Iterative endocytosis of transferrin by K562 cells

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The effect of iron on the exocytosis of transferrin by K562 cells was studied by first allowing the cells to endocytose apotransferrin or diferric transferrin. Subsequent release of the apotransferrin was very rapid with a $t_{\frac{1}{2}}$ of 3.01 min, compared with 5.5 min for diferric transferrin. Release of apotransferrin was slowed by the weak base methylamine, $t_{\frac{1}{2}}$ 8.0 min, but the effect of this agent was substantially greater when iron–transferrin was used, $t_{\frac{1}{2}}$ 18.65 min, suggesting that methylamine affects both iron removal and receptor recycling. Release of iron–transferrin could be accelerated to a rate comparable with that of apotransferrin by addition of the permeant iron-chelator desferrioxamine. The difference in the rates of release of different forms of the protein could be explained by the re-endocytosis of the iron-rich protein, a process detected by the accelerated release of transferrin when the cells were washed in medium at pH 5.5 containing an iron-chelator or treated with a protease-containing medium to digest transferrin accessible at the cell surface. It appears that in cells incubated under control conditions, re-endocytosis of transferrin, which is incompletely depleted of iron, occurs and that a transferrin molecule may make two passes through the cell before all the iron is removed. This mechanism helps to explain why very little iron–transferrin is released from cells and why the efficiency of the iron uptake process is so high.

INTRODUCTION

It is now well established that during the delivery of iron to cells the metal-binding protein transferrin initially binds to a cell-surface receptor [1], undergoes endocytosis [2] and, after a short sojourn in the cell, is exocytosed, iron-depleted [3] but otherwise intact. Kinetic studies have indicated that the transit time for the transferrin molecule is of the order of 16 min in both HepG2 cells [4] and in K562 cells [3,5]. The process is remarkably efficient, with about 85% of the transferrin iron being removed by the cells [3]. Although the average time for release of the molecule is of the order of 10 min [4,5] we have found that the first molecules to be released appear after a much shorter period (2–3 min) and yet the transferrin released under these conditions is still substantially iron-free [3]. Little is known of the mechanisms by which apotransferrin is preferentially released from the cell, although two sites at which control could be exerted are apparent. Apotransferrin molecules generated within the endosome could be selectively removed and enter the exocytic pathway. However, this would require a mechanism to differentiate between receptors occupied by iron-free and iron-loaded transferrin as well as complex intracellular trafficking machinery. A simpler scheme has its origins in our previous observation that apotransferrin binds to the receptor with a relative affinity of 1, monoferric with an affinity of 5–6 and diferric 23 [6]. Selective entry of iron-free molecules into the exocytic pathway would not be required as release of the transferrin molecules at the cell surface would be determined by the relative affinity of the ligand for its receptor and hence its iron content. A requirement of this scheme would be the re-endocytosis of any transferrin molecules carrying iron. Such a process would help to explain both the efficiency of the iron-removal process and the difference between minimum and average transit times observed.

In the present study we have used a number of techniques to test the possibility that transferrin undergoes iterative endocytic cycling in K562 cells and that this process contributes to the efficient removal of iron from the internalized molecule. These include the protease and acid stripping of previously endocytosed transferrin appearing at the cell surface, and the addition to the cells of ^{125}I-labelled chemically prepared apotransferrin which allows for an endocytic cycle uncomplicated by the process of iron removal. We have found that recycling of iron–transferrin does occur, with the average transferrin molecule undergoing two rounds of endocytosis before complete iron removal is achieved and the iron-free molecule is released from the cell.

MATERIALS AND METHODS

Materials

Minimal essential medium (MEM), RPMI 1640 and fetal-calf serum were obtained from Gibco Europe, Paisley, Scotland, U.K. and all plasticware for tissue culture from Sterilin, Teddington, Middx., U.K. BSA (fraction V) and methylamine were from Sigma Chemical Co., Poole, Dorset, U.K. and Pronase B (Calbiochem) was from CP Laboratories, Bishops Stortford, Herts., U.K. Chelex 100 was bought from Bio-Rad Laboratories, Bromley, Kent, U.K. and desferrioxamine mesylate from Ciba Laboratories, Horsham, Sussex, U.K.

