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GFP-nanobody-mCherry as a tool for identifying the topology of GFP-fused membrane proteins in intracellular organelles in cells

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Abstracts: A key characteristic of membrane proteins in various intracellular organelles is their topology. The topology of green fluorescent protein (GFP)-tagged transmembrane proteins in membranous organelles can be elucidated using various methods, including protease protection and fluorescence protease protection assays. However, there is still a lack of a simple method to identify topology without disrupting the membrane of the plasma membrane and intracellular organelles, including endoplasmic reticulum and mitochondria. In this study, we demonstrate that GFP nanobody (GNb) fused to mCherry (GNb–mCherry) can be used to identify the topology of GFP-fused membrane proteins in cells. We first demonstrate that cytosolically expressed GNb–mCherry can discriminate cytoplasmic or extracellular or luminal side localization of GFP in GFP-fused plasma membrane, endoplasmic reticulum, Trans-Golgi network, lysosome or mitochondria-targeting proteins. Next, using this assay, we could determine the topology of PRMT8(N20)-GFP in the plasma membrane and mitochondria. Overall, GNb–mCherry can be a useful tool for identifying the topology of GFP-fused membrane proteins in intracellular organelles in cells.

Key words: GFP nanobody, membrane protein, topology, live cell

Introduction

Membrane proteins play pivotal roles in numerous functions, including adhesion and signaling, as well as intracellular trafficking between different intracellular membranous organelles. Transmembrane proteins can be classified into three main types: Type I, Type II, and multi-spanning transmembrane proteins.[1]

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Type I membrane proteins, also known as single-pass transmembrane proteins (TMPs), have an extracellular (or luminal) N-terminus and a cytoplasmic C-terminus. In contrast, Type II TMPs exhibit the opposite orientation, with a cytoplasmic N-terminus and an extracellular (or luminal) C-terminus. Multi-spanning TMPs trespass through the membrane several times. Therefore, the topology of the transmembrane domain is a key determinant in the classification of transmembrane proteins. The green fluorescent protein (GFP) continues to be widely used as a reporter and nonperturbing tag.[2,3] It is believed that most GFP-tagged proteins retain their endogenous localization and function even in the case of membrane proteins.[4] Various methods are available to elucidate the topology of GFP-tagged transmembrane proteins in membranous organelles, including protease protection and fluorescence protease protection (FPP) assays.[5,6] Nevertheless, these assays require mechanical or chemical disruption of the plasma membrane. Regarding endoplasmic reticulum (ER)-targeting membrane proteins, redox-sensitive or glycosylatable GFP has been used to determine the topology of ER membrane proteins without membrane disruption in live cells.[7,8] Therefore, it would be extremely useful if the topology could be identified without membrane disruption in intracellular organelles other than ER.

An important step in achieving the abovementioned goal was the recent development of nanobodies that can detect GFP or RFP.[9] Several researchers have used GFP-targeting or RFP-targeting nanobodies (GNb or RNb) as a toolkit for immunoprecipitation, immunocytochemistry, and targeted proteasomal degradation, or as fluorescent sensors to visualize various ions and ATP/ADP dynamics.[10-13] Especially, Fridy *et al.*[13] fused GNb with monomeric Cherry fluorescent protein (mCherry), which is less toxic with outstanding photostability and excellent pH resistance. The aim of this study is to achieve easy and rapid determination of the membrane protein topology without disrupting cells by utilizing GNb-mCherry as a tool. If the GFP tag fused to N- or C-termini of membrane proteins is exposed toward cytosol, GNb-mCherry will be recruited to the GFP fusion protein due to picomolar Kd between GNb and GFP, enabling us to observe the translocation of the mCherry signal from cytosol to the membrane and colocalize with the GFP signal. Otherwise, GNb-mCherry is not expected to change its localization in the cell. In this study, we clearly demonstrated that cytosolically expressed GNb–mCherry can be used to determine the topology of GFP-fused membrane proteins targeting various intracellular organelles, including the plasma membrane, ER, lysosome and mitochondria in cells.

