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RESEARCH ARTICLE

A novel fractionation method of the rough ER integral membrane proteins; Resident proteins *versus* exported proteins?

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We treated the high salt-washed canine pancreatic rough ER (KRM) with 0.18% Triton X-100, separated the extract from the residual membrane (0.18%Tx KRM), and processed the extract with SM-2 beads to recover membrane proteins in proteoliposomes. To focus on integral membrane proteins, KRM, 0.18%Tx KRM and proteoliposomes were subjected to sodium carbonate treatment, and analyzed by 2-D gel electrophoresis. Consequently we found that a distinct group of integral membrane protein of KRM preferentially extracted from the membrane and recovered in proteoliposomes did exist, while majority of KRM integral membrane proteins were fractionated in 0.18%Tx KRM, which retained the basic structure and functions of KRM. Protein identification showed that the former group was enriched with proteins exported from the ER and the latter group comprised mostly of ER resident proteins. This result will potentially affect the prevailing view of the ER membrane structure as well as protein sorting from the ER.

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1 Introduction

The ER is a site of lipid and protein biosynthesis. Its membrane is the site of production of all the transmembrane proteins and lipids for most of the cell's organelles. The ER membrane synthesizes nearly all of the major classes of lipids, including both phospholipids and cholesterol. Newly synthesized proteins and lipids enter the secretory pathway connected by vesicular transport. The various organelles along the pathway display striking differences in both their protein components and the composition of their membrane lipids. For example, the plasma membrane has a strongly asymmetric transbilayer lipid distribution and contains high levels of sterols and sphingolipids, in contrast to the situation of the ER, which has symmetric one and contains low levels of sphingolipids [1].

In addition to the heterogeneity of protein and lipid contents from organelle to organelle, eukaryotic cell membranes contain variety of phospholipids, sphingolipids and sterols. This lipid heterogeneity leads to the possible nonrandom mixing and microdomains. Studies in artificial bilayers reveal that lipids have a strong self-organizing capacity; lipid immiscibility can drive phase separation and give rise to domains with unique lipid compositions and properties. Current evidence supports the existence of such phase-separated lipid domains in the cellular membranes [2]. Biophysical studies on model membranes firmly established that mixtures of sphingolipids, unsaturated glycolipids and cholesterol can segregate spontaneously into two fluid phases, where the sphingolipids and part of the cholesterol coalesce into ''liquid-ordered'' domain and break

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Abbreviations: 0.18%Tx KRM, KRM extracted with 0.18% Tx-100 treatment; **KRM,** high salt-washed canine pancreatic rough endoplasmic reticulum; **Sec61-α,** Sec61-α subunit; **TCR-α,** T-cell receptor α subunit; **Tx-100,** Triton X-100

away from the unsaturated glycerolipids in a ''liquid-disordered'' phase [3]. In contrast to cytoplasmic membranes, which are enriched in sphingolipids and sterols, the ER is very low in cholesterol and sphingolipids, and is probably present in liquid-disordered phase [3].

Protein translocation-integration into the ER membrane has been also extensively studied, leading to the discovery of translocation machinery components such as signal-recognition particle receptor, translocon complex, signal peptidase complex and oligosaccharyl transferase complex. Integral membrane proteins are immediately integrated into the lipid bilayer as soon as their stop transfer signals reach translocons [4, 5]. Following the integration, protein transport from the ER along the secretory pathway begins with packaging into COPII-coated transport vesicles, which can be a selective process. The generation of COPII vesicles requires a distinct set of coat proteins and secretory factors. The sequential events of COPII complex assembly start with Sar1p activation caused by membrane-bound Sec12p, which catalyzes GDP-GTP exchange on Sar1p [6]. This activation leads to a functional prebudding formation by recruiting the Sec23/24p complex. COPII-coated buds are formed at the ER exit sites, microdomains continuous with the ER, but ribosomeless, smooth patches of the rough ER. Although Sec12p is presumably one of the most upstream components of the COPII budding process, some sort of a budding domain scaffold is thought to exist from the disruption experiment of the Sec12p function [7]. Little is known about such a scaffold [8].

The exit signals that direct proteins out of the ER for transport to the Golgi and beyond are mostly not understood. Only a few membrane proteins' signals working for COPII recruitment have been known. One is a diacidic motif, Asp-X-Glu (DXE, where X is any amino acid) [9–11] and the other motif is the one binding on Sec23p/24p [12–15]. However, only a small number of integral membrane proteins exported out of the ER have such exit signals, but the rest great majority can get packaged in vesicles without any known exit signals at a much faster rate than ER resident proteins, which slowly leak out of the ER. It is quite interesting to know whether and how these proteins without known signals are recruited into the scaffold prior to or after Sec12p activation.

The process from protein integration at the translocon to COPII formation started with Sec12p activation remains unexploited at all.

We made a hypothesis that there are at least two distinct microdomains in the ER, to which distinct subgroups of ER integral membrane proteins with different fates are integrated into respectively. This hypothesis could potentially provide a clue to elucidate the presence of the correlation between these two topics, ER membrane structure and protein sorting prior to Sec12 activation. As a first step, we treated ER membrane with various concentrations of Triton X-100 (Tx-100), as treatment of cytoplasmic membrane with 1% Tx-100 successfully showed the presence of ''raft'' in the cytoplasmic membrane [3]. We found that by the treatment of high salt-washed canine pancreatic rough ER (KRM) vesicles with Tx-100 of up to 0.24% concentration more than half of KRM vesicles retained their structure and their functions such as protein translocation and core-glycosylation [16]. In this study we treated KRM vesicles with 0.18% Tx-100, recovered membrane components, if any, in the extract to proteoliposomes by SM-2 beads treatment, and analyzed integral membrane protein fractions obtained by sodium carbonate treatment together with the analysis by 2-D gel electrophoresis. This novel combination followed by protein identification by LC-MS/MS led to the first demonstration that a distinct group of ER integral membrane proteins is extracted by 0.18% Tx-100 treatment without solubilizing the KRM membrane.

