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A New Role for the Transferrin Receptor in the Release of Iron from Transferrin†

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ABSTRACT: Iron removal by pyrophosphate from human serum diferric transferrin and the complex of transferrin with its receptor was studied in 0.05 M HEPES or MES buffers containing 0.1 M NaCl and 0.01 M CHAPS at 25 °C at pH 7.4, 6.4, and 5.6. At each pH, the concentration of pyrophosphate was adjusted to achieve rates of release amenable to study over a reasonable time course. Released iron was separated from protein-bound iron by poly(ethylene glycol) precipitation of aliquots drawn from the reaction mixture at various times during the course of a kinetic run. The amount of ^{59}Fe label associated with the protein and pyrophosphate was determined from the radioactivity of precipitate and supernatant, respectively, in each aliquot. Iron removal of 0.05 M pyrophosphate at pH 7.4 from diferric transferrin bound to the receptor is considerably slower than that from free diferric transferrin, with observed pseudo-first-order rate constants of 0.020 and 0.191 min^{-1}, respectively. For iron removal by 0.01 M pyrophosphate at pH 6.4, corresponding rate constants are 0.031 and 0.644 min^{-1}. However, at pH 5.6, iron removal by 0.001 M pyrophosphate is faster from diferric transferrin bound to its receptor than from free transferrin (observed rate constants of 0.819 and 0.160 min^{-1}, respectively). Thus, the transferrin receptor not only facilitates the removal of iron from diferric transferrin at the low pH that prevails in endocytic vesicles but may also reduce its accessibility to iron acceptors at extracellular pH, thereby minimizing the likelihood of nonspecific release of iron from transferrin at the cell surface.

Transferrin, the iron-binding protein of plasma, is the principal or only source of iron for the metabolic needs of most vertebrate cell types [for recent reviews of the physical chemistry of transferrin and the transferrin-cell interaction, see Brock (1985), Baldwin and Egan (1987), and Aisen (1989), and for the structure and function of the transferrin receptor, see Kühn (1989) and Forsbeck (1990)]. The transferrin molecule consists of a single 80-kDa polypeptide chain disposed in two lobes of highly homologous amino acid sequence. Each lobe is arranged in two domains surrounding a cleft bearing its iron-binding site (Anderson et al., 1987). Ligands of each iron-binding site are identical: two phenolic oxygen atoms from tyrosyl residues, one histidyl nitrogen atom, a single aspartyl oxygen atom, and two oxygen atoms from a carbonate anion (Anderson et al., 1987). Without carbonate,

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or other anion capable of replacing carbonate, the protein loses its iron-binding activity.

The iron-donating interaction of transferrin with cells may be complete in as little as 1–3 min (Aisen, 1983), during which time the protein is internalized by the cell to an acidified compartment, relieved of one or both of its iron atoms, and returned to the circulation for another cycle of iron transport. Throughout its sojourn within the cell, transferrin remains complexed to its receptor (Klausner et al., 1983; Dautry-Varsat et al., 1983), a molecule composed of two identical 95-kDa subunits (McClelland et al., 1984). Whether the receptor functions simply to capture transferrin from the circulation and target the protein to iron-releasing compartments of the cell or also to modulate the kinetics of iron release from transferrin has not previously been experimentally addressed. In this paper we consider a new role for the transferrin receptor in the release of iron from transferrin.

Materials and Methods

Radiolabeled Diferric Transferrin. Human apotransferrin was purchased from Calbiochem and purified as previously described to remove extraneous chelating agents used during its preparation (Aisen et al., 1978). Diferric transferrin was prepared from purified apotransferrin in 0.05 M HEPES[1]/0.1 M NaCl buffer, pH 7.4, by adding the appropriate amount of ^{59}Fe as bis(nitrilotriacetato)ferrate(III) (Bali & Harris, 1990). The Amersham Corp. supplied ^{59}Fe as $^{59}FeCl_3$. Released NTA was removed by ultrafiltration with repeated washing on an Amicon minicell Model 3 with a PM 30 membrane. Specific activities of the labeled transferrin ranged from 400 to 4000 cpm/μg of protein.

