Intermediate Steps in Cellular Iron Uptake from Transferrin

DETECTION OF A CYTOPLASMIC POOL OF IRON, FREE OF TRANSFERRIN*

Des R. Richardson‡ and Erica Baker‡

From the Department of Physiology, University of Western Australia, Nedlands 6009, Western Australia

The uptake of transferrin-bound iron by receptor-mediated endocytosis has been the subject of extensive experimental investigation. However, the path followed by iron (Fe) after release from transferrin (Tf) remains obscure. Once Fe is released from Tf within the endosome, it must be transported across the endosomal membrane into the cell. The present investigation describes the presence of a cytoplasmic Tf-free Fe pool which is detectable only when cells are detached from their culture dishes at low temperature, after initial incorporation of diferric transferrin at 37 °C. This cellular iron pool was greatly reduced if incubation temperatures were maintained at 37 °C or if cells were treated with pronase. Human melanoma cells (SK-MEL-28) in culture were prelabeled by incubation with human 125I-transferrin for 2 h, washed, and reincubated at 4 °C or 37 °C in balanced salt solution in the presence or absence of pronase. The cells were then mechanically detached from the plates and separated into "internalized" and supernatant fractions by centrifugation. Approximately 90% of cellular 59Fe and 20% of 125I-Tf remained internalized when this reincubation procedure was carried out in balanced salt solution at 37 °C. However, at 4 °C, cellular internalized iron was reduced to approximately 50% of the initial value. The release of this component of cellular 59Fe (approximately 40% of total cell 59Fe) at 4 °C was completely inhibited in the presence of pronase and other general proteinases at 4 °C and at 37 °C, without affecting internalized transferrin levels. Similar results were obtained in fibroblasts and hepatoma cells, indicating that this phenomenon is not unique to melanoma cells. The characterization of this Tf-free cellular Fe pool which is detectable at low temperature may yield valuable insights into the metabolic fate of iron following its transport across the membrane of the endocytotic vesicle.

The uptake by cells of transferrin-bound iron has become a classic model of the general process of receptor-mediated endocytosis and has been the subject of extensive experimental investigation (Morgan, 1981; Crichton and Charloteaux-Watters, 1987; Morgan and Baker, 1988; Kuhn et al., 1990). Iron (Fe) is an obligatory requirement for cell growth and replication (Aisen and Listowsky, 1980; Weinberg, 1984) and in mammals is transported in the plasma bound to transferrin (Tf).¹ Donation of Fe to cells occurs following the binding of Tf to the transferrin receptor (TfR) on the cell membrane. The Tf-TfR complex is endocytosed, and iron is released into the cell while transferrin remains membrane-bound in endocytic vesicles and is returned to the plasma membrane (Morgan, 1981). It was demonstrated (Morgan, 1981; Klausner et al., 1983; Dautry-Varsat et al., 1983; Kuhn et al., 1990) that Fe is released from Tf after a decrease in intravesicular pH. In addition, recent studies (Bali et al., 1991; Sipe and Murphy, 1991) indicate that the TfR itself facilitates the release of Fe from Tf bound to the receptor.

Other studies suggest that in some cell types release of Fe from Tf may also occur at the plasma cell membrane, where cooperative proton and electron fluxes mediated by an oxidoreductase may result in the release of Fe at the cell surface (Crane et al., 1985; Sun et al., 1987; Thorstensen, 1988; Thorstensen and Romslo, 1988). It appears that some cells such as reticulocytes (Morgan, 1981) and rat hepatoma cells (Baker et al., 1988) rely on receptor-mediated endocytosis of Tf for their Fe requirements, whereas hepatocytes (Young and Aisen, 1981; Page et al., 1984; Trinder et al., 1986; Holmes and Morgan, 1989; Thorstensen, 1988; Thorstensen and Romslo, 1990) and melanoma cells (Richardson and Baker, 1990, 1992a) may use two mechanisms. However, the physiological importance of each mechanism in Fe uptake remains controversial (Thorstensen and Aisen, 1990).

