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Proteomic Analysis of Lipid Raft-enriched Membranes Isolated from Internal Organelles

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Abstract

The mitochondria-associated membrane (MAM) is a sub-region of the endoplasmic reticulum (ER) that facilitates crosstalk between the ER and mitochondria. The MAM actively influences vital cellular processes including Ca^{2+} signaling and protein folding. Detergent-resistant microdomains (DRMs) may localize proteins to the mitochondria/MAM interface to coordinate these events. However, the protein composition of DRMs isolated from this region is not known. Lipid-raft enriched DRMs were isolated from a combined mitochondria/MAM sample and analyzed using two-dimensional reversed-phased tandem mass spectrometry. Strict post-acquisition filtering of the acquired data led to the confident identification 250 DRM proteins. The majority (58%) of the identified proteins are *bona fide* mitochondrial or ER proteins according to Gene Ontology annotation. Additionally, 74% of the proteins have previously been noted as MAM-resident or -associated proteins. Furthermore, ~20% of the identified proteins have a documented association with lipid rafts. Most importantly, known internal LR marker proteins (inositol 1,4,5-trisphosphate receptor type 3, erlin-2, and voltage-dependent anion channel 1) were detected as well as most of the components of the mitochondrial/MAM-localized Ca^{2+} signaling complex. Our study provides the basis for future work probing how the protein activities at the mitochondrion/MAM interface are dependent upon the integrity of these internal lipid-raft-like domains.

Keywords

Ca^{2+} signaling; detergent-resistant membranes; mitochondria; mitochondria-associated ER membranes; protein folding

1. Introduction

Lipid rafts (LRs) are classically defined as cholesterol- and sphingolipid-enriched microdomains found in plasma membranes (PMs) [1]. They are thought to provide a more ordered environment within the cell membrane due to the presence of lipid species that, together with cholesterol, stabilize the structure through close packing [2]. Rafts are characteristically insoluble in cold, non-ionic detergents and can be experimentally isolated as detergent-resistant membranes (DRMs) [3]. A key characteristic of LR-enriched

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Supplementary data is available.

microdomains is that they sequester select proteins and act as organizing scaffolds for numerous processes (e.g. molecule trafficking) [1,4,5,6].

Although LRs were initially thought to reside solely in the plasma membrane, increasing evidence suggests the presence of raft-like regions in internal organelles [7,8,9,10,11]. Membrane rafts derived from within the cell would most likely exhibit different biochemical properties and contain a unique set of proteins since these regions have distinctive lipid compositions and decreased levels of cholesterol [12]. Although rafts from these internal structures have not yet been characterized in a global way, significant progress is evident. Membrane rafts isolated from mitochondria contain the voltage-dependent anion channel 1 (VDAC1) and the fission protein hFis and can recruit other proteins when the cell death program is initiated [10,11]. Raft-like domains in the endoplasmic reticulum (ER) are characterized by the presence of two proteins that only localize to the ER, the prohibitins erlin-1 and erlin-2 [9]. In addition, DRMs isolated from the ER sub-structure known as the mitochondria-associated membrane (MAM) were recently shown to contain the novel ligand-responsive sigma-1 receptor molecular chaperone and the type 3 inositol 1,4,5-trisphosphate receptor (IP3R3) [13].

The MAM has garnered much attention recently as a signaling focal point because it specifically interacts with the mitochondrial membrane in order to integrate and coordinate Ca^{2+} signaling [13,14,15]. Deregulation of Ca^{2+} trafficking can result in improper protein folding, metabolic disruption, and apoptosis [16,17,18,19] and recent studies suggest that membrane rafts are involved in coordinating the protein interactions required for proper Ca^{2+} exchange between the MAM and mitochondria [13,20]. For this reason, we comprehensively characterized a combined mitochondria/MAM DRM sample in order to determine the identity of the proteins localized at this interface within this specialized subdomain. Fortuitously, the MAM is physically distinct from “normal” ER and is tethered to the mitochondrion by protein filaments [21], a feature that allows for its co-purification with mitochondria during enrichment by centrifugation. DRMs were then rapidly isolated from crude mitochondria samples by differential detergent solubility, solubilized and trypsinized using gel-assisted digestion [22], and analyzed via two-dimensional reversed-phased (RP/RP) tandem mass spectrometry (MS/MS). Our analysis allowed for the confident identification of proteins known to reside in the mitochondrion and/or the MAM and that facilitate crosstalk between the two organelles. This new knowledge provides further insight into the biological processes that may be regulated by intracellular LR-enriched DRMs.

