Proc. Schl. Eng. Tokai Univ., Ser. E
44 (2019) 1-6
DOI: 10.18995/24343641.44.1

Membrane Topological Characterization of the Human Alg14 Protein

by

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(Received on Apr. 9, 2019 and accepted on May 9, 2019)

Abstract

The human Alg14 protein (hAlg14) was identified as a homolog of the yeast Alg14p. It is a transmembrane component of β-1,4 *N*-acetylglucosamine (GlcNAc) transferase involved in the assembly of dolichol-linked oligosaccharides (DLOs) which are precursors of *N*-glycan. In this study, we analyzed the membrane topology of hAlg14 using the yeast split-ubiquitin system (YSUS). The obtained data demonstrated that hAlg14 shows characteristics of the type I membrane topology, where its *N*- and *C*- termini are oriented toward the luminal and cytosolic sides, respectively. Using the YSUS, we also demonstrated that hAlg14 physically interacts with hAlg13, which is the catalytic subunit of β-1,4 GlcNAc transferase, via its cytosolic *C*-terminal loop region. This result well coincides with the fact that β-1,4 GlcNAc transferase utilizes UDP-GlcNAc present in the cytoplasm as a donor substrate.

Keywords: Alg14, Dolichol-linked oligosaccharide, Split-ubiquitin system, Membrane topology, Physical interaction

1. Introduction

N-glycosylation is one of the important events for the maintenance of both structure and function of many glycoproteins in higher eukaryotes. The *N*-glycans on glycoproteins are primarily derived from the dolichol-linked oligosaccharides (DLOs), which are biosynthesized on the rough endoplasmic reticulum (rER) membrane[1]. As shown in Fig.1, the assembly process of DLOs is further divided into three stages: first, early assembly of up to $Man_5GlcNAc_2$-PP-Dolichol (Dol) on the cytoplasmic side by UDP-GlcNAc or GDP-Man dependent glycosyltransferases; second, translocation of $Man_5GlcNAc_2$-PP-Dol into the luminal side by a putative flippase; and finally, late assembly of full-sized DLO ($Glc_3Man_9GlcNAc_2$-PP-Dol) by Dol-P-Man or Dol-P-Glc dependent glycosyltransferases[2)-5)]. These highly ordered steps are well conserved all through eukaryotes.

In the early assembly of DLOs, the β-1,4 *N*-acetyl-glucosamine (GlcNAc) transferase (NAGT) is involved in the second GlcNAc addition. Namely, this enzyme catalyzes the β-1,4 transfer of GlcNAc from UDP-GlcNAc to GlcNAc-PP-Dol, forming $GlcNAc_2$-PP-Dol (red arrow in Fig. 1). This reaction occurs in the cytoplasmic side of the rER membrane. Two genes encoding the NAGT were first identified in the budding yeast[6]. The isolated genes were named the *ALG13* and *ALG14*, according to the conventional nomenclature of the yeast genetics. The *ALG13* encodes the Alg13p, a catalytic subunit, while the *ALG14* encodes a transmembrane subunit, the Alg14p, which recognizes for GlcNAc-PP-Dol as an acceptor substrate. Thus, the expression of the enzymatic activity should be achieved only when the Alg14p localized on the rER membrane physically interacts with the Alg13p in the cytosolic side of the membrane[7].

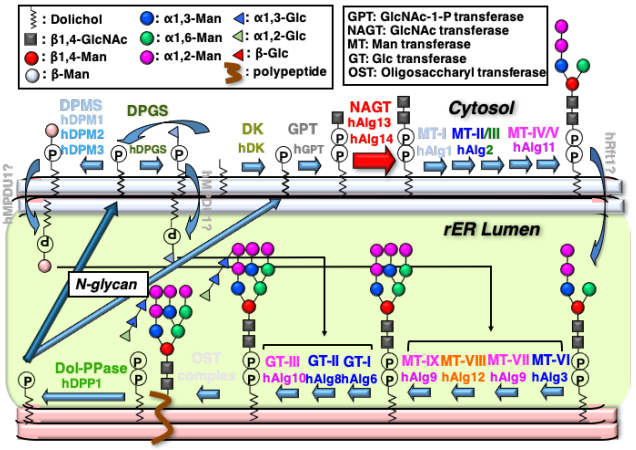

Fig. 1 Assembly of dolichol-linked oligosaccharides (DLOs) and site of action regarding the β-1,4 GlcNAc transferase (NAGT).

