Characterization of an alg2 mutant of the zygomycete fungus *Rhizomucor pusillus*

Kyoko Takeuchi, Haruka Yamazaki, Norihiko Shiraishi, Yasuo Ohnishi, Yoshihisa Nishikawa and Sueharu Horinouchi

Department of Biotechnology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113–8657, and 2Department of Industrial Chemistry, School of Engineering, Tokai University, Kitakaname, Hiratsuka-shi, Kanagawa 259–1292, Japan

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The zygomycete fungus *Rhizomucor pusillus* secretes an aspartic proteinase (MPP) that contains asparagine (N)-linked oligosaccharides at two sites. Mutant strain 1116 defective in N-glycosylation secretes MPP with truncated oligosaccharide chains. Lipid-linked oligosaccharides in mutant 1116 were labeled with [6-3H]glucosamine and [2-3H]mannose, prepared by cycles of solvent extraction, and analyzed by gel filtration chromatography on a Bio-Gel P-4 column after mild acid-hydrolysis. Mutant 1116 accumulated an intermediate, Man₅GlcNAc₂-dolichol pyrophosphate (PP-Dol), whereas wild-type strain F27 synthesized the fully assembled oligosaccharide precursor Glc₁Man₅GlcNAc₂-PP-Dol. Consistent with this, alg2 encoding a mannosyltransferase in the lipid-linked oligosaccharide biosynthetic pathway in mutant 1116 had a 5 bp insertion that generated a stop codon in the middle of the coding sequence. Transformation of mutant 1116 with the intact alg2 gene on a pUC19-derived plasmid generated transformants that contained multicopies of alg2 at the alg2 locus. Glycosylation of the total proteins in the transformants was recovered to the same level as in strain F27, as determined with peroxidase-concanavalin A. These transformants produced MPP mainly with the same N-linked oligosaccharides as that produced by strain F27, but still with truncated oligosaccharides in small amounts. All of these data show that Alg2 is an α-1,3 or α-1,6 mannosyltransferase that elongates Man₅GlcNAc₂-PP-Dol to Man₅GlcNAc₂-PP-Dol. The slower growth of mutant 1116 was significantly recovered on introduction of alg2. The viability of the alg2 mutants of the zygomycete *R.pusillus* makes a contrast with the lethal effect of *ALG2* mutations in the yeast *Saccharomyces cerevisiae*.

**Key words:** alg2/asparagine-linked glycosylation/mannosyltransferase/Rhizomucor pusillus/zygomycete fungus

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**Introduction**

Most secreted and membrane proteins in eukaryotes are modified by the addition of oligosaccharides to specific asparagine residues, which is called N-glycosylation (Herscovics and Orlean, 1993; Kukuruzinska and Lennon, 1994). The oligosaccharide precursor, Glc₃Man₅GlcNAc₂, is synthesized as a lipid-linked form, which is subsequently modified by the action of specific endoplasmic reticulum (ER) and Golgi processing glycosidases and by Golgi glycosyltransferases. Early stages of N-linked glycosylation in the ER are remarkably conserved through evolution of eukaryotes. alg2 mutants of *S.cerevisiae* abnormally accumulate Man₅GlcNAc₂-PP-Dol and Man₅GlcNAc₂-PP-Dol and exhibit a temperature-sensitive lethal phenotype (Huffaker and Robbinson, 1983; Jackson et al., 1993). In these mutants, these two intermediates are transferred to proteins to a significant extent, which suggests that early intermediates move between both faces of the ER (Huffaker and Robbinson, 1983; Jackson et al., 1989).

