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Comparative Proteomics Analysis of Four Commonly Used Methods for Identification of Novel Plasma Membrane Proteins

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Abstract

Plasma membrane proteins perform a variety of important tasks in the cells. These tasks can be diverse as carrying nutrients across the plasma membrane, receiving chemical signals from outside the cell, translating them into intracellular action, and anchoring the cell in a particular location. When these crucial roles of plasma membrane proteins are considered, the need for their characterization becomes inevitable. Certain characteristics of plasma membrane proteins such as hydrophobicity, low solubility, and low abundance limit their detection by proteomic analyses. Here, we presented a comparative proteomics study in which the most commonly used plasma membrane protein enrichment methods were evaluated. The methods that were utilized include biotinylation, selective CyDye labeling, temperature-dependent phase partition, and density-gradient ultracentrifugation. Western blot analysis was performed to assess the level of plasma membrane protein enrichment using plasma membrane and cytoplasmic protein markers. Quantitative evaluation of the level of enrichment was performed by two-dimensional electrophoresis (2-DE) and benzyldimethyl-*n*-hexadecylammonium chloride/sodium dodecyl sulfate polyacrylamide gel electrophoresis (16-BAC/SDS-PAGE) from which the protein spots were cut and identified. Results from this study demonstrated that density-gradient ultracentrifugation method was superior when coupled with 16-BAC/SDS-PAGE. This work presents a valuable contribution and provides a future direction to the membrane sub-proteome research by evaluating commonly used methods for plasma membrane protein enrichment.

Keywords Plasma membrane proteins · Membrane protein enrichment · 2-DE · 16-BAC/SDS-PAGE

Abbreviations

PM	Plasma membrane
MPs	Membrane proteins
PMPs	Plasma membrane proteins
PMAPs	Plasma membrane-associated proteins
PBS	Phosphate-buffered saline
TBS	Tris-buffered saline
HB	Homogenization buffer
CHO	Chinese Hamster Ovary
MS	Mass spectrometry
IEF	Isoelectric focusing
2-DE	Two-dimensional electrophoresis
16-BAC/SDS-PAGE	Benzyldimethyl-*n*-hexadecylammonium chloride/sodium dodecyl sulfate polyacrylamide gel electrophoresis
LC–MS/MS	Liquid chromatography-mass spectrometry

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Introduction

Plasma membrane (PM) serves as a barrier between the inner surface of the cell and its surrounding environment (Helbig et al. 2010; Josic and Clifton 2007; Tan et al. 2008; Wu et al. 2003; Zhao et al. 2004). The PM is composed of lipids, integral proteins embedded in the membrane, and peripheral proteins located at the membrane surface.

Contributions from each component determine the biological functionality of PM. As their critical role in determining cell fate suggests, a relatively large portion of human genome is reserved to encode for plasma membrane proteins (PMPs) (more than 30%) (Baharvand et al. 2007; Wallin and von Heijne 1998). Although some of these proteins are identified and characterized, many of the proteins encoded by these genes remain to be explored. Considering that some of these proteins are novel disease markers and therapeutic targets, their identification and characterization are highly important (Tan et al. 2008; Zhao et al. 2004). However, the implementation of the enrichment and characterization methods commonly used for soluble proteins are not valuable when applied to the PMPs. Behind this difficulty lies the fact that the PMPs are expressed at a very low level in the cell and highly hydrophobic in nature which makes them more prone to precipitation in aqueous solutions (Luche et al. 2003; Rawlings 2016). Even if they are isolated, the absence of charged amino acids such as Arg and Lys hampers their identification by mass spectrometric methods (Helbig et al. 2010).

These challenges in identifying PMPs have led to the search for improved enrichment methods including biotinylation, selective CyDye labeling, temperature-dependent phase partition, and density-gradient ultracentrifugation. In the biotinylation method, cell surface proteins are tagged with biotin and enriched through a streptavidin-agarose column (Gu et al. 2011; Lee et al. 2009; Scheurer et al. 2005). The success of this method is often dependent on the efficiency of tagging and also prone to batch to batch variation. Recently, a novel approach was used for selective labeling of PMPs with CyDyes (Lilley and Friedman 2004). The labeling experiment was then coupled to cell lysis to isolate proteins and subject them to 2-DE for PMP identification. The ability of visualization of low-abundant proteins on 2-DE gels makes this approach appealing in the identification of PMPs (Hagner-McWhirter et al. 2008).

Temperature-dependent phase partition is one of the most commonly used methods for enrichment of PMPs (Hongsachart et al. 2008; Mathias et al. 2011; Qoronfleh et al. 2003; Tanford and Reynolds 1976). Mild detergents such as Triton and CHAPS have been extensively used in this method (Arnold and Linke 2007; Bordier 1981; Gilmore and Washburn 2010; Prive 2007; Qoronfleh et al. 2003). The success of this method is limited and prone to variation.

The density-gradient ultracentrifugation method has been historically the preferred method of choice for the enrichment of PMPs (Tauber and Reutter 1978). In this method, PM can be separated from other cellular components using the differences in physicochemical properties of cellular components (Blonder et al. 2004; Cordwell and Thingholm 2010; Foster et al. 2005; Lund et al. 2009). The gradient facilitates the separation of cellular components and allows enrichment of PMPs. However, differences in media used for gradient formation and the protocols applied cause large variations in outcome of this approach.

In this study, PMPs from CHO cells were enriched using four different methods namely, (1) biotinylation, (2) selective CyDye labeling, (3) temperature-dependent phase partition, and (4) density-gradient ultracentrifugation. The efficiencies of the methods were evaluated using the antibodies against a plasma membrane protein, sodium potassium ATPase (Na^+/K^+-ATPase) and cytoplasmic proteins, beta-actin (β-actin) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative evaluation of the level of enrichment was assessed by 2-DE gels and 16-BAC/SDS-PAGE coupled to MALDI-TOF/TOF analysis. The results demonstrated that PMP enrichment using density-gradient ultracentrifugation approach in combination with 16-BAC/SDS-PAGE was more successful in identification of PMPs.

Materials and Methods

A summary of the experimental workflow used in this study was presented as supplementary material (Supplementary Figure).

Cell Culture

CHO cells were cultured in Dulbecco's Minimum Essential Medium (DMEM-Biochrome) supplied with 10% of FBS, 100 µg/ml Penicillin/streptomycin, and 2 mM L-Glutamine at 37 °C in a humidified incubator with 5% CO_2.

Enrichment of PMPs

Four different methods were used for the enrichment of PMPs. The enrichment methods were performed three times in order to prevent experimental variation.

Biotinylation of PMPs

Biotinylation approach covers the biotinylation of cell surface PMPs in viable cells. The enrichment of proteins was achieved using streptavidin beads. A commercial biotinylation kit (Pierce cell surface protein isolation kit, #89881) provided by Thermo Scientific (USA) was used for the isolation of PMPs and the manufacturer's instructions were followed (deBlaquiere and Burgess 1999; Ellerbroek et al. 2001). Briefly, T75 flasks of 90–95% confluent cells were washed with ice-cold PBS (phosphate-buffered saline) and then incubated with Sulfo-NHS-SS-Biotin for 30 min for biotinylation reaction. Following quenching reaction with Quenching solution, cells were harvested and lysed in TBS (Tris-buffered saline) by sonicating on ice using 1-second

bursts in the lysis buffer and then centrifuged at 10,000×*g* for 2 min to separate supernatant from cell debris. Labeled proteins were isolated by adding cell lysate to NeutrAvidin Agarose column. After wash steps, bound proteins were eluted by SDS-PAGE sample buffer containing DTT.

Selective CyDye Labeling

Based on selective DIGE-labeling, a novel cell surface protein isolation method was proposed by Hagner-McWhirter et al. (Hagner-McWhirter et al. 2008). This method with minor changes was used in this study and the collected data were analyzed in detail. Cells were grown to 80% confluency in four T75 flasks. Cells were detached non-enzymatically and washed in HBSS (Hank's Balanced Salt Solution, Biochrome) buffer (pH8.5) twice and centrifuged at 1500×rpm for 10 min. Resulting pellet was resuspended in HBSS buffer, and centrifuged for 5 min at 800×*g*. Supernatant was discarded and pellet was resuspended in 200 µl HBSS with 1 M urea (pH 8.5). All labeling steps were carried out at dark. 50 µg protein was transferred to a new tube for cell surface protein labeling, and CyDye labeling reactions were carried out as recommended by the manufacturer (CyDye minimal dyes for DIGE, GE Healthcare). The labeled proteins were stored at − 80 °C until use.

