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RND transporters in the living world

Hiroshi Nikaido

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

Abstract

Transporters of the RND superfamily are well-known as the major drug efflux pumps of Gram-negative bacteria. However, they are widespread in organisms ranging from Archaea to Eukaryotes, and perform diverse functions. This review gives a brief overview of these diverse members of the superfamily with emphasis on their structure and functions.

Keywords

Superfamily; Phylogeny; Quaternary Structure

1. Introduction

As is well known, RND (resistance-nodulation-division) transporters function as major drug efflux pumps in many Gram-negative bacteria, but they are also widespread in other branches of life. A summary from M. Saier's laboratory [1, 2] shows that, in addition to the branch containing these efflux pumps (hydrophobe/amphiphile efflux 1, or HAE-1) there are, in bacteria, the heavy metal efflux (HME) family, the nodulation factor exporter (NFE) family and the SecDF family that is involved in protein secretion. In addition, there is an important eukaryotic sterol transporter (EST) family, including the Niemann Pick Type C1 protein. Closely related to this family are also proteins that are involved in developmental hedgehog signaling, whose first recognized member was the dispatched protein in *Drosophila melanogaster*.

All these RND transporters contain 12 (sometimes 13 or 14) transmembrane helices and two large external loops between helices 1 and 2 as well as 7 and 8. In the best-studied representatives, the pump occurs as an asymmetric trimer and the external loops contain binding sites for the exported ligands, while the transmembrane domains mainly function as a conduit for protons that serve as the energy source. This article tries to give a brief overview of these various families in the RND transporter superfamily, whose phylogenetic structure is shown in Fig. 1. Up-to-date information on this superfamily is available on the website maintained by the M. Saier laboratory, Transporter Classification Database (www.tcdb.org), and this article owes much to the information provided therein.

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2. Pumps found in all bacteria (and Archaea)

2.1. The SecDF family

Most secreted proteins in bacteria and archaea are exported across the inner membrane by the translocon SecYEG. However, secretion is aided significantly by the SecDF protein complex [3]. SecD (615 amino acids in *Escherichia coli*) and SecF (323 amino acids), coded by successive genes in an operon, contain only 6 transmembrane segments (TMS) each, and thus correspond to only one-half of a typical RND transporter. As with other members of the superfamily, there are large hydrophilic loops between the first and the second TMS. The SecDF complex from *Thermus thermophilus* was crystallized [4] (Fig. 2, upper panel), and the larger loop domain from SecD (P1) was shown to be separated into two subdomains by a hinge region in the middle. Based on these and other data, it was proposed that the distal subdomain of P1 binds the emerging secreted protein, and that the movement of this subdomain, energized by proton influx, helps in pulling the secreted protein farther out (see Fig. 2, lower panel). Unlike in other families, SecDF function does not require the other components of the tripartite complex, such as members of the membrane fusion protein (MFP) family or outer membrane factor (OMF) family.

3. Pumps found mainly in Gram-negative bacteria

3.1. The HAE1 family

As seen in Fig. 1, the largest number of known RND transporters belong to the HAE1 family. These pumps occur in Gram-negative bacteria, and appear to exist usually as trimers (Fig. 3). As is discussed in other reviews of this series, the three protomers, during export of a broad range of substrates, are ideally thought to undergo consecutive conformational changes called functional rotation through the access, binding and extrusion (or loose (L), tight (T) and open (O)) conformations, and the binding conformer contains two large substrate-binding pockets, the proximal and the distal pockets, the latter with a hydrophobic region rich in Phe residues [5]. However, an AcrB trimer does not always contain one each of these three conformers [5]. This becomes important because an unusual HAE1 transporter, MdtBC of *Escherichia coli*, was found to function only as a B₂C heterotrimer [6], and that only the B protomers appear to bind and extrude substrates [7]. Similarly, an RND pump that appears to function as a dimer will be discussed below in this article.

