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Quantifying the insertion of membrane proteins into lipid bilayer nanodiscs using a fusion protein strategy

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ABSTRACT

A membrane protein's oligomeric state modulates its functionality in various cellular processes. Since membrane proteins have to be solubilized in an appropriate membrane mimetic, the use of classical biophysical methods to analyze protein oligomers is challenging. We here present a method to determine the number of membrane proteins inserted into lipid nanodiscs. It is based on the ability to selectively quantify the amount of a small and robust fusion protein that can be proteolytically cleaved off from a membrane protein after incorporation into lipid nanodiscs. A detailed knowledge of the number of membrane proteins per nanodisc at defined assembly conditions is essential to estimate the tendency for oligomerization, but also for guiding sample optimization for structural investigations that require the presence of a homogenous oligomeric state. We show that this method can efficiently be used to determine the number of VDAC1 channels in nanodiscs at various assembly conditions, as confirmed by negative stain EM. The presented method is suitable in particular for membrane proteins that cannot be probed easily by other methods such as single span transmembrane helices. This assay can be applied to any membrane protein that can be incorporated into a nanodisc without the requirement for special instrumentation and will thus be widely applicable and complementary to other methods that quantify membrane protein insertion in lipid nanodiscs.

1. Introduction

Membrane proteins (MPs) are essential players in cellular signaling [1]. Their functionality can be mediated by conformational changes upon ligand binding, chemical modifications or oligomerization. Robust assays exist to study e.g. downstream signaling of a G-protein coupled receptor leading to G-protein stimulation [2]. In addition, chemical modifications that are triggered by signaling cascades [3] can be probed by mass spectrometry (MS) or NMR. Another important mechanism of membrane protein signal transduction across or in a biological membrane is alterations in their oligomeric state, e.g. in the case of Integrins [4] or Bcl2 [5] proteins. Despite the availability of a vast number of biophysical methods for the analysis of the molecular weight and oligomerization of soluble proteins, the application of those techniques to membrane proteins remains challenging and time-consuming. MS analysis has recently gained attention in monitoring the oligomerization of membrane proteins [6–9]. However, special MS setup and instrumentation is required that is not available in a standard biochemistry laboratory. Phospholipid nanodiscs [10–13], a lipid-based membrane mimetic, where two copies of a so-called membrane scaffold protein, or MSP, wrap around a patch of lipid bilayer membrane, can be used to trap membrane proteins in a defined and controlled manner. Nanodiscs have been used to incorporate different oligomeric states of rhodopsin followed by detection of the oligomer by sucrose gradient centrifugation [14]. Despite the availability of such straightforward protocols, it would be helpful to quantify the insertion numbers of membrane proteins by a spectroscopic assay. The general problem in the determination of the oligomeric state of a membrane protein in lipid nanodiscs by spectroscopic methods is that both, the inserted protein and the MSP contribute to the absorption at 280 nm, which is

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used for quantification. Thus, another independent probe needs to be inserted into the system that reports on the concentration of either the MSP or the inserted membrane protein. It has been shown previously that for proteins containing an intrinsic chromophore, such as Rhodopsin, the absorption of that additional reporter can be utilized for determination of the number of MP molecules relative to the MSP in nanodiscs [15]. However, the absorption properties of such intrinsic chromophores depend on the folding state of the protein and can only be used for quantification if properly folded membrane proteins are obtained. Another strategy would be the chemical attachment of dye molecules to the membrane protein of interest or the MSP, serving as a reporter whose absorption maximum is well separated from the protein. Since many dyes that absorb visible light also contribute to the absorption at 280 nm, slight changes in their spectroscopic properties would complicate the quantification of the modified membrane protein or the MSP. Therefore, it is highly desirable to have access to a robust system that allows for a reliable determination of the number of membrane protein's oligomeric state, or the quantification of the number of inserted membrane proteins in nanodiscs, by using UV/Vis absorption of selected components, without the need for special instrumentation or chemical modifications.

We here present a method that is based on the fusion of a membrane-protein and a highly stable and small soluble protein that does not bind to a membrane surface. Among others, the 56-residue long immunoglobulin-type B1 domain of protein G (GB1) fulfills all required aspects. GB1 has been successfully utilized as a solubility and expression level-enhancing fusion protein for protein production in *E. coli* [16]. The NMR structure of GB1 indicates that this protein is a monomer even at millimolar concentrations [17]. This strategy can also be applied to the production of MPs or transmembrane helices (TMHs), where GB1 leads to a marked increase in expression yields, especially for short transmembrane helices [18]. Membrane proteins fused to GB1 can be easily purified and reconstituted in detergent micelles for further applications. Furthermore, GB1 has been used to assay oligomeric states of transmembrane helices in bicelles using chemical crosslinking [19].

We here show that GB1-MP fusions can be incorporated into nanodiscs successfully followed by complete cleavage of the GB1 tag. After cleavage, the amount of GB1, which is equal to the number of the inserted membrane protein, as well as the amount of MSP in nanodiscs can be quantified by size exclusion chromatography and detection of the absorption at 280 nm. We show that reliable results can be obtained by this method using both the voltage gated anion channel (VDAC1) and two single-span transmembrane helical proteins BclxL and GlycophorinA as model systems. For VDAC1, results obtained with the GB1-cleavage assay could be confirmed by and compared to negative-stain EM data. In addition, the GB1-tag can also be placed on the MSP in cases where GB1-membrane protein fusion constructs cannot be produced. Thus, this method will be generally useful for optimizing the nanodisc assembly conditions to obtain a homogenous membrane protein sample and for an estimation of the oligomeric state of a membrane protein in MSP lipid nanodiscs.

2. Materials and methods

2.1. Protein production

The GB1 fusion construct of human VDAC1 was designed harboring an N-terminal GB1 followed by a Thrombin cleavage site and a short Gly-Ser linker and VDAC1 with a C-terminal His_6-tag in a pET21a plasmid (Novagen). Production in *E. coli* BL21(DE3) and refolding into the detergent LDAO was done as described previously [20,21]. A concentrated stock solution of ~ 400 μM VDAC1 in LDAO was used for nanodisc assembly. BclxL-transmembrane helix (TMH) was produced as a GB1-fusion protein harboring an N-terminal His_6-tag, GB1, a linker and a thrombin cleavage site and solubilized in SDS detergent as described previously [18]. Human GlyA transmembrane helix (UniProt P02724, residues 88–120) was inserted into the same GB1-thrombin plasmid and produced as described for BclxL-TMH [18]. MSP proteins MSP1D1 and MSP1D1ΔH5 were produced as described earlier [12,22,23].

