Low-density lipoprotein particles are taken up by cells and delivered to the lysosome where their cholesterol esters are cleaved off by acid lipase. The released, free cholesterol is then exported from lysosomes for cellular needs or storage. This article summarizes recent advances in our understanding of the molecular basis of cholesterol export from lysosomes. Cholesterol export requires NPC intracellular cholesterol transporter 1 (NPC1) and NPC2, genetic mutations of which can cause Niemann–Pick type C disease, a disorder characterized by massive lysosomal accumulation of cholesterol and glycosphingolipids. Analysis of the NPC1 and NPC2 structures and biochemical properties, together with new structures of the related Patched (PTCH) protein, provides new clues to the mechanisms by which NPC proteins may function.

After endocytosis, LDL2 is delivered to the lysosome, where its cholesterol esters are cleaved by acid lipase to release free cholesterol for cellular use (1). NPC2 protein is a small (~25-kDa) globular protein (2, 3) that is thought to bind the cholesterol released from LDL and transfer it onto NPC1 (4–9), a 1278-residue protein with 13 transmembrane domains (10) (Fig. 1A). Mutations in either NPC2 or NPC1 protein can lead to a severe disorder called Niemann–Pick Type C disease, which leads to massive accumulation of cholesterol and glycosphingolipids in lysosomes of all tissues, especially in the liver, spleen, and brain, and ultimately leads to premature death (11).

Mechanistic analysis of NPC1 protein is challenging for many reasons, including the fact that the cholesterol substrate is hydrophobic and will partition into membranes (or lysosomes) harboring NPC1. Moreover, despite a large number of possible pathogenic mutations, most of these do not provide useful functional information because they instead interfere with proper protein folding in the endoplasmic reticulum and lead to premature protein degradation. Nevertheless, workers in this field have taken diverse and innovative approaches to overcome these challenges, and many of the important milestones in our understanding of cholesterol transport have been published in the Journal of Biological Chemistry and will be summarized here.

NPC1 and NPC2 bind cholesterol

The structures of NPC2 without (2) or with (3) (green in Fig. LA) cholesterol showed that NPC2 binds cholesterol via its iso-octyl chain and undergoes only limited conformational changes adjacent to the cholesterol-binding pocket upon sterol binding (3). The discoveries that NPC1’s N-terminal domain (red in Fig. LA), and likely also its membrane-embedded, so-called sterol-sensing domain, both bind cholesterol have represented significant breakthroughs in our understanding of NPC1 and NPC2 functions.

Ohgami et al. (12) were the first to show binding of a photoactivatable cholesterol analog to NPC1 and noted that P692S and Y635C mutations in the sterol-sensing domain blocked this interaction. For these experiments, the authors used a 7,7-azo-[3H]cholesterol (Fig. 2). Using an entirely different approach, Infante et al. (13) identified NPC1 in a search for membrane-associated hydroxy-cholesterol–binding proteins. NPC1 showed a preference for 24, 25, or 27 hydroxysterols; a hydroxyl group at positions 7, 19, or 20 failed to confer binding. This latter finding suggested that hydroxycholesterol was binding at a site distinct from that detected by Ohgami et al. using the position 7-modified azosterol. Subsequent work by Infante et al. (14) revealed that NPC1 N-terminal domain (residues 25–264) comprises a saturable binding site for cholesterol; determination of the structure of this domain (6) with and without cholesterol confirmed that cholesterol binding occurs via the hydroxyl moiety, with little conformation difference between cholesterol-bound and apo states.

Kwon et al. (6) were the first to propose that the opposite orientation of cholesterol binding to NPC2 compared with NPC1 provided a perfect arrangement for transfer of cholesterol from NPC2 onto the NPC1 N-terminal domain. These researchers wrote, “. . . . In transferring its bound cholesterol to the lysosomal membrane, the N-terminal domain of NPC1 could interact either with its own membrane domain, in which case it might transfer the cholesterol to the putative sterol-sensing domain in transmembrane helices 3–7, or with the membrane domain of a neighboring NPC1 molecule.”

