pits. Moreover, Hsc70 and auxilin are required for the clathrin-exchange. These results support our observations that clathrin and AP2 both exchange on clathrin-coated pits in vivo.

**Materials and Methods**

**Cell culture and transfection**

Chinese hamster ovary (CHO) cells were transfected using Fugene 6 (Roche) with the following enhanced green fluorescent protein (EGFP) constructs (pEGFP, Clontech): clathrin light chain, α adaptin, (Wu et al., 2001; Wu et al., 2003) and epsin (gift of P. DeCamilli, Yale University, New Haven, CT). The GFP constructs of clathrin light chain and α adaptin, which have been used in our previous studies (Wu et al., 2001; Wu et al., 2003), were localized to the clathrin-coated pit and did not affect transferrin internalization. Stable cell lines of the transformants were selected on G418 (1 mg/ml) and cultured in HAM’s F-12K medium (Biosource International) with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 units/ml), and G418 sulfate (0.5 mg/ml) in a humidified incubator with 5% CO2 at 37°C. Stable cell lines of the GFP-constructs were used in all experiments.

**Permeabilization and transferrin internalization**

Cells were grown overnight in either 4-well or 8-well Labtek coverslips (Nunc). Coverslips were placed on ice and treated for 2 minutes with cold permeabilization solution (60 mM PIPES, 25 mM HEPES, 5 mM EGTA, and 3% sucrose) containing 20 µg/ml digitonin. The cells were washed twice with PBS and then incubated in buffer A [20 mM imidazole, 2 mM magnesium acetate, 25 mM KCl, 10 mM (NH4)2SO4 pH 7.0]. To load transferrin, cells were incubated for 30 minutes at 37°C in HAM’s F-12K medium without FBS for depleting serum transferrin, and then 10 µg/ml of Alexa-Fluor-546-conjugated transferrin (Molecular Probes) was loaded at 15°C for 25 minutes. Incubation at this temperature prevents internalization of transferrin, while the transferrin concentrates on the coated pits (Wu et al., 2001). Acid-treatment of transferrin-loaded cells was performed by treating the cells with 0.5% HCl-acetate, 0.5 M NaCl pH 2.5.

**Immunofluorescence**

Cells were fixed with 4% paraformaldehyde before immunostaining. The following antibodies were used: anti-clathrin antibody was either X22, an IgG1 isotype (Affinity BioReagents) or CHC5.9, an IgM (Biodesign International), anti-AP2 antibody was AP.6 (Affinity BioReagents), anti-AP1 was 100/3 (Sigma), anti-GGA3 and anti-GM130 antibodies were purchased from BD Transduction Lab and anti-epsin antibody was from Santa Cruz Biotechnology, Inc. Secondary antibodies used were rhodamine-conjugated goat anti-mouse IgG antibody, rhodamine-conjugated rabbit anti-goat IgM antibody and Cy5-conjugated goat anti-mouse IgM antibody (Jackson Immunoresearch Laboratory Inc.). For the staining of nonpermeabilized cells, 0.02% saponin was added to the antibody dilution-buffer (PBS, 10% FBS, 0.02% NaN3).

**Uncoating of clathrin-coated pits**

Uncoating experiments were performed at room temperature and in buffer A. Uncoating of clathrin-coated pits was carried out by adding preincubated ATP-Hsc70 and auxilin onto the permeabilized cells. Unless otherwise indicated, 1 mM ATP, 2 µM Hsc70 and 0.2 µM auxilin were used. The chemical

![Fig. 1. Effects of permeabilization on clathrin-coated pits. (A-D) Clathrin-coated pits were fixed before (a,b) and after (c,d) permeabilization. Cells were imaged for both GFP (a,c) or Alexa 546 (b,d) fluorescence. Immunostained cells were imaged for clathrin in (A, b,d), for AP2 (B, b,d), and for epsin (C, b,d). In D, cells were loaded with Alexa 546 transferrin and imaged for transferrin (b,d). Notice that, all fluorescence settings used in comparing nonpermeabilized and permeabilized cells were identical. Bars, 10 µm.](image-url)
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Dissociation and rebinding of the clathrin-coated pits by brain cytosol

