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Clathrin Hub Expression Affects Early Endosome Distribution with Minimal Impact on Receptor Sorting and Recycling

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Clathrin-coated vesicles execute receptor-mediated endocytosis at the plasma membrane. However, a role for clathrin in later endocytic trafficking processes, such as receptor sorting and recycling or maintaining the organization of the endocytic pathway, has not been thoroughly characterized. The existence of clathrin-coated buds on endosomes suggests that clathrin might mediate later endocytic trafficking events. To investigate the function of clathrin-coated buds on endosomal membranes, endosome function and distribution were analyzed in a HeLa cell line that expresses the dominant-negative clathrin inhibitor Hub in an inducible manner. As expected, Hub expression reduced receptor-mediated endocytosis at the plasma membrane. Hub expression also induced a perinuclear aggregation of early endosome antigen 1-positive early endosomes, such that sorting and recycling endosomes were found tightly concentrated in the perinuclear region. Despite the dramatic redistribution of endosomes, Hub expression did not affect the overall kinetics of receptor sorting or recycling. These data show that clathrin function is necessary to maintain proper cellular distribution of early endosomes but does not play a prominent role in sorting and recycling events. Thus, clathrin's role on endosomal membranes is to influence organelle localization and is distinct from its role in trafficking pathways at the plasma membrane and trans-Golgi network.

INTRODUCTION

Clathrin-coated vesicles (CCV) mediate the selective transport of integral membrane proteins between some cellular membranes (Kirchhausen, 2000). At the plasma membrane, CCV facilitate receptor-mediated endocytosis (RME), whereby cell surface receptors and their ligands are internalized and delivered to the endocytic pathway (Schwartz, 1995). After RME, the fates of internalized receptors and ligands vary (reviewed by Mukherjee *et al.*, 1997). Some progress to the late endocytic pathway where they are degraded in lysosomes (Carpenter and Cohen, 1976; Fox and Das, 1979). Others, such as transferrin (Tf) and low density lipoprotein (LDL) receptors, are sorted to a recycling compartment (Dunn *et al.*, 1989; Mayor *et al.*, 1993), from which they return to the plasma membrane (Anderson *et al.*, 1982; Hopkins and Trowbridge, 1983). Although it is known that these receptors are endocytosed via CCV (Anderson *et al.*, 1977; Willingham *et al.*, 1979; Bleil and Bretscher, 1982), the degree to which clathrin mediates subsequent sorting and recycling of the proteins has been a subject of debate.

The early endocytic pathway is composed of both sorting and recycling endosomes (Ghosh *et al.*, 1994). Sorting endosomes separate endocytosed material that is to be recycled from material destined for the lysosome. Recycling components proceed to recycling endosomes from which they return to the plasma membrane. Clathrin-coated buds have been observed on early endosomes (Stoorvogel *et al.*, 1996), making it a logical hypothesis that clathrin might mediate receptor sorting in the recycling pathway. A compelling finding in support of a clathrin-dependent sorting/recycling

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Abbreviations used: C_6-NBD-SM, 6-{(*N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-hexanoyl}sphingosyl phosphocholine; CCV, clathrin-coated vesicles; CHO, Chinese hamster ovary; DiI, 3,3′-dioctadecylindocarbocyanine; EEA1, early endosome antigen 1; LDL, low-density lipoprotein; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; RME, receptor-mediated endocytosis; Tf, transferrin; TfR, transferrin receptor.

pathway is that endosomal clathrin-coated buds are enriched for Tf and Tf receptor (TfR; Killisch *et al.*, 1992; Whitney *et al.*, 1995; Stoorvogel *et al.*, 1996). However, not all studies have supported the hypothesis that clathrin is involved in these trafficking events. Under conditions that inhibit clathrin-mediated endocytosis of TfR, recycling occurs normally (Jing *et al.*, 1990; McGraw and Maxfield, 1990; Damke *et al.*, 1994). Kinetic studies comparing the trafficking of TfR to the flow of fluorescent lipid analogues concluded that recycling occurs as part of a bulk flow process (Mayor *et al.*, 1993). These results leave open the question as to what function endosomal clathrin-coated buds serve if they do not participate in receptor sorting or recycling. A finding that might provide a clue to the function of clathrin-coated buds on endosomes is that in polarized Madin-Darby canine kidney (MDCK) cells endosomal clathrin-coated buds were found to mediate polarized recycling of TfR to the basolateral membrane (Odorizzi *et al.*, 1996; Futter *et al.*, 1998). Disruption of these coated buds did not affect the kinetics of recycling but abolished polarized targeting to the basolateral surface, suggesting a morphological role for clathrin in maintaining the directionality of recycling.

We wanted to further investigate the role of clathrin-coated buds on endosomes by studying endosomal function and distribution in cells expressing the dominant-negative clathrin inhibitor Hub. Hub comprises the C-terminal third of the clathrin heavy chain (Liu *et al.*, 1995) and has been shown to act as a dominant-negative clathrin inhibitor by competing for light chain binding (Liu *et al.*, 1998). Previously, Hub has been used to demonstrate clathrin's involvement in sorting at the trans-Golgi network (Liu *et al.*, 1998), in endocytosis of HIV Nef-CD8 chimeras and protease-activated receptor-1 (Lu *et al.*, 1998; Trejo *et al.*, 2000), and in ARF-6–mediated apical internalization in MDCK cells (Altschuler *et al.*, 1999). In the present study, HeLa-T7Hub cells, in which Hub expression is inducible, were used to study the effect of clathrin inhibition on endosomal trafficking processes. A novel effect of clathrin inhibition, the collapse of early endosome antigen 1 (EEA1)-positive early endosomes into the perinuclear region, was observed and was reproduced in Chinese hamster ovary (CHO) cells that were transiently transfected with Hub. In Hub-expressing HeLa cells both sorting and recycling endosomes were found in a tight distribution near the nucleus. Despite this dramatic redistribution of endosomes, we did not observe a significant effect of clathrin inhibition on the overall kinetics of receptor sorting or recycling in these cells. Whereas RME of Tf and LDL was dramatically reduced by the expression of Hub, sorting of LDL from Tf and recycling of Tf occurred normally. These results identify a novel function of clathrin in maintaining the cellular distribution of early endosomes and show that the effect of clathrin inhibition on the kinetics of receptor sorting and recycling is minimal.

MATERIALS AND METHODS

Cells, Antibodies, and Fluorescent Reagents

HeLa-T7Hub cells are permanently transfected with Hub under control of the tetracycline operator sequence such that Hub expression can be induced by doxycycline treatment (Bennett and Brodsky, unpublished results). HeLa cells transfected with an empty vector (lacking the Hub insert) were created as a control. HeLa-T7Hub and control cells were grown in DMEM containing 10% Tet System-approved fetal bovine serum (Clontech, Palo Alto, CA), 0.2 mg/ml G418 (Life Technologies-BRL, Rockville, MD), and 0.4 mg/ml hygromycin (Roche Molecular Biochemicals, Indianapolis, IN). CHO cells were transiently transfected with pCDM8-T7Hub vector, as previously described (Liu *et al.*, 1998). mAb H68.4 (against human TfR) was purchased from Zymed (South San Francisco, CA) and rabbit anti-EEA1 antiserum was a gift from Harald Stenmark (The Norwegian Radium Hospital, Oslo, Sweden). Rhodamine Red X- and fluorescein isothiocyanate-conjugated donkey anti-mouse and fluorescein isothiocyanate-conjugated donkey anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Human Tf (Sigma, St. Louis, MO) was iron loaded, purified by Sephacryl S-300 (Pharmacia LKB, Uppsala, Sweden) gel-filtration chromatography, and conjugated to Alexa488 according to the manufacturer's instruction (Molecular Probes, Eugene OR). For some experiments Alexa488-Tf was purchased directly from Molecular Probes. 6-{(*N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-hexanoyl}sphingosyl phosphocholine (C_6-NBD-SM) was from Molecular Probes. 3,3′-Dioctadecylindocarbocyanine (DiI)-labeled LDL was a gift from Ira Tabas (Columbia University, New York).

