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In vivo interaction between the human dehydrodolichyl diphosphate synthase and the Niemann–Pick C2 protein revealed by a yeast two-hybrid system

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Abstract

Dehydrodolichyl diphosphate (DedolPP) synthase catalyzes the sequential condensation of isopentenyl diphosphate with farnesyl diphosphate to synthesize DedolPP, a biosynthetic precursor for dolichol which plays an important role as a sugar-carrier lipid in the biosynthesis of glycoprotein in eukaryotic cells. During certain pathological processes like Alzheimer's disease or some neurological disorders, dolichol has been shown to accumulate in human brain. In order to understand the regulatory mechanism of dolichol in eukaryotes, we performed a yeast two-hybrid screen using full length human DedolPP synthase gene [Endo et al. BBA 1625 (2003) 291] as a bait to find some proteins specifically interacting with the enzyme. We identified Niemann–Pick Type C2 protein (NPC2) to show a specific interaction with human DedolPP synthase. This interaction was further confirmed by in vitro co-immunoprecipitation experiment, indicating the possible physiological interaction between NPC2 and DedolPP synthase proteins in human.

Keywords: Dehydrodolichyl diphosphate synthase; Niemann–Pick type C2 disease; NPC2; Yeast two-hybrid system

Dolichol has been shown to present ubiquitously in most of all mammalian tissues [1,2]. The levels of dolichol in human tissues are 5–10-fold higher than that in the corresponding tissues from other animals [3,4]. On rough endoplasmic reticulum membrane, dolichyl monophosphate (DolP) plays an indispensable role as a glycosyl carrier lipid in the biosynthesis of *N*-linked glycoprotein, *O*-linked oligosaccharides on yeast glycoproteins, *C*-linked mannose to tryptophan, and glycosylphosphatidylinositol-anchored protein [5–7]. Dolichol has also been shown to be required for embryonic development [8] and affects the fluidity of phospholipids and the stability of membrane [9], suggesting that dolichol is an essential component of animal tissues and participates physiologically or physically in the cellular events. Moreover, recent studies on human pathology indicated that disorder of the dolichol level is associated with some of human diseases. For example, dolichol is accumulated in human brain with aging and in Alzheimer's disease or some other neurological disorders [10–13]. However, little is known at the molecular level about enzymes responsible for the biosynthesis of dolichol as well as the regulatory mechanism in mammalian cells.

The polyprenyl backbone of dolichol, dehydrodolichyl diphosphate (DedolPP), is biosynthesized by DedolPP synthase, which catalyzes successive condensation of isoprene units, isopentenyl diphosphate (IPP), onto farnesyl diphosphate (FPP) with *cis* configuration. In eukaryotes, two *Saccharomyces cerevisiae* cDNAs (*RER2* and *SRT1*) and an *Arabidopsis thaliana* cDNA have been isolated and shown to encode DedolPP synthase responsible for the biosynthesis of dolichol [5,14–16]. Recently, human DedolPP synthase (HDS) has been isolated from a search through databases for amino acid sequences similar to those of undecaprenyl diphosphate synthase from prokaryotes and *S. cerevisiae* DedolPP synthases and characterized [17]. HDS expressed in *S. cerevisiae* catalyzes the formation of polyprenyl

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products with chain lengths of C_{90}–C_{100}, which are consistent with those of known dolichols in human tissues. Moreover, tissue distribution of *HDS* expression is similar to that of the dolichol amount in human tissues [18].

The aim of this study was to find out any regulation factors of DedolPP synthase, which participate in the regulation of dolichol biosynthesis as well as the factors showing physiological relationship with any diseases correlating with dolichol level in vivo. As the first step to detect such factors, proteins that interact with HDS were screened from the human testis cDNA library by a yeast two-hybrid system using the full length HDS as a bait. In this paper, we report the identification of a specific interaction of HDS with the Niemann–Pick Type C2 protein (NPC2/HE1), whose defects cause Niemann–Pick type C2 disease [19].

