Nonglucosylated oligosaccharides are transferred to protein in MI8-5 Chinese hamster ovary cells

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A CHO mutant MI8-5 was found to synthesize Man9GlcNAc2-P-P-dolichol rather than Glc3Man9GlcNAc2-P-P-dolichol as the oligosaccharide-lipid intermediate in N-glycosylation of proteins. MI8-5 cells were incubated with labeled mevalonate, and the prenol was found to be dolichol. The mannose-labeled oligosaccharide released from oligosaccharide-lipid of MI8-5 cells was analyzed by HPLC and α-mannosidase treatment, and the data were consistent with a structure of Man9GlcNAc2. In addition, MI8-5 cells did not incorporate radioactivity into oligosaccharide-lipid during an incubation with tritiated galactose, again consistent with MI8-5 cells synthesizing an unglucosylated oligosaccharide-lipid. MI8-5 cells had parental levels of glucosylphosphoryl-dolichol synthase activity. However, in two different assays, MI8-5 cells lacked dolichol-P-Glc:Man9GlcNAc2-P-P-dolichol glucosyltransferase activity. MI8-5 cells were found to synthesize glucosylated oligosaccharides after they were transfected with Saccharomyces cerevisiae ALG6, the gene for dolichol-P-Glc:Man9GlcNAc2-P-P-dolichol glucosyltransferase. MI8-5 cells were found to incorporate mannose into protein 2-fold slower than parental cells and to approximately a 2-fold lesser extent.

**Key words:** ALG6/CHO cells/glucosyl transferase/N-linked glycosylation

**Introduction**

A major type of carbohydrate linkage on glycoproteins found in the secretory pathway of eukaryotic cells is an N-glycosidic linkage between an GlcNAc residue of an oligosaccharide and an asparagine residue in the sequon Asn-X-Set/Thr of a protein. The synthesis of the oligosaccharide moiety on N-linked glycoproteins has five major steps in mammalian cells. First, the lipid carrier of saccharides in the biosynthetic process, dolichyl phosphate, is synthesized from mevalonate utilizing some of the same steps as are used in cholesterol biosynthesis (Kaiden and Krag, 1991). Second, Glc3Man9GlcNAc2 oligosaccharide is assembled on dolichyl pyrophosphate (Cummings, 1992). Third, the oligosaccharide is transferred en bloc to an asparagine residue of an Asn-X-Set/Thr sequon in a newly synthesized, translocated nascent protein chain by oligosaccharyl transferase (Silberstein and Gilmore, 1996). Next, removal of glucose residues and reglucosylation of the oligosaccharide (Sousa et al., 1992) occurs during folding and oligomerization steps which require interaction of the newly glycosylated proteins with endoplasmic reticular chaperones (Hammond and Helenius, 1995). Finally, more extensive removal of glucose residues and readdition of terminal sugars occurs in the Golgi apparatus (Schachter, 1995).

One fascinating aspect of this process is the potential role of glucose in the assembly, transfer, and initial processing steps of the oligosaccharide, since glucose is not a component of oligosaccharides on mature N-linked proteins. In vitro studies using membranes (Turco et al., 1977), solubilized membranes (Trimble et al., 1980; Sharma et al., 1981), or purified oligosaccharyl transferase (Breuer and Bause, 1995) demonstrated that glucosylated oligosaccharide-P-P-dolichol, and in particular Glc3Man9GlcNAc2-P-P-dolichol (Staneloni et al., 1980; Murphy and Spiro, 1981), is the preferred substrate of the oligosaccharyl transferase, in some cases being preferred by more than an order of magnitude over nonglucosylated oligosaccharide-P-P-dolichols. This dramatic effect of the presence of glucose on preferential transfer of oligosaccharides has not been observed in vivo in Saccharomyces cerevisiae or in protozoan species. Protozoan species have N-linked glycoproteins although they only synthesize nonglucosylated oligosaccharide-lipids (Parodi, 1993). In a mutant alg6 of S. cerevisiae which synthesizes Man9GlcNAc2-P-P-dolichol, nonglucosylated oligosaccharides were transferred to protein (Runge et al., 1984); although underglycosylation of protein did occur (Muñoz et al., 1994; Reiss et al., 1996).