The path followed by Fe after release from Tf has remained obscure in spite of intensive biological and medical investigation. Once Fe is released from Tf within the endosome (or at the cell surface), it must be transported across the endosomal (or plasma) membrane into the cell. Several recent studies have suggested that Fe, probably as Fe(II) ion, is actively transported across the membrane by a membrane-bound protein (Egyed, 1988; Fuchs et al., 1988; Morgan, 1988; Wright et al., 1986, 1988).

The present investigation describes the identification of a cellular Tf-free iron pool which is detectable at low temperature but inaccessible at 37 °C or after treatment with pronase. This suggests the presence of a membrane-bound protein molecule or complex whose activity is temperature-dependent and which may have an important role in the movement of Fe into the cytoplasm after its release from dfferent Tf.

This study has been performed primarily using the human melanoma cell, which is of particular interest as this cell expresses high concentrations of a Tf homologue, p97, or melanotransferrin (Brown et al., 1982; Rose et al., 1986) on the plasma membrane, as well as the TfR (Musgrove et al.,

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¹ The abbreviations used are: Tf, transferrin; TfR, transferrin receptor; MEM, Eagle's modified minimum essential medium; BSA, bovine serum albumin; BSS, balanced salt solution.
1984; Seligman et al., 1986). We have recently described in the human malignant melanoma cell line, SK-MEL-28, two main processes of Fe uptake: 1) a process consistent with receptor-mediated endocytosis and 2) a nonspecific but saturable process compatible with the release of Fe from Tf at the cell surface (Richardson, 1990; Richardson and Baker, 1990, 1991a, 1991b, 1992a). While most of the experiments in the present study were performed on melanoma cells, similar results were obtained in fibroblasts and hepatoma cells, suggesting the phenomenon may be common to both normal and neoplastic cells in culture.

MATERIALS AND METHODS

Chemicals—Iron-59 (as ferric chloride in 0.1 M HCl) and iodine-125 (as sodium iodide) were purchased from Amersham International. Pronase was purchased from Boehringer Mannheim. Eagle’s modified minimum essential medium (MEM) as Autopow and fetal calf serum were supplied by Flow Laboratories. Penicillin (crystall-pen, benzylpenicillin sodium B.P.) was obtained from Glaxo. Bovine serum albumin (BSA, 98% pure), l-glutamine, Heps, collagenase (Type IV; Product No. C-5138; containing 4.5 mg/ml clostripain, nonspecific proteinase, and trypsin), proteinase K (a nonspecific proteinase, Product No. P5380), and trypsin (proteinase K, Sigma) were obtained from Sigma. Radiochemical products Iodine-125 (as sodium iodide) were purchased from Amersham International. Pronase was purchased from Pronase.“C.”

Cell Culture—The human melanoma cell line, SK-MEL-28 (American Type Culture Collection) was used. Cells were grown and subcultured as described previously (Richardson and Baker, 1990). Procedures used to check cell viability and differentiation were also the same as described previously (Richardson and Baker, 1990).

Experimental Procedure—The procedures used initially to investigate the uptake of Fe and Tf by confluent monolayers of melanoma cells were by standard techniques as described previously (Richardson and Baker, 1990). Briefly, confluent cell monolayers were incubated with MEM containing BSA (5 mg/ml), 20 mM Hepes (pH 7.4), and 1% dialyzed TF at a concentration of 1.25 mg/ml (0.1 mg/ml). At the end of the incubation, the cell monolayer was washed four times with ice-cold BSS. The amount of radioactivity internalized by the cells was then measured after incubation with pronase (1 mg/ml in BSS) for 30 min at 4°C to release membrane-bound Tf and Fe (Karin and Mintz, 1981; Iacopetta and Morgan, 1983). The cells were detached mechanically (using a Teflon spatula) from the plates in the pronase solution at 4°C, transferred to ice-cold microcentrifuge tubes, and centrifuged at 14,500 x g for 1 min in a Hereus Christ Biofuge A microcentrifuge to separate “internalized” (pronase-insensitive) radioactivity in the cell pellet from formerly membrane-bound (pronase-sensitive) radioactivity in the supernatant. This fraction will henceforth be referred to as the “membrane” compartment, although it will become evident that under some experimental conditions it may include cytoplasmic Fe. When alterations to this standard protocol were used to investigate the movement of Fe and Tf between cellular compartments, they are described in the text. The data are expressed as mean ± S.E.

