Proteomic Analysis of Apical Microvillous Membranes of Syncytiotrophoblast Cells Reveals A High Degree of Similarity with Lipid Rafts

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Abstract: Brush borders (microvilli) are cell membrane specialized structures that function mainly as high-throughput absortive/secretory areas. It has been well-established that brush borders are particularly rich in membrane lipids characteristic to lipid rafts. Here, we report 57 proteins identified from microvillous membranes (MVM) isolated from human syncytiotrophoblast cells using an experimental method that avoids the use of nonionic detergents. About 60% of the proteins reported here have been described previously as lipid-raft specific. Well-known lipid raft-markers such as Annexin A2 and alkaline phosphatase were identified. Cytoskeleton structural constituents and proteins related with the control and modulation of the cytoskeletal architecture as well as the regulation of the interaction of cytoskeletal constituents with the cell membrane and particularly with lipid raft domains were found (Ezrin, IQGAP1 and 2, EBP50). Other proteins identified include signal transduction molecules, such as Ras-related protein Rab-1B and Rab-7, and ADP-ribosylation factor 1. Several proteins harbor putative post-translational modifications that favor its localization in the lipid-raft environment, such as GPI (alkaline phosphatase and 5′-nucleotidase) and myristoylation (BASP1 and MARCKS). On the whole, this extensive description demonstrates from the protein composition point of view that brush border membranes are indeed highly enriched in lipid raft microdomains.

Keywords: lipid raft • brush border • syncytiotrophoblast • multidimensional chromatography-nano ESI−MS/MS • in-solution digestion

Introduction

Lipid rafts are membrane microdomains highly enriched in cholesterol and glycosphingolipids. As result of the tight packing of the lipid acyl chains, these domains are not readily solubilized in nonionic detergents. This property has been widely used as the purification method of choice to obtain lipid rafts for further study. Intrinsic variability in the lipid raft population is now becoming apparent as an increasing number of extraction and purification methods are developed.[1] Most of these methods make use of different nonionic detergents.

In addition to its characteristic lipid composition, interest has turned on protein composition. However, results published to date remain controversial as the issue of distinguishing lipid-raft specific proteins from possible contaminants remains largely unresolved. Methods to discriminate between "true" lipid raft proteins and contaminants have been published recently.[2]

However, despite the use of different experimental approaches, there is a remarkable degree of coincidence in the protein composition of lipid rafts. This coincidence is particularly interesting after attending to the heterogeneity of the sample sources used in the few systematic descriptions published to date. In fact, comprehensive lipid raft protein descriptions have been published for neutrophils,[3] monocytes,[4,5] liver cells,[6] keratinocytes,[7] Vero cells (kidney epithelium),[8] endothelial cells,[9] HeLa cells (cervix epithelium carcinoma)[2,10] and Jurkat T lymphocytes.[11] From these and other data, lipid rafts are believed to play a role in the major routes of membrane trafficking, transport of GPI-anchored proteins and glycosphingolipids to the cell surface, nutrient absorption, regulated secretion, and transport for the endosomes to the Golgi apparatus and internalization via both caveolae and chlatrin-coated pits.[12,13]

However, significant variability in protein composition requires further explanation. Three major facts contribute to this heterogeneity. First, the intrinsic variability due to the different sources used to obtain the samples. Second, the experimental method used to obtain the sample and finally, as mentioned above, the variability introduced by the intrinsic heterogeneity inside the lipid raft population.[14] In fact, increasing evidence shows that different lipid raft proteins traffic very differently in response to different stimuli.[15] For these reasons, it is of paramount interest to obtain a more detailed description of

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the protein composition of lipid rafts in order to define properly the role of this characteristic structure.

Brush borders (microvilli) are cell membrane specialized structures of epithelial cells that function mainly as high-throughput absortive/secretory areas.[16] Among the different brush-border bearing cells, the best known examples are the intestinal enterocytes, the kidney proximal tubule cells and the placental syncytiotrophoblast although other cell types show similar but less organized features, such as cells of the liver, pancreas and commonly used cell lines (e.g., MDCK). It has been well-established that brush borders are particularly rich in membrane lipids characteristic to lipid rafts,[17,18] which seem to be essential for the maintenance and stability of microvilli. For example, the extraction of cholesterol from the plasma membrane has been shown to reduce the number of microvilli on the surface of MDCK kidney cells.[19,20] Furthermore, some well-known raft markers such as Annexin A2,[21] prominin,[22] and stomatin[23] have been shown to reside in microvillous rafts. However, some interesting differences have also been observed. For example, a significant difference in lipid composition between kidney and intestinal microvillous lipid rafts has been reported,[17,24] while the use of different nonionic detergents has demonstrated the existence of a heterogeneous population of raft domains in the microvilli.[24] Hansen et al. have demonstrated that the cholera toxin entry into pig enterocytes occurs after binding to the brush border and its internalization through a lipid-raft dependent mechanism that, however, was not affected by the cholesterol depleting drug methyl-β-cyclodextrin.[25] Additionally, caveolin-1 is a common marker of "conventional" lipid rafts that is rarely seen or not detected at all in microvilli.[26]

