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Regulation of iron uptake and transport by transferrin in Caco-2 cells, an intestinal cell line

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Abstract

Caco-2 cells grown in bicameral chambers, a model of intestinal epithelial iron transport (Biochim. Biophys. Acta (1991) 1070, 205–208), were used to study the effect of apo-transferrin (apo-Tf) in the basal chamber on ^{59}Fe uptake from the apical surface, intracellular ^{59}Fe distribution, and ^{59}Fe transport into the basal chamber. Caco-2 cells were grown with varying amounts of iron to achieve cells that were either iron-deficient (FeD), of normal iron status (FeN), or iron-loaded (FeH). The effect of apo-Tf was most marked in FeD cells with the transport of ^{59}Fe from 1 μM ^{59}Fe-ascorbate on the apical side to the basal chamber measured as $(22.2 \pm 3.0) \cdot 10^4$, $(8.2 \pm 0.6) \cdot 10^4$, and $(2.7 \pm 0.4) \cdot 10^4$ atoms ^{59}Fe/cell/min in the presence of apo-Tf, BSA, and no added protein, respectively. Unexpectedly in FeD cells total ^{59}Fe uptake (i.e., both ^{59}Fe in the cells and that transported into the basal chamber) was decreased by basolateral apo-Tf with total uptake of $(2.6 \pm 0.3) \cdot 10^5$, $(4.8 \pm 0.6) \cdot 10^5$, and $(4.8 \pm 0.7) \cdot 10^5$ atoms/cell/min with apo-Tf, BSA, and no additions, respectively. Analysis of intracellular ^{59}Fe by isoelectrofocusing in polyacrylamide gels demonstrated ^{59}Fe migrating both with a basic p*I* and with the p*I* values of ferritin (Ft) at a ratio of 200:1 (basic p*I* moiety: ferritin) in FeD cells. The presence of Tf further decreased the small amount of ^{59}Fe in Ft. These studies demonstrate that basolateral Tf affects the apical uptake of ^{59}Fe, the intracellular distribution of ^{59}Fe, and the transport of ^{59}Fe across intestinal epithelium, the latter effect occurring even when cellular content of ferritin is high.

Key words: Intestine; Iron transport; Caco-2; Regulation iron absorption

1. Introduction

Although the physiology of iron uptake across the intestinal epithelium has been well described, the molecular mechanisms of iron transport remain to be fully elucidated. Measurements of iron uptake using whole animals or various gut sac preparations have defined three phases of iron uptake: an uptake phase in which iron enters the apical or lumenal surface; an intracellular phase in which iron is either transported to the basolateral surface for subsequent delivery to the plasma or is stored to be lost when the epithelial cells are sloughed; and a transfer phase by which iron is released from the epithelium to the plasma. The uptake phase appears to involve vesicular transport into the cell [1,2] and may involve binding of Fe to particular iron binding proteins in these vesicles [3–7]. Uptake into the cell is more rapid for Fe(II) than Fe(III) [8]. Both ferritin and transferrin have been suggested to play a role in the intracellular phase [9] but the mechanisms involved in the intracellular and transfer phases remain enigmatic.

Regulation of body iron stores occurs solely at the absorptive site in intestinal epithelium as there is no excretory regulation. The molecular details of the regulatory mechanisms are not known. Kinetic analyses of iron uptake suggest that regulation occurs at either the uptake or intracellular phases [10]. The physiology of the regulation is well described – iron uptake is inversely proportional to iron stores [11,12] and is stimulated by several well defined conditions such as ineffective erythropoiesis [3]. While increased reticulocytes

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appear to stimulate uptake [13], plasma levels of transferrin and ferritin do not appear to influence uptake in the systems studied [9,14,15].

We [7,8] and others [16] have recently described the Caco-2 cell line grown in bicameral chambers as a model for intestinal iron uptake. The utility of the model is that it enables the uptake, intracellular, and transfer phases of iron transport to be studied with greater ease than with animal or organ models. Transport of iron in the Caco-2 system responds to the iron status of the cell with iron-deficient cells having increased and iron-loaded cells exhibiting decreased transport into the basolateral chamber. In the studies presented here we have extended the model to investigate the effect of additions of the plasma proteins transferrin and bovine serum albumin to the basal chamber, studying both the uptake of ^{59}Fe and the transport of ^{59}Fe into the basal chamber as well as the intracellular distribution of ^{59}Fe.

