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Live-cell topology assessment of URG7, $MRP6_{102}$ and SP-C using glycosylatable green fluorescent protein in mammalian cells

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ABSTRACT

Experimental tools to determine membrane topology of a protein are rather limited in higher eukaryotic organisms. Here, we report the use of glycosylatable GFP (gGFP) as a sensitive and versatile membrane topology reporter in mammalian cells. gGFP selectively loses its fluorescence upon N-linked glycosylation in the ER lumen. Thus, positive fluorescence signal assigns location of gGFP to the cytosol whereas no fluorescence signal and a glycosylated status of gGFP map the location of gGFP to the ER lumen. By using mammalian gGFP, the membrane topology of disease-associated membrane proteins, URG7, $MRP6_{102}$, SP-C(Val) and SP-C(Leu) was confirmed. URG7 is partially targeted to the ER, and inserted in C_{in} form. $MRP6_{102}$ and SP-C(Leu/Val) are inserted into the membrane in C_{out} form. A minor population of untargeted SP-C is removed by proteasome dependent quality control system.

1. Introduction

Membrane proteins constitute about 30% of all proteins [1]. Membrane proteins function as receptors and channels in various biological processes, thus are major targets for drugs [2]. Despite their biological importance, membrane protein structures contribute only 1% to the solved protein structures [3]. This is due to the experimental difficulties with overexpression, purification and crystallization of membrane proteins. In most cases, the first step towards understanding structure of membrane proteins is the determination of membrane topology, two-dimensional structural information with a number of transmembrane (TM) segments and relative orientations of the loops connecting the TM segments in the membrane. Membrane topology of proteins in their native state needs to be experimentally determined even for those with known three-dimensional structure, as their orientation in the cellular membrane cannot be assessed with crystallography. Yet, there are a limited number of tools available for assaying membrane protein topology in mammalian cells, and the live cell assessment of membrane topology is rare. Glycosylatable green fluorescent protein (gGFP) is available to serve such purpose in yeast [4]. In mammalian cells, fluorescence protease protection (FPP) assay has been developed to elucidate the membrane protein topology. FPP is similar to yeast gGFP assay in that it utilizes the fluorescence pattern to determine the position of a membrane protein, but it requires an additional protease treatment and in some cases, membrane permeabilization [5]. Here, we report the development and use of gGFP in mammalian cells as a membrane protein topology reporter. Like yeast gGFP, mammalian gGFP is fluorescent in cytosol, but upon N-linked glycosylation in the endoplasmic reticulum (ER) lumen, it becomes non-fluorescent. Three clinically important membrane proteins, URG7, truncated MRP6 and lung surfactant protein C (SP-C), were chosen to validate the assay system.

The up-regulated gene clone 7 (*URG7*), which encodes for the protein URG7 is induced by the hepatitis B virus (HBV) x antigen. This up-regulation is involved in the pathogenesis of chronic

Abbreviations: GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; TM, transmembrane; ER, endoplasmic reticulum; FPP, fluorescence protease protection; URG7, up-regulated gene clone 7; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ILD, interstitial lung disease; Endo H, endoglycosidase H; WT, wild type; DMSO, dimethyl sulfoxide.

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infection and furthermore, in the development of hepatocellular carcinoma (HCC) [6,7]. *In vitro* studies of URG7 support an N_{out}/C_{in} topology [8,9]. The first 74 amino acid residues are identical to the N-terminal residues of the multidrug-resistance protein 6 (MRP6) [6]. The additional C-terminal 28 residues in the truncated MRP6 ($MRP6_{102}$) construct contain a second TM domain, making the protein to adopt an N_{out}/C_{out} topology. The 35 amino acid SP-C is synthesized as a 197 amino acid precursor, proSP-C and integrated in the ER membrane as a type II membrane protein [10]. SP-C functions together with phospholipids to lower the alveolar surface tension in the lungs, thus preventing the lungs from collapsing. The TM domain of SP-C contains mostly valine residues, which can be converted into a β-strand conformation and further to amyloid fibrils in patients with interstitial lung disease (ILD) having mutations in the proSP-C gene [11]. Interestingly, the substitution of valines to leucines abolishes aggregation completely [12,13].

