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Glycosylatable GFP as a compartment-specific membrane topology reporter

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ABSTRACT

Determination of the membrane topology is an essential step in structural and functional studies of integral membrane proteins, yet the choices of membrane topology reporters are limited and the experimental analysis can be laborious, especially in eukaryotic cells. Here, we present a robust membrane topology reporter, glycosylatable green fluorescent protein (gGFP). gGFP is fully fluorescent in the yeast cytosol but becomes glycosylated and does not fluoresce in the lumen of the endoplasmic reticulum (ER). Thus, by assaying fluorescence and the glycosylation status of C-terminal fusions of gGFP to target membrane proteins in whole-cell lysates, the localization of the gGFP moiety (and hence the fusion joint) relative to the ER membrane can be unambiguously determined.

1. Introduction

The topology of a membrane protein – i.e. a two-dimensional model showing the location of the transmembrane (TM) segments and the overall orientation of the protein relative to the membrane – is a basic characteristic that helps guide structure-function studies and provides a necessary starting point for *de novo* structure modelling.

While current topology prediction programs perform quite well [1–4], the combination of even limited experimental data (knowledge of the location of the protein's C terminus relative to the membrane) and bioinformatics prediction greatly improves the reliability of the resulting membrane topology models [5–8]. Even in cases where 3-dimensional structures are available, the structural information does not reveal how these proteins are orientated in the native membrane since they are determined as a membrane solubilized form. Thus, membrane topology still has to be determined by biochemical and/or cell biological experiments. Therefore, efficient experimental tools to determine the topology of a membrane protein are indispensable in the membrane biology toolkit.

Commonly available approaches to determine membrane protein topology include protease protection, antibody/epitope accessibility assays, and reporter protein fusions [9–12]. Most of these methods allow positive identification of soluble loops that are exposed to one side of the membrane only (e.g. cytosol or ER lumen), thus two different experiments have to be carried out to obtain conclusive results. In yeast, a dual-function membrane topology reporter composed of invertase (a protein that contains numerous acceptor sites for N-linked glycosylation) and the catalytic domain of histidinol dehydrogenase has been developed and used successfully [5,7,13]. However, the size of this reporter is very large (~125 kDa) and its usage is limited to yeast.

In an effort to develop a more robust membrane topology reporter for eukaryotic cells, we have engineered a GFP with a strategically placed N-linked glycosylation acceptor site (gGFP). gGFP exhibits high levels of fluorescence when localized in the cytosol. In contrast, gGFP is glycosylated and non-fluorescent when localized in the ER lumen. When fused to the C terminus of a membrane protein of interest, gGFP serves as a positive reporter both for a lumenal localization (glycosylated, non-fluorescent) and for a cytosolic localization (non-glycosylated, fluorescent) of the fusion joint. We have validated the use of gGFP as a topology reporter by fusing it to different membrane proteins of known topology.

Compared to the invertase-histidinol dehydrogenase dual-function reporter, gGFP has the advantages of relatively small size and easy detection by fluorescence in whole cells or whole-cell lysates. gGFP (or variants thereof) further has the potential to be applicable to other eukaryotic cells beyond yeast.

2. Materials and methods

2.1. Plasmid construction

We initially constructed a plasmid carrying genes of *Escherichia coli LepH2-HA* [14] and *yEGFP* [15] by overlap PCR [16]. In the first

Abbreviations: GFP, green fluorescent protein; ER, endoplasmic reticulum; TM, transmembrane.

