

Analyses of the Membrane Topology and Physical Interaction of Human Dolichol Kinase

by

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Abstract

In the biosynthetic pathway of dolichol-linked oligosaccharide (DLO) in the eukaryotic cell, human dolichol kinase (hDK) catalyzes the CTP-mediated phosphorylation of dolichol (Dol), yielding dolichol-phosphate (Dol-P), which is in turn utilized by human GlcNAc-1-P transferase (hGPT), Dol-P-mannose synthase (DPMS) complex and Dol-P-glucose synthase (DPGS) as a substrate. In this study, we cloned the *hDK1* gene encoding hDK into the vectors for the yeast split-ubiquitin system (YSUS) and explored its membrane topology and physical interaction. Our result indicated that both *N*- and *C*- termini of hDK are oriented toward the cytoplasmic side of the rough ER membrane and that it physically interacts with itself. In addition, we refined a region critical for the self-interaction of hDK. Analysis using eight truncated versions of the bait constructs revealed that a region from the fifth hydrophobic region (HR5) to the seventh loop region (LR7) should be critical for homodimerization of hDK.

Keywords: Dolichol kinase, Membrane topology, Physical interaction

1. Introduction

The biosynthesis of dolichol-linked oligosaccharides (DLOs) is essential for the protein *N*-glycosylation in eukaryotes, as basic structure of the *N*-glycans on glycoproteins is derived from penta-saccharide of DLOs localized on the rough endoplasmic reticulum (rER) membrane¹⁾. As shown in Fig. 1, the dolichol-phosphate (Dol-P) produced by the dolichol kinase (DK or DOLK) serves as scaffold for the assembly of an oligosaccharide portion of DLO on the rER membrane. Moreover, it is utilized for generation of Dol-P-mannose and Dol-P-glucose, both of which are required for the late stage of DLO assembly. Apart from *de novo* synthesis by DK, Dol-P is produced from dephosphorylation of dolichol pyrophosphate (Dol-PP) by dolichol pyrophosphatase (DPP) (Fig. 1).

The gene encoding DK was firstly isolated from *Saccharomyces cerevisiae* as *SEC59* gene^{2),3)}. Later, a human orthologous gene of *SEC59* was identified as *hDK1*⁴⁾. Human DK (hDK) protein consists of 538 amino acids⁴⁾ and shows significant homology to yeast Sec59p. The overexpression of the *hDK1* cDNA in yeast *sec59* mutant cells could complement the deficiency of yeast DK⁴⁾. In addition, the

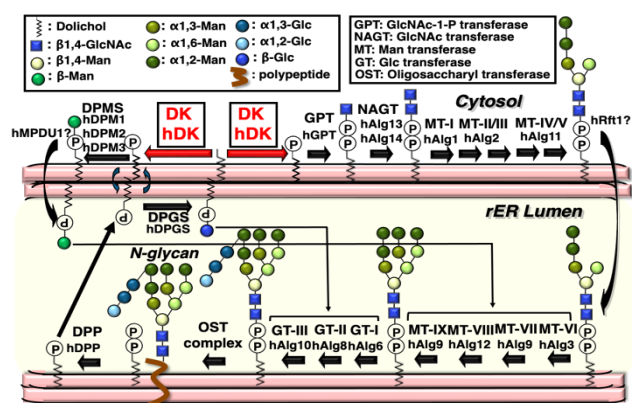


Table I The PCR primers used in this study. Additional sequences containing a *Sfi* I- cleavage site are shown in lowercase letters.

