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PRESENCE OF FREE HYDROPHOBIC PEPTIDES IN THE BRUSH BORDER AND BASOLATERAL MEMBRANES OF PIG ENTEROCYTES

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Short peptides containing approx. 60% of hydrophobic amino acids have been extracted by chloroform/methanol from purified brush border and basolateral membranes of pig enterocytes. These peptides can be separated from membrane lipids by thin-layer chromatography on Kieselgel plates using chloroform/methanol/water as developer. Their molecular weight is approx. 8000 as judged by SDS-gel electrophoresis. But, this value may be overestimated. They are devoid of cystine and methionine. They contain no N-terminal amino acid detectable by the dansyl and Edman degradation techniques. Extraction of papain-treated, right side out brush border vesicles led to mixtures containing the above peptides and the anchors which normally bind a variety of hydrolases to the external surface of the brush border. Peptides and anchors could not be separated by high performance thin-layer chromatography and SDS-gel electrophoresis. Their amino acid compositions were similar. However, several lines of evidence did not support the assumption that the peptides existing in non-treated brush border membranes can be identified to anchors left inside the bilayer after proteolytic cleavage of surface hydrolases. It is not yet known whether these peptides represent other hydrophobic fragments (leader or stop-transfer sequence, for instance) left in the membrane during the co-translational processing of certain proteins or constitute an independent population of molecules.

Introduction

Two morphologically and functionally distinct regions exist in the plasma membrane of enterocytes, the invaginated brush border which develops during cell differentiation in the direction of the lumen, and the rest of the membrane called the basolateral region. The two regions which are separated by the tight junctions are known to contain different types of enzymes.

A variety of hydrolases (disaccharidases, aminopeptidases, alkaline phosphatase, etc. [1–3]) are bound to the brush border. The structure of these enzymes, their biosynthesis and assembly with the bilayer have been extensively investigated in recent years (for a review, see Ref. 3). They are amphiphilic molecules composed of a large hydrophilic and enzymatically active domain which protrudes from the external surface of the membrane in intact cells and right side out brush border vesicles and of a much shorter, predominantly hydrophobic N-terminal segment [3–11]. This segment is assumed to span the membrane and to anchor the hydrophilic domain at the bilayer surface [7]. The region of the molecules between the two domains (the interdomain junction) is strongly exposed to proteolytic attack [12]. Papain digestion of right side out brush border vesicles

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leads to solubilization of the hydrophilic domains while, by analogy with the erythrocyte 'band 3' protein [13], the hydrophobic segments (called below 'the anchors' *) may be assumed to remain in the bilayer. Full purification of the anchors of pig and rabbit aminopeptidases has been achieve after limited proteolysis of detergent-extracted, complete enzyme molecules by papain or trypsin [6,9–11]. Their hydrophobic amino acid content is approx. 60% and their molecular weight derived from SDS-gel electrophoresis data is 8500 [6,9–11]. This latter value has recently been reduced to 3500–4500 using a novel isotope dilution technique [14].

By contrast, little information is available about the mode of integration of the enzymes characterized so far in the basolateral membrane of enterocytes $(Na^+ + K^+)$-ATPase and 5′-nucleotidase). Small amounts of brush border hydrolases are also present in this membrane [15].

The purpose of the present report is to show that the brush border and basolateral membrane purified from pig intestinal mucosa contain substantial amounts of as yet undetected free hydrophobic peptides soluble in chloroform/methanol. Possible relationships between these peptides and the above reported anchors of surface proteins have been investigated by comparing the material extractable from right-side-out brush border vesicles before and after papain digestion. These vesicles are designated in this article 'non-treated' and 'papain-treated', respectively.

Materials

Prosyl 28, an organosilane derivative for glassware coating (see later) was from PBS Research Chemical Inc. Kieselgel 60 plates for preparative thin-layer chromatography and Silica gel 60 F/254 plates for high performance thin-layer chromatography were from Merck. The mixed bed resin used for gel destaining (RG grade RG 501 × 80, 20–50 mesh) was from Biorad. Papain was from Boehringer and the sodium [^{125}I] iodide (15 mCi/μg) from Amersham.

