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Proteomic characterization of lipid rafts markers from the rat intestinal brush border ☆,☆☆

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Abstract

To assess intestinal lipid rafts functions through the characterization of their protein markers, we have isolated lipid rafts of rat mucosa either from the total membrane or purified brush-border membrane (BBM) by sucrose gradient fractionation after detergent treatment. In both membrane preparations, the floating fractions (4–5) were enriched in cholesterol, ganglioside GM1, and N aminopeptidase (NAP) known as intestinal lipid rafts markers. Based on MALDI-TOF/MS identification and simultaneous detection by immunoblotting, 12 proteins from BBM cleared from contaminants were selected as rafts markers. These proteins include several signaling/trafficking proteins belonging to the G protein family and the annexins as well as GPI-anchored proteins. Remarkably GP2, previously described as the pancreatic granule GPI-anchored protein, was found in intestinal lipid rafts. The proteomic strategy assayed on the intestine leads to the characterization of known (NAP, alkaline phosphatase, dipeptidyl aminopeptidase, annexin II, and galectin-4) and new (GP2, annexin IV, XIIIb, $G\alpha_q$, $G\alpha_{11}$, glutamate receptor, and GPCR 7) lipid rafts markers. Together our results indicate that some digestive enzymes, trafficking and signaling proteins may be functionally distributed in the intestine lipid rafts.

Keywords: Intestine; Brush border; Lipid rafts; Proteomic; Marker; GP2

Our recent understanding of the cell membrane suggested that the plasma membranes may be organized into heterogeneous functional microdomains and one type of these microdomains called lipid rafts is stated to be enriched in glycosphingolipids/cholesterol and specific proteins [1,2]. The lipid rafts can be isolated by their resistance to non-ionic detergent extraction at cold and buoyancy on gradient ultracentrifugation. Lipid rafts hypothesis was originally proposed to explain how proteins and lipids are sorted to the apical surface of polarized cells. However, in recent years, several functions including signaling, cholesterol homeostasis, cell trafficking or even docking sites on mammalian cells for certain pathogens and toxins have been attributed to lipid rafts [3,4]. Despite accumulated

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☆☆ *Abbreviations:* DRM, detergent resistant membrane; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol phospholipase C; GPCR, G protein coupled receptor; CD, methyl β-cyclodextrin; TLC, thin-layer chromatography; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS); GC, gas chromatography; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEM, standard error of the mean.

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experimental data from biophysical, biochemical, and fluorescent microscopy studies supporting the fact that lipid rafts may exist in vivo, the lipid rafts hypothesis remains controversial at least for their size and the mechanism of their formation [5].

The BBM of small intestinal enterocytes is a highly specialized epithelial cell surface involved in digestive and absorptive functions. Although the lipid rafts have been presumed to be important in nutrient absorption, digestive enzyme retention, host defense, and signaling platform, their functional role of the lipid microdomains in the intestinal brush border is poorly understood. High interest has been focused on the partitioning of some proteins into lipid rafts of small intestine, which are identified mostly by immunodetection using available antibodies [6,7]. Here, we investigated the proteins present in the Triton X-100 and the Brij 98 insoluble low density membranes of rat small intestine by a proteomic approach using MALDI-TOF/MS analysis. Combined with the biochemical determination, our results show that the approach was able to identify most of the known lipid rafts markers previously reported and also new lipid rafts protein markers. Among these new protein markers, we have characterized the GPI anchored glycoprotein GP2, previously shown as lipid rafts resident protein in the pancreas. GP2 was reported to be secreted in the pancreatic juice after cleavage of the GPI and our results showed that GP2 from BBM was sensitive to the removal of the GPI by the phosphatidylinositol phospholipase C (PI-PLC). Furthermore, using monoclonal antibody, GP2 distribution was shown on the vesicle-like lipid rafts by immunogold labeling. These data suggest that the enterocytes express GP2 but its function remains to be elucidated. The protein markers determined in this study were classified into different protein family groups with potential functions in digestive, signaling, and trafficking in the small intestine.

Experimental procedures

Antibodies and reagents. The polyclonal antibodies to $G\alpha_{q/11}$, annexin II, intestinal alkaline phosphatase (IAP), and galectin-4 were purchased from Santa Cruz Biotechnology, Inc. Antibodies to caveolin-1, $G\alpha_q$, and $G\alpha_{11}$ were kindly provided by Dr. Lylia Nini (NIH/NIDDK, USA). The rabbit polyclonal antibody to pig intestinal NAP was kindly given by Dr. Michael Danielsen (University of Copenhagen, Denmark). The use of antibodies against the intestinal sucrase–isomaltase (8A9), dipeptidyl aminopeptidase IV (4H3), and annexins IV and XIII for immunoblotting has been described elsewhere [8,9]. The rabbit polyclonal anti-GP2 antibody was a kind gift from Dr. Michael Schrader (University of Marburg, Germany). A mouse monoclonal antibody to rat GP2 was kindly provided by Dr. Anson Lowe (Stanford University School of Medicine, USA). The rabbit antibodies to intestinal carboxylesterase (ICE) were a kind gift from Dr. André Moulin (University of Paul Cézanne, France). The mouse monoclonal antibody to β-actin, the sheep anti-mouse IgG (HRP-conjugated), the polyclonal anti-Na^+/K^+-ATPase and goat anti-rabbit IgG (HRP-conjugated), PI-PLC, methyl-β-cyclodextrin (CD), ganglioside GM1, and cholera toxin B (HRP-conjugated) were from Sigma.

