Molecules Internalized by Clathrin-independent Endocytosis Are Delivered to Endosomes Containing Transferrin Receptors

Steen H. Hansen,* Kirsten Sandvig,† and Bo van Deurs* 

*Structural Cell Biology Unit, Department of Anatomy, the Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark; and †Institute for Cancer Research at the Norwegian Radium Hospital, Montebello, 0310 Oslo 3, Norway

Abstract. We have previously demonstrated that the preendosomal compartment in addition to clathrin-coated vesicles, comprises distinct nonclathrin coated endocytic vesicles mediating clathrin-independent endocytosis (Hansen, S. H., K. Sandvig, and B. van Deurs. 1991. J. Cell Biol. 113:731-741). Using K+ depletion in HEp-2 cells to block clathrin-dependent but not clathrin-independent endocytosis, we have now traced the intracellular routing of these nonclathrin coated vesicles to see whether molecules internalized by clathrin-independent endocytosis are delivered to a unique compartment or whether they reach the same early and late endosomes as encountered by molecules internalized with high efficiency through clathrin-coated pits and vesicles. We find that Con A-gold internalized by clathrin-independent endocytosis is delivered to endosomes containing transferrin receptors. After incubation of K+ -depleted cells with Con A-gold for 15 min, ~75% of Con A-gold in endosomes is colocalized with transferrin receptors. Endosomes containing only Con A-gold may be accounted for either by depletion of existing endosomes for transferrin receptors or by de novo generation of endosomes. Cationized gold and BSA-gold internalized in K+-depleted cells are also delivered to endosomes containing transferrin receptors. h-lamp-I-enriched compartments are only reached occasionally within 30 min in K+-depleted as well as in control cells. Thus, preendosomal vesicles generated by clathrin-independent endocytosis do not fuse to any marked degree with late endocytic compartments. These data show that in HEp-2 cells, molecules endocytosed without clathrin are delivered to the same endosomes as reached by transferrin receptors internalized through clathrin-coated pits.

Molecules internalized through clathrin-coated pits and vesicles are generally delivered to acidic early endosomes and sorted for recycling, transport to late endosomes, lysosomes, the TGN, and for transcytosis (7, 10, 17, 27, 32, 45). Although this endocytic pathway by far is the most extensively characterized, it is clearly not the only one. It has thus been found that, in kidney collecting duct principal cells and toad bladder granular cells, vasopressin-sensitive water channels recycle between the apical plasma membrane and nonacidic early endosomes (24, 33, 48) after entry from clathrin-coated pits (4, 5, 41). Furthermore, evidence has accumulated in favor of the existence of endocytic mechanisms that do not require clathrin; for review see van Deurs et al. (45), Watts and Marsh (49). Using Con A-gold as endocytic tracer, we have previously characterized the preendosomal compartment in HEp-2 cells and in other cells and revealed the existence of nonclathrin-coated primary endocytic vesicles (14). Several observations indicated that these vesicles were not derived from uncoating of clathrin-coated vesicles. Thus, the nonclathrin-coated vesicles identified were: (a) in average, smaller (95 nm) than coated vesicles (110 nm, excluding the coat); (b) observed with higher frequency than noncoated vesicles when anti-transferrin receptor (TfR) antibody B3/25 coupled to gold was used as endocytic marker; (c) not recycling vesicles; and (d) present in HEp-2 cells which before addition of Con A-gold were subjected to K+ -depletion in combination with a hypotonic shock, a treatment which in HEp-2 cells removes nearly all clathrin-coated pits from the plasma membrane (26). These vesicles, however, are not the only structures mediating clathrin-independent endocytosis, since results from other studies strongly indicate the existence of larger—macropinocytotic—primary endocytic vesicles in growth factor-stimulated cells which derive from areas of the plasma membrane exhibiting extensive membrane ruffling (12, 29, 34, 52) by a process that may be regulated by ras-like proteins (2, 31). Thus constitutive as well as growth factor-inducible mechanisms of clathrin-independent endocytosis exist. In addition, cadherins from both desmosomes

1. Abbreviations used in this paper: h-lamp-I, human lysosome-associated membrane protein-I; PAG, protein A-gold; TfR, transferrin receptor.

© The Rockefeller University Press, 0021-9525/93/10/89/9 $2.00
The Journal of Cell Biology, Volume 123, Number 1, October 1993 89-97 89
and zonulae adherens are internalized in MDBK cells after removal of extracellular Ca$^{2+}$ (19, 20). Whereas the mechanism involved in internalization of desmosomal cadherins is not known, the cadherins from zonulae adherens seem to be internalized by clathrin-independent endocytosis (19).