Materials and methods

Strains. Yeast strains AH109 (*MAT* a, *trp-901*, *leu2-3*, *112*, *ura3–52*, *his3–200*, *gal4*Δ, *gal80*Δ, *LYS2::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3*, *GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2 URA3::MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ MEL1*) and Y187 (*MAT* α, *ura3-52*, *his3–200*, *ade2–101*, *trp1–901*, *leu2–3*, *112*, *gal4*Δ, *gal80*Δ, *met-*, *URA3::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ MEL1*) were from Clontech, Madison, USA. AH109 or Y187 were grown in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose).

Plasmid construct. The full length HDS cDNA was subcloned into pGBKT7 (Clontech) containing a GAL4 DNA-binding domain for the preparation of bait construct. Briefly, HDS was amplified by PCR from a HDS DNA template using HDS1F primer, 5′-CGACTGGGA ATTCACTATGTCATGG-3′, and HDS1R primer, 5′-AAAGTGGC AGGTCGACAGCCTCATTC-3′. The PCR product was digested with *Eco*RI and *Sal*I and subcloned into pGBKT7 vector digested with the same enzymes to make pGBKT7-HDS. The plasmid was introduced into the yeast strain AH109 using lithium acetate method according to the Yeast Protocols Handbook (YPH, Clontech), and the transformants were selected and maintained on a synthetic medium lacking Trp and Leu.

Yeast two-hybrid system. Yeast two-hybrid screening was performed using the MATCHMAKER two-hybrid system (Clontech) with pGBKT7-HDS as a bait plasmid to screen human testis cDNA library fused with GAL4 activation domain according to the manufacturer's instruction. In brief, a single colony of AH109 harboring pGBKT7-HDS was inoculated into 50 ml SD/-Trp medium and cultured overnight at 30 °C. The cells were collected by centrifugation at 1000*g* for 5 min, resuspended in residual 5 ml medium, and then incubated with 1 ml library culture at 30 °C overnight with gentle swirling. After confirming the zygote formation, the mating mixture was centrifuged at 1000*g* for 10 min. Cells were resuspended in 10 ml of 0.5× YPDA medium and spread on 50 SD/-Trp/-Leu/-His/-Ade plates (150 mm diameter) containing 10 mM 3-AT to suppress the background growth. Colonies that grew successfully in the selective media were further screened for the expression of reporter gene, β-galactosidase. Colonies that showed positive activity for β-galactosidase within 8 h were selected for further analysis. The positive colonies were re-streaked several times on selective media, and the individual isolated colonies were re-screened for β-galactosidase activity, eliminating most of the false positives that initially passed through the quadruple selection.

Assay of β-galactosidase activity. The β-galactosidase assay on membrane filter was carried out using replicas of colonies, which were grown on filter papers at 30 °C for 3 days. Filters were then removed from the plates, frozen in liquid nitrogen, and allowed to thaw. After repeating the freeze–thaw cycle twice, filters were placed onto Whatmann 1 filter papers that had been saturated with Z-buffer (100 mM sodium phosphate, pH 7.4, 10 mM KCl, 2 mM $MgSO_4$, 40 mM β-mercaptoethanol, and 0.33 mg/ml X-gal) and incubated at 30 °C.