2.2. Nanodisc assembly and GB1 assay

The Nanodisc assembly was done as described previously [12,22,23] using the lipid DMPC or DMPC:DMPG (3:1) mixtures. GB1-VDAC1 nanodiscs were assembled at various VDAC1:MSP ratios (1:1,1:2,1:4,1:6, 1:8) using the MSP variants MSP1D1 and MSP1D1ΔH5 in MSP buffer (20 mM Tris/HCl pH 8, 100 mM NaCl, 0.5 mM EDTA) supplemented with 2 mM DTT and a DMPC:DMPG (3:1) lipid mixture solubilized in 100 mM cholate. The VDAC1 concentration was kept constant at 50 μM while the MSP concentration was varied from 50 to 400 μM. After incubating for 1–2 h at room temperature, the nanodisc assembly step was initiated by adding of 0.6 g/mL Biobeads SM-2 for 1.5 h. The assembled nanodiscs were further purified by Ni-NTA in 20 mM Tris/HCl pH 8, 100 mM NaCl, 5 mM BME to remove empty nanodiscs. This sample was injected on a S200 size exclusion column in 20 mM NaPi pH 7.0, 50 mM NaCl, 0.5 mM EDTA and 2 mM DTT using an Äkta Pure chromatography system (GE Healthcare). The main homogenous SEC peak was collected and concentrated to a volume of 1 mL. Thrombin digestion was done overnight at room temperature by adding 20 U/mL Thrombin (Merck). Complete digestion was confirmed by SDS-PAGE and/or ESI-MS. This mix was then directly subjected to another S200a run and the peak integrals of the nanodisc and GB1 peak were quantified with the Unicorn analysis software (GE Healthcare).

The GlyA nanodisc assembly was done with MSP1D1ΔH5 and a DMPC:DMPG (3:1) lipid mixture solubilized in 100 mM cholate in MSP buffer. The concentration of GlyA was 50 μM, while the concentration of MSP was 200 μM, yielding an MSP-to-MP ratio of 4. The Lipid-to-MSP ratio was empirically optimized to 40:1. As described above, the assembled nanodiscs were further purified by Ni-NTA and S200 size exclusion chromatography, subjected to thrombin digestion and analyzed by a final SEC run. An identical procedure was used for the assembly and analysis of the GB1-BclxL-TMH MSP1D1ΔH5 nanodiscs. The GB1-BclxL-TMH MSP1E3D1 nanodiscs were assembled using 200 μM of GB1-BclxL-TMH and 100 μM of MSP1E3D1 resulting in an MSP-to-MP ratio of 1:2. A lipid-to-MSP ratio of 90:1 was used. The amino acid sequence-derived molecular extinction coefficients (obtained with the online tool ProteinCalculator v3.4, http://protcalc.sourceforge.net/cgi-bin/protcalc) that were used for the calculations are: His_6-GB1: 13370 $cm^{-1}M^{-1}$ [1] (14,039 $cm^{-1}M^{-1}$ after calibration, see Fig. 5a), GB1: 9530 $cm^{-1}M^{-1}$ (10,006 $cm^{-1}M^{-1}$ after calibration, see Fig. 5a), VDAC1-His_6: 36840 $cm^{-1}M^{-1}$, GlyA: 1280 $cm^{-1}M^{-1}$, BclxL-TMH: 5690 $cm^{-1}M^{-1}$, MSP1D1/MSP1D1ΔH5: 17780 $cm^{-1}M^{-1}$, MSP1E3D1: 26030 $cm^{-1}M^{-1}$.

2.3. ESI-mass spectrometry (ESI-MS)

The MS experiments were conducted on an LCQ-FLEET (Thermo Scientific) system equipped with a 3D ion trap and using electro spray ionization (ESI). The instrument is connected to a high-performance liquid chromatography (HPLC) system and UV detector (UltiMate 3000 HPLC system, Dionex with a Retain PEP, Drop-in, 10 × 2.1 mm column).

2.4. Negative-stain electron microscopy

5 µl VDAC1 nanodisc samples were adsorbed for 30 min to carbon-coated grids that had been glow discharged on air for 30 mins. Excess solution was blotted off and the samples were negatively stained for 30 s by adding 5 µL 1% uranyl formate solution. Micrographs were recorded with an JEOL 1400 plus microscope utilizing a JEOL Ruby CCD camera at a final pixel size of 3.32 Å. Particles were selected semi-automatically with EMAN2 [24]. All subsequent image processing steps were performed with IMAGIC5 [25]. Particles were centered, band pass filtered (110 nm–1.5 nm) and classified by Multi-statistical analysis (MSA). The number of classes was set to have an average of 30 particles per class. The final statistics on the number of VDAC1 molecules per nanodisc was based on visual inspection of the class averages and considering the number of particles that have been used for each class average.

