Intelectin: A Novel Lipid Raft-Associated Protein in the Enterocyte Brush Border[†]

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Received March 22, 2006; Revised Manuscript Received June 1, 2006

ABSTRACT: Intelectin is a mammalian Ca^{2+}-dependent, D-galactosyl-specific lectin expressed in Paneth and goblet cells of the small intestine and proposed to serve a protective role in the innate immune response to parasite infection. In addition, it is structurally identical to the intestinal lactoferrin receptor known to reside in the enterocyte brush border. To clarify this apparent discrepancy with regard to localization, the aim of this work was to study the cellular and subcellular distribution of small intestinal intelectin by immunofluorescence and immunogold electron microscopy. Secretory granules of lysozyme-positive Paneth cells in the bottom of the crypts as well as goblet cells along the crypt−villus axis were intensively labeled with intelectin antibodies, but quantitatively, the major site of intelectin deposition was the enterocyte brush border. This membrane is organized in stable glycolipid-based lipid raft microdomains, and like the divalent lectin galectin-4, intelectin was enriched in microvillar "superrafts", i.e., membranes that resist solubilization with Triton X-100 at 37 °C. This strategic localization suggests that the trimeric intelectin, like galectin-4, serves as an organizer and stabilizer of the brush border membrane, preventing loss of digestive enzymes to the gut lumen and protecting the glycolipid microdomains from pathogens.

The small intestinal brush border is specifically designed to act both as a high-throughput digestive/absorptive surface and as a permeability barrier preventing lumenal pathogens from gaining access to the organism (*1*, *2*). To accomplish these tasks, the microvillar membrane is organized into stable lipid raft microdomains, mainly by virtue of its high content of glycolipids (*3*, *4*) and galectin-4, a divalent β-galactoside-recognizing lectin able to cross-link glycoconjugates (*5*, *6*). The extraordinary stability of these microdomains has been documented by their resistance to extraction with the detergent Triton X-100 at 37 °C, which induces them to form "superraft" membranes (*7*). Together, these superraft components are able to cluster many of the microvillar digestive hydrolases and thereby minimize their loss to the gut lumen due to proteolytic and lipolytic activities and exposure to bile salts (*8*). Unfortunately, the high-density glycolipid-based microdomains may also serve as portals for a large number of pathogens that specifically recognize glycolipids in their initial contact with a target epithelial cell (*9*−*13*). As the first line of defense against such pathogens, the invaded organism relies on secretion of antibodies (*14*), and "anti-glycosyl" antibodies, i.e., antibodies induced in the host by a glycosyl antigen (*15*), were recently shown to be deposited in the intestinal brush border (*16*). These lectin-like antibodies were thought to offer mucosal protection by competing with pathogens for binding sites at the brush border having terminal galactosyl residues.

In addition to galectin-4 and anti-glycosyl antibodies, other carbohydrate-recognizing proteins may well participate in mucosal brush border protection and pathogen surveillance. Thus, intelectin is a lectin expressed in the digestive tract. Its cloning and sequencing from the mouse revealed it to be homologous to *Xenopus laevis* lectin XL35, a Ca^{2+}-dependent, D-galactosyl-specific lectin (*17*, *18*). By in situ hybridization, intelectin expression was demonstrated specifically in Paneth cells of the small intestinal crypts (*19*). More recently, human intelectin was cloned and shown to have a high level of homology with the mouse protein (*20*). Human intelectin was affinity purified by chromatography on galactose-Sepharose, and its lectin properties also revealed an affinity for D-pentoses and a D-galactofuranosyl residue in the presence of Ca^{2+}. Intelectin has since been shown to exist in two isoforms in the small intestine, intelectin-1 and -2, whose amino acid sequences are 91% identical (*21*, *22*). Interestingly, intelectin-2 is expressed in goblet cells and is induced by infection with the nematode *Trichinella spiralis*, suggesting a protective role for this isoform in the innate immune response to parasite infection (*22*, *23*).

The enterocyte brush border membrane has long been known to harbor a receptor for lactoferrin, the member of the transferrin family of iron-binding proteins principally found in milk (*24*). Interestingly, the cloning and sequencing of the human small intestinal lactoferrin receptor (LfR)[1] have revealed its total identity with human intelectin (*20*, *25*).

[†] This work was supported by grants from the Danish Medical Research Council, the Novo-Nordic Foundation, the Beckett Foundation, and the Augustinus Foundation.

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[1] Abbreviations: DRM, detergent-resistant membrane; GPI, glycosylphosphatidylinositol; LfR, lactoferrin receptor; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi060570x CCC: $33.50
Published on Web 07/06/2006

Whereas some of the non-immune, antimicrobial functions ascribed independently to intelectin and LfR can easily be reconciled, the available data concerning the precise mucosal localization (enterocytes vs Paneth and goblet cells) of the two proteins are conflicting. This work was therefore undertaken to study in greater detail the cellular and subcellular distribution of intelectin in the small intestine. By immunofluorescence and immunogold electron microscopy, intelectin was localized both to Paneth cells in the bottom of the crypts and to goblet cells along the entire crypt−villus axis. Quantitatively, however, the brush border of the epithelium was the predominant site of intelectin deposition in the small intestine. Furthermore, intelectin was found to be a major component of microvillar lipid rafts and superrafts. This observation thus highlights the strategic importance of these glycolipid-rich microdomains in the mucosal defense against microorganisms.

MATERIALS AND METHODS

Materials. The rabbit antibodies to human intelectin (hIntL) were described previously (*20*). Briefly, rabbits were immunized with hIntL-transfected RK-13 cells with complete Freund's adjuvant. Immunoglobulins were isolated from the antiserum by ammonium sulfate fractionation, and the hIntL-specific antibodies were purified by affinity chromatography on recombinant hIntL bound to Affi-Gel 10 (Bio-Rad). Sheep antibodies to lysozyme were obtained from Abcam (Cambridge, U.K.), goat antibodies to annexin A2 from Santa Cruz (Santa Cruz, CA), rabbit antibodies to alkaline phosphatase from Biogenesis (Poole, U.K.), and rabbit antibodies to human lactoferrin and secondary antibodies for immunogold labeling from Dako Cytomation (Glostrup, Denmark). Secondary Alexa 488/594-conjugated antibodies were from Molecular Probes (Eugene, OR), and human lactoferrin and FITC-conjugated antibodies to human lactoferrin were from ICN (Costa Mesa, CA).

