

**Iron uptake by human upper small intestine microvillous membrane vesicles. Indication for a facilitated transport mechanism mediated by a membrane iron-binding protein.**

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**Research Article**

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# Iron Uptake by Human Upper Small Intestine Microvillous Membrane Vesicles

## Indication for a Facilitated Transport Mechanism Mediated by a Membrane Iron-binding Protein

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### Abstract

To investigate the hypothesis that iron absorption in man involves a carrier-mediated cellular uptake mechanism, influx velocity ( $V_o$ ) of  $^{59}\text{Fe}^{3+}$  by isolated human microvillous membrane (MVM) vesicles of the upper small intestine was examined.  $V_o$  revealed saturation kinetics ( $K_m = 315 \text{ nM}$ ;  $V_{\max} = 361 \text{ pmol Fe}^{3+} \times \text{min}^{-1} \times \text{mg protein}^{-1}$ ) was temperature dependent and inhibited by pronase pretreatment of MVM. In the presence of an inwardly directed  $\text{Na}^+$ -gradient a typical overshoot phenomenon with maximal uptake at 30–40 s was observed. The suggestion of an active, carrier-mediated uptake mechanism for iron was pursued by isolation of a 160-kD iron-binding protein from solubilized human MVM proteins.

This glycoprotein was assembled as a trimer composed of 54-kD monomers. A monospecific antibody against the 54-kD subunit inhibited vesicular influx of  $\text{Fe}^{3+}$  into MVM by > 50%. Immunofluorescence and immunoblot analysis confirmed the localization of the protein in brush border plasma membranes. It was detectable in human intestinal mucosa and liver, but not in esophagus. These data indicate that the translocation of  $\text{Fe}^{3+}$  across human MVM represents a facilitated transport mechanism which is, at least in part, mediated by a membrane iron-binding protein. (*J. Clin. Invest.* 1990. 86:2145–2153.)  
Key words: iron absorption • microvillous membranes • carrier-mediated transport • hemochromatosis • iron metabolism

### Introduction

Although the regulation of iron absorption is essential in the homeostasis of body iron levels, the molecular mechanism of iron uptake into mucosal cells is poorly understood. In recent studies from our laboratory, evidence for a carrier-mediated transport system in rat duodenal brush border plasma membranes was obtained (1). It was shown that uptake of  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$  into microvillous membrane (MVM)<sup>1</sup> vesicles revealed a

saturable and temperature-dependent influx component. Moreover, a 52-kD membrane iron-binding protein was isolated from these membranes (1). These observations were in accordance with the early hypothesis of Manis and Schachter who proposed that iron, in physiological amounts, is absorbed by an active transport mechanism consisting of an initial mucosal uptake step followed by intracellular transfer to the basolateral plasma membrane and release into the portal venous blood (2). Subsequently a number of studies focused on the initial brush border permeation step, performing uptake experiments with isolated MVM vesicles of various animal species (2–11). In most of these studies a specific interaction of iron with microvillous membranes involving a facilitated transmembrane translocation mechanism was postulated. Simpson and Peters suggested that one uptake component may be promoted by free fatty acids (12, 13) and a second more important pathway for entry of iron into mucosal cells may involve a membrane potential sensitive transport mechanism (14).

Although the kinetics of iron absorption was intensively studied in various animal species, little is known about this uptake process in humans. However, this is of particular importance in medicine since primary hemochromatosis, a common genetic iron-overload disease, was shown to be associated with increased iron absorption (15). Therefore, it is not unlikely that a defect of the molecular mechanism of iron absorption may be responsible for the iron accumulation in these patients. To address this interesting hypothesis, it is essential to determine whether under physiological conditions the translocation of iron across human duodenal microvillous membranes represents a carrier-mediated uptake process, and to identify the responsible iron-transporting membrane protein.

### Methods

**Preparation of human MVM.** MVM-enriched fractions were prepared from autopsy or resection material of the human upper small intestine. Preferably, freshly obtained duodenum or jejunum from multiorgan donors was used after given consent of the relatives. The tissue was kept on ice and the lumen intensively rinsed with saline (4°C). The lumen was opened by a longitudinal section, adherent mucus was carefully removed with tissues, and the mucosa was scraped off gently with a glass slide. MVM were isolated by an established  $\text{Ca}^{2+}/\text{Mg}^{2+}$  precipitation procedure (1, 16). The final MVM pellets were resuspended in 2 ml of 100 mM D-mannitol, 100 mM NaCl, 40 mM HEPES/Tris (pH 7.4). MVM preparations were stored at  $-70^\circ\text{C}$  until use.

**Characterization of MVM vesicles.** MVM vesicle preparations were characterized by electron microscopy and enzymatic marker determination as previously described in detail from our laboratory (17) using sucrose as marker for MVM (18). Membrane preparations were further analyzed by immunoblot analysis (see below) against antibodies to human (h) cyclo-keratin (Boehringer Mannheim GmbH, Mannheim,

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1. Abbreviations used in this paper: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MVM, microvillous membrane; NTA, nitrilotriacetic acid;  $V_o$ , influx velocity.

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FRG), h-transferrin (Sigma Chemical Co., München, FRG), horse spleen ferritin (Sigma), h-albumin (Dakopatts, Hamburg, FRG), and whole human serum (Sigma) to detect cytoplasmatic and serum protein contamination. Membrane protein concentration was determined according to the method of Bradford et al. (19) employing a protein-assay kit (Bio-Rad Laboratories, München, FRG) with ovalbumin (Sigma) as standard.

**Uptake studies.** All incubations were performed at least in quadruplicate with one batch of pooled MVM vesicle preparations frozen at  $-70^{\circ}\text{C}$ . After thawing at  $4^{\circ}\text{C}$  MVM vesicles were diluted to a protein concentration of 1 mg/ml with 100 mM D-mannitol, 100 mM NaCl, 40 mM Hepes/Tris (pH 7.4) (standard incubation buffer), vesiculated by passage (five times) through a 27-gauge needle, and placed on ice. Storage of vesicles at  $-70^{\circ}\text{C}$  for up to 2 wk resulted in no loss of transport activity compared to freshly prepared vesicles. The absolute amount of iron uptake varied by up to a factor of two among different batches of MVM preparations. Therefore, results of quadruplicate experiments obtained in representative batches of MVM preparations are presented. However, all experiments were repeated with at least three separate lots of membrane preparations with qualitative highly reproducible results.

