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Receptor-mediated endocytosis of transferrin and the uptake of Fe in K562 cells: Identification of a nonlysosomal acidic compartment

(subcellular fractionation/intracellular pH/fluorescein pH probe)

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ABSTRACT **At physiological temperature, the Fe-carrier transferrin is taken up by K562 human erythroleukemia cells through receptor-mediated endocytosis. Both ligand (now minus Fe) and receptor recycle back to the cell surface where the receptor is rapidly reutilized. After endocytosis, transferrin becomes transiently lodged within an acidic compartment inside the cell, as judged by the changed spectral characteristics and quantum yield of fluorescein isothiocyanate-labeled transferrin that is cell-associated at 37°C. Upon binding to transferrin, anti-fluorescein antibody strongly quenches the emission of the fluorescein-labeled residues on the protein and is used to assess whether the transferrin is at the cell surface (incubation at 0°C) or mainly internalized into the cell (incubation at 37°C). Using Percoll gradient fractionation of postnuclear supernatants, we show that the acidic compartment is not the lysosomal compartment.**

All cells require Fe, and the physiologic apparatus for Fe delivery utilizes a serum glycoprotein, transferrin (1). Despite some controversy concerning the pathway of Fe uptake, there is accumulating evidence pointing to receptor-mediated endocytosis of transferrin, with the subsequent transfer of Fe to the cell (2–5). There has been a great deal of interest in this general cellular process, and certain patterns are emerging from a number of endocytic systems (6). Such endocytosis is an energy-dependent process mediated by specific cell surface receptors, which are generally preferentially internalized through coated pits (7). Although the exact cellular path taken by the ligand is yet to be elucidated, most end up in lysosomes in which they are degraded (8, 9). The pathway taken by the receptor is even less clear. However, in several systems, the receptors clearly are not degraded and are reutilized (10, 11). We have studied certain details of the pathway taken by the asialoglycoprotein receptor (12). We showed that the ligand enters the cell attached to the receptor and that both receptor and ligand are internalized. Soon after internalization, the ligand is released from the receptor, and, after an obligate processing time, the ligand is degraded in the lysosomes. The receptor recycles back to the cell surface. The initial replacement for internalized receptor is derived from a cryptic spare receptor pool.

In striking contrast to many other endocytosed ligands, transferrin is not degraded (3, 13). Rather, mono- or diferric transferrin is taken up, the Fe removed, and the apotransferrin is released into the medium. Data from our laboratory (unpublished observations) concerning the transferrin uptake by K562 cells have led to the following conclusions. The K562 cells contain about 2×10^5 high-affinity ($K_d = 1 \times 10^{-9}$ M) transferrin surface-receptors per cell. Transferrin containing one or two ferric ions binds to these receptors and, when the cells are warmed, is rapidly internalized ($t_{1/2} = 3$ min). Upon internalization of the receptor–transferrin complex, the Fe is removed from the transferrin and, in a yet unknown way, shuttled to ferritin. Apotransferrin is released from the cell after a minimum time of about 4 min. Receptors appear to be internalized with the ligand bound to them and rapidly recycle. The result of this recycling is a linear uptake of Fe with a rate coefficient of ≈0.1 min^{-1}. Excess transferrin down-regulates the number of surface receptors to ≈30% of the initial value. Despite this down-regulation, the recycling of receptors and uptake of Fe continues for hours, even in the absence of protein synthesis. In this paper, we describe attempts to localize the transferrin and the Fe after internalization.