Primer name	Nucleotide sequence	Purpose
DK1BPfw	5'-gtgtaatggccattacggccATGACCCGAGAGTGGCCATCTCCGG-3'	Cloning of the full-length and truncated <i>hDK1</i>
DK1BPrv	5'-gcctttggccgagggcgccGCCATCAGCAATATCAGGAGGTAGAG-3'	Cloning of the full-length <i>hDK1</i>
DK1(LR2)BPrv	5'-gcctttggccgagggcgccGTTGCGTGGATGCTCAGCACCA-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR2
DK1(LR3)BPrv	5'-gcctttggccgagggcgccGTTGCGGACATTCGGAAGTGG-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR3
DK1(LR4)BPrv	5'-gcctttggccgagggcgccCTCAAAGAACGGGTTCACAGCAG-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR4
DK1(LR5)BPrv	5'-gcctttggccgagggcgccCACTGGGCGAGTGATGCCGAG-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR5
DK1(LR6)BPrv	5'-gcctttggccgagggcgccCACCTCCCCACGCTCAACG-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR6
DK1(LR7)BPrv	5'-gcctttggccgagggcgccGCAGCGGGCAGCAGGTACAG-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR7
DK1(LR8)BPrv	5'-gcctttggccgagggcgccGTCCACTGGGTCCCCCTGACT-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR8
DK1(LR9)BPrv	5'-gcctttggccgagggcgccGGAGGAGGCCAGGTGCCTG-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR9
DK1(LR10)BPrv	5'-gcctttggccgagggcgccGGTGTCTGTCTGGAAGAGAACTG-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR10
DK1(LR11)BPrv	5'-gcctttggccgagggcgccCTTCGGGCGATGGTGGGGG-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR11
DK1(LR12)BPrv	5'-gcctttggccgagggcgccACATAGAGCAGTGCCGGTCAAA-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR12
DK1(LR13)BPrv	5'-gcctttggccgagggcgccGGTCCACTGTCTCGTTCATCCA-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR13
DK1(LR14)BPrv	5'-gcctttggccgagggCGCCCTGGCTCCTCCCAGG-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR14
DK1(LR15)BPrv	5'-gcctttggccgagggcgccGTCCCTCAAAGTCTTTTTGGTT-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR15
DK1(LR16)BPrv	5'-gcctttggccgagggcgccGTTTAGTCCACTCCACTGTCAAA-3'	Cloning of the truncated <i>hDK1</i> from <i>N</i> -terminus to LR16

this study, we first investigated the membrane topology of hDK using the yeast split-ubiquitin system (YSUS)¹³⁻¹⁷⁾, a yeast two-hybrid system that can specifically detect the physical interaction between two membrane proteins¹⁸⁻²⁰⁾. Subsequently, we examined the physical interaction of hDK using the YSUS.

2. Experimental Methods

2.1 Prediction of the membrane topology of hDK

In order to predict the membrane topology of hDK protein, a WWW algorithms server, TOPCONS²¹⁾ (<https://topcons.cbr.su.se>) was used. On its WEB site, the amino acid sequence of hDK protein consisting of 538 residues was registered and surveyed regarding its potential transmembrane domains (TMDs) and membrane topology.

2.2 Construction of recombinant plasmids for the YSUS

The coding region of *hDK1* gene was amplified from the human cDNA pools derived from the human brain by standard PCR method²²⁾ using specific primers listed in Table 1. It was then digested with *Sfi* I, purified and ligated to the pBT-N or pBT-C plasmid vector for expression of hDK bait protein which has Cub (C-terminal half of ubiquitin

protein) tag prepared in the YSUS, at the *N*- or *C*- terminus, respectively. Then the ligation mix was used for transformation of *Escherichia coli* JM109 strain prepared by SEM method²³⁾. Preparation of each recombinant plasmid from the transformants was conducted by standard cloning method²⁴⁾.

In order to analyze membrane topology of hDK, fifteen pBT-C-based constructs for expression of the *C*-terminally truncated hDK bait proteins in yeast, pBT-C-hDK/LR1 to pBT-C-hDK/LR15, were prepared via PCR cloning described above.

In order to analyze the physical interaction of hDK, the constructs for expression of hDK prey protein having Nub G (N-terminal half of I13G mutational ubiquitin protein) tag prepared in the YSUS, at the *N*- or *C*- terminus, were also prepared by the same procedure, except usage of the pPR-N and pPR-C plasmid vectors instead of the pBT-N and pBT-C vectors, respectively.

2.3 Assays for the membrane topology of hDK

The bait constructs (the pBT-N-hDK, pBT-C-hDK and fifteen truncated pBT-C-hDK/LR series) were used for co-transformation of *Saccharomyces cerevisiae* NMY51 strain, together with the positive or negative control prey