Preparation of BBM from rat small intestine. Male Wistar rats 400–450 g, Elevage Janvier (France), were fasted overnight and sacrificed by cerebral traumatism. Small intestines jejunums were rinsed and cells were scrapped in cold homogenization buffer (HB) consisting of 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 μg leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 1 mM PMSF, and 5% sucrose. Unless otherwise stated, all the experiments were then performed at 4 °C with an appropriate protease inhibitor concentration. For TM fractionation, the mucosa scrapped from the inner side of the small intestine (400 mg) was homogenized in about 8 vol. (w/v) of HB with a Potter–Elvehjem homogenizer as previously reported [10,11]. The homogenate was then filtered through gauze and the pellet was discarded by centrifugation at 1000 rpm for 5 min. The supernatant underwent a second centrifugation at 10,000 rpm for 10 min. One-half of the recovered supernatant was saved as TM and the other half was used to isolate BBM by divalent cation precipitation with $CaCl_2$ or Mg^{2+} at a final concentration of 10 mM for 10 min at 4 °C then centrifugation was performed as described above. The resulting supernatant containing 1–1.5 mg protein was adjusted to 40% sucrose by adding an equal volume of 80% sucrose in 20 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, then sufficient 40% sucrose in the same buffer was added to bring the volume to twice that of the initial. The centrifuge tube was overlaid with HB. After a centrifugation at 35,000 rpm for 1 h at 4 °C in a TH-641 Sorvall rotor, the floating membrane at the interface was collected and suspended in 4 vol. (w/v) HB. The suspension was centrifuged again at 35,000 rpm for 1 h. The final membrane pellet, defined as BBM, was suspended in HB at 1–1.5 mg/ml protein and saved for further studies.

Detergent extraction and DRM isolation. Lipid rafts isolation was carried out as previously described [3]. Briefly, TM or BBM prepared from rat small intestine was incubated in cold HB containing 0.5–1% Triton X-100 for 30 min at 4 °C. Control experiments were run without detergent or membranes were pre-treated with either 1% Brij 98 at 37 °C or 60 mM octyl glucoside (OG) at 4 °C. The extract (2 ml) containing about 1.3 mg/ml protein was mixed in a 12 ml centrifuge tube with an equal volume of 80% sucrose in the same buffer, giving a suspension of 40% sucrose. A discontinuous gradient was prepared by overlaying with 5 ml of 30% sucrose and then 2 ml of 5% sucrose both in 20 mM Tris–HCl pH 7.4, buffer containing protease inhibitors. The tubes were centrifuged at 35,000 rpm for 17 h at 4 °C in a TH-641 Sorvall rotor. Eleven fractions (1 ml each) were harvested gently from the top of the gradient. The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology, Inc.) with bovine serum albumin as the standard (BSA) or the Bio-Rad DC™ (detergent compatible) protein assay with immunoglobulin G as the standard.

Electrophoresis and Western blotting. SDS–PAGE on 4–20% Tris–HCl precast gels (Bio-Rad) or 15% gels was performed under reducing conditions according to Laemmli [12]. For immunoblotting, the separated proteins were transferred to a nitrocellulose membrane and probed with the appropriate primary antibody and a HRP-conjugated secondary antibody as we previously described [13]. For lipid rafts ganglioside GM1, 2.5 μl of each fraction was spotted on a nitrocellulose membrane before incubation with 1 μg cholera toxin B HRP conjugated. Blots were developed by electrochemiluminescence (ECL) detection reagents according to manufacturer's instructions (Amersham Biosciences, France).

Immunogold electron microscopy. The negative-staining procedure was performed as previously described [14]. Briefly collected lipid rafts pellet was suspended in 20 μl phosphate-buffered saline (PBS) and placed on copper grids that were coated with formvar and carbon. Rafts vesicles were adsorbed on grids for 1 min. For immunogold labeling, grids were treated successively with 2% *p*-formaldehyde, 5% BSA, and then with primary antibody in 0.5% BSA for 2 h. After washing, the sample was incubated with protein A conjugated to gold particles (10 nm) and fixed in 1% glutaraldehyde. Grids were negatively stained with uranyl acetate (1%) and observed by Zeiss EM9 transmission electron microscopy (TEM). For Lowicryl labeling, the lipid rafts pellet was fixed in 2% (wt/vol) *p*-formaldehyde with 0.2% (vol/vol) glutaraldehyde in PBS, then dehydrated in dimethylformamide and embedded in Lowicryl K4M. Ultrathin sections were layered on Maxtaform HR 24-Cu/Rh (200 mesh) and then immunolabeled with primary and 10 nm gold-labeled secondary antibodies as described [15].

