The mammalian transferrin-independent iron transport system may involve a surface ferrireductase activity

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Mammalian cells accumulate iron from ferric citrate or ferric nitrilotriacetate through the activity of a transferrin-independent iron transport system [Sturrock, Alexander, Lamb, Craven and Kaplan (1990) *J. Biol. Chem.* **265**, 3139–3145]. The uptake system might recognize and transport ferric-anion complexes, or cells may reduce ferric iron at the surface and then transport ferrous iron. To distinguish between these possibilities we exposed cells to either [^{59}Fe]ferric citrate or ferric [^{14}C]citrate and determined whether accumulation of iron was accompanied by the obligatory accumulation of citrate. In HeLa cells and human skin fibroblasts the rate of accumulation of iron was three to five times greater than that of citrate. Incubation of fibroblasts with ferric citrate or ferric ammonium citrate resulted in an enhanced accumulation of iron and citrate; the molar ratio of accumulation approaching unity. A similar rate of citrate accumulation, however, was observed when ferric citrate-incubated cells were

exposed to [^{14}C]citrate alone. Further studies demonstrated the independence of iron and citrate accumulation: addition of unlabelled citrate to cells decreased the uptake of labelled citrate without affecting the accumulation of ^{59}Fe ; iron uptake was decreased by the addition of ferrous chelators whereas the uptake of citrate was unaffected; reduction of ferric iron by ascorbate increased the uptake of iron but had no effect on the uptake of citrate. When HeLa cells were depleted of calcium, iron uptake decreased, but there was little effect on citrate uptake. These results indicate that transport of iron does not require the obligatory transport of citrate and vice versa. The mammalian transferrin-independent iron transport system appears functionally similar to iron transport systems in both the bacterial and plant kingdoms which require the activities of both a surface reductase and a ferrous metal transporter.

INTRODUCTION

Mammalian cells accumulate iron by either of two transport systems. The first system involves the binding of diferric transferrin [$\text{Tf}(\text{Fe})_2$] to high-affinity receptors. The $\text{Tf}(\text{Fe})_2$ -receptor complex is internalized into an acidic endosome where iron is released from the receptor-bound transferrin (Tf). The release, and subsequent transport of iron out of the endosome, results from the interplay of pH (Klausner et al., 1983; Dautry-Varasat et al., 1983), receptor binding (Bali et al., 1991; Sipe and Murphy, 1991) and possibly a reductase (Nunez et al., 1990). While Tf receptors may be found on all cell types they are most prevalent on dividing cells and erythrocyte precursors. The second iron transport system is a Tf-independent transport system, present on cell surfaces, which mediates the accumulation of low-molecular-mass iron-anion complexes (Wheby and Crosby, 1963; Brissot et al., 1985; Basset et al., 1986; Wright et al., 1986; Richardson and Baker, 1992; Inman and Wessling-Resnick, 1993). In contrast to the Tf-based transport system, the activity of the Tf-independent system in some cell types is increased by the presence of extracellular iron (Kaplan et al., 1991). The regulation and tissue distribution of this system suggests a scavenger function for the removal of non-Tf-bound iron (Craven et al., 1987). Non-Tf-bound iron occurs in a number of iron-overload diseases, most commonly hereditary haemochromatosis (Grootveeld et al., 1989) and the rarer disorder, atransferrinaemia (Craven et al., 1987). This transport system is most active in organs, but is nearly non-existent in erythroid precursors (Craven et al., 1987). Analysis of the chemical form of non-Tf iron present in the plasma of patients

with hereditary haemochromatosis suggested that a significant fraction was in the form of ferric citrate (Grootveeld et al., 1989). Studies using cultured mammalian cells demonstrated that the Tf-independent transport system could mediate iron accumulation when cells were presented with ferric citrate (Basset et al., 1986; Kaplan et al., 1991; Inman and Wessling-Resnick, 1993).