2. Materials and methods

2.1. Cell culture

Caco-2 cells, from American Type Culture Collection No. HTB37 (Rockville, MD), were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD), 1% non-essential amino acids, and antibiotics/antimycotic (100 U/ml Penicillin-G, 100 U/ml Streptomycin and U/ml Fungizone, Gibco). Cells were grown in Transwell bicameral chambers with 3 μm pore size membrane (Costar, Cambridge, MA) coated with collagen. The collagen film was applied to the filter as 50 μl of collagen solution (3 mg/ml, 60% ethanol; rat tail, type I, Boehring Mannheim, Germany) and then the Transwells were inverted and dried under a sterile laminar air flow. Formation of a monolayer was monitored by measuring the transepithelial electrical resistance (TEER) with a Millicell electrical resistance system (Millipore, Bedford, MA). Iron deficient (FeD) and iron loaded (FeH) cells were obtained as previously described [8]. The FeD cells were cultured using Dulbecco's MEM without added iron (Gibco) supplemented with iron depleted FBS prepared by titrating the FBS to pH 4.5 in the presence of 300 gm/l Chelex (Sigma, St. Louis, MO); after 2 h with stirring the pH was increased to pH 7.4 with NaOH and the FBS allowed to sit overnight in the presence of Chelex. The iron content of the iron depleted FBS and medium was always less than 0.1 μM as measured by atomic absorption spectroscopy (AAS) in a Perkin-Elmer AAS equipped with an argon furnace. The FeH cells were obtained by culturing cells under high iron conditions using medium made 50 μM in Fe(III) by the addition of Fe(III)-NTA. Under these conditions the cell iron contents were 23 ± 5, 75 ± 23, and 303 ± 69 pmol Fe/mg protein for the FeD, iron normal (FeN), and FeH cells, respectively, as measured by AAS.

Iron uptake was measured only after TEER had risen to a level indicating the formation of an intact monolayer (TEER at least 250 Ohm cm^2) [8,17–19]. The cell monolayers were depleted of serum proteins prior to testing the effects of Tf by washing the monolayers twice with DMEM followed by overnight incubation with DMEM without FBS.

2.2. ^{59}Fe uptake and transport by Caco-2 cells

^{59}Fe ($^{59}FeCl_3$, 5–85 Ci/g, NEN-DuPont, Boston, MA) was added as Fe(II)-ascorbate at an Fe/ascorbate ratio of 1:1000 [20]. The ^{59}Fe-ascorbate was added at 1 μM Fe to the apical or upper chamber in 50 mM Hepes-buffered saline solution (HBS), pH 7.4, prepared from 193 mM Hepes diluted with an isotonic salt solution consisting of 130 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgSO_4$. The buffer in the basal or lower chamber was HBS made 50 μM in human apo-transferrin (Boehring Mannheim, Germany) or 50 μM in BSA (Sigma). Stock solutions at pH 4.5 of both proteins were passed through a Chelex column to remove metals. Iron uptake was allowed to proceed for 60 min., the basal chamber buffer harvested, the cell monolayers washed three-times with HBS containing 10 mM EDTA, and cells removed from the polycarbonate membranes with 0.5% trypsin. Radioactivity in the cells, apical and basal chambers was determined in a Compugamma LKB gamma counter. In some experiments ^{59}Fe was presented as ^{59}Fe-lactate prepared by diluting $^{59}FeCl_3$ in 0.1 N HCl into a four-fold molar excess of Na lactate and titering to pH 7.0 just prior to addition to the cells. When noted Brefeldin A (Epicenter Technologies, Madison, WI) was added at 5 μg/ml to the basal chamber for 10 min prior to the addition of ^{59}Fe-ascorbate.

2.3. HPLC fractionation of ^{59}Fe transported into basal chambers by Caco-2 cells

The basal chamber buffer (HBS) was fractionated by HPLC (Beckman 334) on tandem Zorbax 450 and 250 (DuPont) size fractionation columns. The samples were made 0.025% in Chaps (Sigma) and then eluted with 20 mM Hepes (pH 7.0), 0.025% Chaps. The non-specific binding of ^{59}Fe by the columns was reduced by saturating the columns with 10 μM Fe-citrate at 1:2 ratio (Fe/citrate). The fractionations were performed at 4°C. to reduce detergent micelle formation. Optical absorbance was both detected at 280 nm (Beckman) and monitored on a diode-array spectrophotometer (Hew-

lett Packard). Fractions were collected and ^{59}Fe radioactivity determined.