Transferrin Receptor. Human transferrin receptor was isolated from both fresh and freshly frozen placentas by reported procedures entailing detergent solubilization and affinity chromatography (Turkewitz et al., 1988). (Use of human placentas for the isolation of the transferrin receptor was approved by the Committee on Clinical Investigation of the Albert Einstein College of Medicine.) The published method was modified only in that diferric transferrin was coupled to Affi-Gel 15 (Bio-Rad) according to the manufacturer's instructions, rather than to cyanogen bromide activated Sepharose. Preparations showed a single band at 95 kDa by SDS–PAGE electrophoresis with the Pharmacia Phast-System or a major band at 95 kDa and a minor component at 190 kDa as would be expected with incomplete reduction of receptor dimer to component subunits. Only a single peak was evident by gel filtration chromatography (Figure 1).

Gel Filtration. Gel filtration chromatography was carried out on a Bio-Rad Model 1330 HPLC system with an analytical Bio-Sil TSK-250 column calibrated with Bio-Rad gel filtration standards. Protein peaks were detected by continuously monitoring absorbance at 280 nm with a Bio-Rad Model 1305A UV monitor (Figure 1). A sample size of 20 μL, flow rate of 1.0 mL/min, and chart speed of 10 mm/min were chosen for analytical chromatography. For estimation of radioactivity in ^{59}Fe-labeled samples, 1-mL fractions were collected and counted in a Searle Model 1195 γ-counter with a channel set for ^{59}Fe (Figure 2).

Receptor Binding Assay. The transferrin-binding activity of the receptor was assayed at pH 7.4 in 0.05 M HEPES/0.1 M NaCl/0.01 M CHAPS and at pH 6.5 and 5.5 in 0.05 M MES/0.1 M NaCl/0.01 M CHAPS buffer. Two to four molar equivalents of ^{59}Fe-labeled diferric transferrin at room temperatures was incubated at room temperature for 30–60 min with 20 μg of purified receptor. Receptor concentration was estimated from absorbance at 280 nm, by use of a molar absorption coefficient of 250 000 M cm^{-1} for purified receptor and 350 000 M^{-1} cm^{-1} for receptor–transferrin complex. These values were estimated with the Pierce Micro-BCA protein assay. Incubation mixtures were chromatographed on the analytical gel filtration column, incubation buffers being used for elution. In each preparation two peaks of radioactivity were detected, the first corresponding in retention time to purified receptor and the second to purified free transferrin. The column provided good separation of transferrin from the receptor, although it did not resolve the transferrin–receptor complex from free receptor in the detergent–buffers used. Relative radioactivity in each peak of chromatograms depended on the ratio of transferrin to receptor in the incubation mixture applied to the column. In the presence of an excess of transferrin (2.5 mol/mol of receptor) the relative peak areas corresponded to the binding of two transferrin molecules to each molecule of receptor.

Transferrin–Transferrin Receptor Complex. For kinetic studies of iron release, the purified complex of [^{59}Fe]transferrin and its receptor was obtained by collecting the first peak of radioactivity after gel filtration chromatography of an incubation mixture of labeled transferrin and purified receptor. Chromatography was performed with an 85 × 1.6 cm column of Sephacryl S-300 HR or with the HPLC system using a preparative Bio-Sil TSK-250 column. Appropriate fractions were combined and concentrated by ultrafiltration. SDS–PAGE electrophoresis of the purified complex revealed two bands at 95 and 80 kDa, corresponding to the receptor subunit and transferrin, respectively. Gel filtration chromatography of the purified complex showed a single peak of radioactivity with the mobility of the transferrin receptor.

Iron Removal. The kinetics of iron removal from free transferrin and the purified receptor–transferrin complex were studied at pH 7.4 in 0.05 M HEPES buffer and at pH 6.4 and 5.5 in 0.05 M MES buffers. In each case the buffer also contained 0.1 M NaCl to achieve a physiological concentration of chloride (Williams et al., 1982) and 0.01 M CHAPS to maintain the receptor–transferrin complex in solution. In order to achieve reasonable rates of iron removal, pyrophosphate was used as an iron acceptor; even at the lowest pH studied, hours were required to remove 50% of the iron from transferrin and the receptor–transferrin complex in the absence of an iron acceptor. At each pH the concentration of pyrophosphate was adjusted to attain reasonable rates of iron removal. Two different diferric transferrin preparations and three different receptor–transferrin complex preparations were used. In each pair of experiments, the concentration of free transferrin was adjusted to be similar to that of transferrin in the receptor–transferrin complex, 1.0 μM. However, the normalized data shown in Figures 3–5 were insensitive to concentration of transferrin over a 4-fold range of concentration. All experiments were carried out at room temperature, 25 ± 1 °C.