A number of non-mammalian species can accumulate iron from ferric citrate. These organisms, which include plants, bacteria and fungi, reduce ferric citrate at the cell surface, generating ferrous iron which is then transported by a ferrous iron transport system (for review see Winkelmann et al., 1987). A ferrireductase may also be present on the surface of mammalian cells (Morley and Bezkorovainy, 1985; Sun et al., 1987), and it has been suggested that mammalian cells do not accumulate ferric iron, but rather accumulate the reduced form, ferrous iron (Thorstensen, 1988). In this report we have tested that hypothesis by determining whether mammalian cells exposed to ferric citrate accumulate both iron and cognate anion. Since citrate is not an $\text{Fe}(\text{II})$ chelator, if ferric iron is reduced before entry into cells then the accumulation of iron would not necessarily be quantitatively accompanied by the accumulation of citrate. Our studies indicate that exposure of cells to ferric citrate results in the differential accumulation of iron relative to citrate. Conditions which increase or decrease the uptake of iron do not correspondingly affect the accumulation of citrate and vice versa; impermeant $\text{Fe}(\text{II})$ chelators inhibit iron uptake without affecting the uptake of citrate. Our results suggest that iron is dissociated from citrate prior to its uptake, supporting the view that accumulation of iron by the Tf-independent transporter requires the activity of a surface ferrireductase.

Abbreviations used: Tf, transferrin; $\text{Tf}(\text{Fe})_2$, diferric transferrin; NTA, nitrilotriacetate; BPS, bathophenanthroline disulphonate; HBSS, Hank's balanced salt solution; FAC, ferric ammonium citrate; MEM, minimal Eagle's medium.

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MATERIALS AND METHODS

Cells

HeLa cells and human skin fibroblasts were cultured and maintained as described previously (Kaplan et al., 1991). Human skin fibroblasts were used between 10 and 14 passages.

Measurement of iron or citrate accumulation

[^{59}Fe]Ferric nitrilotriacetate (NTA) was prepared by addition of [^{59}Fe]Cl $_3$ (specific radioactivity 20–55 mCi/mg; Dupont–New England Nuclear) to a 5-fold molar excess of the disodium salt of NTA. [^{59}Fe]Ferric citrate was prepared by addition of [^{59}Fe]Cl $_3$ to a 5-fold molar excess of sodium citrate, and ferric [^{14}C]citrate by addition of FeCl $_3$ to a 5-fold molar excess of [^{14}C]citric acid (specific radioactivity 110 mCi/mmol; Amersham). To measure uptake of either ^{59}Fe or [^{14}C]citrate, cells grown on 35- or 60-mm-diam. tissue culture dishes were incubated for 1 h in serum-free minimal Eagle's medium (MEM) to deplete cells of Tf. The cells were then washed and incubated in serum-free MEM containing 10 mM Hepes (pH 7.2) with the specified concentrations of ferric citrate. Incubations were performed at either 37 °C or 0 °C for specified periods of time. Uptake measured at 0 °C is subtracted from that measured at 37 °C. In Figure 3 we have included the 0 °C measurements to give an appreciation of what is essentially non-specific uptake. The cells were washed with ice-cold phosphate-buffered saline, and solubilized in 0.1 % Triton X-100. Radioactivity of ^{59}Fe -containing samples was determined in a Packard gamma counter. The radioactivity of ^{14}C -containing samples was determined in a Packard liquid-scintillation counter, using Optifluor (Packard Instrument Co.) as the fluor. Uptake is expressed as femtomoles (fmol) of iron, or citrate/ μg of cell protein. Protein determinations were performed using the procedure of Lowry et al. (1951) with BSA as a standard. All experiments were performed multiple times and the data is expressed as mean $\pm$ S.E.M.