It is unclear how molecules internalized by clathrin-independent endocytosis are routed within the cell. Tran et al. (44) found that cholera toxin which has been suggested to enter exclusively through nonclathrin coated pits (28), was delivered to the same early endosomes as αc-macroglobulin. In contrast, Keller et al. (21) found that whereas the transmembrane form of CD4 transfected into CHO cells was internalized via clathrin-coated vesicles, a glycosylphosphatidylinositol (GPI)-linked form (CD4DAF) apparently entered the cells through caveolae and that the two forms were routed to separate populations of early endosomes but converged in late endocytic compartments. Becich et al. (3) found that internalized galactosyl BSA and WGA followed distinct intracellular pathways. However, no measures were taken in these studies to secure that the markers used for clathrin-independent endocytosis actually entered the cell by such a mechanism and not through clathrin-coated pits. Furthermore, in none of these studies was the extent to which the markers for the two pathways colocalized in endosomes quantitated.

We have therefore studied the fate of the previously identified nonclathrin-coated primary endocytic vesicles in HEp-2 cells. Using ultrastructural immunocytchemistry, we find that molecules internalized by clathrin-independent endocytosis in HEp-2 cells are delivered to endosomes containing TIR and thus are routed similar to molecules internalized by clathrin-coated pits.

**Materials and Methods**

**Cells**

HEp-2 cells were grown as previously described (14). 60–80% confluent cultures in T-25 flasks (Nunc, Roskilde, Denmark) were used for experiments. Repeating experiments were performed with Hep-2 cells from different passages.

**K$^{+}$ Depletion**

Cells were processed according to (23) with some modification. Cultures were rinsed three times with K$^{-}$-free buffer (140 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 1 mg/ml d-glucose), and hypotonically shocked by a brief rinse followed by incubation for 5 min in K$^{-}$-free buffer diluted 1:1 with distilled water. Next, cells were rinsed three times and incubated in K$^{-}$-free buffer for 15 min and finally incubated with endocytic markers in K$^{-}$-free buffer. Controls were processed identically except 10 mM KCl was added to the incubation buffer. All incubations were performed at 37°C.

**Binding and Endocytosis of $^{125}$I-labeled Transferrin**

Internalization of $^{125}$I-Tf was measured essentially as previously described (26) throughout the course of this study to ascertain that K$^{+}$ depletion perturbed endocytosis from clathrin-coated pits in HEp-2 cells as efficiently as previously demonstrated (26). Endocytosis of $^{125}$I-Tf in K$^{+}$-depleted cells was reduced to <10% of control cells in all experiments.

**Endocytic Tracers**

Mouse monoclonal IgGl, K anti-human transferrin receptor antibody, clone B3/25 (Boehringer Mannheim, Mannheim, Germany) was dialyzed for 24 h at 4°C against 1 liter of Hepes (20 mM) buffered DME with 2 mM glutamine without sodium bicarbonate (DME-H; Flow Laboratories, Irvine, Scotland). To tag the TIR with anti-TIR B3/25, HEp-2 cells were rinsed twice with DME-H and incubated with 20 μg/ml anti-TIR B3/25 in DME-H for 90 min at 37°C before processing for K$^{+}$ depletion or control incubations.

Con A coupled to 5 nm colloidal gold OD$_{20,0}$ 50.0 (Sigma Chemical Co., St. Louis, MO) was dialyzed against three changes of 500 ml of K$^{-}$-free buffer for 24 h (2 × 2 h + 1 × 20 h) at 4°C before each experiment and diluted in K$^{-}$-free buffer to OD$_{20,0}$ 0.5 (except where indicated) and the temperature adjusted to 37°C shortly before use. Poly-l-lysine coupled to 10 nm gold (cationized gold; Zymed, San Francisco, CA) OD$_{20,0}$ 30.0 was dialyzed and used at OD$_{20,0}$ 0.75. 10 nm BSA-gold was prepared according to Slot and Geuze (56), dialyzed and diluted to OD$_{20,0}$ 5.0. In controls, KCl was added to 10 mM.

Direct binding experiments between anti-TIR B3/25 and Con A was performed in an ELISA assay with anti-TIR B3/25 coated at 10 μg/ml (or serial twofold dilutions hereof) in 50 mM buffer, pH 9.5, followed by incubation with 10 μg/ml Con A conjugated to HRP (Con A-HRP; Sigma Chemical Co.) and using 3', 3',5', 5'-tetramethylbenzidine substrate solution. Binding of Con A-HRP to wells coated with anti-TIR B3/25 was equally low as binding to wells exposed to buffer only.

