# Extracellular Ferrireductase Activity of K562 Cells Is Coupled to Transferrin-Independent Iron Transport†

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ABSTRACT: The reduction of $Fe^{3+}$ to $Fe^{2+}$ has been established to play a critical role in the uptake of iron by many organisms. Recently, a mechanism of iron transport in the absence of transferrin (Tf) was described for the human K562 cell line and a role for ferrireductase activity was implicated in this process as well [Inman, R. S., & Wessling-Resnick, M. (1993) *J. Biol. Chem. 268*, 8521–8528]. The present report characterizes the extracellular reduction of ferricyanide to ferrocyanide catalyzed by K562 cells. The observation that membrane-impermeant ferricyanide competitively inhibits Tf-independent assimilation of iron from $^{55}Fe$–nitriloacetic acid indicates that this ferrireductase activity is indeed coupled to the transport mechanism. From a series of initial rate experiments, the kinetic parameters for cell surface ferrireductase activity, $V_{max}$ = 0.102 nmol $min^{-1}$ $(10^6$ cells$)^{-1}$ and $K_m$ = 6.13 $\mu$M, were determined. Neither the $V_{max}$ nor the $K_m$ of this reaction is modulated by changes in extra- or intracellular iron levels; thus, similar to Tf-independent transport activity in K562 cells, the ferrireductase activity is not regulated in response to iron levels. Transmembrane oxidoreductase activity is also reportedly involved in the control of cellular growth; however, the K562 cell ferrireductase is unresponsive to insulin and is not inhibited by the antitumor drugs adriamycin, actinomycin D, or *cis*-platin, observations that fail to support a role for this particular activity in cell regulation. Rather, the K562 cell ferrireductase appears to be tightly coupled to the mechanism of Tf-independent transport as demonstrated by its sensitivity to $Cd^{2+}$, a specific inhibitor of non-Tf iron uptake by K562 cells. $Cd^{2+}$ blocks the reduction of ferricyanide catalyzed by K562 cells with an $IC_{50} \sim$ 35 $\mu$M, a value close to that determined for inhibition of iron transport. Moreover, $Cd^{2+}$ inhibits transport in the presence of ascorbate, a chemical reducing agent, suggesting that the substrate binding site for reduction and transport are one in the same and that both of these functions may be carried out by the same molecular complex.

Microorganisms, fungi, plant, and animal cells all acquire iron as an essential nutrient, albeit through several different strategies. Each of these distinct mechanisms must serve to overcome enormous difficulties presented by the chemistry of iron, including the insolubility of $Fe^{3+}$ at physiologic pH and the potential of the more soluble $Fe^{2+}$ to produce oxidative damage. To avoid these coupled problems, many organisms secrete siderophores that chelate $Fe^{3+}$ to maintain the cation in a soluble state for membrane translocation via specific receptors, a process followed by the intracellular reduction to $Fe^{2+}$ (Nielands, 1981). This mechanism is perhaps analogous to the delivery of iron to mammalian cells by the receptor-mediated endocytosis of the serum iron-binding protein, transferrin (Tf). $Fe^{3+}$ is released from the internalized Tf–Tf receptor complex upon delivery to the cell's acidic endosomal compartments (Smythe & Warren, 1991). The subsequent transport of iron from the lumen of the endosome to the cytosol is thought to involve the reduction of $Fe^{3+}$ to $Fe^{2+}$, with a carrier-mediated translocation of the divalent cation across the membrane bilayer (Nunez et al., 1990; Watkins et al., 1992). Other known systems for iron uptake also include the activity of a plasma membrane reductase for the subsequent transport of ferrous iron across the cell surface, a mechanism that has been recently identified for the yeast *Saccharomyces cerevisiae* (Dancis et al., 1990). A critical and universal element in each of these different uptake mechanisms is the apparent requirement for ferrireductase activity, yet few molecular details are known about the enzymatic machinery that catalyzes this reaction or how it is associated with these different membrane transport processes.

Work by Inman and Wessling-Resnick (1993) and Sturrock et al. (1990) has recently established that iron can also be directly imported into certain mammalian cells in a *Tf-independent* manner, similar to the iron transport system identified in *S. cerevisiae*. Iron assimilation in the absence of receptor-mediated endocytosis is characterized by at least two distinct mechanisms: (1) a calcium-dependent, transition metal transport system (Sturrock et al., 1990) and (2) a calcium-independent, Fe/Cd-specific process (Inman & Wessling-Resnick, 1993). A major distinction between the two known Tf-independent transporters is that the more universal transition metal uptake system may be up-regulated in response to extracellular heavy metal salts, allowing for the clearance of potentially toxic elements (Kaplan, 1991). In contrast, the activity of the highly specific, $Cd^{2+}$-inhibitable, iron transporter identified in the human erythroleukemia K562 cell line is not modulated by extracellular iron salts and may be unique to cells of hematopoietic origin (Inman & Wessling-Resnick, 1993).

A cell surface ferrireductase is implicated in the K562 cell Tf-independent transport, suggesting mechanistic similarities with the system for iron import characterized for yeast (Dancis et al., 1990). However, only limited information is available

