COMPARISON OF PROTEIN EXTRACTION CONDITIONS FOR EDEM3 INTERACTORS IN MELANOMA CELLS

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ER-degradation enhancing α-mannosidase-like protein 3 (EDEM3) is a key element of the Endoplasmic Reticulum Associated protein Degradation (ERAD) pathway. Coordination of EDEM3 with other proteins of ERAD is critical in accelerating the disposal of misfolded glycoproteins from ER and therefore, investigating EDEM3 interactome is of highest importance. We report here the effect of various extraction conditions for EDEM3 associated proteins resulted from co-immunoprecipitation experiments. By using mass spectrometry along with complementary methods, we have managed to optimize suitable conditions for extraction and identification using stringent detergents such as Triton-X100 or improved identification of potential interaction partners with mild detergents such as Digitonin.

Keywords: mass spectrometry, immunoprecipitation, EDEM3.

INTRODUCTION

To maintain protein homeostasis, cells developed control mechanisms in key positions to overcome errors in protein synthesis and folding. Most of the membrane and secretory proteins are synthesized on ER-bound ribosomes and are co-translationally folded, process assisted by the ER resident chaperones and enzymes (1, 2). Since folding is an error prone process, the first mechanisms of quality control arise immediately after synthesis. Newly synthesized proteins are translocated to the ER, where they undergo folding, post-translational

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modifications and ultimately are selected either for transport to the secretory pathway or to degradation. Considering the complexity of the process it is justified that proteins have to pass many checkpoints in the ER (3, 4).

For newly synthesized glycoproteins to achieve their native conformation, a large number of chaperones and ER resident enzymes (OST, GI, GII, CNX, CRT, PDI) are required for this process (5, 6). Proteins that pass the quality control check will be packed into vesicles and exported to the Golgi apparatus; if folding fails, the folding cycle can be repeated several times (7-9). However, in many cases the polypeptide chain fails to reach the native conformation due to sequence defects, erroneous post-translational modifications or stress factors that disturb cellular homeostasis. To cope with the increasing amounts of misfolded proteins, ERAD is activated (10-12). Proteins like EDEM1, EDEM2, EDEM3, OS-9, XTP-3B and others are actively involved in selection, transport and dislocation of misfolded polypeptides from ER for proteasomal degradation (13-17).

We focus on the role of EDEM3 in ERAD. According to Hirao *et al*., EDEM3 is a soluble member of G47 hydrolase family proteins located in the ER lumen of mammalian cells that accelerates ERAD of misfolded glycoproteins; it is heavily glycosylated and its 932 amino acids are structured in different domains: a signal peptide sequence (aa 1-41), mannosidase domain (aa 49 to 500), a protease associated domain (aa 674-779) and an ER retention motif (aa 929-932). The mechanism by which EDEM3 exerts its function is likely different from that of EDEM1 or EDEM2, since EDEM3 greatly stimulates mannose trimming *in vivo* (16, 18). However, a recent paper published by Ninagawa *et al.*, proposed that EDEM2 is the key initiator of the de-mannosylation process and EDEM3 functions as a second limiting enzyme responsible for mannose trimming from M8 (mannose) to M7A M6 of M5 (19).

In the recent years, mass spectrometry (MS) has emerged as one of the leading methods to investigate protein pools and protein-protein interactions prompting to the advent of new research fields such as proteomics and interactomics (20).

In this study we aimed to set up a mass-spectrometry based method to identify possible physical partners of EDEM3 in ERAD pathway. Thus, we tested two lysis buffers, previously reported as compatible with MS analysis, and we focused on finding how buffer stringency affected the identification of EDEM3 possible interactors (21). Our studies show that although Digitonin containing buffer preserves even weak interactions between EDEM3 and its partners, an overall low level of extracted membrane proteins was detected. However, when we used lysis buffer containing Triton-X100, an increased number of membrane proteins were extracted, but many weak bonds were not preserved and thus, less interactors were identified.

MATERIALS AND METHODS

Reagents, antibodies, plasmids. Rabbit α-EDEM3 (E8906) antibody, Ammonium Hydroxide, Iodacetamide, MS grade Acetonitrile, Water, Methanol, Isopropanol, Triton-X100 were purchased from Sigma Aldrich. DTT (dithiothreitol), Digitonin from Santa Cruz, Trypsin (V5111) was purchased from Promega and protease inhibitors from Roche, inhibitors from Roche. Protein A-Sepharose and all culture media and supplements were from Invitrogen.

