Postnatal Maturation of Rat Small Intestinal Brush Border Membranes Correlates with Increase in Food Protein Binding Capacity

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To investigate maturation-related changes in membrane food protein binding capacity, we studied binding characteristics of brush border membranes isolated from small intestines of newborn and adult rats. Binding of biotinylated gliadin peptides, cow’s milk proteins (α-casein, β-lactoglobulin, α-lactalbumin, bovine serum albumin) and lectins was assessed by a sensitive chemiluminescence blot assay. We found specific food protein binding with regard to saturation and inhibition. Maximal binding of most food proteins and several lectins to brush border membranes of newborn and adult rats was comparable, whereas binding of β-lactoglobulin was substantially less. Common or adjoining binding sites for the different food proteins tested were indicated by corresponding membrane protein binding patterns and by inhibition properties of unrelated proteins. Compared to newborns, adult membrane vesicles as well as isolated membrane proteins showed higher binding capacities. Thus postnatal maturation of small intestinal brush border membranes correlated with increased food protein binding capacity.

KEY WORDS: brush border membrane; small intestine; maturation; food proteins; lectins.

In addition to their barrier function and participation in digestive, absorptive, and secretory processes, small intestinal enterocytes were proposed to contribute to mucosal immune reactions by presenting antigens coming from the gut lumen (1, 2). Binding of foreign proteins to the apical brush border membrane (BBM) was reported to facilitate uptake and to influence intracellular processing (3–5). Interactions of luminal molecules with the BBM also could result in signal transduction and cellular responses (6, 7).

Rats are a suitable model for studying developmental changes in BBM architecture and enterocytic functions, because profound changes occur during postnatal maturation. At the time of weaning, the shift from milk-based to carbohydrate-rich feeding is accompanied by changes of BBM, in enzyme activities, protein and lipid composition, glycosylation, and membrane fluidity (6, 8–10). Furthermore, the newborn rat mucosal barrier appears to be more leaky, as indicated by higher rates of endocytosis (11, 12). Enhanced interaction of proteins with BBM is supposed to contribute to the increased uptake seen in newborn rats (10).

Conflicting results were obtained earlier in our laboratory using different approaches to study intestinal food protein binding in vitro. In experiments using whole gut sacs, we found a maturation-related increase in binding and uptake of β-lactoglobulin and bovine serum albumin when comparing immature 18-day-old rats with mature adult rats (13). In contrast, a maturational decrease in the binding capaci-
ties of isolated BBM vesicles from newborn to adult age was demonstrated for α-lactalbumin, β-lactoglobulin, bovine serum albumin, and the gliadin peptide B3142 (14). Our aims, therefore, were to clarify whether enhanced BBM food protein binding occurs before weaning and to characterize membrane components that interact with food proteins. Food proteins of clinical importance (gliadin peptides, cow’s milk proteins, ovalbumin) and lectins (controls for specificity of binding and for detection of developmental changes in membrane glycosylation) were used.

MATERIALS AND METHODS

Chemicals. Peroxidase-conjugated streptavidin was obtained from Dianova (Hamburg, Germany). ECL blotting detection reagents were from Amersham (Braunschweig, Germany). Araldite was purchased from Serva (Heidelberg, Germany). All other chemicals of highest purity available were from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Food Proteins and Lectins. Cow’s milk proteins [α-casein (CAS), β-lactoglobulin (BLG), α-lactalbumin (ALA), bovine serum albumin (BSA)] and hen egg ovalbumin (OVA) were obtained from Sigma. Gliadin peptides (PT-GLI), free of contaminating proteases, were produced by peptic–trypsic digestion of gliadin as described elsewhere (15). Biotin-labeled lectins from Sambucus nigra (SNA), Arachis hypogaea (PNA), and Maackia amurensis (MAA) were from Boehringer (Mannheim, Germany). Ulex europaeus I lectin (UEA I) was purchased from Sigma. These lectins are specific for residues containing NeuNAcα2-6 Gal/NeuNAcα2-6 GalNac, Gal/NacGal, NeuNAcα2-3 Gal, and L-Fucα1-4 GlcNac, respectively.

Animals. Adult Sprague-Dawley rats, weighing 200–300 g, and timed-pregnant rats were obtained from Charles-River Wiga (Sulzfeld, Germany). They were kept on a gluten-free and cow’s milk protein-poor rat chow (diet no. C1078, Altromin, Lage, Germany). Newborn rats were allowed to suckle freely and were killed at the age of 24 hr. Adult rats were fasted one day prior to the study.