Iron release was followed by tracing the ^{59}Fe label as a function of time. Precipitation in 20% poly(ethylene glycol) (PEG, MW 8000) was used to distinguish label bound to protein from label bound to pyrophosphate. In control experiments, addition of an equal volume of 40% PEG to 0.001 M labeled diferric transferrin in 0.05 M HEPES/0.1 M NaCl/0.01 M CHAPS precipitated more than 98% of the

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NTA, nitrilotriacetate; MES, 2-(*N*-morpholino)-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PP_i, pyrophosphate; PEG, poly(ethylene glycol).

radioactivity, while ^{59}Fe complexed to pyrophosphate remained in solution in the presence of 20% PEG. Accordingly, at each time point a 20-μL aliquot of reaction mixture was added with mixing to 20 μL of 0.001 M unlabeled diferric transferrin contained in a 400-μL microcentrifuge tube. After addition of 40 μL of 40% PEG to this mixture, the tube was centrifuged for 1–2 min in a Beckman Microfuge and supernatant withdrawn from precipitate. In addition to assuring precipitation of labeled protein, the presence of an excess of unlabeled transferrin facilitated visualization of protein precipitated by PEG.

Kinetic Analysis. Radioactivity of iron bound to PEG-precipitable transferrin as a function of time was converted to a reaction coordinate R_t and normalized:

$$R_t(\text{ppt}) = 100[(A_0 - A_t)/(A_0 - A_\infty)] \quad (1)$$

where A_0 = total radioactivity in transferrin at the start of each experiment, A_t = radioactivity at time t, and A_∞ = background radioactivity. R_t was fit to a simple two-exponential model, eq 2, by nonlinear regression with the Marquardt–Levenberg

$$R_t = 50\exp(-k_1 t) + 50\exp(-k_2 t) \quad (2)$$

algorithm (SigmaPlot Version 4.0, Jandel Scientific). Attempts to fit the experimental data to a rigorous macroscopic rate equation (Baldwin, 1980) were not successful, presumably because the scatter in the data points was too great and the number of data points too small.

When reaction kinetics were evaluated from PEG supernatants, the expression used for fitting data was the complement to eq 1:

$$R_t(\text{spn}) = 100 - 100[(A_0 - A_t)/(A_0 - A_\infty)] \quad (3)$$

The two phenomenological rate constants, k_1 and k_2, are not true macroscopic rate constants but serve simply as descriptors for comparing apparent rates of release of iron from transferrin and the receptor–transferrin complex under equivalent conditions. Each of these constants is a function of true site-specific rate constants characterizing the removal of iron from transferrin under experimental conditions (Baldwin, 1980; Bali & Harris, 1990). Only when there is no cooperativity between the two sites of transferrin in releasing iron and the rates of release from the two sites differ widely does the simple biexponential equation provide estimates of the macroscopic kinetic constants (Bali & Harris, 1989). In some of our studies, experimental curves were better described by fitting to a single exponential, as would be expected if each site of transferrin released iron at the same rate or only one site releases iron during the course of an experiment. Results for three typical kinetic runs are plotted in Figures 3–5; a summary of the constants derived from all experiments is presented in Table I.

Results and Discussion

Transferrin Receptor. Preparations of transferrin receptor obtained from human placentas for use in the present studies were similar in properties to those reported by other laboratories (Enns & Sussman, 1981; Reckhow & Enns, 1988; Turkewitz et al., 1988). Homogeneity was evidenced by single peaks in gel filtration chromatograms (Figure 1) and single 95-kDa bands in SDS–PAGE as expected of the receptor subunit. Transferrin binding activity was assessed by gel filtration chromatography, as indicated in Figure 2. The stoichiometry of transferrin binding by receptor, near 2 mol of transferrin/mol of receptor, is also in accord with earlier work (Enns & Sussman, 1981) and attests to the functional integrity of our preparations. When stored at 4 °C, detergent-solubilized receptor retained transferrin binding activity for at least 10 weeks.

