Phorbol Esters Stimulate Non-transferrin Iron Uptake by K562 Cells*

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Thomas Akompont, Robin S. Inman, and Marianne Wessling-Resnick§

From the Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115

Characterization of non-transferrin (non-Tf) iron transport by K562 cells has revealed unique properties relative to iron uptake mechanisms present in other cell types (Inman, R. S., and Wessling-Resnick, M. (1993) J. Biol. Chem. 268, 8521-8528). Since treatment of K562 cells with phorbol esters promotes stable megakaryocytic differentiation, we examined the uptake of non-Tf iron in response to protein kinase C activation. Treatment of K562 cells with phorbol esters increased the cellular uptake of 55Fe 4-6-fold compared with untreated cells. The phorbol ester–induced stimulation of 55Fe uptake was time- and dose-dependent, with significantly enhanced transport observed only after prolonged administration of 50 nM phorbol 12,13-dibutyrate (-8 h). These effects can be attributed to an increased Vmax of transport (140 ± 5 versus 0.6 ± 0.2 pmol/min/10⁶ cells) as well as a 6-fold increase in the apparent Km (1.2 ± 0.4 versus 0.2 ± 0.06 μM). It is thought that the reduction of Fe³⁺ to Fe²⁺ is required as a first step in the uptake mechanism, and the associated ferric reductase activity of K562 cells is also enhanced with phorbol ester treatment by 5-10-fold (337 ± 53 versus 43 ± 3 pmol/min/10⁶ cells). Bryostatin-1, a protein kinase C activator that fails to induce differentiation of K562 cells, did not promote this effect, indicating that the enhanced transport activity is dependent on the differentiation response. The idea that synthesis of a new class of transporters is responsible for this effect is supported by the observation that actinomycin D blocks up-regulation of non-Tf iron transport. The increased transport and ferric reductase activity induced upon differentiation also correlate with the appearance of saturable iron-binding sites on the surface of K562 cells with Kd ~ 0.4 μM. These results indicate that non-Tf iron transport activity and the expression of cell-surface iron-binding proteins can be controlled by environmental factors that promote megakaryocytic differentiation of K562 cells.

Although the major pathway for the cellular delivery of iron is via receptor–mediated endocytosis of transferrin (Tf), considerable evidence for alternative pathways of non-Tf iron uptake has been established. Properties defined for Tf-independent iron transport systems for many different cell types suggest that there may be two classes: "high" Km (5-30 μM) transport has been described in studies of fibroblasts (1, 2), HeLa cells (1, 2), Chinese hamster ovary cells (1), hepatocytes (3), HepG2 cells (4), and L1210 cells (5), while "low" Km (0.3-0.5 μM) iron transport has been observed for reticulocytes (6, 7) and K562 cells (8). Our knowledge about the factors that regulate the appearance of these alternative pathways for iron acquisition is limited, but it has been reported that non-Tf iron uptake by fibroblasts can be up-regulated upon exposure to heavy metals (2), suggesting that environmental factors can influence iron assimilation through Tf-independent mechanisms. In contrast, modulation of Tf receptor activity is well documented and, in particular, has been found to be associated with cancer cell differentiation in Friend, M1, HL-60, and K562 cell leukemias (9-15). Among other chemical induc-
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RESULTS

To assess the effects of phorbol esters on non-Tf iron uptake, K562 cells were treated with the concentrations of PDBu indicated in Fig. 1 for 16 h. The ability of the cells to transport non-Tf iron was then measured as described under "Materials and Methods." As shown in Fig. 1, the rate of $^{55}$Fe uptake was significantly increased upon exposure to 25 nM PDBu, with the maximum increase in non-Tf transport activity induced by 50 nM PDBu. Treatment with PDBu promoted a maximal stimulation of transport 3-5-fold over that observed for control (untreated) K562 cells in a dose-dependent manner.