Thus, protein composition of microvillous lipid-rafts might be quite different when compared to "classical" or nonmicrovillous lipid rafts. To our knowledge, it has not been published any extensive description of a human microvillous subproteome, although a proteomic analysis of vesicles obtained from the rat renal cortex and highly enriched in brush border membranes has been recently described.[27]

In this paper, we describe up to 57 proteins identified by nanoLC ESI−MS/MS from microvillous membranes purified from human placental syncytiotrophoblast cells using a non-detergent based method recently described.[28] This method allows simultaneous isolation of apical and basal membranes free of mitochondrial membranes. Interestingly, most of the proteins identified have been described previously as lipid raft-specific, including some categorized as lipid-raft markers. These data strongly support previous observations showing that microvilli are highly enriched in lipid-rafts microdomains. Our description of the proteome associated to microvillous membranes purified from syncytiotrophoblast cells is of particular relevance considering that this epithelium composed of large, multinucleated cells forms a polarized epithelial architecture that is the main barrier for maternal-fetal exchange.

Materials and Methods

Preparation of Placental Apical Membrane (MVM). Human placentas obtained from normal pregnancies were collected immediately after delivery from the San José Hospital Maternity Unit and transported to the laboratory on ice. The human placental microvillous membranes (MVM) vesicles were prepared using a method previously described by Riquelme et al.[28] This method allows simultaneous isolation of apical and basal membranes from the same placenta and includes a step to isolate plasma membrane free of mitochondrial membranes (Mp). The purification method involves precipitation of non-microvillous membrane with magnesium ions, differential centrifugations and a sucrose step gradient. All solutions were buffered with 20 mM Tris-HEPES, pH 7.4. An aliquot (2 to 3 mL) of the microvillous-enriched preparation containing about 10 to 15 mg of protein was overlaid on the sucrose gradient. The band at the 37/45% sucrose interface was collected and diluted 10-fold with 20 mM Tris-HEPES, pH 7.4 and centrifuged at 110 000 $\times$ *g* for 30 min. The final pellet was dissolved in 300 mM sucrose, 20 mM Tris-maleate, pH 7.4, and stored at −196 °C (liquid nitrogen).

The purity and enrichment of the MVM membrane fraction was evaluated routinely using enzymatic assays, binding studies and Western blotting. We used alkaline phosphatase as apical membrane marker. Lack of contamination of purified apical membranes from basal and mitochondrial membranes was confirmed by quantification of adenylate cyclase activity and binding of H^3-Dihydroalprenolol to β-adrenergic receptors, both basal membrane markers, and cytochrome-c oxidase or succinate dehydrogenase as mitochondrial markers.

Western Blotting. Isolated MVM fractions from placentas were tested for the presence of specific markers by SDS-PAGE and immunoblotting. 20 μg of vesicle protein were loaded on a 12% SDS-polyacrylamide gel with molecular weight markers (Invitrogen, Germany). Electrophoresis was performed at 100 V and the gel was transferred to a nitrocellulose membrane (BioRad, Hercules, Ca, USA) overnight at 30 V. The nitrocellulose membrane was blocked 1 h at RT with 2% non fat milk in saline buffer-Tween (1.38 mM NaCl, 2.7 mM KCl and Tween 20 0.05% w/v), and washed in saline buffer-Tween. Each membrane was incubated with primary antibodies against placental alkaline phosphatase (SIGMA, St. Louis, MO), or cytochrome-C (BD Biosciences, Palo Alto, Ca, USA) for 2 h at room temperature. After intensive washing, goat anti-mouse IgG conjugated with peroxidase (Amersham Biosciences, Uppsala, Sweden) was used as secondary antibody. Detection was done by chemiluminiscence using ECL Western Blotting Analysis System (Amersham Biosciences, Uppsala, Sweden). Densitometry analysis of western blot bands was done in an UN-SCAN-IT gel 4.1 system (Silk Scientific Corporation, Orem, UT).

