**Lipid bilayer nanodisc platform for investigating polyprenol-dependent enzyme interactions and activities**

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Lipid bilayer nanodisc platform for investigating polyprenol-dependent enzyme interactions and activities

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Contributed by Barbara Imperiali, November 5, 2013 (sent for review September 13, 2013)

Membrane-bound polyprenol-dependent pathways are important for the assembly of essential glycoconjugates in all domains of life. However, despite their prevalence, the functional significance of the extended linear polyprenyl groups in the interactions of the glycan substrates, the biosynthetic enzymes that act upon them, and the membrane bilayer in which they are embedded remains a mystery. These interactions are investigated simultaneously and uniquely through application of the nanodisc membrane technology. The Campylobacter jejuni N-linked glycosylation pathway has been chosen as a model pathway in which all of the enzymes and substrates are biochemically accessible. We present the functional reconstitution of two enzymes responsible for the early membrane-committed steps in glycan assembly. Protein stoichiometry analysis, fluorescence-based approaches, and biochemical activity assays are used to demonstrate the colocalization of the two enzymes in nanodiscs. Isotopic labeling of the substrates reveals that undecaprenyl-phosphate is coincorporated into discs with the two enzymes, and furthermore, that both enzymes are functionally reconstituted and can sequentially convert the coembedded undecaprenyl-phosphate into undecaprenyl-diphosphate-linked di-saccharide. These studies provide a proof-of-concept demonstrating that the nanodisc model membrane system represents a promising experimental platform for analyzing the multifaceted interactions among the enzymes involved in polyprenol-dependent glycan assembly pathways, the membrane-associated substrates, and the lipid bilayer. The stage is now set for exploration of the roles of the conserved polyprenols in promoting protein–protein interactions among pathway enzymes and processing of substrates through sequential steps in membrane-associated glycan assembly.

Cellular membranes accommodate abundant biological activities, including the transport of small molecules and proteins, energy production, and multistep biosynthetic transformations. These functions are crucial for cell viability; however, studying these processes in biophysical and biochemical detail is challenging because of the complexity of working with both integral and peripheral membrane proteins in lipid bilayer systems. An important class of membrane-associated pathways involves the assembly of complex glycoconjugates, which is dependent on extended polyprenyl groups (1–4). The products of these pathways are essential for cellular viability in all domains of life and, intriguingly, the polyprenols that are used in glycan assembly vary between organisms, with considerable differences in the overall length and degrees of unsaturation (2, 5). For example, bacteria generate peptidoglycan components on the membrane-anchored undecaprenyl-diphosphate before cell wall assembly (6), whereas eukaryotes produce glycan Y-linked protein glycosylation via dolichyl-diphosphate–linked intermediates, which feature polyprenols ranging from 14–25 isoprene units (1, 2, 7). Despite the ubiquitous presence of linear polyprenols as glycan carriers in these important biosynthetic pathways, it remains unclear why these structures have been so faithfully conserved throughout evolution and what might be their functional significance, particularly because extended polyprenols have no other known role in cells (2, 4, 5).

The discovery (8) and biochemical investigation (9–11) of N-linked protein glycosylation in the Gram-negative bacterium Campylobacter jejuni has revealed a canonical polyprenol-dependent glycan assembly pathway. The C. jejuni protein glycosylation (pgl) pathway is an appealing subject for in-depth analysis because the component enzymes can be heterologously expressed and purified in good yields, and can be subjected to protein engineering approaches for the introduction of tags and labels. As illustrated in Fig. 1A, in the pgl pathway, a heptasaccharide is assembled onto undecaprenyl-diphosphate (Und-P-P) by the sequential action of five membrane proteins, designated as PglC, PglA, PglJ, PglH, and PglI. Of these proteins, PglC and PglI are predicted to be integral membrane proteins (12), whereas the other glycan assembly enzymes (PglA, PglJ, and PglH) lack discrete transmembrane domains (TMDs) and are peripheral membrane proteins. Both the integral and peripheral membrane Pgl proteins form insoluble aggregates in the absence of detergent, which further supports a model wherein these enzymes are recruited to the membrane to collaborate in the sequential glycan assembly process. After biosynthesis of the undecaprenyl-diphosphate heptasaccharide is complete, a flippase (PglK) translocates the assembled product to the periplasmic face of the inner membrane (13), where the oligosaccharyltransferase (PglB) transfers the assembled glycan to asparagine residues in selected acceptor proteins (10, 14). After translocation through the outer membrane, glycosylated proteins