Pig and mouse small intestines were kindly provided by the Department of Experimental Medicine, Panum Institute, Copenhagen, Denmark. Paraffin-embedded pig kidney cortex was kindly given by C. Ørskov (Department of Medical Anatomy, Panum Institute).

Lactoferrin Binding and Uptake Studies in Organ-Cultured Mucosal Explant. The small intestines of adult mice, fasted overnight, were rinsed in MEM medium, and mucosal explants were excised and cultured in MEM medium essentially as described previously (26). The explants were cultured for 30 min at 4 or 37 °C in the presence of lactoferrin (0.5 mg/mL) in MEM medium, adjusted to pH 6.5. Control explants were cultured in the absence of lactoferrin in parallel.

Fluorescence Microscopy. The mucosal explants were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) (PB) for 2 h at 4 °C. After being rinsed in PB, the explants were frozen in precooled 2-methylbutane and mounted on a precooled cryostat table. Sections were cut in a Leitz cryostat at −20 °C, collected on glass slides, and incubated with a FITC-conjugated antibody to human lactoferrin.

In other experiments, sections of small intestine and kidney cortex were fixed as described above and embedded in paraffin. Rehydrated paraffin sections were incubated with primary antibodies (anti-intelectin, anti-lysozyme, and anti-aminopeptidase N), followed by fluorescent secondary antibodies. Control experiments with omission of primary antibodies were routinely performed in parallel. After being labeled, the sections were mounted in antifade mounting medium (DAKO, Glostrup, Denmark) and finally examined with a Leica DM 4000 B microscope equipped with a Leica DC 300 FX camera.

Immunogold Electron Microscopy. For Epon sectioning, pieces of pig small intestines were fixed in a 3% glutaraldehyde/2% paraformaldehyde mixture in PB for 30 min at 4 °C and membrane pellets of microvillar vesicles and superrafts in 2.5% glutaraldehyde in PB for 2 h at 4 °C.

After being rinsed in PB, the sections were postfixed in 1% osmium tetroxide in PB for 1 h at 4 °C, dehydrated in acetone, and finally embedded in Epon. Ultrathin sections were cut in an LKB Ultrotome III ultramicrotome, and the sections were incubated with anti-intelectin antibodies followed by labeling with 13 nm gold particles, prepared according to the method of Slot and Geuze (*27*) and conjugated to anti-rabbit immunoglobulins, as previously described (*28*). Control experiments with omission of the primary antibodies were performed in parallel. The sections were stained in 1% uranyl acetate in H_2O and lead citrate.

For ultracryosectioning, pieces of pig small intestine were fixed in a 2% paraformaldehyde/0.1% glutaraldehyde mixture in PB for 2 h at 4 °C, and mouse small intestinal mucosal explants cultured for 30 min at 4 or 37 °C in the presence of lactoferrin were fixed in 4% paraformaldehyde in PB for 2 h at 4 °C. After being rinsed in PB, the tissues were immersed in 2.3 M sucrose overnight, mounted on top of a metal pin, and frozen in liquid nitrogen. Ultracryosections were cut in a RMC MT 6000-XL ultracryomicrotome, collected using a sucrose droplet, and attached to Formvar-coated nickel grids. Immunogold labeling was performed using antibodies against intelectin and lactoferrin as previously described (*29*).

Finally, the Epon and ultracryosections were examined with a Zeiss EM 900 electron microscope equipped with a Mega View camera system.

Subcellular Fractionation. Microvillar vesicles were prepared from mouse and pig small intestine by the divalent cation precipitation method (*30*). Briefly, mucosal scrapings were homogenized in 2 mM Tris-HCl and 50 mM mannitol (pH 7.1) containing 10 μg/mL aprotinin and leupeptin by using a manually operated Potter-Elvehjem homogenizer. The homogenate was cleared by centrifugation at 500*g* for 10 min, and $MgCl_2$ was added to a final concentration of 10 mM. After 15 min on ice, the preparation was centrifuged at 1500*g* for 10 min to pellet intracellular and basolateral membranes. The supernatant was collected and centrifuged at 48000*g* for 1 h to obtain a pellet of microvillar membrane vesicles and a supernatant of soluble proteins.

For preparation of lipid rafts ("DRMs"), microvillar membranes were resuspended in 1 mL of HEPES-HCl and 150 mM NaCl (pH 7.1) containing 10 μg/mL aprotinin and leupeptin and extracted with 1% Triton X-100 for 10 min on ice. DRMs were isolated by sucrose gradient ultracentrifugation as described previously (*31*) with the modification that the extract was placed in a 60% sucrose cushion with a 50 to 25% sucrose gradient layered on top. The extract was centrifuged in an SW40 Ti rotor (Beckman Instruments, Palo

Alto, CA) for 20−22 h at 35 000 rpm (g_{max} = 217 000), as described previously (*32*). After centrifugation, the floating DRMs were carefully collected with a pipet, diluted five times with 25 mM HEPES-HCl and 150 mM NaCl (pH 7.1), and centrifuged at 48000*g* for 1 h to obtain a pellet of DRMs. For preparation of superrafts, DRMs were subsequently resuspended in 25 mM HEPES-HCl and 150 mM NaCl (pH 7.1) and extracted with 1% Triton X-100 for 10 min at room temperature, followed by reextraction at 37 °C, as previously described (*7*).

SDS−PAGE and Immunoblotting. SDS−PAGE in 15% gels was performed as described previously (*33*). After electrophoresis and electrotransfer of proteins onto Immobilon PVDF membranes, immunoblotting was performed with antibodies to alkaline phosphatase and annexin A2. The blots were developed with an electrochemiluminescence detection reagent kit according to the protocol supplied by the manufacturer (Amersham Biosciences, Little Chalfont, U.K.). After immunoblotting, total protein was visualized by staining with Coomassie brilliant blue R250 [0.2% dissolved in a methanol/H_2O/acetic acid mixture (50:43:7) for 1 min], followed by destaining in the same solvent for 30 min.

MALDI-TOF Analysis. Superraft membranes, prepared from mouse microvillar membrane vesicles, were subjected to SDS−PAGE in a 15% gel and electrotransferred onto an Immobilon PVDF membrane. After protein staining, a 35−40 kDa band was carefully excised and submitted to commercial MALDI-TOF analysis (Alphalyse, Odense, Denmark). Six peptides were identified, matching the amino acid sequences of residues 51−59, 156−163, 190−198, 230−239, 302−313, and 303−313 of intelectin (molecular mass of 34 995 Da) of *Mus musculus*. In addition, two bands of ∼15−20 kDa were excised that generated peptides matching eight and 10 amino acid sequences, respectively, of galectin-4 (molecular mass of 36 602 Da) of *M. musculus*.