2.4. Isoelectric focusing of ^{59}Fe in Caco-2 cells

The analysis of intracellular ^{59}Fe and detection of ferritin was performed on cell lysates by isoelectrofocusing (IEF) in 2% polyacrylamide (30 acrylamide/0.8 bisacrylamide, w/w) gels in a flat bed IEF apparatus (LKB) using a ampholine gradient of pH 4–6 and containing 2.0% Chaps. Transwell membranes containing cells exposed to ^{59}Fe were removed from the bicameral chambers, placed directly on the polyacrylamide gels, and a drop of 2% Chaps placed on the cells prior to the initiation of focusing. Parallel lanes of the gels were either subsequently electrotransferred onto nitrocellulose paper for detection of ferritin by western blotting, exposed directly to X-ray film or washed in acid-EtOH then exposed for determination of radioactivity by autoradiography with subsequent densitometric scanning, or silver stained (ICN, Costa Mesa, CA) for detection of proteins. Ferritin (Ft) was detected by using a rabbit polyclonal antibody against human liver Ft in western blots. Exposure to acid-EtOH prior to autoradiography solubilized ^{59}Fe from ferriproteins except ferritin and allowed quantification of ^{59}Fe in ferritin by autoradiography.

3. Results

3.1. The effect of apo-Tf in the basal chamber on ^{59}Fe transport

Prior to studying the effects of basal additions on Fe transport, preliminary experiments were performed to determine conditions necessary to deplete the Caco-2 cells either of Tf or BSA acquired during culture. In these experiments cells were cultured for 4 h either with ^{125}I-Tf or ^{125}I-BSA, washed twice with medium, cultured with medium without FBS, and radioactivity assayed in aliquots of the basal chamber for varying times up to 16 h. By 16 h neither ^{125}I-Tf nor ^{125}I-BSA were being released into the medium nor could any residual radioactivity be detected in the cells. Additional experiments were performed on the cells after overnight depletion of Tf to characterize the cellular uptake into and transport of ^{59}Fe from the Caco-2 cells. As seen in Fig. 1, the transport of ^{59}Fe into the basal chamber from 1 μM ^{59}Fe-ascorbate in the apical chamber was linear for at least 2 h. In these experiments transport of ^{59}Fe was markedly temperature dependent. As shown in Fig. 1 in the presence of apo-Tf there was an approx. 50% decrease in ^{59}Fe transport at 25°C. compared to 37°C. In the absence of apo-Tf there was an even greater decrease in transport.

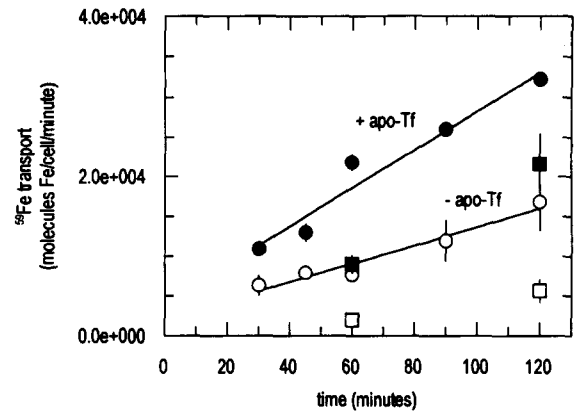


Fig. 1. Caco-2 cells were exposed to 1 μM ^{59}Fe-ascorbate in the apical chamber in the presence (+ apo-Tf, •) or the absence (− apo-Tf, ○) of 50 μM apo-Tf in the basal chamber, and subsequently the radioactivity in the basal chamber was measured at the indicated times as detailed in the Materials and methods. Transport was also measured at 25°C. in the presence (■) and absence (□) of apo-Tf. Shown are the means ± S.D. of triplicates for three experiments for transport at 37°C and two experiments for 25°C. When not apparent the error bars are smaller than the symbols.