To date, the in vivo effect of glucose on oligosaccharide transfer has not been addressed directly in mammalian systems, due to a lack of appropriate mutants. However, mammalian mutants that synthesized primarily Man9GlcNAc2-P-P-dolichol, due to a lack of mannosylphosphoryldolichol synthase activity, transferred glucosylated Man9GlcNAc2 to protein (Kornfeld et al., 1979; Stoll et al., 1992), indicating that the oligosaccharyl transferase had a distinct preference for glucosylated oligosaccharides. In this article we present evidence that the Chinese hamster ovary (CHO) cell line MI8-5 lacks glucosyl transferase activity, synthesizes Man9GlcNAc2-P-P-dolichol as its major oligosaccharide-lipid, and transfers that oligosaccharide to protein. The effect of this defect on the overall mannosylation of proteins is presented.

**Results**

MI8-5 cells synthesize Man9GlcNAc2-P-P-dolichol

MI8-5 cells were isolated by a two-step procedure. First, mutagenized CHO cells were subjected to a mannoside suicide selection technique. Second, survivors of the selection technique were screened as individual colonies grown on polyester discs for...
Fig. 1. HPLC Analysis of the oligosaccharides released from oligosaccharide-lipid of parental and MI8-5 Cells. Parental and MI8-5 cells were incubated with 2-[3H]mannose for 10 or 60 min as described in Materials and methods. There were $3.2 \times 10^6$ parental cells/dish (8 dishes) and $4.6 \times 10^6$ parental cells/dish (2 dishes) for the 10 and 60 min incubations, respectively; there were $2.9 \times 10^6$ MI8-5 cells/dish (8 dishes) and $3 \times 10^6$ MI8-5 cells/dish (2 dishes), respectively for the 10 and 60 min incubations. Oligosaccharide-lipid was extracted (Cacan et al., 1992), hydrolyzed with mild acid, and desalted. Oligosaccharides were separated by HPLC on an amino-derivatized column ASAHIP AK NH2–50 (250 × 4.6 mm) (Asahi, Kawasaki-ku, Japan) with a solvent system of acetonitrile/water from 70/30 to 50/50 by volume at a flow rate of 1 ml/min during 90 min. The separation of the labeled oligosaccharides was followed by continuous-flow detection of the radioactivity with a Flo-one beta detector (Packard) using Luma Flow II (Lumac, the Netherlands) as scintillation fluid. The identity of standards are as follows: 1, Man$_5$GlcNAc$_2$; 2, Man$_9$GlcNAc$_2$; 3, Glc$_3$Man$_9$GlcNAc$_2$. (A) and (C) were parental cells; (B) and (D) were MI8-5 cells. (A) and (B) were 10 min incubations; (C) and (D) were 60 min incubations.

reduced mannose incorporation into protein (J.L.O’Rear, J.R.Scocca, B.K.Walker, A.Kaiden and S.S.Krag, unpublished observations). The incorporation of radioactive mannose for both the selection and the screen was done at 40.5°C instead of the normal growth temperature of 34°C in order to allow for the isolation of mutant cells that had no defect at 34°C, but had a defect at 40.5°C (i.e., cells with a temperature-sensitive lesion). However, the biochemical defect in MI8-5 cells was observed at both 34°C, as described in detail below, and at 40.5°C (data not shown).

Subsequently, MI8-5 cells were shown to die at 40.5°C, although they grew as well as parental cells at 34°C (B.W.Walker, H.Lei and S.S.Krag, unpublished observations). MI8-5 cells were isolated because after an overnight incubation of MI8-5 at 40.5°C, they had a 9-fold lower incorporation of mannose into glycoprotein during a 1 h label compared to parental cells (data not shown).

