# A Transferrin-like GPI-linked Iron-binding Protein in Detergent-insoluble Noncaveolar Microdomains at the Apical Surface of Fetal Intestinal Epithelial Cells

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*Abstract.* A GPI-anchored 80-kD protein was found to be the major component of detergent-insoluble complexes, prepared from fetal porcine small intestine, constituting about 25% of the total amount of protein. An antibody was raised to the 80-kD protein, and by immunogold electron microscopy of ultracryosections of mucosal tissue, the protein was localized to the apical surface of the enterocytes, whereas it was absent from the basolateral plasma membrane. Interestingly, it was mainly found in patches of flat or invaginated apical membrane domains rather than at the surface of microvilli. Caveolae were not found in association with these labeled microdomains. In addition, the 80-kD protein was seen in apical endocytic vacuoles and in tubulo-vesicular structures, suggesting that the apical microdomains are involved in endocytosis of the 80-kD protein. By its $NH_2$-terminal amino acid sequence, iron-binding capacity and partial immunological cross-reactivity with serum transferrin, the 80-kD protein was shown to belong to the transferrin family, and it is probably homologous to melanotransferrin, a human melanoma-associated antigen. The 80-kD iron-binding protein was fully detergent-soluble immediately after synthesis and only became insoluble after gaining resistance to endo H, supporting a mechanism for exocytic delivery to the apical cell surface by way of detergent-insoluble glycolipid "rafts" that fuse with the plasmalemma at restricted sites devoid of microvilli.

D ETERGENT-insoluble complexes are operationally defined as the fraction of membranes that, due to their high content of glycosphingolipids, resist solubilization at low temperature by detergents with a low critical micellar concentration, such as Triton X-100 (van Meer and Burger, 1992). They are thought to reflect the existence of glycolipid microdomains or rafts in the ectoplasmic leaflet of the lipid bilayer and this property of glycolipids has been proposed to be the underlying mechanism responsible for the clustering and sorting in the *trans*-Golgi network of GPI-anchored membrane proteins, destined for targeting to the apical surface of epithelial cells (Brown, 1992; Hannan et al., 1993; Zurzolo et al., 1994). The presence of caveolin in detergent-insoluble complexes has associated them with caveolae at the cell surface (Rothberg et al., 1992; Kurzchalia et al., 1992; Dupree et al., 1993), and a large number of proteins with a variety of functions have subsequently been localized to these complexes in different cell types (Sargiacomo et al., 1993; Chang et al., 1994; Stahl and Mueller, 1995). However, the general identification of caveolae with detergent-insoluble complexes has recently been questioned (Gorodinsky et al., 1995; Mayor and Maxfield, 1995; Kurzchalia et al., 1995).

The brush border membrane of the small intestinal enterocyte is a highly specialized digestive/absorptive surface that ensures an efficient internalization of dietary nutrients (Trier, 1987). We have recently shown that several of the brush border hydrolases, including the transmembrane peptidases and glycosidases, are partially localized in detergent-insoluble complexes of which the small intestine is a rich source despite the fact that few, if any, caveolae are present (Danielsen, 1995). The observation that newly synthesized brush border enzymes cluster in detergent-insoluble complexes already during their intracellular transport lead us to propose that association with glycolipid "rafts" functions as a sorting step in the enterocyte for transmembrane as well as GPI-anchored proteins, targeted to the apical cell surface.

The apical cell surface of the enterocyte gradually develops into a brush border during embryogenesis, but it lacks the glycosidases which are not expressed until around the time of birth or after weaning, and the peptidases, notably aminopeptidase N, are present in only comparatively small amounts (Kedinger et al., 1980; Henning, 1987). Morphologically, the fetal and neonatal enterocyte is typified by

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the development of apical endocytic and vacuolar structures which enables the newborn to absorb and digest milk protein and to acquire passive immunity by transcytosis of maternal immunoglobulins (Kraehenbuhl et al., 1969; Rodewald, 1970; Trier and Moxey, 1979; Wilson et al., 1991). We have recently shown that aminopeptidase N (EC 3.4.11.2) in fetal enterocytes of the pig after synthesis and transport to the brush border is internalized into vacuolar structures where a significant proportion of the steady-state amounts of the enzyme is localized (Danielsen et al., 1995).

In the present work, a 80-kD protein was found to be the predominant polypeptide component of detergent-insoluble complexes, prepared from fetal small intestine. By immunogold electron microscopy, the 80-kD protein was localized mainly to noncaveolar microdomains devoid of microvilli at the apical cell surface and to apical tubulovesicular structures and vacuoles. The 80-kD protein was characterized as a transferrin-like, GPI-anchored iron-binding protein, probably equivalent to the previously described melanoma-associated antigen p97 or melanotransferrin (Woodbury et al., 1980).

# Materials and Methods

## Materials

Equipment for performing organ culture, including Trowell's T-8 medium, culture dishes with grids, and [$^{35}$S]methionine (specific radioactivity >1,000 Ci/mmol) was obtained as previously described (Danielsen et al., 1982). Protein A-gold was purchased from Amersham (Bucks., U.K.), peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins were from DAKO (Glostrup, Denmark), and phosphatidylinositol-specific phospholipase C (from Bacillus cereus) was a product of Sigma Chemical Co. (St. Louis, MO). Anti-cross reacting determinant, which specifically recognizes phospholipase-cleaved GPI-anchors, was obtained from Oxford Glycosystems (Abingdon, U.K.), and endo-β-N-acetyl-glucosaminidase H (endo H, from Streptomyces plicatus) from Boehringer Mannheim GmbH (Mannheim, Germany).

Pig fetuses were kindly provided by De Forenede Andelsslagterier (Ringsted, Denmark). Small intestine from neonatal pigs were kindly given by Dr. Per Sangild (Royal Veterinary and Agricultural University, Copenhagen, Denmark).

## Preparation of Detergent-insoluble Complexes

Flotation of detergent-insoluble complexes by sucrose gradient centrifugation was performed essentially as described by Brown and Rose (1992). Briefly, 2 ml of labeled explant extract, or, in some experiments, Mg$^{2+}$-precipitated -or microvillar membranes in 25 mM Hepes, 150 mM NaCl, pH 7.0, 1% Triton X-100 was mixed in a centrifugation tube with an equal volume of 40% sucrose, made up in the same buffer, and 8 ml of a 5–30% linear sucrose gradient was layered on top of the extract. After centrifugation in a Beckman SW40 Ti rotor (Beckman Instruments, Palo Alto, CA) at 35,000 rpm ($g_{max}$ = 217,000) for 20–22 h at 3°C, the resulting floating light-scattering band was carefully collected by use of a pipette, mixed with 5 vol of 25 mM Hepes, 150 mM NaCl, pH 7.0, and centrifuged at 48000 g for 1 h to obtain a pellet of detergent-insoluble membranes.

## Preparation of Antiserum

Detergent-insoluble complexes were prepared from a microvillar fraction of the small intestine of fetuses with a crown-rump length of 14 cm. 150 μl samples of complexes (~1 mg protein/ml) in 25 mM Hepes, 150 mM NaCl, pH 7.0, were mixed with equal volumes of Freund's incomplete adjuvant and injected intracutaneously into a rabbit at two week intervals. One week after the fourth immunization, the rabbit was bled for 50 ml, and booster injections were subsequently given every sixth week, followed by new bleedings. The antisera from the bleedings were either used di-

rectly for immunoelectrophoresis or the IgG fraction purified by protein A–Sepharose chromatography. This antibody recognized aminopeptidase N and the 80-kD protein in Western blotting and immunoelectrophoresis. To obtain an antibody specific to the 80-kD protein for use in immunogold electron microscopy, reactivity against aminopeptidase N was removed from the IgG fraction by chromatography on a column of Triton X-100–solubilized microvillar membranes from the small intestine of an adult animal, coupled to Sepharose. The removal of aminopeptidase N reactivity was monitored by Western blotting and rocket immunoelectrophoresis, and after two passages through the column, the IgG fraction only exhibited detectable reactivity against the 80-kD iron-binding protein.

