RESEARCH COMMUNICATION

The potential dolichol recognition sequence of β -1,4-mannosyltransferase is not required for enzymic activity using phytanyl-pyrophosphoryl- α -N,N'-diacetylchitobioside as acceptor

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The *ALG1* gene of *Saccharomyces cerevisiae* encodes β -1,4-mannosyltransferase, an essential membrane-associated enzyme involved in the assembly of dolichyl-linked oligosaccharide precursors for N-glycosylation [Albright and Robbins (1990) J. Biol. Chem. 265, 7042–7049], which catalyses the transfer of a mannose residue from GDP-mannose to dolichyl-pyrophosphoryl- α -N,N'-diacetylchitobioside; it also possesses a putative transmembrane domain, bearing an 11-amino-acid consensus sequence, which has been proposed to mediate dolichol recognition. Here we report the construction and bacterial expression of a mutant β -1,4-mannosyltransferase derived from *ALG1*, which carries a 34-amino-acid deletion resulting in the absence of the entire N-terminal transmembrane domain. This truncated enzyme has an apparent K_m value of 17 μ M for

phytanyl-pyrophosphoryl- α -N,N'-diacetylchitobioside, a known acceptor for β -1,4-mannosyltransferase [Flitsch, Pinches, Taylor and Turner (1992) J. Chem. Soc., Perkin Trans. 1, 2087–2093]. The intact enzyme, expressed in the same system, has an apparent K_m value of 25 μ M. These figures are in good agreement with previously reported values for wild-type β -1,4-mannosyltransferase incubated with the natural dolichyl-linked substrate. Gel-filtration chromatography (before and after β -mannosidase digestion) of the products of both forms of the enzyme verifies the formation of $\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc}$. We therefore conclude that the putative dolichol recognition sequence is not necessary for recognition of the phytanyl analogue of its natural dolichol substrate and suggest it probably also is not needed for its natural substrate.

INTRODUCTION

The polyisoprenoid dolichol is well established as being an essential cofactor in the biosynthesis of glycoconjugates. In eukaryotes, the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ precursor to N-linked oligosaccharides is assembled in the endoplasmic reticulum on dolichyl pyrophosphate, either from nucleotide sugars on the cytoplasmic face or from lumenally orientated dolichyl phosphate sugars [1]. Mannose units of fungal O-linked oligosaccharides and glycosylinositol phospholipids are also donated from dolichyl-phosphoryl- α -D-mannose (Dol-P-Man) [2,3]. Since dolichol has a distinct branched structure, it appears attractive to propose that the enzymes which act on dolichyl-linked substrates contain specific binding sites within their transmembrane domains. Some evidence for specific dolichol recognition is indicated by the preference of various enzymes for 3S-dolichol rather than the R-enantiomer [4] and for α -saturated, rather than fully unsaturated, polyisoprenoids, in addition to chain-length dependence [5]. However, the fully saturated and far shorter lipid phytanol can replace dolichol in some enzyme substrates [6]. The existence of a dolichol-binding site was apparently corroborated by protein sequence analysis [7], which revealed a potential peptide motif within the putative hydrophobic domains of enzymes that act on dolichyl-linked substrates. This potential dolichol recognition sequence (PDRS) was first observed in the sequences of four enzymes from *Saccharomyces cerevisiae*: β -1,4-mannosyltransferase (Alg1), N-acetylglucosaminyl-1-phosphotransferase (Alg7), Dol-P-Man synthetase (Dpm1) and dolichyl kinase

(Sec59) [7]. More recently, the PDRS has been noted in the mammalian oligosaccharyltransferase subunit ribophorin I [8], hamster and mouse N-acetylglucosaminyl-1-phosphotransferases [9], yeast α -1,3-mannosyltransferase (Alg2) [10], sialyltransferases from *Escherichia coli* [11] and dolichyl pyrophosphatase from *Sulpholobus* [12]. However, consensus with the PDRS appears to be weak or absent in N-acetylglucosaminyl-1-phosphotransferase from *Leishmania amazonensis* [13] and a putative yeast oligosaccharyltransferase subunit (Swp1) [14].

