Microvillar iron-binding glycoproteins isolated from the rabbit small intestine

Martin W. O'DONNELL and Timothy M. COX*

Royal Postgraduate Medical School, Ducane Road, London W12 0HS, U.K.

(Received 20 July 1981/Accepted 12 October 1981)

Rabbit intestinal microvillus membranes possess high-affinity receptors for iron whose activity reflects homeostatic changes in mucosal iron transport. To isolate and characterize these membrane components, purified microvilli were radiolabelled with $^{59}\text{Fe}(\text{II})$ and solubilized in Triton X-100. ^{59}Fe in 105 000 g supernatants co-eluted with a major broad protein peak (M_r approx. 100 000) on gel-permeation chromatography and was rendered diffusible by Pronase digestion but not mild periodate degradation. Fluorescence studies with castor-bean lectin conjugates showed specific binding of this affinity probe exclusively to brush-border membranes in the intestinal epithelium. Affinity chromatography of solubilized membrane proteins showed binding to columns of immobilized lectin. Elution with D(+)-galactose released glycoprotein-bound ^{59}Fe purified up to sevenfold over initial membrane extracts. The lectin bound up to 82% of protein-bound ^{59}Fe . In contrast polyspecific antisera raised against rabbit microvilli in guinea-pigs precipitated less than 10% of solubilized radioactivity. Significantly more protein-bound ^{59}Fe in detergent extracts of microvilli purified from bled animals interacted specifically with the lectin, suggesting that membrane glycoprotein receptors are involved in the homeostatic control of intestinal iron transport.

Absorption in the mammalian small intestine requires the ordered interaction of several translocation steps across different cellular compartments within the mucosal epithelium. The prime step, uptake from the digestive lumen, involves transfer across the brush border, and discrete modalities that facilitate transport of nutrients have been localized to this membrane (see Crane, 1980). Moreover, membrane-associated binding moieties have also been identified, and are believed to function as receptors at the luminal surface of the mucosa; the vitamin D-dependent Ca^{2+} -binding protein purified from rat intestine is an example of such transport-associated membrane components (Kowarski & Schachter, 1980).

These considerations are pertinent to investigations of regulatory mechanisms in iron homeostasis, since, in the absence of an effective excretory pathway, the intestine controls iron balance uniquely by adjusting absorption to meet body requirements (McCance & Widdowson, 1937). Although the nature of the rate-limiting step controlling iron flux across the absorptive epithelium is obscure, we have recently shown that rabbit intestinal microvilli possess high-affinity receptors for iron whose activity reflects homeostatic changes in mucosal

iron influx (Cox & O'Donnell, 1981*a,b*). Adaptive changes in transport of this nutrient across the intestine may thus be accomplished by modulation of microvillus membrane receptors. Accordingly, we have endeavoured to purify and characterize these iron-binding components from solubilized preparations of microvillus membranes isolated from rabbit intestine. To distinguish related glycosylated constituents in these membranes, we have used lectin- and immuno-affinity techniques in the analysis of solubilized membrane-bound iron.

Experimental

Materials

Sephadex, CNBr-activated Sepharose, Protein A-Sepharose and protein standards for molecular-weight estimations were products of Pharmacia, Uppsala, Sweden. Acrylamide and DEAE-agarose (DEAE-Bio-Gel A) were from Bio-Rad, Richmond, CA, U.S.A. Fluorescamine {Fluram; 4-phenylspiro-[furan-2(3),1'-phthalan-3,3-dione]} was obtained from Hoffman-La Roche, Basel, Switzerland. Triton X-100 was purchased from Koch-Light laboratories, Colnbrook, Bucks., U.K. Pronase was from Calbiochem, London W.1., U.K. Fluoresceinated concanavalin A and wheat-germ lectins were obtained from Vector Laboratories, Burlingame,

* To whom correspondence and reprint requests should be sent.

