LOCALIZATION OF THE MONOGLYCERIDE PATHWAY ENZYMES IN THE VILLUS TIPS OF INTESTINAL CELLS AND THEIR ABSENCE FROM THE BRUSH-BORDER

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1. Introduction

Morphological and physiological data indicate that neutral lipids are primarily absorbed as monoglycerides and fatty acids in the proximal intestine at the levels of the villus tips, where the cells with a differentiated brush-border are localized [1]. The monoglyceride pathway enzymes (acyl-CoA synthetase (EC 6.2.1.3); acyl-CoA: mono- and diglyceride acyltransferase (EC 2.3.1.22 and 20)) are involved in the reesterification process of monoglycerides to triglycerides [2]. Since diglycerides are formed through both the (sn)glycerol-3-phosphate and 2-monoglyceride pathways, and since two pools of diglycerides seem to exist in the intestinal cells [3], the monoglyceride acyltransferase can be considered as the more representative enzyme of the neutral lipids pathway. The intracellular localization of the enzymes involved in this pathway has been subject to conflicting reports [4–8]. Robins et al. suggested that the isolated brush-border fraction owes its activity for glyceride synthesis to a contamination by endoplasmic reticulum [8]; however the monoglyceride pathway enzymes were not assayed for activity. Our results show that the acyl-CoA: monoglyceride and diglyceride acyltransferases are localized in the villus tips where the cells with a differentiated brush-border are concentrated. Therefore, in order to study binding and transport of monoglycerides and fatty acids with purified brush-border vesicles, it became critical to reinvestigate the intracellular localization of the monoglyceride pathway enzymes. Our data demonstrate that these enzymes are absent from the brush-border and, in support to some previous works, likely bound to the endoplasmic reticulum [4,7,8].

Consequently the study of binding and transport of the above-mentionned lipids using brush-border vesicles can be performed without any interference of subsequent metabolic reactions.

2. Materials and methods

Intestinal epithelial cells of rat and pig were obtained as described by Weiser [9]. The subcellular fractionation and the preparation of purified brush-border vesicles were that of Louvard et al. [10], as well as the assays for aminopeptidase (EC 3.4.1.2.), alkaline phosphatase (EC 3.1.3.1.), cytochrome *c* oxydase (EC 1.9.3.1.) and NADPH-cytochrome *c* reductase (rotenone insensitive; EC 1.6.2.3. Acyl-CoA synthetase was assayed as previously described [11].

Acyl-CoA: monoglyceride and diglyceride acyltransferases were assayed [12] using respectively as substrates 2-monoolein or 1,2(2,3)-diolein with palmityl-CoA chemically synthetised [13].

Na^+, K^+-adenosine triphosphatase (Na^+, K^+-ATPase) ouabain sensitive (EC 3.6.1.3.) activity was determined according to Hepner and Hofmann [14] and the assays for P_i determinations according to Chen et al. [15]. *N*-acetylglucosaminyltransferase activity (endogenous acceptor) (EC 2.4.1.51.) was assayed as described by Weiser [16].

Solubilisation of the different subcellular fractions was performed in sodium phosphate pH 7.2 containing 1% sodium dodecylsulfate and the proteins were determined by absorbance readings at 280 and 260 nm [17]. Proteins were also determined by the method of Lowry et al., using bovine serum albumin as standard [18].

L-Alanine-*p*-nitroanilide was a product of Cyclo-

chemicals. Cytochrome *c* and NADPH were from Boehringer. Glycerides were purchased from Hormel and 5,5'-dithiobis- (2-nitro-benzoic acid) from Aldrich. Chromatographically pure CoA was obtained from P-L Laboratories. Ouabain was a product of Merck. [9,10-^{3}H]palmitic acid was purchased from C.E.A. and UDP-*N*-acetyl-[^{14}C]glucosamine from New England Nuclear.

3. Results

3.1. *Localization of acyl-CoA: monoglyceride acyltransferase in the villus tips of intestinal epithelium*

In fig.1 a decreasing gradient in the specific activity of some marker enzymes of the brush-border, that is aminopeptidase and alkaline phosphatase, is observed going from fraction I (villus tips) to fraction 9 (crypts), as already described by Weiser [9]. On the contrary an increasing gradient is observed for UDP-*N*-acetylglucosaminyl transferase, characteristic enzyme of the crypt cells [9]. The diagrams of fig.1 clearly show that the pattern of acyl-CoA: monoglyceride acyltransferase follows those of aminopeptidase and alkaline phosphatase.