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regarding the reduction of $Fe^{3+}$ at the surface of mammalian cells. Past investigations led to the proposal for a plasma membrane ferrireductase activity in the acquisition of iron from Tf by hepatocytes (Thorstensen, 1988; Thorstensen & Romslo, 1988), but more thorough investigation does not support a physiologic role for this activity (Thorstensen & Aisen, 1990). The reduction of iron in the lumen of the gut is thought to play a role in intestinal absorption, and there is evidence for a $Fe^{3+}$-reducing activity present on the mucosal surface of mouse duodenum that is potentially coupled to transport (Raja et al., 1992). Cell surface oxidoreductase activity has also been reported for several types of mammalian cells, but the relationship of this function to iron uptake is obscure, as transmembrane redox activity may be primarily involved in cell growth (Crane et al., 1985). We have undertaken an investigation of the capacity of K562 cells to mediate the reduction of ferricyanide to ferrocyanide in order to understand the first steps in the passage of iron across the plasma membrane. The results presented here characterize the properties of a cell surface ferrireductase activity and how it may play a role in Tf-independent transport. Our observations indicate that the extracellular reduction of $Fe^{3+}$ and transport of $Fe^{2+}$ by K562 cells are tightly coupled functions that may be mediated by the same molecular complex.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Preparation.* Human erythroleukemia K562 cells were cultured as previously described (Inman & Wessling-Resnick, 1993), with $\alpha$-minimum essential medium ($\alpha$-MEM) supplemented with 7% fetal bovine serum (Bio-Whittaker). For growth in serum-free medium, K562 cells were initially collected by centrifugation at 500*g* for 5 min and washed once in $\alpha$-MEM. The cells were then resuspended in $\alpha$-MEM containing 1 mg/mL bovine serum albumin (BSA) and 1 $\mu$g/mL $FeSO_4$, incubated at 37 °C for 30 min, washed one more time, and then grown for 2 days in serum-free $\alpha$-MEM supplemented with the same concentrations of BSA and $FeSO_4$. For some experiments, K562 cells were treated overnight with desferrioxamine (50 $\mu$M) and added to $\alpha$-MEM supplemented with 7% fetal bovine serum. K562 cells cultured under all of these conditions were washed three times in phosphate-buffered saline (PBS) and resuspended in Hanks' buffer (137 mM NaCl, 5.36 mM KCl, 1.3 mM $CaCl_2$, 410 $\mu$M $MgSO_4$, 490 $\mu$M $MgCl_2$, 337 $\mu$M $Na_2HPO_4$, 440 $\mu$M $KH_2PO_4$, 4.17 mM $NaHCO_3$, and 5.55 mM dextrose) at (1–5) × $10^6$ cells/mL in preparation for experiments. Other conditions of cell culture included incubation of K562 cells with $FeNH_4$ citrate as follows. Cells were collected by centrifugation at 500*g* for 5 min and washed three times in PBS. The cells were resuspended in $\alpha$-MEM containing 1 mg/mL BSA and 0.3 mg/mL $FeNH_4$ citrate and incubated for 1 h at 37 °C. In preparation for the ferrireductase assay, these cells were washed four times in ice-cold PBS, followed by one additional wash in Hanks' buffer.

*Ferrireductase Assays.* Extracellular ferrireductase activity was monitored by the production of ferrocyanide catalyzed by K562 cells incubated with ferricyanide. Ferrocyanide was measured as described by Avron and Shavit (1963). Briefly, K562 cells were resuspended in 1 mL of Hanks' buffer at 37 or 4 °C [(1–5) × $10^6$ cells/mL]. The ferrireductase reaction was initiated upon addition of potassium ferricyanide to cells, and after the desired incubation period, the reaction was terminated by chilling the cells on ice. For initial rate measurements, times of incubation were such that less than 10% of substrate had been converted to ferrocyanide. The cells were immediately separated by microcentrifugation and a 700-$\mu$L aliquot of supernatant was transferred into a spectrophotometric cuvette to which was added, in order, 100 $\mu$L of 3 M sodium acetate, pH 6.4, 100 $\mu$L of 0.2 M citric acid, 50 $\mu$L of 3.34 mg/mL bathophenathrolinesulfonate (BPS), and 50 $\mu$L of 3.3 mM $FeCl_3$, prepared in 0.1 M acetic acid. The samples were well-mixed by inverting the cuvette 10 times, and the absorbance at 535 nm was measured after allowing for color development (10–60 min). For some experiments, the reduction of ferricyanide was measured directly by monitoring the change in absorbance at 420 nm from supernatants collected after the initial centrifugation step.

*Iron Transport Measurements.* The uptake of non-Tf iron by K562 cells was measured using an assay previously established (Inman & Wessling-Resnick, 1993). Briefly, $^{55}Fe$ (DuPont–New England Nuclear, >30 mCi/mg) was complexed with a 4-fold excess of nitriloacetic acid (NTA) in 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid–tris(hydroxymethyl)aminomethane (HEPES–Tris), pH 6.0, containing 100 mM HCl, and titrated to neutral pH with NaOH. Assay mixtures of 300 $\mu$L [(1–10) × $10^6$ cells/mL final] were prechilled and appropriate amounts of $^{55}FeNTA$ with additions of $CdCl_2$, freshly dissolved ascorbate, or buffer alone, as indicated in figure and table legends, were added. Uptake of radiolabel was initiated by rapidly warming the reaction mixture to 37 °C. After a 5-min incubation, 250-$\mu$L aliquots were withdrawn and the transport reaction was quenched by addition to 750 $\mu$L of ice-cold buffer (1 mM FeNTA, 25 mM HEPES, and 150 mM NaCl, pH 7.4), followed by incubation on ice for 20 min. Of this mix, 950 $\mu$L was filtered through nitrocellulose filters and washed twice with 3 mL of 150 mM NaCl. The cell-associated $^{55}Fe$ collected on the filters was measured by scintillation counting. Specific uptake was determined as the difference between cell-associated radioactivity measured at 37 and 4 °C in order to control for nonspecific binding.

*Other Methods.* Inhibition studies were performed with the following conditions. K562 cells were prepared as described above, except that adriamycin, *cis*-platin, or $CdCl_2$ was also included in the reaction mixture at indicated concentrations. Ferrireductase activity was also blocked upon alkylation with 5 mM *N*-ethylmaleimide (NEM) for 30 min at 4 °C. In order to study insulin effects, K562 cells were suspended in serum-free medium supplemented with 1 mg/mL BSA and 1 $\mu$g/mL $FeSO_4$ as described above [(1–2) × $10^6$ cells/mL], in the presence of appropriate concentrations of insulin (Gibco-BRL). All experiments were performed on at least three separate occasions, except where indicated, and data from representative individual experiments are shown, except for Figure 4, which presents averaged values from two separate experiments, and Figure 5, which presents the pooled data obtained in four individual experiments. Ferrireductase measurements were also made in duplicate and the determined average values with standard error bars are presented.