Transferrin preparation and radioisotopic labelling

The methods used for purify transferrin from pooled human plasma and to label it with ^{125}I (Amersham International, Amersham, Bucks., U.K.) have been described in full elsewhere [6]. Apotransferrin was prepared as described previously [6], after radiolabelling of diferric transferrin, by dialysis against 0.1 M sodium citrate/acetic acid, pH 4.5. Specific radioactivities of both forms of the protein were in the range 150–350 c.p.m./ng of transferrin.

Abbreviation used: MEM, minimal essential medium.

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Cell culture

K562 cells were obtained from the Imperial Cancer Research Fund, London, U.K. and grown in RPMI 1640 supplemented with 20 mM Hepes, 10% (v/v) fetal-calf serum and antibiotics [penicillin (50 units/ml) and streptomycin (50 mg/ml)]. Cells were cultured at 37 °C in 75 cm^2 tissue culture flasks at densities of 5×10^5 to 1×10^6 cells/ml under 5% CO_2/air. Cells were maintained in exponential phase by dividing 1 to 2 with fresh medium every 48 h. Experiments were performed on cells that had been fed 24 h previously. Before use all cells were incubated three times at 37 °C for 15 min in MEM containing 1% (w/v) BSA to free them of endogenous transferrin [7]. Cell viability was > 98% as assessed by Trypan Blue exclusion.

Uptake of apotransferrin

The specificity of uptake of ^{125}I-labelled apotransferrin by K562 cells was investigated by incubating cells with saturating concentrations of labelled protein in the presence or absence of unlabelled diferric transferrin to block receptor-mediated uptake. K562 cells ($1–2 \times 10^7$ cells/ml) were incubated in three changes of MEM with Hepes, containing 1% (w/v) BSA, for 15 min at 37 °C to free them of bovine transferrin [7]. The cells were then incubated with 6.25 μM ^{125}I-labelled apotransferrin in siliconized glass pots at 37 °C with shaking under 5% CO_2/air in iron-depleted medium prepared as described below. Non-specific uptake was estimated from parallel incubations to which 12.5 μM unlabelled diferric transferrin had been added before addition of the labelled protein. Cells were recovered at intervals by pipetting into ice-cold PBS, centrifugation at 400 $g_{av.}$ and washing three times in this buffer. Cell pellets were counted in an LKB Compugamma γ-counter.

Exocytosis of apo- and diferric-transferrin

To measure transferrin release, washed cells ($1–2 \times 10^7$/ml) were incubated in MEM, containing 1% (w/v) BSA and 25 mM Hepes, at 37 °C under 5% CO_2/air in siliconized glass tubes, with apo- or diferric-transferrin labelled with ^{125}I for 15 min at 37 °C. Saturating concentrations of the ligands were used [6] which were 310 nM for diferric transferrin and 20-fold higher, 6.25 μM, for apotransferrin. Free iron was removed from the medium used in these incubations and for washing the cells by passing MEM, containing 1% (w/v) BSA and 25 mM Hepes, over Chelex 100 (bed volume 50 ml) equilibrated in 25 mM Hepes, pH 7.4. The concentrations of Ca^{2+} and Mg^{2+} were restored to 1.8 mM and 0.8 mM respectively after this treatment. We have previously shown that medium produced in this manner allows apotransferrin to remain iron-free during incubations with cells [3]. After loading with either diferric transferrin or apotransferrin cells were washed three times in MEM containing 1% (w/v) BSA at 4 °C and then rapidly re-warmed to 37 °C in medium containing 310 nM unlabelled diferric transferrin. Cells were recovered by pipetting into cold PBS with centrifugation at 400 $g_{av.}$ and counted in an LKB Compugamma γ-counter. The half-times for transferrin release were derived from the slope of a semi-logarithmic plot of the decrease with time of the cell-associated radioactivity [3,4]. To determine the effect of perturbing intravesicular pH on the exocytic process some incubations were performed in the presence of the lysosomotropic weak base, methylamine [8]. Cells were pre-incubated with 25 mM methylamine for 30 min at 37 °C before loading with labelled transferrin and all washing and incubation media contained methylamine at the same concentration as in the initial incubation. Cell viability, judged by Trypan Blue exclusion, was unaffected by the presence of methylamine. In other experiments cells were pre-incubated with 100 mM desferrioxamine for 30 min at 37 °C before loading with labelled transferrin. Again, cell viability was unaffected by the compound [9].