## Electron Microscopy

Small pieces of fetal intestine were fixed in 2% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, cryoprotected and frozen (Hansen et al., 1993). Ultracryosections were cut and labeled with anti-80-kD protein followed by protein A conjugated to either 5 or 10 nm gold particles. Pellets of Triton X-100–insoluble complexes, prepared from fetal intestine, were fixed, cryoprotected and frozen, and ultracryosections labeled as described above.

## Organ Culture of Mucosal Explants

Pig fetuses were removed from the womb of the sow immediately after it was disemboweled, and their small intestines excised and placed in ice-cold Hanks' buffered salt solution. 0.5–1 cm sections of intestine were cut open, placed on grids in culture dishes and cultured for periods up to 6 h as previously described for mucosal explants from adult animals (Danielsen et al., 1982). After culture, the tissue was quickly frozen and kept at −20°C until further processing.

## Subcellular Fractionation

Fetal small intestine was homogenized, fractionated by the divalent cation precipitation method (Schmitz et al., 1973; Booth and Kenny, 1974) into Mg$^{2+}$-precipitated and microvillar membranes and solubilized by Triton X-100 as previously described (Danielsen, 1982).

## Electrophoretic Methods

Quantitative rocket immunoelectrophoresis and crossed immunoelectrophoresis in 1% agarose gels was performed essentially as described by Weeke (1973). In some experiments, aminopeptidase N-precipitates were visualized by histochemical staining, using alanyl-β-napthylamide as substrate, as previously described (Danielsen et al., 1977). SDS-PAGE in 10% gels under reducing conditions was performed according to Laemmli (1970) and fluorography as described by Bonner and Laskey (1974). Gel tracks were scanned in an Ultroscan XL densitometer (Pharmacia LKB, Bromma, Sweden). For Western blotting, proteins separated by SDS-PAGE were electrotransferred onto Immobilon PVDF membranes (Millipore Corp., Bedford, MA), and visualized by the procedure of Bjerrum et al. (1983).

## NH$_2$-terminal Amino Acid Sequence Determination

Samples (~100 μg of protein) of detergent-insoluble complexes prepared from a microvillar fraction of fetal small intestine were subjected to SDS-PAGE, followed by electrotransfer onto Immobilon PVDF membranes and staining with Coomassie brilliant blue. Membrane areas containing the 80-kD protein were excised and subjected to sequencing as described by Ploug et al. (1989), using a sequencer (model 477A; Applied Biosystems, Foster City, CA), equipped with on-line PTH-amino acid analyzer (model 120A) using the "Normal-1" program supplied by the manufacturer.

## Solubilization with Phosphatidylinositol-specific Phospholipase C

50 ml samples of detergent-insoluble complexes (~2 mg protein/ml) in 25 mM Hepes, 150 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, pH 7.0, were incubated in the presence or absence (control) of 1 U of phosphatidylinositol-specific phospholipase C for 1 h at 37°C. After incubation, the samples were centrifuged at 20,000 g, 30 min. The supernatants (= phospholipase solubilized fraction) were analyzed by crossed immunoelectrophoresis.

The pellets were resuspended in 50 μl of the above buffer and solubilized by 1% Triton X-100 for 10 min at 37°C before centrifugation at 20,000 g, 10 min. The supernatants (= phospholipase-insoluble fraction) were collected and analyzed by crossed immunoelectrophoresis.

## Digestion with Endo H

Labeled explants were homogenized in 0.5 ml of 25 mM Hepes, 150 mM NaCl, pH 7.0, and centrifuged at 20,000 g, 15 min. The pellet was resuspended in 0.5 ml of the above buffer, containing 10 μg/ml of aprotinin, and solubilized by 1% Triton X-100 for 10 min at 37°C. After centrifugation at 20,000 g, 5 min, the supernatant was collected and the 80-kD protein and aminopeptidase N immunoprecipitated by addition of 200 μl antibody raised against detergent-insoluble complexes. After incubation overnight at 4°C, the immunoprecipitates were pelleted by centrifugation at 5000 g, 5 min, washed once in the above buffer and finally resuspended and boiled for 3 min in 50 μl of 100 mM sodium citrate, 0.1% SDS, pH 5.5. 50 mU of endo H was then added, and the samples incubated at 37°C overnight before analysis by SDS-PAGE. Control samples without the addition of endo H were incubated in parallel.

## Results

### A 80-kD Protein Is the Major Component of Detergent-insoluble Complexes from Fetal Small Intestine

Fig. 1 (*upper panel*) shows the polypeptide composition of detergent-insoluble complexes prepared from the $Mg^{2+}$-precipitated fraction and the microvillar fraction of fetal small intestine. The two profiles looked essentially similar with a band of about 80 kD as the most abundant component; by densitometric scanning of the gel tracks, this polypeptide was calculated to represent approx. 25% of the total amount of protein of microvillar-derived complexes and about 15% of the complexes isolated from the $Mg^{2+}$-precipitated fraction. The 166-kD band, representing aminopeptidase N, constituted 7–8% of the protein of the complexes; the minor peaks of lower molecular weight represent actin (43 kD) and unidentified polypeptides of 37–35 and 32 kD that are also found in detergent-insoluble complexes prepared from the small intestine of adult animals (Danielsen, 1995).

A polyclonal antibody against detergent-insoluble complexes prepared from fetal small intestine was raised in rabbits. This antibody reacted with the 166-kD band and, more strongly, with 80-kD band in Western blotting and was able to precipitate both components (Fig. 1, *lower panel*). In crossed immunoelectrophoresis the antibody gave rise to two detectable precipitates of which the minor, faster migrating one was shown by enzyme staining to correspond to aminopeptidase N (Figs. 6 and 7). The antibody reactivity towards the latter was removed by chromatography on a column of solubilized microvillar membranes from adult pig intestine, coupled to Sepharose. After this absorption, the antibody only recognized the 80-kD protein in Western blotting (Fig. 1, *lower panel*) and in immunoelectrophoresis (Fig. 10).

### The 80-kD Protein Is Localized in Noncaveolar Apical Membrane Microdomains and Endocytic Structures

In general, the differentiating brush border of the fetal enterocytes was found to be sparsely populated with short microvilli which often appeared in small bundles sepa-
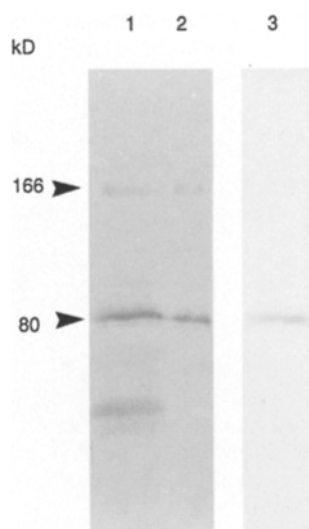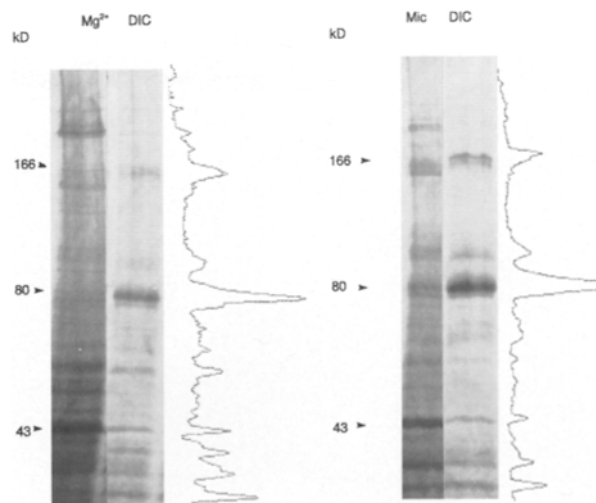