Temperature-Dependent Phase Partition

A commercial kit by GBiosciences (Focus Membrane Isolation Kit, #786249) was used to enrich PMPs (Morre and Morre 1989; Santoni et al. 1999). All steps were performed according to the manufacturer's instructions. In brief, CHO cells were grown in T175 flasks to 80% confluency and the harvested cell pellet was resuspended in MPE-I buffer containing protease inhibitors, and disrupted with sonication. Two phases become visible after adding 500 µl MPE-II buffer. The top layer containing hydrophilic proteins was transferred to a new tube and stored at − 80 °C for WB analysis. The bottom layer was collected and protein concentration was determined using Bradford assay. For 2-DE analysis, 1–100 µg of protein sample was cleaned-up with UPPA-I and UPPA-II buffers based on the recommendations of the kit.

Density-Gradient Ultracentrifugation

A protocol based on flotation through a discontinuous gradient was performed by following the instructions of OptiPrep Application protocol (Li et al. 2006; Wu et al. 2001). Cells were grown to 80% confluency in T175 flasks. Washed cells were homogenized in Homogenization buffer (HB: 0.25 M sucrose, 1 mM EDTA, 2 mM $MgCl_2$, 20 mM Hepes–NaOH, pH 7.4) by repeated passages through a syringe needle (25/26 G) up to 20 times. Homogenate was centrifuged at 2000×*g* for 10 min to pellet the cell debris. Supernatant was harvested and centrifuged at 100,000×*g* for 45 min. Gradient formation was achieved using iodixanol solutions of 2.5%, 10%, 17.5%, 25%, and 30% (30% contains the pellet after centrifugation at 100,000×*g*) in 5-ml ultracentrifuge tubes (Beckman Coulter, #326819) by overlayering technique. The tube was centrifuged at 165,000×*g* for 4.5 h and the gradients were collected in fractions of 0.5 ml by tube puncture. PM fraction was located between 2.5 and 10% gradients.

Determination of Protein Concentration

Protein concentrations were determined using Bradford assay with a spectrophotometer (Nanodrop™ 1000 Spectrophotometer, Thermo Fisher Scientific, USA) (Bradford 1976).

Protein Precipitation

Enriched PMPs were cleaned and concentrated using ReadyPrep 2-DE Cleanup kit (Bio-Rad, USA). All steps were carried out at + 4 °C according to the manufacturer's instructions.

Electrophoretic Separation of PMPs

Enriched PMPs were subjected to 2-DE and 16-BAC/SDS-PAGE for separation. At least two gels were run for each experiment.

Two-Dimensional Electrophoresis (2-DE)

When the eluted proteins were subjected to 2-DE, the elutions were performed with 2-DE rehydration buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris pH 8.5, and 1× protease inhibitor cocktail. 800 µg protein was passively rehydrated to 17 cm pH 3–10 immobilized pH gradient (IPG) strips for overnight. Rehydrated strips were focused using Protean IEF cell (Bio-Rad, USA) applying 250 V (20 min, linear), 10,000 V (2 h, linear), and 50,000 V h (rapid), respectively, for the first dimension separation. Strips were washed with equilibration buffer I and II (Bio-Rad, USA) and placed onto 10%, 1 mm SDS-PAGE gels for the second dimension separation. Gels were run at 180 V.

Benzyldimethyl-*n*-hexadecylammonium Chloride/Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (16-BAC/SDS-PAGE)

A protocol recommended by Hartinger et al. (1996) was performed for the separation of PMPs. Briefly, 6–10% of

gradient separation and 4% stacking gel solution were prepared using 0.75-mm glass plates. Samples were mixed with 16-BAC-SDS/PAGE sample buffer and loaded to 0.75 mm gels. Electrophoresis was carried out in electrode buffer (2.5 mM 16-BAC, 150 mM glycine, and 50 mM phosphoric acid) at 20 mA. At the end of separation, vertical gel strips were generated by cutting with a clean scalpel. The strips were fixed isopropanol:acetic acid solution (35:10 v/v), and equilibrated in 100 mM Tris–HCl (pH 6.8) before loading onto 1 mm SDS-PAGE gels for the second dimension separation (Laemmli 1970).

Gel Staining

Gels were fixed in solution containing 40% methanol and 10% acetic acid for overnight and stained in colloidal Coomassie Brilliant Blue (cCBB) G-250 the following day.

Image Analysis

Spots were visualized by Versa Doc4000 MP using Quantity One software (Bio-Rad, USA-Version 4.6.7). Spots of interest were cut from gels by ExQuest Spot Cutter using PDQuest Advanced 2-D analysis software (Bio-Rad, USA-Version 8.0.1) and stored at + 4 °C until analysis.

In-Gel Digestion, Mass Spectrometry and Protein Identification

Protein identification experiments were performed at DEKART Proteomics Laboratory (Kocaeli, Turkey) using ABSCIEX MALDI-TOF/TOF 5800 system (Applied Biosystems®, Framingham, MA, USA). Spots of interest were cut from the gels and subjected to in-gel tryptic digestion using an in-gel digestion kit following the recommended protocol by the manufacturer (Pierce®, USA). Before deposition onto a MALDI plate, all samples were desalted with a 10 µl ZipTipC18 (Millipore®, USA). Peptides were eluted in a volume of 1 µl using a concentrated solution of α-CHCA in 50% acetonitrile and 0.1% trifluoroacetic acid in water and spotted onto the MALDI target plate. The TOF spectra were recorded in the positive ion reflector mode with a mass range from 400 to 2000 Da. Each spectrum was the cumulative average of 2000 laser shots. The spectra were calibrated with the trypsin autodigestion ion peaks m/z (842.510 and 2211.1046) as internal standards. Ten of the strongest peaks of the TOF spectra per sample were chosen for MS/MS analysis. The PMFs were searched in the MASCOT v.2.5 (Matrix Science) by using a streamline software, ProteinPilot (ABSCIEX®, USA), with the following criteria: National Center for Biotechnology Information non-redundant (NCBInr); species restricted to *H. sapiens*; enzyme of trypsin; at least five independent peptides matched; at most one missed cleavage site; MS tolerance set to ± 50 ppm and MS/MS tolerance set to ± 0.4 Da; fixed modification being carbamidomethyl (Cys) and variable modification being oxidation (Met); peptide charge of 1 + and being monoisotopic. Only significant hits, as defined by the MASCOT probability analysis ($p < 0.05$), were accepted.

WB Analysis

WB analysis was performed as described in (Ozgul et al. 2015), except for anti-alpha 1 Sodium Potassium ATPase (Na^+/K^+-ATPase) antibody, extracts in SDS sample buffer were incubated at 60 °C for 10 min prior to SDS-PAGE analysis. Anti-alpha 1 Na^+/K^+-ATPase mouse monoclonal antibody was from Abcam (ab7671), and β-Actin (sc-81178) and GAPDH (G9) mouse monoclonal antibodies (sc-365062) were from Santa Cruz Biotechnology. HRP-labeled secondary antibody (Bio-Rad, USA) was used as the secondary antibody.

Results

Four different commonly used MP enrichment methods were evaluated to provide a comparative assessment for PMP identification. 2-DE and 16-BAC/SDS-PAGE were used for the separation of enriched proteins. The separated proteins were then identified by MALDI-TOF/TOF (Tables 1 and 2).

Identification of PMPs Enriched by Biotinylation

Biotinylation of cell surface PMPs and their subsequent enrichment with streptavidin column chromatography was performed as described in the "Materials and Methods" section. The extent of PMP enrichment was assessed by using a PM-specific marker, Na^+/K^+-ATPase, and a cytoplasmic protein marker, GAPDH.