**Preparation and Immunolabeling of Cryosections**

The protocol for the Tokuyasu technique (42) was based on modifications devised by Griffiths et al. (11), Slot et al. (37), and Tokuyasu (43). After incubation with endocytic tracers, cultures were rinsed three times with buffer (with or without K$^{+}$) and fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.2 M phosphate buffer overnight at 4°C. Next, cells were rinsed twice, scraped off, pelleted (150 g, 5 min), resuspended in 10% gelatin in PBS and incubated at 37°C for 15 min. The cells were then pelleted again (735 g, 5 min) and placed on ice for 15 min at 4°C for the gelatin to solidify. The tubes were cut open and 4–12 μm pieces of pellets were immersed in PVP-sucrose (1.8 M sucrose containing 10% polyvinylpyrrolidine) for 4 h at 4°C with two changes. Finally, the blocks were mounted on stubs, frozen in liquid nitrogen and sectioned on a RMC MT-7 micromtome.

Cryosections were immunolabeled as previously described (13). Double labeling using protein A-gold (PAG) was performed in principle according to Geuze et al. (8) except that 1% glutaraldehyde as devised by Slot et al. (38) was used to prevent interference between the first and second labeling. Double labeling with species-specific secondary antibodies was carried out with simultaneous incubation with primary and secondary antibodies, respectively. Controls comprised (a) exclusion of either primary antibody alone or (b) in combination with exclusion of secondary antibody specific for the primary antibody present during the primary antibody incubation.

**Primary Antibodies and Secondary Reagents**

Anti-TIR B3/25 was diluted 1:20 (20 μg/ml) for labeling of cryosections. Affinity-purified rabbit anti-mouse immunoglobulins were obtained from Dakopatts (Copenhagen, Denmark) and used 1:50. Rabbit anti-human lysosome-associated membrane protein-1 (h-lamp-1) antiserum (6) kindly provided by Dr. Sven Carlsson (Department of Medical Biochemistry and Biophysics, University of Umeå) was used 1:60.

The following colloidal gold-coupled secondary reagents were obtained from Amersham International (Buckinghamshire, England) and used at the indicated dilutions: PAG$_{1}$, 1:40; PAG$_{10}$, 1:20; PAG$_{15}$, 1:20; goat anti-mouse IgG (Fc)10 nm, 1:20; goat anti-rabbit IgG (H+L)15 nm, 1:20.

**Quantification**

Endosomes from immunolabeled sections were sampled systematically for quantification by moving the specimen horizontally and vertically in a sequence determined at 1,600× that maintained focus over the chosen section. The areas of cells that came into the field of view at 13,000× were multiplied by the number of sections and the area of the objects counted. The number of points and intersections as well as the number of contained gold particles were counted. From these determinations the relative volume and surface/volume ratio and labeling density were calculated. Statistics were performed as previously described (15).

The Journal of Cell Biology, Volume 123, 1993
Figure 1. Schematic presentation of the experimental protocol used to tag TfRs in HEp-2 cells with anti-TfR B3/25 and subsequently perturb endocytosis from clathrin-coated pits by K+ depletion before exposure to Con A-gold (or cationized gold or BSA-gold). All incubations were performed at 37°C.

Results

Con A-gold was used as endocytic tracer in nearly all experiments since we have previously found that this conjugate binds to the entire accessible surface of HEp-2 cells, that it is internalized by clathrin-coated and nonclathrin-coated vesicles, and that it does not induce endocytosis (14). To study whether molecules internalized by clathrin-independent endocytosis are routed to a separate early endosomal compartment or are delivered to endosomes also reached by the clathrin-coated pit pathway, we preincubated HEp-2 cells with anti-TfR B3/25, processed the cells for K+ depletion or control treatment and allowed the cells to internalize Con A-gold for 5–30 min at 37°C. The protocol used in these experiments is summarized in Fig. 1. Next, the rout-

Figure 2. Localization of Con A-gold and TfR tagged by anti-TfR B3/25 in control HEp-2 cells processed as summarized in Fig. 1, and incubated with 5 nm Con A-gold for 15 min at 37°C. Cryosections were immunolabeled to detect antibody-tagged TfRs using rabbit anti-mouse Ig and PAG10 (arrows). Both markers are internalized by the same coated pits (A, cp) and coated vesicular profiles (B, cv). In addition Con A-gold is found in presumptive noncoated preendosomal vesicles (curved arrow in B). A and D–H show the extensive colocalization of Con A-gold and TfRs in spherical endosomes, and I–J, in tubular endosomes of control cells, whereas none of the markers are detected in lysosome-like structures (asterisk in G). No direct binding of PAG10 to anti-TfR B3/25 in sections was detected. Thus, omission of rabbit anti-mouse IgG abolished labeling. Bar, 200 nm.
Table I. Colocalization of Con A-Gold with TfR in Control and K+-depleted HEp-2 Cells