CA, U.S.A.; castor-bean agglutinins were prepared from *Ricinus zanzibariensis*, as described by Nicholson & Blaustein (1972) and coupled with fluorescein isothiocyanate (Sigma Chemical Co.) by the method of Rinderknecht (1962). **(These lectins are extremely toxic and must be handled with great care, using protective materials.)** *Ricinus communis* agglutinin-120 (RCA₁₂₀) was purchased from Boehringer-Mannheim, Lewes, East Sussex, U.K. and lectins were covalently coupled to CNBr-activated Sepharose 4B (Parikh *et al.*, 1974). Aprotinin was obtained from Bayer U.K., Haywards Heath, West Sussex, U.K. and dimethylmaleic anhydride and phenylmethanesulphonyl fluoride were from Sigma Chemical Co., St. Louis, MO, U.S.A. The latter compound was dispensed from a stock solution (0.5M) dissolved in propan-2-ol. Other reagents, apart from those listed previously (Cox & O'Donnell, 1981a), and enzymes were of analytical grade, and were obtained from Sigma or BDH Chemicals, Poole, Dorset, U.K.

Preparation and solubilization of iron bound to microvillus membranes

Microvillus membranes were prepared from the proximal intestinal mucosa of freshly killed rabbits by a slight modification of the method of Schmitz *et al.* (1973). The animals were treated as previously described (Cox & O'Donnell, 1980) and the intestinal homogenates were prepared in the presence of 1mM-phenylmethanesulphonyl fluoride and 100 Kunitz units of aprotinin/ml to inhibit proteolysis. Protein content and sucrase (EC 3.2.1.48) activity in homogenate, membrane pellets and in subsequent fractions was also determined by the methods previously cited.

The membranes, suspended at approx. 1mg of protein/ml of iso-osmotic Hepes [4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid] incubation buffer at pH 7.35, were radiolabelled by incubation at 37°C in the presence of final concentrations of 10–100 μ M-⁵⁹Fe(II) ascorbate prepared as described in detail by Cox & O'Donnell (1981a). After 5 min, the mix was diluted 5-fold with ice-cold buffer and centrifuged at 38 000 *g*_{av} for 30 min. The pellet, containing bound ⁵⁹Fe, was washed carefully with 5 ml of buffer twice and resuspended at 1–2 mg of protein per ml in 0.01M-Hepes, pH 7.9, with or without the addition of 0.15–0.5M-NaCl as indicated. The membrane proteins were then solubilized by adding sufficient aq. 20% (v/v) Triton X-100 to give a final concentration of 4–8mg per mg of protein and the clarified mixture was stirred intermittently while on ice for 15–30 min. Detergent-solubilized membrane radioactivity was obtained after centrifugation at 105 000 *g*_{av} for 60 min at 4°C and this fraction is henceforth termed the ⁵⁹Fe-Triton supernatant. Throughout membrane prepara-

tion, and during all subsequent procedures, this material was maintained below 4°C to minimize degradation.

Membrane antisera

Antisera to three separate preparations of rabbit proximal microvillus membranes were raised in Duncan–Hartley guinea-pigs. Each animal received approx. 100 μ g of antigen solubilized by the addition of Triton X-100 to 1% (v/v), emulsified with an equal volume of Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY, U.S.A.) and inoculated into foot pads and glutei. Three similar booster injections, excluding foot pads, were given over a period of 4 months and the animals were bled by cardiac puncture. After separation of serum, the complement activity was destroyed by heating to 56°C for 30 min. Immunoglobulins were purified partially by repeated precipitation and washing at 0°C with (NH₄)₂SO₄ at a final concentration of 1.75M. Aprotinin (100 Kunitz/ml) was added to inactivate plasmin and the final pellet was dissolved in 0.1M-potassium phosphate at pH 7.0. Immunoglobulin G was isolated by slowly passing this solution twice over a column of Protein A–Sephadex CL6B, washing with 10 bed vol. of buffer and eluting with 2 bed vol. of neutral 3.5M-KSCN. The eluted immunoglobulin G fraction was immediately equilibrated with coupling buffer for activated Sepharose (0.1M-NaHCO₃/0.5M-NaCl, pH 9.0) by desalting on Sephadex G-25. Purified antiserum not subsequently coupled was stored at –20°C without further additions. Fluorescence microscopy of 5 μ m crescentic cryostat sections of duodenal mucosa (removed 10 cm distal to the pylorus) was carried out with fluoresceinated lectins, in the presence or absence of 0.25M appropriate hapten-inhibiting sugars (Etzler & Branstrator, 1974). The specificity of antisera was tested with a double-layer immunofluorescence technique. Sections were air-dried for 10 min and incubated with diluted brush-border antisera for 40 min. The sections were washed in 0.15M-sodium phosphate and 0.004M-potassium phosphate, pH 7.3, containing 0.15M-NaCl. Afterwards they were incubated for a similar period with fluorescein isothiocyanate-conjugated goat anti-(guinea-pig immunoglobulin G) and again washed in phosphate buffer. Control experiments with comparable dilutions of non-immune control guinea-pig sera were performed.