RESULTS

Immunofluorescence Localization of Intelectin. The intelectin gene was originally cloned from mice where it was shown by in situ hybridization to be expressed in intestinal Paneth cells (*19*), and it was proposed to function as a lectin because the deduced amino acid sequence revealed a level of homology with a *Xenopus laevis* oocyte lectin of 61% (*34*).

Figure 1A shows an immunofluorescence localization of intelectin along the crypt−villus axis of pig small intestine, using an immunopurified rabbit anti-human intelectin antibody previously described (*20*). The entire enterocyte brush border surface of the epithelium was distinctly labeled. In addition, an intense intracellular labeling was seen in goblet cells both in villi and in crypts. At higher magnifications, a faint labeling was also seen along the basolateral sides of the enterocytes as well as in a subapical region of the cells. For goblet cells, the intense labeling was confined to the apical pole of the cells (Figure 1B,C).

Paneth cell expression of intelectin was studied by double immunofluorescence labeling with the Paneth cell marker lysozyme (*35*−*37*). Along the epithelium, antibodies to lysozyme labeled only very few cells located near the bottom of the crypts (Figure 2A), but in addition, positive staining

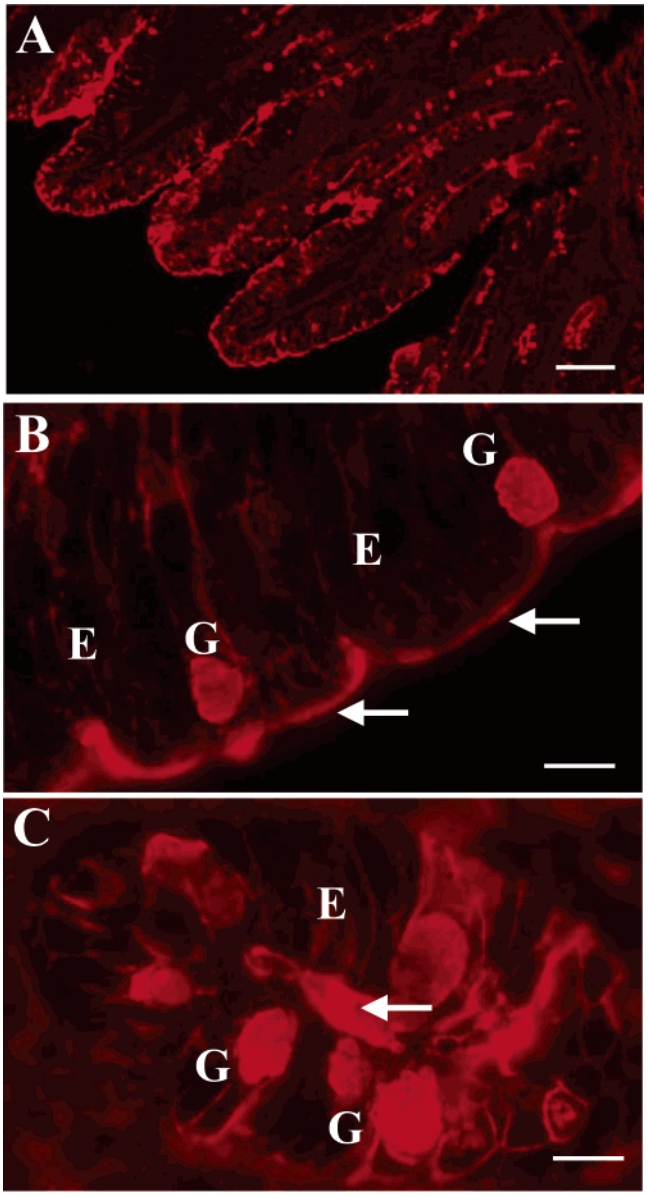

FIGURE 1: Localization of intelectin in the small intestine by immunofluorescence microscopy. (A) Longitudinal section of pig small intestinal mucosa labeled with anti-intelectin. A distinct staining of the brush border surface is seen along villi and crypts. In addition, goblet cells appear as strongly labeled dots along the entire crypt−villus axis. No labeling is present in the lamina propria region of the mucosa. (B and C) Higher-magnification images of the villus and crypt regions, respectively, showing labeling of goblet cells (G), enterocytes (E), and the brush border (arrows). Bars are 100 (A) and 10 μm (B and C).

was also found in a few cells of the lamina propria, as previously reported by others (data not shown) (*35*). As shown (Figure 2B,C), Paneth cells were clearly among the intelectin-positive cells located in the crypts, although they were clearly outnumbered by neighboring goblet cells.

Altogether, these results indicate that not only Paneth cells but also goblet cells, and possibly enterocytes, express intelectin. The complete absence of labeling of the lamina propria and the submucosa is indicative of the specificity of the antibody that was used. The antibody gave only a faint labeling of mouse intestinal epithelial cells (data not shown).

Intelectin expression was also studied by immunofluorescence microscopy in sections of kidney cortex. The kidney