For iron uptake experiments  $^{59}\text{FeCl}_3$  (110–740 MBq  $\times$  mg  $\text{Fe}^{-1}$ ; 99% radio-nuclidic purity; Amersham Corp., Braunschweig, FRG) was used. In previous studies from our laboratory with isolated rat MVM vesicles it was shown that  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  reveal the same specific uptake kinetics (1). This is in agreement with observations of other groups (20, 21) and indicates that the soluble monomeric concentrations of iron and not its valency state is the main determinant for uptake. Optimal solubilization and bioavailability of iron is achieved by complexing  $\text{Fe}^{3+}$  to nitrilotriacetic acid (NTA) (22, 23) which itself is not absorbed by intestinal mucosal cells (24). Therefore, for our experiments the  $^{59}\text{Fe}$ -containing substrate solution was prepared from stock solutions consisting of 15.75  $\mu\text{M}$   $^{59}\text{FeCl}_3$  in 100 mM HCl, 63  $\mu\text{M}$  NTA, and 20 mM Hepes/Tris (pH Hepes/Tris 6.0). After titration to neutral by dropwise addition of 100 mM NaOH, this stock solution was diluted to the desired  $\text{Fe}^{3+}$  concentration with standard incubation buffer. 5 min before the start of the incubation experiments all solutions were warmed to  $37^{\circ}\text{C}$ . The uptake reaction was initiated by addition of 150  $\mu\text{l}$  of the  $\text{Fe}^{3+}$ :NTA solution to 50  $\mu\text{l}$  of the MVM preparation (50  $\mu\text{g}$  protein). Incubations were carried out in Eppendorf cups at  $37^{\circ}\text{C}$  in an Eppendorf thermostat. The reaction was terminated at desired times by addition of 2 ml ice-cold incubation buffer. 2 ml of this mixture was pipetted onto the center of a nitrocellulose membrane filter (Schleicher & Schuell, Inc., Dassel, FRG; pore size 0.2  $\mu\text{m}$ , washed, and prewetted with incubation buffer) under 50-mmHg vacuum pressure using a Bio-Rad filtration apparatus. Filters were then washed with 20 ml ice-cold 0.15 M NaCl/0.02 M  $\text{Na}^+$ -phosphate, pH 7.4 (PBS). Radioactivity remaining on filters was gamma-counted for determination of the amount of  $^{59}\text{Fe}$  associated with MVM vesicles. Nonspecific association of radioactivity to filters was determined in each experiment by adding the 2-ml ice-cold incubation buffer to 200  $\mu\text{l}$  incubation mixture without MVM vesicles. This blank constituted  $< 0.5\%$  of the incubated radioactivity and was subtracted from all determinations.

Studies of  $^{59}\text{Fe}^{3+}$  uptake into MVM vesicles included the following information: effect of various  $\text{Fe}^{3+}$ :NTA molar ratios on uptake; time course of uptake at  $37^{\circ}\text{C}$ ; temperature dependency of  $\text{Fe}^{3+}$  influx into MVM vesicles; and influx rate as a function of the  $\text{Fe}^{3+}$  concentration in the medium.

To determine the effect of pronase pretreatment on vesicular influx of  $\text{Fe}^{3+}$ , MVM vesicle aliquots (50  $\mu\text{g}$  protein) were incubated with 10  $\mu\text{g}$  pronase (6 U/mg; Sigma) in 100  $\mu\text{l}$  standard incubation buffer for 30 min at  $37^{\circ}\text{C}$ , before  $^{59}\text{Fe}^{3+}$ :NTA was added as described. Controls were incubated under identical conditions in the absence of pronase.

For influx studies in presence of an inwardly directed  $\text{Na}^+$ - or  $\text{K}^+$ -gradient, MVM vesicles were prepared in 300 mM D-mannitol, 40 mM Hepes/Tris (pH 7.4) as intravesicular medium. These vesicles were then incubated with  $^{59}\text{Fe}^{3+}$ :NTA in a medium containing 100 mM NaCl or KCl in 100 mM D-mannitol, 40 mM Hepes/Tris (pH

7.4). Vesicular uptake of 0.1 mM D-[1- $^3\text{H}$ ]glucose (555 MBq/mmol; New England Nuclear, Dreieich, FRG) was examined as described before (25), employing an identical incubation buffer as for the iron uptake experiments. To evaluate the effect of medium osmolarity on D-glucose uptake, the incubation buffer was supplemented with increasing concentrations of 0–500 mM Hepes/NaOH; pH 7.4. Moreover, vesicular influx of D-glucose was examined in presence of inwardly directed 100 mM NaCl or KCl gradients.

**Isolation of the human MVM iron-binding protein by an iron chelate gel affinity chromatography column.** To 5 g epoxy-activated sepharose 6B (Pharmacia-LKB, Freiburg, FRG), swollen and washed in  $\text{H}_2\text{O}$ , 6 g iminodiacetic acid in 30 ml 2 M  $\text{Na}_2\text{CO}_3$  was added (26). Coupling was allowed to proceed at  $65^{\circ}\text{C}$  for 24 h. After packing the  $\text{H}_2\text{O}$ -washed gel into a column, an  $\text{FeCl}_3$  solution (1  $\text{g} \times 1^{-1}$  in  $\text{H}_2\text{O}$ ) was passed through the column at a flow rate of 20 ml  $\times$  h $^{-1}$ . After two thirds of the column were loaded with iron, the gel matrix was washed and equilibrated with 0.1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Sigma) in 50 mM NaCl, 20 mM Hepes/Tris (pH 7.0). Aliquots of 400 mg duodenal/jejunal MVM proteins were solubilized with 2% CHAPS in 1 mM PMSF (phenylmethylsulfonyl fluoride), 50 mM NaCl, 20 mM Hepes/Tris (pH 7.0) in presence of 10 mM ascorbic acid to prevent lipid peroxidation. After incubation with CHAPS for 1 h at  $4^{\circ}\text{C}$ , the membrane suspension was centrifuged at 150,000 g for 1 h to remove remaining membrane structures. The clear supernatant was dialyzed against 0.1% CHAPS, 0.1 mM PMSF in 50 mM NaCl, 20 mM Hepes/Tris (pH 7.0). By this procedure 40–50% of all membrane proteins could be solubilized which were subsequently applied at a concentration of 2 mg/ml to the iron chelate affinity-chromatography column with a flow rate of 20 ml  $\times$  h $^{-1}$ . Thereafter, the column was washed with 0.1% CHAPS in 50 mM NaCl, 20 mM Hepes/Tris (pH 7.0) until no further protein (OD 280 nm) appeared in the eluate. The column was eluted with 0.1% CHAPS in 50 mM NaCl, 20 mM Hepes/Tris spanning a continuous pH gradient of pH 7 (adjusted with Tris) down to pH 3 (adjusted with HCl). The resulting protein-containing eluate was neutralized to pH 7.0 and concentrated to 1–5 ml by ultrafiltration (Diaflow membranes PM-10; Amicon Corp., Witten, FRG).

