Regulation of the Transferrin-independent Iron Transport System in Cultured Cells*

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Mammalian cells accumulate iron via the binding of transferrin to high affinity surface receptors, or through a transferrin-independent pathway which involves the uptake of iron-organic anion chelates by a membrane-based transport system. Previously we determined that the transferrin-independent transport system was present on a wide variety of cultured cells (Sturrock, A., Alexander, J., Lamb, J., Craven, C. M., and Kaplan, J. (1990) J. Biol. Chem. 265, 3139–3145). In this communication we demonstrate that the transferrin-independent iron uptake system is regulated differently than the transferrin-mediated pathway. The activity of the transferrin-independent system was unaffected by changes in cellular growth rate, induction of DNA synthesis and cell division, or depletion of cellular iron. Exposure of cells to ferrous or ferric iron, however, resulted in a time-dependent increase in transport activity, due to a change in Vmax, with no change in Km. Increased transport activity was seen in a variety of cultured cell types, occurred in the presence of cycloheximide, and persisted for hours after removal of iron. The ability of other transition metals to induce changes in transport, or to compete with iron for accumulation by the transferrin-independent uptake system, was critically dependent on the composition of the media in which the cells were incubated. Metals such as Cu2+ or Zn2+ but not Cd2+ or Mn2+, when dissolved in a balanced salt solution buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, induced changes in the transferrin-independent iron transport system. The same metals which induced changes in transport were ineffective in media containing amino acids, ascorbate, or N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine. The Vmax of the transferrin-independent iron transport system was also elevated by increases in intracellular Ca2+. The effect of iron on transport activity, however, did not result from an iron-induced release of intracellular Ca2+. These results suggest a novel form of regulation in which the presence of extracellular iron induces the appearance of previously cryptic transporters and thus accelerates the clearance of potentially toxic molecules.

Iron is a required element but is toxic to cells in excessive amounts. Consequently, the concentration of free iron in bodily fluids and in cells is tightly regulated. Iron uptake in most cell types is primarily mediated through the binding of Tf to high affinity surface receptors. Iron uptake, mediated by Tf, is regulated by the modulation of Tf receptor number resulting from an iron-induced alteration in the half-life of Tf receptor mRNA (Owen and Kuhn 1987; Koeller et al., 1989) and by regulation of the rate of transcription (Casey et al., 1988).

In addition to the Tf-mediated iron uptake system, a number of cell types express a transport system capable of accumulating iron-organic anion chelates (Brisot et al., 1985; Wright et al., 1986, 1988; Sturrock et al., 1990). The function of this transport system seems to be the removal of non-Tf iron from plasma. There are a number of circumstances in which the amount of iron absorbed into the body exceeds the binding capacity of circulating apotransferrin (Craven et al., 1987). In iron overload diseases, such as hereditary hemochromatosis or atransferrinemia, massive amounts of iron accumulate in cells resulting in tissue injury. The Tf-independent uptake system has been well characterized compared with the Tf-mediated iron delivery system, but the presence of tissue iron does not preclude iron accumulation, indicating that the activity of the non-Tf iron transport system is not diminished by an increase in cellular iron content (Wright et al., 1986; Craven et al., 1987). This observation suggests that regulation of the non-Tf iron transport system is different from that of the Tf-mediated iron delivery system. Here, we present studies on the regulation of the non-Tf iron uptake system in cultured cells. Our results demonstrate that, in contrast to the Tf-mediated iron uptake system, the transport of non-Tf iron is independent of cellular iron requirements and growth state. We also demonstrate that there is a novel form of regulation of this system in which the number of transporters is increased dramatically when cells are exposed to high concentrations of intracellular calcium, iron chelates, or other selected transition metals.

MATERIALS AND METHODS

Cells—HeLa cells were cultured and maintained as described previously (Sturrock et al., 1990). Human skin fibroblasts obtained from healthy volunteers were initiated and maintained in minimal essential medium (MEM) containing 10% fetal bovine serum, penicillin, and streptomycin as described in Ward et al. (1984a). Cultures were used between the fourth and eighth passage.