Thereafter, the human genes orthologous to the yeast *ALG13* and *ALG14* have been cloned by complementation of the yeast *alg13* and *alg14* mutations, respectively[8,9]. The co-immunoprecipiotation[8] and pull-down[9] assays revealed that

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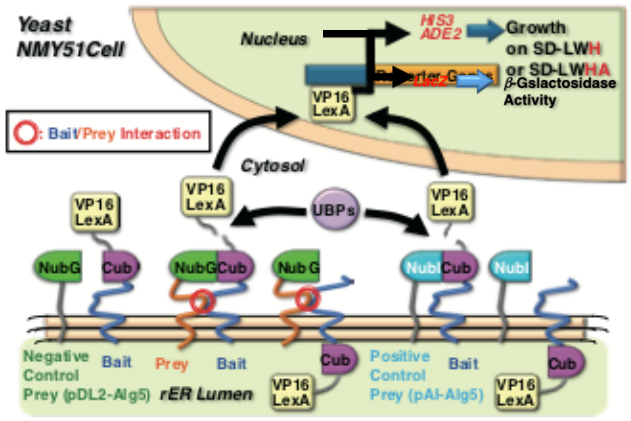

Fig. 2 The yeast split-ubiquitin system (YSUS).

the expressed human Alg13 protein (hAlg13) is specifically and tightly associated with the human Alg14 protein (hAlg14).

We uniquely cloned the human orthologs of the yeast *ALG13* and *ALG14*, by homology cloning method[10]. Although the physical interaction between the hAlg13 and the hAlg14 has been biochemically evidenced as described above, the membrane topology of the hAlg14 remained to be experimentally analyzed in detail. Therefore, in this study, we investigated the membrane topology of the hAlg14 using the yeast split-ubiquitin system (YSUS), a yeast two-hybrid system that can specifically detect cytoplasmic portion of membrane proteins. Also we again demonstrate the interaction between the hAlg13 and the hAlg14 *in vivo*, by this genetic method.

2. The Principal of the Yeast Split-Ubiquitin System (YSUS)

The YSUS was originally developed for detection of physical interaction between two membrane proteins[11)-14)]. Using this methodology, various physical interactions among membrane proteins, including enzymes or subunits of an enzymatic complex residing on the rER membrane, have been revealed[15)-18)].

This system uses *C*-terminal half of ubiquitin (Cub) and *N*-terminal portion of ubiquitin (Nub), which were genetically separated from each other. As shown in Fig. 2, the wild type of Nub (NubI) is able to automatically associate with Cub *in vivo* and form so called "split-ubiquitin". However, mutant type of Nub (NubG; amino acid substitution of isoleucine to glycine at position 13 of the yeast ubiquitin protein) can no longer associate with Cub, spontaneously. But, only if NubG is forced to position near Cub, split-ubiquitin should form. This is the case that the bait fused with Cub interacts with the prey fused with NubG. Unless the bait interacts with the prey, fused NubG cannot associate with fused Cub. While the bait membrane protein is terminally fused with Cub and transcription factors LexA-VP16 (CLV cassette), the prey protein is terminally fused with NubG in the system. If the physical interaction between the bait and prey occurs on the cytosolic side of the membrane, the reconstituted split-ubiquitin should be recognized by the ubiquitin-specific protease (UBP) in the cytosol. As a result, LexA-VP16 released from the bait anchored on the membrane, translocates into nucleus and activates three reporter genes (*HIS3*, *ADE2* and *LacZ*). Therefore, the specific interaction between bait and prey proteins can be detected in the form of the viability of yeast transformed cells where bait and prey proteins are co-expressed, on the synthetic dextrose (SD) medium lacking histidine and/or adenine, and expressed β-galactosidase activity. In other words, only transformants in which the interaction occurs would be able to grow on SD-LWH medium lacking histidine and SD-LWHA medium lacking histidine and adenine, and gain the β-galactosidase activity.