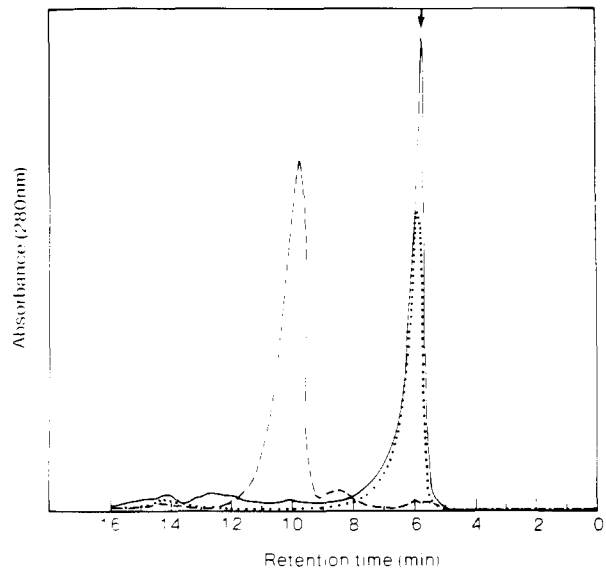

FIGURE 1: Gel filtration chromatograms of transferrins (---), receptor (—), and receptor–transferrin complex (···) on a size-exclusion analytical Bio-Sil TSK-250 column. Sample size, 20 μL; flow rate, 1.0 mL/min; chart speed, 10 mm/min. The buffer used for loading and elution was 0.05 M HEPES/0.1 M NaCl/0.01 M CHAPS. Ordinate is absorbance at 280 nm in arbitrary units. Arrow indicates void volume measured with blue dextran.

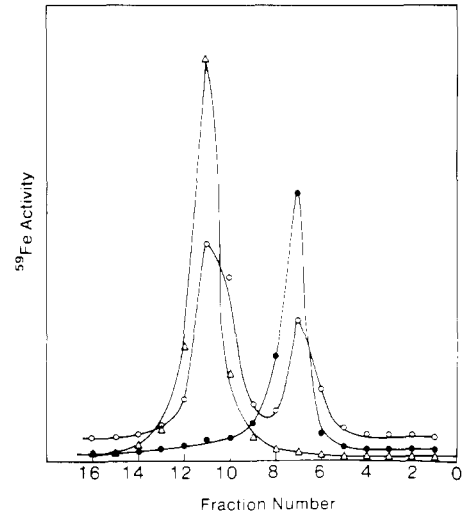

FIGURE 2: Radioactivity of fractions from gel filtration chromatograms of pure transferrin (△), receptor–transferrin complex isolated by chromatography (●), and an incubation mixture of receptor plus transferrin (○). Chromatography as in Figure 1. One-milliliter fractions were collected and counted for ^{59}Fe activity. Ordinate is arbitrary activity units; peak activities were 3600 cpm for transferrin, 2400 cpm for receptor–transferrin complex, and 1600 and 2400 cpm for the mixture of receptor and transferrin.

Kinetics of Iron Release. At the pH of the cell surface, 7.4, the initial rate of release of iron from transferrin to 0.05 M pyrophosphate is nearly 10 times faster from free transferrin than from the complex of transferrin and its receptor (Figure 3 and Table I). Even at this unphysiological concentration of pyrophosphate, however, only 35% of iron initially bound to transferrin is released in 60 min. At pH 6.4 release is much faster than that at pH 7.4, as would be expected from the weakening of the iron–transferrin bonds as pH is lowered (Aisen et al., 1978). No direct comparison of release kinetics at the two pH values is possible, however, because of the

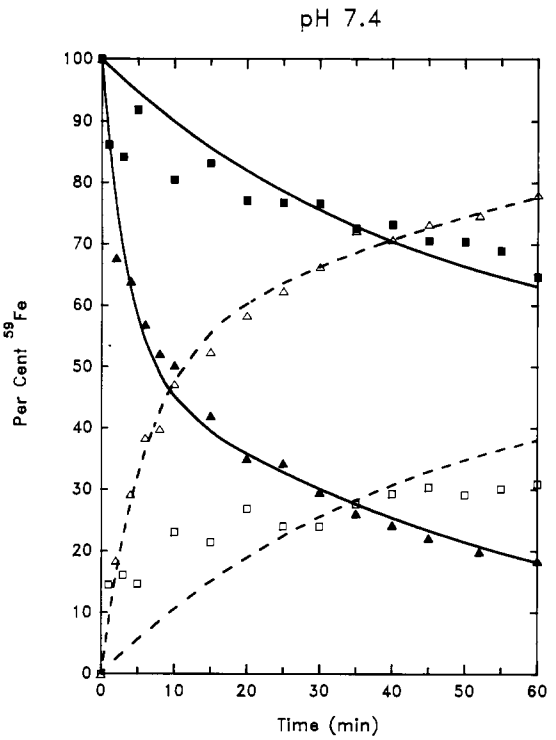

FIGURE 3: Normalized reaction coordinate (% ^{59}Fe) as a function of time for iron removal from transferrin and receptor–transferrin complex by 0.05 M PP_i at pH 7.4 in 0.05 M HEPES/0.1 M NaCl/0.01 M CHAPS. (△, ▲) Transferrin; (□, ■) receptor–transferrin complex; (▲, ■) activity in PEG precipitates; (△, □) activity in supernatants; (—) curve fit to precipitate activities; (---) curve fit to supernatant activities.