Measurement of Iron Uptake—29Fe-nitrilotriacetic acid was prepared by addition of 29FeCl3 (Du Pont-New England Nuclear) to a 5-

The abbreviations used are: Tf, transferrin; Tf-R, transferrin receptor; NTA, nitrilotriacetate; HBSS, Hanks’ balanced salt solution; MEM, minimal essential medium; FAC, ferric ammonium citrate; BAPTA, bathophenanthrolinedisulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine.


d-5-
fold molar excess of the disodium salt of NTA. "Fe-citrate was prepared as described (Craven et al., 1987). "Fe-Tricine ascorbate was prepared by adding "FeC13 to medium containing 10 mM Tricine plus 0.3 mM ascorbate. To measure uptake of iron, cells grown on 35-

mm tissue culture dishes were incubated for 1 h in serum-free medium to deplete cells of Tf and then incubated in serum-free medium with specified concentrations of "Fe-citrate or "Fe-NTA. For most experi-
ments the cells were incubated in serum-free MEM (Hanks’ salts) containing 10 mM Hepes (pH 7.2). Incubations were performed at either 37 or 0 °C for specified periods of time. The cells were washed with ice-cold phosphate-buffered saline, solubilized in 1% sodium dodecyl sulfate, and radioactivity determined in a Packard y counter. Nonspecific uptake was determined by incubating cells with "Fe-
NTA, "Fe-citrate, or "Fe-Tricine ascorbate in the presence of a 1000-
fold excess of Fe-NTA. This background accumulation of radioactiv-
ity, which was less than 5%, was subtracted from total radioactivity yielding specific accumulation. Uptake was expressed as femtomoles of iron/μg of cell protein. All experiments were performed multiple times, and the data are presented as the mean ± S.E. Radioactive metals of the highest specific activity available were obtained from Du Pont-New England Nuclear.

Uptake of Tf-bound Iron—Human Tf was purified from plasma using the procedure of Sawatzki et al. (1981). The Tf was saturated with either Fe⁺⁺ or with Fe⁺⁺ as described by Larrick and Cresswell (1979). Accumulation of 59Fe by cells incubated with Tf(Fe⁺⁺) was measured as described above, except that nonspecific uptake was determined using 1000-fold excess of nonradioactive Tf (Fe⁺⁺).

Measurement of Intracellular Ca²⁺—Cells grown on glass coverslips in standard culture medium were incubated in serum-free MEM for 60 min. Cells were incubated with 5 μM of the calcium-sensitive fluorescent dye Indo 1/acetoxyethyl ester (Molecular Probes) for 30–60 min at room temperature in serum-free MEM. Cells were extensively washed with HBSS containing 1 mg/ml glucose and 10 mM Hepes (pH 7.2). The cells were perfused with this buffer in the chamber of a standard spectrofluorometer (Peretors et al., 1987) and fluorescence intensity was measured (excitation 360 nm; emission 410 and 480 nm) using the emission ratio procedure (Gryniewicz et al., 1985). Autofluorescence of cells not loaded with Indo 1/acetoxy-
ethyl ester was adjusted to zero with the DC offset. The measurements were performed using an SLM 8000/800 OS spectrofluorometer (SLM Instruments).

Additional Procedures—Protein determinations were performed using the procedure of Lowry et al. (1951) with bovine serum albumin (Sigma fraction V) as a standard. The valency of the iron solutions employed was determined using BAPTA as described by Laskey and Zak (1958). Ferritin concentrations were measured by radio-immune assay as described in Dadone et al. (1982).

RESULTS

Effect of Cell Growth on Non-Tf Iron Transport—Studies on the regulation of Tf-R number have identified two mecha-
nisms which regulate Tf-R biosynthesis. First, transcription of the Tf-R gene is accelerated by a variety of growth hormones (Casey et al., 1988). Second, the half-life of the mRNA is regulated by the intracellular free iron pool. Message half-
life is drastically shortened under conditions in which the free iron pool is increased (Owen and Kuhn, 1987; Koehler et al., 1988). Based on these observations, we examined the uptake of non-Tf bound iron in cells in which either cell growth or intracellular iron content is subject to experimental manipu-
lation.

Exposure of human peripheral lymphocytes to phytoha-
magglutinin results in T-cell proliferation and an increase in Tf-R number (Table I and Ward et al., 1984b). The increase in Tf-R number reflects an increased demand for iron (Laskey et al., 1988). In contrast, there was a decrease in the uptake of 59Fe-NTA in phytohemagglutinin-stimulated cells (Table I). The relatively low level of non-Tf iron transport activity in phytohemagglutinin-stimulated cells provides an explana-
tion for the observation of why the iron needs of these cells can be met by Tf(Fe⁺⁺) or lipophilic iron-containing compo-
unds but not by iron salts (Laskey et al., 1988). The rate of uptake of non-Tf iron was also assayed in fibroblasts plated at different densities. Human skin fibroblasts exhibit a den-
sity-dependent inhibition of growth. Although cell division was radically affected when the cells reached their saturation density (1.09 × 10⁶ cells/mm²), there was little change in the rate of uptake of non-Tf-bound 59Fe, suggesting that the rate of transport was unaffected by growth stage (data not shown).