Tryptic Digestion and Strong Cationic Exchange (SCX) Fractionation. About ~35 μg of sample was dissolved in 50 μL of digestion buffer (50 mM ammonium bicarbonate, 0.05% SDS, pH 8.3). Proteins were denatured at 99 °C for 10 min and then chilled on ice for 20 min. 1.5 μg of recombinant trypsin (Roche, Mannheim, Germany) were then added and sample incubated overnight at 37 °C. Tryptic peptides were dried and dissolved in 100 μL of SCX Buffer A (5 mM KH_2PO_4, 25% acetonitrile, pH 3) and fractionated by SCX chromatography using a MonoS PC 1.6/5 column (Pharmacia LKB, Uppsala, Sweden) in a HP1050 chromatographic system (Hewlett-Packard, Waldbron, Germany). After 30 min of washing with 100% SCX Buffer A, a gradient of 0−30% of SCX buffer B (5 mM KH_2PO_4, 600 mM KCl, 25% acetonitrile, pH 3), 50 min, flow 100 μL/min, was applied. Fractions were collected every 2 min and dried. ZipTip C18 (Millipore Co., Billerica, MA) was used to wash the salts. Finally, peptides were dissolved in ESI Buffer A (0.5% acetic acid in water) for further analysis by Mass Spectrometry.

Mass Spectrometry Analysis. LC−ESI−MS/MS analysis was done as follows: SCX fractions were individually loaded in a 100 mm $\times$ 100 μM I.D. IntegraFrit capillary column (New Objective, Woburn, MA) packed in-house with Kromasil 5 μM

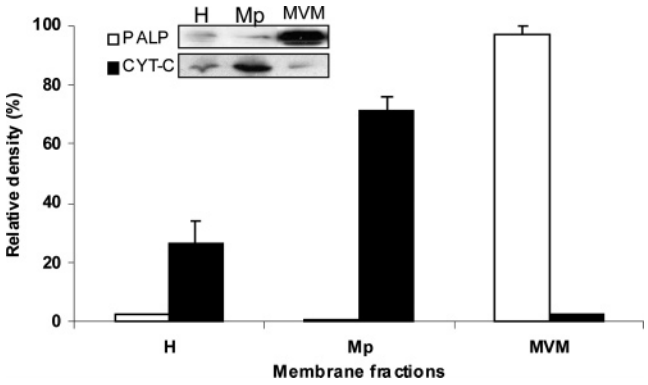

Figure 1. Enrichment of apical microvillous membrane (MVM) from human placental syncytiotrophoblast. Relative density was calculated from fractions obtained by differential and sucrose gradient centrifugation (Homogenate, H; Mitochondrial pellet, Mp; Microvillous membrane, MVM) using Western Blot analysis. Membrane proteins were resolved by 12% SDS-PAGE and blotted onto nitrocellulose. Monoclonal primary antibodies were obtained from commercial sources; anti-Placental Alkaline Phosphatase antibody was used at a final concentration of 5 μg/mL, and anti-Cytochrome C antibody at 2 μg/mL. Blots were developed using a chemiluminescence kit.

C18 beads (EKA Chemicals, Bohus, Sweden) and fractionated in a Famos-Switchos-Ultimate chromatographic system (LCPackings, The Netherlands) with a linear gradient of 5−30% ESI Buffer B (90% acetonitrile, 0.5% acetic acid in water), 60 min, at 500 nL/min. Peptides eluting from the column were directly analyzed on a Esquire 3000^{Plus} ion trap mass spectrometer (Brucker Daltonics, Bremen, Germany). Data dependent MS/MS spectra were acquired by automatic switching between MS and MS/MS mode using dynamic exclusion to avoid obtaining repetitive data of the most abundant peptides. Searches using home licensed MASCOT software (http://www.matrixscience.com/home.html) were launched against the latest version of MSDB databases (http://csc-fserve.hh.med.ic.ac.uk/msdb.html). Positive hits were individually analyzed to confirm sequence assignations.