Effects of protein kinase C activation by phorbol esters can be manifested within several minutes after administration; however, treatment of K562 cells with phorbol esters is known to induce cellular differentiation, which requires several hours to days before observable changes occur (18). To establish the earliest time point for induction of the increase in non-Tf iron uptake, K562 cells were treated with 50 nM PDBu for up to 16 h and then assayed for $^{55}$Fe transport activity. The results presented in Fig. 2 demonstrate that increased transport rates are not observed until after exposure to PDBu for >8 h. The increase in $^{55}$Fe uptake could result from changes in the $V_{\text{max}}$ of uptake, the apparent $K_m$, or both of these kinetic parameters. We therefore determined these values for treated and control K562 cells as described previously (8). Double-reciprocal plots of initial transport rate as a function of [FeNTA] were employed to establish the $V_{\text{max}}$ and apparent $K_m$. As shown by data presented in Fig. 3, both kinetic parameters were altered upon overnight treatment with PDBu. Table I presents a summary of the results from these experiments. While control cells display a $V_{\text{max}}$ comparable to previously reported activity (8), PDBu-treated cells have a $V_{\text{max}}$ that is 20-fold greater (Table I). The $K_m$ of transport was also increased 4-6-fold compared with control K562 cells.

In contrast to the observed changes induced by phorbol esters, treatment of K562 cells with bryostatin-1 fails to induce any significant alteration in the properties of non-Tf iron uptake (Table I). Like phorbol esters, bryostatin-1 is an activator of protein kinase C; however, the latter agent does not induce megakaryocytic differentiation of K562 cells (21). Thus, our observations suggest that up-regulation of non-Tf iron transport activity is specifically associated with phorbol ester-mediated differentiation of K562 cells and the concomitant expression of phenotypic markers of megakaryocytic lineage. Since actinomycin D, an inhibitor of mRNA synthesis, blocks phorbol ester-stimulated transport activity (Fig. 4), we conclude that transcriptional activation associated with megakaryocytic differentiation must be involved in the observed increase in the $V_{\text{max}}$ and $K_m$ of non-Tf iron transport. The simplest interpretation of these results is that synthesis of a new class of transporters is induced under these conditions, although it is not possible to rule out other post-translational effects that may

![Fig. 1. Dose response of PDBu in the stimulation of non-Tf iron uptake by K562 cells. K562 cells were treated with the indicated concentrations of PDBu overnight. $^{55}$Fe uptake was then assayed as described under "Materials and Methods." Briefly, 30 μl of 6 μM $^{55}$FeNTA was added to 270 μl of PBS-washed K562 cells in uptake buffer (4.7–6.1 × 10⁶ cells/ml). After a 5-min incubation, the 250-μl reaction mixture was quenched with 750 μl of ice-cold buffer containing 1 mM unlabeled FeNTA to displace any nonspecific surface-bound radioactivity. The cell-associated radioactivity measured in the same manner at 4°C was subtracted from that measured at 37°C to obtain the specific rate of $^{55}$Fe transport. Shown are the averaged values from triplicate transport measurements (± S.E.) as a function of [PDBu] for a single experiment. These data are representative of similar results obtained on three separate occasions.

![Fig. 2. Time course of induction of PDBu-stimulated $^{55}$Fe uptake. K562 cells were incubated with 50 nM PDBu at 37°C for 0, 2, 4, 8, and 16 h and then collected by centrifugation, washed with PBS, and resuspended in uptake buffer. Transport activity was assayed exactly as described for Fig. 1 with 5.0–6.6 × 10⁸ K562 cells/ml. Shown are the averaged values (± S.E.) for $^{55}$Fe transport rates determined in triplicate for K562 cells incubated with PDBu for the indicated times. Data are from a single experiment that was repeated three times. ***, significantly different from control (p < 0.001).]
regulate the activity of the existing iron uptake system.