Indirect Immunofluorescence

Cells grown on coverslips and treated with 2 μg/ml doxycycline for 48 h were fixed for 20 min in phosphate-buffered saline (PBS) containing 4% formaldehyde. Cells were permeabilized in 0.04% saponin for 15 min and then blocked in PBS containing 1% cold fish gelatin, 0.1% bovine serum albumin, 0.02% SDS, 0.1% Nonidet P-40, and 0.02% azide for at least 1 h. Cells were incubated with appropriate antibodies in blocking buffer for at least 1 h, followed by incubation with fluorescent-labeled secondary antibodies for at least 1 hour. Cells were washed with PBS containing 0.008% saponin and 10% blocking buffer after each incubation. Coverslips were mounted onto glass slides with Vectashield (Vector Laboratories, Burlingame, CA).

Fluorescent Labeling of Cells

Cells were grown on coverslips affixed beneath holes in the bottom of 35-mm Petri dishes and treated with 2 μg/ml doxycycline for 48 h. To study steady-state Tf distribution, cells were incubated at 37°C in serum-free DMEM containing 20 mM HEPES and 5 μg/ml Alexa488-Tf for 60 min followed by fixation. To study Tf and LDL trafficking, cells were pulse labeled at 37°C in serum-free DMEM/HEPES containing 5 μg/ml Alexa488-Tf and/or 5 μg/ml DiI-LDL for 3–5 min. Cells were washed with ice-cold M2 buffer (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM HEPES, pH 7.4) and incubated in chase medium (DMEM containing 0.1 mg/ml unlabeled Tf and 0.1 mM deferoxamine mesylate [Sigma]) for various lengths of time followed by fixation. For some experiments residual Tf and LDL were stripped from the cell surface before incubation in chase medium. To remove surface Tf and LDL cells were incubated in ice-cold pH 4.6 citrate buffer (25.5 mM citric acid, 24.5 mM sodium citrate) with 280 mM sucrose and 0.01 mM deferoxamine mesylate. Cells were washed once quickly with citrate buffer, followed by two 2-min incubations. Cells were then washed multiple times in ice-cold M2 buffer containing 0.01 mM deferoxamine mesylate (Ghosh *et al.*, 1994). Lipid labeling was accomplished by incubating cells at 37°C in M2 buffer containing 5 μM C_6-NBD-SM and 0.2% glucose for 3 min, followed by washing with ice-cold M2 buffer. Cell surface C_6-NBD-SM was removed with six 10-min washes in ice-cold PBS containing 5% fatty acid-free bovine serum albumin (Sigma; Mayor *et al.*, 1993).

Widefield and Confocal Microscopy

Indirect immunofluorescence samples mounted on glass slides were viewed with an Axiophot fluorescence microscope (Carl Zeiss,

Thronwood, NY). For coverslip-bottomed dishes, widefield fluorescence microscopy was performed on a DMIRB inverted microscope (Leica, Deerfield, IL). Confocal images were collected on an LSM510 laser scanning confocal unit (Zeiss) attached to an Axiovert 100M inverted microscope (Zeiss). Excitation on the LSM510 laser was with a 25-mW argon laser emitting 488 nm and a 1.0-mW helium/neon laser emitting at 543 nm. Emissions were collected with the use of a 505- to 530-nm band pass filter to collect Alexa488 and a 585-nm-long pass filter to collect DiI emission. For confocal images, reduced excitation light was applied for control of photobleaching. Cross-talk of the fluorophores into the wrong detectors was negligible.

^{125}I-Tf Internalization and Recycling

Cells were grown in 12-well plates and treated with 2 μg/ml doxycycline for 48 h. Cells were serum starved in serum-free DMEM containing 20 mM HEPES for 60 min at 37°C. To study a single round of ^{125}I-Tf endocytosis and recycling, cells were incubated on ice in 500 μL of serum-free DMEM/HEPES containing 0.2 μCi/ml ^{125}I-Tf (NEN, Boston, MA) for 1 h, washed with ice-cold medium, and then incubated at 37°C for various lengths of time. At each time point cells were washed twice with 500 μL of ice-cold PBS. The medium and both PBS washes were combined in the "released" fraction. Surface ^{125}I-Tf was removed by acid stripping at room temperature in 50 mM MES, pH 5, 0.15 M NaCl, 280 mM sucrose (Dunn *et al.*, 1989). Cells were incubated in 500 μL of stripping buffer for 1 min, followed by 500 μL of fresh buffer for an additional 3 min. Cells were then washed three times with PBS. Both acid washes and all three PBS washes were combined in the "surface-bound" fraction. Cells were lysed in 1 ml of 1% Triton X-100, 0.1 M NaOH (the "internal" fraction). The amount of radioactivity in each fraction was determined with the use of a CliniGamma gamma counter (Wallac, Gaithersburg, MD). The percentage of radioactivity in each fraction was calculated as the number of counts in that fraction divided by the total number of counts recovered. To study ^{125}I-Tf recycling after continuous uptake, cells were grown and serum starved as described above and then incubated at 37°C in 500 μL of serum-free DMEM/HEPES containing 0.2 μCi/ml ^{125}I-Tf for 30 or 60 min. After acid stripping, 500 μL of DMEM/HEPES containing 0.5 mg/ml unlabeled Tf (Calbiochem, La Jolla, CA) were added to each well, and cells were incubated at 37°C for various lengths of time. At each time point released and internal fractions were collected, and the percentage of radioactivity in each fraction was determined as described above.