MATERIALS AND METHODS

Cells and Ligands. K562 Cells were grown in RPMI 1640 medium containing 10 mM Hepes and 10% fetal bovine serum. Cells were grown to a density of 5×10^5 cells/ml. Before use, cells were washed with phosphate-buffered saline (P_i/NaCl) at 0°C. Incubations were performed either in P_i/NaCl (for fluorescence experiments) or RPMI medium (for gradient experiments). Human transferrin (Calbiochem) was passed over an S-200 (Pharmacia) column to remove any aggregated protein. This material showed a single peak in HPLC. Diferric transferrin was used in all experiments. Saturation of transferrin with Fe was performed as follows. Transferrin (6 mg) was dissolved in 1 ml of 0.25 M Tris chloride, pH 8.0/10 μM $NaHCO_3$, and 20 μl of 100 mM disodium nitrilotriacetate/12.5 mM $FeCl_3$ was added. The sample was incubated at 37°C for 30 min and then passed over a PD-10 column (Sephadex G-25, Pharmacia) that had been equilibrated with 0.15 M NaCl/0.02 M Tris chloride, pH 7.4. The amount of Fe bound by the transferrin was estimated from the A_{465nm}/A_{280nm} ratio, which was routinely found to be ≈0.046, consistent with full saturation.

Preparation of [^{59}Fe]Transferrin. Transferrin was first treated with sodium ascorbate to remove unlabeled Fe. Transferrin (5 mg) was dissolved in 0.5 ml of 0.1 M Tris chloride (pH 8), 4 mg of sodium ascorbate was added, and 0.5 M sodium phosphate (pH 5.1) was then added to bring the final pH to 5.8. After incubation at 37°C for 30 min, the sample was passed over a PD-10 column equilibrated with 0.25 M Tris chloride, pH 8.0/10 μM $NaHCO_3$. $^{59}FeCl_3$ [100 μl at 1 mCi/ml (1 mCi = 37 MBq); 70 μg ^{59}Fe/ml, Amersham] was added to 500 μl of 100 mM disodium nitrilotriacetate, and this solution was combined with the transferrin solution and incubated at room temperature for 1 hr. The [^{59}Fe]transferrin solution then was passed over a PD-10 column equilibrated with 0.15 M NaCl/0.02 M Tris chloride, pH 7.4, to remove nitrilotriacetate and unincorporated ^{59}Fe.

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Abbreviations: FITC, fluorescein isothiocyanate; P_i/NaCl, phosphate-buffered saline; ^{125}I- and ^{131}I-transferrin, ^{125}I- and ^{131}I-labeled transferrin.

Iodination of Transferrin. Diferric transferrin in 0.15 M NaCl/0.02 M Tris chloride, pH 7.4 was combined with 50 μl of lactoperoxidase/glucose oxidase suspension immobilized on agarose beads (Enzymobeads; Bio-Rad). $Na^{125}I$ (10 μl; 100 mCi/ml, Amersham) or $Na^{131}I$ (40 μl; 400 mCi/ml, Amersham) followed by 50 μl of 1% β-D-glucose were added to this mixture. The sample was incubated at room temperature for 30 min and then passed over a PD-10 column equilibrated with 0.15 M NaCl/0.02 M Tris chloride, pH 7.4. The specific activities of ^{125}I- and ^{131}I-labeled transferrin (^{125}I- and ^{131}I-transferrin) were both ≈600–900 cpm/ng.

Labeling of Transferrin with Fluorescein Isothiocyanate (FITC). Diferric transferrin (8 mg) was treated with 1.3 mg of FITC I (10% wt/wt on celite) (Calbiochem) in 1.1 ml of 0.1 M sodium borate (pH 9.3) for 30 min at 0°C with continuous stirring. The reaction was stopped by removing the FITC-celite particles from the mixture by centrifugation for 5 min at top speed in an Eppendorf centrifuge. The protein-FITC conjugate was subsequently freed from noncovalently bound FITC by dialysis (Spectrapor no. 3) against three changes of a 500-fold excess of 0.15 M NaCl/0.02 M Tris·HCl, pH 7.3, at 4°C. The mole ratio of FITC to protein in the conjugate was assessed by the method of Jobbagy and Kiraly (14). Under the conditions given above, the conjugate was found to contain—on the average—8 mol of FITC per mol of protein.