## RESULTS

*K562 Cells Exhibit an Extracellular Ferrireductase Activity.* Several past investigations have been directed toward identifying extracellular $Fe^{3+}$ reduction catalyzed by mammalian cells (Thorstensen, 1998; Thorstensen & Romslo, 1988); however, problems created by the method of analysis compromise conclusions about the presence of functional reductase activity (Thorstensen & Aisen, 1990). In particular, the use of the $Fe^{2+}$-chelating agent ferrozine to monitor production of ferrous iron is problematic since the presence

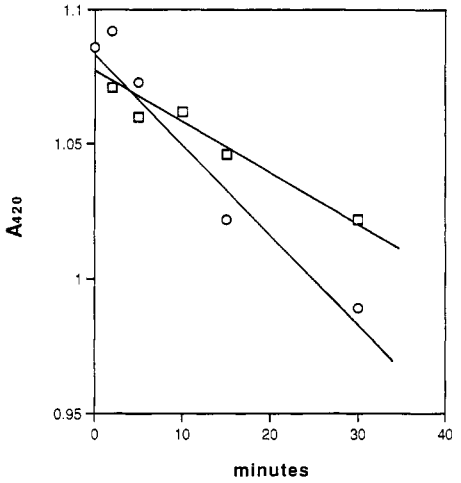


FIGURE 1: Time course of ferricyanide reduction. K562 cells were incubated with 1.0 mM $K_3Fe(CN)_6$ at 37 °C in 25 mM HEPES and 150 mM NaCl, pH 7.4, with 1 mg/mL dextrose and 1.3 mM $CaCl_2$. At the indicated times, 0.8-mL aliquots were withdrawn from the 5.0-mL reaction mixture and chilled on ice. Cells were then pelleted by centrifugation at 14000*g* for 5 min and the supernatant was collected. Reduction of ferricyanide is monitored by the decrease in absorbance at 420 nm of the supernatant due to extracellular reductase activity present in samples containing either $1.0 \times 10^6$ cells/mL (□) or $5.0 \times 10^6$ cells/mL (○).

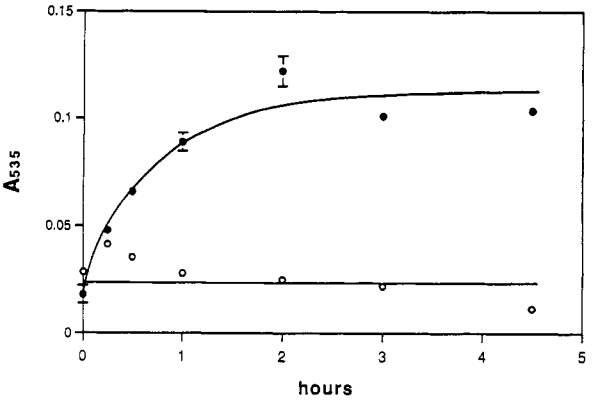


FIGURE 2: Time course of ferrocyanide production. K562 cells ($1.62 \times 10^6$ cells/mL) were suspended in an 8-mL volume of Hanks' buffer, with the addition of 10 μM $K_3Fe(CN)_6$ at 37 °C (●) or 4 °C (○). At the indicated times, 1-mL aliquots were withdrawn, chilled on ice, and immediately centrifuged at 500*g* for 5 min. The appearance of ferrocyanide upon reduction of ferricyanide was assayed as described by Avron and Shavit (1963) and detailed under Experimental Procedures. Shown are mean values for the absorbance at 535 nm (due to the BPS complex of resultant $Fe^{2+}$), obtained for duplicate samples incubated at the different temperatures.

of the chelating agent itself alters the iron reducing potential, therefore driving the reaction. For these reasons, we sought alternative approaches to address the question of whether K562 cells could mediate the extracellular reduction of $Fe^{3+}$ as an early step of Tf-independent uptake of iron. Other studies have relied on ferricyanide as a substrate to monitor $Fe^{3+}$ reduction by following the decrease in absorbance at 420 nm (Thorstensen & Aisen, 1990; Crane et al., 1985; Clark et al., 1981). Because both ferricyanide and its product ferrocyanide are membrane-impermeant, the latter method provides a convenient measure for extracellular ferrireductase activity and is compatible with our concerns regarding the role of iron reduction in the Tf-independent transport mechanism. In fact, a time-dependent reductase activity is observed when K562 cells are incubated with 1 mM ferricyanide at 37 °C, as shown by the experimental results of Figure 1. The rate of ferricyanide reduction increases in a manner proportional to the number of K562 cells present in the assay. Shown is the decrease in $A_{420}$ catalyzed by $1 \times 10^6$ cells/mL (squares) compared to a parallel incubation with $5 \times 10^6$ cells/mL (circles). The determined rates for $Fe^{3+}$ reduction are 1.6 and 3.2 nmol/min, respectively. This experiment confirms the presence of an activity associated with K562 cells that catalytically reduces ferric iron.

Using this method, initial attempts were made to assess the substrate concentration dependency for ferricyanide reduction catalyzed by K562 cells. However, the molar extinction coefficient for ferricyanide at 420 nm ($\epsilon$ = 1000 $cm^{-1}$ $M^{-1}$) did not permit accurate determination of $K_m$ values, which were estimated to be below the limit of sensitivity that could be achieved in our hands. Moreover, as indicated by the results of Figure 1, activity was not linear with respect to cell concentration. Thus, an alternative strategy was chosen in which the amount of product, ferrocyanide, was followed upon the catalyzed reduction of ferricyanide. This approach relies on a rapid and sensitive assay to determine ferrocyanide, modified as detailed under Experimental Procedures. Briefly, cells are incubated in suspension with ferricyanide for appropriate times and then removed by centrifugation. The supernatant is collected and the amount of product is then determined under conditions outlined by Avron and Shavit (1963), including the addition of ferric ion, which reduces to ferrous ion in the presence of ferrocyanide. The latter is measured by the complex formed in the presence of bathophenanthrolinesulfonate (BPS). Since the BPS complex has a molar extinction coefficient of 22 000 $cm^{-1}$ $M^{-1}$ at 535 nm, nanomole amounts of ferrocyanide can be accurately determined. Moreover, ferricyanide does not produce any color in the assay, therefore allowing the amount of ferrocyanide produced to be analyzed in the presence of excess ferricyanide.