2. Experimental

2.1. DNA constructs

N3-C4orf52-pEGFP was generated by PCR using primers 52-D3-S and C4orf52-EcoRI-A, and subcloned into the pEGFP-N3 vector via HindIII/EcoRI. N3-mPRMT8(N20)-EGFP was amplified with mPRMT8-S and mPRMT8-A and inserted into pEGFP-N3 using HindIII/KpnI. C1-EGFP-PLCδ1 was obtained by PCR with PLCδ1-ApaI(stop)-A and S(N30)-koM-NheI-S, and ligated into the pEGFP-C1 vector via ApaI/NheI. pcDNA3.1(+)-TOM20-GFP, N3-SP-GFP-KDEL, and N3-SP-GFP-CD52 were PCR-amplified using the following primer sets: pTOM20-NheI-S / pTOM20-EcoRI-A (TOM20); SP-S2 / SP-S1 / SP-A (SP); GFP-BamHI-S / GFP-KDEL-Stop-NotI-A (SP-GFP-KDEL); and SP-NheI-Ass-S / GFP-EcoRI-Ass-A (Sp-GFP-CD52); CD52-ex-Ass-S1 / CD52-ex-S2 / CD52-ex-Ass-NotI-A (CD52), respectively, and inserted into pcDNA3.1(+)-GFP or pEGFP-N3 using HindIII, XbaI, NheI, EcoRI, KpnI, BamHI, and NotI as appropriate. C1-EGFP-Sec61 was inserted into the eGFP-C1 vector using BglII/EcoRI. The following plasmids were obtained from Addgene (Cambridge, MA, USA): GFP-GalT (#11929), GFP-PH-FAPP1 (#161986), Cytochrome c-GFP (#41182), LetM1-GFP (#191937), and GNb–mCherry (#128769). Primers information used in this study is shown in *Table* 1.

2.2. Cell culture, transfection, confocal microscopy

HEK 293T cells were maintained in DMEM

Table 1. Primer sequences used for this study

Construct	Primer sequences (5'-3')
C4ORF52	Forward: CGCCCAAGCTTATGTCCCGGAACCTGCGC Reverse: GCAGAATTCCATTTCCTGCCAAATGGATC
mPRMT8	Forward: CGCCCAAGCTTGCCATGGCGGAGAATGCAGTC Reverse: GACGGTACCTGCATTCTCCGCCATTTT
PLCδ1	Forward: CTAGGGCTAGCGCCACCATGCAGAAGCTGAATTTC Reverse: CGTAGGGCCCTTACTGGATGTTGAGCTC
TOM20	Forward: CTAGGGCTAGCGCCACCATGGTGGGTCGGAACAGC Reverse: GCAGAATTCCCTTCCACATCATCTTCA
SP	Forward1: AAGCTTTTTTATTTCTGCTGGCAGGTTTTGCAGCAAAGAT Forward2: CTAGGGCTAGCGCCACCATGTTATTGCAAGCTTTTTTATTTCTGC Reverse: GACGGTACCAGAGGCAGAAATCTTTGCTGCAAAACCT
GFP-KDEL (SP-KDEL)	Forward: CGCGGATCCATGGTGAGCAAGGGCGAG Reverse: ATAAGAATGCGGCCGCTCACAGCTCGTCCTTCTTGTACAGCTCGTCCAT
SP-GFP (SP-CD52)	Forward: AGTGAACCGTCAGATCCGCTAGCGCCACCATGTTATTGCAAGCTTTT Reverse: GGATGCTGAGGGGCTGCTGAATTCCTTGTACAGCTCGTCCAT
CD52	Forward1: AGCAGCCCCTCAGCATCCAGCAACATAAGCGGAGGCATTTTCCTTTTCT Forward2: GGCATTTTCCTTTTCTTCGTGGCCAATGCCATAATCCACCTCTTCTGCT Reverse: ATTATGATCTAGAGTCGCGGCCGCTCAACTGAAGCAGAAGAGGTGGAT

supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin/streptomycin in a humidified atmosphere of 5 % (v/v) CO_2 at 37 °C. The cells were incubated in a sticky-slide 8-well system (#80828; ibidi GmbH, Martinsried, Germany) to obtain 40 %–60 % confluence on the day of imaging and in 10-cm cell culture dishes (#20100, Spl Life Science, Pocheon, South Korea). Then, the cells were transfected with plasmid DNA constructs using Lipofectamine™ 2000 (Life Technologies, Carlsbad, CA, USA) 14–16 h before imaging. The relative amount of each construct was determined empirically based on the relative expression of each construct combination.