Brush Border Membrane Vesicles. For isolation of brush border membrane vesicles, whole small intestines of newborn rats and proximal halves of adult small intestines were removed and washed with cold physiological saline. Starting with homogenates of neonatal intestines or scrapings of adult intestinal mucosa, BBM vesicles were obtained by a modified Ca2+-precipitation technique as described before (16). For each preparation, small intestines of several newborn rats or mucosa scrapings of two adult rats were pooled. Activities of the BBM enzymes lactase, sucrase, alkaline phosphatase, and total maltase activity were determined according to Dahlqvist (17) and Bowers and McComb (18). One unit was defined as 1 μmol substrate hydrolyzed per minute under the experimental conditions. Specific enzyme activities were expressed as units per milligram of protein. Protein concentrations were measured using bovine serum albumin as standard (19).

Electron Microscopy. BBM vesicles were fixed in 2% (v/v) glutaraldehyde, 100 mM cacodylate buffer, pH 7.4, for 1 hr at 4°C and collected by centrifugation (30 min at 30,000 g). Pellets were washed three times with cacodylate buffer, pelleted, and postfixed in 1% (v/v) osmium tetroxide in cacodylate buffer for 1.5 hr at room temperature. After washing, the membranes were dehydrated stepwise in ethanol and treated with saturated uranyl acetate for contrast enhancement. Ultrathin sections were prepared from membrane vesicles embedded in Araldite, stained with lead citrate, and analyzed with a Zeiss EM10 electron microscope.

Biotinylation. Food proteins were labeled with biotin, using biotinamidocaproate-N-hydroxysuccinimide ester for cow’s milk proteins and biotinamidocaproate-N-hydroxysulfosuccinimide ester for gliadin peptides as described previously (15).

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Mini-Protean II electrophoresis system (Bio-Rad, Munich, Germany) according to Laemmli (20) under non-reducing conditions.

Western Blot. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed by tank blotting as described before (21), using the Mini Trans-Blot electrophoresis transfer cell from Bio-Rad. To enhance transfer, SDS was added in a final concentration of 0.05% (w/v) to the electrode buffer.

Chemiluminescence Binding Assay. Intact BBM vesicles or electrophoretically separated membrane proteins were immobilized on nitrocellulose sheets by the dot blot (22) or western blot technique. Free binding sites of nitrocellulose were blocked with 2% (v/v) Tween 20 in phosphate-buffered saline (PBS), pH 7.4. To diminish nonspecific adherence, 0.05% (v/v) Tween 20 in PBS was used in each washing or incubation step. After incubation with biotinylated proteins overnight at 4°C, bound probes were detected using peroxidase-conjugated streptavidin and ECL blotting detection reagents. Chemiluminescence signals were quantitated densitometrically with the videodensitometer system Bio-profile (LTF Labotechnik, Wasserburg, Germany), using the program Bio-ID version 5.08 (Vilber Lourmat Biotechnology, Marne La Vallée, France). Binding intensity was expressed as densitometric units per microgram BBM protein in relation to biotinylated standard proteins (Bio-Rad), which were applied directly to nitrocellulose. Saturation and inhibition of protein binding were assessed as criteria for binding specificity. Inhibition of protein binding was measured by addition of nonlabeled proteins to solutions of biotinylated probes. Changes in binding were calculated in relation to binding of biotinylated proteins alone. Maximal binding intensities were calculated from results of saturation experiments.

Statistical Analysis. All data are expressed as mean ± standard deviation (SD). Student’s t test was used for statistical analysis of differences of BBM binding capacities between newborns and adults.

RESULTS

Characterization of BBM Vesicles. BBM vesicles were isolated from rat small intestines, forming closed
vesicles of varying sizes. Cytoskeleton proteins of the brush border appeared as electron-dense material inside the vesicles (Figure 1). Enrichment of BBM marker enzymes indicated sufficient purity of membrane preparations (Table 1). Characteristic developmental changes of enzyme activities included an increase in maltase and alkaline phosphatase activities, a decrease of lactase, and the appearance of sucrase activity at the time of weaning.

**Effect of Concentration on BBM Protein Binding.**
In dot blots, saturation of binding of all food proteins investigated could be demonstrated. Binding of food proteins to newborn BBM was saturated at concentrations of 0.1 μg/ml (CAS), 5 μg/ml (BLG, BSA), and 10 μg/ml (PT-GLI, ALA, OVA). With adult BBM, saturation was observed at 0.05 μg/ml (CAS), 5 μg/ml (ALA, BSA), and 10 μg/ml (PT-GLI, BLG, OVA) (Figure 2). Saturation of lectin binding started at 0.5 μg/ml (SNA, UEA I) or 1 μg/ml (PNA) in case of newborn BBM and at 0.1 μg/ml (SNA), 0.5 μg/ml (UEA I), and 1 μg/ml (PNA) in case of adult BBM.