2 Materials and methods

2.1 Tx-100 extraction of KRM

Rough microsomes were prepared from the canine pancreas, as described by Walter and Blobel [17]. The Oita University animal experiment committee approved the experiment, which was carried out according to the University and national guidelines and regulations. Microsomes were nuclease-treated after EDTA washing and subsequent high salt washing by the procedure of Walter and Blobel [17], and are referred to as KRM. The extraction mixture contained 3–5 mg/mL KRM protein, 100 mM KCl, and 0.18% of Tx-100. The mixture was incubated on ice for 20 min, applied on a cushion (0.4 M sucrose, 500 mM KOAc, and 5 mM $Mg(OAc)_2$) and centrifuged for 20 min at 424 000g_{av} in the Beckman TLA100.3 rotor. After centrifugation, the supernatant and the pellet were separated. The membrane vesicles pelleted were resuspended in the membrane buffer (0.25 M sucrose, 50 mM triethanolamine acetate pH 7.5, and 1 mM DTT) and are referred to as 0.18%Tx KRM (KRM extracted with 0.18% Tx-100 treatment).

2.2 Proteoliposome reconstitution

The supernatant prepared as described above was incubated on ice with SM-2 beads (Bio-Rad) by batch method for 30 min. The amount of beads used depended on the amount of Tx-100 and was determined according to the manufacturer's instruction. After incubation, the flow-through was diluted with an equal volume of ice-cold water and centrifuged for 10 min at 424 000 $\times$ g_{av} in the Beckman TLA100.3 to collect proteoliposomes. The supernatant containing unreconstituted materials was incubated with trichloroacetic acid with a final concentration of 10% to yield the unreconstituted materials.

2.3 Analysis of 2-D Electrophoresis

Membrane vesicles were resuspended in the 2-D sample buffer (8 M urea, 2.5 M thiourea, 4% CHAPS, 100 mM DTT, 0.2% w/v Bio-Lyte 3/10, and 0.001% bromophenol blue). The resuspended sample was applied on an IPG ready strip pH 3–10 (Bio-Rad) and processed using Bio-Rad Protean-IEF Cell (Bio-Rad) according to the manufacturer's instruction. After IEF, the IPG strip was subjected onto 10–20% SDS-PAGE (Bio-Rad). The gel was stained with CBB R-250. Three independent experiments were carried out, and each spot of the proteoliposome fraction was quantitatively compared with the corresponding spot of 0.18% Tx KRM by PDQuest version 8.0 (Bio-Rad). Spots that were two or more times larger than the corresponding spots from 0.18% Tx KRM were selected and were considered to be proteins specifically fractionated in the proteoliposome fraction. All such spots were excised from the gel, pooled, and sent to Hitachi Science Systems (Hitachinaka Ibaraki, Japan) for LC-MS/MS analysis. After sodium carbonate treatment 0.18% Tx KRM was also sent to Hitachi Science Systems. The subsequent processing of samples was provided by them as described below.

2.4 Methods of sample preparation for LC-MS/MS

Isolated protein spots from proteoliposomes stained with CBB were excised from the gel, pooled, washed with 50 mM NH_4HCO_3 in 50% v/v ACN twice, and dehydrated in 100% ACN. Reduction of protein sample was achieved with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 56°C and alkylation was performed with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 45 min at room temperature in the dark. Subsequently, the gel pieces were washed twice for 5 min alternatively with 100 mM ammonium carbonate and ACN, and then completely dried up under reduced pressure. Appropriate volumes of trypsin solution (25 μ/μl in 50 mM NH_4HCO_3) were added to the dried gel pieces. After incubation overnight at 37°C, the supernatant containing digested peptides was transferred to a new tube. The gel pieces were washed with 20 mM NH_4HCO_3 and 50% v/v ACN in 5% v/v formic acid. The supernatants were collected to the new tube and dried up under reduced-pressure up to 10 mL.

One molar of DTT was added to 0.18% Tx KRM to a final concentration of 10 mM, incubated at 37°C for 1 h, and alkylation was performed by adding 0.5 M iodoacetamide to a final concentration of 25 mM and incubated for 30 min at room temperature followed by the incubation at 100°C for 10 min. After the sample was cooled, appropriate volumes of trypsin solution (25 μg/μl in 50 mM NH_4HCO_3) were added and incubated at 37°C for 2 h. The reaction was stopped by adding equal volume of 0.1% TFA.

The solution contained peptides mix was analyzed with an LC-ESI-MS/MS LCQdecaXP (Thermo Fisher Scientific K.K., Yokohama, Japan) in positive ion mode. The spectrum data were submitted for protein identification and database searching was performed with MASCOT ver1.9 (Matrix Science, London, UK) in NCBInr. The searching parameters were as indicated below, mammals as taxonomy, enzyme of trypsin, one miss cleavage, fixed modification of carbamidomethyl (C), variable modification of oxidation (M), peptide tolerance of 2.0 Da, MS/MS tolerance of 0.8 Da, peptide charge of 1+, 2+and 3+, monoisotopic. Only significant hits, as defined by the MASCOT probability analysis ($p<0.05$), were accepted.

2.5 Integration of T-cell receptorα subunit (TCR-α) synthesized *in vitro* into KRM membrane and fractionation in proteoliposomes

TCR-α or Hsp47 were synthesized in Rabbit Reticulocyte Lysate System (Promega) and subsequently subjected to an integration assay, as described [17]. KRM vesicles harboring TCR-α or Hsp47 were collected and resuspended into the membrane buffer (0.25 M sucrose, 50 mM triethanolamine acetate pH 7.5, and 1 mM DTT). Carrier KRM vesicles were added to the suspension to adjust the protein concentration of the extraction mixture to 3–5 mg/mL. The extraction and the reconstitution experiments were performed as described above.