Acid stripping of surface iron–transferrin

Cells were loaded with ^{125}I-labelled diferric transferrin at 37 °C as described before and were then washed in the cold. They were then suspended in 1 ml of ice-cold 25 mM acetate/150 mM NaCl, pH 5.5, containing 100 mM desferrioxamine to render surface transferrin iron-free [10]. After 5 min 5 ml of ice-cold MEM, containing 25 mM Hepes and 1% (w/v) BSA, was added to restore the pH to 7.4 and so release the iron-free transferrin from the cell surface [10,11]. The cells were centrifuged down. The supernatants were collected and the cells were suspended in 1 ml of warm MEM and incubated at 37 °C for 2 min. Ice-cold PBS (2 ml) was added and the cells were again centrifuged and resuspended in the same sequence of buffers: i.e. the acid buffer followed by MEM. The sequence of buffers was repeated four times. A suspension of control cells was taken through the same procedure but with the substitution of PBS (pH 7.3) for the acid/desferrioxamine buffer. Cell viability, judged by Trypan Blue exclusion, was shown to be unaffected over the duration of the experiment by this acid-washing procedure.

Protease stripping of surface transferrin

In addition to the acid-stripping method used to detect previously endocytosed transferrin appearing at the cell surface, a second procedure, using protease digestion, was employed [2]. Cells (8×10^7), freed of bovine transferrin as described above, were incubated with 310 nM ^{125}I-labelled diferric transferrin for 15 min at 37 °C in MEM containing 1% (w/v) BSA and Hepes under 5% CO_2/air. The cells were then washed three times in cold medium and divided into two aliquots. One was resuspended in cold MEM containing Pronase B (100 mg/ml) and incubated at 4 °C for 20 min, while the second sample was incubated in MEM alone. The cells were then centrifuged at 4 °C and the transferrin-containing supernatant harvested. The cell pellets were then resuspended in warm MEM containing 1% BSA (3 ml) and incubated for 2 min at 37 °C to allow exocytosis to proceed. Transferrin release was halted by the addition of ice-cold MEM (5 ml) and the cells were pelleted by centrifugation at 4 °C. This sequence of buffers and temperature shifts, providing protease digestion of surface ^{125}I-transferrin followed by a period of exocytosis at 37 °C, was repeated four times. At the end of the experiment the cell pellets and all the buffers and wash media were made up to the same volume (5 ml) and radioactivity counted in the γ-counter. Viability of the protease-treated and control cells was assessed by Trypan Blue exclusion at the conclusion of the experiment and was found to be > 95%.

RESULTS

Transferrin exocytosed from cells is predominantly in the form of apotransferrin with only minor amounts of the monoferric and diferric species [3]. This suggests that some mechanism, to allow specific release of the iron-free molecule, is operating and thus ensuring the efficiency of the iron-uptake process. If this is the case then the release of apotransferrin might occur more rapidly than that of iron–transferrin. K562 cells were therefore loaded with chemically prepared ^{125}I-apotransferrin and its rate of release was compared with that of iron–transferrin. As this

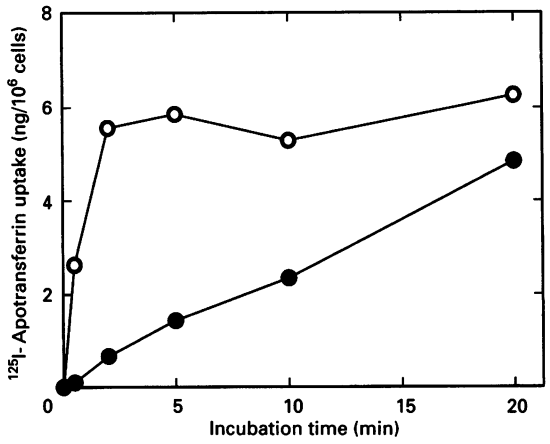

Figure 1 Uptake of ^{125}I-apotransferrin by K562 cells

Shown are the non-specific (●) and specific (○) uptake with time. At the 15 min point > 70% of the uptake could be accounted for by specific receptor-mediated endocytosis.