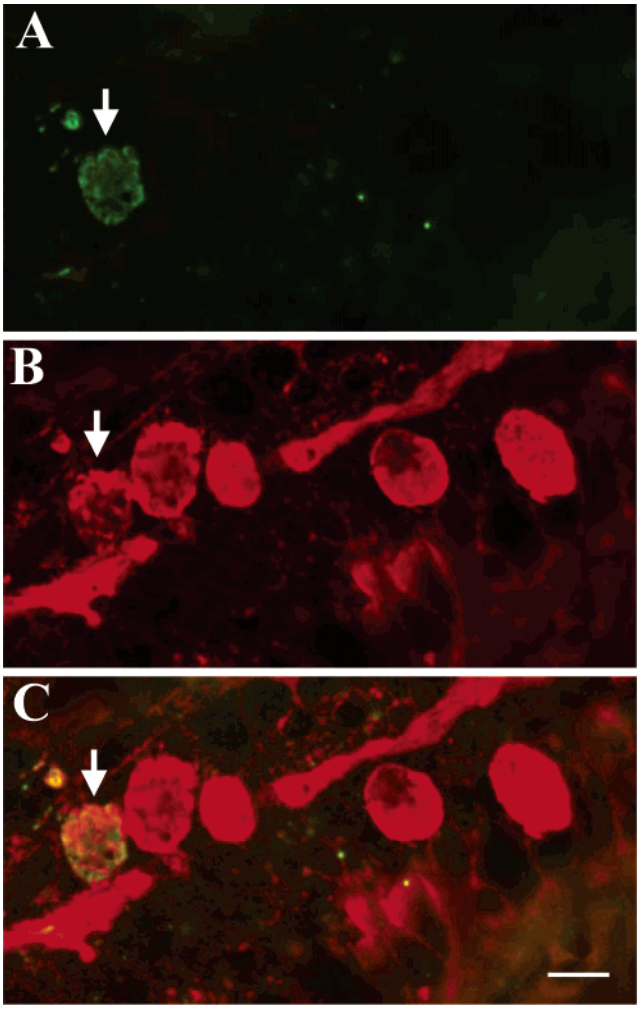

FIGURE 2: Double immunofluorescence labeling of lysozyme and intelectin. Crypt region labeled for lysozyme (A) and intelectin (B). Only one cell (arrow) in the section is lysozyme-positive, identifying it as a Paneth cell, whereas several cells as well as the luminal brush border are intelectin-positive. The merged image (C) shows that the Paneth cell is also labeled with the intelectin antibody. The bar is 10 μm.

proximal tubule cell resembles the enterocyte morphologically and expresses many of the intestinal brush border enzymes, including aminopeptidase N, and intelectin expression was likewise observed apically in this cell type (Figure 3). A similar labeling pattern for intelectin/LfR was previously demonstrated in mouse kidney by immunohistochemistry (*42*), and since gobletlike cells are not present in the kidney proximal tubule epithelium, this indicates that the proximal tubule cell itself synthesizes the protein.

Immunogold Localization of Intelectin in Enterocytes and Goblet Cells. A high-resolution subcellular localization of intelectin in the small intestine was performed by immunogold electron microscopy. Intense labeling was observed over the secretory granules, but endoplasmic reticulum membranes adjacent to the granular regions were also labeled (Figure 4A). Figure 4B shows the intense labeling of the secretory granules clustered in the apical pole of a goblet cell. The gold particles were evenly distributed inside the lumen of the granules, indicating that goblet cells express and secrete a soluble form of intelectin. A micrograph of the same cell taken at a higher magnification revealed an ongoing degranulation with microvilli from neighboring

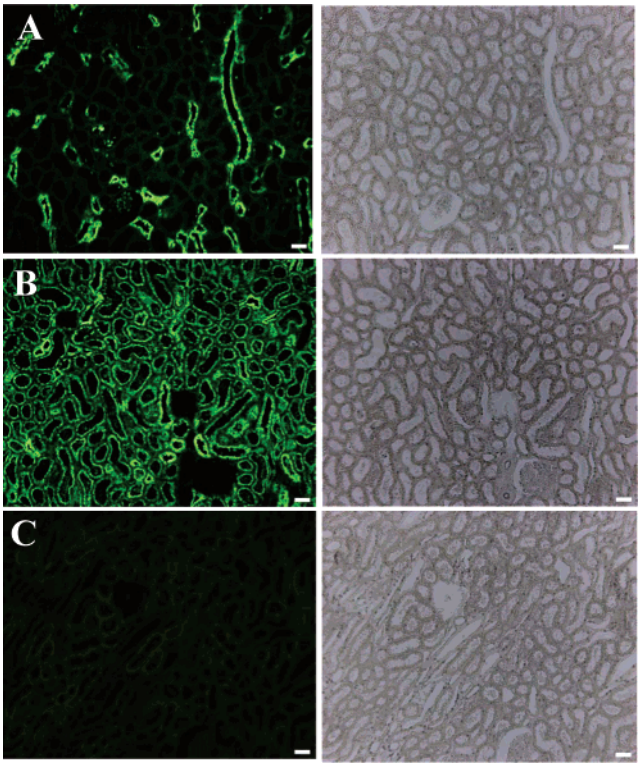

FIGURE 3: Localization of intelectin in the kidney. Paraffin-embedded sections of pig kidney cortex labeled for intelectin (A) and aminopeptidase N (B). For both proteins, the labeling was confined to the apical region of the proximal tubule cells, but unlike aminopeptidase N, intelectin was expressed in only a subset of the tubules. No labeling was detected when the primary antibody was omitted (C). The panels to the right show the corresponding sections recorded by incident light microscopy. The bar is 50 μm.

enterocytes in the proximity becoming decorated with intelectin in the process (Figure 4C).

The brush border membrane of enterocytes in both the crypt and villus region was also labeled with the anti-intelectin antibody (Figure 5), and a considerable variation in labeling intensity between neighboring enterocytes was occasionally observed (Figure 5A). Generally, labeling was seen along the entire length of the microvilli, and frequently, large aggregates of gold particles localized to invaginations between microvilli and to vesicle-like structures just below the apical surface (Figure 5B). Aggregates of gold particles were also present in multivesicular bodies in the subapical region, implying an ongoing internalization of intelectin (Figure 5C). A much weaker labeling was observed in the Golgi complex deeper in the cytoplasm of enterocytes (Figure 5D). No large aggregates of gold particles were observed in this compartment, and the labeling was confined to the membranes rather than to the lumen of the Golgi cisternae. Finally, a relatively faint labeling was observed along the basolateral surface of the enterocytes (data not shown).

In conclusion, the immunogold localization of intelectin confirmed its presence in both goblet cells and enterocytes. Although the granules of the goblet cells were by far the most intensely labeled structures, the enterocyte brush border membrane is a major site of intelectin deposition in the intestinal epithelium. The brush border intelectin most likely originates mainly from neighboring degranulating goblet cells. However, the faint but distinct labeling of the Golgi complex and basolateral surface of the enterocytes suggests

