Current Topics

Recent Progress in Biophysical Research of Biological Membrane Systems

Review

Nanodiscs for Structural Biology in a Membranous Environment

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The structures of many membrane proteins have been analyzed in detergent micelles. However, the environment of detergent micelles differs somewhat from that of the lipid bilayer, where membrane proteins exhibit physiological functions. Therefore, a more membrane-like environment has been awaited for structural analysis of membrane proteins. Nanodiscs are "hockey-puck"-shaped lipid bilayer particles that distribute in a monodispersed manner in aqueous solution. We review how nanodiscs or protein-reconstituted nanodiscs are prepared and how they are utilized to analyze protein structure, dynamics, and interactions with lipid molecules using solution NMR and cryo-electron microscopy.

Key words nanodisc; membrane protein; structural analysis; functional analysis; solution NMR; cryo-electron microscopy

1. Introduction

Membrane proteins, such as G-protein-coupled receptors (GPCRs), ion channels, and transporters, are involved in signal transduction and/or the transport of ions, small molecules, and macromolecules across the membrane. Since many membrane proteins function by changing their conformations or shifting the conformational equilibrium in response to ligand binding or other stimuli such as changes in membrane potential, it is important to reveal their functional structure and dynamics for understanding their functional mechanisms. The structures of many membrane proteins have been determined at atomic resolution, where most proteins are solubilized in detergent micelles. However, the environment of micelles differs somewhat from that of the lipid bilayer, where membrane proteins function (Fig. 1). For the analysis of the physiological structure and function of membrane proteins, a number of technologies have been developed for their *in vitro* reconstitution in a membranous environment such as bead-linked proteoliposomes,[1] bicelles,[2,3] salipro,[4] styrene maleic acid lipid particles (SMALPs),[5] and nanodiscs.[6] Among these methods, nanodiscs are now widely used for structural and functional analyses of membrane proteins and/or those in a lipid bilayer-like environment.

A nanodisc is composed of discoidal lipid bilayers of phospholipids surrounded by two pairs of amphipathic membrane scaffold proteins (MSPs)[7,8] (Fig. 1C). Since both the top and bottom of the bilayer surfaces are exposed to aqueous solution, it is possible to analyze the interactions of ligands in solution, such as membrane-associating proteins, with the surface of the lipid bilayer. Reconstitution of integral membrane proteins in nanodiscs enables the analyses of the structure and dynamics of membrane proteins in lipid bilayers using solution NMR and cryo-electron microscopy (cryo-EM). Hydrophobic ligands, such as cholesterol and fatty acids, regulating protein functions can also be incorporated in the lipid bilayer in nanodiscs, which contributes to revealing the functional structure and dynamics of membrane proteins.

Here, we review recent progress in structural and functional analyses using solution NMR and cryo-EM of proteins with nanodiscs as a membranous environment.

2. Preparation of Nanodiscs

The general protocol for MSP production and forming nanodiscs can be found in previously published papers[7–12] or online (http://sligarlab.life.uiuc.edu/nanodisc/protocols.html). In brief, detergent-solubilized phospholipids, membrane proteins, and MSPs are mixed, followed by removal of detergent molecules by dialysis or detergent absorbent beads such as Bio-Beads SM-2 (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), resulting in the formation of nanodiscs by self-association.

After the removal of the detergent, nanodiscs are separated by size-exclusion chromatography (SEC). Any composition of bilayer-forming phospholipids can be used for making

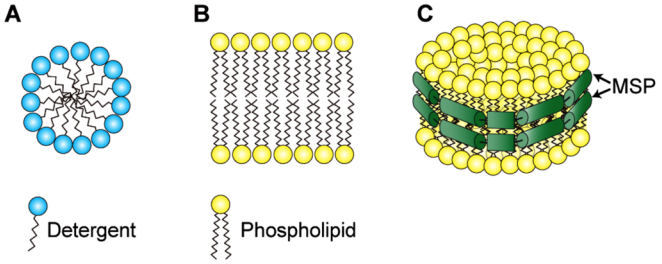

Fig. 1. Schematic Drawings of Membranous Environments

(A) Detergent micelle, (B) lipid bilayer, (C) nanodisc. (Color figure can be accessed in the online version.)

