Secretion of signal peptides via extracellular vesicles

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Abstract

Signal peptides (SPs) consist of short peptide sequences present at the N-terminal of newly synthesizing proteins and act as a zip code for the translocation of the proteins to the endoplasmic reticulum (ER). It was thought that the SPs are intracellularly degraded after translocation to the ER; however, recent studies showed cleaved SPs have diverse roles for controlling cell functions in auto- and/or intercellular manners. In addition, it still remains obscure how SP fragments translocate away from the site where they are produced. Extracellular vesicles (EV) are important for intercellular communication and can transport functional molecules to specific cells. In this study, we show that SPs are involved in EV from T-REx AspALP cells that were transfected with a human APP SP-inducible expression vector. There was no difference in the average particle size or particle concentration of EV collected from T-REx AspALP cells and T-REx Mock cells. When the SP content in the EV was examined by mass spectrometry, the C-terminal fragment of APP SP was identified in the exosomes (SEV) of T-REx AspALP cells. In our preparation of SEV fractions, no ER-specific proteins were detected; therefore, SPs may be included in SEV but not in the debris of degraded ER. This is the first indication that SPs are secreted from cells via EV.

Keywords: signal peptide, extracellular vesicles, exosomes, intercellular communication

Introduction

Signal peptides (SPs) are short peptide sequences present at the N-terminal of newly synthesizing proteins and play an important role by acting as a zip code for the translocation of proteins to the endoplasmic reticulum (ER). SPs are removed from the synthesizing proteins at the surface of the ER by signal peptidases [1] and are further processed to two peptide fragments by signal peptide peptidases [2]. Initially, it was thought that cleaved SPs are degraded intracellularly [3]; however, it has become clear that some SPs and/or their fragments have diverse functions for intra- and inter-cellular communications [4]. SPs of viral proteins have various functions such as infection-related control and escape from the immune system during the infection cycle [4]. Ovalbumin SP modulates cell adhesion and the differentiation of mouse embryonic fibroblasts [5]. A SP fragment of eosinophil cationic protein cleaved by signal peptide peptidases gives rise to an increase of TGF-α protein expression [6]. PAR-1 SP plays a role in cardioprotection after ischemia and reperfusion injury [7]. B-type natriuretic peptide (BNP) SPs are present in human circulation and serve as biomarkers to accelerate the clinical diagnosis of cardiac ischemia and myocardial infarction [8]. Despite the above physiological roles of SP fragments, it remains unknown how SP fragments are secreted out of a cell.

Recently, intercellular communication via extracellular vesicles (EV) has attracted much attention. EV are classified into apoptotic vesicles, microvesicles (large EV; LEV), and exosomes (small EV;

SEV), each of which has a different secretory pathway [9]. EV are produced by buddings from the plasma membrane and have a diameter of about 100 nm to 1 μm. SEV are formed within the intracellular multivesicular endosome and range in size from about 30 nm to 200 nm in diameter. Although LEV and SEV are surrounded by a cell membrane, there is a major difference in their formation. EV encapsulate biofunctional molecules such as proteins, peptides and nucleic acids. The encapsulated molecules are delivered to other cells without being degraded and act in the receiving cells. Therefore, we hypothesized that SPs and their fragments are secreted by vesicles such as EV.

In this study, we report that the C-terminal part of SP fragment, but not the full SP of human APP, is present in EV from genetically modified T-REx AspALP clonal cells.

Materials and methods

T-REx AspALP cells

The T-REx System (Thermo Fisher Scientific, Waltham, MA) was used for the experiments. Tetracycline (Tet) regulation in the T-REx System is based on the binding of Tet to the Tet repressor and depression of the promoter controlling the expression of the gene of interest [10]. T-REx-293 (T-REx) cells stably express the Tet repressor. The SP sequence of human placental secreted alkaline phosphatase (SEAP) was replaced with the SP sequence of APP. The APP SP-SEAP sequence was inserted into multiple cloning sites of pcDNA 4/TO vector, and the vector was then genetically transferred into T-REx cells. T-REx AspALP cells were picked up from the transferred cells as a stable mutant strain. T-REx Mock cells were generated as a stable strain by the gene transfer of unmodified pcDNA 4/TO vector.