Precipitation studies

Two-dimensional gel immunodiffusion analyses were carried out in Ouchterlony plates with 1% agarose containing 0.02M-Hepes/0.15M-NaCl (pH 7.3), and 1% (v/v) Triton. Diffusion was carried out for 72 h in a moist atmosphere at 4°C. Crossed immunoelectrophoresis was carried out with 1%

agarose gel in Tris/glycine/sodium barbital buffer at pH 8.6 with the addition of Triton X-100 to 0.2% (v/v), as described by Mayer & Walker (1980). After pressing and washing, gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R250 in methanol/acetic acid/water (9:2:9) and de-stained with the same solvent mixture. Tube precipitation experiments with purified antibodies and lectins were carried out in 3 ml polycarbonate tubes: (100 μ l) of membrane fractions [2% (v/v) Triton] containing 10–100 μ g of protein were mixed with an equal volume of lectin or immunoglobulin G solution (2–5 mg/ml) in 0.02 M-Hepes/0.5 M-NaCl (pH 7.35) and 10 Kunitz units of aprotonin and left for 1 h at room temperature. Sufficient aq. 20% (w/v) poly(ethylene glycol) 6000 was added to give a final concentration of 4% (w/v) and the mix was chilled to 4°C for 48 h. In the case of antibody experiments, a second antibody [donkey anti-(guinea pig serum); Wellcome Reagents, Beckenham, Kent, U.K.] was added in excess after the first 24 h. Proteins precipitated by antibody or lectin solutions were sedimented after centrifugation at 4000 g_{av} for 25 min and washed twice with 1 ml of Hepes buffer containing 1% (v/v) Triton after careful decantation of the supernatants. Control experiments with non-immune sera or lectins with added 0.25 M-hapten-inhibiting sugar were carried out simultaneously. Radioactivity due to γ -emission from ^{59}Fe was assayed at 6–10% efficiency in an automatic NaI crystal counter at constant geometry.

Autoradiography of dried unstained Ouchterlony gels was carried out in some cases before treatment with Coomassie Blue to preserve acid-labile bound iron. Plates were wrapped in plastic sheets and placed in a film holder at –70°C with light-activated Kodak X-Omat H film and a Haminex intensifying screen (Fujimex), and surrounded by lead sheeting during exposure (Laskey & Mills, 1975).

Pronase and periodate degradation

^{59}Fe -Triton supernatant (approx. 1 mg of protein/ml) was digested with Pronase in the presence of 2 mM- CaCl_2 at 37°C in tubes (0.3 cm $\times$ 5 cm) under toluene as described by Spiro (1966). Control incubations in 0.02 M-Hepes/0.15 M-NaCl (pH 7.4) were also run in parallel. Mild periodate degradation was carried out for 20 h at 4°C in the dark at a final concentration of 10 mM-sodium metaperiodate (Andersen *et al.*, 1966). ^{59}Fe supernatant (0.54 mg of protein/ml) in sodium Hepes buffer at pH 7.0 and containing 1% (v/v) Triton X-100 was used and the reaction was stopped with ethylene glycol.

Treatment with dimethylmaleic anhydride

Radiolabelled membranes were suspended in 0.02 M-sodium Hepes/0.15 M-NaCl at 0.5 mg of protein/ml. Solid dimethylmaleic anhydride was

added with stirring to give a concentration of 2 mg/ml, and the pH of the solution was maintained between 8.0 and 8.5 by manual titration with 0.2 M-NaOH until evolution of acid ceased. The pH was then restored to 7.9 with dilute HCl.

Gel-permeation chromatography

Triton supernatant in 0.02 M-Hepes/0.15 M-NaCl (pH 7.35) was applied to a column of Sepharose CL 6B equilibrated in the same buffer containing 1% (v/v) Triton X-100 and eluted at 4.8 ml/h. Fractions (0.75 ml) were collected and assayed for protein and radioactivity. The column was calibrated with thyroglobulin, ferritin, catalase and ovalbumin.

