PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2009, 36(11): 1458~1468
www.pibb.ac.cn

Aqueous Polymer Two-phase Partition for The Proteomic Analysis of Plasma Membrane From Rat Dorsal Root Ganglion Neurons*

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Abstract Dorsal root ganglion (DRG) neurons are primary sensory neurons that conduct neuronal impulses related to pain, touch and temperature senses. To comprehensively identify proteins of plasma membrane (PM) from small amount of dorsal root ganglion (DRG) neurons, a proteomics strategy that utilizes aqueous polymer two-phase partition in combination with differential velocity centrifugation was adopted to enrich the PM, followed by SDS-PAGE, CapLC-MS/MS and bioinformatics analysis. Western blot analysis showed that the concentration of PM in purified plasma membrane(PPM) was 2.3 times higher than that in crude plasma membrane(CPM), 15 times higher than that in whole tissue lysate (WTL). By searching against the rat IPI protein sequence database, a total of 729 non-redundant proteins were identified from the PM preparation, of which 547 had a gene ontology (GO) annotation indicating a cellular component, and 159 (21.8%) were unambiguously identified as PM proteins. A data set of plasma membrane proteins of DRG as well as a tool to study PM proteins were provided in a small amounts of sample.

Key words dorsal root ganglion, plasma membrane, aqueous two-phase partition, mass spectrometry, proteomics
DOI: 10.3724/SP.J.1206.2009.00276

Dorsal root ganglion (DRG) neurons are primary sensory neurons that conduct neuronal impulses related to pain, touch and temperature senses. These primary sensory neurons send their axons peripherally to the body surface, muscles and viscera. They enter the central ascending pathways carrying information to the brain, and encode sensory messages in the form of a series of action potentials. Plasma membrane(PM) is a selectively permeable barrier and communication interface of cells owing to the presence of specific membrane proteins, which play important biological roles in intercellular communication, cellular development, cell migration and drug resistance. Furthermore, more than half of all membrane proteins are predicted to be pharmacological targets[1]. Systematical characterization of plasma membrane proteins that participate in those functions will contribute to a better understanding of the mechanism of DRG neurons. PM proteins are usually of hydrophobic nature, and are present in low abundance compared with other membranes in the cell. Systematic proteomic analysis for this class of proteins has been less reported due to the very low amount of DRG neurons. For the proteomic analysis of PM, purification of PM is the crucial first step. Traditionally, protocols for PM purification combine differential velocity centrifugation with density gradient centrifugation. However, due to the overlapping densities, PM prepared by density gradient centrifugation are often heavily contaminated with mitochondrial and other cellular inner membranes. In addition, density gradient centrifugation is a time- and material-consuming method [2, 3]. For PM purification from DRG neurons that are generally difficult to isolate in large amounts, we need other methods that

*This work was supported by grants from The Special Program for Key Basic Research of the Ministry of Science and Technology of China (2004CCA00300), Hi-Tech Research and Development Program of China (2006AA02Z141) and The National Natural Science Foundation of China (30370260, 90408017).

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Received: April 29, 2009 Accepted: August 25, 2009

are efficient enough to solve the problems. Aqueous two-phase system consists of polymers polyethylene glycol (PEG) and dextran. Two-phase partition method was widely used in 1980's because it was believed to be able to separate mitochondria and other cellular inner membranes from PMs on the basis of their different surface properties [4]. As PMs have a higher affinity to the PEG-enriched phase than all other membranes, they preferentially enter into the top phase [5]. In the present paper, the aqueous polymer two-phase partition in combination with differential velocity centrifugation was employed to enrich the PM from rat DRG neurons for proteomic analysis. The efficacy of this technique has been verified by the use of antibodies against specific PM marker proteins. Using this purification method in combination with SDS-PAGE and CapLC ion trap mass spectrometry, we have identified 729 proteins, including 159 plasma membrane proteins.

1 Materials and methods

1.1 Materials

Trypsin (proteomics sequencing grade), dithiothreitol (DTT), iodoacetamide (IAA), 4- (2-hydroxyethyl)-1- piperazine ethanesulfonic acid (HEPES), phenylmethylsulfonyl fluoride (PMSF) and sucrose were purchased from Sigma-Aldrich (St. Louis, MO). Acrylamide, bis-acrylamide, thiourea, urea, glycine, Tris and sodium dodecyl sulfate (SDS) were from Amresco (Solon, OH, USA). Water was obtained with a Milli-Q Plus purification system (Millipore, Bedford, MA). Other reagents were domestic products of the highest grade available. Sprague-Dawley rats (weighting 125 ～200 g) were from Hunan Academy of Traditional Chinese Medicine (Changsha, China).

1.2 Crude plasma membrane preparation

Dorsal root ganglia (DRGs) were obtained from Sprague-Dawley rats according to the method described by Wang *et al* [6]. Briefly, the rats were anesthetized with ether and killed by decapitation. Their spinal columns were isolated and placed in Dulbecco's modified Eagle's medium (DMEM) saturated with oxygen. The spinal columns were cut out lengthways and the DRGs were separated from the spinal cords and other tissues. Isolated DRGs were homogenized with a mortar and pestle in liquid nitrogen. The homogenate was suspended in a solution containing 50 mmol/L HEPES (pH 7.4) 1 mmol/L $CaCl_2$ and 0.1 mmol/L PMSF, and centrifuged at 600 *g* for 10 min at 4℃ (Beckman SW28 rotor) to remove nuclei and unbroken cells. The pellet was homogenized and centrifuged again under the same conditions. The supernatants were combined and centrifuged at 10 000 *g* for 10 min at 4℃ to pellet out partial subcellular organelles including mitochondria and peroxisomes, *etc*. The resulting supernatant was transferred to another tube and centrifuged at 100 000 *g* for 1 h at 4℃ (Beckman, Ti 70 rotor) to obtain a plasma membrane-enriched fraction, which was named crude plasma membrane (CPM). After washing twice, CPM was resuspended in 0.2 mol/L K_3PO_4 (pH 7.8) for further purification using aqueous polymer two-phase partition.