*Figure 1. (Upper panel)* $Mg^{2+}$-precipitated ($Mg^{2+}$) and microvillar (*Mic*) membranes were prepared from fetal small intestine and solubilized by 1% Triton X-100 at 0°C. Detergent-insoluble complexes (*DIC*) were prepared from each of these fractions by sucrose density gradient centrifugation and samples (~100 μg protein) analyzed by SDS-PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue, and the gel tracks of the detergent-insoluble complexes were densitometrically scanned. Molecular mass values (kD) are indicated. (*Lower panel*) Western blotting, using as primary antibody either the "crude" antiserum to detergent-insoluble complexes (lanes *1* and *2*) or the antibody specific to the 80-kD protein (lane *3*). (Lane *1*) 200 μl of detergent-insoluble complexes, solubilized by 1% Triton X-100 at 37°C, and immunoprecipitated by overnight incubation with 100 μl of antiserum to detergent-insoluble complexes. The immunoprecipitate was pelleted by centrifugation at 5,000 g, 3 min. (Unspecific staining of a 50-kD band, representing the precipitating antibody, is visible). (Lanes *2* and *3*) 100 μl of detergent-insoluble complexes. Molecular mass values (kD) are indicated.

rated by patches of flat membrane surfaces, in particular close to the intercellular spaces (Fig. 2) (Louvard et al., 1992). Caveolae (i.e., small regularly shaped invaginations of about 50–60 nm in diameter) were not observed. Tight junctions and desmosomes between neighboring cells were well developed (not shown). Immunogold labeling on ultracryosections of fetal intestine with the anti-80–kD protein antibody revealed the 80-kD protein to be present in large amounts at the apical surface of the enterocytes; no labeling at the basolateral plasma membrane was observed. Most interestingly, the protein was found mainly in patches of variable density in the flattened or slightly in-
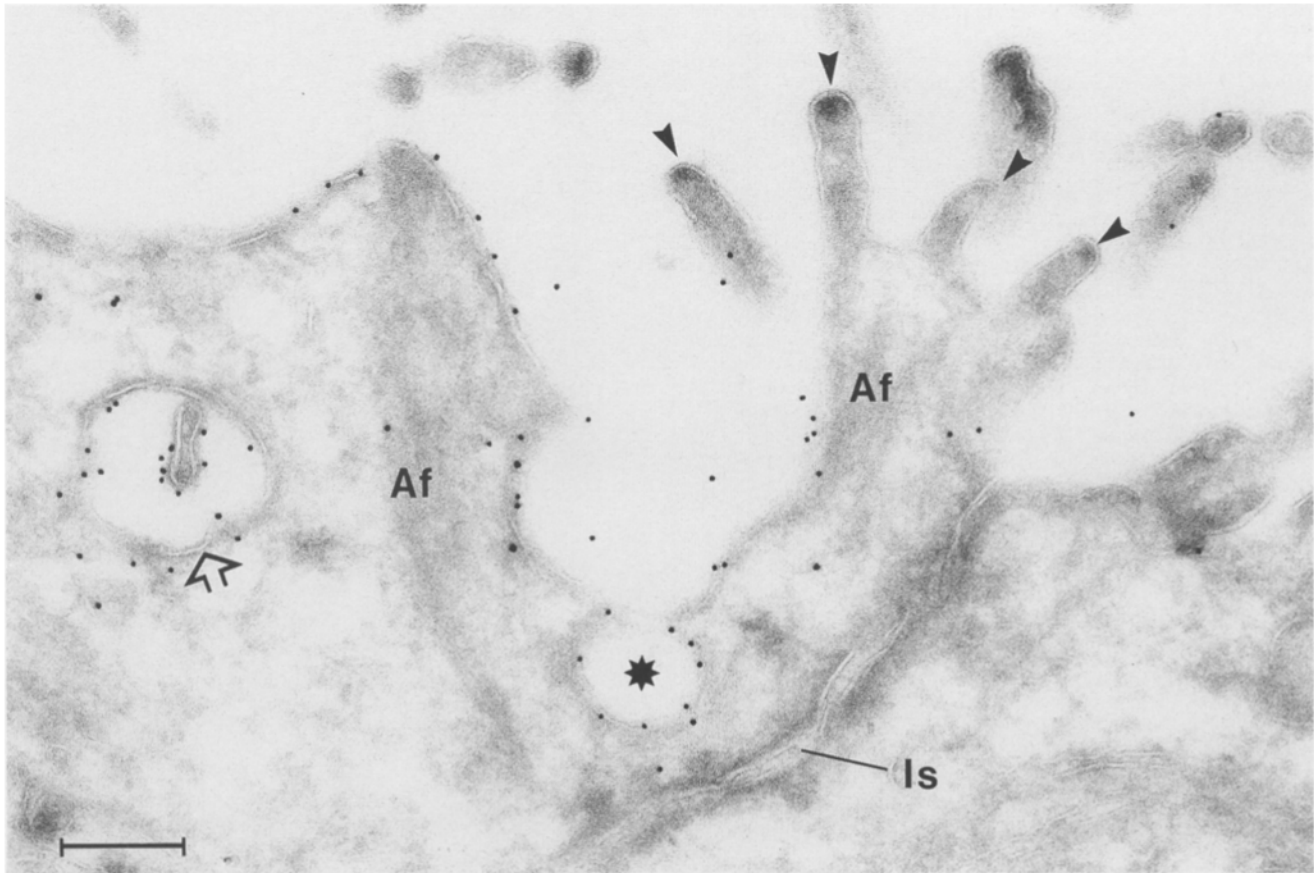
*Figure 2.* Electron micrograph of an ultracryosection showing typical features of the apical surface of the developing fetal enterocyte and the localization of the 80-kD protein. The section was incubated with anti-80-kD protein followed by protein A conjugated to 10 nm gold (PAG 10 nm). Note the irregular, poorly developed brush border, and the bundles of actin filaments (*Af*). The 80-kD protein is confined to the apical membrane, but is only rarely present at the microvilli (*arrowheads*); the intercellular space (*Is*) is unlabeled. A vesicular invagination is indicated by an asterisk and a vacuolar profile, which could represent a cross-sectioned surface invagination, is also seen (*open arrow*). Bar, 0.25 μm.

ward-bulging apical membrane domains between the tufts of microvilli while only little or scattered labeling was found at the microvillar membrane itself (Figs. 2 and 3). Vesicular pits or invaginations (70–250 nm in diameter) were often found to be associated with the 80-kD protein-labeled apical membrane domains. At least some of these invaginations must be involved in endocytosis of the 80-kD protein since the protein was localized in apical, large endosome-like vacuoles (Fig. 4), structures previously reported to be engaged in endocytosis (Wilson et al., 1991; Kömüves and Heath, 1992; Danielsen et al., 1995). Moreover, characteristic tubulo-vesicular structures in the apical cytoplasm were labeled by the anti-80-kD protein antibody (Fig. 4). Such structures have been proposed to be active in membrane recycling in enterocytes of neonatal piglets (Kömüves and Heath, 1992). Labeling of distinct Golgi stacks was not observed, but the TGN was often labeled (not shown).

Electron microscopy of ultracryosections of the detergent-insoluble complexes revealed frequent membrane-rich structures including numerous vesicular profiles with a diameter range of 50–150 nm. These structures labeled distinctly with the anti-80–kD protein antibody (Fig. 5).