The YSUS is also able to detect the location of the terminus of bait membrane protein. Transformants co-expressing the CLV cassette-fused bait and the positive control prey can survive on the SD-LWH and SD-LWHA media and possess the β-galactosidase activity only when the CLV cassette is oriented toward the cytosolic side. Because, if it is oriented toward the luminal side, split-ubiquitin does not occur (Fig. 2) and consequently three reporter genes are not activated. Using this reactivity of the bait with the positive control prey, it is possible to know whether the termini or internal regions of transmembrane protein are located in the cytosol or rER lumen.

3. Experimental Methods

3.1 *Prediction of the membrane topology of the hAlg14*

For prediction of the membrane topology of hAlg14 protein, four WWW servers, TMHMM[19)] (http://www.cbs.dtu.dk/services/TMHMM/), SOSUI[20)] (http://harrier.nagahama-i-bio.ac.jp/sosui/), TMAP[21)] (http://bioweb.pasteur.fr/seqanal/interfaces/tmap.html) and PSORTII[22)] (https://psort.hgc.jp) were used. On each WEB site the amino acid sequence of the hAlg14 protein, composed of 216 residues, was registered and surveyed regarding transmembrane region and membrane topology.

3.2 *Construction of plasmids for the YSUS*

The coding region of *hAlg14* gene was amplified from cDNA pools derived from the human brain by standard PCR method[23)] 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 hAlg14 bait protein whose *N*- or *C*- terminus is fused with CLV cassette, respectively. Preparation of each recombinant plasmid was conducted by standard cloning method[24)] with the *Escherichia coli* JM109 strain. The prey constructs for *hAlg13* gene were also prepared by the same procedure, except usage of the pPR-N and pPR-C plasmid vectors instead of pBT-N and pBT-C.

3.3 *Assays for the membrane topology of the hAlg14 with the control preys*

The bait constructs, pBT-N-A14 and pBT-C-A14, obtained from PCR cloning were used for co-transformation of the *Saccharomyces cerevisiae* NMY51 strain, together with the positive or negative control prey construct, pAI-Alg5 or pDL-Alg5. The transformation of the yeast cells was carried out by the standard method[25)]. The co-transformants obtained on the SD medium lacking leucine and tryptophan (SD-LW)

Table I The PCR primers used in this study. Additional sequences are shown in lowercase letters.

Primer name	Nucleotide sequence	Purpose
A14BN-F	5'- tgtaatggccattacggccATGGTGTGCGTTCTCGTTCTA -3'	PCR cloning of *hAlg14* into pBT-N
A14BN-R	5'- gcctttggccgaggcggccTGTCAAACAATTCGCCCAAGG -3'	PCR cloning of *hAlg14* into pBT-N
A14BC-F	5'- aatctagaaaaATGGTGTGCGTTCTCGTTCTAG -3	PCR cloning of *hAlg14* into pBT-C
A14BC-R	5'- gcctttggccgaggcggccACAATTCGCCCAAGGTACACC -3'	PCR cloning of *hAlg14* into pBT-C
A13PN-F	5'- tgtaatggccattacggccATGAAGTGCGTGTTTGTTACC -3'	PCR cloning of *hAl3* into pPR-N
A13PN-R	5'- ctagtcgacTTGCTTTTAAAGTGTTGAGAG -3'	PCR cloning of *hAl3* into pPR-N
A13PC-F	5'- aaggatccaaaaATGAAGTGCGTGTTTGTTACCG -3'	PCR cloning of *hAl3* into pPR-C
A13PC-R	5'- gcctttggccgaggcggccTTTTGTAATCCAACAACTTTA -3'	PCR cloning of *hAl3* into pPR-C

