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Special Contribution

Niemann-Pick C1-Like 1: A Key Player in Intestinal Cholesterol Absorption

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Niemann-Pick C1-Like 1 (NPC1-L1), as its name indicates, was identified in 2000 as a homolog of NPC1. Its major physiological function was clarified by a research group in Shöring's laboratory who had long been searching for a target of ezetimibe, a cholesterol-lowering drug. They published a paper on 2004 in Science, reporting a reduction of intestinal cholesterol absorption and a lack of effects of ezetimibe in NPC1-L1 knockout mice. With subsequent studies that confirmed their findings, it is now clear that NPC1-L1 is a key player in one of the major pathways of intestinal cholesterol absorption and that it is the target of ezetimibe. This review summarizes what has been shown up to now about the structure and function of NPC1-L1. This review also refers to ABCG5/G8, a member of ABC family transporters, which co-localizes with NPC1-L1 on the intestinal epithelial cells and appears to work in a closely related manner.

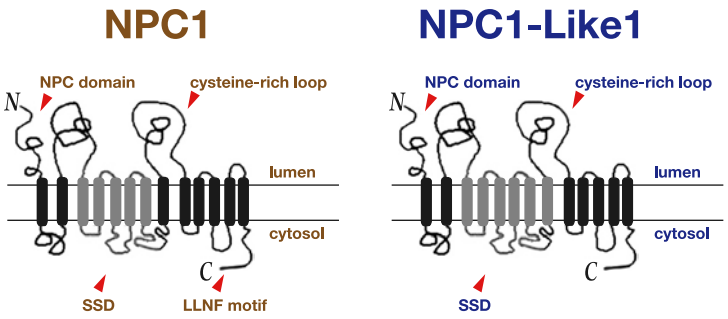
Key words: ABCG5/G8; cholesterol; low-density lipoprotein; Niemann-Pick; Niemann-Pick disease type C1-like 1

Structures of NPC1 and NPC1-L1

Let us start with Niemann-Pick disease type C1 (NPC1) which was identified on 1997 (Fig. 1), because it is the prototype of these two proteins and hence much of what we know about NPC1-Like 1 (NPC1-L1) originates in studies on NPC1. It is a glycoprotein with a molecular mass of 170 to 200 kDa and is predicted to contain 13 transmembrane domains. Its primary structure is featured by a signal peptide on its *N*-terminus and a dileucine motif on the *C*-terminus, the latter of which is believed to be a targeting signal to the endosome (Davies et al., 2000). The N-terminal extracellular loop contains an amino acid sequence of approximately 100 amino acids long (55–165), which is of high conservation between species, and is called an Niemann-Pick disease type C (NPC) domain. Recent studies have shown that cholesterol directly binds to this domain (Infante et al., 2008). The middle part of this molecule (transmembrane domain 5–8) constitutes a sterol-sensing domain (SSD). The SSD is shared by other molecules involved in the control of cholesterol homeostasis, including hydroxymethyl-glutaryl-CoA reductase and sterol regulatory element-binding protein cleavage-activating protein. NPC1 is one of the house-keeping genes, expressed in every tissue and cell.

Loss of function of NPC1 results in NPC, an autosomal recessive lipid storage disease (Patterson et al., 2001). Although heterogeneous, NPC is typically a childhood disease with an onset in the infancy and an eventual demise in the second decade of life. The NPC kids exhibit a variety of neurological symptoms including ataxia, cataplexy, vertical gaze palsy and so on. At the cellular level, the most prominent feature of NPC is accumulation of

Abbreviations: ABC, ATP-binding Cassette; GFP, Green Fluorescent Protein; LDL, low-density lipoprotein; NPC, Niemann-Pick disease type C; NPC1, Niemann-Pick disease type C1; NPC1-L1, NPC1-Like 1; SSD, sterol-sensing domain



genome	18q11	7p13
tissue distribution	ubiquitous	enterocyte/hepatocyte
intracellular localization	late endosome	cell surface/endosome
functional partner	NPC2	?
function	intracellular transport of cholesterol	cholesterol uptake from diet/bile
loss of function phenotype	NPC	reduced choleserol uptake
inhibitor	none	ezetimibe