2.6 Materials

Chemicals and solvents were of analytic grade and obtained from Wako Pure Chemical Industries, (Tokyo, Japan). Anti-dog C-terminal calnexin rabbit polyclonal antiserum was obtained from Stressgen Bioreagents; anti-mouse ERp72 rabbit polyclonal antiserum, from GENETEX; anti-human Sec61 α subunit (Sec61-α) rabbit polyclonal antiserum, from Upstate USA; and ECL Western Blotting Detection System, from GE Healthcare.

3 Results

3.1 A novel fractionation of microsomal proteins

We treated KRM with 0.18% Tx-100 and separated the extracted materials (extract in Fig. 1A) from the residual membranes (extracted membranes; 0.18% Tx KRM) by ultracentrifugation through a high-salt sucrose cushion. In order to examine which cell components were extracted by 0.18% Tx-100 treatment, we further fractionated the extracted materials by SM-2 bead treatment into two fractions: a proteoliposome fraction to which extracted membrane proteins, if any, were expected to be incorporated and a fraction of proteins not recovered in proteoliposomes which were expected to be ER luminal proteins. A membrane

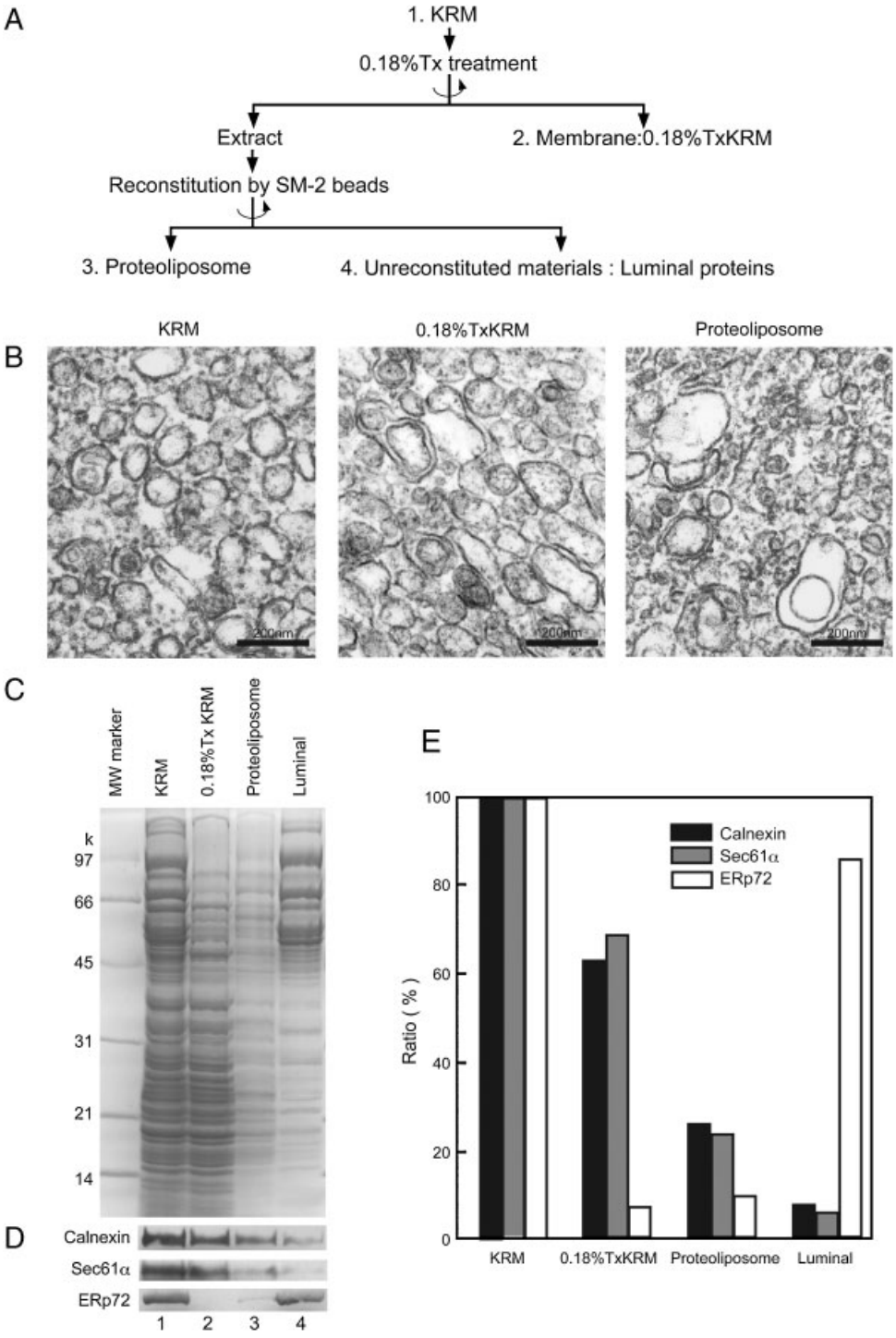

Figure 1. Fractionation of KRM vesicular protein. (A) Scheme of KRM vesicular protein fractionation. After KRM was incubated with 0.18% Tx-100, the extract and residual membrane (0.18% Tx KRM) were separated by ultracentrifugation. The extract obtained was processed using SM-2 beads to reconstitute proteoliposome, and the proteins not recovered in the proteoliposome (luminal proteins) were separated by ultracentrifugation. (B) Electron Microscopic picture of KRM (left panel), 0.18% Tx KRM (middle panel), and proteoliposomes (right panel) (C) Protein profile of each fraction. KRM (lane 1) was fractionated into 0.18% Tx KRM (lane 2), proteoliposome (lane 3) and luminal proteins (lane 4) as shown in panel A. (D) Immunoblotting of KRM fractions shown in panel B by anti-calnexin, anti-Sec61-α, and anti-ERp72 antisera. (E) Quantitation of the results of the immunoblotting results shown in panel D.

floatation experiment confirmed that the proteoliposome fraction was composed of membrane vesicles (unpublished data). This extraction–proteoliposome reconstitution treatment resulted in the fractionation of KRM proteins into three fractions: 0.18% Tx KRM, proteoliposomes, and a luminal protein fraction (Fig. 1A).

