Rapid Endocytosis of the Transferrin Receptor in the Absence of Bound Transferrin

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ABSTRACT The rate of endocytosis of transferrin receptors, occupied or unoccupied with transferrin, was measured on the cell line K562. At 37°C, receptors, radioiodinated on the cell surface at 4°C, were internalized equally rapidly in the presence or absence of transferrin. In both cases, 50% of the labeled receptors became resistant to externally added trypsin in 5 min. An antitransferrin antibody was used to show directly that the receptors had entered the cells without bound transferrin. The distribution of the receptors on the cell surface was revealed by antibody and protein A–gold staining after prolonged incubation in the presence or absence of transferrin. The receptors were concentrated in coated pits under both conditions. The data suggest that endocytosis of transferrin receptors is not "triggered" by ligand binding and raise the possibility that ligand-induced down-regulation of surface receptors may not occur by this mechanism. Instead receptors may be recognized as being ligand-occupied, not at the cell surface, but at some other site in the recycling pathway such as the endosome.

The last few years have seen a rapid increase in our understanding of the mechanisms used by cells to bind and capture macromolecular nutrients and growth factors from the external environment. Specialized regions of the cell surface called coated pits are able to accumulate receptors together with their bound ligands whilst excluding other cell surface proteins (1–3). What role, if any, does the binding of ligand play in localizing its receptor in a coated pit and thus ensuring it will be endocytosed? Surprisingly, there is little information on this important point. Most investigations have depended on the use of either the ligand itself or an antibody to the receptor; the behavior of the unoccupied receptor cannot be observed so easily.

The question of whether receptors are clustered in coated pits in the absence of ligand has been tackled in the case of the low density lipoprotein (LDL) receptor. Fibroblasts grown without LDL were fixed in formaldehyde and then detergent-extracted before being exposed to LDL-ferritin. Under these conditions, which should ensure that no lateral redistribution by ligand takes place, the receptors were concentrated in coated pits (4). Similar experiments were performed on cells that were formaldehyde-fixed, though not detergent-extracted, to investigate the distribution of the asialoglycoprotein receptor (5) and the $\alpha_2$-macroglobulin receptor (6). Again, the receptors appeared to be clustered in coated pits in the absence of ligand.

It still remains to be shown that receptors actually enter cells as readily in the absence of their bound ligand as in its presence. Data are presented here which demonstrate that this is indeed the case for the transferrin receptor. The experiments measure the rate of endocytosis of the receptor by an assay that does not require the use of either transferrin or antibodies (7).

MATERIALS AND METHODS

Cell Growth and Transferrin Depletion: K562 cells were grown in RPMI-1640 medium 5% fetal calf serum plus antibiotics under a 5% CO$_2$ atmosphere. The cells were harvested by centrifugation when they had reached a density of 4–6 x 10$^5$/ml. After washing once in growth medium without serum, they were resuspended in citrate-buffered saline (137 mM NaCl, 3.5 mM KCl, 20 mM Na-citrate, pH 5.0), containing 0.1% ovalbumin and 5 mM nitrilotriacetic acid (an iron-chelating agent). At pH 5.0 surface-bound ferri-transferrin is converted to apotransferrin, which rapidly dissociates at neutral pH (8, 9). The cells were collected by centrifugation, resuspended in RPMI-1640 medium, 0.1% ovalbumin, 5 mM nitrilotriacetic acid, and incubated at 37°C for 30 min with gentle rotation (4 rpm). Transferrin trapped within the cells should thus be recycled during this time and be released from the cells as apotransferrin. The cells were then washed twice in cold Dulbecco's phosphate-buffered saline (DPBS) and finally resuspended in DPBS plus 20 mM glucose at ~4 x 10$^7$ cells/ml.

Cell Surface Iodination: Iodination was performed at 4°C by addition of 40 $\mu$g/ml lactoperoxidase, 200 ng/ml glucose oxidase (Sigma Chemical...
Co., Poole, England), and 1 mM/l (carrier-free [Amersham International, Amersham U.K.]) (10). After 30 min at 4°C, labeling was terminated by the addition of 1 ml cold DPBS. 20 mM KCl and the cells were washed twice in the same buffer before being resuspended in RPMI-1640 medium, 0.1% ovalbumin, 25 mM HEPES, pH 7.5, at ~10⁶ cells/ml.