The β-galactosidase assay in tube was done using *o*-nitrophenol β-D-galactopyranoside (ONPG) as a substrate. Yeast transformants were grown overnight in synthetic medium lacking Trp and Leu and subcultured to mid-log phase. As the positive control, yeast cells harboring pBD-p53+ and pAD-SV40 T-Antigen were used. The overnight cultures were subcultured in YPD medium and incubated for 3–5 h until the cell densities were between 0.8 and 1.0 of A_{600}. The cells were harvested and washed with 1.5 ml Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_4$, pH 7) and resuspended in 0.3 ml Z-buffer, and 0.1 ml of the cell suspension was frozen in liquid nitrogen and allowed to thaw. After cell lysis by the freeze–thaw cycle twice, 0.7 ml of Z-buffer containing 0.27% (v/v) β-mercaptoethanol was added to the tube and mixed well, followed by 0.16 ml ONPG in Z-buffer (4 mg/ml, pH 7). The tube was incubated at 30 °C until yellow color developed. The reaction was terminated by the addition of 0.4 ml of 1 M Na_2CO_3 and the reaction time was recorded. The tubes were centrifuged for 10 min and the absorbance of the supernatant at 420 nm was measured. The β-galactosidase unit was defined as the activity, which hydrolyzes 1 μmol ONPG to *o*-nitrophenol and D-galactose per min per cell. The following equation was used to calculate the β-galactosidase units:

$$\beta\text{-galactosidase units} = 1000 \times OD_{420}/(t \times V \times OD_{600}),$$

where t is the elapsed time (in min) of incubation, $V = 0.1\,\text{ml} \times$ concentration factor, and $OD_{600} = A_{600}$ of 1 ml culture.

In vitro co-immunoprecipitation. Full-length cDNA encoding human NPC2 protein was kindly gifted by Professor Peter Lobel (Robert Wood Johnson Medical School, USA). pACT2-full-length NPC2 was digested with *Nde*I/*Xho*I and ligated into the pGADT7 vector digested with the same restriction enzymes to give pGADT7-NPC2 for production of NPC2 protein tagged with HA. The plasmids pGBKT7-HDS for the production of HDS protein tagged with c-Myc and pGADT7-NPC2 were applied for in vitro transcription and translation using TNT T7 Coupled Reticulocyte Lysate System according to the instruction manual (Promega, Madison, USA). For the positive control, pGBKT7-p53 and pGADT7-SV40 T-antigen were used. These genes were transcribed and translated in the presence of [^{35}S]Met (Amersham, New Jersey, USA). The products were mixed and incubated at room temperature for 1 h. Then, either anti-c-Myc monoclonal antibody or anti-HA-tag polyclonal antibody was added and incubated at room temperature for 1 h. After the additional incubation for 1 h with protein A beads, the bead complex was gently washed five times in CoIP buffer (Clontech) and boiled in SDS sample buffer. Released proteins were analyzed by 10% SDS–PAGE followed by autoradiography.

Results

Yeast two-hybrid screening of proteins interacting with human dehydrodolichyl diphosphate synthase

To identify proteins which can associate with the HDS, the full-length ORF of HDS was cloned as a translational fusion of a GAL4 DNA-binding domain and used as a bait for screening of a human testis cDNA library fused with the GAL4 activation domain by a yeast two-hybrid system. Human testis cDNA library was chosen because the level of dolichol has been shown

Table 1
HDS interaction in two-hybrid system

pGBKT7 fusion (BD)	pACT2 fusion (AD)	Filter β-galactosidase assay	β-Galactosidase activity (U)
Vector	Vector	White	3
HDS	Vector	White	4
p53	T-antigen	Blue	19
HDS	Clone 1	Blue	24
HDS	Clone 2	Blue	18
HDS	Clone 3	Blue	20
HDS	Clone 4	Blue	29

The interactions were determined by X-gal filter assays and liquid assays using ONPG as a substrate for β-galactosidase activity (activation of *LacZ* reporter gene).