3. Results

3.1. GB1 fusion protein assay for the quantification of membrane protein insertion in nanodiscs

In order to determine the number of membrane proteins inserted in phospholipid nanodiscs, we used a fusion protein consisting of GB1 and a membrane protein (MP) (Fig. 1). By using phospholipid nanodiscs we are able to trap MPs in a confined lipid bilayer patch of defined size for subsequent quantification. The MP is refolded and/or extracted in detergent micelles and purified for further use. In detergent micelles, it is difficult to quantify the oligomeric state of a MP using standard methods such as light scattering or simple size exclusion chromatography. We here make use of the fact that two MSP proteins wrap around a patch of a lipid bilayer to form a nanodisc, as shown in a recent NMR structure [26]. Thus, the determination of the stoichiometry between the inserted MP and the MSP will directly yield the average number of the MP in a nanodisc. Furthermore, quantification of MP insertion in a lipid bilayer environment can be assayed at various MP-to-MSP ratios in order to screen for cooperative effects that would provide useful hints for specific interactions between individual MP monomers. Consequently, our workflow consists of three basic steps. First, the MP of interest is purified as a GB1 fusion protein. Second, nanodisc assembly is conducted with the fusion protein and purified by Ni-NTA and SEC to only retain homogenous MP-loaded nanodiscs. Third, GB1 is cleaved off by a protease and the resulting mixture is analyzed by SEC while detecting the absorption at 280 nm. In order to obtain absolute concentrations, the fractions containing the nanodiscs or GB1 would need to be pooled and quantified by UV absorption. However, this usually leads to loss of material, thus introducing additional errors. Therefore, we rather use the A_{280} integrals of the corresponding SEC peaks for further analysis. As these numbers cannot directly be used to obtain absolute concentrations of each species, we use the molar extinction coefficient at 280 nm, ε_{280}, to obtain pseudo particle numbers that enable us to derive the ratio of the two components in the nanodisc system. This analysis consists of three calculation steps. 1: quantification of GB1 (n_{GB1}) (Eq. (1)), which can be set to be equal to the number of the inserted MP (n_{MP}), 2: back-calculation of the contribution of the inserted MP to the A_{280} value of the loaded nanodiscs followed by the determination of a pseudo particle number of the nanodiscs (n_{ND}) (Eq. (2)). After this step, the MP-to-ND ratio (R) can be determined by dividing n_{MP} by n_{ND} (Eq. (3)).

$$n_{GB1} = Int_{GB1}/\varepsilon_{GB1}; n_{MP} = n_{GB1} \quad (1)$$

$$n_{ND} = \left(Int_{ND+MP} - \left(n_{GB1} \bullet \varepsilon_{MP}\right)\right) / \left(2 \bullet \varepsilon_{MSP}\right) \quad (2)$$

$$R = n_{MP}/n_{ND} \quad (3)$$

Trace amounts of residual protease can be neglected because only very low concentrations are used and its SEC elution volume is well separated from the nanodisc or GB1 peaks.

3.2. Insertion numbers of VDAC1 in different nanodiscs suggest a tendency for cooperative co-assembly

Next, we wanted to apply this methodology to a membrane protein system of interest, for which the homogeneity of the nanodisc preparation is crucial for structural biology methods such as NMR and EM. We chose to use the voltage dependent anion channel VDAC1 as a model system (Fig. 2a). Due to its large pore diameter of ~2 nm, the number of VDAC1 channels in nanodiscs can be readily visualized with negative-stain EM [27,28] to have access to an inde-

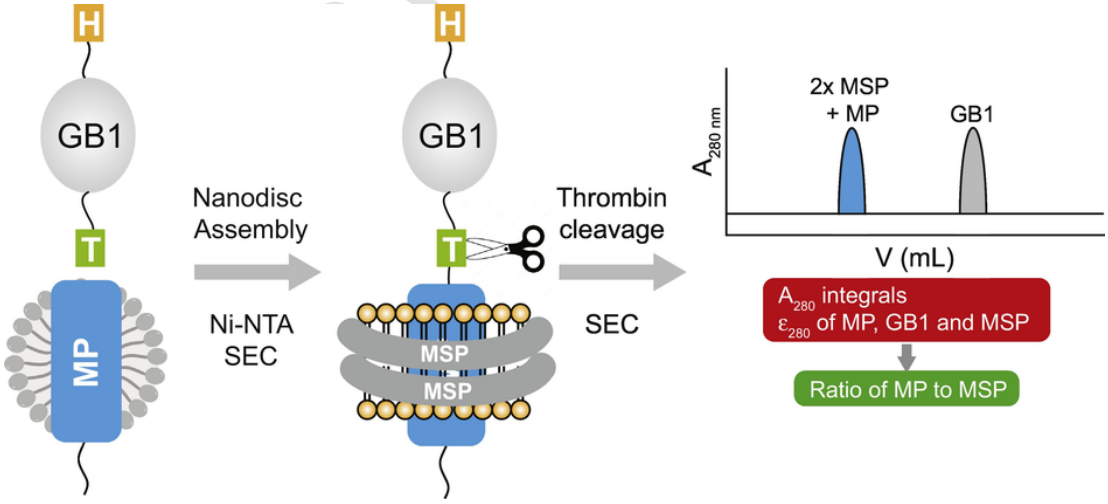

Fig. 1. Principle of the presented GB1 assay for the quantification of the insertion of membrane proteins in nanodiscs. A membrane protein (MP) is produced as a GB1 fusion construct harboring a Thrombin cleavage site and is purified in detergent. After nanodisc assembly, only MP-loaded nanodiscs are retained by Ni-NTA affinity purification followed by size exclusion chromatography (SEC). The homogenous nanodisc preparation is then cleaved by Thrombin to remove the GB1 tag, followed by another SEC run. The integrals of the absorption at 280 nm of the nanodisc and GB1 peak are then used for further calculations together with the calculated molar extinction coefficients at 280 nm (ε_{280}) of all components. MSP: membrane scaffold protein; T: thrombin cleavage site; H: His_6 tag.

