Function of Integrin in Duodenal Mucosal Uptake of Iron

By Marcel E. Conrad, Jay N. Umbreit, Raymond D.A. Peterson, Elizabeth G. Moore, and Kimberly P. Harper

A mechanism for the absorption of inorganic iron in the small intestine is described in which integrins appear to play an important role in the passage of iron across microvillous membranes. Biochemical isolates from microvillous preparations of duodenum from rats dosed with radioiron showed radioactivity concentrated in integrins. The presence of integrins on mucosal surfaces of duodenal cells was confirmed by immunofluorescence microscopy using anti-integrin monoclonal antibodies. Immunoprecipitation methods were used to show that microvillous radioiron was precipitated with anti-integrin antibodies and that mobilferrin, a 56-Kd cytosol iron-binding protein, coprecipitated with integrins. We postulate from these data that the mucosal uptake of iron from the gut lumen is mediated via an integrin-mobilferrin pathway.

eer. 1993 by The American Society of Hematology.

IRON IS PRIMARILY absorbed in the proximal small intestine where the mucosa remains attuned to the body requirements for iron. For many years, ferritin was believed to be the mucosal regulator of iron absorption. It was postulated that iron-poor apoferritin was present in iron deficient mucosa to encourage absorption. Immunologic studies refuted this hypothesis and showed that absorptive cells of iron-deficient animals contained little ferritin because iron was required to induce ferritin synthesis. Currently, it is believed that ferritin is primarily a storage protein that protects cells from oxidative damage. A more recent viewpoint was that mucosal transferrin acts as a shuttle protein by being secreted into the intestinal lumen to bind iron and enter the mucosal cell to facilitate absorption. This hypothesis was challenged because humans with transferrinemia become iron-overloaded rather than iron-deficient, the gene for transferrin is not expressed in duodenal mucosal cells, and neither transferrin nor transferrin receptors can be identified in the apical portion of intestinal mucosal cells where iron binding substances are identified. This led to a search for proteins that mediated the uptake of inorganic iron by the intestinal mucosal cells. Mucins were shown to bind soluble acidified iron and keep it soluble and available for absorption in the small intestine. A 56-Kd cytosol iron binding protein was identified in apical portions of intestinal cells and was called mobilferrin. A water-insoluble, detergent-soluble membrane radioiron-labeled protein was identified in microvillus preparations of rat duodenal mucosa. This report describes purification of the membrane protein and shows it belongs to the integrin superfamily of transmembrane proteins. We postulate that it explains the mucosal uptake of iron from the lumen of the gut.

MATERIALS AND METHODS

Fifty Wistar rats were obtained and kept on an iron-deficient casein diet (iron-deficient test diet with vitamin mix AOAC for rats; Teklad, Madison, WI) for 2 weeks. Under pentobarbital anesthesia (4 mg/100 g), a celiotomy was performed and duodenal loops were isolated with umbilical ligatures. Radioiron (10 μCi, 10 μmol/L FeCl₃, pH 2.0) was injected into each duodenal loop (pylorus to ligament of Treitz). Ten minutes later, the rats were killed by infusion with cold 0.15 mol/L NaCl into the left ventricle after transection of the hepatic veins. The gut was excised, opened along the greater curvature, and washed in two changes of ice-cold 0.15 mol/L NaCl, 0.15 mol/L NaCl into the left ventricle after transection of the hepatic Treitz). Ten minutes later, the rats were killed by infusion with cold water from an Oster blender (model 5028; Milwaukee, WI) for 2 minutes at high speed. Intestinal brush border membranes were sequentially isolated using the calcium precipitate procedure as described by Kessler et al. The final precipitate was resuspended in 15 mL 2% Triton X-100, 10 mmol/L HEPES (pH 7.4) and placed in a rotator for 16 hours at 4°C. The suspension was centrifuged at 20,000g in a Sorvall RC-SB refrigerated centrifuge (DuPont de Nemours, Irving, TX) for 30 minutes. The supernatant was subsequently treated with 0.2% protamine sulfate and recentrifuged as above. The supernatant was placed on a DE-52 ion exchange column with a 0.5 mol/L NaCl, 0.1% Triton X-100, 10 mmol/L HEPES (pH 7.4) gradient. Aliquots of the fractions were taken for radioactivity determination and protein was measured by the BCA method (Pierce, Rockford, IL). For antigenicity, 100 μL was applied to the wells of a Bio-Dot apparatus (BioRad, Melville, NY) and attached to Immobilon (Millipore, Bedford, MA) membrane under vacuum. This was incubated with a 1: 100 dilution of α₃ anti-integrin (human) monoclonal antibody (MoAb; Oncogene Science, Manhasset, NY) as the primary antibody. This was developed with a goat antimouse antibody with alkaline phosphatase according to the manufacturer’s instructions (BioRad). Bovine serum albumin and mouse sera were used as a negative control in each experiment. The relative reactivity of dot-blot was quantified with a Zenith Video Densitometer (Biomed Instruments, Fullerton, CA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of reduced and denatured samples was performed in slab gel using equipment as described by Laemmli. Certain gels were performed under incompletely dissociating conditions without heat denaturation because heating interfered with antigen-antibody reactivity. Gels were stained with Coomassie stain (BioRad), a3 Molecular mass standards for SDS-PAGE were obtained from BioRad. In certain studies, protein on SDS-PAGE was transferred to Immobilon (Millipore) by semidyde electrophoresis (BioRad) and the Western blot was developed with anti-integrin MoAbs to human integrin, using goat antimouse antibody conjugated to alkaline phosphatase (BioRad). Immunofluorescent light microscopy was performed on frozen sections of rat duodenum using similar antibodies conjugated to fluorescein.