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nanodiscs. Optimization is necessary for other variables in the nanodisc assembly step, such as nanodisc size, lipid/MSP ratio, and membrane protein/MSP ratio. Nanodisc size is solely defined by the MSP length. The optimal ratios of lipid/MSP as well as membrane protein/MSP are dependent on the MSP length, lipid composition, and membrane proteins of interest, and they should be empirically determined by a series of test assembly reactions and subsequent investigation of the homogeneity of the nanodiscs by SEC.

An MSP is an amphipathic helical protein engineered based on the amino acid sequence of apolipoprotein A-I (apoA-I), a major protein component of high-density lipoprotein (HDL) particles. In order to control the diameter of nanodiscs, a series of MSP variants, possessing helices with different numbers and/or sequences, have been developed[8,13] (Table 1). Truncated MSPs were also developed to make smaller nanodiscs for their easy application to solution NMR.[14,15] Recently, covalently circularized MSPs have been developed to prepare more stable, homogeneous nanodiscs, which also enables the making of nanodiscs larger than those prepared by conventional linear MSPs.[15–17] Peptide-based nanodiscs composed of amphipathic helix-forming peptides derived from apoA-I are another potent approach to modulate the size of nanodiscs by only changing the lipid/peptide ratio using a single peptide, instead of changing the MSP length.[18–20]

3. Application of Nanodiscs to Solution NMR Analyses

Since nanodiscs are soluble in aqueous solution, they are readily applicable for solution NMR analyses of integral membrane proteins, membrane-anchored proteins, and membrane-binding proteins. While the molecular weight of nanodiscs is large for conventional solution NMR, valuable, unique information on protein function can be obtained by combining it with improved NMR techniques.

The stability of membrane proteins is markedly improved by reconstitution into a nanodisc. CCR5 is a member of the GPCR family and plays important roles in the inflammatory response; however, the instability of CCR5 in detergent micelles had hampered its application to structural and functional analyses. Reconstitution of CCR5 in a nanodisc enabled CCR5 to maintain its functions for longer than 24 h and to undergo structural and functional analyses.[25]

Membrane proteins exhibit physiologically dynamic features in the native-like membrane environment of nanodiscs. KcsA, a pH-dependent K^+ channel, exhibits structural equilibrium between permeable and impermeable conformations of the selectivity filter under acidic conditions. Methyl-detected NMR analyses revealed that the structural equilibrium in nanodiscs was significantly different from that in detergent micelles[22] (Fig. 2). The transition between permeable and impermeable conformations in nanodiscs is well correlated with the open probabilities revealed by electrophysiological assays,

Table 1. MSPs and Membrane Scaffold Peptides Utilized for Preparing Nanodiscs

Conventional MSPs[a]			
Name	No. of apoA-I helices	Diameter of nanodisc (method)	Reconstituted membrane protein
MSP1	10	9.8 nm (SAXS)[8]	CD4 (1 TM),[21] KcsA (8 TMs)[22]
MSP1E1	11	10.6 nm (SAXS)[8]	Coxsackievirus and adenovirus receptor (1 TM)[23]
MSP1E2	12	11.9 nm (SAXS)[8]	Bacteriorhodopsin (bR) trimer (21 TMs)[24]
MSP1E3	13	12.9 nm (SAXS)[8]	bR trimer (21 TMs),[24] CCR5 (7 TMs),[25] Kv1.2–2.1 chimera (24 TMs)[26]
MSP1D1	10	9.7 nm (SAXS)[8]	ABCG2 (12 TMs)[27]
MSP1E3D1	13	12.1 nm (SEC)[8]	Ryanodine receptor (24 TMs)[28]
MSP2N2	20	~17 nm (SAXS)[13]	TRPV1 (24 TMs)[29]
Truncated MSPs			
Name	No. of apoA-I helices	Diameter of empty disc (method)[14]	Reconstituted membrane protein
MSP1D1ΔH4	9	7.8 nm (EM)	[b]
MSP1D1ΔH5	9	8.2 nm (EM)	[b]
MSP1D1ΔH4H5	8	6.9 nm (EM)	[b]
MSP1D1ΔH4-H6	7	6.3 nm (EM)	[b]
Covalently circularized MSPs (cMSPs)			
Name	No. of apoA-I helices	Size[17]	Reconstituted membrane protein
cNW9	9 (cMSP1D1ΔH5)	~8.5 nm (EM)	NTR1 (7 TMs)[30] VDAC-1 (β-barrel)[30]
cNW11	10 (cMSP1D1)	~11 nm (EM)	VDAC-1 (β-barrel)[30]
cNW30	28	~15 nm (EM)	[c]
cNW50	46	~50 nm (EM)	[c]
ApoA-I-mimicking peptides			
Name	Amino acids (aa)	Diameter of disc (method)/reconstituted membrane protein	
NSP	37 aa	11.7 nm (DLS)/no membrane protein[18]	
NSPr	37 aa	11.7 nm (EM)/MalFGK$_2$ (14 TMs)[20]	
Beltide-1, Beltide-2, Beltide-3	36 aa (Beltide-1), 38 aa (Beltide-2, Beltide-3)	~11–12 nm (SAXS)/bR (7 TMs)[19]	