**Characterization of the MVM iron-binding protein.** The protein composition of the column eluate was analysed by SDS-PAGE under reducing (presence of 5%  $\beta$ -mercaptoethanol) and nonreducing conditions (27). For protein detection the silver stain kit from Amersham and for staining of carbohydrate components a glycan detection kit from Boehringer were employed. Isoelectric focusing was performed as described by the manufacturer (application note 250; Pharmacia-LKB).

**Cochromatography studies.** Aliquots of the iron MVM-binding protein as well as of human transferrin and serum albumin (both from Sigma) as positive and negative controls, respectively, were incubated with tracer amounts of  $^{59}\text{Fe}^{3+}$ :NTA (1:4) in 0.1% CHAPS/0.05% SDS in 20 mM Hepes/Tris (pH 7.0) for 30 min at room temperature. Samples containing 10  $\mu\text{g}$  protein in a total vol of 200  $\mu\text{l}$  were subjected to gel filtration over an UltraPac TSK G3000 SW HPLC column (Pharmacia-LKB) equilibrated with 50 mM Na-phosphate (pH 6.8). Elution was carried out with the same buffer at a flow rate of 0.5 ml  $\times$  min $^{-1}$ . Eluted fractions were monitored for protein (OD 214 nm) and radioactivity.

**Preparation of an antibody to the MVM iron-binding protein.** After SDS-PAGE the 54-kD iron-binding protein was cut out of the gel and electroeluted by a standard procedure according to the instructions of the manufacturer (model 422 electroeluter; Bio-Rad). Then outbred New Zealand white rabbits were intradermally injected with twenty 25- $\mu\text{g}$  doses of the purified 54-kD subunit of the MVM iron-binding protein in complete Freund's adjuvant. Animals were boosted by intradermal and intramuscular injections with 200  $\mu\text{g}$  of the antigen in complete Freund's adjuvant after 6 and 10 wk, and bled 7 and 9 d later (28). The presence of antibody was monitored by immunoblot analysis with total MVM proteins, separated by SDS-PAGE, and blotted onto nitrocellulose sheets (Schleicher & Schuell) (29). The antiserum to the

MVM iron-binding protein or the preimmune serum (control) were added, both in dilutions of 1:200 with PBS. A peroxidase-coupled swine anti-rabbit Ig (Dakopatts) (1:200 diluted in PBS) served as second antibody. For staining 120 mg 4-chloro-1-naphthol (Sigma) and 150  $\mu$ l 30% H<sub>2</sub>O<sub>2</sub> (vol/vol) in 200 ml 20% (vol/vol) methanol/PBS were used.

**Antibody inhibition studies.** To examine the effect of the antibody to the membrane iron-binding protein on the interaction of iron with brush border plasma membranes, uptake studies with MVM vesicles were performed. First the total IgG fraction of the antiserum and preimmune serum were prepared over a Sepharose-protein A column (Pharmacia-LKB) (30). Then MVM vesicles (50  $\mu$ g protein/50  $\mu$ l 100 mM D-mannitol, 100 mM NaCl, 40 mM Hepes/Tris; pH 7.4) were incubated with 100  $\mu$ g of either IgG fraction in 50  $\mu$ l of the same buffer at room temperature for 30 min. After warming to 37°C, <sup>59</sup>Fe<sup>3+</sup>:NTA (1:4) complexes were added and uptake was determined as described above. As control, initial uptake of D-[<sup>3</sup>H]glucose was measured in presence of either antibody under Na<sup>+</sup>-gradient conditions (Na<sub>out</sub><sup>+</sup> > Na<sub>in</sub><sup>+</sup>) as described above.

**Immunofluorescence studies.** Frozen 5- $\mu$ m sections of human duodenum were air-dried and then incubated for 30 min at room temperature with 1:200 dilutions of rabbit antibody against the 54-kD iron-binding MVM protein. Sections were then washed with PBS for 30 min and incubated with fluorescein isothiocyanate-conjugated swine anti-rabbit antiserum (Dako Corp., Copenhagen, Denmark) diluted 1:50 in PBS. After thorough washing with PBS for 60–90 min, sections were mounted with aquamount (Shandon Ltd., Cheshire, UK). Controls consisted of incubations with preimmune serum also diluted 1:200 in PBS. Sections were examined with a fluorescence microscope (Carl Zeiss, Inc., Oberkochen, FRG) using the filter combinations PB485, FT510, LP520.

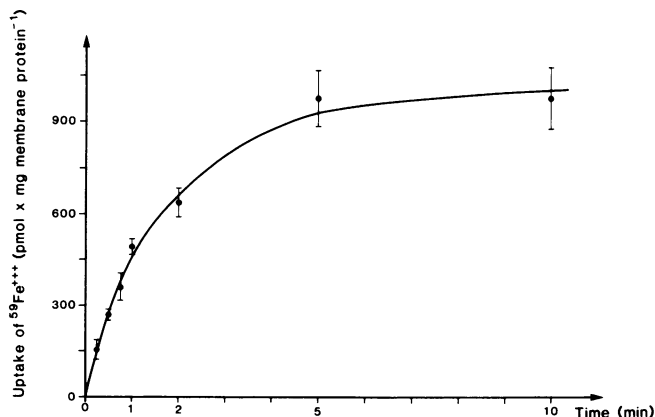
**Statistical analysis.** Results are given as means $\pm$ SD. The *t* test was used to test for significant differences among means (31). *P* values < 0.05 were considered significant. The kinetics of Fe<sup>3+</sup> uptake rates as a function of the iron concentration in the medium was determined by fitting the weighted uptake data by computerized least-square regression as described (32). All reagents were of analytical grade and doubly distilled, deionized water was used in all experiments. Glassware was acid washed.

## Results

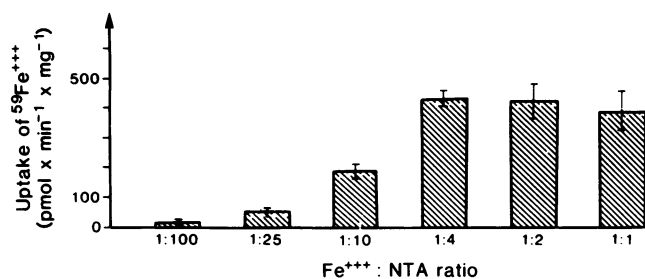
**Characterization of human MVM fractions.** In numerous randomly analyzed electron micrographs of the MVM fractions employed, only one kind of mostly vesiculated membranes was seen. The pictures resembled those of rat MVM preparations which were previously prepared in our laboratory (17). Some of the vesicles were filled with electron-dense material which presumably originated from the core of the microvilli. Contaminating membranes from organelles other than brush borders could not be detected. Determination of sucrase activity as specific marker enzyme for MVM revealed 1.83 $\pm$ 0.21 U/mg protein sp act in the MVM fraction as compared with 0.07 $\pm$ 0.01 sp act in the homogenate, i.e., an enrichment of 25-fold. About 40 $\pm$ 8% of the original sucrase activity in the homogenate was recovered in the final preparation. There was no significant contamination by basolateral membranes as judged by the sp act of Na<sup>+</sup>/K<sup>+</sup>-ATPase (0.34 $\pm$ 0.11  $\mu$ mol P<sub>i</sub>  $\times$  mg protein<sup>-1</sup>  $\times$  30 min in the MVM fraction vs. 0.76 $\pm$ 0.24 in the homogenate). Impurities with microsomes, lysosomes, mitochondria, or Golgi organelles were negligible as assessed by enzymatic marker determination. Cytoplasmatic and serum protein contamination were not detectable by immunoblot analysis of the MVM-enriched fraction with antibodies to human cyclo-keratin, human transferrin, albumin, ferritin, and whole human serum.