Isolation of EV from conditioned medium

Cells (1 x 10^6) of T-REx Mock and T-REx AspALP were plated on a 100 mm collagen-coated dish (AGC Techno Glass Co., Ltd., Haibara-gun, Shizuoka, Japan) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum, from which EV were removed by centrifugation at *110,000 x g* for 24 h, 5 μg/ml blasticidin (InvivoGen), 1 μg/ml doxycycline (Dox) (Takara Bio Inc., Kusatsu, Shiga, Japan) and Penicillin-Streptomycin (Thermo Fisher Scientific) for 72 h at 37℃ in 5% CO_2/95% humidified air.

Conditioned medium and cells were collected from 12 dishes. The conditioned medium was centrifuged at *300 x g* for 5 min at 4℃ to remove live cells, and the supernatant was centrifugated at *2000 x g* for 20 min to remove apoptotic vesicles. LEV were collected by centrifugation at *10,000 x g* for 60 min. SEV were prepared from the supernatant by centrifugation at *110,000 x g* for 60 min. LEV and SEV were re-suspended with 100 μl of PBS. The cells were washed in PBS twice after cell counting and stocked as cell pellets.

Measurement of SEAP activity

SEAP activity in the conditioned medium was measured using QUANTI-Blue Solution (InvivoGen, CA). In brief, 20 μl of conditioned medium was mixed with 180 μl of QUANTI-Blue Solution and incubated at 37℃ for 30 min in a 96-well plate. Absorbance at 620 nm was measured using a microplate reader.

Nanoparticle tracking analysis

The number and average size of EV were measured using NanoSight NS300 (Malvern Panalytical Ltd, Malvern, UK). LEV and SEV were diluted at 1:100 in degassed water to a final volume of 600 μl and applied through a syringe for measurement. The camera level was increased until all particles were distinctly visible without exceeding a particle signal saturation over 20% (level 14-16). Automatic settings for the maximum jump distance and blur settings were utilized. The detection threshold was 5. For each measurement, five 60-s videos were captured under the following

conditions: cell temperature, 25℃; syringe pump speed, 100 (instrument-specific unit); camera, sCMOS; laser, 488 nm blue. After capture, the number and size of EV were analyzed on NanoSight NTA 3.2 software build 3.2.16. Released EV (particles per cell) were calculated using data from the NTA and cell counting.

Isolation of ER fraction

T-REx Mock and T-REx AspALP cells were seeded in 10-cm dishes at a concentration of 1 x 10^6 cells and cultivated with the medium in the presence of 1 μg/ml Dox for 3 days. T-REx Mock and AspALP cells (3 x 10^7 cells) were collected and washed with PBS twice. The ER fraction was isolated from both cell types using the Endoplasmic Reticulum Enrichment Extraction Kit (Novus Biologicals, Centennial, CO). In brief, to the cells was added 1 ml of 1 x Isosmotic Homogenization Buffer followed by 10 μl of 100 x Protease Inhibitor Cocktail. The cells were then homogenized by moving them in and out of a pipette 100 times. The homogenate was centrifuged at *1,000 x g* for 10 min at 4 ℃, and the supernatant was centrifuged at *12,000 x g* for 15 min at 4 ℃. The supernatant was then centrifuged at *90,000 x g* for 70 min at 4 ℃ and discarded. The pellet was suspended in 200 μl of 1 x Suspension Buffer supplemented with 2 μl of 100 x Protease Inhibitor Cocktail as the total ER fraction.

SP identification with MALDI-TOF MS/MS

LEV and SEV (10 μl each) were dissolved in 90 μl of 8 M urea. Peptides were concentrated from the

solution with GL-Tip SDB and GC columns (GL Sciences Inc., Tokyo, Japan) and eluted with 10 μl of 80% acetonitrile supplemented with 0.1% trifluoroacetate. The peptide solution was mixed at 1:1 with 10 mg/ml CHCA in 50% acetonitrile supplemented with 0.1% trifluoroacetate, and 1 μl of the mixture was plated on MTP 384 target plate ground steel (Bruker, Billerica, MA). After the plate was air-dried, peptides were measured using ultraflextreme MALDI-TOF MS (Bruker) and analyzed using Flex analysis software (Bruker). In the MS/MS analysis, amino acid sequences were determined within an error of 0.7 Da.