Figure 2 shows data typical of results obtained by this method, demonstrating the time course for the appearance of ferrocyanide due to the reduction of ferricyanide mediated by K562 cells at 37 °C (closed circles). At 4 °C, no accumulation of product is observed (open circles). In order to verify that the observed reductase activity was not due to damaged or leaky cells, cell viability was assessed based on trypan blue exclusion and was found to be greater than 90% for both control and cells exposed to 100 μM ferricyanide for 60 min at 37 °C. Furthermore, the cellular ATP levels were measured under these conditions and were found to be identical. Finally, to test whether some secreted product of intact (or damaged) cells was actually responsible for this activity, medium that was conditioned by K562 cells at 37 °C was added to the assay mixture but was found to lack any detectable reductase activity. The latter control experiment is important since a mechanism for the transmembrane reduction of ferricyanide by red blood cells has been described that is ascorbate-mediated (Orringer & Roer, 1979). In the latter studies it was observed that ascorbate released by erythrocytes could reduce extracellular iron; however, we do not find any evidence of a similar mechanism that would account for the K562 cell-mediated ferricyanide reductase activity. This is most likely due to the fact that this cell line is ascorbate-deficient under the culture conditions employed. Finally, the idea that the catalytic reduction is cell-mediated is also supported by the data of Figure 3, which demonstrate that ferricyanide reductase activity is linear with the number of K562 cells in the assay mixture. Because neither ferricyanide nor ferrocyanide is permeant to cells (Orringer & Roer, 1979), it is reasonable to assume that this reaction occurs extracellularly. We conclude that the K562 cells have a surface-associated

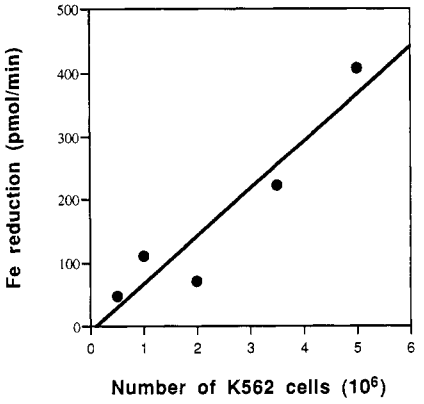


FIGURE 3: Ferrireductase activity measured as a linear function of cell density. The indicated numbers of K562 cells [(0.5–5) × $10^6$ cells/mL] were incubated in a 1-mL volume of Hanks' buffer containing 100 μM $K_3Fe(CN)_6$ at 37 and 4 °C for appropriate periods of time to obtain initial rates of the reductase reaction. The assay mixtures were immediately chilled on ice, the cells were then pelleted by centrifugation, and the supernatants were assayed for ferrocyanide as described under Experimental Procedures. The rate of ferrocyanide production is determined as the difference between measurements made for samples held at 37 versus 4 °C. The calculated rates (picomoles per minute) are shown as a function of cell concentration ($10^6$ cells per milliliter) for an individual experiment; comparable results were obtained on two separate occasions.

ferrireductase activity and that our assay provides a reliable method to measure and characterize this membrane function.

Using this approach, initial rates of ferrocyanide production were determined as a function of ferricyanide concentration in the reaction catalyzed by K562 cells. The top panel of Figure 4 presents the Michaelis–Menten curve for the K562 cell ferrireductase activity, demonstrating saturation kinetics. The lower panel presents the double-reciprocal plot of this initial rate data, which displays linearity with respect to reciprocal substrate concentration. From a series of experiments, values for $V_{max}$ and $K_m$ were obtained and are summarized in Table 1.

*Ferrireductase Activity Is Not Modulated by Changes in Iron Levels.* Many investigations have now established the biosynthetic regulation of proteins involved in iron metabolism in response to environmental iron levels, including transcriptional and translational control of the synthesis of ferritin and the transferrin receptor (O'Halloran, 1993). The Tf-independent transport mechanism in fibroblasts can also be up-regulated when cells are exposed to iron or other transition metal salts (Kaplan et al., 1991). The latter effect is due to rapid alterations in the $V_{max}$ of transport thought to reflect the recruitment of an intracellular pool of transporters to the cell surface. In contrast, Tf-independent transport in K562 cells is not up-regulated either biosynthetically or in response to extracellular concentrations of iron due to relocalization of cryptic transporters (Inman & Wessling-Resnick, 1993). Therefore, we investigated whether the K562 cell ferrireductase activity could be modulated with respect to different levels of iron.

K562 cells were grown either in the absence of Tf and in the presence of serum-free medium supplemented with 1 μg/mL $FeSO_4$ or in the presence of medium supplemented with serum and the addition of 50 μM desferrioxamine. Initial rates of reduction were determined as a function of ferricyanide concentration to obtain $V_{max}$ and $K_m$ values presented in Table 1. By comparison to the kinetic parameters determined under normal growth conditions, the supplementation of medium with excess iron salt or with the iron chelator had little effect on the $K_m$ or $V_{max}$ of reductase activity associated with the cell

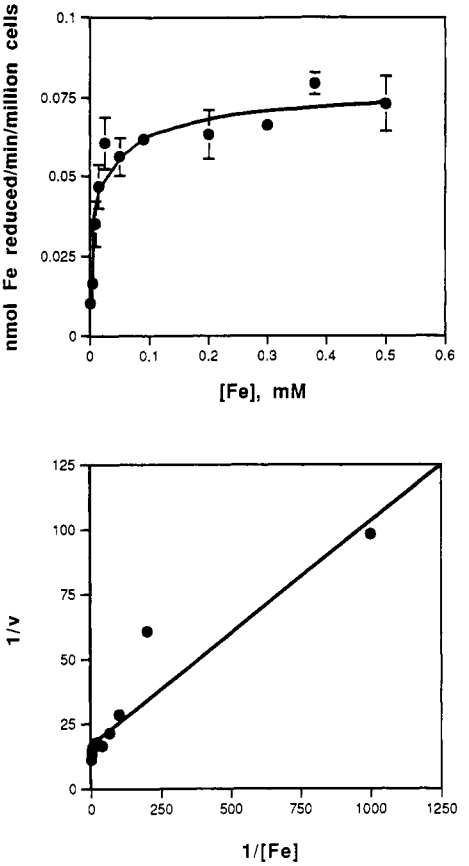


FIGURE 4: Saturation kinetics of ferrireductase activity. Top panel: K562 cells were incubated in a 1-mL volume of Hanks buffer containing the indicated concentrations of $K_3Fe(CN)_6$ (0.001–0.5 mM) at 37 and 4 °C. Reactions were terminated on ice, cells were removed by centrifugation, and the collected supernatants were assayed for ferrocyanide as described for Figure 2 and under Experimental Procedures. The rate of ferrocyanide production is determined as the difference between measurements made for samples held at 37 versus 4 °C; the rates determined in two independent experiments were averaged and are plotted ± SD as a function of ferricyanide concentration in the assays. Bottom panel: Shown is the reciprocal of data from the top panel presented as the Lineweaver–Burk plot.