To test for resealing of the membranes as vesicles, their transport competence for the Na<sup>+</sup>-gradient-driven D-glucose uptake was determined (25). At 37°C incubation of 0.1 mM D-[<sup>3</sup>H]glucose in presence of a 100-mM NaCl gradient (extravesicular > intravesicular) revealed a rapid influx phase leading to a 5.6-fold accumulation of D-glucose after 30 s (267 $\pm$ 19 pmol  $\times$  mg protein<sup>-1</sup>) compared to the equilibrium situation after 30 min (48 $\pm$ 4 pmol  $\times$  mg protein<sup>-1</sup>). The initial intravesicular accumulation of glucose above equilibrium values in presence of an inwardly directed Na<sup>+</sup>-gradient (overshoot phenomenon) can only be observed with intact vesicles. Another criterion for the functional integrity of the vesicles relates to the variation of glucose uptake under ion equilibrium conditions in response to the osmotic modulation of the intravesicular space. After an incubation period of 30 min at 37°C equilibrium uptake of 0.1 mM D-[<sup>3</sup>H]glucose yielded 95 $\pm$ 27 pmol  $\times$  mg protein<sup>-1</sup> under isosmotic conditions. With increasing osmolarity of the medium and thus decreasing intravesicular space, equilibrium uptake decreased linearly with virtually no vesicle associated D-[<sup>3</sup>H]glucose at infinite medium osmolarity (calculated by least-square analysis of the regression line back to theoretical zero intravesicular volume). From equilibrium uptake of D-[<sup>3</sup>H]glucose a mean apparent intravesicular volume of 0.95 $\pm$ 0.27  $\mu$ l/mg protein was calculated. Marker enzyme determination and variation of glucose uptake in response to the osmotic modulation of the intravesicular space was performed before each series of experiments with one batch of pooled MVM preparations. Only intact vesicles were used for the incubation experiments.

**Uptake of <sup>59</sup>Fe<sup>3+</sup> by MVM vesicles.** To determine the time course of uptake, vesicular accumulation of Fe<sup>3+</sup> was examined over a total period of up to 30 min. For all Fe<sup>3+</sup>:NTA concentrations employed, uptake at 37°C was maximal and linear over the initial 45-s incubation period representing cellular influx (Fig. 1 illustrates a representative experiment). Thereafter, the uptake curve declined. Whether this is due to a vesicular efflux component resulting in equilibrium of influx and efflux at about 5 min or whether it represents a reduced influx rate due to the limited iron-loading capacity of the vesicles remains unclear and was not further examined in this study. Accordingly, all subsequent experiments focused on ve-



**Figure 1.** Time course of uptake. MVM vesicles (50  $\mu$ g protein) were incubated with 1.575  $\mu$ M Fe<sup>3+</sup> in the presence of a fourfold molar excess of NTA at 37°C in a total volume of 200  $\mu$ l standard incubation buffer. Values are means $\pm$ SD of four replicate experiments.



**Figure 2.** Effect of various concentrations of NTA on the initial uptake rate of Fe<sup>3+</sup>. MVM vesicles (50 μg protein) were incubated with 1.575 μM <sup>59</sup>Fe<sup>3+</sup>, complexed to various concentrations of NTA such that the molar ratio of Fe<sup>3+</sup>:NTA varied from 1:100 to 1:1. Initial uptake rates were determined from the linear slope of each individual uptake curve over the first 45 s. Values are means±SD of four replicate experiments.

sicular influx ( $V_o$ ) which was determined from the slope of the initial uptake phase over the first 45 s.

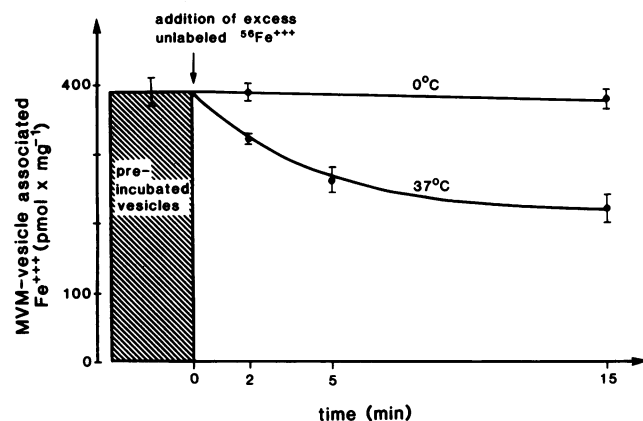
To evaluate the optimal presentation of <sup>59</sup>Fe<sup>3+</sup> and to avoid formation of ferric hydroxide polymers due to the insolubility of ferric salts in aqueous media, Fe<sup>3+</sup> solutions were prepared by adding the iron-chelating agent NTA to the substrate solution (33). For determination of the optimal concentration of NTA in the incubation system, the effect of various molar ratios of Fe<sup>3+</sup>:NTA on vesicular uptake of iron was examined. At Fe<sup>3+</sup>:NTA molar ratios of 1:1, 1:2, and 1:4, the initial uptake rate was identical at 37°C and 1.575 μM <sup>59</sup>Fe<sup>3+</sup> incubated (Fig. 2). However, unspecific binding of Fe<sup>3+</sup> to the MVM surface and filters increased with decreasing NTA concentration (1:4 < 1:2 < 1:1 Fe<sup>3+</sup>:NTA). At a molar ratio of 1:4, binding to filters was < 0.5% of total counts incubated. Further increase of the NTA concentration (1:10, 1:25, 1:100 Fe<sup>3+</sup>:NTA) resulted in a significant reduction of  $V_o$ . This is due to the high affinity of this nonmembrane-permeable chelator for iron, rendering iron not available for uptake. From these results it was concluded that a molar ratio of 1:4 (Fe<sup>3+</sup>:NTA) is optimal for membrane translocation studies.

Next the temperature dependency of influx was evaluated. It was shown that <sup>59</sup>Fe uptake was optimal at 37°C, whereas at 0°C no transport was detectable (Table I).