Electron microscopic examination showed that the proteoliposomes were indeed formed and remarkably different in appearance either from the KRM or from 0.18% Tx KRM (Fig. 1B, compare right panel with left and middle panels), indicating that the solubilization–reconstitution treatment yielded vesicles which varied considerably in size.

On the other hand, there was no significant difference between KRM and 0.18% Tx KRM (Fig. 1B, compare middle panel with left panel), while no membrane structure was observed when treated by 1% Tx-100 to solubilize the KRM membrane completely [16]. This suggested that KRM vesicles maintained the basic membrane structure in spite of 0.18% Tx-100 treatment and were sedimented in a pellet as 0.18% Tx KRM by ultracentrifugation. Protein profiles of these fractions showed that the majority of KRM proteins were recovered in the 0.18% Tx KRM (Fig. 1C, compare lane 2 with lane 1) and a great part of the extracted proteins from KRM, especially those of higher molecular range, were recovered in the luminal proteins fraction (Fig. 1C, compare lane 4 with lane 1). The results of these electron microscopy and protein profiles suggested that by 0.18% Tx-100 treatment, the majority of KRM vesicles retained their basic membrane structure and membrane proteins, but were left in a leaky state, releasing luminal proteins. Under the conditions of our experiment, the effect of the 0.18% Tx-100 treatment, which is higher than the CMC, probably occurred because the ratio of detergent to membrane lipids was too low. This was consistent with the result of our previous functional study, in which we showed that 0.18% Tx KRM still retained protein translocation and core-glycosylation activities when included in an *in vitro* protein synthesis system [16]. The functional study suggested that the majority of integral membrane proteins involved in those activities were retained in the 0.18% Tx KRM membrane, but also kept their structures to retain their functions in spite of the treatment.

In contrast with 0.18% Tx KRM, only a small amount (less than 15%) of KRM proteins was recovered in the proteoliposome fraction (Fig. 1C, lane 3). When each band was compared between KRM or 0.18% Tx KRM, and proteoliposomes (Fig. 1C, compare lane 3 with lane 1 or lane 2), the amounts of almost all bands of the proteoliposome fraction were considerably decreased from those of corresponding bands of KRM or of 0.18% Tx KRM. From this small amount of recovery of each protein to proteoliposomes and the electron microscopic picture, we considered that a small population of KRM vesicles had been nonspecifically solubilized, and membrane proteins together with membrane lipids of solubilized vesicles formed proteoliposomes by SM-2 beads treatment. Nonspecifical solubilization of KRM vesicles inevitably occurred when 0.5% Tx-100 was added to the extraction mixture to obtain a final concentration of 0.18%.

Immunoblotting analysis showed that Sec61-α, a component of translocation machinery, and calnexin, both of which were representatives of KRM membrane proteins, mainly were not extracted, then were recovered in the 0.18% Tx KRM fraction (Fig. 1D, compare lane 1 with lane 2). The relatively small amounts (one-third at most) of both of the proteins that were extracted were mostly recovered by incorporating into the proteoliposome fraction (Fig. 1D, lane 3), and trace amounts (less than 5%) of the proteins were detected in the luminal protein fraction (Fig. 1D, lane 4), probably because of incomplete reconstitution efficiency. Quantitation of the band in each fraction showed that practically neither of the proteins was lost in this reconstitution process by binding to SM beads (unpublished data). In contrast to these integral membrane proteins, ERp72 – an ER luminal protein – was almost entirely extracted and recovered in the luminal protein fraction (Fig. 1D, compare lane 1 with lane 2 and lane 4). These results supported that most of the membrane proteins of KRM were fractionated in 0.18% Tx KRM. Their small portions contained in the extract were recovered in the proteoliposomes, and most of the luminal proteins were extracted from the KRM and fractionated in the luminal protein fraction. The quantitation data are shown in Fig. 1E.

Close inspection of the protein profile, however, suggested that some proteins appeared to be specifically extracted from KRM membrane and recovered in the proteoliposome fraction (Fig. 1C, compare lane 3 with lane 2 and lane 4), in contrast to those that were expected to be released from the small, nonspecifically solubilized population of KRM vesicles, and recovered in proteoliposomes, as observed in Sec61-α and calnexin fractionation (Fig. 1D and E).

3.2 Demonstration of integral membrane proteins specifically fractionated in proteoliposomes by 2-D gel analysis

To focus on the integral membrane proteins of each fraction, we treated each fraction, except the luminal protein fraction, with sodium carbonate. This treatment is a well-established method for eliminating soluble and peripheral membrane proteins from ER membrane preparations [18]. The extraction–reconstitution followed by sodium carbonate treatment enabled the fractionation of KRM integral membrane proteins into two fractions: 0.18% Tx KRM and proteoliposomes. The fractions obtained were subjected to 2-D gel electrophoresis. As expected from the protein profile and immunoblotting results shown in Fig. 1, most of the spots obtained from the KRM fraction were also detected in the 0.18% Tx KRM fraction (Fig. 2, compare panel A with panel B), although the spots appeared to be decreased in size to some extent. Many of the spots obtained from both the KRM and 0.18% Tx KRM did not have sharp, well-defined shapes but were streaky or fuzzy. This was considered to be a result of their hydrophobic membrane-spanning domains; the more hydrophobic membrane-spanning domains they have, the streakier are their shapes. In our experiment, the presence of numerous spots such as these suggested that proteins with several membrane-spanning domains were solubilized well and visualized in the 2-D gel. This ill-defined spot characteristic resulted in the overlap of these spots, as well as overlap with the well-defined spots (see Fig. 2A and B), making spot by spot quantitative