The looseness of the PDRS consensus and its absence from the sequences of some enzymes that utilize dolichyl-linked substrates raise doubts as to the requirement for the PDRS (as it is presently defined). Recent experiments by two different research groups have used recombinant genetic techniques to mutate portions of the putative transmembrane domains of *S. cerevisiae* Dol-P-Man synthetase and hamster N-acetylglucosaminyl-1-phosphotransferase. In the former case, enzyme activity was significantly decreased *in vitro* after deletion of the PDRS, but the PDRS in this enzyme was not required for yeast viability [15]. The latter study concluded that mutation of the PDRS of either enzyme abolished enzyme activity [16]. Since in both these cases enzymes which utilize Dol-P-Man as the acceptor were investigated, perturbation of the transmembrane domains may have affected the conformation of the active sites, so reducing catalytic activity. In this context yeast β -1,4-mannosyltransferase, which contains the PDRS motif, has been examined. It uses dolichyl-pyrophosphoryl- α -N,N'-diacetylchitobioside, which has a larger hydrophilic portion than Dol-P-Man, as its acceptor, and hence

Abbreviations used: ALG1, asparagine-linked glycosylation gene 1 (yeast β -1,4-mannosyltransferase gene); Alg1 Δ 2, β -1,4-mannosyltransferase with a Phe $\rightarrow$ Val substitution at residue 2; Alg1 Δ TM, β -1,4-mannosyltransferase with residues 2–35 deleted; Dol-P-Man, dolichyl-phosphoryl- α -D-mannose; PDRS, potential dolichol recognition sequence; PPGn2, phytanyl-pyrophosphoryl- α -N,N'-diacetylchitobioside.

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Figure 1 Construction of expression vectors pLR22 and pLR23

The *ALG1Δ2* PCR fragment generated from plasmid pRA106 was cloned into the unique *NcoI/PstI* sites of phagemid pCE830 to produce the in-frame *trc-ALG1Δ2* expression vector pLR22. This phagemid was further treated as shown to yield pLR23, bearing a 102-base deletion mutant *ALG1ΔTM* under the same promoter, without disrupting the reading frame. *P_{trc}* *trc* promoter; *amp^R*, ampicillin-resistance gene (β -lactamase).

the active site of the enzyme may be some distance from the membrane. As a soluble form of this enzyme would also aid in the overexpression of this enzyme for mechanistic and synthetic studies, it was decided to construct a β -1,4-mannosyltransferase lacking the entire putative transmembrane domain.

EXPERIMENTAL

Materials

Restriction endonucleases, DNA-modifying enzymes and Vent_R polymerase for PCR procedures were purchased from New England BioLabs (Beverly, MA, U.S.A.). GDP-[2-³H]mannose (19 Ci/mmol) was obtained from New England Nuclear (Stevenage, Herts., U.K.). Plasmid pRA106 [17] was the kind gift of Professor P. W. Robbins. Phagemid pCE830 [18] was generously provided by Dr. J. D. Sutherland. *Escherichia coli* strain TG1 [*supE*, *hsdΔ5*, *thi*, Δ (*lac-proAB*), *F'*:*traD36*, *proAB*⁺, *lacI*^Q, *lacZΔM15*] was chosen as the host strain for plasmid construction and expression, using standard molecular biological techniques [19]. Recombinant bacterial strains were grown at 37 °C with vigorous aeration (250 rev./min) in Luria-Bertani medium supplemented with 100 μ g/ml ampicillin. Cultures were induced for

1 h by the addition of 1 mM isopropyl β -D-thiogalactopyranoside. *Helix pomatia* β -mannosidase was obtained from Oxford Glyco-Systems (Abingdon, U.K.). The modified Lowry reagent kit (Sigma) was used for protein assays. All other reagents were of the highest grade commercially available.