Ion-exchange chromatography

Triton supernatant in 0.01 M-sodium Hepes (pH 7.9) was applied to a column of DEAE-Bio-Gel A equilibrated in the same buffer containing 1% Triton X-100. The gel was washed with 1 bed vol. of equilibration buffer; this eluted a considerable proportion of the applied protein. Protein-bound radioactivity was then eluted with a linear gradient (500 ml) of 0–0.5 M-NaCl at 10 ml/h and collected in 5 ml fractions.

Lectin affinity chromatography

^{59}Fe -Triton supernatant or protein-bound ^{59}Fe (0.3–3 mg) eluted from DEAE-Bio-Gel A was applied slowly to small columns of RCA₁₂₀-Sepharose 4B (1–6 ml packed-gel volume) equilibrated in 0.01 M-sodium Hepes (pH 7.4), containing 0.5 M-NaCl and 1% (v/v) Triton X-100. The sample proteins were allowed to interact for 45 min and unbound material was removed by washing with a further 3 bed vol. of buffer. Specifically bound glycoprotein was eluted on applying 0.25 M-D(+)-galactose in the same buffer. A linear flow rate of 10 cm/h was used for specific elution, after allowing 30 min for the inhibiting sugar to interact with the upper layer of gel.

Immunoaffinity chromatography

Solubilized protein (0.2–1 mg/ml) in 0.01 M-sodium Hepes/0.5 M-NaCl (pH 7.9), containing 1% (v/v) Triton X-100, was applied to columns of guinea-pig immunoglobulin-Sepharose 4B (2–5 mg/ml of gel) and allowed to interact for 30 min at room temperature and for a further 12 h at 4°C. The gel bed was washed with 10 column vol. of buffer. Specifically-bound material was eluted at room temperature with 2% (w/v) sodium dodecyl sulphate in the same buffer. Control experiments with Sepharose linked identically to non-immune immunoglobulin were run in parallel to correct for non-specifically adsorbed material.

Analytical polyacrylamide-gel electrophoresis

Discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of native membranes and fractions of solubilized membrane proteins was carried out in 9% polyacrylamide tube gels (4.5 cm × 150 mm) by the method of Neville & Glossman (1971), under reducing conditions. Solubilized protein fractions in Triton X-100 were precipitated on ice with trichloroacetic acid at a final concentration of 10% (w/v). After centrifugation at 4000 g_{av} for 25 min, the pellets were washed thrice with diethyl ether at -20°C to remove detergent and acid. Gels were stained for sugar residues by the periodate/Schiff reagent or for protein with Coomassie Blue exactly as in the source reference.

Results

Preparation and solubilization of membrane-bound radioactivity

The membrane pellets prepared by the calcium precipitation method of Schmitz *et al.* (1973) were considerably purified since they were enriched 15–20-fold in sucrase activity when compared with starting homogenate. After incubation with $^{59}\text{Fe}(\text{II})$ ascorbate, centrifugation and careful washing, less than 5% of the bound radioactivity could be released by extraction with diethyl ether or chloroform/methanol (2:1, v/v). Acylation of the aqueous membrane suspension with dimethylmaleic anhydride, an agent used to solubilize predominantly extrinsic membrane proteins (Pillon *et al.*, 1980), released less than 10% of the bound radioactivity, but 40–50% of the membrane protein into the 105 000 g supernatant. Sodium dodecyl sulphate (1%, w/v) released 80% of the ^{59}Fe and all the measurable protein into the supernatant; 80–90% of the protein and 40–60% of the membrane radioactivity was extracted into the high-speed supernatants by treatment with Triton X-100. ^{59}Fe in the residue was presumably bound to extraneous core material, since it was released by disruption of the brown pellet with 1 M-Tris at pH 7.6 (Eichholz & Crane, 1965). More than 95% of the radioactivity in detergent extracts was protein bound as judged by gel filtration of Sephadex G-25 or precipitation with cold acetone.

Fluorescence microscopy

The affinity of several lectins for cellular structures within the intestinal mucosa from proximal rabbit intestine was examined in fresh cryostat sections after incubation with fluorescein conjugate of lectin protein. Representative results of ultraviolet fluorescence microscopy of concanavalin A and castor-bean lectins are shown in Plate 1. Concanavalin A fluorescence was restricted to the vascular core of the villi and examination with high-

power objectives showed a distribution in both endothelial cells and blood components. Wheat-germ agglutinin conjugates produced a strikingly widespread membrane fluorescence involving both basolateral and microvillus membranes of epithelial cells and including goblet-cell mucus and cellular elements in the lamina propria. In contrast, the distribution of tissue fluorescence after treatment with castor-bean agglutinin conjugates was restricted exclusively to the luminal microvillus membranes of both crypt and villous epithelial cells. Fluorescence was abolished in the presence of 0.25 M-D(+)-galactose.