| Time of incubation | No. of endosome profiles sampled | No. of endosomes with both markers | No. of endosomes with TfR only | No. of endosomes with Con A-gold only | Percent endosomes colocalized with Con A-gold | Percent of TfR colocalized with Con A-gold |
|--------------------|----------------------------------|-----------------------------------|-----------------------------|-------------------------------------|---------------------------------------------|------------------------------------------|
| 5 min              | Control                          | 31                                | 27                          | 3                                   | 1                                           | 88                                       | 89                                       | 98                                       |
|                    | K+-depleted                      | 32                                | 12                          | 18                                  | 2                                           | 38                                       | 47                                       | 76                                       |
| 15 min             | Exp. I: Control                 | 40                                | 34                          | 3                                   | 3                                           | 85                                       | 96                                       | 93                                       |
|                    | Exp. I: K+-depleted             | 48                                | 30                          | 8                                   | 10                                          | 63                                       | 84                                       | 74                                       |
|                    | Exp. II: Control*               | 51                                | 45                          | 1                                   | 5                                           | 88                                       | 98                                       | 97                                       |
|                    | Exp. II: K+-depleted*           | 46                                | 32                          | 2                                   | 12                                          | 70                                       | 95                                       | 72                                       |

The data were obtained as described in Materials and Methods.

* Con A-gold: OD_{520} = 1.25

ing of Con A-gold was studied in cryosections with respect to the localization of antibody-tagged TfR detected by use of rabbit anti-mouse IgG and PAG.

Internalized Con A-gold Colocalizes with the TfR in Endosomes of HEp-2 Cells

In control cells preincubated with anti-TfR B3/25 and incubated with Con A-gold for 5 or 15 min both markers were detected in clathrin-coated pits and vesicles (Fig. 2, A and B). Con A-gold was also found in nonclathrin-coated invaginations of the plasma membrane and in vesicles probably derived from clathrin-independent endocytosis as well as uncoating of clathrin-coated vesicles (Fig. 2 C). Extensive colocalization of TfR and Con A-gold was detected in endosomes of cells that were densely tagged with Con A-gold (Fig. 2, D-J). The majority of endosomes were basically spherical organelles containing some internal vesicles and comprising short tubular extensions (Fig. 2, D-H). In addition colocalization of the two markers was found in tubular endosomes (Fig. 2, I and J). Quantitative data have revealed that tubular endosomes are rare in HEp-2 cells (46). Accordingly, tubular endosomes occurred with low frequency in thin cryosections (estimated <100 nm, based on interference color) and were hardly seen in thicker cryosections (>150 nm and thicker), probably obscured since the cytoplasm of HEp-2 cells in cryosections is rather dense. To estimate the extent to which the two markers colocalized, we quantified the number of systematically sampled endosomal profiles containing one or both markers and the number of contained gold particles of either specificity. To reduce the probability of underestimating the number of endosomes containing Con A-gold, the quantification was performed on sections ~150 nm thick. As shown in Table I, in these sections, the colocalization of Con A-gold and the TfR approached 100% in endosomes of cells that were densely tagged by Con A-gold. This shows that the clathrin-independent endocytic mechanism, previously evidenced to operate in nonperturbed HEp-2 cells (14), delivers Con A-gold to endosomes containing TfRs.

Table II. Relative Dimensions of, and TfR-Density in Endosomes of Control HEp-2 Cells and Cells K+-depleted for 30 min (Corresponding to 15-min Incubation with Con A-Gold)

| Endosome population | Preincubation with 20 μg/ml anti-TfR B3/25 | Relative volume (points/endosome) Mean ± SEM | Surface/volume ratio (intersections/point) Mean ± SEM | Relative TfR-density in endos. (gold/intersect.) Mean ± SEM |
|---------------------|--------------------------------------------|---------------------------------------------|------------------------------------------------------|----------------------------------------------------------|
| Exp I: Control (n = 40) | Yes                                        | 6.5 ± 0.9                                   | 2.9 ± 0.2                                            | 0.73 ± 0.09                                              |
| Exp I: K+-depleted (n = 48) | Yes                                        | 6.0 ± 0.8†                                  | 3.0 ± 0.2‡                                           | 0.34 ± 0.04                                              |
| Exp IIa: Control (n = 51) | Yes                                        | 5.2 ± 0.6†                                  | 2.7 ± 0.11                                           | 0.49 ± 0.05                                              |
| Exp IIa: K+-depleted (n = 46) | Yes                                        | 4.5 ± 0.7†                                  | 3.1 ± 0.2**                                          | 0.28 ± 0.04                                              |
| Exp IIb: Control (n = 40) | No                                         | 5.3 ± 0.5‡                                  | 2.4 ± 0.11                                           | 0.005 ± 0.003                                            |
| Exp IIb: K+-depleted (n = 41) | No                                         | 4.8 ± 0.5‡                                  | 2.7 ± 0.2**                                          | 0.02 ± 0.01                                              |
| Control vs K+-depleted (exp IIa/IIb) |                             |                                              |                                                      |                                                          |
| Unpaired two-tailed t test* |                             |                                              |                                                      |                                                          |

The data were obtained as described in Materials and Methods.