**Table 1. Brush Border Membrane Enzyme Activities and Their Enrichment in Final BBM Preparations**

|          | Newborn |          | Adult |          |
|----------|---------|----------|-------|----------|
|          | Specific activity (units/mg) | Enrichment factor | Specific activity (units/mg) | Enrichment factor |
| Lactase  | 0.78 ± 0.32 | 13.4 ± 2.3 | 0.53 ± 0.09 | 9.1 ± 2.7 |
| Maltase  | 1.60 ± 0.36 | 7.1 ± 1.6  | 7.00 ± 1.35 | 13.4 ± 3.5 |
| Sucrase  | 0        |          | 1.08 ± 0.42 | 19.1 ± 2.5 |
| ALP      | 0.18 ± 0.01 | 8.7 ± 2.3  | 0.29 ± 0.11 | 16.4 ± 5.7 |

*Enrichment factors were calculated in relation to initial mucosal homogenates. Mean values ± sd are given (N = 5–12); ALP, alkaline phosphatase.

**Fig 1.** BBM from newborn (NB) and adult (AD) rat intestines formed closed vesicles during isolation (bar = 110 nm).
MAA binding was not saturated in the concentration range tested (Figure 3).

Food protein binding to adult BBM reached 18.6 to 27.4 densitometric units/μg BBM protein except BLG, which was significantly less bound to BBM (Table 2). Adult BBM bound significantly more PT-GLI, CAS, BLG, ALA, BSA, and OVA than newborn membranes. Lectin binding reflected known changes in membrane glycosylation during postnatal maturation. According to the shift from sialylation to fucosylation, newborn BBM bound more PNA, MAA, and SNA, but less UEA I (Table 2).

**Inhibition of Binding.** Besides saturation, specificity of food protein binding to BBM was studied by inhibition experiments. Binding of PT-GLI, CAS, BLG, and ALA was inhibited by a 10³-fold excess of nonlabeled protein. However, the observed aggregation of BSA and OVA, defined as the ability of the biotinylated protein to bind to the immobilized unmarked counterpart, was not compatible with this kind of experiment (Figure 4). Only in the case of weak binding due to low concentrations of biotinylated proteins was a 10³-fold excess of nonlabeled probe able to achieve 100% inhibition (data not shown). With concentrations above 0.01 μg/mL, assay sensitivity did not allow 100% inhibition of specific lectin binding, too, using either nonlabeled lectins or monosaccharides (Figure 4).

**Food Protein Binding to Isolated Membrane Proteins.** Binding characteristics of isolated membrane proteins were further analyzed in western blots to define maturational differences. In accordance with results obtained with whole BBM in dot blots, isolated membrane proteins of adult rats showed higher binding capacities for PT-GLI, ALA, BLG, and OVA than obtained with whole BBM (Figure 3).

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**Table 2. Maturational Increase of Food Protein Binding to Brush Border Membranes**

| Intensity of saturated binding (densitometric units/μg BBM protein)* | Newborn | Adult |
|-------------------------------|---------|-------|
| **Food proteins**              |         |       |
| PT-GLI                        | 20.34 ± 0.86 | 22.18 ± 1.23 c |
| CAS                           | 24.42 ± 1.25 | 27.40 ± 1.99 c |
| BLG                           | 6.20 ± 0.89  | 6.79 ± 0.60 a  |
| ALA                           | 17.11 ± 0.78 | 18.56 ± 1.67 b |
| BSA                           | 16.30 ± 1.98 | 20.54 ± 2.11 b |
| OVA                           | 20.40 ± 0.49 | 21.27 ± 1.63 a |
| **Lectins**                   |         |       |
| UEA I                         | 21.23 ± 1.46 | 22.91 ± 1.37 c |
| PNA                           | 29.78 ± 1.58 | 26.88 ± 1.76 c |
| MAA                           | 21.37 ± 1.20 | 17.80 ± 1.15 c |
| SNA                           | 49.75 ± 2.78 | 35.06 ± 2.13 c |

*Significant differences of binding capacities between newborn and adult BBM are indicated (a, P < 0.05; b, P < 0.01; c, P < 0.001). Mean values ± SD are given (N = 8–24).
and more binding sites than newborn rats (Figure 5). This maturational difference was also seen with BSA and CAS (densitometric profiles not shown). Common or at least adjoining binding sites for food proteins were detected on newborn membrane proteins of 124–131 kDa, 117–120 kDa, and 66 kDa. Adult membrane proteins of 128–138 kDa, 115–120 kDa, 103–105 kDa, 90–92 kDa, 85–86 kDa, 65–67 kDa, and 45–46 kDa shared food protein binding abilities. To exclude that these maturational differences were due to methodical errors, eg, failure to isolate newborn BBM proteins, lectin binding was studied in western blots, too. Newborn membrane proteins had more binding sites and enhanced binding capacity for SNA, PNA, and MAA than adults, whereas UEA I binding was lower in newborns compared to adults (data not shown).