Incubation and Fixation of Cells. Cells (10^7 cells/ml; washed three times with P_i/NaCl) were incubated with FITC-transferrin at 0°C for 10 min or at 37°C for 30 min in RPMI 1640 medium supplemented with 1% fetal bovine serum. The cells were then washed three times with P_i/NaCl at 0°C and subsequently fixed (overnight at 0°C) in 2.5% paraformaldehyde in 0.15 M sodium cacodylate, pH 7.3. Control incubations, in which the uptake of ^{125}I-transferrin and FITC/^{125}I-transferrin by cells was compared, showed that the presence of eight fluorescein residues on the protein did not alter its uptake characteristics.

Anti-fluorescein Antibody. Anti-fluorescein antibody (affinity-purified on FITC-bovine serum albumin-substituted Sepharose) was a gift from P. Henkart. The protein concentration in the antibody preparation (as used) was 250 μg/ml in P_i/NaCl.

Microscopy. Prior to microscopy, cells were washed three times with ice-cold P_i/NaCl to remove the fixer solution and were taken up in P_i/NaCl (final density, 4×10^7 cells per ml). Fluorescence microscopy was carried out with a Zeiss standard microscope equipped with a high and low cutoff filter combination for fluorescein. To test the effect of anti-fluorescein antibody on the microscopic image of the fluorescent cells, 50 μl of antibody solution was added to 250 μl of cell suspension (equivalent to 10^7 cells). The mixture was allowed to sit at 0°C for 1 hr prior to microscopy.

Fluorescence Spectroscopy. All fluorescence measurements were done with a Perkin–Elmer MPF 44B spectrofluorometer interfaced to a Bascom–Turner microcomputer. Temperature was maintained at 4°C with a Haake circulating water bath. For calibration curves, FITC-transferrin in P_i/NaCl or 10 mM acetate-buffered saline was adjusted to the desired pH with NaOH or HCl, and the pH in the cuvette was checked. Cells at 1×10^7/ml were incubated with either FITC-transferrin or unlabeled transferrin in P_i/NaCl (final concentration, 100 μg/ml) at either 0°C or 37°C. The cells were then washed three times with 50 ml of ice-cold P_i/NaCl, resuspended (final concentration, 1×10^7 cells per ml), and kept on ice; 2 ml of cell suspension, placed in a 1-cm-path-length cuvette and mixed with a Pasteur pipette, was used for each measurement. The fluorescence intensity at 520 nm was measured separately for the excitation wavelengths of 490 and 460 nm. Three time-averaged readings with a 5-sec signal integrator were recorded. The cells were then mixed again, and a further reading was made. There was no sedimentation of the cells in the cuvette over this time period as judged by the absence of a systematic change of the fluorescence signal. Immediately after the readings on FITC-transferrin-treated cells, the blank values were obtained by making identical readings with transferrin-treated cells. We noted that autofluorescence was low in these cells compared to fibroblasts or hepatocytes. For cells labeled with FITC-transferrin at 0°C and 37°C, the background signals were <10% and <17%, respectively, of the peak intensities at 490 nm.

Gradients. After incubation with the appropriate ligand at either 0°C or 37°C, cells (2.5×10^7 cells/ml) were washed thoroughly free of unbound ligand at 0°C. They were then taken up in homogenization buffer (0.25 M sucrose/10 mM Tris/1 mM EDTA, pH 8.0) at 1.25×10^7 cells/ml and homogenized in a tight-fitting Dounce ("B" pestle) glass homogenizer with 40 up and down strokes. This resulted in disruption of more than 95% of the cells, as estimated by phase microscopy. The homogenates were spun at $1,000 \times g$ for 10 min in a Sorvall SS34 rotor. The supernatants were mixed with an appropriate amount of a stock solution of 40% Percoll in homogenization buffer (see figure legends). The final volume was adjusted to 12 ml with homogenization buffer. Gradients were run in a type 65 rotor at 12,500 rpm at 4°C. Running time (≈45 min) was determined by a preset $\omega^2 t$ limit of 4.55×10^9. Gradients were collected at 4°C with a Hoeffer gradient puncture device and a Gilson fraction collector; ≈40 fractions were collected per gradient. Radioactivity in samples was determined with a Beckman gamma 5000 counter. ^{131}I and ^{125}I double-label assay was carried out with a Beckman gamma 8000 counter with a program mode for correction of ^{131}I spillover into the ^{125}I channel.