Assay of Transferrin Receptor Endocytosis: In a typical experiment, the freshly iodinated cells were divided into equal 0.25-ml portions and ferritin transferrin (0.5 mg/ml) was added back to half of these. After a 20-min incubation period at 4°C, duplicate tubes were warmed to 37°C to initiate endocytosis. After various time periods, the cells were resuspended with the addition of 10 vol of ice-cold 0.15 M NaCl, 20 mM Na-citrate, pH 5.0. The cells were pelleted and following resuspension in 0.25 ml 0.15 M NaCl, 1 mM EDTA, 20 mM HEPES, pH 7.5 (digestion buffer), 25 μl trypsin solution (5 mg/ml) was added to selected tubes. Proteolysis was terminated after 1 h at 4°C by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). 2 ml of digestion buffer and 1 ml of 5% ovalbumin were added, and, after dispersing any cell aggregates, the cells were pelleted and resuspended in 0.25 ml digestion buffer, 1 mM PMSF in 1.5 ml Eppendorf tubes. Each cell suspension was sonicated briefly with the fine probe of an MSE 150 W sonicator (Measuring and Scientific Equipment Ltd., Crawley, England) and portions were either frozen at ~70°C or mixed with electrophoresis sample buffer (final concentrations, 0.4 M sucrose, 1% SDS, pH 8.8 for 2-3 d.

Gel Electrophoresis: SDS gel electrophoresis was performed essentially as described by Laemmli (11) in 7% acrylamide gels at 35 mA constant current. For two-dimensional, nonreducing/reducing analysis (7) the samples were prepared by mixing 0.7 ml protein A-Sepharose (Pharmacia Fine Chemicals, Upsala, Sweden) gel with 0.25 ml rabbit anti-human transferrin serum (a gift from S. Green, Medical Research Council) in a final volume of 2 ml 0.5% Nonidet P-40, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 1% PMSF, 10 mM Tris-Cl, pH 7.5. Aliquots (50 μl) of sonicated cells (see above) were mixed with 5 vol of IP buffer (pH 5.5) and after standing on ice for 10 min were centrifuged at 15,000 g at 4°C. The supernatant was transferred to a Gilson P20 tip stuffed with glass wool and the adsorbed proteins eluted with 50 μl of electrophoresis sample buffer including 50 mM dithiothreitol.

The immunoglobulins in a rabbit anti-human transferrin receptor serum (a gift from J. Bleil, Medical Research Council) were purified on protein A-Sepharose as described above. The gel was washed in a small glass column with 10 vol of 137 mM NaCl, 3.5 mM KCl, 10 mM Tris-Cl, pH 8.0, and immunoglobulins were eluted in 2 vol of 0.1 M Na-citrate, pH 2.5, and collected in a tube containing 0.5 ml 1 M Tris-Cl, pH 8.6. Protein was precipitated by the addition of an equal volume of saturated (NH₄)₂SO₄ solution and collected by centrifugation. The pellet was resuspended in 0.2 ml phosphate-buffered saline. 0.02% Na-azide at ~2 mg/ml protein, and stored at 4°C.

Cell surface transferrin receptors on K562 cells were saturated by this antibody preparation at a 1:40 dilution as determined by fluorescence microscopy using donkey anti-rabbit Ig conjugated to Texas Red (Amersham Corp.) as a second antibody diluted 1:100.

Localization of Surface Transferrin Receptors by Electron Microscopy: K562 cells were depleted of transferrin as described above, washed once in serum-free RPMI-1640 medium, 0.1% ovalbumin, 5 mM nitritotritiatric acid, and resuspended in serum-free RPMI-1640 medium, 0.1% ovalbumin, flushed with 5% CO₂. Incubation at 37°C was continued for 40 min with or without the addition of 1 mg/ml freshly prepared ferritin transferrin. The cells were quickly chilled, washed twice in cold DPBS, 0.1% ovalbumin, and resuspended in the same buffer with or without transferrin receptor antibody at 1:40 dilution. Incubation was continued at 4°C for 30 min followed by washing three times in DPBS, 0.1% ovalbumin. Protein A-gold (Janssen, 5 nm) was diluted 15-fold in DPBS, 0.1% ovalbumin, and the cells were resuspended in this at ~2 × 10⁶/ml at 4°C for 30 min. Unbound protein A-gold was removed by washing the cells twice in DPBS, 0.1% ovalbumin, and twice in IP buffer (pH 5.5).