Mass spectrometry analysis. Proteins in excised gel plugs were digested as described previously [16] using sequencing grade modified porcine

trypsin (12.5 ng/ml, Promega, Madison, WI). The peptides were extracted, dried in a vacuum, centrifuged, and redissolved in 10–20 μl of 0.1% TFA. The peptide mixture resulted from protein digestion was analyzed using an Ettan pro MALDI time-of-flight mass spectrometer (Amersham Biosciences, Uppsala, Sweden) in positive ion reflector mode. 0.3 μl of the peptide mixture was co-crystallized on the MALDI target with an equal amount of matrix solution (3 mg/ml of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile) in the presence of 0.5% TFA. Alternatively, peptide mixtures derived from proteins were desalted and concentrated using zip tips (Millipore Bedford, MA) and deposited onto the MALDI target by elution with the matrix solution. Proteins were identified by the Profound (ProteoMetrics, LLC, New York, NY) and the Mascot (Matrix science Ltd., London, UK) software that query comprehensive sequence databases. The presented data are representative of at least four experiments with similar results.

Total lipid extraction and analysis. The total lipid extraction was carried out as described previously [17]. For standard lipid extraction, 0.5 ml membrane aliquot containing about 0.25 mg protein was used. The total lipid extracts were subjected to TLC analysis on 0.25 mm silica gel 60 plates (Merck). Quantitative determination of fatty acids composition by GC was carried out as we previously described [18]. The cholesterol concentration of lipid raft fractions and membrane preparations was enzymatically determined by using the colorimetric method from Boehringer–Mannheim.

Miscellaneous procedures. For cholesterol depletion, CD (2%) was applied to the mucosa explants or the BBM as previously reported [19]. GPI removal on GP2 by PI-PLC was performed as previously described [20] except that BBM were used.

Results

Purification of the DRM fractions from the small intestine

In our attempt to further characterize lipid rafts markers from the small intestine by a dual biochemical and proteomic approach, we have performed the lipid rafts isolation from either the TM or purified BBM as indicated in Fig. 1A. NAP was enriched in the pooled low density fractions (4–5) from DRMs of the BBM pre-treated with 0.5% Triton X-100 at 4 °C or 1% Brij 98 at 37 °C. By screening several intestinal proteins under the same conditions, we identified the ICE as a membrane protein which was not found in lipid rafts fractions by immunoblotting (Fig. 1B). The ICE was previously shown tightly associated with the BBM [21]. We therefore defined the ICE as the non-lipid raft marker for intestinal mucosal cells. As shown in Fig. 2A, when plotted as μg cholesterol/mg protein, the light density fractions (4–5) were significantly enriched in this sterol as expected in the lipid rafts membrane [1]. Additionally, low total protein content was quantified in the raft fractions and the bulk of the protein was found at the bottom of the gradient. Furthermore, NAP and GM1 are distributed in the floating