When MI8-5 cells were incubated at 34°C with [3H]mevalonolactone to achieve steady state labeling of polyisoprenyl lipids, the prenol in these cells was found to be dolichol (data not shown; Rosenwald et al., 1993).

Parental and MI8-5 cells were incubated at 34°C with tritiated mannose for 10 or 60 min, oligosaccharide-lipid was extracted, and labeled oligosaccharides were released from oligosaccharide-lipid by mild acid treatment. The released oligosaccharides were analyzed by HPLC. As shown in Figure 1, parental cells labeled primarily Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol during 10 and 60 min of incubation with [2–3H]mannose whereas MI8-5 cells labeled primarily Man$_9$GlcNAc$_2$-P-P-dolichol. The major labeled oligosaccharides from both parental and MI8-5 cells were treated with α-mannosidase in order to test whether the oligosaccharide in MI8-5 cells was completely sensitive to digestion, as would be expected of a non-glucosylated species. As seen in Figure 2, the major oligosaccharide released from parental cells, Glc$_3$Man$_9$GlcNAc$_2$, was partially resistant to digestion by α-mannosidase, while the major oligosaccharide released from MI8-5 cells, Man$_9$GlcNAc$_2$, was
MI8-5 CHO cells lack glucosyltransferase activity

Fig. 2. Analysis of oligosaccharides from oligosaccharide-lipid from parental and MI8-5 cells by treatment with α-mannosidase. Parental and MI8-5 cells were incubated with [2–3H]mannose for 1 h as described in Materials and methods. Oligosaccharide-lipid was extracted (Cacan et al., 1992), oligosaccharides were released by mild acid, and oligosaccharides were separated by chromatography on Biogel P4. Radioactivity eluting as the largest labeled oligosaccharide from each cell was pooled, desalted, and resuspended in α-mannosidase buffer (Oxford Glycosystems.). One-half of the sample was treated with jack bean α-mannosidase (Oxford Glycosystems), while the other half was untreated. Both samples were incubated overnight at 37°C. The reaction mixtures were then applied to a Biogel P4 column. The majority of each fraction was analyzed for radioactivity, while a small portion of appropriate fractions were analyzed for internal standards (Krag, 1979). Dex, Dextran; Man, mannose; N1–N6, oligomers of GlcNAc. (A) parental oligosaccharide untreated; (B) parental oligosaccharide treated; (C) MI8-5 oligosaccharide untreated; (D) MI8-5 oligosaccharide treated.

sensitive to α-mannosidase digestion, yielding mannose and a small oligosaccharide eluting in a position consistent with ManGlcNAc2 (Figure 2).

No radioactivity was found in oligosaccharide-lipid extracted from MI8-5 cells labeled with [3H]galactose (Figure 3). Under identical conditions, two glucosylated oligosaccharide-lipids, presumably Glc3Man9GlcNAc2-P-P-dolichol and glucosylated Man9GlcNAc2-P-P-dolichol, were observed in parental cells (Figure 3). As has been observed before (Hubbard and Robbins, 1979), glucose moieties in oligosaccharide-lipids are labeled during incubation with tritiated galactose due to the epimerization of UDP-[3H]galactose to UDP-[3H]glucose.
Fig. 3. Analysis of oligosaccharides of oligosaccharide-lipid labeled by parental and MI8-5 cells during an incubation with tritiated galactose. Parental and MI8-5 cells were incubated with D-[1–3H]galactose for 30 min as described in Materials and methods. Oligosaccharide-lipid was extracted (Cacan et al., 1992) and hydrolyzed by treatment with mild acid. The resultant oligosaccharides were analyzed by gel filtration chromatography as described in Materials and methods and in the Figure 2 caption. (A) Oligosaccharides from parental cells; (B) Oligosaccharides from MI8-5 Cells.

**MI8-5 cells lack glucosyltransferase activity**

One possibility to account for these observations was that MI8-5 lacked glucosylphosphoryldolichol synthase activity, thus being unable to synthesize the glucosyl donor responsible for glucosylating oligosaccharide-lipid (Cummings, 1992). Specific activities of 34.6 ± 6.6 and 35.2 ± 5.8 pmol/min/mg were observed for parental and MI8-5 membranes, respectively. These specific activities were determined in eight separate assays performed using three independent membrane preparations. Thus, it appears that MI8-5 cells synthesize glucosylphosphoryldolichol.