a) These plasmids are available through Addgene (http://www.addgene.org). *b*) Among these truncated MSP variants, the MSP1D1ΔH5 nanodisc was optimal for OprG and OprH, which are β-barrel outer membrane proteins.[31] The solution structure of OmpX (β-barrel) was determined in the MSP1D1ΔH5 nanodisc using NMR.[14] *c*) The interaction of poliovirus with CD155-decorated nanodiscs was detected by cryo-EM.[30] SAXS: small-angle X-ray scattering; TM: transmembrane helix.

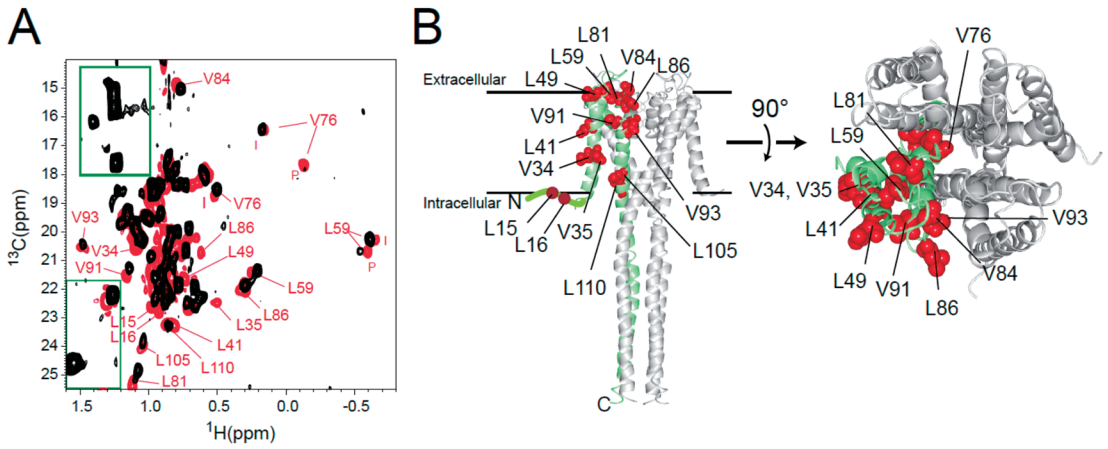

Fig. 2. Functional Equilibrium of the KcsA Structure in a Nanodisc

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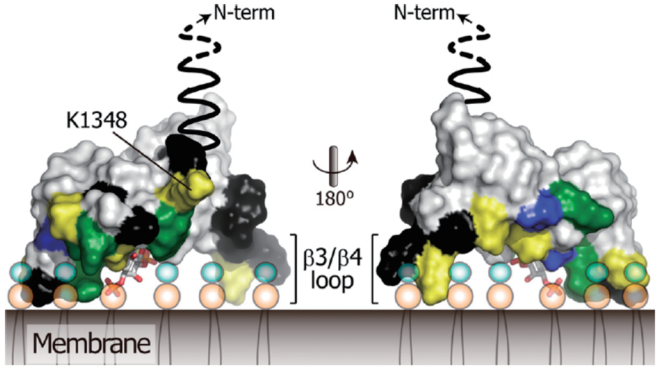