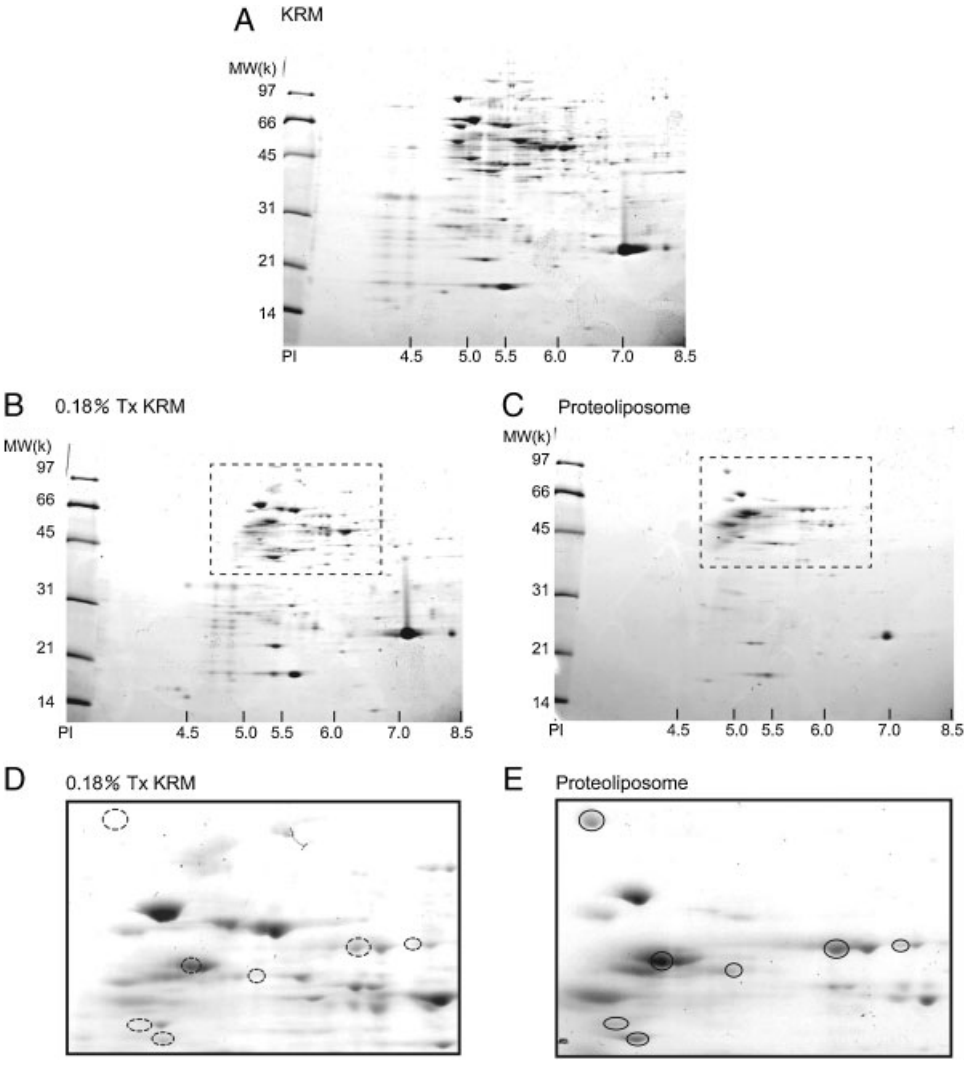

Figure 2. Analysis of KRM integral membrane protein fractionation by 2-D gel electrophoresis. KRM (A) and its fractions 0.18% Tx KRM (B) and proteoliposome (C) were treated with alkaline solution, and analyzed by 2-D gel electrophoresis. Dashed rectangles in panels B and C are enlarged in panels D and E, respectively. In panels D and E, the spots from proteoliposome (E), that were two or more times larger than the corresponding spots (dashed circle) from 0.18% Tx KRM (D), are circled.

assessment very difficult. Consequently, after sodium carbonate treatment, the 0.18% Tx KRM fraction was directly subjected to the shotgun analysis by LC-MS/MS without 2-D gel analysis. This led to the overestimation of 0.18% Tx KRM proteins, since even if the larger portion is extracted and recovered in the proteoliposomes, the small amount of a protein left in the 0.18% Tx KRM membrane, might be sufficient for its identification.

In contrast with those proteins that were retained mainly in the 0.18% Tx KRM fraction, 2-D gel analysis demonstrated that the other types of proteoliposome spots larger than those corresponding to the spots in 0.18% Tx KRM existed (Fig. 2, compare panel D with panel E). In other words, they were specifically fractionated in the proteoliposome fraction. The presence of such proteins indicated that the proteins recovered in the proteoliposomes were composed of two distinct groups; one derived from the nonspecifically solubilized minor population of KRM vesicles; the other specifically extracted from the majority of unsolubilized KRM vesicles. In order to select proteins that were primarily recovered in the proteoliposome fraction, we had to quantitatively assess each spot that was obtained from proteoliposomes in 2-D gel, comparing them with the corresponding spot from the 0.18% Tx KRM. However, 2-D gel electrophoresis of the proteoliposome fraction revealed that many protein spots were streaky and did not show well-defined patterns like those obtained from KRM or from 0.18% Tx KRM. In this study, we focused on quantitative assessment of the well-defined spots, in spite of the risk of contamination by neighboring streaky spots. We selected well-defined protein spots obtained from proteoliposomes, especially those that were at least two or more times larger than the corresponding spots from 0.18% Tx KRM (semiquantitative information by PDQuest version 8.0 (Bio-Rad) in Fig. S1 in the Supporting Information), excised them from the gel, pooled them, and subjected them to the shotgun analysis by LC-MS/MS, as described in Section 2.