Further support for this observation comes from experiments on fibroblasts in which the effect of mitogenic hormones was examined. Fibroblasts incubated in serum-free medium sup-
plemented with epidermal growth factor were stimulated to divide, as assayed by an increase in [³H]thymidine incorpora-
tion. Increases in cell division were not accompanied, how-
ever, by increases in the uptake of 59Fe-NTA (Table II). These results suggest that, in contrast to Tf receptors, the Tf-
dependent iron transport system was not increased by in-
creases in mitogenic activity.

To determine the effect of intracellular iron content on iron uptake, fibroblasts were incubated in medium containing deferoxamine, a low molecular weight iron chelator which gains access to cytoplasmic compartments and chelates free intracellular iron. Exposure of fibroblasts to deferoxamine results in cellular iron depletion and an increase in Tf-R number, mediated through an increase in Tf-R mRNA half-life (Rao et al., 1986). While increases in Tf-R number were observed, no increase was seen in the uptake of 59Fe-NTA (data not shown).

Exposure of Cells to Iron-containing Compounds—The above experiments demonstrate that the rate of non-Tf iron transport was minimally affected by changes in cell growth rate or iron depletion. Very different results were observed when the effect of increased cellular iron content was examined. Incubation of fibroblasts with FAC, a compound used to increase cellular iron content (Ward et al., 1984a), resulted in a sustained increase in non-Tf iron accumulation (Fig. 1).

<table>
<thead>
<tr>
<th>Condition</th>
<th>59Fe-NTA uptake</th>
<th>³H]Thymidine incorporation</th>
<th>fmol/μg protein</th>
<th>cpm/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.5</td>
<td>150 ± 31</td>
<td>115 ± 12</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>8.1 ± 0.2</td>
<td>6,115 ± 442</td>
<td>50 ± 8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Non-Tf iron uptake</th>
<th>³H]Thymidine incorporation</th>
<th>fmol/μg protein</th>
<th>cpm/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0 h</td>
<td>-EGF</td>
<td>123.7 ± 12</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>+EGF</td>
<td>124.1 ± 11</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>-EGF</td>
<td>145.9 ± 9</td>
<td>43.7 ± 7</td>
<td></td>
</tr>
<tr>
<td>+EGF</td>
<td>108.2 ± 10</td>
<td>731 ± 23</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Non-Tf iron uptake</th>
<th>³H]Thymidine incorporation</th>
<th>fmol/μg protein</th>
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</tr>
</thead>
<tbody>
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<td></td>
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<td>PHA</td>
<td>8.1 ± 0.2</td>
<td>6,115 ± 442</td>
<td>50 ± 8</td>
<td></td>
</tr>
</tbody>
</table>

Table I

Effect of phytomagglutinin on Tf receptor number, [³H]thymidine incorporation, and 59Fe-NTA uptake in human lymphocytes

Lymphocytes were obtained from human plasma and cultured as described in Ward et al. (1984b). Aliquots of cells were incubated with phytohemagglutinin (PHA) (171 μg/ml) for 3 days. Samples of cells, both before and after the addition of phytomagglutinin, were assayed for binding of 125I-Tf, uptake of 59Fe-NTA, and incorporation of [³H]thymidine. The data have been normalized to the protein content of the cultures.

Table II

Effect of epidermal growth factor on 59Fe-NTA uptake in fibroblasts

Fibroblasts were incubated in serum-free medium for 24 h. Epider-
mal growth factor (10 ng/ml) was added to cells and aliquots of cells were assayed for 59Fe-NTA uptake (15 min, 3 μM 59Fe-NTA) and [³H] thymidine incorporation 6 and 24 h later.
The increase in iron transport was a function of the time of exposure. Fibroblasts were incubated in serum-free medium supplemented with FAC (0.291 mg/ml) overnight. The cells were then incubated for specified times with FAC (0.291 mg/ml). Cells were washed and the activity of the Tf-independent iron transport system assayed by incubating cells with 1 μM 59Fe-NTA for 15 min and determining specific uptake.