**Inhibition Properties of Unrelated Proteins.** Inhibition experiments with unrelated proteins indicated common binding of food proteins because cow’s milk proteins, especially CAS, were able to reduce BBM binding of an unrelated protein, whereas OVA interfered to a lower extent with cow’s milk protein and gliadin peptide binding (Table 3). It was not possible to discriminate whether interference was due to protein interactions in solution, to nonspecific membrane binding, or to the presence of common binding sites. On the other hand, cow’s milk proteins and OVA did not interfere with lectin–BBM interactions and did not disturb the chemiluminescence reaction. Addition of a $10^4$-fold excess of nonlabeled food proteins to solutions of biotinylated lectins at a concentration of 0.1 µg/ml did not result in significant changes of lectin binding. Due to its strong adherence to BBM, only CAS was able to reduce lectin binding by 19.4–37.7%.

**DISCUSSION**

Different approaches have been reported to study BBM–protein interactions and to characterize developmental changes in BBM structure. Beside histochemical studies (23, 24) and utilization of isolated enterocytes (25) and of gut sacs (3, 13, 26, 27), BBM vesicles have been used. As the smallest unit for studying membrane structure and function (28), BBM vesicles allow description of enterocytic binding structures in detail, at the same time reducing the complexity of the investigated system. Binding characteristics of rat BBM vesicles for food proteins and lectins have been assessed in centrifugation and filtration binding assays (14, 16, 29–33). A considerable drawback of these methods was their low sensitivity in detecting weak food protein binding.

To improve sensitivity, we used a chemiluminescence binding assay in this study. Food protein binding to BBM of newborn and adult rats was found to
be specific with regard to saturation and inhibition. Overall food protein binding capacity of BBM was comparable to lectins, except for BLG. Saturation of food protein binding was only reached at 10- to 100-fold higher molar concentrations compared to lectins and CAS. This difference between food proteins and lectins is in accordance with former studies (16, 33). In contrast, the former centrifugation binding assay did not show any saturation and inhibition of food protein binding to BBM vesicles (13, 14, 16). In our opinion, results of nonspecific food protein binding to BBM were obscured by methodological difficulties in the centrifugation binding assay, which did not allow washing of ligand–BBM complexes without concurrent loss of binding signals.

While Colyer et al (25) reported up to 50% inhibition of gliadin peptide binding to isolated enterocytes, Farré Castany et al (34) demonstrated nonspecific binding of a peptic–trypptic digest of gliadin to the colon carcinoma epithelial cell line HT-29. The authors studied binding properties of ovalbumin-blocked and glutaraldehyde-fixed HT-29 cells, which might be an explanation for the observed concentration-dependent, but nonspecific, gliadin binding. Nevertheless, gliadin is able to associate with many proteins, mainly by hydrophobic interactions (35). To exclude weak hydrophobic interactions between food proteins and BBM, we studied binding phenomena in presence of the detergent Tween 20.

Experiments with BBM vesicles in dot blots gave a marked increase in food protein binding capacities from newborn to adult rats. This finding contradicts previous results of a maturational decrease of food protein binding to rat BBM (14, 16) and might be due to the more sensitive and specific binding assay used in this study. However, our findings are in accordance with data on increased binding and uptake of BLG and BSA obtained in gut sacs of adult rats compared to juvenile rats (13).

For measurement of strong and highly specific lectin binding to BBM, the binding assay system was not found as critical. The same maturational changes in BBM glycosylation were observed using either the chemiluminescence binding assay in this study or other methods as described elsewhere (29, 30, 32). Nevertheless, minor differences indicated enhanced sensitivity of the chemiluminescence assay. Binding of SNA to adult BBM and of UEA I to newborn membrane proteins was measurable, in contrast to the earlier results of Taatjes and Roth (24) and Lenoir et al (36), which were based on a different methodology.