RESULTS

Incubation of HeLa cells (Figure 1a) or human skin fibroblasts (Figure 1b) with [^{14}C]citrate, ferric [^{14}C]citrate or [^{59}Fe]ferric citrate resulted in a time-dependent accumulation of radioactivity. The accumulation of both molecules was temperature-dependent and linear with respect to concentration (results not shown). Addition of iron to citrate resulted in an increase in [^{14}C]citrate uptake above that observed when cells were incubated with [^{14}C]citrate alone. Based on the stoichiometry of chelation we would expect a ratio of uptake of iron to citrate of either 1:1 or 1:2 if ferric citrate was accumulated as a complex (Hussein et al., 1981). In addition, the preparation of ferric citrate required that cells be exposed to a concentration of citrate that is 5-fold higher than that of iron (cf. Materials and methods section). In HeLa cells the molar stoichiometry of accumulation was 5 Fe/1 citrate. In fibroblasts at early time points twice as much iron was accumulated as citrate. At later times ^{59}Fe uptake appeared to saturate while [^{14}C]citrate accumulation still continued. The uptake of ^{59}Fe (presented as [^{59}Fe]ferric NTA or [^{59}Fe]ferric citrate) by HeLa cells or fibroblasts (results not shown) was markedly inhibited by simultaneous incubation with ferric citrate. Citrate was capable of inhibiting iron accumulation, but at a 20–40-fold higher concentration (Figure 2). At these concentrations citrate may be forming ferric citrate complexes with trace quantities of iron present in MEM, allowing these complexes to compete for uptake with the radiolabelled complexes.

Previously we reported that exposure of human skin fibroblasts to ferric ammonium citrate (FAC) resulted in a marked increase in the activity of the Tf-independent iron transport system (Kaplan et al., 1991). We therefore examined the ability of FAC-exposed fibroblasts to accumulate [^{14}C]citrate. As expected, FAC-exposed cells showed an enhanced accumulation of ^{59}Fe (compare Figure 1b to Figure 3; cf. Kaplan et al., 1991). There was, however, a marked increase in the uptake of [^{14}C]citrate. On a molar basis the accumulation of both molecules was almost equivalent. The increased rate of citrate accumulation, however, occurred whether FAC-incubated cells were exposed to either ferric [^{14}C]citrate or [^{14}C]citrate. This observation suggested that iron citrate was not taken up as a complex. To confirm this result we examined the ability of either iron or citrate to compete with the uptake of the radiolabelled molecules. At similar concentrations, citrate and ferric citrate were capable of inhibiting

Figure 1 Time dependency of iron and citrate uptake in HeLa cells and fibroblasts

Cultured HeLa cells (a) and fibroblasts (b) were incubated in serum-free medium for 1 h. The cells were incubated in MEM containing either [^{14}C]citrate (○), ferric [^{14}C]citrate (●), or [^{59}Fe]ferric citrate (□). The concentration of citrate was 25 μM and the concentration of iron was 5 μM . The cells were incubated at 37 °C, for the designated times, washed, solubilized and the radioactivity and cell protein were determined.

Figure 2 Effect of increasing concentrations of citrate or ferric citrate on ^{59}Fe accumulation by cultured HeLa cells

Cultured HeLa cells were incubated in serum-free medium for 1 h. The cells were incubated in MEM containing the specified concentrations of citrate (●) or ferric citrate (○) and ^{59}Fe -NTA ($1 \mu\text{M}$ ^{59}Fe : $5 \mu\text{M}$ NTA). Cells were incubated at 37°C for 30 min, washed, solubilized and radioactivity determined. The amount of radioactivity accumulated by cells incubated with ^{59}Fe -NTA alone is defined as 100%.

Table 1 Effect of citrate on the accumulation of ^{59}Fe or [^{14}C]citrate by FAC-exposed fibroblasts

Human skin fibroblasts were exposed to FAC for 48 h. The cells were washed and incubated in serum-free medium for 1 h. The cells were washed again and then incubated in MEM buffered with 10 mM Hepes (pH 7.2) containing either [^{59}Fe]ferric citrate, ferric [^{14}C]citrate or [^{14}C]citrate in the presence or absence of $100 \mu\text{M}$ citrate for a further hour at 37°C . The concentration of citrate in the ferric citrate chelate solution was $5.0 \mu\text{M}$ and the concentration of iron was $1.0 \mu\text{M}$. The cells were washed and specific uptake determined.

Sample	Citrate ($100 \mu\text{M}$)	Specific uptake (fmol/ μg of protein)
[^{14}C]Citrate	—	183 (45)
	+	37 (0)
Ferric [^{14}C]citrate	—	199 (2)
	+	44 (0)
[^{59}Fe]Ferric citrate	—	95 (6)
	+	93 (23)

the accumulation of [^{14}C]citrate. At the highest concentration tested ($100 \mu\text{M}$) there was an 80% reduction in accumulation (Table 1). Citrate even at $100 \mu\text{M}$ had no effect on ^{59}Fe uptake.