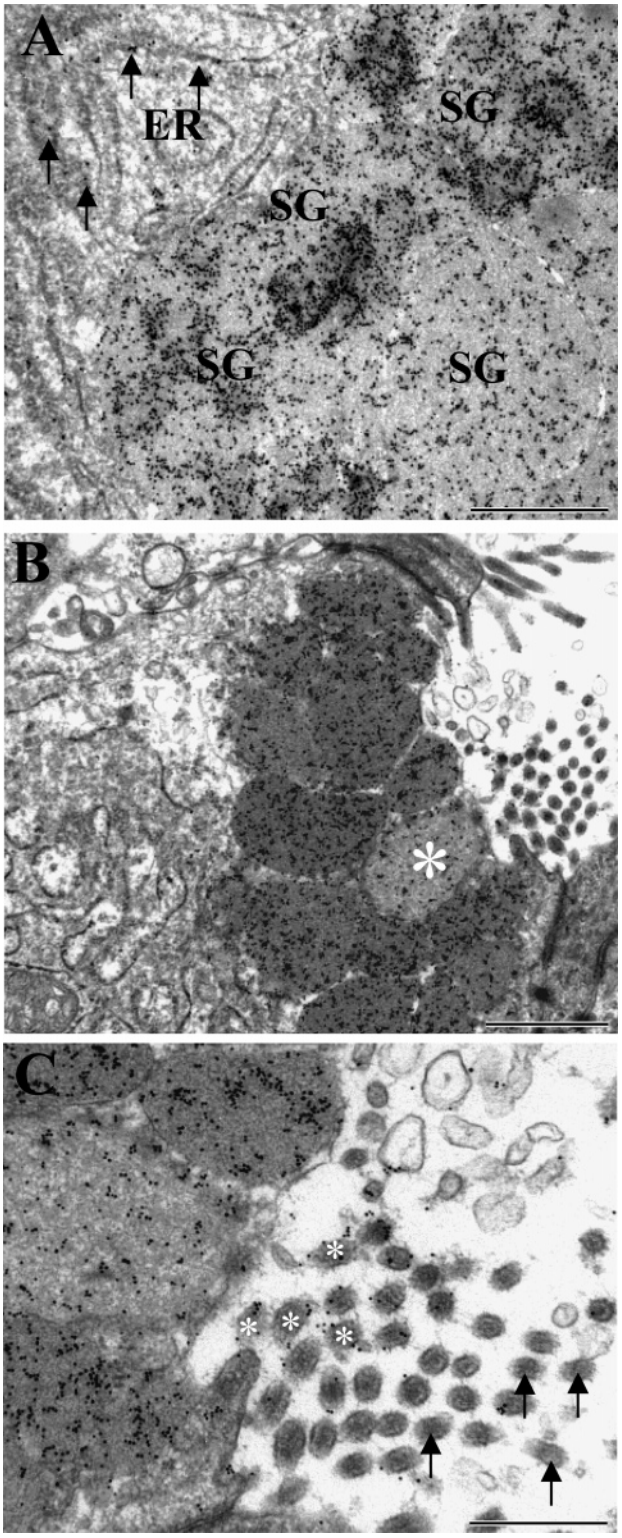

FIGURE 4: Immunogold localization of intelectin in goblet cells. (A) Electron micrograph showing labeling of endoplasmic reticulum (ER) membranes (indicated by arrows) adjacent to a heavily labeled region of secretory granules (SG). (B) Apical region of a goblet cell in the crypt region, containing several heavily labeled secretory granules. One granule at the cell surface (marked with an asterisk) is lighter in color than its neighboring granules and contains much less labeling, indicating an ongoing degranulation. (C) The microvilli of enterocytes in the proximity of the degranulating goblet cell (marked with asterisks) are decorated with gold particles, indicating a deposition of intelectin. By comparison, microvilli farther from the goblet cell show no or little labeling (arrows). Bars are 0.5 (A and C) and 1 μm (B).

that intelectin may also, at least in part, be synthesized and exocytosed by the enterocytes themselves.

Identification of Intelectin in Microvillar Superrafts. A superraft analysis of microvillar membrane vesicles was next performed to characterize the brush border association of intelectin. We have previously characterized a superraft fraction derived from pig intestinal microvillar DRMs by sequential extractions with Triton X-100, first at room temperature and then at 37 °C, finally yielding a membranous fraction that resists solubilization at physiological temperature (*7*). This microvillar subfraction was particularly enriched in glycolipids and the β-galactoside-recognizing lectin galectin-4, and the latter was proposed to function as a core lipid raft stabilizer/organizer for other, more loosely raft-associated proteins.

Figure 6 shows a superraft analysis of mouse microvillar membranes, prepared by the divalent cation precipitation method that yields a homogeneous population of closed, outside-out membrane vesicles (*30*). Two bands of ~15−20 kDa were by far the most predominant components of the superraft fraction, and both were identified by MALDI-TOF analysis as fragments of galectin-4 (36 kDa). Galectin-4 is a divalent lectin that contains two separate carbohydrate recognition domains each of ~130 amino acids, connected by a smaller linker peptide (*6*, *38*). None of the sequence-matching peptides obtained for either of the two bands (18 peptides in all) were localized to the central linker region, indicating that the full-length galectin-4 in the mouse is effectively cleaved into two separate carbohydrate recognition domains (data not shown). Mouse microvillar glycosylphosphatidylinositol-anchored alkaline phosphatase (~70 kDa) was highly enriched in the superraft fraction, as previously observed in the pig (*7*). Annexin A2 is another raft-associated protein that is present in intestinal microvillar as well as intracellular membranes (*39*), and although clearly detectable in superrafts, it was not enriched in this fraction relative to the microvillar- and Mg^{2+}-precipitated membranes (Figure 5), indicating that it does not belong to the core components of the lipid raft microdomains. In contrast, a band of 35−40 kDa was highly enriched in the superraft fraction, and by MALDI-TOF analysis, six sequence-matching peptides identified this protein as intelectin (peptide molecular mass of 35 kDa). Previously, a comparison of molecular mass values of hIntL under reducing and nonreducing conditions has indicated that the native intelectin is a homotrimer (*20*). The presence of intelectin in the microvillar superraft fraction therefore implies that it may serve as a lipid raft organizer/stabilizer like galectin-4 by cross-linking lipid and protein glycoconjugates at the brush border membrane.

Immunogold Localization of Intelectin in Microvillar Vesicles and Superrafts. Morphologically, an intestinal microvillar fraction appears as closed, outside-out membrane vesicles with a diameter of ~100 nm (Figure 7A). Superrafts also form vesicle-like structures, albeit of a more heterogeneous composition, and in addition, tubular or rodlike membranes are seen (Figure 7B), as previously described (*7*). Both microvillar vesicles and superrafts were prominently labeled by the intelectin antibody, confirming the presence of intelectin in the enterocyte brush border as well as the biochemical identification of intelectin as a major component of superrafts (Figure 6). The labeling density of