Fig. 1. Structure and function of NPC1 and NPC1-L1. See text for details. NPC, Niemann-Pick disease type C; NPC1, Niemann-Pick disease type C1; NPC2, Niemann-Pick disease type C2; NPC1-L1, NPC1-Like 1; SSD, sterol-sensing domain.

free cholesterol in the endosome/lysosome. Cholesterol is supplied to peripheral cells mainly by low-density lipoprotein (LDL). Lysosomal hydolyses convert cholesterol esters in LDL to the free form, which then must be delivered to other cellular compartments such as the plasma membrane and the endoplasmic reticulum. NPC1 localizes mainly in the late endosomes. Cells without NPC1 function fail to transport cholesterol out of the endosomes and develop hybrid structures containing both the marker molecules of late endosomes and lysosomes. These abnormal endosomes contain multivesicular bodies within their lumen. Because of these loss-of-function phenotypes, it is clear that NPC1 takes part in the cholesterol transport out of the endosomal system. Although the precise mechanism of action remains to be clarified, it has been shown that the protein "moves" within the cell, being contained in transport vesicles that shuttle between cellular compartments (Zhang et al., 2001).

NPC1-L1 has the amino acid sequence that is 40% identical to that of NPC1 and is predicted to have a similar secondary structure. Like NPC1, it has a signal peptide on its *N*-terminus, an NPC domain on its *N*-terminal extracellular loop, and an SSD in its middle part. A clear difference is that it lacks the *C*-terminal dileucine motif (Davies et al., 2000). The tissue distribution of NPC1-L1 is also quite different from that of NPC1; both in mice and humans, its expression is tissue-specific and is specifically high in the small intestine. According to an analysis in mice, its expression is heterogeneous in the intestine, being high in the duodenum and jejunum, and low in the ileum (Davis et al., 2004). This distribution provides a good explanation for the fact that dietary cholesterol is absorbed mainly in the duodenum and jejunum. It should be noted that its expression level in the liver is clearly different between mice and humans; in humans, its level in the liver is as high as in the intestine whereas it

is barely expressed in mouse livers. Histochemical studies using human tissues demonstrated its sequestration on the brush border membrane of the intestinal epithelial cells and on the bile canaliculi of hepatocytes (Sané et al., 2006; Temel et al., 2007). Figure 2 depicts its cellular localization in a scheme.

Intestinal absorption of dietary cholesterol in NPC1-L1 knockout mice is reduced to approximately half the level of that in wild-type mice (Altmann et al., 2004). Feeding of the knockout mice with a high-cholesterol diet failed to induce hypercholesterolemia and resultant fatty liver (Davies et al., 2005).

Its selective tissue distribution and the phenotypes of gene knockout mice made it clear that NPC1-L1 is required for intestinal absorption of dietary cholesterol. Following studies focused on how this protein takes part in the absorption process at the molecular level. Although its localization in the hepatic canaliculi suggests its role in cholesterol uptake from bile, the following discussion focuses on its role in the intestine, because of space limitation.

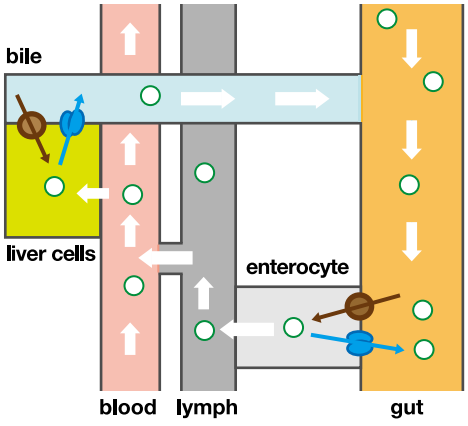


Fig. 2. Cholesterol circulation and NPC1-L1 localization. In humans, NPC1-L1 resides on the apical membrane of intestinal absorptive cells and in bile canaliculi of hepatocytes, where it facilitates cholesterol uptake. ABCG5/G8 resides on the same sites and facilitates cholesterol flow in the opposite direction. NPC1-L1, Niemann-Pick disease type C1-Like 1.

