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REVIEW ARTICLE

Proteins that bind and move lipids: MsbA and NPC1

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Abstract

Membrane proteins that bind and transport lipids face special challenges. Since lipids typically have low water solubility, both accessibility of the substrate to the protein and delivery to the desired destination are problematical. The amphipathic nature of membrane lipids, and their relatively large molecular size, also means that these proteins must possess substrate-binding sites of a different nature than those designed to handle small polar molecules. This review considers two integral proteins whose function is to bind and transfer membrane lipids within or across a membrane. The first protein, MsbA, is a putative lipid flippase that is a member of the ATP-binding cassette (ABC) superfamily. The protein is found in the inner (cytoplasmic) membrane (IM) of Gram-negative bacteria such as *E. coli*, where it is proposed to move lipid A from the inner to the outer membrane (OM) leaflet, an important step in the lipopolysaccharide biosynthetic pathway. Cholesterol is a major component of the plasma membrane in eukaryotic cells, where it regulates bilayer fluidity. The other lipid-binding protein discussed here, mammalian NPC1 (Niemann-Pick disease, Type C1), binds cholesterol inside late endosomes/lysosomes (LE/LY) and is involved in its transfer to the cytosol as part of a key intracellular sterol-trafficking pathway. Mutations in NPC1 lead to a devastating neurodegenerative condition, Niemann-Pick Type C disease, which is characterized by massive cholesterol accumulation in LE/LY. The accelerating pace of membrane protein structure determination over the past decade has allowed us a glimpse of how lipid binding and transfer by membrane proteins such as MsbA and NPC1 might be achieved.

Keywords: Lipid A, ABC transporter, lipid flippase, lipid binding pocket, cholesterol, sterol trafficking, Niemann-Pick Type C disease, NPC2 (Niemann-Pick disease, Type C2)

Introduction

Complex interactions exist between membrane lipids and membrane-associated proteins, which may be integral, peripheral or lipid-anchored. These proteins are either embedded in the bilayer itself, or interact closely with the membrane surface, so lipids play an essential role, both as modulators of their function, and as substrates for their enzymatic/transport activity. Membrane proteins that use lipids as their substrates face special challenges. Membrane lipids typically have very low water solubility, so accessibility of the substrate to the protein and delivery to the desired destination are always an issue, especially when the lipid substrate or product may have to cross the barrier presented by the membrane. The amphipathic nature of membrane lipids, and their relatively large molecular size, means that proteins which bind lipids, and then either transport or transform them, must possess substrate-binding sites of a different nature than those designed to bind polar molecules such as sugars. The accelerating pace of membrane protein structure determination over the past decade has allowed us a glimpse of how lipid binding and transfer by membrane proteins might be achieved.

This review considers two integral proteins (one bacterial, one mammalian) whose function is to bind and transfer membrane lipids. Despite this functional resemblance, the proteins themselves appear at first glance to be quite different. The first protein, MsbA, is found in the inner (cytoplasmic) membrane (IM) of Gram-negative bacteria such as *E. coli*, and is proposed to move an acylated lipid with a sugar headgroup from the inner to the outer membrane (OM) leaflet; in other words, it is a putative lipid flippase. MsbA is a member of the large ATP-binding cassette (ABC) superfamily, and has been the subject of much controversy. Although crystal structures for the protein have been determined, there is

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Abbreviations:

ABC	ATP-binding cassette
AMP-PNP	adenosine 5′-(β,γ-imido)triphosphate
CMC	critical micelle concentration
DEER	double electron-electron resonance
EPR	electron paramagnetic resonance
ER	endoplasmic reticulum
ERC	endocytic recycling compartment
Fos-choline 13	tridecylphosphocholine
ICL	intracellular loop
IM	inner membrane
Kdo	D-manno-octulosonic acid
LBPA	lyso-bis-phosphatidic acid
LDL	low density lipoprotein
LE/LY	late endosomes/lysosomes
LPS	lipopolysaccharide
NBD	nucleotide-binding domain
NPC	Niemann-Pick Type C
NTD	N-terminal domain
OM	outer membrane
OSBP	oxysterol-binding protein
ORP	OSBP-related protein
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
SCAP	sterol regulatory element binding protein [SREBP] cleavage-activating protein
SSD	sterol-sensing domain
TGN	trans-Golgi network
TM	transmembrane
TMD	transmembrane domain
V_i	ortho-vanadate

limited information about its biochemical properties, or the mechanism by which it binds and potentially moves its substrate, lipid A. Lipid flippases play a very important role in all organisms, both prokaryotic and eukaryotic, but it has proved very difficult to study them. MsbA provides an excellent opportunity to determine the mechanisms by which flippases bind and translocate lipids.

The other lipid-binding protein discussed in this review, NPC1, is mammalian in origin, although homologs exist in flies, worms, and yeast. Mutations in its amino acid sequence lead to a devastating neurodegenerative condition, Niemann-Pick Type C disease, which is characterized by intracellular cholesterol accumulation leading to cell death. Mammalian NPC1 binds sterol, which is a major component of the plasma membrane in eukaryotic cells, and is involved in its transfer out of a membrane-bound compartment as part of an intracellular cholesterol-trafficking pathway. The structure of the portion of NPC1 that binds cholesterol has been determined, but there is little known about the remainder of the molecule, and how the protein carries out its function at a biochemical level remains a mystery. One of the goals of this review will be to highlight similarities that exist between these two proteins, which arise from their common need to bind and transport lipid molecules, and contrast this with the differences in their molecular structure.

MsbA – a bacterial lipid A flippase

Biological role of MsbA

MsbA is an integral protein found within the cytoplasmic membrane of Gram-negative bacteria, including important pathogens such as *Escherichia coli*, *Salmonella typhimurium* and *Vibrio cholerae*. It is a component of the biological pathway that produces the lipopolysaccharide (LPS) molecules that coat the OM of such bacteria. Over the last two decades, MsbA has risen to prominence largely due to its homology with clinically important mammalian drug pumps, such as ABCB1, which have been implicated in the phenomenon of multidrug resistance. A large body of evidence points to MsbA being a flippase protein involved specifically in the movement of the membrane-bound component of LPS across the bacterial membrane. Therefore, before progressing to a discussion of our current understanding of the structure and function of MsbA, we will briefly review the organization of membranes in Gram-negative bacteria.

The envelope of Gram-negative bacteria is a layered arrangement of two membranes, between which lie the periplasmic space and a third layer composed of peptidoglycan. Like other biological membranes the IM and OM both have a bilayer structure composed primarily of amphipathic lipid molecules arranged as two monolayers or leaflets. The IM, which is the functional equivalent of the plasma membrane found in other cells, is composed mainly of glycerol phospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and cardiolipin (Huijbregts et al., 2000). How these lipids are distributed between the two leaflets of the IM is unknown due to technical complications presented by the presence of the OM (Huijbregts et al., 2000). However, for Gram-positive bacteria, which possess only a single membrane, lipids are found to be distributed asymmetrically between the two leaflets, with the inner and outer leaflets showing higher levels of PE and PG, respectively (Rothman and Kennedy, 1977). It seems reasonable, therefore, to expect that this is also the case for the IM of Gram-negative bacteria. Certainly, it is known that the OM exhibits an asymmetric distribution of lipids between its leaflets. The spontaneous movement of lipids between leaflets of membrane bilayers ("flip-flop") is slow, on the order of hours to weeks in reconstituted systems. This is likely due to the high energetic cost of moving the hydrophilic headgroup across the hydrophobic core of the bilayer, which would need an energy input of ~15–50 kcal mol^{-1} relative to available thermal energy of only 1 kcal mol^{-1}. Therefore, cells require a mechanism to enhance the flip-flop of lipids between leaflets. A group of proteins has been identified, termed flippases, that facilitate the rapid transbilayer movement of lipids with timescales of

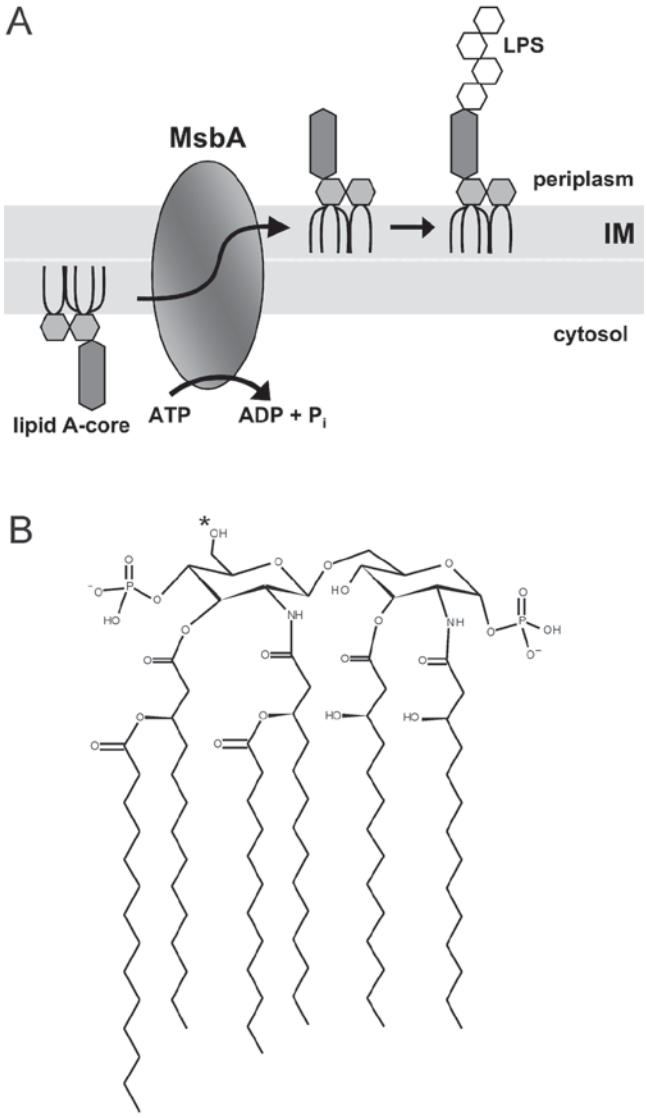

Figure 1. (A) The flippase activity of MsbA transports lipid A-core (composed of Kdo_2-lipid A and the core oligosaccharide) from the inner leaflet to the outer leaflet of the IM of Gram-negative bacteria. The process is powered by hydrolysis of ATP, catalyzed by the C-terminal NBDs. Lipid A-core is subsequently glycosylated with O-antigen to form LPS, which is then transported to the OM. (B) Structure of bacterial hexa-acylated disaccharide Lipid IVA, which is the membrane-bound moiety of the LPS molecule. The hydroxyl group marked with a * is the site for addition of the two Kdo residues, followed by the oligosaccharide core, to form the putative substrate for MsbA.

tens of seconds or less (Sanyal and Menon, 2009; Sharom, 2011). MsbA is proposed to be just such a protein operating within the IM of Gram-negative bacteria; it is part of the LPS biosynthesis pathway that generates the polysaccharide coating of the OM.

The inner leaflet of the OM is made up mostly of various glycerophospholipids, whereas the outer leaflet is composed primarily of the large amphipathic molecule lipid A (Figure 1B), which acts as the membrane anchor for LPS (Barb et al., 2007; Raetz et al., 2007). LPS is a complex molecule made up of three parts: lipid A, the core oligosaccharides, and O-antigen repeats. Prior to assembly of lipid A with the core oligosaccharides, two 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) residues are added to lipid A (to form Kdo_2-lipid A), which in *E. coli* is the minimal structure required for cell growth (Wang et al., 2004). Furthermore, lipid A is known to be responsible for the toxic effects of infections by Gram-negative pathogens (Galanos et al., 1985).