Table 1: Kinetic Parameters for K562 Cell Ferrireductase Activity[a]

| growth condition | $K_m$ (μM) | $V_{max}$ [nmol $min^{-1}$ ($10^6$ cells)$^{-1}$] |
|---|---|---|
| control ($n$ = 4) | 6.13 ± 2.25 | 0.102 ± 0.001 |
| desferrioxamine (50 μM) ($n$ = 3) | 3.80 ± 0.97 | 0.119 ± 0.002 |
| serum-free medium supplemented with BSA and $FeSO_4$ ($n$ = 4) | 4.54 ± 1.10 | 0.116 ± 0.029 |
| preincubation with $FeNH_4$ citrate for 1 h ($n$ = 3) | 3.57 ± 0.52 | 0.0904 ± 0.025 |

[a] Rates of ferrireduction were assayed as a function of ferricyanide concentration as described for Figure 4 to obtain values for the kinetic parameters $K_m$ and $V_{max}$. Compared are those values determined for control cells versus values determined under different conditions of cell growth, as detailed in Experimental Procedures. For each condition tested, $K_m$ and $V_{max}$ values were not statistically different from those measured for control cells, indicating that the K562 cell ferrireductase is not responsive to environmental iron levels.

surface. To verify that intracellular reductases were not rapidly recruited to the cell surface, we also studied the influence of short-term (1-h) exposure to 0.3 mg/mL $FeNH_4$ citrate, a condition that promotes the relocalization of Tf-independent transporters to the fibroblast plasma membrane. As our results demonstrate, under all of the conditions tested the kinetic parameters of ferrireductase activity remain fairly constant. In particular, one would anticipate any change in

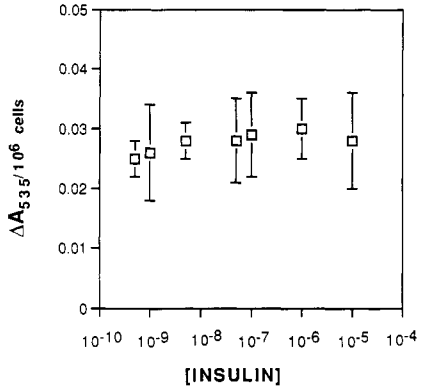


FIGURE 5: Ferrireductase activity is not responsive to insulin. K562 cells were suspended in serum-free medium supplemented with 1 mg/mL BSA and 1 μg/mL $FeSO_4$ [(1.0–2.0) × $10^6$ cells/mL]. The cells were incubated in 5-mL volumes in the presence of indicated concentrations of insulin (0.5 nM–10 μM) for 60 min at 37 °C. Following two washes in PBS and one wash in Hanks' buffer, the cells were resuspended in Hanks' buffer (1 mL) and incubated with 0.3 mM $K_3Fe(CN)_6$ for 30 min at 37 and 4 °C. Reactions were chilled on ice and samples of supernatants were assayed for ferrocyanide as described for Figure 2. The difference in absorbance at 535 nm measured for duplicate samples held at 37 versus 4 °C is presented as a function of insulin concentration employed during the preincubation of these samples. Shown are the mean values of data collected from four separate experiments.

the number of surface ferrireductases to be reflected in alterations in $V_{max}$. Values for this kinetic parameter were determined to be between 0.090 and 0.119 nmol $min^{-1}$ ($10^6$ cells)$^{-1}$ with no obvious trend pointing to the idea that the activity is modulated by cellular or environmental iron levels. Therefore, the ferrireductase is not regulated in response to iron levels, consistent with the notion that this activity may be coupled to Tf-independent transport in K562 cells, which is also unaltered by changes in extra-or intracellular iron levels (Inman & Wessling-Resnick, 1993). It should be noted that, for yeast, a ferrireductase activity linked to iron transport appears to be regulated independent of the transporter itself (Eide et al., 1992).

*Ferrireductase Activity Is Not Involved in Growth Control.* A trans-plasma membrane redox reaction has also been described for many different cell types, and ferricyanide has been used as a substrate for detection of this activity [reviewed by Crane et al. (1985)]. The trans-plasma membrane electron transport system has been described to function in growth and development, and many studies suggest that its activity is subject to hormonal control mechanisms including either stimulation or inhibition by insulin. However, as demonstrated by the results of Figure 5, exposure of K562 cells to insulin does not affect the rate of ferricyanide reduction. The idea that the K562 cell ferrireductase is not involved in growth control is also supported by the fact that extracellular ferricyanide does not stimulate growth of these cells. Ellem and Kay (1983) have reported that, under conditions where serum is limiting, the presence of ferricyanide will promote melanoma cell growth. Similar observations have been made for the stimulation of HeLa cell growth by ferricyanide, and the effect of impermeable oxidants as stimulators of growth has been related to the control of cell growth by trans-plasma membrane redox activity (Crane et al., 1985). However, when the growth of K562 cells in α-MEM with 0.75% serum was monitored in the presence or absence of ferricyanide, there was no detectable change in cell proliferation. Thus, a role for the K562 cell ferricyanide reductase in redox activity contributing to growth control appears unlikely.

Table 2: Inhibition of Ferrireductase Activity[a]

| experimental condition | ferrireductase activity ($\Delta A_{535}/10^6$ cells) |
|---|---|
| control | 0.029 |
| adriamycin, 1 μM | 0.032 |
| actinomycin D, 5 μM | 0.032 |
| control | 0.024 |
| *cis*-platin, 0.1 μM | 0.030 |
| control | 0.029 |
| NEM, 5 mM | 0.009 |

[a] Ferrireductase activity was determined as described in Experimental Procedures. Briefly, K562 cells [(2.0–4.0) × $10^6$ cells/ml] were washed and resuspended in Hanks' buffer containing the indicated concentrations of various inhibitors. Ferrireduction was initiated upon addition of ferricyanide, and reaction mixtures were held at 37 and 4 °C. The reaction was terminated after 30 min of incubation and the supernatant, collected after centrifugation to separate the cells, was assayed for ferrocyanide by the method of Avron and Shavit (1963). Shown is the difference in the measured absorbance at 535 nm (due to the resultant BPS complex), for samples held at 37 and 4 °C. Data are mean values of duplicate measurements from individual experiments. Each set of experiments were performed on at least three separate occasions with similar results.