Cell lines. A375 cell line was stably transfected to express heterologous EDEM3 using an Amphotropic retroviral packaging system. For this, the gene encoding for the full length EDEM3 with an HA tag at the C-terminus was cloned into pLPCX retroviral vector and the fidelity of the sequence was verified by DNA sequencing. HEK 293T grown in DMEM supplemented with 10% fetal bovine serum were co-transfected with pLPCX-EDEM3 and the packaging vector pCL-Ampho. Virus containing media of HEK 293T was further used to infect the A375 for 24 h. Selection of infected cells was made with 5μg puromycin for 24-48 h, after the cells were grown in the same media with a lower concentration of puromycin (1μg/ml).

Western blotting. Pelleted cells were washed with PBS and lysed in buffer containing 50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 5 mM EDTA, protease inhibitors, and either 1% (wt/vol) Digitonin or 1% (vol/vol) Triton-X100, and incubated on ice for 30 min. The lysates were cleared by centrifugation at 20,000 g for 15 min at 4°C. Samples prepared under reducing conditions were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked overnight (ON) at 4ºC in 10% milk in PBS and the next day were probed with rabbit α-EDEM3 antibodies (1:1000) followed by incubation with HRP coupled goat α-rabbit (1:10.000) in 5% milk in PBS-Tween20 0.1%. Following incubation with luminol substrate (ECL) for 1 min, the membranes were exposed to chemiluminescent sensitive films and developed.

Immunoprecipitation and LC-MS/MS. A375 cells that stably express the transduced EDEM3 along with the control A375 cells that express only endogenous EDEM3 were grown in 75 cm^2 dishes and harvested at 90% confluence. Cell lysates corresponding to each lysis conditions were immunoprecipitated with EDEM3 antibodies ON, captured on protein A sepharose (1:2) for 2 h at 4^{o}C and eluted with the corresponding solutions SEB (soft elution buffer 0.2% SDS, 0.1% Tween20) and NH_4OH pH 12, 150 mM. The eluted samples were separated by SDS-PAGE and prepared for MS analysis using in-gel digestion protocol (22). Briefly, the gel pieces were subjected to reduction with 10 mM DTT in 50 mM ABC (ammonium bicarbonate) for 1 h at 56^{o}C, alkylation with 100 mM Iodacetamide in 50 mM ABC for 1h at room temperature and trypsinization for 16 h at 37^{o}C (8-10 ng trypsin/protein). Before each step two washes were made with 50 mM ABC in 50% ACN and 100% ACN, respectively.

Peptides were extracted with two buffers (buffer 1:5% HCOOH in 50% ACN and buffer 2:5% HCOOH in ACN) followed by complete drying. Samples were resuspended in mobile phase A (0.1% HCOOH and 2% ACN) and subject to nanoLC-MS/MS analysis. The peptides were separated using an Easy nanoLC II (Proxeon Biosystems) connected online to an Orbitrap Velos Pro mass spectrometer. The instrument was operated in data dependent mode, by automatically selecting top 20 most abundant peptides eluting from an EASY-Column, 10 cm, ID 75 μm, 3 μm, C18-A2 (SC200, Thermo Fisher Scientific) under a gradient of 2–30% mobile phase B (0.1 % HCOOH, 98 % ACN). The instrument resolution was set at 30000 (m/z 400). MS/MS spectra were searched against human sequences from UNIPROT KB database (13 August 2013) using SEQUEST HT implemented in Proteome Discoverer v1.4. To estimate the false positive results a decoy database containing the reversed sequences was searched in parallel. Only peptides with an estimated confidence of 99% (high confidence) and a mass deviation of less the 5 ppm were accepted as a positive identification. For the protein group filtering at least two unique peptides were required to validate the identifications.

RESULTS AND DISCUSSION

To identify EDEM3 interactors we have immunoprecipitated endogenous and overexpressed EDEM3 protein from A375 melanoma cells, respectively. A375 amelanotic melanoma cells transduced or not to express EDEM3 were grown in 75 cm^2 dishes at 90% confluence, harvested and lysed using different stringency buffers (*e.g.*, CHAPS, TritonX100, Digitonin). The cell lysates were immunoprecipitated with an antibody against EDEM3 by overnight incubation at 4°C. Antigen-antibodies complexes were immobilized on protein A/G washed and eluted in different conditions (low pH, increased salt concentration or detergent). The eluted samples were separated by SDS-PAGE followed by in-gel tryptic digestion as described in materials and methods chapter.

Two types of lysis buffers were tested to compare the protein extraction efficiency. The first buffer contained 1% Triton-X100, a frequently used non-ionic detergent capable to efficiently extract transmembrane and hydrophobic protein, but it has the disadvantage of disrupting labile protein complexes. The second buffer used by us contained 1% Digitonin – a non-ionic detergent capable to preserve the interaction partners more efficiently than Triton-X100 (21).