LPS biosynthesis and transport across the complex cellular envelope have been studied extensively in *E. coli*, and much progress has been made in identifying the components of this pathway. Numerous proteins are involved in the process, which can only be summarized briefly here. Further details of LPS biosynthesis can be found in recent review articles (Wang and Quinn, 2010; Sperandeo et al., 2009). The biosynthesis of LPS begins at the inner leaflet of the IM with the separate manufacture of the LPS components, lipid A-core and O-antigen. These components are then translocated, or flipped, to the outer leaflet of the IM, where O-antigen is polymerized and ligated to the lipid A-core molecule (Figure 1A). The nascent LPS molecule must then journey across the periplasmic space and peptidoglycan layer to the OM by a process that is not clearly understood. Several proteins are proposed to be involved in this stage, including the IM proteins LptF and LptG, along with the cytoplasmic ATPase LptB, which is thought to actively extract LPS from the outer leaflet of the IM (Ruiz et al., 2008). The last step is incorporation of LPS into the outer leaflet of the OM by a process that has not been clearly defined but is thought to involve the LptDE (Imp-RlpB) complex (Wu et al., 2006).

Early genetic studies were important in identifying the protein components of LPS synthesis. A significant step forward was the identification of a gene product that was capable of restoring normal growth to mutants defective in LPS biosynthesis. One of the proteins involved in the biosynthesis of lipid A in *E. coli* is a lauryltransferase termed HtrB (Clementz et al., 1996). Mutations in the *htrB* gene were shown to result in overproduction of phospholipids and the accumulation of under-acylated derivatives of lipid A in the IM, thereby rendering cells unable to grow above 32°C (Karow et al., 1992; Karow et al., 1991; Karow and Georgopoulos, 1991). Two multicopy suppressors of the *htrB* gene mutations were subsequently found to restore cell viability; these were termed *msbA* and *msbB* (Karow and Georgopoulos, 1993; Karow and Georgopoulos, 1992). The gene product of *msbB* was shown to be an acyltransferase similar in function to HtrB (Clementz et al., 1997; Karow and Georgopoulos, 1992).

Numerous studies have shown that MsbA, the gene product of *msbA*, is directly involved in the trafficking of lipid A across the IM, suggesting that it acts as a flippase. In eukaryotes, a number of proteins in two major protein families (the P_4-ATPase and ABC superfamilies) have been identified as flippases for various classes of lipids (Sharom, 2011). In contrast, MsbA is one of only a few proteins in prokaryotes with a role in lipid translocation (Sharom, 2011), highlighting the significance of this protein for increasing our understanding of flippase activity.

MsbA was initially identified as a member of the ABC transporter family (Polissi and Georgopoulos, 1996; Karow and Georgopoulos, 1993; Zhou et al., 1998). The integral protein was subsequently implicated in the movement of lipid A-core molecules from the inner leaflet to the outer leaflet of the IM (Doerrler et al., 2004; Zhou et al., 1998). Depletion of MsbA or loss of MsbA function was found to result in the accumulation of LPS and phospholipids in the IM of *E. coli* (Doerrler et al., 2004; Doerrler et al., 2001). MsbA ATPase activity was highly stimulated by hexa-acylated LPS/lipid A species, suggesting that they might be substrates (Doerrler and Raetz, 2002). For cells in which MsbA function was impaired, PE and lipid A were found to be accessible from the inner leaflet of the membrane rather than the outer leaflet (Doerrler et al., 2004). These results therefore strongly indicated that MsbA functions as an ATP-dependent flippase within the IM and facilitates the movement of lipid A (and possibly phospholipids) from the inner to the outer leaflet. Phospholipid translocation by MsbA remains controversial, largely as a result of studies in *Neisseria meningitides*, which showed that while MsbA had a definite role in lipid A translocation, it was not absolutely necessary for the translocation of glycerophospholipids (Tefsen et al., 2005). This conclusion was further supported by the work of Kol and co-workers (Kol et al., 2003a; Kol et al., 2003b) in which phospholipid flippase activity mediated by a variety of bacterial peptides and proteins was studied in reconstituted lipid systems. While transmembrane (TM) peptides were found to mediate the flipping of PG and PE phospholipids, MsbA did not, even in the presence of ATP. This conclusion has however been challenged by the recent work of Eckford and Sharom (2010) who demonstrated that MsbA reconstituted into proteoliposomes of *E. coli* lipids could flip fluorescently labeled derivatives of the phospholipids PE, PG, PC, phosphatidylserine (PS), phosphatidylcholine (PC), and sphingomyelin (SM) in an ATP-dependent manner. Using purified protein reconstituted into liposomes, MsbA was shown to have a maximal flippase activity of 7.7 nmol of lipid translocated per mg of protein over a 20 min period for an acyl chain-labeled PE derivative (Eckford and Sharom, 2010). Finally, multiple copies of homologous MsbA genes have been identified in many bacterial genomes, whereas, intriguingly, lipid A is found only in Gram-negative bacteria (Zhou et al., 1998), suggesting that MsbA may have a different role in these organisms that is possibly related to the movement of phospholipids.

MsbA is the only essential ABC family member in Gram-negative bacteria, making it a potentially attractive antibiotic target. Many proteins in this family have been extensively studied because of their involvement in the phenomenon of multidrug resistance in clinical medicine. Prominent ABC superfamily members include the mammalian multidrug transporter ABCB1, also known as P-glycoprotein (Eckford and Sharom, 2009), and the bacterial multidrug transporters LmrA of *Lactococcus lactis* (van Veen et al., 1996) and Sav1866 of *Staphylococcus aureus* (Dawson and Locher, 2006). MsbA shares ~30% sequence identity with LmrA and ABCB1 (van Veen et al., 1998; van Veen et al., 1996), while the nucleotide-binding domains (NBDs) of MsbA and ABCB1 share 51% identity. Consequently, MsbA is one of the closest bacterial homologues to mammalian ABC transporters, and provides a valuable model for these proteins, which historically have proven technically challenging to study. This close link between ABCB1 and MsbA is supported by functional studies which suggest that MsbA is also a transporter for multiple drugs. For example, MsbA expression in *L. lactis* causes resistance to certain antibiotics, and confers H33342 and ethidium transport capability that can be inhibited by lipid A and vinblastine (Woebking et al., 2005; Reuter et al., 2003). The results from these and other studies (which will be discussed further below) strongly suggest that MsbA is an excellent target for furthering our understanding of multidrug transport.

Biochemical properties of MsbA

As a member of the ABC superfamily, MsbA possesses a characteristic NBD, which couples the energy derived from ATP hydrolysis to the active transport of substrates up their concentration gradients. The biochemical properties of MsbA have been characterized over the last decade or so. MsbA was consistently found to have a K_M for ATP hydrolysis of ~0.9 mM (Eckford and Sharom, 2008; Doerrler and Raetz, 2002) which is comparable to that of the mammalian ABC transporter ABCB1 (Sharom et al., 1995). In contrast, there is a significant disparity in the specific ATPase activities reported in the literature (Buchaklian et al., 2004; Doerrler and Raetz, 2002; Eckford and Sharom, 2008; Zou and McHaourab, 2009) with values ranging from ~10 to ~1000 nmol.mg^{-1}.min^{-1}, the latter value being obtained in some recent studies. While this variation may be attributed to differing conditions of protein isolation and solubilization, which are known to be significant factors for other ABC proteins, the disparities have so far not been explained satisfactorily. As is typical for ABC proteins, the ATPase activity of MsbA is inhibited up to 80% by the phosphate analogue vanadate (V_i) (Doerrler and Raetz, 2002; Eckford and Sharom, 2008). Following ATP hydrolysis, V_i is able to displace P_i in the active site of the NBD and thus acts as a trapping agent that stalls the conformation of ABC proteins in what is assumed to be the post-hydrolysis state. Like ABCB1, MsbA is also sensitive to inhibition by the trapping agents AlF_x and BeF_x (Eckford and Sharom, 2008).

The ATPase activity of MsbA can be modulated by lipids and lipid-like molecules. Significantly, MsbA was shown to be a lipid-activated ATPase whose activity was stimulated up to four- to five-fold by the presence of 3-deoxy-D-manno-2-octulosonic acid-lipid A (Doerrler and Raetz, 2002). This initial observation was confirmed by the recent work where ATPase activities and equilibrium dissociation constants (K_d) were determined for a range of compounds, including nucleotides and putative substrates, using intrinsic tryptophan fluorescence

quenching measurements (Eckford and Sharom, 2008). This work revealed that the ATPase activity of purified MsbA can be modulated by a variety of lipids, lipid-based molecules, and phospholipid-based amphipathic drugs. The ATPase specific activity of MsbA in detergent solution was shown to increase up to two-fold in the presence of egg PE and *E. coli* lipids (Eckford and Sharom, 2008), which are a mixture of PE, PG, PS and small amounts of other species. In the presence of lipid A alone the ATPase activity of MsbA was increased more than two-fold, whereas the LPS precursors RaLPS (LPS from the Ra mutant of *E. coli*) and ReLPS (deep rough chemotype LPS), increased activity by only 20% and inhibited activity as their concentrations increased further (Eckford and Sharom, 2008). Other researchers have shown 2.5- to 3.5-fold stimulation of MsbA ATPase activity by lipid A (Doerrler and Raetz, 2002; Buchaklian et al., 2004).

MsbA has been shown to confer multidrug resistance by its ability to act as a drug efflux pump, and displays overlapping substrate specificity with mammalian ABC drug efflux pumps (Reuter et al., 2003; Woebking et al., 2005; van Veen et al., 1998). However, there is some disagreement over the specific effects of drugs on the ATPase activity of MsbA. Reuter et al. (2003) reported that the known ABCB1 substrates daunorubicin, vinblastine, H33342 and azidopine stimulated MsbA activity in bacterial membrane vesicles. In contrast, Doerrler and Raetz (2002) reported no stimulation by daunorubicin or vinblastine, as well as colchicine, doxorubicin and verapamil, in reconstituted proteoliposomes. Eckford and Sharom (2008) demonstrated stimulation of the ATPase activity of MsbA in detergent solution by verapamil, inhibition at high concentrations of vinblastine, and both stimulation and inhibition by H33342. Colchicine and daunorubicin yielded no stimulation or inhibition, but were indeed able to interact with MsbA as shown by tryptophan quenching. MsbA also confers resistance to erythromycin and ethidium (Woebking et al., 2005), can be photolabeled by the ABCB1/LmrA substrate [^{3}H]azidopine, and transports H33342 across membrane vesicles in an energy-dependent manner (Woebking et al., 2005). Homologous function between MsbA and LmrA is also evidenced by studies showing that LmrA can substitute for a temperature-sensitive MsbA mutant in *E. coli* WD2 at non-permissive temperatures (Reuter et al., 2003). Woebking et al. (2005) further characterized the transport of ethidium and H33342 by MsbA over-expressed in *Lactococcus lactis*, and showed that transport could be inhibited by lipid A, the likely primary substrate of MsbA.