It is interesting to note that the higher $K_m$ of iron transport by phorbol ester-treated K562 cells correlates well with apparent $K_m$ values reported for several other cell types. This may suggest that upon differentiation, K562 cells express an uptake mechanism akin to that observed for hepatocytes, fibroblasts, and Chinese hamster ovary and HeLa cells (1-3). In the latter studies, supraphysiological concentrations of calcium have been shown to promote significant increases in non-Tf iron uptake. Moreover, depletion of intracellular $Ca^{2+}$ also correlates with decreased transport activity. To establish whether the changes in transport induced by PDBu may reflect an altered response of non-Tf iron uptake to intra- or extracellular $Ca^{2+}$ levels, treated and control K562 cells were washed with 1 mM EGTA to deplete intracellular $Ca^{2+}$ or exposed to extracellular $Ca^{2+}$ (1 mM). As shown by the results presented in Table II, neither treatment had a significant effect on the transport activity measured for PDBu-treated or control cells. These observations are consistent with previous data from our laboratory indicating that the non-Tf iron uptake system in K562 cells is calcium-independent (8); most important, the results demonstrate that stimulation of transport activity by PDBu does not reflect an altered $Ca^{2+}$ response of the non-Tf iron uptake mechanism. The specificity of the non-Tf iron transporter in K562 cells with respect to other transition metals is also distinct from other uptake systems that have been characterized. Whereas the transporter in K562 cells may be specific only for iron and cadmium (8), the non-Tf iron transporter in HeLa cells may be able to transport cadmium, manganese, copper, and zinc as well (1, 2). Since the evidence presented above indicates fundamental changes in transport characteristics, we further examined the effects of 200 $\mu$M Cd$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, and Mn$^{2+}$ on the uptake assay. As shown by the results presented in Table II, only Cd$^{2+}$ and Cu$^{2+}$ significantly inhibited non-Tf iron uptake by control K562 cells, consistent with previous observations (8). None of the other cations were potent inhibitors of transport, unlike observations made for non-Tf iron uptake by HeLa cells, Chinese hamster ovary cells, and fibroblasts (1). The stimulated transport activity of PDBu-treated K562 cells was also resistant to inhibitory effects of these metals; moreover, the efficacy of Cd$^{2+}$ and Cu$^{2+}$ to inhibit uptake was reduced (Table II). This result may reflect the increase in apparent $K_m$ of transport upon phorbol ester treatment. Thus, the specificity of non-Tf iron transport by K562 cells does not appear to be altered upon differentiation.

A cell-surface ferrireductase activity has been associated with non-Tf iron uptake by K562 cells (8). Reduction of Fe$^{3+}$ to Fe$^{2+}$ is thought to be the first step in the uptake mechanism, and inhibition of the ferrireductase activity corresponds to a block in non-Tf iron uptake (22). Thus, the observed increase in non-Tf iron uptake could be associated with a parallel stimulation of the ferrireductase activity. To test this hypothesis, cell-surface ferrireductase activity was measured in PDBu-treated and control K562 cells, with the results shown in Fig. 3. Extracellular reduction of ferricyanide by PDBu-treated cells

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**TABLE I**

Kinetic parameters of non-Tf iron transport by K562 cells

| Condition         | Apparent $K_m$ | $V_{max}$ |
|-------------------|----------------|-----------|
|                   | $\mu$M          | pmol/min/10$^6$ cells |
| Untreated         | 0.22 ± 0.07     | 0.58 ± 0.24 |
| 50 nM PDBu, 16 h  | 1.36 ± 0.50     | 14.00 ± 5.18 |
| 50 nM bryostatin-1| 0.24 ± 0.13     | 0.72 ± 0.28 |

$^a$ Significantly different from control ($p < 0.001$).
Figure 5. Ferricyanide reductase activity is stimulated in PDBU-treated K562 cells. K562 cells were treated with 50 nM PDBU overnight (16 h) and then assayed for cell-surface ferricyanide reductase activity as described under "Materials and Methods." Briefly, the capacity of cells to reduce membrane-impermeant ferricyanide was measured by the production of ferrocyanide upon incubation at 37°C. Background activity was measured at 4°C in parallel reactions, and this value was subtracted to yield specific cell-associated ferricyanide reductase activity. Data are the average activity ± S.E. from four independent experiments expressed as pmol/min/10^6 cells.