The maturational decrease in the lipid/protein ratio of BBM (10, membrane protein profiles in Figure 5) is accompanied by an increase in food protein binding to isolated BBM proteins. In western blots, membrane proteins of adult rats had more binding sites and higher binding capacities than BBM proteins of newborn rats. Although membrane proteins are denatured during SDS-PAGE, their binding properties are not necessarily destroyed, as has been shown for several receptor–ligand interactions in western blots (37, 38).

Our data of multiple binding sites do not support the hypothesis of distinct receptors for food proteins comparable to the enzyme aminopeptidase N as a receptor for coronavirus (39). Moreover, protein–oligosaccharide interactions leading to membrane attachment could be excluded because food protein

### Table 3. Interference of Unrelated Protein with BBM Binding of Biotinylated Food Protein*

| Biotinylated ligand | Inhibition (%) with added nonlabeled protein in 10³-fold excess |
|---------------------|---------------------------------------------------------------|
|                     | CAS | BLG | ALA | OVA |
| Newborn             |     |     |     |     |
| PT-GLI             | 100 ± 0 | 100 ± 0 | 100 ± 0 | 28.8 ± 7.1 |
| CAS              | 100 ± 0 | 46.4 ± 2.3 | 29.6 ± 5.3 | 11.2 ± 4.1 |
| BLG              | 35.2 ± 21.9 | 35.1 ± 12.4 | 26.4 ± 15.2 | 15.1 ± 18.4 |
| ALA            | 51.6 ± 5.0 | 41.0 ± 6.9 | 53.5 ± 2.5 | 4.8 ± 5.9 |
| OVA            | 55.0 ± 0.8 | 44.9 ± 4.2 | 53.2 ± 2.7 | 11.5 ± 5.6 |
| Adult            |     |     |     |     |
| PT-GLI             | 37.7 ± 4.0 | 27.1 ± 3.0 | 24.5 ± 5.8 | 19.4 ± 4.7 |
| CAS              | 73.3 ± 2.2 | 23.5 ± 6.1 | 14.5 ± 6.4 | 3.6 ± 1.4 |
| BLG              | 68.8 ± 6.7 | 75.7 ± 8.4 | 73.6 ± 16.3 | 15.9 ± 17.0 |
| ALA            | 60.1 ± 1.9 | 54.7 ± 2.7 | 58.9 ± 3.7 | 24.5 ± 20.3 |
| OVA            | 55.2 ± 1.6 | 44.1 ± 3.1 | 56.0 ± 1.2 | 9.5 ± 7.9 |

*Biotinylated ligands were used in a concentration of 1 μg/ml or 0.1 μg/ml in case of CAS (mean ± sd, N = 6). Boldface type indicates inhibition by nonlabeled counterparts.
binding patterns did not show any similarity to lectin patterns in western blots. Lectin-like binding characteristics of BSA and ALA binding to rat BBM vesicles and of gliadin to model glycoproteins were ruled out earlier by other investigators (27, 40). In our opinion, protein–protein interactions are responsible for food protein attachment to BBM. This kind of interaction was obviously not influenced by the different composition and physicochemical characteristics of the investigated cow’s milk proteins, gliadin peptides, and ovalbumin. On the contrary, common or at least adjoining binding sites exist for the different food proteins, as indicated by western blot findings. Inhibition by unrelated food proteins supports this possibility. Control experiments could exclude artifacts caused by biotinylation of food proteins.

Vesicle destruction leading to release of brush border cytoskeleton proteins could possibly influence binding characteristics recorded in Western blots. For example, actin as a major component of adult brush border might have caused the positive signals in the area of 45 kDa. Therefore further experiments have to be conducted to identify structures in membrane binding and their orientation towards the intestinal lumen in vivo.

In conclusion, contrary to earlier reports, BBM food protein binding was found increased during enterocytic maturation of rat small intestine. It has to be kept in mind that any experimental design using isolated BBM vesicles in vitro lacks important parts of the mucosal barrier (41). Differences in protein resistance to intestinal hydrolysis (gliadin, BLG in contrast to CAS) (42) influence their access to small intestinal epithelial cells in vivo. For evaluation of consequences of changes in food protein binding capacity caused by alterations in BBM composition, it should be investigated whether processes of uptake and intracellular processing or signal transduction occur. It is well-known that endocytosis is stimulated by prior membrane adsorption (43, 44). Moreover, differences of intracellular processing after uptake of membrane-bound proteins or pinocytosis of fluid-phase molecules have been reported for small intestinal epithelial cells (4, 5, 45). Our results clearly indicate that BBM composition influences its capacity for specific food protein binding. In this regard, studies of enterocytic membrane alterations possibly involved in the development of food-sensitive enteropathies would be worthwhile.

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