Western blotting

The cells, ER fraction, and LEV and SEV from T-REx Mock and AspALP cells were lysed in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, pH 7.5) (Cell Signaling Technology, Danvers, MA) by sonication in iced water. The lysates were stored at -80 ℃ prior to the western blot analysis. The BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL) was performed to determine protein concentrations. Briefly, 1 μl of total protein per sample was aliquoted, mixed with loading buffer supplemented with or without dithiothreitol (Cell Signaling Technology), boiled for 5 min, and separated by SDS-PAGE on mini-gels (Oriental Instruments, Kanagawa, Japan). The separated proteins were then transferred to PVDF (polyvinylidene difluoride) membranes on an iBlot Gel Transfer Device (Thermo Fisher

Scientific) and blocked in TBS (Tris-buffered saline) supplemented with 5% nonfat dry milk (Cell Signaling Technology) and 0.1% Tween 20 for 1 h at room temperature. Membranes were then incubated in the presence of primary antibodies diluted in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo, Osaka, Japan) at a 1:6000 dilution overnight at 4°C. Primary antibodies directed against CD63 (Novus Biologicals, Centennial, CO) and CD81 (Novus Biologicals) for nonreducing conditions, and against Grp78 (GeneTex, Irvine, CA) and ATF6 (Proteintech, Rosemont, IL) for reducing conditions were used. The following day, the membranes were washed 4 times for 8 min with TBS-T (TBS supplemented with 0.1% Tween 20) and incubated in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo) containing HRP-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG, Cell Signaling Technology) at a 1:10000 dilution for 1 h at room temperature. The proteins were visualized by chemiluminescence using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) and Light Capture II (Atto, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using one-way ANOVA and Tukey post hoc tests. Differences were considered significant when the p value was less than 0.05.

Results

To confirm the expression of transfected gene products including APP SP by treatment with Dox, the activity of SEAP, a mature product protein derived from the transfected gene, was measured with the conditioned medium (Fig. 1A). T-REx Mock cells expressed SEAP protein, and the activity was not altered by treatment with Dox. On the other hand, the SEAP activity from T-REx AspALP cells was markedly increased by Dox. These results indicated that our model system expressing APP SP efficiently worked. To examine the properties of EV released from Dox-treated cells, the number and average size were determined by the NTA method and no difference was observed between the Mock and AspALP groups (Fig. 1B and C).

To clarify whether EV from T-REx AspALP cells contained APP SP, we examined APP SP in LEV and SEV using MALDI-TOF MS. We found the peak size at *m/z* 859, which was increased by Dox treatment, which differs from the peak size for full length APP SP (MLPGLALLLLAAWTARA, *m/z* 1781) in the SEV fraction (Fig. 2A). Neither peak was found in the LEV fraction of Dox-treated T-REx AspALP cells or T-REx Mock cells. MS/MS analysis identified the peak at *m/z* 859 +/- 4 as the C-terminal fragment of APP SP (LAAWTARA, MW 859), which contains a -TAR- amino acid sequence (Fig. 2B).

To exclude the possibility of contamination of ER derived from dead cell remnant, we examined the viability of T-REx mock and AspALP cells. T-REx mock and AspALP cells did not show any

difference in viability when stimulated with Dox (Fig. 3A). For further confirmation, we examined whether the EV fraction contained ER-associated proteins, such as Grp78 and ATF6 by western blotting. Although tetraspanins, such as CD63 and CD81, two SEV markers, were detected in SEV, Grp78 and ATF6 were not in EV fractions (Fig. 3B). Finally, we examined APP SPs (full length and C-terminal fragment) in the ER fraction by MALDI-TOF MS, but did not detect APP SPs (Fig. 3C), suggesting SPs in the ER rapidly degrades. These results indicated that SEV contained APP SP and did not contaminate the ER fraction.