Table I: Pseudo-First-Order Rate Constants for the Release of Iron to Pyrophosphate from Transferrin and Receptor–Transferrin Complex

	pH		
	7.4 ([PP_i] = 0.05 M)	6.4 ([PP_i] = 0.01 M)	5.6 ([PP_i] = 0.001 M)
transferrin			
k_1[a]	0.19 ± 0.03	0.64 ± 0.03	0.16 ± 0.013
k_2	0.016 ± 0.001	0.027 ± 0.001	[b]
receptor–transferrin complex			
k_1	0.020 ± 0.002	0.031 ± 0.002	0.819 ± 0.0077
k_2	[b]	[b]	0.021 ± 0.002

[a] n = 4 for all values. [b] Indeterminate.

difference in concentrations of pyrophosphate. The effect of receptor in retarding release of iron from transferrin is amplified at pH 6.4, with an initial rate some 20 times greater from free transferrin than from the receptor–transferrin complex.

At a pH of 5.6, a value approaching that attained in the acidified endosome bearing the complex of transferrin and its receptor within the iron-requiring cell (Klausner et al., 1983; Dautry-Varsat et al., 1983), the effect of receptor is reversed. Now, the receptor facilitates release of iron from transferrin, with an apparent initial rate of release from the receptor–transferrin complex five times faster than that from free transferrin. The concentration of pyrophosphate at pH 5.6, 0.001 M, approximates that of intracellular ATP, a molecule suggested to be a carrier of iron within the cell (Weaver & Pollack, 1989). Furthermore, more than 50% of iron is released within 3 min by the receptor–transferrin complex, a rate likely to be even faster at physiological temperature and almost sufficient to account for iron release from transferrin within cells.

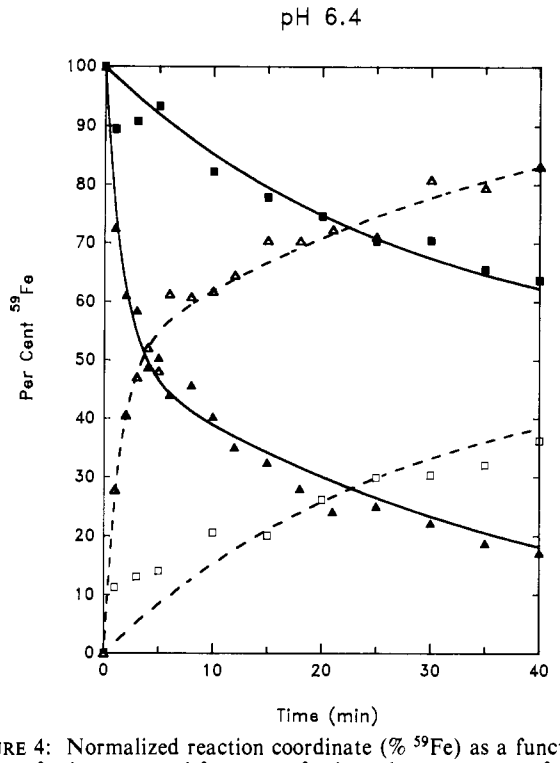

FIGURE 4: Normalized reaction coordinate (% ^{59}Fe) as a function of time for iron removal from transferrin and receptor–transferrin complex by 0.01 M PP_i at pH 6.4 in 0.05 M MES/0.1 M NaCl/0.01 M CHAPS. Symbols and lines as in Figure 3.