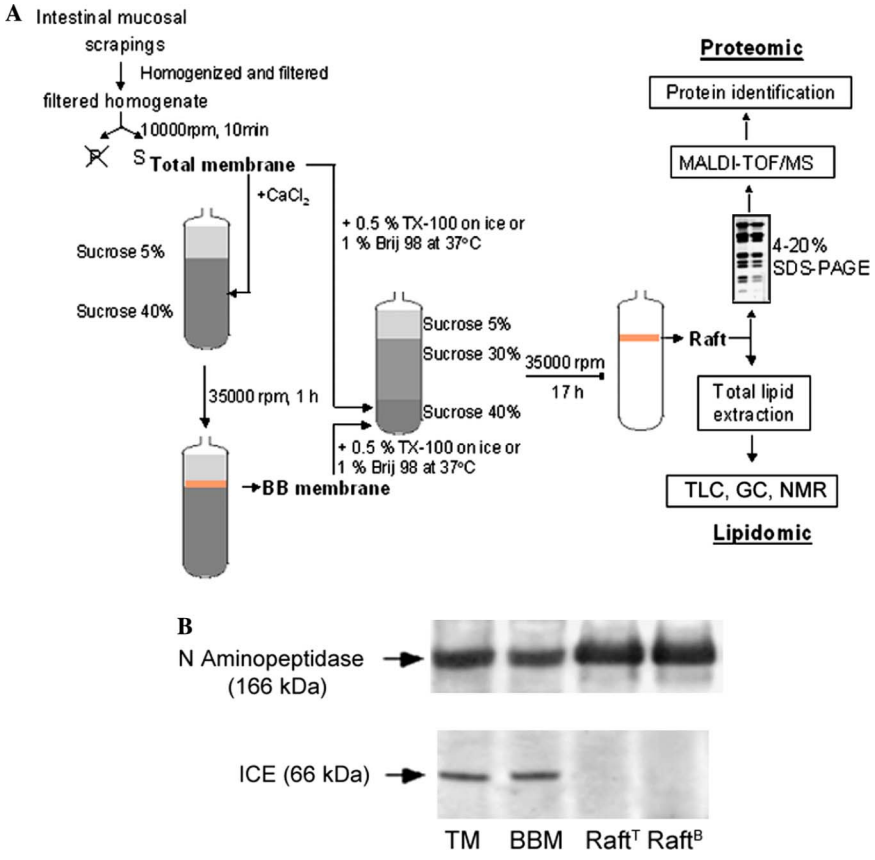

Fig. 1. Schematic overview of methodology approach and purification of lipid rafts from the rat small intestine. (A) The flowchart illustrating the lipid rafts isolation procedures. The TM, BBM, and rafts fractions were isolated by sub-cellular fractionation as described under Experimental procedures. (B) NAP and ICE localization in membranes from the rat mucosa by immunoblotting. After ultracentrifugation of the discontinuous sucrose gradient, fractions (1 ml) were collected starting from the top (fraction 1) to bottom (fraction 11). The low density fractions (4–5) were pooled and considered as "lipid rafts." $Rafts^{T}$ and $Rafts^{B}$ were DRMs from BBM pre-treated with 0.5% Triton X-100 at 4 °C and 1% Brij 98 at 37 °C, respectively. Samples of TM, BBM, and lipid rafts containing 25 μg total protein were separated by 10% SDS–PAGE and immunoblotted with the antibody against NAP or ICE.

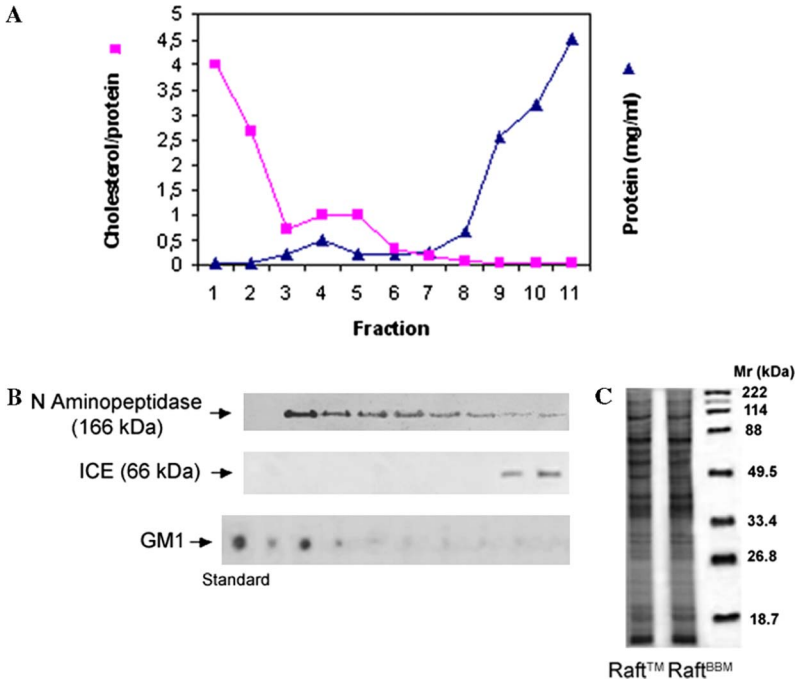

Fig. 2. N aminopeptidase co-purifies with cholesterol-rich DRM in low density fractions. (A) BBM were treated with 0.5% Triton X-100 at cold and after the sucrose gradient fractionation, fractions were collected as described in legend Fig. 1. The content in each fraction of the total protein and cholesterol was determined. (B) The total protein in each fraction of the gradient was separated by electrophoresis and immunoblotted with antibodies to NAP, ICE. For ganglioside GM1 distribution, equal volume (2.5 μl) of each fraction was separately spotted on a nitrocellulose membrane before incubation with 1 μg cholera toxin B HRP conjugated. The data are of representative from four experiments. (C) Lipid rafts under Triton X-100 solubilization conditions from TM and BBM were referred as, Raft™ and Raft^{BBM}, respectively. Total protein (10 μg) of Raft™ and Raft^{BBM} was separated by 4–20% SDS–PAGE and the gel was visualized by silver staining. Molecular weight standards are indicated (right). The major protein bands were excised for MALDI-TOF/MS analysis.

fractions, whereas, the non-raft membrane protein ICE was only detected in the high density fractions 10–11 (Fig. 2B). The lipids composition analysis by TLC and GC indicated that the lipid rafts isolated after treating with Triton X-100 or Brij 98 exhibited high content of cholesterol and glycolipids but showed low phosphoglycerolipids compared to the TM (data not shown). The analysis of acyl chain compositions by GC from BBM and lipid rafts showed that palmitic (16:0) and stearic (18:0) are the dominating acyl chains in both membrane preparations, and the proportion of saturated acyl chains was increased in the lipid rafts compared to the BBM, whereas monounsaturated and PUFAs were decreased (supplemental Table 1). These results were consistent with previous work showing that lipid rafts contain preferentially saturated fatty acids [22]. Taken together our data strongly support that the lipid rafts isolated here exhibited most of the characteristics expected for the lipid microdomains. For proteomic investigation, the protein bands of lipid rafts from TM and BBM defined as Raft™ and Raft^{BBM}, respectively, were silver stained (Fig. 2C). The protein bands from four experiments with a similar protein profile were cut and submitted to MALDI-TOF/MS analysis.

