BIOCHEMISTRY

Lipopolysaccharide is transported to the cell surface by a membrane-to-membrane protein bridge

David J. Sherman,[1]* Ran Xie,[1]* Rebecca J. Taylor,[1]* Alexander H. George,[1]* Suguru Okuda,[1]* Peter J. Foster,[2,3,4]* Daniel J. Needleman,[2,3,4] Daniel Kahne[1,4,5]†

Gram-negative bacteria have an outer membrane that serves as a barrier to noxious agents in the environment. This protective function is dependent on lipopolysaccharide, a large glycolipid located in the outer leaflet of the outer membrane. Lipopolysaccharide is synthesized at the cytoplasmic membrane and must be transported to the cell surface. To understand this transport process, we reconstituted membrane-to-membrane movement of lipopolysaccharide by incorporating purified inner and outer membrane transport complexes into separate proteoliposomes. Transport involved stable association between the inner and outer membrane proteoliposomes. Our results support a model in which lipopolysaccharide molecules are pushed one after the other in a PEZ dispenser–like manner across a protein bridge that connects the inner and outer membranes.

The cell envelope of Gram-negative bacteria consists of an outer membrane (OM) and an inner membrane (IM). The outer membrane has an asymmetric structure, with the outer leaflet composed of lipopolysaccharide (LPS) and the inner leaflet composed of phospholipid. LPS is a large glycolipid with six fatty acyl chains and numerous sugars (Fig. 1A) (*1*). LPS must be transported from its site of synthesis at the inner (cytoplasmic) membrane across the aqueous space between the two membranes (the periplasm) to the cell surface (*2*–*4*). Lipid transport is fundamental to cellular physiology; however, it is not known how this membrane-to-membrane LPS transport is accomplished or how transport against a concentration gradient is achieved given that there is no adenosine 5′-triphosphate (ATP) in the periplasm (*5*–*9*).

LPS transport requires seven lipopolysaccharide transport (Lpt) proteins (*10*, *11*). At the inner membrane, the heteromeric ATP-binding cassette transporter (LptBFG) associates with a membrane-bound protein (LptC) to form an inner membrane complex. This complex uses ATP hydrolysis to extract LPS from the outer leaflet of the inner membrane and transfer it to LptC and then to a periplasmic protein, LptA (*11*–*16*). LPS is then transported across the periplasm to an outer membrane translocon, LptDE, a large β-barrel protein with a separate luminal protein plug. LptDE inserts LPS into the outer leaflet of the outer membrane (*17*–*21*).

[1]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138, USA. [2]John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA. [3]FAS Center for Systems Biology, Harvard University, Cambridge, MA 02138, USA. [4]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA. [5]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA.
*These authors contributed equally to this work.
†Corresponding author. Email: kahne@chemistry.harvard.edu

Indirect evidence suggests that LptA associates with LptC and LptD to form a protein bridge that spans the periplasm, but the existence of such a bridge has not been established (Fig. 1A) (*22*–*25*).

To investigate whether LPS is transported via a protein bridge, we sought to reconstitute LPS transport using purified components. First, to monitor LPS transfer to LptC, we purified LptBFGC with the photocrosslinkable amino acid *p*-benzoylphenylalanine (*p*BPA) incorporated in LptC at an LPS binding site (fig. S1) (*24*, *26*). We incorporated the complex into liposomes containing LPS and confirmed adenosine triphosphatase (ATPase) activity (fig. S2). We incubated the proteoliposomes with ATP for various lengths of time before exposing them to ultraviolet (UV) light. We observed increasing cross-linking of LPS to LptC over time and in a manner that was dependent on ATP (Fig. 1B).

Next, we wanted to observe LPS transfer to LptA in our purified system and test whether this transfer depends on LptC. We incubated proteoliposomes containing either LptBFG or LptBFGC with LptA containing *p*BPA (*24*). We also prepared liposomes containing the inactive mutant LptB-E163Q in the LptBFGC complex (*15*). We observed that LPS cross-linked to LptA, and the extent of cross-linking was strongly stimulated by LptC (Fig. 1C). This is consistent with earlier in vivo experiments that suggest that LPS is transferred from LptC to LptA (*24*).

With the goal of achieving transport to a second membrane, we generated proteoliposomes containing the outer membrane LptDE translocon with *p*BPA incorporated in LptD (fig. S3) (*27*). Previous work suggests that LptA preferentially associates with the outer membrane (*22*). Therefore, we preincubated the outer membrane proteoliposomes with excess LptA, isolated the LptA-associated outer membrane proteoliposomes, and incubated them with LptBFGC proteoliposomes containing LPS. LPS cross-linked strongly to LptD in the presence of ATP (Fig. 2A and fig. S4). We did not observe cross-linking without LptBFGC or LptA. Thus, all seven Lpt proteins and ATP are necessary and sufficient for membrane-to-membrane transport to occur.

We tested if the rate of LPS transport was affected by ATP concentration using two concentrations flanking the Michaelis constant (K_m) for ATP hydrolysis (fig. S2). Cross-linking intensity was greater at the low ATP concentration and persisted for a longer period (Fig. 2B). At high ATP concentrations, the LPS likely moves more quickly through the site where *p*BPA is incorporated in LptD. This would decrease the probability of cross-link formation because cross-linking efficiency of a ligand to a protein increases with ligand residence time (*28*). These observations are consistent with a process that is powered by ATP hydrolysis.

We wanted to monitor flux of LPS through the pathway at two sites simultaneously, so we incorporated *p*BPA into both LptC and LptD and monitored cross-linking over time. Cross-links between LPS and LptC were observed at the same time as cross-links between LPS and LptD (Fig. 2C). This suggests that release from the inner membrane is coincident with arrival at LptD. Although this is not direct evidence, this result is consistent with rapid transit across a bridge.

If the bridge model is correct, it should be possible to observe an LptA-dependent association between inner and outer membrane Lpt complexes. To allow analysis by flow cytometry, we labeled inner membrane and outer membrane proteoliposomes with different fluorophores and confirmed that transport activity was maintained (Fig. 3A and figs. S5 to S7) (*29*, *30*). Inner membrane or outer membrane proteoliposomes analyzed alone showed fluorescence only in a single channel, as expected (fig. S8). Next, we mixed inner and outer membrane proteoliposomes in the presence or absence of LptA. Without LptA, flow cytometry revealed two populations of fluorescent particles corresponding to either inner or outer membrane proteoliposomes (Fig. 3B, top left panel). Addition of LptA, in contrast, resulted in a large increase in particles with signal in both fluorescence channels. Most of these two-color particles localized to a distinct population (population B) located approximately along the diagonal between the individual IM and OM populations, but there was also a region of more disperse particles located between the IM population and population B, termed population A (Fig. 3B, top right panel).

We wanted to know whether the two-color particles were the result of either liposome fusion or nonspecific association. A fluorescence dequenching assay showed that fusion did not occur in our system (fig. S9) (*31*). Furthermore, we repeated the flow cytometry experiments with a blocked LptA variant containing a protein tag that impedes interaction with LptC or, in a separate experiment, with a truncated LptD variant that is incapable of interaction with LptA. LptA-dependent proteoliposome association was not observed in either case (Fig. 3B, lower panels, and fig. S8). In toto, these data demonstrate that LptA drives a

Downloaded from http://science.sciencemag.org/ on April 9, 2019