Note that the only significant difference between K+-depleted and control cells detected in two independent experiments with cells from different passages was in the density of TfRs in endosomes which was significantly reduced by K+ depletion. This finding was made in both experiments whereas no other significant differences between the two experiments were recorded.

* Exp I: 87 degrees of freedom. Exp IIa: 96 degrees of freedom. Exp IIb: 80 degrees of freedom.
†† 90 degrees of freedom.
‡‡ 90 degrees of freedom.
§§ 90 degrees of freedom.
¶¶ 90 degrees of freedom.
+ 94 degrees of freedom.
++, 94 degrees of freedom.
nsd, not significantly different.
Figure 3. Localization of Con A-gold and TfR in K+-depleted HEp-2 cells processed as summarized in Fig. 1, and incubated with 5 nm Con A-gold for 5 min (A and B) or 15 min (C–J) at 37°C. Cryosections were labeled to detect antibody-tagged TfRs using rabbit anti-mouse Ig and PAG	n (arrows). A, B, and E–J reveal that Con A-gold and TfR are colocalized in endosomes of K+-depleted HEp-2 cells, whereas none of the markers are detected in lysosome-like structures (asterisks in C, D, and I). Examples of endosomes containing Con A-gold but not TfRs are shown in C and D. Whereas the labeled endosomes are typically spherical, they are sometimes provided with numerous processes (J). Bar, 200 nm.

Molecules Internalized by Clathrin-independent Endocytosis in K+-depleted Cells Are Delivered to Endosomes Containing TfRs

Whereas K+ depletion of HEp-2 cells in general did not affect basic morphological parameters of endosomes such as relative volume and surface/volume ratio, it significantly reduced the content of TfRs in endosomes (Table II). Yet, after 5-15 min of incubation, 75% of internalized Con A-gold still colocalized with the TfR in endosomes as shown in Fig. 3 (A, B, and E–J) and Table I. Concerning the quantitative data summarized in Table I, it should be noticed that the fraction of endosomes with TfR and/or Con A-gold as well as the fraction of TfRs in endosomes colocalizing with Con A-gold all are values which depend on the amount of bound and internalized Con A-gold. Therefore, to obtain reproducible data, it is necessary to perform quantifications on cells from experiments where the accessible cell surface has been densely tagged with Con A-gold. However, the fraction of internalized Con A-gold in endosomes colocalizing with TfRs is not sensitive to variations in binding of Con A-gold to the cell surface. In two additional K+-depletion experiments with a 15-min incubation with Con A-gold including one where low amounts of conjugate were bound, 77% (1,641 of 2,126 particles; 53 endosomes) and 75% (205 of 273 particles; 35 endosomes) respectively, of internalized Con A-gold in endosomes colocalized with TfR. There was no correlation between the TfR-labeling and number of Con A-gold in cells preincubated with anti-TfR B3/25, neither in K+-depleted cells nor in control cells in any of the experiments quantified (range of correlation coefficient, r², for K+-depleted and control cells 0.0001 < r² <0.052). This result shows that the targeting of Con A-gold occurred randomly with respect to the endosomes containing TfR. Also cationized gold and BSA-gold internalized by K+-depleted cells colocalized with TfRs in endosomes (data not shown). These data thus show that in HEp-2 cells, molecules internalized by clathrin-independent endocytosis are delivered to endosomes containing transferrin receptors. Interestingly, after 15 min of incubation with Con A-gold, a population of endosomes emerged which did not label for the TfR (Fig. 3,
Table III. Relative Dimensions of Endosome Populations in K⁺-depleted HEp-2 Cells

| Endosome population          | Relative volume (points/endosome) Mean ± SEM | Surface/volume ratio (intersections/point) Mean ± SEM |
|-----------------------------|---------------------------------------------|-----------------------------------------------------|
| Only Con A-gold (n = 22)    | 2.5 ± 0.6                                   | 4.0 ± 0.3                                           |
| TfR ± Con A-gold* (n = 72)  | 6.1 ± 0.6                                   | 2.8 ± 0.2                                           |

Unpaired two-tailed t test

|                               | p < 0.01†                               | p < 0.01‡                                          |

The sampling procedure is representative of the relative frequencies of endosomes containing one or both of the two markers providing their size is similar. It is biased, however, with respect to volume, since large endosomes will occur in more sections and therefore have a higher probability of being sampled than small ones. Thus, the data summarized in this table probably underestimates the frequency of endosomes containing Con A-gold but not TfR.

The data were obtained as described in Materials and Methods.

* TfR ± Con A-gold signifies endosomal profiles containing either TfRs only or TfR and Con A-gold.
† 93 degrees of freedom.