The kinetics of the loss of transport activity in fibroblasts which occurred in the first 3-4 h and a slower decline which occurred over a time course of days. Increased rates of non-Tf iron transport activity back to basal levels. The kinetics of the loss of transport activity in fibroblasts were complex. There was a rapid decline in transport activity which occurred in the first 3-4 h and a slower decline which occurred over a time course of days. Increased rates of non-Tf iron transport could be observed up to 48 h later (Fig. 4).

Analysis of the Change in Transport Activity—Several mechanisms might explain the change in the rate of iron accumulation, including nonspecific effects on cell permeability, changes in the intracellular metabolism of iron, or alterations in the kinetic properties of the non-Tf iron transporter. Analysis of the concentration-dependent uptake of 59Fe-NTA in FAC-loaded cells indicates that changes in non-Tf iron uptake were due to an increase in Vmax with no measurable affect on Ks (Fig. 5). Thus, once cells were exposed to FAC, changes in non-Tf iron transport activity were seen at all concentrations of iron employed. Elevated uptake at high concentrations of radiolabeled iron rules out the possibility that increased iron transport results from exchange diffusion (trans-stimulation).

The increase in non-Tf iron transport activity is not a result of a generalized alteration in cell permeability but represents a specific cellular response. First, incubation of cells at 0 °C for 1 h with FAC did not affect transport activity.
does not reflect a general alteration in intracellular iron metabolism. Results indicate that the increase in non-Tf iron transport was either no effect (fibroblasts) or a decrease (HeLa) in Tf-mediated \( ^{59}\)Fe accumulation (Table III). The decrease in Tf-mediated \( ^{59}\)Fe uptake was seen in HeLa cells that had been exposed to FAC (10 pg/ml in Fe) for 6 h. At the end of the incubation period the cells were extensively washed and the specific uptake of \( ^{59}\)Fe-NTA was determined (Table IV). Values are mean ± S.E.

### Table III

**Effect of FAC treatment on the uptake of Tf\(^{(59)}\)Fe, or \(^{59}\)Fe-NTA by cultured HeLa cells or fibroblasts.**

<table>
<thead>
<tr>
<th></th>
<th>( ^{59})Fe-NTA</th>
<th>( ^{59})Fe-NTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fmol/µg protein</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>380 ± 51</td>
<td>210 ± 21</td>
</tr>
<tr>
<td>FAC</td>
<td>123 ± 23</td>
<td>390 ± 69</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11 ± 2</td>
<td>254 ± 67</td>
</tr>
<tr>
<td>FAC</td>
<td>10 ± 1</td>
<td>462 ± 25</td>
</tr>
</tbody>
</table>

### Table IV

**Effect of cycloheximide on the FAC-induced change in non-Tf iron transport activity.**

Inhibition of protein synthesis by cycloheximide did not prevent the FAC-induced increase in \(^{59}\)Fe uptake (Table IV). The concentration of cycloheximide employed resulted in a greater than 85% inhibition of \(^{[35]}\)Smethionine incorporation into trichloroacetic acid-precipitable material. This level of inhibition prevents the synthesis of ferritin, suggesting that the increase in transport activity was independent of ferritin content. This conclusion was confirmed by exposing fibroblasts to different iron-containing compounds and determining changes in both ferritin content and non-Tf iron uptake. As expected, incubation of cells with FAC resulted in an increase in ferritin content and an increase in iron uptake (Table V). Incubation of cells with heme, resulted in a greater increase in ferritin accumulation than with FAC, yet there was no increase in \(^{59}\)Fe-NTA uptake. Additionally, after exposure of cells to FAC, changes in non-Tf iron transport rate occurred well before detectable changes in intracellular ferritin levels (data not shown). These results lead to two conclusions: First, the increase in non-Tf iron uptake is not due to an increase in the cellular retention of iron as a result of storage in ferritin. Second, if ferritin content is a measure of the absolute amount of iron in cells, then the increase in uptake is independent of the absolute cellular iron content.

### Metal Specificity of the Effect—It is possible that the effect of FAC might be due to trace metals present as contaminants in the FAC preparation. We examined the effect of iron chelators on cells incubated with iron salts. Addition of apo-

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**Fig. 4.** Effect of removal of FAC on the iron-induced change in the Tf-independent transport system. Fibroblasts were incubated overnight in serum free MEM with FAC (iron concentration of 40 µg/ml). The cells were then washed and incubated in MEM in the absence of FAC for specified times. The specific uptake of \(^{59}\)Fe-NTA (1 µM, 15 min) was determined at each time point for control cells (○) or cells exposed to FAC (△). The data are expressed as a Lineweaver-Burk plot.