Frances Sharom and colleagues (15) crosslinked 7,7-azo-cholesterol (Fig. 2) to purified FLAG-tagged NPC1 protein and also characterized the binding of fluorescent sterols to NPC1. They found that upon addition of NBD-cholesterol (Fig. 2),
NPC1’s intrinsic tryptophan fluorescence was quenched and the protein displayed sensitized fluorescence emission at 520 nm. NPC1 binding to NBD-cholesterol was competed by cholesterol, 25-hydroxycholesterol, dihydroergosterol, and to a lesser but significant extent, the cationic sterol U18666A, but not epicholesterol. This suggested that NPC1 distinguishes the orientation of the cholesterol hydroxyl group (consistent with Refs. 6, 13, and 14), and importantly, that U18666A might block cholesterol export by direct interaction with NPC1. Note, however, that while the NPC1 N-terminal domain would have been able to accommodate the NBD-cholesterol used in this study, the N-terminal domain would not likely be able to interact with U18666A (Fig. 2), as shown by Infante et al. (14). Thus, it is probable that these authors were monitoring binding to two distinct cholesterol-binding sites in these experiments.

Strong evidence for a cholesterol-binding site located outside of the NPC1 N-terminal domain came from Ohgane et al. (16) in their studies of the trafficking of NPC1 carrying the most common pathogenic mutation, I1061T. The presence of this mutation slows NPC1 folding in the endoplasmic reticulum, and little of the NPC1 is delivered to lysosomes. Ohgane et al. found that a number of oxysterols enhance NPC1I1061T folding and export to lysosomes; interestingly, oxysterol-mediated trafficking enhancement was also seen for NPC1 missing the cholesterol-binding, N-terminal domain. Direct photoaffinity sterol crosslinking was also possible with an N-terminal domain-deleted NPC1, demonstrating the presence of a second site for sterol binding.

Finally, Lu et al. (17) showed that a U18666A derivative could be crosslinked directly to NPC1 in a manner that was independent of the N-terminal domain and sensitive to the integrity of the sterol-sensing domain, reminiscent of the findings of Ohgami et al. (12). Similarly, Trinh et al. (18) showed that a photoactivatable triazole inhibitor of NPC1 could also be crosslinked to NPC1 independent of the N-terminal domain, and may also bind to the sterol-sensing domain. Altogether, these studies reveal at least two cholesterol-binding sites: one within the N-terminal domain and a second binding site that may, in fact, represent the sterol-sensing domain.

NPC structures into focus

The recent determinations of the structures of NPC1 luminal domains 2 (Fig. 1A, blue) and 3 (Fig. 1A, yellow) (19–22) and of the full-length NPC1 by cryo-EM (23) have catapulted our understanding of these proteins to a new level (Fig. 1). The prediction that the protein would show structural similarity to bacterial RND transporters was very prescient (cf. Refs. 10, and 12). Moreover, the co-crystal structure of NPC2 bound to NPC1’s second luminal domain (24) provides a valuable starting point for thinking about how cholesterol is likely to be transferred onto NPC1 protein. Despite these important breakthroughs, we still know little about how NPC1 actually transfers cholesterol across the lysosome membrane after receiving it from NPC2.

Li et al. (22) proposed that the NPC1 N-terminal domain utilizes the flexibility of a polyproline linker to transfer cholesterol to a cavity detected on the other side of the protein, at the so-called sterol-sensing domain, as originally proposed by Goldstein and colleagues (6). This pocket lies at the boundary between the inner leaflet of the lysosome membrane and the lumen, which is advantageous because it would be available to both receive cholesterol from NPC1’s N-terminal domain and transfer it to the adjacent membrane. Consistent with this model, Trinh et al. (25) recently reported that NPC1’s N-termi-
nal domain can transfer cholesterol (albeit relatively inefficiently) to an adjacent NPC1 molecule for membrane transfer.

NPC1 is a very tall molecule, and domains that receive cholesterol from NPC2 are located 8 nm from the membrane bilayer (21, 23). This corresponds well with the height of the glycocalyx that is thought to line the limiting membrane of the lysosome (26). The relevance of the glycocalyx to the mechanism of cholesterol transfer was supported by data showing that gentle thinning of the membrane protein glycans decreased the requirement for NPC1 protein in cholesterol export from lysosomes (27). Given the presence of the glycocalyx, it is hard to imagine how NPC1’s N-terminal domain gains access to the membrane interface to deliver cholesterol to the membrane, unless the glycocalyx is not uniform in terms of membrane coverage and/or thickness. In considering the established mechanisms of transporters for amino acids, sugars, and hydrophobic small molecules, it is also important to consider models in which cholesterol may pass through NPC1 protein. Recent structures of the related Patched protein support this possibility (28–31).