As also shown in fig.1, lactate dehydrogenase is present in all fractions (but fraction 1) at similar levels, and thus does not behave as a specific marker.

A similar localization in the villus tips of acyl-CoA: monoglyceride acyltransferase has also been obtained for pig intestine (not shown). Since the acyl-CoA: monoglyceride acyltransferase activity was found in intestinal cells presenting a differentiated apical membrane and since some authors have found that the enzymes of the monoglyceride pathway are localized in the brush-border fraction, it became critical to reexamine the problem of their subcellular localization.

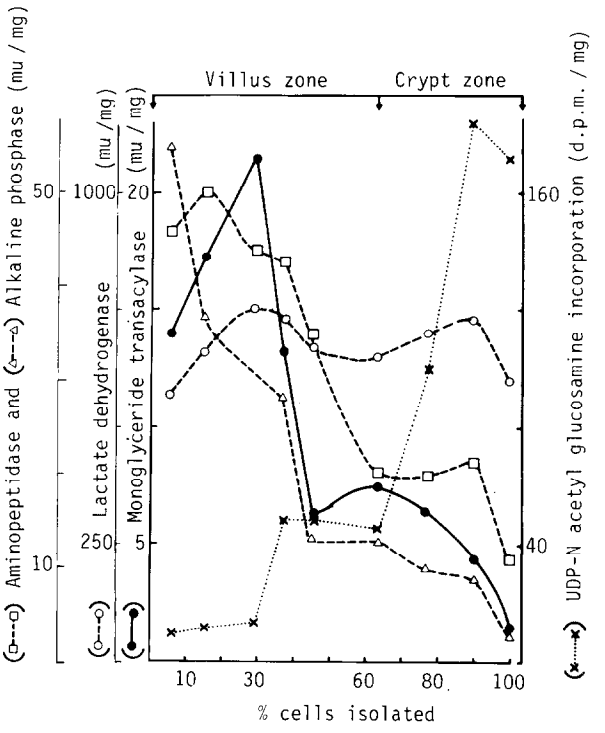

Fig.1. Localization of the acyl-CoA: monoglyceride acyltransferase in the villus tip cells of rat intestinal mucosa. Each of the nine fractions of cells was collected according to the technical procedure described by Weiser [9], which allows a differential separation of cells from the villus tips to the crypts. The 100% cells isolated corresponds to the sum of the nine fractions expressed in protein amounts. The percentage of cells isolated in each successive fraction was determined by the proportions of cell protein isolated in a given fraction. The specific radioactivity of UDP-*N*-acetyl[^{14}C]glucosamine was 56.5 mCi/mmol.

3.2. *Subcellular localization of the monoglyceride pathway enzymes from duodenum, jejunum and ileum from pig intestine*

The fractionation scheme of cells homogenates from different parts of pig intestine is given in table 1. The presence of Ca^{++} and Mg^{++} in the medium used for homogeneization brings a heavy precipitation of the endoplasmic reticulum at the first step (C_I pellet). Therefore low percentages of NADPH-cytochrome *c* reductase and monoglyceride acyltransferase were found in S_2 supernatant and C_3 pellet. The bulk of the acyl-CoA synthetase (60–80%) sediments similarily to NADPH-cytochrome *c* reductase used as marker for the endoplasmic reticulum (75–83%) and also (not shown) to (Na^+, K^+) dependent-ATPase (77%) and to cytochrome *c*-oxydase (75%), the markers of the basolateral membranes and of the mitochondria respectively. An enrichment factor (2–3-fold) was observed for NADPH-cytochrome *c* reductase and acyl-CoA: monoglyceride acyltransferase in the C_I pellet. Under the same conditions 65 to 85% of aminopeptidase (and alkaline