Mutagenesis and expression vector construction

Point-mutated *ALG1* DNA, termed *ALG1Δ2*, with a mutation in codon 2 (changing phenylalanine to valine) was generated from the pRA106 template by PCR. Two heterogeneous primers, 5'-CAATTGGTGACCATGGTTTTGGAAATTCCTCGGTGG-3' (upstream) and 5'-GGCTGAGTTACCCTGCAGTCAATGAATTAGCTTCAAATCTC-3' (downstream) were used to introduce unique *NcoI* and *PstI* sites respectively (underlined) at the 5'- and 3'-ends of the sense strand. The amplified fragment was subcloned into the *EcoRV* site of a Bluescript vector (Stratagene, La Jolla, CA, U.S.A.) and the sequence verified by a standard dideoxy chain-termination method (CircumVent thermal-cycle sequencing kit, New England BioLabs). Digestion of this plasmid with *NcoI/PstI* yielded an *ALG1Δ2* fragment, which was cloned into the complementary sites of the *trc* expression phagemid pCE830, resulting in phagemid pLR22.

The transmembrane-deleted *ALG1* mutant, *ALG1ΔTM* (lacking codons 2–35), was conveniently generated by digestion of pLR22 with *NcoI*/*SaI* followed by sequential treatment of the backbone fragment with DNA polymerase I (Klenow) and T4 DNA ligase, yielding the phagemid pLR23 (Figure 1).

Synthesis of the lipid substrate phytanyl-pyrophosphoryl- α -*N,N'*-diacetylchitobioside (PPGn2)

The lipid substrate PPGn2 was synthesized by a modification of the procedure for the preparation of the naturally occurring dolichyl-pyrophosphoryl- α -*N,N'*-diacetylchitobioside [6,20]. Commercially available chitobiose octa-acetate (Dextra Laboratories, Reading, Berks., U.K.) was regioselectively deacetylated at the anomeric position using hydrazine acetate in dimethylformamide, at 50 °C. Phosphorylation was accomplished by deprotonation of this disaccharide with lithium di-isopropylamide in tetrahydrofuran, followed by addition of the phosphorylating reagent tetrabenzyl pyrophosphate. Subsequent deprotection by hydrogenolysis gave the monophosphate monoester, which, after conversion into the triethylammonium salt, was coupled to the triethylammonium salt of phytanyl phosphate [6] in the presence of the coupling reagent, carbonyl di-imidazole. The penta-*O*-acetylated phytanyl-pyrophosphoryl- α -*N,N'*-diacetylchitobioside was deprotected under Zemplén conditions [7% (w/v) sodium methoxide in methanol], followed by treatment with triethylamine to give the di-triethylammonium salt of the unnatural lipid-linked disaccharide, PPGn2. The n.m.r. and m.s. data were in agreement with those published previously [6].

Assay of recombinant *E. coli*

Pellets of recombinant *E. coli* were stored at –80 °C. After thawing and addition of DNAase I and Triton X-100, mannosyltransferase activity was typically assayed at 37 °C by incubation of the cell lysate with final concentrations of 10 μ M PPGn2, 20 μ M GDP-[2-³H]mannose (0.2 μ Ci/ml), 1% (v/v) Triton X-100 in 50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 10 mM 2-mercaptoethanol (final volume of 1 ml). Incubations were performed in Reactivials with continuous stirring in a Pierce ReactiTherm heating/stirring module. Aliquots (100 μ l) were removed at appropriate time intervals and the reaction quenched with an equal volume of chloroform/methanol (1:1, v/v). The aqueous phase was removed after centrifugation, and the organic phase and particulate material at the interface were washed twice with 1 vol. of water and 1 half-vol. of chloroform/methanol (1:1, v/v). The chloroform phase was retained and the particulate material washed with chloroform/methanol/water (10:10:3, by vol.). The organic phases were combined and the incorporated label measured by scintillation counting (Ultima Gold scintillation fluid; LKB scintillation counter). After this procedure minimal counts were retained in the pellet.

Analysis of mannosyltransferase assay products

Phytanyl-linked oligosaccharides were released by mild acid hydrolysis [20 mM HCl in 40% (v/v) propanol, 60 min, 100 °C]. After partitioning with chloroform, the aqueous phase was passed through resins, filtered and subject to gel-filtration chromatography on a Bio-Gel P4 column as described previously [21]. Fractions were pooled and subjected to digestion with *Helix*

pomatia β -mannosidase (0.2 unit/ml, 50 μ l, 100 mM sodium citrate/phosphate, pH 4.0) for 18 h in a toluene atmosphere.