RESULTS

Hub Induces Perinuclear Aggregation of Early Endosomes

To determine the role of clathrin-coated buds on endosomes, the effect of Hub expression on the early endosomal compartment was characterized in HeLa-T7Hub cells that were treated with doxycycline. In these cells, drug treatment induces Hub expression from a stably transfected episomal vector encoding Hub under the control of the tetracycline promoter. Hub has an inhibitory effect on RME, substantially reducing the rate and amount of Tf internalized by Hub-expressing cells (Liu *et al.*, 1998; see below). However, there is a low level of residual endocytosis in Hub-expressing cells, which makes it possible to accumulate ligand in these cells over time to study the effect of Hub expression on post-RME–trafficking events. When uptake of Alexa488-Tf was used to label sorting and recycling endosomes in induced HeLa-T7Hub cells, a striking alteration in the distribution of Tf-containing vesicles was observed relative to their localization in control-transfected HeLa cells. After a 60-min incubation, Tf-containing vesicles in control cells were dispersed throughout the cytosol (Figure 1A). However, in HeLa-T7Hub cells, which had been induced to express Hub for 48 h before Tf uptake, intracellular vesicles were concentrated in the perinuclear area, with hardly any vesicles in the periphery (Figure 1B). The perinuclear aggregate did not represent clustered Tf trapped on the cell surface, because an acid strip of the cell surface failed to eliminate it (Bennett and Brodsky, unpublished results) and Tf that was trapped on the cell surface appeared to rim the cells uniformly (Figure 1B). When Hub-expressing cells were stained with an anti-TfR antibody, the staining pattern was identical to the pattern of Alexa488-Tf localization, indicating that the receptor and its ligand were both present in the perinuclear aggregate or trapped on the cell surface (Figure 1D). After a 3-min incubation and a 5-min chase, DiI-LDL was also heavily concentrated in the center of induced HeLa-T7Hub cells (Figure 1F), whereas LDL-containing endosomes in control cells were dispersed throughout the cytosol (Figure 1E). C_6-NBD-sphingomyelin is a fluorescent lipid analogue that labels sorting endosomes and recycling endosomes (Mayor *et al.*, 1993), and the distribution of these lipid-labeled endosomes was also highly condensed in induced HeLa-T7Hub cells (Figure 1, G and H), indicating that the redistributed endosomes are part of the normal endocytic/recycling pathway not specific to the Tf or LDL markers used. Both Tf and LDL arrived in the perinuclear accumulation of endosomes rapidly after endocytosis. As shown below, the LDL and Tf concentrated near the nucleus did sort from one another (Figures 4 and 5). Therefore, we conclude that the perinuclear vesicles in HeLa-T7Hub cells represent both sorting and recycling early endosomes. These data demonstrate that clathrin inhibition can lead to a redistribution of endosomes and endosomal clathrin-coated buds play a role in mediating the intracellular distribution of early endosomes.

Hub Inhibits TfR Endocytosis with Little Impact on Receptor Recycling

In light of the redistribution of endosomes in induced HeLa-T7Hub cells, it was of interest to investigate whether Hub expression has any effect on the sorting and recycling functions of the early endosomal compartment. First, the effect of Hub expression on receptor recycling was analyzed with ^{125}I-Tf to monitor the flow of material through the endocytic/recycling pathway. Tf remains bound to its receptor throughout endocytosis and recycling, making it a useful marker for monitoring the trafficking of TfR (Octave *et al.*, 1981; Bleil and Bretscher, 1982). As expected, a Hub-induced reduction of Tf internalization was observed. Whereas control cells showed a rapid internalization of ^{125}I-Tf (Figure 2B, open triangles) accompanied by a disappearance of ^{125}I-Tf from the cell surface (Figure 2A, open squares) within 5 min, there was an obvious delay in the internalization of ^{125}I-Tf in HeLa-T7Hub cells that had been induced to express Hub for 48 h before ^{125}I-Tf exposure (Figure 2B, closed triangles). This delay was mirrored by a prolonged lifespan of ^{125}I-Tf on the cell surface (Figure 2A, closed squares). By 10 min control cells began to recycle ^{125}I-Tf to the cell medium (Figure 2B, open circles), whereas there was not much de-

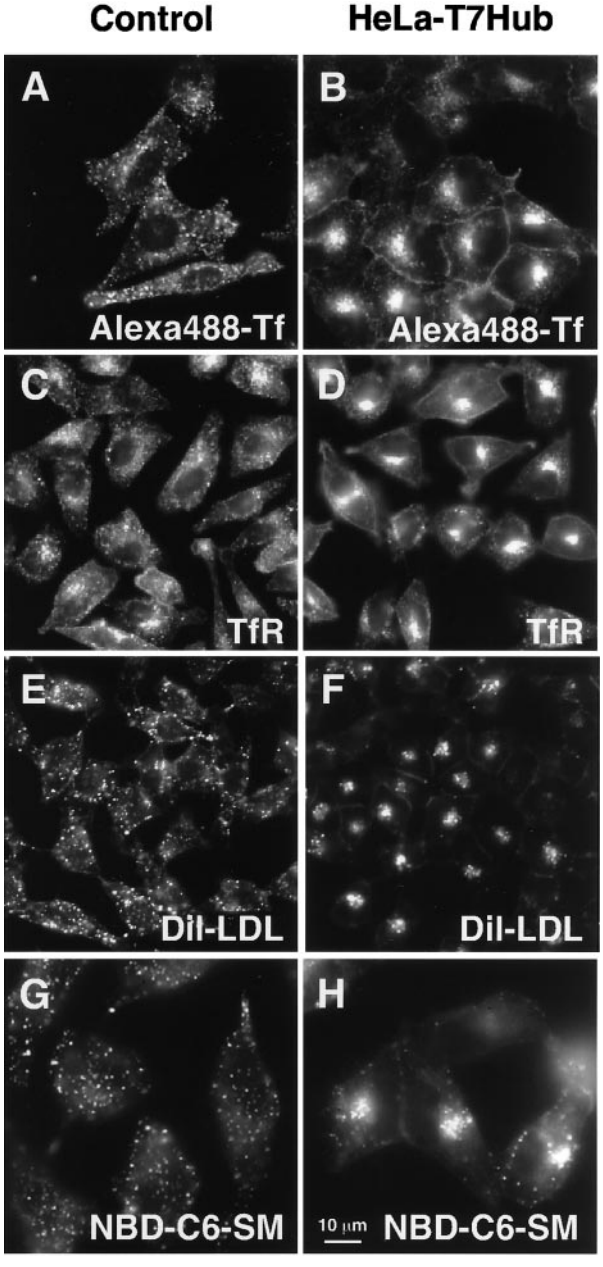

Figure 1. Hub induces perinuclear aggregation of early endosomes. HeLa-T7Hub cells (right) and control cells transfected with an empty vector lacking the Hub insert (left) were grown on coverslips and treated with 2 μg/ml doxycycline for 48 h. (A and B) Cells were incubated at 37°C for 60 min in medium containing Alexa488-Tf and immediately photographed. (C and D) Cells were processed for immunofluorescence and stained with mAb H68.4, which recognizes TfR, followed by Rhodamine Red X-conjugated goat anti-mouse secondary antibody. (E and F) Cells were incubated at 37°C for 3 min in medium containing DiI-LDL, followed by a 5-min chase before photographing the cells. (G and H) Cells were incubated at 37°C for 3 min in M2 buffer containing C_6-NBD-SM, and surface C_6-NBD-SM was back exchanged on ice before photographing the cells.

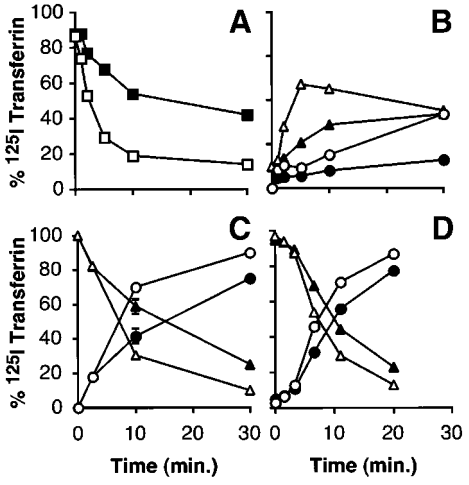

Figure 2. Effect of Hub on endocytosis and recycling of ^{125}I-Tf. (A and B) HeLa-T7Hub cells (closed symbols) and control cells transfected with an empty vector lacking the Hub insert (open symbols) were grown in 12-well dishes and treated with 2 μg/ml doxycycline for 48 h. Cells were serum starved and then incubated on ice in medium containing ^{125}I-Tf. After washing, cells were incubated in chase medium at 37°C for various times. At each time point surface-bound (squares), internal (triangles), and released (circles) fractions were collected as described in MATERIALS AND METHODS, and the percentage of radioactivity in each fraction was determined. A and B show data from the same experiment. All points are the average of three wells. (C and D) HeLa-T7Hub cells (closed symbols) and control cells transfected with an empty vector lacking the Hub insert (open symbols) were grown in 12-well dishes and treated with 2 μg/ml doxycycline for 48 h. Cells were serum starved and then incubated at 37°C in medium containing ^{125}I-Tf for 30 (C) or 60 (D) minutes. Surface ^{125}I-Tf was removed by acid stripping, and cells were reincubated at 37°C in chase medium for various times. At each time point internal and released fractions were collected as described in MATERIALS AND METHODS, and the percentage of radioactivity in each fraction was determined. All points are the average of three wells. Symbols are as for A and B. Error bars are indicated on all graphs.

tectable recycling in induced HeLa-T7Hub cells even after 20 min (Figure 2B, closed circles).