Results and Discussion

Samples are Highly Enriched in Apical Microvillous Membranes and Essentially Devoid of Contaminants. A detergent-free method previously described[28] was used here to obtain highly enriched apical microvillous membranes (MVM) from human placental syncytiotrophoblast. The method involves precipitation of nonmicrovillous membranes with Mg^{2+}, differential centrifugation and a sucrose step gradient. To determine its effectiveness we assessed the relative enrichment of both apical and basal fractions using a set of well-known fraction specific markers. Alkaline phosphatase is a well-known marker for syncytiotrophoblast apical membranes as well as for other apical membranes. Alternatively, adenylate cyclase and β-Adrenergic receptor have been described as useful markers for the syncytiotrophoblast basal membrane.[29,30] Finally, mitochondrial membranes contamination was addressed using an antibody against the mitochondrial marker Cytochrome C. As shown in Figure 1, apical membranes (MVM) were enriched over 20-fold in alkaline phosphatase activity relative to the homogenate and were essentially free of mitochondrial membranes. Samples from the initial homogenate (H), the purified MVM and the mitochondrial fraction (Mp) were tested by Western Blot analysis using anti-Placental Alkaline Phosphatase (PLAP) and anti-Cytochrome C (Cyt-C) monoclonal primary antibodies as apical and mitochondrial membrane markers, respectively. In each case, the sum of the densitometric quantification of the three fractions tested was considered as 100% relative density. The mitochondrial marker Cyt-C was concentrated in the Mp, as depicted by the Western blot, and represents 72% ($n = 2$) of the total mark obtained for all the fractions tested, decreasing close to zero in the MVM fraction. Similar results were obtained when an antibody recognizing a second mitochondrial marker, succinate dehydrogenase, was used (Data not shown). Interestingly, some authors have reported the presence of mitochondrial proteins such as ATP synthase, NADH dehydrogenase and cytochrome *c* oxidase in the plasma membrane.[31] Other mitochondrial proteins such as Voltage-dependent anion channel (VDAC) have been described as lipid-raft specific proteins.[32] In contrast, the relative intensity of PLAP in the MVM fraction represents 97% ($n = 2$) compared with 2.5% in the homogenate. A Western blot specific for Adenylate cyclase was used to demonstrate the absence of detectable contamination of MVM membranes with basal membranes. While clearly detected in basal membranes, adenylate cyclase could not be detected in the MVM fraction (Data not shown). A second specific marker for basal membranes, such as the binding of ^{3}H-dihydroalprenolol (^{3}H-DHA) to β-adrenergic receptors yielded similar results (Data not shown).

In conclusion, the samples obtained using the method described above are highly enriched in MVM (apical) and basal membranes, respectively. Therefore, we reasoned that the MVM fraction represent an excellent sample source for the analysis of the microvillous specific subproteome.

Tryptic Digestion and MS Analysis. Our first approach to the characterization of the microvillous proteome included 1D reversed-phase (RP) nanoLC−ESI−MS/MS of 10 μg of a tryptic peptide mixture. Although 14 proteins were identified (data not shown), it soon became clear that further fractionation of the sample was required to obtain a more complete map of the microvillous proteome. Thus, 30 μg of MVM sample were trypsin-digested and fractionated by SCX chromatography (see Materials and Methods). SCX fractions were collected and individually analyzed by RP nanoLC−ESI−MS/MS. Figure 2 shows the base peak chromatogram of a representative RP nanoLC−ESI−MS/MS experiment along with representative MS/MS spectra and their corresponding sequence assignments. This strategy allowed for the identification of a number of proteins ranging between 57 and 70 (Table 1) through the confident assignment of 166 peptides. The uncertainty in the total number of proteins identified is explained by the fact that some peptide sequences do not match unequivocally with a single protein. This fact affects specially to highly homologous protein families, as is the case for the Actin, alpha-actinin and tubulin families. For example, 9 out of 15 tryptic peptides identified for alpha-actinin 4 (see Additional Data) were specific for this protein and hence, this member of the alpha-actinin family was unequivocally identified. Nevertheless, the remaining 6 peptides could also have been processed from the rest of the members of the family, namely alpha-actinin 1, 2, and 3. Thus, the presence of these members could not be unequivocally established. From now on, the minimum number of proteins identified (57) will be used. The number of peptides identified per protein ranged from 15 and 12 for alpha-actinin 4 and Annexin A2, respectively, to one single peptide for more