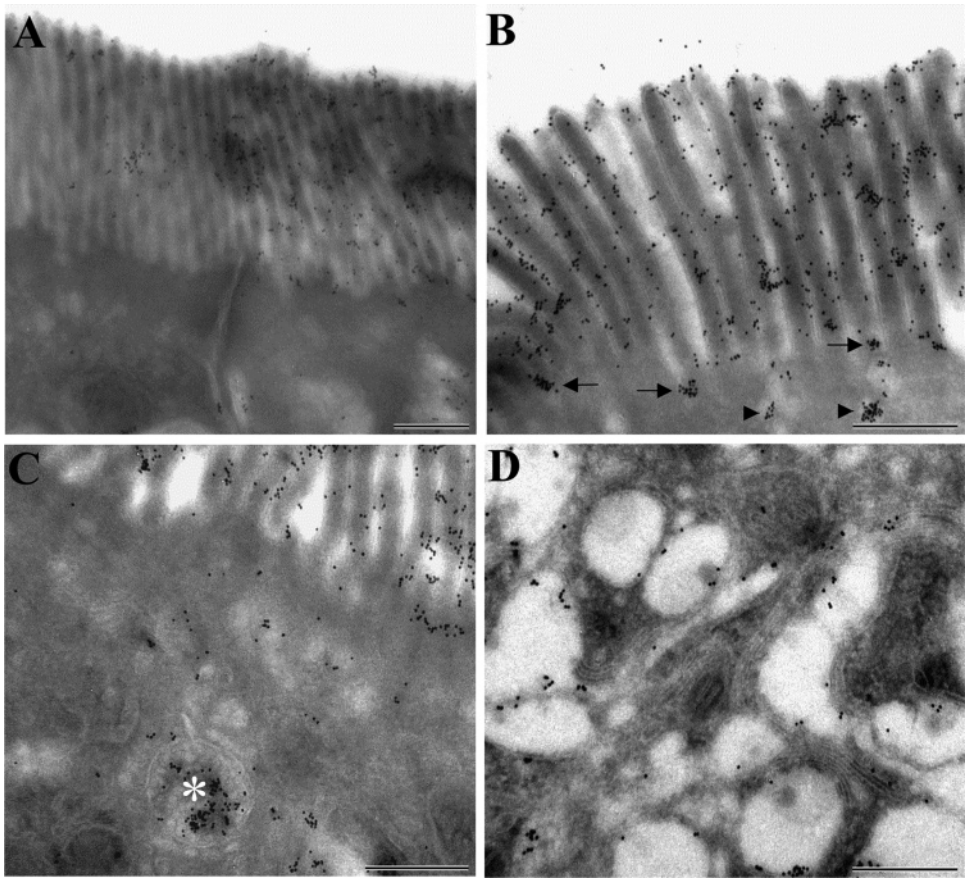

FIGURE 5: Immunogold localization of intelectin in enterocytes. (A) Electron micrograph showing the brush border of two neighboring enterocytes of which the one to the right is intensely labeled and the one to the left only weakly labeled. (B) Aggregates of gold particles are frequently seen in invaginations between microvilli (arrows) and in vesicle-like structures in the proximity of the apical cell surface (arrowheads). (C) A multivesicular body in the subapical region (asterisk) heavily labeled by gold particles. (D) Labeling of membranes in the Golgi complex region. Bars are 1 (A) and 0.5 μm (B−D).

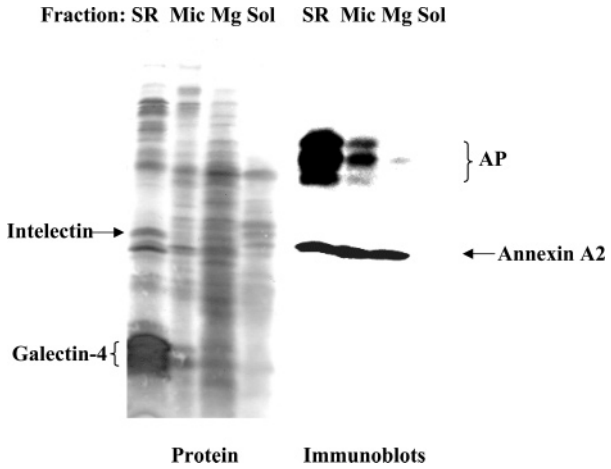

FIGURE 6: Intelectin is enriched in superrafts from microvillar membranes. Mouse intestinal mucosa was fractionated into microvillar membranes (Mic), Mg^{2+}-precipitated (basolateral and intracellular) membranes (Mg), soluble proteins (Sol), and microvillar superraft (SR) membranes prepared as described in Materials and Methods. After SDS−PAGE and transfer onto Immobilon, alkaline phosphatase (AP) and annexin A2 were visualized by immunoblotting. After protein staining, a distinct band of 35−40 kDa and two intense bands of 15−20 kDa were carefully excised and submitted to MALDI-TOF analysis. The former was identified as intelectin and the latter as fragments of galectin-4.

the superraft membranes was not much different from that of the microvillar membranes, despite the enrichment seen in SDS−PAGE. Most likely, this is explained by a previous observation that raft lipids (cholesterol and glycolipids) are only moderately enriched in superrafts (~1.2−1.5-fold), indicating that it is mainly proteins and not lipids that are removed from the membranes by the sequential detergent extractions at increasing temperatures (*7*).

Binding and Uptake of Lactoferrin. Human lactoferrin is known to bind to the mouse LfR (*40*), and as shown in Figure 8A, this ligand bound to the lumenal surface of intestinal explants cultured for 30 min at 4 °C. The entire brush border was labeled with lactoferrin with punctate strong labeling occasionally seen at the tip of the microvilli. When the explant was cultured for 30 min at 37 °C, a temperature that permits endocytosis, the punctate labeling was much more pronounced, and some of the clustering appeared below the apical surface (Figure 8B). No labeling below the subapical region of the enterocytes was detectable.

Immunogold electron microscopy confirmed the binding of lactoferrin to the enterocyte brush border with clustering at the tip of the microvilli at 4 °C (Figure 9A). No intracellular labeling was detected at this temperature. At 37 °C, the clusters of gold particles typically appeared at the base of the microvilli (Figure 9B) and in subapical endosomal structures (Figure 9C). This pattern of labeling resembles that of intelectin (Figure 5), suggesting that lactoferrin, after binding to the brush border, is clustered and eventually internalized together with its receptor.