Parallel immunofluorescence studies with dilutions of pooled guinea-pig antisera showed fluorescence exclusive to the microvillus membranes of the mucosal epithelium at titres from 1:5 to 1:250. The appearance of these sections under u.v. microscopy closely resembled sections exposed to the castor-bean agglutinin conjugates. Control studies employing similar dilutions of normal guinea-pig sera showed no fluorescence.

Treatment with Pronase and periodate

The effects of Pronase digestion and periodate degradation of native Triton supernatants are shown in Fig. 1. Pronase progressively rendered high-molecular-weight bound radioactivity diffusible across dialysis membrane when compared with control supernatants incubated in the absence of enzyme. Exposure to periodate for 20 h, sufficient

Fig. 1. Diffusible radioactivity after Pronase or periodate treatment

Samples of ^{59}Fe -Triton Supernatant were digested with 50 μg of Pronase for 45 h (▲) and 90 h (△) or treated with periodate (◇) for 20 h, as described in the Experimental section. After incubation, 0.5 ml portions were placed in dialysis sacs (8/32 in; Visking) and tumbled in tubes containing 50-fold volume excess of iso-osmolar Hepes buffer (pH 7.35) at 4°C for a further 20 h. Samples of buffer were removed at intervals for determination of radioactivity. The results of dialysis of samples of control incubations for 20 h (●) and 45 h (○) are also shown.

EXPLANATION OF PLATE 1

U.v. fluorescence micrograph of rabbit duodenal mucosa after exposure to fluoresceinated concanavalin A lectin conjugate (a) and to fluoresceinated castor-bean lectin-conjugate (b)
The horizontal bars represent 50 μ m.

to oxidize sugar residues in glycoproteins extensively (Andersen *et al.*, 1966), did not significantly release bound iron. Further evidence for the efficacy of these agents is presented below.

Gel-permeation and anion-exchange chromatography

The behaviour of membrane radioactivity, solubilized, and in the presence of detergent, on gel filtration is shown in Fig. 2. The elution profile of native

Fig. 2. Gel-permeation chromatography on Sepharose CL 6B

^{59}Fe -Triton supernatant (0.75 ml) was applied to a column (1.2 cm $\times$ 60 cm) of gel equilibrated in Hepes buffer and eluted in the presence of 1% (v/v) Triton as described in the Experimental section. Fractions (0.75 ml) were analysed for radioactivity (Δ) and protein by the fluorescamine method (—; arbitrary units of fluorescence). ——— shows the profile of eluted radioactivity obtained from a sample previously digested for 45 h with Pronase. The arrows indicate elution peaks of molecular-weight markers (left to right): 669 000, 232 000, 49 000 and 18 500.

supernatant on Sepharose CL 6B shows a major broad peak of protein-bound radioactivity included within the gel with M_r approx. 100 000. Minor peaks of radioactivity occur at the void volume and associated with a low-molecular-weight fraction. Also shown is the elution profile of supernatant after Pronase digestion for 45 h, confirming a reduction in apparent molecular size of bound radioactivity after proteolysis.

Anion-exchange chromatography of ^{59}Fe supernatant on DEAE-agarose is illustrated in Fig. 3. A substantial proportion of the applied membrane protein, but no associated radioactivity, failed to bind to the gel under these conditions. Protein-bound iron was eluted in a major peak at approx. 0.1 M Cl^- and no further material could be removed at concentrations up to 2 M. The protein-bound radioactivity was enriched 2–4-fold after ion-exchange chromatography.

Affinity chromatography on RCA_{120} -Sepharose 4B

As indicated by studies with fluoresceinated lectins, chromatography of solubilized membranes on affinity columns of immobilized lectins provided a valuable method for investigating the role of membrane glycoconjugates as microvillus iron receptors. ^{59}Fe incorporated by microvillus membrane constituents was bound to, and could be specifically eluted from, columns of RCA_{120} -Sepharose 4B (Fig. 4). As predicted by the fluorescence studies in whole mucosa, similar experiments employing immobilized concanavalin A failed to show specific binding of either protein or radioactivity; results with wheat-germ lectin-Sepharose were variable, and recoveries or purifications of bound material were generally poor. The specific radioactivity of ^{59}Fe -Triton supernatants was enriched 3–5-fold on

Fig. 3. Anion-exchange chromatography on DEAE-Bio-Gel A

^{59}Fe -Triton supernatant (3 ml) in 0.01 M-Hepes buffer (pH 7.9) was applied to a column (0.9 cm $\times$ 15 cm) of DEAE-Bio-Gel A equilibrated and eluted, as described in the Experimental section, with a salt gradient. Fractions were analysed for radioactivity (Δ), osmolality (—; [NaCl]) and protein (—; fluorescence) by the fluorescamine method.