2. Material and Methods

2.1 Materials

Standard laboratory chemicals were obtained from Thermo Fisher Scientific (Rockfold, IL). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of the following: FBS was from Atlanta Biologicals (Lawrenceville, GA); protease inhibitor cocktail was from Roche Applied Science (Indianapolis, IN); sequencing-grade modified-trypsin was from Promega (Madison, WI). Primary antibodies (anti-flotillin-2 and anti-histone H3) and all secondary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) except for the anti-IP3R3 primary antibody, which was obtained from BD Sciences (Franklin Lakes, NJ).

2.2 Cell Culture

NG108-15 cells (ATCC, Manassass, VA) were cultured at 37 °C, 5% CO2 in MEM (pH 7.4) containing 10% FBS, 0.1 mM hypoxanthine, 400 nM aminopterin, and 0.016 mM thymidine. Cells were considered confluent at 80% flask coverage.

2.3 Whole Cell Lysate Preparation

Attached cells were dissociated with 1.5 mM EDTA solution, washed twice with PBS, and resuspended in 0.32 M sucrose and 10 mM Tris-HCl (pH 7.4) containing a cocktail of protease inhibitors (Roche). Cells were manually lysed by 40 strokes of a Dounce homogenizer. Nuclear debris was removed from the whole cell lysate by low speed centrifugation (500 × g, 5 min, 4 °C).

2.4 DRM Enrichment

Bulk DRMs from the crude mitochondrial fraction (P2, Fig. 1) were isolated using a modified version of a published protocol [3,13]. Care was taken to maintain a homogeneous solution of Triton X-114 throughout the isolation procedure by keeping the temperature at 4°C at all times [23]. Briefly, mitochondria/MAMs were obtained from whole cell lysates by centrifugation at 16,000 × g for 45 min. The pellet was resuspended in 150 mM NaCl, 0.5% TritonX-114 in 10 mM Tris (pH 7.4). After a 1 h end over end rotation period, the sample was centrifuged at 12,000 × g for 30 min to reduce plasma membrane contamination. The supernatant was removed and then centrifuged at 100,000 × g for 1 h. The resulting pellet was taken as the DRM fraction. DRM proteins were solubilized in a mixture of 2% SDS and 10 mM Tris (pH 7.4). Aliquots of each fraction from each stage of the enrichment procedure were analyzed by Western blotting.

2.5 SDS-PAGE and Western Blot Analysis

Protein samples separated by SDS-PAGE (NuPAGE Novex 10% Bis/Tris gels; Invitrogen Corp., Calsbad, CA) were electrophoretically transferred to a PVDF membrane (0.2 μm, Millipore, Billerica, MA) at 4 °C for 90 min (45 V) using the Invitrogen XCell II Blot Module. HRP -conjugated secondary antibodies allowed for detection and visualization of specific antigens by ECL (SuperSignal Chemiluminescent Substrate; Thermo Fisher Scientific). Blots were imaged using a Syngene GeneGnome (Frederick, MD) bio-imaging system.

2.6 Gel-assisted Digestion

DRM proteins were digested with trypsin using a gel-assisted method [24]. Briefly, a polyacrylamide gel was created by adding 18.5 μL of a 19:1 mixture of 40% (v/v) acrylamide solution/bis-acrylamide, 2.5 μL of 10% (w/v) ammonium persulfate, and 1 μL of 100% TEMED to 50 μg of protein in a Eppendorf tube. After polymerization, the gel/protein matrix was cut into small pieces and washed twice with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate (pH 8.0), followed by a single wash with neat acetonitrile. Dried gel pieces were rehydrated with trypsin solution and incubated at 37 °C for 18 h. Peptides were extracted using 0.1% acetic acid, 50% acetonitrile in 0.1% acetic acid, and neat acetonitrile sequentially. Extracts were pooled and concentrated by vacuum centrifugation to ~2 μL.