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round of PCR, two reactions, one to amplify the sequences carrying *LepH2-HA* and the other to amplify *yEGFP* with the 5′ overhang nucleotides complementing the end of the HA sequence, were set up. One reaction was carried out using the construct carrying *E. coli LepH2-HA* [14] as a PCR template and primers A and B. Primer A (5′TCGACGGATTCTAGAACTAGTGGATCCCCCATGGCGAATTCCACCAGC3′) contains the homologous recombination sequence to the vector, p424GPD [17], and the start of the *LepH2-HA* gene (underlined). Primer B (5′AACACCAGTGAATAATTCTTCACCTTTAGAATTACATAGCTCGAGGAG3′) contains the end of the *LepH2-HA* sequence excluding the stop codon and the *yEGFP* sequence except the initiation Met codon. In a parallel reaction, pDD1-2 [18] was used as a PCR template with primers C and D. The primer C (5′CCAGATTACGCTCTCCTCGAGTCATGTAATTCTAAAGGTGAAGA3′) contains the complementary sequence of primer B, and primer D (5′ATCGATAAGCTTGATATCGAATTCCTGCAGTTTGTACAATTCATCCAT3′) contains the end of *yEGFP* sequence (underlined) and the homologous recombination sequence to p424GPD [17]. After checking the correct sizes of the two PCR fragments (~1100 bp of *LepH2-HA* and ~700 bp of *yEGFP*), the second round of the PCR was carried out using the first two PCR reactions as templates and primers A and D. The correct size of *LepH2-HA-yEGFP* recombinant gene (~1800 bp) was confirmed by agarose gel electrophoresis. For plasmid construction by homologous recombination, this PCR fragment was transformed into a *Saccharomyces cerevisiae* strain, W303-1α (*MAT α, ade2, can1, his3, leu2, trp1, ura3*) together with *Sma I*-linearized p424GPD. Yeast transformants were selected on −Trp plates, plasmids were isolated, and the gene coding sequence was confirmed by DNA sequencing. The sequence was correct except that the stop codon at the end of the *yEGFP* was missing. We found out that primer D did not contain the stop codon of *yEGFP*. Nevertheless, this plasmid (designated as pJM1) was used to construct the following plasmids as it contained the correct *HA-yEGFP* sequence.

To construct the plasmid carrying the genes encoding *HA-yEGFP*, pJM1 was used as a PCR template with primers E (5′GTTTCGACGGATTCTAGAACTAGTGGATCCATGCCATCTTACCCATACGATG3′) which contains the initiation Met codon for translation of *HA* (underlined), and F (5′ATCGATAAGCTTGATATCGAATTCCTGCAGTTATTTGTACAATTCATCCAT3′) that includes the stop codon at the end of the *yEGFP* sequence (underlined). The PCR fragment was then transformed into W303-1α strain, selected on −Trp plate, plasmids were isolated and sequenced. The correct sequence was confirmed and the plasmid was named as pHL1. For construction of a plasmid carrying the gene encoding an ER version of *HA-yEGFP*, a signal peptide of *SUC2* was amplified by PCR using a vector carrying the genes of the *SP-Lep-H-segment* [19] and primers G (5′GTTTCGACGGATTCTAGAACTAGTGGATCCATGCTTTTGCAAGCTTTC3′) that contains the start of the *SUC2* signal peptide (underlined) and H (5′CATCGTATGGGTAAGATGGCATTGATGCAGATATTTTGGC3′) that contains the end of the *SUC2* signal peptide and the overhang complementing the start of the *HA-yEGFP* sequence. The PCR fragment was subcloned into p424GPD vector by homologous recombination as described above, and the correct sequences were confirmed by DNA sequencing, and the plasmid was named as pHL2.

Following constructs were prepared by overlap PCR and were subcloned by yeast homologous recombination as described above. The PCR fragment of *HA-GFP(I123T)* was obtained using pHL1 as a