RESULTS

Fluorescence Microscopy. Incubation of cells with FITC-labeled diferric transferrin at 0°C for 30 min formed a faint peripheral fluorescence on top of a diffuse fluorescence over the cell body (Fig. 1*A*). The large mass of the nucleus was very poorly distinguished in the fluorescent cells, indicating that the label most likely resides at the cell surface, not in the cytoplasm. Upon focusing up and down, the peripheral fluorescence was seen to follow the contours of the cell. This supports the impression of surface staining as the predominant specific fluorescence over the background. In contrast, cells that were incubated with the fluorescent transferrin at 37°C for 30 min fluoresced mainly from numerous brilliant dots; in the majority of the cells, a large single cluster of fluorescent dots was seen (Fig. 1*B*).

Binding of anti-fluorescein antibody to FITC-substituted proteins results in a dramatic quenching of the FITC quantum yield (15). Addition of anti-fluorescein antibody to cells incubated at 0°C resulted in a nearly complete loss of cell-associated fluorescence, leaving behind only a rapidly fading fluorescence that appeared to be identical to the weak autofluorescence observed in cells treated with unlabeled transferrin. The appearance of cells incubated with FITC-transferrin at 37°C did not change upon addition of the antibody. These observations indicate that, in cells incubated at 0°C, the vast majority of cell-associated transferrin molecules are located at the cell-surface, whereas, with cells incubated at 37°C, they are inaccessible to the antibody and, thus, are most likely internalized by the cells. After incubation of cells with the fluorescent ligand at either 0°C or 37°C, the level of cell-associated fluorescence did not vary greatly from cell to cell (Fig. 1 *A* and *B*). At present, we do not know the meaning or nature of the clusters of fluorescent dots seen inside cells upon incubation with FITC-transferrin at 37°C. When unfixed cells, incubated with FITC-trans-

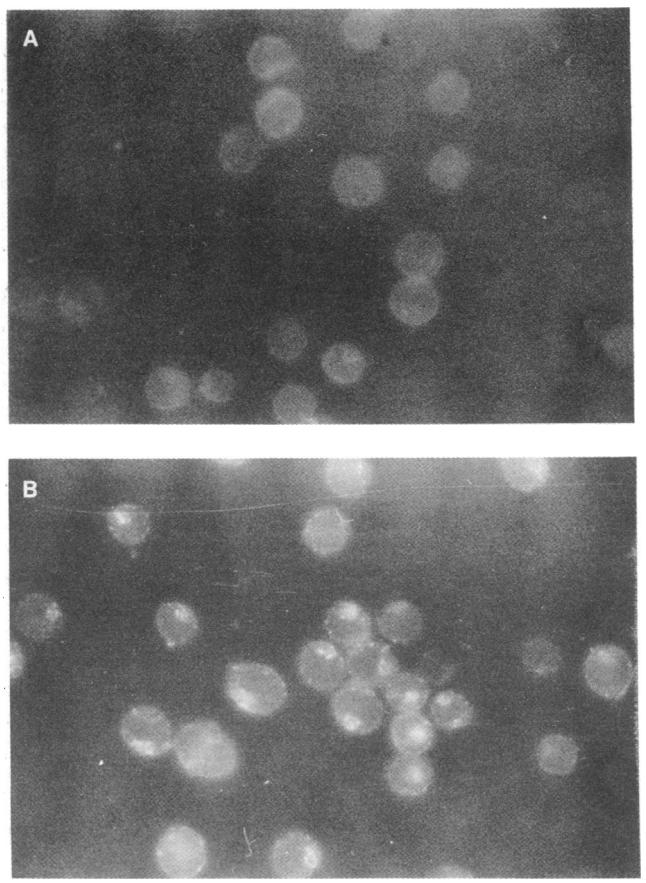

FIG. 1. Fluorescence micrographs of K562 cells after an incubation with FITC-transferrin (final concentration, 0.5 mg/ml) at either 0°C for 30 min (*A*) or 37°C for 30 min (*B*). A Leitz standard microscope, equipped with a IV FL fluorescence accessory and a ×65 Neofluar oil immersion objective, was used.

ferrin at 0°C, were allowed to warm to room temperature during microscopy, the evenly distributed fluorescence first became "grainy"; thereafter, a large part of the grains started to cluster.