The cells were fixed in 2.5% glutaraldehyde, 0.1 M Na cacodylate, pH 7.2, for 20 min at room temperature. After washing in 0.1 M Na-cacodylate, pH 7.2, three times, the cells were resuspended in 2% osmium tetroxide, 0.1 M Na-cacodylate, pH 7.2, for 1 h at 4°C. After washing in water, the pellets were immobilized in 1% agarose, cut into 1-mm cubes and stained in 1% uranyl acetate for 1 h at 37°C. The samples were dehydrated through graded ethanol, infiltrated, and embedded in Araldite. Thin sections were cut and cell surfaces examined at high magnification for the presence of 5-nm gold particles.

RESULTS

Receptor Internalization Proceeds without Transferrin

K562 cells were depleted of transferrin by incubating at 37°C in transferrin-free medium containing a chelating agent. This treatment should allow dissociation of apotransferrin from receptors on the cell surface and from those returning to the cell surface during this time. The binding of apotransferrin to the receptor, though tight at pH 5.5, is readily reversible at pH 7.0 (8, 9). After chilling the cells to 4°C, receptors present on the cell surface were labeled at 4°C with [125I] by the lactoperoxidase/glucose oxidase method (10). A saturating level of ferritin transferrin (0.5 mg/ml) was added back to one half of the cells and, after a further 20-min incubation at 4°C, the cells were divided into aliquots and either kept at 4°C or warmed to 37°C to initiate endocytosis.

At different times the incubations were terminated by the addition of ice-cold citrate-buffered saline, pH 5.0, and the cells were collected by centrifugation. The cells were resuspended and digested for 1 h at 4°C with trypsin (500 μg/ml). After termination of the digestion, the cell suspension was sonicated and portions were solubilized and electrophoresed on 7% SDS-acrylamide gels, first under nonreducing conditions, and then, in the second dimension, under reducing conditions (7). The abundance of transferrin receptors on these cells permitted the direct analysis of equal numbers of cells from each condition without having to prepare a membrane fraction and so contend with variable yields.

Data representative of several experiments are shown in Fig. 1. All of the radiolabeled transferrin receptors were trypsin-sensitive when digestion was performed without any prior warming up of the cells. In contrast, warming the labeled cells at 37°C resulted in a time-dependent increase in the proportion of receptors that were trypsin-resistant, indicating that they had been cleared from the cell surface by endocytosis. The amount of radiolabeled receptor protected from trypsin was measured at each time point by excision of the receptor spot and gamma counting (Fig. 2). In the presence of transferrin, ~50% of the surface-labeled receptor becomes trypsin-resistant in 5 min, as previously shown by Bleil and Bretscher (7). In the absence of transferrin, the receptor still entered the cells at the same rate (Figs. 1 and 2). In four separate experiments the presence of bound transferrin did not influence the rate of endocytosis of this receptor.

A second type of experiment was performed to show directly that transferrin receptors had been internalized with or without complexed transferrin. Transferrin remains bound to its receptor during passage through the acidic environment of the endosome (8, 9), so it seemed probable that the (trypsin-resistant) receptors internalized with bound transferrin would be immunoprecipitable with antitransferrin antibodies, whereas those internalized without transferrin would not be.
Figure 1. Endocytosis of cell-surface transferrin receptors. Transferrin-depleted cells were surface labeled with $^{125}$I at 4°C and warmed to 37°C for the times indicated with (a) and without (b) 0.5 mg/ml ferritransferrin. The cells were rechilled and incubated with or without 0.5 mg/ml trypsin as indicated. After proteolysis, aliquots from each condition (~2 x 10⁶ cells) were dissolved in sample buffer and electrophoresed on 7% acrylamide gels, first under nonreducing and then under reducing conditions. Autoradiographs of the dried gels are shown. The transferrin receptor is the only major disulphide-linked surface protein and migrates as a distinct spot away from the diagonal in the second dimension.

Figure 2. Rate of endocytosis of cell surface transferrin receptors. The transferrin receptor spots in Fig. 1 and in three similar experiments were excised from the dried gel and counted for 10 min in a gamma-counter (LKB Instruments). The number of counts in each spot is expressed as a percentage of those in the spot in the leftmost panels of Fig. 1, i.e., from cells unwarmed and undigested. Means ± SEM from four separate experiments are plotted against time at 37°C. • and ●, minus and plus transferrin, respectively.