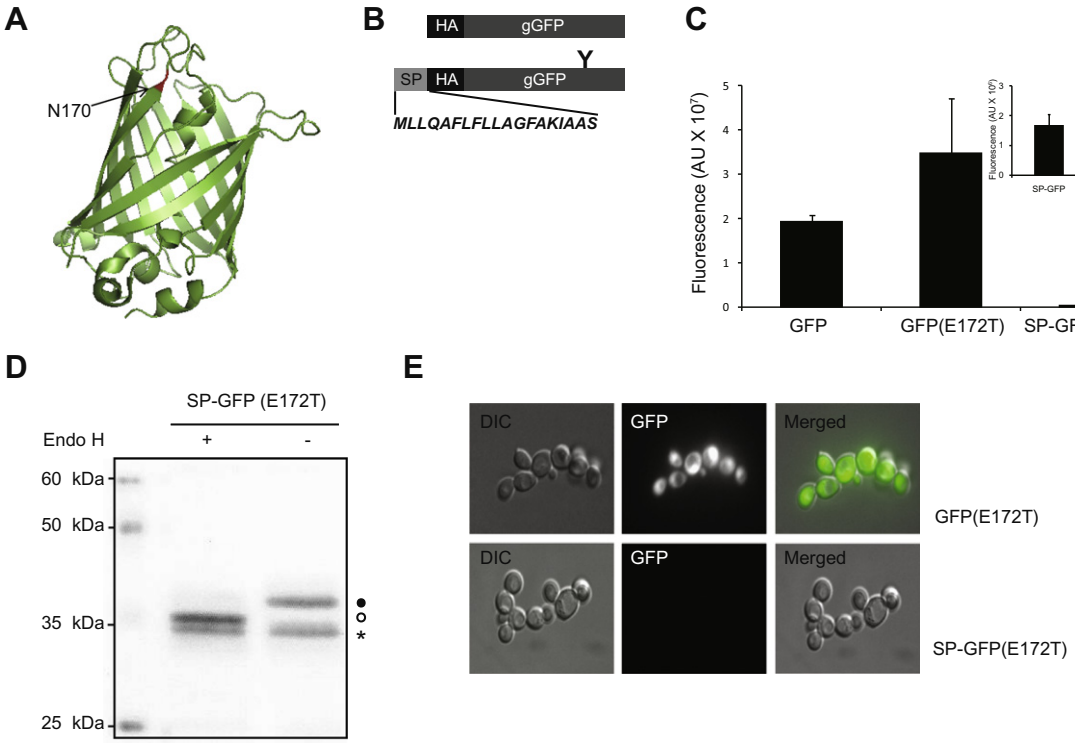

Fig. 1. Engineering a glycosylatable GFP (gGFP). (A) Structure of yEGFP (PDB 3AI4) [23]. The red label indicates the Asn (N170) residue that becomes glycosylated in gGFP. (B) Schematic representation of GFP constructs. The glycosylation site is labeled as Y. HA denotes a triple hemagglutinin tag. The sequence of the invertase signal peptide (SP) is shown. (C) Fluorescence measurements. Whole-cell lysates were prepared from yeast transformants expressing GFP, GFP(E172T), SP-GFP or SP-GFP(E172T). Fluorescence was measured with a 460 nm excitation and 535 nm cut-off filter, and relative fluorescence units were normalized by subtracting the values from the whole-cell lysate of a yeast transformant carrying an empty vector. Averages of at least three independent measurements are shown with standard errors. In the inset, GFP fluorescence of SP-GFP and SP-GFP(E172T) is compared. (D) SP-GFP(E172T) is glycosylated. Whole-cell lysates prepared from yeast transformants expressing GFP(E172T) or SP-GFP(E172T) were subjected to Endo H digestion, SDS–PAGE, and Western blot analysis. ● denotes a glycosylated form, ○ denotes a non-glycosylated form. *indicates an uncharacterized background band. (E) Fluorescence microscopy of yeast cells expressing GFP(E172T) or SP-GFP(E172T). Cells were viewed with DIC and GFP filters, and the images were merged.

PCR template and primers A, I (5′TTTTAATTCGGTTCTATTAA CTAAGGTATC3′), J (5′GTTAATAGAACCGAATTAAAAGGTATTG3′) and F. Similarly, the PCR fragment of the *HA-GFP(E172T)* was obtained using pHL1 as a PCR template and primers A, K (5′AGA-ACCATCTGTAATGTTGTGTCTAATTTTG3′), L (5′CACAACATTACAGAT GGTTCTGTTCAATTAG3′) and F. For the E172T mutation in the ER version of *HA-yEGFP* (*SP-HA-GFP(E172T)*), pHL2 as a PCR template and primers A, K, L and F were used.

For construction of plasmids carrying the gene coding sequences of *Lep-HA-GFP(E172T)*, the *E. coli* Lep sequences were subcloned using plasmids used in [14] and primers A, p183FOR (ACCTACTCAAACGTGGAACCGAGC), p183REV (GCTCGGTTCCAC GTTTGAGTAGGT) and F. The Lep-C_{in} construct contains the P2 sequence (GGPGDKQEGEWPTGLRLSRIGGIGPGG) in place of the second hydrophobic segment (H2 segment) of *E. coli* Lep protein whereas the Lep-C_{out} construct contains the [6L/13A] sequence (GGPGAAAALALALALALALAAAAGPGG) in place of the H2 segment [14].