**Table I.** Temperature Dependency of the Initial Uptake Rate

Temperature (°C)	Initial uptake rate (pmol × min <sup>-1</sup> × mg protein <sup>-1</sup> )
0	3.9±4.93
15	41.0±7.8
25	113.0±13.0
30	160.0±14.5
37	219.0±12.5
42	199.0±13.8
47	138.0±10.6
56	63.4±17.7

MVM vesicles (50 μg protein) were incubated with 1.575 μM Fe<sup>3+</sup>:NTA (1:4) at indicated temperatures. The initial rates of uptake were determined from the linear slopes of the cumulative uptake curves over the initial 45-s incubation period. Values are means±SD of four replicate experiments.

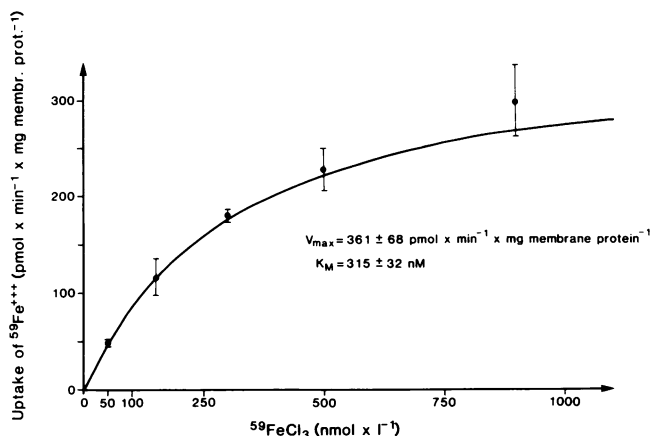


**Figure 3.** Exchange of MVM vesicle associated labeled iron by excess of unlabeled iron. MVM vesicles (50 μg protein) were preincubated with 1.575 μM <sup>59</sup>Fe<sup>3+</sup>:NTA (1:4) for 15 min at 37°C. Thereafter vesicles were chilled on ice for 5 min before a 100-fold molar excess of unlabeled <sup>56</sup>Fe<sup>3+</sup>:NTA was added and incubation was continued at 0°C, or the chase solution was added immediately after the preincubation period and incubation was continued at 37°C. Values are means±SD of four replicate experiments.

The observation that at 0°C no transmembrane movement of Fe<sup>3+</sup> occurs, was used to determine whether iron accumulation by MVM vesicles is due to intravesicular uptake or alternatively to binding at the outer surface of the plasma membrane (Fig. 3). For these experiments vesicles were first incubated with 1.575 μM <sup>59</sup>Fe<sup>3+</sup>:NTA (1:4) for 15 min at 37°C (equilibrium uptake conditions). These iron-loaded vesicles were then exposed to a 100-fold molar excess of unlabeled <sup>56</sup>Fe<sup>3+</sup>:NTA (1:4) for 60 min at 37°C or 0°C. At 37°C 41% of the vesicle-associated <sup>59</sup>Fe was released into the medium, representing an exchangeable vesicular iron pool. In contrast, at 0°C only a nonsignificant fraction of <sup>59</sup>Fe was removed from the vesicles. In the absence of transmembrane movement this small fraction represents iron attached to the outer surface of the vesicles accessible for displacement by excess unlabeled iron.

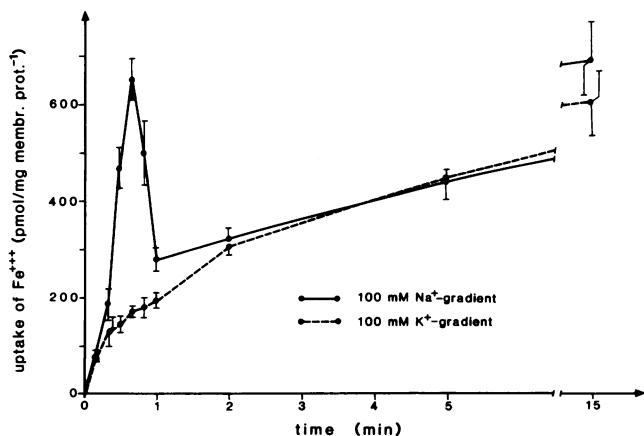
For determination of the influx kinetics of <sup>59</sup>Fe<sup>3+</sup> by MVM vesicles, the slopes of individual uptake curves over the initial 45 s ( $V_o$ ) were analyzed as a function of the iron concentration in the medium. It revealed saturation kinetics of uptake with a  $K_m$  of 315±33 nM and a  $V_{max}$  of 361±68 pmol iron × min<sup>-1</sup> × mg protein<sup>-1</sup> (Fig. 4). Pretreatment of MVM vesicles with the protease pronase lead to a significant inhibition of the iron influx velocity to 49% (160±48 pmol Fe<sup>3+</sup> × min<sup>-1</sup> × mg protein<sup>-1</sup>) of the control level obtained in native vesicles (328±46 pmol Fe<sup>3+</sup> × min<sup>-1</sup> × mg protein<sup>-1</sup>). This suggests that a protein might be involved in the iron uptake process.

Furthermore Fe<sup>3+</sup> influx in the presence of ion gradients across the plasma membrane was analyzed. All of the foregoing experiments were performed under ion-equilibrium conditions in the extra- and intravesicular space. When uptake was examined in the presence of Na<sup>+</sup>- and K<sup>+</sup>-gradients across the plasma membrane, a different pattern was observed. Inwardly directed Na<sup>+</sup>- and K<sup>+</sup>-gradients were achieved by incubation in media with 100 mM of these cations but none in the vesicular space which was isosmotically adjusted with D-mannitol. In the presence of an inwardly directed Na<sup>+</sup>-gradient a rapid initial phase of iron uptake was observed with a maximal



**Figure 4.** Initial uptake rates of  $\text{Fe}^{3+}$  by MVM vesicles as a function of the iron concentration in the medium. MVM vesicles ( $50 \mu\text{g}$  protein) were incubated with increasing concentrations of  $^{59}\text{Fe}^{3+}$ :NTA (1:4). The initial uptake rates were determined from the linear slopes of the cumulative uptake curves over the initial 45-s incubation period. Values are means  $\pm$  SD of four replicate experiments. The kinetic parameters were generated from a weighted least-squares fit of the individual data points from each experiment to a rectangular hyperbola.

accumulation of  $\text{Fe}^{3+}$  at 40 s (Fig. 5). This was followed by a significant decline of the uptake curve as iron effluxed from the vesicles for the next 20 s. Thereafter, a slowly increasing uptake curve was detectable, reflecting a gradual net accumulation of iron in the intravesicular space. This might be due to precipitation of iron at the inner site of the plasma membrane with time (8). The initial rapid transient accumulation of iron represents an overshoot phenomenon which is typical for an active, carrier-mediated uptake process stimulated by an inwardly directed  $\text{Na}^+$ -gradient. In contrast,  $V_0$  in presence of an inwardly directed  $\text{K}^+$ -gradient was slow compared to the presence of a  $\text{Na}^+$ -gradient and did not show an overshoot phenomenon. The later slow accumulating uptake phase was also