If iron is dissociated from citrate by reduction of Fe(III) to Fe(II) at the cell surface prior to uptake, then two predictions can be made. First, impermeable ferrous chelators should inhibit ^{59}Fe accumulation without affecting the accumulation of [^{14}C]citrate. The data in Figure 4 confirms that prediction. Addition of bathophenanthroline disulphonate (BPS) inhibits iron accumulation without affecting the accumulation of citrate (results not shown). Addition of preformed Fe-BPS chelates had no effect on ^{59}Fe uptake. This result suggests the inhibitory effect of BPS resulted from the ability of the chelator to bind iron, as opposed to the chelator-iron complex affecting the transporter. Incubation of BPS with ferric citrate at 37°C for up to 4 h does not result in the formation of a BPS-ferrous chelate. The formation of BPS-Fe(II) chelates requires the presence of cells, does not occur at 0°C but does occur at 37°C . Analogous results were obtained using ferrozine, which is another impermeable

Figure 3 Time dependency of iron and citrate uptake in fibroblast cells exposed to iron salts

Cultured fibroblasts were incubated in medium containing FAC (2.1 mg/ml) for 48 h. Cells were then washed with serum-free medium for 1 h. The cells were then incubated in MEM containing either [^{14}C]citrate (●, ○), ferric [^{14}C]citrate (▲, △) or [^{59}Fe]ferric citrate (■, □). The concentration of citrate was $25 \mu\text{M}$ and the concentration of iron was $5 \mu\text{M}$. The cells were incubated at either 37°C (closed symbols) or 0°C (open symbols), for the designated times, washed, solubilized and levels of radioactivity and cell protein were determined.

Figure 4 The effect of the impermeable Fe(II) chelators BPS and ferrozine on the accumulation of ^{59}Fe in cells exposed to ferric citrate

Fibroblasts were incubated with FAC for 48 h. The cells were washed, incubated in serum-free medium for 1 h and then incubated in MEM with 10 mM Hepes (pH 7.2) containing [^{59}Fe]ferric citrate. The cultures were also incubated with either PBS (□) or Fe-BPS (●, 1:2 molar ratio of Fe:BPS) or ferrozine (■). The cultures were incubated at 37°C and specific uptake determined. The amount of uptake determined in the absence of the chelators was defined as 100%.

Fe(II) chelator. The inhibition of Fe(II) accumulation was much lower with ferrozine than with BPS, consistent with the much lower activity of ferrozine as an Fe(II) chelator (Berczi et al.,

Table 2 Effect of ascorbate on the accumulation of iron and citrate by cultured fibroblasts

Cultures of both control and FAC-incubated fibroblasts (2.1 mg/ml FAC, 48 h) were washed and incubated in serum-free medium for 1 h. The cells were then incubated with either ^{59}Fe -NTA, ferric $[^{14}\text{C}]$ citrate, or $[^{59}\text{Fe}]$ ferric citrate in the presence or absence of 300 μM ascorbate. The cells were incubated for 30 min at 37 °C and specific uptake determined.

Sample	Incubation	Specific uptake (fmol/ μg of protein)	
		— ascorbate	+ ascorbate
$[^{59}\text{Fe}]$ ferric citrate	Control	12 (1)	62 (5)
	FAC	203 (6)	528 (30)
^{59}Fe -NTA	Control	14 (1)	68 (9)
	FAC	229 (15)	465 (44)
Ferric $[^{14}\text{C}]$ citrate	Control	7 (1)	6 (1)
	FAC	294 (39)	201 (24)

Table 3 Effect of Ca^{2+} depletion on accumulation of iron and citrate

Suspension-grown HeLa cells were washed and incubated for 1 h in either HBSS or HBSS — Ca^{2+} , containing 10 mM Hepes and 1.0 mg/ml glucose. The cells were then incubated in either Ca^{2+} -replete or Ca^{2+} -depleted media with $[^{59}\text{Fe}]$ ferric citrate, ferric $[^{14}\text{C}]$ citrate, or $[^{14}\text{C}]$ citrate. The citrate concentration was 25 μM and the concentration of iron was 5 μM . The cells were incubated for 1 h at 37 °C, washed and specific uptake determined.