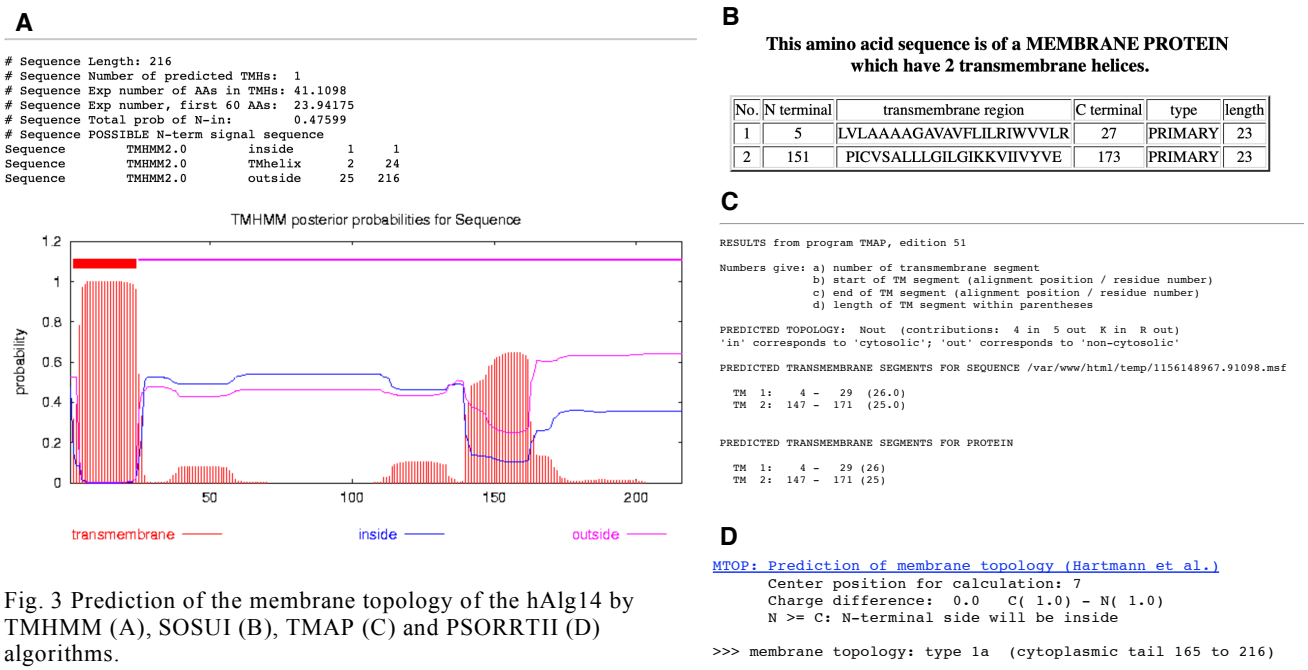

Fig. 3 Prediction of the membrane topology of the hAlg14 by TMHMM (A), SOSUI (B), TMAP (C) and PSORRTII (D) algorithms.

were then subject to the growth examination on the SD-LWH and SD-LWHA media, and *β*-galactosidase assay according to the instructional manual prepared for the YSUS supplied by Dualsystems Biotech (www.dualsystems.com).

3.4 *Assays for the membrane topology of the hAlg14 with the hAlg13 preys*

Using the prey constructs, pPR-N-A13 and pPR-C-A13, obtained from PCR cloning were used for co-transformation of the yeast NMY51 strain together with the bait constructs, pBT-N-A14 and pBT-C-A14. After the co-transformation, the resultant co-transformants on SD-LW medium were subject to growth examination with SD-LWH and SD-LWHA media, and *β*-galactosidase assay, for detection of reporter expression. The procedure is the same as described above.

4. Results and Discussion

The hAlg14 protein is made up with 216 amino acid residues. First, we started from predicting the membrane topology of the hAlg14 protein with several algorithms freely available on WEB sites. One of them, TMHMM server ver.2.0, predicted one transmembrane helical domain corresponding to the amino acid residues 2-24 at *N*-terminus of the hAlg14 (Fig. 3A). This prediction well agreed with the previous prediction on the yeast Alg14p, in which it was estimated to possess one transmembrane helix comprising residues 5-24 at *N*-terminus[7]. On the other hand, SOSUI and TMAP servers predicted one additional transmembrane domain (residues 151-171 and 147-171, according to predictions by SOSUI and TMAP, respectively) near *C*-terminus (Fig. 3B and Fig. 3C, respectively). Although PSORTII server did not indicate the transmembrane region, it predicted that the hAlg14 would belong to type Ia membrane protein with a cytosolic tail consisting of residues 165-216 (Fig. 3D). As the Alg14 orthologous protein members all need to associate with the Alg13 members in the cytosolic face of rER membrane, they must contain at least one cytosolic loop domain. Hence, it seems that the prediction of the cytosolic tail by PSORTII would be rational. Taken together, as well as the yeast Alg14p, it is estimated that the hAlg14 could be a type I membrane protein whose *N*- and *C*- termini are orientated toward luminal and cytosolic sides of the rER membrane, respectively.

