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Determination of the biosynthetic pathway of a novel glycolipid involved in preprotein translocation and membrane protein integration

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Research aims

Molecular mechanisms underlying preprotein translocation across, and membrane protein integration into biological membranes are conserved in all organisms at fundamental levels. The understanding of the mechanisms is not only one of the most important subjects in cell biology, but also important for applications such as secretory production of useful proteins and functional analysis of membrane proteins with unknown function as target of drug designs. We developed a reconstitution system of protein translocation and integration in *E. coli*, which reflects the *in vivo* reactions precisely[1,2]. By means of this system, we found that not only the known proteinaceous translocation/integration factors but also a novel complex glycolipid are necessary for the reactions. A subset of membrane proteins was found to be integrated in a manner dependent on only this glycolipid. After this function, we named this glycolipid MPIase (Membrane Protein Integration)[2]. This research was aimed to clarify the biosynthetic pathway of MPIase. To achieve this, we determined the structure of MPIase (Fig. 1), studied the structure-activity relationship, and clarified the mechanisms for MPIase function[3]. Based on these results, we predicted the synthetic pathway of MPIase, and then isolated factors involved in the pathway.

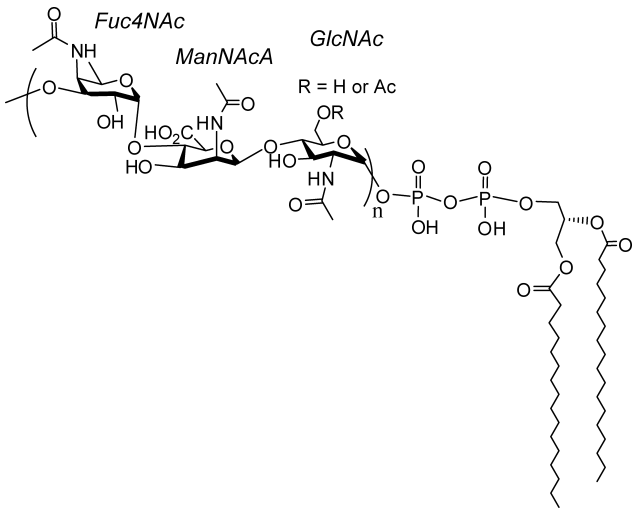

Figure 1. Structure of MPIase. The lipid part possesses fatty acids of C16 or C18. In the glycan chain, MPIase has a repeating unit consisting of 4-acetamide-fucose (Fuc4NAc), *N*-acetylmannosaminuronic acid (ManNAnA) and *N*-acetylglucosamine (GlcNAc), where n=9–11. About 1/3 of R is acetylated.

Methods

Purification and detection of MPIase

Inner membrane vesicles, prepared from *E. coli* MC4100, were successively extracted using urea, sodium cholate and trichloroacetic acid. Highly purified MPIase was obtained by anion exchange and partition chromatographies. MPIase was detected either by anisaldehyde-H_2SO_4 on TLC or by silver on SDS-PAGE[2].

Reconstitution of liposomes and assaying protein integration

MPIase was mixed with phospholipids supplemented with diacylglecerol (DAG) in a solvent (chloroform : ethanol : water=3/7/4), followed by evaporation. Liposomes were then formed in aqueous buffer by sonication. The protein integration activity was measured using 3L-Pf3 coat[4] as substrate, which was synthesized *in vitro* in the presence of liposomes. The integrated materials were detected as proteinase K-resistant bands.

Anti-MPIase antibody

MPIase was crosslinked to KLH, a carrier protein, by means of EDAC, a crosslinker, followed by immunization in rabbits.

Results

1. Structure determination of MPIase

The structure of MPIase was determined by a combination of NMR and MS analyses of natural MPIase and its acid- or alkaline-hydrolysates. Partially synthetic compounds were used to confirm the structure. As a result, the MPIase structure was concluded to be that shown in Fig. 1. Surprisingly, the glycan chain was similar to that of ECA (enterobacterial common antigen), an outer membrane constituent. The differences between MPIase and ECA were that the repeating number in MPIase was n=9–11 while that in ECA was n=18–55, and that the linker to the lipid in MPIase was a pyrophosphate while that in ECA was a

phosphate.