RESULTS

We have obtained evidence for the transport of transferrin-free iron from the endocytic vesicle (or cell membrane) to a cytoplasmic compartment which is detectable after reincubation at 4°C, but not at 37°C or in the presence of proteases. This iron pool comprises approximately 40% of the total cellular iron in melanoma cells, fibroblasts, and hepatoma cells.

Release of Iron and Transferrin from Cells Treated with BSS Alone or with Pronase

Initial experiments investigated whether the proteolytic enzyme pronase had any effect on the release of Fe and Tf from cells. Melanoma cells were reincubated for 2 h with radioactive Tf (0.1 mg/ml) at 37°C to trace-label cellular Fe and Tf, washed, and reincubated for 30 min at 4°C in the presence of BSS alone or BSS with pronase. After this incubation period, the cells were removed mechanically from the plates in their respective solutions at 4°C and centrifuged to separate “membrane-bound” (supernatant) and internalized (cellular) Fe and Tf. Surprisingly, the cellular distribution of Fe and of Tf was very different in the two incubation media (Fig. 1 and Table I). In the presence of BSS alone, the amount of internalized Fe decreased to approximately one-half that observed using pronase and BSS (Fig. 1a). This decrease in Fe level resulted in the reduction of the molar ratio of internalized Fe:Tf from about 60 down to about 30, with a corresponding decrease in the percentage of Fe internalized (Table I). This did not appear to be due to cell damage caused by

FIG. 1. The effect on cellular 59Fe (a) and 125I-transferrin (b) distribution in prelabeled melanoma cells (2-h incubation, 37°C, 0.1 mg/ml 59Fe-125I-Tf) of reincubation for 30 min at 4°C in the presence of BSS with pronase or BSS alone. When melanoma cells were removed from the culture plates in the presence of BSS alone (open columns), internalized Fe decreased to approximately 50% of that found when cells were detached from the plates in the presence of BSS and pronase (hatched columns). However, there was little change in the distribution of Tf. Results are mean ± S.E. (five determinations).
release, cells were preincubated for 2 h with doubly labeled presence of BSS alone at 
were used, probably because in the absence of pronase mem-
plates in the presence of BSS alone resulted in about a 50% decrease 
that found when pronase was present, without an increase in 
the proportion of Fe internalized compared to 10% when the cells 
were removed in BSS and pronase. Results are mean ± S.E. (three 
to four determinations).

mechanical removal of cells from the plates as there was not 
a corresponding change in internalized Tf (Fig. 1b and Table I). Indeed, there was a slight increase in the proportion of internalized Tf in BSS compared to when pronase and BSS were used; probably because in the absence of pronase membrane-bound Tf was not removed by the proteolytic activity of the enzyme.

Conversely, when the cells were incubated and removed from the plates in BSS alone, the amount of Fe in the membrane (i.e. supernatant) had increased to about four times that found when pronase was present, without an increase in Tf (Fig. 1a and Table I). The total amount of Fe or Tf present was similar for all cells (Table I). These observations suggest that when the cells were removed from the plates in the presence of BSS alone at 4 °C, an Fe pool comprising approximately 40% of the total cellular iron-59 diffused out of the cells. There was little variation in the size of this pool in replicate experiments. The osmolarity of BSS in the presence and absence of pronase was approximately the same (300 mosm/liter).