Cell images were acquired using an inverted Zeiss LSM-700 laser scanning confocal microscope and processed using the ZEN software (Carl Zeiss, Oberkochen, Germany). GFP was excited using the 488 nm laser line, and emission signals were collected using a 500–550 nm bandpass filter. mRFP was excited using the 561 nm laser line, and emission signals were collected using a 575–625 nm bandpass filter. The theoretical emission ranges for the fluorophores are approximately 508–543 nm for GFP and 578–649 nm for mRFP, which are adequately captured by the respective filters. Most images were obtained with live cells. MitoTracker® Red was purchased from Invitrogen (Catalog #: M7512; Thermo Fisher Scientific, Waltham, MA, USA) and was used at 37 °C.

3. Results and Discussion

3.1. GNb-mCherry as a tool for identifying the orientation of N- or C-termini of GFP-fused plasma membrane proteins

Because cytosolic GNb–mCherry can bind to GFP localized to the cytoplasmic side but not the extracellular side in the plasma membrane, it can easily identify the orientation of N- or C-termini of GFP-fused membrane proteins in the plasma membrane (*Fig.* 1A). However, if GFP fusion protein and GNb-mCherry are co-expressed simultaneously, strong interaction between GFP and GNb might affect the localization of GNb-mCherry. To exclude this possibility, GNb-mCherry was transfected 1 day after transfection of GFP-fused protein. When GNb–mCherry alone was expressed in HEK293T cells, it was diffusely localized in the nucleus and cytosol (*Fig.* 1B). Then, GNb–