The zygomycete fungus *Rhizomucor pusillus* (previously called *Mucor pusillus*) has been used for industrial production of a milk-clotting enzyme, *Rhizomucor* pepsin (MPP) (Arima et al., 1967, 1968). MPP produced by the wild-type strain contains two N-linked glycosylation sites: Man₅GlcNAc₂ to Asn-79 and Man₅GlcNAc₂ to Asn-188 (Murakami et al., 1994). We previously isolated and characterized a mutant strain of *R.pusillus* defective in N-linked glycosylation (Murakami et al., 1994). This mutant, designated 1116, secretes a mixture of MPP molecules, some of which contain no sugar chain and some of which contain truncated N-linked oligosaccharide chains such as Man₇GlcNAc₂. In addition, the mutant is viable, although the growth is slightly slower than the wild-type strain. These phenotypes led us to assume that the mutation point in strain 1116 was in *ALG2*. We cloned a genomic DNA and cDNA encoding an *ALG2* homolog from *R.pusillus* (Yamazaki et al., 1999). The cloned cDNA complemented the temperature-sensitive growth of the *alg2-1* mutant of *S.cerevisiae*, indicating that it represented a functional *ALG2* homolog of *R.pusillus*. We named this homolog gene *alg2* in accordance with the nomenclature of fungal genes. The nucleotide sequence of *alg2* cDNA from *R.pusillus* has predicted that *alg2* encodes a 455-amino-acid protein showing end-to-end similarity in amino acid sequence to yeast Alg2 and containing a dolichol-binding consensus sequence (Val/Ile-x-Phe-x-x-Ile, where x is any amino acid) very near its C-terminus.

In the present study, we analyzed the lipid-linked oligosaccharide accumulated in the *R.pusillus* 1116 by gel filtration chromatography and determined the mutation point by gene cloning. Consistent with the finding that strain 1116 accumulates Man₅GlcNAc₂-PP-Dol, *alg2* in this strain contains a 5 bp insertion in the middle of the coding sequence, which results in generation...
of a termination codon. Since strain 1116 is viable, it makes a contrast to yeast where alk2 mutations cause a lethal effect.

Results

Incorporation of [3H]glucosamine and [3H]mannose into the lipid-linked oligosaccharides

We previously found that R. pusillus 1116 secreted a mixture of MPP molecules with no sugar chain and with Man1GlcNAc2 at two asparagine residues (Murakami et al., 1994). Furthermore, analysis of glycosylation in strain 1116 by using concanavalin A and wheat germ agglutinin lectins suggested that not only MPP but also all other N-linked glycoproteins had truncated oligosaccharide chains exposing GlcNAc residues. For determination of lipid-linked oligosaccharides that were accumulated in strain 1116, we first examined incorporation of [6-3H]glucosamine and [2-3H]mannose into the lipid-linked oligosaccharide fraction. When [3H]glucosamine was used, almost no accumulation of radioactivity in the “lipid” fraction was observed in the wild-type strain F27, whereas [3H] was rapidly incorporated and accumulated in the “oligosaccharide-lipid” fraction (Figure 1). This means that lipid-linked oligosaccharide intermediates with short sugar chains (the lipid fraction) are rapidly converted to those with longer sugar chains (the oligosaccharide-lipid fraction). On the other hand, [3H] was rapidly incorporated and accumulated in the lipid fraction in strain 1116, suggesting that lipid-linked precursors were accumulated. Significant radioactivity in the oligosaccharide-lipid fraction in this mutant strain was also detected. Since strain 1116 was found to accumulate Man1GlcNAc2-PP-Dol (see below), this is probably due to the extraction procedure: the lipid and the oligosaccharide-lipid fractions are not strictly separated. The results from experiments with [3H]mannose were similar (data not shown).

Identification of lipid-linked precursor oligosaccharides

The lipid and oligosaccharide-lipid fractions labeled with [3H]glucosamine for 2 h prepared from strains F27 and 1116 were combined and subjected to mild acid-hydrolysis and the resultant free oligosaccharides were resolved by chromatography on a Bio-Gel P-4 (Figure 2). The wild-type strain F27 accumulated the fully assembled precursor oligosaccharide, Glc2Man1GlcNAc2. On the other hand, the major lipid-linked oligosaccharide observed in strain 1116 was Man1GlcNAc2, as expected from the above-described incorporation experiments with [3H]glucosamine and [3H]mannose.