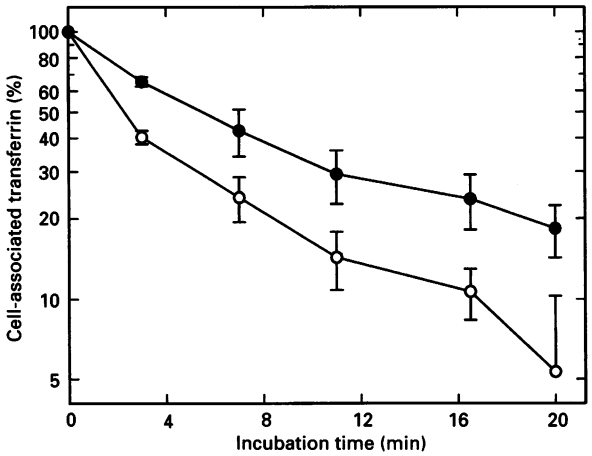

Figure 2 Release of diferric transferrin and apotransferrin from K562 cells

K562 cells (8–10 × 10^6 cells/ml) were pre-incubated in iron-free MEM containing 1% (w/v) BSA at 37 °C for 15 min with either ^{125}I-apotransferrin (6.25 μM; ○) or ^{125}I-diferric transferrin (310 nM; ●) to allow endocytosis to occur. Cells were then washed three times in MEM containing 1% BSA at 4 °C and then re-warmed to 37 °C in medium containing 310 nM unlabelled diferric transferrin. The 100% point was in the range of 2.0–4.1 × 10^4 c.p.m. Cells were recovered by pipetting into ice-cold PBS followed by centrifugation at 400 $g_{av.}$ Cell-associated ^{125}I was measured by γ-counting. Shown are the means ± S.D. of three independent experiments.

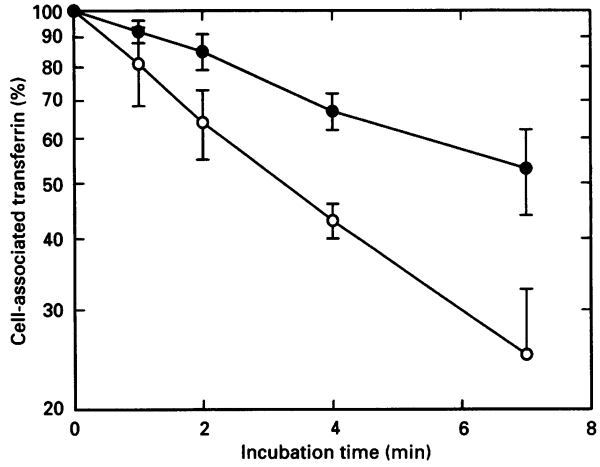

Figure 3 Effect of methylamine on exocytosis of apotransferrin from K562 cells

K562 cells (8–10 × 10^6 cells/ml) were pre-loaded with apotransferrin by incubation for 15 min in iron-free MEM containing 1% (w/v) BSA at 37 °C and ^{125}I-labelled apotransferrin (6.25 μM). The ^{125}I radioactivity initially associated with the aliquot of cells was 1.3–2.2 × 10^4 c.p.m. A duplicate sample was also prepared by pre-incubating cells with methylamine (25 mM) before loading with apotransferrin. This was treated in the same way as the control cells in subsequent steps but with buffers also containing methylamine. After washing in cold iron-free medium cells were resuspended (2 × 10^6 cells/ml) in medium containing unlabelled diferric transferrin (310 nM). Samples (0.4 ml) were removed at intervals into ice-cold PBS and after centrifugation at 400 $g_{av.}$ cell pellets were counted in a γ-counter. Results for cells with (●) and without (○) methylamine are shown.