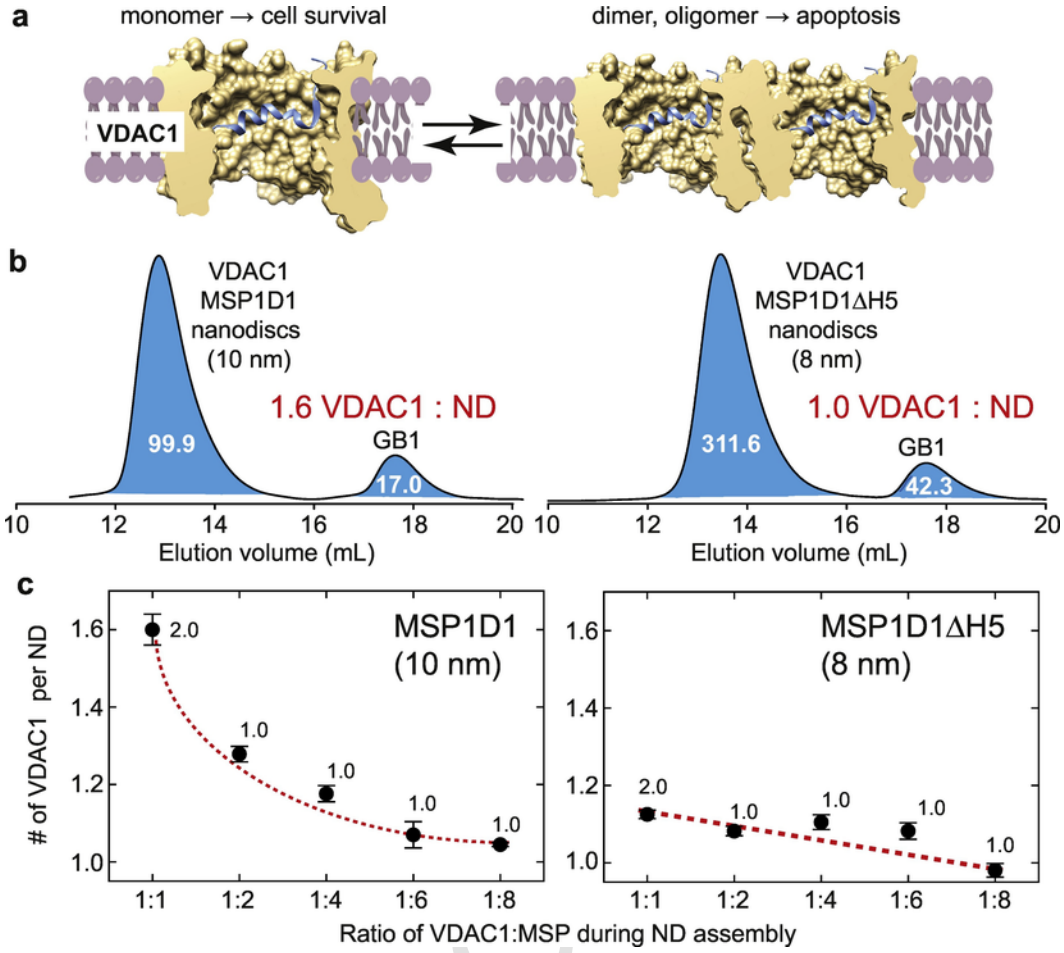

Fig. 2. Insertion numbers of the VDAC1 anion channel in nanodiscs as probed by the GB1 assay. (a) VDAC1 is located in the outer mitochondrial membrane and is responsible for the passage of metabolites. VDAC1 can be present in different oligomeric states that might be connected to functional features. (b) GB1-assay data with VDAC1 in nanodiscs of 10 nm (MSP1D1, left) or 8 nm (MSP1D1ΔH5, right) in diameter. Nanodisc assembly conditions favoring dimerization have been used for the 10 nm discs, i.e. using a VDAC1:MSP ratio of 1:1, whereas for the smaller nanodiscs a ratio of 1:8 was used to favor the insertion of a monomer. As a result, the GB1 assay indicates that on average 1.6 VDAC1 monomers are inserted in the larger nanodiscs, whereas the smaller nanodiscs harbor just one monomer. The integrals for each peak are shown in bold white letters. (c) Dependence of the VDAC1 oligomeric state trapped in nanodiscs on the molar ratio between VDAC1 and MSP during nanodisc assembly using MSP1D1 and MSP1D1ΔH5 nanodiscs. In 8 nm nanodiscs, the oligomeric state of VDAC1 is always close to 1, whereas it can adopt values of up to 1.6 in 10 nm discs. The dotted red line highlights the different insertion behavior of VDAC1 in these two cases. ND: nanodisc. Values and error bars represent the average and standard deviation obtained with 3 independent SEC experiments. The numbers next to the data points indicate the expected amount of VDAC1 in each nanodisc preparation based on the chosen assembly conditions. Only nanodiscs that contain at least one copy of VDAC1 are retained during initial purification, thus, the lowest possible insertion number is 1.

pendent readout. In order to probe the power of the GB1 assay to detect different numbers of VDAC1 in nanodiscs, we assembled MSP1D1 nanodiscs (10 nm diameter) using a 1:1 ratio between VDAC1 and MSP, which would result in an expected average number of two VDAC1 molecules in one nanodisc. As a comparison, we used smaller nanodiscs (8 nm diameter) composed of MSP1D1ΔH5 and adjusted the VDAC1:MSP ratio to 1:8, thus enforcing the incorporation of just one VDAC1 per nanodisc. As shown in Fig. 2b, we obtained an average number of VDAC1 in MSP1D1 nanodiscs of 1.6 and a value of 1.0 for the MSP1D1ΔH5 nanodiscs, confirming the initial assumption that the number of VDAC1 molecules in a nanodisc can be adjusted by alteration of the VDAC1-to-MSP ratio and MSP variant during assembly. In order to further investigate how the number of VDAC1 insertion per nanodisc is influenced by changing the assembly conditions, we performed a systematic screen of assembly conditions, where the VDAC1-to-MSP ratio (1:1 to 1:8) and the nanodisc size (10 or 8 nm) have been varied (Fig. 2c). The VDAC1-to-MSP ratios in each case are shown in Table 1. We calculated the lipids per nanodiscs, taking into account that the inner diameter of MSP1D1 and MSP1D1ΔH5 nanodiscs is 8 and 6 nm, respectively, the surface

Table 1
VDAC1 nanodisc assembly conditions.