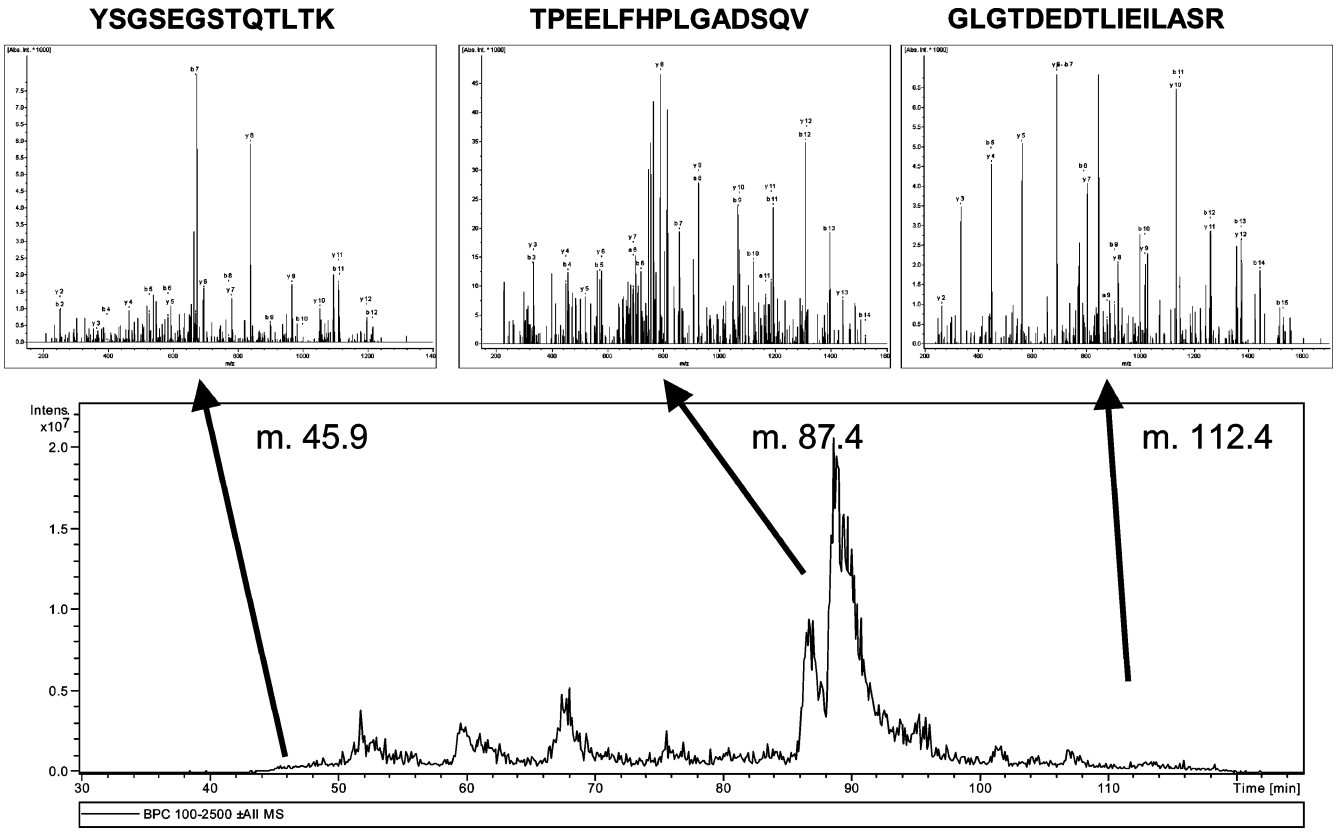

Figure 2. Base Peak ion chromatogram (BPC) of a RP nanoLC−ESI−MS/MS experiment corresponding to SCX fraction #7. 72 different tryptic peptides corresponding to 38 different proteins were identified in this fraction. Three representative MS/MS spectra along with their assigned peptide sequences are shown.

than 30 proteins (see Additional data). Each positive assignment was individually analyzed and confirmed to avoid misidentifications. Protein molecular masses ranged from ~512 kDa for the Ciliary dynein heavy chain 9 to less than ~8 kDa for the Guanine nucleotide binding protein γ12 (Table 1). Eleven out of 57 proteins (~20%) harbor transmembrane (TM) domains, including four proteins with 12, 11, 10, and 8 TM domains, respectively. These results point out the suitability of our experimental strategy over gel-based approaches for the solubilization, digestion and identification of proteins with extreme molecular weight or highly hydrophobic. According to the Human Protein Reference Database (http://www.hprd.org).[33] Thirty-two out of 57 (~56%) of the proteins identified are primarily located in the cytoplasm, 13 (~23%) are plasma membrane proteins and 3 (~5%) and 4 (~7%) are primarily targeted to the endoplasmic reticulum (ER) and the nucleus, respectively. The five remaining proteins (~9%) are found primarily in the extracellular medium, the Golgi complex and the endosome. In one case, (Transgelin 2) neither the location nor the function has been clearly established although its Calponin homology domain could interact with members of the actin family in the cytoplasm. However, it is important to note that ~54% of the proteins present alternative localizations. For example, several members of the Annexin family translocate from the cytoplasm to the cytosolic face of cellular membranes in response to elevations in intracellular Ca^{2+} levels.[34] In addition, some primary localizations require further explanation. For example, several proteins described as cytosolic (Villin, ERBP50 or 14-3-3, among others) are actually attached to the inner face of the plasma membrane. Finally, according to the biological process, the most important categories are: signal transduction and cell communication (34%); cell growth and/or maintenance (30%) and transport (16%) (Figure 3).