Methods

Preparation of membranes and digestion with papain. Right-side-out brush border vesicles were purified from pig small intestinal mucosa by several centrifugations under different conditions. The preparations were freed from microsomes in the presence of calcium [16]. Only 11% of the total aminopeptidase N activity originally present in the intestine at the death of the animals were found to be dissolved in the aqueous phase of the lumen. The proportions were 13% for aminopeptidase A, 12% for alkaline phosphatase, 17% for sucrase-isomaltase and 21% for maltase. The rest of the activities was bound to the mucosa and small membrane fragments in the lumen [17]. Moreover, only 10% of the activities bound to the mucosa were lost during the purification of the vesicles.

In other assays, the brush border and basolateral membranes were simultaneously purified by a procedure recently worked out in the Laboratory for rat [18] and rabbit [15] mucosa. An important step of the procedure is a 25 min centrifugation at 18000 × g of pre-purified mucosa homogenates in a 10 mM Tris-HCl buffer (pH 7.4) containg 0.15 M sucrose, 3 mM $MgCl_2$ and various salts (5 mM monosodium phosphate, 30 mM sodium succinate and 1 mM $MnCl_2$) used to load mitochondria. Under these conditions, the basolateral membranes are spun down while the brush borders remain in supernatant. Both were further purified as already described until maximal specific activity of their respective enzyme markers.

Papain digestion of purified right side out brush border vesicles [4] was performed at 37°C and pH 6.2 for 30 min in the presence of an amount of enzyme equal to 10% of the membrane proteins. Solubilized hydrolases were separated from membrane by a 15-min centrifugation at 18000 × g in the angular rotor of a Heraeus-Chris centrifuge.

Extraction of membrane fractions by chloroform/methanol. Glassware was rinsed with Prosyl-28 in order to minimize adsorption of hydrophobic components. In a typical assay, a membrane sample corresponding to 30 mg of protein

* For the sake of simplicity, the hydrophobic peptide fragments split off from brush border hydrolases by limited proteolysis are called 'anchors' But, they may differ from the anchors proper by some additional hydrophilic residues of the interdomain junction if the proteolytic cleavages do not occur exactly at the membrane surface

before papain digestion was suspended in 1 ml phosphate-buffered saline (10 mM sodium potassium phosphate (pH 7.4) containing 0.15 M NaCl) mixed with 20 ml of chloroform/methanol (2:1, v/v) and stirred overnight at room temperature. Denatured proteins were removed at $10000 \times g$ for 10 min and 4 ml of 0.1 M KCl were added to the supernatant. After shaking, the two phases were separated at $10000 \times g$ for 10 min. The upper aqueous phase and any insoluble material at the interphase were discarded. The organic phase (9.5 ml) was washed three times with its volume of chloroform/methanol/0.1 M KCl (3:40:40, v/v), cleared by passage through a 0.45 μm Millipore filter and dried by rotoevaporation. The residue was taken up in 1 ml of chloroform.

^{125}I-labeling of the detergent form of aminopeptidase N and of the corresponding anchor. The detergent form of porcine aminopeptidase N was extracted by incubation of brush border vesicles in 2% Emulphogen and purified as previously reported (11). The protein was iodinated with the aid of sodium [^{125}I] iodide in the presence of lactoperoxidase [19]. The hydrophobic anchor of the labeled material was split off by trypsin and purified as described in Ref. 6.

SDS-gel electrophoresis. Slab gel electrophoresis in the presence of 0.1% SDS were carried out according to Blobel and Dobberstein [20]. Radioactive material was revealed by autoradiography using XS-5X Omat Kodak films. Proteins were stained with 2.5% Commassie blue in methanol/acetic acid/water (50:10:40, v/v). Gels were destained in methanol/acetic acid/water (5:7:88, v/v) using a Biorad mixed bed resin for continuous adsorption of the dye.