## The 80-kD Protein Is a Transferrin-like Iron-binding Protein

The finding of the 80-kD protein being the most prominent polypeptide component of detergent-insoluble complexes from fetal small intestine prompted a further characterization of this protein. By microsequence analysis, the following NH$_2$-terminal amino acid sequence of the 80-kD protein was obtained: Gly-Met-Glu-Val-Arg-Trp-Xxx-Thr-Ile-Ser-Asp-Pro-Glu-Gln-Gln. In database searches, this sequence was found to share the highest homology with proteins of the transferrin family of iron-binding proteins; thus it was 47% homologous with positions 3–17 of mature porcine transferrin (Baldwin and Weinstock, 1988), 40% homologous with positions 22–36 of porcine pre-lactoferrin (Lydon et al., 1992) and 73% homologous with positions 20–34 of human pre-melanotransferrin (Rose et al., 1986). The antibody raised against the detergent-insoluble complexes reacted in Western blotting with a 80-kD protein present in porcine serum but could not precipitate it, suggesting a resemblance, but not an identity with, serum transferrin (data not shown). Finally, the iron-binding ability of the 80-kD protein from small intestinal detergent-insoluble complexes was directly visualized by crossed immu-
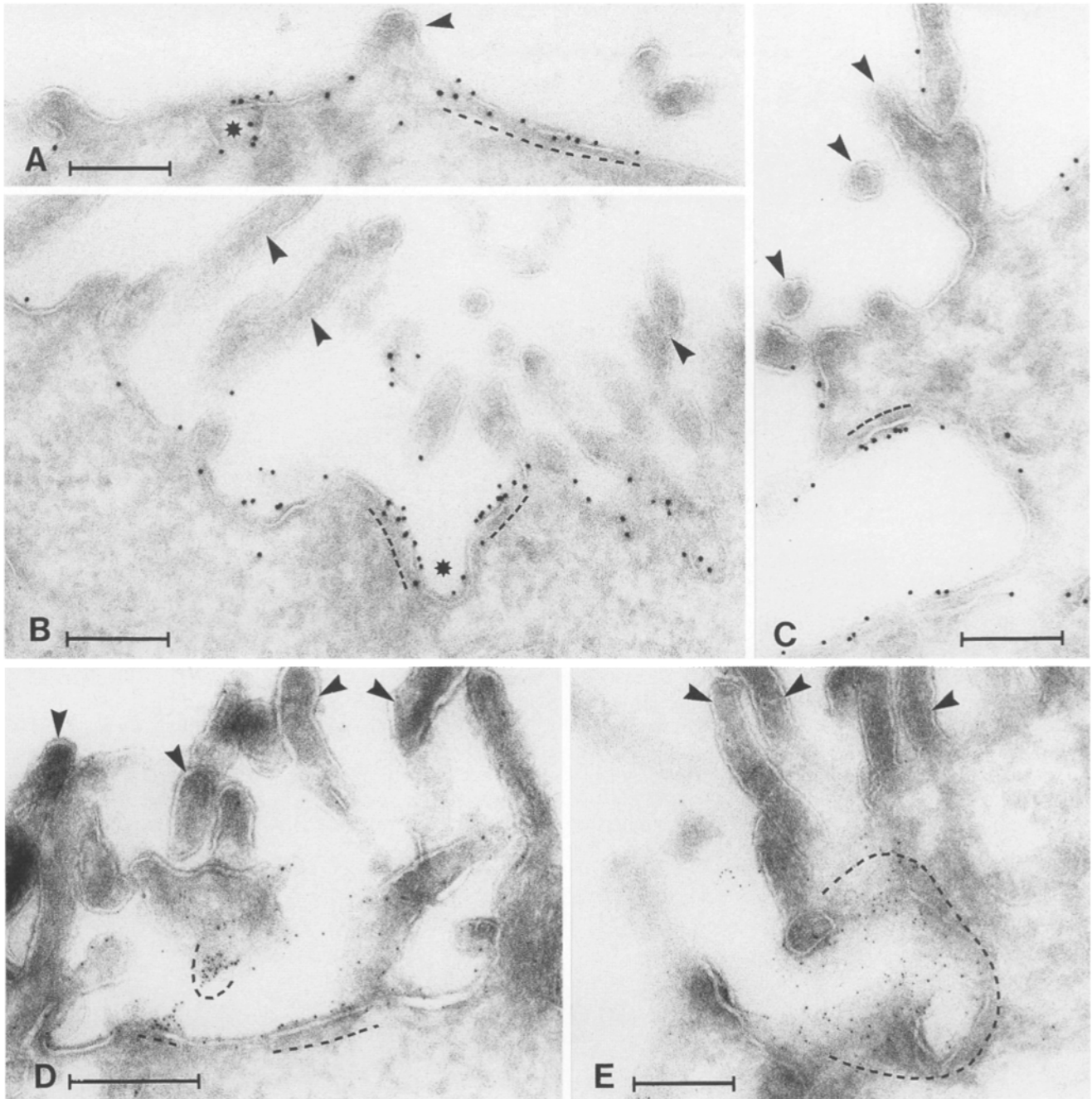
*Figure 3.* This figure shows more details of the characteristic apical localization of the 80-kD protein in the fetal enterocyte. The cryosections were labeled with anti-80-kD protein, followed by PAG 10 nm (*A–C*) or PAG 5 nm (*D–E*). The 80-kD protein is mainly localized to nonmicrovillar microdomains at the apical surface (*dashed lines*). Largely unlabeled microvilli are indicated by arrowheads. Two apical membrane invaginations are indicated with asterisks in *A* and *B*. Bars, 0.25 μm.

noelectrophoresis in the presence of $^{59}Fe^{3+}$. As shown in Fig. 6 *A*, the slower migrating of the two precipitates was labeled by the radioactive iron. In a parallel experiment with porcine serum electrophoresed against rabbit anti-porcine serum IgG, an immunoprecipitate with a relative mobility corresponding to serum transferrin was specifically labeled by the radioactive iron (Fig. 6 *B*). It can therefore be concluded that the 80-kD protein from fetal small intestinal detergent-insoluble complexes is an iron-binding protein that belongs to the transferrin family.

## The 80-kD Iron-binding Protein Is a GPI-linked Integral Membrane Protein

More than 90% of the 80-kD iron-binding protein in a fetal mucosal homogenate could be pelleted by centrifugation for 15 min at 20,000 g, indicating that the bulk mass of the protein is membrane-associated, and this association resisted carbonate extraction at pH 11, showing that the 80-kD protein is an integral membrane protein (data not shown). The overall detergent-insolubility at low tempera-
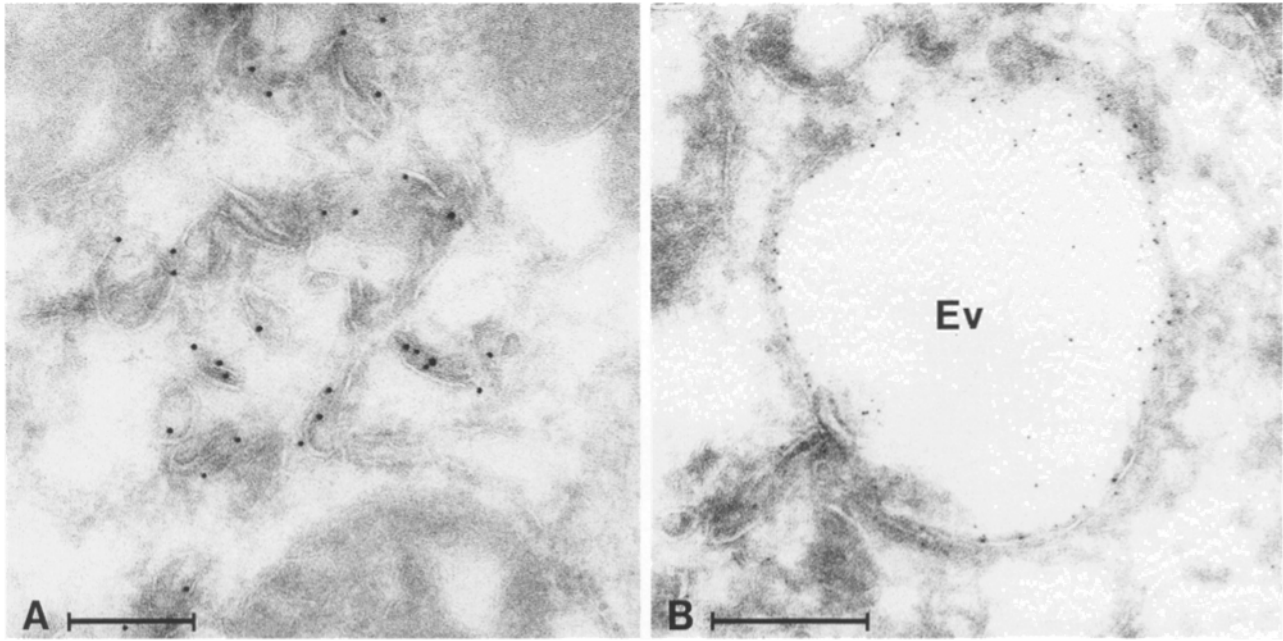
*Figure 4.* *A* shows apical tubulo-vesicular structures containing the 80-kD protein, and *B* shows an endocytic vacuole (*Ev*) labeled for the protein. *A* is labeled with PAG 10 nm, *B* with PAG 5 nm. Bars, 0.25 μm.