Table 1
Subcellular fractionation of the different parts of hog intestine

CELL FRACTIONS AND FRACTIONATION SCHEME	AMINOPEPTIDASE			NADPH Cyt C REDUCTASE			ACYL-CoA SYNTHETASE			MONOGLYCERIDE ACYLTRANSFERASE		
	D	J	I	D	J	I	D	J	I	D	J	I
HOMOGENATE	100 (100)	100 (150)	100 (106)	100 (16)	100 (13.5)	100 (6.1)	100 (4.5)	100 (3)	-	100 (14)	100 (17)	100 (11.4)
10 min 600×g (D) or 3000×g (J and I) → C_1 PELLET	30 (50)	15 (60)	18 (58)	75 (23)	80.6 (18.3)	83 (9.5)	81 (4.2)	60 (2.9)	-	68 (40)	86 (27)	86 (20.4)
S_1 SUPERNATANT	65 (170)	83.5 (335)	85 (145)	23 (6.8)	16.25 (5.4)	14 (1.4)	12 (1)	15 (1.2)	-	27 (7)	13 (5)	10 (2)
10 min 10 000×g, then 5 min 10 000×g and 5 min 12 000×g → C_2 PELLET												
S_2 SUPERNATANT	48 (200)	60.5 (350)	52 (210)	-	5 (2.4)	10 (1.3)	-	4 (0.5)	-	-	8 (4.5)	-
45 min 105 000×g → S_3 SUPERNATANT	-	44.5 (1030)	49 (660)	-	4.8 (7.5)	9 (7.7)	-	n.d	-	-	4 (10.3)	7 (11.5)
C_3 PELLET → Alkaline treatment, then 5 min 10 000 g, 45 min 105 000 g, pellet resuspended and 5 min 10 000 g → C_4 PELLET												
S_4 SUPERNATANT	18 (500)	40 (1800)	44 (1380)	-	0.44 (1.6)	2 (3.6)	n.d	n.d	-	-	2.3 (8)	3.3 (11.35)
Discontinuous Sucrose gradient → FRACTION I	-	1.2 (450)	1.5 (350)	-	0.4 (8)	-	n.d	n.d	-	-	1 (40)	-
FRACTION II	10 (1000)	27 (2200)	18.5 (1580)	1 (10)	0.1 (0.75)	0.5 (2)	n.d	n.d	-	2 (12)	1 (8.5)	1.5 (11.3)

Subcellular fractions from duodenum (D) jejunum (J) or ileum (I) were obtained as shown on the left column, after homogeneization in 10 mM Tris–Cl pH 7.3 containing 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.25 M sucrose. The yields are calculated in each fraction with 100% taken from the homogenate. All specific activities (in parenthesis) are expressed in nmoles of product formed/min (mU) or reduced/min per mg of proteins. The results are the mean of 2, 5 and 3 experiments for D, J and I respectively. n.d.: no detectable activity.

phosphatase, not shown), characteristic enzymes of the brush-border membrane, do not sediment (supernatant S_1). Therefore a further purification of the brush-border fraction from duodenum, jejunum and ileum was pursued. Based on the specific activity of aminopeptidase, an enrichment factor of 10, 15 and 15-fold as compared to homogenates was obtained for duodenum (D), jejunum (J), and ileum (I) respectively with corresponding yields of 10, 27 and 18.5%. By comparison the percentages of recovery for acyl-CoA: monoglyceride acyltransferase are 2, 1 and 1.5% and those of NADPH-cytochrome *c* reductase 1, 0.1 and 0.5%, with specific activities similar or even lower than those observed for homogenates; the acyl-CoA synthetase became undetectable. The pattern of fractionation of the acyl-CoA: diglyceride acyltransferase (not shown) strictly follows that of the acyl-CoA: monoglyceride acyltransferase, with a ratio of mono-versus diglyceride acyltransferase equal to 3 in all fractions. The products formed from 2-monoolein and radioactive palmityl-CoA were also the same in all fractions (not shown). However Fraction I, arising from discontinuous sucrose gradient performed in order to obtain the fraction of brush-border vesicles (called Fraction II), was enriched approximately 3-fold in the acyltransferase and in the aminopeptidase activities as compared to the homogenate. Therefore a comparison was made between fractions I and II on a linear sucrose gradient [19] to see whether or not both activities co-sediment.