RESULTS AND DISCUSSION

The PCR fragment derived from pRA106 and containing the entire coding region of the yeast *ALG1* gene was introduced as described above (hence replacing the *gdhA* gene) into the expression phagemid pCE830 (a derivative of pTrc99A from Pharmacia). This vector has previously been successfully employed to express soluble enzymes in *E. coli* [18]. In order to afford efficient expression of *ALG1* under the *trc* promoter of pCE830, an *NcoI* site overlapping the initiator ATG was introduced during construction of pLR22. In the process, the second codon of *ALG1* had to be altered from TTT to GTT, thus conservatively substituting valine for phenylalanine, and so generating *ALG1Δ2*. It was then possible to construct pLR23 by deleting the region encoding the entire putative transmembrane domain (residues 2–35 inclusive) without affecting the reading frame, due to the presence of the conveniently located unique *NcoI* and *SaI* sites in the pLR22, thus generating *ALG1ΔTM*. As noted by Albright and Robbins [17], residues 2–35 constitute the most hydrophobic region as identified by the Goldman–Engelman–Steitz scale and includes the PDRS (residues 19–32). The constructs pLR22 and pLR23 are shown in Figure 1.

E. coli TG1 cells transformed with either phagemid were assayed alongside the untransformed host. While no activity was detected in the untransformed TG1 strain or with cells transformed with vector alone (results not shown), TG1 transformed with either the *ALG1Δ2* (pLR22) or *ALG1ΔTM* (pLR23) contained significant activity. The K_m values for the PPGn2 substrate were measured for Alg1Δ2 and Alg1ΔTM enzymes. PPGn2 has previously been shown to be a substrate for pig and yeast mannosyltransferases and was used because it can be conveniently obtained by chemical synthesis [6]. Rates of incorporation were measured over the first 2 min of incubation using *E. coli* lysates (0.44 mg for *E. coli* harbouring pLR23 and 1.7 mg for *E. coli* harbouring pLR22) with substrate concentrations of 100 μ M GDP-[2-³H]mannose (0.4 μ Ci/ml) and 5, 10, 15, 20 or 25 μ M PPGn2. The resultant Hanes plot (Figure 2) indicated that the apparent K_m values were 25 μ M and 17 μ M for Alg1Δ2

Figure 2 Hanes plot for β -1,4-mannosyltransferase acceptor PPGn2

The assays were carried out as described in the text with various concentrations of PPGn2 at a fixed GDP-mannose concentration using lysates of *E. coli* expressing Alg1Δ2 (●) and Alg1ΔTM (○).

Figure 3 Analysis of [2-³H]mannosylated products by Bio-Gel P4 gel-filtration chromatography

Saccharides released after mild acid hydrolysis of incubation products of PPGn2 with GDP-mannose and lysates of *E. coli* expressing Alg1Δ2 and Alg1ΔTM were analysed as described in the text. Fractions were taken at 6 min intervals and aliquots subjected to scintillation counting. The positions of glucose oligomers (internal standard) are indicated. Solid lines represent radioactivity corresponding to 20% of the saccharides released by mild acid hydrolysis. Dashed lines represent radioactivity corresponding to 50% of pooled saccharides after treatment with β-mannosidase.

and Alg1ΔTM respectively. These values are akin to those obtained for the native enzyme with the natural substrate: yeast and soybean β-1,4-mannosyltransferases have apparent K_m values for dolichyl-pyrophosphoryl- α -*N,N'*-diacetylchitobioside of 17 μ M and 9 μ M respectively [22,23]. The K_m value of the AlgΔTM enzyme for GDP-Man was found to be 2.1 μ M (results not shown), which is comparable with the value of 1.7 μ M reported for the soybean enzyme [23]. It is interesting to note the lower specific activity of cells harbouring pLR22 (Alg1Δ2; 0.12 nmol/mg per h) compared with cells harbouring pLR23 (Alg1ΔTM; 1.1 nmol/mg per h) under standard assay conditions.