3.3 Proteins identified

The proteins identified after subtracting contaminant soluble proteins and mitochondrial proteins are summarized in Table 1. A total of 32 integral membrane proteins were identified in the 0.18% Tx KRM fraction. Twenty-four of 32 proteins that were identified as resident integral membrane proteins of the ER, and more than half of them are involved in the protein translocation process, such as signal-recognition particle receptor, translocon

Table 1. Integral membrane proteins identified in 0.18%Tx KRM and in proteoliposomes

gi	Protein	Score	Subcellular location
0.18%Tx KRM			
50978924	Ribosome-binding protein 1	224	ER
73991908	Ribophorin II precursor isoform 3	200	ER
73984484	Dolichyl-diphosphooligosaccharide—protein glycosyltransferase 67 kDa subunit	186	ER
50979164	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase	169	ER
57112873	Translocon-associated protein, delta subunit precursor (TRAP-delta)	131	ER
73963665	Transmembrane trafficking protein isoform 1	91	Transport vesicle
50979076	Signal sequence receptor, alpha	88	ER
73969959	Cytoskeleton-associated protein 4	375	Transport vesicle
73945996	DC2 protein	54	ER
47523726	Ribophorin I	261	ER
73957446	Transmembrane emp24 protein transport domain containing 6	158	Transport vesicle
11345462	Signal peptidase complex subunit 3	156	ER
5531849	SURF-4 isoform	116	ER
50979098	Glycoprotein 25L	116	Transport vesicle
73954525	Oligosaccharyl transferase STT3 subunit homolog (B5)	138	ER
50979162	Signal peptidase complex subunit 2 homolog	72	ER
57101164	Microsomal signal peptidase 12 kDa subunit	118	ER
73990468	Signal sequence receptor, gamma isoform 2	70	ER
73962567	Defender against cell death 1	67	ER
57112339	Progesterone receptor membrane component	62	ER
73996823	FK506-binding protein 11 precursor	76	ER
57087817	Vitamin K epoxide reductase complex, subunit 1 isoform 1	56	ER
76639219	Protein KIAA0152	77	Transport vesicle
50979014	Calnexin	240	ER
50979146	Sec61 alpha 1 subunit	77	ER
73945930	ERGIC-53 protein precursor	60	ER-golgi intermediate
57098751	SEC22 vesicle trafficking protein-like 1	104	Transport vesicle
73990585	Signal recognition particle receptor beta subunit	77	ER
73980965	Mannosyl-oligosaccharide glucosidase	47	ER
21759777	ENTPD4 protein	28	ER
57112869	B-cell receptor-associated protein 31	39	ER
50979070	Translocation associated membrane protein 1	35	ER
Proteoliposomes			
73959043	Nodal modulator 2 isoform 2	75	ER
27696122	DOK7 protein	22	Plasma membrane
50979076	Signal sequence receptor, α	63	ER
73994752	Protein KIAA0152 precursor isoform 1	29	Transport vesicle
73970263	Prenylcysteine oxidase 1	521	Lysosome
50979234	Zymogen granule membrane glycoprotein 2	293	Zymogen granule
73945930	ERGIC-53 protein precursor	234	ER-golgi intermediate
73989836	Adipocyte plasma membrane-associated protein	437	Secretary vesicle
73963665	Transmembrane trafficking protein isoform 1	209	Transport vesicle
57112873	Translocon-associated protein, delta subunit precursor	229	ER
73957263	ATPase, H+transporting, lysosomal, V0 subunit D isoform 1	119	Lysosome
73994418	Coated vesicle membrane protein isoform 3	116	Transport vesicle

KRM was fractionated into proteoliposomes and 0.18%Tx KRM. After sodium carbonate treatment, 0.18% Tx KRM was directly subjected to shot gun analysis by LC-MS/MS. Also after sodium carbonate treatment, proteoliposomes and 0.18% Tx KRM were analyzed by 2-D gel, and proteoliposome spots the amounts of which were twice or more than corresponding those of 0.18% Tx KRM were selected, excised, pooled, and subjected to shot gun analysis by LC-MS/MS. The results are summarized in table after soluble proteins and mitochondorial proteins were eliminated as contaminations.

complex, signal peptidase complex, and oligosaccharyl transferase complex and its related proteins. So that our experiment successfully identified main members of rough ER integral membrane proteins in the 0.18% Tx KRM was also consistent with our previous functional assay [16]. Only eight proteins in this fraction were identified as proteins exported out of the ER. No glycosylphosphatidylinositol-anchored protein was identified. In contrast, 9 of the 12 proteins that were identified in the proteoliposome fraction were exported out of the ER. Only three ER resident proteins were identified in this fraction. The ratio of proteins exported out of the ER increased significantly from 25% for 0.18% Tx KRM to approximately 75%, for the proteoliposome fraction, indicating that our extraction–reconstitution method successfully enriched the proteoliposome fraction with integral membrane proteins exported out of the ER. Conversely, the ratio of ER resident proteins decreased remarkably from 75% for 0.18% Tx KRM to approximately 25% for the proteoliposome fraction. These results indicated that two distinct groups of KRM integral membrane proteins exist according to varying sensitivity to 0.18% Tx-100 treatment. Other information such as number of peptides used to identify a protein, the sequence and charge state of each peptide, and MS/MS spectra of some low score proteins are in the Supporting Information Table S1 and the Supporting Information data.

3.4 Confirmation by an *in vitro* system

To confirm integral membrane protein extraction from the KRM membrane by 0.18% Tx-100 treatment we used an *in vitro* system, wherein synthesized integral membrane proteins were indeed integrated into KRM vesicular membranes included in the system. It is well known that only interaction between the membrane-spanning domain and membrane lipids determines whether the protein is integrated into the membrane. TCR-α is an integral membrane protein that has one membrane-spanning domain. We used TCR-α as a representative of the integral membrane proteins exported out of the ER. We previously showed that TCR-α synthesized in the *in vitro* system was translocated into KRM membrane and core-glycosylated [16]. We performed a sodium carbonate treatment to test whether the TCR-α synthesized in the system, in the presence of KRM, was integrated into the vesicular membranes. As a control, Hsp47, a luminal protein, was synthesized. Hsp47 synthesized in the presence of KRM was core-glycosylated at translocation through the KRM membrane. In fact, two products moving slower than the precursor in SDS-PAGE (Fig. 3A, compare lane 2 with lane 1 in the upper panel) were confirmed to be the core-glycosylation products by an EndoH digestion experiment (unpublished data). After the synthesis, vesicles harboring translocated Hsp47 were sedimented in neutral pH condi-