Clues from the related Patched structures

The Patched protein (PTCH) is the receptor for the Hedgehog ligands (Hh) that are critical regulators of developmental signaling. In the absence of ligand, PTCH inhibits smoothened; ligand binding to PTCH removes this inhibition. PTCH is closely related to NPC1 and has 12 transmembrane domains instead of 13; it lacks the cholesterol-binding N-terminal domain. Transmembrane domains 2–6 comprise a sterol-sensing domain analogous to transmembrane domains 3–7 of NPC1.

Using cryo-EM, Nieng Yan and colleagues (28) detected two sterol molecules in the PTCH structure: one in a cavity located between the two extracellular domains and the second, adjacent to the sterol-sensing domain. In comparing the PTCH structure with and without a non-lipidated Hh ligand, the authors noted significant shifts in the orientations of the extracellular domains: they move closer together with no change in the transmembrane domains. Moreover, the presence of cholesterol hemisuccinate influenced the ability of sonic Hh to bind PTCH. Strikingly, comparison with the structure of a triple mutant PTCH protein suggested that cholesterol binding causes significant conformational changes, with an untwisting of the interactions between the extracellular domains and reorientation of certain TMs. The triple mutant protein structure was of somewhat lower resolution (4.1 Å), and additional experiments will add credence to this very exciting possibility.

Xiaochun Li and colleagues (29) reported the structure of PTCH in complex with palmitoylated Hh ligand. Interestingly, the orientation of the palmitoylated ligand was different from that observed for the non-modified ligand by Yan and colleagues (28); the palmitoyl group was buried in between the two extracellular domains as if plugging a channel for possible sterol transport. Indeed, the PTCH structures are consistent with the possibility that cholesterol passes through the PTCH protein, en route at least as far as the pocket adjacent to the sterol-sensing domain. The most recent findings of Li and colleagues (30) indicate that in the presence of physiological (1 mM) calcium, two PTCH molecules may engage a single Hh ligand, anchored together by N-terminal palmitoyl binding in one PTCH monomer and via the polypeptide portion binding to the other. Here, two sterol-like ligand densities were detected in the transmembrane domain of each PTCH molecule, one in the sterol-sensing domain and the other near transmembrane domain 12. Importantly, the structural analysis is consistent with a tunnel in the PTCH protein, through which a sterol could pass; the channel would be blocked by the Hh palmitoyl moiety. A puzzle for those studying PTCH is how the asymmetric interaction of the Hh ligand with the PTCH receptor accomplishes signaling: if the palmitate forms a plug to block transport through the molecule, the plug would not be present in the other partner.

Cholesterol passage through NPC1 protein

Does NPC1 also bind cholesterol in a cavity between its extracellular domains, analogous to PTCH? Does NPC1 contain a similar channel through which cholesterol might pass? Important hints come from the work of Cravatt and colleagues (32), who carried out a proteome-wide analysis of cholesterol-binding proteins using a cholesterol derivative that contains a photoactivatable diazirine group at the 6 position of the steroid core. He and his colleagues identified 250 cholesterol-binding proteins including NPC1; the peptides they identified as having crosslinked to cholesterol are shown in red in the high resolution NPC1 crystal structure (22) (Fig. 1, center). Note that none of these peptides are localized to the N-terminal domain or the sterol-sensing domain, suggesting that cholesterol may indeed interact more broadly with the NPC1 extracellular domain.

The availability of new structures for NPC1 and PTCH proteins can now guide the next steps toward our understanding of cholesterol export from lysosomes. Analogous to PTCH (28), does NPC1 also undergo significant conformational changes in the presence of cholesterol that may be important for its transport functions? Does cholesterol binding to the sterol-sensing domain regulate these distinct conformations or actually permit cholesterol transfer to the adjacent membrane? These are
among many important questions next to be tackled by workers in the field.

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