Microvillous Membranes (MVM) are Highly Enriched in Lipid-Raft Specific Proteins. Microvillous or brush border membranes are particularly rich in membrane lipids characteristic to lipid rafts such as glycosphingolipids and cholesterol.[17,18] We hypothesized that a significant percentage of the proteome identified here should have been described previously as lipid-raft associated or specific. Thus, we sought for similarities between the proteome described here and those systematic descriptions of lipid-raft proteomes published to date. As depicted in Table 1, 34 out of 57 proteins (~60%) have been previously reported as lipid raft specific. Several proteins identified here are common constituents of the lipid-raft proteome (Chlatrin heavy chain, calnexin, Facilitated glucose transporter, BASP1, actin gamma and beta, G3PDH and HSP27, among others), according to the number of articles that report their presence. Interestingly, 7 out of 57, and 8 out of 57 proteins were described as raft-core and raft associated proteins, respectively, according to a quantitative experimental approach aimed to distinguish raft-core or raft-associated proteins from contaminants or method-specific byproducts.[2]

Table 1 includes several well-known lipid raft markers, such as Annexin A2 and alkaline phosphatase (placental type). Annexin A2 has been demonstrated to localize in lipid rafts (see Table 1) in a process regulated/modulated by Ca^{2+} in some cases.[35] Annexin A2 interacts with actin and actin-binding proteins such as α-actinin, ezrin and moesin, and thus func-

Table 1. Identified Proteins Copurifying with Microvillous Membranes (MVM) Isolated from Human Syncityotrophoblast Cells