ture of the 80-kD protein was determined by quantitative rocket immunoelectrophoresis against the antibody described above. As shown in Fig. 7, the main portion (67% in a series of three experiments) of this protein in a whole tissue homogenate was indeed insoluble in Triton X-100 at 0°C (but soluble at 37°C). By comparison, aminopeptidase N was found to be 55% insoluble at low temperature, a value close to that previously reported for this enzyme

from adult tissue (Danielsen, 1995). Fig. 8 *A* shows that the 80-kD protein could be solubilized from the detergent-insoluble complexes by digestion with phosphatidylinositol-specific phospholipase C, indicating that it is linked to the membrane via a GPI anchor. Notice that the electrophoretic mobility of the 80-kD iron-binding protein was significantly increased after incubation with the phospholipase. As a control, the transmembrane aminopeptidase N
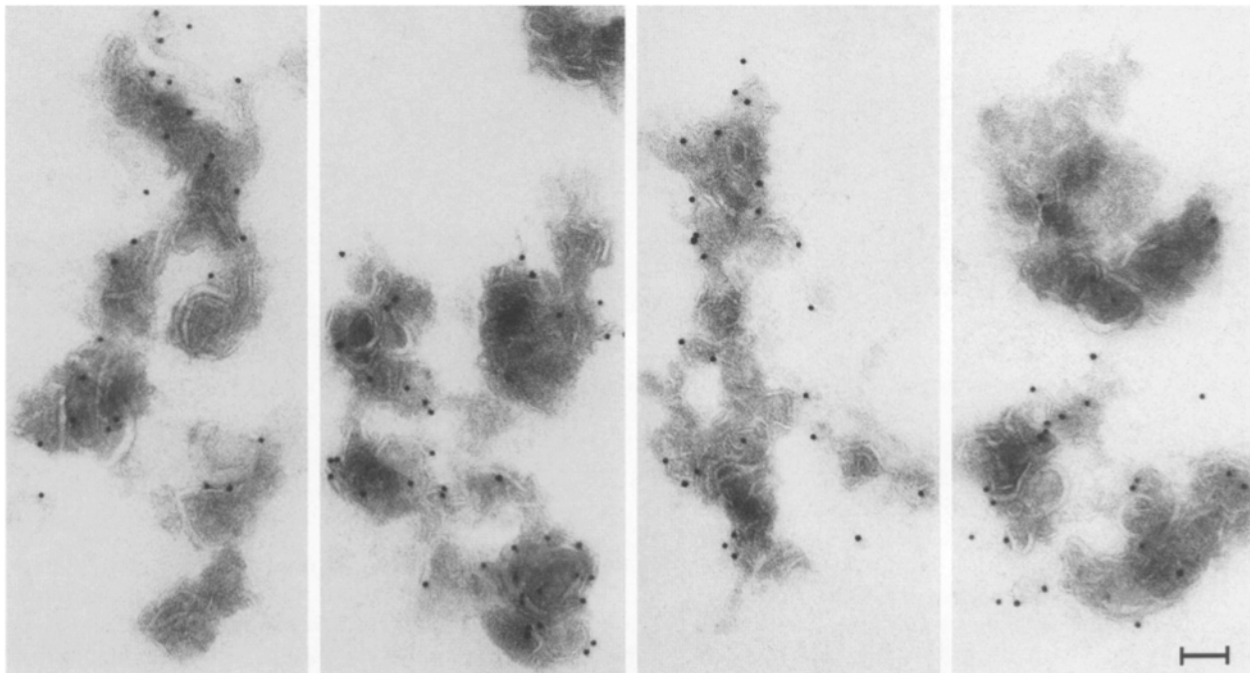


*Figure 5.* Ultracryosections of the Triton X-100-insoluble complexes, labeled with anti-80-kD protein followed by PAG 10 nm. In the preparation, many vesicular profiles are seen, around 100 nm in diameter. Bar, 100 nm.
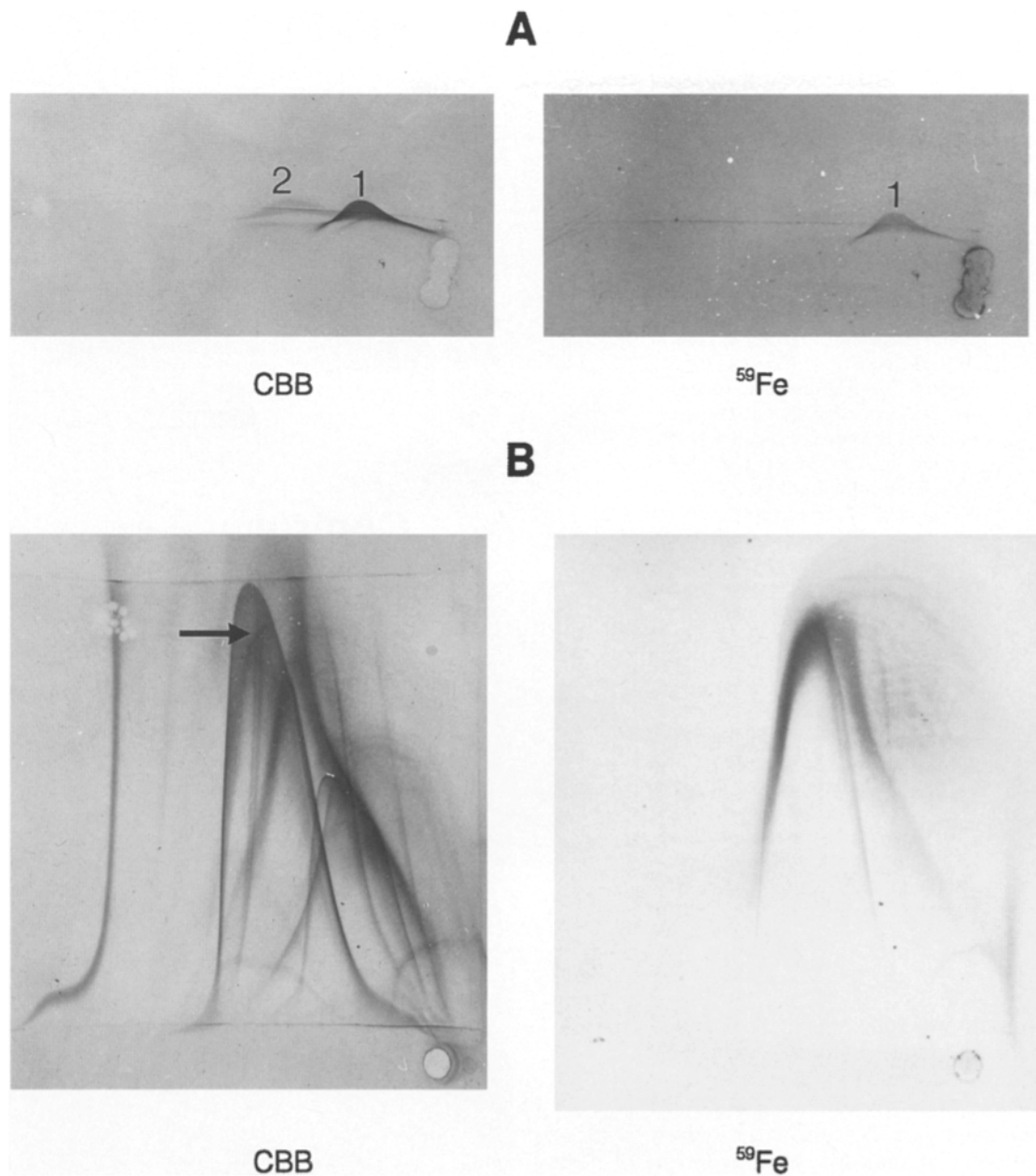
**A**