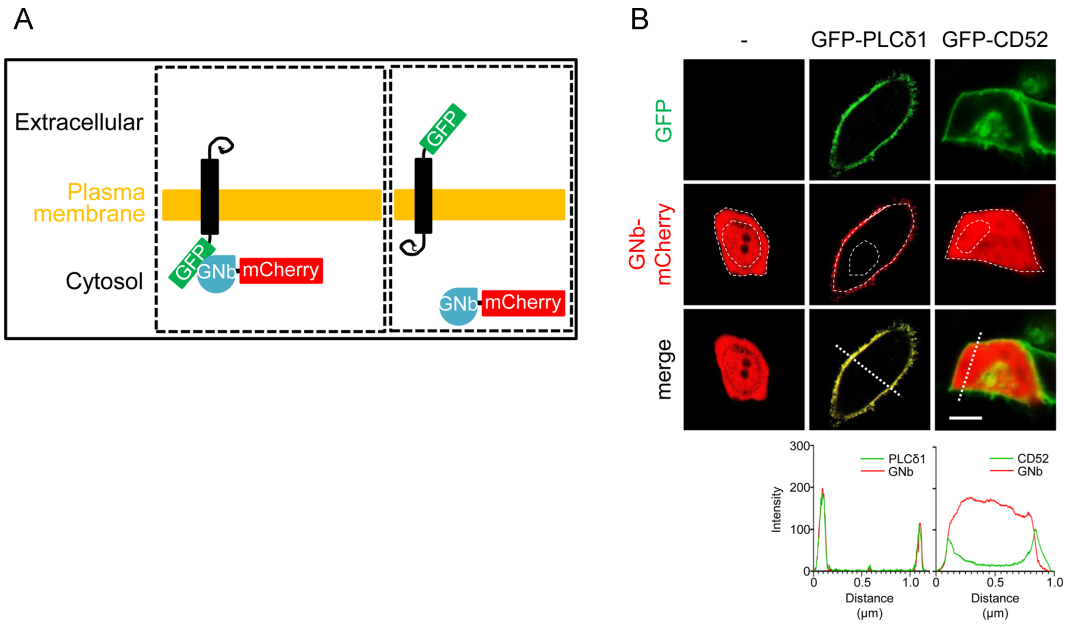

Fig. 1. Identification of cytoplasmic or extracellular side localization of plasma membrane-targeted proteins using GNb–mCherry. (A) Schematic diagram illustrating the discrimination between cytoplasmic or extracellular side localization of plasma membrane-targeting proteins using GNb–mCherry. Left panel: when GFP tag is exposed to the cytosolic part, GNb-mCherry is recruited to the inner leaflet of the membrane, showing co-localization signal. Right panel: when GFP tag is exposed to the extracellular space, GNb-mCherry cannot be recruited and stayed in the cytoplasm. Therefore, the topology of the membrane protein can be determined by monitoring the subcellular localization of GNb-mCherry. (B) Confocal images revealing the cellular localization of GNb–mCherry in HEK293T cells expressing no GFP, GFP-PLCδ1, or GFP-CD52. GNb–mCherry is localized to the plasma membrane in cells expressing GFP-PLCδ1 but not GFP-CD52, whereas GNb–mCherry alone is diffusely localized to the cytosol and nucleus. The pixel intensity trace chart for GFP-fusion proteins and GNb-mCherry is drawn from the dotted line in the merge panels. Scale bar: 20 μm.

mCherry was transfected into cells expressing the GFP-fused plasma membrane-associated protein GFP-PLCδ1 or GFP-CD52. GFP-PLCδ1 is generally localized to the cytoplasmic leaflet of the plasma membrane via PI(4,5)P_2 binding,[14] whereas CD52 is a glycosylphosphatidylinositol (GPI)-linked glycoprotein localized to the outside leaflet of the plasma membrane.[15] As illustrated in *Fig.* 1B, GNb–mCherry is colocalized with GFP-PLCδ1 in the plasma membrane, whereas it is diffusely localized to the nucleus and cytosol of cells expressing GFP-CD52. These findings indicate that GFP in GFP-PLCδ1 is localized to the cytoplasmic side in the plasma membrane, whereas GFP in GFP-CD52 is localized to the extracellular side in the plasma membrane. Therefore, our results obviously confirmed that GNb–mCherry can clearly discriminate between cytoplasmic or extracellular side localization of GFP protein in the plasma membrane.

3.2. GNb-mCherry as a tool for identifying the orientation of N- or C-termini of GFP-fused intracellular membrane proteins

We next investigated whether GNb–mCherry can discriminate between the cytoplasmic and luminal side of intracellular organelles, including ER, trans-Golgi network (TGN), or lysosome (*Fig.* 2A). First, GNb–mCherry was expressed in cells expressing GFP-Sec61β (transmembrane protein) or the signal peptide (Sp)-GFP-KDEL, which is localized to the ER membrane or lumen, respectively. As depicted in *Fig.* 2B, GNb–mCherry is colocalized with GFP-Sec61β in the ER membrane, whereas it is diffusely localized in the cytosol and nucleus of Sp-GFP-KDEL-expressing cells. These data suggest that GFP