Determination of the mutation point in strain 1116R3

All the above data suggested that strain 1116 had a defect in the mannosyltransferase that transfers a mannose onto Man1GlcNAc2-PP-Dol to form Man2GlcNAc2-PP-Dol. In the yeast S. cerevisiae, this enzyme is encoded by ALG2 (Kukuruzinska et al., 1987; Jackson et al., 1993). We previously cloned an ALG2 homolog from strain F27 that complemented the yeast alk2-1 mutation (Yamazaki et al., 1999). Five introns intervene R. pusillus alk2 that encodes 455-amino-acid protein with a dolichol-binding consensus sequence at its C-terminus. On the basis of the nucleotide sequences of the genomic alk2, we cloned the genomic alk2 region from strain 1116R3. The nucleotide sequence of the genomic alk2 revealed an insertion of 5 bp, GAAGA, in the coding region, which results in generation of a stop codon, TAG (Figure 3). Because this sequence is repeated at this site, the mutation seems to be generated by duplication of the 5 bp sequence. No other changes in the nucleotide sequence were found. The mutated alk2 gene would direct the synthesis of a truncated 277-amino-acid protein that lacks the dolichol-binding sequence.

Complementation of the defect of strain 1116R3 in N-linked glycosylation by alk2

Since the alk2 mutation of mutant 1116 was recessive to the wild-type F27 strain, as determined by genetic studies with forced primary heterokaryons (Murakami et al., 1994), we introduced
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![Fig. 3. Insertion of 5 nucleotides into the coding sequence of \textit{alg2} of \textit{R. pusillus} 1116R3. \textit{alg2} of the wild-type strain F27 encodes a 455-amino-acid protein with a dolichol (Dol)-binding sequence very near the C-terminus (the DDBJ, AB015054). Five nucleotides, GAAGA, were inserted between A-1149 and A-1150, which resulted in generation of a stop codon, as shown.](https://academic.oup.com/glycob/article-abstract/9/12/1287/611822)

Three species are visible on SDS–polyacrylamide gel electrophoresis (Murakami \textit{et al.}, 1994), although these are not very apparent in the photograph in Figure 5B. Western blotting analysis of the MPP produced by transformants RA1 and RA2 showed that most MPP molecules are the same size as that produced by strain F27, indicating that the defect in \textit{N}-glycosylation of strain 1116R3 was complemented by \textit{alg2}. However, smaller amounts of MPP with Man\textsubscript{3},Glc\textsubscript{N}Ac\textsubscript{2}, and no sugar were still produced. A difference in the \textit{N}-glycosylation of MPP in strain 1116R3 and the transformants is that almost no detectable amount of the MPP molecule with Glc\textsubscript{N}Ac\textsubscript{2} (corresponding to band b in Figure 5B) is produced in the latter, the reason of which is unclear at present. The glycosylation patterns of MPP produced by RA3 to RA5 were almost the same as those produced by RA1 and RA2. The glycosylation pattern was also the same when the transformants were grown in YPD liquid medium (data not shown). All of these data show that the defect in \textit{N}-glycosylation in strain 1116R3 is complemented by \textit{alg2}, but still not to the full level as in the wild-type strain F27 in some unknown way.

Strains 1116 and 1116R3 grew more slowly than strain F27 in the range of 20°C to 48°C both in liquid and on solid media. The growth of transformants RA1 to RA5 was significantly recovered, but still slightly slower than in strain F27. The slow growth of the mutants strains as well as the transformants may be caused by some additional mutation(s), since strain 1116 was derived through multiple cycles of mutagenesis from strain F27 for the purpose of isolating mutants producing a larger amount of MPP. It is also possible that the slow growth reflects the partial complementation of the defect in \textit{N}-glycosylation by \textit{alg2} in strain 1116R3.