study was critically dependent on apotransferrin entering the cells by receptor-mediated endocytosis rather than fluid-phase endocytosis, preliminary experiments were performed to determine the relative proportion of the protein entering the cells via the two pathways. The results of an experiment to investigate the specificity of uptake of apotransferrin by K562 cells are shown in Figure 1. During the first 10 min of the incubation there was a rapid increase in the amount of ^{125}I-apotransferrin associated with the cells, after which time there was a period during which uptake was linear. Non-specific uptake, determined in the presence of excess diferric transferrin, increased in a linear fashion throughout the time course. Specific or receptor-mediated uptake of ^{125}I-apotransferrin, calculated by subtracting non-specific from total uptake, very rapidly reached a steady state and this shows that although apotransferrin binds to the receptor with relatively low affinity, this interaction can still lead to specific endocytosis of the iron-free protein. After 15 min specific uptake accounted for more than 70% of the total and so this time was used to load cells with ^{125}I-apotransferrin for experiments on the release of apotransferrin.

Apotransferrin was exocytosed very rapidly from the cells with a $t_{\frac{1}{2}}$ for the process of 3.01 ± 0.23 min (mean ± S.D., n = 3) in the series of experiments shown in Figure 2. This compares with a $t_{\frac{1}{2}}$ for transferrin, originally presented to the cells as diferric transferrin, of 5.9 ± 0.87 min (mean ± S.D., n = 3) (Figure 2), a figure which is in agreement with a number of other published values [3,4]. Methylamine, an agent known to inhibit removal of iron from transferrin by cells [3,4], slowed the exocytosis of the apotransferrin, in spite of there being no iron to remove from the protein. The $t_{\frac{1}{2}}$ for the release of apotransferrin in the presence of methylamine was 8.0 ± 2.1 min (mean ± S.D., n = 3) (Figure 3) compared with a control value of 2.8 ± 0.2 min (mean ± S.D., n = 3) in these experiments. The exocytosis of iron–transferrin was slowed to a much greater extent by methylamine, with a $t_{\frac{1}{2}}$ of 20.9 min in the experiment shown (Figure 4), and a mean value of 18.65 ± 3.2 min (mean ± S.D., n = 4) in a series of independent experiments. This difference in the magnitude of the effect of methylamine suggests that this agent has two sites of action. First, an inhibitory action on the process of iron removal from transferrin and, secondly, a slowing of the exocytosis of the receptor and thus the release of apotransferrin. The different rates of exocytosis of apotransferrin and iron–transferrin suggest that the process of iron removal by the cell influences, or controls, the rate of release of the protein. This could be demonstrated by incubating the cells in the presence of the permeant iron-chelator desferrioxamine, which is known to bind

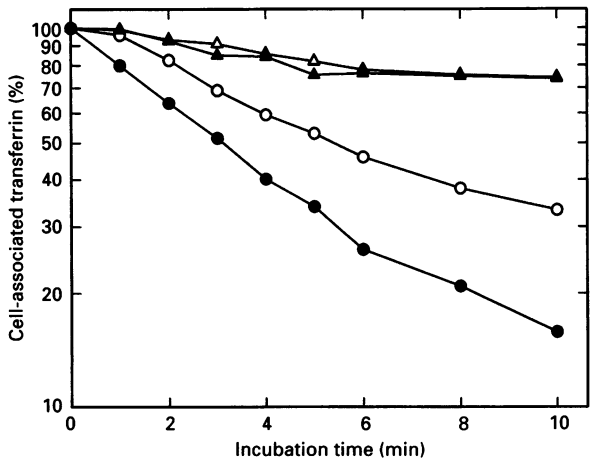

Figure 4 Effects of methylamine and desferrioxamine on release of diferric transferrin from K562 cells

Cells were pre-incubated for 30 min at 37 °C with methylamine (25 mM; △), desferrioxamine (100 μM; ●), both of these (▲), or neither (○) before loading with transferrin by incubation with ^{125}I-labelled diferric transferrin (310 nM) as described in the legend to Figure 2. After 15 min at 37 °C, cells were rapidly cooled, washed and resuspended in warm medium containing the same additives and release was monitored.