VDAC:MSP ratio	VDAC1 in MSP1D1 nanodiscs					VDAC1 in MSP1D1ΔH5 nanodiscs				
	1:1	1:2	1:4	1:6	1:8	1:1	1:2	1:4	1:6	1:8
[VDAC1] (μM)	50	50	50	50	50	50	50	50	50	50
[MSP] (μM)	50	100	200	300	400	50	100	200	300	400
[DMPC/DMPG] (3:1) (mM)	1.5	4	8	12	20	1	3	6	9	16
Lipid:MSP ratio	30	40	40	40	50	20	30	30	30	40

Buffer: 20 mM Tris pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 2 mM DTT and 20 mM cholate.

area for each DMPC or DMPG lipid is 57 Å^2 [11,12], and the diameter of the VDAC pore is 4 nm, as derived from its solution structure [20]. This resulted in 60 and 40 lipids for the VDAC1 monomer or dimer in MSP1D1 nanodiscs, respectively, and in 30 lipids per bilayer leaflet for VDAC1 monomer in MSP1D1ΔH5 nanodiscs. It has been shown that assembly conditions can affect the oligomeric state of bacteriorhodopsin [29]. Thus, we took special care to optimize these parameters for every VDAC1-to-MSP ratio. The number of inserted VDAC1 molecules approaches a value of 1 in both cases, if an excess of MSP is used during assembly (1:8 ratio). However, there are also distinct differences between the 10 and 8 nm nanodiscs. The maximum number of VDAC1 in smaller nanodiscs is only 1.15 whereas the larger discs can accommodate up to 1.6 VDAC1 molecules on average, if the VDAC1-to-MSP ratio is increased to 1:1. The 10 nm discs apparently provide enough space for two VDAC1 channels. In addition, the insertion number of VDAC1 into nanodiscs steadily increased at assembly ratios of 1:4 or lower, even though at this ratio only a monomer would be expected theoretically. This observed increase in insertion number suggests positive cooperative effects between VDAC1 monomers, which could be caused by a direct interaction or a higher nanodisc stability if two VDAC1 monomers are present. The 8 nm lipid nanodiscs do not show this behavior due to a lack in surface area that can only harbor one VDAC1 molecule. These data suggest that VDAC1 is not forming a high-affinity dimer. For a tight dimer or oligomer, the number of co-assembled VDAC1 monomers would be expected to be higher than 1, in particular at assembly conditions that would favor the insertion of a monomer, i.e. with a large excess of MSP.

3.3. Validation of the GB1 assay by negative-stain EM

Since VDAC1 can be easily visualized in nanodiscs by negative-stain EM [27,28], we set out to use this method for a thorough validation of the results obtained in the GB1 assay. For EM, we prepared nanodiscs with MSP1D1 or MSP1D1ΔH5 and untagged VDAC1 using a VDAC1-to-MSP ratio of 1:6 in both cases (Fig. 3a,b). Looking at the GB1 assay, we would expect a ratio of around 1.1 in each case. After processing of the negative-stain images and compiling class averages, we inspected the resulting images manually and used the number of particles for each class average for the final calculations. This was necessary because lipid surfaces tend to stain in regions that contain hollow areas. Thus, only clearly stained dots were considered to originate from a VDAC1 channel. With that procedure we came up with a VDAC1-to-ND ratio of 1.19 for MSP1D1 and 0.94 for MSP1D1ΔH5 nanodiscs, respectively. These values take into account the detected empty nanodiscs that tend to co-purify with loaded nanodiscs in small amounts (~5%). The obtained numbers are very close to the ones obtained in the GB1 assay. Slight deviation between both methods might be caused by errors in the class averaging procedure, where dimers are sorted into monomeric classes. In the GB1 assay, incomplete integration of the SEC peaks, in particular of the nanodisc peak would cause slight errors in the analysis. Overall, the deviation between these two methods is below 10%. This excellent agreement also demonstrates that GB1 does not affect the nanodisc insertion properties of VDAC1.

In contrast to the favorable staining properties of VDAC1 in nanodiscs, most membrane proteins cannot easily be visualized by negative-stain EM. Thus, the presented GB1 assay can be used to determine the nanodisc insertion numbers of a larger range of membrane proteins in a reliable and rapid manner.

3.4. Quantification of nanodisc-inserted BclxL-TMH and GlycophorinA

Single-spanning transmembrane helical proteins are highly abundant in the cell and fulfill important roles in cellular signaling. Due to their high biological significance, we also wanted to demonstrate the applicability of our assay to this protein class. As a benchmarking model system, we used GlycophorinA (GlyA) that is known to form a stable dimer even in harsh detergents [30,31]. Since the TMH cannot be easily observed by SDS-PAGE, we identified the assembled components in nanodiscs by ESI-MS (Fig. 4a). The correct mass for the GB1-GlyA fusion protein and the MSP1D1ΔH5 could be detected. After Thrombin cleavage (Fig. 4b), we observed three distinct signals, corresponding to GlyA, GB1 and MSP1D1ΔH5, confirming quantita-

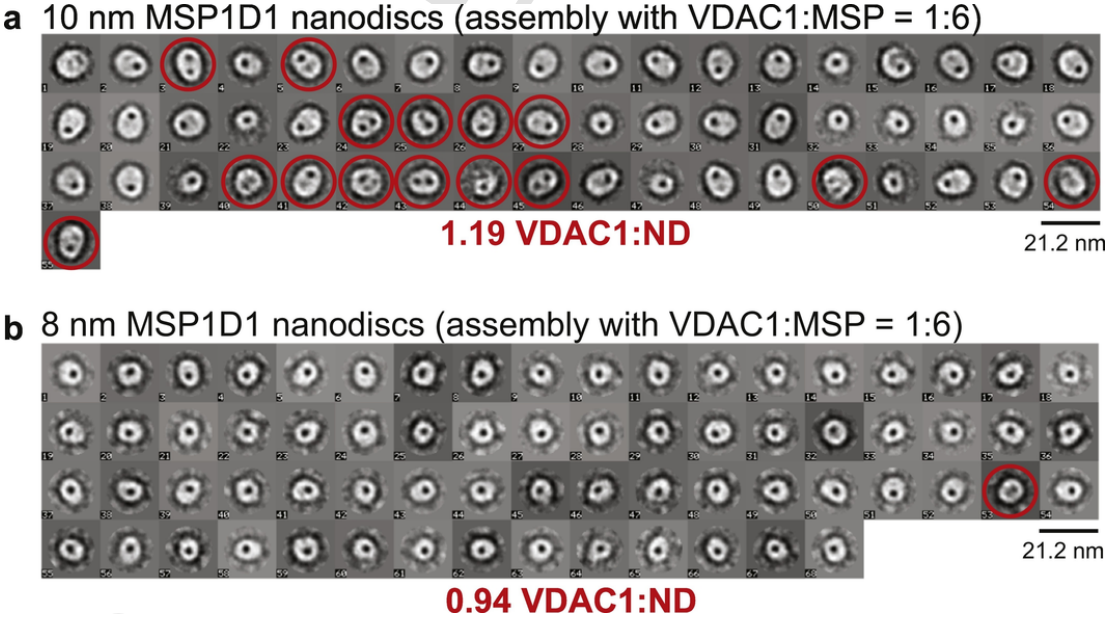

Fig. 3. Nanodisc insertion of VDAC1 probed by negative-stain EM. (a) EM class averages of VADC1 in 10 nm nanodiscs assembled with MSP1D1 show an average number of VDAC1 per nanodisc of 1.19. (b) In contrast, the EM analysis of VADC1 in 8 nm nanodiscs assembled with MSP1D1ΔH5 results in an average value of 0.94. These number have been obtained by counting the number of particles for each class average and manual inspection of the images, including empty nanodiscs that were co-purified. Boxsize = 21.2 nm. ND: nanodisc.