Molecular structure of MsbA

Sequence and domain structure

MsbA is a 64.5 kDa protein of 582 amino acids with six putative TM helices and one ABC-type NBD at the C-terminal end. It is often termed a "half-transporter" by comparison to mammalian ABC proteins, since each MsbA polypeptide chain provides only half the number of TM helices and NBDs required for a typical ABC transporter. MsbA is therefore assumed to function as a homodimer. Purified MsbA from *E. coli* has been shown to form homodimers in detergent solution by gel filtration chromatography, and SDS-stable homodimers are

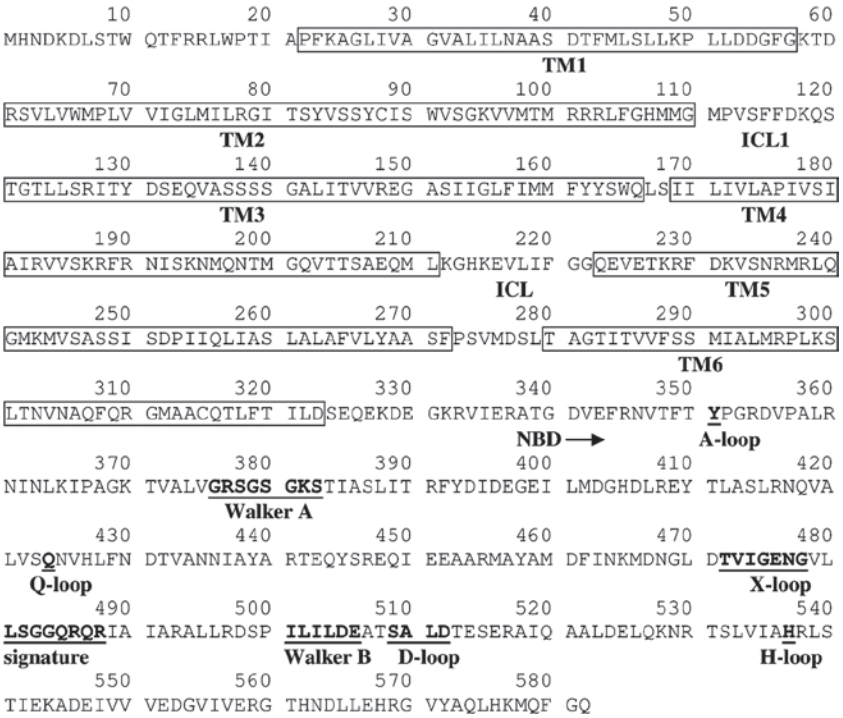

Figure 2. Primary sequence of *E. coli* MsbA protomer (UniProt: P60752) highlighting the 6 TM helices (boxed), ICLs and conserved ABC protein motifs in the C-terminal NBD (residues 341–582). Two protomers associate to form a functional MsbA dimer with 12 TM helices and two NBDs.

present on SDS-PAGE, indicating a strong interaction between monomers (Eckford and Sharom, 2008; Borbat et al., 2007; Dong et al., 2005). Furthermore, as will be presented below, X-ray crystallography studies confirm that MsbA polypeptides associate to form dimers.

Multiple conserved sequences are found within MsbA that identify it as a member of the ABC superfamily of proteins (consensus sequences are given in parentheses): the Walker A (GxxGxGKS where x is any amino acid) and Walker B (ΦΦΦΦDE, where Φ is any hydrophobic residue) motifs, the C or signature sequence (LSGGQxQR), the D-loop (SALD), as well as single residue motifs termed the A-loop (Y), Q-loop (Q), and H-loop (H) (Karow and Georgopoulos, 1993). The annotated primary sequence of MsbA, highlighting the location of the transmembrane domains (TMDs) and the conserved ABC motifs in the C-terminal NBD, is shown in Figure 2. The signature and Walker B motifs along with the Q- and H-loops from one monomer come together with the Walker A motif from the opposite monomer to bind and/or hydrolyse ATP (Davidson and Chen, 2004) in what has been described as a nucleotide sandwich dimer (Smith et al., 2002). The Q-loop and the Walker B motif are involved in ATP binding and hydrolysis, by hydrogen bonding to Mg^{2+} and the attacking water (Schmitt et al., 2003). The H-loop is believed to be involved in binding ATP by hydrogen bonding to the γ-phosphate. The role of the D-loop (SALD) is less well understood, although recent work has suggested that the L residue is necessary for efficient ATP hydrolysis and the D residue is essential for conformational rearrangements (Schultz et al., 2011). The X-loop (TxVGExG) was first identified in the Sav1866 structure and its name reflects its likely role in cross-linking the intracellular loops (ICLs) (Dawson and Locher, 2006). The X-loop precedes the signature motif, and may respond to ATP binding and hydrolysis by transmitting conformational changes to the ICLs by alternately engaging and releasing the cross-link.

X-ray crystal structures

As shown in Table 1, X-ray crystal structures have been determined for MsbA from *E. coli*, *V. cholerae* and *S. typhimurium*, the latter in the presence of the non-hydrolysable ATP analogue adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP) (Ward et al., 2007). Earlier structures determined by the same research group were inconsistent with the available biochemical data and also with the later, widely accepted structure for the bacterial ABC protein Sav1866 (Dawson and Locher, 2006). The MsbA structures were subsequently found to be incorrect due to a data processing error and subsequently retracted. The re-released structures were found to agree much more closely with that of Sav1866. Unfortunately, the level of resolution for these structures is not high (4.2–5.5 Å), thus little information on side-chain orientation is available. Computational methods for enhancing resolution were applied to the previous incorrect structures (Campbell et al., 2003) but attempts to apply this approach to the re-released structures have not been reported. Perhaps the most significant finding from the X-ray structures was MsbA's potential for elaborate conformational changes, which raised interesting questions about the mechanism by which this protein transports lipid A. It is important to note at this point that the usual caveats for crystal structures apply. These include the possibility of capturing structural aberrations arising from the solubilization conditions, or through the formation of crystal contacts that favor non-native conformers. Furthermore, it was recently discovered that MsbA contains a structural feature that interferes with its ability to crystallize (Terakado et al., 2010). Through analysis of the crystal contacts of the MsbA structures, a C-terminal helix of 21 residues was identified and deleted, enabling MsbA from *E. coli* bound

Table 1. Summary of biophysical data on MsbA structure and conformational dynamics in the presence and absence of nucleotides.

	Ligand	TMD conformation	NBD conformation	Resolution (Å)	Reference	PDB code
X-ray crystallography in detergent						
E. coli	-	inward	open	5.3	Ward et al., 2007	3B5W
E. coli	AMP-PNP	outward	closed	4.5	Terakado et al., 2010	-
V. cholerae	-	inward	closed[a]	5.5	Ward et al., 2007	3B5X
S. typhimurium	AMP-PNP	outward	closed	4.5	Ward et al., 2007	3BY5
S. typhimurium	AMP-PNP	outward	closed	3.7[b]	Ward et al., 2007	3B60
S. typhimurium	ADP-V_i	outward	closed	4.2	Ward et al., 2007	3B5Z
EPR/DEER in liposomes						
E. coli	-	inward	n.a.	n.a.	Zou et al., 2009	n.a.
E. coli	AMP-PNP	outward	n.a.	n.a.	Zou et al., 2009	n.a.
Cryo EM in liposomes						
S. typhimurium	AMP-PNP	outward	closed	~20	Ward et al., 2009	n.a.
S. typhimurium	ADP-V_i	outward	closed	~20	Ward et al., 2009	n.a.
V. cholerae	ADP-AlF_x	outward	closed	~20	Ward et al., 2009	n.a.

[a]NBDs are closed but there is no association of the Walker A motif with the signature C motif.
[b]Includes side-chain atoms. All other MsbA structures listed are Cα backbone only.
n.a., data not available.

to AMP-PNP to be crystallized. The resulting structure was found to have an outward-facing closed conformation. This approach may prove successful for crystallizing MsbA with other ligands in the future.

Collectively, the X-ray structures of MsbA determined by Ward et al. (2007) show a homodimer with a typical TMD formed from a bundle of non-covalently interacting α-helices, followed by a canonical NBD (Figure 3). The TM4 and TM5 α-helices from one polypeptide chain "cross over" to form inter-helical contacts with the second MsbA polypeptide chain, and it is presumably this interaction that is responsible for maintaining MsbA as a dimer. With the exception of TM1, the TMD helices are elongated beyond lengths typical for membrane-spanning regions (~35 Å). With an average length of ~44 residues and spanning a distance of around 70 Å, these helices likely extend a substantial way from the membrane surface into the cytoplasm under native conditions. The termini of these elongated helices form multiple contacts with the NBD. TM6 immediately precedes the NBD in the sequence of MsbA (see Figure 2), and is thus directly linked to the NBD via ICL3. The intracellular loops ICL1 (which connects TM2 and TM3) and ICL2 (which connects TM4 and TM5) appear to form α-helices that interact non-covalently with residues on the top surface of the NBD. Furthermore, while the ICL1 helix contacts the NBD within its own MsbA polypeptide, the ICL2 helix contacts the NBD of the other monomer in a region that includes residues near the Walker A and X-loop motifs. This cross-over of the chains may be responsible for communicating conformational changes between the NBD and the TMD following substrate binding or ATP hydrolysis.

The conformational changes that ABC transporters undergo in order to perform their functions are not fully understood. Fortunately, MsbA is proving to be a highly amenable model for elucidating the structural dynamics that take place during the ATP hydrolysis cycle. Multiple conformational states of MsbA in the presence and absence of nucleotides have been determined by X-ray crystallography and cryo-electron microscopy (EM), and its structural dynamics have been explored using electron paramagnetic resonance (EPR) techniques. The most important results of these studies are summarized in Table 1.

The X-ray crystal structures present MsbA in three different conformations, which have been proposed to represent the states through which the protein progresses during the "lipid flipping" cycle (Ward et al., 2007). As shown in Figure 3A, in the absence of nucleotide (i.e. the apo state) the NBDs of *E. coli* MsbA are separated by a substantial distance (~50 Å) while the TMD bundle has split and splayed apart to accommodate this extensive motion, thus creating a large central cavity. Under native conditions this cavity would be centered within the membrane and face inwards towards the cytoplasm, and it is therefore assumed to represent the binding site for lipid A. This structure is therefore said to represent an open-inward conformation of MsbA. In contrast, the apo structure determined for MsbA from *V. cholerae* (Figure 3B) shows that the TMDs and NBDs are much more closely associated, although the NBDs are not in the classic dimer configuration, since there is no interaction between the Walker A and the signature motifs. This has been termed the closed-inward conformation, and can be formed from the open-inward conformation by a relative simple inward movement of the two wings of the protein around a hinge point centered in the extracellular loops (Ward et al., 2007). The third MsbA structure from *S. typhimurium* (Figure 3C) was solved with two molecules of AMP-PNP bound to the NBDs. In this conformation the NBDs are fully closed to form the classic sandwich dimer. Remarkably, the TMD has undergone significant rearrangement relative to the open-inward structure,

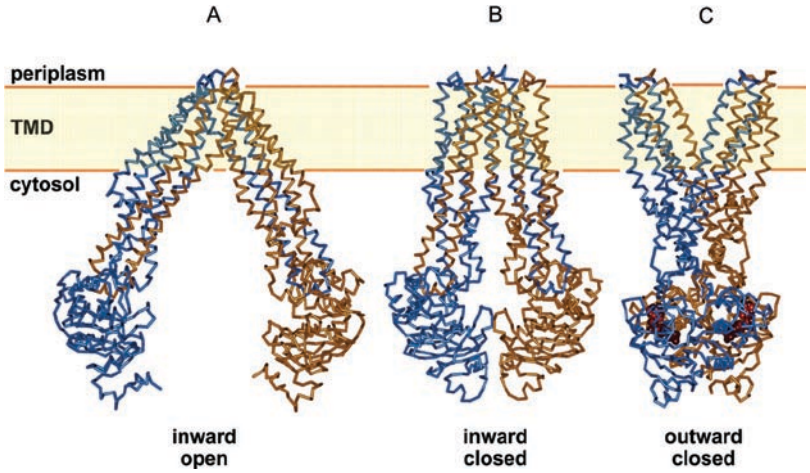

Figure 3. Conformational states of MsbA determined by X-ray crystallography from detergent solubilized samples (Ward et al., 2007). The two MsbA polypeptides making up the dimer are shown in blue and red, respectively. The Cα backbones of the proteins are shown for (A) apo inward-open MsbA from *E. coli* (PDB: 3B5W) (B) apo inward-closed MsbA from *V. cholerae* (PDB: 3B5X) (C) nucleotide-bound outward-closed MsbA from *S. typhimurium* with the bound AMP-PNP rendered in space-filling format (PDB: 3B60). The yellow bar represents the expected position of the membrane, with the extracellular and intracellular sides at the top and bottom, respectively.

with TM1 and TM2 moving away from TM3 and TM6 on the extracellular side, thus creating a central cavity that under native conditions would face the periplasm. This has therefore been termed the outward-closed conformation for MsbA, and is similar to that observed for Sav1866 (Dawson and Locher, 2006). Since these structures imply motions of the NBDs and TMDs in response to nucleotide binding, they have been interpreted as possible intermediate states in the functional cycle of MsbA. The large conformational changes implied by these structures seem to be unique to MsbA, since in ABC importers these changes are more subtle, with the NBDs remaining in contact throughout the cycle. One possible explanation for this discrepancy is that the large size of MsbA's lipid A substrate may necessitate high flexibility in its structure.