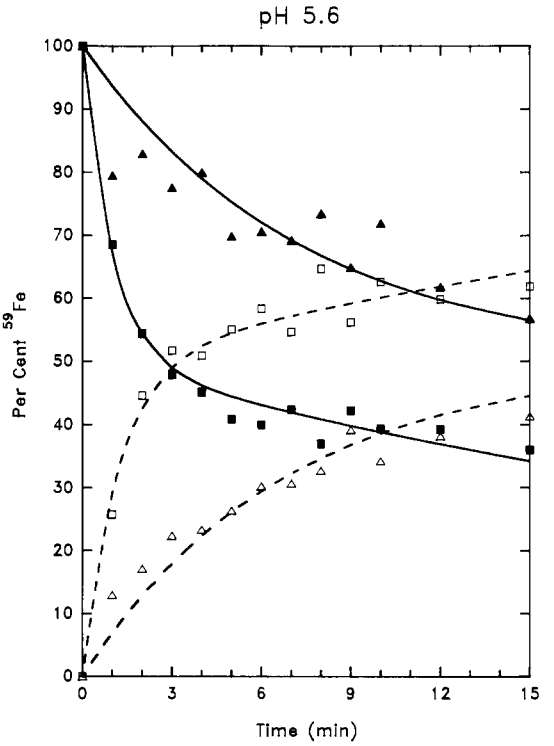

FIGURE 5: Normalized reaction coordinate (% ^{59}Fe) as a function of time for iron removal from transferrin and receptor–transferrin complex by 0.001 M PP_i at pH 5.6 in 0.05 M MES/0.1 M NaCl/0.01 M CHAPS. Symbols and lines as in Figure 3.

In each of these experiments one measure of the quality of the data is provided from the total radioactivity found in supernatants and precipitates. The graphs of Figures 3–5 show that at each time the measured radioactivity in supernatant and precipitate totals close to 100% of the radioactivity in transferrin at the start of the experiment. This relation is even clearer in the fitted curves of these graphs, lending credence

to the reliability of our observations. However, the data should be taken as indicating the role of the receptor in modulating release of iron from transferrin, rather than as providing true kinetic constants characterizing such release.

Possible Mechanisms Underlying the Effect of Receptor on Iron Release from Transferrin. The mechanism of iron release from transferrin to acceptor chelating molecules is not yet well understood. One possibility is that transferrin exists in two conformational states, one "closed" and unable to release iron and the other "open" and facile in releasing iron (Cowart et al., 1982). This view, supported by other kinetic studies (Kretchmar & Raymond, 1986), is consistent with comparative X-ray crystallographic studies of diferric lactoferrin and apolactoferrin (Anderson et al., 1990), a protein homologous in primary and three-dimensional structure to serum transferrin (Bailey et al., 1988). Both sites of lactoferrin assume a closed structure in the presence of iron. In the absence of metal the N-terminal site changes to a wide-open configuration due to a jaw-like pivoting of the surrounding domains about their hinge, with the C-terminal site remaining in a closed conformation. We suggest, therefore, that at low pH the transferrin receptor forces one or both lobes of transferrin into the open conformation, thereby facilitating release of iron. Consistent with this view is the finding that receptor binds transferrin even more strongly at pH 5.0 than at pH 7.4 (Ecarot-Charrier et al., 1980), despite the progressive loss of iron from transferrin at this low pH. Conversely, at extracellular pH, where the receptor has low affinity for apotransferrin (Tsunoo & Sussman, 1983; Young et al., 1984), it may lock iron-bearing transferrin into the closed state and may impede iron release as well by restricting access to the iron-binding cleft.

Functions of the Transferrin Receptor. A well-established function of the transferrin receptor is to sequester iron-bearing transferrin for internalization by iron-requiring cells, while ignoring iron-free transferrin. Transferrin is thereby able to donate iron to cells even in the presence of a preponderance of apotransferrin in the circulation, as in iron deficiency. The present studies point to another biological function of the transferrin receptor in modulating iron release from transferrin. At the pH of the cell surface, 7.4, formation of a complex between transferrin and its receptor impedes release of iron from transferrin. Although spontaneous release of iron at this pH is negligible in terms of cellular needs for iron, even trace amounts of iron release promoted by phosphate groups of the cell membrane may be sufficient to cause peroxidation of membrane lipids (Gutteridge, 1987). Thus, the transferrin receptor may serve to protect the cell membrane against locally released trace iron. Perhaps more importantly, the receptor facilitates freeing of iron from transferrin in the pH range achieved by the endosome to which transferrin is internalized by the cell. Reductive release of iron (Kojima & Bates, 1979) or involvement of specialized iron acceptors (Weaver & Pollack, 1989) has previously been invoked to account for the rapidity of iron uptake from transferrin by cells. The present results suggest that the interaction of transferrin with its receptor has a key role in the delivery of iron from transferrin to cells, accounting in whole or in large part for physiological rates of iron release.

Added in Proof

Our attention has been called to an abstract reporting that binding of transferrin to its receptor raises by 0.9 unit the pH at which 50% of iron is dissociated from the C-terminal site in release experiments (Sipe et al., 1988). This observation appears to be in substantial accord with our results.

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