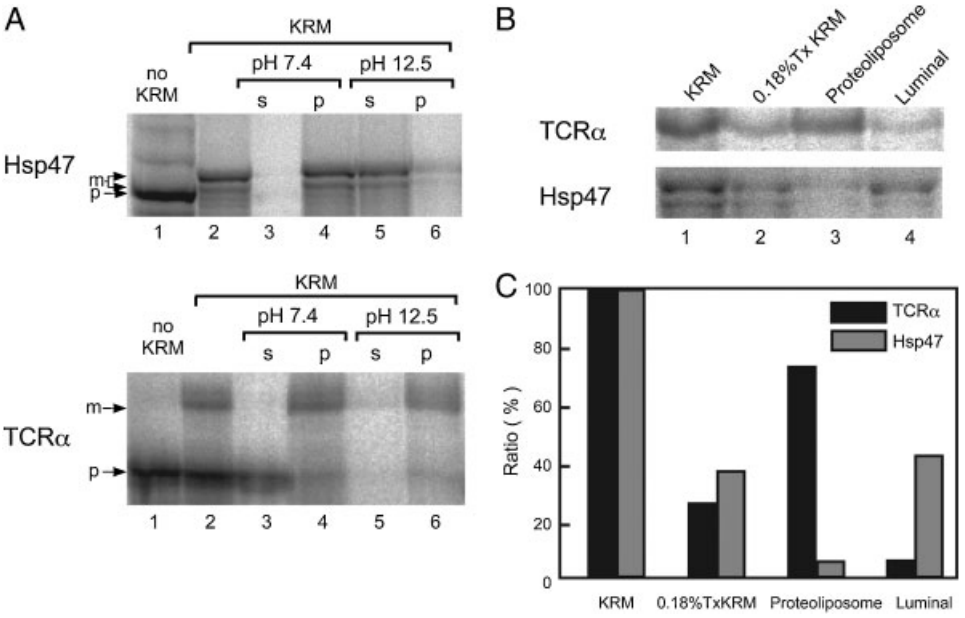

Figure 3. Integration of TCR-α synthesized in an *in vitro* system into KRM and its fractionation into proteoliposome. (A) Integration of TCR-α synthesized in an *in vitro* system into KRM membrane. Hsp47 mRNA (upper panel of A) and TCR-α mRNA (lower panel of B) was translated in an *in vitro* system in the absence (lane 1) or presence of KRM (lanes 2–6). After the translation, membrane vesicles were sedimented at pH 7.5 to separate the supernatant (lane 3) from the pellet (lane 4). The pellet was resuspended, and treated with alkaline solution (pH 12.5), and then sedimented to separate the supernatant (lane 5) from the pellet (lane 6). m; mature form, p; precursor. (B) Fractionation of TCR-α into proteoliposomes KRM vesicles harboring TCR-α or Hsp47 (lane 1 of B) were fractionated into 0.18% Tx KRM (lane 2), proteoliposome (lane 3), and luminal fractions (lane 4). (C) Quantitation of the fractionation results is shown in panel B.

tions and sodium carbonate treatment was subsequently performed. Mature forms of products were sedimented with KRM vesicles at a neutral pH (Fig. 3A, lane 4 in upper panel), but extracted from the vesicles at alkaline pH (Fig. 3A, lane 5 in upper panel), showing that the sodium carbonate treatment acted as expected. On the other hand, TCR-α synthesized in the presence of KRM was glycosylated to a single mature product that was probably fully glycosylated at its putative glycosylation sites (Fig. 3A, compare lane 2 with lane 1 in the lower panel). Further, the resistance of this product to sodium carbonate treatment (Fig. 3A, lane 6 in the lower panel) indicated that this product was indeed integrated into the KRM vesicular membranes.

KRM vesicles harboring either TCR-α or Hsp47 synthesized using the *in vitro* system were collected and subjected to the extraction–reconstitution experiment carried out in the manner illustrated in Fig. 1. Approximately 80% of TCR-α was extracted from the KRM vesicular membrane (Fig. 3B, compare lane 2 with lane 1 in the upper panel) and recovered in the proteoliposome fraction (Fig. 3B lane 3), while approximately 70% of Hsp47 was recovered in the luminal fraction (Fig. 3B lane 4). This showed that a large portion of TCR-α, once integrated into the KRM membrane, was extracted by 0.18% Tx-100 treatment, strongly supporting the possibility that a group of integral membrane proteins was preferentially extracted from the KRM membrane itself. In addition, no obvious loss of TCR-α by binding to SM-2 beads was observed. The quantitation data are shown in Fig. 3C.

4 Discussion

To our knowledge, this is the first 2-D gel analysis of rough ER integral membrane proteins. This enabled us to evaluate our novel fractionation method of rough ER integral membrane proteins, leading to the successful demonstration of preferential extraction of a distinct group of integral membrane proteins from KRM vesicular membrane by 0.18% Tx-100 treatment. In addition, the group of extracted proteins was interestingly enriched with proteins exported from the ER.