A strong Na^+/K^+-ATPase band was detected in the enriched protein fractions. However, we also detected a strong GAPDH band in the same enriched protein fractions indicating that the enriched protein fractions also contain notable amount of cytoplasmic proteins. Quantitative evaluation of the level of enrichment was performed by running 2DE gels from which protein spots were cut and identified. Approximately 120 ± 20 protein spots were detected on the gels and 106 of these were excised and identified by MALDI-TOF/TOF. Only 23 proteins were reliably identified. However, the identified proteins corresponded to 46 different spot positions on the 2-D gels. Cellular localizations of the identified proteins were determined using UniProt database. The identified proteins did not localize to the PM (Fig. 1a). They were either PM-associated (39%),

Table 1 List of identified proteins by 2-DE

No	AC no	Protein NAME	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
Nuclear proteins									
1	P62827	GTP-binding nuclear protein Ran	Nucleus, cytoplasm, melanosome, nucleus envelope	342	1.60E−30	11	7.01	34	Bio-PMPs, Sel-CdL, T-dPP
2	Q61656	Probable ATP-dependent RNA helicase DDX5	Nucleus	64	0.0094	5	9.06	9	Sel-CdL
3	Q99K48	Non-POU domain-containing octamer-binding protein	Nucleus, nucleolus	199	3.20E−16	18	9.01	23	T-dPP
4	P48679	Lamin-A	Nucleus, nucleus envelope, intermediate filament	307	5.00E−27	31	6.54	28	D-gUc
5	P24452	Macrophage-capping protein	Nucleus, cytoplasm	98	2.80E−06	10	6.73	18	T-dPP
Cytoplasmic proteins									
6	Q8BTM8	Filamin-A	Cytoplasm, cytoskeleton	354	1.00E−31	35	5.68	15	Bio-PMPs
7	P31000	Vimentin	Cytoplasm, intermediate filament	946	6.40E−91	40	5.06	64	Bio-PMPs, Sel-CdL, T-dPP, D-gUc
8	P68365	Tubulin alpha-1C chain	Cytoplasm, cytoskeleton	526	6.40E−49	17	4.96	35	Bio-PMPs
9	P63260	Actin, cytoplasmic 2	Cytoplasm, cytoskeleton	630	2.50E−59	22	5.31	39	Bio-PMPs
10	P48975	Actin, cytoplasmic 1	Cytoplasm, cytoskeleton	748	4.00E−71	23	5.22	37	Bio-PMPs, Sel-CdL, T-dPP, D-gUc
11	Q9JKY1	Peroxiredoxin-1	Cytoplasm, melanosome	235	8.00E−20	14	8.22	47	Bio-PMPs
12	Q63610	Tropomyosin alpha-3 chain	Cytoplasm, cytoskeleton	88	4.00E−05	15	4.75	40	Bio-PMPs
13	P68361	Tubulin alpha-1B chain	Cytoplasm, cytoskeleton	164	1.00E−12	9	4.94	21	Bio-PMPs
14	P69893	Tubulin beta-5 chain	Cytoplasm, cytoskeleton	300	2.50E−26	13	4.78	20	Bio-PMPs, Sel-CdL, T-dPP
15	P50543	Protein S100-A11	Cytoplasm	88	3.90E−05	5	5.28	24	T-dPP
16	P99027	60S acidic ribosomal protein P2	Cytoplasm, nucleus	101	2.00E−06	7	4.42	48	T-dPP
17	Q99PT1	Rho GDP-dissociation inhibitor 1	cytoplasm	266	6.40E−23	18	5.12	45	T-dPP
18	P62259	14-3-3 protein epsilon	Cytoplasm	182	1.60E−014	22	4.63	48	T-dPP
19	P62976	Ubiquitin	Cytoplasm	376	6.40E−34	13	6.56	77	T-dPP
20	P15991	Heat shock protein beta-1	Cytoplasm	496	6.40E−046	19	6.23	46	T-dPP
21	P48500	Triosephosphate isomerase	cytoplasm	293	1.30E−25	16	6.89	39	T-dPP
22	Q9DBJ1	Phosphoglycerate mutase 1	Cytoplasm	168	3.20E−13	12	6.67	34	T-dPP

Table 1 (continued)

No	AC no	Protein NAME	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
23	P11980	Pyruvate kinase isozymes M1/M2	Cytoplasm, nucleus	558	4.00E−52	32	6.63	39	Bio-PMPs, T-dPP, D-gUc
24	P04642	L-lactate dehydrogenase A chain	Cytoplasm	70	2.40E−03	13	8.45	17	T-dPP
25	P09445	Elongation factor 2	Cytoplasm, nucleus	257	5.00E−22	32	6.31	23	Bio-PMPs, T-dPP
26	P68040	Guanine nucleotide-binding protein subunit beta-2-like 1	Cytoplasm (perinuclear region)	84	9.80E−05	11	7.6	28	T-dPP
27	P60843	Eukaryotic initiation factor 4A-I	Cytoplasm, nucleus	235	8.00E−20	26	5.32	37	T-dPP
28	P50310	Phosphoglycerate kinase 1		124	1.00E−08	20	8.02	36	T-dPP
ER, ER membrane, and golgi apparatus proteins									
29	Q01853	Transitional endoplasmic reticulum ATPase	ER, cytoplasm, nucleus	256	6.40E−22	20	5.14	27	Sel-CdL
30	Q9CPW5	Translocon-associated protein subunit beta	ER membrane	68	3.60E−03	7	7.93	12	Sel-CdL
31	P19324	Serpin H1	ER, ER lumen	160	2.50E−12	26	8.9	36	Sel-CdL, D-gUc
32	P11598	Protein disulfide-isomerase A3	ER, ER lumen	336	6.40E−30	28	5.88	39	D-gUc
33	P08113	Endoplasmin	ER lumen, melanosome	260	2.50E−22	32	4.74	23	D-gUc
34	Q60432	Hypoxia up-regulated protein 1	ER, ER lumen	361	2.00E−032	39	5.09	27	D-gUc
35	P38660	Protein disulfide-isomerase A6	ER lumen	540	2.50E−50	24	5.04	40	D-gUc
36	Q8K3H7	Calreticulin	ER lumen	476	6.40E−44	21	5.57	54	T-dPP, D-gUc
37	Q8BHN3	Neutral alpha-glucosidase AB	ER, Golgi apparatus, melanosome	195	8.00E−16	29	5.67	24	D-gUc
38	Q9R1J8	Prolyl 3-hydroxylase 1	ER lumen, secreted, basement membrane	121	2.00E−008	22	5.02	20	D-gUc
39	O08795	Glucosidase 2 subunit beta	ER lumen	80	2.50E−04	15	4.41	21	D-gUc
40	Q66H94	Peptidyl-prolyl *cis–trans* isomerase FKBP9	ER lumen	177	5.00E−14	23	4.93	22	D-gUc
41	Q8K297	Procollagen galactosyltransferase 1	ER lumen	170	2.50E−13	22	6.83	23	D-gUc
42	O35887	Calumenin	ER lumen, melanosome, secreted	122	1.60E−08	15	4.49	39	D-gUc

Table 1 (continued)

No	AC no	Protein NAME	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
43	Q8BH97	Reticulocalbin-3	ER lumen	60	2.80E−02	6	4.74	16	D-gUc
44	P38659	Protein disulfide-isomerase A4	ER lumen	99	3.30E−06	16	4.98	18	D-gUc
45	P56395	Cytochrome b5	ER membrane, microsome membrane (single-pass, cytoplasmic)	350	2.5E−031	9	4.96	36	T-dPP
46	P35279	Ras-related protein Rab-6A	Golgi apparatus membrane (lipid-anchor)	506	6.4E−047	30	5.42	59	T-dPP
47	P84078	ADP-ribosylation factor 1	Golgi apparatus	169	3.20E−13	14	6.32	42	T-dPP
48	Q8CFN2	Cell division control protein 42 homolog	Cell membrane (lipid-anchor, cytoplasmic side), extracellular	162	1.6E−012	8	6.15	29	T-dPP
49	P46638	Ras-related protein Rab-11B	Recycling endosome membrane, synaptic vesicle membrane (lipid-anchor, cytoplasmic side)	297	5.00E−26	13	5.64	40	T-dPP
50	P36536	GTP-binding protein SAR1a	ER, Golgi apparatus	122	1.60E−08	8	6.08	23	T-dPP
51	Q921E2	Ras-related protein Rab-31	early endosome, trans Golgi network membrane, phagosome membrane (transport to cell membrane)	162	1.6E−012	14	7	54	T-dPP
52	Q9DBH5	Vesicular integral-membrane protein VIP36	Golgi apparatus membrane (single-pass, type-I)	337	5.00E−30	18	6.46	28	T-dPP
53	P51150	Ras-related protein Rab-7a	Cytoplasmic vesicle; phagosome membrane, late endosome membrane (peripheral membrane protein-cytoplasmic side)	475	8.00E−44	29	6.4	66	Sel-CdL
Mitochondrial proteins									
54	P18687	60 kDa heat shock protein, mitochondrial	Mitochondrion matrix	769	3.2E−73	29	5.83	34	Sel-CdL, D-gUc

Table 1 (continued)