Thin-layer chromatography. Experimental details about preparative and analytical thin-layer chromatography are reported in the next Section. After development, the plates were air-dried and submitted to autoradiography as indicated above for electrophoresis assays. Peptides were stained with 0.2% ninhydrin in acetone. Phospholipids and other lipids of the membrane were revealed by exposure to iodine vapours.

Determination of N-terminal amino acid residues. N-terminal amino acids in hydrophobic peptides and anchors were identified either by the dansyl technique of Hartley or by automated Edman degradation in a Beckman liquid phase Sequencer 890 C using the 0.1 M Quadrol program. In this latter case, the peptides were taken up in 300 μl of 98% formic acid and 4 mg of poly(β-alanine) was added as carrier. Conversion of anilinothiazolinones into the corresponding thiohydantoins was carried out in 20% trifluoroacetic acid at 55°C for 30 min. Thiohydantoins were identified by high performance liquid chromatography using a Waters equipment and a Merck RP 18 column.

Results

Characterization of hydrophobic peptides in non-treated pig intestinal brush border

Right-side-out brush border vesicles purified from pig intestine were supplemented with trace amounts of the ^{125}I-labeled anchor of aminopeptidase N (specific radioactivity, $4 \cdot 10^5$ cpm per nmol; total amount added, 20000 cpm for 30 mg of proteins in the original membrane sample) which served as marker for fractions containing hydrophobic peptides. Then, the membranes were extracted with chloroform/methanol (2:1, v/v) as described in Methods and the extracts were submitted to preparative thin-layer chromatography on Kieselgel plates. As indicated by Fig. 1 (lanes A1 and B1), the patterns corresponding to non-treated membranes showed a radioactive band which remained at the origin. Phospholipids, sterols and other membrane lipids were displaced, thus allowing an easy separation from the radioactive material. The presence of peptides in the radioactive band was suggested by ninhydrin staining. Two iodine positive bands attributed, respectively, to phosphatidylserines and phosphatidylethanolamines were also revealed by ninhydrin.

When applied to papain-treated membranes, the procedures led to qualitatively similar results (Fig. 1, lanes A2 and B2). In both cases, the radioactive band was scraped off prior to lipid revelation and extracted with 98% formic acid. The extracts were dried under nitrogen. Average radioactivity recovery was 95% after chloroform/-methanol extraction, 45% after washing of the extracts, drying and redissolution in chloroform, and 27% after preparative thin-layer chromatography followed by formic acid extraction.

When the radioactive anchor marker was re-

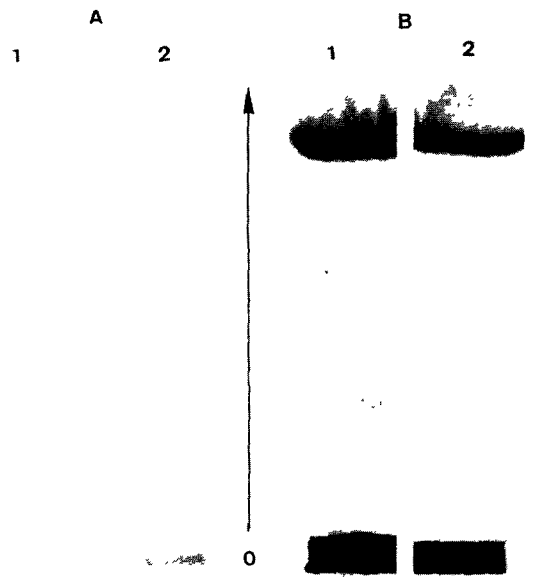

Fig 1 Preparative thin-layer chromatography of chloroform-methanol extracts of right-side-out porcine intestinal brush border vesicles before (1) and after (2) papain treatment The extracts of an amount of membranes containing 15 mg of proteins before papain treatment, were applied as 6 cm long streaks on 20 cm×20 cm 2 mm thick Kieselgel 60 plates The places were developed with chloroform/methanol/water (65 25 4, v/v) In A, autoradiographic patterns showing in both cases a radioactive band at the origin In B, lipid staining with iodine after removal of the radioactive band The scraped areas appear as black rectangles at the bottom of the plates in patterns B