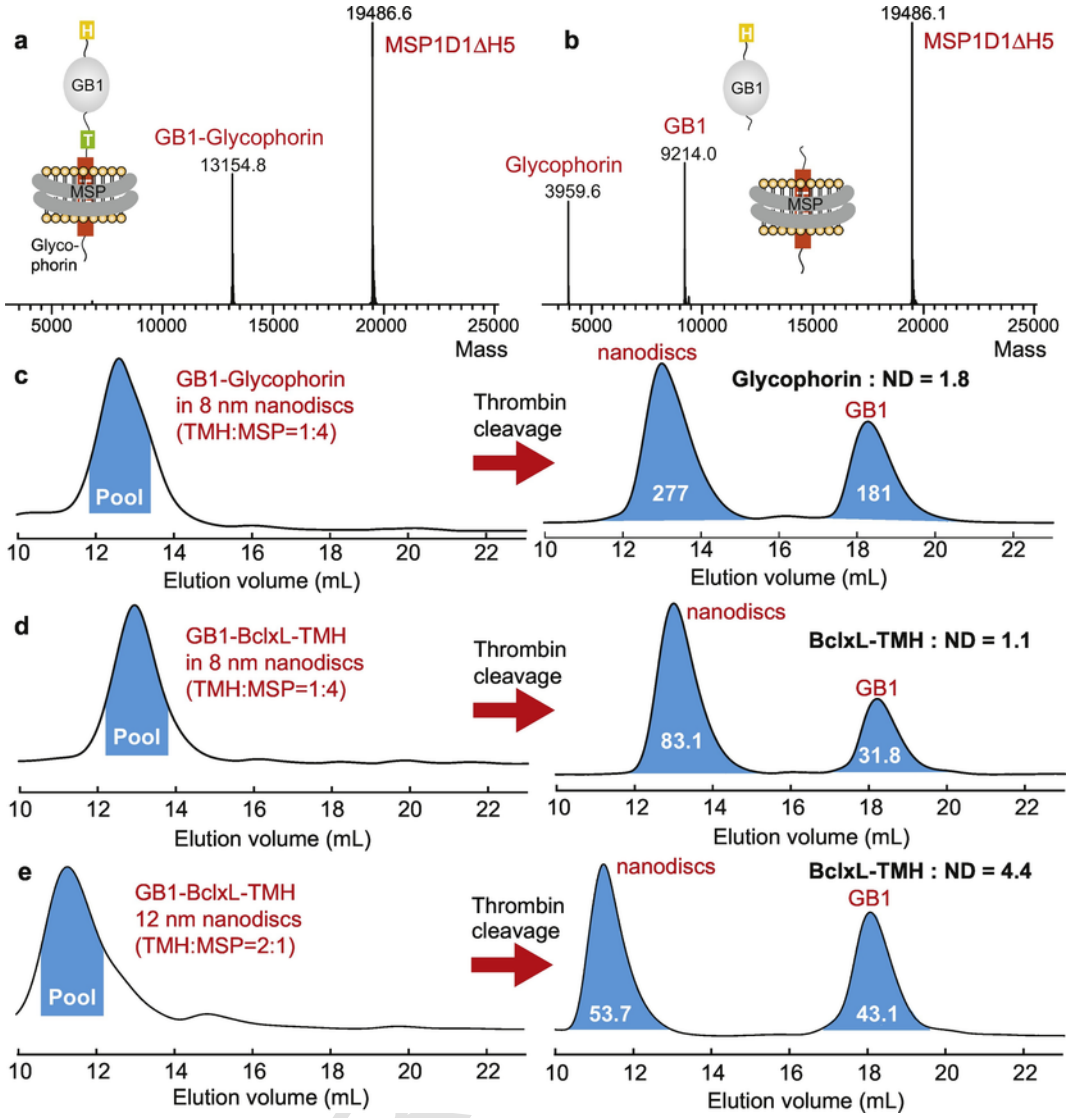

Fig. 4. Analysis of the oligomeric state of transmembrane helices by the GB1 assay. (a,b) Mass spectrometry (MS) analysis of nanodiscs containing the dimeric transmembrane helical protein glycophorinA (GlyA). After nanodisc assembly (a) as well as after Thrombin cleavage (b), the correct mass for the MSP and the inserted GlyA-GB1-fusion protein, GB1 and free GlyA could be detected. (c) Determination of the insertion number of GlyA in nanodiscs yields a value of 1.8 at an assembly ratio of 1:4 (GlyA:MSP). (d) As a comparison, the assay reports a value of 1.1 for the transmembrane helix of the anti-apoptotic protein BclxL in small nanodiscs if a low TMH-to-MSP ratio is used during assembly. (e) A value of 4.4 is obtained in larger 12 nm nanodiscs (assembled with MSP1E3D1) and if using a TMH-to-MSP ratio of 2:1 (leading to an expected number of 4 TMHs per nanodisc). In the BclxL-TMH case, TMH insertion is governed by the assembly conditions suggesting that no dimerization but rather co-assembly takes place.