Proteomic analysis of the lipid rafts from rat small intestine

For protein band identification, several criteria were considered [23]. The more important of these were the coverage full length, the expectation values and the peptide number. When the coverage exceeded 10% and/or the expectation value was less than 0.20, the identification was considered to be sufficient unless there were some obvious conflicts such as discrepancies in the molecular weight or the tissue specificity. Importantly, whenever the expectation value of a target protein was higher than 0.20, the selection was confirmed by its immunodetection. Proteomic analysis of protein bands of lipid rafts from the intestinal TM exhibited high mitochondrial protein contaminants, thus, the BBM rafts preparations were defined as the most accurate source for the proteomic characterization. Combining proteomic identification by MALDI-TOF/MS and immunoblotting as independent approaches, 15 lipid rafts proteins from the BBM treated with Triton X-100 at cold were selected (Table 1). Most of them are membrane-associated proteins, either as GPI-anchored (# 1–3), acyl chain modified proteins (# 4, 5, and 8) or transmembrane (# 9, 11–13) proteins. Among these proteins, 12 (# 1–12) were defined as intestinal lipid rafts markers. Consistent with previous work [7], the β-actin (# 13) was identified in our rafts preparation by the proteomic analysis. However, immunoblotting using equal total proteins indicated that the bulk of β-actin was found in high density fractions. Additionally in contrast to the intensive clusters of NAP observed, immunogold labeling of β-actin showed low level of labeling in the lipid raft vesicle (data not

Table 1
MALDI-TOF/MS identification of the lipid rafts protein markers from the rat small intestinal BBM

Protein #	Protein ID	gi accession	Peptides%	Expectation[a]	MW (kDa)	Rafts
1	N Aminopeptidase	gi/47523628	16.4	0.003	109.19	√
2	Intestinal alkaline phosphatase-I	gi/7109285	16	1.47	58.590	√
3	Granule membrane glycoprotein GP2	gi/599245	27.3	0.002	60.44	√
4	Guanine nucleotide binding protein α q (Gαq)	gi/40254462	19.8	1.13	42.41	√
5	Guanine nucleotide binding protein α 11 subunit (Gα11)	gi/13591951	22.5	1.1	42.29	√
6	Annexin II	gi/9845234	45.1	0.001	38.939	√
7	Annexin IV	gi/55742832	43	0.028	36.213	√
8	Annexin XIIIb	gi/21218387	28.3	0.001	39.529	√
9	Dipeptidyl peptidase IV	gi/408716	10.2	1.749	91.93	√
10	Galectin-4	gi/6981152	28	0.000	36.44	√
11	Glutamate receptor	gi/1169962	14.2	0.027	108.62	√
12	G-protein coupled receptor 7	gi/12667796	11.3	0.89	40.98	√
13	β-Actin	gi/2318133	21.3	0.005	42.04	
14[b]	Sphingosine 1-phosphate receptor Edg-8	gi/7025517	8.0	0.794	43.26	
15[b]	Zinc-finger protein (ZNFpT3)	gi/2136419	30.2	0.049	11.28	

Lipid rafts protein bands from BBM were resolved on a 4–20% gradient SDS–PAGE and silver stained. The proteins bands were cut, subjected to trypsin digestion, and analyzed by mass spectrometry as described under Experimental procedure. The detailed of peptide spectrum of each band is available under the Supplemental data. The data are representative of three experiments with similar results.

[a] The simple interpretation of an expectation value is the number of matches that would be expected to have a particular score if the matches were completely random. An expectation value of 0.0001 means that a similar match would be found approximately once in every 10,000 similar sized databases that did not contain the sequence that truly matches our MS data. This system of risk estimation was applied to the most conventional sequence homology matching systems, such as BLAST and SWISS-PROT.

[b] Successively identified from the intestinal lipid rafts sample but not tested by immunoblotting.

shown). Together these findings suggest that actin may not be a lipid rafts protein marker in the intestine. It should be noted that the sphingosine 1-phosphate receptor (# 14[b]) and the zinc-finger protein (# 15[b]) were successively found during our proteomic identification, but their immunoreactivity was not tested in this study. Annexin II, annexin XIIIb, GP2, NAP, and galectin-4 were the more accurate probes found in lipid rafts by MALDI-TOF/MS as shown in Table 1 and by peptide mass fingerprints (supplemental figures).

Immunoblotting lipid rafts distribution of GP2, annexin II, and galectin- 4

Western blot analysis distribution of the selected proteins (Table 1) indicated that most of them were enriched in the low density fractions 4–5 defined as lipid rafts (data not shown). Only a representative lipid rafts markers distribution is given in Fig. 3 and the detail of *m/z* peptides profile of these markers is shown in supplemental Figure 1. Endogenous GP2 in the small intestine brush border partitioned to the DRM fraction 4 but was also detected in high density fractions 9–11. In contrast, both galectin-4 and annexin II were detected predominantly in the floating fractions 4–6 (Fig. 3). Pretreating the mucosa with CD, a cholesterol depleting agent, and then isolating the DRMs from BBM showed that the distribution of GP2 and galectin-4 in DRMs was not affected even after 55% the cholesterol depletion (data not shown). The result was consistent with previous work suggesting that cholesterol-independent raft microdomains may exist in the intestinal brush border [24].