By taking advantage of gGFP's selective loss of fluorescence upon N-linked glycosylation in the ER lumen, the membrane topology of URG7, $MRP6_{102}$, SP-C(Leu/Val) was confirmed *in vivo*. $MRP6_{102}$ and SP-C(Leu/Val) are inserted into the membrane with their C-terminus translocated to the ER lumen. A minor population of SP-C(Leu/Val) was untargeted and removed by proteasome dependent quality control system. URG7 is partially targeted to the ER, and the targeted URG7 is inserted with its N-terminus in the ER lumen.

2. Materials and methods

2.1. Plasmid construction

pEGFP-N1 plasmid (Clontech) was a template to construct SP-EGFP(WT), EGFP(E173T), SP-EGFP(E173T), EGFP(N147T/E173T) or SP-EGFP(N147T/E173T) using a site-directed mutagenesis kit (Toyobo) following the manufacturer's protocol. For the ER targeting and retention, a signal peptide of yeast invertase, MLLQAF LFLLAGFAAKISAS, was added at the N-terminus and the ER retention signal, KDEL at the C-terminus of EGFP variants. For construction of plasmids encoding gGFP fusion proteins, gGFP was amplified from pEGFP-N1 encoding EGFP(N147T/E173T) and were tagged to the C-terminus of LepH3 proteins, URG7, $MRP6_{102}$, SP-C(Leu) and SP-C(Val) by overlap PCR. The stitched PCR products were cloned into pcDNA3.1 (Invitrogen) by a standard cloning procedure with restriction enzymes and ligases (Taraka). In order to introduce the N5 glycosylation acceptor site in the SP-C sequence, MDVGSKEVLM was substituted to MDVGNKTVLM with the Asn of