HA-GFP(E172T) with an additional glycosylation site was made using a site-directed mutagenesis kit (Toyobo) following the manufacturer's protocol.

2.2. Protein preparation and Western blot analysis

Yeast transformants expressing gGFP fusion constructs were grown overnight at 30 °C in 5 ml of −Trp medium. Whole-cell lysates were prepared as previously described [5]. Lysates were incubated with or without endoglycosidase H (Roche) for 2 h at 37 °C to remove N-linked oligosaccharides. SDS–PAGE and Western blot analysis with an anti-HA antibody were followed. Western blots were developed with Amersham Bioscience Advanced ECL kit on a Biorad Chemi-doc-XRS+ system (Biorad).

2.3. Fluorescence measurements

Yeast transformants expressing gGFP fusion constructs were grown in 10 ml of −Trp medium at 30 °C overnight. Cells were harvested at 0.3–0.6 OD_{600} by centrifugation at 3,000g and resuspended in 200 µl of YSB buffer (50 mM Tris–HCl, pH 7.6, 5 mM EDTA, pH 8, 10% (w/v) Glycerol and 1× Protease Inhibitor Cocktail). Cells were transferred to a 96-well microplate (Nunc) and the fluorescence measurements were taken on Perkin Elmer Envision 2102 Multilabel reader with excitation band pass filter at 460 nm and cut-off FITC filter at 535 nm. All fluorescence measurements were subtracted by fluorescence from whole-cell lysates of yeast transformants carrying an empty vector. Averages of at least three independent measurements were plotted with standard errors.

2.4. Fluorescence microscopy

Yeast transformants expressing gGFP fusion constructs were grown overnight in 5 ml of −Trp medium at 30 °C. 100 µl of cells were taken from 0.7 OD_{600} culture. Cells were transferred to a 96 well plate for fluorescence assessment using a Zeiss Axiovert 200 M inverted microscope with a Plan-NeoFluar 100×/1.30 NA oil-immersion objective lens. Fluorescence images were taken as described in [20] using a standard fluoresceinisothiocyanate (FITC) filter set (excitation band pass filter, 450–490 nm; beam splitter, 510 nm; emission band pass filter, 515–565 nm). The pictures were taken with an exposure time of 0.2 ms.