CBB



⁵⁹Fe

**B**



CBB



⁵⁹Fe

*Figure 6.* (*A*) 20 ml (~1 mg protein/ml) of fetal detergent-insoluble complexes were solubilized by 1% Triton X-100 at 37°C, mixed with 0.5 μCi of ⁵⁹FeCl₃ and analyzed by crossed immunoelectrophoresis. (*B*) 2 μl of swine serum, diluted with 10 μl of 25 mM Hepes, 150 mM NaCl, pH 7.0, was mixed with 0.5 μCi of ⁵⁹FeCl₃ and analyzed by crossed immunoelectrophoresis. After electrophoresis, immunoprecipitates were stained with Coomassie brilliant blue (*CBB*), and visualized by autoradiography (⁵⁹*Fe*). (*1*) 80-kDa protein; (*2*) aminopeptidase N. (The *arrow* indicates the position of the iron-binding precipitate representing serum transferrin).

was not solubilized and its mobility was unaffected by the treatment. Finally, the presence of a GPI anchor on the 80-kD iron-binding protein was directly demonstrated by its reaction in Western blotting with anti-cross reacting determinant which specifically recognizes phospholipase-cleaved GPI-anchors (Cardoso de Almeida and Turner, 1983; Zamze et al., 1988). As shown in Fig. 8 *B*, the 80-kD band reacted with this antibody only after lipolytic cleavage which exposes the inositol 1, 2-cyclic monophosphate epitope. The transmembrane aminopeptidase N did not react with this antibody. It can therefore be concluded that

the 80-kD iron-binding protein is an integral membrane protein, linked to the lipid bilayer by a GPI anchor.

## Biosynthesis of the 80-kD Iron-binding Protein

Fig. 9 *A* shows that immediately after a 30-min pulse of [³⁵S]methionine in cultured fetal mucosal explants, both the 80-kD iron-binding protein and aminopeptidase N were fully soluble in Triton X-100, and that after 6 h of chase, a significant proportion of both labeled proteins had become detergent-insoluble. Furthermore, by 30 min, the

S     I



S     I

*Figure 7.* Quantitative rocket immunoelectrophoresis of detergent-soluble (*S*) and detergent-insoluble (*I*) fractions of a crude membrane preparation of fetal mucosa. Approximately 0.2 g of small intestine was homogenized in 2 ml 25 mM Hepes, 150 mM NaCl, pH 7.0, and centrifuged at 500 g, 2 min. The supernatant was centrifuged at 20,000 g, 30 min and the resulting pellet resuspended in 250 μl of the above buffer and solubilized by 1% Triton X-100 at 0°C. After centrifugation at 20,000 g, 30 min, the supernatant of detergent-soluble proteins was collected. The pellet was resuspended as above and solubilized by 1% Triton X-100 at 37°C. After centrifugation at 5,000 g, 10 min, the supernatant of (solubilized) detergent-insoluble protein was collected. (*Top*) 20 μl of both fractions was applied. After electrophoresis, the precipitates were stained with Coomassie brilliant blue. (*Bottom*) 30 μl of both fractions was applied. After electrophoresis, the aminopeptidase N-precipitates were visualized by enzyme staining.

## A  +PI-PLC



Sol        Mem

## Control



Sol        Mem

**B**



80-kD iron-binding protein was fully sensitive to endo H (as was the 140-kD form of aminopeptidase N) (Fig. 9 *B*). After 6 h, endo H treatment resulted in a blurred band, indicating a partial resistance to the glycosidase. The 170-kD mature form of aminopeptidase N appeared similarly blurred after endo H treatment, indicating that the Golgi-associated trimming and attachment of "complex" carbohydrate is not fully developed in the fetal enterocyte. These results thus show that association of the 80-kD iron-binding protein with detergent-insoluble complexes only occurs posttranslationally at a time when the protein has acquired (partial) endo H resistance.

*Figure 8.* (*A*) 50 μl samples (∼2 mg protein/ml) of fetal detergent-insoluble complexes in 25 mM Hepes, 150 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, pH 7.0, were incubated in the absence (*Control*) or presence (*+PI-PLC*) of 1 U phosphatidylinositol-specific phospholipase C for 1 h at 37°C. After incubation, the samples were centrifuged at 20,000 g, 30 min, and the supernatants (*Sol*) collected and analyzed by crossed immunoelectrophoresis. The pellets were resuspended in 50 μl of the above buffer and solubilized with 1% Triton X-100 at 37°C, followed by centrifugation at 20,000 g, 10 min. The resulting supernatants of detergent-solubilized membranes (*Mem*) were analyzed by crossed
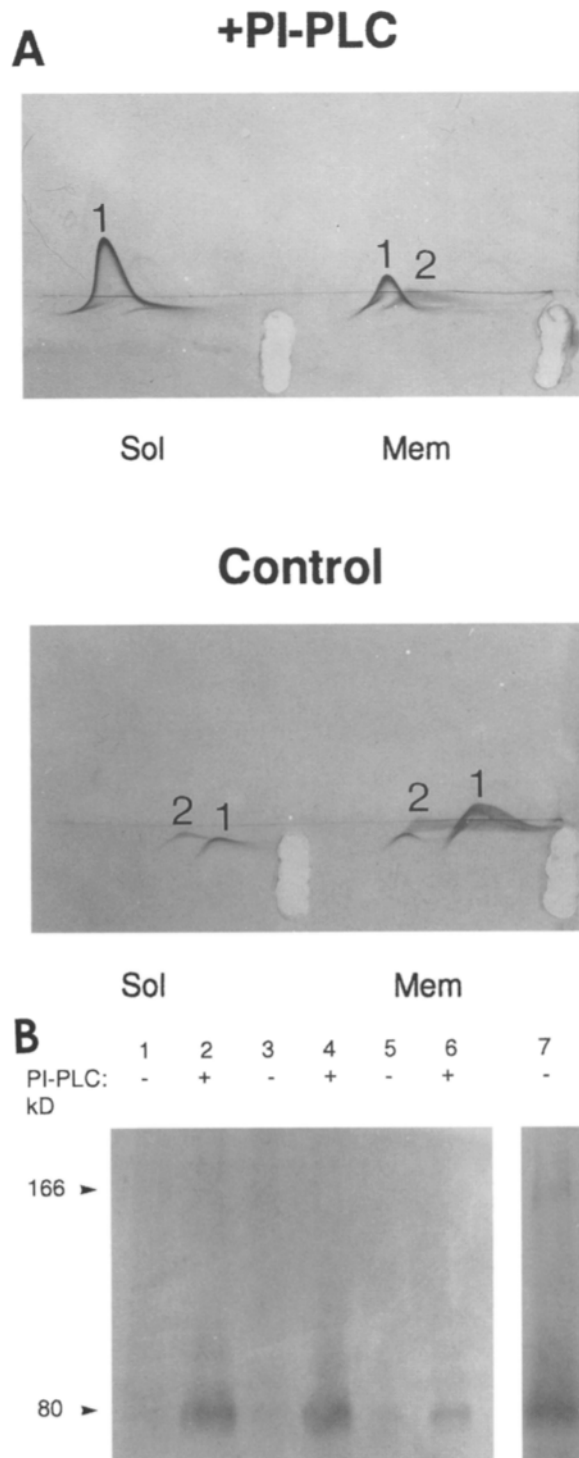
immunoelectrophoresis. After electrophoresis, immunoprecipitates of aminopeptidase N were identified by histochemical staining, followed by staining with Coomassie brilliant blue. (*1*) 80-kD protein. (*2*) aminopeptidase N. (*B*) Western blot of detergent-insoluble complexes, incubated in the presence (+) or absence (−) of PI-PLC as described above and subjected to SDS-PAGE. The primary antibody was either anti-cross reacting determinant, which specifically recognizes phospholipase-cleaved GPI-anchors (lanes *1–6*), or anti-detergent-insoluble complexes (lanes *7* and *8*). Samples of 25 μl (lanes *1* and *2* , *7* and *8*), 10 μl (lanes *3* and *4*), and 5 μl (lanes *5* and *6*) were applied to the gel. Molecular mass values (*kD*) are indicated.