1.3 Further purification of CPM

The resuspended CPM was loaded onto an aqueous polymer two-phase system prepared from stock solution of 20% (*w*/*w*) Dextran T500 and 40% (*w*/*w*) polyethylene glycol 3350. The 16 g (6.4%) two-phase system was constituted of 5.12 g of 20% Dextran T500, 2.56 g of 40% poly (ethylene glycol) 3350, 0.4 ml of 0.2 mol/L K_3PO_4 (pH 7.8), 1.6 ml of 1 mol/L sucrose. The weight of the system was brought to 14 g with distilled water [5]. Resuspended CPM was added to the two-phase system to a final weight of 16 g with distilled water and mixed 40 inversions. Phase separation was accelerated by a 5 min centrifugation at 750 *g* at 4℃. After phase separation, the system was divided into two phases: top phase and bottom phase. The top phase was removed to another tube, and the bottom phase was re-extracted with a fresh top phase (obtained from a two-phase system without adding CPM). The obtained two top phases were combined and re-extracted with fresh bottom phase. For pelleting out plasma membranes, the re-extracted top phase was diluted 5-fold with 1 mmol/L $NaHCO_3$ and centrifuged at 100 000 *g* for 1 h at 4℃. The obtained plasma membrane fraction was named purified plasma membranes (PPM), whose protein content was determined by Bradford method with bovine albumin as standard[7].

1.4 SDS-PAGE and Western blot analysis of PPM proteins

SDS-PAGE of the PPM proteins was performed on a 4.8% stacking gel and a 10.5% separation gel. Prior to electrophoresis, PPM containing 100 μg of proteins was resuspended in 0.2 ml sample loading buffer (0.5 mol/L Tris-HCl pH6.8, 4% SDS, 0.1 mol/L

DTT, 20% glycerol and a trace of bromophenol blue). After centrifugation at 12 000 *g* for 12 min, the supernatant was loaded into gel wells. The SDS-PAGE was run at 20 mA on the polyacrylamide stacking gel and at 40 mA on the separating gel. After completion of electrophoresis, the separated protein bands were visualized using Coomassie brilliant blue G250. A low molecular mass calibration Kit (Bio-Rad) was used as the standard molecular mass marker proteins.

Western blot analysis was performed after SDS-PAGE of PPM proteins following the manufacturer's instructions. Na^+/K^+ ATPase (Abcam, UK) was used as the plasma membrane marker. After separation by SDS-PAGE, the protein bands were electrotransferred to a nitrocellulose membrane, which was blocked with 5% (*v*/*v*) non-fat dry milk in TBST (150 mmol/L NaCl, 0.1% Tween-20, 25 mmol/L Tris, pH7.5) for 1 h at room temperature, followed by incubation with anti-Na^+/K^+ ATPase antibody diluted 1∶2 000 in the same TBST solution for 1 h at 4℃. After washing with TBST extensively, the membranes were incubated for 45 min at room temperature with the HRP-conjugated secondary antibody. After exposure to Hyperfilm ECL (Amersham), the enrichment of plasma membrane was confirmed using the Quantity One 4.6.2(Bio-Rad).

1.5 Trypsin digestion and CapLC-MS/MS analysis

For in-gel digestion, protein bands were excised from the SDS-PAGE gel and subjected to successive destaining and dehydration with 50% acetonitrile (ACN). The proteins in gel slice were reduced with 10 mmol/L DTT at 57℃ for 1 h and alkylated by 55 mmol/L iodoacetamide in the dark at room temperature for 45 min *in situ*. The gel slices were thoroughly washed with 50% methanol and 25 mmol/L ammonium bicarbonate (NH_4HCO_3) in ACN/water (1∶1, *v*/*v*) and freeze-dried. Proteins were digested in 10 μl of modified trypsin solution (20 mg/L in 50 mmol/L NH_4HCO_3) by incubation overnight at 37℃. The peptides released were extracted three times with 50% ACN by sonication for 15 min. The pooled extracts were concentrated to about 2 μl in a SpeedVac and stored at –80℃ until further use[8].

Tryptic peptides prepared as described above were analyzed by CapLC ion trap mass spectrometry (Bruker Daltonics) coupled with an automated Agilent 1200 LC system equipped with an autosamlper and a C18 reverse-phase capillary column (PepMap, 180 μm i.d., 15 cm long, LC-packings). Before separation on the reverse-phase capillary column, the sample was pre-concentrated on a C18 precolumn (500 μm i.d., 3.5 cm long, Bruker). When the sample was separated on the C18 PepMap column, the flow rate of eluting solution was 3 μl/min. For the chromatography, the following solvents were used: solvent A (98% H_2O, 1.9% ACN, and 0.1% formic acid), solvent B (95% ACN, 4.9% H_2O, and 0.1% formic acid). The peptides eluted from the column were online directed into the mass spectrometry. The LC-MS system was controlled using Chemstation B01 (Agilent) and EsquireControlTM 6.1 (Bruker Daltonics) softwares. The nebulizer pressure was 10 psi. Drying gas flow rate was 5 L/min. Drying gas temperature was 300℃. Capillary voltage was 4 000 V. The full MS scan mode was standard enhanced (*m*/*z* 350～1 600). Peptide ions were detected in MS scan, and seven most abundant in each MS scan were selected for collision-induced dissociation (MS/MS), using data-dependent MS/MS mode over the *m*/*z* range of 100～2 000.