No	AC no	Protein NAME	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
55	Q9D6R2	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Mitochondrion	267	5.00E−023	13	6.27	25	Sel-CdL, T-dPP
56	P56480	ATP synthase subunit beta, mitochondrial	Mitochondrion, mitochondrion inner membrane	1130	2.50E−109	35	5.19	47	Sel-CdL, D-gUc
57	P38647	Stress-70 protein, mitochondrial	Mitochondrion	732	1.60E−69	29	5.91	32	Sel-CdL, D-gUc
58	Q8BFR5	Elongation factor Tu, mitochondrial	Mitochondrion	254	1.00E−021	9	7.23	16	Sel-CdL
59	P81155	Voltage-dependent anion-selective channel protein 2	Mitochondrion outer membrane	261	2.00E−22	22	7.44	51	D-gUc
60	P10860	Glutamate dehydrogenase 1, mitochondrial	Mitochondrion matrix	330	2.5E−029	33	8.05	37	D-gUc
61	Q8CIZ7	Dihydrolipoyl dehydrogenase, mitochondrial	Mitochondrion matrix	139	3.20E−10	13	7.96	14	D-gUc
62	Q5XHZ0	Heat shock protein 75 kDa, mitochondrial	Mitochondrion, mitochondrion inner membrane	272	1.6E−023	27	6.56	24	D-gUc
63	Q8K2B3	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	Mitochondrion inner membrane (peripheral membrane protein-cytoplasmic side)	174	1.00E−13	25	7.06	34	D-gUc
64	P12787	Cytochrome c oxidase subunit 5A, mitochondrial	Mitochondrion inner membrane	122	1.60E−08	8	6.08	23	T-dPP
65	P19536	Cytochrome c oxidase subunit 5B, mitochondrial	Mitochondrion, mitochondrial envelope	62	0.017	4	8.69	8	T-dPP
66	Q9CQX2	Cytochrome b5 type B	Mitochondrion outer membrane	212	1.60E−17	9	4.79	23	T-dPP
67	P20070	NADH-cytochrome b5 reductase 3	Isoform 1: ER membrane, mitochondrion outer membrane (lipid-anchor-cytoplasmic side). İsoform 2: cytoplasm.	212	1.6E−017	11	8.56	26	T-dPP

Table 1 (continued)

No	AC no	Protein NAME	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
68	P52825	Carnitine O-palmitoyltransferase 2, mitochondrial	Mitochondrion inner membrane (peripheral, matrix side)	169	3.2E−013	19	8.46	17	T-dPP
69	Q8CAQ8	Mitochondrial inner membrane protein	MITOCHONDRIAL inner membrane (multi-pass)	246	6.40E−21	29	6.18	26	T-dPP
70	Q8BGH2	Sorting and assembly machinery component 50 homolog	Mitochondrion outer membrane (multi-pass)	226	6.40E−19	25	6.34	29	T-dPP
Exosomal proteins									
71	Q61753	D-3-phosphoglycerate dehydrogenase	Extracellular exosome, myelin sheath	250	2.50E−21	18	6.12	31	Bio-PMPs
72	Q6IFZ9	Keratin, type-II cytoskeletal 74	Cytoplasm, extracellular exosome	101	2.00E−06	14	5.51	23	Sel-CdL
73	Q6TMK6	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	Cell body, extracellular exosome	217	5.00E−18	26	5.61	43	T-dPP, D-gUc
Plasma membrane proteins									
74	P31977	Ezrin	Apical cell membrane (peripheral membrane protein-cytoplasmic side), microvillus membrane	238	4.00E−20	29	5.83	31	D-gUc
75	O35763	Moesin	Cell membrane, cytoskeleton, apical cell membrane, microvillus membrane	230	2.50E−19	24	6.22	41	D-gUc
76	Q61490	CD166 antigen	Cell membrane (single-pass, type I), cell projection	94	0.000011	12	5.85	12	T-dPP
77	P05943	Protein S100-A10	Extracellular exosome, extrinsic component of plasma membrane	68	0.0044	6	6.28	27	T-dPP
78	Q9QUI0	Transforming protein RhoA	Cell membrane (lipid-anchor, cytoplasmic side), cytoplasm	84	0.00011	13	5.83	29	T-dPP
79	P62835	Ras-related protein Rap-1A	Cell membrane, cytoplasm, cell junction	146	6.40E−11	12	6.38	45	T-dPP

Table 1 (continued)

No	AC no	Protein NAME	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
80	P62492	Ras-related protein Rab-11A	Cell membrane (lipid-anchor), endosome membrane	180	2.50E−14	9	6.12	21	T-dPP
81	P09055	Integrin beta-1	Cell membrane (single-pass, type-I), recycling endosome	216	6.40E−18	23	5.68	16	T-dPP
82	Q99JB2	Stomatin-like protein 2	Cell membrane (peripheral), mitochondrion	83	0.00012	15	8.95	24	T-dPP
83	Q9Z2L0	Voltage-dependent anion-selective channel protein 1	Isoform 1: mitochondrion outer membrane (multi-pass). İsoform 2: cell membrane, membrane raft (multi-pass)	339	3.20E−30	14	8.62	32	T-dPP
84	Q03265	ATP synthase subunit alpha, mitochondrial	Cell membrane (peripheral, extracellular side). Mitochondrion inner membrane.	650	2.50E−61	34	9.22	41	T-dPP
Plasma membrane-associated proteins									
85	Q8VDD5	Myosin-9	Cytoplasm, cytoskeleton, cell–cell adherens junction	327	5.00E−29	34	5.54	16	Bio-PMPs
86	P07823	78 kDa glucose-regulated protein	ER, melanosome, cytoplasm, cell surface, plasma membrane	1360	2.5E−132	42	5.07	52	Bio-PMPs, Sel-CdL, T-dPP, D-gUc
87	P05064	Fructose-bisphosphate aldolase A	Cytoplasmic, membrane	437	5.00E−40	23	8.31	47	Bio-PMPs
88	P17244	Glyceraldehyde-3-phosphate dehydrogenase	Cytoplasmic, cytoskeleton, nucleus, membrane	420	2.50E−38	16	8.49	33	Bio-PMPs, T-dPP
89	P47953	Galectin-3	Cytoplasm, nucleus, secreted, cell surface	218	4.00E−18	12	8.97	26	Bio-PMPs
90	P62629	Elongation factor 1-alpha 1	Cytoplasmic, nucleus, membrane	233	1.30E−19	15	9.1	27	Bio-PMPs, Sel-CdL
91	P46633	Heat shock protein HSP 90-alpha	Cytosol, melanosome, cell membrane	80	0.00023	6	4.96	7	Bio-PMPs
92	P03995	Glial fibrillary acidic protein	Cytoplasm, cell body, intermediate filament, membrane	61	0.019	2	5.27	2	Bio-PMPs
93	Q8R4U2	Protein disulfide-isomerase	ER, cell membrane	590	2.50E−55	21	4.78	30	Sel-CdL, T-dPP, D-gUc

Table 1 (continued)

No	AC no	Protein NAME	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
94	P67778	Prohibitin	Mitochondrion inner membrane, cytoplasm, nucleus, cell surface	537	5.00E−50	22	5.57	51	Sel-CdL, T-dPP
95	P07356	Annexin A2	Secreted; extracellular space, extracellular matrix, basement membrane	396	6.40E−36	31	7.55	52	D-gUc
96	Q99068	Alpha-2-macroglobulin receptor-associated protein	ER, cytoplasm, cell surface	275	8.00E−24	23	6.85	37	D-gUc
97	Q5U367	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	Rough ER membrane (peripheral membrane protein-lumenal side)	149	3.20E−11	16	5.82	12	D-gUc
98	P57759	Endoplasmic reticulum resident protein 29	ER lumen, melanosome, cell surface	64	0.0098	8	5.9	16	D-gUc
99	P19378	Heat shock cognate 71 kDa protein	Cytoplasm, melanosome, nucleolus, cell membrane	600	2.50E−56	35	5.24	39	Bio-PMPs, T-dPP, D-gUc
100	Q61598	Rab GDP-dissociation inhibitor beta	Cytoplasm, cell membrane (peripheral)	154	1.00E−11	27	5.93	31	T-dPP
101	P07150	Annexin A1	Cytoplasm, cell membrane (peripheral), apical cell membrane	173	1.30E−13	15	6.97	20	Sel-CdL, T-dPP
102	P17182	Alpha-enolase	Cytoplasm, cell membrane	273	1.3.E-023	24	6.37	35	T-dPP
Other proteins (secreted, etc.)									
103	Q6KDN2	Multimerin-1	Secreted	55	0.078	19	7.74	10	Bio-PMPs
104	P07338	Chymotrypsinogen B	Secreted (extracellular)	96	6.1.E-06	8	4.9	18	Sel-CdL, T-dPP
105	Q6P7A9	Lysosomal alpha-glucosidase	Lysosome, lysosome membrane	86	6.2.E-06	12	5.45	7	D-gUc
106	P24270	Catalase	Peroxisome	269	3.20E−23	21	7.72	32	D-gUc
107	P02468	Laminin subunit gamma-1	Secreted, extracellular space, basement membrane	230	2.50E−19	35	5.08	21	D-gUc
108	P48538	Galectin-1	Secreted (extracellular)	314	1.00E−027	16	5.52	61	T-dPP

pI isoelectric point, *Bio-PMPs* biotinylation of plasma membrane proteins, *Sel-CdL* selective CyDye labeling, *T-dPP* temperature-dependent phase partition, *D-gUc* density-gradient ultracentrifugation