![Ferricyanide reductase activity graph](image)

Table II

| Iron transport | Untreated K562 cells | PDBu-treated K562 cells |
|----------------|---------------------|------------------------|
| 1 mM EGTA (wash) | 101 ± 7 | 90 ± 7 |
| 1 mM CaCl_2 | 133 ± 3 | 104 ± 6 |
| 200 μM MnCl_2 | 134 ± 6 | 126 ± 5 |
| 200 μM NiCl_2 | 89 ± 12 | 100 ± 1 |
| 200 μM CoCl_2 | 70 ± 6 | 68 ± 14 |
| 200 μM CuSO_4 | 42 ± 18 | 79 ± 8 |
| 200 μM CdSO_4 | 35 ± 11 | 54 ± 13 |

was stimulated 5-10-fold compared with control K562 cells, with measured activities of 337 ± 53 and 43 ± 3 pmol/min/10^6 cells, respectively (n = 4). The enhanced ferricyanide reductase activity correlates well with the observed increase in non-Tf iron uptake after overnight PDBU treatment and is compatible with the idea that this function is closely associated with the uptake system. Since reduction of Fe^{3+} to Fe^{2+} is presumably a prerequisite for non-Tf iron transport (22, 23), it is possible that the observed stimulation of uptake is a functional consequence of enhanced ferricyanide reductase activity, although direct evidence that the latter step is rate-limiting for transport is lacking.

To identify proteins potentially involved in the phorbol ester-induced transport activity, iron binding studies were performed. As indicated by the results of Fig. 6, K562 cells do not display a significant number of 55Fe-binding sites under basal conditions. However, upon megakaryocytic differentiation, saturable binding of 55Fe to the surface of phorbol ester-treated K562 cells can be measured. Scatchard analysis of binding data reveals a K_d for 55Fe binding of 0.37 ± 0.06 nM, with a single class of ~5.4 × 10^7 binding sites/cell. Although the functional relationship with the observed stimulation of non-Tf iron uptake remains unknown, it is possible that the iron-binding proteins are components of a new class of transporters expressed by K562 cells upon megakaryocytic differentiation.

**DISCUSSION**

TF-mediated iron delivery is known to be modulated by iron-induced changes in the half-life of receptor mRNA, as well as by regulation of the rate of transcription (24). In contrast, non-Tf iron uptake does not appear to be influenced by intracellular iron levels (8), indicating fundamental differences in the regulation of these two pathways for iron assimilation. Regulation of TF receptor activity has also been correlated with leukemia cell differentiation and the cessation of cellular proliferation (9–15). When human erythroleukemia K562 cells are exposed to phorbol esters, a rapid down-regulation of surface TF receptors to intracellular compartments is observed (14, 15), and prolonged exposure will diminish receptor synthesis (17). In contrast, our results show that the non-Tf iron transport system is up-regulated by phorbol esters. Combined, these observations suggest that the cellular expression of these two different iron uptake mechanisms may be coordinately regulated. It is interesting to note that despite the down-regulation of the K562 cell TF receptors under these conditions, the TF-mediated iron delivery is only slightly suppressed (14, 25), supporting the idea that the coordinate regulation between TF-dependent and -independent pathways does not reflect changes in intracellular iron content.

Phorbol esters promote other changes in the phenotype of K562 cells as they cease to divide and begin to differentiate, expressing megakaryocytic markers (18). The fact that actinomycin D blocks the PDBU-induced stimulation of TF-independent transport indicates that mRNA and most likely protein synthesis are both required for this effect. Thus, altered transport activity appears to be the consequence of changes in cellular function due to the differentiation program. In fact, bryostatin-1, which activates protein kinase C but does not induce K562 cell differentiation (21), fails to stimulate non-Tf iron transport activity. Two potential explanations can account for stimulation of non-Tf iron transport. 1) Expression of regula-
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