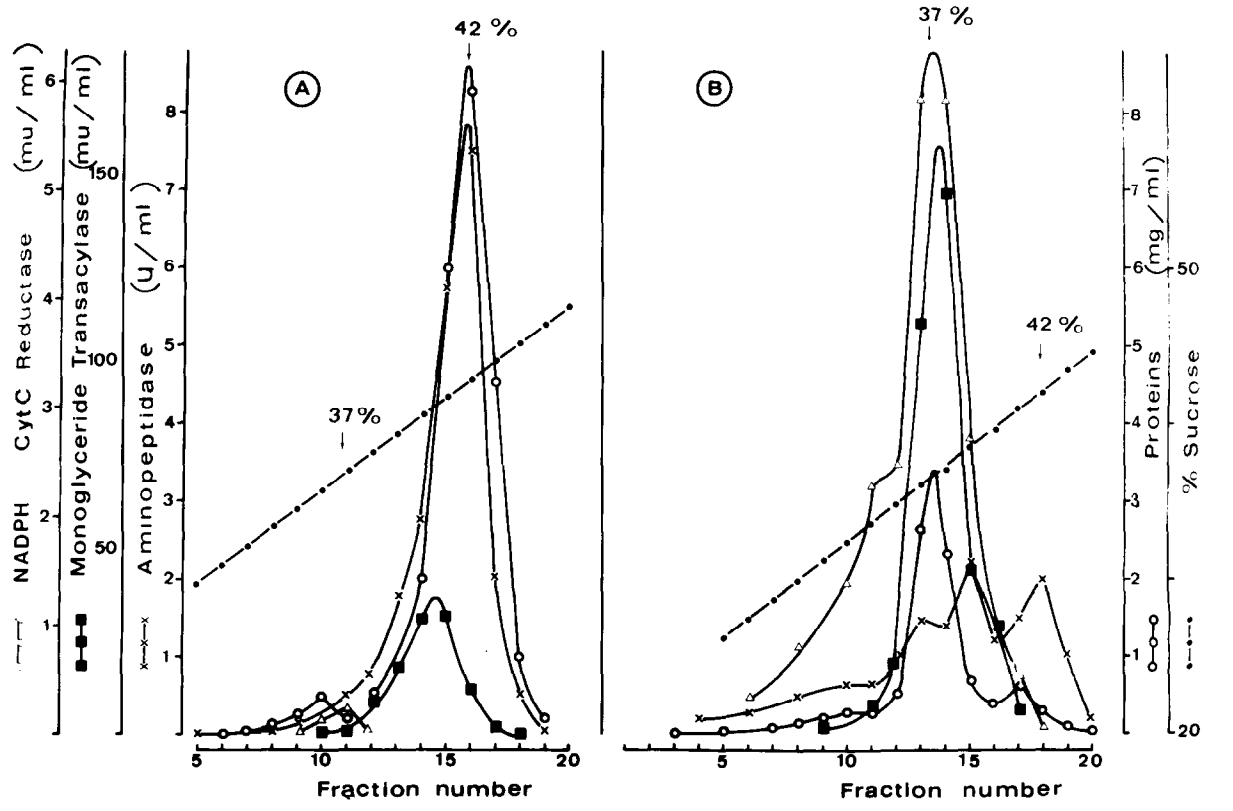

Fig.2. Centrifugations on linear sucrose gradient of Fraction II (brush-border vesicles) (A) and of Fraction I (B) from hog jejunum. 30 mg of protein (A) and 12 mg of protein (B) were first obtained after a 5 hr centrifugation at 22 000 rev/min in a Beckman SW 27 rotor on a discontinuous sucrose gradient formed with: 4 ml of 50%; 8 ml of 44%; 10 ml of S_4 supernatant adjusted at 43%; 8 ml of 42%; 6 ml of 39% and 2 ml of 10% sucrose (w/w) in 10 mM Tris–HCl buffer pH 7.3 containing 10 mM $MgCl_2$. Fraction II (A) corresponds to the purified brush-border vesicles fraction sedimenting at 43% sucrose and Fraction I (B) corresponds to the enriched endoplasmic reticulum fraction floating at the top of the 39% sucrose layer. Each collected fraction, diluted in buffer without sucrose and centrifuged at 105 000 *g* for 45 min., was resuspended in 10 mM Tris–Cl buffer pH 7.3 containing 0.154 M NaCl and 10 mM $MgCl_2$ and loaded on a 32 ml linear 20–50% (w/w) sucrose gradient in 10 mM Tris–Cl buffer containing 10 mM $MgCl_2$. The centrifugation was performed at 1°C for 18 hr at 22 000 rev/min using a SW-27 Beckman rotor. 1.2 ml fractions were collected and aliquots were used for enzymatic and protein determinations.

3.3. *Continuous sucrose gradients of fractions I and II*

The results are presented in fig.2 (A) for Fraction II (brush-border vesicles) and in fig.2 (B) for Fraction I.

The use of the same scale in both figures clearly indicate the higher specific activity for aminopeptidase in Fraction II as well as for acyl-CoA: monoglyceride acyltransferase and for NADPH-cytochrome *c* reductase in Fraction I; a small residual activity to the latter enzyme is apparent in Fraction II.