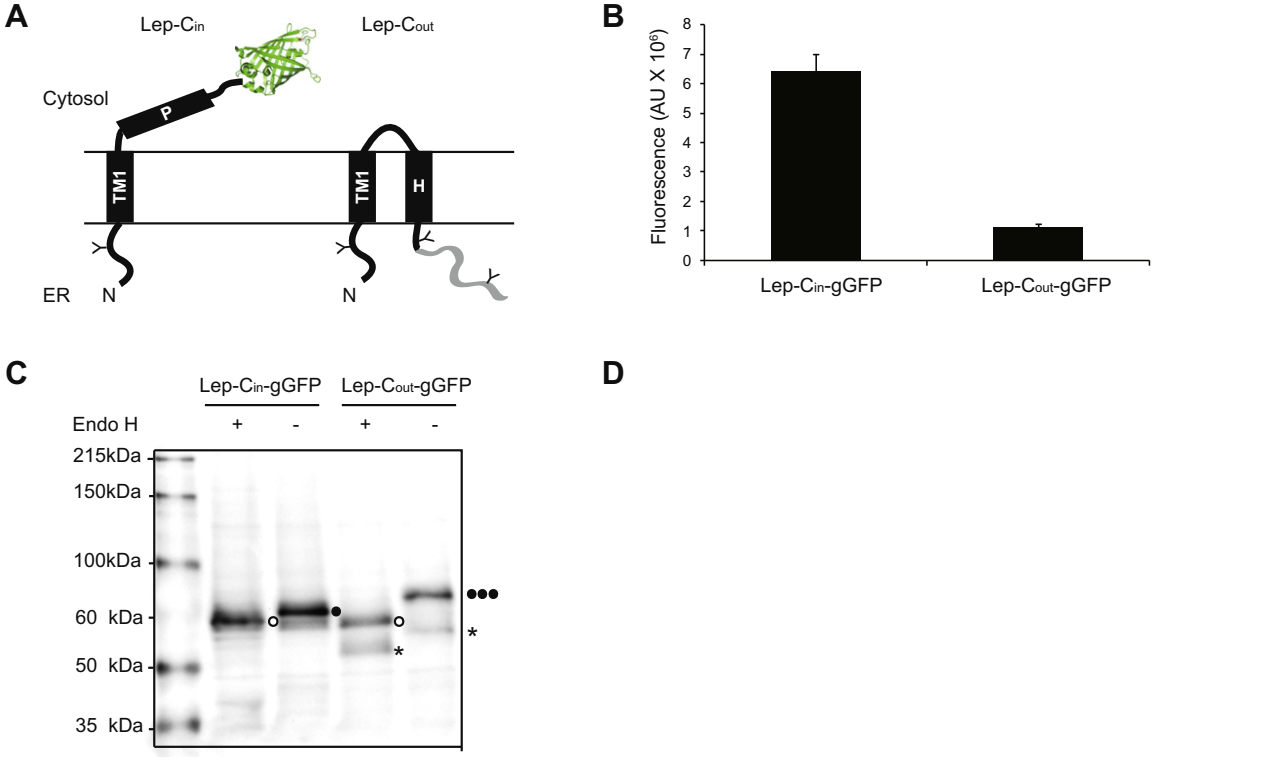

Fig. 2. gGFP can be used as a membrane topology reporter. (A) Schematic representation of model proteins, Lep-C_{in} and Lep-C_{out} [14]. The glycosylated sites are labeled as Y. TM1 indicates TM segment 1 of *E.coli* Lep [22], P stands for a polar segment, and H stands for a hydrophobic segment. (B) Fluorescence measurements. Whole-cell lysates were prepared from yeast transformants expressing Lep-C_{in}-gGFP or Lep-C_{out}-gGFP. Fluorescence was measured as in Fig. 1C. (C) Endo H digestion analysis of yeast transformants expressing Lep-C_{in}-gGFP or Lep-C_{out}-gGFP. ●●● indicates a triply glycosylated form, ● indicates a singly glycosylated form, ○ indicates a non-glycosylated form, and *indicates a cleaved product.

3. Results

3.1. Engineering of a glycosylatable gGFP

The rationale behind our design of gGFP was the assumption that the presence of an N-linked glycan near the GFP fluorophore might interfere with protein folding and maturation of the fluorophore, rendering the protein non-fluorescent. Based on the X-ray structure of GFP [21], we initially targeted two Asn residues at positions 121 and 170, near the fluorophore (Fig. 1A). To generate an N-linked glycosylation acceptor site, Asn-X-Thr/Ser-Y (where X, Y can be any amino acid except proline), Ile^{123} and Glu^{172}, two residues downstream of the Asn residues at positions 121 and 170, were individually changed to Thr in yeast enhanced GFP (yEGFP) [15]. To facilitate detection by Western blotting and immunofluorescence, a hemagglutinin (HA) tag was introduced directly upstream of GFP (Fig. 1B). We found that GFP fluorescence was abolished when Ile^{123} was changed to Thr (data not shown), and this mutant was therefore not further studied.

To obtain a version of gGFP that is localized to the lumen of the ER, the signal peptide (SP) from invertase was fused to the N terminus of GFP (Fig. 1B). Three constructs each carrying *GFP*, *GFP(E172T)* or *SP-GFP(E172T)* were transformed into the *S. cerevisiae* strain W303-1α. Whole-cell lysates were prepared and fluorescence was measured. While fluorescence of GFP(E172T) was comparable to that of wildtype GFP, fluorescence of SP-GFP(E172T) was not significantly different from the whole-cell lysate prepared from a yeast transformant carrying an empty vector (Fig. 1C). We noticed that the version of GFP used in the study, yEGFP [15], in itself has much lower fluorescence when localized to the lumen of the ER. When comparing the fluorescence of SP-GFP and SP-GFP(E172T), approximately a twofold reduction in fluorescence for SP-GFP(E172T) was observed (Fig. 1C, inset). Hence, the addition of the glycan moiety reduces the residual fluorescence of lumenally located SP-GFP(E172T) to background levels.