In the case of cell lysis with Triton-X100 buffer we tested two elution buffers subsequently: ammonium hydroxide (NH_4OH) at a concentration of 150 mM and a pH of 12 and soft elution buffer (SEB-0.2% SDS, 0.1% Tween20) (23), respectively. Ammonium hydroxide has a major advantage because it is volatile and completely evaporates, therefore the samples can be resuspended directly in a

MS compatible buffer. The NH_4OH elution was made in a volume of 500 µL at 4°C for 15 min in a head over head mixer. This step was repeated three times, and the collected elutions were reunited. For SEB elution, the samples were vortexed in 60 µL of elution buffer for 10 min at room temperature. As observed in Fig. 1, both overexpressed and endogenous EDEM3 were efficiently immunoprecipitated from cell lysates and an increased amount of EDEM3 was recovered using the elution with NH_4OH, although it was made after the SEB elution.

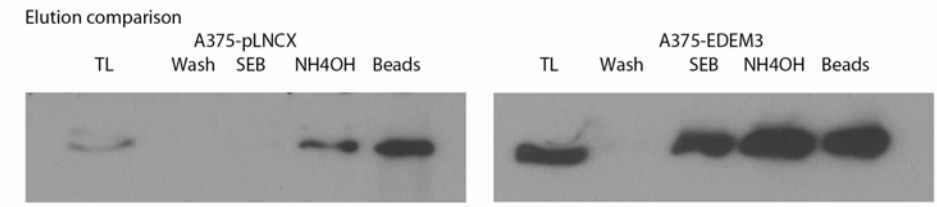

Fig. 1 – Western blotting identification of EDEM3 after IP and elution with different buffers. A375-pLNCX and A375-EDEM3 were harvested at above 90% confluence, lysed in 1 mL lysis buffer (50 mM HEPES pH 7.4, 50 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton-X100), imunoprecipitated with rabbit α-EDEM3 overnight, eluted with Soft Elution Buffer in 60 µL for 10 min and NH_4OH in 500 µL for 10 min. It can be observed that the ammonium hydroxide is more efficient in eluting the protein of interest, although a relatively high amount of protein still remains bound. Multiple elutions are required for a more efficient recovery of EDEM3.

A similar experiment was performed to test the efficiency of different detergents (Triton-X100 and Digitonin) for cell lysis and identification of EDEM3 interactors by LC-MS/MS. When using A375-EDEM3 cell line for Digitonin extraction, we have identified 1630 proteins compared to 836 proteins identified in case of Triton-X100 extraction. Similar results were obtained for the control cell line A375-pLNCX (Fig. 2A). When we compared the control and the overexpressed possible protein interactors, it is observed that for Digitonin three quarters of the identified proteins were found in both samples (1333 from a total of 1630). For the samples prepared with Triton-X100, less than half of the identified proteins were common for endogenous and overexpressed EDEM3.

As seen in Table 1 we were able to imunoprecipitate EDEM3 from non-transfected cells possibly because of the high expression level of endogenous EDEM3 in A375 melanoma cells. Upon analysis of the MS spectra and data interpretation we observed that in the case of protein extraction with Digitonin the interactions of EDEM3 with its ER partners were better conserved compared to the case when proteins were extracted with Triton-X100, an observation justified by the higher number of proteins identified with an increased sequence coverage. Therefore, for the following experiments we chose Digitonin as detergent for lysis.

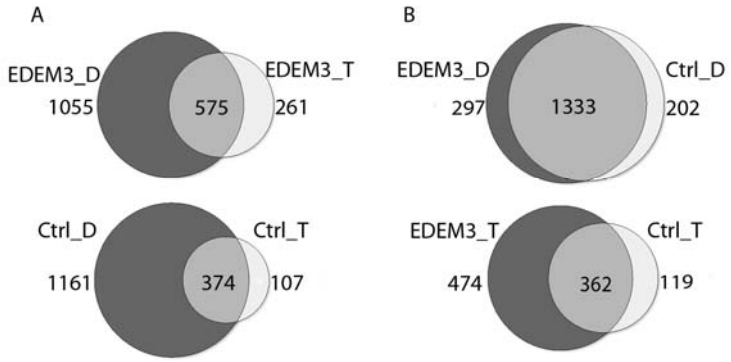

Fig. 2 – Number of identified proteins by MS analysis. (A) Depicts the total number of proteins identified for the two conditions, respectively the two detergents used for lysis. (B) A comparison of the number of proteins identified for each sample CTRL (A375 non-transduced cells) and EDEM3 (A375 stably expressing EDEM3).