Cryo-EM structures

One criticism of using detergents to solubilize proteins in structure determination studies is that detergent micelles are not wholly representative of the biochemical and mechanical stresses the protein is subjected to in a native biological membrane. Cryo-EM approaches have therefore been applied to gathering structural information for MsbA reconstituted into lipid bilayers (Ward et al., 2009). MsbA was shown to crystallize differently depending on whether it was in the substrate-bound state, with bound AMP-PNP or trapped ADP-AlF_x, compared to the product-bound state with trapped ADP-V_i. Differences were also observed between micellar and membrane-embedded MsbA. This is in contrast to the X-ray crystallography studies where crystals of MsbA bound to AMP-PNP or ADP-V_i were found to be isomorphous. Moderate resolution maps (~20 Å) were calculated for each state, which revealed MsbA to be a dimer with a large channel between the membrane-spanning domains about 5 nm (~50 Å) deep and 1.5 nm (~15 Å) wide, similar to the outward-facing crystal structures of MsbA and Sav1866. Unfortunately, the resolution of the maps was not good enough to examine the differences between the crystals, since the altered symmetry of the helical crystals prevented rigorous difference mapping. This suggests that while there are likely structural differences between the different nucleotide-bound and trapped states, membrane-embedded MsbA remains in an outward-facing conformation while nucleotide is bound.

EPR spectroscopic studies

The technique of EPR spectroscopy is increasingly being applied for better understanding the structure and dynamics of MsbA. Of particular interest is the apparently large physical separation of the NBDs in the X-ray structure of the apo form of MsbA from *E. coli* (Figure 3A), which was unexpected, and considerable efforts have been made to verify the existence of this conformation using EPR techniques. The magnitude and direction of the changes in distances between the NBDs during the transition from the apo form to the ADP-V_i trapped state have been studied by Borbat et al. (2007) for MsbA reconstituted in liposomes. The direction of the motional change was found to agree with that observed in the three crystal structures reported by Ward and co-workers. In particular, the magnitude of change for three of the four residues examined correlated extremely well with transitions from the closed apo *V. cholerae* structure to the V_i-trapped *S. typhimurium* structure. However, for the open apo conformation this study reported a separation distance for the NBDs of only 30 Å, which is substantially shorter than the 50 Å found in the X-ray structure. This may indicate that a restriction is imposed on the MsbA conformational changes by the mechanical properties of the bilayer.

The solvent accessibility of various MsbA residues has also been explored using spin labeling, and in some cases the results from these studies conflict with the X-ray structures. EPR quenching studies on the signature and H-loop regions (Buchaklian and Klug, 2006) reported that the LS residues within the signature sequence LSGGQ were buried and inaccessible to the solvent. In contrast, in the *E. coli* apo inward-facing structure these same residues appear to be readily accessible to solvent. This conflict is only slightly lessened for the structures from *V. cholerae* and *S. typhimurium*. Furthermore, the same EPR study suggested that this sequence motif was inaccessible in the post-hydrolysis V_i-trapped state, and maintained tertiary contacts throughout hydrolysis, which conflicts with the *S. typhimurium* structure. Using EPR in conjunction with 112 spin-labeled mutants of MsbA, Dong and co-workers (2005) undertook a detailed analysis of the structural dynamics of MsbA reconstituted into liposomes, in an attempt to map conformational changes during the ATP hydrolysis cycle. MsbA was trapped in four intermediate states, including apo and AMP-PNP-bound. This work found that residues 284–296 from the N-terminal half of TM6 presented low accessibility to the solvent for all intermediates, whereas residues 300 and 303 of the same TM were accessible throughout the transport cycle. The results of this study are therefore at odds with the X-ray structures and the EM images, which suggest that residues 284–296 should be solvent-accessible. A possible explanation for the disparity between the X-ray and EPR studies is that the low resolution of the X-ray structures forces the analysis of the EPR data to rely on predicted side-chain accessibilities which may not be entirely reliable. Perhaps most significantly, the EPR data, in contrast to the X-ray structures, suggest that major conformational changes occur in MsbA following nucleotide hydrolysis.

In summary the results from X-ray crystallography and EPR studies suggest that MsbA has an inverted V-like structure in the absence of nucleotide, with a large chamber open to both the cytoplasm and the inner leaflet of the bilayer. In contrast, the presence of AMP-PNP results in this chamber being closed at the cytoplasmic side and open to the extracellular side of the membrane. However, it remains unclear whether either of the nucleotide-free states of MsbA observed by EPR and X-ray crystallography represents a physiologically relevant state (which would likely only be present transiently in an intact cell), and the scale of the conformational transitions remains controversial.

Substrate-binding site(s) of MsbA

Based on the X-ray crystal structures of MsbA, it is believed that the central cavity between the two monomers is the location where lipid A binds. Recent studies have sought to further understand the molecular architecture of the substrate-binding site(s) of the transporter. Results revealed that lipid and amphipathic drug molecules may bind at distinct sites within the TMD of the protein (Siarheyeva and Sharom, 2009; Smriti et al., 2009; Zou and McHaourab, 2009; Woebking et al., 2008). Smriti and co-workers (2009) reported direct evidence for daunorubicin interaction with MsbA, and attempted to map the residues involved in binding of the drug. They found that in the absence of nucleotide, amino acids interacting with the drug were clustered at the cytoplasmic end of TM helices 3 and 6 at a site accessible from the membrane/water interface, extending into an aqueous chamber within the TMD. Furthermore, in the presence of the non-hydrolysable analogue, AMP-PNP, it was suggested that MsbA adopts an outward-facing conformation consistent with the X-ray structures and EPR spectroscopy data. Woebking et al. (2008) further showed the importance of TM6 in drug binding. Mutation of residues within TM6 on the periplasmic side was linked to substrate specificity, since the proteins showed reduced binding and transport of ethidium and taxol, whereas interactions with H33342 and erythromycin were unaffected. Zou and McHaourab (2009) carried out an extensive mutagenesis study on MsbA using spin labeling and EPR. They concluded that in the V_i-trapped state, spin labels on the cytoplasmic side experienced increased movement restrictions and reduced water accessibility, while those on the extracellular side displayed increased water penetration, consistent with alternating access of the substrate-binding cavity. Using fluorescence quenching methods, Siarheyeva and Sharom (2009) showed that lipid A and daunorubicin bound to MsbA simultaneously and that binding was not ordered, implying that they occupy different sites in the protein. Furthermore, the affinity of lipid A binding was found to be reduced by prior binding of daunorubicin, and also by nucleotide binding, implying that MsbA contains two substrate-binding sites capable of communicating with each other and with the NBD. A high-resolution X-ray crystal structure of MsbA with a bound substrate (preferably lipid A-core) would be invaluable in showing the molecular details of how this putative flippase interacts with its lipid substrates.

Mechanism of action of MsbA

How does MsbA transport its substrate lipid A across the bacterial membrane? At present, we do not know the mechanism by which any flippase moves lipids between leaflets of the membrane bilayer (Sharom, 2011). MsbA represents perhaps the best target for delineating a mechanism for one protein, since it is readily available via homologous expression (Eckford and Sharom, 2008), several crystal structures have been determined (Ward et al., 2007), and it displays flippase activity in reconstituted lipid systems (Eckford and Sharom, 2010). It is possible to speculate on the flippase mechanism by considering the conditions that would be imposed by the amphipathic nature of the substrates. In particular it would seem likely that the hydrocarbon chains remain within the hydrophobic core of the bilayer during the flipping process, likely by being dragged through the bilayer interior. In contrast, the movement of the large polar headgroup is likely facilitated by interactions within the flippase with something resembling an aqueous channel or a hydrophilic binding domain. In the case of MsbA, if the large sugar headgroups of the lipid A-core were to bind to the protein rather than follow an aqueous channel, then we might expect significant conformational changes to occur in the protein during translocation. It is interesting, therefore, that the results of X-ray and EPR studies do reveal the potential for large conformational changes in MsbA. Given the significant size of most transport substrates (e.g. lipid A-core in the case of MsbA) and the complicated mechanics of accommodating the rotation of large amphipathic molecules, it seems unlikely that flippases completely enclose their substrates as they travel between membrane leaflets. The alternating access model for flippases follows these principles, and describes a central cavity that binds only to the polar region of the substrate and opens alternately to the inner and outer leaflets of the bilayer. This model is widely accepted for other ATP-driven flippases, and has been proposed to apply to MsbA based on the available structural data (Zou and McHaourab, 2009).

One current model for MsbA flippase activity, presented schematically in Figure 4, has the following

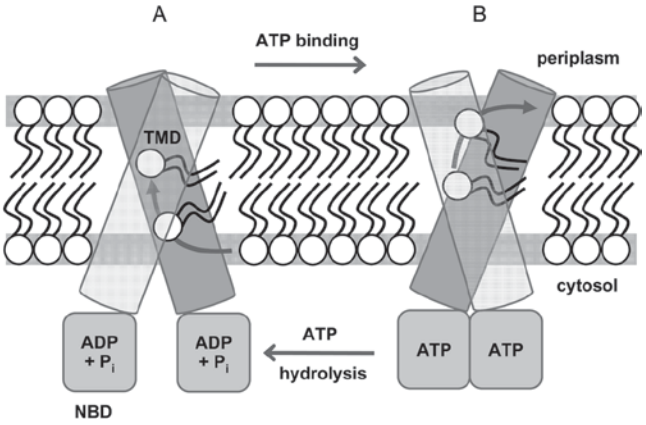

Figure 4. Alternating access model for the flippase mechanism of MsbA. (A) Lipid A-core molecules in the inner leaflet bind to the central cavity of MsbA via interactions between the headgroup and the protein while the protein is in the inward-facing apo conformation. (B) NBD dimerization is induced by ATP binding, and a conformational change in the TMDs leads to closing of the cavity at the inner leaflet and opening at the outer leaflet. The non-polar tails of the substrate are dragged through the hydrophobic interior of the bilayer, while the polar headgroup is stabilized by interactions with the interior chamber of the protein. Following the transition to the outward-facing conformation, a decrease in binding affinity results in release of the lipid A-core molecule to the outer leaflet of the membrane. ATP hydrolysis either resets the structure to begin the cycle again, or drives the conformational change.

features. The flippase cycle is triggered by the lipid substrate entering the binding site of MsbA from the inner leaflet of the membrane. This occurs while MsbA is in a nucleotide-free inverted V-shaped conformation with the NBDs potentially separated by a distance in the range of 30–50 Å. Although it is yet to be confirmed, the binding site is likely to be located within the large chamber formed from the splaying of the two TMD wings (Smriti et al., 2009). The dimer is stabilized by the cross-over of two TM helices from each polypeptide chain. This chamber is lined with hydrophobic, aromatic, and polar amino acids that allow for a variety of interactions with a range of substrates at distinct regions. MsbA's interior chamber is a high dielectric environment able to stabilize the polar headgroup moieties of amphipathic substrates, while their hydrophobic regions remain in direct contact with the bilayer. The large separation of the NBDs may facilitate the binding of the large polar group of the lipid A-core substrate. The proportion of MsbA in the apo state conformation is expected to be low, since the K_M for ATP is below the typical intracellular concentration. However, since MsbA displays a high basal ATPase activity it is unclear how the cell reconciles this seemingly wasteful consumption of cellular energy. In the nucleotide-bound state the NBDs come together, and a rearrangement of the TMD helices takes place. TM3 and TM6 move away from TM1 and TM2 in a twisting motion, and MsbA makes the transition from an inward- to an outward-facing conformation as evidenced in the X-ray structures. In the MsbA homologs ABCB1 and LmrA, it has been suggested that ATP binding occludes the cytoplasmic binding sites thereby lowering the apparent affinity for substrate (Higgins and Linton, 2004) which can be explained by the closing of the TM chamber. The polarity of the chamber becomes reversed by the conformational change, reducing the affinity of the substrate binding site, and thus releasing the substrate to the outer leaflet. The conformational change in the TMDs may be driven either by ATP binding (Higgins and Linton, 2004), in which case hydrolysis of ATP subsequently resets MsbA to the inward-facing conformation (Figure 4), or by the energy released on ATP hydrolysis.