The trimers then form tripartite complexes together with the members of MFP and OMF families. The structure of these complexes [8] allows Gram-negative bacteria to move the substrates out into the external medium, from periplasm or locations that are in rapid equilibrium with periplasm. Thus, they often play important roles in the efflux of antimicrobial agents. Some of these pumps, such as AcrB of *E. coli*, and MexB of *Pseudomonas aeruginosa*, are constitutively expressed and thus affect the baseline resistance levels of these organisms to a wide range of antibiotics. These constitutive transporters pump out compounds that contain at least a hydrophobic patch, but there are also pumps (AcrD of *E. coli* and MexY of *P. aeruginosa*) known to handle completely hydrophilic ligands such as aminoglycosides [9, 10]. Interestingly, it was shown that the capacity of AcrD to pump out

dianionic β -lactams depends on the residues in the proximal, not distal binding pocket [11]. The role of Gram-negative RND pumps in drug resistance has been reviewed recently [12].

3.2. The HME family

In contrast to HAE1, the members of this family pump out heavy metal ions. The tripartite cobalt-zinc-cadmium efflux system of *Cupriavidus metallidurans* CH34, CzcCBA, has been studied extensively in terms of biochemistry and genetics [13], but no structure is available. The first crystal structure of a member of this family, CusA of *E. coli* [14], in 2010, was thus important. It crystallized as a symmetric trimer (Fig. 4, left), unlike AcrB, and Cu^+ was found to bind to a pocket similarly located as the distal binding pocket of AcrB. However, the sequence in this particular area is radically altered, so that the pocket no longer contains Phe and other hydrophobic amino acid residues. Instead, there are three methionine side-chains protruding into the pocket, and the metal ion is thought to be coordinated by the three sulfur atoms of these side-chains. Curiously, there are multiple methionine residues in the transmembrane domain, but it seems unlikely that these are involved in the direct uptake of Cu^+ from the cytosol, because an efficient efflux of copper from intact cells requires the P-type ATPase CopA, which moves the ion from cytosol to periplasm [15].

More recently the crystal structure of ZneA, a Zn^{2+} -specific RND pump from *C. metallidurans* CH34, was elucidated [16]. Its trimeric structure is reminiscent of AcrB, but the distal binding pocket contains two glutamate and one aspartate residue that bind the metal ion.

3.3. The NFE family

In 1991, disruption of genes in the nod box 4 of *Rhizobium meliloti* was reported to impair the production of a class of nodulation signal, N-acetylglucosamine oligosaccharides in which the acetyl group of the terminal sugar is replaced by a fatty acid. Since *nolG*, *nolH*, and *nolI* did not appear to code for the production of the nodulation signal, they were proposed to be involved in its export [17]. Saier and associates found that these three genes were actually one gene coding for an RND transporter, preceded by gene *nolF* coding for a smaller protein [18]. The transporter then became the prototype for a nodulation factor exporter (NFE) family, and contributed to the name of the parent superfamily, resistance-nodulation-division or RND. An NFE transporter and associated proteins in the plant pathogen *Pseudomonas syringae* were shown to be involved in the export of the lipodepsipeptides syringopeptin and syringomycin [19], compounds not very dissimilar from the lipooligosaccharide nodulation factors. Another NFE family member, YerP, was shown to enhance the secretion of another lipodepsipeptide, surfactin, this time in Gram-positive *Bacillus subtilis* [12].

However, during the nearly three decades following the initial report, there has been no direct confirmation that the NolG transporter actually pumps out nodulation signals, and a critical study is sorely needed here. A computational study [20] also predicts that NolG is close to the drug pumps such as MexF of *P. aeruginosa* in terms of its predicted substrate specificity. Indeed, some recently identified members of the NFE family contain drug exporters, such as CmeF of *Campylobacter jejuni* [21], BesB of *Borrelia burgdorferi* [22],

and HefA of *Helicobacter pylori* [23]. Genes coding for these pumps occur together with those coding for MFP and OMF, but the MFP in *Borrelia* apparently lacks the coiled-coil region needed for interaction with OMF [22].