Fig. 3. Model of the Complex of EEA1 FYVE and PI(3)P Embedded in a Membrane

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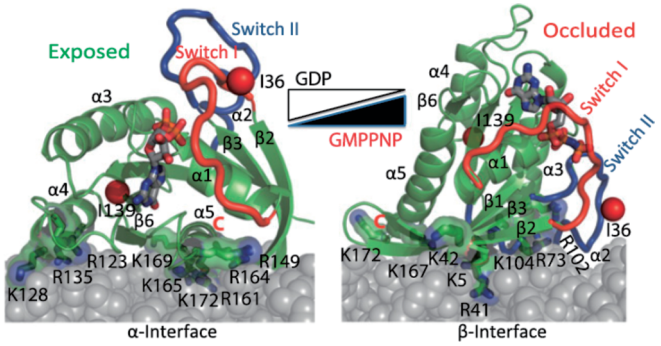

Fig. 4. Models of Functional Dynamics of Lipid-Anchored K-RAS4B on a Membrane

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but that in micelles is not. This result indicates that KcsA in nanodiscs is functionally similar to that in lipid bilayers, and that the equilibrium of KcsA conformations is artificially affected by detergent micelles.

NMR analyses of protein–membrane interactions using nanodiscs are useful for identifying residue-specific interactions in a lipid bilayer environment composed of the desired lipid compositions. Phosphoinositides (PIs) are crucial lipid components of membranes and are involved in various cellular processes through interactions with their effector proteins. Interaction between PI-binding protein and PI using a PI-containing nanodisc indicated the need for a lipid bilayer environment for their interactions,[32] and the relative orientation of PI-binding protein to the PI-embedded membrane under physiologically related lipid bilayer conditions was successfully determined using the FYVE domain of EEA1 and a PI(3)P-containing nanodisc[33] (Fig. 3).

The structural mechanism of membrane-dependent protein activity was elucidated, and the interaction between membrane and protein was proposed as a candidate drug target. The oncogenic protein K-RAS4B is a membrane-anchored protein and the protein–membrane interaction defines its relative orientation to the membrane. NMR investigation of the interaction between K-RAS4B tethered to a nanodisc and the effector protein RAS-binding domains indicated that lipid bilayer-anchored K-RAS4B is in equilibrium between the occluded and exposed conformations at the effector-binding region[34] (Fig. 4). The protein–membrane interaction defines the orientation of membrane-anchored K-RAS4B, which is in equilibrium and important for signal induction activity. A more recent investigation elucidated that a small molecule inhibitor binds to the membrane–protein interface and stabilizes the occluded state of K-RAS4B, which leads to the inhibition of K-RAS4B.[35]

4. Application of Nanodiscs to Single-Particle Cryo-EM

Single-particle cryo-EM is one of the most powerful methods developed recently in the structural biology field. Since the structure of TRPV1, a member of the transient receptor potential cation channels, was reported in 2013 as the first atomic-level cryo-EM structure of a membrane protein,[36] a number of other structures have been determined at atomic or subatomic resolution using single-particle cryo-EM analyses. For such analysis, the quality of protein samples is one of the most important factors. Nanodisc-reconstituted membrane proteins are often monodispersed and stable.[25,28,37] Furthermore, the detergent-free solvent enables reduction of the background noise in EM images. Publications in last 5 years have demonstrated that nanodiscs are a highly versatile technique