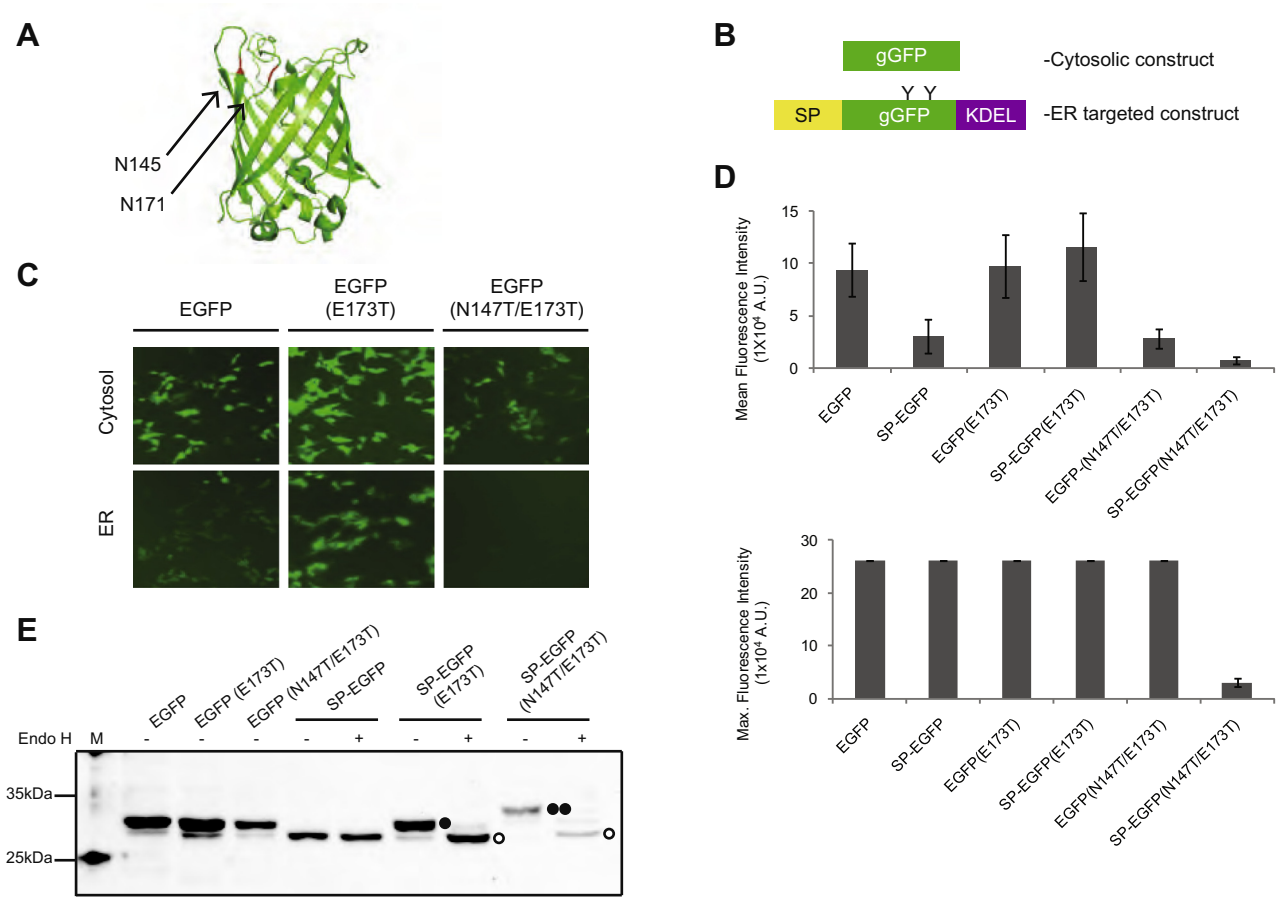

Fig. 1. Glycosylatable GFP for mammalian cells. (A) The structure of EGFP (PDB 2Y0G) [20] with engineered glycosylation sites, N145 and N171, marked in red. (B) Glycosylatable GFP constructs. SP denotes a cleavable signal peptide of an invertase and KDEL is the ER retention sequence. The engineered glycosylation sites are labelled with Ys. (C) Fluorescence microscopy of HEK-293T cells expressing EGFP(WT), EGFP(E173T), EGFP(N147T/E173T) in the cytosol and SP-EGFP, SP-EGFP(E173T) or SP-EGFP(N147T/E173T) in the ER. Cells were viewed with a GFP filter for GFP fluorescence after 24 h of transfection under Juli cell imager. (D) The cells were analyzed by FACS Canto. Mean (top) and maximum (bottom) fluorescence of EGFP(WT), EGFP(E173T), EGFP(N147T/E173T), SP-EGFP, SP-EGFP(E173T) or SP-EGFP(N147T/E173T) are plotted with standard deviations. (E) SP-EGFP(N147T/E173T) is efficiently glycosylated. Whole-cell lysates prepared from HEK-293 cells expressing EGFP(WT), EGFP(E173T), EGFP(N147T/E173T), SP-EGFP, SP-EGFP(E173T) or SP-EGFP(N147T/E173T) were subjected to Endo H digestion, SDS–PAGE and Western blot analysis. ●● Denotes doubly glycosylated, ● singly glycosylated and ○ non-glycosylated form. M marks the standard molecular weight marker loaded for analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the sequence Asn-Lys-Thr at position 5. Site-specific mutagenesis was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) and the mutants were confirmed by sequencing at Eurofins MWG Operon.

2.2. Mammalian cell culture and transfection

HeLa or HEK-293T cells were grown in medium (10% FBS in DMEM with antibiotics) at 37 °C with 5% CO_2. Cells were transiently transfected with plasmids encoding either gGFP or gGFP fusion constructs using Attractene (Qiagen) following the manufacturer's protocol.

2.3. Protein preparation and Western blot analysis

Lysates of HEK-293T expressing either gGFPs or gGFP fusion proteins were prepared using lysis buffer (1% NP-40 in 1× PBS with Protease Inhibitors). Endoglycosidase H (Endo H) treatment (Roche), SDS–PAGE and Western blot analysis were performed as described previously [4]. HRP conjugated GFP antibody (Rockland) was used to detect GFP and GFP fusion proteins (1:5000).

2.4. Fluorescence microscopy

Transiently transfected cells were assessed under JuLi fluorescence cell imager (Digital Bio) for fluorescence measurement. Where indicated, cells were cultured on a cover glass for direct imaging on Axioimager A1.

2.5. FACS analysis

Transiently transfected cells were washed with 1× PBS and collected by trypsin–EDTA treatment. The collected cells were resuspended in 3.7% formaldehyde in 1× PBS and analyzed with FACS Canto (BD).