Significance

Linear polyprenols are recurring molecular components in the biosynthetic pathways responsible for the assembly of essential glycoconjugates, including peptidoglycan and N-linked glycoproteins. Despite their highly conserved presence in all domains of life, the role of the extended linear polyprenyl groups in the dynamics of membrane-bound glycan assembly pathways remains a mystery. Here we apply the nanodisc model membrane platform to simultaneously assess the interactions and activities of the polyprenyl-linked substrates, enzymes, and lipid bilayer by investigating initial steps from the Campylobacter jejuni N-linked glycosylation pathway. This work represents a proof-of-concept demonstrating that nanodiscs can be used for the precise manipulation and study of polyprenol-dependent pathways.

Author contributions: M.D.H., P.E.S., and B.I. designed research; M.D.H. and P.E.S. performed research; M.D.H. and P.E.S. contributed new reagents/analytic tools; M.D.H., P.E.S., and B.I. analyzed data; and M.D.H., P.E.S., and B.I. wrote the paper.

The authors declare no conflict of interest.

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for the preparation of and lipid ex-
(P-DiNAcBac) to undecaprenyl-
PglC is a phosphoglycosyltransferase contain-
Canonical reaction pathway of bacterial N-linked glycosylation in
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With these parameters in mind, we have turned to nanodiscs,
To gain insight into the interactions among the C. jejuni
glycan assembly pathway enzymes and the specific roles of the poly-
renyl-linked substrates, an ideal model system would allow for re-
constitution of the entire biosynthetic pathway in a native mem-
brane environment. Importantly, the system should be flexible
eough to permit structure/activity studies of enzymes with dif-
ferent membrane components and polyrenyl-linked substrates in a
systematic manner. In addition, the system should be ame-
able to detailed biophysical analysis without the constraints
imposed by the application of detergent micelles or polydisperse
liposome structures.

With these parameters in mind, we have turned to nanodiscs,
a valuable and emerging membrane mimetic technology intro-
duced by the Sligar laboratory (25). In nanodiscs, a membrane
scaffold protein (MSP), derived from the cholesterol transport
protein apolipoprotein A1 (ApoA-1), was engineered to produce
stable self-assembled structures. Two MSPs form a lariat, which
solubilizes the hydrophobic edge of a flat bilayer of lipids to
afford soluble assemblies with diameters ranging from 7 nm to
12 nm. Larger nanodiscs, with diameters ranging from 16–25 nm,
have also been prepared (26), although the current versions of
these discs have been shown to exhibit greater polydispersity.
A variety of synthetic lipids have been used for nanodisc prepara-
tion, including variants of phosphatidylcholine (PC), phosphati-
dylethanolamine (PE), and phosphatidylglycerol (PG), as well as
Escherichia coli lipid extracts (25, 27–29). Diverse membrane
proteins have been incorporated into nanodiscs and studies on
these systems have revealed fascinating nuances of protein struc-
ture, protein oligomeric state, protein–protein interactions, sub-
strate binding, and enzyme activity (30–36). In addition, particularly
noteworthy for the current study, the effects of lipid composition
on enzymes have been probed in a highly reproducible manner
relative to other membrane mimetic systems (37, 38).

To address the fundamental challenge of defining the physical
and biochemical roles of polyrenyls in membrane-associated
pathways, we present the development and validation of an
experimental strategy to reconstitute the first two membra-
re-committed steps of the N-linked glycosylation pathway of C. jejuni
(9, 11) in membrane bilayer nanodiscs (28). The approach in this
study renders the simultaneous and controlled manipulation of
all three determining elements in polyrenol-dependent glycan
assembly at the membrane bilayer a tangible objective.