Brain cytosol was prepared by homogenizing bovine brains into an equal volume of homogenization buffer [0.1 M MES pH 6.5, 1 mM EGTA, 0.5 mM magnesium acetate, 3 mM NaN₃, 0.2 mM phenylmethylsulphonylfluoride (PMSF)]. The homogenate was then spun at 5000 g for 1 hour and the supernatant was respun at 100,000 g for 1 hour. The supernatant was then dialyzed into buffer A, pH 7.0. The cytosol, which was used at a concentration of 10 mg/ml unless otherwise indicated, contained Hsc70, auxilin, clathrin, AP2 and many other proteins. In the dissociation experiments, the various pure proteins or cytosol were preincubated with 1 mM ATP and then added to the permeabilized cells at room temperature. Recombinant yeast YDJ1 protein and human HDJ1 protein were made according to King et al. (King et al., 1997) and the dominant-negative Hsp70 ATPase mutant (K71E) was purified according to Rajapandi et al. (Rajapandi et al., 1998). Hsc70 was prepared according to Greene and Eisenberg (Greene and Eisenberg, 1990) and auxilin was prepared as described in Greener et al. (Greener et al., 2000).

Results

To compare the fluorescence intensity of GFP-clathrin before and after permeabilization, we used CHO cells stably transfected with GFP-clathrin so that we could follow uncoating with real-time imaging. We first made certain that digitonin treatment permeabilized the cells and released the cytosol within the cells by showing that, in cells transfected with GFP-Hsc70, more than 90% of the GFP-Hsc70 dispersed into the surrounding solution following digitonin treatment (data not shown). We then tested whether GFP-clathrin-coated pits were preserved on the plasma membrane following digitonin treatment. Fig. 1 shows the distribution of clathrin, AP2, epsin and transferrin on the plasma membrane of cells before and after permeabilization. Clathrin was visualized by both antibody staining and GFP fluorescence (Fig. 1A). AP2 and epsin were visualized by antibody staining, and transferrin was loaded on the pits before permeabilization (see Materials and Methods). The images were produced using identical scan-settings that allowed direct comparison of fluorescence intensities. The permeabilized cells have almost as many clathrin puncta as the intact cells and showed only a slight decrease in the overall fluorescence intensity of clathrin and AP2 on the puncta.

Fig. 2. Uncoating of clathrin-coated pits requires Hsc70, auxilin and ATP. Factors known to affect the uncoating of CVs were incubated with the permeabilized cells for 5 minutes, in an attempt to uncoat permeabilized cells. Conditions were buffer A plus ATP, 1 mM; Hsc70, 2 µM; auxilin, 0.2 µM as indicated. Bars, 10 µm.

Confocal microscopy

GFP-expressing cells were imaged on a Zeiss LSM 510 confocal microscope using argon laser to excite at 488 nm with a 40X, 1.4 NA objective. Rhodamine and Cy5 were imaged using the helium-neon laser by exciting at 543 nm and 633 nm, respectively. Image analysis was done using the LSM 510 confocal program, which measures the relative intensity of our fluorescence in a given area. Images were always checked to insure that the photomultiplier tube was not saturated.

Fig. 3. Kinetics of uncoating of clathrin-coated pits by Hsc70-auxilin-ATP mixture. (A) Uncoating mixture composed of Hsc70 (2 µM), auxilin (0.2 µM) and ATP (1 mM) was added to the permeabilized cells and imaged at 30-second intervals. (B) The decrease in fluorescence intensity of three different regions of interest (ROI) (circles, numbered 1-3) for the time course in A is plotted against time. Data were normalized to the maximal initial fluorescence in each plot. Bar, 10 µm.
demonstrating that the clathrin-coated pits remained intact after permeabilization. As expected, AP2 and epsin colocalized with the GFP-clathrin (Fig. 1B,C). Moreover, the transferrin on the pits was not significantly decreased following permeabilization (Fig. 1D), showing that the pits have not yet pinched-off from the membrane.