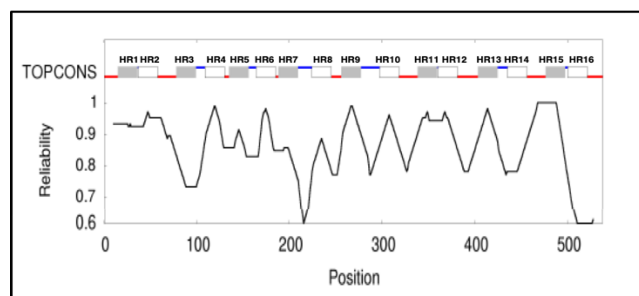


Fig. 2 Prediction of the membrane topology of hDK by TOPCONS algorithm server (<http://topcons.cbr.su.se/>). The sixteen hydrophobic regions (HR1 to HR16) are indicated with boxes, as candidates of transmembrane domains (TMDs), and the seventeen loop regions (LR1 to LR17) are indicated with red or blue lines, as cytoplasmic or luminal loops, respectively.

construct, pAI-Alg5 or pDL-Alg5. The transformation of the yeast cells was carried out by the standard method²⁵.

The co-transformants obtained on the synthetic dextrose (SD) medium lacking leucine and tryptophan (SD-LW) were then subject to the growth examination on the SD medium lacking leucine, tryptophan and histidine (SD-LWH) and SD medium lacking leucine, tryptophan, histidine and adenine (SD-LWHA) according to the manual supplied by Dualsystems Biotech (www.dualsystems.com).

2.4 Assays for the physical interaction of hDK

The bait construct, pBT-N-hDK or pBT-C-hDK was combined with the prey construct, pPR-N-hDK or pPR-C-hDK, and then they were used for co-transformation of yeast NMY51 strain. After the co-transformation, the co-transformants grown on SD-LW medium were subject to growth examination with SD-LWH and SD-LWHA media, according to the same procedure as described above.

3. Results and Discussion

3.1 The membrane topology of hDK

The hDK protein is composed of 538 amino acid residues. First, we started from predicting the membrane topology of hDK protein with TOPCONS algorithms, a server freely available on WEB sites. As shown in Fig. 2, TOPCONS algorithm consequently predicted seventeen loop regions (LRs 1 to 17) and sixteen hydrophobic regions (HRs 1 to 16) corresponding to potential TMDs in hDK protein (Fig. 2). It also predicted that both *N*- and *C*- termini would be orientated toward cytoplasmic sides of the rER membrane (red lines in Fig. 2).

In order to examine the predictions, we applied the YSUS to our analysis of hDK. The negative and positive control prey constructs (pDL-Alg5 and pAI-Alg5, respectively) prepared in the YSUS are designed to express Nub (*N*-terminal half of ubiquitin protein) tag terminally fused to hDK protein in cytoplasmic side of the rER, and readily available to ascertain whether the terminal Cub tag of the bait protein expressed on the rER membrane is located in the cytoplasm or lumen²⁶⁻³². Therefore, we prepared the two bait constructs, pBT-N-hDK and pBT-C-hDK, which express

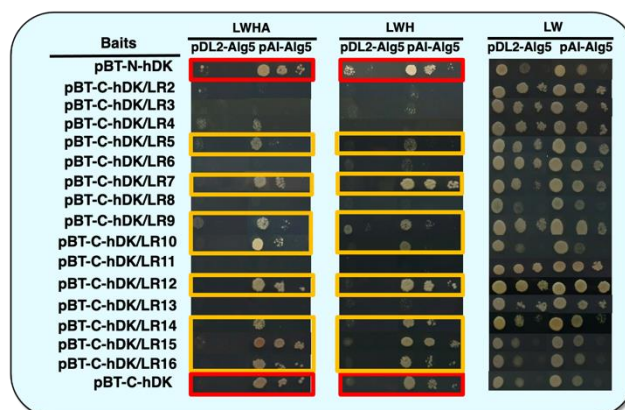


Fig. 3 Growth examination of co-transformants with hDK baits/control prey constructs. After the selection of colonies derived from the co-transformants on the SD-LW medium, their suspensions were diluted with sterilized water and adjusted to the OD₆₀₀ values of 1.0, 0.1 and 0.01 (from left to right). These diluents were orderly spotted on the SD-LWH and SD-LWHA media for reporter detection, and SD-LW media for growth control, and then incubated at 30 °C for 2~4 days.

a full-length hDK protein with *N*- and *C*-terminal Cub tags, respectively. We also prepared the fifteen additional bait constructs, pBT-C-hDK/LR1 to pBT-C-hDK/LR15, each of which expresses a truncated hDK, which has Cub tag at *C*-terminus. Each bait construct was combined with the pDL-Alg5 or pAI-Alg5 control prey, and then they were used for co-transformation of yeast NMY51 cells.

