Intracellular processing of transferrin and iron by isolated rat hepatocytes

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Transferrin bound by isolated rat hepatocytes is rapidly endocytosed and enters a compartment of low density. Little was found associated with the lysosomes, even though the protein was subsequently lost from the cells. Iron entering the cells on transferrin was subsequently found in a number of intracellular components: transferrin, haem, ferritin and a residual fraction. After 2 h incubation with ^{59}Fe -transferrin almost 70% of the iron was in ferritin, and this proportion increased to 80% during a 'chase' experiment. Residual iron, because of its rapid increase at the start of the incubation and its decline during the 'chase', probably represents an intracellular transit pool, which at steady state was present at 23 pg/10⁶ cells.

INTRODUCTION

About 10% of iron bound to plasma transferrin is destined for the liver (Polycove & Mortimer, 1961), and so it appears that transferrin is a major source of the metal for the hepatic store. With many cell types it has been shown that the acquisition of transferrin iron requires the expression of cell-surface transferrin receptors to which the iron-laden protein binds, leading to its endocytosis (Karin & Mintz, 1981; van Renswoude *et al.*, 1982; Iacopetta & Morgan, 1983; Dautry-Varsat *et al.*, 1983; Ciechanover *et al.*, 1983). After the removal of iron within the cell, in a vesicle of low pH (van Renswoude *et al.*, 1982; Iacopetta & Morgan, 1983), the iron-depleted protein is released undegraded from the cell. Although this process has been shown to occur in a hepatoma-derived cell line with many of the characteristics of the hepatocyte (Ciechanover *et al.*, 1983), little information is available on the mechanism by which the hepatocyte itself acquires transferrin iron or on the fate of that iron within the cell. Evidence from Young & Aisen (1980, 1981) has shown that the hepatocyte expresses the transferrin receptor, and this has been confirmed by a number of other studies (Cole & Glass, 1983; Thorstensen & Romslo, 1984), and the presence of this receptor implies that a similar endocytic cycle occurs in the hepatocyte. However, one study has attempted to identify endocytosed transferrin within the liver with little success (Milsom & Batey, 1979), and a number of groups have concluded that receptor-mediated endocytosis does not occur in the hepatocyte, and that iron uptake occurs by an alternative process such as pinocytosis (Page *et al.*, 1984; Grohlich *et al.*, 1979), diffusion (Grohlich *et al.*, 1979) or removal of iron from transferrin at the plasma membrane (Cole & Glass, 1983). If this is the case, either transferrin will not be found in the cell or, after uptake by a non-specific process, then its most likely fate would be transfer to the lysosomes. Karin & Mintz (1981), Morgan (1981) and Octave *et al.* (1981) have suggested that lysosomes may be involved in the removal of iron from transferrin in a number of cell types, and so such a process could occur in the hepatocyte. The purpose of the present investigation was

to study the processing of transferrin and its iron by the isolated rat hepatocyte to determine whether transferrin is endocytosed and to investigate the intracellular location of transferrin and the fate of the iron. We have compared the processing of transferrin with that of asialo-orosomucoid, a protein known to be processed in the lysosomes after endocytosis.

MATERIALS AND METHODS

Proteins

Human transferrin was prepared as described elsewhere (Bomford *et al.*, 1983). Human transferrin was used throughout the study in order to distinguish between endogenous transferrin synthesized in the hepatocytes and that added to the cells. The two proteins have similar affinities for the rat transferrin receptor (S. P. Young, S. Roberts & A. Bomford, unpublished work). Human orosomucoid (Sigma Chemical Co., Poole, Dorset, U.K.) was desialylated, and the proteins were radiolabelled with ^{125}I with the use of lactoperoxidase or with ^{59}Fe as described previously (Young *et al.*, 1983). Antiserum to human transferrin (Leibman & Aisen, 1979) and to rat liver ferritin (Bomford *et al.*, 1977) were raised in rabbits. An IgG fraction of the anti-transferrin serum was further purified on DEAE-cellulose (Whatman Biochemicals, Maidstone, Kent, U.K.) and linked to CNBr-activated Sepharose (Pharmacia, Milton Keynes, Bucks., U.K.) (Hudson & Hay, 1980). The capacity of this gel for transferrin, determined with ^{125}I -transferrin, was found to be 0.44 mg/ml (Conlon-Hollingshead *et al.*, 1982).