An Internal Acidic Compartment. Cells were washed extensively in P_i/NaCl and incubated with FITC-transferrin at either 0°C for 10 min or at 37°C for 30 min. Both preparations were thoroughly washed free of any unbound ligand at 4°C and suspended in P_i/NaCl at 1×10^7 cells per ml. Cells treated with nonlabeled transferrin served as a control for autofluorescence and scatter corrections. At 0°C, the fluorescence could be completely quenched by anti-fluorescein antibodies. In contrast, at 37°C only about 25% of the fluorescence was quenched (Fig. 2). Upon addition of 0.5% (final concentration) Triton X-100, complete quenching was attained. At this concentration, the detergent did not affect the fluorescence of FITC-transferrin. These findings are consistent with all of the label being accessible to antibody at 0°C in contrast to the majority being internalized after incubation at 37°C. The difference in the accessibility of the FITC-transferrin to externally added antibody after incubation at 0°C and 37°C correlates well with two other methods used to distinguish surface from internal transferrin. After binding of transferrin to cells at 0°C, >95% of the ligand could be removed by a 5-sec wash with 0.25 M acetic acid/0.25 M NaCl. A comparable amount could be removed by externally added proteases. After incubation of transferrin with cells at 37°C for 30 min, only ≈10–15% of the cell-associated transferrin could be removed by these two techniques (Fig. 2*A*).

Fluorescein is an excellent pH indicator because of the al-

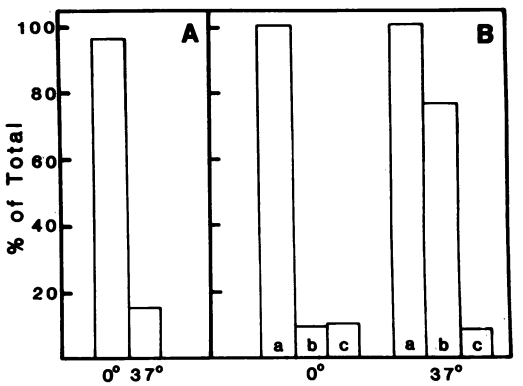

FIG. 2. (*A*) Cells (10^7 cells per ml) were incubated with ^{125}I-transferrin (10 μg) for 20 min at either 0° or 37°C. The cells were washed for 5 sec with 0.25 M acetic acid/0.25 M NaCl and spun through dibutylphthalate to separate cell-associated from acid-released transferrin. After incubation at 0°C, 96% was releasable by acid, whereas only 17% was releasable after incubation at 37°C. (*B*) Cells labeled with FITC-transferrin at either 0°C or 37°C and washed free of unbound ligand. The cell suspension was then placed in the cuvette of the fluorometer and kept at 4°C. Bars: a, fluorescence of cells; b, fluorescence intensity after the addition of anti-fluorescein antibody; and c, fluorescence intensity after addition of anti-fluorescein antibody and Triton X-100.

terations in fluorescence that accompany the titration of the carboxyl group. The shift in its excitation spectrum provides the most specific measure of pH (16). Calibration curves were obtained with FITC-transferrin and can be represented accurately by the ratio of fluorescence intensities at 520 nm when excitation was at either 490 or 460 nm (Fig. 3).