Fig. 4. Lectin affinity chromatography on RCA₁₂₀-Sephacrose 4B

⁵⁹Fe-Triton supernatant (1 ml), containing approx. 1 mg of solubilized membrane protein, was applied to a 1 ml column of packed affinity gel in a plastic syringe barrel. After equilibration and washing, the specifically bound glycoproteins were eluted on addition of D(+)-galactose to 0.25 M in the buffer, as described in the Experimental section and indicated by the left-hand arrow. Fractions (0.25 ml) were collected. Subsequent addition of 1 M-D(+)-galactose is indicated by the right-hand arrow. ●, Fluorescence; △, radioactivity.

elution from RCA₁₂₀-Sephacrose, and further enrichments of material already purified by ion-exchange chromatography were also achieved.

Interactions with antibodies and soluble lectins

Precipitation experiments including two-dimensional diffusion gels confirmed that both antisera and castor-bean lectins interacted with solubilized membrane proteins in ⁵⁹Fe-Triton supernatants (Fig. 5). Periodate treatment for 20 h substantially reduced this reactivity, especially precipitation by the lectins. The immunodiffusion gels indicated precipitin lines at titres of antisera up to 1:32 when tested against supernatant containing 1 mg of protein/ml; further gel precipitin experiments showed that immunoreactivity was largely confined to the specific eluates from RCA₁₂₀-Sephacrose columns. Material passing unbound through the gel failed to produce visible precipitates. Quantitative immunoprecipitation of membrane disaccharidase activity in supernatant extracts was demonstrated (Fig. 6), but only 5–10% of the radioactivity was sedimented at optimal proportions in these immunoprecipitates,

Fig. 5. Interactions between antisera, lectins and solubilized membranes

(a) Two-dimensional crossed immunoelectrophoresis. Immunoprecipitates have been stained, after washing, with Coomassie Blue. (b) and (c) Gel-diffusion plate stained for protein (b) and radioautographed (c). The centre well contained 15 μ l (2000 d.p.m.) of ⁵⁹Fe-Triton supernatant; antisera (1:2) were placed in the left-hand wells and 25 μ g of castor-bean lectins were placed in the right-hand wells.

even after addition of a second antibody. Crossed immunoelectrophoresis in two-dimensional gels showed, as expected, that the membrane antibodies were polyspecific and at least four immunoprecipitin lines were detected.

Although the antibodies and lectins reacted specifically with several membrane components (Figs. 5 and 7), their affinity for the iron-binding moiety was different. In simple tube reactions, 35–85% of the radioactivity in Triton supernatants was precipitated by castor-bean lectins and this was abolished by addition of D(+)-galactose. The autoradiograph of the gel-diffusion plate (Fig. 5c) illustrates marked differences in the amounts of ⁵⁹Fe incorporated by lectin- and immuno-precipitates. Moreover, the results of precipitation experiments and lectin-affinity chromatography of ⁵⁹Fe supernatants prepared identically from matched normal and iron-deficient animals are set out in Table 1. These data confirm that the increased biological activity of microvillus iron receptors in conditions of iron deficiency may be extracted into Triton supernatants and may then interact with both soluble and immobilized castor-bean lectins. Application of Triton supernatant or RCA₁₂₀-Sephacrose eluate to immunoabsorbent columns resulted in up to 2-fold

enrichments in the radioactivity of the unbound material, thus effecting a further, 'negative', purification by removing components not involved in membrane iron-binding.