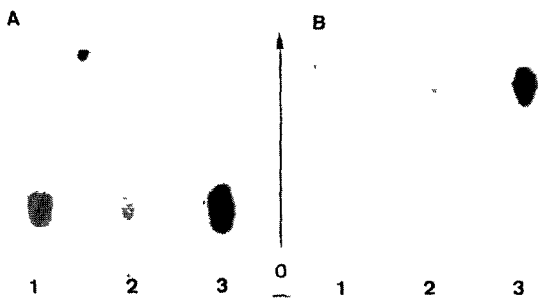

Fig 2 Analytical high performance thin-layer chromatography on Kieselgel 60 F/254 plates of the material in the radioactive bands (Fig 1) The chromatograms were developed in A with chloroform/methanol/water (65 25 4, v/v) and, in B, by formic acid/ethanol (5 95, v/v) Lanes 1 and 2, respectively, radioactive material from papain-treated and non-treated brush border vesicles Lane 3, radioactive marker alone (^{125}I-labeled anchor of porcine aminopeptidase N) Spots were revealed by ninhydrin in lanes 1 and 2, and by autoradiography in lane 3

placed in the analyzed mixtures by the complete ^{125}I-labeled detergent form of aminopeptidase N (200000 cpm of which 20000 cpm in the anchor (see above)), no radioactivity was detected in the chloroform-methanol extracts, thus proving the stability of the interdomain junction of brush border hydrolases during chloroform-methanol extraction. This point is further discussed in the next Section.

Analytical thin-layer chromatography and gel electrophoresis of peptide material soluble in chloroform-methanol

The peptide material thus separated from membrane lipids by preparative thin-layer chromatography was further tested by analytical high performance thin-layer chromatography and gel electrophoresis in the presence of SDS. The Kieselgel 60 F/254 plates used for analytical chromatography were developed with chloroform/methanol/water or formic acid/water. As shown in Fig. 2, the material extracted from non-treated and papain-treated vesicles yielded in both solvents a single spot migrating with the radioactive marker.

Size distribution and apparent molecular weight of peptides and anchors were also compared by gel electrophoresis in the presence of SDS. As shown by Fig. 3, all extracted material irrespective of its origin had the same electrophoretic mobility and this mobility was similar to that of the radioactive peptide marker. The position of the bands was consistent with an apparent molecular weight of 8000 as already known for the anchors under the same conditions [6,9–11].

Amino acid composition and N-terminal residues of brush border peptides and anchors

As shown by Table I, the radioactive band extracts from non-treated brush border yielded substantial amounts of amino acids upon acid hydrolysis, thus giving the final proof of the presence in this membrane of free peptides extractable by chloroform/methanol. The amino acids were approximately doubled after papain treatment, sug-

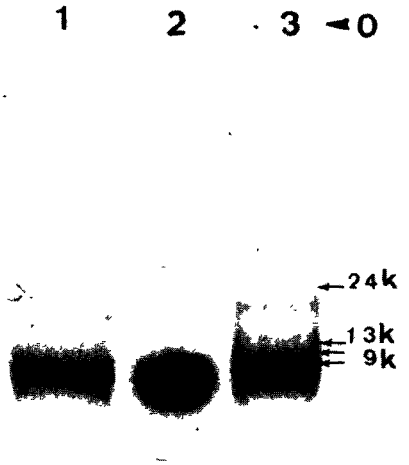

Fig 3 Gel electrophoresis of peptide material in the radioactive bands of Fig 1 obtained from non-treated and papain-treated brush border vesicles (lanes 1 and 3, respectively) Applied samples corresponded to 1 5 mg of protein in untreated membrane Lane 2, radioactive marker (5000 cpm). Electrophoresis was performed in polyacrylamide slab gels (13–23% concentration gradient) at 10 V/cm for 3 h in the presence of 0 1% SDS Spots were revealed by Coomassie blue staining in lane 1 and 3, and by autoradiography in lane 2. Gels were calibrated with reduced-carboxymethylated bovine trypsinogen (M_r, 24000 (24 K)), the B and C chains of bovine α-chymotrypsin (13000 (13 K) and 11000 (11 K), respectively) and porcine colipase II (9000 (9 K)) The migration of reference proteins are indicated by arrows The minor bands corresponding to an apparent M_r of approx 17000 in lane 1 and 3 are probably dimers since similar bands are also observed in lane 2 with higher marker concentrations