Hepatocytes

Male Wistar rats (250–300 g) were anaesthetized with diethyl ether, and the hepatocytes were isolated by perfusion of the liver with collagenase as described previously (Young & Aisen, 1981). The cell suspension was centrifuged four times at 50 *g*_{av} for 2 min at room temperature to minimize contamination of the hepatocytes with debris and non-parenchymal cells (Doolittle & Richter, 1981).

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Incubations

Hepatocytes (5×10^6 /ml) were incubated in Eagle's minimal essential medium with 25 mM-Hepes buffer, pH 7.3 (Gibco-Europe, Paisley, Scotland, U.K.), containing 1% (w/v) bovine serum albumin (fraction V; Sigma Chemical Co.) in a shaking water bath at 37 °C under an atmosphere of 5% CO_2 in air. Radiolabelled (^{59}Fe and ^{125}I) human diferric transferrin (20 $\mu\text{g}/\text{ml}$) was added, and samples were removed at intervals into ice-cold Dulbecco phosphate-buffered saline (Dulbecco & Vogt, 1954). After the incubation the cells were washed three times in cold phosphate-buffered saline by centrifuging for 2 min at 100 g_{av} in the cold. Cells were then taken for γ -radiation counting or for further fractionation.

For the measurement of transferrin or asialo-orosomucoid endocytosis by hepatocytes, the cells were incubated in ice-cold medium with ^{125}I -labelled diferric transferrin (20 $\mu\text{g}/\text{ml}$) or ^{125}I -labelled asialo-orosomucoid (2.5 $\mu\text{g}/\text{ml}$) for 45 min. They were then washed three times in the cold and then rapidly warmed to 37 °C. Samples were taken into cold phosphate-buffered saline, the cells were centrifuged down and washed before further fractionation.

Fractionation of cells

After incubation with ^{59}Fe -labelled diferric transferrin the washed samples of cells were taken up in 1% Triton X-100/1% sodium deoxycholate/25 mM-Hepes buffer, pH 7.4 (1 ml). One-third of the supernatant was diluted to 2 ml, then made 1 M in HCl, and haem was extracted by the addition of 2 ml of butan-2-one (Teale, 1959).

Fig. 1. Endocytosis of surface-bound diferric transferrin by hepatocytes

Cells (5×10^6 /ml) were incubated for 45 min at 4 °C with 20 μg of ^{125}I -labelled diferric transferrin/ml. They were then washed three times in the cold, resuspended in cold medium, sampled and then warmed rapidly to 37 °C. Samples were taken into cold phosphate-buffered saline, and the cells were sedimented and then resuspended in phosphate-buffered saline containing 100 μg of Pronase/ml. After 30 min the cells were recovered by centrifugation. Radioactivity released from the cells on warming (Δ) and that associated with the cells that was accessible ($\bullet$) and inaccessible ($\circ$) to Pronase was determined by γ -radiation counting.

After vigorous shaking the phases were separated by centrifugation at 2000 g_{av} , the organic layer was removed and the extraction was repeated. The pooled organic fractions and the aqueous residues were subjected to γ -radiation counting. Control extractions with erythrocytes showed that this procedure extracted over 96% of the haem into the organic phase. The insoluble residue was sedimented from the rest of the solubilized cells by centrifugation at 9000 g_{av} in an Eppendorf microcentrifuge. One-half of the cleared supernatant was passed down a small column (0.2 ml) of anti-transferrin serum linked to CNBr-activated Sepharose. The column was washed with 10 column volumes of phosphate-buffered saline, at which point the radioactivity in the eluate had fallen to background, and transferrin retained on the gel was determined by γ -radiation counting. Anti-ferritin serum (20 μl) was added to another portion of the solubilized cell supernatant along with carrier rat liver ferritin (20 μg). After overnight incubation in the cold the precipitate formed was sedimented at 9000 g_{av} and subjected to γ -radiation counting. Repetition of the ferritin immunoprecipitation led to no further precipitation of radioactivity.