tive digestion by the protease. This sample was then injected on a S200 column and quantified as described above, yielding a GlyA-to-ND ratio of 1.8. This is very close to the expected value of 2 for this dimeric protein. In order to find a fast way to distinguish unspecific co-assembly from dimerization or oligomerization, we compared the insertion numbers of GlyA with the ones obtained with the monomeric transmembrane helix of BclxL. In case of a monomeric MP that does not have a pronounced homo-oligomerization tendency, one would expect one MP per nanodisc for assemblies using a large excess of MSP, since the loaded nanodiscs are selectively pulled out by NiNTA chromatography, leading to the removal of empty nanodiscs. With a dimeric MP like GlyA, a 1:4 ratio of MP to MSP1D1ΔH5 still gives an average number of almost two copies in one nanodisc. This suggests that the GB1 assay that monitors the number of co-assembled MPs in a nanodisc can be used to estimate the oligomeric state of a membrane protein if assembly conditions are employed that would favor the incorporation of a monomer, i.e. with an excess of MSP compared to the MP. For GlyA, an insertion number of 1.8 in nanodiscs assembled with excess MSP clearly reflects the dimeric state of this well-known protein. As a comparison, we then applied the same strategy to the TMH of BclxL (Fig. 4d) and detected an average number of 1.1 BclxL-TMHs per nanodisc at a TMH:MSP assembly ratio of 1:4, suggesting that BclxL-TMH is most likely a monomer. In order to further corroborate the assumption that the insertion of such a monomeric TMH is simply governed by the TMH:MSP ratio during assembly, as well as the available size in the nanodisc, we increased the size of the nanodisc to 12 nm (with MSP1E3D1 [10]) and used a BclxL-TMH-to-MSP ratio of 2:1, which would lead to four BclxL-TMH copies in each nanodisc. As shown in Fig. 4e, we obtain a value of 4.4 BclxL-TMH in each MSP1E3D1 nanodisc, which nicely matches the expected number. This behavior, where only one TMH is present in the nanodisc in the case of a low TMH-to-MSP ratio during assembly and, at a higher TMH-to-MSP ratio, the number of inserted TMHs is solely determined by the assembly conditions and the available space in the nanodisc, can be considered typical for a monomeric TMH or membrane protein. Thus,

our GB1-based quantification method that reports on the number of co-assembled MPs in lipid nanodiscs can be used to obtain information on the oligomeric state of a MP if suitable assembly conditions with different MP-to-MSP ratios are chosen.

3.5. Nanodisc assembly and quantification with GB1-tagged membrane scaffold protein

Since not every MP can be produced as a fusion protein with GB1, we wondered whether the GB1-tag could also be fused to the MSP instead. By this, the amount of the MSP in MP-loaded nanodiscs can be set equal to GB1. The amount of the MP can subsequently be calculated as described in Eqs. (1)–(3). In order to prove this strategy, we designed GB1-MSP constructs (GB1-Thrombin-MSP1D1 and GB1-Thrombin-MSP1D1ΔH5) harboring an N-terminal His_6-tag, a TEV cleavage site, GB1, a thrombin site followed by the MSP sequence, yielding a GB1-Thrombin-MSP construct that lacks the N-terminal His_6 tag after purification and TEV cleavage. As shown in Fig. 5a, nanodiscs can be assembled with the GB1-MSP fusion constructs just like with the MSP variants without an attached GB1. The resulting larger size of the GB1-MSP nanodiscs can be readily monitored by the altered SEC elution volume (12.7 mL versus 13.6 mL without GB1). Furthermore, VDAC1-loaded GB1-nanodiscs can be purified, cleaved by Thrombin and used for the SEC-based assay (Fig. 5b). By this, we were able to obtain an average insertion number for VDAC1:MSP1D1DH5 (1:8) of 1.08, which is very close to the value that has been obtained with GB1-tagged VDAC1 using MSP1D1ΔH5 (8 nm) nanodiscs (Fig. 2b,c). Thus, we can conclude that the location of the GB1 tag does not influence the nanodisc insertion process of a membrane protein. Furthermore, placing the GB1 tag on the MSP will be very useful for membrane proteins that cannot be easily produced as a GB1 fusion.

4. Conclusions

Here, we report on a method for the quantification of the insertion number of membrane proteins in lipid nanodiscs. We also show that nanodiscs can be used as an efficient tool to control the insertion number of a membrane protein in a native lipid environment. Compared to soluble proteins, determining the oligomeric state of membrane proteins cannot be achieved in a straightforward manner. Among many other obstacles, the presence of a membrane mimicking environment is the main reason for these problems. For soluble proteins, light scattering, detection of diffusion constants or a simple size exclusion chromatography is often sufficient to obtain reliable information. The presence of a detergent micelle or even a nanodisc environment of defined size renders such approaches very difficult if not impossible. Mass spectrometry was developed to characterize lipid-dependent oligomerization of membrane proteins [6,7]. However, such experiments require specialized setup and instrumentation. Thus, methods that rely on absorption spectroscopy are more accessible for any biochemistry laboratory. The quantification of a membrane protein in a membrane mimicking environment requires a frame of reference to determine the relative number of MP per particle. The benefit of the MSP nanodisc system is to take advantage of the MSP that wraps around the lipid bilayer to selectively quantify the amount of inserted MP and the MSP by simple UV absorption at 280 nm. However, since both components are proteins, selective detection of each one is not possible. Recently, it has been shown that membrane proteins containing an intrinsic chromophore with defined spectral properties can be selectively quantified [15]. However, in most cases such chromophores are not available, rendering chemical modification of a membrane protein with extrinsic fluorescence dyes necessary. Unfortunately, the quantification of an extrinsic dye heavily depends on its spectral properties that are governed by its chemical environment. This issue becomes relevant in presence of a