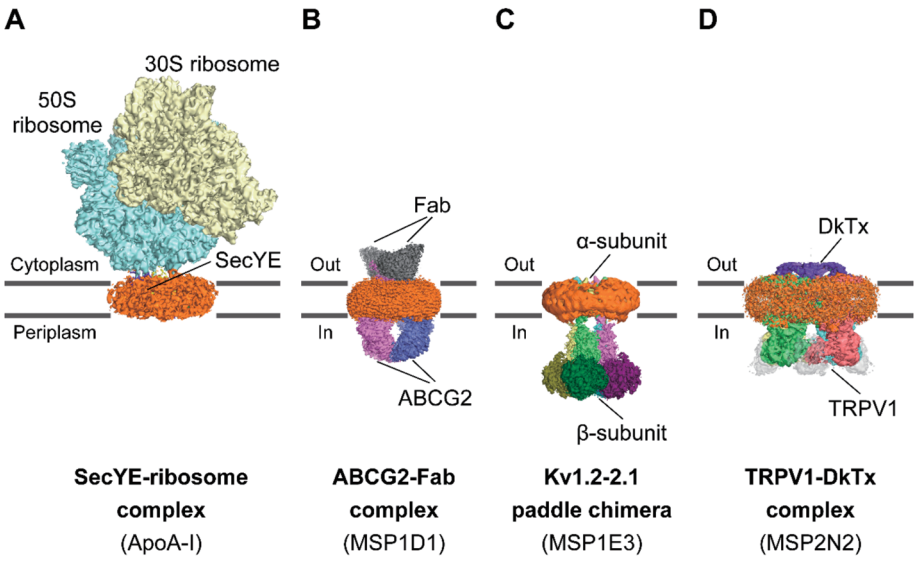

Fig. 5. Cryo-EM Structures of Membrane Proteins Reconstituted in Nanodiscs

(A) Bacterial ribosome-SecYE complex (7.1 Å resolution, EMD-1858). (B) Mammalian multidrug exporter ABCG2 in complex with Fab fragments (3.1 Å resolution, EMD-3953). (C) Mammalian voltage-dependent potassium channel Kv1.2–2.1 paddle chimera (3.3 Å resolution, EMD-9024). (D) Mammalian transient receptor potential cation channel TRPV1 in complex with a double-knot toxin DkTx (2.95 Å resolution, EMD-8117). The density of nanodiscs is shown in orange. The MSPs used for nanodisc reconstitution are shown in parentheses.