To further determine whether SP-GFP(E172T) was translocated to the lumen of the ER, whole-cell lysates were treated with endoglycosidase H (Endo H) for removal of N-glycans, followed by SDS–PAGE and Western blotting. The sample treated with Endo H showed faster migration on the Western blot, indicating that GFP(E172T) was glycosylated, and thus correctly translocated to the lumen of the ER (Fig. 1D). Next, fluorescence from the cells expressing either GFP(E172T) or SP-GFP(E172T) was assessed by fluorescence microscopy. Only the cells expressing the cytosolically localized GFP(E172T) exhibited a detectable fluorescence signal (Fig. 1E). These results demonstrate that GFP(E172T) is unglycosylated and fluorescent in the cytosol but is glycosylated and non-fluorescent when localized in the ER lumen. We call this version of GFP, glycosylatable GFP (gGFP).

3.2. gGFP is a robust topology reporter

To test whether gGFP can be used as a topology reporter, we fused it to the C terminus of two membrane protein constructs based on the Lep protein from *E. coli*, an inner membrane protein with two TM helices (TM1, TM2) near the N terminus [22]. In these

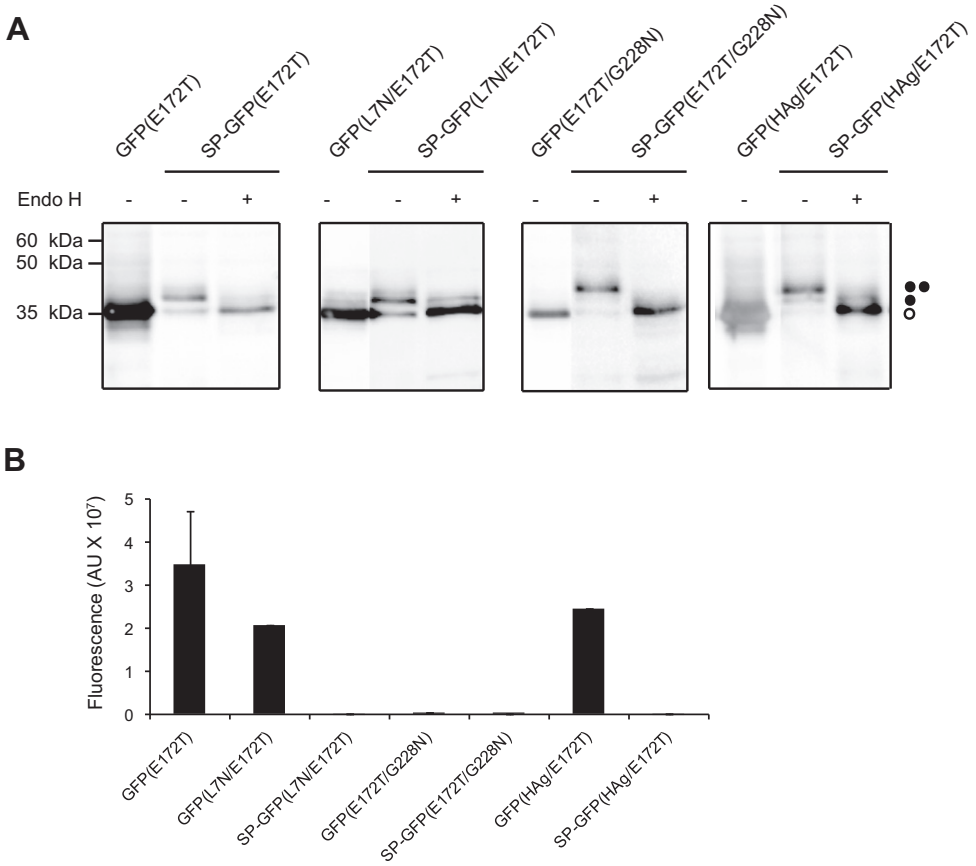

Fig. 3. Engineering an additional glycosylation site into gGFP. (A) gGFP with an additional glycosylation site can be doubly glycosylated. Endo H digestion analysis of yeast transformants expressing SP-GFP(E172T), SP-GFP(L7N/E172T), SP-GFP(E172T/G228N) or SP-GFP(HAg/E172T). GFP(E172T), GFP(L7N/E172T), GFP(E172T/G228N) and GFP(HAg/E172T) were loaded as controls for an unglycosylated gGFP. ●● indicates a doubly glycosylated form, ● indicates a singly glycosylated form, ○ indicates a non-glycosylated form. (B) Fluorescence measurements. Fluorescence was measured as described in Fig. 1C. Averages of two measurements are shown except for GFP(E172T). Average of three measurements is shown for GFP(E172T) as in Fig. 1C.