After incubation of 10 nmol of PPGn2 with *E. coli* expressing either Alg1Δ2 (3.4 mg total protein) or Alg1ΔTM (0.88 mg) and 20 nmol of GDP-mannose, the reaction products were subjected to mild acid hydrolysis and subsequent gel-filtration chromatography. As shown in Figure 3, the ³H-labelled oligosaccharide products generated by both forms of the enzyme had a hydrodynamic volume corresponding to 5 glucose units. This is the same elution position as is expected for unreduced Manβ1 →

4GlcNAcβ1 → 4GlcNAc [24]. In order to confirm the anomeric configuration of mannose in the oligosaccharide, β-mannosidase digests were performed: this resulted in a shift of radioactivity to an elution position of 0.9 glucose unit (Figure 3).

From the present results, it is possible to draw two major conclusions. First, yeast β-1,4-mannosyltransferase can be successfully overexpressed in *E. coli* in a soluble, transmembrane deleted form, despite the fact that it occurs *in vivo* as a membrane-associated protein. The full sequence of the mannosyltransferase had already been expressed in *E. coli* and had been demonstrated to catalyse the formation of the Manβ1-4GlcNAc linkage [25]. The present study is the first to demonstrate bacterial expression of a glycosyltransferase from the endoplasmic reticulum, other than Dol-P-Man synthetase, in a soluble form. Also, to our knowledge, the only other eukaryotic glycosyltransferases previously to have been overexpressed successfully in *E. coli* are β-1,4-galactosyltransferase (in one instance renaturation was required) [26, 27] and yeast α-1,2-mannosyltransferase [28]. Eukaryotic systems appear to be more commonly used for expression of Golgi glycosyltransferases (e.g. [29,30]) and frequently the transmembrane domain is deleted so as to simplify expression and purification in both eukaryotic and prokaryotic systems [26–29].

Secondly, it is apparent that the PDRS of β-1,4-mannosyltransferase is not required for efficient enzyme activity when using the phytanyl analogue as acceptor. Apparent K_m values for the phytanyl substrate are obtained which are similar to those reported in the literature, even though the PDRS is absent and the diastereoselectivity of the enzyme is retained as demonstrated by glycosidase digestion analysis of the product. In contrast with the results obtained with sequential deletion mutants of Dol-P-Man synthetase [15], transformants expressing Alg1ΔTM have a higher specific activity than transformants expressing the conservatively point-mutated Alg1Δ2. However, even though with Dol-P-Man synthetase mutants deletion of the PDRS caused a 92% or greater reduction in activity per mg of protein, two of these mutants were still capable of rescuing a yeast strain in which the *DPM1* gene had been disrupted [15]. Key residues of the PDRS of Dol-P-Man synthetase have been substituted (Phe-250 → Val, Ile-253 → Phe, Ile-253 → Asn and Tyr-257 → Val) with little effect except for an increased K_m for dolichyl phosphate with the Ile-253 → Asn mutant when assayed in Nonidet P-40 [31]. Also, the present results can be contrasted with those obtained with *N*-acetylglucosaminyl-1-phosphotransferase. In one construct, the first PDRS was scrambled, while in another, key residues in the second PDRS were point-mutated. Expression of both mutants did not increase microsomal enzyme activity of Chinese hamster ovary cells over that of controls, thus indicating a requirement for the PDRS in this enzyme [16]. Thus, it is possible that the PDRS may be more important for some enzymes than others. In the case of β-1,4-mannosyltransferase, it may be that residues 2–35 do not have a role in substrate recognition, but function as a 'hairpin' transmembrane domain. To be compatible with the loop model of signal-sequence insertion [32], this region may contain both the signal sequence required for correct targeting to the endoplasmic reticulum during translation and a second hydrophobic sequence required to cross the membrane so that the correct enzyme topography is attained. The topography is significant as the donor GDP-mannose is only found in the cytoplasm and so the early stages of biosynthesis of dolichyl-linked oligosaccharides must take place on the cytoplasmic face of the endoplasmic reticulum membrane [33]. Experiments are now in progress to examine further the role of sequences within β-1,4-mannosyltransferase and to optimize expression of the enzyme in a soluble form.

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