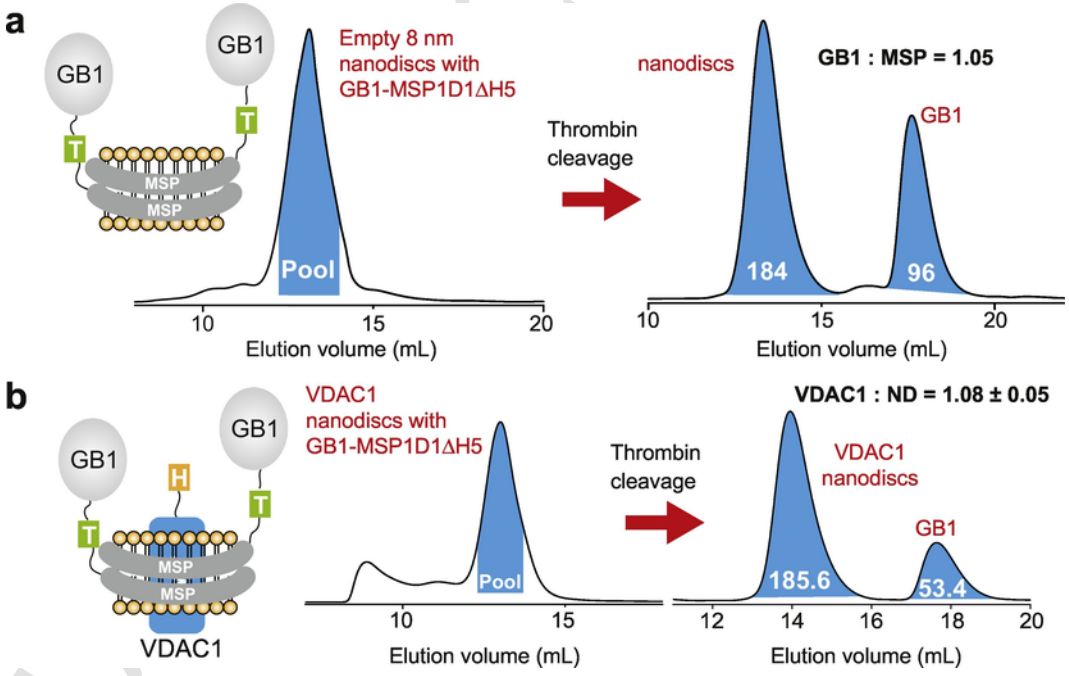

Fig. 5. Fusion of GB1 to MSP yields proper nanodisc assembly and correct insertion numbers. (a) The use of GB1-MSP1D1ΔH5 yields properly assembled nanodiscs (left panel) that can be cleaved with thrombin for the determination of the molar ratio of GB1 and MSP (right). In case of GB1-MSP fusion proteins, this ratio must be 1. Thus, such samples can be used to adjust the calculated molar extinction coefficient (ε_{280}) of GB1 by the offset from the expected 1:1 ratio. (correction by 5%, resulting in an ε_{280} of 10,006 $M^{-1}\,cm^{-1}$ instead of the calculated value of 9530 $M^{-1}\,cm^{-1}$). This value was used for all calculations in this study. In this setup, GB1 reports on the amount of MSP. (b) Using VDAC1-loaded nanodiscs, a calculation similar to Eqs. (1)–(3) can be utilized to derive the VDAC1:nanodisc ratio, which is shown for VDAC1 in GB1-MSP1D1ΔH5 nanodiscs to be 1.08 ± 0.05 (average and standard deviation derived from 3 independent experiments). T: thrombin cleavage site; H: His_6-tag.

membrane, where hydrophobic dyes tend to interact and/or insert. Furthermore, these dyes have a contribution to the absorption at 280 nm, interfering with the absorption and quantification of the protein component. A similar situation is encountered with fluorescent proteins whose absorption properties highly depend on their folding state and the chemical environment complicating such quantitative analyses. With our GB1-fusion protein system, we found a very robust method that can be used for any MP of interest that can be produced as a GB1 fusion protein. In cases where this might not be possible, e.g. if an endogenous protein is used, the GB1 tag can be fused to the MSP instead and used in a similar manner for quantification. The main prerequisite for this system is the ability to assemble and purify MP-loaded nanodiscs right before GB1 cleavage. We usually use Ni-NTA affinity purification followed by SEC to separate empty from loaded nanodiscs, but similar results can also be obtained by using other chromatographic methods, such as ion exchange, which works very well for GPCRs [32].

This study reports on a robust method for the determination of the insertion number of MPs in nanodiscs. For reasonably large membrane protein pores, such as VDAC1, negative-stain EM can be applied to characterize the number of channels in nanodiscs [27,28]. However, in contrast to biochemical methods, this requires access to EM infrastructure and is rather time-consuming. Moreover, the values obtained by negative-stain EM and subsequent figure processing by class averaging are biased by the averaging procedure, i.e. the chosen reference images and the overall heterogeneity of the sample. Even though the correlation is very good in the VDAC1 case, errors in the numbers obtained by EM might be caused by incorrect clustering of monomeric or dimeric/oligomeric species. Furthermore, EM can only provide information on a rather small potentially not representative subset of particles.

Our assay relies on successful and proper nanodisc assembly which needs to be optimized in each case and which can be time consuming. In cases where the MP of interest is forming large and unspecific oligomeric arrays, nanodisc assembly would not work, rendering this method not suitable. However, since in many cases, structural and functional investigations of membrane proteins are conducted in nanodiscs, this issue needs to be addressed anyway and is often already solved before. Then, the presented workflow can be nicely incorporated into the nanodisc assembly and purification procedure. Another critical point for obtaining reliable information with the GB1 assay is the careful calibration of the extinction coefficients of the proteins in the assembly reactions. We found that a deviation of 5–10% would lead to a change in the calculated ratio of up to 0.2. For most applications, this is not a serious problem, however, to obtain accurate numbers, a calibration is necessary. For doing so, we make use of the GB1-MSP nanodiscs where the amount of cleaved-off GB1 is equal to the amount of MSP. We usually scale the extinction coefficient of GB1 to obtain equal numbers for both components. Another option is to determine the extinction coefficient of each component using chemical unfolding [33]. If, in addition, efficient cleavage by the chosen protease and accurate integration of the SEC peaks is ensured, our method provides reliable numbers.

In summary, we have developed a versatile and robust method for the determination of the number of membrane proteins in lipid nanodiscs that can be used to estimate its tendency to form oligomers. MSP nanodiscs of various sizes can be employed to populate a desired oligomeric state of a membrane protein for functional and structural studies. In these cases, the presented GB1 assay can be used to quickly detect the number of inserted MPs in order to optimize assembly conditions. This method is another example for the wide applicability of the MSP nanodisc technology which offers the opportunity for protein engineering [12,22,27,34] and other chemical modifications in a highly specific manner.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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