In our growth examination of resultant co-transformants grown on SD-LW plates, co-transformants of the pBT-N-hDK or pBT-C-hDK bait with the pAI-Alg5 positive control prey have well grown on SD-LWH and SD-LWHA plates (right side in red frames of Fig. 3), indicating that the terminal Cub tag fused to hDK protein specifically interacted with NubI (normal type of Nub) tag of pAI-Alg5 located in cytoplasm. On the contrary, those with the pDL-Alg5 negative control prey exhibited no growth on both media (left side in red frames of Fig. 3), because NubG (mutational type of Nub) tag of pDL-Alg5 located in cytosol is not able to spontaneously interact with Cub tag located in cytosol¹⁹. From these results, it was concluded that both *N*- and *C*- termini of hDK should be located in the cytoplasmic side of the rER membrane (Fig. 5).

In the fifteen kinds of co-transformants where the truncated hDK bait protein and the pAI-Alg5 positive control prey protein are co-expressing, those with the pBT-C-hDK/LR5, pBT-C-hDK/LR7, pBT-C-hDK/LR9, pBT-C-hDK/LR10, pBT-C-hDK/LR12, pBT-C-hDK/LR14, pBT-C-hDK/LR15 and pBT-C-hDK/LR16 displayed growth activities on SD-LWH and SD-LWHA media (orange frames of Fig. 3), while those with the others did not (Fig. 3). These observations suggest that the LR5, LR7, LR9, LR10, LR12, LR14, LR15 and LR16 of hDK might be located in the cytoplasmic side of the rER membrane (Fig. 5).

Taken together, our membrane topological analysis of hDK demonstrated that it has at least ten TMDs with *N*- and *C*- termini oriented towards cytosol, as a topological

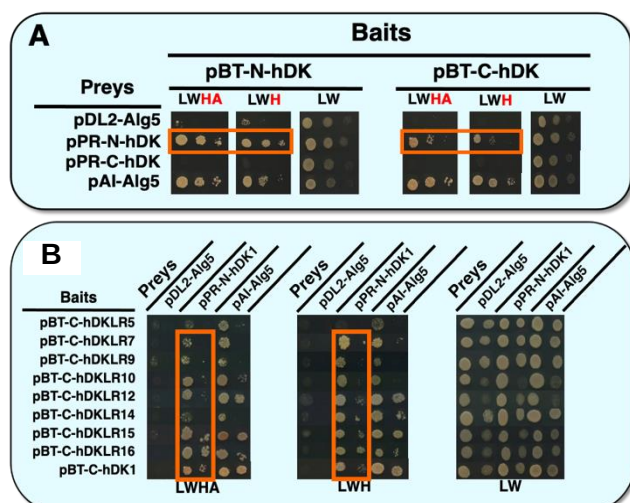


Fig. 4 (A) Growth examination of co-transformants of two full-length hDK baits with two full-length hDK preys. (B) Growth examination of co-transformants of eight truncated hDK baits with a full-length hDK prey derived from pPR-N-hDK. The experiments were carried out according to the same procedure as described in the legend of Fig. 3.

model proposed in Fig. 5. This model differs from that predicted in Fig. 2 and that originally proposed⁴⁾, especially with regard to the number of TMD. Although the possibility that the HR2, HR3, HR9, HR14, HR15 and HR16 of hDK would be a potential membrane-embedded domain cannot be excluded, our model is so far the only one which were exhibited based on the experimental evidence.

The hDK possesses a KTxE motif, which might bind CTP or be a putative catalytic domain⁴⁾, in the LR15 (a red frame in Fig. 5). Therefore, the LR15 should be located within the cytoplasm, because CTP is biosynthesized in the cytoplasm and addition of GlcNAc-1-P to the produced Dol-P by the GlcNAc-1-P transferase (GPT) in DLO assembly is also occurred in the cytoplasmic side of the rER membrane (Fig. 1). Hence, our model is consistent with this fact.