C and D, and Table I). Since these were very rare in control cells they are unlikely to represent a unique endosomal compartment. More likely, they are generated either by depletion of existing endosomes for TfRs or by de novo formation of endosomes while endocytosis of anti-TfR B3/25 is perturbed by K⁺ depletion. Supporting the latter possibility was the finding that the Con A-gold containing TfR-negative endosomes had smaller volume and higher surface/volume ratio than the TfR-positive ones (Table III). As a consequence of their smaller size, the frequency of the Con A-gold containing TfR-negative endosomes is underestimated due to the bias of the sampling method used (see Materials and Methods).

Localization of Internalized Con A-Gold with Respect to h-lamp-1-enriched Compartments in K⁺-depleted and Control HEp-2 Cells

As a marker of late endocytic compartments in HEp-2 cells, we used h-lamp-1 which in these cells is present in low amounts on the plasma membrane and in early endosomes but found mainly in late endosomes, and in particular in spherical lysosomal structures characterized by their content of numerous disintegrating vesicles and/or myelin figures (46). In these h-lamp-1-enriched compartments we rarely detected Con A-gold internalized for 15–30 min in neither K⁺-depleted (Fig. 4) nor in control HEp-2 cells (Fig. 5). These data show that Con A-gold internalized for up to 30 min in K⁺-depleted cells like in control cells does not gain access to late endocytic compartments to any appreciable ex-
Figure 5. Localization of Con A-gold, TfR and h-lamp-1 in control HEp-2 cells processed as summarized in Fig. 1, and incubated with 5 nm Con A-gold for 15 min (A–C) or 30 min (D and D') at 37°C. (A–C) Cryosections were labeled with rabbit anti-h-lamp-1 and PAG$_{10}$ (arrowheads). (D and D') Cryosections were double-labeled to detect antibody-tagged TfR (arrows) and h-lamp-1 (arrowheads). Sections were first incubated with rabbit anti-mouse Ig and PAG$_{0}$, stabilized with 1% glutaraldehyde, and then exposed to rabbit anti-h-lamp-1 and PAG$_{5}$. A–D reveal that Con A-gold which is delivered to endosomes containing transferrin receptors (D and D') essentially does not reach h-lamp-1-enriched compartments ($l_e$) in control cells within 30 min of incubation. D and D' furthermore show the reciprocal distribution of antibody-tagged TfR and h-lamp-1. The order of labeling of cryosections could not be reversed since exposure to 1% glutaraldehyde abolished the reactivity of rabbit anti-mouse Ig to anti-TfR B3/25. In contrast, h-lamp-1 labeling is only moderately reduced. $m$, mitochondrion; $nu$, nucleus; $pm$, plasma membrane. Bars, 200 nm.

Preincubation with anti-TfR B3/25 Does Not Cause Artificial Colocalization of TfR and Con A–Gold

Preincubation with anti-TfR has been shown to retard recycling of transferrin receptors from early endosomes (22, 48). This side effect of using anti-TfR as a marker of the clathrin-coated pit pathway is useful in the present study since it reduces depletion of TfRs from endosomes following perturbation of receptor internalization. Furthermore, the use of an indirect assay allowed us to use polyclonal antibodies to detect the TfR and resulted in increased and reproducible...
labeling specific for the TfR. It also resulted in negligible nonspecific labeling as determined by labeling of cells not preincubated with anti-TfR B3/25 (Table II). However, it has also been found that tagging the TfR with antibody reduces half-life of the receptor to 50% of normal in K562 cells (51) and increases the volume of early TfR-containing endosomes twofold (22) in chicken erythroblasts transformed by a temperature-sensitive mutant of avian erythroblastosis virus concomitant with a block of erythroid differentiation. We were thus concerned with the possibility that tagging the TfR in HEp-2 cells with antibody would affect the use of the TfR as marker molecule in the present study. With the concentration of anti-TfR B3/25 used and within the time-frame studied this did not appear to be the case neither in control cells nor in K+-depleted HEp-2 cells. First, there was no marked difference in the morphology of compartments labeled by the indirect assay and by direct labeling of TfR using anti-TfR B3/25 on cells not preincubated with anti-TfR B3/25. Second, the antibody-tagged TfR was very rarely detected in compartments with a typical lysosome-like morphology. Accordingly, double labeling of Hep-2 cells to detect anti-TfR B3/25 and h-lamp-1 revealed a reciprocal distribution with sparse colocalization of the two molecules (Fig. 5). It should be noted that there was also sparse colocalization of h-lamp-1 and TfR when both markers were detected directly using species specific secondary antibodies (data not shown).