2.7 High pH Reversed-phase (RP) HPLC

Peptide digests were reconstituted in 0.1% acetic acid and loaded onto an Agilent ZORBAX 300Extend-C18 column (150 × 2.1 mm i.d., 3.5 μm) using an Agilent 1200 binary HPLC system. Peptides were separated by gradient elution from 0–80% mobile phase B in 20 min with a constant flow rate of 0.1 mL/min. Mobile phase A was composed of 1% methanol in 20 mM ammonia (pH 10.5) while mobile phase B consisted of a 90/10 mix of acetonitrile/

1% methanol in 20 mM ammonia. UV absorbance was monitored at 214 nm. Fractions were collected every minute and concentrated by vacuum centrifugation to ~2 μL.

2.8 LC-MS/MS and Data Analysis

Each offline HPLC fraction was reconstituted in 50 μL of 0.1% acetic acid and analyzed by nanoLC-MS/MS using a Tempo MDLC system coupled to a QSTAR Elite hybrid quadrupole time-of-flight mass spectrometer (ABSciex, Foster City, CA) with an ionization voltage setting of 1800 V. The peptides were eluted at a flow rate of 70–80 nl/min onto analytical columns (50 μm ID, 10 cm length of Monitor 100Å-Spherical Silica C18; Column Engineering Inc., Ontario, CA) using the following gradient: 0–30% solvent B in 40 minutes; 30–60% B in 40 minutes; and 60–95% B in 20 minutes. Solvent A consisted of 0.1% formic acid and 2% acetonitrile in water and solvent B contained 0.1% formic acid and 2% water in acetonitrile. MS data were acquired in information-dependent acquisition mode with Analyst QS 2.0 (ABSciex) with Smart IDA enabled. MS cycles were comprised of one full scan (m/z range = 300–2000, 1 sec accumulation) followed by sequential MS/MS scans of the four most abundant ions (+2 to +4 charge state, minimum ion count = 100, collision energy = 40.0 V, exclusion time = 15 sec, maximum accumulation time = 2 sec). The Paragon algorithm within ProteinPilot 2.0.1 (ABSciex) and a combined mouse (ver. 3.84) and rat (ver. 3.84) International Protein Index (IPI) database was used to search the MS/MS files. A 95% confidence threshold for protein matches was used to filter the data, which corresponded to an unused protein score ≥1.3.

3. Results and Discussion

3.1 Isolation and analysis of DRM proteins from a combined Mitochondria/MAM Sample

Novel lipid microdomains differ from classically-defined LRs in that they can localize within internal organelles [9,10,11,25], be cholesterol-independent [26,27], remain stable over extended timeframes [28,29], and/or exhibit a higher density on sucrose gradients [30]. Because of this anticipated variability, we chose to isolate a bulk DRM sample that ***contains*** LRs. Our rapid method to enrich for internal organelle DRMs used differential centrifugation in combination with differential detergent extraction (Fig. 1A). We specifically chose to use Triton X-114 because DRMs derived from internal organelles are preserved in its presence [13,31]. Initially, a series of centrifugation steps were performed to deplete the cell lysate of the nucleus (P1 pellet) and microsomes (S2 supernatant) [13]. The crude mitochondrial pellet (P2 pellet) was then solubilized with Triton X-114, cleared of debris, and centrifuged to obtain DRMs (P4 pellet). As a final step, the DRM pellet was resuspended in 2% SDS to ensure complete dissolution.

DRM samples generated using similar approaches to ours are enriched in lipid microdomains as verified in published studies [3,32,33,34]. Our Western blot data indicated that the DRM fraction we obtained is enriched in flotillin-2, a LR protein marker, and IP3R3, a protein that is an MAM DRM component [13] (Fig. 1B). Histone H3, a biomarker for the nucleus, is essentially absent in the insoluble fraction suggesting minimal contamination of DRMs by nuclear material (Fig. 1B). Collectively, these data confirm that LR-enriched DRMs were isolated from the P4 pellet.