	protein name	entry name[a]	primary accesion no.[b]	protein MW (kDa)	peptide N#[c]	reference	TM/PTM[d]
1	Ciliary dynein heavy chain 9	DYH9_HUMAN	Q9NYC9	511931	1	(2)	
2	Spectrin alpha chain, erythrocyte	SPTA1_HUMAN	P02549	280884	2	(3, 11)	
3	chlatrin heavy chain 1	CLH1_HUMAN	Q00610	191483	1	(2, 7, 8, 11)	
4	Ras GTPase-activating-like protein IQGAP1	IQGA1_HUMAN	P46940	189252	5	(2, 7)	
5	Ras GTPase-activating-like protein IQGAP2	IQGA2_HUMAN	Q13576	180482	6		
6	Integrin alpha-5 precursor	ITA5_HUMAN	P08648	114536	1		1 TM
7	Sodium/potassium-transporting ATPase alpha-1 chain precursor	AT1A1_HUMAN	P05023	112896	1	(5, 7, 8)	10 TM
	Sodium/potassium-transporting ATPase alpha-2 chain precursor	AT1A2_HUMAN	P50993	112265	1		
	Sodium/potassium-transporting ATPase alpha-3 chain	AT1A3_HUMAN	P13637	111735	1		
8	Short transient receptor potential channel 4	TRPC4_HUMAN	Q9UBN4	112101	1		8 TM
9	9 alpha-actinin 4	ACTN4_HUMAN	O43707	104854	15	(3, 4, 7)	
	alpha-actinin 2	ACTN2_HUMAN	P35609	103854	2		
	alpha-actinin 3	ACTN3_HUMAN	Q08043	103294	1		
	alpha-actinin 1	ACTN1_HUMAN	P12814	103058	6		
10	Band 3 anion transport protein	B3AT_HUMAN	P02730	101792	3		11 TM
11	Transferrin receptor protein 1	TFR1_HUMAN	P02786	84901	6		1 TM
12	Protein-glutamine gamma-glutamyltransferase	TGM2_HUMAN	P21980	77329	1		
13	annexin A6	ANXA6_HUMAN	P08133	75742	10	(2, 7, 9)	
14	calpain 6	CAN6_HUMAN	Q9Y6Q1	74576	2		
15	Serum albumin precursor	ALBU_HUMAN	P02768	69367	3		
	Alpha-fetoprotein precursor	FETA_HUMAN	P02771	68678	2		
16	Villin 2 (Ezrin)	EZRI_HUMAN	P15311	69199	8	(2, 7)	
	Radixin	RADI_HUMAN	P35241	68564	1		
	Moesin	MOES_HUMAN	P26038	67689	1		
17	calnexin precursor	CALX_HUMAN	P27824	67568	1	(2, 4, 5, 7, 8)	1 TM
18	Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	65886	3	(8)	
19	5′-nucleotidase precursor	5NTD_HUMAN	P21589	63368	1	(2, 8, 9)	1 TM; GPI
20	Keratin, type I cytoskeletal 9	K1C9_HUMAN	P35527	61987	1	(8)	
21	Alkaline phosphatase, placental type precursor	PPBI_HUMAN	P05187	57954	11	(2, 10)	GPI
22	4F2 cell-surface antigen heavy chain	4F2_HUMAN	P08195	57909	2	(10)	1 TM
23	Annexin A11	ANX11_HUMAN	P50995	54390	1		
24	Solute carrier family 2, facilitated glucose transporter, member 1	GTR1_HUMAN	P11166	54118	4	(2, 4, 5, 7, 8,47)	12 TM
25	Keratin, type II cytoskeletal 8	K2C8_HUMAN	P05787	53543	1	(8)	
26	Tubulin alpha-1 chain	TBA1_HUMAN	P68366	49924	1	(5, 8, 11)	
	Tubulin alpha-3 chain	TBA3_HUMAN	Q71U36	50136	1		
	Tubulin alpha-2 chain	TBA2_HUMAN	Q13748	49960	1		
	Tubulin alpha-6 chain	TBA6_HUMAN	Q9BQE3	49895	1		
27	Protein kinase C and casein kinase substrate in neurons protein 3	PACN3_HUMAN	Q9UKS6	48487	1		
28	Actin like protein 3- Human	ARP3_HUMAN	P61158	47341	1	(11)	
29	chloride intracellular channel protein 5	CLIC5_HUMAN	Q9NZA1	46469	2	(46)	
30	3beta-hydroxy-Delta5-steroid dehydrogenase multifunctional protein I	3BHS1_HUMAN	P14060	42094	1		1 TM
31	Actin, aortic smooth muscle (alpha actin 2)	ACTA_HUMAN	P62736	42009	6	(8)	
32	Actin, cytoplasmic 2 (actin gamma)	ACTG_HUMAN	P63261	41793	9	(4, 5, 9, 10,11)	
	Actin cytoplasmic 1 (actin beta)	ACTB_HUMAN	P60709	41737	8	(2, 4, 5, 7, 8, 10, 11)	
33	Ezrin-radixin-moesin binding phosphoprotein 50	NHERF_HUMAN	O14745	38868	2		
34	annexin A1	ANXA1_HUMAN	P04083	38583	11		
35	annexin A2	ANXA2_HUMAN	P07355	38473	12	(2, 4, 5, 7, 8, 9)	
36	Glyceraldehyde-3-phosphate dehydrogenase	G3P2_HUMAN	P04406	35922	1	(2, 4, 5, 7, 8)	
37	Annexin A5	ANXA5_HUMAN	P08758	35806	6		
38	annexin A4	ANXA4_HUMAN	P09525	35752	5		
39	Estradiol 17-beta-dehydrogenase 1	DHB1_HUMAN	P14061	34849	1		
40	PDZ and LIM domain protein 2	Q9H4L9_HUMAN	Q9H4L9	34826	1		
41	myristoylated alanine-rich C-kinase substrate	MACS_HUMAN	P29966	31413	1	(44)	Myristoylation
42	Basigin precursor	BASI_HUMAN	P35613	29397	1	(5, 8)	1 TM
43	14−3−3 protein zeta/delta	1433Z_HUMAN	P63104	27745	1	(2, 5, 7)	
44	Chorionic somatomammotropin hormone precursor	CSH_HUMAN	P01243	25004	1		
45	Ras-related protein Rab-7	RAB7_HUMAN	P51149	23490	1	(4, 7, 8)	
46	Heat-shock protein beta-1 (heat shock protein 27)	HSPB1_HUMAN	P04792	22768	3	(2, 4, 5, 7, 8)	
47	Brain acid soluble protein 1	BASP_HUMAN	P80723	22562	2	(5, 8, 9,40,41)	Myristoylation
48	Cysteine-rich protein 2	CRIP2_HUMAN	P52943	22493	2	(8)	
49	transgelin-2	TAGL2_HUMAN	P37802	22391	2		
50	Ras-related protein Rab-1B	RAB1B_HUMAN	Q9H0U4	22171	1		
51	ADP-ribosylation factor 1	ARF1_HUMAN	P84077	20566	1	(8)	
52	calmodulin	CALM_HUMAN	Q13942	17152	1	(11)	
53	Myosin light polypeptide 6	MYL6_HUMAN	P60660	16799	2		
54	Hemoglobin gamma-1 chain	HBG1_HUMAN	P69891	16009	2		
	Hemoglobin gamma-2 chain	HBG2_HUMAN	P69892	15995	2		
55	Calgizzarin (S100 calcium-binding protein A11)	S10AB_HUMAN	P31949	11740	1		
56	S-100P protein	S100P_HUMAN	P25815	10400	1		
57	Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-12 subunit	GBG12_HUMAN	Q9UBI6	7875	1	(2, 7, 8)	

[a] UniProt/SwissProt Entry name. [b] UniProt/SwissProt Primary Accession Number. [c] Number of individual peptides identified. [d] TM, transmembrane domains; PTM, postranslational modifications.