1.6 Data analysis and bioinformatics

Raw mass spectrometry data were processed, and Mascot compatible mgf files were created using Data AnalysisTM 3.3 software (Bruker Daltonics) with the following parameters: compounds (autoMS) threshold 10 000, number of compounds 300, retention time windows 1.0 min. Searches were performed using Mascot software 2.0 (Matrixscience, London, U.K.), and the international protein index (IPI) rat database was used for protein identification. Search parameters were set as follows: enzyme, trypsin; allowance of up to one missed cleavage; mass tolerance, 1.2 u and MS/MS mass tolerance, 0.6 u; fixed modification, carbamidomethyl (C); variable modification, oxidation (at Met); auto hits allowed (only significant hits were reported); results format as peptide summary report. The theoretical molecular mass and isoelectric point of identified proteins were retrieved from Mascot output files. The grand average hydropathy (GRAVY) values for identified proteins and peptides were analyzed using the ProtParam program (http://tw.expasy.org/tools/protparam.html). Mapping of transmembrane (TM) regions for the identified proteins was conducted using the TMHMM 2.0 program based on transmembrane hidden Markov model (http://www.cbs.dtu.dk/services/TMHMM) by submitting the FASTA files. The subcellular localization and function of identified

proteins were retrieved by gene ontology (GO) prediction and function annotations, respectively. Text-based annotation files were available for download from GO database ftp site at ftp://ftp.geneontology.org/pub/go.

We confirmed candidate proteins according to the probability-based Mowse scores. Proteins were identified on the basis of distinct peptides whose ions scores exceeded the threshold, $P < 0.05$, which indicates identification at the 95% confidence level for these matched peptides. In the study most of the candidate proteins with mascot scores above the threshold was based on at least two identified peptides, with the peptide score above 15. If a protein was identified by a single peptide, MS/MS spectrum of the peptide was manually inspected. For a protein to be confirmed, (1) the masses of all the major peaks (typically more than 7 peaks) in the MS/MS spectrum had to match those of the theoretically calculated fragment ions; (2) the assignment had to be based on successive four or more b- or y-series ions; (3) the molecular mass of the matched proteins were in reasonable agreement with the gel migration data[9~11]. In addition, the protein validation adopted MIAPE standards (minimum information about proteomics experiment, http://www.mcponline.org). If several proteins were identified based on the same set peptides, only the one with more complete GO annotation was chosen.

2 Results

2.1 Preparation of rat DRG neuron PMs

In this study, a method is described that allows enrichment of PM proteins with low contamination by non-PM proteins. In the method PMs were enriched by differential velocity centrifugation combined with aqueous two-phase partitioning. The evaluation of PM purification was made by Western blotting analysis of its marker, Na^+/K^+ ATPase (Figure 1). Figure 1 showed that the relative concentration of PMs in the PPM was 2.3 times higher than that in the CPM, 15 times higher than that in the WTL.

2.2 Separation and identification of DRG neuron PM proteins

After the proteins in PPM preparation were dissolved they were separated by one-dimensional SDS-PAGE (Figure 2). Following in-gel tryptic digestion and CapLC-MS/MS analysis, a total of 729

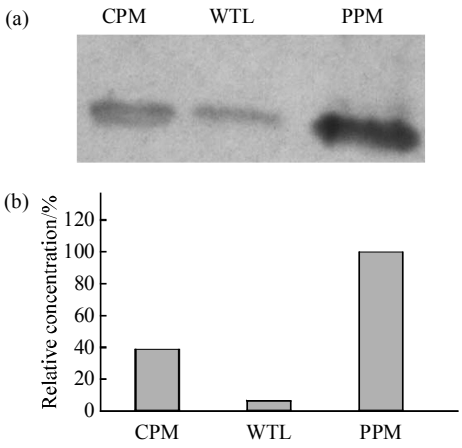

Fig. 1 Western blotting analysis of Na^+/K^+ ATPase in various rat DRG neuron membrane fractions

(a) Western blotting analysis. (b) Densitometric quantitation of Na^+/K^+ ATPase Western blotting results. PPM: Purified PM preparation; CPM: Crude PM preparation; WTL: Whole tissue lystate.

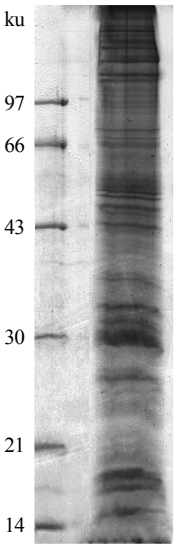

Fig. 2 One-dimensional SDS-PAGE image of the purified plasma membrane (PPM) proteins

Molecular mass marker showed on the left.

proteins were identified using gene ontology and the literature search. The complete list of the proteins identified in this analysis, along with the peptides on the basis of which they were identified, is provided in Table 1. Although some proteins were identified by only a single peptides, 83% of the proteins listed in the Table 1 were identified by two or more unique peptides. In order to further assess the efficacy of the developed protocol for the enrichment of PM and to

estimate contamination by other cellular organelles, including mitochondria and endoplasmic reticulum (ER), *etc*, we classified the 729 identified proteins according to GO annotation and other currently available data including published literature. Of course, this classification was not strict due to that many proteins exist at more than one site in a cell. Of those identified proteins, 547(75%) had gene ontology for cellular component or cellular location, and 182(25%) proteins were not yet categorized in the rat database and therefore had no GO annotation terms. 159(21.8%) of the 729 proteins were unambiguously confirmed as PM proteins and 51(7%) were categorized as PM-associated proteins. Of the remainder proteins, 105(14.4%) were annotated as mitochondrial, 136(18.7%) as cytoplasmic, 45(6.2%) as endoplasm reticulum, and 18 (2.5%) as nuclear. In addition, 33 (4.5%) were annotated in general terms as membrane proteins (Figure 3a). These data indicate that the PM in the preparation was enriched to a certain extent by using our PM purification protocol, and however, there was still contaminations from other subcellular compartments particularly mitochondria and cytoplasm.