**Results**

**Incorporation and Characterization of PglC and PglA in Membrane Bilayer Nanodiscs.** PglC is a phosphoglycosyltransferase contain-
ing one predicted N-terminal TMD (11). PglC catalyzes the
transfer of phospho-N,N′-diacetylbacillosamine from UDP-N,
N′-diacetylbacillosamine (UDP-diNAcBac) to undecaprenol-
linked substrates (Und-P) (Fig. 1B). For these studies a GB1 (domain
of Ig binding protein G)–PglC fusion construct was engineered
in which a His 

**Fig. 1.** Canonical reaction pathway of bacterial N-linked glycosylation in C. jejuni. (A) Scheme of the membrane-bound enzymatic conversions that produce the polyrenyl-diphosphate–linked glycan, which is used in protein glycosylation. (B) The reactions of the first two enzymes in the C. jejuni pathway, PglA and PglC, are shown.
Ni-NTA–purified nanodiscs showed an elution profile consistent with a PglC-ND assembly (Fig. 3B and Fig. S2). Nanodiscs were also prepared in the presence of PglC and PglA at an MSP:PglC:PglA ratio of 10:1:1 (5:1:1 ND/PglC/PglA) and subjected to affinity chromatography on Ni-NTA. In this case, the only nanodisc species binding to the stationary phase are those containing His6–GB1–PglC, because the affinity tags of all other components were removed previously. At this stage, two possible outcomes were anticipated: Either there would be a statistical distribution of PglA among all of the discs, and therefore only 20% of the discs isolated after Ni-NTA purification would contain both PglC and PglA, or alternatively, a positive interaction between PglC and PglA in the discs would bias the distribution. When the purified nanodiscs were analyzed by gel densitometry, the PglC:PglA ratio was 1:0.7 (Fig. 3D). This result implies that PglA is colocalized with PglC in ~70% of the population.

Dynamic light scattering (DLS) was applied to estimate the hydrodynamic radii of the various nanodisc assemblies (Fig. 3C). The measurements show particles with average hydrodynamic radii of 6.6 nm for empty nanodiscs, 12.9 nm for PglC-NDs, and 15.4 nm PglC/PglA-NDs, which is consistent with the effect of systematically adding proteins to the nanodisc assemblies. As a control, PglC and MSP in the absence of phospholipids did not affect detectable particles, and it is likely that the protein components precipitated under detergent withdrawal conditions. We note that the measured polydispersities are somewhat high, which may indicate the presence of a population of aggregates, which has been documented previously with DLS measurements on nanodisc assemblies (33).

Fluorescently labeled proteins were reconstituted into nanodiscs using the approaches developed for the unlabeled species. Fluorescent PglC/PglA-NDs were adjusted to the same concentration labels (Fig. 4A) to probe the cofacial organization of the two reconstituted proteins in the nanodisc assembly using FRET. Based on estimated distances in the PglC/PglA-ND (Fig. 4B), the known tetramethylrhodamine (TAMRA) and Cyanine5 (Cy5) FRET-pair (R0 ~ 6.5 nm) was selected. For fluorophore labeling, PglC lacks native Cys residues and therefore single Cys mutations were introduced to generate two mutants for site-specific thiol-directed modification using a Cy5-maleimide derivative (SI Text and Fig. S3). The labeling sites were selected such that the Cy5 would modify either the globular domain of PglC [PglC(Cy5-globular)] or the N terminus [PglC(Cy5-terminal)], which would be localized on the distal side of the membrane bilayer (Fig. 4B and Fig. S3). The chemical labeling proceeded to ~50% conversion and the precise labeling efficiency was quantified following reconstitution of the PglC mutants into nanodiscs. With respect to PglA, the native sequence includes six Cys residues, and therefore an alternate labeling strategy was adopted. Labeling of PglA (Fig. 4A) with the TAMRA fluorophore was achieved by sortase-mediated ligation (40); this method guaranteed quantitative labeling of the donor species, which is beneficial in a static ensemble FRET experiment. For this process, a PglA variant with a C-terminal sortase A recognition peptide [GGGYK(TAMRA)KG] were reacted to yield the modified PglA (TAMRA). The C terminus was chosen for labeling because addition of an N-terminal peptide tag had been found to disrupt the association of PglA with PglC-NDs (Fig. S4). Fluorescence labeling was carried out as summarized in Fig. 4A and presented in the SI Text.