Electron microscopic examination, protein profiles, and immunoblotting results illustrated in Fig. 1, suggested that the majority of KRM vesicles retained their basic membrane structure and membrane proteins in spite of 0.18% Tx-100 treatment, and were recovered in the pellet as 0.18% Tx KRM by ultracentrifugation. This was consistent with our previous functional assay of 0.18% Tx KRM [16]. On the other hand, KRM membrane appeared to be in a leaky state, as a result of 0.18% Tx-100 treatment, releasing their luminal proteins recovered as the extract in the supernatant after ultracentrifugation. To examine whether any membrane components besides soluble components were contained in the extract, we treated the extract with SM-2 beads to recover the former ones in proteoliposomes. As shown in Fig. 1, proteoliposomes were formed from the extract, indicating that the extract obviously contained enough membrane lipids and proteins to form proteoliposomes without affecting the structure and functions of the residual membrane, 0.18% Tx KRM, so much. Thus, our concern lay in determining what type of lipids and proteins were extracted. Some portion of the extract came from the nonspecifical solubilization of the minor portion of KRM vesicles as already described. But in this case, each spot obtained from proteoliposomes in the 2-D gel should be smaller than the corresponding one from the 0.18% Tx KRM. However, we showed that the other kind of spots, each of which was larger than the corresponding one from 0.18% Tx KRM, did exist in the 2-D gel obtained from the proteoliposomes. Based on numerous experiments under various conditions to confirm the reproducibility of the occurrence of these spots, we picked up and subjected them to the protein identification by LC-MS/MS as described in Section 2. These spots never come from the minor subpopulation of KRM vesicles that became low density by 0.18% Tx-100 treatment and were recovered in the supernatant, and thereby in proteoliposomes.

Another problem was whether the proteins recovered in the proteoliposome fraction originated from the KRM or from contaminant intracellular membrane systems other than KRM. The main contaminants were expected to be mitochondria, because KRM was prepared from post-mitochondrial supernatants by sedimentation through a 1.35 M sucrose cushion [17] that could almost completely eliminate low-density intracellular membrane systems especially of secretory pathway. We estimated that contaminants other than mitochondria, if any, were confined to a small percentage of the KRM. Proteins identified in the proteoliposome fraction were detected as Coomassie-stained spots, and they were unexpectedly large to have originated from contaminants. In fact, no peroxisomal, proteins not part of the secretory pathway, were identified in 0.18% Tx KRM or in the proteoliposome fraction. At the very last moment of this paper preparation we succeeded in showing that the majority of glycoproteins recovered in proteoliposomes were of high mannose type, sensitive to EndoH digestion (unpublished data). This indicated that membrane proteins recovered as proteoliposomes were derived from KRM itself rather than the contaminated intracellular membrane system. In addition, the *in vitro* system provided strong supporting evidence that a protein, once integrated into the KRM membrane, could be extracted from the membrane by 0.18% Tx-100 treatment, and recovered in proteoliposomes. Thus, we concluded that we did establish a novel fractionation method to divide KRM integral membrane proteins into two distinct groups, based on whether or not they can be extracted by low-concentration Tx-100 attack.

In addition, we found that the subgroup of integral membrane proteins specifically fractionated in the proteoliposomes was enriched with integral membrane proteins exported from the ER. The amount of total proteins in 0.18% Tx KRM was considerably greater than that in the proteolipososmes; hence, the former was a rate-limiting amount applied on the 2-D gel, and it limited the latter to a small quantity. The amount of each protein exported from the ER is generally expected to be very small because it stays in the ER only transiently. In spite of these unfavorable aspects, the successful identification of ten proteins exported from the ER in the proteoliposome fraction was estimated to be unexpectedly significant. Furthermore, because of the aforementioned difficulties in the 2-D gel analysis of integral membrane proteins and recovery of the vast majority of KRM proteins in the 0.18% Tx KRM, we subjected 0.18% Tx KRM directly to LC-MS/MS. Thus, it may be possible to interpret as follows: the eight proteins exported out of the ER in 0.18% Tx KRM might have been identified due to their small portions retained in this fraction, although their major portions were recovered in the proteoliposomes. Note the presence of many streaky spots in the proteoliposome fraction which were larger than the corresponding ones in 0.18% Tx KRM (Fig. 2C and E). This suggested that proteins with several membrane-spanning domains were also recovered in the proteoliposome fraction. However, proteins identified in the proteoliposome fraction have only one or two membrane-spanning domains, probably because we selected clear spots for the purpose of comparative quantitation reason. Although 2-D gel analysis was indispensable to show that a group of integral membrane proteins was specifically fractionated in the proteoliposomes, a systematic immunoblotting analysis of numerous candidate proteins is required. This procedure must serve to show much more proteins specifically recovered in the proteoliposomes and allow detection of proteins showing streaky or poorly circumscribed spots. It will also prove that our method fractionates the rough ER integral membrane proteins into clear-cut fractions (such as resident protein fraction *versus* a fraction of proteins exported from the ER).

It remains to be elucidated how the group of integral membrane proteins is extracted from KRM membrane by 0.18% Tx-100 treatment without affecting its basic structure and functions. Some may argue that proteins with a few membrane-spanning domains are easily extracted from KRM membrane even by 0.18% Tx-100 treatment, and recovered in proteoliposomes. Nevertheless, this is unlikely, because 24 out of 32 identified proteins in the 0.18% Tx KRM fraction have one or two membrane-spanning domains. The preferential extraction would be attributed to the presence of at least two types of microdomains into which integral membrane proteins were selectively integrated as soon as they were released from the translocons in the rough ER. By 0.18% Tx-100 treatment one microdomain was extracted from the ER membrane as a Triton-extractable soluble structure, and the other was inextractable and remained in the ER membrane. Thus, our findings will potentially raise a serious doubt regarding the prevailing view of the ER membrane structure, which has been considered to be in a homogeneous ''liquid-disordered'' state because of its low sphingolipid and cholesterol concentrations [3]. Another significant result of this study was the successful enrichment of the proteoliposome fraction with integral membrane proteins exported out of the ER. The protein segregation into the Triton-extractable microdomain might correspond to the segregation into the scaffold prior to Sec12p activation [8]. Both protein integration into the ER membrane at the translocon and protein exit from the ER by COPII vesicle formation (that has been considered to be the first selection step of proteins exported out of the ER) have been extensively studied. The area between those steps remains unexplored.

That a distinct group of integral membrane proteins was extracted from the rough ER membrane without affecting the basic structure and the function of the membrane, and that the group was enriched with proteins exported out of the ER are totally unexpected and very interesting results *per se*. Furthermore, they will potentially have a huge impact on ER membrane biogenesis and protein trafficking.

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5 References

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