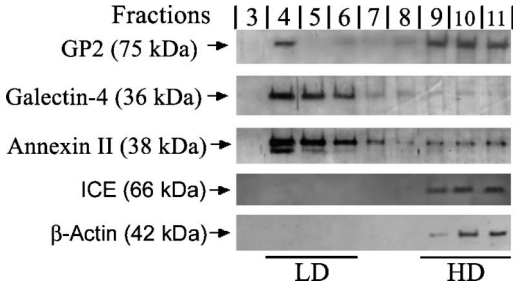

Fig. 3. DRMs rafts distribution of GP2, galectin-4, annexin II, and β-actin. The density gradient fractions from the BBM were collected as indicated in legend Fig. 1. Equal total protein 10 μg of each fraction was separated by 12.5% SDS–PAGE for immunoblotting with appropriate antibodies to the indicated proteins. The control was performed in the same conditions by using antibodies against ICE or β-actin.

Based on the subcellular localization, presumed interactions or/and functions, we propose that the proteins identified from rafts may fall into at least three representative categories including the GPI-anchored proteins, annexins subfamily, and guanidine binding associated proteins. Remarkably galectin-4, a lectin binding protein was identified in lipid rafts proteome with an expectation value of about 0.000% and 28% peptide coverage (Table 1). These results indicate that galectin-4 was identified in lipid rafts with high accuracy. By screening non-ionic detergents of BBM insolubility and immunogold electron microscopy studies, our results showed that Triton X-100 extraction at cold preserved more the vesicle structure than the Brij 98 membrane extraction at physiologic temperature. However as indicated in Fig. 4B, galectin-4 was observed in both vesicles and membrane sheets. Interestingly,

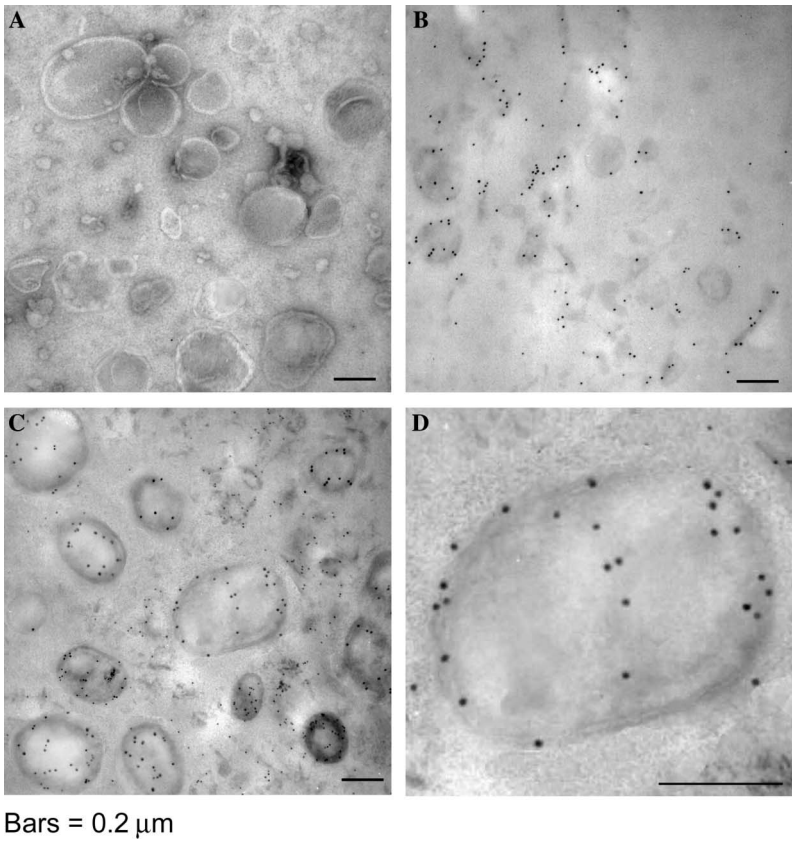

Fig. 4. Galectin immunogold labeling of lipid rafts isolated from small intestinal BBM. Electron micrographs of lipid rafts fractions prepared under extraction with 0.5% Triton X-100 at 4 °C (A, C, and D) or 1% Brij 98 at 37 °C (B) were followed by the Lowicryl labeling as described under Experimental procedures. (A) Control experiment without antibody, no labeling. Electron micrographs show rafts vesicle-like with a diameter in the range of 200–400 nm. Galectin-4 immunogold labeling (B–D). (B) Pleiomorphic membrane fragment and a few rafts vesicle-like immunogold labeled for galectin-4 are shown. (C) The rafts vesicle-like are highly immunogold labeled for galectin-4. (D) Single lipid rafts vesicle with more labeling on the membrane structure. Bars, 0.2 μm.

galectin-4 was seen in the lining of the membrane vesicles (Fig. 4C). Similar findings were reported with NAP localization on rafts like vesicles [24]. In addition by testing sequential detergent extractions of isolated lipid rafts under different temperatures (20 and 37 °C), galectin-4, IAP, and NAP were shown to be co-localized in the remaining high temperature Triton X-100-insoluble membrane referred to as superrafts. Among these proteins galectin-4 was the more prominent and the stability of the superrafts under lactose elution was dependent this protein [25]. Although the function of galectin-4 in the intestinal gut is poorly understood, several biologic activities have been assigned to galectin-4 including cell adhesion, inflammatory activation, and host defense.