Fluorescently labeled proteins were reconstituted into nanodiscs using the approaches developed for the unlabeled species. Fluorescent PglC/PglA-NDs were adjusted to the same concentration

**Fig. 2.** Protein reconstitution in nanodiscs. (A) Nanodisc self-assembly is initiated by removal of detergent. Nanodiscs are purified using Ni-NTA chromatography, which binds the unique His6-tag on PglC-NDs. (B) SDS/PAGE analysis of Ni-NTA fractions (E, elution fractions; FT, flow through; W, wash) from a PglC-NDs preparation (Upper) and a PglC/PglA-NDs preparation (Lower).

**Fig. 3.** Characterization of nanodiscs. (A) Quantification of PglC-NDs and PglC/PglA-NDs by gel densitometry using quantified protein standards of MSP, PglC, and PglA. The estimated ratios were MSP/PglC = 11.461 pmol and MSP/PglC/PglA = 14.089/96.1 pmol. (B) SEC analysis of PglC-NDs. The void volume is indicated. (C) DLS analysis of (left to right): empty nanodiscs, PglC-NDs, PglC/PglA-NDs, and a nanodisc preparation mixture (PglC-NDs) lacking the lipid components (negative control). The average particle radius (r), the percentage of the total analyzed mass, and the polydispersity (Pd) are displayed in each panel.
with respect to the PglA(TAMRA) donor using the unique absorption signal of the fluorophore at 557 nm. PglC/PglA ensembles contained unlabeled PglC (~50%), which led to a disproportional overestimation of unquenched donor for the two nanodisc ensembles. To account for this high donor background, the labeled fraction of reconstituted PglC in the nanodisc ensembles was quantified by UV spectroscopy to determine the number of possible FRET states (see Methods and SI Text for details).

To perform the FRET experiment, TAMRA was excited at a low wavelength (515 nm) to minimize cross-excitation of the Cy5 acceptor. The fluorescence emission showed defined signals for the donor emission and excitation of the acceptor attributable to FRET (Fig. S5). The resulting spectra of the nanodisc ensembles with different PglC mutants were corrected for the number of non-FRET states in the donor fluorescence based on UV quantification and for background contributions stemming from direct excitation of the Cy5 fluorophore in the acceptor fluorescence (SI Text), as determined by analysis of acceptor-only samples of equal concentration (Fig. 4 C and D and Fig. S5).

The donor signal was quenched for donor/acceptor nanodisc ensembles with both PglC mutants, revealing a higher FRET efficiency in the presence of the PglC(Cy5) acceptor labeled within the globular protein segment (E = 0.57) compared with the terminally labeled PglC(Cy5) (E = 0.43). This trend was also observed in the intensity of the acceptor signals, revealing a stronger signal for the PglC(Cy5-globular) species (Fig. 4 C and D). FRET efficiencies were estimated based on donor signal decay upon quenching, thereby avoiding the use of acceptor standards and circumventing errors arising from endogenous acceptor quenching processes, such as those caused by different probe environments. Assuming the free rotation of the appended fluorophores, the stronger energy transfer for PglC(Cy5-globular) relative to PglC(Cy5-terminal) is good evidence for a model in which the soluble globular domains of PglC and PglA obtain a cofacial arrangement in the nanodiscs, as illustrated in Fig. 4B.

**Functional Reconstitution of PglC and PglA.** Radioactivity tracer studies were used to assess the activities of the enzymes in the nanodisc assemblies. Specifically, undecaprenyl-[33]P phosphate (Und-[33]P) allowed for quantification of Und-P incorporation and also provided an orthogonal radiolabel that was used in concert with [14]C- or [3H]-labeled UDP-sugars (UDP-[14]CdNAcBac and UDP-[3H]GalNAc) for precise tracking of the Und-P-linked species. To establish that Und-P incorporated into the nanodiscs, PglC/PglA-NDs were prepared from PE/PG lipids (3:1) and 0.5 mol percent Und-[33]P. The assemblies were purified by Ni-NTA chromatography, and the radioactivity present in the flow-through, wash, and elution fractions was quantified by liquid scintillation counting, which showed that ~18% of the total radioactivity was associated with nanodiscs (Fig. 5A). Because only 20% of the nanodisc assemblies included PglC, this level of Und-P incorporation was consistent with a statistical distribution of Und-P.