The events that occur at the NBDs during the transport cycle of ABC transporters are not clearly understood, but two major mechanistic models have come to the fore in recent years, based largely on studies of ABCB1; namely the occlusion-induced switch model and the ATP switch model (recently reviewed by Seeger and van Veen, 2009). In the occlusion-induced switch model the NBDs are separated in the nucleotide-free state. The cargo substrate binds to the TMD chamber, followed by binding of two ATP molecules to the NBDs. The tightened binding or occlusion of one of the ATP molecules, which can occur randomly at either site, induces the inward- to outward-facing transition and release of the cargo substrate. The occluded ATP molecule is committed to hydrolysis yielding ADP and P_i. One of two series of steps may then occur: the ADP and P_i are released and exchanged for ATP, and the protein resets to an inward-facing configuration enabling a new cargo substrate to bind. The transport process is then repeated with the cost of only a single ATP molecule per substrate molecule transported. Alternatively, ADP and P_i are released without resetting the protein, and the second ATP molecule becomes occluded and hydrolyzed, followed by release of ADP and P_i and the resetting of the structure. The latter event would result in the hydrolysis of two ATP molecules per transport cycle. The ATP switch model is similar to the latter option, and requires that the two ATP molecules are sequentially hydrolyzed in order to destabilize the NBD dimer before the cycle can begin again. While there are numerous studies to support either model, there are controversies within the literature. For example, the current biochemical evidence for MsbA is insufficient to determine whether the hydrolysis of the two ATP molecules occurs randomly, alternately or sequentially. However, it does seem clear that they are likely not hydrolyzed simultaneously, since for ABCB1, V_i trapping at both ATP-binding sites is not observed (Urbatsch et al., 1995).

How the proposed mechanisms account for both lipid transport and drug transport is unclear, but the latter activity may be a side-effect of the flippase mechanism. Clues to the lipid-drug duality of the flippase mechanism come from ABCB1, which is primarily a drug pump but also displays lipid flippase activity. There is some evidence that translocation of lipids by ABCB1 is closely related to that of its drug substrates, which suggests that a similar mechanism is likely involved (Romsicki and Sharom, 2001; Eckford and Sharom, 2005). This process may involve the incidental movement of lipids from the cytoplasmic leaflet of the membrane into the binding cavity within the protein, and then to a partially hydrophilic region on the extracellular face of the protein (van Meer et al., 2006). The lipids, rather than being expelled into the aqueous environment (which would incur a high energetic cost) instead partition into the outer leaflet of the bilayer. Thus the protein would appear to act as a lipid flippase when it is merely a side-effect of its drug transport mechanism. Conversely, the drug transport exhibited by MsbA may be a side-effect of its main proposed function as a lipid translocator.

NPC1 – a mammalian protein that binds and transfers cholesterol

Cholesterol trafficking and Niemann-Pick Type C disease

Cholesterol is an essential component of vertebrate plasma membranes, where it plays a critical role in the regulation of membrane fluidity (Mesmin and Maxfield, 2009). Yet it is present in only small amounts in intracellular membranes such as the endoplasmic reticulum (ER), indicating that cells actively maintain dissimilar levels of cholesterol in different compartments. Trafficking of cholesterol between intracellular membranes takes place

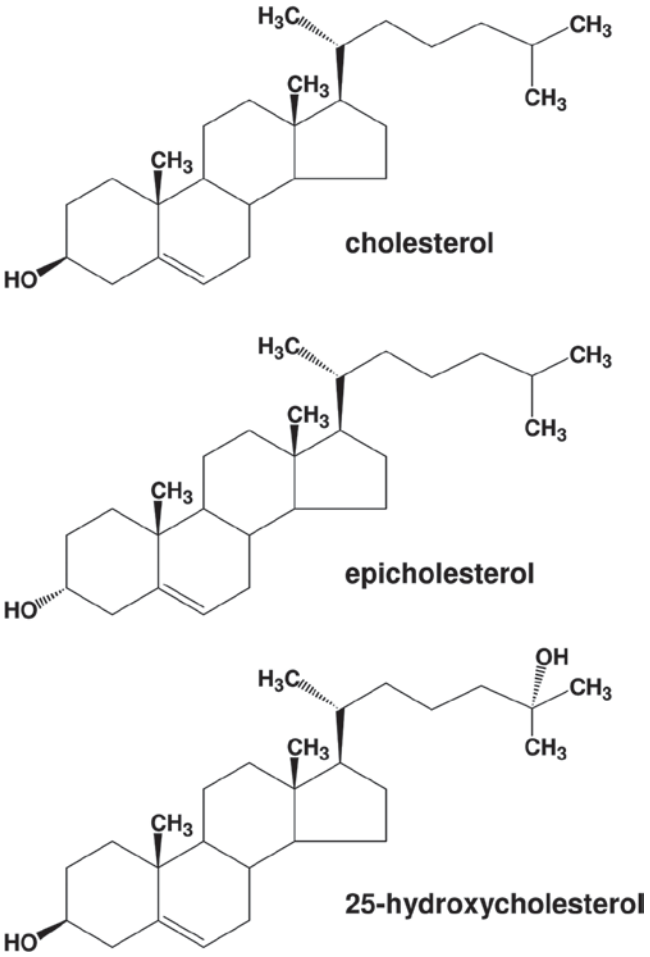

Figure 5. Structures of some sterols that bind to NPC1.

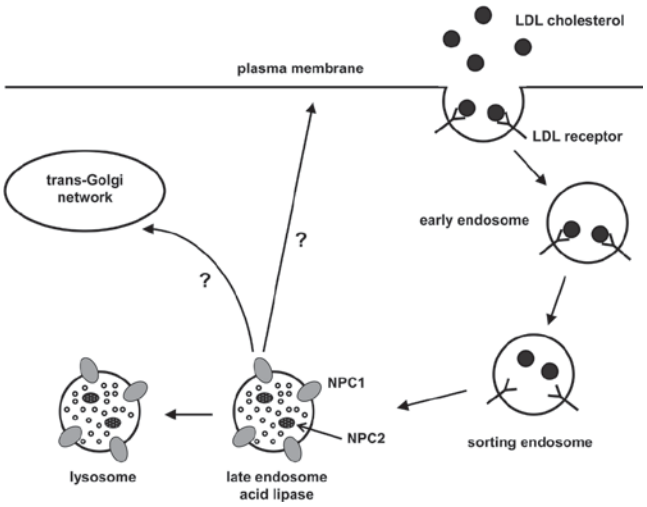

Figure 6. Cartoon showing selected pathways for trafficking of LDL-derived cholesterol. LDL particles bind to LDL receptors, and are taken into the cell by endocytosis via the coated pits. LDL and its receptors are separated in the sorting endosomes, and cholesterol esters are hydrolysed to free cholesterol by lysosomal acid lipase in the LE/LY. The cholesterol is present in LE/LY internal vesicles, and soluble NPC2 is found in the lumen. The combined actions of NPC1 in the LE/LY limiting membrane and NPC2 result in movement of free cholesterol from the interior of the LE/LY to other cellular destinations.

by both vesicular transport and non-vesicular (protein carrier-mediated) mechanisms (Ikonen, 2008; Maxfield and Menon, 2006), however, establishing the molecular basis of these processes and how they are regulated has proved elusive. One important property of cholesterol and related sterols (Figure 5) is their hydrophobicity and extremely low aqueous solubility. This feature greatly restricts the mechanisms by which sterols can be transferred at a meaningful rate between compartments inside the cell, and also complicates all experimental investigations involving cholesterol, especially those carried out *in vitro* using purified components.

Peripheral tissues can obtain the cholesterol they need to maintain their membrane structure in one of two ways; either by exogenous delivery from low density lipoprotein (LDL) in the blood, or *de novo* biosynthesis in the ER. Cholesterol homeostasis (uptake, processing and synthesis) is tightly regulated at the cellular level by a sensing system that involves several key membrane-bound proteins (Sato, 2010). LDL binds to its receptors on the cell surface, and the particle, which contains cholesterol esters, enters the cell by receptor-mediated endocytosis through clathrin-coated pits (Figure 6). After delivery to the late endosomes/lysosomes (LE/LY), cholesterol esters are hydrolyzed by lysosomal acid lipase, thus liberating free cholesterol in the LE/LY lumen. LDL-derived free cholesterol is then rapidly trafficked out of the LE/LY to other cellular compartments, including the plasma membrane and the ER (Figure 6).

Niemann-Pick Type C (NPC) disease is a rare fatal autosomal recessive neurodegenerative condition characterized by massive cellular accumulation of cholesterol and other lipids in the LE/LY (Vanier, 2010). The devastating disease symptoms generally develop in childhood, and include hepatosplenomegaly and central nervous system degeneration caused by the death of Purkinje neurons. There is no cure for the disease, although mobilization of excess stored cholesterol using 2-hydroxypropyl-β-cyclodextrin was recently found to greatly ameliorate symptoms and dramatically extend lifespan in an *npc1*$^{-/-}$ mouse model (Liu et al., 2009a). The cyclodextrin appeared to re-establish the normal movement of sterol (and possibly other lipids) out of LE/LY, however, the molecular mechanisms of its action remain unclear. Cyclodextrins thus represent new potential therapeutic agents for treating NPC disease (Vance and Peake, 2011; Rosenbaum and Maxfield, 2011), and are now being tested on human patients.

Over 95% of NPC disease is caused by mutations in the gene encoding NPC1, a 190 kDa integral protein localized in the limiting membrane of the LE/LY (Figure 6). The other 5% of NPC patients carry a mutation in NPC2, a 22 kDa soluble cholesterol-binding protein found in the LE/LY lumen (Figure 6) (Vanier and Millat, 2004). Cells with the NPC phenotype show LE/LY accumulation of unesterified cholesterol and other lipids, including sphingosine, sphingomyelin and gangliosides (Chang

et al., 2005; Mukhejee and Maxfield, 2004; Lloyd-Evans et al., 2008). Both NPC1 and NPC2 clearly play a crucial role in the movement of LDL-derived cholesterol from LE/LY to the plasma membrane and trans-Golgi network (TGN). Despite the fact that the NPC1 and NPC2 proteins are quite different at the molecular level, the phenotypes displayed by NPC patients carrying mutations in the *NPC1* and *NPC2* genes are virtually indistinguishable. For this reason, it was assumed early on that the NPC1 and NPC2 proteins cooperate in some way in cholesterol efflux, likely operating sequentially within the same pathway. Additional genetic evidence from *npc1*- and *npc2*-deficient mouse strains also supports this hypothesis. Although the human *NPC1* and *NPC2* genes were identified some time ago (Carstea et al., 1997; Naureckiene et al., 2000), only in the past few years has it been possible to study these two proteins at the molecular level. One major hurdle has been the difficulty of purifying sufficient human NPC1 for biochemical studies, which was overcome only recently (Infante et al., 2008a; Liu et al., 2009b).