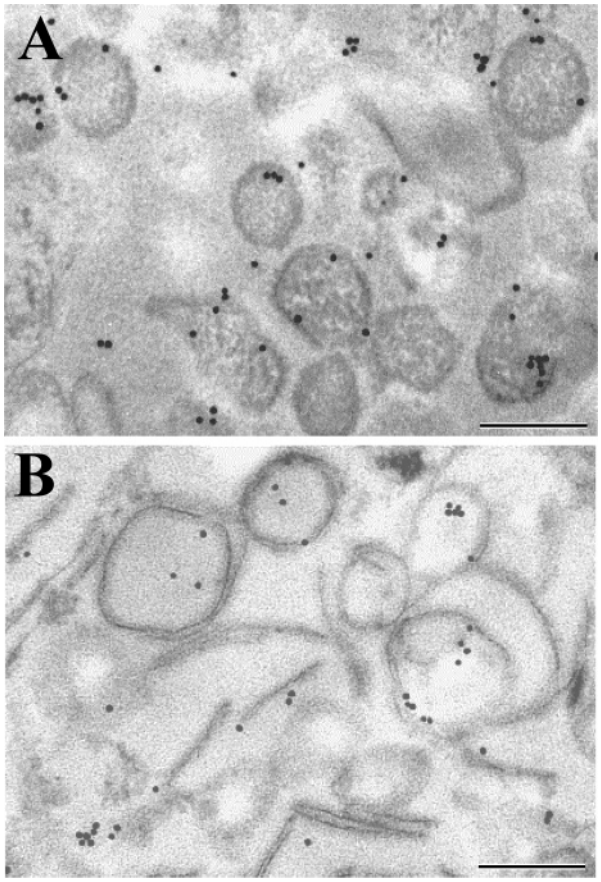

FIGURE 7: Immunogold localization of intelectin in microvillar vesicles and superrafts. Electron micrographs showing intelectin labeling of microvillar membrane vesicles (A) and superraft membranes (B). Bars are 100 nm.

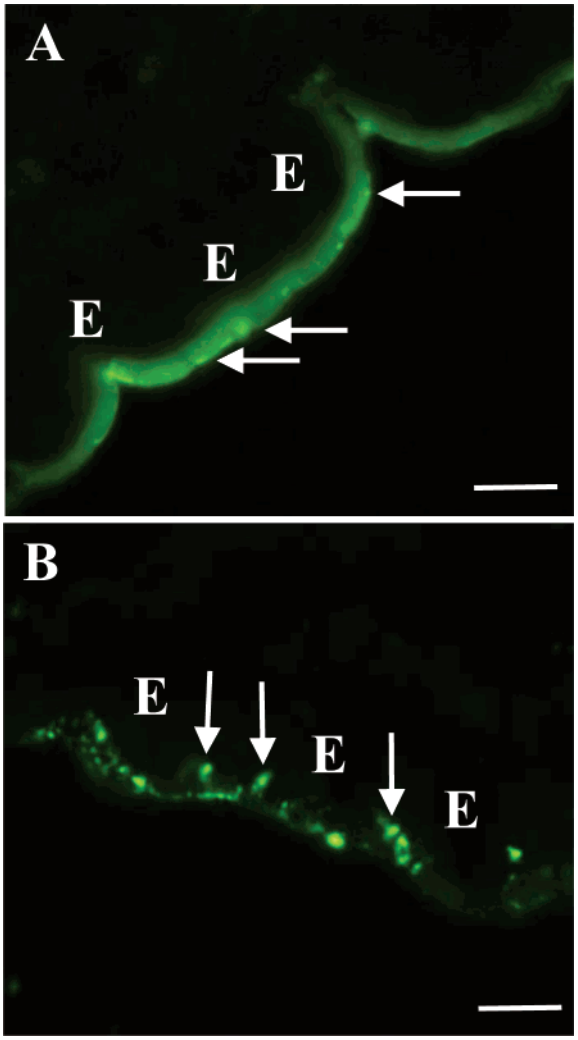

FIGURE 8: Binding and uptake of lactoferrin by enterocytes. Mouse small intestinal explants were cultured for 30 min in the presence of 0.5 mg/mL lactoferrin at 4 (A) or 37 °C (B). After being washed in fresh MEM medium, the explants were fixed in 4% paraformaldehyde and prepared for immunofluorescence microscopy as described in Materials and Methods. At 4 °C, lactoferrin bound to the entire lumenal surface with punctate of bright fluorescence aligned at the rim of the brush border (arrows). At 37 °C, the punctate lactoferrin labeling was more pronounced and in some cases appeared below the apical surface (arrows). E denotes enterocytes. Bars are 10 μm.

DISCUSSION

The results of this work demonstrate that intelectin is present in both Paneth and goblet cells as well as in the enterocyte brush border. The intense immunolabeling observed in the secretory granules of the former two cell types agrees well with the initial identification of intelectin in Paneth cells of the crypts (*19*) and the observed inducible expression of the intelectin-2 isoform in goblet cells of the BALB/c mouse (*21*, *22*), but to our knowledge, localization of intelectin in the enterocyte brush border has not previously been reported. In contrast, LfR has long been recognized as a brush border protein (*40*−*42*), whereas its secretion from neither Paneth nor goblet cells, to our knowledge, has been reported. The immunofluorescence labeling pattern indicated that the brush border is indeed the major site of intelectin deposition, but most likely, the brush border intelectin originates, at least in part, from goblet cells. Like lumenally secreted lectin-like anti-glycosyl antibodies (*16*), soluble goblet cell-derived intelectin most likely associates with lipid rafts in the enterocyte brush border due to the high density of its carbohydrate ligands in these microdomains. This interpretation is supported by a mouse in situ hybridization database showing the level of intelectin expression to be highest in the crypt region, which harbors the largest population of goblet cells, although expression in villi is also detectable (*43*). However, previous work on LfR indicates that the enterocytes themselves syntesize and transport the protein to the apical surface. Thus, in the enterocyte-like Caco-2 cell line which expresses low levels of LfR, a major part of the protein was released by phosphatidylinositol-specific phospholipase C, indicating a membrane insertion by a GPI anchor (*25*). In support of this observation, a hydrophobic consensus sequence for attachment of a GPI anchor is present in the C-terminus of the molecule (*25*). Enterocytes synthesize a number of brush border GPI-anchored proteins, including alkaline phosphatase (*44*) and melanotransferrin (*45*), showing that this cell type harbors the enzymes required for anchorage of GPI in the endoplasmic reticulum, but whether they synthesize a GPI-anchored form of the lactoferrin receptor remains to be investigated further. The distinct immunogold labeling over the Golgi complex membranes observed in this work at least suggests that enterocytes themselves contribute to the overall expression of intelectin in the brush border. Interestingly, melanotransferrin, expressed in the intestine only during fetal and neonatal life and presumably involved in uptake of iron, was