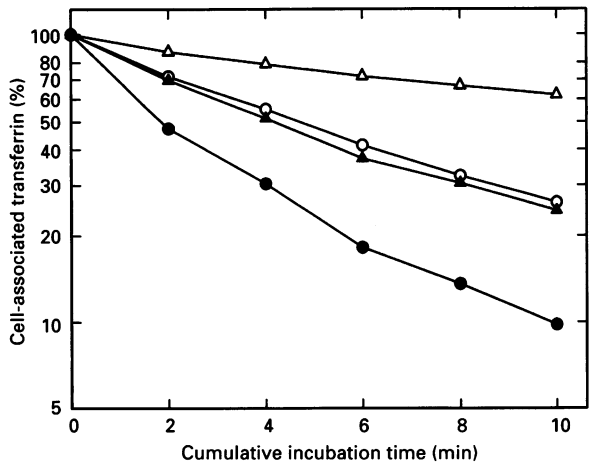

Figure 5 Acid stripping of surface iron–transferrin from K562 cells

K562 cells were pre-loaded with ^{125}I-labelled diferric transferrin as described in the legend to Figure 2. They were then suspended in ice-cold acetate (25 mM)/NaCl (150 mM) buffer at pH 5.5 containing desferrioxamine (100 μM) to render surface transferrin iron-free. After 5 min ice-cold MEM containing 1% (w/v) BSA, pH 7.4, was added to release the non-iron-free transferrin from the receptors. Cells were pelleted by centrifugation at 400 $g_{av.}$ and re-suspended in warm MEM (1 ml) and re-incubated at 37 °C for 2 min, at which point the exocytosis was stopped by the addition of ice-cold iron-free PBS. After pelleting the cells were again acid-stripped as before. This cycle of incubation/acid-stripping was repeated five times. Supernatants were collected for γ-counting. Control cells were put through the same procedure but with PBS substituting for the acetate buffer. Cells with (△,▲) and without (○,●) methylamine, washed in acetate buffer (▲, ●) or in PBS (△,○).

transferrin iron in these cells [9]. When cells were preloaded with this agent and diferric transferrin the rate of exocytosis of the transferrin was increased so that the $t_{\frac{1}{2}}$ value (3.0 min in Figure 4; 3.53±0.41 min, mean±S.D.; $n = 3$) approached that of apotransferrin (means of 3.01 and 2.8 min in Figures 2 and 3 respectively). Figure 4 also illustrates that desferrioxamine had no effect on the rate of release when the iron removal was blocked by methylamine. The release in the presence of methylamine alone proceeded with a $t_{\frac{1}{2}}$ of 18.65±3.2 min (mean±S.D., $n = 4$), while in the presence of both agents the $t_{\frac{1}{2}}$ value was 17.85±2.6 min (mean±S.D., $n = 4$). Desferrioxamine alone had no effect on the release of apotransferrin (results not shown) and so would appear to accelerate the release of transferrin by enhancing the removal of iron within the cells. The removal of iron therefore must be the rate-limiting step in the overall process.

Apotransferrin presented to the cell, and apotransferrin generated by the cell in the presence of desferrioxamine, are lost at a higher rate than iron–transferrin in control cells, and when the latter are exocytosed 80% of the iron has been removed [3]. Two possible mechanisms to ensure that only iron-free transferrin is exocytosed are apparent. Transferrin could remain in the endosome until all of the iron has been removed and then exocytosis of the iron-free protein would occur. Alternatively, continuous cycling of the ligand–receptor complex could occur with re-internalization of transferrin molecules bearing iron and loss from the surface of only the apotransferrin molecules, which have a low affinity for the receptor [6,10,11] but with re-internalization of transferrin molecules bearing iron. Experiments to test the latter were devised. Cells were preloaded with ^{125}I-labelled diferric transferrin and then subjected to sequential washes in an acidic medium containing desferrioxamine which, by removing surface transferrin [10], allowed the distribution of the transferrin to be ascertained in a non-destructive way. Cells incubated under control conditions, i.e. with washes in PBS, released 50% of the protein by 4 min; however, when methylamine was present release was markedly slowed with only 38%