Table 2 List of identified proteins by 16-BAC/SDS-PAGE

No	AC no	Protein name	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
Nuclear proteins									
1	P14733	Lamin-B1	Nucleus inner membrane (lipid-anchor, nucleoplasmic side)	141	2.00E−10	25	5.11	34	T-dPP
2	Q8VIJ6	Splicing factor, proline- and glutamine-rich	Nucleus matrix, cytoplasm	120	2.50E−008	26	9.45	23	T-dPP
3	P0C0S6	Histone H2A.Z (J/1C)	Nucleus	73	0.0014	9	10.58	42	D-gUc
4	P48679	Lamin-A	Nucleus	60	0.025	19	6.54	23	D-gUc
Cytoplasmic proteins									
5	P63260	Actin, cytoplasmic 2	Cytoplasm, cytoskeleton	285	8.00E−25	18	5.31	38	T-dPP, D-gUc
6	P48975	Actin, cytoplasmic 1	Cytoplasm, cytoskeleton	438	4.00E−40	24	5.22	57	T-dPP, D-gUc
7	Q9D8N0	Elongation factor 1-gamma	Cell–cell adherens junction, cytoplasm	84	0.0001	17	6.31	25	T-dPP
8	P20152	Vimentin	Cytoplasm, intermediate filament	304	1.00E−26	37	5.06	51	T-dPP, D-gUc
9	P14659	Heat shock-related 70 kDa protein 2	Cytoplasm, cytoskeleton	127	5.00E−09	12	5.51	15	T-dPP, D-gUc
10	P18708	Vesicle-fusing ATPase	Cytoplasm	145	8.00E−11	24	6.52	24	T-dPP
ER, ER membrane, and Golgi apparatus proteins									
11	P62821	Ras-related protein Rab-1A	Golgi, ER membrane, early endosome, membrane, cytosol	185	8.00E−15	8	5.93	22	T-dPP
12	P51150	Ras-related protein Rab-7a	Cytoplasmic vesicle; phagosome membrane, late endosome membrane (peripheral membrane protein-cytoplasmic side)	260	2.50E−022	16	6.4	52	T-dPP
13	Q9D1G1	Ras-related protein Rab-1B	Cytoplasm, membrane, preautophagosomal structure membrane (lipid-anchor, cytoplasmic side),	174	1.00E−13	9	5.55	33	T-dPP
14	P35279	Ras-related protein Rab-6A	Golgi apparatus membrane (lipid-anchor)	198	4.00E−16	17	5.42	37	T-dPP
15	Q9DBH5	Vesicular integral-membrane protein VIP36	ER-Golgi compartment membrane (single-pass, type-I)	125	8.00E−09	13	6.46	18	T-dPP

Table 2 (continued)

No	AC no	Protein name	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
16	O54734	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa subunit	ER membrane (single-pass, type-I)	284	1.00E−024	16	5.52	23	T-dPP, D-gUc
17	P07687	Epoxide hydrolase 1	Microsome membrane (single-pass, type-II), ER membrane (single-pass, type-II)	151	2.00E−11	19	8.59	21	T-dPP
18	Q7TQ95	Protein lunapark	ER membrane (multi-pass)	82	0.00018	11	5.24	18	T-dPP
19	Q91YQ5	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1	ER membrane (single-pass, type-I)	74	0.0011	11	6.02	15	T-dPP
20	P35565	Calnexin	ER membrane (single-pass, type-I), melanosome	143	1.30E−010	16	4.49	19	T-dPP
21	P08113	Endoplasmin	ER lumen, melanosome	57	0.047	15	4.74	15	T-dPP
22	Q9DCF9	Translocon-associated protein subunit gamma	ER membrane (multi-pass)	70	0.0023	3	9.61	12	T-dPP
23	Q3ZT31	Sorting nexin-25	Endosome membrane (peripheral)	78	0.00043	13	5.83	10	D-gUc
Mitochondrial proteins									
24	Q60930	Voltage-dependent anion-selective channel protein 2	Mitochondrion outer membrane	128	4.00E−09	12	7.44	30	T-dPP
25	P20070	NADH-cytochrome b5 reductase 3	Isofrom 1: ER membrane, mitochondrion outer membrane (lipid-anchor, cytoplasmic side). İsoform 2: cytoplasm.	90	2.50E−005	11	8.56	29	T-dPP
26	P10719	ATP synthase subunit beta, mitochondrial	Mitochondrion, mitochondrion inner membrane.	444	1.00E−40	22	5.19	43	T-dPP, D-gUc
27	P18687	60 kDa heat shock protein, mitochondrial	Mitochondrion matrix	85	8.80E−005	10	5.83	12	T-dPP
28	P52825	Carnitine O-palmitoyltransferase 2, mitochondrial	Mitochondrion inner membrane (peripheral, matrix side)	137	5.00E−10	17	8.46	16	T-dPP
29	Q8CAQ8	Mitochondrial inner membrane protein	Mitochondrial inner membrane (multi-pass)	113	1.30E−007	17	6.18	14	T-dPP

Table 2 (continued)

No	AC no	Protein name	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
Plasma membrane proteins									
30	Q99JI6	Ras-related protein Rap-1b	Cell membrane, cytoplasm, cell junction	75	0.00078	12	5.65	36	T-dPP
31	P53994	Ras-related protein Rab-2A (2B)	Cell membrane (lipid-anchor, cytoplasmic side), ER membrane.	157	5.00E−12	11	6.08	46	T-dPP
32	P35278	Ras-related protein Rab-5C (5A/5B)	Cell membrane (lipid-anchor, cytoplasmic side), early endosome membrane (lipid-anchor), melanosome, cytoplasmic vesicle	72	0.0018	9	8.64	26	T-dPP
33	Q9Z2L0	Voltage-dependent anion-selective channel protein 1	Isoform 1: mitochondrion outer membrane (multi-pass). İsoform 2: cell membrane, membrane raft (multi-pass)	194	1.00E−15	12	8.62	34	T-dPP
34	Q03265	ATP synthase subunit alpha, mitochondrial	Mitochondrion inner membrane. Cell membrane (peripheral, extracellular side)	350	2.50E−031	37	9.22	40	T-dPP
35	Q63355	Myosin-Ic	Cytoplasm, cell membrane (peripheral-cytoplasmic side)	144	1.00E−10	26	9.45	23	D-gUc
36	P26041	Moesin	Cell membrane, cytoskeleton, apical cell membrane, microvillus membrane	178	4.00E−14	17	6.22	25	D-gUc
37	P26043	Radixin	Cell membrane (peripheral-cytoplasmic side), cytoplasm	148	4.00E−11	14	5.85	21	D-gUc
38	P26040	Ezrin	Apical cell membrane (peripheral membrane protein-cytoplasmic side), microvillus membrane	143	1.30E−10	9	5.83	10	D-gUc
39	O54724	Polymerase I and transcript release factor	Membrane (caveola), cell membrane	108	4.00E−007	13	5.43	21	D-gUc
40	Q91ZX7	Prolow-density lipoprotein receptor-related protein 1	Cell membrane (single-pass, type-I; peripheral), cytoplasm	211	2.00E−17	57	5.14	11	D-gUc

Table 2 (continued)