Purifications, as judged by specific radioactivity, per mg of membrane protein, up to 7-fold over original Triton supernatants were achieved when ion-exchange and lectin affinity chromatography

Fig. 6. Immunoprecipitation of solubilized membrane disaccharide activity

Triton supernatant (100μl; 77μg of protein) was mixed with increasing amounts of sera from immunized (▲) or non-immunized (△) guinea-pigs in a total volume of 500μl; buffered to pH 7.0 with 0.1 M-phosphate. After 16 h at 4°C maltase activity in supernatants after centrifugation at 4000g_{av} was assayed by the method of Dahlqvist (1968). The untreated supernatant contained 24.8 units/ml.

Fig. 7. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of fractions of solubilized membrane proteins

The following fractions (20–30μg of protein) were analysed on 9% (w/v) tube gels as described in the Experimental section. (a) Native membranes; (b) Triton supernatant; (c) DEAE-Bio-Gel eluate; (d) RCA₁₂₀-Sepharose eluate; (e) material bound to immune immunoglobulin G-Sepharose; (f) Total material bound to non-immune immunoglobulin G-Sepharose. Migration of marker proteins is indicated, from top to bottom (10⁻³ × molecular weight): phosphorylase *b* (*M*_r 94 000); bovine serum albumin (67 000); carbonic anhydrase (30 000); α-lactalbumin (14 400).

Table 1. Interactions of ⁵⁹Fe supernatants with castor-bean lectins

Portions of high-speed supernatants of radiolabelled intestinal microvilli purified from two matched pairs of normal control and chronically bled animals were allowed to interact in tubes with castor-bean lectins, or were pooled and subjected to affinity chromatography directly on RCA₁₂₀-Sepharose, as described in the Experimental section. Specific radioactivity of protein-bound iron was determined in 4000g pellets and pooled column eluates.

	Normal controls	Bled animals
Triton supernatants (d.p.m./μg of protein)	54.6; 34.8	103; 64.2
Lectin precipitates (d.p.m./μg of membrane protein)	23.8; 16.3	60.0; 30.6
Column eluates (d.p.m./μg of protein)	162	290
Purification factor (fold)	3.3	3.7

procedures were annexed serially. In view of the 15–20-fold enrichments of microvillus marker enzymes we obtain on preparing the membranes (Cox & O'Donnell, 1981a) from mucosal homogenate, a considerable purification of the iron-binding components was achieved. Indeed, analytical polyacrylamide-gel electrophoresis of the various fractions confirmed radical alterations of the profile of membrane proteins during these chromatographic steps, especially after lectin affinity chromatography (Fig. 7). There are remarkable similarities in the material bound to this gel and to the immunoabsorbent gels. The few high-molecular-weight bands near the gel origin (M_r 150 000–250 000), enriched during purification and absent from the immunoabsorbed material, may thus represent iron-binding components. Periodate/Schiff reagent stained five to six bands and the affinity-absorbed material represents most of these high-molecular-weight glycoproteins as detected in polyacrylamide-gel electrophoresis of native membranes.

Discussion

The intestinal microvillus membrane contains carriers that facilitate the vectorial transfer of different nutrients from the gut lumen to the absorptive epithelium. In eukaryotic cells a functional polarity is maintained across such plasma membranes by the asymmetrical insertion of integral proteins that subserve specific receptor and transport modalities (Singer & Nicholson, 1972).

We present evidence that intestinal microvilli, which possess a high affinity for iron, contain such specific high-molecular-weight iron-binding glycoprotein receptors, which are released from the membrane into micellar solutions of Triton X-100. Removal of only trivial amounts of bound iron by treatment of the membranes with the acylating agent dimethylmaleic anhydride, and the resistance to extraction with lipid solvents, suggests that the iron is bound to hydrophobic integral membrane glycoprotein. The successful digestions with Pronase support this contention, whereas the failure of mild periodate degradation to materially remove iron also suggests that the binding component is related to protein rather than the sugar moiety.

The specificity of these iron-binding glycoprotein components is indicated by the affinity studies involving antisera and lectins, both of which were shown to interact exclusively with intestinal microvilli and to overlap to some extent in their interactions with membrane proteins. Lectin-binding material without incorporated ^{59}Fe could be demonstrated also in the unbound material washed from the DEAE-agarose columns, and the differences in

interactions between antibodies and lectins with the iron-binding material, together with the purifications achieved, is further evidence for specificity of the iron-binding moiety. Disparities of a similar type have been shown in adipocyte membranes, where antibodies against intrinsic membrane proteins fail to react with the glucose-transport system or insulin receptors (Pillon *et al.*, 1980). Lectins have been shown to bind to the insulin receptor in such membranes (Cuatrecasas & Tell, 1973).