**Fig. 5.** Concentration dependence of \(^{59}\)Fe-NTA in control and FAC-exposed cells. Fibroblasts were washed and incubated in serum free MEM for 60 min. Cells were then incubated in FAC (Fe concentration of 2.0 µg/ml) for 75 min. The cells were washed and incubated in the absence of FAC for an additional 30 min. Control cells (○) and FAC-treated cells (△) were incubated with different concentrations of \(^{59}\)Fe-NTA for 15 min, washed, and the amount of cell-associated radioactivity and protein was measured. To determine nonspecific uptake, cells were incubated with \(^{59}\)Fe-NTA in the presence of 2.5 mM FeNTA. The data are expressed as a Lineweaver-Burk plot.
TABLE V

**Effect of different iron compounds on ferritin accumulation and on the activity of the Tf-independent iron transport system**

Fibroblasts were grown for 2 days in MEM + 10% fetal bovine serum with different concentrations of FAC, hemin, or Fe-NTA. At the end of the incubation period cells were washed and divided into 2 aliquots. One set of cells was washed, solubilized with (1.6%) Triton X-100, and the content of ferritin determined by RIA. The other set of cultures was incubated in serum-free medium and uptake of \(^{59}\)Fe-NTA determined as described under "Materials and Methods." The concentrations of the additives were normalized to their iron content. Values are mean ± S.E.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Ferritin</th>
<th>Iron uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng ferritin/µg protein</td>
<td>fmol/µg protein</td>
</tr>
<tr>
<td>None</td>
<td>ND</td>
<td>240 ± 20</td>
</tr>
<tr>
<td>Hemin (µg Fe/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>0.22 ± 0.07</td>
<td>160 ± 20</td>
</tr>
<tr>
<td>3.0</td>
<td>0.47 ± 0.07</td>
<td>150 ± 30</td>
</tr>
<tr>
<td>FeNTA (µg Fe/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>ND</td>
<td>280 ± 20</td>
</tr>
<tr>
<td>3.0</td>
<td>ND</td>
<td>350 ± 60</td>
</tr>
<tr>
<td>9.0</td>
<td>0.13 ± 0.01</td>
<td>400 ± 70</td>
</tr>
<tr>
<td>FAC (µg Fe/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>ND</td>
<td>400 ± 20</td>
</tr>
<tr>
<td>6.0</td>
<td>ND</td>
<td>610 ± 70</td>
</tr>
<tr>
<td>9.0</td>
<td>0.27 ± 0.03</td>
<td>1300 ± 10</td>
</tr>
</tbody>
</table>

TABLE VI

**Effect of apo transferrin (apoTf) on the FAC-induced change in the Tf-independent iron transport system**

Fibroblasts were incubated in serum-free medium for 1 h. The cells were then incubated for 30 min, either alone or in combination with FAC (2 µg Fe/ml), apoTf (2.0 × 10^{-5} M), or Tf(Fe)2 (2.0 × 10^{-5} M). At the end of the incubation period the cells were washed and incubated with \(^{59}\)Fe-NTA (2 µM, 15 min) to measure the activity of the Tf-independent iron transport system. Values are mean ± S.E.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Non-Tf iron uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fmol Fe/µg protein</td>
</tr>
<tr>
<td>Control</td>
<td>111 ± 4</td>
</tr>
<tr>
<td>FAC</td>
<td>615 ± 93</td>
</tr>
<tr>
<td>Tf(Fe)2</td>
<td>122 ± 18</td>
</tr>
<tr>
<td>apoTf</td>
<td>117 ± 4</td>
</tr>
<tr>
<td>apoTf + FAC</td>
<td>134 ± 12</td>
</tr>
<tr>
<td>Tf(Fe)2 + FAC</td>
<td>813 ± 75</td>
</tr>
</tbody>
</table>

transferrin simultaneously with FAC abolished the FAC induced increase in iron transport, whereas addition of Tf(Fe)2 to FAC had no effect. These results suggest that the effect of apo transferrin was due to its ability to bind iron (Table VI). A variety of other iron chelators also prevented the iron-induced increase in transport activity. Among these chelators were deferoxamine, α',α'-dipyridal, and Tricine. Exposure of cells to these reagents in the absence of iron had no effect on transport activity. Iron chelators such as EDTA, EGTA, or BAPTA did not prevent the iron-induced increase in iron transport (data not shown).