To determine the specificity of cellular uptake and transport of ^{59}Fe non-specific transport was determined by dilution of 1 μM ^{59}Fe ascorbate with a 100-fold excess of ^{56}Fe ascorbate. Measured in this manner non-specific transport was $(0.18 \pm 0.04) \cdot 10^4$ molecules of ^{59}Fe/cell per min compared to total transport into the basal chamber of $(2.57 \pm 0.7) \cdot 10^4$ molecules of ^{59}Fe/cell per min (mean ± S.D. of three experiments with three replicates each). In these same experiments non-specific ^{59}Fe cell uptake was $(1.65 \pm 0.35) \cdot 10^4$ compared to total cellular uptake of $(3.05 \pm 1.5) \cdot 10^5$ molecules of ^{59}Fe/cell per min (mean ± S.D. of three experiments with three replicates each). To further ascertain that ^{59}Fe transport was specific, Caco-2 cells depleted of Tf were incubated with Brefeldin A, an inhibitor of exocytosis [29,30], and ^{59}Fe transport measured. At concentrations of 5 μg/ml Brefeldin A and in the absence of apo-Tf, transport decreased from $1.9 \cdot 10^4$ and $1.1 \cdot 10^4$ molecules of ^{59}Fe/cell per min in the absence to $0.32 \cdot 10^4$ and $0.16 \cdot 10^4$ molecules of ^{59}Fe/cell per min in the presence of Brefeldin A (mean of two experiments with three replicates each). It is not known if the transport detected in the presence of Brefeldin A represents non-specific transport. Further experiments are being conducted to determine if the residual transport represents residual exocytosis still involved in the transport of ^{59}Fe or a non-specific transport process. Finally, specificity was also documented by measuring the cell uptake and transport of ^{59}Fe presented as ^{59}Fe-lactate. Cell uptake from 1 μM ^{59}Fe-lactate was $(1.25 \pm 0.35) \cdot 10^4$ molecules ^{59}Fe/cell per min compared to uptake from ^{59}Fe-ascorbate of $(3.5 \pm 0.3) \cdot 10^5$ molecules

Table 1
The effect of basolateral apo-transferrin on ^{59}Fe transport and uptake

	Transport	Total cellular uptake
Low Fe cells		
Transferrin	$(22.2 \pm 3.0) \cdot 10^4$ [a]	$(2.6 \pm 0.3) \cdot 10^5$ [a]
BSA	$(8.2 \pm 0.6) \cdot 10^4$ [a]	$(4.8 \pm 0.6) \cdot 10^5$
Nothing	$(2.7 \pm 0.4) \cdot 10^4$	$(4.8 \pm 0.7) \cdot 10^5$
Normal Fe cells		
Transferrin	$(3.2 \pm 1.0) \cdot 10^4$ [b]	$(1.3 \pm 0.3) \cdot 10^5$ [a]
BSA	$(3.4 \pm 0.6) \cdot 10^4$ [c]	$(4.5 \pm 0.8) \cdot 10^5$
NTA	$(2.4 \pm 0.3) \cdot 10^4$	$(2.9 \pm 0.6) \cdot 10^5$
Nothing	$(2.1 \pm 0.7) \cdot 10^4$	$(3.4 \pm 1.2) \cdot 10^5$

Transport of ^{59}Fe into the basal chamber and cellular ^{59}Fe uptake were determined by incubation of Caco-2 cells for 60 min with 1 μM ^{59}Fe ascorbate as described in the Materials and methods. The total cellular uptake, which represents the sum of the ^{59}Fe transported into the basal chamber and the cellular ^{59}Fe uptake, and the ^{59}Fe transported are expressed as molecules of ^{59}Fe/cell per min (mean $\pm$ S.D. of three experiments with three replicates each). Transferrin as apo-Tf and BSA were added to the basal chamber at 50 μM and NTA at 10 μM. Non-specific transport determined as noted in the Results was $(0.18 \pm 0.04) \cdot 10^4$ and $(1.65 \pm 0.35) \cdot 10^4$ molecules of ^{59}Fe/cell per min for transport and cellular uptake, respectively (mean $\pm$ S.D. of three experiments with three replicates each).
The mean values labelled with same letter differ significantly as determined by Student's t-test from the no addition controls as follows: [a] $P < 0.0001$; [b] $P = 0.02$; [c] $P = 0.006$. In addition, in the presence of transferrin cellular iron uptake and basolateral transport differed significantly between Low Fe cells and normal Fe cells at $P = 0.006$ and $P = 0.0005$, respectively.

^{59}Fe/cell per min (mean $\pm$ S.D. of two experiments each in triplicate). Transport of ^{59}Fe presented from ^{59}Fe-lactate in these experiments was barely detectable at $(2.0 \pm 0.8) \cdot 10^2$ molecules ^{59}Fe/cell per min or approx. 1% of that observed with ^{59}Fe-ascorbate.