No	AC no	Protein name	Cellular location	Protein score	Expect value	The # of matched peptides	Calc. pI	Seq. cov. (%)	Methods of enrichment
Plasma membrane-associated proteins									
41	P67778	Prohibitin	Mitochondrion inner membrane, cytoplasm, nucleus. Cell surface (direct assay)	72	0.0014	16	9.83	45	T-dPP
42	P17182	Alpha-enolase	Cytoplasm, cell membrane	63	0.013	11	6.37	17	T-dPP
43	P62629	Elongation factor 1-alpha 1	Cytoplasmic, nucleus. Membrane (direct assay)	73	0.0012	10	9.1	14	T-dPP, D-gUc
44	P19378	Heat shock cognate 71 kDa protein	Cytoplasm, melanosome, nucleolus, cell membrane.	229	3.20E−019	27	5.24	37	T-dPP, D-gUc
45	P07823	78 kDa glucose-regulated protein	ER, melanosome, cytoplasm. cell surface, plasma membrane (direct assay)	285	8.00E−25	27	5.07	38	T-dPP, D-gUc
46	P34058	Heat shock protein HSP 90-beta	Cytosol, melanosome, cell membrane	101	2.00E−06	22	4.97	27	T-dPP
47	P17244	Glyceraldehyde-3-phosphate dehydrogenase	Cytoplasmic, cytoskeleton, nucleus. Membrane (direct assay)	143	6.40E−011	17	8.49	31	T-dPP, D-gUc
48	Q07936	Annexin A2	Secreted; extracellular space, extracellular matrix, basement membrane	110	2.50E−007	10	7.55	28	D-gUc
Other proteins (secreted, etc.)									
49	Q99ML5	Prenylcysteine oxidase	Lysosome	80	0.00027	12	6.61	18	T-dPP
50	Q3URK1	Coiled-coil domain-containing protein C1orf110 homolog	Secreted	57	0.052	11	9.2	28	T-dPP

pI isoelectric point, *T-dPP* temperature-dependent phase partition, *D-gUc* density-gradient ultracentrifugation

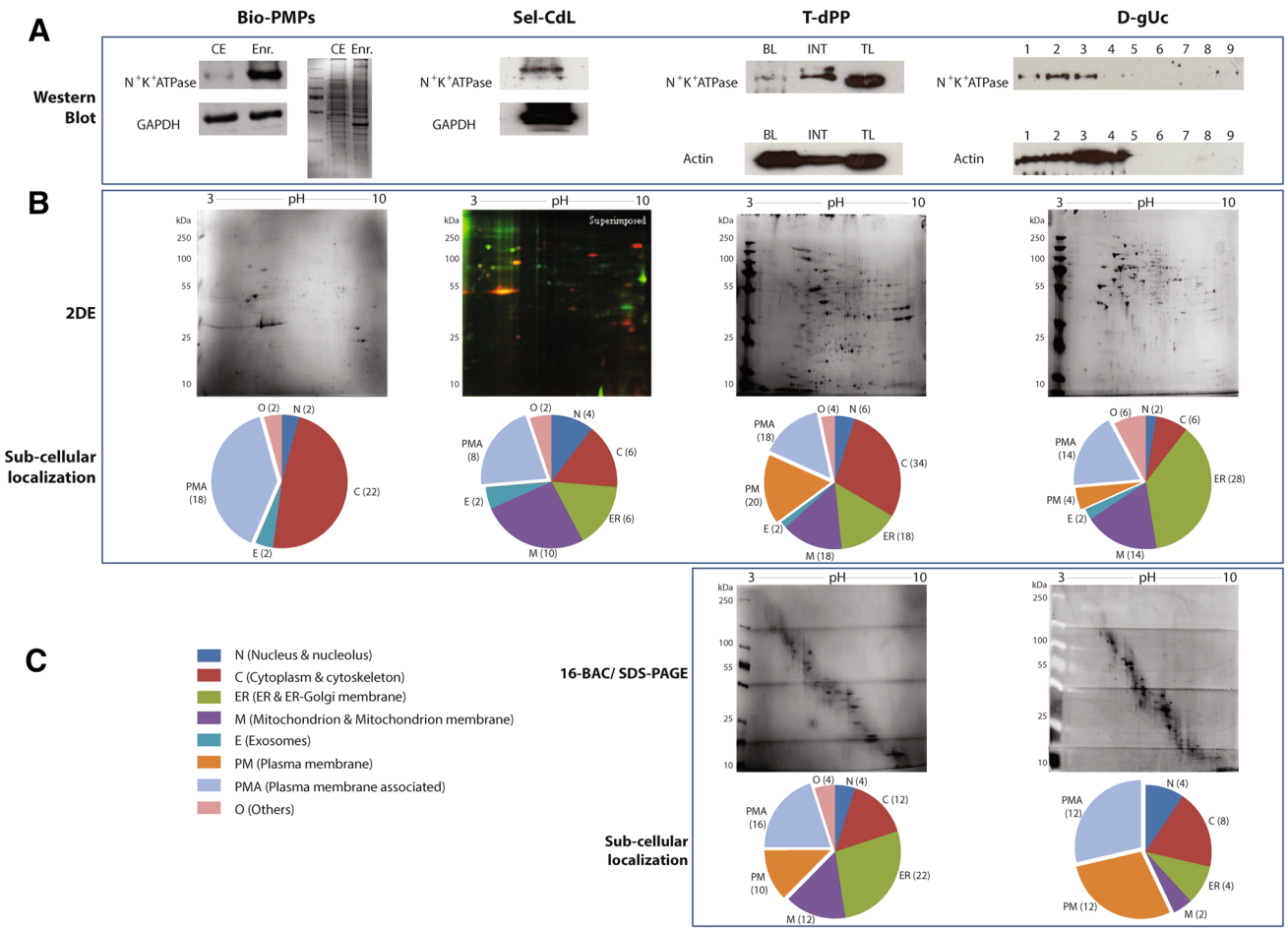

Fig. 1 Representative images summarizing the major findings of the study. **a** Western Blot analysis of the enriched membrane protein fractions. For Bio-PMPs approach, a cell-free protein extract (CE) prepared from CHO cells were included in the WB analysis to demonstrate the degree of enrichment. In addition, an image of an SDS-PAGE gel was used to show equal loading from the CE and the enriched fraction (Enr.). Monoclonal antibodies against Na^+/K^+-ATPase, β-Actin, and GAPDH were used to assess the level of PMP enrichment. **b** Images of 2-DE gels from which the putative PMPs were cut and identified. The pie-charts were used to present subcellular localization. **c** Images of 16-BAC/SDS-PAGE gels from which the putative PMPs were cut and identified. The pie-charts were used to present subcellular localization. *Bio-PMPs* biotinylation of plasma membrane proteins, *Sel-CdL* selective CyDye labeling, *T-dPP* temperature-dependent phase partition, *D-gUc* density-gradient ultracentrifugation, *N* nucleus and nucleolus, *C* cytoplasm and cytoskeleton, *ER* ER and ER membrane, *M* mitochondrion and mitochondrion membrane, *E* exosomes, *PM* plasma membrane, *PMA* plasma membrane-associated, *O* others. The number of identified proteins was given in parenthesis in the pie chart

or organelle-specific membrane proteins or contaminating cytoplasmic proteins.

Identification of PMPs Enriched by Selective CyDye Labeling

Two consecutive labeling experiments were performed; the initial labeling with Cy3 targeting PMPs of the whole cells and the subsequent second labeling with Cy5 targeting total proteins after cell lysis. The total protein extracts were subjected to WB analysis to demonstrate the presence of Na^+/K^+-ATPase and GAPDH. Imaging of the gels for Cy3 revealed the presence of 80 ± 10 protein spots representing putative PMPs and 350 ± 20 protein spots for Cy5 representing the soluble proteome. Among those Cy3-labeled protein spots, 31 of them were reliably identified. Some of the identified spots belonged to the same protein thus causing a decrease in the total number of identified proteins. Classification of the identified proteins based on subcellular locations revealed that none of the identified proteins were PMPs. We were able to identify PMAPs (21%) indicating that *selective CyDye labeling* approach was not effective in selective enrichment of the PMPs (Fig. 1b). Most of the identified proteins were cytoplasmic and organelle-associated.

Identification of PMPs Enriched by Temperature-Dependent Phase Partition

Three different phases—namely sediment, inter-phase, and upper-phase—were generated and the bottom layer (sediment plus inter-phase) containing the hydrophobic proteins was collected. WB analysis revealed the presence of PM marker Na^+/K^+-ATPase in the inter-phase fraction, but also at the top layer which was expected to have only hydrophilic proteins. To examine the proteomic profiling of enriched proteins, 2-DE analysis was performed. Sixty spots were identified and classified based on their subcellular location (Fig. 1b). We were able to identify PMPs (17%) and PMAPs (15%) along with the proteins belonging to mitochondrion and mitochondrion membrane (15%). Overall, *temperature-dependent phase partition* approach did not provide sufficient enrichment of PMPs despite some success over the other enrichment methods.

Identification of PMPs Enriched by Density-Gradient Ultracentrifugation

Ten fractions were collected and subjected to WB analysis using anti-Na^+/K^+-ATPase antibody. Fractions positive for Na^+/K^+-ATPase were used in 2-DE analysis. Thirty eight spots were successfully identified and their subcellular localizations were assigned (Fig. 1b). We were able to identify PMPs (5%) and PMAPs (18%) along with the proteins belonging to ER and ER membrane (37%). In overall, *density-gradient ultracentrifugation* approach did not provide sufficient enrichment of PMPs despite some success over the other enrichment methods.