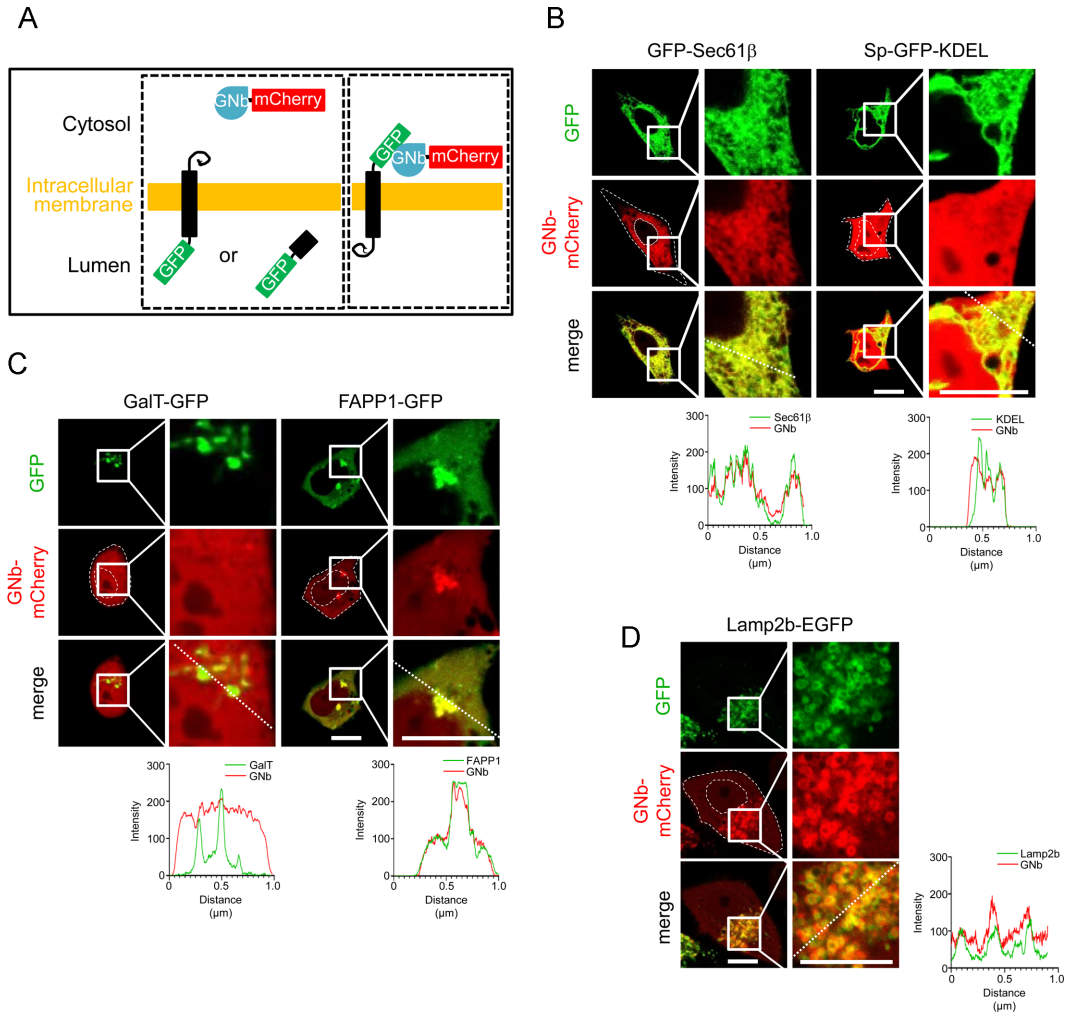

Fig. 2. Identification of cytoplasmic or luminal side localization of various intracellular organelle-targeting proteins using GNb–mCherry. (A) Schematic diagram illustrating the discrimination between cytoplasmic or luminal side localization of intracellular organelle-targeting proteins using GNb–mCherry. (B) Confocal images revealing the cellular localization of GNb–mCherry in HEK293T cells expressing GFP-Sec61β or SP-GFP-KDEL. GNb–mCherry is localized to the ER in cells expressing GFP-Se61β, whereas it is diffusely localized to the cytosol and nucleus in cells expressing Sp-GFP-KDEL. (C) Confocal images showing the cellular localization of GNb–mCherry in HEK293T cells expressing GalT-GFP or FAPP1-GFP. GNb–mCherry is localized to the TGN in cells expressing FAPP1-GFP, whereas it is diffusely localized to the cytosol and nucleus in cells expressing GalT-GFP. (D) Confocal images showing the cellular localization of GNb–mCherry in HEK293T cells expressing Lamp2b-GFP. GNb–mCherry is localized to the lysosome in cells expressing Lamp2b-GFP. The pixel intensity trace chart for GFP-fusion proteins and GNb-mCherry is drawn from the dotted line in the merge panels. Scale bar: 20 μm.

in GFP-Sec61β but not in Sp-GFP-KDEL is exposed on the cytoplasmic side in ER. Our findings are consistent with previous reports that GFP fused to the N-terminus of Sec61β was exposed on the cytoplasmic side of the ER membrane,[16] and Sp-GFP-KDEL was expressed in the ER lumen.[17]