A series of inhibitors were also employed to characterize the K562 cell ferrireductase and its relationship to other trans-plasma membrane redox activities. The results of these experiments are presented in Table 2 as the percent inhibition of ferrireductase activity compared with that of untreated K562 cells. Antitumor drugs such as adriamycin and actinomycin D are potent inhibitors of plasma membrane redox activity and it has been suggested that these drugs may exert their effects, in part, due to this action (Crane et al., 1985; Sun et al., 1983). However, neither of these agents blocked the activity of the K562 cell ferricyanide reductase. Moreover, *cis*-platin also did not inhibit the reductase, although it has been reported to be a potent inhibitor of ferricyanide reduction by HeLa cells (Crane et al., 1985). The lack of effect of these agents on the K562 cell ferricyanide reductase activity points to a characteristic nature that is different from that previously described for other trans-plasma membrane oxidoreductases. It should be noted that this evidence does not argue against the idea that plasma membrane oxidoreductases are not involved in growth control—only that a ferrireductase with unique properties is available at the K562 cell surface to catalyze this reaction. Whether this activity is cell-type-specific or not remains to be determined; the presence of multiple (5–7) distinct activities has been observed within a single membrane preparation, suggesting that plasma membrane oxidoreductases are in fact heterogeneous in nature (Dreyer, 1990; Zurbriggen & Dreyer, 1994).

In addition to the inhibitors discussed above, we also tested whether the K562 cell ferrireductase activity was inactivated by the alkylating agent *N*-ethylmaleimide (NEM). As shown by the results of Table 2, the ferricyanide reduction was blocked after treatment of the cells on ice for 30 min with 5 mM NEM. The Tf-independent iron uptake by K562 cells is also blocked under identical conditions (Inman & Wessling-Resnick, 1993), further suggesting a relationship between these two activities. Although this does not constitute strong evidence of a link between reduction and transport, if the opposite were true, that is, if uptake were blocked while reduction was not inhibited, one could clearly dissociate the two activities on the basis of sensitivity to the alkylating agent. Thus, the fact that NEM does inhibit the ferricyanide reductase is consistent with the idea that this activity is potentially involved in the transport process, although the role of sulfhydryl groups in this function remains obscure.

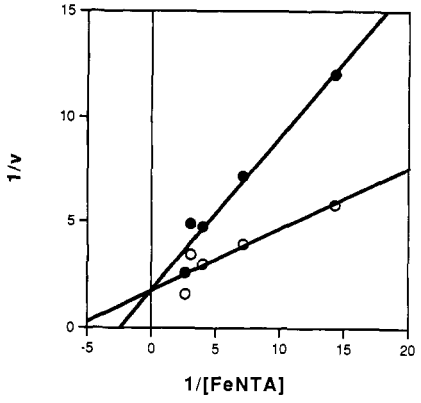


FIGURE 6: Ferricyanide competitively inhibits non-Tf iron transport by K562 cells. K562 cells ($9.4 \times 10^7$ cells/mL) were incubated in uptake buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, and 1 mg/mL dextrose) containing indicated concentrations of $^{55}$FeNTA (0.07–0.40 $\mu$M) in the absence (○) or presence (●) of 10 $\mu$M $K_3Fe(CN)_6$ for 5 min at 37 or 4 °C. Uptake of $^{55}$Fe was terminated by placing 250-$\mu$L aliquots from the 300-$\mu$L assay mixtures into 750 $\mu$L of ice-cold quench buffer (1 mM FeNTA, 25 mM HEPES, pH 7.4, and 150 mM NaCl). After 20 min of incubation on ice, 950-$\mu$L aliquots of the quenched samples were filtered through nitrocellulose discs and washed twice with 3 mL of 150 mM NaCl, and the amount of cell-associated radioactivity collected on the filters was measured by scintillation counting. Mean values of duplicate samples were used to calculate transport rates, based on the difference in counts per minute measured for samples incubated at 37 versus 4 °C. Shown is the Lineweaver–Burk plot of the reciprocal values obtained for the rate of transport (picomoles per minute per $10^6$ cells) measured at indicated $^{55}$FeNTA concentration (micromolar).

*Ferrireductase Activity Is Involved in Tf-Independent Iron Transport.* In order to directly ascertain whether the cell surface ferrireductase activity was indeed coupled to the mechanism of Tf-independent transport, we performed kinetic studies of the assimilation of iron from $^{55}$FeNTA in the presence or absence of ferricyanide. Earlier studies indicated that the reduction of $Fe^{3+}$ to $Fe^{2+}$ precedes K562 cell uptake of iron from $^{55}$FeNTA (Inman & Wessling-Resnick, 1993). We reasoned that if the ferrireductase was involved in the transport mechanism, then the presence of ferricyanide would competitively block $^{55}$Fe uptake. The results presented in Figure 6 confirm our hypothesis—ferricyanide is a competitive inhibitor of non-Tf uptake. This fact is demonstrated by the intersection of Lineweaver–Burk plots generated by initial rate data measured for transport in the absence or presence of 10 $\mu$M ferricyanide. All of our data are therefore consistent with the idea that the extracellular ferrireductase activity of K562 cells is tightly coupled to Tf-independent iron transport.