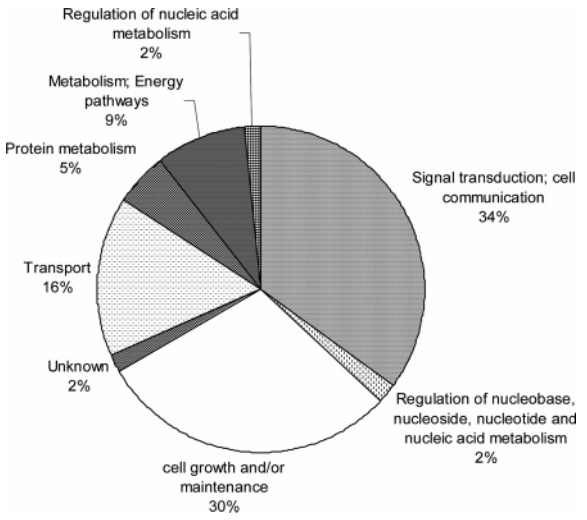

Figure 3. Functional classification of 57 proteins identified from microvillous membranes purified from human syncytiotrophoblast cells. Proteins were classified according to the Human Protein Reference Database (http://www.hprd.org/).[33]

tions as an interface between lipid raft membranes and the actin cytoskeleton.[36,37] Moreover, Annexin A2 plays a crucial role in routing vesicles transporting lipid-raft specific proteins to the apical membrane of MDCK polarized epithelial cells.[38]

Several proteins described in Table 1 harbor structural features of interest. For example, alkaline phosphatase and 5'-nucleotidase are modified by the covalent attachment of a glycosylphosphatidylinositol (GPI) moiety at the C-terminus. GPI-anchored proteins have been shown to target specifically to sphingolipid and cholesterol-enriched domains (lipid-rafts).[39] *N*-myristoylation, exemplified here by BASP1 and MARCKS, also represents a type of posttranslational modification required for certain proteins to be associated to lipid rafts.[39] BASP1 (NAP-22) is a Ca^{2+}-dependent calmodulin-binding protein that has been found in neuronal[40] and natural killer cells[41] lipid rafts. BASP1 binding to raft-like domains in model membranes[42] and induced formation of cholesterol rich domains[43] have been demonstrated. MARCKS is a calmodulin and actin-binding protein that has been identified in detergent-resistant membranes obtained from cultured cerebellar granule cells although its presence in these domains follows a sequentially regulated process.[44]

Several proteins have not been described as lipid-raft constituents but interact directly with proteins present in these microdomains. For example, EBP50 connects the membrane raft and the cytoskeleton by binding to Cbp (exclusively localized in the lipid raft) through its PDZ domain and to the complex ezrin-radixin-moesin through the C terminus.[45] Finally, some proteins deserve further attention according to their tissue-specific characteristics, as exemplified by CLIC5 and GLUT1. The former has been isolated from extracts of placental microvilli as a component of a multimeric complex that includes several proteins identified here, such as actin, ezrin, α-actinin and IQGAP1.[46] Interestingly, the authors found that CLIC5, in contrast with other members of the CLIC family, is enriched in isolated placental microvilli and is associated with the detergent-insoluble fraction. GLUT1 (Solute carrier family 2, member1) is responsible for the constitutive glucose uptake in mammalian cells and is a common constituent of lipid rafts[39] (see Table 1). Glucose deprivation increases the GLUT1 content in these subdomains. Interestingly, GLUT1 has been identified in the syncytiotrophoblast, where its distribution is asymmetric: the microvillous membrane contains markedly more GLUT1 than the basal membrane.[47]

Conclusion

Our data demonstrate that the method used for obtaining microvillous (and basal) membranes free of cross-contaminants is reliable and that microvillous membranes are highly enriched in lipid raft specific proteins. About 60% of the proteins described here have been found previously associated to lipid rafts obtained from different sources. This is in agreement with previous results showing that microvillous membranes contain lipids characteristic to lipid rafts such as cholesterol and sphingolipids. This and previous descriptions have depicted a fairly complete picture of the lipid raft proteome. However, further experiments are needed to understand its highly dynamic nature.

Abbreviations: ESI−MS/MS, electrospray tandem mass spectrometry; GPI, glycosylphosphatidylinositol; MVM, microvillous membranes; SCX, strong cationic exchange.

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Supporting Information Available: Additional Table 1 information. This material is available free of charge via the Internet at http://pubs.acs.org.

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