To validate that PglC was functionally reconstituted into the nanodiscs, PglC-NDs with Und-[33]P were treated with soluble UDP-[14]CdNAcBac. After incubation of the reaction for 1 h, an organic-aqueous extraction of the reaction mixture was performed to assess incorporation of [14]C, representing diNAcBac, into Und-[33]P-P-P-[14]CdNAcBac, which would extract into the organic phase. The organic extract was analyzed by normal-phase HPLC and elution fractions were collected and analyzed by liquid scintillation counting. The [33]P signal corresponding to Und-[33]P eluted first, followed by [33]P and [14]C signals that eluted in a single fraction and corresponded to Und-[33]P-P-P-[14]CdNAcBac (Fig. 5B).

The PglC/PglA-NDs were prepared with Und-[33]P and activity was assessed in a similar fashion, but using first unlabeled UDP-diNAcBac and UDP-[3H]GalNAc (Fig. 5C), and then both...
lipid extract, the nanodiscs were pre-
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studies on the
the potential of the approach, we have focused the current
long-chain polyprenols in glycan assembly pathways. To establish
Discussion
the formation of nanodiscs containing stable cofacially oriented
and the glycosyltransferase PglA, and have investigated coin-
the same for all dilutions (Fig. 6
reaction rates re-
tions. Assuming that nanodiscs are stable macromolecular com-
plexes containing cofacially oriented PglC, PglA, and Und-P, then
the observed activity rate should not depend on the concentration
for enzyme activity, the enzymatic reaction rates were determined
in nanodiscs and in detergent-disrupted nanodiscs. Two identical
nanodisc samples were diluted with either buffer or detergent and
for enzyme activity, the enzymatic reaction rates were determined
in nanodiscs and in detergent-disrupted nanodiscs. Two identical
nanodisc samples were diluted with either buffer or detergent and
then assayed for activity. The reaction rate of detergent-disrupted
nanodiscs was \(~12.5\text{−}15\) times slower than the reaction rate of the
undisrupted sample (Fig. 6B).

Finally, evidence for a static colocalization of the PglC and
PglA enzymatic reactions at the nanodisc surface was obtained by
evaluating the reaction rates at different PglC/PglA-ND dilu-
tions. Assuming that nanodiscs are stable macromolecular com-
plexes containing cofacially oriented PglC, PglA, and Und-P, then
the observed activity rate should not depend on the concentration
of PglC, PglA, or Und-P, but rather the absolute amount of these
components. To test this theory, an equimolar amount of nanodiscs
was assayed at three different dilutions and the concentra-
tions of the soluble UDP-sugars were kept constant. The initial
reaction rates reflected in the slopes of the reaction turnover are
the same for all dilutions (Fig. 6C). This finding is consistent with
the formation of nanodiscs containing stable cofacially oriented
PglC and PglA in tandem with available Und-P substrate.

**Discussion**

We present an experimental platform that exploits membrane
bilayer nanodiscs for investigating the functional roles of linear
long-chain polypropenols in glycan assembly pathways. To establish
the potential of the approach, we have focused on the current
studies on the two membrane-committed steps in the *C. jejuni*
Pgl pathway carried out by the phosphoglycosyltransferase PglC
and the glycosyltransferase PglA, and have investigated coin-
corporation of the enzymes into nanodiscs using multiple bio-
chemical and biophysical methods.

**Protein Interactions at the Membrane Interface.** We demonstrate
that PglC incorporates into nanodiscs as a functional monomer
{(Figs. 2 and 5, and Fig. S1)}, which is unique in showing the
oligomeric state of a phosphoglycosyltransferase being probed
in a lipid bilayer. Furthermore, we show that PglC and PglA
through the application of triple isotopic-labeling approaches using the uniquely labeled substrates Und-[3-3H]P, UDP-[14C] \( \text{diNacBac} \), and UDP-[\(^{14}C\)]GalNac (Fig. 5D). Importantly, the ability to carry out quantitative analysis of activities, for example in the disc disruption and disc dilution assays (Fig. 6B and C), reveals the impact of enzyme colocalization in a 2D membrane. The results from such studies are crucial for deepening our understanding about how membrane-bound enzymes in multistep pathways function in vivo.