**Effect of Other Metals on the Tf-independent Iron Transport System**—The effect of other transition metals on the Tf-independent iron transport system varied dramatically depending upon the composition of the medium in which the cells were incubated when exposed to the metals. Cells incubated in a simple physiological salt solution (HBSS/Hepes) containing Cu^{2+} or Zn^{2+} showed an increase in the activity of the Tf-independent iron transport system (Fig. 6A), whereas Cd^{2+} and Mn^{2+}, in the same medium, were without effect. Addition of amino acids prevented Cu^{2+} and Zn^{2+} from increasing non-Tf iron transport (Fig. 6B). Addition of either Tricine (Fig. 6C) or Tricine ascorbate (data not shown) to simple physiological salt solutions inhibited the ability of transition metals to alter transport activity. In the presence of Tricine, however, transport of \(^{59}\)Fe-NTA was severely reduced. The ability of Cu^{2+} to inhibit non-Tf iron transport was affected by the presence of reductants. In an amino acid-containing medium Cu^{2+} did not interact with the Tf-independent iron transport system; it neither increased the activity of transport system nor blocked \(^{59}\)Fe-NTA uptake by the transport system. Addition of ascorbate to amino acid-containing medium allowed Cu^{2+} to inhibit \(^{59}\)Fe-NTA uptake, but the addition of ascorbate still did not allow exposure of cells to Cu^{2+} to increase non-Tf iron transport (data not shown).

**Effect of Increased Intracellular Ca^{2+} on Ionic Iron Transport**—The Tf-independent iron uptake system exhibits a Ca^{2+} dependence, being severely reduced in the absence of extracellular Ca^{2+} (Wright et al., 1988; Sturrock et al., 1990). Exposure of HeLa cells or fibroblasts to supraphysiological levels of Ca^{2+}, for brief periods, resulted in a 2–3-fold increase in non-Tf iron transport activity (data not shown). Incubation of cells in high Ca^{2+} at 0 °C does not alter transport activity. The change in transport activity resulted from a change in \(V_{max}\) with little or no change in \(K_m\) (data not shown). The effect of increased extracellular Ca^{2+} may result from in-
increased intracellular Ca\(^{2+}\). Direct measurement of intracellular Ca\(^{2+}\) levels using the chromophore Indo 1 demonstrated that incubation of cells with supraphysiological concentrations of extracellular Ca\(^{2+}\) resulted in an increase in intracellular free Ca\(^{2+}\) (Fig. 7). Addition of the ionophore ionomycin to buffers containing physiological Ca\(^{2+}\) resulted in an increase in intracellular Ca\(^{2+}\) and a change in non-Tf iron transport. The increase in transport activity was unaffected by the presence of cycloheximide, persisted in the absence of the ionophore, and was a result of an increase in \(V_{\text{max}}\) (data not shown).

The Effect of FAC Exposure on Non-Tf Iron Uptake Is Not Due to a Change in Intracellular Ca\(^{2+}\)—Smith et al. (1989) recently reported that fibroblasts contain a surface receptor for transition metals which, when occupied, stimulated the release of inositol phosphate, resulting in an increase in intracellular Ca\(^{2+}\) concentration. That report and our observations on the effect of Ca\(^{2+}\) on non-Tf iron transport suggested that the effect of iron on the non-Tf iron transport activity system might result from an iron-induced change in intracellular Ca\(^{2+}\) concentration. To examine this possibility we measured the effect of FAC exposure on intracellular Ca\(^{2+}\). Incubation of cells with FAC did not result in any detectable change in cytosolic free Ca\(^{2+}\) as assayed by changes in Indo 1 fluorescence (Fig. 7). Epidermal growth factor, a hormone capable of increasing cytosolic Ca\(^{2+}\) in a physiologically relevant manner (Moolenaar et al., 1984), had no effect on iron transport rate (cf. Table II). Additionally, incubation of cells with 100 \(\mu\)M solutions of the permeable ester of EGTA or BAPTA had no inhibitory effect on the FAC-induced increase in non-Tf iron transport activity.