Table 1 Selected plasma membrane proteins from rat dorsal root ganglion

	ID	Protein name	Scores/Peptides	*M*/u	TMD	GRAVY	p*I*
Ion channel and transport proteins	IPI00197878	Isoform 1 of kv channel-interacting protein 4	38/1	28 965	0	–0.4588	5.03
	IPI00359734	Similar to potassium channel tetramerisation domain containing protein12	114/5	47 077	0	–0.6501	8.949
	IPI00211012	Voltage-gated potassium channel subunit beta-2	38/1	41 280	0	–0.2692	9.10
	IPI00198327	Voltage-dependent anion-selective channel protein 2	223/5	32 353	0	–0.2210	7.18
	IPI00188119	Isoform long of potassium-transporting ATPase alpha chain 2	264/5	115 656	8	0.0337	6.08
	IPI00421874	Voltage-dependent anion-selective channel protein 1	580/11	32 513	0	–0.4229	8.61
	IPI00326305	Sodium/potassium-transporting ATPase alpha-1 chain precursor	1 913/37	114 316	10	0.0020	5.26
	IPI00231451	Sodium/potassium-transporting ATPase alpha-3 chain	1 389/28	113 480	8	–0.0066	5.22
	IPI00192160	Plasmolipin	218/4	19 934	4	0.9538	9.46
	IPI00558343	Na, K-ATPase alpha-1 subunit	93/2	27 339	0	–0.1592	6.77
	IPI00390795	ATPase, Na^+/K^+ transporting, alpha 4 polypeptide	398/8	115 260	10	0.0337	5.55
	IPI00231462	Isoform short of potassium-transporting ATPase alpha chain 2	169/6	115 656	8	0.1807	5.80
	IPI00365705	Similar to potassium-transporting ATPase alpha chain 1	229/4	115 573	8	0.0644	5.57
	IPI00476086	ATPase, H^+ transporting, v0 subunit d isoform 1	290/9	40 731	0	–0.0925	4.84
	IPI00421888	Annexin a6	1 446/40	57 315	0	–0.3817	4.78
	IPI00339124	Sodium/potassium-transporting ATPase subunit beta-1	258/6	35 762	1	–0.5241	8.82
	IPI00193983	Clathrin heavy chain	349/10	193 187	0	–0.2396	5.48
	IPI00567919	Similar to Ap-2 complex subunit alpha-1	56/3	106 703	0	–0.0639	7.71
	IPI00209247	Isoform 1 of lin-7 homolog a	41/1	25 963	0	–0.4745	8.82
	IPI00471901	Adaptor protein complex Ap-2, alpha 2 subunit	127/5	105 020	0	–0.0966	6.31
	IPI00231742	Catalase	260/5	60 062	0	–0.6385	6.96
	IPI00203747	Low-density lipoprotein receptor precursor	95/3	100 037	1	–0.4006	4.77
	IPI00196530	Ap-2 complex subunit mu-1	190/7	49 965	0	–0.2726	9.57
	IPI00190701	Apolipoprotein e precursor	42/1	35 788	0	–0.7073	5.15
	IPI00782735	83 kda protein.	717/14	114 293	7	0.0709	5.53

Continued

	ID	Protein name	Scores/Peptides	*M*/u	TMD	GRAVY	p*I*
Ion channel and transport proteins	IPI00191391	Solute carrier family 2, facilitated glucose transporter member 1	109/3	54 270	12	0.5170	8.93
	IPI00515821	Membrane copper amine oxidase.	311/10	184 955	1	–0.1705	5.98
	IPI00194873	Isoform d of plasma membrane calcium-transporting ATPase 1	131/3	139 601	7	–0.1685	5.67
	IPI00780645	112 kda protein	1 078/21	113 457	7	–0.0287	5.38
Signaling proteins and receptor	IPI00231012	Isoform flip of glutamate receptor 1 precursor	37/2	102 289	3	–0.1882	7.73
	IPI00202168	Isoform 2 of sigma 1-type opioid receptor	100/3	21 652	1	0.2942	5.39
	IPI00203575	Ras-related protein ral-a	41/1	23 709	0	–0.6208	6.10
	IPI00422067	Guanine nucleotide-binding protein subunit beta 4	181/4	38 080	0	–0.1873	5.59
	IPI00230868	Guanine nucleotide binding protein, alpha q polypeptide	585/15	42 416	0	–0.4473	5.38
	IPI00192495	Isoform 1 of nicastrin precursor	62/1	79 035	1	–0.1307	5.44
	IPI00192313	Isoform alpha of caveolin-1	176/4	20 710	1	0.0286	5.15
	IPI00204843	Guanine nucleotide-binding protein g(o) subunit alpha 2	754/15	40 568	0	–0.3468	5.58
	IPI00231925	Guanine nucleotide-binding protein g(i), alpha-2 subunit	1 001/21	41 043	0	–0.3715	5.20
	IPI00360645	Guanine nucleotide binding protein, alpha 14	135/4	41 871	0	–0.3625	5.81
	IPI00201699	Transforming protein rhoa precursor	136/5	22 110	0	–0.3663	5.57
	IPI00192337	Calcium/calmodulin-dependent protein kinase type Ⅱ alpha chain	455/8	54 651	0	–0.3857	6.52
	IPI00361924	Annexin a7	174/5	50 272	0	–0.4855	5.68
	IPI00777883	Proteolipid protein	129/3	27 646	2	0.4078	7.74
	IPI00325763	23 kda protein	75/2	23 759	0	–0.3153	7.26
	IPI00230866	Guanine nucleotide-binding protein alpha-12 subunit	86/2	44 208	0	–0.3562	9.84
	IPI00231965	ADP-ribosylation factor 5	116/2	20 631	0	–0.2044	5.95
	IPI00778633	Apolipoprotein h	102/3	39 743	0	–0.2539	8.58
	IPI00187747	Ras-related protein rap-1a precursor	198/4	21 316	0	–0.3750	5.70
	IPI00231102	Myelin proteolipid protein	410/8	30 855	4	0.5501	8.70
	IPI00201792	Protein kinase c alpha type	111/3	77 883	0	–0.4793	6.53
	IPI00371187	Ras-related protein rab-35	148/2	23 759	0	–0.4726	8.51
	IPI00199872	Isoform gnas-1 of guanine nucleotide-binding protein g(s) subunitalpha isoforms short	185/5	46 091	0	–0.5972	5.58
	IPI00231001	Guanine nucleotide-binding protein g(t), alpha-3 subunit	116/3	40 781	0	–0.3344	5.50
	IPI00231726	Guanine nucleotide-binding protein g	365/8	41 066	0	–0.3601	5.40
	IPI00200437	Guanine nucleotide-binding protein alpha-11 subunit	182/6	42 285	0	–0.3509	5.78
	IPI00208784	Isoform 1 of flotillin-1	710/14	47 755	0	–0.3558	6.51
	IPI00209150	Ras-related protein rab-3c	60/2	23 759	0	–0.5229	4.99
	IPI00210733	Small GTP-binding protein rab5	116/3	23 828	0	–0.4167	8.29
	IPI00555185	Rab10, member ras oncogene family	240/4	23 759	0	–0.3300	8.57
	IPI00368431	103 kda protein	325/12	89 417	0	–0.8004	5.22
	IPI00199224	Ras-related protein rab-4b	87/2	23 759	0	–0.2272	5.53
	IPI00366719	Similar to guanine nucleotide-binding protein g (t), alpha-1 subunit	118/3	40 383	0	–0.2885	5.31
	IPI00231733	Guanine nucleotide-binding protein g(i), alpha-1 subunit	414/11	40 889	0	–0.3203	5.57
	IPI00331955	Guanine nucleotide-binding protein g	36/1	41 367	0	–0.3757	7.35