3.4. The HAE3 family

Another small family HAE3 includes some putative pumps in Archaea, but also contains HpnN, a pump in Gram-negative eubacteria, first identified in *Rhodopseudomonas palustris*, apparently needed for pumping out hopanoids to the outer membrane [24]. A recent crystallization of HpnN from *Burkholderia multivorans* [25] surprisingly showed that the protein exists as a dimer (Fig. 4, right), rather than a trimer found with many other RND pumps so far.

3.5. The APPE family

The small, brominated aryl polyene pigment extrusion family was first identified as a component of the pigment locus in *Xanthomonas oryzae* and was shown to function in exporting the synthesized aryl polyene pigment to the outer membrane [26]. Its external loops are relatively small, resulting in a rather small protein with only 807 amino acids (in comparison with 1,049 amino acids in AcrB).

4. Pumps Found in Gram-positive bacteria

Members of the HAE2 family, which is closely related to the HAE1 family (Fig. 1), are all expressed in Gram-positive bacteria. Noteworthy among them are the MmpL proteins in *Mycobacterium tuberculosis* (see Fig. 5), which include MmpL7 that is involved in the export of phthiocerol dimycocerosate [27]. The details of this export pathway are not yet clear, because the biosynthetic gene cluster, which includes the *mmpL7* gene, also includes genes for an ABC transporter, which is also known to affect the transport of the lipid [28]. In any case, the substrate that is exported across the inner membrane is likely to be taken up by the lipoprotein LppX, which contains 11 antiparallel β -strands forming a hydrophobic pocket that can accommodate the substrate [29]. When the *mmpL7* gene of *M. tuberculosis* was introduced into *M. smegmatis* on a plasmid, isoniazid resistance was increased apparently due to its efflux [30].

Similarly, MmpL10 was shown to be essential in the transport of diacyltrehalose and pentaacyltrehalose [31], and MmpL8 was shown to be involved in the export of sulfolipid-1 [32]. In various species of mycobacteria, genes for MmpL4a and MmpL4b are found within cluster(s) coding for the glycopeptidolipid synthesis and are suspected to function in its export [33]. Recently, in *M. abscessus*, inactivation of MmpL4a was shown to abolish the export of glycopeptidolipids [34]. Interestingly the export becomes less efficient when the MFP component, MmpS4, is deleted [35]. MmpS4/MmpL4 and MmpS5/MmpL5 are also involved in the secretion of a siderophore, mycobactin [36].

Mycolate, a characteristic component of the mycobacterial cell wall (or outer membrane), is synthesized as trehalose monomycolate and then gets exported by a mechanism that had remained unknown [37]. A breakthrough in our understanding came when the new antimycobacterial compounds AU1235 and SQ109 were found to exert their action by

inhibiting mycolate export through inhibition of MmpL3 [38, 39]. This function of MmpL3 also fits with the observation that it is an essential gene in *M. tuberculosis* [40]. MmpL3 is now known to exist in the form of trimers in detergent solutions and its homology models have been built [41]. Interestingly, the inhibitors mentioned above appear to bind not to the extramembranous domain, but to the region involved in proton relay in the transmembrane domain [41]. Finally, a clever use of the spheroplast system showed that MmpL3 acts as a trehalose monomycolate flippase [42].

One of the extramembranous domains of MmpL11 has been crystallized, and was shown to fold in a pattern similar to that found in HAE1 pumps [43]. The functions of MmpL transporters have been reviewed [28, 44].

5. Pumps in eukaryotes

5.1. The EST (eukaryotic sterol transporter) family

This family, typified by the Niemann-Pick Type C protein 1 (NPC1), was discovered by sequencing of the gene causing this hereditary disease, resulting in the defective trafficking of cholesterol [45](Fig. 6). Cholesterol esters are components of low density lipoprotein (LDL), which is brought into the cells of various tissues by targeted endocytosis. Within the endosomes, cholesterol esters are hydrolyzed, releasing free cholesterol, which is finally exported out of the endosome by NPC1 with assistance from a soluble protein NPC2.