to be the highest in testis among human tissues [4] and DedolPP synthase gene is highly expressed in human testis [17]. Among 4×10^6 transformants screened, 395 clones grew on SD medium without Trp, Leu, His, and Ade. Then, 24 clones further survived on medium supplemented with 10 mM 3-AT and showed strong β-galactosidase activity signals on medium containing X-gal. These 24 positive clones were further tested for β-galactosidase expression. Among them, seven clones showed strong β-galactosidase activity in the X-gal filter assay as well as in the liquid assay using ONPG as a substrate (Table 1). The results for DNA sequencing and homology search of seven positive clones obtained from the two-hybrid screening are summarized in Table 2. In these positive clones, one cDNA encoding partial NPC2 protein (550 bp including 200 bp coding sequence and 3′-UTR), 4 cDNAs encoding partial lysyl oxidase (500 bp), one cDNA encoding partial NADH dehydrogenase I (400 bp), and one cDNA encoding an unknown protein were included. Among them, the clone encoding NPC2 protein was chosen for further study because the clone showed strong positive signal to β-galactosidase activity (Table 1).

Co-transformation and vector swapping

To confirm the interaction between HDS and NPC2, pGBKT7-HDS and pACT2-partial-NPC2 isolated from testis cDNA library were simultaneously co-transformed into yeast AH109 strain and allowed to grow in SD/-Leu/-Trp/X-gal medium. Since the full-length NPC2/HE1 is 132 amino acid protein with 19 amino acid signal peptide at the N-terminal region, interaction between HDS and full-length NPC2 was also analyzed.

Table 2
Proteins found to interact with HDS in yeast two-hybrid system

Clone	Gene	Fragment size (bp)	No. of colony
1	Niemann–Pick type C2 (NPC2)	500	1
2	NADH dehydrogenase I (NADH1)	400	1
3	Lysyl oxidase (LO)	550	4
4	Unknown	1000	1

At the same time, in order to check the sequence-dependent activation of transcription, vector-swapping analyses were performed. Although the cells harboring HDS fused with GAL4 DNA-binding domain and GAL4 activation domain could not show any β-galactosidase activity (Fig. 1, panel 1), cells harboring HDS fused with GAL4 DNA-binding domain and partial- or full-length-NPC2 fused with GAL4 activation domain showed strong β-galactosidase activity comparably to that of positive control (Fig. 1, panels 3–5). These results indicate that the transactivation of the reporter genes in two-hybrid system occurred by co-expression of HDS and NPC2 and that the interaction domain of NPC2 is included in the 66 amino acids from the C-terminal of NPC2 protein. Moreover, cells harboring vector-swapped constructs, i.e., HDS fused with GAL4 activation domain and full-length NPC2 fused with GAL4 DNA-binding domain, also showed obvious β-galactosidase activity (Fig. 1, panel 2), indicating that the transactivation of the reporter genes was a specific event for the interaction between HDS and NPC2.

In vitro co-immunoprecipitation

In order to confirm the in vivo interaction between HDS and NPC2 identified from the yeast two-hybrid assay, direct interaction of these proteins was analyzed with the method of in vitro co-immunoprecipitation. HDS tagged with c-Myc and NPC2 protein tagged with HA were transcribed and translated in vitro and labeled with [^{35}S]Met. After immunoprecipitation from the mixture of HDS and NPC2 with each specific antibody for HA-tag and c-Myc-tag, precipitated proteins were analyzed by autoradiography. In vitro transcription and translation from HDS-c-Myc and NPC2-HA constructs gave the corresponding protein bands of 40 and 20 kDa, respectively (data not shown). When anti-c-Myc antibody was used for precipitation, not only the major band of 40 kDa corresponding to HDS-c-Myc but also a minor band of 20 kDa, which corresponds to NPC2-HA, was detected (Fig. 2, lane 1). On the contrary, the immunoprecipitation with anti-HA antibody resulted in the major precipitated protein of 20 kDa and the minor protein of 40 kDa (Fig. 2, lane 2). These results clearly