One explanation for the delay in Tf recycling in HeLa-T7Hub cells is that clathrin is directly involved in recycling of TfR and its ligand. However, a second possibility is that the delay in recycling is simply due to the initial delay in Tf internalization. Substantial recycling was not observed in control cells until ~80% of the Tf was endocytosed from the cell surface. This level of Tf internalization was never reached in induced HeLa-T7Hub cells, and therefore the observed delay in recycling might indicate that Tf simply never progressed far enough along its intracellular route to recycle. To distinguish between these two possibilities, cells were incubated with ^{125}I-Tf for 30 min at 37°C to saturate the entire endocytic/recycling pathway before measuring recycling. Figure 2C shows the kinetics of Tf recycling in HeLa-T7Hub and control cells after 30 min of continuous ^{125}I-Tf uptake. A slight delay was observed in ^{125}I-Tf recycling in induced HeLa-T7Hub cells (closed symbols). Although the delay was not extreme, it was consistent and reproducible. Interestingly, however, the delay in recycling became even

less dramatic when cells were allowed to internalize ^{125}I-Tf for 60 min before measuring recycling (Figure 2D). Thus the longer cells were allowed to internalize ^{125}I-Tf, the less of an effect Hub had on Tf recycling. These data indicate that, once the recycling endosome is loaded with Tf, recycling can occur with little interference from Hub, implying that the farther along its intracellular pathway Tf progresses, the less of a role clathrin plays in directing its trafficking. Furthermore, the effect of Hub on ^{125}I-Tf recycling seen in Figure 2, C and D, was not nearly as striking as the effect on Tf endocytosis seen in Figure 2, A and B. These results indicate that, whereas clathrin directly mediates RME at the plasma membrane, its role in receptor recycling is less prominent.

These findings are further supported by immunofluorescence pulse-chase experiments. HeLa-T7Hub and control cells were pulsed with Alexa488-Tf and then chased for various times. Again, a delay in Tf internalization was observed in induced HeLa-T7Hub cells (Figure 3, E vs. A), with Tf rimming the cell surface longer in Hub-expressing cells (Figure 3, G vs. 3 C). However, despite the inhibition of Tf endocytosis, by 30 min of chase the majority of labeled Tf recycled from both control and HeLa-T7Hub cells (Figure 3, D and H). This result is striking because previous work had shown that recycling through the endocytic recycling compartment is, in fact, a slower process than endocytosis (Bleil and Bretscher, 1982; Hopkins and Trowbridge, 1983; Presley *et al.*, 1993). That recycling from HeLa-T7Hub cells can nearly catch up to control cells despite the initial inhibition of Tf internalization further indicates that, although inhibition of clathrin affects the kinetics of early endocytic steps of TfR trafficking, it does not substantially affect later recycling events.

To confirm that trafficking of Tf accurately represents that of TfR, we followed the endocytosis and recycling of TfR directly with the use of a disulfide-linked biotin reagent to label cell surface proteins and then monitored the amount of glutathione-sensitive TfR. The results correlated with those shown in Figure 2. The kinetics of TfR recycling to the cell surface in induced HeLa-T7Hub cells were the same as in control cells (Bennett and Brodsky, unpublished results). Thus, the effect of Hub expression on Tf trafficking accurately represents the effect on trafficking of TfR.

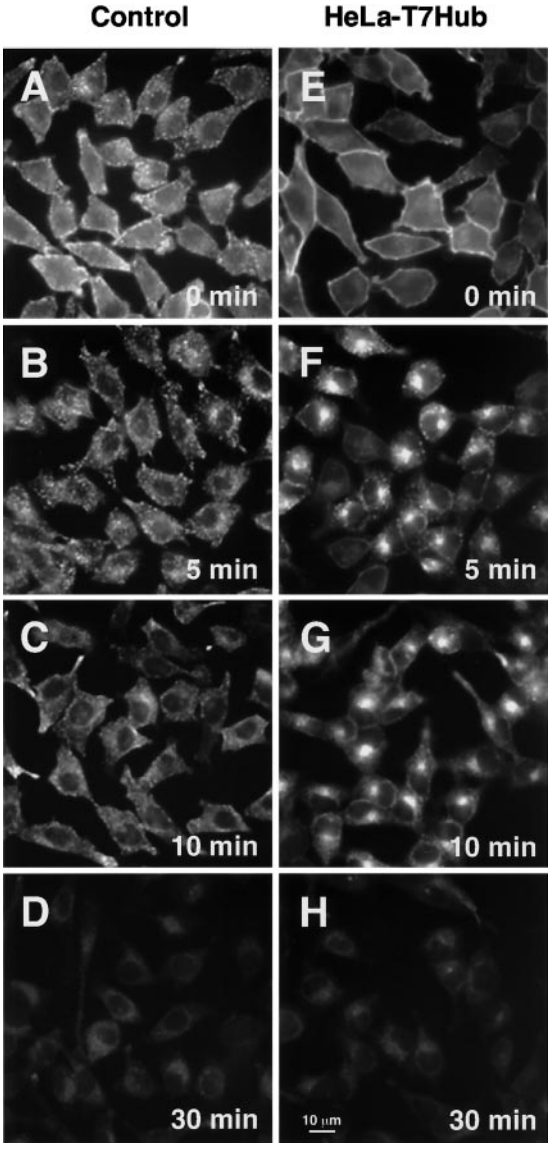

Figure 3. Endocytosed Tf visibly recycles in HeLa-T7Hub cells. HeLa-T7Hub cells (right) and control cells transfected with an empty vector lacking the Hub insert (left) were grown in coverslip-bottom dishes and treated with 2 μg/ml doxycycline for 48 h. Cells were incubated at 37°C for 3 min in medium containing Alexa488-Tf, followed by ice-cold washes. Cells were reincubated in chase medium at 37°C for 0 (A and E), 5 (B and F), 10 (C and G), or 30 (D and H) minutes. At each time point, cells were washed, fixed, and photographed.