NPC1 is a large protein containing at least 13 transmembrane helices, and in addition to the usual two large extramembrane loops (called MLD (middle luminal domain) and CTD (C-terminal domain) in Fig. 7), contains a large N-terminal luminal domain (NTD in Fig. 7). In a minority of patients with Niemann-Pick disease, defects are found in a soluble protein NPC2 [46], which was known to bind cholesterol. It was found early on that the transmembrane helices in the N-terminal half (labeled SSD, sterol-sensing domain) of NPC1 had homology with the putative sterol-binding domains of HMG-CoA reductase, as well as the regulator of cholesterol-regulated transcription activation, SCAP [45]. The domain that binds cholesterol with the highest affinity, within NPC1, however, is the NTD [47].

Cryo-EM studies [48] showed that, except for the extreme N-terminal region, the NPC1 protein followed the typical RND transporter folding pattern, and the CTD domain (called Domain C in Fig. 8) is close to the membrane surface and the MLD domain (called Domain I in Fig. 8) is stacked on top of CTD. NTD protrudes the farthest distance into the lumen. These results were recently confirmed and refined by an x-ray crystallographic study of NPC1 lacking the NTD [49], which proposes that cholesterol is first bound by NPC2 in the lumen, and the liganded NPC2 becomes attached to the open surface of MLD in NPC1, resulting in the transfer of cholesterol to the NTD domain of NPC1. The final export of cholesterol out of the endosomes is thought to be preceded to its movement from NTD to SSD in the transmembrane domain, but how this occurs is still not known [49].

Regardless of the detailed mechanism, it should be emphasized that the substrate transport in NPC1 occurs in the reverse direction from that found in the usual bacterial RND transporters. In the bacterial transporters, substrates are captured by the extramembranous

loops, usually at a location close to the surface of the inner membrane, and then transported farther away from the membrane. In the NPC2-NPC1 complex, the substrate is captured at a location far away from the membrane by NPC2, and then is brought to a location close to the membrane surface (NTD of NPC1), and is finally moved to the intramembranous region of NPC1. How it is released from the SSD of NPC1 remains a future topic of study.

Since the endosome lumen is more acidic than the cytosol, NPC1 appears to function, unusually among RND transporters, as a proton/ligand symporter, rather than an antiporter.

5.2. The Dispatched family

An RND transporter, Dispatched, is known [50] to export the amino-terminal portion (19 kDa) of the Hedgehog, whose C-terminus becomes covalently linked to cholesterol during autoproteolysis of the 45 kDa precursor [51]. The intracellular movement of this Hedgehog signal is quite complex, and is now thought to involve the insertion into the apical cell membrane, followed by endocytosis, and then secretion at the basolateral membrane by Dispatched [52]. Dispatched shows a typical RND protein folding pattern, although its overall sequence homology to Patched (see the paragraph below) or NPC1 is low. However, it contains the typical sterol sensing domain, which is similar to those found in NPC1 as well as in Patched [50]. Interestingly, the Hedgehog anchored by glycosylphosphatidylinositol, rather than cholesterol, was not exported by Dispatched, suggesting that the cholesterol anchor is recognized by the transporter [50]. Finally, the secretion of Hedgehog by Dispatched across the membrane requires help by other proteins, including a secreted soluble protein called Scube [53]

Before the discovery of this family, Patched was known to be an important component of Hedgehog signaling in organisms from *Drosophila* [54] to humans [55], sometimes long before the RND transporters became known (for example [54]), and displays a typical RND folding pattern. Although Hedgehog appears to bind to Patched, there does not seem to be not much evidence that it catalyzes its import; thus, it is generally thought that Patched functions as a Hedgehog receptor, not as a transporter. The two extramembranous loops of Patched are thought to face the exterior [56], and possibly play a role in the binding of the signal protein. However, there is some evidence suggesting that Patched indeed functions as a transporter, possibly of small molecules [57].