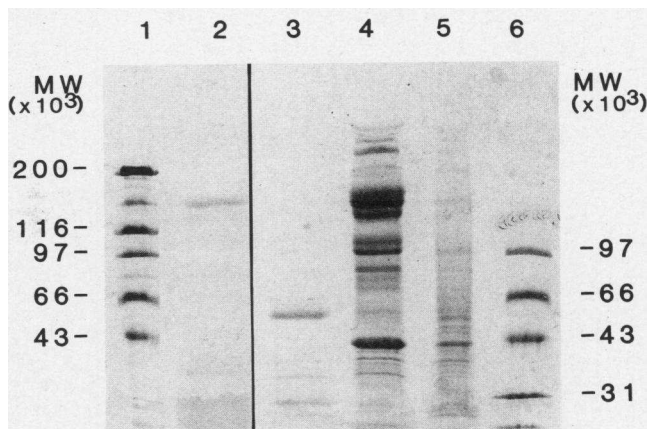
**Figure 5.** Time course of  $\text{Fe}^{3+}$  uptake by MVM vesicles in presence of an inwardly directed  $\text{Na}^+$ -vs.  $\text{K}^+$ -gradient. MVM vesicles prepared in 300 mM mannitol, 40 mM Hepes/Tris (pH 7.4) were incubated with  $1.575 \mu\text{M}$   $^{59}\text{Fe}^{3+}$ :NTA (1:4) in an extra-vesicular medium containing 100 mM mannitol, 40 mM Hepes/Tris (pH 7.4) supplemented with either 100 mM NaCl or 100 mM KCl at  $37^\circ\text{C}$ . At the times indicated uptake was terminated as described. Values are means  $\pm$  SD of four replicate experiments.

observed in presence of an inwardly directed  $\text{K}^+$ -gradient and not significantly different from the one observed in presence of the  $\text{Na}^+$ -gradient.

Saturability of uptake, temperature dependency, inhibition of influx after pretreatment of MVM vesicles with pronase, and an overshoot phenomenon in the presence of an inwardly directed  $\text{Na}^+$ -gradient indicate a facilitated transmembrane uptake process.

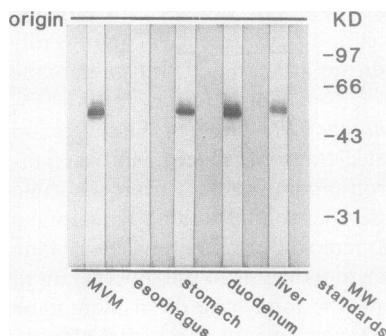
**Isolation of an iron-binding MVM protein.** The suggestion of an active carrier-mediated transport mechanism was pursued by isolation of an iron-binding protein from the same human MVM preparations. After solubilization of membrane proteins with 2% CHAPS, removal of remaining membrane structures by centrifugation, and dialysis of the supernatant to remove excess of detergent, the total solubilized membrane protein mixture was passed over an iron chelate gel affinity-chromatography column. Only proteins with high affinity for iron were retained in the column and thereafter eluted by a pH(7-3)-gradient. This procedure resulted in the isolation of a protein which migrated under nonreducing conditions on SDS-PAGE as a single 160-kD band. After reduction with 5%  $\beta$ -mercaptoethanol, the protein appeared after SDS-PAGE as an intense 54-kD band. This indicates that the native protein represents a trimer of 54-kD subunits linked by disulfide bonds (Fig. 6). Isoelectric focusing revealed a pI of 4.7 for the 160-kD complex and a pI of 6.1 for the 54-kD monomer. The protein was shown to contain carbohydrate components, employing an enzyme immunoassay for the detection of sugars in glycoconjugates. After Western blotting the protein revealed no immunologic reactivity with antibodies to human transferrin or ferritin.

In its native form this membrane protein has affinity for iron as determined by cochromatography studies on gel-permeation HPLC. However, in these experiments a presumably



**Figure 6.** Isolation of an iron-binding protein from human MVM by iron affinity chromatography. 200 mg CHAPS-solubilized human MVM proteins were applied to a sepharose 6B-iminodiacetic acid chromatography column loaded with  $\text{FeCl}_3$ . Proteins with affinity to iron were eluted as described in Methods. A Coomassie stained gradient gel (4–15%) after SDS-PAGE is illustrated. (Lanes 1 and 6) molecular weight standards; (lane 2) eluted proteins from the iron affinity-chromatography column under nonreducing conditions; (lane 3) eluted proteins from the iron affinity-chromatography column in presence of 5%  $\beta$ -mercaptoethanol; (lane 4) purified human microvillous membrane proteins; (lane 5) protein-homogenate of intestinal mucosa.





**Figure 7.** Distribution of the MVM iron-binding protein in intestinal human tissues determined by immunoblot analysis. Tissues were homogenized in 10 mM Na-phosphate/1 mM PMSF (pH 7.0). 100  $\mu$ g protein of each sample was subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and probed with the antiserum to the MVM iron-binding protein.