Cells incubated with ^{125}I -transferrin as above were resuspended in phosphate-buffered saline containing 100 μg of Pronase (Calbiochem, CP Laboratories, Cambridge, U.K./ml at 4 °C. After 30 min of incubation with intermittent shaking, the cells were recovered by centrifugation and were taken for γ -radiation counting along with the incubation supernatant and the Pronase solution.

Percoll fractionation of the cells

Cells previously incubated with ^{125}I -labelled transferrin or asialo-orosomucoid were suspended in 0.25 M-sucrose/mM-EDTA/10 mM-Tris/HCl buffer pH 7.4, at 4 °C. They were then homogenized on ice by 40 strokes of a Dounce homogenizer with a tight pestle (clearance 0.025–0.076 mm), and unbroken cells and nuclei sedimented by centrifugation at 3000 g_{av} for 15 min. The supernatants (0.5 ml) were applied to the top of a 20% (w/v) solution of Percoll (8 ml) in homogenization buffer above a cushion of 50% (w/v) sucrose (0.5 ml). The tubes were then centrifuged at 30000 g_{av} for 30 min in a Beckman 65 angle rotor in an L8-70 centrifuge at 4 °C. After this the self-generated gradients were fractionated (0.2 ml) by piercing the bottom of the tube and pumping out the contents into a fraction collector. Radioactivity in each fraction was counted, and 5'-nucleotidase (EC 3.1.3.5) (Michell & Hawthorne, 1965) and acid phosphatase (EC 3.1.3.2) (Trouet, 1974) activities were determined in alternate fractions.

RESULTS

When hepatocytes were incubated with diferric transferrin at 4 °C, and unbound ligand had been washed away, about 4 ng/ 10^6 cells remained bound to the cells, and of this 88% could be dissociated from the cells with Pronase (Fig. 1). When the cells were rapidly warmed to 37 °C, this cell-surface transferrin was rapidly internalized, and after 5 min only 51% was accessible to the proteinase and this proportion continued to decline. There was little increase over the first 5 min in the amount of transferrin in the medium, suggesting that endocytosis had occurred, but after this initial period there was a rapid increase in

this fraction, which was balanced by a decline in both the surface-bound and intracellular transferrin.

When cells from such an experiment were homogenized and fractionated on a Percoll density gradient, the cellular distribution of the transferrin was revealed. Before the cells were warmed much of the cell-associated transferrin was found in solution above the gradient, representing transferrin displaced from its receptor during homogenization (Fig. 2a). A proportion had entered the gradient and was associated with a low-density fraction representing plasma membrane, as judged by the distribution of 5'-nucleotidase (Fig. 2e). Upon warming to 37 °C the amount of ligand associated with this low-density fraction increased (Fig. 2b) and remained elevated for 15 min (Fig. 2c), after which it declined and by 40 min returned to a level similar to that at time zero (Figs. 2d and 2a). At no time was a significant amount of transferrin found with the lysosomes in the denser part of the gradient, which were recognized by the distribution of acid phosphatase (Fig. 2f).

Contrasting with this distribution of transferrin was that of asialo-orosomucoid. Initially all of this ligand was found above the gradient (Fig. 3a), because its binding to the asialoglycoprotein receptor on the hepatocyte membrane is Ca^{2+} -dependent (Pricer & Ashwell, 1971) and, since the homogenization buffer used contained EDTA, the ligand was lost from the surface. After 5 min at 37 °C, however, little of the asialo-orosomucoid was lost from the cells, and most of it was associated with a low-density fraction in the gradient of a density similar to that of the transferrin-containing fractions (Fig. 3b). As the incubation progressed the ligand migrated down the gradient, until by 40 min it was largely associated with the high-density acid-phosphatase-rich lysosomal fraction (Fig. 2f).