In order to ascertain our hypothesis that the hAlg14 would possess one transmembrane domain with *N*- and *C*-termini respectively located in lumen and cytoplasm, we applied the YSUS to the analysis of membrane topology of this protein. Two bait constructs, pBT-N-A14 and pBT-C-A14, were prepared, which express the hAlg14 proteins whose *N*- and *C*- termini were tagged with CLV cassette, respectively.

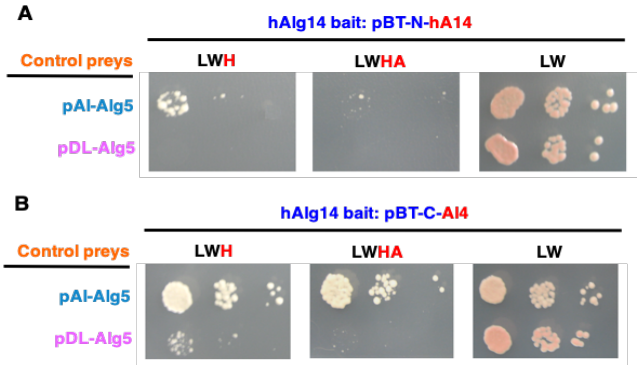

Fig. 4 Growth examination of co-transformants with hAlg14 bait / control prey constructs. After the selection of colonies derived from the co-transformants on the SD-LW medium, their suspensions were diluted with sterilized water and adjusted to the OD_{600} values of 1.0, 0.1 and 0.01 (from left to right). These diluents were orderly spotted on the SD-LWH, SD-LWHA for reporter detection, and SD-LW media for growth control, and then cultured at 30 ºC for 2~4 days.

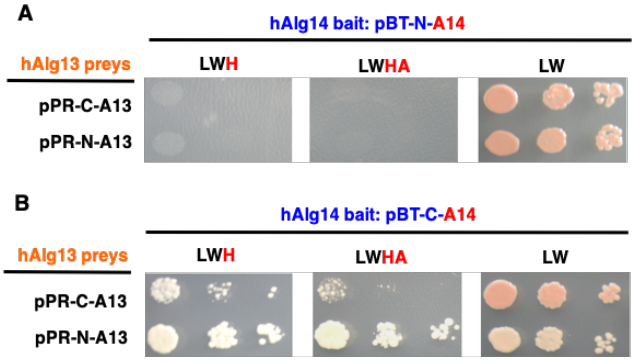

Fig. 5 Growth examination of co-transformants with hAlg14 bait / hAlg13 prey constructs. After the selection of colonies derived from the co-transformants on the SD-LW medium, their suspensions were diluted with sterilized water and adjusted to the OD_{600} values of 1.0, 0.1 and 0.01 (from left to right). These diluents were orderly spotted on the SD-LWH, SD-LWHA for reporter detection, and SD-LW media for growth control, and then cultured at 30 ºC for 2~4 days.

Two control prey constructs, pDL-Alg5 and pAI-Alg5, are designed to express the yeast Alg5p proteins whose cytosolic *C*-terminal loops are tagged with NubG and NubI, respectively (Fig. 2). In growth examination, co-transformants with pBT-N-A14 and pAI-Alg5 has poorly grown on the SD-LWH medium and exhibited no growth on the SD-LWHA medium (Fig. 4A). In contrast, co-transformants with pBT-C-A14 and pAI-Alg5 has well grown on both SD-LWH and SD-LWHA media (Fig. 4B). These observations strongly suggest that the *N*-terminus of the hAlg14 would be located mainly within the rER lumen, whereas that the C-terminus be in the cytoplasm.