The effects of the basal additions of apo-Tf and BSA on the transport of ^{59}Fe by Caco-2 cells during a 1-h period following the addition of ^{59}Fe-ascorbate to the apical surface are shown in Table 1. The iron content of the cells as noted in Materials and methods was 23 ± 5, 75 ± 23 pmol/mg protein in FeD and FeN cells, respectively. In FeD cells apo-Tf stimulated ^{59}Fe transport into the basal chamber producing about an 8-fold increase over the absence of added protein. BSA stimulated transport about 3-fold. In the FeN cells both Tf and BSA stimulated transport about 1.5-fold. To determine if the effect of apo-Tf was merely to shift the 'equilibrium' of the system to the basal chamber the following studies were undertaken: First, the rates of transport of ^{59}Fe into the basal chamber were measured at varying times up to 60 min in the presence and absence of 50 μM apo-Tf (Fig. 1). The linear transport in both instances suggests that the apo-Tf is not acting as an iron chelator to shift equilibrium but is changing the rate of ^{59}Fe transport. Second, ^{59}Fe transport was examined at concentrations of apo-Tf from 1–50 μM apo-Tf. No differences were noted from the results shown in Table 1 (data not shown). Third, the effect of 10 μM NTA in the basal chamber on ^{59}Fe transport was studied and, as shown in Table 1, transport was not significantly different from no addition in both the FeN and FeD cells.

A paradoxical observation was the effect of the addition of apo-Tf on total uptake of ^{59}Fe by the Caco-2 cells. As shown in Table 1, the cellular ^{59}Fe uptake (i.e., the sum of ^{59}Fe transported to the basal chamber and ^{59}Fe within the cells) was decreased nearly 40% in FeD cells and nearly 60% in FeN cells exposed to apo-Tf despite the greater transport of ^{59}Fe into the basal chamber in the presence of apo-Tf. Although BSA increased transport in FeD and FeN cells there was no effect of BSA on cellular uptake. The presence of 10 μM NTA did not stimulate cellular ^{59}Fe uptake in FeN cells.

3.2. Analysis of ^{59}Fe binding proteins in the basal chamber

The contents of the basal chambers were analyzed after 1 h exposure to ^{59}Fe-ascorbate in the apical chamber. To determine if the ^{59}Fe was indeed bound to either Tf or BSA and to characterize the ^{59}Fe binding moiety in the chambers without plasma proteins, the contents of the basal chambers were analyzed by HPLC molecular sieve columns. As shown in Fig. 2, ^{59}Fe from basal chambers either with apo-Tf (panel B) or BSA (panel A) chambers could be demonstrated to elute with the same time as either Tf or BSA. No other ^{59}Fe moiety could be detected when either plasma protein was present. However, in the absence of a protein addition (panel C), ^{59}Fe was detected both on a high and a low molecular weight moiety. The retention time of the larger moiety was not the same as Ft, Tf or BSA while the retention time of the smaller moiety was not the same as Fe complexed either to ATP or ADP.

3.3. The effect of basal additions on intracellular distribution of ^{59}Fe

To determine if the basal additions affected the intracellular distribution of ^{59}Fe, cells were lysed after the 1 h incubation with ^{59}Fe-ascorbate by placing the cell monolayers on the Costar filters directly on the isoelectrofocusing gels in the presence of 2.0% Chaps, and the lysates analyzed following isofocusing by autoradiography. In parallel lanes Western blot analysis with rabbit anti-human ferritin antibodies was used to determine the position of ferritin while ^{125}I-Tf was used to determine Tf migration. The autoradiograph patterns of a typical profile are shown in Fig. 3. The lanes in Fig. 3 panel A were not-fixed prior to exposure to the X-ray film while the parallel lanes in panel B

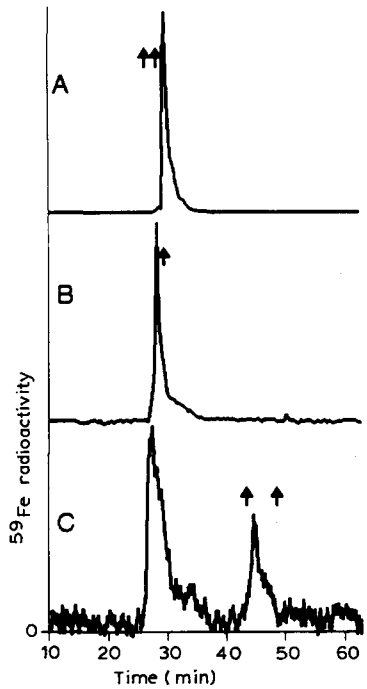


Fig. 2. Caco-2 cells were exposed to ^{59}Fe-ascorbate for 1 h as described in the Materials and methods with the basal media containing either BSA (panel A), Tf (panel B), or no protein addition (panel C). Medium from the basal chambers was subsequently fractionated on tandem 450-250 Zorbax HPLC columns, the ^{59}Fe radioactivity determined in the resulting fractions, and normalized radioactivity expressed as a function of elution time. The molecular weight markers used were rabbit ferritin (elution time [Et] = 26 min), BSA (Et = 28 min), Tf (Et = 29.5 min), Phenol red (Et = 43 min), and ^{125}I (Et = 49 min). Shown is a representative experiment of three such experiments.