Perinuclear Congregation of Early Endosomes Does Not Inhibit Sorting of Tf from LDL

The Hub-induced reorganization of early endosomes was then investigated for its effect on endosomal sorting processes. In contrast to Tf, LDL dissociates from its receptor in the sorting endosome and progresses to the lysosome (Davis *et al.*, 1987). Because Tf and LDL are both internalized via RME to sorting endosomes and then separate from one another, these two ligands can be used simultaneously as markers to monitor receptor endocytosis, sorting, and recycling. Induced HeLa-T7Hub and control cells were given a short pulse with Alexa488-Tf (Figure 4, shown in green) and DiI-LDL (shown in red) and chased for various times to determine whether the two ligands colocalized to the same vesicles (indicated by yellow) or whether they had properly sorted from one another. In control cells some sorting occurred by 5 min of chase (Figure 4B), and by 10 min almost all Tf and LDL were sorted to distinctly separate vesicles (Fig. 4C). As expected, there was an inhibition of internalization of Tf and LDL in induced HeLa-T7Hub cells (Figure 4D), and residual plasma membrane staining was evident throughout the chase (Figure 4, E and F). Tf and LDL that were endocytosed in HeLa-T7Hub cells proceeded rapidly to the perinuclear aggregate (Figure 4E). For this reason we conclude that sorting endosomes are present in the perinuclear accumulation of endosomes. However, despite the altered distribution of Tf- and LDL-containing vesicles in Hub-expressing cells, the ligands sorted from one another with kinetics similar to control cells. By 10 min of chase, induced HeLa-T7Hub cells contained a substantial number of vesicles that stained only for Tf, despite the fact that these vesicles

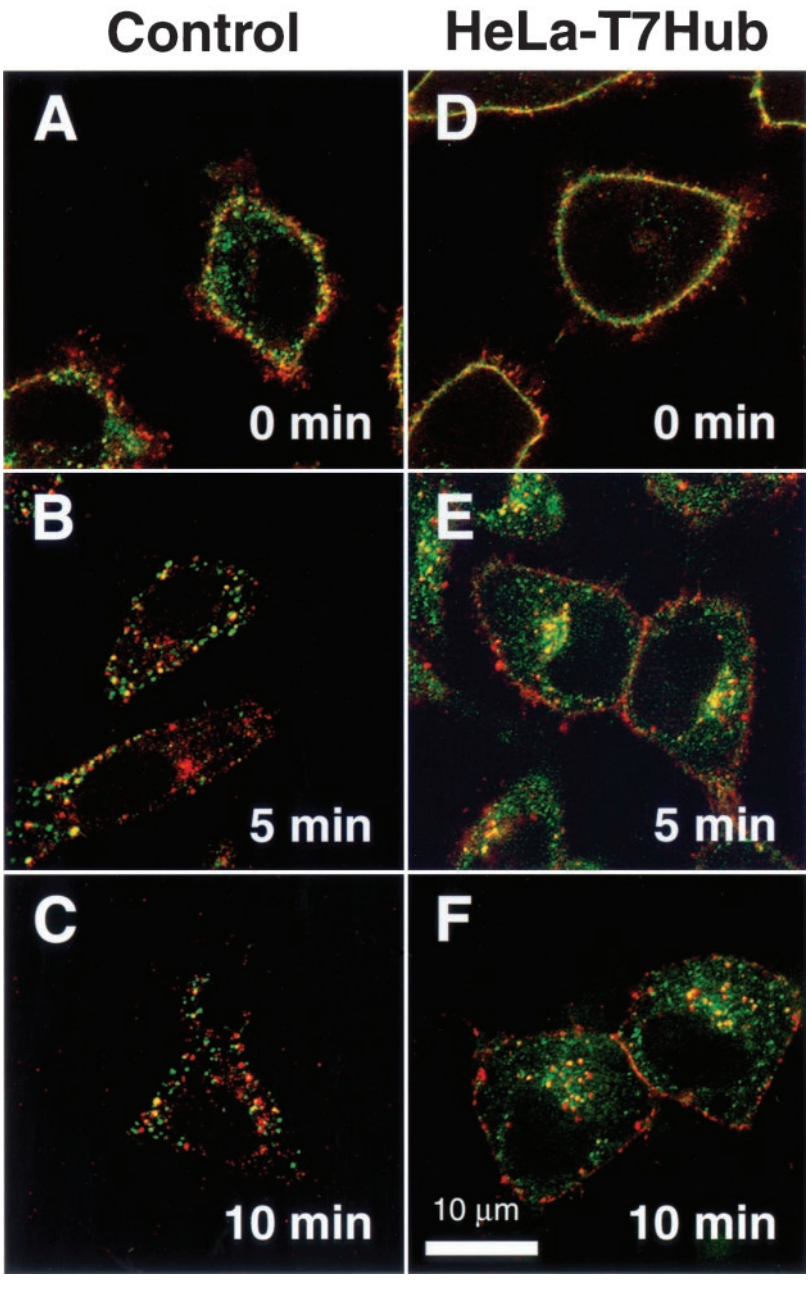

Figure 4. Effect of endosomal aggregation on sorting of Tf and LDL. HeLa-T7Hub cells (right) and control cells transfected with an empty vector lacking the Hub insert (left) were grown in coverslip-bottom dishes and treated with 2 μg/ml doxycycline for 48 h. Cells were incubated at 37°C for 3 min in medium containing Alexa488-Tf (green) and DiI-LDL (red), followed by ice-cold washes. Cells were then incubated in chase medium at 37°C for 0 (A and D), 5 (B and E), or 10 (C and F) minutes. At each time point cells were washed, fixed, and photographed with the use of a confocal microscope. Each panel shows a single plane of focus.

remained clustered in the perinuclear area (Figure 4F). This result indicates that proper sorting of Tf from LDL did occur in the presence of Hub and that recycling vesicles (which stain only for Tf) are also included in the perinuclear aggregate.

The appearance of green (Tf-containing) vesicles by 5–10 min of chase indicates that Tf properly sorts from LDL in induced HeLa-T7Hub cells. Interestingly, despite the appearance of Tf-containing vesicles, there was not a corresponding appearance of vesicles staining only for LDL, as is seen in control cells (Figure 4, F vs. C). The majority of LDL-containing vesicles in induced HeLa-T7Hub cells continued to costain for Tf, at least partially, throughout the 10-min chase. This finding could indicate that LDL is not progressing to late endosomes/lysosomes or that the maturation of these latter compartments is disrupted by the expression of Hub. However, an alternative explanation for the continued existence of yellow-staining vesicles in HeLa-T7Hub cells is that because of inhibition of endocytosis, material from the cell surface continues to arrive in the early endocytic compartment throughout the chase. To test this possibility, cells were pulsed with labeled Tf and LDL, and residual surface label was stripped with an acid wash before incubation in chase medium. Under these conditions, sorting of Tf and LDL occurred identically in induced HeLa-T7Hub cells and control cells (Figure 5). Thus, despite the effect of clathrin inhibition on the distribution of early en-