We subsequently investigated the physical interaction of the hAlg14 with the hAlg13 proteins. To do this, two prey constructs, pPR-N-A13 and pPR-C-A13, were prepared. They are able to express hAlg13 proteins in yeast cells, N- and C-termini of which are respectively tagged with NubG. Results of growth examination demonstrated that the *N*-terminus of the hAlg14 exhibit no reactivity with the hAlg13 protein (Fig. 5A), again suggesting that its *N*-terminus would not located within the cytosol. On the contrary, the observations obtained using co-transformants with pBT-C-A14 indicated that the *C*-terminus of the hAlg14 should physically interact with hAlg13 protein in the cytosol (Fig. 5B). Interestingly, growth of co-transformants with pPR-N-A13 on SD-LWH and SD-LWHA media were clearly better that that of co-transformants with pPR-C-A13 (Fig. 5B). This result suggests that an *N*-terminal domain of hAlg13 would be accessible to the *C*-terminal loop domain of hAlg14 protein, but other not. This idea would be supported by the fact that the *Escherichia coli* MurG transferase[26], a prokaryotic ortholog to the eukaryotic NAGT, was encoded by just one *Murg* gene, in which the coding region homologous to the *C*-terminus of Alg14 is directly followed by that to the *N*-terminus of Alg13 *in frame*[5,6].

Assays of *β*-galactosidase activity were also conducted about a series of co-transformants. The similar results were obtained with this enzymatic assay. The highest activity was

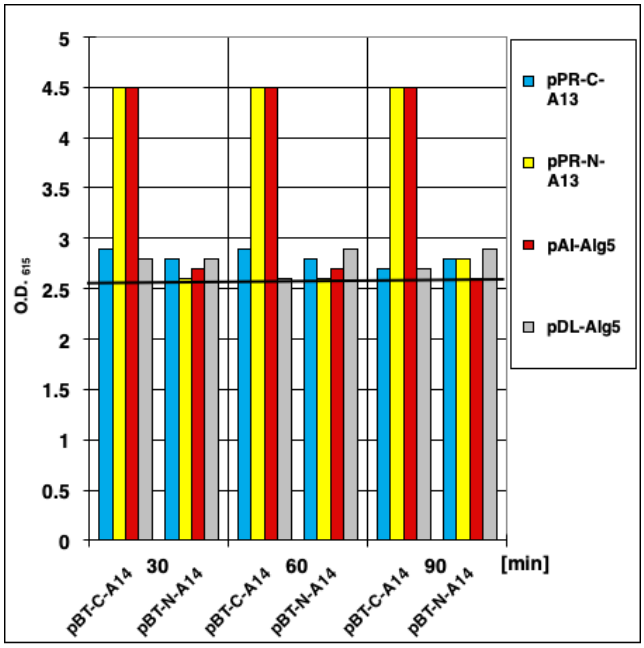

Fig. 6 Assay of *β*-galactosidase activity. Whole cell lysates were prepared from eight types of co-transformants and parental NMY 51 cells. They were incubated with the substrate-containing solution at 37 °C for 30, 60, and 90 min. The measurement of $O.D._{615}$ was then carried out on each sample. The black horizontal solid line around $O.D._{615}$ value of 2.5 indicates the background activity which the parental NMY51 cells possess.

achieved by lysates derived from co-transformants with pBT-C-A14 and pPR-N-A13 and those with pBT-C-A14 and pAI-Alg5 (Fig. 6). Other six types of co-transformants all exhibited background level of $O.D._{615}$ values (approximately 2.5 in Fig. 6). These observations again imply that the *C*-terminal region of the hAlg14 could be located in cytosol and physically interact with *N*-terminal region of the Alg13.

5. Conclusion

In order to determine the membrane topology of the hAlg14 protein, which is a transmembrane subunit of the human β-1,4 GlcNAc transferase involved in the early assembly of DLO, the YSUS was utilized. Growth test and assay of β-galactosidase activity revealed that the hAlg14 protein contains one transmembrane domain around *N*-terminus, and that it has a *C*-terminal cytosolic loop domain, which could physically interact with the *N*-terminal region of the hAlg13 protein in the cytoplasm. In the near future, the the regions or the motifs on the hAlg14 and hAlg13 proteins, which are required for the physical interaction, should be investigated and refined by the further analyses using the YSUS in combination with various mutants for *hAlg13* or *hAlg14* gene.

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