2. Structure-activity relationship of MPIase

MPIase derivatives were obtained chemically and enzymatically for the analysis of structure-activity relationship. A pyrophosphatase cleaved at the center of the pyrophosphate to yield PP-MPIase. On the other hand, both the center of pyrophosphate and acetyl groups in GlcNAc(-R) were digested by NaOH(NaOH-MPIase). PP-MPIase was found to be more active than authentic MPIase, while NaOH-MPIase was inactive as to protein integration. These indicate that the lipid part of MPIase was not essential, but that acetyl groups in GlcNAc were essential.

Next, we examined whether or not ECA was inactive, since ECA possesses a glycan similar to that in MPIase. ECA was found to be completely inactive, suggesting that the glycan length is important for activity.

By means of PP-MPIase, which is soluble and active, we examined whether or not the coupling of protein synthesis with protein integration is necessary. Protein integration occurred after protein synthesis, if PP-MPIase was present during translation, indicative of the post-translational integration. Furthermore, PP-MPIase formed a soluble complex with a membrane protein, indicating that MPIase possesses an integration-dedicated chaperon activity.

3. Isolation of biosynthetic factors of MPIase

A subcellular localization of MPIase was examined using anti-MPIase antibody. As expected, MPIase was localized exclusively in inner membrane of *E. coli*. This antibody also reacted with mature and precursor forms of ECA, because of the similarity of glycan. On the other hands, in the *wecA* and *wecF* mutants, defective in the ECA biosynthesis, no ECA-derived bands were detected, while MPIase was detected, indicating that the biosynthetic pathways of both glycolipids are different.

The first step of ECA biosynthesis is the attachment of GlcNAc to undecaprenyl pyrophosphate. On the other hand, it is assumed that GlcNAc is directly attached to phosphatidic acid (PA) or DAG. For these reasons, we mixed UDP-GlcNAc with either PA, DAG or CDP-DAG in the presence of *E. coli* cytosol or inner membranes. We obtained a possible GlcNAc-PP-DAG compound by mixing UDP-GlcNAc, PA and inner membranes, in a manner dependent on each factor. We purified this activity as the Cdh protein (CDP-DAG hydrolase). However, even in the *cdh* mutant, MPIase was normally expressed, indicating that there is a factor other than Cdh.

Conclusion

In this research, we determined the complete structure of MPIase and studied the structure-activity relationship. We found that MPIase possesses not only the integration-dedicated chaperone activity but also an integrase activity which subsequently functions. We have reported that MPIase functions catalytically[2]. These observations reveal that MPIase possesses an enzyme-like activity, to be called ‘glycolipozyme’.

While the structures of MPIase and ECA are similar, the subcellular localization and biosynthetic pathways are quite different. We isolated Cdh as a first enzyme involved in MPIase biosynthesis, however, the overall biosynthesis of MPIase was not affected by disruption of the *cdh* gene. It is suggested that multiple enzymes are involved in a same step of biosynthesis, consistent with the important function of MPIase *in vivo*. In near future, it is expected that another factor could be isolated using a *cdh* mutant.

References

1) Nishiyama, K. *et al.* (2006) A derivative of lipid A is involved in signal recognition particle/SecYEG-dependent and -independent membrane integrations. *J. Biol. Chem.* **281**: 35667–35676.
2) Nishiyama, K. *et al.* (2010) A novel complete reconstitution system for membrane integration of the simplest membrane protein. *Biochem. Biophys. Res. Commun.* **394**: 733–736.
3) Nishiyama, K. *et al*. (2012) MPIase is a glycolipozyme essential for membrane protein integration. *Nature Communications*. 3, 1260. DOI: 10.1038/ncomms2267
4) Kawashima, Y., Miyazaki, E., Muller, M., Tokuda, H. & Nishiyama, K. (2008) Diacylglycerol specifically blocks spontaneous integration of membrane proteins and allows detection of a factor-assisted integration. *J. Biol. Chem.* **283**: 24489–24496.