Table 1

Identification of EDEM3 using the software Proteome Discover 1.4

		Unique Peptides		Score		Coverage (%)		PSM	
Description		Triton X100	Digitonin	Triton X100	Digitonin	Triton X100	Digitonin	Triton X100	Digitonin
EDEM3	A375-pLNCX	18	27	360.84	862.25	22.96	38.73	107	205
	A375-EDEM3	51	29	3942.15	2754.77	45.17	42.49	1330	673

In Table 1 we identified EDEM3 and the corresponding number of unique peptides, score and peptide spectrum match (PSM) for each experiment is indicated. A peptide-spectrum match is a scoring function that assigns a numerical value to a peptide-spectrum pair (P, S) expressing the likelihood that the fragmentation of a peptide with sequence P is recorded in the experimental mass spectrum S.

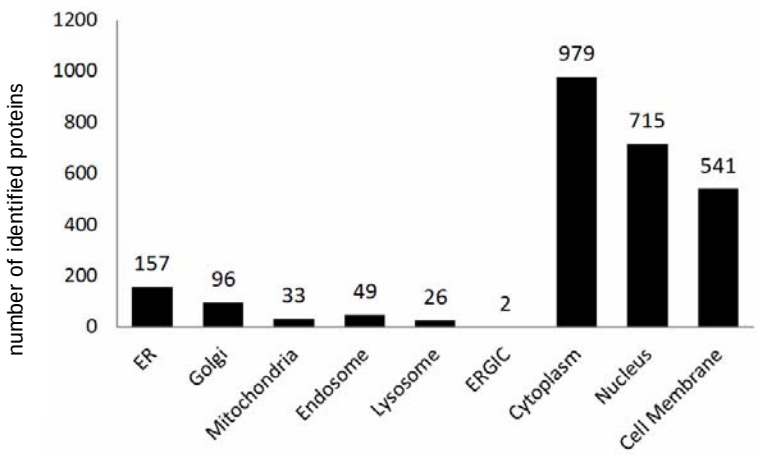

Fig. 3 – Number of proteins count observed in each subcellular compartment. Because a mild detergent for lysis as well as washing was used, a large number of unspecific proteins remained bound to our protein of interest.

The drawback of Digitonin usage for protein extraction is its low solubility in aqueous solutions, being solubilized only in ethanol at temperatures over 90^{o}C. In addition, Digitonin is a mild non-ionic detergent, which is not able to wash out all the nonspecific bound proteins. This can be observed in Fig. 3 where contaminants from the cytoplasm, nucleus and cell membrane represented more than 75% of the proteins identified.

We also analyzed the proteins known to be involved in the processes affected by EDEM3 as folding, ERAD, transport to Golgi and proteasomal degradation (Fig. 4). Apparently, we found that proteins involved in ERAD (*i.e.*, EDEM 1, 2, 3, Sel1L) and proteasomal degradation (*i.e.*, ubiquitin activating or conjugating enzymes) are more abundant than proteins involved in protein folding (calnexin, calreticulin, glucosidase) and ER to Golgi transport (coatomer, protein ERGIC-53). This could be explained by the active involvement of EDEM3 in dislocating proteins form the ER and less in protein folding or transport from the ER.

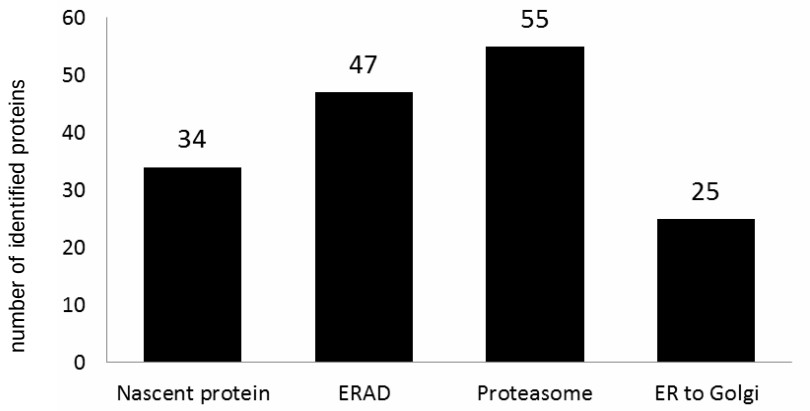

Fig. 4 – Number of ER resident proteins identified in specific ER processes.

Based on our results we can conclude that both lysis buffers containing either Digitonin or Triton-X100 can be efficiently used for protein extraction and further LC-MS/MS analysis. Triton-X100 provides a more powerful extraction and removes better the non-specific proteins, but less interactors are identified since labile complexes are disrupted by this detergent. When a mild extraction with Digitonin was used an increased number of possible interactors were identified, however specificity is reduced in this case due to low stringency washing.

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