2.6. Proteasome inhibition assay

Six hours after transfection, the proteasome inhibitor, MG132 was added to the cells together with fresh media at a concentration of 10 μM in DMSO. Equal amount of DMSO was added to control populations.

3. Results

3.1. Development of glycosylatable green fluorescent protein (gGFP) in mammalian cells

To test the applicability of yeast gGFP in mammalian cells, yeast gGFP was cloned into pcDNA3.1 and transfected into HeLa cells. However, yeast gGFP was not expressed in HeLa cells, which may have been due to the differences in codon usage between yeast and mammalian systems.

To overcome problems in gGFP expression in HeLa cells, pEGFP-N1 plasmid encoding mammalian EGFP was used to introduce a glycosylation site by engineering an E173T substitution (equivalent to E172T in yEGFP, which was reported to abolish fluorescence upon N-linked glycosylation) (Fig. 1A) [4]. Mammalian EGFP contains an additional valine residue at position 2 compared to yEGFP, thus the residue number is one higher for mammalian EGFP. To check whether the engineered N-linked glycosylation site (N^{171}- I^{172}-T^{173}) is utilized in the ER lumen, the cleavable signal peptide (SP) of secreted yeast invertase was fused to EGFP variants at their N-terminus for translocation into the ER lumen. Previously, SP of yeast invertase was shown to be functional in mammalian cells [14]. In addition, the ER retention signal, KDEL sequence,