TABLE I

AMINO ACID COMPOSITION OF MEMBRANE PEPTIDES

Strongly hydrophobic residues are underlined

Residues (nmol) [a] per 1 mg of membrane protein [b]			
	Brush border		Basolateral (non-treated)
	Non-treated	Papain treated	
Ala	2.3	5.5	5.6
Arg	1 1	2.8	2.3
Asx	2 0	4 5	3 1
Cys [c]	0 0	0 0	0 0
Glx	2 6	5.9	4.1
Gly	4.3	8 7	7.8
His	0 6	0 9	0.9
Ile	1 5	3 4	3 7
Leu	3 6	7 2	8 7
Lys	1 9	4 5	3 7
Met	0.0	0 0	0 0
Phe	2 2	3 6	4 3
Pro	1.3	2 7	2 4
Ser	2 3	4.8	5 8
Thr	1.4	3 5	2 0
Trp [d]	–	–	–
Tyr	0 6	0.9	1 3
Val	2 6	6 3	6 0

[a] After a 48 h hydrolysis in boiling 6 M HCl.
[b] Calculated from the radioactivity
[c] As cystine
[d] Not determined

gesting that the amount of anchors left behind in the membrane during papain digestion is similar to that of the peptides pre-existing in the non-treated material. The proportions of strongly hydrophobic amino acids in peptides was approx. 60%, as already observed for anchors [9–11]. Cystine and methionine were absent in all cases.

No N-terminal residues were found by the dansyl technique in peptides derived from non-treated membranes. By contrast, detectable amounts of Dns-alanine and Dns-leucine, already known to be N-terminal in anchors [6], were identified after papain treatment. Much lower quantities of other Dns-amino acids (isoleucine, phenylalanine, valine, glycine and aspartic acid) were also characterized in this latter case. No special search was made for Dns-arginine and Dns-lysine.

More quantitative data were obtained by submitting the peptides to Edman degradation. As reported in Table II, the thiohydantoins generated during the first cycle were not significantly higher than the background, except again for alanine and leucine in peptides extracted from papain treated vesicles. The yield of alanine and leucine thiohydantoins was approx. 10% assuming equal proportions of peptides and anchors in the mixtures submitted to degradation.

TABLE II

THIOHYDANTOINS (IN PMOL OF RESIDUE) YIELDED BY AUTOMATED EDMAN DEGRADATION (FIRST CYCLE) OF BRUSH BORDER PEPTIDES (5 NMOL IN EACH CASE)

	Non-treated membrane	Papain-treated membrane
Ala	35	199
Asn	21	42
Glu	38	66
Gly	31	58
Leu	46	164
Lys	–	12
Pro	26	45
Tyr	32	56
Val	22	57

Free hydrophobic peptides in the basolateral membrane

Chloroform-methanol extracts followed by preparative thin-layer chromatography also revealed the presence of free hydrophobic peptides in the non-treated basolateral membrane of pig enterocytes. As shown by Table I, the amino acid composition of these peptides was not significantly different from that of brush border peptides and, anchors. When calculated on a protein basis, the peptide content of the basolateral membrane was found to be similar to that of the papain-treated brush border and, consequently, to be twice as high as in the non-treated brush border. However, since the protein density is lower in the basolateral region, the two types of membrane may be assumed to contain about the same quantity of hydrophobic peptides compared to the lipid phase.