Incubation of the cells with ${}^{59}\text{Fe}$ -labelled transferrin was followed by fractionation of the cells into transferrin, ferritin, haem and residual iron components. Initially, 95% of the ${}^{59}\text{Fe}$ was recovered in the transferrin fraction (Fig. 4). However, this percentage rapidly fell proportionally as the cells accumulated iron (Fig. 4), although the fraction changed little in absolute terms (4.5–6.5 pg of $\text{Fe}/10^6$ cells) over the period from 30 to 120 min (Fig. 5). This corresponds to the amount of iron that could be bound by 2.4×10^4 – 4.5×10^4 transferrin molecules per cell, when the protein is saturated with iron. Total cell ${}^{59}\text{Fe}$ increased in approximately linear fashion, as did ferritin iron (Fig. 5). The increase in haem iron was also linear, but represented only a small proportion (2–4%) (Fig. 4) of the total. Residual iron increased as a proportion over the first hour, but then reached a steady level (Fig. 4). After 2 h 68.3% of the ${}^{59}\text{Fe}$ was in ferritin,

Fig. 2. Distribution of hepatocyte-associated transferrin on a Percoll gradient

Cells surface-labelled with ${}^{125}\text{I}$ -labelled diferric transferrin were warmed to 37 °C and taken into cold phosphate-buffered saline. After sedimentation the cells were homogenized, nuclei and unbroken cells were sedimented, and the supernatant was layered on to 20% Percoll. After centrifugation the gradients were fractionated and each fraction subjected to γ -radiation counting and assayed for marker enzymes. The panels represent times after warming for (a) 0 min, (b) 5 min, (c) 15 min and (d) 40 min; (e) 5'-nucleotidase; (f) acid phosphatase.

Fig. 3. Distribution of hepatocyte-associated asialo-orosomucoid on a Percoll gradient

The experiment was similar to that described in Fig. 2 legend except that ^{125}I -labelled asialo-orosomucoid was used. The panels represent the time after warming for (a) 0 min, (b) 3 min, (c) 15 min and (d) 40 min.

6.6% on transferrin and 2.3% in haem, and the residue (22.8%) was unidentified.

After 2 h incubation the cells were washed, and the incubation was continued for a further 60 min. Total ^{59}Fe declined slightly during the re-incubation, and much of this decrease was accounted for by the decrease in residual iron and transferrin iron, whereas ferritin remained steady (Fig. 5). In consequence ferritin increased as a proportion of the total during the re-incubation, from 68% to 79% of cellular ^{59}Fe (Fig. 4). Haem iron remained steady at 2.1% (2.3 pg/ 10^6 cells) at the end of the re-incubation, transferrin declined from 6.6% to 5% (6.7 to 4.0 pg/ 10^6 cells) and the residue declined from 22.8% to 13.9% (23 to 11 pg/ 10^6 cells).

Fig. 4. Proportional distribution of ^{59}Fe in the hepatocyte

Cells ($5 \times 10^6/\text{ml}$) were incubated with ^{59}Fe -labelled diferric transferrin (20 $\mu\text{g}/\text{ml}$). Samples were removed, and the cells were washed and fractionated as described in the Materials and methods section. After 120 min the remaining cells were cooled to 4 °C, washed and resuspended at 37 °C. Sampling was then continued. The lines represent ^{59}Fe associated with transferrin ($\blacktriangle$), ferritin ($\bullet$), haem ($\circ$) and none of these ($\triangle$).