A similar variation in the distribution of Fe and Tf in the presence and absence of pronase was found using a rat hepa-
toma cell line (McA7777) and a normal human fibroblast cell line (Fig. 2). This suggested that the observed phenomenon was not due to an intrinsic property of melanoma cells alone.

It was concluded that the addition of pronase to BSS prevented the release of a Tf-free Fe pool from the cells at 4 °C.

**Kinetics of Iron Release from Melanoma Cells**

To determine the effect of pronase on the kinetics of Fe release, cells were preincubated for 2 h with doubly labeled Tf (0.1 mg/ml), washed, and then incubated with BSS alone or BSS with pronase, for up to 3 h at 4 °C. The cells were then mechanically removed from the plate in their respective solutions and centrifuged. The inhibitory effect of pronase, preventing the release of internalized Fe without altering the partitioning of Tf, was apparent within 5 min of incubation with the enzyme (Fig. 3). As observed previously (Table I), in the absence of pronase, internalized Fe dropped to approximately 50% of the total, resulting in a decrease in the molar ratio of internalized Fe to Tf to approximately one-half that found in the presence of pronase. Pronase also inhibited the release of internalized Fe from cells that were labeled for 2 h with citrate-Fe complexes (molar ratio of citrate:Fe = 100:1; [Fe] = 2.5 μM; data not shown).

**Studies Investigating the Mechanism of Action of Pronase on Iron Release from Melanoma Cells**

**Effect of Varying the Pronase Incubation Protocol**—If the prelabeled cell monolayer was reincubated with BSS alone (at 4 °C for 30 min) and then the supernatant was removed using a Pasteur pipette, the molar ratio of Fe:Tf in the supernatant remained about 2, suggesting the release only of diferric Tf (molar ratio Tf:Fe = 2:0) from the cell membrane, without release of intracellular Fe. When the cells were incubated in BSS with pronase for 30 min at 4 °C and the supernatant was then removed in the same manner, the molar ratio of Fe to Tf in the supernatant was greater than 2 (2.66; Richardson and Baker, 1990), suggesting the presence of membrane non-Tf-bound Fe, which may correspond to a membrane Fe-binding molecule (Richardson and Baker, 1990; Chakraborty et al., 1992). However, if the cells were detached from the plates mechanically (at 4 °C), removed simultaneously with the supernatant, and then separated by centrifugation into cell pellet and supernatant, the molar ratio of Fe:Tf in the supernatant increased from 2 to about 12 in BSS, but did not change in BSS with pronase.

These data suggest that the Fe release observed in the presence of BSS alone may occur when cells are detached mechanically and that pronase prevents this effect. This hypothesis was confirmed, firstly by incubating prelabeled cells in BSS alone (30 min at 4 °C), then removing the BSS and replacing it with either fresh BSS or BSS with pronase (at 4 °C), in which media the cells were detached from the plates and centrifuged. Removing cells from the plates in pronase and BSS prevented the release of internalized Fe which occurred when cells were removed in BSS alone. Secondly, when prelabeled cells were incubated in pronase plus BSS (30 min, 4 °C), Fe release was inhibited whether cells were detached from the plates in BSS or pronase plus BSS. This experiment demonstrated that pronase altered the plasma membrane before cells were removed from the plate, preventing the release of Fe.

**Effect of Proteins on the Release of Iron and Transferrin from Melanoma Cells**—It was possible that the ability of
pronase to inhibit the release of internalized Fe from cells was due to some property other than its proteolytic activity. To test this hypothesis, the effect of various proteinases and other proteins on Fe release was assessed. Both trypsin and protease (a nonspecific proteinase) had the same effect as pronase (at both 1 and 10 mg/ml), preventing the release of internalized Fe (Fig. 4). However, collagenase had little inhibitory effect, resulting in a decrease in internalized Fe to 57% of the total (Fig. 4). Hence, only slightly more Fe was left internalized than in cells in BSS alone (52% of the total).