A coupled glucosylphosphoryldolichol synthase/glucosyltransferase assay was performed by incubating exogenous unlabeled oligosaccharide-lipid prepared from MI8-5 cells with UDP-[3H]glucose, dolichyl phosphate, and membranes prepared from either parental or MI8-5 cells as the source of enzyme. The addition of exogenous oligosaccharide-lipid from MI8-5 cells stimulated the activity in parental cells 2-fold. As shown in Figure 4, MI8-5 membranes had no detectable activity, while parental membranes had a specific activity of 0.2 pmol/min/mg protein. These results suggested that the defect in MI8-5 cells was in the glucosyltransferase that catalyzes glucose transfer from glucosylphosphoryldolichol to Man9GlcNAc2-P-P-dolichol. It also showed that the oligosaccharide synthesized by MI8-5 cells was a substrate for the glucosyltransferase.

Next, an assay developed by D'Souza-Schorey and Elbein, 1993, was used to directly determine whether MI8-5 membranes had detectable glucosyltransferase activity. An average specific activity of 0.3 pmol/min/mg was observed using parental membranes as determined from six assays using three independent membrane preparations. No detectable activity was observed when using MI8-5 membranes in four assays performed using two independent membrane preparations. An upper limit on the...
**Fig. 5.** Transient transfection of parental and MI8-5 cells with *S. cerevisiae ALG6*. Parental and MI8-5 cells were transfected with either pcDNAI-PyT and pcDNAI-ALG6 or pcDNAI-PyT and pcDNAI-β-gal and then labeled 3 days later with D-[1-3H]galactose as described in Materials and methods. Oligosaccharide-lipid was extracted and treated with mild acid. Released oligosaccharides were analyzed on Biogel P4 as described in Materials and methods. The majority of each fraction was used for determination of radioactivity, while the remainder was analyzed for internal standards (Krag, 1979). (A) Parental cells (1.3 × 10^6 cells) transfected with pcDNAI-β-gal; (B) parental cells (1.3 × 10^6 cells) transfected with pcDNAI-ALG6; (C) MI8-5 cells (2.2 × 10^6 cells) transfected with pcDNAI-β-gal; (D) MI8-5 cells (2.2 × 10^6 cells) transfected with pcDNAI-ALG6. The amount of radioactivity per million cells associated with oligosaccharide (after subtraction of background) in each panel was 7070, 5660, 0, and 1510 d.p.m., respectively.

Possible specific activity in MI8-5 membranes was calculated to be 0.075 pmol/min/mg.

**ALG6 expression complements MI8-5 in vivo**

A genomic clone that encodes the *S. cerevisiae* glucosyltransferase enzyme, *ALG6*, has been isolated (Reiss et al., 1996). This gene was inserted into an expression vector pcDNAI and its ability to complement the lesion in MI8-5 cells was assessed during transient transfection. Seventy-two hours after transfection of parental and MI8-5 cells with pcDNAI containing either *ALG6* or a control insert, cells were labeled with tritiated galactose, oligosaccharide-lipid was extracted, and labeled oligosaccharide was released from oligosaccharide-lipid by mild acid treatment. The labeled oligosaccharide was then analyzed by gel filtration chromatography. As seen in Figure 5, MI8-5 incorporated label into oligosaccharide after transfection by *ALG6*, indicating that the defect in MI8-5 cells was the glucosyltransferase. In this experiment, the transfection efficiency was about 30% for all conditions.