**Discussion**

The zygomycete fungus \textit{R. pusillus} mutant strain 1116 defective in \textit{N}-glycosylation accumulates Man\textsubscript{3},Glc\textsubscript{N}Ac\textsubscript{2}-PP-Dol as the lipid-linked oligosaccharide. This is consistent with the previous observation that the largest \textit{N}-glycans on MPP produced by this mutant is Man\textsubscript{3},Glc\textsubscript{N}Ac\textsubscript{2} at either Asn-79 or Asn-188 (Murakami \textit{et al.}, 1994). In the budding yeast \textit{S. cerevisiae}, \textit{alg2}-1 mutants accumulated lipid-linked Man\textsubscript{3},Glc\textsubscript{N}Ac\textsubscript{2} at the nonpermissive temperature and the expected precursor Glc\textsubscript{3},Man,Glc\textsubscript{N}Ac\textsubscript{2} at the permissive temperature (Jackson \textit{et al.}, 1989, 1993). These observations prompted us to determine a possible mutation point(s) in the \textit{alg2} gene in \textit{R. pusillus} 1116R3 and to introduce an \textit{ALG2} homolog of \textit{R. pusillus} into the mutant. As expected, \textit{alg2} was found to contain a 5-bp insertion in the coding region, which results in translation of a truncated protein lacking the dolichol-binding sequence. Because the defect in \textit{N}-glycosylation of mutant 1116R3 was complemented by \textit{alg2} from the wild-type strain of \textit{R. pusillus}, it is solely due to the mutation in \textit{alg2}. In mutant 1116R3, Man\textsubscript{3},Glc\textsubscript{N}Ac\textsubscript{2}-PP-Dol remains without further conversion due to complete destruction of \textit{alg2}. This is a contrast to the \textit{S. cerevisiae} \textit{alg2}-1 mutant which accumulates a small amount of Man\textsubscript{3},Glc\textsubscript{N}Ac\textsubscript{2}-PP-Dol in addition to Man\textsubscript{3},Glc\textsubscript{N}Ac\textsubscript{2}-PP-Dol.

We assume that the temperature-sensitive \textit{alg2}-1 mutation in yeast allows addition of a mannose to Man\textsubscript{3},Glc\textsubscript{N}Ac\textsubscript{2}-PP-Dol to a small extent under the conditions examined, which results
in accumulation of a small amount of Man$_1$GlcNAc$_2$-PP-Dol. Because of a null mutation, but not temperature-sensitive mutation, in alg2 in mutant 1116, it accumulates Man$_1$GlcNAc$_2$-PP-Dol alone. Thus alg2 of R. pusillus encodes a mannosyltransferase that elongates Man$_1$GlcNAc$_2$-PP-Dol to Man$_2$GlcNAc$_2$-PP-Dol. At present, it is unclear whether Alg2 shows both the $\alpha$-1,3 and $\alpha$-1,6 mannosylation activity, as is postulated for Alg2 in S. cerevisiae (Jackson et al., 1989; Herscovics and Orlean, 1993).

The R. pusillus alg2 gene encoding a protein showing end-to-end similarity in amino acid sequence, including a dolichol-binding sequence very near their C-termini, to yeast Alg2 complemented the temperature-sensitive growth of the yeast alg2-1 mutant. In addition, an amino acid replacement at Gly-368 of the R. pusillus Alg2, generated by site-directed mutagenesis on the basis of the mutation points (Gly-377 to Arg) in yeast alg2-1 and alg2-2, resulted in generation of a temperature-sensitive enzyme (Yamazaki et al., 1999). Both alg2-1 and alg2-2 contain
The *S.cerevisiae ALG2* gene, in addition to *ALG7* and *ALG1*, all of which function early in the dolichol pathway of N-glycosylation, are essential for cell viability and perturbation in their expression causes G1-specific cell cycle arrest (Lennon *et al.*, 1995). This is a contrast with the viability of the *R.pusillus* mutant 1116 having a null mutation in *alg2*. Small lipid-linked glycans that accumulate in the yeast *alg1* and *alg2* mutants at the nonpermissive temperature are transferred to proteins (Jackson *et al.*, 1989, 1993), as in *R.pusillus* 11116 (Murakami *et al.*, 1994). Since MPP is efficiently secreted from mutant 1116 in a large amount, the translocation of smaller lipid-linked oligosaccharides like Man$_{1-}$GlcNAc$_{2}$-PP-Dol across the ER membrane into the lumen and the subsequent transfer of them to proteins are rather efficient. It is unclear why the *R.pusillus alg2* mutant is viable but those of yeast are not. It may be related to the distant phylogeny and to totally different morphogenesis (Bartnicki-Garcia, 1968; Lipke and Ovalle, 1998).