For cells labeled with FITC-transferrin at 0°C, the ratio of intensity (490 nm/460 nm) was 3.62 ± 0.11, consistent with the pH of the external buffer. After addition of detergent, the ratio was 3.52 ± 0.06. For cells at 37°C, the ratio was 2.03 ± 0.22. The mean ratio corresponds to a pH of 5.5, and the error bars correspond to a pH range of <5.0–5.8. After addition of detergent, the excitation ratio was 3.64 ± 0.1.

Localization of the Internal Transferrin and Fe on Density Gradients. We attempted to localize internalized transferrin on density gradients to determine (*i*) whether it resides either within a vesicular compartment inside the cells or remains

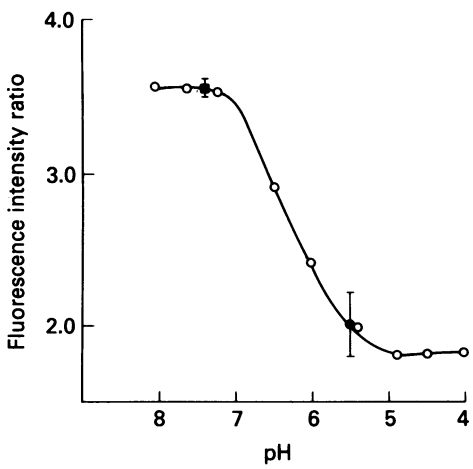

FIG. 3. The data from the FITC-transferrin calibration spectra are plotted (○) as the ratio of the fluorescence intensity at 490 nm to that at 460 nm. This ratio is shown (mean ± SEM) for FITC-transferrin bound to cells at 0°C (■) and for FITC-transferrin associated with cells after incubation at 37°C (●).