Intracellular sequestration of NPC1-L1 and cholesterol transport: findings in cultured cells

Immediately following its identification, the intracellular localization of NPC1-L1 was examined using cultured cells and epitope-tagged recombinant proteins. Initial two reports were obviously contradictory to each other. Shöring's group reported localization of NPC1-L1 tagged with Flag epitope or Green Fluorescent Protein (GFP) on the cell surface of Chinese hamster ovary cells (Iyer et al., 2005) whereas Mt. Sinai's group demonstrated localization of Yellow Fluorescent Protein-tagged NPC1-L1 in the intracellular vesicles of COS cells (Davies et al., 2005). The latter group also showed sequestration of endogenous NPC1-L1 in cytosolic vesicles of HepG2 cells. They claimed that these vesicles represent early endosomes and recycling endosomes that emanate from them.

A later report appeared to reconcile the discrepancy of these initial studies. Yu et al. studied intracellular localization of GFP-tagged NPC1-L1 in rat hepatoma cells and observed that this protein shuttles between the cell surface and cytosolic vesicles (Yu et al., 2006). This movement appeared to be regulated by the cellular level of cholesterol: the protein resided on the cell surface in a low-cholesterol condition and on the cytosolic vesicles in a high-cholesterol condition. The cytosolic vesicles appeared to be recycling endosomes because they contained fluorescent transferrin. Subsequently, several groups made a similar observation that NPC1-L1 "moves" within the cell in a cholesterol-dependent manner (Ge et al., 2008; Petersen et al., 2008). Our observation using a confocal microscopy is depicted in Fig. 3. It has also been shown that the movement from the cell surface to the endosome depends on clathrin/AP2 complexes (Ge et al., 2008).

This intracellular movement of NPC1-L1 is reminiscent of that of NPC1. In the case of NPC1, it has been shown that a steroid-derivative U18666A that inhibits its function apparently blocks its intracellular movement. Likewise, the intracellular movement of NPC1-Like1 is blocked by its inhibitor

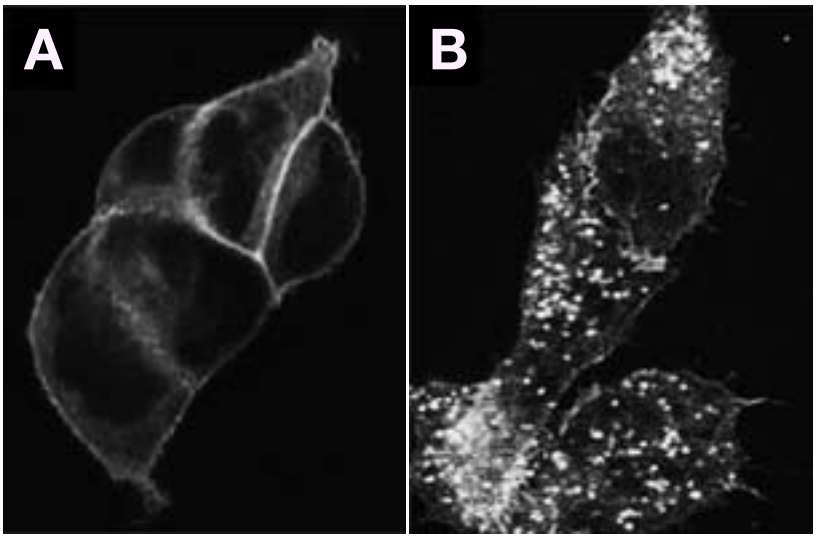


Fig. 3. Intracellular localization of Yellow Fluorescent Protein-tagged NPC1-L1 expressed in Human Embryonic Kidney 293 cells.
A: Low-cholesterol condition. Cells cultured in lipoprotein-deficient serum were treated with methyl-β-cyclodextrin to reduce cellular cholesterol contents.
B: High-cholesterol condition. Cells in condition **A** were loaded with free cholesterol.
NPC1-L1, Niemann-Pick disease type C1-Like 1.

ezetimibe. These pharmacological findings suggest that these proteins must move between the cellular compartments to operate cholesterol transport.

Function of NPC1-L1 in intestinal epithelial cells

There has been no report yet that examined intracellular localization of NPC1-L1 in the intestinal epithelium in situ. On ordinary immunohistochemical examinations of fixed tissues, it appears to be sequestrated on the apical membrane of absorptive cells; however, immunoelectron microscopy revealed that in addition to the apical membrane, it was also localized on the limiting membrane of intracellular vesicles (Sané et al., 2006). From morphological features, these vesicles appear to be lysosomes.