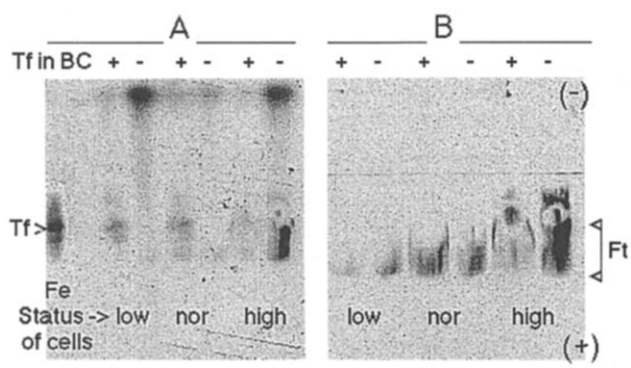
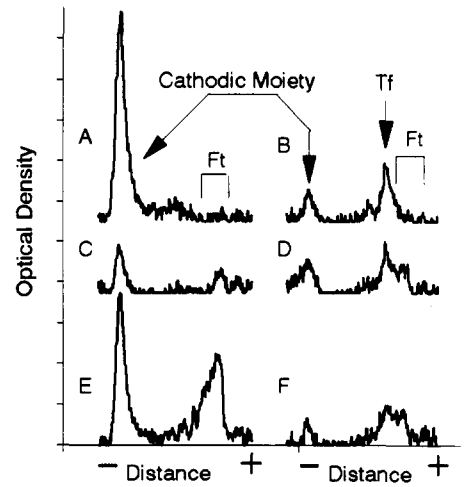


Fig. 3. Transwell membranes containing either iron-deficient (low), iron-repleted (nor), or iron-overloaded (high) cells exposed to ^{59}Fe were removed from the bicameral chambers, placed directly on isoelectric focusing polyacrylamide gels, and a drop of 2% Chaps placed on the cells prior to the initiation of focusing. Parallel lanes were either dried and exposed for autoradiography (panel A) or fixed in acetic acid-ethanol and then exposed (panel B). The lower chamber (BC = basolateral chamber) contained either no added protein (−) or 50 M (+) apo-transferrin (Tf). The position of ferritin (Ft) was determined by Western blot analysis as described in the Materials and methods while Tf was detected using ^{125}I-Tf. The cathode (−) and anode (+) are indicated. Shown is a representative experiment of three such experiments.



Figs. 4 and 5. Transwell membranes containing either iron-deficient (A and B), iron-replete (C and D), or iron-overloaded (E and F) cells exposed to ^{59}Fe were removed from the bicameral chambers, placed directly on isoelectric focusing polyacrylamide gels, and a drop of 2% Chaps placed on the cells prior to the initiation of focusing. Parallel lanes were either dried and exposed for autoradiography (Fig. 4) or fixed in acetic acid-ethanol and then exposed (Fig. 5) prior to densitometric analysis. The lower chamber contained either no added protein (densitometer traces A, C and E) or 50 M Tf (traces B, D and F). The position of ferritin (Ft) was determined by Western blot analysis as described in the Materials and methods while Tf was detected using ^{125}I-Tf. The cathode (−) and anode (+) are indicated. Shown is a representative experiment of three such experiments.

were fixed in acid-EtOH prior to exposure. Fixation removed ^{59}Fe from all but ferritin and allowed the longer development times necessary to detect ^{59}Fe in ferritin in the iron-deficient cells and to distinguish radioactivity from ^{59}Fe in Tf and ferritin in the iron-replete and iron-overloaded cells. In similar experiments in which the Caco-2 cells were incubated with a 100-fold excess of ^{56}Fe ascorbate prior to analysis by isoelectric

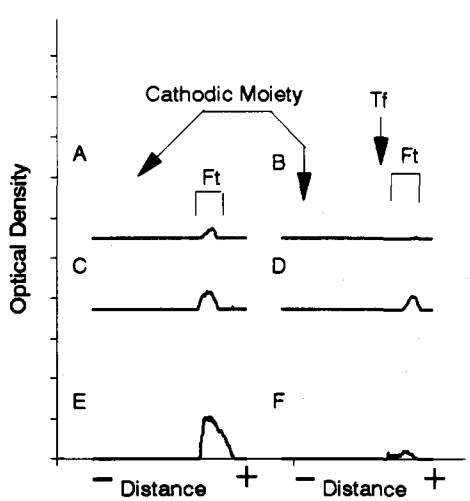


Fig. 5.