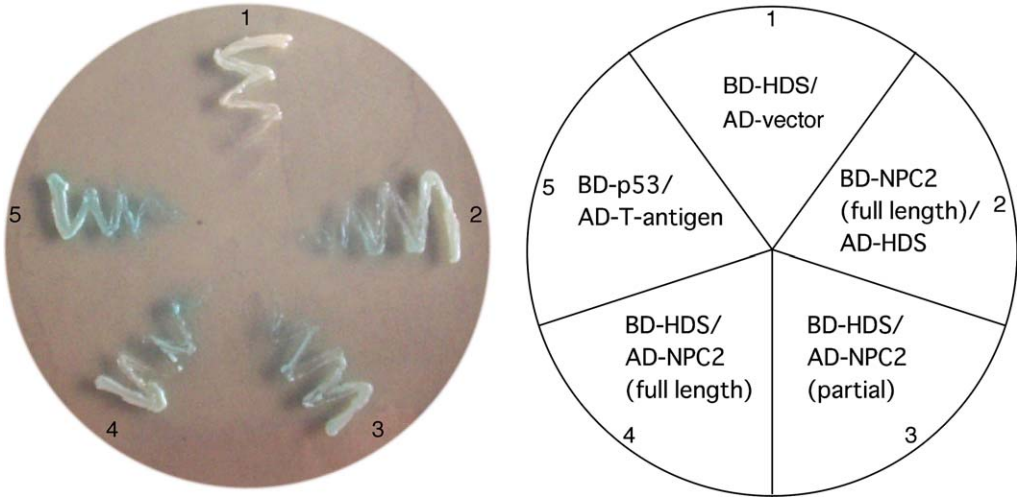

Fig. 1. In vivo association between HDS and NPC2. The yeast strain AH109 was transformed with the indicated plasmids and grown on SD medium lacking Trp for 4 days at 30 °C. Each colony was restreaked on SD medium lacking Trp, Leu, His, and Ade containing 5 mM 3-AT and X-gal.

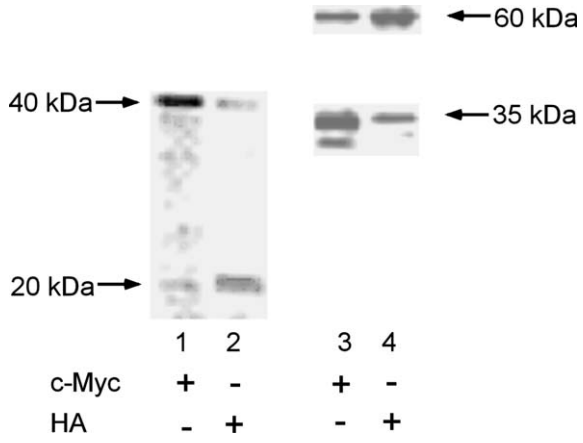

Fig. 2. HDS interaction with NPC2 in vitro. In vitro co-immunoprecipitation of HDS with NPC2 (lanes 1 and 2) and p53 with T-antigen (lanes 3 and 4, positive control). Tagged proteins HDS-c-Myc and HA-NPC2 were transcribed and translated with [^{35}S]Met incorporation (lanes 1 and 2). After HDS-c-Myc and HA-NPC2 were immunoprecipitated with c-Myc monoclonal or HA-polyclonal antibody, the antibody–protein complex was resolved in 10% SDS–PAGE and subjected to autoradiography.

indicate that HDS can interact specifically with NPC2 not only in vivo but also in vitro.

Discussion

Long-chain polyisoprenoids with *cis*-configuration, such as dolichol and polyprenol, are found ubiquitously as one of the membrane components. Contents of these polyisoprenoids are not abundant compared to those of glycerol-based phospholipids [20–22]. However, dolichol plays an indispensable role in protein glycosylation, such as N-glycosylation and O-mannosylation. In pathological studies, disorder of the level of dolichol and its relative metabolites in many tissues of patients with some diseases, such as Alzheimer's disease [11] and some other neurological disorders [10,12,13], has been reported. These facts indicate that the *cis*-prenyltransferase might have some physiological relationship with these diseases and that the *cis*-prenyltransferase activity could be regulated by some factors directly or indirectly.