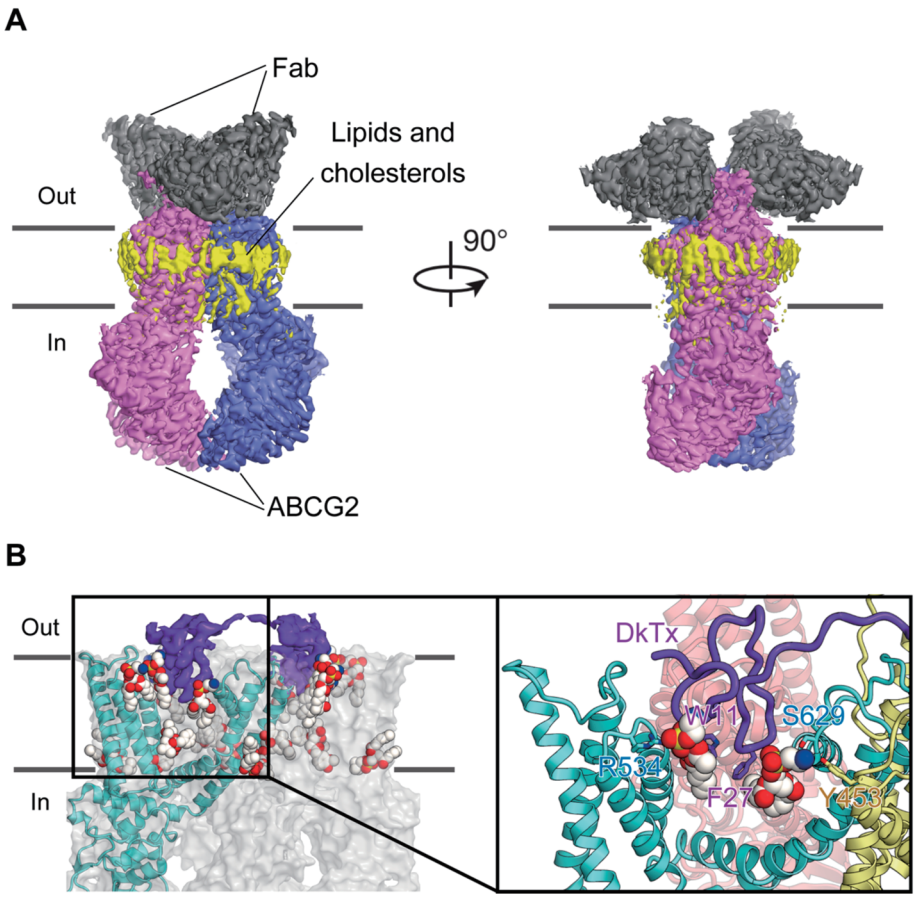

Fig. 6. Lipid–Protein Interactions in Nanodisc-Reconstituted Membrane Proteins

(A) Structure of the ABCG2 transporter (blue and magenta) in complex with Fab (gray) (EMD-3953). Annular lipids and cholesterols bound to ABCG2 are shown in yellow. (B) DkTx-TRPV1 channel–lipid complex (PDB-ID: 5IRX, EMD-8117). Lipid molecules (CPK representation) mediate the interaction between channels (ribbon representation) and toxins (purple). The surface of the channel is shown in gray (left panel). The side chains cooperating in lipid interactions are shown in the ball-stick model (right panel).

for high-resolution cryo-EM analysis of membrane proteins and membrane protein complexes[26–29,37–42] (Fig. 5).

Nanodiscs enable high-resolution structural determination of membrane proteins in the lipid bilayer, successfully visualizing the interactions of the proteins with phospholipids and/or hydrophobic molecules partitioned in the lipid bilayer, such as cholesterol. In the cryo-EM structure of the nanodisc-embedded human multidrug ATP binding cassette (ABC) transporter ABCG2, which is also known as the breast cancer-resistance protein, the density of the phospholipid and/or cholesterol molecules tightly bound to the transmembrane domains was observed[27,43] (Fig. 6A). Cholesterols, which are mainly localized in cholesterol-rich lipid rafts of the plasma membrane, are essential for the function of ABCG2.[44] The structure of the nanodisc-reconstituted ABCG2 suggested the functional importance of these lipid–protein and cholesterol–protein interactions.

Another example is the cryo-EM structure of the nanodisc-reconstituted TRPV1 channel, in which the locations of annular and regulatory phospholipids were well resolved[29] (Fig. 6B). This structure revealed the mechanism for channel activation by bioactive lipids bound in the allosteric regulatory site. Moreover, specific toxin–lipid-channel interactions were observed in this structure. The Trp11 side chain in the toxin, DkTx, interacts with the aliphatic tail of a phospholipid (Fig. 6B). The head group of the same lipid molecule forms a polar interaction with Arg534 in TRPV1. Furthermore, the side chain of Phe27 is stabilized by direct interaction with an aliphatic tail of another lipid molecule, of which the head group interacts with the side chains of Ser629 and Tyr453 in TRPV1. These specific lipid interactions mediate the binding of DkTx to the TRPV1 channel.[29]

Lipid molecules were also resolved in other cryo-EM structures of nanodisc-reconstituted membrane proteins, such as TMEM16[45] and PKD2.[38] Although there are still many unclear points in the regulatory mechanisms and dynamics of membrane proteins in the lipid bilayer, nanodiscs have great potential to allow visualization of lipid/cholesterol–protein interactions in a near-native environment.

5. Conclusion and Outlook

The dispersion of nanodiscs as homogeneous particles in aqueous solution opened new avenues for the structural analysis of membrane proteins in the lipid bilayer and their interactions using solution NMR and cryo-EM. Although conventional nanodiscs are mostly monodispersed, the homogeneity in size is further improved by circularizing MSPs,[16,17,30] which improves the quality of the NMR spectra and EM images.

On the other hand, short lipid-binding peptides are also effective in forming lipid bilayer nanoparticles, as reported for salipro,[4] in which laborious optimization for the selection from several MSPs is unnecessary. Other techniques, such as SMALP,[5] where a styrene maleic acid co-polymer is used to surround lipid bilayer particles, have also been developed.

By utilizing these techniques, not only the physiological structure and dynamics of membrane proteins but also their interactions with regulatory molecules such as phospholipids and/or cholesterols could be further characterized, leading to more complete understanding of the regulation of membrane proteins by lipid-partitioning ligands.

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Conflict of Interest The authors declare no conflict of interest.

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