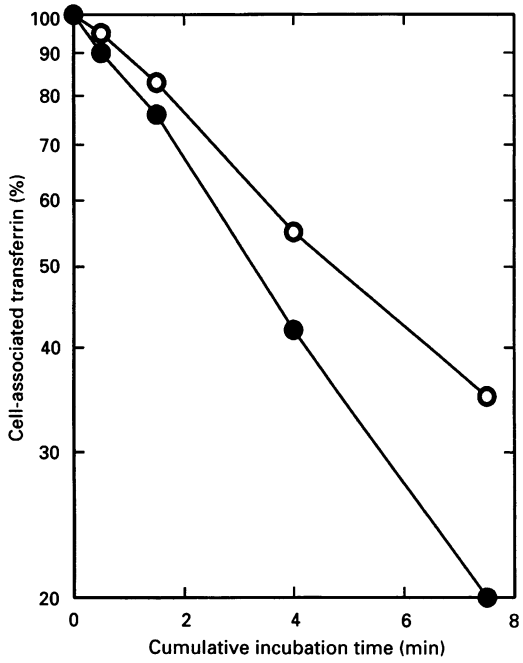

Figure 6 Acceleration of iron–transferrin release from K562 cells by proteolysis steps

Shown is the release from cells repeatedly treated with PBS (○), or with Pronase B (●) to remove surface-exposed transferrin.

released by the end of the 10 min sequence of incubations (Figure 5). When acid/desferrioxamine washing was used 50% of the transferrin was released within 2 min (Figure 5). In the presence of

methylamine the release remained rapid but there was a measurable slowing with about 4 min required to release 50% of the protein (Figure 5). To corroborate these results an alternative method was used, in which Pronase B, instead of the acid/desferrioxamine, removed the surface-accessible transferrin. Very similar results were obtained using this technique, and in the experiment shown (Figure 6) the $t_{\frac{1}{2}}$ of release of diferric transferrin was decreased from 5.0 min in control cells to 3.1 min in Pronase-treated cells.

DISCUSSION

Most ligands undergoing receptor-mediated endocytosis are destined for destruction in the lysosome. Transferrin is unique in that it escapes this fate and is released undegraded from the same membrane through which it was internalized. The property that confers this ability to evade the lysosome appears to be its retention of a high affinity for its receptor at low pH, which allows it to re-surface with its receptor [10,11]. A major factor influencing the stability of the transferrin–receptor complex at neutral pH is the iron content of the protein molecule [6,10], and from the studies presented here it appears that the cell makes use of this to allow the selective loss from the cell of transferrin molecules which have been completely depleted of iron.

Apotransferrin release occurred very rapidly with a $t_{\frac{1}{2}}$ of between 2.8 and 3.01 min. The $t_{\frac{1}{2}}$ for the loss of apotransferrin from the surface receptors at 4 °C has been shown to be approx. 17 s [4,10], and, because this is so rapid, the rate of release of apotransferrin probably approximates to the rate of exocytosis of the cycling receptor. The receptor appears to be endocytosed even in the absence of ligand [12,13] and since under normal conditions the steady-state distribution of receptors between the interior and the surface appears to be constant [5] then the rates of endocytosis and exocytosis should be equal. We have shown previously that in K562 cells endocytosis of diferric transferrin occurs with a $t_{\frac{1}{2}}$ value of about 2 min [3], a figure close to our estimates for the release of apotransferrin, thus providing further support for the concept of a steady-state distribution of receptors.

Apotransferrin release was slowed by methylamine but was not completely abrogated. Evidence from studies of the asialoglycoprotein/receptor system in cultures of hepatocytes [14] and hepatoma cells [15] suggest that weak bases which perturb intracellular pH act both to prevent the dissociation of the ligand from the receptor, with the accumulation of the ligand in a pre-lysosomal compartment, and also to prevent, in a ligand-independent manner, the return of the receptor to the plasma membrane. Thus, the effect on the transferrin receptor appears to be different in that receptor recycling continues albeit at a lower rate. We found that iron transferrin, once endocytosed, became accessible on the surface of the cells and could be made to leave the receptor by conversion of the protein into apotransferrin using an acid/desferrioxamine buffer. As desferrioxamine does not penetrate into the cell at 4 °C [9] (the temperature during these incubations) it must have been exerting its effect in this way at the extracellular face of the plasma membrane. Published work has shown that the transferrin receptor continues to be cycled through the endocytic cycle regardless of whether it is occupied by its ligand [12,13]. This evidence, together with that presented here, suggests that iron–transferrin binding to this continually cycling receptor is rapidly endocytosed ($t_{\frac{1}{2}}$ 2.2–2.8 min) [3], the endosome is acidified and some iron is removed from the binding sites of the protein. The receptor is then exocytosed ($t_{\frac{1}{2}}$ 2.80–3.01 min) and at the surface of the cell those molecules containing no iron detach from the receptor, but an appreciable proportion still have one or two iron atoms and so are re-endocytosed. In the presence of methylamine nearly all of the iron is retained by the transferrin [3,4] and so the majority of the protein remains bound to the receptor, and therefore recycles, leading to the greatly prolonged residence time in the cell.