The curves of fig.2 (B) show that the monoglyceride acyltransferase pattern is closely parallel to that of the NADPH-cytochrome C reductase and to the proteins, while the bulk of the *residual* aminopeptidase will sediment above 37% sucrose that is at 39% and at 42%. On the contrary the pattern of sedimentation of the brush-border membrane vesicles (fig.2A) shows that the peak of aminopeptidase and that of proteins are found at 42% sucrose, while the peak of residual NADPH-cytochrome *c* reductase sediments at 37% sucrose. Some monoglyceride acyltransferase does sediment highly reproducibly at 41% sucrose, that is below 42% where the peak of aminopeptidase is found.

From the results of table 1 as well as from fig.2 (A) and (B), it seems likely that the very small activity responsible for the monoglyceride esterification in the brush-border fraction, merely represents a contamination by membraneous structures of different origin. Thus immunoprecipitation of the brush-border vesicles with antibodies directed against aminopeptidase was performed to assess this point.

3.4. *Precipitation of brush-border membrane vesicles by aminopeptidase antibodies*

The aminopeptidase activity remaining at a given time in the supernatant decreases by increasing the concentration of immunoserum (table 2, 1B compared to 1A) in the incubation mixture. As expected, the percentage of alkaline phosphatase closely follows the percentage of aminopeptidase while twice more (54 and 40%) of the acyltransferase remains in the supernatant after immunoprecipitation (table 2).

Similar results with purified antibodies were found (experiment 2). At a given concentration of antibodies, the percentage of precipitated aminopeptidase increases with time while the acyltransferase is almost not precipitated under the same conditions. Thus it is clear that the small esterifying activity still present in the brush-border fraction *does not* belong to the differentiated structures of these apical periplasmic membranes.

4. Discussion

The finding that in pig and rat (fig.1) the acyl-CoA: monoglyceride acyltransferase is localized in the villus

Table 2
Precipitation of the brush-border fraction by aminopeptidase antibodies

EXPERIMENTS	RESIDUAL ACTIVITY IN THE SUPERNATANT (%)		
	AMINOPEPTIDASE	ALKALINE PHOSPHATASE	MONOGLYCERIDE:ACYL–CoA ACYLTRANSFERASE
1 A	34	27.5	54
1 B	17.5	14	40
2 A	93	97	100
2 B	78	75	90

Experiment I: after a preincubation of 15 min. at 56°C, 0.2 ml (1A) and 0.3 ml (1B) of the rabbit immunoserum directed against homogeneous hog intestinal brush-border aminopeptidase (kindly supplies by Dr Louvard, Centre de Biologie Moléculaire, C.N.R.S., Marseille), were incubated with purified brush-border vesicles from hog jejunum (1 mg of protein; 2200 mU of aminopeptidase; 0.5 ml final volume). After 2 hr at 37°C followed by centrifugation 1 min at 2000 *g*, aliquots of the supernatant were withdrawn for assays.
Control experiments performed in the presence of non-immune rabbit serum showed no precipitation under these conditions.
Experiment II: purified brush-border vesicles (same conditions as experiment I) were incubated 2 hr at 37°C followed by 15 hr (experiment 2A) or 35 hr (experiment 2B) at 4°C with 0.3 mg of purified rabbit aminopeptidase antibody (corresponding to 0.05 ml of the initial immunoserum). The supernatants were obtained and assayed as in experiment I.

tip cells demonstrate that this enzyme can be, as well as the brush-border enzymes, used as a marker of differentiation for the intestinal cells. The same is true for the acyl-CoA: diglyceride acyltransferase [20].

The subcellular localization of the esterifying enzymes of monoglycerides has been the subject to much controversy, since different reports suggested that the brush-border fraction may be the major site for the synthesis of triglycerides [4–8].

The fractionation procedure used for duodenal and jejunal cells [10] allowed us a purification of 15-fold of the brush-border membrane vesicles from ileal cells which look as closed vesicles, as shown by electron microscopy.

The results of table 1 and fig.2 clearly indicate that the acyl-CoA: monoglyceride acyltransferase, the specific marker of the monoglyceride pathway, does not belong to the brush-border fraction.

Further proof was obtained by immunoprecipitation of brush-border vesicles. The results of table 2 demonstrate that the membraneous structures to which the monoglyceride acyltransferase is attached are different from the brush-border membranes and could possibly be some smooth endoplasmic reticulum in continuity with the plasmic membrane.

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