**DISCUSSION**

Our observation that a Tf-independent iron transport system is present in cultured cells (Sturrock et al., 1990) allowed us to study the factors that regulate transport activity. The activity of the transport system was unaffected by changes in growth rate or by iron depletion. We did observe, however, that the Tf-independent transport system was affected by very specific conditions of iron loading. Incubation of cells with either ferric citrate or FAC resulted in a time and concentration dependent increase in iron accumulation. The onset of the increase could be seen within 1 h of exposure to the agents, was temperature-dependent, and was independent of protein synthesis. The rapidity of the increase and its independence from protein synthesis indicate that it is a post-translational effect.

Analysis of the change in transport activity suggests that iron exposure results in the appearance of new transporters rather than altering the kinetic properties of pre-existing transporters. Exposure of cells to iron resulted in either no change or, depending upon length of incubation, the expected decrease in Tf-mediated iron transport. The changes in the rate of transport of non-Tf iron do not result from changes in intracellular iron accumulation since they are independent of ferritin accumulation and occur in the absence of protein synthesis.

At first glance, our results are discrepant with those of Basset et al. (1986). They observed that incubation of L-cells with increasing concentrations of ferric citrate resulted in a change in the kinetic properties of the Tf-independent iron transport system. At increased iron concentrations the velocity of iron transport exhibited a Hill coefficient of 2, leading to the suggestion that iron had a positive co-operative effect on transport activity. Our observations suggest a different interpretation, that the concentration of iron which appears to change the kinetic properties of the transporter actually induces the appearance of previously cryptic transporters. Under such conditions, the increase in uptake velocity would appear to be a change in kinetic properties. The difference between the two alternatives (change in \(V_{\text{max}}\) versus change in \(K_m\)) was not easy to distinguish at first. Exposure of cells to most forms of iron resulted in the change in transport activity. For example, exposure of cells to the concentrations of ferric citrate required to measure the affect of iron concentration on the velocity of uptake resulted in increased transport activity. Thus, the same conditions that are used to analyze the transport system alter the system. Two observations allowed us to resolve the issue. First, as opposed to most low molecular weight chelates used to assay transport activity, Fe-NTA does not readily induce changes in transport activity. Second, the iron chelate-induced change in transport activity exhibits a cell-specific variation in the length of incubation before changes in transport activity are seen. In fibroblasts, changes in transport activity can be seen with incubation periods as short as 15 min, while in HeLa cells hour-long incubations are required. This result explains why, in part, in our initial studies on HeLa cells (Sturrock et al., 1990) we did not see changes in transport activity using ferric citrate to assess the kinetic properties of the transport system. In those
studies the incubation periods employed were insufficient to induce the change in transport activity.

The other reason we did not see increased iron transport was that our previous experiments were performed using Tricine ascorbate as a buffer system to maintain iron in solution. As demonstrated in Fig. 3 iron under these conditions, Fe(II), was much less efficient in inducing changes in transport activity than was Fe(III). This last observation brings up a significant point of caution regarding studies on iron transport, either in terms of inducing changes in transport activity or in terms of defining the specificity of the transporter, depends on the buffer and composition of the medium in which cells are incubated. For example, we (Sturrock et al., 1990) and Wright et al. (1986) reported that Cu²⁺ could block the uptake of non-Tf iron in perfused rat liver and in cultured HeLa cells. These studies further suggested that uptake of iron by the Tf-independent system was the result of a carrier. Basset et al. (1986) using L-cells reported that Cu²⁺ did not compete with ⁵⁹Fe citrate for transport. Thorstenson (1988), using isolated rat hepatocytes showed that Cu²⁺ had a limited ability to inhibit iron uptake. He further suggested that the ability of a transition metal to compete with ⁵⁹Fe for uptake was a function of its ionic radius. From that observation he concluded that uptake of non-Tf iron was mediated by a channel as opposed to a carrier. It is our contention that, rather than reflecting cell-specific metal transport systems, all of these disparate results can be explained by differences in experimental protocols. In Basset’s study, cells were incubated in amino acid-rich medium which, as demonstrated here, in the absence of ascorbate eliminates the ability of Cu²⁺ to compete with non-Tf iron for uptake. Thorstenson (1988) used cells incubated in an amino acid-free media which contained 30 mM Tricine, 10 mM Hepes as a buffer system. Our studies indicate that uptake of non-Tf iron in this buffer system is dramatically reduced relative to uptake of iron in buffers in the absence of Tricine. Furthermore, we observed that Cu²⁺ or Mn²⁺ dissolved in Tricine were essentially unable to prevent the uptake of ⁵⁹Fe-NTA. The implication of these results is that the metal specificity of the non-Tf iron uptake system is unclear, particularly under physiological conditions.