To further confirm whether GNb–mCherry can discriminate between the cytoplasmic and luminal side of the Golgi complex, we used GalT-GFP and FAPP1-GFP, which are frequently used for the TGN marker. As shown in *Fig.* 2C, GNb–mCherry is diffusely distributed in the nucleus and cytosol of

GalT-GFP-expressing cells, whereas it is localized to the TGN in FAPP1-GFP-expressing cells. GalT is a type II membrane protein, and GFP fused to the C-terminus of GalT might be exposed on the luminal side of TGN.[18] FAPP1 is a PI4P- and ARF1-binding probe expressed on the cytoplasmic surface of TGN, and GFP in FAPP1-GFP might be exposed on the cytoplasmic surface of TGN.[19] Hence, GNb–mCherry can discriminate between cytoplasmic and luminal side localization of the GFP sequence in GFP-fused proteins within intracellular organelles, including the ER or TGN.

We also confirmed whether GNb–mCherry can discriminate between the cytoplasmic and luminal side of lysosome. To do this, we used Lamp2b-GFP, a well-known lysosomal marker. As shown in *Fig.* 2D, GNb–mCherry is co-localized to Lamp2b-GFP. C-terminus of Lamp2 belongs to Type I membrane

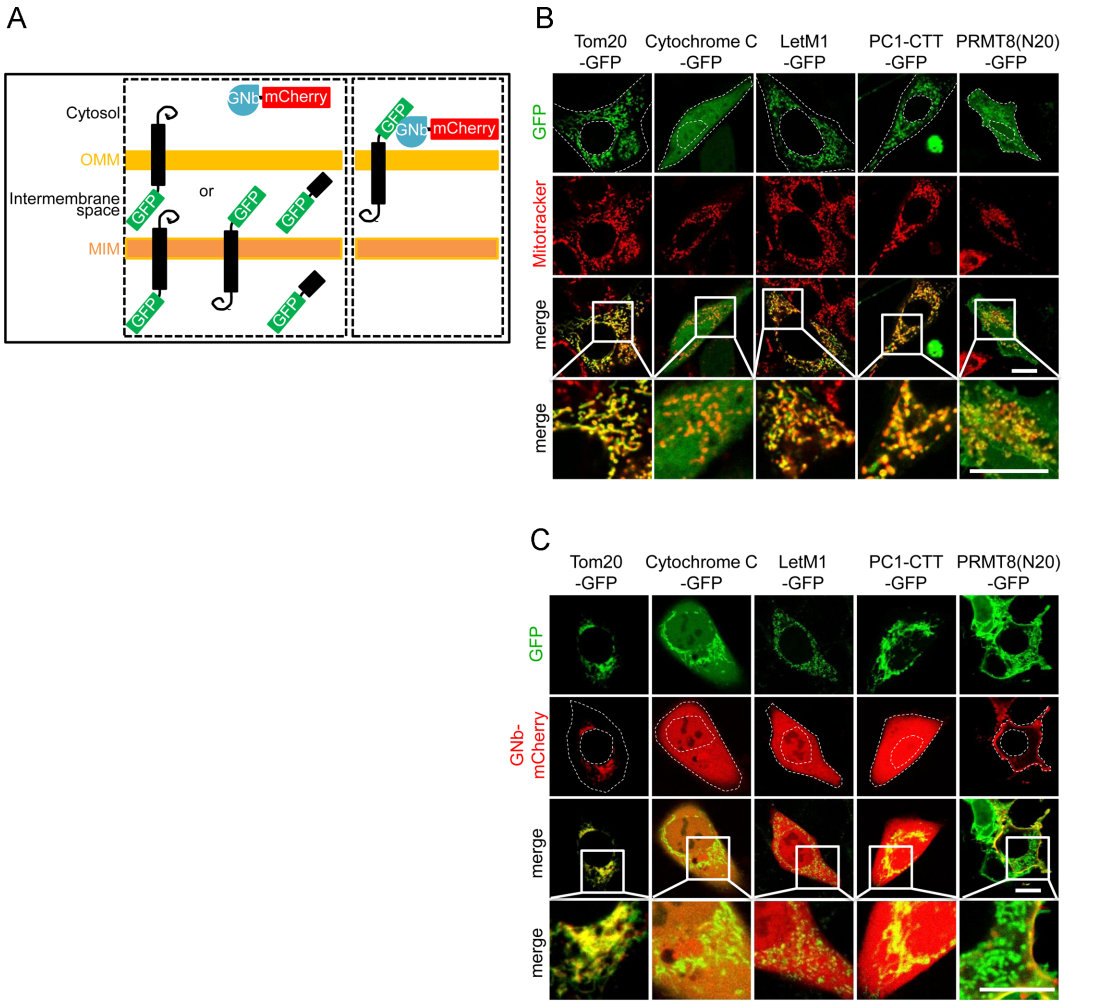

Fig. 3. Identification of cytoplasmic surface and inner side localization of mitochondria outer membrane (MOM) of various mitochondria-targeting proteins. (A) Schematic diagram illustrating the discrimination between cytoplasmic or inner side of mitochondria outer membrane of mitochondria-targeting proteins using GNb–mCherry. (B) Confocal images of HEK293T cells showing mitochondria-targeting of various GFP-fused proteins. TOM20-GFP, Cytochrome C-GFP, LetM1-GFP, PC1-CTT-GFP or PRMT8(N20)-GFP is co-localized to mitochondria marker, Mitotracker. (C) Confocal images of HEK293T cells showing the cellular localization of GNb–mCherry in cells expressing TOM20-GFP, Cytochrome C-GFP, LetM1-GFP, PC1-CTT-GFP or PRMT8(N20)-GFP. GNb–mCherry is co-localized to Tom20-GFP and PRMT8(N20)-GFP but not to others. Scale bar: 20 μm.