Table 2
Relative distribution of intracellular iron determined by isoelectric focusing

^{59}Fe moiety	Iron status of cells					
	Fe deficient		Normal Fe		Fe overload	
Addition:	Tf	–	Tf	–	Tf	–
Cathodic	28%	99.5%	35%	64%	10%	46%
Transferrin	71%	0 %	46%	0%	56%	0%
Ferritin	1%	0.5%	19%	36%	34%	54%

The peaks in the densitometric scans of the autoradiographs from Figs. 4 and 5 corresponding to ^{59}Fe in the cathodic moiety, transferrin, and ferritin were quantitated and expressed as the percentage of total radioactivity.

focusing no radioactivity could be detected for the same exposure times used for the gels in Fig. 3A. The autoradiograms were subjected to densitometric analysis as shown in Figs. 4 and 5 and were subsequently quantified, as shown in Table 2. In the absence of any Tf addition to the basal chamber ^{59}Fe radioactivity was detected primarily with a very basic p*I* in the FeD and FeN cells. With the longer development time used in the fixed gels (Fig. 5) ^{59}Fe was detected in ferritin in the FeD cells. The ratio of ^{59}Fe in the cathodic moiety (from Fig. 4, panel A) to ^{59}Fe in ferritin (Fig. 5, panel A) was about 200:1 (cathodic moiety/ferritin). Similar analysis in the FeN cells demonstrated a ratio of about 2:1 in the FeN cells, and 1:1 in the FeH cells. Both by Western blot analysis and by densitometric tracings of the autoradiographs multiple isoferritins could be detected (data not shown); the isoferritins in the FeD cells were more acidic than in the FeH cells.

The addition of apo-Tf to the basal chamber altered the isoelectric focusing patterns in two ways. First, ^{59}Fe-Tf was detected despite extensive washing of cells prior to isoelectric focusing. In the absence of added apo-Tf Western blot analysis could not detect any Tf in the lysates. Secondly, in the presence of basal chamber apo-Tf, the ^{59}Fe in ferritin and in the basic moiety was decreased.

4. Discussion

The Caco-2 cell line is a useful model for studying intestinal iron absorption [7,8,16] allowing in prior studies delineation of various aspects of the uptake and transfer phases of intestinal iron transport. In the present studies it has been possible to demonstrate that (1) the transfer phase is facilitated by the presence either of apo-Tf or BSA in the basal chamber, (2) that apo-Tf on the basal surface of the cell regulates the flux of ^{59}Fe across the apical surface into the cell, and (3) that basal chamber apo-Tf alters the intracellular distribution of ^{59}Fe.

The form of iron that is released from enterocytes has been enigmatic with suggestions that Tf may [9] or may not [15,21,22] act as a carrier of iron from mucosal cells to the portal blood with iron being released in the later case in a low molecular weight form. Recent experiments with the hypotransferrenemic mouse [14,15] suggest that Tf is not required for iron absorption. However, in these mice a plasma level of 0.5–0.9 μM Tf [15] was detected and in preliminary studies of ours the stimulatory effects of Tf occurs at levels as low as 0.03 μM (data not shown), at least 15–30 fold lower than the plasma Tf concentration of hypotransferrinemic mice. Release of iron from isolated enterocytes labeled with ^{59}Fe [22] supports a constitutive release of non-transferrin iron that in the presence of bicarbonate is rapidly transferred to transferrin. These later findings are supported by our observation of non-transferrin ^{59}Fe moieties on HPLC analysis of basal chamber contents. It has not yet been possible to identify either the high or low molecular weight forms released in the absence of Tf in the basal chamber. While the higher molecular weight moiety may represent a polymerized form of $Fe(OH)_3$, the lower molecular weight moiety may represent either the entity that transfers iron to transferrin [22] or the low molecular weight form that passes through intestinal capillary walls [21]. The absence of both of these moieties in the presence of either Tf or BSA suggests that released iron is quickly bound to the proteins or that the proteins bind to the basolateral surface and bind iron prior to the release of iron into the medium.