RESULTS

Mucosal homogenates were prepared from the duodena of rats that were dosed with radioiron in vivo. Microvilli were

From the Department of Medicine, Pathology and Biochemistry, University of South Alabama, Mobile, AL.

Submitted April 7, 1992; accepted September 15, 1992.

Supported by Grant No. 2 R01 DK36112 from the National Institutes of Diabetes, Digestive and Kidney Diseases of the National Institutes of Health, Bethesda, MD.

Address reprint requests to Marcel E. Conrad, MD, University of South Alabama, USA Cancer Center, Room 414, 307 University Blvd, Mobile, AL 36688.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.

0006-4971/93/8102-002$3.00/0

Blood. Vol 81, No 2 (January 15), 1993: 517-521

517
isolated from homogenates by standardized methodology; purity was verified in ultrastructural preparation. The microvilli isolates reacted with both an MoAb against integrin and a polyclonal antibody against mobilferrin using fluorescent microscopy. Similar studies with antiferritin and antitransferrin were not reactive. A membrane-associated iron-binding protein was solubilized from purified microvilli in Triton X-100 and purified by DE-52 ion exchange chromatography. The final isolate showed a retained protein fraction with coincident protein and radioactivity. This peak reacted with anti-integrin antibody (Fig 1).

The integrins are usually considered to be components of either cell-cell or cell-matrix adhesion complexes and their association with a gut lumenal transport system in microvilli might be viewed as anomalous. However, their isolation and identification in purified preparations of microvilli and by fluorescent microscopy demonstrated their presence on the absorptive surface of duodenal mucosal cells (Fig 2).
INTEGRINS IN IRON ABSORPTION

SDS-gel electrophoresis was performed on the isolated fractions under two conditions. Incomplete dissociation was obtained by not heating the sample before electrophoresis. This showed a broad doublet of Coomassie staining protein (Fig 3). Western blots of this material showed strong reactivity with anti-α3 and anti-α5 integrin MoAbs. Complete dissociation, obtained by heating the sample at 100°C for 5 minutes, resulted in the dissociation of the bands into molecular masses of 150,000 and 90,000 kDa. Under fully dissociating conditions, the anti-integrin antibodies did not associate with their antigens. A similar loss of reactivity to anti-integrin antibodies was observed on heated materials tested using the Dot-Blot method.

Labeled, purified, membrane protein was incubated with anti-α3 and anti-α5 integrin MoAbs and then the immune complex was absorbed onto beads with either protein A or protein G. Immune complex between the radiolabeled iron binding protein and the anti-integrins were formed (Fig 4). The immune precipitation isolates on protein A and G were then heated with SDS and the eluted proteins electrophoresed on SDS gels. The bands at 150,000 and 90,000 kDa were those anticipated for integrins. The other bands were those expected for either protein A or G and the heavy and light chains of the antibodies added to the mixtures.

Fig 3. Gel electrophoresis and Western blots show that the membrane iron-binding protein belongs to the integrin superfamily of proteins. SDS-gel electrophoresis was performed under incompletely dissociating conditions without heat denaturation. SDS gels performed according to the method of Laemmli17 with 7.5% acrylamide demonstrated a broad doublet of protein staining with Coomassie blue (left). This material was transferred to Immobilon by semi-dry electroblotting and the Western blot was developed with α5 anti-integrin MoAb using goat antimouse antibody conjugated to alkaline phosphatase (right). The immunoblot showed that the iron-binding protein shared antigenic determinants with integrin. Similar results were observed using a biotinylated primary antibody against integrin. SDS-gel electrophoresis under fully denaturing conditions showed that the iron-binding protein consisted of two polypeptide chains of molecular mass 150,000 and 90,000 kDa corresponding to the α and β chains of integrin (not shown).