With respect to the reported $K_m$ values for reduction and transport of iron, it is important to bear in mind differences between the substrates studied and the conditions employed in these separate investigations. We have previously reported the $K_m$ of iron uptake from $^{55}$FeNTA, using the chelating agent to stably introduce $Fe^{3+}$ to monitor the uptake of radioactivity by K562 cells (Inman & Wessling-Resnick, 1993). It is anticipated that the kinetic parameters of transport will reflect the presence of NTA, although the chelating agent is used in limiting amounts (Teichmann & Stremmel, 1990). In contrast, ferricyanide is studied as a substrate for the cell surface ferrireductase since the product of this reaction (ferrocyanide) is known to be membrane-impermeant (Orringer & Roer, 1979). This is an important distinction between the two forms of iron as presented to the K562 cells and most likely accounts for the difference in $K_m$ values measured for these two reactions (0.2 $\mu$M for Fe transport from FeNTA and 6 $\mu$M for ferricyanide reduction). Clearly, the redox

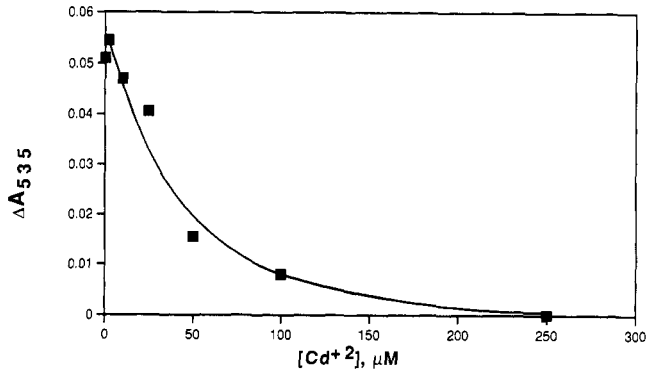


FIGURE 7: Inhibition of ferrireductase activity by cadmium. K562 cells ($1.37 \times 10^6$ cells/mL) were incubated in Hanks' buffer (1 mL) containing 0.3 mM $K_3Fe(CN)_6$ and the indicated concentrations of $CdSO_4$ (2–250 $\mu$M) for 30 min at 37 or 4 °C. Reactions were chilled on ice and cells were immediately centrifuged to collect supernatants for the ferrocyanide assay in order to measure ferrireductase activity. The results from an individual experiment are presented as the difference in absorbance at 535 nm for average duplicate samples held at 37 versus 4 °C. Comparable results were obtained on six separate occasions.

potential of these two very different iron complexes would contribute to interactions with the cell-associated ferrireductase activity that is reported here and that we theorize is involved in transport activity. Consistent with this is the fact that the nontransported substrate, ferricyanide, has a higher $K_m$ value with respect to the ferrireductase activity. Unfortunately, since iron is transported into K562 cells presented with FeNTA, we are unable to use this same approach to accurately determined the action of the ferrireductase on this complex.

*Cadmium, an Inhibitor of Iron Transport, Also Blocks Ferrireductase Activity.* A second line of evidence confirming the relationship between the ferricyanide reductase activity and the Tf-independent transport mechanism is the fact that the divalent cation, $Cd^{2+}$, inhibits the reduction reaction. Figure 7 presents the dose–response curve for the inhibition of ferrireductase activity in the presence of increasing concentrations of cadmium. An $IC_{50}$ of ~35 $\mu$M $Cd^{2+}$ was determined in three separate experiments and is nearly identical to that found for inhibition of iron uptake, ~50 $\mu$M $Cd^{2+}$ (Inman & Wessling-Resnick, 1993). These earlier studies identified that, among other divalent cations and transition metals examined, only cadmium specifically blocked entry of iron from FeNTA into K562 cells. Other potential inhibitors, $Cu^{2+}$ and $La^{3+}$, which were previously shown to block transport (Inman & Wessling-Resnick, 1993), directly interfere with the colorimetric assay for ferrocyanide production and thus cannot be employed in inhibition studies of ferrireductase activity.

The specificity of $Cd^{2+}$ action on ferrireductase activity was also examined. Inhibition of the surface-associated activity required the continued presence of the divalent cation: cells pretreated with 50 $\mu$M $Cd^{2+}$ for 15 min and subsequently washed prior to the assay failed to show effect. This argues against a nonspecific action of the divalent cation on protein sulfhydryl groups, for example. Instead, as shown by the results of Figure 8, the presence of $Cd^{2+}$ competitively inhibits the K562 cell ferrireductase activity, indicated by the intersection of the Lineweaver–Burk plots generated in the absence or presence of 10 $\mu$M $Cd^{2+}$.

Previous studies of the uptake of non-Tf iron by various cells have indicated that extracellular $Fe^{2+}$ is the cationic form of iron that appears to be translocated across the plasma

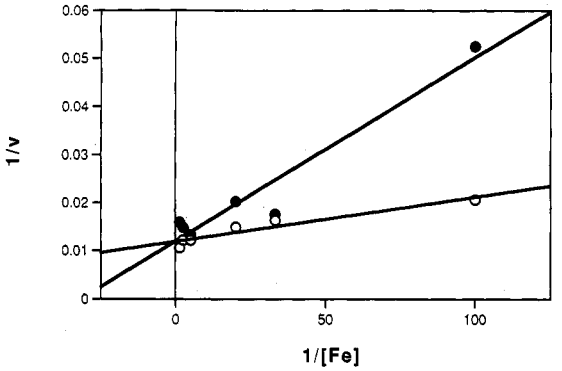


FIGURE 8: Cadmium is a competitive inhibitor of the K562 cell ferrireductase. K562 cells ($1.35 \times 10^6$ cells/mL) were incubated in a 1-mL volume of Hanks' buffer containing the indicated concentrations of $K_3Fe(CN)_6$ (0.01–0.75 $\mu$M) in the absence (○) or presence (●) of 10 $\mu$M $CdSO_4$ at 37 and 4 °C. Reactions were chilled on ice, cells were removed by centrifugation, and the collected supernatants were assayed for ferrocyanide. The rate of ferrocyanide production (picomoles per minute per $10^6$ cells) is based on the difference between measurements made for samples held at 37 versus 4 °C. Shown is the Lineweaver–Burk plot of reciprocals for the values obtained as the rate of ferrireduction (picomoles per minute per $10^6$ cells) as a function of $[Fe(CN)_6^{3-}]$. Note that the intersection of these lines is indicative of competitive inhibition in the presence of $CdSO_4$.