DISCUSSION

The results shown here demonstrate that, following the binding of diferric transferrin to the surface of the isolated rat hepatocyte, the protein is endocytosed. After a short delay the transferrin started to re-appear in the extracellular medium as a result of exocytosis (Fig. 1). We have previously shown (Young *et al.*, 1983) that holotransferrin, which is endocytosed via the transferrin receptor, is released from hepatocytes intact, only a small proportion being degraded within the cells. This contrasts with rat asialotransferrin, which under certain experimental conditions (Young *et al.*, 1983) can be endocytosed via the hepatic asialoglycoprotein receptor and is degraded. This difference can be explained by the observation made here that holotransferrin is not routed via the lysosomes (Fig. 2), whereas asialo-orosomucoid (Fig. 2) (and presumably rat asialo-transferrin) does appear in a dense intracellular vesicle. The transferrin associated with the cells remained in a light fraction of the Percoll gradient after the cells were warmed to 37 °C, even though most of the transferrin was intracellular. This suggests that the endocytic vesicle into which the transferrin is taken by the hepatocyte is of similar density to the plasma membrane and so no significant shift in the position of the transferrin on the gradient was observed. This seems to be the case with other cell types, for Dickson *et al.* (1983) found only a small difference between the density of plasma membrane and transferrin-containing endocytic vesicles isolated from KB carcinoma cells on a Percoll gradient. Other work has failed to find transferrin within the liver (Milsom & Batey, 1979), but this was based in experiments performed *in vivo* and it is possible that insufficient radiolabel was used to detect the relatively small proportion of circulating transferrin taken up by the liver. The hepatocyte has only about 40000 transferrin receptors (Young & Aisen, 1980), which, for the size of the cell, is a low density compared with, for instance, erythroid cells (Iacopetta & Morgan, 1983), and the detection of this small amount of endocytosed transferrin may be difficult with experiments

Fig. 5. Distribution of ^{59}Fe in hepatocytes

The experiment is the same as that described in Fig. 4 legend, but the absolute amounts in the fractions are shown. The lines represent ^{59}Fe associated with transferrin (▲), ferritin (●), haem (○) and none of these (△); the total is also shown (□).

performed *in vivo*. Uptake of transferrin into hepatocytes by receptor-independent endocytosis has also been suggested (Grohlich *et al.*, 1979; Page *et al.*, 1984), and the appearance in the lysosomes of ligand taken up by this route might be expected. We found little transferrin in the lysosomes (Fig. 2), and other work suggests that transferrin taken up via this route represents that which is non-specifically bound to the plasma membrane in protein-free medium (Thorstensen & Romslo, 1984). This non-specific mechanism may also explain the occurrence of lysosomal transferrin observed in cultured fibroblasts (Octave *et al.*, 1981). It appears, then, that the receptor-mediated endocytosis of transferrin by the rat hepatocyte has many features of the process that has been shown to occur in a variety of cells ranging from the reticulocyte (Morgan, 1981), an erythroleukaemia cell line K562 (van Renswoude *et al.*, 1982) and, of greater relevance, a human hepatoblastoma cell line HepG2 (Ciechanover *et al.*, 1983).

Intracellular transferrin was also detected when hepatocytes were solubilized in detergent and initially represented the major cell ^{59}Fe -labelled component (Figs. 3 and 4). As the cells accumulated iron the transferrin iron content remained steady, and so diminished as a proportion of the total as iron was incorporated into other constituents. The principal of these was ferritin, which represented 60–80% of the ^{59}Fe . This finding is in keeping with the role of the hepatocyte as an iron store and with other work showing that *in vivo* much of the transferrin iron cleared by the liver is incorporated into ferritin (Milsom & Batey, 1979). The liver also has substantial concentrations of haem enzymes, and so it was no surprise that about 2–3% of the ^{59}Fe was found to be extractable by organic solvent. The other major component observed within the cells was not characterized apart from its exclusion from any one of the other three pools of iron investigated. This component may represent the so-called 'labile' or 'chelatable' iron pool, which may exist in most cells and may be that iron in transit between

the other intracellular compounds (Jacobs, 1977). Since its concentration rose even more rapidly than that of ferritin at the start of the incubation, reached a steady-state level after 30–60 min and then fell when the cells were re-incubated, its role as an intermediate pool seems most likely. Baker *et al.* (1985) have recently shown that a fraction of intracellular iron is more easily chelated soon after its uptake from transferrin by cultured hepatocytes. After several hours it becomes less available, which would agree with the finding here that residual iron decreases after removal of the extracellular source of transferrin iron. At steady state this pool of iron is present at about 23 pg/10⁶ cells.

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