In order to explore the physiological relationship of the *cis*-prenyltransferase, HDS, with any disease related proteins in human and to elucidate the regulation mechanisms of HDS activity, which affects the metabolic pathway of dolichol, HDS-specific interaction proteins were searched by yeast two-hybrid system. On screening of a human testis cDNA library, seven putative clones were identified as the associated proteins with HDS, which included Niemann–Pick type C2 protein, lysyl oxidase, NADH dehydrogenase I, and an unidentified protein. Among them, NPC2 was considered to be the most possible interacting partner of HDS from the results of X-gal filter assay, β-galactosidase activity, and co-immunoprecipitation assay in vitro.

Niemann–Pick disease causes accumulation of lysosomal cholesterol and impaired low density lipoprotein cholesterol esterification [23]. Recent studies have shown that in Niemann–Pick type C2 disease patients, some altered levels of dolichol and of DolP have been detected [24,25]. These studies on Niemann–Pick type C2 disease patients show that the decreased amount of dolichol was due to the reduction of the *cis*-prenyltransferase (DedolPP synthase) activity. However, this disease is caused by a deficiency in HE1/NPC2, a ubiquitously expressed lysosomal 132 amino acid protein shown to bind to cholesterol specifically with a 1:1 stoichiometry and submicromolar affinity [26,27]. Recently, it has been shown that mutations of NPC1 and NPC2 cause the increase of cellular cholesterol level, but do not affect the

LDL receptor activity and cholesterol biosynthesis [23]. These results suggest that mutation of NPC2 could not suppress the initial step of dolichol biosynthetic pathway, FPP or IPP biosynthesis, because the initial mevalonate pathway to synthesize IPP and the following FPP biosynthetic pathway is common to those of cholesterol and dolichol. Taken together, the change of the *cis*-prenyltransferase activity or other dolichol biosynthetic pathway may cause the increased amount of dolichol and DolP in NPC2 protein deficient patient. This hypothesis is consistent with the result that HDS can interact with NPC2 protein. Although we still do not know about the physiological meaning of the interaction at this time, it is reasonable to hypothesize that NPC2 may play a role to regulate the human DedolPP synthase activity to keep the level of dolichol and DolP constant in the cell. The disordered DedolPP synthase activity due to the impaired NPC2 protein may cause an increased amount of dolichol and DolP in the NPC2 deficient patients. Further analysis of precise interacting domain between NPC2 and HDS, co-localization of these two enzymes in mammalian cells to ensure the interaction, and the effect of NPC2 protein on enzymatic activity of HDS will be needed for elucidation of the role of NPC2 in dolichol biosynthesis. Moreover, understanding the correlation between the regulation of HDS activity and the pathology of Niemann–Pick disease will help in the development of a new approach to the treatment for NPC2 disease.

The biosynthetic pathway of dolichol is proposed to be composed by three steps: (1) biosynthesis of allylic substrates (FPP) and homoallylic substrates (IPP), (2) formation of liner polyisoprene chain by DedolPP synthase, and (3) the final step including dephosphorylation and α-saturation. In this pathway, the initial steps, (1) and (2), are common to the biosynthesis of other isoprenoids, such as cholesterol and ubiquinone, and studied well. The final biosynthetic pathway (3) still remains to be clarified. A few hypothetical mechanisms have been proposed; i.e., (i) DedolPP is converted to DolP by dephosphorylation followed by α-saturation; (ii) DedolPP is converted to DedolOH by dephosphorylation and reduced to dolichol by α-saturation [28,29]. In both pathways, a specific phosphatase and an α-saturase which can recognize the long hydrocarbon chain might be essential for the conversion of the terminal group of DedolPP. The other proteins except for NPC2 that show some interaction with HDS as listed in Table 2 may participate in the final step of dolichol biosynthesis by forming a protein complex with HDS.

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