Having obtained a method of permeabilization that provides a lawn of GFP-clathrin-coated pits, we examined in real-time imaging the dissociation of clathrin from these pits. Fig. 2 shows that no significant uncoating occurred unless we added a combination of Hsc70, auxilin and ATP. In an earlier study, Heuser and Steere used freeze-etch electron microscopy to examine clathrin uncoating by Hsc70, but did not observe uncoating of coated pits (Heuser and Steere, 1989). However, at the time of their study auxilin was not yet identified (Ungewickell et al., 1995), so the absence of auxilin might account for the lack of uncoating in their experiments. Our results suggest that, auxilin, and also Hsc70 and ATP are essential for the uncoating of clathrin-coated pits just as we observed for the uncoating of clathrin baskets (Ungewickell et al., 1995). Fig. 3 shows the time course for the uncoating of GFP-clathrin puncta by 2 μM Hsc70 and 0.2 μM auxilin. After normalizing to the maximum fluorescence, the change in fluorescence intensity averaged over the region of interest (ROI) was measured using three different regions in Fig. 3B. In all cases, the decrease in fluorescence roughly followed the same time course with a half-life of about 3 minutes. This includes region 1, which contains clathrin at the TGN, as confirmed by immunostaining the Golgi proteins GGA3 and GM130 (data not shown). Unlike clathrin-coated pits, there was much more variability in the preservation of clathrin on the TGN after permeabilization. Nevertheless, our results showed that the uncoating of this region occurs at a similar rate as that observed for clathrin in the other regions.

We were particularly interested in examining the time course of clathrin dissociation from individual clathrin-coated pits. The plot in Fig. 3B is the fluorescence intensity of GFP-clathrin averaged over the entire region, so it is not possible to distinguish from this graph whether individual clathrin pits are catastrophically dissolving at different times or whether clathrin is gradually dissociating from all the pits. Therefore, we imaged at higher magnification to determine the time
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Fig. 6. Hsc70-auxilin-ATP mixture does not dissociate adaptors or transferrin from clathrin-coated pits. GFP-clathrin cells were uncoated with Hsc70, auxilin, and ATP for 10 min, at which time they were fixed. Cells were immunostained for clathrin (a,b), AP2 (c,d) and epsin (e,f) both before (a,c,e) and after uncoating (b,d,f). The GFP-clathrin cells were preloaded with transferrin, permeabilized, and imaged before (g) and after uncoating (h). Bars, 10 μm.

Fig. 7. Bovine brain cytosol dissociates and exchanges clathrin on the clathrin-coated pits and active Hsc70 is required for clathrin uncoating. (A) Permeabilized GFP-clathrin cells loaded with transferrin were incubated with cytosol and nucleotide for the indicated times. GFP-clathrin dissociates (a-c), whereas there is no change in transferrin (d-f). (B) In cells expressing GFP-clathrin, clathrin uncoating by cytosol (∗) was established by measuring GFP-clathrin fluorescence at 20-second intervals. To establish whether Hsc70 is responsible for clathrin uncoating in the cytosol, two Hsc70 inhibitors were used, YDJ1 (∗), and the dominant-negative mutant Hsp70(K71E) (∗). The permeabilized cell membranes and also the bovine brain cytosol were treated with 1 μM YDJ1, and the decrease in fluorescence was measured in 20-second intervals (∗). Bovine-brain cytosol was treated with 5 μM Hsp70(K71E) and the decrease in fluorescence was measured in 20-second intervals (∗). Measurements were carried out by using a confocal microscope. (C) Clathrin-coated pits uncoated by cytosol also show rebinding of clathrin. Cells were treated with bovine-brain cytosol and 1 mM ATP for 5 minutes and then fixed and immunostained. Permeabilized GFP-clathrin cells shown before (a-c) and after (d-f) cytosol treatment. Fluorescence intensity of the GFP signals was increased to clearly distinguish the puncta (inset in d). The same area is shown in the immunostained cells (inset in e) with no manipulation of fluorescence intensity. The merged image (inset in f) shows that clathrin rebinds to the same pits. Bar, 10 μm.

course of uncoating by measuring puncta no larger than 0.3 μm (Fig. 4A). At this resolution, we should be only looking at individual pits or two pits adjacent to one another. Fig. 4B shows a gradual decrease in fluorescence intensity of such puncta. This was substantiated when we measured the time course of disappearance of 200 clathrin puncta from 5 cells. More than 80% of the pits took longer than 1 minute to disappear, whereas less than 5% of the pits disappeared within 30 seconds in the presence of Hsc70, auxilin and ATP (Fig. 4C). Thus, Hsc70 and auxilin are slowly dissembling the clathrin lattice, rather than causing catastrophic collapse of the pits. These findings are consistent with our previous observations that, individual triskelions are able to be uncoated from clathrin baskets when a chimera of AP180 and auxilin is used (Ma et al., 2002).