Collagenases are enzymes which specifically degrade collagen (Stryer, 1981). Hence, the inability of collagenase to prevent the release of intracellular Fe was not unexpected. The slight inhibitory effect observed may be due to the presence of contaminating nonspecific proteinases which were found in this preparation (assayed by Sigma). BSA (1 mg/ml) also did not inhibit the release of internalized Fe from the cells (Fig. 4). These experiments suggested that the proteolytic action of pronase was responsible for its ability to inhibit Fe release from cells. Since high molecular weight proteinases such as pronase cannot penetrate the cell membrane, a peripheral membrane protein(s) may be their site of action.

**Effect of Temperature on Iron Release in BSS**—The effect of incubation temperature on the release of cellular Fe from melanoma cells was also investigated (Fig. 5). Cells were labeled with $^{59}$Fe-$^{125}$Tf, and the cell monolayer was washed with ice-cold BSS. Then, BSS at 37 °C or 4 °C was added, and the cell plates were incubated at these temperatures for 30 min. The BSS was then aspirated and replaced with BSS at 37 °C or 4 °C. The cells were then detached from the plates mechanically at these temperatures and centrifuged. The results are shown in Fig. 5 (iron release in BSS and pronase is included for comparison). Removing the cells from the plates in BSS at 4 °C resulted in a large decrease in internalized Fe, as described previously. This occurred irrespective of whether the first incubation in BSS had been at 37 °C or 4 °C. In contrast, if the cells were removed from the plates in BSS at 37 °C, internalized Fe was retained at a level similar to that observed in pronase, irrespective of whether the first incubation in BSS was at 37 °C or 4 °C (Fig. 5). It was concluded that the temperature at which the cells were removed from the plates in BSS was a crucial factor in determining the amount of Fe remaining internalized. In contrast, Fe release in BSS containing pronase was blocked at both 37 °C and 4 °C.

It was possible that the release of internalized Fe from cells with BSS was due to some intrinsic property of BSS. This was tested by using MEM instead. Identical results were found for both BSS and MEM (results not shown).

**Fig. 3.** The effect of time of reincubation at 4 °C in BSS with pronase (BSS + P) compared to BSS alone on the proportion of total cellular $^{59}$Fe- and $^{125}$I-transferrin remaining internalized (Int.) in melanoma cells that had been prelabeled for 2 h at 37 °C with $^{59}$Fe-$^{125}$I-transferrin (0.1 mg/ml). The inhibitory effect of pronase on the release of internalized Fe was evident within 5 min of the start of incubation in the presence of the protease, with little change over 3 h of incubation. Pronase had little effect on the partitioning of Tf. Results are means of duplicates.

**Discussion**

These experiments have demonstrated the presence of a cellular iron pool which is detectable after cell detachment at 4 °C. Release of iron-$^{59}$ from this compartment was not accompanied by a change in the distribution of transferrin-$^{125}$I and was far in excess of the iron binding capacity of the transferrin molecule. Thus, this compartment may be the pool of cytoplasmic iron which has dissociated from Tf in the endocytotic vesicle (or at the cell surface) and then been transferred across the vesicle (or plasma) membrane. These data also indicate that the iron is not released from the extracellular matrix or extracellularly from between overlapping cells (McKinley and Wiley, 1988) as it is not bound to transferrin-$^{125}$I. In addition, the mechanism of iron release is not related to the high MTf concentration on melanoma cells as a similar phenomenon was observed with fibroblasts and hepatoma cells, which express little or no MTf (Brown et al., 1982; Rose et al., 1986).

It is possible iron release was due to cell damage during removal from the culture dishes, with release of cytoplasmic Fe but retention of the larger organelles and endocytotic vesicles (with their enclosed Tf-Fe). The adherence of the cell monolayer to the plates was slightly reduced in the presence of the proteases. However, release of Tf-free iron was observed in BSS alone at 4 °C, but not at 37 °C, at which temperature cells would be more adherent (McNeil et al., 1984), despite using the same technique of mechanical detachment. There was also no release of iron at 37 °C or at 4 °C when cells were detached in the same way in the presence or after preincubation with pronase. In addition, there was good agreement between replicates, which would not be anticipated with a variable factor such as cell damage during detachment. Hence, alternative explanations for the effect of temperature in BSS must also be considered.