Solubilized DRM proteins were subjected to our multiplexed analysis strategy that includes gel-assisted digestion [22] and RP/RP-MS/MS (Y. Cao, H. M. Johnson, and C. R. Bazemore-Walker, under review). During sample processing, detergent is removed from the sample, proteins are in-gel digested with trypsin, peptides are fractionated offline by RP-HPLC at pH 10 and peptide fractions are analyzed by RP-LC-MS/MS at pH 2. A total of 4 analyses were conducted: two technical replicates for each of two biological replicates. The

MS/MS spectra were searched using ProteinPilot software and a combined rat/mouse IPI database and 2,033 unique peptides representing 447 proteins were identified. We required that each protein: be substantiated by the detection of 2 or more unique peptides at the 95% confidence level; have a protein unused score of 2.6 or greater; and have a protein confidence level of >95%. Spectra were also searched against a decoy database and an FDR of 1% was determined. Applying these filters resulted in the identification of 250 high-confidence proteins.

3.2 Identification and categorization of Mitochondria/MAM DRM Proteins

The majority of our 250 high-confidence proteins (146 proteins) are *bona fide* mitochondrial or ER proteins (Fig. 2A; Table S1). Proteins without a reported location comprise the third largest group followed closely by PM proteins. The proteins annotated as residing in the PM may actually have multiple subcellular locations that are not yet described in the GO database, a problem noted in other publications [35,36]. The low percentage of protein identifications from the Golgi, nucleus and other subcellular locations suggests that these proteins are residual contamination. Almost 52% of the 250 high confidence identifications are membrane proteins, mostly of mitochondrial, ER, or PM origin (Fig. 2B; Table S1). Our manual review of the literature indicates that ~20% (49 proteins) of the 250 proteins have been described as components of LRs or DRMs previously (Table S1). Importantly, we detected the known internal LR marker proteins IP3R3 (specific for the MAM) [13], erlin-2 (specific for the ER) [9], and VDAC1 (specific for the mitochondria) [10]. Additionally, 74% (184 proteins) of the 250 proteins have previously been noted as MAM-resident or -associated proteins (Table S1). In sum, these results provide strong evidence that our isolation method preferentially recovered DRM proteins from mitochondria and MAMs and that the sample was enriched in LR proteins.

Further classification of the 250 proteins according to biological process and molecular function revealed that the proteins segregate into categories congruous with the central role of mitochondria in metabolism and the transport/protein processing activity of the MAM (Fig. 3; Table 1). Protein processing (24%), metabolic processes (22%), and transport (10%) are the top three biological activities represented (Fig. 3A). Proteins localized to the MAM that participate in different aspects of protein processing (Table 1) as well as components of the mitochondrial electron transport chain (ETC) and the tricarboxylic acid (TCA) cycle were detected (Table S1). In addition, enzymes tasked with cholesterol, glucose, lipid, and glycerophospholipid metabolism were found (Table S1). Particularly noteworthy is the detection of members of the Ca^{2+} macromolecular complex that resides at the ER-mitochondrion interface and exerts control over Ca^{2+} signaling and transport between the two organelles [14,37]. The proteins in this complex that were detected include IP3R3 (gene symbol Itpr3), VDAC1 (Vdac1), Grp75 (Hspa9), Bip/Grp78 (Hspa5), ERp57 (Pdia3), calnexin (Canx), calreticulin (Calr), and the adenine nucleotide transporters ANT1 (Slc25a4) and ANT2 (Slc25a5) (Table 1). Furthermore, the functional classification of the 250 proteins separate into roughly two categories – enzymes and binding proteins (Fig. 3B). This ‘big picture’ view of molecular function supports the roles of the identified proteins in the biological processes that occur in the mitochondrion and the MAM regions, respectively.

The significance of DRMs in processes, such as signal transduction and small molecule transport, is an emerging theme in the literature [4,5]. Here, we describe biologically relevant proteins isolated from LR-enriched DRM samples obtained from crude mitochondria membranes. Our gel-assisted shotgun proteomic method identified MAM residents that assist protein processing at the ER and regulate Ca^{2+} trafficking from the ER to the mitochondrion. Our method also revealed that Triton X-114-resistant DRMs are present in mitochondria and contain proteins that facilitate ATP production and export from the mitochondrion. Interestingly, glycolytic and TCA enzymes as well as components of the

ETC and ATP synthases have been detected in the MAM recently [38]. This fact combined with their presence in our data suggests that it is plausible that DRMs exert influence on these disparate processes as well.