Previous work from this laboratory has indicated that microvillar iron-binding receptors may be functionally related to regulatory membrane components involved in the control of mucosal iron transport (Cox & O'Donnell, 1981b). In the present experiments, enhanced ^{59}Fe binding was demonstrated in solubilized membranes prepared from bled animals, in whom overall iron absorption is adaptively stimulated. Moreover, this material behaved identically on affinity matrices (but at higher specific activity) with glycoproteins in supernatants prepared from intestinal membranes of normal control animals. This indicates that galactose-terminal glycoproteins are involved in the adaptive response occurring in the membrane as a result of biological stress, and additionally links these iron-binding glycoproteins to the microvillus iron transport system.

After solubilization with non-ionic detergent, membrane-incorporated iron remained protein-bound in high-speed supernatants. Solubilized but unlabelled membranes were shown by gel filtration to bind ^{59}Fe and binding was enhanced in glycoprotein fractions eluted from affinity gels, but no increases in iron-binding were demonstrable in material extracted from intestinal membranes obtained from bled animals (Cox & O'Donnell, 1981a,b; T. M. Cox & M. W. O'Donnell, unpublished work). Further, although subtle differences in staining patterns between native membranes from normal and bled animals were seen on sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoretograms, no consistent modifications of the 30–40 bands of membrane polypeptides were identified. Previous work involving equilibrium dialysis of membranes against ^{59}Fe chelates, and membrane iron determinations by atomic-absorption spectrophotometry (Cox & O'Donnell, 1980), has indicated no differences in iron content that could account for observed changes in subsequent membrane ^{59}Fe binding activity induced reversibly by chronic haemorrhage. It would thus appear that the adaptive changes in specific iron binding in response to body iron deficiency can only be observed if the integrity of membrane proteolipid is preserved. Further work involving re-constitution of iron transport with isolated microvillus components is necessary for detailed analysis of these membrane control processes at the molecular level.

This work was supported by the Wellcome Trust. We thank Mr. N. Amos for assistance with the immunoelectrophoretic studies and Mrs. B. Owens for typing the manuscript.

References

- Andersen, B. R., Abele, D. C. & Vannier, W. E. (1966) *J. Immunol.* **97**, 913–924
- Cox, T. M. & O'Donnell, M. W. (1980) *Biochem. Int.* **1**, 446–454
- Cox, T. M. & O'Donnell, M. W. (1981a) *Biochem. J.* **194**, 753–759
- Cox, T. M. & O'Donnell, M. W. (1981b) *Biochem. Soc. Trans.* **9**, 157–158
- Crane, R. K. (1980) *Biochem. Soc. Trans.* **8**, 688–690
- Cuatrecasas, P. & Tell, G. P. E. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 485–489
- Dahlqvist, A. (1968) *Anal. Biochem.* **22**, 99–107
- Eichholz, A. & Crane, R. K. (1965) *J. Cell Biol.* **26**, 687–691
- Etzler, M. E. & Branstrator, M. L. (1974) *J. Cell Biol.* **62**, 329–343
- Kowarski, S. & Schachter, D. (1980) *J. Biol. Chem.* **255**, 10834–10840
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- Mayer, R. J. & Walker, J. H. (1980) *Immunochemical Methods in the Biological Sciences: Enzymes and Proteins*, pp. 64–73, 139–144, Academic Press, London and New York
- McCance, R. A. & Widdowson, E. M. (1937) *Lancet* **233**, 680–684
- Neville, D. M. & Glossman, H. (1971) *J. Biol. Chem.* **246**, 6339–6346
- Nicholson, G. L. & Blaustein, J. (1972) *Biochim. Biophys. Acta* **266**, 543–547
- Parikh, I., March, S. & Cuatrecasas, P. (1974) *Methods Enzymol.* **34**, 77–102
- Pillon, D. J., Carter-Su, C. A., Pilch, P. F. & Czech, M. P. (1980) *J. Biol. Chem.* **255**, 9168–9176
- Rinderknecht, H. (1962) *Nature (London)* **193**, 167–168
- Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B. K., Cerda, J. J. & Crane, R. K. (1973) *Biochim. Biophys. Acta* **323**, 98–112
- Singer, S. J. & Nicholson, G. L. (1972) *Science* **175**, 720–731
- Spiro, R. G. (1966) *Methods Enzymol.* **8**, 27–30