Structure of NPC1

NPC1 is a member of the RND permease superfamily, a ubiquitous group of proteins found in all the major kingdoms (Tseng et al., 1999). Bacterial RND proteins are generally proton symporters involved in cellular efflux of substrates such as amphipathic drugs, fatty acids, detergents and antibiotics. Eukaryotic RND proteins are poorly characterized, but fall into two major subclasses; proteins that have an internal duplication, and likely function as transporters, and those that do not, and probably act as sterol-modifying enzymes or sterol sensors (Tseng et al., 1999). Thus, phylogenetic analysis predicts that NPC1 may function as a proton-coupled transporter, compatible with a proposed role as an export pump for sterols and/or other amphipathic molecules. Early studies of NPC1 expressed in *E. coli* indicated that it facilitated the import of acriflavine in intact bacterial cells (Davies et al., 2000), and NPC1 expressed in mammalian cells appears to require the acid interior of endosomes/lysosomes to remove cholesterol, compatible with the idea that it might be a proton symporter. However, whether NPC1 functions as a true transporter remains unknown.

Human NPC1 has 1278 residues, with 13 predicted TM helices 3-7 form and three large soluble domains located on the luminal side of the membrane (Figure 7) (Davies and Ioannou, 2000). The large N-terminal luminal domain of NPC1 (NTD, residues 25–264) contains several conserved cysteine residues involved in intra-domain disulfide links, and a leucine zipper motif. The NTD, also known as the NPC1 domain, is highly conserved in all NPC1 homologs. Five of the TM helices form a so-called sterol-sensing domain (SSD), a motif that is conserved in six human proteins involved in cholesterol homeostasis and metabolism, including Niemann-Pick C1-like 1 (NPC1L1), Patched and SCAP (sterol regulatory element binding protein [SREBP] cleavage-activating protein) (Nohturfft et al., 1998). The SSD appears to be essential for cholesterol binding by some of these proteins. Lysosomal targeting sequences include a dileucine motif present at the C-terminus of NPC1, and another signal within the SSD (Scott et al., 2004). Over 100 "loss of function" mutations have been reported in patients with NPC disease. They are scattered throughout the protein, with a small cluster in a conserved cysteine-rich luminal loop in the C-terminal half (Loop 4; see Figure 7) (Greer et al., 1999). About half the NPC disease mis-sense mutations are localized to this region, including the most common mutation in

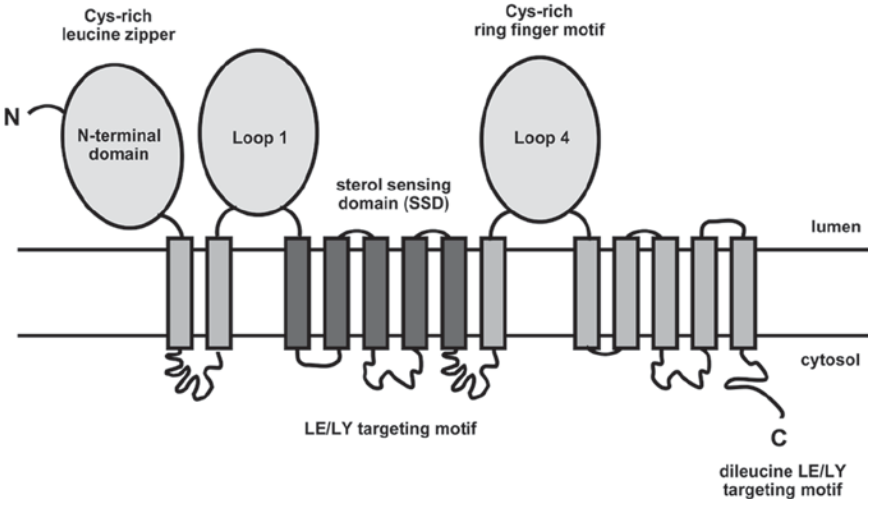

Figure 7. Proposed membrane topology of NPC1. The protein comprises 13 predicted membrane-spanning α-helices, and three large soluble domains located on the luminal side of the membrane. The 240-amino acid soluble N-terminal domain (NTD), characterized by the presence of five potential N-glycosylation sites and nine disulfide links between cysteine residues, has been shown to bind cholesterol. TM helices three–seven form a 5-helix bundle known as the sterol SSD, which is also found in several other proteins involved in cholesterol sensing and metabolism. LE/LY targeting motifs are found at the C-terminus and within the SSD, the NTD has a leucine zipper, and Loop 4 contains a ring finger motif.

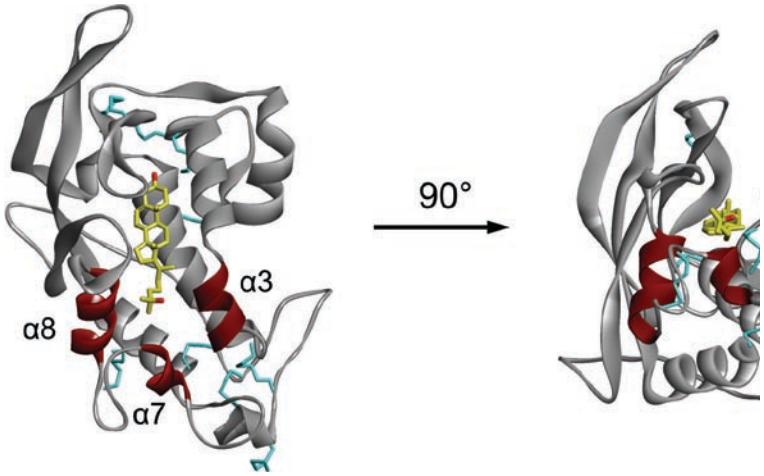

Figure 8. X-ray crystal structure of the soluble NTD of NPC1 with bound 25-OH-cholesterol (PDB: 3GKJ). The protein is represented as a ribbon diagram in grey, with α-helices 3, 7 and 8 shown in red, and the disulfide bonds in green. The sterol molecule is shown as a stick diagram in yellow. A 90° rotation of the protein shows a view along the long axis of the sterol-binding pocket.

Western European populations, I1061T, which causes ~20% of all NPC cases.

In 2008, Infante and co-workers isolated a 25-OH-cholesterol-binding protein from rabbit liver, which analysis showed was NPC1 (Infante et al., 2008a). Systematic deletion analysis of recombinant NPC1 showed that the sterol-binding site was contained exclusively within the NTD of the protein, a 240-amino acid region that projects into the lumen of the LE/LY (Infante et al., 2008b). This was confirmed by recombinant expression and purification of the NTD as a separate protein construct. The NTD domain (residues 23–252) with three of the five putative N-glycosylation sites mutated was then purified and crystallized in the absence (apo form) and presence of both cholesterol and 25-OH-cholesterol (Kwon et al., 2009). The high resolution X-ray structures (1.6–1.8 Å) showed that the protein comprises eight α-helices flanked by a mixed 3-strand β-sheet (see Figure 8). Eighteen cysteine residues are present, all of which are involved in disulfide bonds. The apo and sterol-bound forms displayed almost identical structures. Bound sterol is located in a binding pocket, with the 3β-OH group adjacent to a very small opening (the W-opening), just sufficient to allow passage of a single water molecule. The side-chain end of the bound sterol is located close to a larger opening to the outside (the S-opening), but this is not of sufficient size to allow entry/exit of the sterol without a change in protein conformation to expand it. The S-opening could be enlarged to allow sterol to enter or leave by displacement of a loop connecting α-helix 8 with strand 7, and/or by movement of α-helices 3, 7 or 8 (Figure 8). The sterol would then exit the NTD with its hydrophobic iso-octyl side-chain leading the way. The proposal that sterol gains access to the binding pocket through the S-opening rather than the W-opening is supported by site-directed mutagenesis data (Kwon et al., 2009).

The sterol-binding pocket of the NTD is lined with primarily hydrophobic residues (Trp, Leu, Ile, Phe, Pro), whereas the region close to the 3β-OH group includes two polar residues (Asn and Gln) which are engaged in H-bonds with this group. The substrate-binding pocket encloses the sterol very tightly, and likely determines the binding specificity of the NTD. Changes in the configuration of the OH group (e.g. to a 3α-OH group in epi-cholesterol) do not allow binding within the pocket, and bulky -OH modifications to the tetracyclic ring or C20 residue of the side-chain also preclude binding. Alanine-scanning mutagenesis studies of the NTD showed that mutations abolishing sterol binding were located in residues lining the sterol-binding pocket, as expected.

NPC1L1, a close relative of NPC1 (Yu, 2008), mediates intestinal cholesterol absorption. A recent high-resolution X-ray crystal structure of the NTD of NPC1L1 (residues 22–284) in a cholesterol-free form (Kwon et al., 2009) showed the presence of a large central cavity similar to the cholesterol-binding pocket of NPC1. The NTDs of the two proteins share the same overall folding topology, but the cholesterol-binding pocket of NPC1L1 appears to be slightly larger, and is effectively completely closed off from contact with the solvent at the surface of the protein, i.e. it appears to represent a "closed" form.

Biochemical properties of NPC1

To date, only two research groups have reported the purification of full-length human NPC1 that is functional in terms of sterol binding (Infante et al., 2008a; Liu et al., 2009b). Infante et al. originally isolated NPC1 serendipitously during a search for high affinity 25-OH-cholesterol-binding proteins in rabbit liver, and then expressed and purified a recombinant version in mammalian cells (Infante et al., 2008a). They developed a sterol binding assay using [^{3}H]-labeled sterols in detergent solution, recovering His-tagged

NPC1 and bound sterol using a Ni-chelate affinity column. A similar assay had been developed previously during their work on the sterol-binding protein, SCAP (Radhakrishnan et al., 2004). Full-length NPC1 was shown to bind 25-OH-cholesterol saturably with high affinity, but binding of unmodified cholesterol could not be demonstrated at first.

The extremely low solubility of cholesterol and related sterols makes binding experiments very challenging. These molecules move at extremely low rates as monomers in the aqueous phase between proteins or membrane-bound compartments. The true critical micelle concentration (CMC) of cholesterol has been estimated to be as low as 25–40 nM (Haberland and Reynolds, 1973), so all experiments using concentrations higher than this risk complications from micelle/aggregate formation. Fos-choline family detergents, such as Fos-choline 13 (tridecylphosphocholine), have proved to be very useful in solubilizing cholesterol at higher concentrations for delivery to sterol-binding proteins such as SCAP and NPC1 (Radhakrishnan et al., 2004; Liu et al., 2009b). They mimic phospholipids, and appear to maintain membrane proteins in their native conformation. However, one caveat regarding their use is that sterols in these detergent solutions are subject to very slow monomer-micelle equilibria (Radhakrishnan et al., 2004). Under conditions where detergent is present at levels above the CMC, sterol binding to proteins such as NPC1 and SCAP is observed to be inhibited, likely because the added sterol is distributed into detergent micelles, making equilibration with the protein extremely slow (Radhakrishnan et al., 2004; Infante et al., 2008a; Liu et al., 2009b).

When Infante and co-workers lowered the detergent concentration below the CMC, they observed high affinity cholesterol binding to NPC1, with a K_d of 100 nM and an apparent stoichiometry of 1 mole of sterol per 4 moles of protein (Infante et al., 2008a). The affinity of 25-OH-cholesterol binding also increased as the detergent concentration was lowered below the CMC, from 80 nM at 1% (w/w) Nonidet P-40 to 10 nM at 0.004% (w/w). Epi-cholesterol (which has a 3α-OH group) did not compete for cholesterol binding, whereas 25-OH- and 27-OH-cholesterol were able to do so, indicating that the NPC1 binding pocket cannot accommodate changes in stereochemistry at the -OH group end of the sterol molecule. This observation is in accordance with the crystal structure, which showed that the -OH group was buried at the bottom of the sterol-binding pocket. When Infante et al. expressed the NTD of NPC1 as a separate soluble protein, they found that it was also able to bind cholesterol and 25-OH-cholesterol with high affinity, at a stoichiometry of 1 mole of sterol per 2 moles of protein (Infante et al., 2008b). The discrepant stoichiometry values (<1) obtained for the both full-length NPC1 and the NTD likely reflect the difficulties associated with the cholesterol-binding assay, which involved extensive washing of the bound complexes in the presence of detergent.