Discussion

In this study, we demonstrated the presence of SPs in EV. We detected the C-terminal but not N-terminal fragment of APP SP in SEV from T-REx AspALP cells. SPs are digested by signal peptidases on the ER membrane [1] and then by signal peptide peptidases [2]. SPs have the dual function of targeting proteins and membrane anchoring proteins at the ER [11]. SPs can insert into the membrane in either type I or type II orientation. SPs of type I membrane proteins penetrate the N-terminus across the membrane while the C-terminus remains in the cytoplasm. By contrast, SPs of type II membrane proteins transfer the C-terminus of the protein across the membrane while the N-terminus remains on the cytoplasmic side. The determination of type I and type II orientation is dependent on the features of the SP sequences [12,13]. Among cleaved SP fragments, some migrate to the cytoplasmic side and others to the ER side. The N-terminal side of APP SP may be inserted at the ER lumen as type I orientation. In an analysis of SPs from other cells, we found N-terminal fragment of SPs in EV with type II orientation (manuscript in preparation).

We could not detect the C-terminal fragment of APP SP in peptides extracted from ER in MALDI-TOF MS analysis. This observation suggested that cleaved SP was rapidly transferred to the cytoplasm or degraded in the ER. The fate of SP fragments released into the cytoplasm is not clear, but SP fragments derived from mammal preprolactin and HIV-1 gp160 have been reported to bind to calmodulin in a calcium-dependent manner [14]. Calmodulin is an important modulator involved in

a number of signal transduction and cellular functions. In addition, calmodulin translocates between intracellular organelles, the cytoplasm, and nucleus depending on its activation state [15]. Furthermore, calmodulin has been reported as a constituent of EV in the EV database Vesiclepedia (http://microvesicles.org). Therefore, it is possible that SP fragments released into the cytoplasm are bound to signaling molecules, such as calmodulin, and translocated to EV. How the C-terminal SP fragment is incorporated into SEV without being degraded after the C-terminal fragment is transferred to the cytoplasm needs further investigation.

In this study, T-REx AspALP cells, in which the SP of SEAP was artificially exchanged with that of APP, were used to examine whether SPs are present in EV. Whether SPs of endogenous proteins are contained in EV when various cells are stimulated to induce transcription and translation also needs further investigation. In addition, it is important to clarify what kinds of SPs are present in EV.

Since some SPs are detected in plasma, they are expected to be new biomarkers. Fragments derived from BNP SP were not only detectable in the cytosolic extracts of explant human heart tissue but also secreted from the heart into the circulation of healthy individuals [8]. In addition, the plasma level of BNP SP fragments in patients with acute ST elevation myocardial infarction rose to peak values significantly earlier than currently used biomarkers such as myoglobin and creatin kinase-MB. Further, SP fragments of prepro-A-type natriuretic peptide and C-type natriuretic peptide as well as BNP SP were present in human circulation and have potential as novel biomarkers [16,17].

Assuming that cells secrete SPs into plasma via EV, collecting EV from plasma may enable more sensitive biomarker detection.

Recent studies showed that full-length SPs and/or SP fragments have various physiological functions such as differentiation, secretion of humoral factors and protection from myocardial injury [5–7]. In addition, SPs acted on autocrine [6] and endocrine/paracrine signals [7]. Moreover, SPs were detected in the circulation of healthy individuals as well as patients [8,16,17]. These studies suggested that SPs are secreted as substances with physiological functions in vivo. In this study, we show for the first time that SPs are secreted via EV. Therefore, SPs may serve as functional peptides for intercellular communication via EV.

Declaration of competing interest

We have no conflict of interest to disclose.