Continued

	ID	Protein name	Scores/Peptides	*M*/u	TMD	GRAVY	p*I*
Signaling proteins and receptor	IPI00285606	Isoform 1 of cell division control protein 42 homolog precursor	129/4	21 587	0	−0.1570	5.78
	IPI00231615	Annexin a1	636/13	39 147	0	−0.4306	6.67
	IPI00206703	Ras-related protein ral-b	43/1	23 417	0	−0.5844	5.91
	IPI00325146	Isoform short of annexin a2	1 401/29	38 939	0	−0.5262	7.33
	IPI00471889	Annexin a5	1 423/28	35 648	0	−0.3219	4.85
	IPI00207292	Isoform 4 of flotillin-2	595/13	41 953	0	−0.1617	5.16
	IPI00196751	Heat shock 70 kda protein 1a/1b	154/3	70 427	0	−0.3951	5.52
	IPI00212655	Guanine nucleotide-binding protein g(i)/g(s)/g(t) subunit beta 1	502/10	38 151	0	−0.2311	5.46
	IPI00212658	Guanine nucleotide-binding protein g(i)/g(s)/g(t) subunit beta 2	356/7	38 048	0	−0.1770	5.46
	IPI00210381	Ras-related protein rab-11b	158/3	24 588	0	−0.4353	5.40
	IPI00231134	Guanine nucleotide-binding protein subunit beta 2-like 1	202/5	35 853	0	−0.2503	7.44
	IPI00422092	Ras-related c3 botulinum toxin substrate 1 precursor	204/6	21 835	0	−0.1005	8.77
Catalytic proteins	IPI00231610	Calpain-1 catalytic subunit	40/1	82 752	0	−0.3904	5.41
	IPI00470288	Creatine kinase b-type	213/5	42 983	0	−0.4661	5.32
	IPI00211448	Eh-domain containing 2	218/8	61 370	0	−0.3182	6.03
	IPI00326412	Gamma-enolase	60/1	47 510	0	−0.1827	4.97
	IPI00327143	Alkaline phosphatase, tissue-nonspecific isozyme precursor	100/2	57 965	0	−0.3322	6.35
	IPI00411230	Glutathione s-transferase mu 2	82/2	25 857	0	−0.5018	6.54
	IPI00231502	Isoform 2 of Ap-2 complex subunit beta-1	78/3	106 537	0	−0.0884	5.15
	IPI00189554	CDP-diacylglycerol--inositol 3-phosphatidyltransferase	359/9	23 939	3	0.6042	8.54
	IPI00326462	Ectonucleotide pyrophosphatase/phosphodiesterase 3	318/12	100 946	1	−0.3931	5.93
	IPI00190555	Glutamate carboxypeptidase 2	504/14	84 715	1	−0.3367	6.93
	IPI00206434	Leucyl-cystinyl aminopeptidase	298/7	117 649	1	−0.2279	5.32
	IPI00210097	Isoform 2b of GTPase kras	67/2	21 709	0	−0.5808	8.22
	IPI00476292	121 kda protein	384/12	121 458	0	−0.4901	5.35
	IPI00197568	Rab GDP dissociation inhibitor beta	341/11	51 018	0	−0.3044	5.78
	IPI00198080	Prenylcysteine oxidase precursor	287/6	56 480	0	−0.0994	6.47
	IPI00464815	Alpha-enolase	237/7	47 440	0	−0.1928	6.00
	IPI00208422	Dipeptidyl peptidase 4	114/4	88 774	1	−0.3543	5.86
Binding and structural proteins	IPI00777130	94 kda protein	144/4	95 398	1	−0.4158	4.47
	IPI00212868	Laminin subunit beta-2 precursor	43/1	203 474	0	−0.4812	6.32
	IPI00210536	Isoform l-mag of myelin-associated glycoprotein precursor	336/8	70 164	1	−0.1142	4.93
	IPI00214435	Integrin beta-4 precursor	241/6	198 633	2	−0.4229	5.57
	IPI00476991	Neural cell adhesion molecule 1, 140 kda isoform precursor	273/8	95 411	1	−0.4328	4.81
	IPI00191681	Integrin beta-1 precursor	161/5	91 687	1	−0.3738	5.70
	IPI00365286	Similar to vinculin	40/1	117 112	0	−0.4139	5.77
	IPI00206054	Contactin-1 precursor	156/4	114 278	0	−0.3094	5.71
	IPI00195173	Cd59 glycoprotein precursor	141/3	14 465	2	0.3420	8.89
	IPI00363849	Similar to laminin gamma-1 chain precursor	115/3	182 975	1	−0.6028	5.08
	IPI00372839	Similar to procollagen, type Ⅵ, alpha 2	85/4	99 294	0	−0.6487	6.12