Site-directed mutagenesis of the NTD identified Gln79 as being important in sterol binding (Infante et al., 2008b), and the NTD X-ray crystal structure later showed that this residue is involved in a direct H-bond with the 3β-OH group of cholesterol (Kwon et al., 2009). However, when a Q79A mutation that abolished sterol binding to the NTD was introduced into full-length NPC1, the protein was still able to restore normal cholesterol trafficking in NPC1-deficient cells. Thus, it is not clear what precise role the sterol-binding site located on the NTD plays in the context of cholesterol binding and trafficking *in vivo*.

Sharom and co-workers isolated full-length NPC1 from mammalian cells over-expressing the protein (Liu et al., 2009b). The purified protein was found to exist as oligomers, depending on the detergent concentration, and could be photolabeled by an azido-cholesterol derivative. Several fluorescent cholesterol analogues, including dehydroergosterol and cholestatrienol, bound to the protein with a large enhancement of their fluorescence emission, compatible with binding in a hydrophobic cavity, with K_d values in the 0.5–6 μM range. The bound fluorescent sterol molecule was inaccessible to dynamic quenchers, again indicating that it is deeply buried and shielded from the aqueous medium. The intrinsic tryptophan fluorescence properties of NPC1 were characterized and used to monitor sterol binding by saturable fluorescence quenching. This approach avoided the problems associated with separation of sterol-bound protein complexes. Various sterols, including cholesterol and 25-OH-cholesterol competed for binding. As expected, the affinity and kinetics of sterol binding were greatly affected by detergent concentrations above the CMC.

Molecular role of the SSD of NPC1

As described above, the NTD of NPC1 contains a sterol-binding site, and this domain must clearly play a role in interacting directly with cholesterol, however, the function of the rest of the NPC1 protein is unknown. In particular, the involvement of the highly conserved SSD motif of 5 TM segments remains a mystery. The SSD is known to be essential for NPC1 function *in vivo*, so it clearly plays a critical role in cholesterol trafficking. Watari and co-workers created point mutations in P691 and Y634, residues which are conserved in the SSDs of other sterol-regulated proteins (NPC1L1, Patched and SCAP), and also generated the mutation Y634C, which renders SCAP resistant to hydroxysterol inhibition (Watari et al., 1999). All of these mutations abolished the cholesterol-mobilizing functions of NPC1. In contrast, Ory and co-workers identified two gain of function (activating) mutations in the SSD, D787N and L657F, which resulted in increased rates of cholesterol trafficking (Millard et al., 2005). Finally, photolabeling of NPC1 in intact cells by an azido-cholesterol analogue was shown to require an intact SSD (Ohgami et al., 2004). The introduction of SSD

mutations known to abolish cholesterol-trafficking function also abolished labeling, suggesting that an intact SSD is necessary for NPC1 interactions with cholesterol. The mechanism by which the SSD mediates such interactions may be direct, i.e. the SSD may itself be a sterol-binding domain, or alternatively it may be indirect, where the SSD is required to maintain the correct folded conformation of a separate sterol-binding domain, in this case, the NTD of NPC1.

Recent studies carried out on SCAP may help to shed some light on these possibilities. The N-terminal membrane-bound domain of SCAP, comprising TM segments 1–8 and including the SSD, was shown to bind cholesterol with high affinity some years ago (Radhakrishnan et al., 2004), and it appeared at first that the SSD itself might interact directly with the sterol. However, recent work has shown that in fact the first luminal loop of SCAP, a large domain (residues 40–284) containing an N-linked glycosylation site, forms the cholesterol-binding site, not the SSD (Motamed et al., 2011). This loop binds sterols with the same high affinity (apparent K_d of 50–100 nM) and specificity as the entire TM domain. Given the conservation of the SSDs between SCAP, NPC1 and NPC1L1, it remains to be seen whether the cholesterol-binding site contained within the first luminal loop of SCAP bears any resemblance structurally to the cholesterol-binding pockets of the other two proteins.

Binding of cholesterol to the binding site in Loop 1 of SCAP causes a conformational change in Loop 6 (at the C-terminal end of the SSD), which promotes binding of SCAP to the ER-localized protein INSIG, in turn leading to ER retention of SCAP. The Y234A mutation in Loop 1 resulted in Loop 6 assuming the cholesterol-bound conformation constitutively, in the absence of sterol (Motamed et al., 2011). Thus, it appears that the five TM segments of the SSD of SCAP are not involved in direct binding of sterol.

Liu et al. used fluorescence methods to show that full-length NPC1 bound fluorescent sterol with a stoichiometry of 1 mole of sterol per mole of protein (Liu et al., 2009b). This suggests that the SSD indeed does not bind sterol directly, and the NTD of NPC1 is the only available sterol-binding site in the protein under these conditions. When expressed as a soluble protein in the absence of the TM regions, the NTD of NPC1 binds cholesterol constitutively. However, in the case of the full-length protein, the SSD may regulate the conformation of the NTD, allowing it to adopt the correct structure for sterol binding. Thus it is possible that mutations within the SSD of NPC1 may abolish sterol binding to the NTD via a conformational change. By analogy to SCAP, the SSD of NPC1 may also regulate the conformation of NPC1 in a sterol-dependent fashion to allow binding to its downstream protein partner(s), possibly ORP5 (see below). This might be an essential step in transfer of cholesterol out of the LE/LY compartment.

Transfer of cholesterol from NPC2 to NPC1

It seems highly likely that the NPC1 and NPC2 proteins cooperate in the intracellular cholesterol trafficking pathway (Mukhejee and Maxfield, 2004; Sleat et al., 2004; Storch and Xu, 2009; Vance and Peake, 2011). The small soluble NPC2 protein displays high affinity cholesterol binding, with 1:1 stoichiometry and K_d values in the range 30 nM to 2 μM (Infante et al., 2008b; Okamura et al., 1999; Liou et al., 2006). The molecular details of cholesterol binding to NPC2 are known from the X-ray structures of the apoprotein (Friedland et al., 2003) and the protein bound to cholesterol-3-O-sulfate (Xu et al., 2008). The sterol is bound in a deep hydrophobic pocket with the sulfate substituent exposed to the solvent at the entrance, and the iso-octyl side-chain buried at the bottom. For this reason, NPC2 cannot bind to cholesterol derivatives with hydroxyl substitutions on the side-chain, such as 25-OH- and 27-OH-cholesterol, since it would be energetically unfavorable for such hydrophilic groups to be deeply buried in a hydrophobic pocket.

Fluorescence dequenching assays showed that purified NPC2 can transfer cholesterol from donor to acceptor lipid bilayer vesicles *in vitro* (Babalola et al., 2007) via a mechanism that involves direct contact with the membrane rather than aqueous diffusion of monomeric sterol (Xu et al., 2008; Cheruku et al., 2006). The unique negatively charged lipid lyso-bis-phosphatidic acid (LBPA) is enriched in interior LE/LY membranes, and may play a role in fusion events (Kobayashi et al., 2002). The rate of cholesterol transfer by NPC2 to lipid vesicles was greatly accelerated when they contained LBPA (Cheruku et al., 2006; Babalola et al., 2007; Xu et al., 2008).

Since NPC2 is a soluble cholesterol-binding protein in the LE lumen (Vanier and Millat, 2004), it represents the most likely candidate as the immediate upstream (or possibly downstream) partner of NPC1. Thus the two proteins may work in tandem. Bi-directional exchange of sterol between the separately expressed water-soluble NTD of NPC1 and liposomes was studied using an *in vitro* system (Infante et al., 2008c). NPC2 was observed to greatly accelerate (>100-fold) cholesterol transfer to/from liposomes and the binding site of the NTD, which was otherwise very slow, even at 37°C. NPC2 may thus be a cholesterol chaperone, transferring it from the internal membranes of the LE/LY to NPC1 in the limiting membrane (Figure 9). However, it was not possible to infer the directionality of cholesterol transfer *in vivo* from these *in vitro* experiments, and sterol transfer from the NTD of NPC1 to NPC2 is an alternative scenario, although less likely.

The orientation of the cholesterol molecule during transfer from the inner LE/LY membranes to NPC2, then to NPC1 (or the reverse), and finally to the limiting membrane of the LE/LY is of considerable interest. Assuming that cholesterol binds to NPC2 in the same way as cholesterol sulfate, the 3β-OH group would be

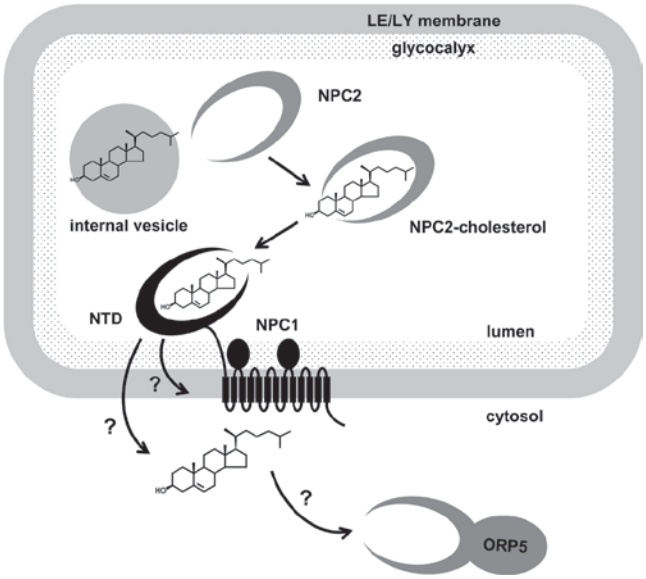

Figure 9. Proposed roles of NPC2, NPC1 and ORP5 in export of free cholesterol across the limiting membrane of the LE/LY. Cholesterol from internal vesicles in the lumen is extracted by NPC2, where it binds with the iso-octyl side-chain at the bottom of the binding pocket and the OH group exposed to the aqueous surroundings. The sterol is then transferred from NPC2 to the NTD of NPC1 in the limiting membrane, likely by transient contact between the two proteins. The sterol is bound to NPC1 in the "reverse" orientation, with the OH group at the bottom of the pocket, and the iso-octyl side-chain exposed. The fate of cholesterol past this point is unknown. NPC1 may transfer the sterol into the LE/LY limiting membrane, thus bypassing the thick luminal glycocalyx, or it may be transferred to a cytoplasmic acceptor such as ORP5 at specialized membrane contact sites.

exposed to the hydrophilic solvent, and the iso-octyl side-chain would be located at the bottom of the binding pocket (Figure 9). Exactly how NPC picks up cholesterol from the membrane in such an orientation is not clear, since the 3β-OH group would normally be positioned at the membrane surface. The sterol would thus need to "reverse" its orientation as it leaves the bilayer and enters the binding pocket of NPC2. It is possible that the transfer to NPC2 may take place as cholesterol rapidly flips from one bilayer leaflet to the other (Rosenbaum and Maxfield, 2011). Transfer of bound cholesterol from NPC2 to NPC1 (or the reverse) will be facilitated by the fact that the bound sterol has a reverse orientation in the binding cavities of these proteins, as shown by their sterol-bound crystal structures (Kwon et al., 2009; Xu et al., 2007). The surface exposed 3β-OH group of cholesterol bound to NPC2 will readily slide into the binding pocket of the NTD of NPC1, where it is positioned at the bottom (Figure 9). The last group to transfer from NPC2 to NPC1 would be the iso-octyl side-chain, which would then be located at the opening of the binding pocket in the NTD. This process has been referred to as a "hydrophobic handoff" (Wang et al., 2010), since it avoids contact of the hydrophobic cholesterol molecule with the aqueous phase.