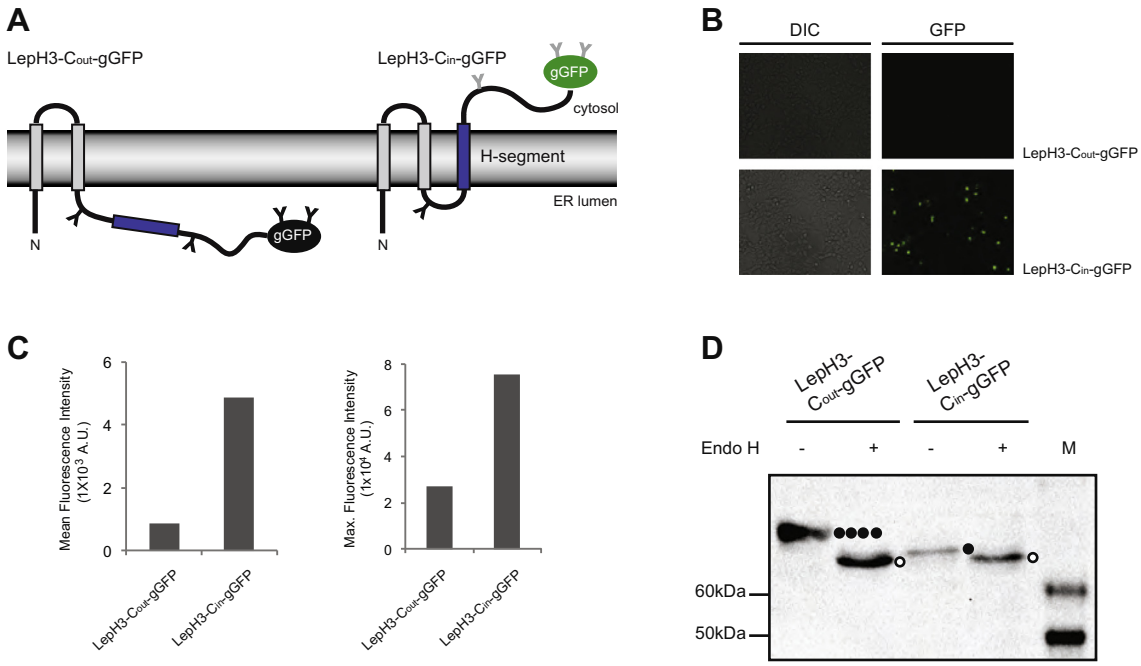

Fig. 2. Glycosylatable GFP fused to model membrane proteins. (A) Schematics of LepH3-C_{out}-gGFP and LepH3-C_{in}-gGFP in the ER membrane. Glycosylation sites are marked with Ys. Utilized glycosylation sites are colored in black and unutilized sites are colored in grey. (B) Fluorescence microscopy of HEK-293T cells expressing Lep-H3-C_{out}-gGFP and Lep-H3-C_{in}-gGFP. Cells were viewed with a GFP filter for GFP fluorescence after 24 h of transfection under Juli cell imager. Brightness and contrast were adjusted for clearer pictures. (C) Mean (left) and maximum (right) fluorescence of LepH3-C_{out}-gGFP and LepH3-C_{in}-gGFP. The cells were analyzed by FACS Canto and the average of two independent measurements is plotted. (D) Whole-cell lysates prepared from HEK-293T cells expressing LepH3-C_{out}-gGFP or LepH3-C_{in}-gGFP were subjected to Endo H digestion, SDS–PAGE and Western blot analysis. ●●●● Denotes quadruply glycosylated (C_{out}), ● singly glycosylated (C_{in}) and ○ non-glycosylated form. M marks the standard molecular weight marker loaded for analysis.

was added to the C-terminus to prevent a gGFP fusion protein from being secreted [15] (Fig. 1B). The plasmid encoding EGFP(WT) or EGFP(E173T) with or without the SP was transfected into HEK-293T cells and their fluorescence pattern was examined by fluorescence microscopy and FACS analysis. Both EGFP(WT) and EGFP(E173T) exhibited fluorescence in the cytosol (Fig. 1C and D). The fluorescence from EGFP(E173T) was more intense compared to that of the wild type EGFP. SP-EGFP(E173T) fluoresced in the ER lumen, indicating that the E173T mutation alone did not abolish fluorescence even though being glycosylated (Fig. 1E).

Recently, another version of glycosylatable GFP was developed for clinical use [16]. This version of GFP carries an engineered N-linked glycosylation site at position 145. Once this site is glycosylated, GFP loses fluorescence in mammalian cells. Therefore, we prepared EGFP(WT) with two N-linked glycosylation sites, N145 and N171, by introducing an N147T substitution in EGFP(E173T) (Fig. 1A). With two N-linked glycosylation sites in the EGFP sequence, the size difference between glycosylated and unglycosylated EGFP would be more prominent, thus enhance the applicability as a potential membrane topology reporter when fused to larger membrane proteins. The protein EGFP(N147T/E173T) was tested for fluorescence and glycosylation patterns. EGFP(N147T/E173T) exhibited fluorescence in the cytosol whereas its ER version, SP-EGFP(N147T/E173T), showed no fluorescence under a fluorescence microscopy (Fig. 1C). FACS analysis offered more detailed fluorescence measurements of EGFP variants (Fig. 1D). Mean fluorescence was reduced in SP-EGFP(N147T/E173T) compared to the cytosolic version but we noticed a significant difference in maximum fluorescence between the cytosolic and the ER version of EGFP(N147T/E173T), as well as between detectable and non-detectable fluorescent EGFP variants under fluorescence microscopy (Fig. 1C and D). While mean fluorescence may differ depending on protein concentration, maximum fluorescence may more accurately indicate changes in fluorescence intensity due to N-linked glycosylation, thus more reliable to assess glycosylation-dependent fluorescence changes of gGFP. Endo H digestion of whole-cell lysates showed that SP-EGFP(N147T/E173T) was efficiently glycosylated in the ER lumen (Fig. 1E). As EGFP(N147T/E173T) exhibits the characteristics of yeast gGFP whose fluorescence is selectively lost only upon glycosylation in the ER lumen, we refer to EGFP(N147T/E173T) as a mammalian gGFP.

3.2. The gGFP is an efficient membrane topology reporter in mammalian cells

To validate the applicability of mammalian gGFP as a membrane protein topology reporter, gGFP was fused to a set of model membrane proteins derived from *Escherichia coli* Leader peptidase (Lep) of known membrane topology. The derived Lep variant (LepH3) contains 3 TM domains, where the last TM domain is the test segment of varying hydrophobicity made of leucines and alanines [17]. It has N-linked acceptor at two sites; in loop 2 and in the C-terminus (Fig. 2A). LepH3 with 3 leucines and 16 alanines in the test segment (LepH3-C_{out}) is expected to have its C-terminus translocated to the ER lumen whereas LepH3 with 19 leucines (LepH3-C_{in}) is expected to have its test segment inserted into the membrane, thereby leaving its C-terminus in the cytosol. The gGFP was fused to the C-terminus of LepH3-C_{out} and LepH3-C_{in}, then expressed in HEK-293T cells. When the fusion proteins were