Sample	Incubation ($\pm \text{Ca}^{2+}$)	Specific uptake (fmol/ μg of protein)
$[^{14}\text{C}]$ citrate	+	48 (1)
	—	104 (8)
Ferric $[^{14}\text{C}]$ citrate	+	175 (6)
	—	332 (0)
$[^{59}\text{Fe}]$ ferric citrate	+	1189 (246)
	—	355 (191)

1991; Cowart et al., 1993). These results further suggest that ferric citrate is reduced before the accumulation of iron. Secondly, if iron and citrate are taken up as a complex, then chemical reduction of ferric citrate should decrease the uptake of iron. As demonstrated in Table 2 addition of ascorbate markedly increases the uptake of iron and reduces the uptake of citrate.

We, and others, have described a number of conditions which affect the activity of the Tf-independent iron transport system. If there is a co-ordinate accumulation of citrate with iron, then we predict that treatments which affect the accumulation of iron should similarly affect the accumulation of citrate. Accumulation of iron by the Tf-independent iron transport system, particularly in perfused rat liver and cultured HeLa cells, is dependent on the presence of extracellular Ca^{2+} (Wright et al., 1986; Sturrock et al., 1990). Incubation of HeLa cells in Ca^{2+} -free medium (Hanks' balanced salt solution, HBSS) with $[^{59}\text{Fe}]$ ferric citrate results in a decreased rate of accumulation of ^{59}Fe (Table 3). There was, however, an increase in the uptake of $[^{14}\text{C}]$ citrate both in the presence or absence of iron.

DISCUSSION

Studies have suggested the presence of a plasma membrane ferrireductase activity on mammalian cells. This activity has been reported to reduce extracellular ferricyanide and extracellular $\text{Tf}(\text{Fe})_2$, and may be involved in an internalization-independent

iron accumulation pathway (Sun et al., 1987; Thorstensen, 1988). The interpretation of these experiments has been challenged on both experimental and theoretical grounds (Thorstensen and Aisen, 1990). To determine if the Tf-independent transport system requires a surface reductase activity, we examined whether cells exposed to ferric citrate exhibited obligatory uptake of iron and citrate. Because of the low affinity of citrate for $\text{Fe}(\text{II})$ (Hamm et al., 1954), if ferric citrate is reduced at the cell surface, then due to the low affinity of $\text{Fe}(\text{II})$ for citrate the iron-chelator complex should dissociate at the cell surface and the stoichiometry of accumulation of radiolabelled iron and citrate would not reflect the molar ratio of the two in the chelate. Similar approaches were used to demonstrate the presence and function of surface ferrireductase activity in bacteria (Hussein et al., 1981) and plants (Romheld and Marschner, 1983).

If iron citrate was accumulated as a complex we expected to see an accumulation of iron: citrate of either 1:1 or 1:2, based on the reported stoichiometry of ferric citrate chelation (Hussein et al., 1981). The method of preparation of ferric citrate resulted in the exposure of cells to a greater concentration of citrate than iron. In HeLa cells and fibroblasts, iron was accumulated at a rate that was 3–5-fold greater than that of citrate. This result is consistent with the involvement of a reductase but other alternatives are possible. One possibility is that ^{59}Fe is accumulated by a ligand-transfer reaction: $\text{Fe}(\text{III})$ is transferred from citrate (or NTA) to an $\text{Fe}(\text{III})$ -binding moiety. Membrane proteins or lipids with $\text{Fe}(\text{III})$ -binding capacity have been reported (Raja et al., 1987; Teichman and Stremmel, 1990; Conrad et al., 1990). The effect of BPS or ferrozine, impermeant $\text{Fe}(\text{II})$ chelators, makes this alternative unlikely: they inhibit ^{59}Fe accumulation without affecting the accumulation of $[^{14}\text{C}]$ citrate. Addition of preformed BPS-Fe complexes had little effect on the uptake of ^{59}Fe , indicating that it is the chelating activity of BPS that is responsible for inhibition of uptake rather than BPS-Fe affecting the transporter *per se*. Further, the ability of BPS or ferrozine to chelate iron is dependent upon cells being incubated at 37 °C. The rate of formation of BPS- $\text{Fe}(\text{II})$ or ferrozine- $\text{Fe}(\text{II})$ complexes in the absence of cells, or in the presence of cells incubated at 0 °C, was not detectable during the measurement period. If a ligand-transfer reaction were involved it is unlikely that the $\text{Fe}(\text{II})$ -chelating activity of BPS would inhibit iron transport. These results are more consistent with $\text{Fe}(\text{III})$ being reduced to $\text{Fe}(\text{II})$ on the cell surface, prior to transport, where it is accessible to the action of BPS.