We detected 250 proteins that were identified across four replicate analyses with at least 2 unique peptides at the 95% confidence level, making our identifications robust. Based on our findings, we postulate that several activities specific to the mitochondria and ER are (at least partially) dependent on DRMs. This study provides the basis for further investigation probing the activity and association of the Ca^{2+} signaling complex with LRs after physiological perturbation. We also note that we did not detect other well known proteins involved in regulating the physical contact between the mitochondrion and the ER such as PACS-2 and mitofusin-2. It is possible that our proteomic analysis was limited to the more highly abundant mitochondrial proteins found in the sample. However, the detection of numerous MAM marker proteins argues against this. Consequently, it is feasible that the 'physical contact' proteins, and the interaction that they create between the mitochondrion and ER, are not modulated by lipid rafts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DRMs	detergent-resistant membranes
ETC	electron transport chain
ER	endoplasmic reticulum
IP3R3	inositol 1,4,5-trisphosphate receptor type 3
LRs	lipid rafts
MAM	mitochondria-associated ER membrane
MS/MS	tandem mass spectrometry
PM	plasma membrane
RP/RP	reversed-phase reversed-phase
TCA	tricarboxylic acid
VDAC1	voltage-dependent anion channel 1

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Highlights

- Identified 250 proteins from mitochondria/MAM detergent-resistant membranes.
- Identified proteins primarily involved in metabolic and protein processing activities.
- Detected known ER/MAM/mitochondria lipid-raft markers (erlin-2, IP3R3, and VDAC1).
- Detected most of the members of the MAM-localized Ca^{2+} macromolecular complex.

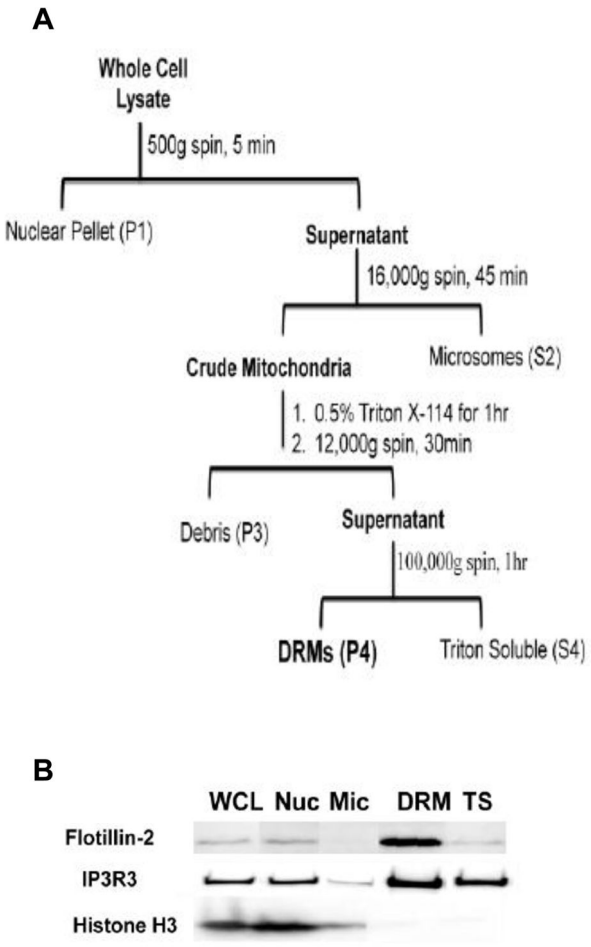

Figure 1. Flowchart and validation of enrichment procedure

(A) Overview of the isolation procedure. A crude mitochondrial pellet was obtained by differential centrifugation from NG 108-15 whole cell lysates. MAM/Mitochondrial DRMs were isolated from crude mitochondria by differential detergent solubility using Triton X-114. (B) Western blot analysis of protein markers. Select fractions from the DRM enrichment procedure were assayed for biomarkers for lipid rafts (flotillin-2), MAM (IP3R3), and nuclei (histone H3). An equal amount of protein, as determined by BCA assay, was loaded in each lane. The experiment was performed at least 3 times with similar results. Abbreviations used: WCL, whole cell lysate; Nuc, nucleus; Mic, microsomes; DRM, detergent-resistant membranes; TS, triton X-114 soluble.