Figure 3. Presence and absence of transferrin bound to the endocytosed receptors. TF/E and TF/I refer to the presence or absence of transferrin during endocytosis or immunoprecipitation, respectively. Surface iodinated cells, warmed to 37°C for 10 min in the presence or absence of transferrin, were trypsinized and equal portions (about 5 x 10⁶ cells) were solubilized for immune precipitation (see Materials and Methods). 100 μl of a 50% suspension of protein A-Sepharose coated with antitransferrin antibodies were added to cell lysates with or without 0.1 μg/ml ferritransferrin. The samples were incubated with gentle agitation at 4°C for 3 h. The Sepharose beads were washed four times and adsorbed proteins were eluted in gel sample buffer and run on a 7% gel under reducing conditions. An autoradiograph of the dried gel is shown together with molecular weight markers.

Radiolabeled receptors were allowed to enter the cells by warming to 37°C for 10 min in the presence and absence of transferrin. The cells were chilled, washed free of transferrin, and then trypsinized as described above to remove remaining surface radiolabeled receptors (and transferrin). Immunoprecipitations were performed with an antitransferrin antibody pre-bound to protein A-Sepharose. The immunoprecipitation was performed at pH 5.5 to maintain any apotransferrin-receptor complexes. The immune complexes were analyzed on SDS acrylamide gels. The result, shown in Fig. 3, demonstrates that internalized receptors on "minus transferrin" cells could not be recovered with antitransferrin antibodies, whereas those internalized with bound transferrin could be. As a control, transferrin was added back to parallel samples from both conditions. The internalized receptors on "minus transferrin" cells now became precipitable though not as efficiently as those which were internalized with bound transferrin (Fig. 3). Evidently these receptors had indeed entered the cells without bound transferrin though at the same rate as those complexed with transferrin.

Unoccupied Transferrin Receptors Accumulate in Coated Pits

An indirect index of the ability of the transferrin receptor to continue cycling over longer time-periods without transferrin was obtained by measuring the proportion of receptors, located in coated pits. Cells were depleted of transferrin as before and incubation continued at 37°C for 30 min with and without added transferrin as described in Materials and Meth-
The cells were rapidly chilled, washed, and incubated with an antitransferrin receptor antibody at 4°C. The antibody was first purified by affinity chromatography on protein A-Sepharose to remove serum transferrin. The sites of antibody binding on the cell surface were revealed by a second incubation with 5-nm colloidal gold particles coated with protein A.

The cells were processed for electron microscopy and thin sections examined. The 5-nm gold particles were observed in coated pits whether or not the cells had been incubated with transferrin. No gold particles were observed when the antireceptor antibody was omitted. Patterns of labeling seen on transferrin-free cells can be seen in Fig. 4. Although the receptor was concentrated in coated regions of the membrane (Fig. 4, b and c, and Table I) particles were also seen outside of coated pits (Fig. 4a), often in rows, ~10 nm apart, indicating a patch of receptors. Since serial sections were not examined, it was often difficult to judge whether or not a particle or set of particles was in a coated region or not. Nevertheless, a rough estimate of the proportion of receptors in coated pits was made. The transferrin receptor appeared to concentrate in coated pits at least as well in the absence of transferrin as in its presence (Table I). This finding strongly suggests that the receptor continues to cycle into and out of the cell without bound transferrin.

DISCUSSION

Experiments were described recently in which human hepatoma cells expressing the asialoglycoprotein receptor were allowed to accumulate asialoorosomucoid conjugated to lactoperoxidase. This conjugate accumulated in endosomes and was used to radioiodinate proteins of the endosome membrane (12). The transferrin receptor was one of the most strongly labeled proteins and accounted for a much higher proportion of the label incorporated into the endosome than it did when the cell surface was radioiodinated. This enrichment of the transferrin receptor in endosomes occurred without transferrin being present during the accumulation of the asialoorosomucoid-lactoperoxidase conjugate, which suggested that selective capture of transferrin receptors by coated pits might occur equally well with or without transferrin. Three different types of experiment were performed to examine this question.

First, the rate of entry of surface-iodinated receptors into K562 cells was measured directly without the use of either transferrin or antireceptor antibodies (7). Second, the presence or absence of transferrin bound to the endocytosed receptors was assessed with antitransferrin antibodies. Third, the extent of transferrin receptor clustering in coated pits was examined by electron microscopy. The results show that transferrin receptors enter these cells equally rapidly with or without bound transferrin and that the receptor clusters in coated pits under both conditions.