constructs, TM2 was replaced by two different 19-residue long segments; one composed of polar residues (P) that does not insert into the inner membrane (construct Lep-C_{in}-gGFP) and the other composed of a very hydrophobic [6 Leu/13Ala] segment (H) that is known to insert efficiently into the ER membrane (Lep-C_{out}-gGFP) [14] (Fig. 2A). Whole-cell lysates from yeast transformants expressing Lep-C_{in}-gGFP exhibited high fluorescence, whereas Lep-C_{out}-gGFP transformants showed only weak fluorescence (Fig. 2B).

Next, to assess the glycosylation status of the two constructs, whole-cell lysates from yeast transformants expressing either Lep-C_{in}-gGFP or Lep-C_{out}-gGFP were prepared and treated with Endo H. Two glycan acceptor sites are present in the Lep part, one in the lumenally located N-terminal tail and the other just downstream of the P or H segment, hence molecules with a single glycan (1G) have an N_{out}–C_{in} membrane topology, whereas triply glycosylated molecules (3G) have an N_{out}–C_{out} orientation (Fig. 2A). As expected, Lep-C_{in}-gGFP was singly glycosylated and Lep-C_{out}-gGFP was triply glycosylated (Fig. 2C). A cleaved and glycosylated form of Lep-C_{out}-gGFP was also detected. Previously, it was shown that this form is generated by signal peptidase cleavage in the [6Leu/13Ala] segment, releasing the C-terminal domain of the protein to the lumen [14]. These results demonstrate that gGFP fusion neither interferes with correct protein targeting nor with the membrane topology, thus can be used for topology mapping.

3.3. Additional glycosylation site in gGFP

For large membrane proteins, the difference in molecular weight between the presence or absence of a single N-linked glycan (~2 kDa) in gGFP might be too small to be detected on SDS-gels. To overcome this difficulty, an additional glycosylation site was engineered at positions 7 or 228 in gGFP, or in the HA tag. The positions of these sites were chosen such that they would least disrupt folding and maturation of the fluorophore. To test the efficiency of N-linked glycosylation at the three sites, whole-cell lysates of yeast transformants expressing GFP(L7N/E172T), GFP(E172T/G228N), GFP(HAg/E172T), SP-GFP(L7N/E172T), SP-GFP(E172T/G228N), or SP-GFP(HAg/E172T) were prepared and subjected to Endo H digestion. SP-GFP(E172T/G228N) and SP-GFP(HAg/E172T) were efficiently glycosylated on both sites (Fig. 3A). In comparison, Asn^7 in SP-GFP(L7N/E172T) did not get efficiently glycosylated as about equal amounts of singly and doubly glycosylated proteins were apparent. Whole-cell lysates from yeast transformants expressing these constructs were then tested for fluorescence. GFP(E172T/G228N) did not fluoresce, whereas GFP(L7N/E172T) and GFP(HAg/E172T) showed comparable levels of fluorescence as GFP(E172T) (Fig. 3B). Since the additional glycosylation site was efficiently glycosylated and the fluorescence pattern of gGFP was maintained in GFP(HAg/E172T), this version of gGFP can be used to assay the topology of membrane proteins of relatively large size.

4. Discussion

We have developed a glycosylatable version of GFP (gGFP) as a reporter for membrane topology and subcellular localization in the secretory pathway in eukaryotic cells. An N-linked glycosylation site was engineered close to the fluorophore in yEGFP. This change did not interfere with maturation of the fluorophore when gGFP was expressed in the cytosol, but led to efficient glycosylation and reduction of fluorescence when the protein was targeted to the ER. By fusing gGFP to a membrane protein with known membrane topology, we demonstrated that gGFP is a robust topology reporter. gGFP provides a dual-function topology reporter that can be easily analyzed in intact cells by fluorescence microscopy and by Western blotting and fluorescence measurements with whole-cell lysates. We also provide a version of gGFP with two engineered glycosylation acceptor sites that can be used to determine the topology of larger membrane proteins.

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