Acknowledgements

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Figure legends

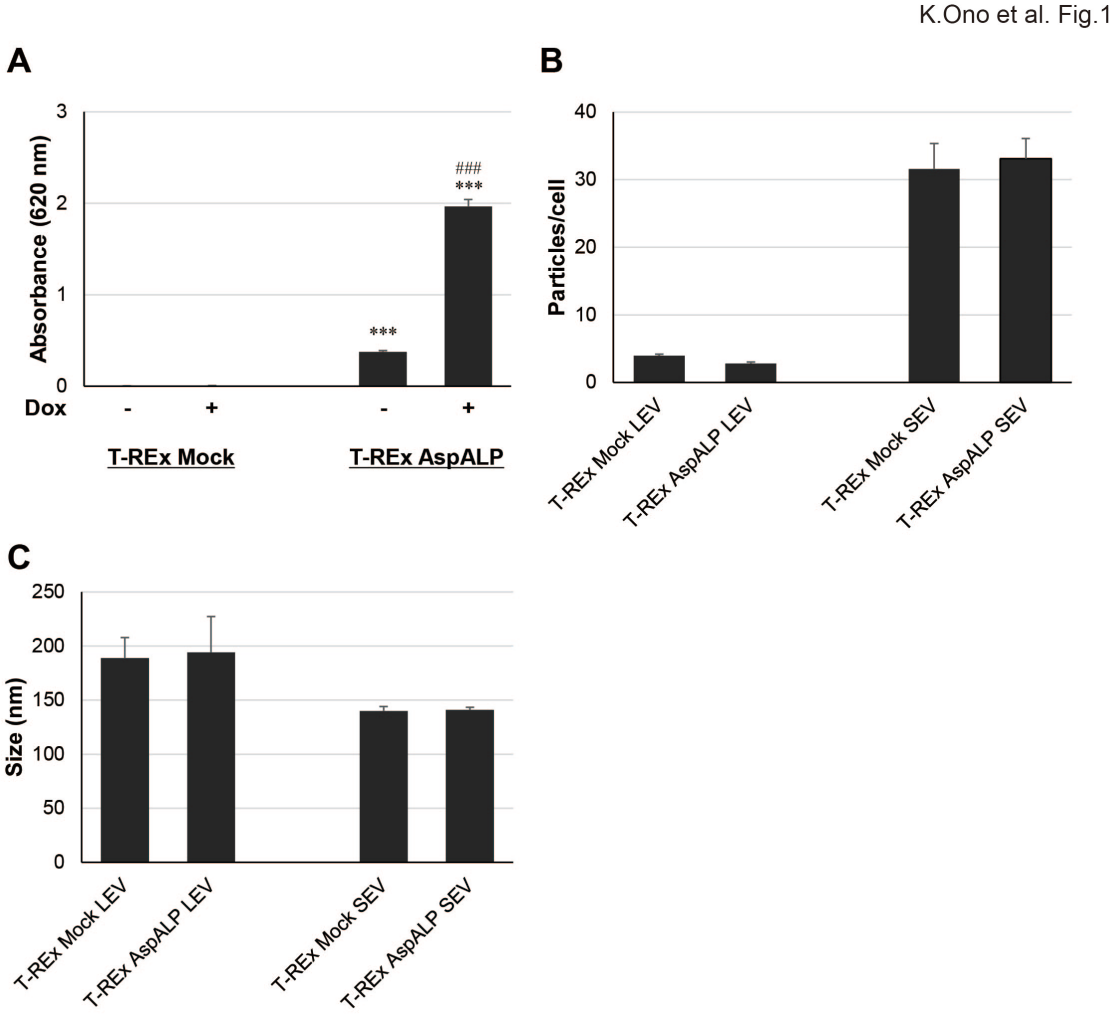
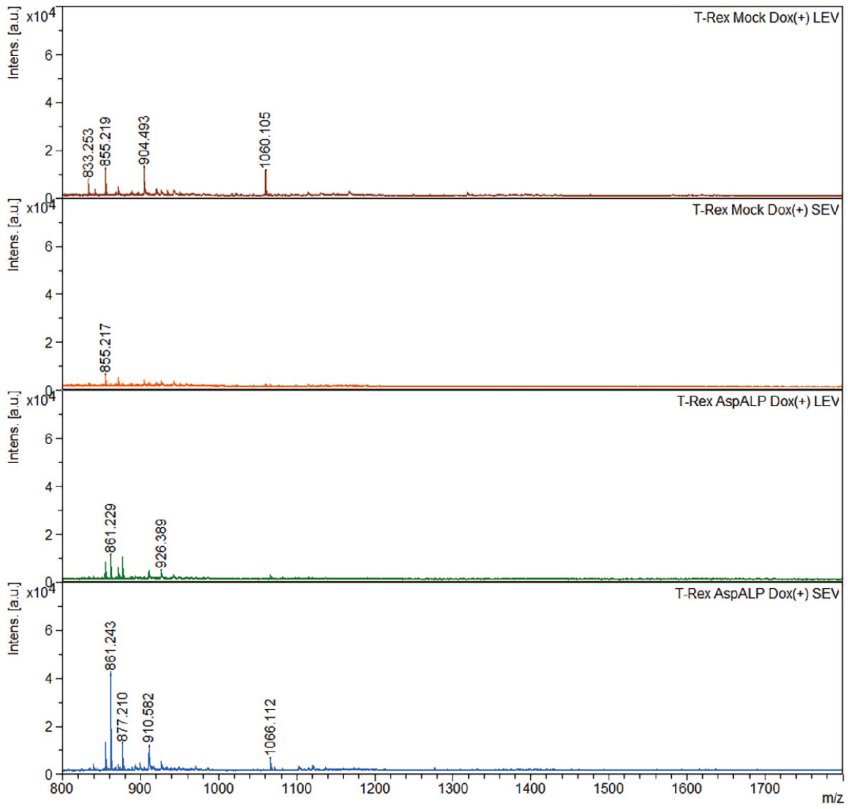