The rate of the uncoating of individual coated pits is similar to the rate observed for the uncoating of CVs in vitro at comparable concentrations of Hsc70 and auxilin (Greene and Eisenberg, 1990). However, we determined that the time course of uncoating depends, as expected, on both the concentration of auxilin and Hsc70 (Fig. 5). At low concentrations of Hsc70 and auxilin, the rate of uncoating is so slow that over the 5-minute interval, there was only a small decrease in the fluorescence intensity of clathrin. The rate further increased when we raised the concentration of Hsc70 to 10 μM, whereas
the maximum rate of uncoating occurred at 0.2 µM auxilin. The uncoating-rate obtained at 10 µM Hsc70 is similar to the rate we previously measured for clathrin-exchange in vivo (Wu et al., 2001). This suggests that there are high concentrations of Hsc70 in vivo, at least locally, which agrees with the possibility that Hsc70 might be directly associated with the plasma membrane. In addition, other events, such as phosphorylation of AP2 (Smythe and Ayscough, 2003; Conner and Schmid, 2002) that regulates the affinity of clathrin for the membrane, might also contribute to the high rate of exchange in vivo.

Having established that Hsc70, auxilin and ATP remove clathrin from clathrin-coated pits in permeabilized cells, we examined whether dissociation of clathrin is accompanied by the dissociation of AP2 and epsin. Immunostaining of these proteins shows no apparent loss of either AP2 or epsin (Fig. 6c-f) when the clathrin had dissociated (Fig. 6a,b). As expected, transferrin also remained on the pits after clathrin uncoating (Fig. 6g,h).

If the uncoating activity of Hsc70 and auxilin is physiologically relevant, brain cytosol should have the same ability to carry out clathrin uncoating as Hsc70 and auxilin. Therefore, we tested whether brain cytosol can uncoat GFP-clathrin from the membranes of permeabilized cells in the presence of either ATP alone or ATP and GTP. Real-time imaging (Fig. 7A, panel a-c) showed that, in the presence of ATP, cytosol uncoats GFP-clathrin from clathrin-coated pits, just like it uncoats CVs (King et al., 1997). Regardless of whether GTP was present or not, the cytosol (which was diluted about 5-fold during preparation) uncoated the pits at about the same rate as observed with 2 µM Hsc70 and 0.2 µM auxilin (compare Fig. 7B and Fig. 3B). Treatment with cytosol did not lead to dissociation of the transferrin (Fig. 7A, panels d-f), which would occur if coated vesicles were budding from the membrane. Rather, the transferrin on the pits was still accessible to acid-wash or a chase by non-fluorescent transferrin after treatment with cytosol. The clathrin-coated pits from this preparation were expected not to bud because permeabilization was carried out by digitonin, which removes cholesterol from the plasma membrane (Boesze-Battaglia et al., 1990; deDiego et al., 2002; Hogenboom et al., 2004). This depletion of cholesterol has been shown to inhibit clathrin-mediated endocytosis in tissue-culture cells by preventing the budding of the clathrin-coated pits (Rodal et al., 1999; Subtil et al., 1999).

To be certain that the uncoating activity of cytosol is due to Hsc70, we added two different J-domain proteins, either recombinant yeast YDJ1 or human HDJ1, that have been previously shown to inhibit the uncoating of CVs by Hsc70 in vitro (King et al., 1997). Clathrin uncoating was almost completely inhibited when the permeabilized cells and cytosol were pre-treated with the J-domain protein, YDJ1 (Fig. 7B). Similarly to the results of King et al. (King et al., 1997), treatment with HDJ1 gave similar results (data not shown). In addition, a reduction in uncoating was also obtained when we added the ATPase-deficient mutant of Hsp70, Hsp70(K71E) to the cytosol (Fig. 7B). This mutant has been shown to have a dominant-negative effect on Hsc70 uncoating activity (Newmyer and Schmid, 2001; Rajapandi et al., 1998), and we can therefore conclude that Hsc70 in the cytosol was responsible for the clathrin uncoating.

Surprisingly, while the GFP-clathrin was uncoated by cytosol when we immunostained for clathrin, the total clathrin on the puncta remained constant (Fig. 7C). We used the same settings throughout to measure the GFP- and rhodamine-fluorescence. Furthermore, when raising the fluorescence intensity of GFP to look at the residual GFP-clathrin that remained on the pits, the rebinding of clathrin occurred on exactly those pits that had previously been uncoated (Fig. 7C inset). This suggests that the clathrin-exchange on the pits happens while cells are treated with cytosol.