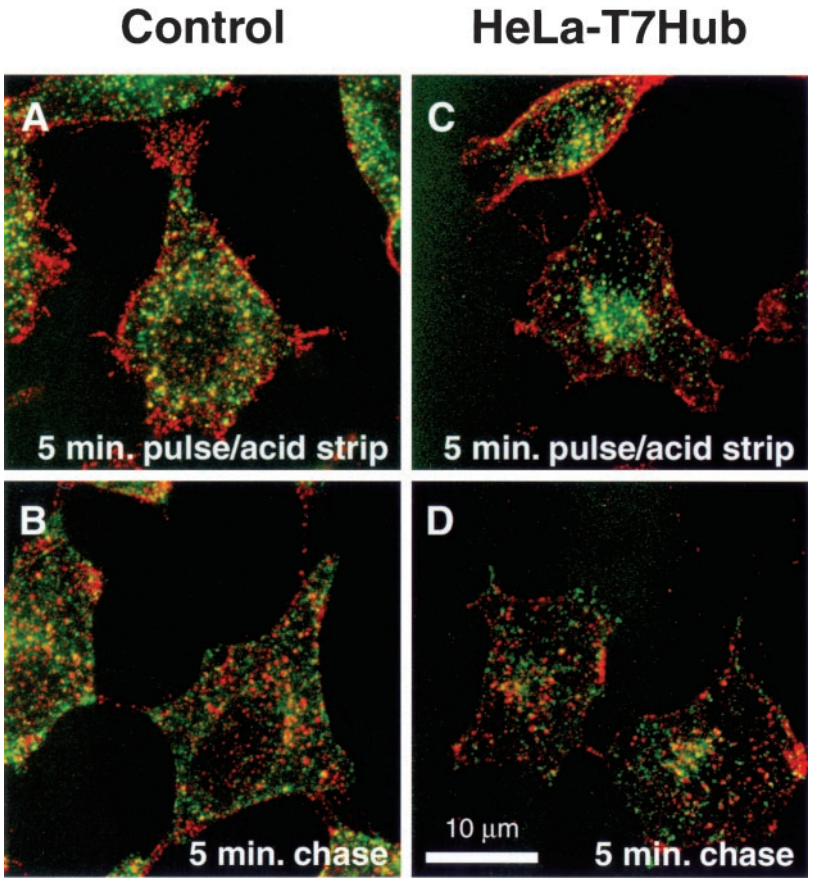

Figure 5. Clathrin inhibition does not inhibit sorting of Tf and LDL. HeLa-T7Hub cells (right) and control cells transfected with an empty vector lacking the Hub insert (left) were grown in coverslip-bottom dishes and treated with 2 μg/ml doxycycline for 48 h. Cells were incubated at 37°C for 5 min in medium containing Alexa488-Tf (green) and DiI-LDL (red), followed by ice-cold washes. Residual Tf and LDL were stripped from the cell surface with pH 4.6 citrate buffer. Cells were then incubated in chase medium at 37°C for 5 min at which time cells were washed, fixed, and photographed with the use of a confocal microscope. A and C show cells after the acid stripping step; B and D show cells after the 5-min chase. Each panel shows a projection of multiple planes of focus.

dosomes, there is not a corresponding effect on early endosomal sorting or recycling functions.

Hub-induced Redistribution of EEA1-positive Endosomes Observed in Two Cell Lines

The observation that receptor recycling and sorting were not substantially affected in induced HeLa-T7Hub cells suggests that the compartments that mediate these functions were still intact in Hub-expressing cells. The EEA1 marker of early endosomes is characteristic of both sorting and recycling endosomes (Mu *et al.*, 1995). To confirm their presence in the perinuclear aggregate of endocytic vesicles in Hub-expressing cells, these cells were labeled with Alexa488-Tf (internalized for 60 min) and an antibody against the EEA1 protein (Figure 6, A and B). EEA1-positive vesicles were seen in the perinuclear aggregate of endosomes in Hub-expressing cells, colocalized with internalized Tf. EEA1-positive vesicles were observed colocalized with internalized Tf in control cells but were more dispersed in the periphery. Note that, in the experiment shown, the expression of Hub in the induced HeLa-T7Hub cells varied from cell to cell and the degree of perinuclear aggregation of endosomes varied correspondingly, but the aggregate was consistently EEA1-positive. In both Hub-expressing cells and control cells, vesicles labeling independently for each marker were also observed. This is likely because, to label the endocytic pathway with Alexa488-Tf, cells were allowed to internalize the Tf continuously over a period of 60 min without washing. Under these conditions some Tf is internalized independent of RME and may therefore progress to late endosomes and lysosomes (EEA1-negative compartments).

To determine whether the effect of Hub on early endosome distribution is specific to HeLa cells or reflects a general role of clathrin in influencing localization in the endocytic pathway, CHO cells were transiently transfected with Hub and the distribution of endosomes was analyzed (Figure 6, C and D). Again, the level of Hub expression varied in the population of transiently transfected CHO cells, but perinuclear aggregation of endosomes was observed in ~50% of Hub-expressing cells. In all CHO cells in which Hub-induced perinuclear aggregation of Tf-containing endosomes was observed, the aggregated endosomes were EEA1 positive.

DISCUSSION

The role of clathrin-coated buds on endosomes was analyzed with the use of the dominant-negative clathrin inhibitor Hub to disrupt clathrin function, and a novel effect of clathrin inhibition on the distribution of early endosomes was observed. The steady-state distribution of EEA1-positive endosomes was much more condensed in HeLa or CHO cells expressing Hub than in control cells. When clathrin was