**Materials and methods**

**Strains and media**

*R.pusillus* strains F27, 1116, and 1116R3 were our laboratory stock strains (Murakami *et al.*, 1994). Strain 1116 was a mutant defective in N-linked glycosylation. Because strain 1116 appeared to be an auxotrophic mutant, strain 1116R3 that grew on minimal medium and showed the same glycosylation pattern as strain 1116 was derived spontaneously (Murakami *et al.*, 1994). pUC19 (Yanisch-Perron *et al.*, 1985) was used for DNA manipulation in *Escherichia coli* JM109 (Yanisch-Perron *et al.*, 1985). Fungal strains were cultured on Koji medium (10% (w/v) koji extract (obtained from Meito Sangyo, Tokyo), 2% agar); PD medium (2.4% potato dextrose broth (Difco), 2% agar); and YNB medium (0.05% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.15% sodium glutamate, 0.15% (NH$_4$)$_2$SO$_4$, 1% glucose, 2% agar) for uracil auxotroph strains. For the liquid culture, YPD medium (1% yeast extract (Difco), 2% bacto peptone (Difco), 2% glucose) and YNB medium were used. For uracil auxotroph strains, 400 µg/ml uracil was supplemented. For MPP production, wheat bran medium (1 g wheat bran (obtained from Meito Sangyo), 0.01 g (NH$_4$)$_2$SO$_4$, 0.8 ml distilled water per a 10 ml test tube) and YPD medium were used. LB broth (0.5% yeast extract, 1% bacto peptone, 1% NaCl) was used for *E.coli*.

In vivo labeling of lipid-linked oligosaccharides

Freshly prepared spores were inoculated into glucose-restricted YPD medium (1% yeast extract, 2% bacto peptone, 0.2% glucose) in a shaking flask. The low concentration of glucose was confirmed not to affect the growth and glycosylation. Spores had been cultured at 30°C until the germ tubes started branching. Portions (3 ml) were then transferred to L-shape tubes containing 120 µCi of [3H]glucosamine (D-[6-3H]glucosamine hydrochloride, American Radiolabeled Chemicals, Inc.) or [3H]mannose (D-[2-3H]mannose, Amersham) and incubated at 30°C. Portions (150 µl) were taken out at intervals and radioactivity incorporated into all mycelia (TCA-precipitated fraction), smaller-sized precursors (lipid fraction), and larger-sized precursors (oligosaccharides-lipid fraction) were measured. Protein concentrations were determined.

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**Fig. 5.** Glycosylation of proteins in *R.pusillus* strains. (A) Lectin binding analysis of intracellular (In) and extracellular (Ex) fractions prepared from *R.pusillus* strains F27 and 1116R3, and transformant RA2. Each fraction was subjected to SDS–polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (CBB) and analyzed by lectin blotting using peroxidase-concanavalin A (HRP-ConA). (B) Western blot analysis of MPP produced by *R.pusillus* strains F27 and 1116R3, and transformants TP5, RA1 and RA2. The wild-type strain F27 produces MPP with Man$_{1-}$GlcNAc$_{2}$ at Asn-79 and Asn-188. Mutant 1116R3 produces MPP with three species of N-glycans which is seen as three bands (a, b, and c). Transformants RA1 and RA2 produce the same size of MPP as strain F27, as the major species of MPP, but still produce small amounts of MPP corresponding to bands a and c.
by the method of Lowry (1951). Protocols for extraction of these lipiddlinked precursors were described by Nishikawa (Nishikawa, 1984, 1991). Briefly, mycelia were first extracted with CHCl3/CH3OH/H2O (1:1:1). The lower layer was saved as the lipid-fraction. The remaining materials were extracted with CHCl3/CH3OH/H2O (10:10:3) (the oligosaccharide-lipid fraction).

Identification of lipid-linked oligosaccharides accumulated in\ R.pusillus

The lipid fraction and the oligosaccharides-lipid fraction, obtained after labeling for 120 min, were combined and dried. After mild acid-hydrolysis in n-propanol and HCl, the sample was evaporated to dryness and subjected to a Bio-Gel P-4 (-400 mesh, Bio-Rad) gel filtration column (1.0 cm diameter and 115 cm height). The standards used were Glc3Man9GlcNAc2, Man5GlcNAc2, Man3GlcNAc2, Man2GlcNAc2, and GlcNAc2. Protocols for these procedures were described by Nishikawa (1991).