Discussion

In this work we have provided evidence that molecules internalized by clathrin-independent endocytosis in K+-depleted HEp-2 cells are delivered to endosomes containing TfRs and only rarely reach h-lamp-1 enriched compartments within 30 min of incubation. Colocalization of internalized Con A-gold with TfRs in endosomes was lower in K+-depleted cells than in control cells but this could be accounted for either by depletion of TfR from preexisting endosomes or perhaps more likely by biogenesis of endosomes in K+-depleted cells. The occasional delivery of Con A-gold to h-lamp-1-enriched compartments which was observed within 30 min of incubation in both perturbed cells and control cells is in agreement with data showing that late endocytic compartments are fusion-accessible to endocytic vesicles (40, 46).

Several molecules have been claimed to enter cells by a nonclathrin pathway based on their localization in "micropinocytic invaginations" or caveolae (1, 9, 16, 18, 21, 25, 28, 30, 44). However, it remains unsettled if and to what extent caveolae are involved in endocytosis, mainly because the numerous studies ascribing endocytosis of specific molecules to caveolae do not include a serial sectioning analysis to show that identified nonclathrin coated vesicular profiles are actually free vesicles and not cross-sectioned invaginations of the plasma membrane (35, 47). Due to the uncertain existence of specific markers for clathrin-independent endocytosis, we have chosen to use nonelective endocytic markers, mainly Con A-gold, in combination with K+-depletion to remove clathrin-coated pits, and preincubation with anti-TfR B3/25 to label endosomes reached by clathrin-dependent endocytosis. Each of these steps pose a potential source of inducing artifacts. We have previously shown that Con A does not induce endocytosis in HEp-2 cells (14). Control experiments in this work revealed that, within the time-frame studied, preincubation with anti-TfR B3/25 did not interfere with the use of TfR as a marker of early endosomes reached by clathrin-mediated endocytosis (see Results). It might be argued that the colocalization of Con A-gold and TfRs in endosomes of K+-depleted cells could result from internalization of TfRs by clathrin-independent endocytosis. However, as explained below, the putative uptake of TfR by this pathway is too small to account for the colocalization with Con A. The content of TfRs in endosomes after 15 min of incubation with Con A-gold is only reduced to ~50% (Table II), ~90% of which colocalizes with Con A-gold in endosomes (Table I). Colocalization due to uptake from nonclathrin-coated plasma membrane domains would therefore require (a) that ~45% of the TfRs present in endosomes of control cells were internalized by clathrin-independent endocytosis in K+-depleted cells; and (b) that essentially all TfRs in endosomes reached by the clathrin-coated pit pathway were recycled despite the tagging with anti-TfR, while (c) the TfRs assumed to enter the cell by clathrin-independent endocytosis accumulated. Evidently, the quantitative data obtained in the present study exclude that the extensive colocalization of Con A-gold and TfRs observed in endosomes of K+-depleted cells could result from antibody-tagged TfRs internalized by the clathrin-independent endocytosis. Our results thus show that molecules internalized by clathrin-independent endocytosis are delivered to the same early endosomes as reached by the clathrin-mediated mechanism and are consistent with a model where the two pathways converge in early endosomes.

We are very grateful to Henrik Clausen, Department of Oral Diagnosis, the Panum Institute, for performing the ELISA assays, and to Gert H. Hansen, Department of Biochemistry, The Panum Institute for access to his Zeiss EM 900 electron microscope. We thank Marianne Lund, Annette Øhlsen, Keld Ottosen, and Kirsten Pedersen for expert technical assistance. This work was supported by the Danish Cancer Society, the Danish Medical Research Council, the Novo Nordic Foundation, the Marshall Foundation, the Madsen Foundation, the Carlsberg Foundation, the Davidsenske-Præstens Fond and the Danish Civilingeniør Frode V. Nyegaard og Hustru Foundation, the Madsen Foundation, the Carlsberg Foundation, the Novo Nordic Foundation, the Marshall Foundation, the Madsen Foundation, the Carlsberg Foundation, the Civilingeniør Frode V. Nyeaard og Hustru Foundation, the Direktør Ib Henriksen Foundation, Højmosegårde Legat, Fonden til Sygdomsbeæmpe- pelsen uden Dyreforsøg, and a NATO Collaborative Research Grant (CRG 900517).

Received for publication 26 January 1993 and in revised form 29 June 1993.
References

1. Bamezai, A., V. S. Goldmacher, and K. L. Rock. 1992. Internalization of glycosyl-phosphatidylinositol (GPI)-anchored lymphocyte proteins II. GPI-anchored and transmembrane molecules internalize through distinct pathways. *Eur. J. Immunol.* 22:15–21.

2. Bar-Sagi, D., and J. R. Feramisco. 1986. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. *Science* (Washington, DC.) 233:1061–1067.