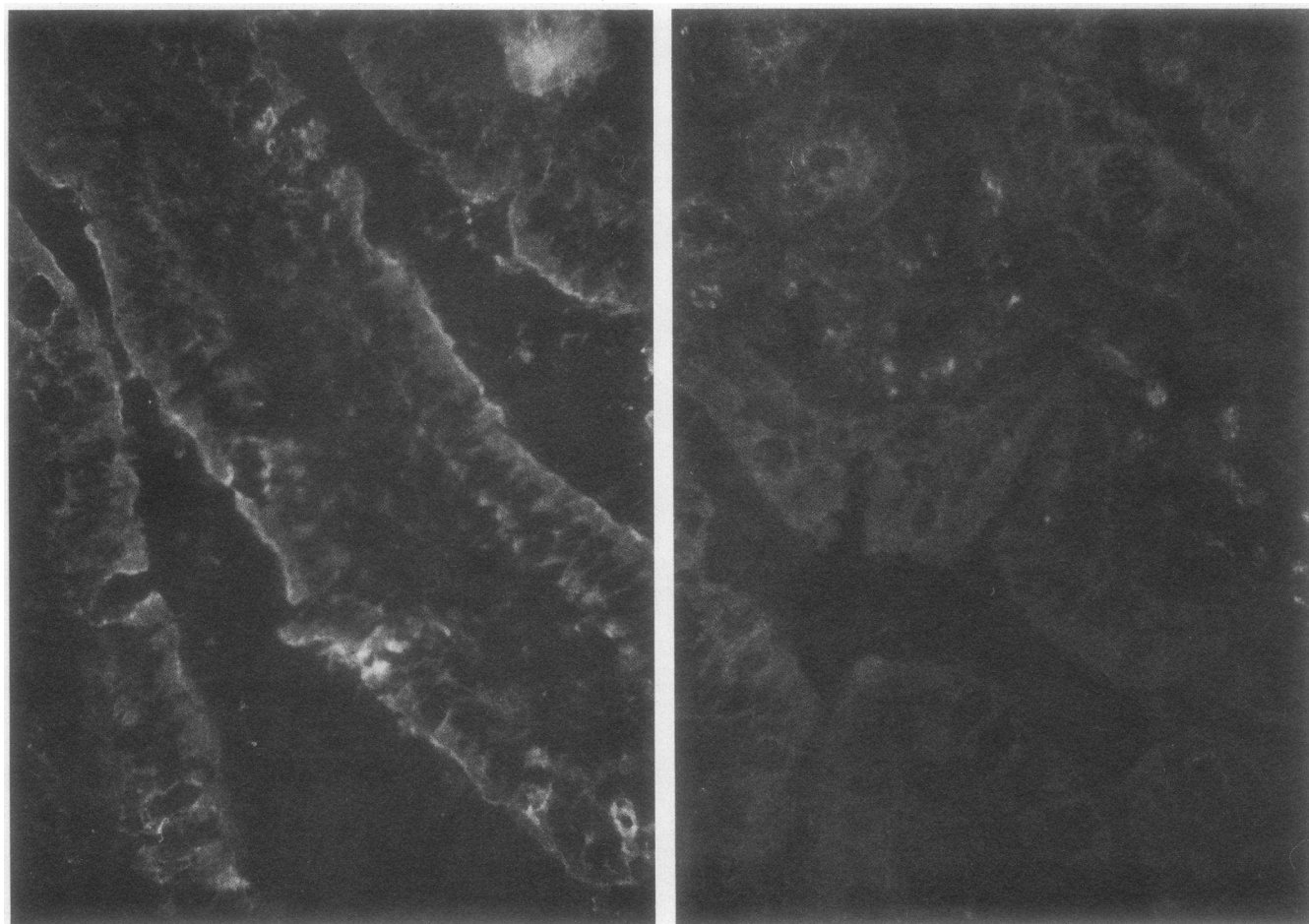
dimeric form of the 160-kD membrane iron-binding protein coeluted with  $^{59}\text{Fe}^{3+}$ . Collection of the  $^{59}\text{Fe}$ -containing protein peak and application to SDS-PAGE in the presence of 5%  $\beta$ -mercaptoethanol revealed again the single 54-kD protein band.

For further characterization, antibodies to the 54-kD monomer of this protein were raised in rabbits. Western blot analysis revealed that this antibody reacted with the authentic

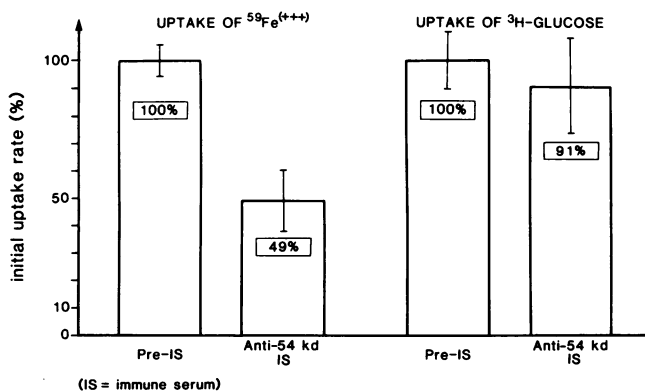
160-kD native protein as well as with the 54-kD monomer. In total MVM protein extracts and homogenates of human intestinal mucosa and human liver only reactivity with the membrane iron-binding protein was detectable (Fig. 7). It was not present in homogenates of human esophagus. The antibody did not show cross-reactivity to the 52-kD rat MVM iron-binding protein, previously isolated in our laboratory (1).

With antibodies to the 54-kD subunit of the MVM iron-binding protein immunofluorescence studies were performed. In intact human duodenal mucosa a predominant staining of the brush border plasma membrane was apparent (Fig. 8). This staining was not observed in control sections pretreated with the preimmune serum.

To evaluate the physiologic significance of the isolated MVM iron-binding protein, the effect of the monospecific antibody to this protein on transmembrane transport of  $\text{Fe}^{3+}$  was examined. Preincubation of human MVM vesicles with the IgG fraction of the antiserum to this protein inhibited iron influx significantly compared to controls pretreated with the IgG fraction of the preimmune serum (Fig. 9). As expected, the antibody did not affect equilibrium uptake of  $^{59}\text{Fe}^{3+}$  after a 15-min incubation period at 37°C (IgG antiserum:  $857 \pm 32$  pmol  $\times$  mg protein $^{-1}$ ; IgG preimmune serum:  $841 \pm 79$  pmol  $\times$  mg protein $^{-1}$ ). For comparison, initial as well as equilibrium uptake of D- $^3\text{H}$ ]glucose was not altered by the antibody.



**Figure 8.** Indirect immunofluorescence staining of the apical plasma membranes of human duodenal mucosa cells after incubation with rabbit antiserum to the MVM iron binding protein (right) or preimmune serum (left).



**Figure 9.** Effect of monospecific antibodies to the MVM iron-binding protein on  $\text{Fe}^{3+}$  and D-glucose uptake by MVM vesicles. MVM vesicles (50  $\mu\text{g}$  protein) were preincubated with 100  $\mu\text{g}$  of the IgG fractions of the antiserum to the MVM iron-binding protein or of the preimmune serum (control) for 30 min at room temperature. Thereafter, uptake studies were performed with 1.575  $\mu\text{M}$   $^{59}\text{Fe}^{3+}$ :NTA (1:4) or 0.1 mM D- $^3\text{H}$ ]glucose at 37°C. Initial rates of uptake were determined from the linear slopes of the cumulative uptake curves over the initial 45-s incubation period. In the presence of the antibody to the MVM iron-binding protein  $^{59}\text{Fe}^{3+}$  uptake was reduced to  $134 \pm 17 \text{ pmol} \times \text{mg}^{-1} \times \text{min}^{-1}$  compared with  $275 \pm 45 \text{ pmol} \times \text{mg}^{-1} \times \text{min}^{-1}$  in control incubations. Initial uptake of D- $^3\text{H}$ ]glucose in the presence of an inwardly directed  $\text{Na}^+$ -gradient was not significantly different in antibody pretreated ( $544 \pm 99 \text{ pmol} \times \text{mg}^{-1} \times \text{min}^{-1}$ ) and control preparations ( $596 \pm 65 \text{ pmol} \times \text{mg}^{-1} \times \text{min}^{-1}$ ). Illustrated are the relative differences in control (100%) and antibody-pretreated vesicles. Values represent means  $\pm$  SD of four replicate experiments.