Determination of a mutation point in alg2 of \R.pusillus\ 1116R3

Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co., Kyoto. General techniques for DNA manipulation in E.coli were as described by Maniatis et al. (1982). On the basis of the nucleotide sequence of the alg2 region in strain F27 (Tomouchi et al., 1986; Yamazaki et al., 1998), two primers (5′-ttgacgctgactttccttcccggctgTACTGAC-3′ (the underline indicates a SacI site; capitals represent the sequence from nucleotide positions –124 to –99, when the A residue of the translational initiation codon is taken as +1) and 5′-GcAAGAACAAGAATACGACAGGCGC-3′ (representing the sequence from nucleotide positions 1951 to 1978) were synthesized and used for amplification of the whole alg2-coding sequence by the polymerase chain reaction (PCR) under the standard conditions. A 2.1 kb fragment was amplified as expected from the nucleotide sequence of the alg2 region. Because of the presence of a SacI site in one of the primers and at nucleotide positions from 1755 to 1760, digestion of the 2.1 kb fragment with SacI yielded a 1.9 kb fragment, which was then cloned in the SacI site of pUC19. The cloned 1.9 kb SacI fragment was digested with BglII and the resulted three fragments were cloned in pUC19. The nucleotide sequences of the three fragments were determined by the dideoxynucleotide method (Sanger et al., 1977) using the thermo sequenase fluorescent labeled primer cycle sequencing kit (Amersham) in an Automated Fluorescence DNA sequencer (Li-Cor model 4000L). The whole nucleotide sequence was determined with two independently cloned fragments to avoid errors in PCR.

Construction of pAP4 and transformation of \R.pusillus\ 1116U17

Chromosomal DNA of \R.pusillus\ was isolated by the method of van Heeswijk and Roncerco (1984) and purified by equilibrium centrifugation in CsCl-ethidium bromide gradient. The genomic alg2 sequence together with its 5′- and 3′-flanking regions about 2 kb each in length was cloned from strain F27 by the standard DNA manipulation including Southern hybridization and colony hybridization with the previously cloned alg2 cDNA sequence as the probe (Yamazaki et al., 1999). DNA fragments for hybridization probes were labeled with [α-32P]dCTP (110 TBeq/mmol, Amersham Japan) and a BeqBEST labeling kit (Takara Shuzo). The 2.9 kb PvuII fragment thus cloned was introduced by transformation into strain 1116U17 by the use of the host-vector system we established (Yamazaki et al., 1999). Briefly, the PvuII fragment was inserted into the Smal site of pRPPr4, resulting in pAP4 (see Figure 4), pRPPr4 is a uC19-derived plasmid containing the R.pusillus pr4 gene as a selection marker. pAP4 was introduced by transformation into an ura1 auxotroph mutant, 1116U17, derived by UV-mutagenesis from strain 1116R3. Among the Ura+ transformants, we chose five colonies. After two cycles of single spore isolation, we named these transformants RA1 to RA5. Integration of the pAP4 sequence into the chromosome was checked by Southern hybridization with a 1.4 kb SacI–Xbal fragment containing the whole alg2 cDNA sequence, a 1.1 kb HincII–EcoRI fragment containing the whole pr4 sequence, and the full sequence of EcoRI-digested pUC19.

Immunoblotting

Spores (1×105) of \R.pusillus\ strains were inoculated on wheat bran medium and incubated at 37°C for 3 days. Five milliliters of distilled water was added, and the mixture was left overnight at 4°C to extract MPP. The mixture was centrifuged and the supernatant was filtered through a 0.45 µm membrane filter. An appropriate volume of the filtrate was subjected to SDS–polyacrylamide gel electrophoresis for Western blotting. The antibody specific to MPP (Aikawa et al., 1990) was used for immunological detection of MPP by the method of Burnett (1981) with anti-rabbit (goat) antibodies conjugated with peroxidase (Bio-Rad) as secondary antibodies. A polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore) was used for Western blotting.

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Abbreviations

MPP, Rhizomucor pusillus pepsin; PP-Dol, dolichol pyrophosphate; ER, endoplasmic reticulum.

References


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