3. Becich, M. J., S. Mahlknauf, and J. U. Baenziger. 1991. Wheat germ agglutinin is selectively transported to multivesicular bodies. *Eur. J. Cell Biol.* 55:83–93.

4. Brown, D., and L. Orci. 1983. Vasopressin stimulates formation of coated pits in rat kidney collecting ducts. *Nature (London).* 302:255–257.

5. Brown, D., P. Weyer, and L. Orci. 1988. Vasopressin stimulates endocytosis in kidney collecting duct principal cells. *Eur. J. Cell Biol.* 46:336–341.

6. Carlsson, S., J. Roth, F. Piller, and M. Fukada. 1988. Isolation and characterization of human lysosomal membrane proteins, b-lamp-1 and b-lamp-2. *J. Biol. Chem.* 263:18911–18919.

7. Compans, R. W. 1991. Dissection of endosomes. In Intracellular Trafficking of Proteins, C. Steer and J. Hanauer, editors. Cambridge University Press, Cambridge, England. 103–156.

8. Geuze, H. J., J. W. Slot, P. A. v. d. Ley, R. T. Scheffer, and J. M. Griffiths. 1986. Use of doubly gold particles to double-label immunoelectron microscopy of ultrathin frozen tissue sections. *J. Cell Biol.* 99:653–665.

9. Goldberg, R. I., R. M. Smith, and L. Jarett. 1987. Insulin and ox-macroglobulin-methylamine undergo endocytosis by different endocytic pathways in rat adipocytes: I. Comparison of cell surface events. *J. Cell Physiol.* 133:203–212.

10. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, and W. J. Schonider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* 1:1–39.

11. Griffiths, G., A. McDowell, R. Back, and D. Dubochet. 1983. On the preparation of cryosections for immunocytochemistry. *J. Ultrastruct. Res.* 85:53–67.

12. Haigler, H. T., J. A. McKenna, and S. Cohen. 1979. Rapid stimulation of pinocytosis in human carcinoma cells A-431 by epidermal growth factor factor. *J. Cell Biol.* 83:82–90.

13. Hansen, S. H., K. Sandvig, and B. van Deurs. 1993. Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium and cytosol acidification. *J. Cell Biol.* 121:61–72.

14. Hansen, S. H., K. Sandvig, and B. Deurs. 1991. The preendosomal compartment comprises distinct coated and uncoated endocytic vesicle populations. *J. Cell Biol.* 113:731–741.

15. Hansen, S. H., K. Sandvig, and B. van Deurs. 1992. Internalization efficiency of the transferrin receptor. *Exp. Cell Res.* 199:19–28.

16. Hopkins, C. R., K. Miller, and J. M. Beadmore. 1985. Receptor-mediated endocytosis of transferrin and epidermal growth factor receptors: A comparison of constitutive and ligand-induced uptake. *J. Cell Biol.* 109:2731–2739.

17. Hubbard, A. 1989. Endocytosis. *Curr. Opin. Cell Biol.* 1:675–683.

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

19. Kartenbeck, J., M. Schmelz, W. W. Franke, and B. Geiger. 1991. Endocytosis of junctional cadherins in bovine kidney epithelial (MDCK) cells cultured in low Ca²⁺ ion medium. *J. Cell Biol.* 113:881–892.

20. Kartenbeck, J., E. Schmid, W. W. Franke, and B. Geiger. 1982. Different modes of intracellular transport of proteins associated with adhesions functions and desmosomes: experimental separation of lateral contacts induces endocytosis of desmosomal plaque material. *EMBO (Eur. Mol. Biol. Organ.)* 1:725–732.

21. Keller, G.-A., M. W. Siegel, and J. W. Caras. 1992. Endocytosis of glycoprophosphatidylanchored and transmembrane forms of CD4 by different endocytic pathways. *EMBO (Eur. Mol. Biol. Organ.)* 11:863–874.

22. Killisch, I., P. Steinlein, K. Rönisch, R. Hollinshead, H. Beug, and G. Griffiths. 1992. Characterization of early and late endocytic compartments of the transferrin cycle. Transferin receptor antibody blocks erythroid differentiation by trapping the receptor in the early endosome. *J. Cell Sci.* 103:211–222.

23. Larkin, J. M., S. M. Brown, J. L. Goldstein, and R. G. W. Anderson. 1983. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell.* 35:273–285.

24. Lencer, W. I., A. S. Verkmen, M. A. Arnaout, D. A. Ausiello, and D. Brown. 1990. Endocytic vesicles from renal papilla which retrieve the vasopressin-sensitive water channel do not contain a functional H₂ATPase. *J. Cell Biol.* 111:379–389.

25. Lotti, L. V., C. D. Lazzaro, C. Zompetta, L. Frati, and M. R. Torrisi. 1989. Different endocytic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J. Cell Biol.* 109:2731–2739.