The solubility of ionic iron is extremely limited and it is unlikely that iron, or other transition metal exists as a non-chelated species in any sort of a physiological buffer. It is most probable that cells accumulate transition metals in the context of, or in association with, other molecules. An example of this is that in the presence of amino acids cells accumulate Cu²⁺-histidine complexes and not Cu²⁺ (Schmitt et al., 1983). It is difficult to determine what iron-anion chelates are recognized by the Tf-independent transport system, and what form of iron is able to increase transport activity. It is clear, however, that high molecular weight chelates of iron such as Tf(Fe)₂ are unable to alter transport activity. Lower affinity, strongly anionic-iron chelates, such as FAC or ferric citrate, can activate the transport system. However, strong iron chelators such as NTA, EDTA, EGTA, or BAPTA had little affect on increasing non-Tf iron transport. The relationship between the the ability of a metal to be recognized by the transport system and be transported, or to effect changes in transport activity, is unclear. For example, Cu²⁺ dissolved in amino acid-containing media was incapable of both competing with ⁵⁹Fe-NTA for transport by the non-Tf iron transport system and of increasing the activity of that transport system. The apparent reduction of Cu²⁺ to Cu⁺ by the addition of ascorbate allowed the metal to compete for transport but still did not allow it to activate the transport system. Similar rates of ⁵⁹Fe-NTA transport are seen in cells incubated in physiological salt solutions as in amino acid-containing media (Fig. 6). Yet there is a striking increase in non-Tf iron uptake by FAC in cells incubated in amino acid-containing media. These results lead to the suggestion that the structural features necessary for transport recognition may be different from those that result in regulation of the transport system. We have not been able to correlate specific structural features or affinity constants with an ability to altering iron transport, confounding attempts to determine the effect of these metals on the activation of the Tf-independent iron transport system.

How do iron-containing compounds induce changes in transport activity? We considered that the affect of iron was mediated through changes in intracellular Ca²⁺. Smith et al. (1989) observed that fibroblasts had surface receptors for transition metals, which when occupied resulted in liberation of inositol triphosphate and increased intracellular Ca²⁺. A number of experiments, however, rule out a direct relationship between this putative transition metal surface receptor, increased intracellular iron, and the increase in the Tf-independent iron transport system. First, Cd²⁺, the transition metal shown by Smith et al. (1989) to be the most efficient in inducing changes in intracellular Ca²⁺, was unable to induce changes in the Tf-independent iron transport system at reasonable physiological concentrations (1–50 μM). Second, we observed that Fe⁺⁺ was a more efficient inducer of changes in the transport system than Fe³⁺. Smith et al. (1989) observed that activation of inositol phosphate hydrolysis was specific for ferrous iron and that ferric iron was without effect (although the chemical form of ferric iron employed was not mentioned). Third, direct measurement of changes in intracellular Ca²⁺ levels using Indo 1 revealed that exposure of cells to varying concentrations of FAC did not alter intracellular Ca²⁺. Fourth, incubation of cells with epidermal growth factor, a hormone demonstrated to induce changes in intracellular Ca²⁺, had no affect on the Tf-independent iron transport system. These results suggest that while extreme changes in intracellular Ca²⁺ may induce changes in non-Tf iron transport activity the affect of iron on the transport system is not mediated by changes in intracellular free Ca²⁺. Resolution of the mechanism by which iron affects the Tf-independent pathway will require the isolation and biochemical characterization of the transport system.

Studies in vivo have demonstrated the accumulation of non-Tf iron by a variety of cell types (Craven et al., 1987). Recent studies have demonstrated that non-Tf iron in the plasma of individuals with hemochromatosis (a condition in which Tf is highly saturated) is present as ferric citrate (Grooteveldt et al., 1989). The concentrations of ferric citrate (8.0–21.0 μM) measured in the plasma of individuals with hemochromatosis is well within the range we have shown to increase non-Tf iron transport. We feel that the activation of iron transport activity mimics an in vivo event. Thus, the presence of organic iron chelates in plasma induces cells to express a transport system which accelerates the clearance of this potentially toxic molecule.

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REFERENCES


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Regulation of Non-Tf Iron Uptake