Lowering the temperature may cause conformational changes in membrane lipids or proteins that facilitate diffusion of low molecular weight components from endocytic and other cellular membrane compartments. The partitioning of other molecules needs to be examined to test for "leakage." Another possibility is the temperature-dependent gating of an iron "channel," allowing diffusion of iron out of the cell down a concentration gradient when this mechanism is inhibited at low temperature. This may be a protein channel because it is blocked by pronase at 4 °C and 37 °C. Iron release only occurred after mechanical perturbation at 4 °C, suggesting that both metabolic energy and membrane integrity may be essential for the gating process. Possible mechanosensitive iron ion channels are involved (Morris, 1990).

An iron transport channel is suggested as all naturally occurring membrane iron transport systems appear to be channels
McNeil et al. (1989) who demonstrated that basic fibroblast growth factor, which lacks the signal peptide required for the normal secretory pathway, was released when endothelial cells were detached mechanically from their substratum, apparently via transient plasma membrane disruptions which mimic the plasma membrane wounds observed in cells subject to mechanical forces in vivo (McNeil et al., 1989; Muthukrishnan et al., 1991). However, in contrast to the present study on the partitioning of Fe in melanoma cells, removing endothelial cells from culture plates by scraping at 37 °C resulted in more basic fibroblast growth factor being released from the cells than at 4 °C. Also, again in contrast to melanome cells, proteolytic enzymes such as trypsin apparently caused disruptions of the cell membrane in a large proportion of endothelial cells, allowing the release of cytoplasmic molecules (McNeil et al., 1989).

These inconsistencies suggest that the release of internalized Fe may not be due simply to cell membrane disruptions caused by mechanical perturbation at 4 °C.

1. If Fe release in BSS at 4 °C was due to a loss of cellular integrity during detachment of the cells from the substratum, a similar loss would be expected when the cells were removed in BSS at 37 °C. In addition, no decrease in internalized Fe was noted when the cells were preincubated in pronase and BSS and removed from the plates in BSS alone. This suggests that Fe release from cells was due to some property of the cells which could be affected by pronase.

2. Despite the release of 40% of cellular iron in BSS at 4 °C, there was no decrease in the amount of internalized Tf.

3. When cells were removed from the plates in BSS (in the absence of pronase) at 37 °C instead of 4 °C, the distribution of Fe was similar to that found when the cells were treated with pronase (Fig. 5).

4. When the cells were detached in BSS with pronase by gentle aspiration using a Pasteur pipette, the distribution of Fe and Tf between the membrane and intracellular compartments was very similar to when the cells were removed from the plate using a spatula (data not shown).

5. It is difficult to reconcile the high degree of reproducibility in replicate experiments with such a variable parameter as membrane disruption.

The nature of the cellular iron pool released at 4 °C is also uncertain and deserves further investigation. It appears to be of limited size and must be almost entirely Tf-free iron. It may represent the "transit iron pool" (Jacobs, 1977) or the "chelatable iron pool" (Baker et al., 1980; Pippard et al., 1982). Iron is never found uncomplexed in mammalian cells. This
iron pool may be composed of low molecular weight iron complexes or it may be protein-bound, for example, to ferritin or the low-spin non-ferritin iron complex identified in melanoma and hepatoma cells using $^{57}$Fe Mossbauer spectroscopy (St. Pierre et al., 1992). If so, it may be depleted using iron chelators, or with longer incubation times, or traced using a pulse-chase protocol or metabolic inhibitors. The study of this cellular Fe pool which is only detectable at low temperature may yield valuable insights into the metabolic fate of iron following its transport across the membrane of the endocytic vesicle.

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