## A

Chase (h):    0        6

| | S | I | S | I |

kD

166 ►
140 ►

80 ►

## 80-kD protein



1  2  3  4  5  6  7

## Aminopeptidase N



1  2  3  4  5  6  7

## B

Chase (h):    0       6

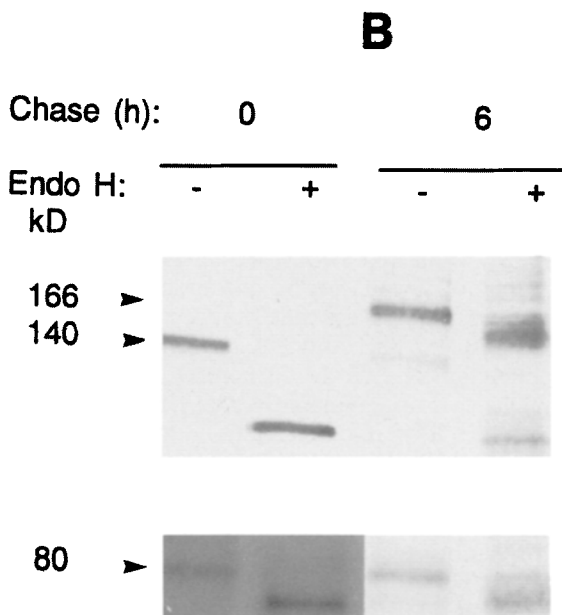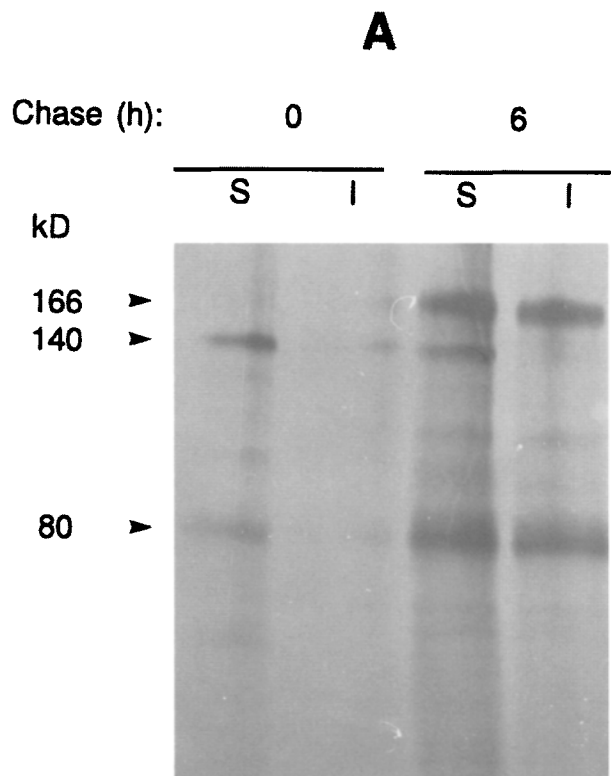Endo H:  −   +   −   +
kD

166 ►
140 ►

80 ►

*Figure 9.* Fetal mucosal explants in organ culture were labeled for 30 min with 400 μCi/ml of [$^{35}$S]methionine, followed by a chase of 0 or 6 h. (*A*) After culture, the 80-kD iron-binding protein and aminopeptidase N were immunopurified from the detergent-soluble (*S*) -and detergent-insoluble (*I*) membrane fractions of the explants by immuno-electrophoresis as described in the legend to Fig. 7. After electrophoresis, the immunoprecipitates were excised from the agarose gel and analyzed by SDS-PAGE. (*B*) After culture, the 80-kD iron-binding protein and aminopeptidase N were immunopurified and incubated in the presence (+) or absence (−) of endo H as described in Materials and Methods.
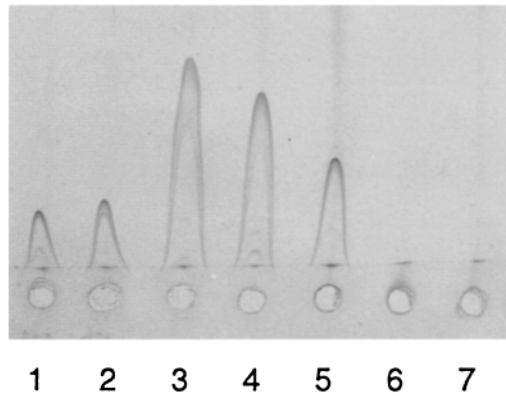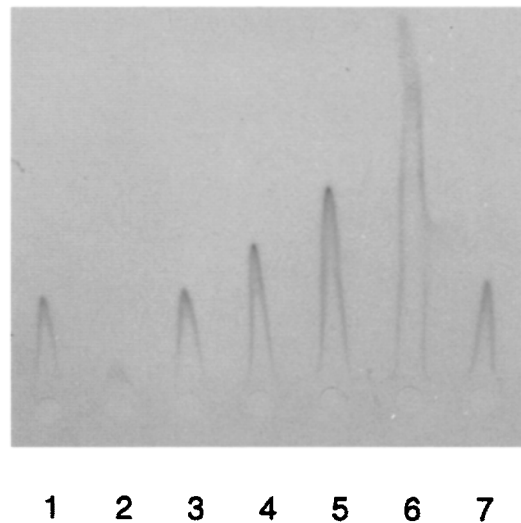
*Figure 10.* Pieces of fetal, neonatal and adult small intestine (200–400 mg wet weight) were extracted in 400 μl of 25 mM Hepes, 150 mM NaCl, 5% Triton X-100, 10 μg/ml aprotinin, pH 7.0, for 10 min at 37°C. After centrifugation at 5,000 *g*, 5 min, the cleared tissue extracts were diluted fourfold with the above buffer and analyzed by quantitative rocket immunoelectrophoresis against antibodies specific to the 80-kD iron-binding protein (*A*) or antibodies to detergent-insoluble complexes (*B*). Samples of 8–13 μl (each containing 1 mg of tissue extract) were applied to each well: *1* and *2*, fetuses with a crown-rump length of 14 cm or 25 cm, respectively; *3*, newborn piglet, delivered 7 d before term; *4*, newborn piglet delivered at term; *5*, 7-d-old piglet; *6*, 4.5-wk-old piglet, postweaned for 5 d; *7*, 3-mo-old pig. (*A*) The immunoprecipitates of the 80-kD iron-binding protein were stained with Coomassie brilliant blue. (*B*) The immunoprecipitates of aminopeptidase N were visualized by histochemical staining.