Although the current studies have focused on PglC and PglA, the native Pgl pathway comprises five glycan assembly enzymes (PglC, PglA, PglJ, PglH, and PglI, Fig. 1A), and intriguingly only PglC and PglI are predicted to be integral membrane proteins by sequence analysis (12). Based on this established foundation of methods, the nanodisc platform should be readily applicable for investigating the specific roles of PglC and PglI in recruiting the entire cluster of enzymes to the membrane bilayer interface where they function. Additionally, the opportunities for orthogonal labeling of substrates and intermediates will enable studies to examine whether substrates are transferred directly between sequential enzymes in the pathway. Because substrate processivity in glycan assembly pathways is poorly understood, this approach promises to provide the experimental system for addressing this key issue.

**Structure/Function Relationships at the Lipid Bilayer.** It is well documented that nanodiscs can be assembled from a variety of phospholipids, which may reflect native or nonnative compositions. In these studies we demonstrated that disc preparations made with native *E. coli* lipids result in a measurable background activity in the absence of added Und-P, presumably because the native *E. coli* lipid extract included a small amount of endogenous Und-P (Fig. 6A). This background activity is completely abolished with discs assembled with synthetic phospholipid components. Importantly, addition of dosed quantities of Und-P restores robust activity that can be readily quantified by using isotopically labeled substrates. These studies enable us to make measurements that are simply inaccessible through convention methods, the nanodisc platform should be readily applicable for investigating the specific effects of lipid bilayer composition on the functional efficiency of the sequential membrane enzyme activities.

The current studies take the versatility of nanodiscs one step further by showing the opportunity to investigate glycan assembly enzymes that require a membrane-bound substrate, in this case a polyisoprenyl-phosphate derivative. To our knowledge, this demonstration of enzymes acting on coincorporated membrane-bound substrates in nanodiscs is unique. Previously, the activity of a series of diverse polyisoprenyl-phosphate substrates was investigated using PglC as a detergent micelle preparation (16). The studies revealed that although unsaturation of the α-isoprene and a particular combination of E and Z isoprene units were key features determining enzyme activity, the length of the polyisoprenyl chain had significantly less impact on protein activity. However, in a detergent-based assay, the effects of polyisoprenol length on membrane partitioning and dynamics would have been taken out of consideration. With the nanodisc approach, we are now in a unique position to quantitatively assess structure/function relationships of native and nonnative polyisoprenyl-linked substrates and phospholipids directly in a membrane bilayer, which will enable us to make measurements that are simply inaccessible through any other in vitro or in vivo system. Furthermore, we will be able to assess the impact of capturing the substrates and enzymes in a 2D bilayer, which is likely to have profound implications for substrate availability and propensity of the turnover (42–44).

**FRET Analysis of Protein Interactions in Nanodiscs.** In contrast to vesicular membrane model systems such as liposomes, both faces of the nanodisc are accessible for interactions with substrates and enzymes. Therefore, we developed orthogonal labeling strategies to prepare fluorescently modified variants of PglC and PglA to enable a FRET analysis to assess the topological relationship between the globular domains of the enzymes in the nanodiscs. The studies clearly reveal a preference for a cofacial relationship.
of the globular domains; FRET efficiency is higher when PglC is labeled on the globular domain compared with N-terminal labeling, which places the label on the distal side of the membrane (Fig. 4 C and D). At the current time, further quantitative analysis of the FRET efficiency extracted from the labeled nanodiscs is not yet available on either PglC or PglA. The finding that the globular domains are cofacially oriented is corroborated by activity analyses and the functional reconstitution of the PglC/PglA-ND. In particular, in experiments using precisely quantified Und-[33]P-P, all of the available of Und-P is converted to final product (Fig. 5 B–D). If the major population of discs did not include PglC and PglA oriented with cofacial active sites, the extent of conversion would be lower because assemblies with the two enzymes oriented on opposite faces would not be able to process two sequential reactions to afford Und-PP-diNAcBac-GalNAc.

Although the FRET analyses in the present studies were carried out with an ensemble of discs including only two protein species, the opportunities for fluorescent labeling of other enzymes in the glycan assembly phase of the pathway (PglJ, PglH, and PglI) using similar orthogonal labeling approaches suggests that the nanodisc platform can be extended to the entire pathway. In this context, it should be noted that although the analysis of nanodisc species as an ensemble was relatively complicated because of incomplete labeling of one of the two enzymes, single-molecule approaches would avoid this complication and render analysis of more than two differentially labeled species feasible. Single-molecule approaches have already been applied to nanodisc systems and, therefore, all of the components are in place for taking the current inquiries on the pgl pathway and the roles of polyprenols in general to the next level of complexity (45).