Fig 4. Formation of immune complexes between antibody to integrins and the membrane iron-binding protein. One hundred microliters of purified membrane protein containing 0.5 mg/mL protein was incubated with 20 μL of anti-α3 or anti-α5 integrin MoAbs. After incubation at 37°C for 2 hours, 20 μL of protein A acrylic beads (Sigma, St Louis, MO) or protein G on Sepharose beads (Pharmacia LKB Biotechnology, Piscataway, NJ) was added as a suspension. These were incubated for 2 hours and then centrifuged (Beckman Microfuge) at 12,000 rpm for 15 minutes at 4°C. The pellet was washed in 0.5 mL, phosphate-buffered saline, recentrifuged, and the radioactivity in the pellet was measured in a gamma detector. The antibody (Oncogene Science, Manhasset, NY) was used as a 1:100 dilution. Results are presented as nanograms of iron above background and showed concentration of radioiron on beads in specimens containing anti-integrins by comparison to control incubations without antibody (buffer). The protein-coated beads were boiled in SDS (10%, 100°C for 5 minutes) and the supernatant was prepared and electrophoresed on 7.5% acrylamide SDS gels as described by Laemmli.17 The gel was stained with a silver stain (BioRad). Similar reactivity with different anti-integrin antibodies is probably due to cross-reactivity because of the homology of the integrins.
Radiolabeled mobilferrin was incubated with nonradioactive purified membrane protein. Then, \( \alpha_3 \) anti-integrin antibody was added to the mixture. The immune complex was absorbed onto beads containing protein A and the beads were repeatedly washed with buffered saline. Markedly greater radioactivity was associated with the beads in mixtures containing integrin than in control specimens containing bovine serum albumin. This indicates that there was coprecipitation of mobilferrin and integrin (Fig 5).

**DISCUSSION**

Iron is delivered to most body cells by transferrin and entry into cells occurs via transferrin receptors located on the cell surface.\(^9\) Intestinal absorptive cells are different from other cells that acquire iron from their milieu because there are no identifiable transferrin receptors on the mucosal surface.\(^1\)

This led to a search for other mechanisms in intestinal cells that facilitated intestinal mucosal uptake and transport of iron.

A search was initiated to identify previously undiscovered iron-binding substances in duodenal mucosal homogenates. A water-soluble 56-kD cytosol protein was identified and characterized in homogenates from both rats and humans and was named mobilferrin.\(^13\)\(^,\)\(^14\) The cytosol protein did not explain membrane transport of iron similar to transferrin receptors in other cells, although it probably serves an important role in the intracellular metabolism of iron.

During preparation of whole duodenal homogenates, half the radioiron from an intraluminal test dose was recovered in water-insoluble precipitates. This was almost equally divided between triton-soluble and -insoluble fractions. The latter was identified as mucin and it appeared to function by keeping iron soluble within the intestinal lumen for absorption by the gut.\(^1\)

This report shows that the triton-soluble iron-binding fractions are integrins and can be immunologically identified on the mucosal surface of absorptive cells within microvilli. Integrins are members of a superfamily of transmembrane proteins and could serve as a mechanism for mucosal transport. A divalent cation-binding site for calcium is known on the \( \alpha \) chain of integrins, but this may not be the only or even the primary iron-binding site.\(^19\) The \( \alpha \) chain is also known to contain a binding site for a 60-kD protein on the cytoplasmic side of the receptor\(^21\) and calreticulin (Ross-A associated protein) binds this site.\(^22,\)\(^23\) The recent demonstration that calreticulin and mobilferrin are homologues indicates that the major cytoplasmic iron-binding protein binds to the \( \alpha \) chain of the integrin receptor.\(^24\) The integrin would therefore dock the soluble mobilferrin to a perimembrane location. Whether the integrin is itself the transmembrane transport protein or functions to localize mobilferrin adjacent to a membrane transport site is yet to be determined (Fig 6).

**REFERENCES**


---

**Fig 5.** Coprecipitation of integrin and mobilferrin using anti-\( \alpha_3 \) monoclonal integrin antibody. One hundred microliters of nonradioactive purified membrane protein and a similar amount of mobilferrin each containing about 0.5 mg/mL of protein were incubated at 37°C for 30 minutes. Subsequently, 20 \( \mu L \) of anti-\( \alpha_3 \) integrin MoAb was added and incubated for 2 hours. Protein A acrylic beads (20 \( \mu L \)) were added and placed on a rotator overnight at 4°C. The beads were recovered by centrifugation in a microfuge and washed four times in buffer containing 0.01 mol/L NaCl, 0.2% Triton X-100, 10 mmol/L HEPES, pH 7.4. The radioactivity on the beads was measured in a gamma counter. Comparison was made with control specimens in which bovine serum albumin was used rather than purified membrane protein. Similarly, anti-integrin antibodies were unable to precipitate mobilferrin in the absence of integrin using this methodology.

**Fig 6.** Postulated small intestinal mucosal uptake of iron. Dietary nonheme iron is solubilized at the acidic \( p \)H of the stomach.\(^12\) At acid \( p \)H, it combines with mucins that maintain the iron soluble and available for absorption in the more alkaline small intestine. Integrins that traverse the luminal surface of the absorptive cell may facilitate the uptake of iron into the mucosal cell in association with mobilferrin.\(^21\) Mobilferrin is a cytosol protein that is probably important in the regulation of iron absorption.\(^13\)\(^,\)\(^14\)

---

From www.bloodjournal.org by guest on May 30, 2017. For personal use only.
Function of integrin in duodenal mucosal uptake of iron

ME Conrad, JN Umbreit, RD Peterson, EG Moore and KP Harper