Fig. 1 SEAP and EV released from T-REx AspALP cells.

The SEAP activity from T-REx AspALP cells (A). ***$p<0.001$ vs. T-REx Mock Dox (-) or (+) cells. $^{###}p<0.001$ vs. T-REx AspALP Dox (-) cells. The number of released EV per cell (B). The average size of EV (C).

K.Ono et al. Fig.2

A



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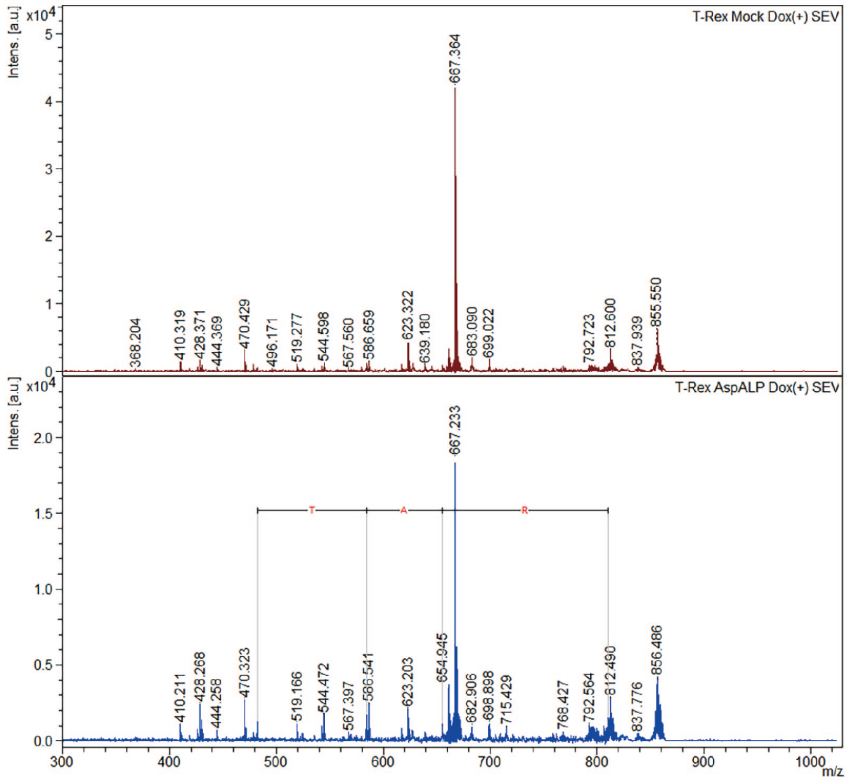

Fig. 2 SEAP SP in EV released from T-REx AspALP cells.

The peptide solution extracted from LEV and SEV of T-REx Mock and AspALP cells 3 days after Dox treatment was analyzed by MALDI-TOF MS (A). MS/MS analysis of peaks at *m/z* 859 +/- 4 was performed (B).