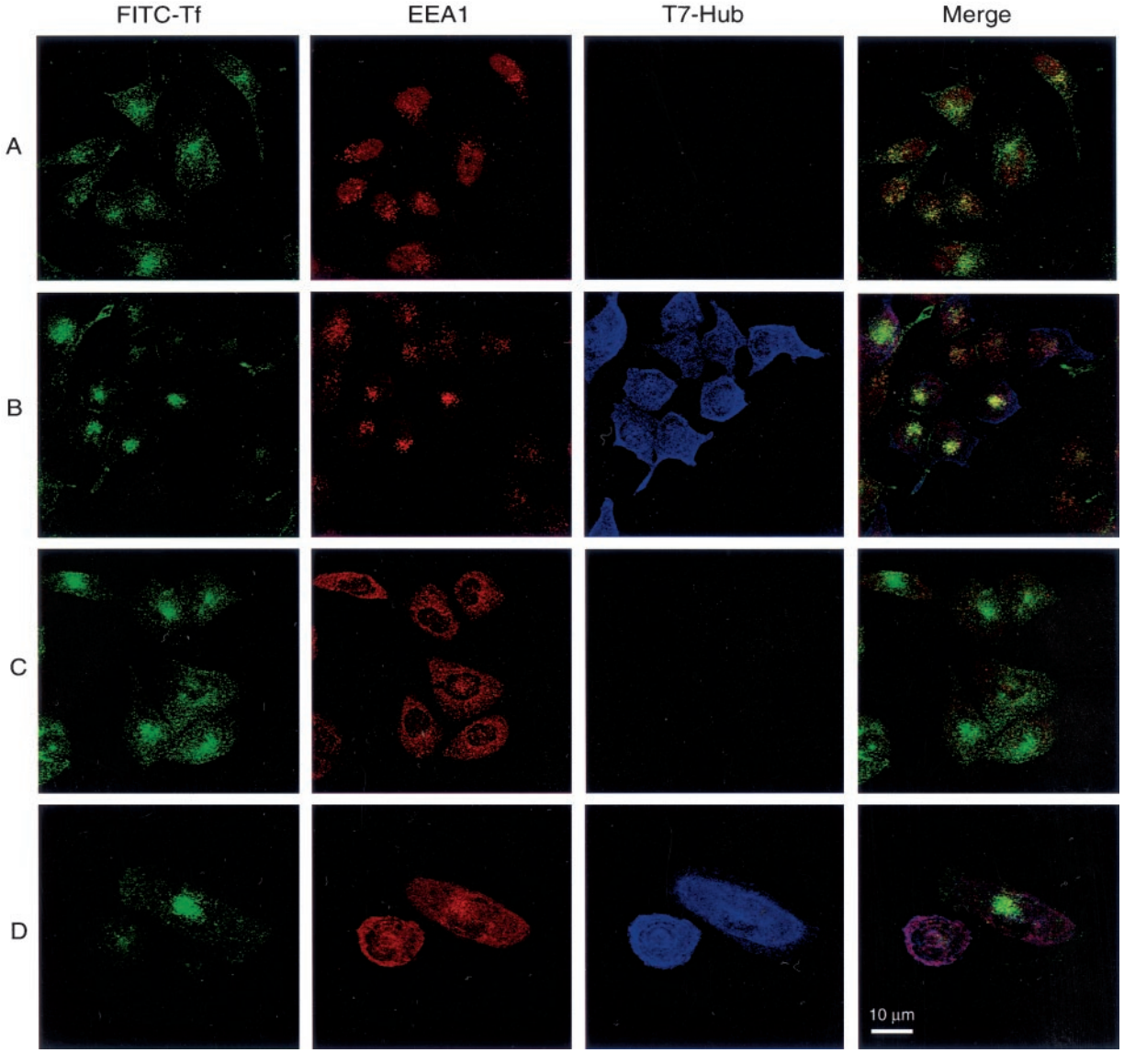

Figure 6. Hub induces perinuclear aggregation of EEA1-positive endosomes in both HeLa and CHO cell lines. HeLa-T7Hub cells (B) and control cells transfected with an empty vector lacking the Hub insert (A) were grown on coverslips and treated with 2 μg/ml doxycycline for 48 h. CHO cells expressing the human TfR were grown on coverslips and mock transfected (C) or transiently transfected with pCDM8-T7Hub, which was allowed to express for 48 h (D). All cells were incubated at 37°C for 60 min in medium containing Alexa488-Tf and processed for immunofluorescence. Cells were stained with a mAb that recognizes the T7-Tag (Novagen, Madison, WI) to identify cells with significant Hub expression. The early endosomal marker (EEA1) was detected with the use of a rabbit polyclonal antibody. Bound antibodies were visualized with the use of secondary antibodies conjugated to Rhodamine Red X (EEA1, red) or Cyanine-5 (T7Hub, blue). Note that, for both the HeLa-T7Hub cells and the CHO cells, the effect of Hub on endosomal localization varied with level of Hub expression, but in cells with dramatic endosome redistribution, EEA1 and internalized Tf were generally colocalized in the perinuclear aggregate. Cells representative of low and high Hub expression are shown.

inhibited by Hub expression, early endosomes were transported rapidly into the perinuclear region. Based on the early appearance of perinuclear vesicles that stain for both Tf and LDL and the later appearance of vesicles in the same region containing only Tf (Figures 4 and 5), we concluded that both sorting and recycling endosomes exist in this perinuclear aggregate. These data suggest that endosomal clathrin-coated buds play a role in maintaining the cellular organization of the early endocytic compartment.

The link between clathrin and early endosome distribution remains unclear, but it may be that clathrin is needed either to remove or retain certain membrane components to achieve proper endosomal attachment to or movement along microtubules. In migrating fibroblasts, TfR preferentially recycles to the leading lamella, suggesting that recycling occurs along the direction of a polarized microtubule cytoskeleton (Hopkins *et al.*, 1994). Early endosomes have been shown to associate with microtubules (Marsh *et al.*,

1995), and an intact microtubule cytoskeleton is needed to maintain the pericentriolar organization of the recycling compartment in CHO cells (McGraw *et al.*, 1993). Additionally, polarized recycling in MDCK cells is dependent on the cytosolic domain of TfR and the presence of clathrin-coated buds on endosomes (Odorizzi *et al.*, 1996; Futter *et al.*, 1998), suggesting that clathrin mediates the directionality of recycling. Another possibility is that clathrin promotes an association of endosomes with the actin cytoskeleton that is necessary for maintaining the initial dispersed distribution of early endosomes. Hub expression interferes with the interaction between clathrin-coated pits and actin at the plasma membrane (Bennett and Brodsky, unpublished results), and inhibition of this interaction may facilitate movement along microtubules, resulting in accumulation of early endosomes in the perinuclear area as shown here. The work presented here and the prior studies just cited suggest that the morphology of the early endosomal compartment is organized, at least in part, by the microtubule and actin cytoskeleton. Clathrin could mediate the relationship between both the microtubule and the actin cytoskeleton and early endosomes either through links between clathrin and cytoskeletal components or through CCV controlling the transport of proteins that interact with cytoskeletal elements.

Although we did not find evidence of a direct role for clathrin in the trafficking events of protein sorting or recycling, the suggestion that clathrin might mediate receptor sorting and recycling is not ill-founded. The existence of clathrin-coated buds on endosomes enriched for Tf and TfR (Killisch *et al.*, 1992; Whitney *et al.*, 1995; Stoorvogel *et al.*, 1996) and the requirement for clathrin in proper basolateral targeting of recycling TfR in polarized MDCK cells (Futter *et al.*, 1998) are strongly suggestive of a role for clathrin in the postendocytic trafficking of TfR. However, in accordance with prior kinetics studies (Jing *et al.*, 1990; McGraw and Maxfield, 1990; Mayor *et al.*, 1993; Damke *et al.*, 1994) we were unable to detect a significant effect of clathrin inhibition on the sorting or recycling of Tf. We conclude that, although clathrin plays a direct role in the trafficking of proteins at the plasma membrane and trans-Golgi network, it plays a different role in receptor sorting and recycling. Clathrin seems to be required for the localization of compartments that mediate these processes but not for their sorting or recycling functions. The actual mechanics of protein sorting and recycling are most likely controlled by physical sorting mechanisms such as iterative membrane budding (Dunn *et al.*, 1989; reviewed by Mukherjee *et al.*, 1997).

That Hub can so drastically alter the distribution of early endosomes without greatly affecting the kinetics of receptor sorting and recycling is surprising but not without precedent. The distribution of recycling endosomes varies among cell types (Hopkins *et al.*, 1990; Tooze and Hollinshead, 1991; McGraw *et al.*, 1993; Apodaca *et al.*, 1994; Ghosh *et al.*, 1994; Marsh *et al.*, 1995; Daro *et al.*, 1996), indicating that the overall organization of the recycling compartment is not crucial to its function. This fact is further illustrated by the observation that reorganization of the recycling compartment does not result in altered receptor recycling kinetics (McGraw *et al.*, 1993; Johnson *et al.*, 1996; Futter *et al.*, 1998). Thus, under conditions that disrupt the distribution of early endosomes, trafficking through this compartment occurs with normal kinetics. These results raise the issue as to why there is any organization of the early endosomal compartment if sorting and recycling can occur normally when the compartment is disrupted. It is likely that, in the context of an organized tissue, there is a much greater need for polarized sorting and recycling functions than in the tissue culture cells used in these experiments. Therefore, clathrin's role in maintaining endosomal organization is probably of greater importance in complex tissue.

The results presented here, in combination with prior work, point to a role for clathrin on endosomes that is characteristically distinct from its role at the plasma membrane and is quite different from the protein trafficking roles of clathrin that are so well studied. The role for clathrin-coated buds on endosomes appears to be to maintain organelle localization rather than directly transporting or sorting receptors as CCV do in RME and at the trans-Golgi network.

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REFERENCES

Altschuler, Y., Liu, S.-H., Katz, L., Tang, K., Hardy, S., Brodsky, F.M., Apodaca, G., and Mostov, K.E. (1999). ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. J. Cell Biol. *147*, 7–12.

Anderson, R.G.W., Brown, M.S., Beisiegel, U., and Goldstein, J.L. (1982). Surface distribution and recycling of the low density lipoprotein receptor as visualized with antireceptor antibodies. J. Cell Biol. *93*, 523–531.