Conclusion

The application of nanodiscs to the well-characterized C. jejuni pgl pathway provides an exciting experimental platform for addressing the evolutionary purpose of linear polyprenols in glycan biosynthetic pathways. The present study presents key steps in establishing the platform and demonstrates that the system delivers robust quantitative measurements of activity that will be valuable for investigating the specific roles of polyprenyl-linked substrates in the integrated function of glycan assembly enzymes typified by components of the bacterial pgl pathway. The study highlights the interaction of PglC and PglA, which are responsible for the first two membrane-committed steps in the pgl pathway. Concorporation of these enzymes into nanodiscs is characterized by multiple biophysical methods that support the co- and cofacial localization of the proteins. In tandem with PglC and PglA, the undecaprenyl-phosphate substrate is also incorporated into nanodiscs and the functional reconstitution of a PglC-PglA complex is verified through biochemical assays. This work represents a proof-of-concept demonstrating that nanodiscs can be used for the precise manipulation and study of polyprenol-dependent pathways. The study is also unique in providing a set of evidence supporting the hypothesis that enzymes dependent on polyprenyl-phosphates associate into bio-synthetic macromolecular complexes.

Methods

Protein Expression, Purification, and Site-Specific Modification of PglC and PglA Variants. Standard techniques of molecular biology and biochemistry were used to clone, express, and purify His6-G810-TEV-PglC, the accompanying single Cys mutants (C7 and S145C), His6-TEV-PglA, and PglA-LPETGG-His6-Pgl single Cys mutants were modified using Cys maleimide and PglA was modified using a sortase-mediated ligation (40) with GGGY(K/TAMARA)KGG. Detailed protocols are included in SI Text. See Tables S1 and S2 for primers used in preparation of PglC and PglA DNA constructs.

Materials. All phospholipids, radioactive compounds, amino acid derivatives and fluorescent labeling agents were purchased from commercial vendors. UDP-[33]P-P, UDP-[33]C]diNAcBac, and phosphoroylated using chemical or enzymatic methods (SI Text) Und-[33]P-P was prepared by using Streptococcus mutans kinase and [γ-33]P]ATP (46). UDP-diNAcBac was prepared using a chemoenzymatic approach exploiting PglF, PglE, and PglD of the pgl pathway (47). For the preparation of UDP-[^33]P]Cl-dNicBac, [[^33]P]acetyle-CoA was applied in the PglD-catalyzed step.

Nanodisc Preparation and Characterization. Nanodisc assembly methods were based on established protocols (27, 29), and involved incubation of the protein components (MSP and PglC in the presence and absence of PglA with membrane lipids containing Und-P at defined ratios. Self-assembly of nanodiscs was initiated by detergent removal with hydrophobic adsorbants (e.g., biobeads). Nanodisc assemblies are purified using the unique His6-tag handling, which places the label on PglC by using Ni-NTA affinity chromatography. Nanodisc assemblies were characterized using SDS/PAGE, SEC, and DLS. Specific quantification of proteins was carried out using Coomassie blue staining followed by gel densitometry. Detailed protocols are described in SI Text.

FRET Analysis. Nanodiscs for FRET analysis were prepared by creating nanodiscs containing the following reconstituted variants of PglC and PglA: (i) PglA(TAMRA) and unlabeled PglC; (ii) PglA(TAMRA) and PglC(Cy5-terminal); (iii) PglA(TAMRA) and PglC(Cy5-globular); (iv) unlabeled PglA and PglC(Cy5-terminal); (v) unlabeled PglA and PglC(Cy5-globular). The concentrations of the PglA(TAMRA) containing nanodiscs were adjusted to identical concentration on basis of the donor TAMRA UV-absorption signal. Acceptor-only samples iv and v were adjusted in concentration to match samples ii and iii, respectively, applying the Cy5-absorption signals. FRET data were acquired by excitation at 515 nm to minimize direct acceptor excitation. Because of the different labeling strategies, the PglA construct was quantitatively labeled, whereas labeling of PglC was incomplete. To minimize errors resulting from different degrees of labeling between the two species, the fractions of labeled versus nonlabeled PglC were stringently quantified after nanodisc assembly to account for overestimation of the donor fluorescence because of the population of non-FRET PglC/PglA-NDs with unlabeled PglC. These non-FRET states were represented by a fraction of 45% in the case of the PglC-globular species and 56% in case of PglC-terminal. To account for this, the “donor-only” emission spectrum (i) was scaled according to the amount of the non-FRET states in each sample. The two resulting donor-only curves were used to correct the spectra of the donor/acceptor FRET samples (i and iii). Subsequently, acceptor signal contributions stemming from direct excitation of the acceptor were subtracted using data from acceptor only samples (iv and v; full analysis is presented in Fig. S5 and SI Text).