16-BAC/SDS-PAGE Analysis of PMPs Enriched by Temperature-Dependent Phase Partition

PMPs were enriched and the enrichment was assessed by WB analysis using the plasma membrane marker, Na^+/K^+-ATPase, and the cytoplasmic protein marker, β-actin (Fig. 1a). The enriched proteins were subjected to separation by 16-BAC/SDS-PAGE, and 40 different proteins were identified. Classification of the identified proteins based on cellular localization revealed that 12.5% were PM, and 20% were PMAPs (Fig. 1c). Majority of the PMAPs and organelle-based proteins originated from ER and Golgi membranes (Table 2).

16-BAC/SDS-PAGE Analysis of PMPs Enriched by Density-Gradient Ultracentrifugation

Fractions collected after density-gradient ultracentrifugation were subjected to WB analysis to assess the PMP enrichment using Na^+/K^+-ATPase and β-actin as the markers (Fig. 1a). Fractions positive for Na^+/K^+-ATPase were used in 16-BAC/SDS-PAGE analysis, and 21 proteins were identified (Table 2). Classification of the identified proteins based on cellular localization revealed that 28.6% were PMPs, 28.6% were PMAPs, and 9.5% were associated with ER and ER membrane (Fig. 1c). List of identified proteins by 16-BAC/SDS-PAGE is given in Table 2.

Discussion

PMPs have important roles in cellular functions and can be potential drug targets. Thus, their identification is highly important. For successful identification of PMPs, they have to be highly enriched and the problem of contamination with soluble proteins and subcellular organelles must be overcome. Therefore, this study aimed at evaluating the efficiency of four different commonly used PMP enrichment methods to provide a comparison for the improvement of novel PMP identification.

The extent of PMPs extracted determines the success of the enrichment methods, which rely on the type of protein, lipid content, and the number of transmembrane domains in proteins. There is a continuing effort to enrich and identify PMPs, but only a few of them found a place in practice. One of the methods relies on biotinylation of PMPs and more often used than the others (Busch et al. 1989; Sabarth et al. 2002; Shin et al. 2003). The biotinylation approach was first utilized to identify surface proteins from *Helicobacter pylori* (Sabarth et al. 2002). This approach was then adopted by the others to study eukaryotic cell surface proteins. Jang et al. were able to enrich cell surface proteins from leukemia cell lines by biotinylation and identified them by 2-DE coupled to mass spectrometry (Jang and Hanash 2003). Although they were able to identify cell surface proteins, most of the identified proteins were cytoplasmic or ER-associated. In another study, Nunomura et al. combined biotinylation with ultracentrifugation, and identified 324 proteins of which 24% were MPs (Nunomura et al. 2005). Similar to these two studies, others also identified MPs by this approach but they were not able to avoid contamination from soluble proteins. In this study, we were not able to enrich PMPs using biotinylation approach despite several attempts and optimization efforts. The reasons for the inadequate enrichment could be (1) low biotinylation efficiency of PMPs, (2) contribution of endogenously biotinylated cellular proteins to the observed contamination, and (3) low purification efficiency of hydrophobic proteins from the streptavidin column.

Selective CyDye labeling was used as a promising approach to label cell surface proteins and identify them through 2-DE coupled to MALDI-TOF/TOF (Hagner-McWhirter et al. 2008). Hagner-McWhirter et al. were able to identify a large number of cell surface proteins, of which

82% were membrane associated. Their results also showed minimal labeling of intracellular proteins indicating a leakage of CyDye into the cytoplasm. We adopted this approach and performed several experiments. To our surprise, we mostly identified cytoplasmic (16%) and organelle-specific proteins (42%). Only 21% of the identified proteins were PMAPs. Similar to our findings, Sidibe et al. (2007) performed integrated biotinylation and Cydye labeling approach and reported identification of 228 proteins. Only 6% of their proteins were integral MPs and 23% of them were PMAPs. These findings implied that either there was a leakage of CyDye into the cytoplasm or the cells were unintentionally lysed during labeling experiments causing identification of contaminating intracellular proteins.

Qoronfled et al. used temperature-dependent phase partition approach for PMP isolation, and showed that hydrophilic proteins remained in aqueous phase while hydrophobic membrane proteins partitioned in the detergent phase (Qoronfleh et al. 2003). In another study using the same approach, Hongsachart et al. evaluated the efficiency of three extraction methods. They used three different detergents and identified the putative PMPs employing 2-DE-based proteomic approach coupled to MALDI-TOF. They found that 20% of the identified proteins were MPs (Hongsachart et al. 2008). Phase separation approach using solutions of Triton X-114 was applied to isolate mouse liver microsomal membrane proteins. 50% of the proteins identified by LC–MS/MS contained at least one TM domain (Mathias et al. 2011). Our results indicated that detergent-containing buffer that we used was not effective enough in separating hydrophilic and hydrophobic proteins. This observation was confirmed by WB analysis. Antibodies against PM-specific proteins generated signals in both hydrophobic and hydrophilic fractions.

Subcellular fractionation by ultracentrifugation method is one of the most preferred strategies used in PMP enrichment. This method has long been the choice of many researchers (Blonder et al. 2004; Foster et al. 2005; Kjeldsen et al. 1999; Pionneau et al. 2005). A good example to the use of ultracentrifugation method was provided by Foster et al. who monitored the changes in expression levels of PMP markers of mesenchymal stem cells (Foster et al. 2005). The researchers identified 463 proteins by LC–MS/MS, and reported that 66% were PMPs. In another study, which aimed to identify cell surface proteins of keratinocytes using gradient centrifugation coupled to LC–MS/MS analysis, a similar success of PMP enrichment was achieved (57.3%) (Blonder et al. 2004). We used this approach and performed several experiments. Unfortunately, only 5.3% of the identified proteins were PMPs and 18.4% were PMAPs. Although we were able to achieve a relative success in identifying PMPs, our success was limited when compared with the literature. This might be due to the limitations resulting from 2-DE-based MS approach, which is less superior to LC–MS/MS approach in PMP identification. The percentage of PMPs increased to 28.6% when 16-BAC/SDS-PAGE was used as the separation method, showing the relative success of the diagonal gel system.

One of the challenging aspects of enriching PM is to overcome the contamination caused by other organelles. The contamination is an expected consequence since the density of membranes of other organelles are within the vicinity of PM (The rough endoplasmic reticulum = 1.20 g/cm^3, Golgi vesicles = 1.14 g/cm^3, and plasma membrane = 1.12 g/cm^3). For instance, Pionneau et al. (2005) emphasized ER and Golgi apparatus contamination in membrane proteome fractions of a breast cancer cell line. In another study, Orsburn et al. indicated that the use of centrifugation to remove soluble proteins and other organelle membranes was not sufficient to obtain the PM in a pure form and with high yield (Orsburn et al. 2011). Our results of density-gradient ultracentrifugation method also showed that PMPs were contaminated by membrane proteins of ER and Golgi apparatus (36.8%), and mitochondria (18.4%). In addition to close-densities' problem, another major source of contamination is inappropriate cell lysis. Vigorous cell lysis may end up in undesired disruption of organelle membranes. It is our experience that standardization of lysis protocol demands extreme caution. If it is not done properly, it is inevitable to observe batch-to-batch variations.

MPs have a hydrophobic core that causes them to precipitate together with lipids during extraction and solubilization procedures. A number of methods can be used to prevent precipitation of MPs such as the use of high concentrations of detergents like SDS, CHAPS, or Triton X-100 in solubilization buffers to break intra- and inter-protein–protein interactions. However, the presence of high concentrations of these detergents inhibit the enzymatic cleavage during protein digestion and can influence the mass spectrometric analysis. Although in some studies it is reported that the addition of surfactants instead of high concentrations of detergents can improve solubility and promote the amount of identified PMPs (Donoghue et al. 2008; Rabilloud 2009), our results demonstrated the otherwise indicating that only a minor additional contribution was achieved. 2-DE has been used as the standard approach for the separation of protein mixtures for decades (Chevalier 2010; Rabilloud et al. 2010). However, 2-DE has many drawbacks especially in the separation of proteins with hydrophobic fractures (Chevalier 2010; Santoni et al. 2000). Hydrophobic proteins can precipitate on the IPG strips preventing them to be visualized on the second dimension gels. As an alternative, 16-BAC, a cationic detergent, was proposed for electrophoretic separation. Wenge and colleagues have revealed that the use of 16-BAC may dramatically increase the separation of PMPs (Wenge et al. 2008). Our results confirmed the Wenge's findings. The number of identified PMPs in density-gradient

ultracentrifugation approach was five-fold higher when 16-BAC/SDS-PAGE was used instead of 2-DE (Fig. 2).