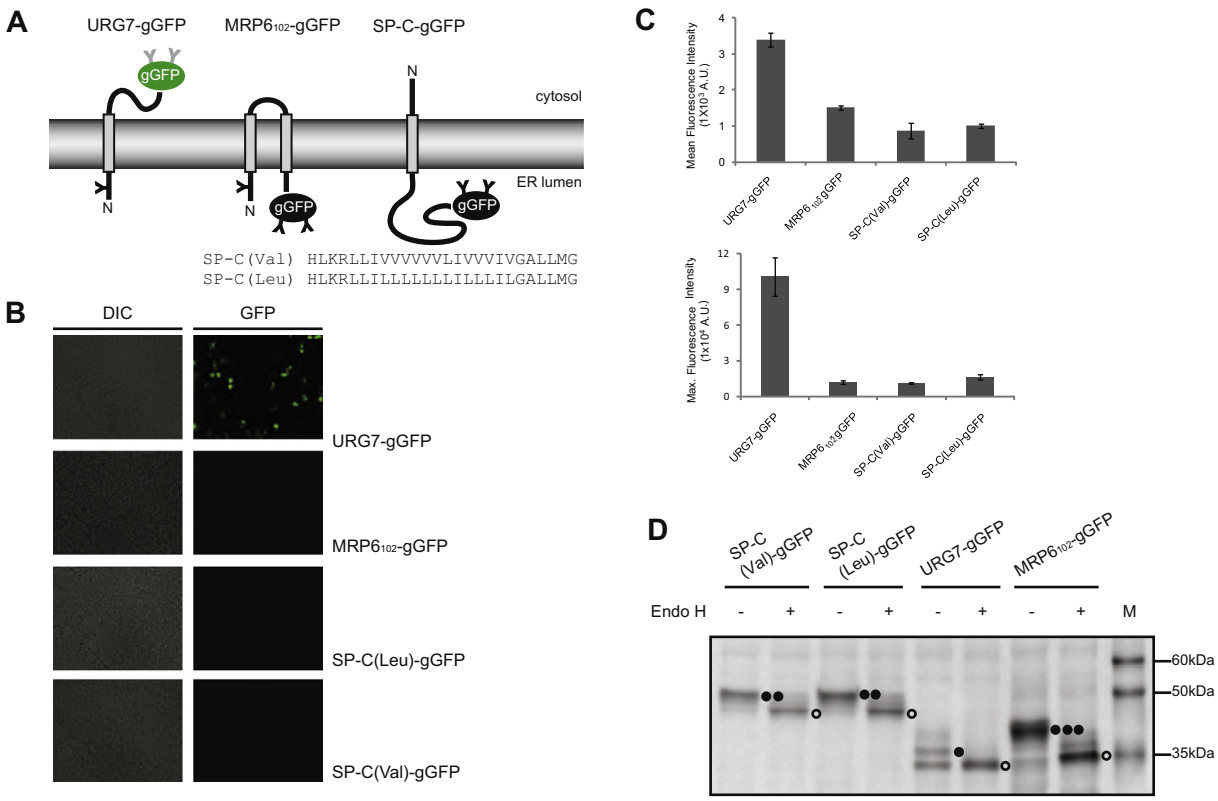

Fig. 3. *In vivo* topology assessment of URG7, $MRP6_{102}$, SP-C(Val) and SP-C(Leu). (A) Schematics of URG7, $MRP6_{102}$, SP-C(Val) and SP-C(Leu) with gGFP tag in the ER membrane. The transmembrane segment sequences of SP-C(Val) and SP-C(Leu) are shown. (B) Fluorescence microscopy of HEK-293T cells expressing URG7-gGFP, $MRP6_{102}$-gGFP, SP-C(Leu)-gGFP and SP-C(Val)-gGFP. Cells were viewed as described in Fig. 2B. (C) Mean (top) and maximum (bottom) fluorescence of URG7, $MRP6_{102}$, SP-C(Val) and SP-C(Leu) with gGFP. The cells were analyzed by FACS Canto and the average of three independent measurements plus the standard deviations is shown. (D) Whole-cell lysates prepared from HEK-293 cells expressing URG7, $MRP6_{102}$, SP-C(Val) and SP-C(Leu) with gGFP were subjected to Endo H digestion, SDS–PAGE and Western blot analysis. ●●● Denotes triply glycosylated form of $MRP6_{102}$-gGFP, ●● doubly glycosylated form of SP-C(Leu/Val)-gGFP, ● singly glycosylated form of URG7-gGFP and ○ non-glycosylated form (N_{out}/C_{in}). M marks the standard molecular weight marker loaded for analysis.