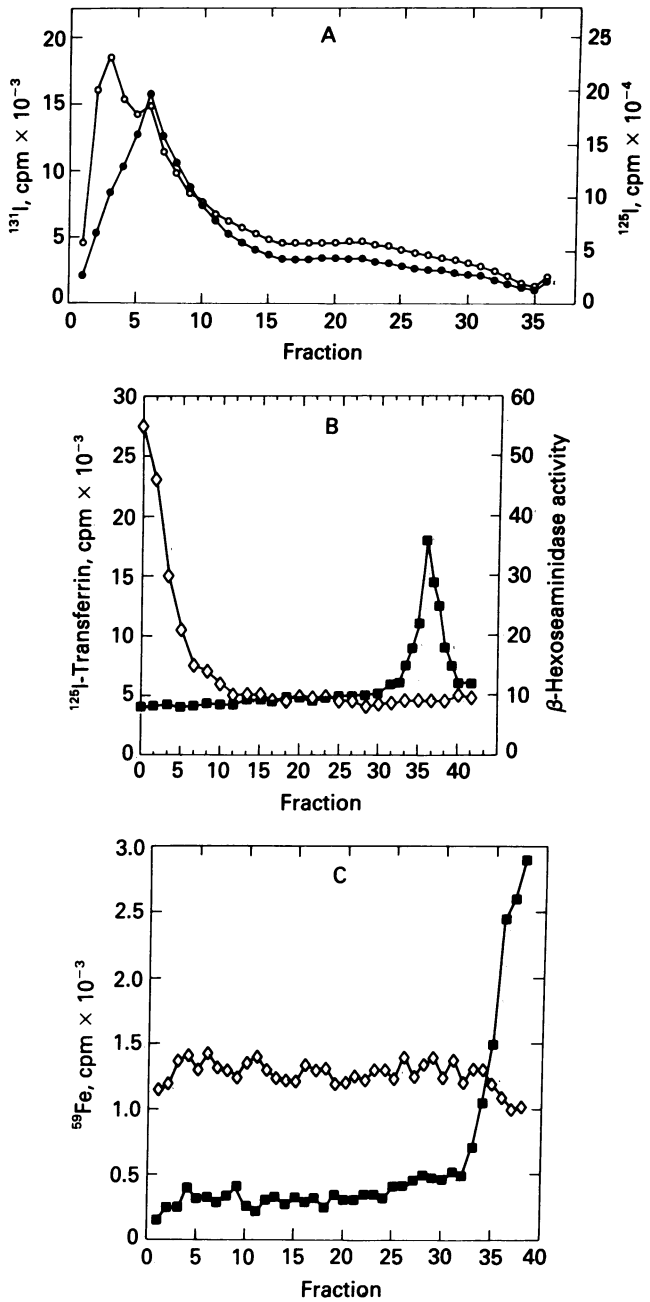

FIG. 4. (*A*) Localization of ^{131}I-transferrin (●) that had been bound to cells at 0°C and of ^{125}I-transferrin taken up by cells at 37°C. The cells from either type of incubation were mixed and homogenized. The ^{131}I-transferrin and ^{125}I-transferrin-containing homogenates were then combined and mixed with Percoll to give a final Percoll concentration of 8%. (*B*) Homogenate of cells that had taken up ^{125}I-transferrin at 37°C for 30 min, mixed with Percoll to give a final concentration of 15%. ■, Localization of the ^{125}I-transferrin; ◇, localization of the lysosomal marker enzyme β-hexoseaminidase. (*C*) Cells were loaded for 30 min with Fe^{59}-labeled diferric transferrin. After homogenization, the cell homogenate was either mixed with 7% Percoll (◇) or layered over 7% Percoll (■). The peak of Fe^{59} radioactivity when the homogenate was layered over the gradient represents the location of the initial 2-ml overlay. Thus, none of this Fe^{59} entered the gradient. β-hexoseaminidase activity is shown in arbitrary units.

bound to a membrane and (*ii*) whether the internalized transferrin (taken up at 37°C) can be separated from the plasma membrane-bound material (bound at 0°C). Cells were loaded at 0°C with ^{131}I-transferrin or at 37°C with ^{125}I-transferrin. After incubation at 0°C, >95% of the transferrin could be released by a wash with 0.25 M (final concentration) acetic acid/0.25 M NaCl. In contrast, after a 37°C incubation for 30 min, only about 15% of all cell-associated ^{131}I-transferrin was releasable in acid. After incubation at each temperature with the respective label, cells were mixed, homogenized at 4°C, and added to Percoll for gradient centrifugation. The profile of the gradient (Fig. 4*A*) showed that the two labels were separated. The surface bound ^{131}I was localized to a single peak at a density of 1.02 g/ml. The majority of the ^{125}I-transferrin bands were at a density of 1.03 g/ml, but a clear shoulder was present at the density of the surface-bound material. Because the compartment containing the internal transferrin was acidic, we examined whether the low-density (1.03 g/ml) compartment contained the lysosomal enzyme β-hexosaminidase. Fig. 4*B* shows that this compartment contained none of this lysosomal enzyme and that all lysosomes traveled as a much more dense compartment. Virtually all of the hexosaminidase activity was found at the bottom of the tube, assuring us of minimal lysosomal breakage during homogenization. We also attempted to localize the Fe that was delivered to the cells by the transferrin. Because Fe is being continuously deposited in the cells, we monitored the position of ^{59}Fe in gradients loaded with homogenates of cells that had been incubated with [^{59}Fe]transferrin at 37°C for times ranging from 30–150 min. Thorough mixing of the homogenate with the Percoll resulted in a uniform distribution of the ^{59}Fe cpm throughout the gradient, consistent with the Fe being in the cytosol. To check whether the Fe was not migrating to many different densities by virtue of being localized to many different vesicular compartments, we overlayered gradients of 7% Percoll with the cell homogenates. Under these circumstances, essentially all of the ^{59}Fe counts failed to enter the gradient (Fig. 4*C*).