protein, which is exposed to cytoplasmic side in lysosome.[20] Therefore, GFP fused to C-terminus of Lamp2 is also expressed in cytoplasmic side in lysosomal membrane. Overall, GNb-mCherry could discriminate between the cytoplasmic and luminal side of intracellular organ-targeting proteins.

3.3. Determining the orientation of N- or C-termini of mitochondria-targeting GFP-fused proteins using GNb-mCherry

Mitochondria have the double-membrane structure including outer membrane and inner membrane, thereby dividing into two inner spaces including intermembrane space and inner matrix. We next examined whether GNb–mCherry can discriminate cytoplasmic surface and inner sides of mitochondria outer membrane (MOM). To do this, we used four different mitochondria targeting proteins: Tom20-GFP (MOM-targeting protein), Cytochrome C-GFP (intermembrane space-targeting protein), Leucine zipper-EF-hand containing transmembrane protein 1 (LetM1-GFP; mitochondria inner membrane (MIM) protein), and the C-terminal tail (CTT) of the polycystin-1 (PC1) protein (PC1-CTT-GFP; inner matrix-targeting protein). As shown in *Fig.* 3B, all GFP-fused proteins are co-localized to Mitotracker, showing that all the constructs are expressed and targeted to the expected destination well. However, GNb–mCherry was only co-localized to Tom20-GFP, but not to others (*Fig.* 3C). Tom20 belongs to an N-terminally anchored transmembrane protein to the MOM.[21] Therefore, GFP fused to C-terminus of Tom20 is exposed to cytoplasmic side in MOM. On the other hand, Cytochrome C, LetM1, and PC1-CTT are well expressed, but their sub-mitochondrial localization is the intermembrane space, inner membrane, and matrix, respectively, which GNb-mCherry was not supposed to access. These results indicate that GNb-mCherry can detect GFP exposed to cytoplasmic side of MOM, but not to other mitochondria areas including intermembrane space and matrix (*Fig.* 3B and C).