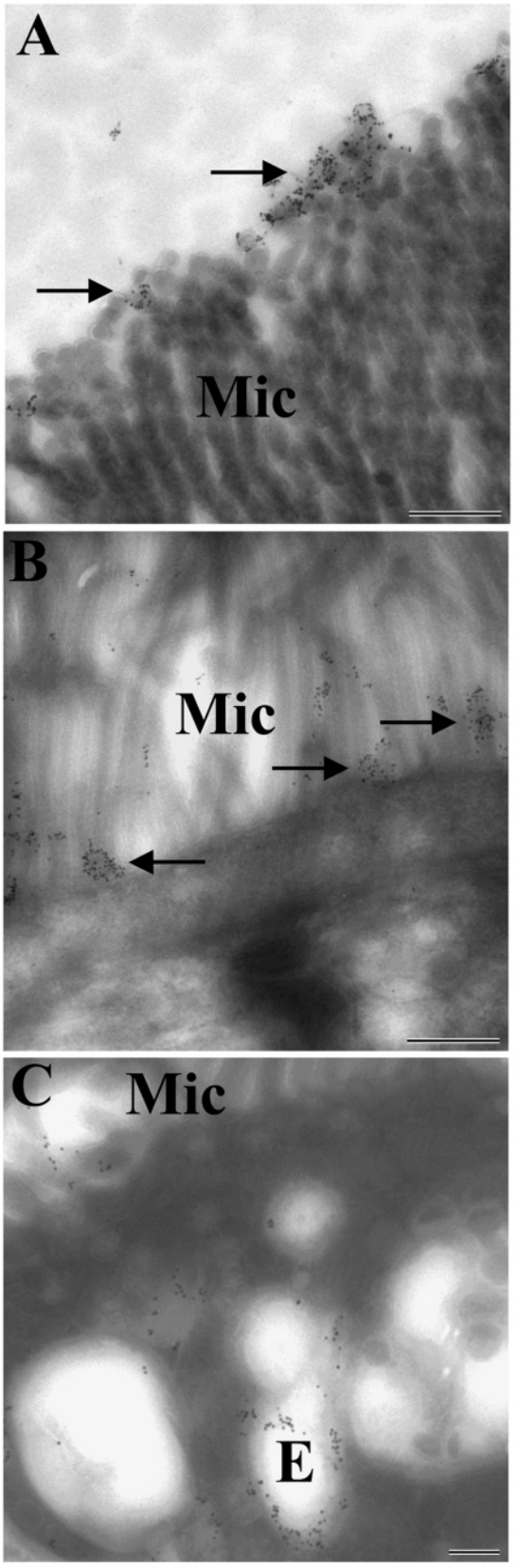

FIGURE 9: Immunogold localization of bound and endocytosed lactoferrin. Immunogold electron micrographs of the lactoferrin binding and uptake experiments shown in Figure 8. (A) After 30 min at 4 °C, clusters of gold particles (arrows) were seen at the tip of the microvilli (Mic). (B and C) After 30 min at 37 °C, the clusters of particles were present near the base of the microvilli (arrows) and in subapical endosomes (E). Bars are 0.5 (A and B) and 0.2 μm (C).

observed in apical endocytic vacuoles and tubulo-vesicular structures, suggesting that it undergoes internalization from the apical surface (*45*). The LfR also exhibits an age-dependent expression profile (*25*), but there are contradictory reports about whether it is endocytosed from the brush border. Thus, whereas lactoferrin was not internalized from the surface of HT 29-D4 cells (*46*), specific uptake from the apical surface of Caco-2 cells was demonstrated (*47*). Interestingly, the lactoferrin that is taken up is localized to the nuclei, in contrast to transferrin which accumulated in the cytoplasm (*47*). This work strongly argues that lactoferrin binds to the enterocyte brush border, and that at least a fraction of it concentrates in hot spots that are taken up by the absorptive cell. Clearly, further work is needed to characterize this lactoferrin internalization in greater detail, but we speculate that the apparently conflicting results may reflect the presence of two different molecular forms of intelectin/LfR: a GPI-anchored, internalization-competent form and a soluble secretory form that is capable of binding lactoferrin and other ligands but is unable to mediate cellular uptake. A putative role of internalized lactoferrin in the nucleus is to act as a transcription factor, possibly stimulating synthesis of proinflammatory cytokine interleukin 18 in the enhancement of the immune system (*24*, *48*, *49*), and at the cell surface, lactoferrin is able to exert a bacteriostatic effect by withholding iron (*50*, *51*). Along these lines, a differential expression of a GPI-anchored form of LfR made by enterocytes, and a soluble form, made by Paneth and goblet cells, could be a simple mechanism for ensuring the delivery of lactoferrin to its two different sites of function.

A common functional theme in the roles ascribed to intelectin and LfR is the mucosal protection against lumenal pathogenic microorganisms and parasites. Here, the essential lactoferrin-independent property seems to be the ability to bind a broad range of carbohydrate structures, including galactofuranose, which is contained in bacterial carbohydrate chains but is lacking in the mammalian host organism (*20*). Soluble defense lectins are generally thought to function as agglutinins, but intelectin reportedly appears to lack agglutination properties, possibly because this lectin does not form noncovalent multimers (*20*). However, the prominent anti-intelectin labeling of the brush border demonstrated in this work is suggestive of an alternative mucosal defense mechanism. Thus, glycolipids with terminal galactose residues, including galactosylceramide, lactosylceramide, and GM_1, are plentiful at the lumenal surface (*3*, *7*), and as shown in this work, much of the soluble intelectin secreted by goblet and Paneth cells is deposited here rather than released freely into the gut lumen. As observed recently for anti-glycosyl antibodies (*16*), intelectin may protect the brush border glycolipids from acting as pathogen receptors. In addition, like divalent galectin-4, trivalent intelectin should be capable of cross-linking lipid and protein glycoconjugates and thereby contribute to formation of stable microdomains, as exemplified by superrafts. In addition, this architecture helps to minimize the loss of digestive enzymes to the gut lumen (*7*), an important rescuing function for enzymes with reported half-lives as short as 5−8 h (*52*).

Very recently, the genomic structure of human omentin, a putative, new adipocytokine expressed in omental adipose tissue, was reported and revealed to be 100% identical to that of human intelectin (*53*). Interestingly, omentin expres-

sion was weak or absent in the majority of tissue probes obtained from patients suffering from Crohn's disease, suggesting a role for the protein in the defense against intestinal bacterial translocation in the context of this disease. This observation is consistent with the multiple defense functions attributed to intelectin/lactoferrin receptor and underscores the general importance of this protein.

ACKNOWLEDGMENT

Lise-Lotte Niels-Christiansen and Lissi Immerdal are thanked for expert technical assistance.

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BI060570X