DISCUSSION

It has been proposed that ligands which enter the cell by a process of receptor-mediated endocytosis rapidly move to a vesicular compartment termed the receptosome (17). Recently, α_2-macroglobulin has been shown to enter an internal compartment that has a pH of about 5.0 (18). Upon internalization by K562 cells, transferrin enters a compartment with a pH of 5.4 (±0.4–0.5 pH units). This measurement includes a contribution from the fluorescent transferrin still on the cell surface. Any FITC-transferrin in a nonacidic environment (whether surface or internal) will raise the measured average pH and, in fact, because of the greater quantum yield at higher pHs, will disproportionately raise the 490/460 fluorescence ratio. Thus, the pH measured is likely to represent an upper limit of the pH in the acidic compartment. We cannot absolutely rule out factors other than pH that might account for the altered excitation spectrum. We found (data not shown) that the excitation spectra were not altered over a 100-fold concentration range of FITC-transferrin. Okhuma and Poole (16) showed that a variety of buffers failed to alter the pH-dependent excitation spectral changes. We can compare the pH calibration curve that was obtained with FITC-transferrin to that obtained by Okhuma and Poole for FITC-dextran (16). Extrapolation from their published spectra shows that the 490/460 ratio ranges from 4.1 to 1.55, whereas ours goes from 3.65 to 1.78 over the same pH range.

In most receptor-mediated endocytic systems, the ligand is delivered to the lysosomes. Transferrin internalization does not lead to degradation; after the deposition of Fe in the cells, the transferrin is released from the cell (3). The endocytic system for transferrin seems to be shunted away from the lysosomal pathway. In this paper, we show that transferrin is delivered to a nonlysosomal acidic compartment. At no point in its cellular

journey is transferrin found in lysosomes. The low pH within the compartment harboring the internalized transferrin may play a role in destabilizing the Fe–transferrin complex, allowing Fe removal (1). The fact that ammonium chloride inhibits Fe transfer to cells by transferrin (19), lends support to this view. Recent studies in our laboratory have revealed that the acceptor for the Fe is ferritin (unpublished data). The data given in this paper suggest that this ferritin is located in the cytosol and that it is not associated with any membranous subcellular compartment. A caveat to this statement is that if ferritin originally was contained within vesicular structures, it could have been liberated during homogenization. However, complete rupture of such vesicular structures would seem unlikely because lysosomes appear to remain fully intact during homogenization.

For many years, it was uncertain whether Fe was taken up by cells from transferrin by a transport mechanism across the plasma membrane or whether the transferrin along with the Fe was internalized. Our results suggest that both an endocytic and a transport mechanism function in this system. First the transferrin–Fe complex is internalized through the transferrin receptor. The combined transferrin–Fe–receptor complex enters the nonlysosomal acidic compartment, where the Fe is removed from the transferrin. The Fe is then transported across the vesicular (not the plasma) membrane and delivered to cytosolic ferritin. This scenario is similar to the uptake of enveloped viruses in which the virus is endocytosed into an acidic compartment, which allows the fusion of the envelope with the vesicular membrane and the extrusion of the nucleocapsid into the cytosol (20).

Many questions remain concerning the acidic compartment described in this paper—e.g., how is the pH maintained, from which membrane is it derived, and is this compartment used by many different receptors? It is now clear that the lysosome is not the only acidic subcellular organelle. As reported for α-macroglobulin (18) and demonstrated here, there exists a nonlysosomal acidic compartment that occupies a central position in the pathway of receptor-mediated endocytosis. The low pH within the compartment harboring the internalized transferrin may play a role in destabilizing the Fe–transferrin complex, allowing Fe removal (1). The fact that ammonium chloride inhibits Fe transfer to cells by transferrin (19) lends support to this view. The role of the acid environment inside the compartment is unknown, but we can speculate that in receptor systems other than transferrin, it serves to separate ligand from receptor, allowing the receptor to be salvaged from the lysosomal destruction that is the fate of many ligands (20). In contrast to other systems, transferrin remains bound to its receptor, even at a pH of 3.5 (unpublished observations). Why transferrin does not reach the lysosome and is not degraded is unclear. The failure of transferrin to be released from its receptor at the pH of this compartment may be the reason. Most likely the low internal pH is central to the role of this organelle—e.g., in facilitating the removal of Fe from transferrin in this system and in dissociating ligands from receptors in other systems.

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