Previously we demonstrated that exposure of cells, particularly fibroblasts, to iron salts resulted in an increase in iron accumulation (Kaplan et al., 1991). We observed that fibroblasts exposed to iron citrate also demonstrated an induction in citrate accumulation but several lines of evidence indicate that ferric citrate is not accumulated as a complex: (a) enhanced uptake of citrate occurred in the absence of added iron; (b) uptake of $[^{14}\text{C}]$ citrate was inhibited by unlabelled citrate whereas the uptake of ^{59}Fe was unaffected; (c) the ferrous chelator BPS inhibited ^{59}Fe uptake without inhibiting the uptake of $[^{14}\text{C}]$ citrate; (d) addition of ascorbate, which reduces ferric citrate, markedly increased the uptake of iron but decreased the uptake of citrate.

The mechanism by which exposure to ferric citrate results in an increase in citrate accumulation is unknown. Little is known about the characteristics of citrate accumulation/transport in cultured cells. Preliminary studies indicate that citrate accumulation in cultured fibroblasts and HeLa cells is independent of bivalent cations and sodium and was not inhibited by bi- or tricarboxylic acids such as succinate or pyruvic acid. It may well be that the increased accumulation of citrate reflects increased metabolism rather than transport, increased metabolism resulting

in an increased retention of citrate metabolites. Citrate is an allosteric regulator of several key enzymes in the glycolytic and oxidative metabolic pathways: increases in citrate result in increased oxidative metabolism. An attractive hypothesis is that increased intracellular iron results in increased activity of *cis*-aconitase, the iron-replete form of the iron-responsive-element-binding protein (O'Halloran, 1993). Increased *cis*-aconitase activity would result in an increase in isocitrate and other citrate metabolites. Studies are in progress to determine if incubation with ferric citrate increases citrate transport or citrate metabolism.

The studies presented here suggest that uptake of iron by the Tf-independent transport system requires at least two steps; reduction at the cell surface and accumulation of ferrous iron. In this regard the mammalian Tf-independent transport systems is analogous to iron-transport systems described in bacteria, plants and fungi. Throughout the phylogenetic tree there appear to be two conserved iron accumulation systems, one of which involves the secretion and re-accumulation of iron chelators, secretion of siderophores being the paradigm. The second mechanism involves the reduction of ferric to ferrous iron, which is then transported across cell membranes. Reduction of Fe(III) to Fe(II) can occur by either of two routes: secretion of molecules with reducing potential, such as phenols, or the direct addition of an electron. We have not been able to demonstrate that fibroblasts secrete molecules with reducing potential, which suggests that Fe(III) reduction is mediated by direct electron transfer. This conclusion leads to the inference that transmembrane iron transport may involve movement of iron through an aqueous channel. The requirement for iron in the ferrous form might result from the near insolubility of ferric iron in aqueous solutions.

In the Tf-dependent iron-uptake system, iron is released from internalized Tf(Fe)₂ within the endosomal apparatus. While ferric iron may be released from Tf, reduction of iron may be required for transport out of the endosome (Nunez et al., 1990). Based on these observations the Tf-mediated transport system may represent an adaptation of a reduction-based ferrous transport system. These results further suggest that while iron may be transported in biological fluids as Fe(III), chelated to either citrate or Tf, iron transport across membranes requires reduction to Fe(II).

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