The intestinal absorptive cells receive cholesterol from biliary micelle on its apical membrane and package it in chylomicron, which in turn is released from the basolateral membrane. Almost all of the cholesterol molecules in biliary micelle are in the free form whereas they are in the esterified form in chylomicron. These conversion and transport processes consist of multiple steps as shown in Fig. 4. Depending on the observations in cultured cells, the site of action of NPC1-L1 is thought to be step 2, in which the cholesterol-enriched plasma membrane domains are internalized to the endosomal compartment.

The total cholesterol content of intestinal epithelial cells from NPC1-L1 knockout mice was reduced compared with wild-type mice, which does not contradict the role of this protein in step 2 (Davies et al., 2005). Knopfel et al. found no difference in the rate of in vitro cholesterol uptake between the apical membrane vesicles prepared form the wild-type and knockout mice (Knopfel et al., 2007). Accordingly, ezetimibe failed to reduce the capacity of these vesicles to take up cholesterol. These negative data indicate that step 1 (i.e., transfer of micellar cholesterol to the apical membrane) does not depend on NPC1-L1 function.

As described above, the cholesterol transport out of the endosomal system (steps 3 and 4 in Fig. 4) depends on NPC1 function and its deficiency

leads to endosomal free cholesterol accumulation. This is also true for intestinal epithelial cells, as evidenced by positive filipin staining of these cells from NPC1 knockout mice. However, the rate of intestinal cholesterol absorption was not at all reduced in these mice compared with wild-type mice (Dixit et al., 2007). Thus it appears that cholesterol molecules derived either from biliary micelle or from LDL take different transport pathways in these cells.

NPC1 and NPC1-L1 as chaperons for cholesterol-enriched membranes

NPC1 and NPC1-L1 share the amino acid sequences of no more than 40% identity and they have quite different tissue specificity and intracellular sites of action. However, the above-mentioned studies have shed light to some common aspects of the functions of these two proteins.

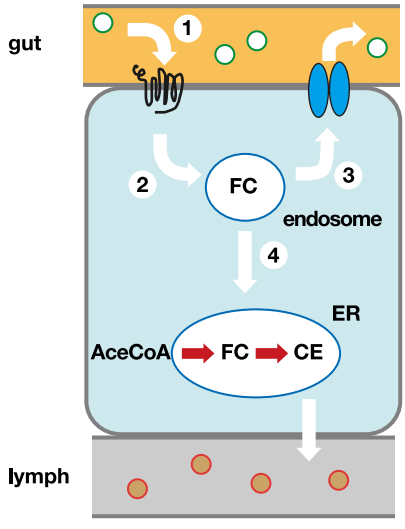


Fig. 4. Intracellular cholesterol flow in intestinal absorptive cells. Free cholesterol (FC) contained in biliary micelle is incorporated into the apical side membrane (①) and then transported to the endosomes (②). A part of internalized FC is recycled back to the apical side and is excreted by ABCG5/G8 (③). Another part is transported to the endoplasmic reticulum (④) where it is esterified and packaged in chylomicron. FC is also supplied by endogenous synthesis from acetylCoA. Step ② depends on Niemann-Pick disease type C1-Like 1 function while others do not.

Cholesterol molecules take either free or esterified form in our body. Biochemically, the free form possesses both hydrophilic and hydrophobic parts and is thus amphiphilic whereas the esterified form is totally hydrophobic. Owing to its amphiphilic nature, the free form always resides within a membrane, i.e., on a two-dimensional surface, whereas the esterified can form a three-dimensional particle.

Most of the cholesterol molecules delivered to peripheral cells by LDL are in the esterified form. To utilize these molecules for structural or metabolic purposes, cells hydrolyze them to the free form in the endosomes and lysosomes. This conversion requires a massive amount of membranes, because the molecules tightly packed in a three-dimensional particle must be spread to a two-dimensional membrane. In the endosomal system, this amount of membranes is supplied by multivesicular bodies, which form three-dimensional particles of stratified membranes. NPC1 is required to transport these membrane structures out of the endosomal system.

Cholesterol molecules are delivered to the intestinal epithelial cells mostly in their free form, because of hydrolysis of dietary cholesterol by digestive enzymes. These molecules enter the apical side of the cells where a massive amount of membranes is equipped by forming microvilli. NPC1-L1 is required to transport the membranes from here to the endosomal system. Thus in both cases, an elaborate membrane system was prepared to deal with free cholesterol. Both NPC1 and NPC-L1 work as a chaperon to transport the membranes out of these compartments.