3.2 The physical interaction of hDK

In order to test whether hDK physically interacts with itself, each of the two bait constructs pBT-N-hDK and pBT-C-hDK, was combined with each of the two prey constructs, pPR-N-hDK and pPR-C-hDK, and then transformation of the NMY51 cells were conducted. Our results of growth examination of co-transformants are shown in Fig. 4A. Co-transformants of pBT-N-hDK and pPR-N-hDK could grow on SD-LWH and SD-LWHA media (a left red frame of Fig. 4A), as well as those of pBT-C-hDK and pPR-N-hDK (a right red frame of Fig. 4A). These results indicate that both termini of the bait hDK would be proximal to N-terminus of the prey hDK. On the other hand, co-transformants with pPR-C-hDK did not grow on two selective media (Fig. 4A), suggesting that C-terminus of the prey hDK would not be accessible to both termini of the bait hDK, probably due to conformational reason.

Interestingly, co-transformants of pBT-N-hDK and pPR-N-hDK apparently grew on two selective media better

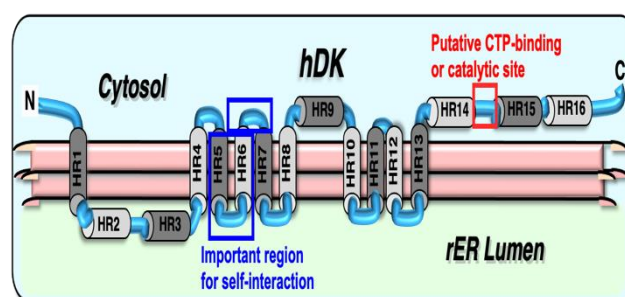


Fig. 5 The model of membrane topology of hDK based on the YSUS analyses indicated in Fig. 3. A region critical for self-interaction of hDK, which is evidenced in Fig. 4B is represented in blue frames.

than those of pBT-C-hDK and pPR-N-hDK (two red frames of Fig. 4A), indicating that N-terminus of hDK bait, rather than C-terminus of hDK bait, preferentially interacts with N-terminus of hDK prey. This fact might reflect the three-dimensional arrangement of two hDKs in homodimer formed on the rER membrane.

Next, we tried to investigate a hDK region important for the self-interaction using the eight additional bait constructs (pBT-C-hDK/LR5, pBT-C-hDK/LR7, pBT-C-hDK/LR9, pBT-C-hDK/LR10, pBT-C-hDK/LR12, pBT-C-hDK/LR14, pBT-C-hDK/LR15 and pBT-C-hDK/LR16) expressing truncated hDKs. These were selected on the basis of the obtained result shown in Fig. 3, because a C-terminal Cub tag portion expressed by pBT-C-based bait constructs needs to be located in cytosol for the analysis of the physical interaction²⁰⁾. Our results of growth examination of co-transformants with each of these bait constructs and pPR-N-hDK are shown in Fig. 4B. Except co-transformants with pBT-C-hDK/LR5, other seven were all able to grow on two selective media (two red frames of Fig. 4B). These observations suggest that a region from HR5 to LR7 of the hDK should be important for the homodimerization of the hDK (a blue frame of Fig. 5). It seems that the region of the bait hDK might play a role in targeting to prey hDK. As it can be divided into four sub-regions (HR5, LR6, HR6 and LR7), we are now investigating which of them is the most critical for the interaction, using pBT-N-based truncated bait constructs.

4. Conclusion

In order to determine the membrane topology of hDK which produces the Dol-P required for the assembly of DLOs, the YSUS was utilized. Results obtained by analyses by the YSUS demonstrated that hDK protein contains at least ten TMDs, with both N- and C-termini located in the cytoplasmic side of the rER membrane (Fig. 3 and Fig. 5).

With respect to the physical interactions of hDK, we obtained two novel informations via analyses by the YSUS ; First, hDK might homodimerize on the rER membrane (Fig. 4A). Secondly, the region from HR5 to LR7 of hDK might be critical for homodimerization of hDK (Fig. 4B and Fig. 5).

The physical interaction between two membrane enzymes could play a role in positively or negatively altering

their enzymatic activities. We are now investigating the physical interactions of hDK bait with preys of the other enzymes involved in DLO assembly, especially the hGPT, two subunits of hDPMs (hDPM2 and hDPM3), hDPGS and hDPP. Our previous researches using them as baits have detected the physical interactions with hDK prey^{(26),(27),(29),(30)}, strongly suggesting that hDK bait could interact with their preys.

On the other hand, in this study, we displayed the data demonstrating that hDK physically interacts with hDK itself. This fact raises the possibility that its enzymatic activity might be regulated by the status of homo- oligomerization of hDK at protein level. In order to establish the importance of this enzyme in Dol-P metabolism, further biochemical and stoichiometric analyses need to be carried out.

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