**Time course of synthesis of mannose-labeled proteins in MI8-5**

MI8-5 cells synthesized Man9GlcNAc2-P-P-dolichol. The next question was whether MI8-5 cells transferred that oligosaccharide to protein. Figure 6 illustrates the data from a time course from 5 to 60 min of incorporation of mannose into oligosaccharide-lipid and protein in parental and MI8-5 cells. Both parental and MI8-5 incorporated comparable amounts of mannose into oligosaccharide-lipid, and that incorporation was rapid and leveled off at 20 min (Figure 6, left panel). The incorporation of mannose into protein began at 20 min and continued linearly in parental cells. The incorporation of mannose into protein in MI8-5 cells began slightly later and occurred at a slower rate (Figure 6, right panel). However, labeled oligosaccharide was clearly transferred to protein in MI8-5 cells.

**Discussion**

MI8-5 is the first reported mammalian cell mutant that synthesizes nonglucosylated oligosaccharide-lipid, Man9GlcNAc2-P-P-dolichol, rather than Glc3Man9GlcNAc2-P-P-dolichol. Glc3Man9GlcNAc2-P-P-dolichol is the major oligosaccharide-lipid synthesized by normal cells, and Glc3Man9GlcNAc2 is thought to be the preferred substrate for the oligosaccharyl transferase (Cummings, 1992; Silberstein and Gilmore, 1996). MI8-5 appears to have the same biochemical defect as the *alg6* *S. cerevisiae* mutant and, as shown in the data in Figure 5, MI8-5 was complemented by transfection with *ALG6*. In this transient transfection experiment, we observed a 30% transfection efficiency. We also observed about 30% of the level of incorporation of labeled glucose into oligosaccharide-lipid as would be expected for a culture of parental cells.
Fig. 6. Kinetics of incorporation of mannose into oligosaccharide-lipid and protein in parental and MI8-5 cells. Parental and MI8-5 cells (1–2 × 10^6 cells per 60 mm dish) were incubated with [2–3 H]mannose for varying periods of time as described in Materials and methods except that the medium on the cells was changed to labeling medium without radioactivity 30 min prior to the labeling period. The amount of radioactivity incorporated into oligosaccharide-lipid and protein was determined. Oligosaccharide-lipid was extracted (Cacan et al., 1992), hydrolyzed, and the entire water-soluble fraction was counted (left panel). Protein after extraction was solubilized by treatment with 5 mg/ml of Pronase overnight, and then the solubilized peptides were counted (right panel). Results of two determinations from each of two different experiments are shown. Open symbols, parental cells; solid symbols, MI8-5.

From numerous in vitro experiments, the presence of glucose on the oligosaccharide-lipid appeared to be necessary for the transfer of the Man<sub>9</sub>GlcNAc<sub>2</sub> oligosaccharide to protein by the oligosaccharyl transferase (Turco et al., 1977; Staneloni et al., 1980; Trimble et al., 1980; Murphy and Sprio, 1981; Sharma et al., 1981; Breuer and Bause, 1995). The differences seen in these in vitro experiments were greater than 10-fold. However, these dramatic order-of-magnitude differences do not appear to be observed in vivo. Experiments in alg6 cells show that Man<sub>9</sub>GlcNAc<sub>2</sub> can be transferred efficiently to protein (Runge et al., 1984) although underglycosylation of specific proteins was observed (Muñoz et al., 1994; Reiss et al., 1996). Interestingly, the extent of underglycosylation varied depending on the protein examined. Our data show that MI8-5 can glycosylate protein, with only 2-fold reductions in the extent and rate of glycosylation. Current experiments are underway to assess underglycosylation of specific proteins.

The isolation of MI8-5 is important as it will allow an assessment of the role of glucose on the oligosaccharide-lipid in the transfer of oligosaccharides by oligosaccharyl transferase and the initial steps in the processing of oligosaccharides on protein in mammalian systems. It will be important to compare the initial steps in the processing of oligosaccharide on protein between MI8-5 and alg6 cells since mammalian cells have UDP-Glc:glycoprotein glucosyltransferase activity (Trombetta et al., 1989; Sousa et al., 1992) that adds a glucose moiety onto protein-bound oligosaccharides but S.cerevisiae do not (Fernandez et al., 1994; Jakob et al., 1998). Preliminary experiments show the presence of Glc<sub>1</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> on protein (data not shown). Importantly, no Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> was observed when the protein-bound glycan was analyzed from cells labeled in the presence of castanospermine (data not shown).