Cholesterol-specificity of NPC1-L1 function

Neither the serum concentrations of free fatty acids nor lipid-soluble vitamins were altered in NPC1-L1 knockout mice (Altmann et al., 2004). When administered to healthy humans, ezetimibe reduced the serum concentration of LDL but failed to alter

those of other lipid molecules including triglyceride. These findings suggested that the function of NPC1-L1 was specifically required for the transport of cholesterol but not for that of other lipids. Given the role as chaperones for membrane transport, however, a speculation persists that NPC1-L1 is involved in the transport of other membrane lipids.

Narushima et al., by using an expression system in Coca2 cells, found that NPC1-L1 facilitated cellular absorption of α-tocofenol (vitamin E) (Narushima et al., 2008). Labonte et al. reported that in mice, NPC1-L1 facilitated intestinal absorption of saturated fatty acids, and that its inhibition lead to suppression of insulin resistance and weight gain (Labonte et al., 2008). If the latter findings are also true for humans, eztimibe may have dream effects in prevention of diabetes mellitus and obesity. It is of regret to note that in humans, there has been no report yet of weight loss caused by administration of this drug. In addition, the rate of intestinal cholesterol absorption had no relation to the body mass index values (Cohen et al., 2006).

Co-operation by NPC1-L1 and ABCG5/G8

ABCG5/G8, a heterodimer of G5 and G8 subunits, is a member of the ATP-binding Cassette (ABC) transporter family. Similar to NPC1-L1, this protein localizes on the apical membrane of intestinal absorptive cells, but in quite contrary to NPC1-L1, it operates cholesterol transport from the cells to the intestinal lumen: thus NPC1-L1 and ABCG5/G8 work in an opposite direction in the regulation of intestinal cholesterol flow. In humans, these two proteins also co-localize in bile canaliculi. Loss of function of either G5 or G8 proteins results in hereditary sitosterolemia characterized by increased serum and tissue levels of sterols derived from plants.

In general, co-operation of two proteins that function in an opposite direction to and independently from each other enables a regulation in a wider range and in a faster speed. As for the range, the net intake of intestinal cholesterol is determined by summation of addition by NPC1-L1 and subtraction by ABCG5/G8. As related to the speed, NPC1-L1 appears to be the major determinant. This is because upon cellular loading of cholesterol, NPC1-L1 is internalized within minutes, whereas there is no known regulatory mechanism for ABCG5/G8 that operates within this time lag.

Apart from the efficiency of regulation, the set of NPC1-L1 and ABCG5/G8 is implicated in selection of sterol species. Our diet contains sterols from both animals and plants, the latter of which (plant sterols or phytosterols) cannot be utilized by our body. Although NPC1-L1 facilitates absorption of phytosterols (Yamanashi et al., 2007), it is relatively selective for animal-derived sterols. ABCG5/G8 has clear preference to phytosterols. Thus for the selective absorption of animal-derived sterols, the intestinal cells adopt a dual filtering system that preferentially takes in useful molecules and discards useless ones. NPC1-L1 gene knockout in ABCG5/G8-deficient mice prevented them from developing hyperlipidemia (Tang et al., 2009), suggesting that a major portion of phytosterols discarded by ABCG5/G8 is taken up by NPC1-L1. This is in accordance with the observed effects of ezetimbe to suppress symptoms and signs of patients with hereditary sitosterolemia.

Human genetics of NPC1-L1

Besides basic studies on cultured cells or experimental animals, studies on humans are in progress. One of the major subjects of these studies is the genotype-phenotype relationship of NPC1-L1. The efficacy of intestinal cholesterol absorption varies between individuals, in a range from as low as 30 up to 80%. Given the role of NPC1-L1 as a critical player in this process, one would expect to see whether the genomic variation of this gene could explain the individual variation of the absorption efficacy. Studies are going on to analyze this relationship (Cohen et al., 2006). Clinically, one of the disadvantages of ezetimibe is the individual

variation in responsiveness to this drug. Studies are also going on to reveal a relationship between ezetimibe efficacy and NPC1-L1 genotype (Simon et al., 2005). These lines of studies may in the future make it possible to treat patients with hyperlipidemia in an "order-made" fashion, depending on their genetic haplotype of NPC1-L1.

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