### Expression of the 80-kD Iron-binding Protein during Development

Fig. 10 shows a determination by quantitative rocket immunoelectrophoresis of the overall amounts of the 80-kD iron-binding protein present in fetal, neonatal, postweaned,

and adult intestine. The protein is present early in the fetal development (a crown-rump length of 14 cm corresponds to a gestational age of about 70 d), but reaches a maximum around the time of birth. It is still present in relatively high amounts in the intestine of the 7-d-old piglet, but is not detectable by this assay in mucosal extracts from the 4.5-wk-old piglet (postweaned for 5 d) or the 3-mo-old animal, showing that the expression declines sharply shortly after weaning. Like the 80-kD iron-binding protein, aminopeptidase N is present already in the fetal intestine, but in contrast to the former, expression of the peptidase persists after weaning (Fig. 10).

## Discussion

Glycolipids are essential components of detergent-insoluble complexes (van Meer and Berger, 1992), and in the microvillar membrane of porcine enterocytes, they constitute more than 30% of the total membrane lipid. The main components are dihexosylceramides containing galactose and pentohexosylceramides composed of fucose, galactose, glucose and hexosamines (Christiansen and Carlsen, 1981). The preparation of detergent-insoluble complexes from fetal intestine, involving extraction with Triton X-100 at low temperature followed by a density gradient centrifugation as the essential steps, lead to a remarkably high enrichment of a 80-kD protein, not seen in similar preparations from the small intestine of adult animals (Danielsen, 1995). Morphologically, the detergent-insoluble complexes resembled similar complexes previously isolated from MDCK cells by the same procedure (Brown and Rose, 1992), and subcellularly they must derive from the two locations where the 80-kD protein was principally found: the non-microvillar patches of the apical plasma membrane and the tubulo-vesicular structures and vacuoles in the apical cytoplasm.

It is likely that at least some of the microdomains at the apical cell surface, labeled by the anti-80-kD protein antibody, must represent target sites of the exocytic membrane traffic. In support of this interpretation, it has previously been shown by lateral mobility analysis that apically targeted GPI-anchored proteins in MDCK cells transiently reside in clusters before they achieve an even steady-state distribution (Hannan et al., 1993).

The apical tubulo-vesicular structures and vacuoles labeled by the anti-80–kD antibody are characteristic endocytic compartments of the fetal and neonatal enterocytes (Wilson et al., 1991; Kömüves and Heath, 1992; Danielsen et al., 1995). This, taken together with the frequently observed noncaveolar plasma membrane invaginations labeled by the anti-80–kD protein antibody suggests to us that some of these patches may represent restricted areas of the apical membrane involved in endocytosis. Until recently, detergent-insoluble complexes were commonly thought to be derived from caveolae which are not usually associated with endocytosis; according to the potocytic mechanism of internalization, generally ascribed to caveolae, the membrane invaginations never pinch off from the cell surface to form endocytic vesicles (Anderson et al., 1992; van Deurs et al., 1993). In spite of this, caveolae have nevertheless been reported to undergo regulated internalization (Parton et al., 1994).

However, we consistently failed to detect any caveolae at the 80-kD protein-containing microdomains of the apical cell surface. A similar lack of correlation between detergent insolubility and caveolae has been reported for a GPI-anchored protein, Thy-1, at the surface of lymphocytes; this cell type lacks caveolae and does not express caveolin either at the protein, nor the mRNA level (Fra et al., 1994). Along these lines, detergent-insoluble complexes isolated from a mouse neuroblastoma cell line were recently observed to contain GPI-anchored proteins as well as heterotrimeric G proteins and tyrosine kinases, despite a lack of morphologically identifiable caveolae and caveolin expression (Gorodinsky et al., 1995). Mayor and Maxfield (1995), studying the cell surface localization of GPI-anchored proteins, observed that these redistribute after detergent-treatment and cautioned that their association with caveolae and signalling proteins must be critically reexamined. A likely conclusion to draw from this is that glycolipid microdomains are a more general and widespread phenomenon than previously thought.

The transmembrane-anchored aminopeptidase N was also significantly enriched in the detergent-insoluble complexes although overall, it was found to be markedly less insoluble than the 80-kD protein. In fetal enterocytes, aminopeptidase N is also internalized and can be co-localized with cationized ferritin in plasma membrane invaginations and seen as well in the apical tubulo-vesicular structures and vacuoles (Danielsen et al., 1995). Unlike the 80-kD protein, however, aminopeptidase N was found evenly distributed over the entire apical cell surface including the microvilli. This difference probably reflects a lower affinity of a transmembrane-anchored protein for the glycolipid-rich microdomains.

The NH$_2$-terminal amino acid sequence, iron-binding capacity, partial immunological cross-reactivity with serum transferrin and molecular weight of the 80-kD protein showed it to belong to the transferrin family of iron-binding proteins. Within this family, it shared the highest sequence homology with human melanotransferrin, also known as the melanoma-associated antigen p97 (Woodbury et al., 1980). Based on its mRNA sequence, this protein was originally predicted to be anchored to the membrane by a stretch of 25 predominantly uncharged and hydrophobic residues near the COOH terminus (Rose et al., 1986). Recently, however, melanotransferrin was proposed to be GPI-anchored, rather than by a transmembrane amino acid sequence, based on its solubilization with phosphatidylinositol-specific phospholipase C, biosynthetic labeling with radioactive ethanolamine and partitioning in Triton X-114 (Food et al., 1994). The resistance of the 80-kD iron-binding protein to carbonate extraction at pH 11 and its solubilization by phospholipase likewise bears the hallmarks of a GPI-anchored integral membrane protein, and - since melanotransferrin is the only known member of the transferrin family bearing a glycolipid anchor, we take the porcine small intestinal 80-kD iron-binding protein described in this work to be homologous to human melanotransferrin. Like serum transferrin, melanotransferrin is composed of two homologous NH$_2$- and COOH-terminal domains (46% amino acid sequence homology) which have evolved by gene duplication, but unlike the other members of the transferrin family, only its NH$_2$-ter-

minal domain harbors a functional iron-binding site (Baker et al., 1992). Its overall sequence homology to other members of the transferrin family (human serum transferrin, human lactoferrin and chicken ovotransferrin) is only 37–39%, suggesting that phylogenetically melanotransferrin diverged from serum transferrin some 390 million years ago, before the divergence of the mammalian and avian lineages (Baldwin, 1993).

A functional role as translocator of iron was proposed for melanotransferrin when its structural resemblance with transferrin was first discovered (Brown et al., 1982). Apart from neoplastic tissues, however, most normal adult tissues only express low amounts of melanotransferrin; significant levels of the protein were only reported in a few fetal organs, in particular in the colon, but also in the umbilical cord and the heart (Brown et al., 1981). The finding of the present work that the 80-kD iron-binding protein is abundantly expressed in the small intestine particularly around the time of birth raises the possibility that it might be involved in the neonate's uptake of iron from mothers milk. Although a great deal is known about factors affecting dietary iron absorption and iron metabolism in general, the exact molecular mechanism(s) responsible for iron transport across the intestinal epithelium is not well understood (Conrad, 1987). Whatever the basic mechanism of iron uptake might be, the 80-kD iron-binding protein seems well suited to act as an enhancer of the neonate's absorption of iron from mother's milk which only contains low concentrations of iron (Iyer and Lönnerdal, 1993): it is strategically located in abundant amounts in microdomains at the apical surface of the enterocyte, and its significant presence in the apical vacuoles testifies to an ongoing internalization.

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