expressed in HEK-293T cells, LepH3-C_{in}-gGFP was fluorescent whereas LepH3-C_{out}-gGFP was not (Fig. 2B and C). Endo H digestion of lysates confirmed that the C-terminus of LepH3-C_{in}-gGFP remained in the cytosol whereas that of LepH3-C_{out}-gGFP was translocated to the ER lumen (Fig. 2D). In sum, these results demonstrate that mammalian gGFP is a convenient *in vivo* membrane topology reporter.

3.3. Assessment of URG7, $MRP6_{102}$, SP-C(Val) and SP-C(Leu) membrane topologies with gGFP

Mammalian gGFP was tagged to the C-terminus of URG7, $MRP6_{102}$, SP-C(Val) and SP-C(Leu) to assay their orientation in HEK-293T cells (Fig. 3A). Three out of four test proteins, $MRP6_{102}$, SP-C(Val) and SP-C(Leu), exhibited no fluorescence (Fig. 3B and C). Endo H digestion analysis revealed that the proteins were efficiently glycosylated, thereby assaying the fusion joint (the localization of gGFP) to the ER lumen (Fig. 3D). In contrast, URG7 exhibited fluorescence, suggesting that its C-terminus is located in the cytosol (Fig. 3B and C). The C-terminus of URG7 can reside in the cytosol in two different forms, untargeted and membrane inserted form in an N_{out}/C_{in} orientation. Endo H digestion of URG7 lysate showed that both glycosylated and unglycosylated forms are present *in vivo* (Fig. 3D). Hence, it is concluded that URG7 targeting *in vivo* is not efficient, leaving about 50% in the cytosol, but once targeted, it is oriented with an N_{out}/C_{in} orientation.

3.4. SP-C(Val) and SP-C(Leu) topogenesis

In contrast to our gGFP study of SP-C where the protein is inserted in an N_{in}/C_{out} form, the *in vitro* topology study with microsomes revealed that SP-C is embedded in the membrane in two different orientations (unpublished data). To test whether the discrepancy between the results from two systems arises from the presence or absence of the quality control system, HEK-293T cells were treated with proteasome inhibitor MG132. While the majority of SP-C was inserted into the membrane in an N_{in}/C_{out} form, the unglycosylated SP-C was also detected in the presence of MG132 (Fig. 4A). These results suggest that SP-C is either inserted in two different membrane orientations or some were not targeted to the membrane, and the cells remove the incorrectly inserted, N_{out}/C_{in} form, or an untargeted form by the proteasome *in vivo*. To distinguish these two possibilities, an additional N-linked glycosylation site was engineered at the N-terminus of SP-C and the protein was expressed in HEK-293T cells. If the unglycosylated SP-C was incorrectly inserted as N_{out}/C_{in} form, with an N-linked glycosylation site at the N-terminus, it would be glycosylated. However, unglycosylated product was still detected, thus suggesting that a small fraction of SP-C is untargeted *in vivo* and removed by the proteasome (Fig. 4A and B).

4. Discussion

We have demonstrated that a glycosylatable GFP (gGFP) is suitable for assaying membrane protein topologies in mammalian cells *in vivo*. The gGFP fused to a C-terminus of a protein is fluorescent and not glycosylated when located in the cytosol, but glycosylated upon translocation into the ER lumen and loses its fluorescence. The distinct fluorescence and glycosylation characteristics of the gGFP allow an easy live cell assessment of membrane protein topology. Once membrane proteins are tagged with gGFP, fluorescence measurements after transfection and Western blotting allow unambiguous assessment of the membrane protein topology.