## Discussion

In this study it was shown that uptake of iron by human MVM vesicles reveals saturation kinetics, is temperature-sensitive with optimal uptake at 37°C, and is significantly inhibited by pronase pretreatment of the vesicles. These are criteria of a facilitated, protein-mediated membrane translocation process. Another argument for carrier-mediated transport was the observation of an overshoot phenomenon in presence of an inwardly directed  $\text{Na}^+$ -gradient. This initial transient peak of iron accumulation was followed by efflux of vesicular iron until a second slow accumulation process of vesicular iron (possibly intravesicular precipitation) was predominant. Such an overshoot phenomenon represents an active uphill transport component stimulated by  $\text{Na}^+$ . This may be compatible with an  $\text{Na}^+$ -iron cotransport system. Alternatively, the presence of an inwardly directed  $\text{Na}^+$ -gradient induces a more negative intravesicular potential (depolarization) compared with a  $\text{K}^+$ -gradient. Therefore, the observed overshoot phenomenon may also be due to a potential dependent influx process stimulated by negative intravesicular charge. This is in agreement with earlier studies also showing membrane potential sensitivity of iron uptake in intestine (14) and liver (34). Further studies to elucidate the driving forces of iron uptake by MVM vesicles are currently performed in our laboratory. Moreover, the specificity of this transport system is still being investigated. The problem of these studies is the competitive interaction of some metals with iron at the binding sites for NTA which interferes with the interpretation of uptake data by MVM vesicles.

The analysis of membrane translocation kinetics as a function of the iron concentration in the medium reveals a  $K_m$  value of 315 nM which indicates that this transport system has a high affinity for  $\text{Fe}^{3+}$ . This is in contrast to a previous study of Cox and Peters on iron uptake by human biopsy specimens (35). They reported a  $K_T$  value of 127  $\mu\text{M}$ , suggesting a significant lower affinity for iron. To explain the difference between both values, one has to consider the different experimental approaches. Compared with the complex system of biopsy specimens, in our study highly purified microvillous plasma membrane vesicles were used which allows examination of transport characteristics across a defined membrane. Moreover, for preparation of MVM vesicles we initially removed the mucus adherent to the brush border plasma membrane. This is of significance, since the mucus barrier may affect the presentation of iron to the surface plasma membrane, e.g., by absorption of iron to certain mucus proteins (36).

Delivery of iron to the mucosal cell is determined by its penetration of the mucus and the unstirred water layer close to the microvillous membrane as well as by its physicochemical state in the lumen of the upper intestine. Interaction of iron with bile salts, peptides, amino acids, and various dietary chelating agents serves to keep iron soluble in the intestinal environment, but may render it difficult for absorption. Therefore, a high affinity mucosal uptake system may be required to provide a sufficient amount of iron being absorbed from chyme. However, our data of a protein carrier-mediated absorption mechanism do not exclude the presence of other facilitated diffusion uptake processes promoted by free fatty acids, as suggested by Simpson and Peters (12, 13) or an oxygen radical-mediated uptake, as assumed by Marx et al. (37). It is conceivable that the heterogeneity of iron compounds in chyme (i.e., valency state, chelation state) may lead to different strategies for the passage of iron across the microvillous membrane.

In this study MVM vesicular uptake kinetics revealed a  $V_{\text{max}}$  value of 361  $\text{pmol iron} \times \text{min}^{-1} \times \text{mg membrane protein}^{-1}$ . Such a membrane translocation capacity for iron across the brush border plasma membrane, distributed over the large surface area of the intestinal mucosa, provides a sufficient absorption capacity for iron at physiological intestinal transit times. Based on our own observation that a duodenal segment of 100-cm length and 3-cm diameter contains 900 mg MVM proteins and assuming a mean transit time of 30 s/cm, a total absorption of 910  $\mu\text{g}$  iron would be possible under  $V_{\text{max}}$  (i.e., ideal) conditions. This is highly sufficient to provide the daily iron requirement of a normal adult (38).

A major aim of this study was the identification of the responsible iron carrier protein in human brush border plasma membranes. For this purpose the one step isolation procedure was used which was earlier applied for the isolation of the rat MVM iron-binding protein (1). This technique is based on the moiety of this protein to bind with high affinity to iron which is immobilized to a sepharose iminodiacetic acid matrix (affinity chromatography). In fact, a single 160-kD protein was identified after SDS-PAGE under nonreducing conditions. After reduction of the protein with 5%  $\beta$ -mercaptoethanol a single monomer with an apparent mol wt of 54,000 was detectable. This indicated that the native protein represents a trimer with disulfide linkage between each monomer, most likely forming a transmembrane iron-transporting system.



Cochromatography studies confirmed that the isolated protein has high affinity to  $\text{Fe}^{3+}$ . It is a glycoprotein with a pI of 4.7 which, by its physicochemical characteristics and its immunologic reactivity, is clearly distinct from other iron-binding proteins such as transferrin and ferritin, as well as from the rat MVM iron-binding protein. Whether this protein has any relationship to the 56-kD iron-binding protein recently described by Conrad et al. remains to be established (39).

As soon as a monospecific antibody to this protein became available, it was possible to determine the subcellular localization of this protein (Western blot, immunofluorescence). In duodenal mucosa it was predominantly detectable at the brush border plasma membranes but not in other cell compartments or other components of the mucosal tissue. Although a systematic analysis of the distribution of this protein throughout the organism is not completed yet, the protein could already be identified in mucosal cells of the stomach and intestine as well as in liver. It was not detectable in esophagus.

To determine the functional significance of this protein in the membrane-binding and translocation process of iron, the effect of the monospecific antibody to this protein on influx of  $\text{Fe}^{3+}$  in MVM vesicles was examined. In the presence of the antibody vesicular influx was reduced by more than 50% compared with controls pretreated with the IgG fraction of the preimmune serum. In contrast, uptake of D-[ $^3\text{H}$ ]glucose, another substrate which is known to be actively transported by MVM vesicles (25), was not affected by the antibody. Incomplete inhibition of iron influx may be due to the characteristics of the employed antibody (binding site at the carrier protein, affinity, titer) or may indicate the presence of a second, e.g., noncarrier-mediated uptake system. Nevertheless, the significant and selective inhibition of vesicular iron influx strongly suggests that the MVM iron-binding protein is of physiologic significance in the transmembrane movement of iron across the brush border plasma membrane. Therefore, it is concluded that at least a component of iron uptake by human mucosal cells represents a facilitated transport mechanism which is mediated by a 160-kD membrane iron-transporting protein.

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