K.Ono et al. Fig.3

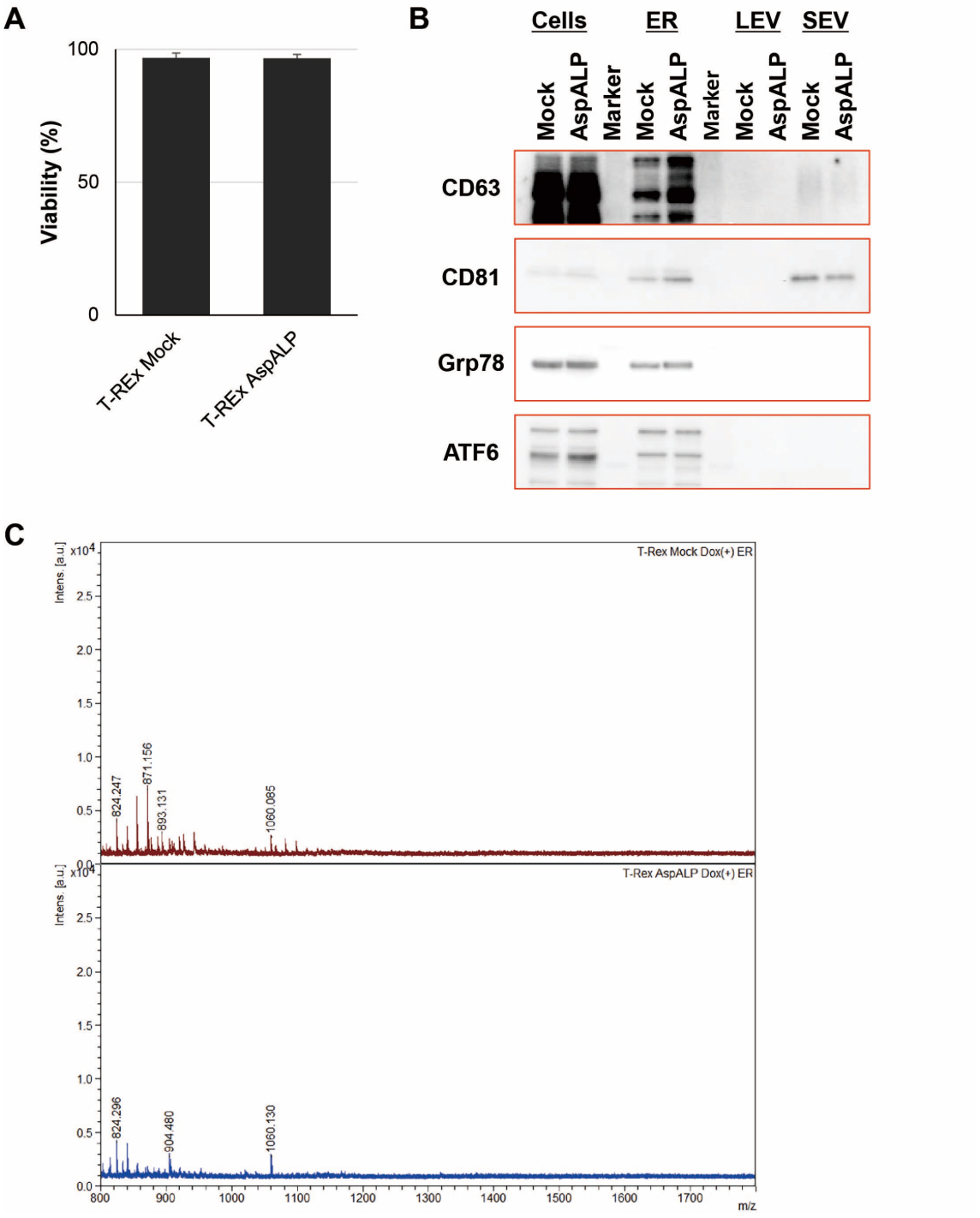

Fig. 3 Confirmation that the EV fraction did not contain the ER fraction.

The viability of T-REx Mock and T-REx AspALP cells 3 days after Dox treatment (A). Western blot analysis of EV and ER markers in cells and in ER, LEV and SEV collected from T-REx Mock and

T-REx AspALP cells (B). The ER fraction from T-REx Mock and AspALP cells after Dox treatment was analyzed by MALDI-TOF MS (C).