Anderson, R.G.W., Brown, M.S., and Goldstein, J.L. (1977). Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. Cell *10*, 351–364.

Apodaca, G., Katz, L.A., and Mostov, K.E. (1994). Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. J. Cell Biol. *125*, 67–86.

Bleil, J.D., and Bretscher, M.S. (1982). Transferrin receptor and its recycling in HeLa cells. EMBO J. *1*, 351–355.

Carpenter, G., and Cohen, S. (1976). ^{125}I-labeled human epidermal growth factor. J. Cell Biol. *71*, 159–171.

Damke, H., Baba, T., Warnock, D.E., and Schmid, S.L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. J. Cell Biol. *127*, 915–934.

Daro, E., Van Der Sluijs, P., Galli, T., and Mellman, I. (1996). Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. Proc. Natl. Acad. Sci. USA. *93*, 9559–9564.

Davis, C.G., Foldstein, J.L., Südhof, T.C., Anderson, R.G.W., Russell, D.W., and Brown, M.S. (1987). Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. Nature *326*, 760–765.

Dunn, K.W., McGraw, T.E., and Maxfield, F.R. (1989). Iterative fractionation of recycling receptors from lysosomally destined ligands in an early sorting endosome. J. Cell Biol. *109*, 3303–3314.

Fox, F.C., and Das, M. (1979). Internalization and processing of the EGF receptor in the induction of DNA synthesis in cultured fibroblasts: the endocytic activation hypothesis. J. Supramol. Struct. *10*, 199–214.

Futter, C.E., Gibson, A., Allchin, E.H., Maxwell, S., Ruddock, L.J., Odorizzi, G., Domingo, D., Trowbridge, I.S., and Hopkins, C.R. (1998). In polarized MDCK cells basolateral vesicles arise from clathrin-γ-adaptin–coated domains on endosomal tubules. J. Cell Biol. *141*, 611–623.

Ghosh, R.N., Gelman, D.L., and Maxfield, F.R. (1994). Quantification of low density lipoprotein and transferrin endocytic sorting in HEp2 cells using confocal microscopy. J. Cell Sci. *107*, 2177–2189.

Hopkins, C.R., Gibson, A., Shipman, M., and Miller, K. (1990). Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. Nature *346*, 335–338.

Hopkins, C.R., Gibson, A., Shipman, M., Strickland, D.K., and Trowbridge, I.S. (1994). In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. J. Cell Biol. *125*, 1265–1274.

Hopkins, C.R., and Trowbridge, I.S. (1983). Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. J. Cell Biol. *97*, 508–521.

Jing, S., Spencer, T., Miller, K., Hopkins, C., and Trowbridge, I.S. (1990). Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. J. Cell Biol. *110*, 283–294.

Johnson, A.O., Ghosh, R.N., Dunn, K.W., Garippa, R., Park, J., Mayor, S., Maxfield, F.R., and McGraw, T.E. (1996). Transferrin receptor containing the SDYQRL motif of TGN38 causes a reorganization of the recycling compartment but is not targeted to the TGN. J. Cell Biol. *135*, 1749–1762.

Killisch, I., Steinlein, P., Römisch, K., Hollinshead, R., Beug, H., and Griffiths, G. (1992). Characterization of early and late endocytic compartments of the transferrin cycle. J. Cell Sci. *103*, 211–232.

Kirchhausen, T. (2000). Clathrin. Annu. Rev. Biochem. *69*, 699–727.

Liu, S.-H., Marks, M.S., and Brodsky, F.M. (1998). A dominant-negative clathrin mutant differentially affects trafficking of molecules with distinct sorting motifs in the class II major histocompatibility complex (MHC) pathway. J. Cell Biol. *140*, 1023–1037.

Liu, S.-H., Wong, M.L., Craik, C.S., and Brodsky, F.M. (1995). Regulation of clathrin assembly and trimerization defined using recombinant triskelion hubs. Cell *83*, 257–267.

Lu, X., Yu, H., Liu, S.-H., Brodsky, F.M., and Peterlin, B.M. (1998). Interactions between HIV1 Nef and vacuolar ATPase facilitate the internalization of CD4. Immunity *8*, 647–656.

Marsh, E.W., Leopold, P.L., Jones, N.L., and Maxfield, F.R. (1995). Oligomerized transferrin receptors are selectively retained by a lumenal sorting signal in a long-lived endocytic recycling compartment. J. Cell Biol. *129*, 1509–1522.

Mayor, S., Presley, J.F., and Maxfield, F.R. (1993). Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. J. Cell Biol. *121*, 1257–1269.

McGraw, T.E., Dunn, K.W., and Maxfield, F.R. (1993). Isolation of a temperature-sensitive variant Chinese hamster ovary cell line with a morphologically altered endocytic recycling compartment. J. Cell. Physiol. *155*, 579–594.

McGraw, T.E., and Maxfield, F.R. (1990). Human transferrin receptor internalization is partially dependent upon an aromatic amino acid in the cytoplasmic domain. Cell Regul. *1*, 369–377.

Mu, F.-T., Callaghan, J.M., Steele-Mortimer, O., Stenmark, H., Parton, R.G., Campbell, P.L., McCluskey, J., Yeo, J.-P., Tock, E.P.C., and Toh, B.-H. (1995). EEA1, an early endosome-associated protein. J. Biol. Chem. *20*, 13503–13511.

Mukherjee, S., Ghosh, R.N., and Maxfield, F.R. (1997). Endocytosis. Physiol. Rev. *77*, 759–803.

Octave, J.-N., Schneider, Y.-J., Crichton, R.R., and Trouet, A. (1981). Transferrin uptake by cultured rat embryos: the influence of temperature and incubation time, subcellular distribution and short-term kinetic studies. Eur. J. Biochem. *115*, 611–618.

Odorizzi, G., Pearse, A., Domingo, D., Trowbridge, I.S., and Hopkins, C.R. (1996). Apical and basolateral endosomes of MDCK cells are interconnected and contain a polarized sorting mechanism. J. Cell Biol. *135*, 139–152.

Presley, J.F., Mayor, S., Dunn, K.W., Johnson, L.S., McGraw, T.E., and Maxfield, F.R. (1993). The *end2* mutation in CHO cells slows the exit of transferrin receptors from the recycling compartment but bulk membrane recycling is unaffected. J. Cell Biol. *122*, 1231–1241.

Schwartz, A.L. (1995). Receptor cell biology: receptor-mediated endocytosis. Pediatr. Res. *38*, 835–843.

Stoorvogel, W., Oorschot, V., and Geuze, H.J. (1996). A novel class of clathrin-coated vesicles budding from endosomes. J. Cell Biol. *132*, 21–33.

Tooze, J., and Hollinshead, M. (1991). Tubular early endosomal networks in AtT20 and other cells. J. Cell Biol. *115*, 635–653.

Trejo, J., Altschuler, Y., Fu, H.W., Mostov, K.E., and Coughlin, S.R. (2000). Protease-activated receptor-1 down-regulation: a mutant HeLa cell line suggests novel requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits. J. Biol. Chem. *275*, 31255–31265.

Whitney, J.A., Gomez, M., Sheff, D., Kreis, T.E., and Mellman, I. (1995). Cytoplasmic coat proteins involved in endosome function. Cell *83*, 703–713.

Willingham, M.C., Maxfield, F.R., and Pastan, I.H. (1979). α_2Macroglobulin binding to the plasma membrane of cultured fibroblasts. J. Cell Biol. *82*, 614–625.