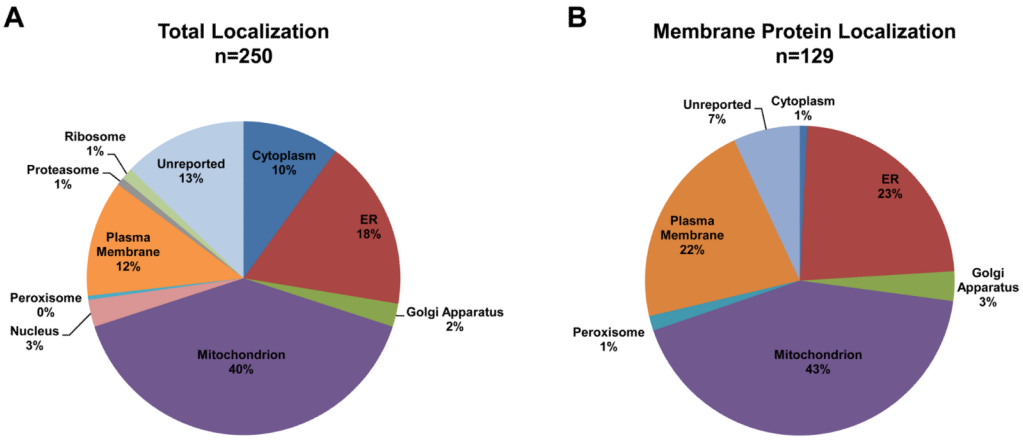

Figure 2. Subcellular localization of the identified proteins based on GO annotation

(A) Proteins were distributed according to their annotated subcellular location based on the number of proteins identifications per category. The total number of protein identifications (n) is shown above each pie chart. (B) Nearly 52% of the identified proteins are known membrane proteins. The organelle association is shown based on protein identifications per organelle.

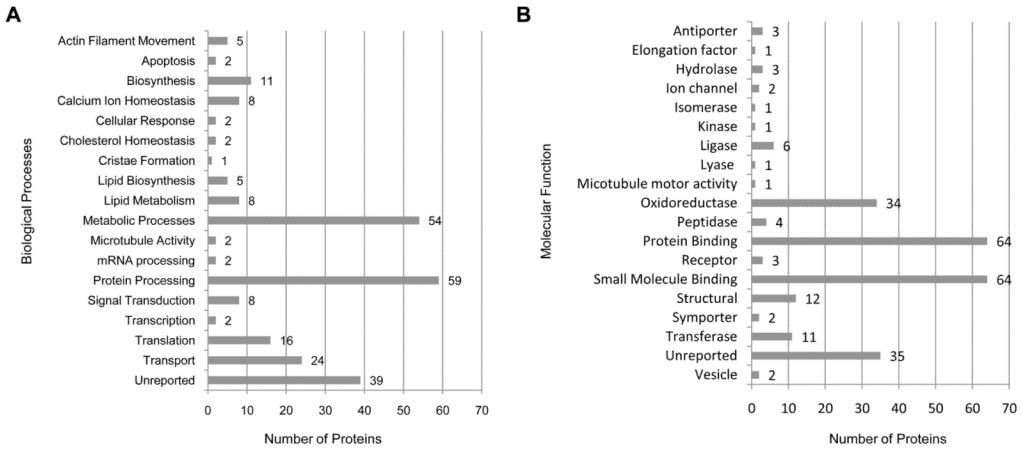

Figure 3. Classification of identified proteins according to biological process and molecular function

(A) Biological processes and (B) molecular functions associated with identified proteins.