To follow this exchange in greater detail, we examined concomitantly imaged GFP-clathrin and clathrin immunostaining at much higher resolution, to enable imaging of single pits or two adjacent pits. We incubated the cells with cytosol for varying times, using fixation to stop the reaction,
and then immunostained for clathrin (Fig. 8A). The permeabilized cells were analyzed for both GFP-fluorescence intensity and rhodamine-conjugated antibody staining. Then, using the same settings, we imaged the GFP-fluorescence intensity (a measure of the dissociation of the initially bound GFP-clathrin) and rhodamine-conjugated antibody staining (a measure of the total amount of bound clathrin). The pits slowly lost the GFP-fluorescence intensity as the clathrin dissociated over a 5-minute period, whereas the total amount of clathrin remained unchanged. Fig. 8B shows the distribution of disappearance time of the GFP-clathrin measured for the uncoating of clathrin-coated pits by cytosol. Data were obtained from 200 puncta (five cells). More than 80% of the pits needed longer than 1 minute to disappear, whereas less than 5% of the pits disappeared within 30 seconds. As we observed with Hsc70 and auxilin (Fig. 4C), the majority of these puncta do not disappear by a catastrophic dissolution. The exchange of clathrin on clathrin-coated pits that we previously obtained in vivo (Wu et al., 2001) has now been reconstituted in this in-vitro system.

We next tested whether cytosol was able to dissociate AP2 and epsin. Real-time imaging of cells expressing GFP-AP2 and GFP-epsin showed that cytosol dissociated these proteins (Fig. 9A). Their rate of dissociation is roughly the same as that observed for clathrin but, unlike the dissociation of clathrin, that of AP2 and epsin was only slightly affected by the addition of YDJ1 to the cytosol (compare Fig. 7B and Fig. 9B). Therefore, the mechanism of dissociation of AP2 and epsin from the pits is very different from that of clathrin.

Because cytosol treatment of the pits caused clathrin to exchange, we next examined whether AP2 and epsin also exchange. We previously showed that AP2 exchanges on clathrin-coated pits and similarly, we now find that by photobleaching epsin puncta, epsin also exchanges in vivo with a half-life of exchange of about 15 seconds (supplementary material, Fig. S1). Our permeabilized preparation shows that, on the coated pit in the presence of cytosol, exchange of AP2 and epsin occurred in addition to clathrin (Fig. 9C). We employed the same technique as used for clathrin of using constant GFP-fluorescence intensity as a measure of the initial protein and using antibody staining as a measure of total protein.

We next checked that clathrin and AP2 rebound to the same clathrin-coated pits. Cells expressing GFP-clathrin were uncoated by cytosol and then immunostained for both clathrin and AP2. As shown in Fig. 10, clathrin and AP2 in the cytosol replaced the bound clathrin and AP2 on the same pits. However, no significant formation of new pits was observed, possibly for the same reason why no endocytosis occurred. Therefore, just as it has been observed in vivo (Wu et al., 2001; Wu et al., 2003), both clathrin and AP2 exchange on the same clathrin-coated pits in permeabilized cells treated with cytosol.

Discussion
In this article, we describe a permeabilization method that preserves the structure of the clathrin-coated pits on the plasma membrane; we utilized this method to further investigate the mechanism of clathrin-exchange on clathrin-coated pits. By using cells that stably express GFP-clathrin, we were able to monitor the dissociation of clathrin from clathrin-coated pits
following permeabilization. Digitonin was used to permeabilize the cells because it resulted in a more reproducible permeabilization than saponin. We also tried to rip off the plasma membrane, but this technique had the problem of permeabilizing only a limited number of cells. Also, in those cells that were permeabilized, diffusion of cytosol or Hsc70 and auxilin to the basal plasma membrane was extremely slow. Having developed a method for preserving the clathrin-coated pits, we were able to establish that Hsc70, auxilin and ATP together not only uncoat CVs but also uncoat clathrin-coated pits. Our results also show that a dramatic reduction in the rate of uncoating is caused by inhibiting Hsc70 activity in the cytosol, showing clearly that, Hsc70 and auxilin are responsible for the dissociation of clathrin by cytosol. In summary, our results are consistent with the view that Hsc70 and auxilin are the major players in the clathrin-exchange that occurs in vivo.