Using gGFP, we have confirmed the topology of URG7, $MRP6_{102}$, SP-C(Val) and SP-C(Leu), disease associated proteins *in vivo*. Interestingly, there was a discrepancy between *in vitro* and *in vivo* topology studies on SP-C. SP-C was inserted with dual topology in microsomes, but it was inserted only in N_{out}/C_{in} form *in vivo*.

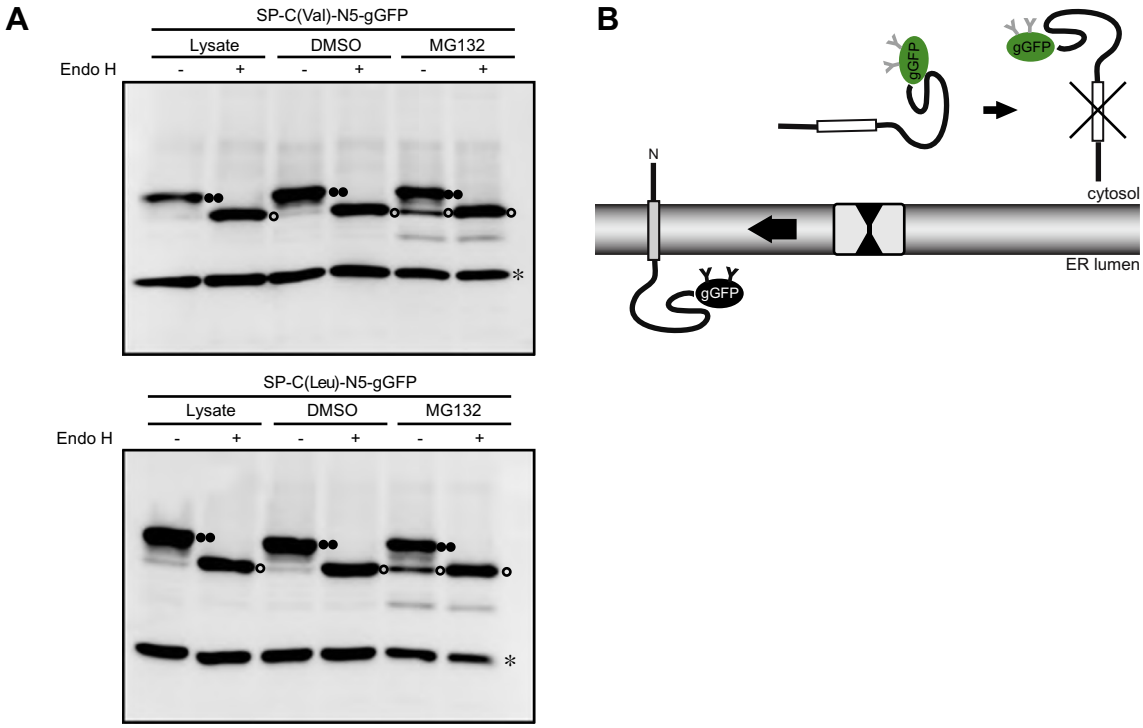

Fig. 4. SP-C is inserted into the membrane in N_{in}/C_{out} orientation *in vivo* upon targeting to the membrane. (A) Whole-cell lysates prepared from HEK-293T cells expressing SP-C(Leu/Val)-N5-gGFP in the presence or absence of MG132 were subjected to Endo H digestion, SDS–PAGE and Western blot analysis. SP-C(Leu/Val)-N5-gGFP contains an additional glycosylation site at the N-terminus compared to SP-C(Leu/Val)-gGFP used in Fig. 3. for detection of proteins in N_{out}/C_{in} form. ●● Denotes a doubly glycosylated form (N_{in}/C_{out}) and ○ a non-glycosylated form (Untargeted). * Denotes an unspecific band. (B) Schematic representation of SP-C biogenesis. Untargeted SP-C is degraded via the proteasome dependent pathway *in vivo* leaving only the N_{in}/C_{out} form in the membrane.

In sum, a mammalian glycosylatable GFP developed in this study can be implemented to other model organisms for membrane topology studies. Further, similar to the proteomics studies done in *E. coli* [18] and *Saccharomyces cerevisiae* [19] this method can be used for global membrane topology mapping in mammalian cells.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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