Activity Analysis. Enzyme function in the nanodisc assemblies was assessed using isotopically labeled substrates (Und-[33]P-P, UDP-[33]P]Cl-dNicBac, and UDP-[33]P-H]GalNAc). For all concentrations and specific activities, see SI Text. For product analysis by HPLC, four nanodisc preparations containing PglC alone or PglC and PglA were prepared with 0.5 mol percent Und-[33]P-P. PglC-NDs were incubated with UDP-[33]P]Cl-dNicBac. PglC/PglA-NDs were incubated with unlabeled UDP-diNAcBac and UDP-[33]P-H]GalNAc, or UDP-[33]P]Cl-dNicBac and UDP-[33]P-H]GalNAc. As a control reaction, PglC/PglA-NDs were incubated with UDP-[33]P-H]GalNAc in the absence of UDP-diNAcBac.

After 1 h, the reactions were quenched in chloroform:methanol (2:1, 1 mL) and the organic layer was washed with chloroform:methanol: 0.1 M aqueous potassium chloride (3:48:3, 3× 400 μL). The organic layer was dried and resuspended in chloroform:methanol (4:1, 100 μL) for analysis by NP-HPLC. Fractions of 1 mL were collected and each fraction was dried under an N2 stream. Radioactivity was determined by liquid scintillation counting.

Activity analysis for assessing the effects of lipid composition and detergent-based nanodisc disruption was carried out using PglC/PglA-NDs prepared with unlabeled Und-P. Reactions were initiated by the addition of UDP-diNAcBac and UDP-[33]P-H]GalNAc, and aliquots were quenched at various time points and then extracted to isolate organic soluble radiolabeled products (Und-P-P-diNAcBac-[33]P-H]GalNAc). For nanodisc disruption, we used Triton X-100 as a nonembedding detergent at a concentration of 0.175% (vol/vol), which is sufficient to completely disrupt the nanodiscs as verified by Ni-NTA chromatographic analysis (Fig. S6 and SI Text). Additionally, measurements of the coupled PglC/PglA reaction show that activity is maximal at 0.175% (vol/vol) Triton X-100 (Fig. S6).

For the dilution assays, Und-[33]P-P was used to enable accurate quantification of the disc-loaded substrate and the rates of enzyme-catalyzed conversions were measured by quantifying [33]P]GalNAc incorporation into the Und-[33]P-P-glycan products. Nanodiscs were assayed at 0.05 μM Und-[33]P-P, 0.025 μM Und-[33]P-P, or 0.017 μM Und-[33]P-P, in volumes of 50 μL, 100 μL, and 150 μL such that all three reactions contained equimolar amounts of the disc preparation at different dilutions. The concentrations of UDP-diNAcBac and UDP-[33]P-H]GalNAc remained constant. Aliquots corresponding to 15% of the total starting volume were quenched and extracted, as described above.
9. Janas T, Chojnacki T, Swiezewska E, Janas T (1994) The effect of undecaprenol on membrane skeleton protein for our initial nanodisc studies. Also, the authors are grateful to Dr. Karen Allen, Dr. Angelyn Larkin, and Vinita Lukose for their valuable feedback regarding the manuscript. This work was supported by the National Institute of Health Grant GM039334 (to B.I.); an American Chemical Society award of a Fellowship in Medicinal Chemistry (to M.D.H.) and a Leopoldina Fellowship Program LPDS 2009–38 (to P.E.S.). The Massachusetts Institute of Technology Biophysical Instrumentation Facility is also acknowledged.