Discussion

Substantial amounts of short hydrophobic peptides have been characterized for the first time in the brush border and basolateral membrane of pig enterocytes. These peptides are extractable by chloroform/methanol (2:1, v/v) and they are readily separated from membrane lipids by preparative thin-layer chromatography on Kieselgel plates using chloroform/methanol/water (65:25:4, v/v) as developer.

Another example of hydrophobic peptides inserted in membranes is given by the so-called proteolipids of mitochondria [21,22], chloroplasts [23] and bacteria [24,25] where they represent one of the subunits of the H^+-ATPase complex. These compounds pass spontaneously into the organic phase during chloroform-methanol extraction of isolated membranes and intact cells. They contain 72–81 amino acids of which more than 80% are hydrophobic (for a review, see Refs. 26 and 27). The hydrophobic amino acid content of the peptides extracted from non-treated enterocyte membranes does not exceed 60% without imparing their solubility in chloroform/methanol. Their molecular weight determined by SDS-gel electrophoresis is similar to that of the proteolipids (approx. 8000). But, this value has recently been shown to be overestimated about 2-fold for several aminopeptidase anchors [9–11], due probably to an abnormally high detergent adsorption on the fragments compared to the hydrophilic proteins serving as reference.

An important aspect of the present work would be to know whether the peptides thus characterized in non-treated brush border and basolateral membranes actually constitute an independent population of molecules or whether they are 'remnants', i.e., hydrophobic fragments which remain inside the membranes after degradation of other protein components. Since a characteristic feature of the brush border is the abundance of surface hydrolases bound to the bilayer by hydrophobic anchors, the first idea was to compare the peptides pre-existing in non-treated samples with the anchors set free by papain digestion of right-side-out vesicles. No separation between peptides and anchors could be achieved either by preparative and high performance analytical thin-layer chromatography, or by SDS gel electrophoresis. But, this latter technique may fail to reveal actual molecular weight difference if the above reported detergent effect is weaker for the peptides than for the anchors, or even inexistant as in the case of the proteolipids. Similarly, the analogies noted in the amino acid composition of peptides and anchors is of questionable significance because of the chemical heterogeneity of the analyzed mixtures. The analyses only show that peptides and anchors have not widely different compositions and that all

contain about 60% of a hydrophobic amino acids, no cystine and no methionine.

However, the hypothesis that the peptides in non-treated membrane really differ from anchors is supported by the following observations: (a) Extensive cleavage of surface hydrolases in vivo (for instance by pancreatic elastase [28,29]) is unlikely in our assays since most of the activities found in the intestinal lumen at the death of the animals are not dissolved in the aqueous phase, but attached to the mucosa or membrane fragments [17]. (b) The possibility for hydrolases to be extensively degraded in vitro is also not supported by the fact that most of the enzymes originally present in the intestine are recovered in the purified vesicles and that the ^{125}I-labeled detergent form of aminopeptidase N added as marker is perfectly stable during chloroform-methanol extraction of the vesicles. (c) No neutral N-terminal residues were found in the peptides from non-treated membranes while leucine and alanine, known to be N-terminal in anchors [6] were identified after papain treatment. (d) Substantial amounts of hydrophobic peptides are also present in the basolateral membrane where no surface proteins attached by hydrophobic anchors have ever been characterized, except for very small amounts of brush border hydrolases [15]. (e) The possibility for anchors to accumulate inside the membrane is not consistent with the recently reported observation that the hydrophobic and hydrophilic moieties of plasma membrane proteins are degraded at approximately the same rate in canine renal tubular epithelial cells as well as in rat liver [30].

In conclusion, the presence of free hydrophobic peptides in the enterocyte membrane is now well documented. The brush border peptides are not likely to be identical to anchors of surface hydrolases. However, it cannot yet be decided whether the peptides in both brush border and basolateral membrane constitute an independent population of molecules or other hydrophobic fragments of protein components. In this latter respect, stop-transfer and leader sequences may remain in the bilayer if certain proteins of the enterocyte membrane are assumed to be inserted according to the recently proposed helical hairpin mechanism [31].

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