Annexin subfamily proteins

The first interesting category of selected lipid rafts markers belongs to the annexin subfamily. The identified annexins II, IV, and XIIIb exhibited a high peptide coverage of 45.1%, 43%, and 28.3%, respectively, and the expectation values almost close to zero (Table 1). Annexins are calcium binding proteins widely expressed and 13 annexins (I–XIIIs) have been identified in mammals. In polarized cells, annexins have been implicated in several functions including the regulation of membrane trafficking and signaling [26]. Although annexins II, IV, and XIIIb were reported to be localized in enterocytes of rabbit [9], only annexin II has been described to localize in lipid rafts from the whole intestine by immunoblotting [25]. The rafts targeting of these proteins may be in relation with their acylation (annexin XIIIb) and/or the calcium-dependent binding to specific phospholipids [13,26]. Our results suggest that in addition to annexin II, annexin IV, and XIIIb are the new lipid rafts markers of the small intestine.

Guanidine binding associated proteins

The next significant group of proteins found in lipid rafts was the guanidine binding, and associated proteins. This group includes the $G\alpha_q$ and $G\alpha_{11}$ of G proteins, and one may extend this category to the GPCR-like GPCR 7 and glutamate receptor. Numerous signaling molecules such as G protein subunits, RGS proteins, and GPCR have been reported to be enriched in lipids rafts [27]. Our previous work showed that $G\alpha_i$ and endogenous RGS16 were

preferentially distributed in lipid rafts. Interestingly, the disruption of RGS16 from rafts microdomains by the mutation at the palmitoylation sites was critical for its rafts association and GTPase activity [13]. The spatial and temporal concentration of specific set of proteins in rafts may increase the efficiency and specificity of signaling by facilitating interactions between proteins [28]. The guanidine binding proteins found in the intestinal lipid rafts may be involved in the signaling processes in the enterocytes.

GPI anchored protein group

Although NAP and IAP were previously described to be localized in intestinal rafts, GP2 was only in our knowledge reported to be distributed in rafts from zymogen membrane [7,29]. In addition to MALDI-TOF/MS and immunodetection analysis, GP2 determination in intestinal lipid rafts was confirmed by MS/MS analysis (data not shown). Furthermore, by using the monoclonal (4A9) GP2 antibody, a prominent polypeptide of 75 kDa corresponding to GP2 was found to be distributed in BBM (Fig. 3). Since it was postulated that GP2 secreted by the pancreas is released from the membrane by a phospholipase C which hydrolyzes the phosphodiester bond linking to diacylglycerol anchor [30], we tested if the GP2 protein found in intestinal rafts may have been originated from the pancreatic juice. For that we extracted GP2 from the BBM with non-ionic detergents including 1% Brij 98 at 37 °C, 0.5% Triton X-100, and 60 mM OG at 4 °C (Fig. 5A). After detergent extraction, more than 90% and 60% of GP2 polypeptide was insoluble in Brij 98 and Triton X-100, respectively. As expected, OG completely solubilized GP2 from the BBM. These results indicated that GP2 is associated to the BBM and the binding is stable to generate its DRMs enrichment. Furthermore, when treated in vitro with specific PI-PLC, GP2 was partly shifted to the soluble fraction, suggesting that the cleavage of the GPI anchor may release GP2 from the membrane. In contrast, the distribution between the insoluble and the soluble membrane fraction of annexin II (without a GPI anchor) was not modified (Fig. 5B). Our results give a strong support that the GP2 isolated from intestinal rafts is localized in the membrane domains that fulfilled the lipid rafts features and originated from the enterocytes. Similar findings were reported for syncollin and ZG 16p, also pancreatic zymogen proteins which were shown to be expressed in the rat enterocytes and goblet cells, respectively [31,32]. Also suggestive as a strong evidence for the presence of GP2 in the BBM, we showed by immunogold labeling that GP2 was clustered on the intestinal membrane