A standardized workflow covering PMP solubilization, enrichment, separation, digestion by proteases, and identification by MS has not been developed yet. One of the main reasons for the lack of such a workflow is because there are many options for each step that may create many different combinations for identification of PMPs (Fig. 3). The preferred choice in each step has advantages as well as disadvantages over the other choices. For example, the use of non-ionic detergents over the ionic ones eases identification of PMPs via mass spectrometry but causes insufficient solubilization and separation. Similarly, biotinylation of PMPs and their subsequent enrichment via avidin purification offers advantages over phase partitioning or density-gradient centrifugation but cannot cope with the limitations arising from inefficient biotinylation process. In overall, two bottlenecks should at least be dealt with to successfully identify PMPs at high efficiency. The first bottleneck is the use of appropriate PMP separation approaches, which would ease their subsequent identification via mass spectrometry. The main choice of PMP separation in this study was 2DE. However, a limited success was obtained with 2DE technology despite all our efforts. PMPs were most likely depleted due to aggregation during first dimension separation. Our efforts to replace 2DE with 16-BAC electrophoresis also did not yield a noticeable increase in the number of identified PMPs. A variation of gel-based approaches is used in the literature

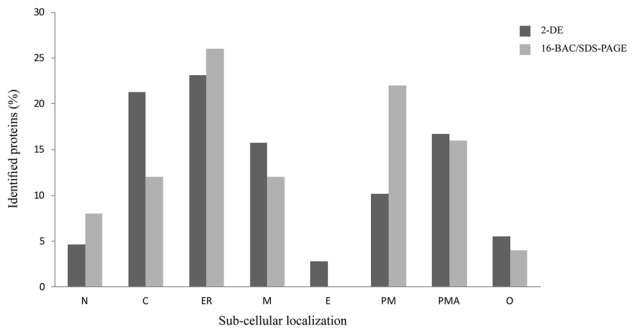

Fig. 2 Bar graph demonstrating the overall comparison of subcellular locations of the identified proteins by 2-DE and 16-BAC/SDS-PAGE. The abbreviations represent *Bio-PMPs* biotinylation of plasma membrane proteins, *Sel-CdL* selective CyDye labeling, *T-dPP* temperature-dependent phase partition, *D-gUc* density-gradient ultracentrifugation, *N* nucleus and nucleolus, *C* cytoplasm and cytoskeleton, *ER* ER and ER membrane, *M* mitochondrion and mitochondrion membrane, *E* exosomes, *PM* plasma membrane, *PMA* plasma membrane-associated, *O* others

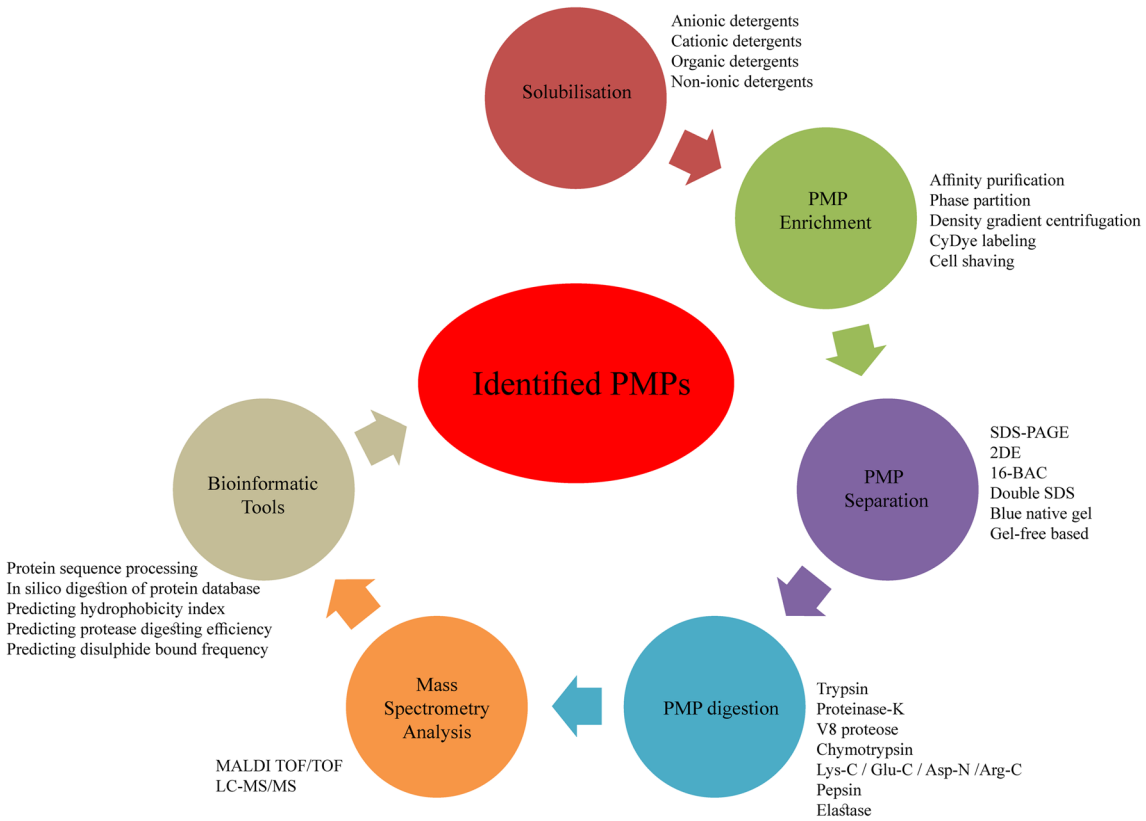

Fig. 3 Graphical representation of the main PMP enrichment and identification methods. For each method, the use of alternative approaches is also given. It is important to note that there are many options that may create many different workflow combinations for identification of PMPs

in which the enriched PMPs are separated by SDS-PAGE and the resulting gel is sliced into equal bands for PMP identification via LC–MS/MS (Cordwell and Thingholm 2010; Ozlu et al. 2010; Smolders et al. 2015). Although this method has the advantage of utilizing SDS for efficient solubilization of hydrophobic proteins it does not allow comparative quantification among biological samples. The most advantageous technique that eliminates the disadvantages of gel-based separation technologies is gel-free IEF which enables both protein and peptide fractionation in solution (Islinger and Weber 2008; Schiffer et al. 2006; Simpson and Smith 2005). However, due to the collection of high number of fractions, the amount of workload in gel-free IEF is tremendous making it less desirable. The second bottleneck is to find a protease that efficiently digests PMPs prior to mass spectrometry analysis. PMP digestion has been largely monopolized by the use of a single enzyme, trypsin due to the fact that trypsin is very efficient, and specific protease at a relatively reasonable cost (Tsiatsiani and Heck 2015). However, the relative high hydrophobicity of PMPs limits their digestion with trypsin. The hydrophobic α-helical segments in PMPs are poor in the charged lysines (K) and arginine (R) residues that are the targets for trypsin (Vit and Petrak 2016). Alternative proteases, e.g., chymotrypsin, Lys-C, Glu-C, Asp-N, and Proteinase-K are available to increase the coverage of peptides to improve identification of PMPs (Tsiatsiani and Heck 2015). These enzymes suffer from non-specificity, low efficiency, and target charged amino acids like trypsin. Cymotrypsin appears to be particularly useful for covering transmembrane regions of membrane proteins because of its preference for hydrophobic amino acids (C-terminal of Phe, Tyr, Lys, Trp, and Met) at its cleavage site. However, the efficiency of chymotrypsin toward different hydrophobic amino acid residues varies and results in quite a few missed cleavages (Giansanti et al. 2016). In our study, trypsin was the main protease used for in-gel digestion of proteins. In several instances, we tested the use of chymotrypsin but did not see any improvement in PMP identification (data not shown). Perhaps, the ameliorating effect of chymotrypsin can be best observed in gel-free systems.

Conclusion

Based on our evaluation, biotinylation and selective CyDye labeling approaches failed to enrich PMPs effectively. Relatively, temperature-dependent phase partition and density-gradient ultracentrifugation approaches were partly successful in PMP enrichment. In addition, in comparison to 2-DE approach, 16-BAC/SDS-PAGE was more successful in separating PMPs. Alternative methods are urgently needed to overcome the problems and generate more coverage for PMPs. Recent advances in LC–MS/MS technology appear to help, but still lack the power to fully gratify the scientists. What is needed is not only a strong identification method, but also a effective enrichment method.

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