Alanine scanning mutagenesis involving one, two, or three sequential amino acids of the NTD of NPC1 was used to establish which residues are involved in cholesterol transfer via NPC2 to acceptor liposomes. Proteins with five double amino acid mutations and one point mutation were identified that showed normal cholesterol-binding affinity and kinetics, but were deficient in their ability to transfer cholesterol to NPC2 (Kwon et al., 2009). All of the 11 altered residues clustered on a surface sub-domain of the NTD comprising residues 162–200, and showed some conservation among mammalian species. Similar experiments using NPC2 identified residues in this protein that were essential for cholesterol transfer to the NTD of NPC1 (Wang et al., 2010). Again, NPC2 mutant proteins were chosen that retained normal cholesterol-binding affinity and kinetics, followed by screening for their ability to participate in cholesterol transfer from the NTD of NPC1 to liposomes. Three amino acids were identified as being required for the transfer process; all were located in a region surrounding the entrance to the sterol-binding pocket of NPC2 (Wang et al., 2010).

These results suggest that the two surface patches on NPC2 and the NTD of NPC1 may interact in order for cholesterol to move between the two proteins. In the case of the NTD, the interaction could also be responsible for opening of the entrance to the sterol-binding pocket. However, despite the use of a large array of different approaches (gel filtration, surface plasmon resonance/Biacore, Amplified Luminescent Proximity Homogeneous Assay/AlphaScreen, chemical cross-linking; (Wang et al., 2010)), a direct physical interaction between the NTD and NPC2 has not yet been demonstrated, either in the absence or presence of sterol. The handoff of cholesterol between NPC2 and NPC1 bears a resemblance to the substrate channeling that has been proposed for enzymes acting sequentially in a metabolic pathway. In these cases, it has also proved impossible to detect the formation of complexes between the two participating proteins, most likely because such interactions are only transient.

Cellular role of NPC1 in cholesterol trafficking and NPC disease

NPC1 has been the object of intense interest ever since it became clear that it carries out a key "housekeeping" function in intracellular cholesterol trafficking. However, its exact role remains enigmatic and controversial. NPC1 may be involved in direct molecular transfer of cholesterol, or vesicular transfer, or both. Finding out precisely how NPC1 functions in sterol trafficking at the molecular level is an important goal in understanding and potentially treating NPC disease. One proposed scenario is that cholesterol esters from LDL are delivered to the LE/LY compartment, where free cholesterol is liberated by the action of acid lipase and subsequently binds to the small soluble protein NPC2 (Figures 6 and 9). The NTD

of NPC1 then accepts cholesterol from NPC2 in the LE/LY lumen. The fate of cholesterol beyond this point is unknown, although it seems likely that it is ultimately transferred from the NTD of NPC1 to an acceptor protein or membrane on the cytosolic side. Such an acceptor is required because of the very low aqueous solubility of cholesterol.

Active investigation of the downstream protein partners of NPC1 is in its infancy. The oxysterol-binding protein (OSBP) and its relatives (OSBP-related proteins, ORPs) constitute a large family of conserved proteins in eukaryotes (Beh et al., 2009; Raychaudhuri and Prinz, 2010), with 12 members in humans and seven in yeast, and seem to be likely candidate acceptors. The ORPs are cytoplasmic proteins that are proposed to bind cholesterol and other sterols via their Osh (OSBP homology) domains and transfer them between cytoplasmic compartments by a non-vesicular route. Indeed a recent report implicates a previously uncharacterized member of this family, ORP5, in interacting with NPC1 and accepting cholesterol from it (Du et al., 2011). ORP5 is a tail-anchored protein, and it is proposed that sterol transfer from NPC1 to ORP5 takes place at transient membrane junctions (or contact sites) between the limiting membrane of the LE/LY and the ER (Du et al., 2011).

How cholesterol is transferred from the NTD of NPC1 in the lumen of the LE/LY to an acceptor such as ORP5 on the cytoplasmic side of the membrane is unknown, although it seems likely that more than one step is involved. The transverse diffusion of cholesterol between membrane leaflets is known to be very fast, in the range of milliseconds to seconds (Hamilton, 2003), so this should not present a kinetic barrier to transbilayer sterol transfer. The simplest proposal suggests that the NTD of NPC1 transfers its bound cholesterol into the limiting membrane of the LE/LY, thus effectively bypassing the thick glycocalyx present at the luminal surface, which may act as a diffusion barrier to prevent NPC2 from transferring cholesterol into the membrane directly (Figure 9) (Kwon et al., 2009). However, there is no direct evidence that such a glycocalyx in fact presents a significant diffusion barrier to NPC2-mediated membrane transfer of cholesterol. Indeed, the presence of gangliosides in the acceptor bilayer either had no effect or increased the rate of NPC2-mediated cholesterol transfer (Xu et al., 2008). Also, specificity for NPC1 is presumably required to allow a cytoplasmic carrier such as ORP5 to accept cholesterol from the limiting membrane, especially since this acceptor protein is also membrane-bound. It thus seems likely that the cellular functions of NPC1 may also involve more complex docking events on the cytoplasmic surface of the LE/LY, in order to mediate cholesterol transfer at the contact sites.

Again, some insights may be gained by examining a protein that is a close relative of NPC1. NPC1L1 is expressed at the apical surface of intestinal epithelial cells, where it plays a major role in cholesterol absorption (Altmann et al., 2004; Davis, Jr. et al., 2004). However, the protein has also been found in the endocytic recycling compartment (ERC) (Brown et al., 2007; Yu et al., 2006). NPC1 and NPC1L1 share 51% amino acid similarity, and have very similar NTD structures (Kwon et al., 2011; Kwon et al., 2009), so it seems likely that they also function in a similar way to transfer cholesterol. Recent work suggests that NPC1L1 imports cholesterol by internalization via clathrin/AP2-mediated endocytosis, and carries it in vesicles along microfilaments to the ERC (Ge et al., 2008). The NPC1L1 protein moves between the plasma membrane and the ERC in a cholesterol-dependent fashion. Ezetimibe, a clinically-used inhibitor of dietary cholesterol absorption, blocks internalization and translocation of NPC1L1 by binding to an extracellular loop (Weinglass et al., 2008), equivalent to Loop1 of NPC1 in Figure 7.

By analogy, trafficking events may also play an essential role in NPC1-mediated cholesterol transfer out of the LE/LY compartment. Chang and co-workers recently reported that SNARE-mediated vesicular trafficking is involved in transport of 50–70% of LDL-derived cholesterol from the LE/LY to the ER and TGN (Urano et al., 2008). Using a permeabilized cell system, they showed that cholesterol transport required ATP, cytosolic factors and functional NPC1, although the exact role of the protein again remained enigmatic. It is possible that these trafficking events come into play at the membrane junctions where cholesterol is transferred to the cytoplasmic acceptor ORP5.

Comparison of MsbA and NPC1

Proteins that bind and transfer lipids face special challenges, and must share some unique properties. This review takes a close look at two different integral membrane proteins that bind lipids and are proposed to transfer them across a membrane. MsbA, a putative prokaryotic lipid flippase, is thought to translocate lipid A between leaflets of the bacterial IM, whereas the mammalian sterol-binding protein NPC1 binds cholesterol, and is somehow involved in its transfer across the LE/LY membrane from the lumen to the cytoplasmic side. The substrates for both proteins are membrane lipids with low water solubility; lipid A is unique to Gram-negative bacterial outer membranes, whereas cholesterol is found only in vertebrate cell plasma membranes. Both MsbA and NPC1 are involved in vital "housekeeping" roles in their respective cell types, illustrating the crucial importance of their lipid substrates for cellular function. The LPS export pathway in which MsbA plays a central role is essential for survival of the Gram-negative bacterial cell, as is the NPC1-mediated pathway for trafficking of cholesterol out of the LE/LY in mammalian cells. Crystal structures are known for either the complete protein (MsbA), or one of its domains (NPC1), and have helped to shed

light on the possible modes of action of these two proteins. However, in both cases, the structures have raised questions in addition to providing answers, and a direct role in cellular lipid transport for these two proteins is still not strictly proven. In the case of MsbA, such a function is quite likely, given that many other members of the ABC superfamily are also involved in lipid transfer/transport (Quazi and Molday, 2011). However, the transport/transfer role played by NPC1, beyond its ability to bind cholesterol with high affinity, still remains somewhat mysterious.

MsbA and NPC1 are very different proteins structurally, reflecting the fact that they belong to two quite different protein families, the ABC superfamily (MsbA) and the RND permease superfamily (NPC1). This reinforces the point that there are many ways for proteins to carry out similar functions at the molecular level in terms of binding and handling lipids; for example, members of several different protein families have been implicated as lipid flippases (Sharom, 2011). MsbA and NPC1 also possess very different types of lipid-binding sites, although both are designed to sequester their hydrophobic substrates away from contact with water. The MsbA binding site is located within the membrane bilayer and undergoes a switch between outward- and inward-facing conformations according to an alternating access mode. It is large and appears very flexible, with minimal steric restraints. However, the lack of a substrate-bound crystal structure limits the information we can glean about this site. In contrast, the sterol-binding site within the soluble NTD of NPC1 is a relatively tight pocket, with a closed end that restricts substrate entry and exit to a single narrow opening. These features, which are known in detail from the crystal structure of the sterol-bound form, control the type of sterol molecules that can fit into the site. They also dictate that substrates must slide into and out of the pocket in reverse directions, leading to the concept of the "hydrophobic handoff".

For both MsbA and NPC1, comparisons with related proteins in the same family have provided some very useful clues about the relationship of their molecular structure to their putative function, and will likely continue to do so. The mechanism of action of other ABC proteins, both bacterial and mammalian, is under intensive scrutiny, and this will likely prove helpful in understanding the mode of action of MsbA, since it is assumed that they must share common features. Ongoing work on SCAP and the closely related protein, NPC1L1, both of which bind sterols, should result in more pieces of the puzzle falling into place regarding the molecular mechanism of NPC1.

Conclusions and future directions

The molecular details of exactly how MsbA and NPC1 bind and potentially transfer their different lipid substrates are now beginning to unfold. In the case of MsbA, the determination of medium resolution X-ray crystal structures has provided some clues as to the mechanism of lipid transfer across the membrane, although the nature of the lipid-binding site(s) in the protein remains unknown. Determination of the structure of MsbA in the lipid-bound state is key to resolving this issue. Continued study of MsbA at the structural and biochemical level should be directed to answering fundamental questions about the mode of action of this protein, which represents a potential new target for antibiotics, since it is an essential protein in *E. coli*. The transporter may also be employed as a useful model for understanding how flippases interact with and translocate their lipid substrates. The availability of a reconstituted system containing purified, functional flippase protein is unique in the field, and will allow the detailed exploration of, for example, which protein regions and amino acid residues contribute to lipid binding and translocation. Since we do not currently know the mechanism of action of any lipid flippase, such information would move the field forward.

Although there is still much to be learned about the biochemical mechanism by which NPC1 is involved in cholesterol transfer out of the LE/LY, our knowledge of this key cellular process has advanced dramatically in the past five years. In contrast to MsbA, the structural basis of cholesterol binding by NPC1 is now clear, and together with structural and biochemical information on related sterol-binding proteins, has allowed the proposal of various mechanisms for sterol transfer. It now seems likely that cholesterol flows through NPC2 to NPC1 and from there to ORP5. Testing the validity of these schemes experimentally, and understanding their molecular basis at the biochemical level provides significant challenges for the future. The ultimate goal would be reconstitution of NPC1-mediated cholesterol transfer using purified proteins and subcellular fractions.

Declaration of interest

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