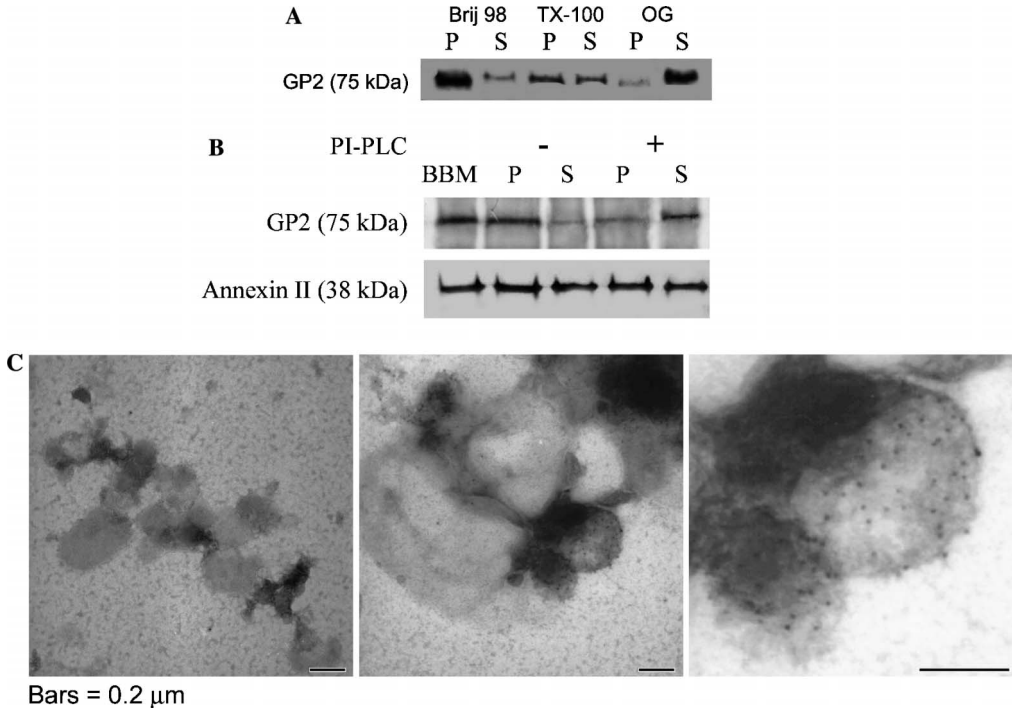

Fig. 5. GP2 solubilization by non-ionic detergents and its binding to BBM after PI-PLC treatment. (A) GP2 association to membrane was tested by incubating equal total protein of BBM with 1% Brij 98 at 37 °C, 1% TX-100, and 60 mM OG at 4 °C for 30 min. After centrifugation at 14,000 rpm, the pellet (P) and supernatant (S) were collected. The distribution of GP2 in the P and S fractions for each detergent was determined by immunoblotting using the GP2 antibody. (B) Shift of GP2 to soluble fraction by PI-PLC. BBM were treated with PI-PLC as described under Experimental procedures. The samples were immunoblotted for GP2 or annexin II (control without GPI anchor). The data are representative of four experiments with similar results. (C) GP2 immunogold labeling of lipid rafts isolated from small intestinal BBM was followed the negative-staining procedure as described under Experimental procedures. Control experiment is shown in the left without labeling. The cluster of GP2 on the lipid rafts membrane structure is shown in the middle. The right electron micrograph shows GP2 clusters at higher magnification. Bars, 0.2 µm.

(Fig. 5C). The GP2 protein was previously suggested to be involved in zymogen formation but it was recently demonstrated that pancreas secretion function was not affected in GP2 knock-out mouse. However, it was reported in the same study that GP2 may have an antimicrobial function, acting in host defense [33]. These data indicate that the lipid rafts isolated from the small intestines contain GPI-APs including GP2 which appears to be a new rafts protein in the intestine.

Discussion

The identification of the intestinal lipid rafts proteins has been limited by the use of a few specific antibodies [7]. The proteomic approach applied here on the lipid rafts was preceded by the isolation of DRMs that fulfilled most of the characteristics expected for mucosa lipid rafts. One major concern with the isolation of lipid rafts from biological membranes is the use of non-ionic detergents, in particular Triton X-100 at 4 °C [34]. Because the degree of membrane insolubility depends on the stability of the membrane molecule interactions and the reported property of Triton X-100 to enhance the formation of lipids complex at cold, membrane extractions by non-ionic detergents (Lubrol, Brij 98, Triton X-100, and OG) were optimized as previously indicated [13,35]. Therefore, from TM or BBM, DRMs were prepared under 0.5% Triton X-100 at 4 °C and 1% Brij 98 at physiologic temperature. The unique band isolated after flotation was further determined as lipid rafts by the enrichment of cholesterol, GM1, NAP and low content of total protein as well as the absence of the non-rafts marker (IEC). Although the detergent methods were determined here as the good tool for lipid rafts isolation, further studies including non-invasive approaches in intact cells are required for more lipid rafts characterization.

The systematic identification of the lipid rafts proteins combined with their immunodetection leads to the identification of several proteins. In agreement with previous works [6], these proteins include enzymes such as NAP, DPPIV, and IAP and also galectin-4 and annexin II. Interestingly, different proteins including GP2, annexin IV, XIIIb, $G\alpha_q$, and $G\alpha_{11}$ are documented for the first time to our knowledge to be distributed in rafts from the intestine. Although the role of GP2 in the intestine was not investigated, the determination of this glycoprotein in the intestine rafts may contribute to define its function. In agreement with several studies on the microvillar lipid rafts, caveolin-1 known as lipid raft marker for many cell types [2] was not identified in the lipid rafts from BBM either by the proteomic or immunoblotting analysis. Except for sucrase–isomaltase [6] the validation of our proteomic strategy was very interesting since most of the reported lipid rafts markers from the intestine were identified. Using a polyclonal antibody against the sucrase–isomaltase, the enzyme was detected in our isolated lipid rafts (data not shown). This discrepancy could be due to the low abundance of the sucrase–isomaltase and/or inaccurate MALDI-TOF/MS analysis. Further proteomic studies by the development of liquid chromatography combined with tandem mass spectrometry LC–MS/MS may improve the characterization of the lipid rafts biomarkers [36].

In summary, since many functions of the brush border are based on membrane dynamics, elucidation of the lipid rafts biomarkers is of great importance to understand the molecular mechanism of the small intestine. Through the characterized lipid rafts proteome, our results indicate that they may be implicated in digestive, signaling, and trafficking functions of the rat small intestine. Additionally, the paradigm used to characterize the intestinal lipid rafts should be generally applicable for other tissues.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.01.141.

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