Continued

	ID	Protein name	Scores/Peptides	*M*/u	TMD	GRAVY	p*I*
Binding and structural proteins	IPI00358406	Catenin (cadherin-associated protein), alpha 1	51/2	100 858	0	−0.3742	5.84
	IPI00187902	Cd9 antigen	293/7	25 647	4	0.5371	6.61
	IPI00188956	Thy-1 membrane glycoprotein precursor	288/6	18 389	0	0.0192	9.41
	IPI00360766	Similar to procollagen, type XⅣ, alpha 1	119/3	194 364	1	−0.3132	5.04
	IPI00231136	Similar to nidogen-1 precursor	46/2	141 070	1	−0.3813	5.21
	IPI00206951	Isoform alpha-7x1b of integrin alpha-7	110/2	125 371	1	−0.2102	5.57
	IPI00215235	Isoform 2 of septin-9	130/5	46 668	0	−0.6058	6.93
	IPI00371853	Similar to collagen alpha-1(Ⅵ) chain precursor	74/2	132 161	0	−0.5358	5.17
	IPI00192409	Glyceraldehyde-3-phosphate dehydrogenase type 2	80/1	47 362	0	−0.3201	8.15
	IPI00361301	Similar to laminin alpha-2 chain precursor	43/1	352 944	0	−0.4309	5.82

We also categorized the identified PM proteins according to their functions described in universal GO annotation. The identified PM proteins include 35% binding and structural proteins, 30% signal proteins and receptors, 18.9% ion channels and transporters, 10.7% catalytic proteins. Besides, there were some proteins (5.4%) involved in biological activities such as protein folding, trafficking and so on, which were grouped into “Other” (Figure 3b).

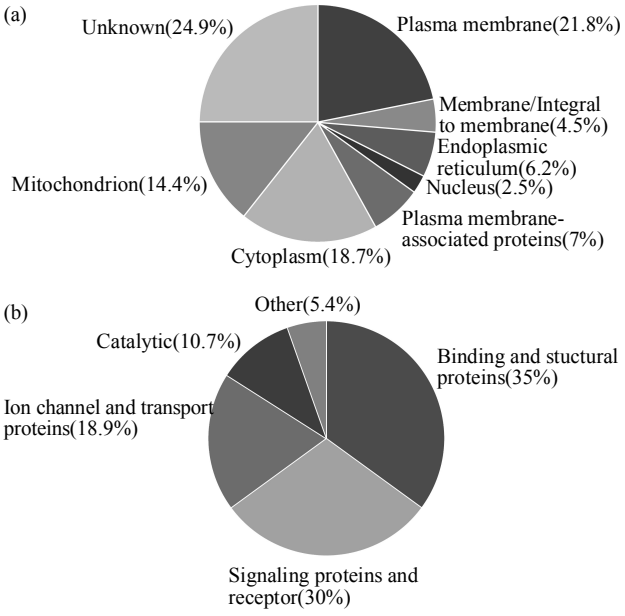

Fig. 3 Classification of identified proteins

(a) Classification based on subcellular localization information. (b) Classification based on functions of the identified PM proteins.

2.3 Characterization of identified proteins from PPM

We compared various physicochemical characteristics —— molecular mass, p*I* in order to detect any possible analytical bias in protein identification using our method. Totally, 513 (70.4%) of the 729 identified proteins were in the range of 10～60 ku (Figure 4a), which was the molecular mass distribution typically seen with 2DE based methods [12]. 93 (12.8%) had a molecular mass >100 ku. The identified proteins were distributed across a wide p*I* range (4.23～11.78), mostly in the range of 5.0～7.0 and 8.0～10.0 (Figure 4b). It can be found that the molecular mass and p*I* distribution profiles of identified PM proteins were similar to those of identified total proteins.

We also analyzed the identified PM proteins on the basis of calculated average GRAVY (grand average of hydrophobicity) values and predicted transmembrane domains (TMDs). The GRAVY values of identified rat DRG PM proteins ranged from −1.122～0.9538. 19 proteins (12.3%) of 159 identified PM proteins had positive GRAVY values and could be considered to be hydrophobic (Figure 4c). 45 (28.3%) identified PM proteins were integral membrane proteins with 1～4 and ≥7 predicted TMDs (Figure 4d).

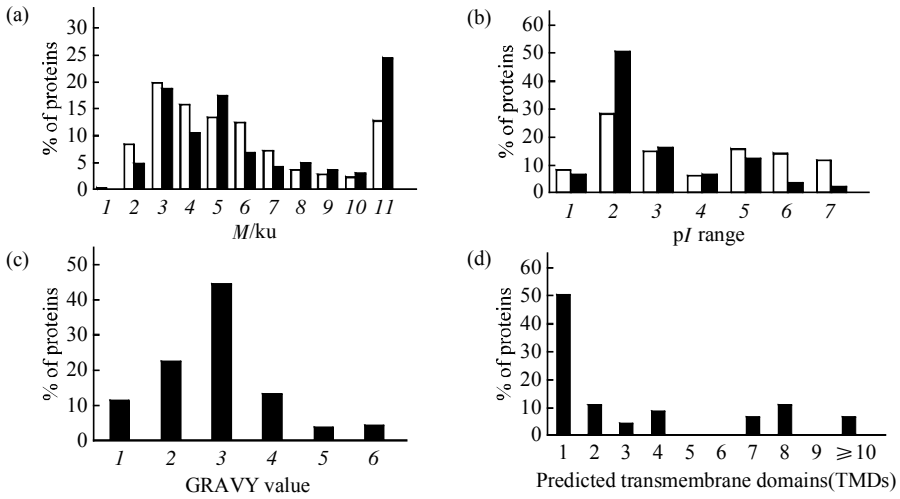

Fig. 4 Distribution of identified proteins

(a) Calculated molecular mass. *1*: ≤10; *2*: 10～20; *3*: 20～30; *4*: 30～40; *5*: 40～50; *6*: 50～60; *7*: 60～70; *8*: 70～80; *9*: 80～90; *10*: 90～100; *11*: ≥100. □: Total proeins; ■: Plasma membrane proteins. (b) Calculated isoelectric point. *1*: 4～5; *2*: 5～6; *3*: 6～7; *4*: 7～8; *5*: 8～9; *6*: 9～10; *7*: ≥10. □: Total proeins; ■: Plasma membrane proteins. (c) GRAVY values calculated using ProtParam algorithm. *1*: ≥0; *2*: 0～–0.25; *3*: –0.25～–0.5; *4*: –0.5～–0.75; *5*: –0.75～–1; *6*: <–1. (d) TMDs predicted by TMHMM.