6. Perspectives

After this brief survey of various members of RND superfamily transporters, we would be tempted to ask what characterizes this group of pumps. Perhaps one characteristic that stands out is that practically all the members are involved in pumping out, or carrying out efflux of, diverse substrates, which typically start their journey from the transmembrane domain or from the membrane-proximal part of the extramembranous domain, and then move out through the membrane-distal part of the latter domain. (One exception here is NPC1, in which the direction of the substrate travel seems to be the opposite. However, NPC1 is located in the endosomal membrane, which has an inverted orientation due to the endocytosis process that created the endosome. In any case, the substrate is pumped out of the vesicle even in this case.) In contrast, other large superfamilies of transporters, ABC and

MF (Fig. 3), contain both importers and exporters. What makes RND members exceptionally versatile exporters? One feature that may be relevant at least for the typical drug efflux pumps of HAE1 family is that they occur as asymmetric trimers, making the binding and extrusion of an extremely wide range of compounds possible by synergy among conformers within the trimeric unit [5]. Although other classes of pumps may occur as dimers, a trimeric construction appears to be rare. Another factor may be the very large size of the extramembranous domains of RND transporters, which obviously facilitates their interaction with other proteins such as MFP family members and construction of a tripartite structure spanning both the inner and outer membranes in Gram-negative bacteria, although MF and ABC transporters with similar construction do exist, as seen with EmrB and MacB in *Escherichia coli*. The large size also makes it possible to have very large substrate-binding sites, needed for the broad specificity of these pumps. Finally, the wide separation between the pathway traversed by protons and that followed by substrates may be beneficial for antiporters. In other PMF-energized transporters [58, 59], the substrate-binding and proton-binding sites often are very close to, or actually overlap, each other, making the construction of antiporters somewhat difficult or limiting the range of substrates, as with SMR [58] and MATE [60] family transporters.

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Fig. 1.
Current view of the phylogenetic relationship among members of the RND superfamily.
From Yen et al [1].

Fig. 2.

The structure of *T. thermophilus* SecDF. The upper panel shows the X-ray crystallographic structure. The lower panel shows a hypothetical mechanism by which SecDF enhances the efficiency of secretion through the SecYEG translocon, by pulling the nascent polypeptide via the relative translocation of P1 head vs. P1 base subdomains. From [4].

Fig. 3.

Crystallographic structure of an HAE1 family RND transporter AcrB (at right) compared with other classes of efflux transporters belonging to ABC, major facilitator (MF), MATE and SMR families. The bound ligands are shown as purplish pink spheres. The structure of AcrB shows ligands bound to the proximal pocket (left) as well as to the deep binding pocket (right). From [59].

Fig. 4. Crystallographic structures of an HME family RND transporter CusA (left, from [14]) and an HAE3 family RND transporter HpnN which crystallizes as a dimer (right, from [25]).

Fig. 5.

A. Phylogenetic relationship between MmpL transporters in *M. tuberculosis*. These transporters are divided into two clusters. B. Schematic folding pattern of MmpL exporters, where D1 and D2 denote periplasmic domains and D3 a cytosolic domain. From Chim et al. [43].

Fig. 6.

Main routes of intracellular cholesterol movement. Cholesterol esters, a major component of low density lipoprotein (LDL) arrives at the surface of cells of various tissues and are internalized by the process of receptor-mediated endocytosis. Within the endosome, the cholesterol esters are split to generate free cholesterol, which becomes exported out of the late endosomes by NPC1 (in a process involving the participation of soluble cholesterol-binding protein, NPC2) to organelles such as ER and Golgi. Excess cholesterol can also be esterified again by ACAT (acyl-coenzyme A: cholesterol acyltransferase) and stored in intracellular lipid droplets. From [61].

Fig. 7. Comparison of the NPC1 structure (below) with that of HAE1 family RND transporters (above). In the NPC1 structure, the three major extramembrane domains, the N-terminal domain (NTD), the middle luminal domain (MLD) and the C-terminal domain (CTD) are indicated, as well as the sterol-sensing domain (SSD) composed of five transmembrane helices. From Ioannou [61].

Fig. 8. Structure of the NPC1 protein revealed by Cryo-EM. The three extramembrane domains NTD, MLD and CTD of Fig. 7 are here called Domains A, I and C. The transmembrane helices corresponding to the sterol-sensing domain (SSD) are colored in yellow. From [48].