Table 1

Select listing of identified proteins involved in relevant biological processes

UniProt	Name	Gene	MAM[a]	Lipid Raft[b]	Biological Process[c]
P05141	**ADP/ATP translocase 2**	**Slc25a5**	Yes	Yes	Calcium signaling
P21796	**Isoform Mt-VDAC1 of Voltage-dependent anion-selective channel protein 1**	**Vdac1**	Yes	Yes	
P48962	**ADP/ATP translocase 1**	**Slc25a4**	Yes	Yes	
Q14573	**Inositol 1,4,5-trisphosphate receptor type 3**	**Itpr3**	Yes	Yes	
O00116	Alkyldihydroxyacetonephosphate synthase, peroxisomal precursor	Agps	Yes	No	Lipid Biosynthesis
P28288	Golgi resident protein GCP60	Acbd3	Yes	No	
P49327	Fatty acid synthase	Fasn	Yes	No	
Q9DB73	NADH-cytochrome b5 reductase 1	Cyb5r1	Yes	No	
E7EQR6	Isoform 1 of T-complex protein 1 subunit alpha	Tcp1	No	No	Protein Folding
O14967	Calmegin	Clgn	No	Yes	
O60613	15 kDa Selenoprotein	Sep15	No	No	
O60884	DnaJ (Hsp40) homolog, subfamily A, member 2, isoform CRA_b	Dnaja2	Yes	Yes	
P07237	Protein disulfide-isomerase	P4hb	Yes	Yes	
P08238	Heat shock protein HSP 90-beta	Hsp90ab1	Yes	Yes	
P27824	**Calnexin**	**Canx**	Yes	No	
P30101	**ERp57**	**Pdia3**	Yes	Yes	
P38646	**Stress-70 protein, mitochondrial**	**Hspa9**	Yes	Yes	
P48643	T-complex protein 1 subunit epsilon	Cct5	Yes	No	
P50990	T-complex protein 1 subunit theta	Cct8	Yes	No	
P80317	Chaperonin subunit 6a	Cct6a	Yes	Yes	
Q12931	80 kDa protein	Trap1	Yes	No	
Q13724	ManNosyl-oligosaccharide glucosidase	Mogs	Yes	No	
Q14318	Isoform 1 of Peptidyl-prolyl cis-trans isomerase FKBP8	Fkbp8	Yes	No	
Q14697	Isoform 1 of Neutral alpha-glucosidase AB	Ganab	Yes	No	
Q9NVH1	59 kDa protein	DNAJC11	Yes	No	
Q9NYU2	UDP-glucose:glycoprotein glucosylrransferase 1	Uggt	No	No	
Q9UBS4	DnaJ homolog subfamily B member 11	DNAJB11	Yes	No	

Biochem Biophys Res Commun. Author manuscript; available in PMC 2012 November 18.

UniProt	Name	Gene	MAM[a]	Lipid Raft[b]	Biological Process[c]
Q9UGP8	Translocation protein SEC63 homolog	Sec63	Yes	No	
P04843	Dolichyl-diphosphooligosaccharide--protein glycosylTransferase subunit 1	Rpn1	Yes	Yes	Protein Processing in the ER
P04844	Dolichyl-diphosphooligosaccharide--protein glycosylTransferase subunit 2	Rpn2	Yes	No	
P05198	Eukaryotic translation initiation factor 2 subunit 1	Eif2s1	Yes	No	
P11021	**78 kDa glucose-regulated protein**	**Hspa5**	Yes	No	
P27797	**Calreticulin**	**Calr**	Yes	Yes	
	Dolichyl-diphosphooligosaccharide--protein glycosylTransferase 48				
P39656	kDa subunit	Ddost	Yes	Yes	
P51571	Translocon-associated protein subunit delta	Ssr4	Yes	No	
P55072	Transitional endoplasmic reticulum ATPase	Vcp	Yes	No	
Q07065	Cytoskeleton-associated protein 4	Ckap4	Yes	No	
Q9Y4L1	Hypoxia up-regulated protein 1	Hyou1	Yes	No	

[a]Proteins associated with or localized in the MAM based on manual literature review;

[b]Proteins previously identified in lipid rafts based on manual literature review;

[c]Biological processes based on Gene Ontology annotation. Proteins shown in **bold** print are known members of the MAM Ca^{2+} macromolecular signaling complex.