The results of two of the experiments suggest that in cells incubated under control conditions some transferrin returns to the cell surface incompletely stripped of its iron, becomes accessible and would normally be re-internalized. First, when cells were loaded with diferric transferrin the $t_{\frac{1}{2}}$ for release could be accelerated by the acid/desferrioxamine wash to 2 min (Figure 5) which is comparable with the rate found for chemically prepared apotransferrin. Secondly, incubating the cells with desferrioxamine before loading with diferric transferrin also accelerated release to a rate comparable with that of apotransferrin (Figure 4). If desferrioxamine can maximize the rate of iron removal from internalized transferrin then the disparity in the $t_{\frac{1}{2}}$ values of release can be explained if iron-rich transferrin makes two passes through the endocytic cycle.

We would argue that desferrioxamine increases the efficiency of iron release from transferrin by acting as a powerful exogenous chelator either within the endosome or at the endosomal membrane [9,16], which allows the molecule to be rendered iron-free during a single round of endocytosis. An additional site of action of desferrioxamine could be at the plasma membrane where it has been suggested that the extracellular chelator scavenges free iron in the incubation medium and so prevents iron binding by receptor-bound apotransferrin, which would then fail to dissociate from the receptor [17].

It could be argued that the desferrioxamine present in the acid-wash buffer was acting in this way and that the release of transferrin could be enhanced in the absence of iterative cycling. For this reason transferrin recycling was further investigated by sequentially exposing the cells to Pronase B-containing buffer [3–5]. The results of this experiment (Figure 6) corroborate those in which the acid/desferrioxamine wash was used (Figure 5) in that the rate of release of diferric transferrin was increased.

Iterative cycling of transferrin as a means of achieving complete removal of iron must also be considered in the light of the recent report that the minimum pH achieved by endosomes in K562 cells is 5.4 [18]. At this pH iron is only released from the N-terminal binding site of transferrin free in solution [19] and yet the majority of transferrin released by these cells is clearly apotransferrin [3]. One explanation for this paradox is provided by the results of two recent reports in which the effect of receptor binding on the pH dependency of iron release from transferrin was studied. In the first, Aisen and colleagues found in a study *in vitro* [20] using pyrophosphate as an iron acceptor that at pH 7.4, the pH at the cell surface, formation of a complex between transferrin and its receptor impeded release of iron from transferrin, while at pH 5.6, close to that achieved in the endosome [18,21,22], the converse was true and receptor binding facilitated iron release with a 5-fold increase in the rate constant for release from receptor-bound ligand as compared with ligand free in solution. Similar results were reported by Sipe and Murphy [23] who used urea/gel electrophoresis [24] to examine the molecular species of transferrin, either receptor-bound on the surface of K562 cells or free in solution. They found that release of iron occurred from both binding sites at mildly acidic pH values (5.6–6.0). Iterative cycling would prolong the time for which ligand and receptor are associated, which would facilitate this mechanism.

In conclusion, the rate at which the transferrin receptor recycles in K562 cells is clearly very rapid and the transferrin residency time too short to permit complete stripping of iron

during a single round of endocytosis. A number of strategies including selective loss of iron-free molecules, re-endocytosis of iron-bearing molecules, and alteration in the pH dependency of iron release from receptor-bound molecules appear to have a role in ensuring the process of cellular iron uptake is efficient.

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