Materials and methods

Cells

Parental cells (K1–2) were pro–CHO cells (K1) (Thompson and Baker, 1973) and were obtained from ATCC (Rockville, MD) and recloned by limiting dilution. MI8-5 was isolated from K1–2 using a mannose suicide selection/screening procedure described elsewhere (J.L.O’Rear, J.R.Scocca, B.K.Walker, A.Kaiden and S.S.Krag, unpublished observations).

Metabolic labeling of oligosaccharide-lipid and protein

Cells were plated on 100 mm dishes at 34°C 2 days prior to the labeling experiment. At 15 and 5 min prior to the labeling period, the medium on the cells was changed to labeling medium without radioactivity. Cells were incubated for varying lengths of time from five to sixty min with 50 µCi/ml D-[2–3 H]mannose (2 Ci/mmol, Amersham Corp.) in α-Minimal Essential Medium lacking glucose but supplemented with 0.5 mM glucose, 0.1 mM mannose, 1 mM pyruvate, and 10% dialyzed fetal bovine serum. Cells were incubated for 30 min with 125 µCi/ml D-[1–3 H]galactose (9.3 Ci/mmol, New England Nuclear) in α-Minimal Essential Medium lacking glucose but supplemented with 0.1 mM glucose, 5 mM pyruvate, and 5% dialyzed fetal bovine serum. Two milliliters of labeling medium was used in each labeling.

Extraction and hydrolysis of oligosaccharide-lipid

Termination of labeling and extraction of oligosaccharide-lipid was performed as described by Cacan et al. (1992). Treatment of the oligosaccharide-lipid with mild acid was performed as described by Stoll and Krag (1988).
Analysis of oligosaccharides from oligosaccharide-lipid

Size analysis of oligosaccharides was performed by HPLC as described by Cacan et al. (1992). Gel filtration of the oligosaccharides was performed by Biogel P-4 chromatography as described by Krag (1979).

Glucosylphosphoryldolichol synthase assay

Membranes were prepared from parental and MI8-5 cells as described in Krag and Robbins, 1977. In vitro assays of glucosylphosphoryldolichol synthase activity in parental and MI8-5 membranes were performed as described previously (McLachlan and Krag, 1994).

Determination of radioactivity

Radioactivity was determined in a Beckman LS 5000 TD liquid scintillation counter using SafetySolve (Research Products International Corp.) as the scintillant.

Preparation of oligosaccharide-lipid substrate

Unlabeled Man\textsubscript{9}GlcNAc\textsubscript{2}-P-P-dolichol was isolated by extracting large numbers of MI8-5 cells according to the procedure described by Cacan et al. (1992). Sixteen to 32 subconfluent plates of cells were sequentially harvested on ice in 1 day. Extracts were stored on ice until all plates could be harvested. The extract of four plates were pooled in one 40 ml glass conical tube, and the extractions were scaled up 4-fold. Purified oligosaccharide-lipid was stored at -20°C in chloroform:methanol:water 10:10:3. Prior to use, the oligosaccharide-lipid was sequentially dried, concentrated to the bottom of the tube, and resuspended in DMSO or 1% NP-40 by sonication.