In their studies on transferrin receptor endocytosis on K562 cells, Klausner et al. (13) concluded that transferrin binding did trigger the endocytosis of the receptor in contrast to the results presented here. The reasons for this discrepancy are not immediately obvious but a potential problem arises in their use of a monoclonal antibody binding assay to identify receptors localized on the cell surface. 30% of the receptors present on the cell surface could not be detected by this assay (13) in contrast to the >95% accessibility to trypsin described here.

Accessibility to trypsin was equally good on cells exposed or unexposed to transferrin and was evidently unaffected by receptor clustering in coated pits (Figs. 1 and 4). On the other hand, the accessibility of the antigenic determinant recognized by the monoclonal antibody might easily be perturbed either

![Figure 4](image-url)
by the presence of bound transferrin or by receptor clustering in coated pits, making interpretation of the results rather difficult.

Whatever the reasons for the discrepancy, the assay used here is simple, quantitative, and direct. Under the conditions described here the binding of ligand is not required for receptor endocytosis. Since the receptors for LDL (4), asialoglycoproteins (5), and \( \alpha_2 \)-macroglobulin protease complexes (6) were also observed to be clustered in coated pits when ligand conjugates were bound to pre-fixed cells, it is likely that these are also continually cycling in the absence of ligand. Using \( ^{125} \)I-labeled epidermal growth factor (EGF) and autoradiography at the electron microscopic level, Gorden et al. (14) observed that, on human fibroblasts, at 0°C 34% of EGF receptors were in coated pits, similar to the proportion of transferrin receptors observed in this study (Fig. 4 and Table 1), which suggests that the EGF receptor may also recycle in the absence of EGF. Further indirect evidence for recycling of the LDL (15), \( \alpha_2 \)-macroglobulin (16), asialoglycoprotein (17), and EGF (18) receptors in the absence of ligand comes from the finding that, in each case, the number of surface receptors falls substantially when the cells are exposed to weak bases or ionophores in the absence of these ligands. Apparently, unoccupied receptors could enter the cells but not recycle properly from an endosome with perturbed pH and ionic composition.

If it is generally true that occupied and unoccupied receptors enter cells equally well, how does this tie-in with the many reports that exposure of cells to various ligands often produces a substantial fall in the number of cell surface receptors (13, 19-23)? This is usually taken as evidence that ligands do induce the clearance of their receptors from the cell surface by endocytosis. However, it has been pointed out that it is very difficult to determine at which point the ligand exerts its effect in the endocytic/exocytic cycle of a receptor (2). It might be return to the cell surface that is slowed down (or abolished), rather than entry that is speeded up. The net result would be the same: a transient (19) or more sustained drop (20-23) in the number of cell surface receptors.

Discrimination between ligand occupied vs. free receptors might occur in the earliest endosome compartment. By increasing the length of time that occupied receptors spend in the acidic environment of the endosome, the probability of ligand-receptor dissociation occurring would be maximized. This would seem to be desirable in the light of Townsend et al.'s (24) recent demonstration that a substantial fraction of internalized asialoglycoprotein-receptor complexes are returned to the cell surface with ligand still bound.

Mellman and colleagues (25, 26) showed recently that the valency of ligands bound to the Fc receptor on macrophages drastically affected the subsequent fate of the endocytosed receptor. Receptors bearing univalent ligands were able to recycle to the cell surface from the endosome whilst those bearing polyvalent ligands could not and were redirected to lysosomes (25, 26). It seems quite possible that receptor fate after endocytosis might also be influenced by the presence or absence of bound ligand.

It might seem pointless to endocytose unoccupied receptors and return them to the cell surface, but this is to view membrane recycling as being solely a means of delivering macromolecular ligands. It might be important to keep the endocytic/exocytic cycle going for other reasons. For example, it was recently shown that endocytosed receptors on substrate-attached cells are not returned to the cell surface uniformly but to the spreading margins of the cell (27, 28). This, together with other findings, led to the suggestion that the cycle might be used to achieve locomotion of the cell over a substrate (29). It would not be desirable to have such an important cellular process as this contingent on the continuous presence of ligands to trigger the endocytic/exocytic cycle.

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