3 Discussion

A proteomic analysis of PM of neurons would contribute to in-depth understanding of neural system development, function and related diseases. Unfortunately, this is made difficult by the small amount of tissues obtainable, low abundance of PM proteins, or obscuration by an overload of high-abundance proteins. Therefore, isolation and enrichment of neuron PM are essential to its proteomic analysis. Due to overlapping densities of organelles and the PM, PM prepared by classical sucrose density centrifugation is often heavily contaminated by other cellular membranes such as mitochondria and ER. For the purification of PMs, aqueous polymer two-phase partition, as an alternative to density gradient centrifugation, is a simple, rapid, relatively inexpensive and highly reproducible method that can be carried out to efficiently reduce the contamination of PM preparation by other subcellular components, without the use of sophisticated equipment. Another advantage of the used method was that small amount of tissues can be used for purification of PMs[3,13,14]. The rat DRG is especially small-size nerve tissue and it is difficult to sample on a large scale. However, using aqueous two-phase partition, the DRG (about 75 mg, wet weight) from an average of 12.5 rats can yield about 100 μg PM proteins, which are enough to run one SDS-PAGE. Therefore, it is reasonable for the DRG neuron PM to be purified by using aqueous two-phase partition as a preferred method.

For the separation and identification of proteins in complex proteome mixtures, the proteins are analyzed by 2D-PAGE followed by MS or MS/MS, or by 2D-LC-MS/MS. The bias of 2D-PAGE against proteins with extreme isoelectric points and molecular mass, as well as its difficulty to resolve membrane proteins, has been well documented[15]. The resolution of the SDS-PAGE gel is sufficient to fractionate the PM proteins and permit the identification of hundreds of PM proteins.

In the present study, we have adopted a proteomics strategy that utilizes aqueous polymer two-phase partition in combination with differential velocity centrifugation to enrich the PMs, followed by SDS-PAGE and mass spectrometry for proteomics analysis. With the strict identification criteria and the specialized rat proteins database, we identified a total of 729 proteins, of which 547 have been characterized to some extent based on the current rat database and GO information. Of the identified proteins, 90% have a mascot score above 43.

Analysis of the distribution of molecular mass, p*I*, predicted transmembrane domain (TMD), and grand average of hydrophobicity (GRAVY) value of PM proteins identified using aqueous two-phase method from rat DRG neuron PM preparation gives an overall picture of these PM proteins. The 729 proteins identified showed a typical molecular mass distribution

(10～60 ku) with 70.5% between 10 ku and 60 ku (Figure 4a), whereas 0.2% of proteins have a molecular mass < 10 ku and 12.8% of proteins > 100 ku, which fall outside the typical limits of protein resolution by 2D-PAGE. The smallest and the largest molecular masses seen are 3.1 ku and 3729.7 ku, respectively. The 729 proteins are distributed across a wide p*I* range from 4.23 to 11.78; 84 (11.6%) of these proteins have p*I* > 10 and 29 (4%) have p*I* < 4.8(Figure 4b), values that are beyond the 2D-PAGE separation capability of 4.5～8.5. 90(12.3%) hydrophobic proteins(with positive GRAVY values up to 0.9538) were identified. Among all of the identified plasma membrane proteins, 45 are integral membrane proteins with at least one predicted TMD.

In the nerve cell membrane, some proteins, such as ion channels, receptor, are still comparatively low-abundant and many other high abundant membrane proteins such as myelin basic proteins and guanine nucleotide-binding proteins, and a large number of membrane-associated proteins such as actins and neurofilament triplet L, M protein can easily cover up them so as to lead to their unsatisfactory identification, especially on small amount of DRG. Of the proteins identified from the PM preparation of rat DRG neuron, there are a number of special PM proteins that play important roles in DRG neuron functions. For example, several ion channel proteins and transporters were identified in our analysis，such as voltage-gated potassium channel subunit beta-2, isoform 1 of kv channel-interacting protein 4, voltage-dependent anion-selective channel protein 1, *etc*. These proteins in plasma membrane mediate the movement of ions and molecules across the membrane bilayer, and some of them may be involved in the production of action potentials. Another group of identified PM proteins with function in accordance with DRG cell's special roles are receptors or the proteins related to signal regulation, including guanine nucleotide-binding protein g (o) subunit alpha 2, annexin a7, Ras-related protein ral-a, *etc* (Table 1). Flotillin-1 as a PM-specific protein [16] and flotillin-2 were also identified in the study. Flotillin has been regarded as a lipid raft marker and a constitutive element in different signaling cascades[17].

Besides the PM proteins, there were some other proteins, mainly the mitochondrial and cytoskeletal proteins, being identified in the PM preparation. It is possible that the aqueous two-phase partition did not separate other membranes from PM completely. And some internal membranes or cytoplasmic proteins are in close proximity to plasma membrane, sometimes it is almost impossible to unambiguously distinguish the true endogenous partners of plasma membranes from the artificial associations induced by cell disruption or incomplete purification. It is not unusual for proteins initially categorized as organelle-specific to be later discovered elsewhere in the cell. For example, protein disulfide isomerase that was commonly classified as an ER protein was recently detected at the PM of platelets and liver[18]. Hsp47 also classified as an ER protein was later discovered to be transported to the PM [19]. Besides, there were reports indicating that mitochondrial proteins can exported to various cellular locations[20].

In summary, taking rat DRG neuron PM as model material, we have evaluated the application of aqueous polymer two-phase partition to the analysis of PM proteome. Western blotting and mass spectrometric analyses indicated that the aqueous two-phase partition is rapid and high reproducibility and the method was well suitable for the purification of PM from small amount of obtainable cells like DRG neurons. 159 PM proteins, accounting for 21.8% of total identified proteins, were detected in the PM preparation, which not only validated the efficiency of the PM purification method, but also helped to our understanding of DRG neuron PM proteome underlying fundamental DRG cellular functions. In view of that the aqueous polymer two-phase system works based on the partition principle, repeating extraction of top-phase with fresh bottom-phase contributes to the increase in the purity of PM preparation, which, however, would decrease the yield. Additionally, for further improvement of the purity of PM preparation obtained with aqueous polymer two-phase partition, some other purification methods such as extensive washing with high-pH and high-salt solutions, careful pre-fractionation and /or affinity two-phase partition may be additionally employed.