Table 3: Transferrin-Independent $^{55}Fe$ Uptake[a]

| experimental condition | cell-associated $^{55}Fe$ (pmol/$10^6$ cells) | |
|---|---|---|
| | −ascorbate | +ascorbate |
| control | 2.64 ± 0.31 | 1.21 ± 0.08 |
| 250 $\mu$M $Cd^{2+}$ | 1.10 ± 0.11 | 0.21 ± 0.06 |

[a] The uptake of non-Tf iron by K562 cells was measured as previously described (Inman & Wessling-Resnick, 1993). Briefly, cells were incubated at 37 or 4 °C in uptake buffer containing 157 nM $^{55}$FeNTA with or without 250 $\mu$M $CdCl_2$. After 5 min of incubation, the transport reaction was immediately filtered and washed twice with 3 mL of 25 mM HEPES, pH 7.4, and 150 mM NaCl. The amount of cell-associated radiolabel was determined by scintillation counting. Specific uptake was taken as the difference between cell-associated $^{55}Fe$ at 37 and 4 °C. In order to identify whether cadmium blocks uptake due to its action on the K562 cell ferrireductase, transport was also measured in the presence of 3.14 mM ascorbate (freshly dissolved in transport reaction mixture). For these samples, some hydrolysis of iron in solution most likely occurs, given that the 4 °C control measurements are significantly higher than those determined in the absence of ascorbate. This would effectively lower the amount of radiolabel available for uptake. However, inhibition of non-Tf iron uptake by $Cd^{2+}$ was observed even in the presence of the chemical reducing agent. Shown are mean values of uptake expressed as picomoles per $10^6$ cells ± SD from three individual experiments.

membrane (Kaplan et al., 1991; Morgan, 1988; Raja et al., 1992). Our studies on the mechanism of iron import mediated by K562 cells suggested that the uptake mechanism discriminates between divalent metal ions to translocate $Fe^{2+}$ and $Cd^{2+}$ specifically across the membrane. In light of our new findings, it is also possible that the inhibition of iron uptake by $Cd^{2+}$ is exerted via its action on the ferrireductase, thereby interfering with the prerequisite reduction of $Fe^{3+}$ for transbilayer transport. In order to experimentally discriminate how $Cd^{2+}$ inhibits iron uptake, Tf-independent transport of $^{55}Fe$ was measured in the presence and absence of $Cd^{2+}$ but with the addition of ascorbate to the incubation medium. Ascorbate will chemically reduce iron, bypassing the requirement of a cellular reductase activity for transport across the plasma membrane (Inman & Wessling-Resnick, 1993). If $Cd^{2+}$ blocks uptake only at the level of iron reduction, then the presence of ascorbate should overcome this effect. However, as the results of Table 3 demonstrate, the presence of $Cd^{2+}$ continues to block $^{55}Fe$ uptake even in the presence of ascorbate. In these experiments, the K562 cells are incubated with radiolabel and immediately filtered to stop the uptake reaction. However, despite the short times of incubation (5 min), significant hydrolysis of iron in the presence of ascorbate occurs, detected by the increased precipitation of iron onto the filters in the absence of cells or at 4 °C. The latter value is actually subtracted from measurements at 37 °C to provide a determination for specific uptake; however, this unavoidable difficulty results in less radioactive iron available for uptake by the K562 cells under these conditions. This problem is exacerbated by the fact that commercial preparations of ascorbate are contaminated with iron, such that concentrations potentially on the order of $10^{-7}$ M are diluting the radiolabel added to the reaction. This explains the difference between control uptake (2.64 pmol/$10^6$ cells) and that measured in the presence of ascorbate (1.21 pmol/$10^6$ cells) in identical reactions that are run in parallel and which might be expected to yield similar values for iron uptake. Nonetheless, the important observation is that $Cd^{2+}$ inhibits specific uptake both in the absence and in the presence of ascorbate, indicating that the divalent cation not only is a potent inhibitor of the reduction of $Fe^{3+}$ to $Fe^{2+}$ but also can independently block the translocation of $Fe^{2+}$ into K562 cells.

## DISCUSSION

Our investigation characterizes the ability of K562 cells to catalyze the extracellular reduction of iron. The K562 cell ferricyanide reductase does not function in the regulation of growth control but is instead tightly coupled to Tf-independent iron transport. Thus, unlike reports of ferrireductase activity in other cell types (Crane et al., 1985), the K562 cell line does not appear to be insulin-responsive and is resistant to the action of antitumor agents. The idea that the K562 cell ferrireductase is responsible for the uptake of non-Tf iron is supported by the observation that activities of the K562 cell Tf-independent iron transporter and ferrireductase are both unaffected by intra- or extracellular iron levels, unlike the yeast iron uptake system, wherein a cell surface reductase is regulated independent of the plasma membrane iron transporter itself (Eide et al., 1992).

Our hypothesis that the extracellular ferrireductase activity of K562 cells is coupled to the Tf-independent transport system is based on three independent observations. First of all, inhibitors of K562 cell Tf-independent transport also block extracellular reduction of ferricyanide. Second, ferricyanide, acting as a membrane-impermeant substrate for the ferrireductase, competitively inhibits uptake of iron from $^{55}$FeNTA. Finally, the critical observation that cadmium inhibits both iron reduction and transport suggests that the substrate binding sites for both functions are one and the same and that these tightly coupled processes are carried out by the same molecular complex to mediate Tf-independent iron transport. An alternative explanation, that the two functions are independently carried out at distinct sites, requires that both the reductase and the membrane transporter recognize cadmium with similar affinities and imposes the restriction that, once reduced, $Fe^{2+}$ must be transferred to the carrier site under conditions that limit the potential for oxidative damage. For these reasons, the simplest model is that elements mediating the reduction and transport of iron reside in the same molecular complex, an idea that is consistent with all of the properties of the K562 cell ferrireductase characterized by our results.

Although our study provides mechanistic details regarding the function of the Tf-independent iron transporter, the physiologic significance of the K562 cell iron uptake system

remains to be defined. However, recent discovery of the transcription factor NF-E2 provides an important link between intestinal and erythroid transport of iron (Andrews et al., 1993). Mice that are defective in the gene for NF-E2 (the *mk* allele) display microcytic anemia promoted by the lack of iron absorption by both duodenum and erythroid tissues (Peters et al., 1993), an observation that suggests the expression of a unique iron transporter is regulated by this DNA-binding protein. The fact that NF-E2 is also expressed in the K562 line (Andrews et al., 1993) supports the idea that elements of Tf-independent iron uptake observed for these cells are highly related to those of the intestinal transport system. The latter mechanism is also thought to be mediated by a coupled iron reduction/transport system (Raja et al., 1992), compatible with the properties we have defined for K562 cell iron uptake. Further studies are necessary to complete our understanding of the relationship between the erythroid and intestinal iron transport mechanisms and how the functional activity of these uptake systems is regulated by NF-E2.

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