The later consideration is supported by the demonstration of transferrin receptors on the basolateral surface of enterocytes [23–25] and the endocytosis of these receptors in Caco-2 cells [26]. Our data suggest that Tf may interact with the Caco-2 cells to acquire transported ^{59}Fe. First, the rate of release of ^{59}Fe from the cells is accelerated in the presence of Tf, an observation that would be unexpected if Tf were acquiring iron from an already released intermediate. That is, while apo-Tf could shift the equilibrium of Fe to an extracellular compartment, the rate of release should be unaffected unless Tf interacted with the release compartment. Secondly, the unexpected observation that apo-Tf effects uptake of ^{59}Fe into the cells also suggests that Tf interacts with the basolateral surface of the Caco-2 cells. Without an interaction of Tf with the cells it is difficult to envision a mechanism by which the presence of Tf could decrease total cellular ^{59}Fe uptake but increase the observed efficiency of transport. As a similar effect was not seen with BSA it is possible that BSA, in contrast to Tf, does acquire ^{59}Fe only after the ^{59}Fe is released from the cells. The Tf-cell interaction could either signal the apical surface to decrease Fe uptake or involve the binding of an intracellular Fe carrier which would then be unavail-

able to return to the apical surface to acquire more Fe. Finally, the observation that NTA, a strong metal chelator, had no effect on ^{59}Fe transport again suggests that the effect of Tf is not merely to shift an equilibrium but requires, instead, the interaction of Tf with the cell to increase the rate of ^{59}Fe release. The mechanism of Fe release from the cells is still obscure but the initial results with Brefeldin A suggest the involvement of exocytosis of an Fe containing compartment.

The finding of ^{59}Fe-Tf within the cell lysate suggests that Tf gains access to the cell by endocytosis [26]. As the TfR is regulated by the iron status of cells, the FeD cells would be able to acquire more Tf than the FeN and FeH cells. Tf interacts with the TfR, gaining access to the cell through endocytosis with subsequent delivery of Fe to the endosome for acquisition by Tf in a reverse of the process described in other cells. This scenario would require that the intestinal TfR be able to bind apo-Tf. The affinity of TfR for apo-Tf is usually considered to be too low to allow significant interaction. Further, given the high affinity of Tf for Fe it is likely that the added apoTf contains some iron. If ferric Tf were bound a mechanism would have to exist to prevent exchange of cellular Fe with Fe from Tf which could limit the net transfer of Fe to the basal chamber. In unpublished studies we have observed that the rate of ^{59}Fe uptake from ^{59}Fe-Tf in the basal chamber is less than 1% the rate of transport ^{59}Fe from the apical to the basal chamber. Additionally, as Tf has no affinity for Fe(II), it is requisite that the ^{59}Fe(II) offered on the apical surface must be oxidized to Fe(III) at some point prior to binding to Tf.

Basolateral apo-Tf also markedly affected the intracellular distribution of ^{59}Fe. As the cells were analyzed by direct placement on the polyacrylamide gel, analysis of all cell-associated ^{59}Fe was assured. In the absence of Tf, ^{59}Fe was found in two compartments, one with a very basic p*I* and the other with the p*I* of ferritin. The intracellular distribution of iron in other cells includes a low molecular weight pool as well as ferritin and other ferriproteins which may be distributed amongst several organelles. In enterocytes iron binding proteins have been described both in the apical membrane [25] and in the cytosol [4]. While the p*I* of these entities is not known, we have previously shown that a plasma membrane iron binding protein from reticuloctyes has a basic p*I* [28]. However, under iron-deficient conditions with more Fe transported to the basal chamber, the ^{59}Fe at the basic p*I* appears increased. This observation suggests that the basic moiety may be an intermediate for the delivery of Fe to the basal surface. Previous studies using a gut sack model have shown that the ratio of intracellular Tf to Ft in gut mucosa correlates with iron absorption although there was not a direct relation of either of these proteins alone to iron absorption [9]. Our studies confirm that the relative intracellular distribution of ferritin and Tf are important in determining the transport of ^{59}Fe: In the FeD cells under conditions of minimal cell ferritin (cf., Table 1 in Ref. [8]) and in the presence of intracellular Tf, ^{59}Fe transport is greater than in the FeN or FeH cells with increased amounts of ferritin even in the presence of intracellular Tf. The studies with the Caco-2 cells have the advantage over the gut sac model as in the gut sac it is not possible to distinguish Ft and Tf in cells of the lamina propria versus the epithelium; hence, the direct effect of one protein or the other might be obscured.

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