Coupled glucosylphosphoryldolichol synthase/glucosyltransferase assay

Membranes were prepared from parental and MI8-5 cells as described in Krag and Robbins, 1977. The assay mixture, in a final volume of 100 μl, contained 25 mM HEPES pH 7.0, 10 mM MgCl\textsubscript{2}, 5 mM CaCl\textsubscript{2}, 0.01% NP-40, 10% DMSO, 85 μM dolichyl phosphate, oligosaccharide-lipid from 10 million MI8-5 cells, and 200 μg membrane protein. The reaction mixtures lacking protein were first prewarmed in a 37°C shaking bath for 1 min before starting the assay with the addition of protein. After 10 min of reaction time at 37°C, 2 ml of chloroform/methanol 2:1 was added to terminate the reaction. The terminated reaction mixture was reconstituted to chloroform/methanol/water 3:2:1 by the addition of 1.33 ml chloroform/methanol 1:1 and 567 μl of a mixture of 0.1M Tris-Cl, pH 7.4, 0.156 M NaCl, 4 mM MgCl\textsubscript{2}, 3 mg/ml gamma globulins. After an extraction as described by Cacan et al. (1992), incorporation into the oligosaccharide-lipid fraction was determined by counting the chloroform:methanol:water 10:10:3 fraction.

Preparation of tritiated glucosylphosphoryldolichol

Multiple glucosylphosphoryldolichol synthase assays were performed as described above with parental membranes. After isolating the labeled lipid by extraction (Folch et al., 1957), the labeled lipid was purified by DEAE chromatography (D’Souza-Schorey et al., 1994).

Glucosyltransferase assay

Assays were performed as described previously (D’Souza-Schorey and Elbein, 1993) except about 60,000 d.p.m. of [3H]glucosyl-phosphoryldolichol and the oligosaccharide-lipid from 10 million MI8-5 cells were used. The concentrations of substrates were approximately 0.36 μM and 0.15 μM, respectively, assuming that CHO cells contain roughly 3.75 pmol of oligosaccharide-lipid per million cells (Kaiden and Krag, 1992). The assay was entirely dependent on the presence of exogenous oligosaccharide-lipid (data not shown). The oligosaccharide-lipid was extracted as described by Cacan et al. (1992) after the incubation. The oligosaccharide portion of the reaction product (released by mild acid treatment) eluted from a Biogel P4 column at a position consistent with Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2} (data not shown).

Plasmids and transfection

A genomic clone containing S.cerevisiae ALG6 (pALG6–4) was obtained from M. Aebi and S. te Hessen (Reiss et al., 1996). The gene was removed by restriction digest with EcoRI and XbaI and inserted into a similarly cut expression vector pcDNAI (Invitrogen) using standard methods (Sambrook et al., 1989) forming the plasmid pcDNAI-ALG6. The plasmid pcDNAI-βgal was similarly created by ligating the E.coli β-galactosidase gene into the same commercial vector cut with BamHI and HindIII, those which were used to remove the gene from the Pharmacia vector pCH110. For the pcDNAI-PyT plasmid, the cDNA encoding the polypoma virus large T antigen was cloned into pcDNAI from the plasmid pPyLT-1 (ATCC 41043; Treisman et al., 1981; Rassoulzadegan et al., 1982; Zhu et al., 1984) using the BamHI restriction sites. The orientation of the insert was verified by treatment of plasmid DNA from transformants with the restriction enzyme PvuII. Transfections were performed using the LipofectAMINE reagent according to manufacturer’s directions (Life Technologies) and using freshly ethanol-precipitated DNA previously purified by Midi-prep kits (Qiagen).

Transient transfection

Parental and MI8-5 cells were transfected on 100 mm dishes with a mixture of 10 μg pcDNAI-βgal and 10 μg pcDNAI-PyT or a mixture of 10 μg pcDNAI-ALG6 and 10 μg pcDNAI-PyT. Cells were replated the next day at roughly 1/4. On the third day posttransfection, duplicate plates from each transfection were incubated with [3H]galactose as described above. Oligosaccharide-lipid was isolated from harvested cells as described in Cacan et al., 1992, and treated with mild acid to release the oligosaccharide. The resultant oligosaccharide was analyzed by Biogel P4 chromatography as described above. A third plate of each set was counted; numbers from the same cell lines were averaged. A fourth plate from each cell line transfected with the β-galactosidase gene was also fixed with paraformaldehyde and stained with X-gal as described previously (Ausubel et al., 1997) to determine transfection efficiency.

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Abbreviations

CHO, Chinese hamster ovary cells; HEPES N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]; NP-40 Nonidet P-40.

References