References

1 Wu C C, Yates III J R. The application of mass spectrometry to membrane proteomics. Nat Biotechnol, 2003, **21**(3): 262～267

2 Morré D M, Sammons D W, Yim J, *et al*. Isolation by preparative free-flow electrophoresis and aqueous two-phase partition from rat adipocytes of an insulin-responsive small vesicle fraction with glucose transport activity. J Chromatogr B Biomed Appl, 1996, **680**(1～2): 201～212

3 Schindler J, Lewandrowski U, Sickmann A, *et al*. Proteomiic analysis of brain plasma membrane isolated by affinity two-phase partitioning. J Proteome Res, 2006, **5**(2): 390～400
4 Walter H, Walter H, Brooks D E, *et al*. Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses and Applications to Biotechnology. Academic Press Orlando, FL: 1985. 327
5 Morré D J, Morré D M. Preparation of mammalian plasma membranes by aqueous two-phase partition. Biotechniques, 1989, **7**(9): 946～948
6 Wang Q W, Li Q, Li Z W. Inhibitory effects of clonidine on GABA-activated currents in DRG neurons. Acta Physiol Sin, 1998, **50**(1): 19 ～27
7 Bradford M M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-bye binding. Anal Biochem, 1976, **72**: 248～254
8 Chen P, Li X W, Sun Y L, *et al*. Proteomic analysis of rat hippocampal plasma membrane: characterization of potential neuronal-specific plasma membrane proteins. J Neurochem, 2006, **98**(4): 1126～1140
9 Clauser K R, Baker P K, Burlingame A L. Roles of accurate mass measurement (± 10 ppm) in peptide identification strategies employing MS or MS/MS and database searching. Anal Chem, 1999, **71**(14): 2871～2872
10 Zhao Y X, Zhang W, Kho Y J, *et al*. Proteomic analysis of integral plasma membrane. Anal Chem, 2004, **76**(7): 1817～1823
11 Forner F, Foster L J, Campanaro S, *et al*. Quantitative proteomic comparison of rat mitochondria from muscle, heart, and liver. Mol Cell Proteomics, 2006, **5**(4): 608～619
12 Jiang X S, Zhou H, Zhang L, *et al*. A high-throughput approach for subcellular proteome: identification of rat liver proteins using subcellular fractionation coupled with two-dimensional liquid chromatography tandem mass spectrometry and bioinformatic analysis. Mol Cell Proteomics, 2004, **3**(5): 441～455
13 Morré D M, Morré D J. Aqueous two-phase partition applied to the isolation of plasma membranes and Golgi apparatus from cultured mammalian cells. J Chromatogr B, 2000, **743**(1～2): 377～387
14 Schindler J, Lewandrowski U, Sickmann A, *et al*. Aqueous polymer two-phase systems for the proteomic analysis of plasma membranes from minute brain samples. J Proteome Res, 2008, **7**(1): 432～442
15 Ceils J E, Gromov P. Proteomics in translational cancer research: Toward an integrated approach. Cancer Cell, 2003, **3**(1): 9～15
16 Nebl T, Pestonjamasp K N, Leszyk J D, *et al*. Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes. J Biol Chem, 2002, **277**(45): 399～ 409
17 Blonder J, Hale M L, Lucas D A, *et al*. Proteomic analysis of detergent-resistant membrane rafts. Electrophoresis, 2004, **25** (9): 1307～1318
18 Honscha W, Ottallahm M, Kistner A, *et al*. A membrane-bound form of protein disulfide isomerase (PDI) and the hepatic uptake of organic anions. Biochim Biophys Acta, 1993, **1153**(2): 175～183
19 Hebert C, Norris K, Della C R, *et al*. Cell surface colligin/Hsp47 associates with tetraspanin protein CD9 in epidermoid carcinoma cell lines. J Cell Biochem, 1999, **73**(2): 248～258
20 Soltys B J, Gupta R S. Mitochondrial proteins at unexpected cellular locations: export of proteins from mitochondria from an evolutionary perspective. Int Rev Cytol, 2000, **194**: 133～196

大鼠背根神经节细胞质膜的双水相法纯化及其蛋白质组学研究 *

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摘要 大鼠背根神经节(dorsal root ganglion, DRG)细胞是一种初级感觉神经元，能传导触觉、痛觉、温觉等神经冲动．为了对少量的 DRG 组织细胞进行质膜蛋白质组学分析，综合利用差速离心与双水相相结合的方法富集 DRG 质膜．然后通过 SDS-PAGE、CapLC-MS/MS 和生物信息学方法对其中的蛋白质进行鉴定和分析．Western blotting 图谱扫描后经过 Quantity One 软件分析，双水相纯化后的质膜与差速离心后得到的粗质膜相比相对浓度增加了 2.3 倍，与匀浆液相比增加了 15 倍．经过大鼠 IPI 数据库以及相关文献检索， 有 729 个蛋白质得到鉴定， 其中 547 个蛋白质具有 GO (gene ontology)注释信息，有 159 (21.8 %)个蛋白质定位在质膜上．通过对大鼠 DRG 质膜的蛋白质组学研究，得到了大鼠 DRG 的质膜蛋白质的分析数据，且提供了一种适用于少量样品的蛋白质组学的分析路线．

关键词 背根神经节，质膜，双水相，质谱，蛋白质组学
学科分类号 G633.91，Q5 **DOI:** 10.3724/SP.J.1206.2009.00276

* 科技部重大基础研究前期研究专项基金(2004CCA00300), 国家高技术研究发展计划(863)(2006AA02Z141)和国家自然科学基金(30370260, 90408017)资助项目.
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收稿日期：2009-04-29，接受日期：2009-08-25