Next, we applied this tool to dual targeting proteins in cells. It has been previously reported that PRMT8(N20)-GFP is localized to the plasma membrane and mitochondria.[22] As shown in *Fig*. 3B, PRMT8(N20)-GFP was localized to the plasma membrane and punctate structure in the cells. These punctate structures are co-localized to MitoTracker®, indicating mitochondria targeting. To discriminate between the cytosolic and luminal side of intracellular organelles, GNb–mCherry was expressed in cells expressing PRMT8(N20)-GFP. As depicted in *Fig.* 3C, GNb–mCherry is colocalized to the plasma membrane but not mitochondria in PRMT8(N20)-GFP-expressing cells, indicating that the GFP sequence in PRMT8(N20)-GFP is located on the cytoplasmic leaflet of the plasma membrane and inside the MOM. Consistent with these findings, a previous study reported that PRMT8(N20)-GFP is localized to the plasma membrane through N-terminal lipid modification and to the mitochondrial matrix through potential mitochondrial signal sequences.[22] Therefore, GNb–mCherry can be used as a discriminating tool to identify the orientation of N- or C-termini of dual targeting GFP-fused proteins in live cells.

Our developed assay has several advantages over the conventional western blot-based assay such as protease protection assay or the fluorescence protease protection (FPP) assay. First, a primary advantage of our assay is that it can be conducted in live cells without the need for membrane disruption. Conventional methods typically require mechanical or chemical disruption of the plasma membrane to allow proteases access to intracellular compartments. Such procedures can perturb the physiological state of the cell. In contrast, the GNb-mCherry system allows for the determination of topology by simply assessing the accessibility of the nanobody to the GFP tag, thereby maintaining cellular integrity. Second, this method can be applied to any intracellular membranous organelle, including the ER, mitochondrion, endosome, and lysosome without the need for organelle isolation. Traditional biochemical methods often necessitate the labor-intensive process of subcellular fractionation to isolate specific organelles with high purity. Our results confirm that GNb-mCherry can directly elucidate the topology of proteins within these diverse organelles in situ. Third, our assay is capable of distinguishing the topology of dual or multiple targeting proteins

within the same cell. As demonstrated with PRMT8(N20)-GFP, which localizes to both the plasma membrane and mitochondria, we were able to spatially discriminate the topology at each specific location. This represents a significant improvement over fractionation-based methods, where cross-contamination between organelle fractions can obscure the true topology of multi-localized proteins. Fourth, this tool is highly useful for studying proteins for which specific antibodies are not available. Unlike Western blotting, which relies on the availability of high-quality antibodies for detection, our system requires only the fusion of a GFP tag, making it a versatile tool for analyzing novel or less-characterized membrane proteins. Fifth, because topology is determined at the single cell level, our assay is particularly advantageous for cells that are difficult to transfect. Western blot-based assays typically require a large population of cells to generate sufficient protein lysate, which can be a limiting factor when transfection efficiency is low. The GNb-mCherry system overcomes this limitation by allowing for the analysis of individual cells via fluorescence microscopy. Nonetheless, a limitation of our assay is the requirement of tagging the GFP, which is a large protein composed of ~240 amino acids, i.e., 24 kDa. However, despite this limitation, if GFP-tagged proteins retain their endogenous localization, our assay may be useful to identify the topology of GFP-fused proteins in cells without any additional modification steps.

In summary, the GNb-mCherry based topology assay overcomes several methodological limitations of existing techniques, providing a robust and versatile tool for investigating membrane protein structure in live cells.

4. Conclusions

In this study, we developed a versatile and robust tool to determine topology of trans-membrane or membrane associated protein. The three main conclusions of this study are i) GNb-mCherry can be used to distinguish the topology of transmembrane proteins, ii) GNb-mCherry can be used to distinguish the topology of membrane protein in cellular organelles such as ER, Golgi, lysosome, and mitochondria; iii) GNb-mCherry can distinguish the topology of the dual targeting protein.

Validation of membrane protein topology is crucial to predict the function of the protein and drug-binding sites. Our developed tool successfully distinguished membrane topology in the plasma membrane, ER, Golgi, lysosome, and mitochondria. Although GNb-mCherry did not respond to luminal proteins of the cellular organelles as expected, it is not enough to suggest sub-organelle localization of proteins by the lack of binding between GNb-mCherry and the protein of interests. Therefore, development of probes that can go inside the lumen of ER or Golgi, and penetrate to sub-mitochondria region will be helpful to determine the localization or topology of proteins in the organelles.

Conflict of Interest Statement

No authors have potential conflicts of interest to disclose.

Acknowledgements

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