Whereas Hsc70 and auxilin can dissociate clathrin from the clathrin-coated pits in an ATP-dependent reaction, they cannot dissociate AP2. Even after most of the clathrin was uncoated from the pits, both AP2 and transferrin remained associated with the same pits they were associated with before the clathrin dissociated. Similarly, neither AP1 nor GGA dissociated with the clathrin from the TGN. These results are consistent with the observation of Hannan et al., who suggested that, Hsc70 is in some way necessary for dissociation of AP2 from CVs, but it is not sufficient (Hannan et al., 1998). A cofactor in cytosol, which has recently been identified by Ghosh et al. to be a phosphatase, has been shown to be required for dissociation of AP1 from CVs (Ghosh et al., 2003). Consistent with their observations, we found that cytosol dissociated both clathrin and AP2 from the clathrin-coated pits in the permeabilized cells. Similarly, cytosol dissociated AP1 and GGA from the TGN.

Interestingly, whereas cytosol dissociated both AP2 and clathrin from the clathrin-coated pits, it did not simultaneously dissociate transferrin from the pits. Because dissociation of AP2 on the membrane by high salt caused the release of transferrin, these data indicated that clathrin and AP2 from the cytosol might be replacing the clathrin and AP2 on the pits, while being dissociated by the cytosol. This was confirmed by antibody staining which determined the total clathrin and AP2 bound to the pits. Because in the presence of ATP, the cytosol removed GFP-clathrin and GFP-AP2, they were indeed replaced by the clathrin and AP2 present in the cytosol and this replacement occurred on the same pits that had just been uncoated. This result is particularly interesting in regard to clathrin because this was the first time we were able to observe the rebinding of clathrin to membranes in vitro. The rebinding of clathrin to uncoated CVs was not previously observed, whether or not Hsc70, or nucleotide was present with the free clathrin. In fact, even when cytosol was added to the uncoated vesicles in the presence of ATP, there was no significant rebinding of clathrin in vitro (our unpublished observations). Therefore, the permeabilized cell preparation allowed us to observe not just dissociation of clathrin by Hsc70, but also rebinding of clathrin and AP2, just as it occurs in vivo during clathrin-mediated endocytosis. Moreover, actual exchange is occurring: individual cytosolic proteins are rebinding when the individual clathrin and AP2 proteins dissociate from the pits. Therefore, this in-vitro system shows the exchange of clathrin and AP2 we previously observed in vivo.

The ability of clathrin to exchange might enable the clathrin lattice to readjust its curvature to form a coated vesicle. Recently, Ehrlich et al. suggested that clathrin-coated pits initially form on the membrane as nascent pits that can dissolve before they reach maturity (Ehrlich et al., 2004). Only after binding their cargo are they committed to maturing and developing into CVs. Because the size of the cargo determines the size of the mature vesicle, the nascent pit must adjust its curvature to fit the cargo that binds to it. For example, it must adjust its size to accommodate both low density lipoprotein, which has a diameter of about 27 nm, and reovirus with a 3-fold larger diameter (Ehrlich et al., 2004). This change in size could occur solely through distortion. However, rearrangement of the distorted clathrin-lattice through clathrin-exchange might provide a more energetically favorable way to change the curvature, compared with maintaining a distorted region of the pit after cargo has bound. Interestingly, a recent electron-cryomicroscopy study of the auxilin-clathrin basket (Fotin et al., 2004) showed that, the binding of auxilin caused a global distortion of the clathrin basket. Distortion of the clathrin basket has also been observed in electron cryomicroscopy reconstruction of clathrin baskets that polymerized with a chimera of AP180 and auxilin upon the binding of Hsc70 at pH 6.0 (Heymann et al., 2005). Although these structural studies certainly show that the basket can undergo global distortion, they do not show whether this distorted state is just a transient step in the uncoating process.

We demonstrated clathrin and AP2 exchange on clathrin-coated pits when cytosol and nucleotide were added to permeabilized cells; however, we did not observe a significant number of pits budding off from the membrane or detected the formation of new pits. A different method of permeabilization might preserve the membrane better than digitonin; and perhaps with a different method, the actual budding-off of clathrin-coated pits from the plasma membrane and also reformation of new pits could be observed. Nevertheless, because our present assay reconstitutes clathrin- and AP2-exchange in vitro, we should be able to identify the cytosolic
factors that are required for both uncoating and rebinding of AP2 to the plasma membrane, and also the factors that facilitate the rebinding of clathrin to the uncoated pits.

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