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Chinese Hamster Ovary Cells With Reduced Hexokinase Activity Maintain Normal GDP-Mannose Levels

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Abstract Parental Chinese hamster ovary (CHO) cells were mutagenized and subjected first to a mannose suicide selection technique and second to a screen of individual colonies grown on polyester discs for reduced mannose incorporation into protein. The incorporation of radioactivity for the selection and the screen was conducted at 41.5°C instead of the normal growth temperature of 34°C in order to allow for the isolation of temperature-sensitive lesions. This selection/screening procedure resulted in the isolation of MI5–4 cells, which had three- to five-fold lower incorporation of [2-^{3}H]mannose into mannose 6-phosphate, mannose 1-phosphate, GDP-mannose, oligosaccharide-lipid, and glycoprotein at 41.5°C. We detected no difference in the qualitative pattern of mannose-labeled lipid-linked oligosaccharides compared to parental cells. MI5–4 cells synthesized dolichol. The defect of MI5–4 cells was determined to be in hexokinase activity; crude cytosolic extracts were eight- to nine-fold lower in hexokinase activity in MI5–4 cells compared to parental cells. As a result of this defect, incorporation of labeled mannose from the medium was significantly decreased. However, the level of GDP-mannose in MI5–4 cells was 70% of normal. The phenotype of MI5–4 was a lower specific activity of labeled GDP-mannose, not a substantial reduction in the level of GDP-mannose. Consistent with these results, no alterations in the glycosylation of a model glycoprotein, G protein of vesicular stomatitis virus, were observed. These cells grew slower than parental cells, especially in low-glucose medium. J. Cell. Biochem. 72:56–66, 1999.

Key words: screen/selection mutant isolation procedure; N-linked glycosylation; G protein of vesicular stomatitis virus

Some of what is known about N-linked glycosylation has been elucidated using mammalian cell mutants. N-linked glycosylation involves a polyisoprenoid lipid carrier, dolichyl phosphate, and approximately 60 reactions occurring in three cellular compartments. First, dolichyl phosphate is synthesized from mevalonate in a series of steps occurring in the cytoplasm or endoplasmic reticulum, and many of which are in common with cholesterol biosynthesis [Kaiden and Krag, 1991]. Next, an oligosaccharide is assembled on dolichyl pyrophosphate [Cummings, 1992]. This oligosaccharide-lipid is synthesized by membrane-associated enzymes of the rough endoplasmic reticulum (RER) using both sugar nucleotides such as GDP-mannose and mono-glycosylated dolichyl phosphate as glycosyl donors. Dolichyl pyrophosphate oligosaccharide intermediates and monoglycosylated dolichyl phosphate translocate from the cytoplasmic face of the RER to the lumenal face of the RER [Hirschberg and Snider, 1987], where synthesis of the oligosaccharide is completed. The completed oligosaccharide, $Glc_3Man_9GlcNAc_2$, is transferred as a unit to nascent protein by the oligosaccharyl transferase [Gilmore, 1994] in the RER. Finally, the transferred oligosaccharide is shortened and then terminal sugars are added to produce the myriad of different oligosaccharide moieties present on N-linked glycoproteins [Kornfeld and Kornfeld, 1985]. These final processing enzymes are localized in Golgi membranes.

Thus far, lectin selections have been used most commonly to isolate various mutants in this pathway, especially mutants in the later processing steps [Stanley, 1984; Zeng and Lehr-

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man, 1991]. Other procedures, for example, a screen for lower mannose incorporation [Stoll and Krag, 1988] and a colony screen for cells with lowered lysosomal enzyme activity [Hall et al., 1986; Stoll et al., 1990] have been utilized in order to find mutants in the earlier biosynthetic steps.

In this paper, we report a new procedure to isolate glycosylation mutants in CHO cells. We have combined a mannose suicide procedure, originally developed using tritiated fucose to isolate glycosylation mutants in CHO cells [Hirschberg et al., 1981], with a colony screen for lowered incorporation of mannose into glycoprotein. In this procedure the incorporation of radioactivity for the selection and the screen was done at 41.5°C, rather than the normal growth temperature of 34°C, to allow for the isolation of temperature-sensitive mutants.

Although this procedure was designed to isolate glycosylation mutants, it is not specific for mutants in N-liked glycosylation. Indeed, any mutant whose lesion results in lower incorporation of labeled mannose into protein would be identified by this procedure. One mutant isolated by this procedure, MI5–4, was found to be defective in hexokinase activity. MI5–4 cells incorporated reduced levels of [^{3}H]mannose into mannose 6-phosphate, mannose 1-phosphate, GDP-mannose, oligosaccharide-lipid, and glycoproteins. However, the level of intracellular GDP-mannose was found to be near normal. Therefore, although there was a significant reduction in the specific activity of tritiated mannose, MI5–4 cells were able to normally glycosylate an N-linked viral glycoprotein. A preliminary report of this work was presented [O'Rear et al., 1995].

MATERIALS AND METHODS

Cell Culture and Media

CHO cells were propagated attached to tissue culture dishes in α-Minimal Essential Medium (α-MEM; KC Biologicals, Lenexa, KS) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). Normal growth conditions were at 34°C in 5% CO_2, 95% humidity in a forced-air incubator. The cells were passaged using 0.1% trypsin/0.04% EDTA, pH 7.4. Cell counts were determined using a Coulter counter (Coulter Corp., Hialeah, FL). In some experiments, cells were incubated at 41.5°C.

In some experiments, α-MEM medium without glucose and glutamine with 10% dialyzed fetal bovine serum was supplemented as follows: 10 mM glucose, 4 mM glutamine; 0.5 mM glucose, 4 mM glutamine; 10 mM glucose, 0.3 mM glutamine; or 0.5 mM glucose, 0.3 mM glutamine. Generation times were calculated from at least five determinations of cell number during logarithmic growth.

Isolation of MI5–4

Parental cells were pro$^-$ CHO cells (K1) [Thompson and Baker, 1973] obtained from ATCC (Rockville, MD) and were recloned by limiting dilution (K1–2). K1–2 cells were mutagenized by treatment for 18 h with 300 μg/ml ethyl methanesulfonate (Sigma, St. Louis, MO) followed by growth at 34°C in fresh medium for two days. Cells were then incubated at 41.5°C for 16 h followed by incubation with [2-^{3}H]mannose (500 μC_i/2.5 ml/dish) for 2 h at 41.5°C. Labeled cells were frozen at -80°C for 1 month, thawed, and grown for 2 days at 34°C. This cycle of growth at high temperature and mannose labeling was repeated five times. After the last thaw, cells were plated at 3,000 cells/dish and grown at 34°C onto polyester discs [Stoll and Krag, 1988]. Colonies on the polyester disc were preincubated at 41.5°C for 16 h then labeled with [2-^{3}H]mannose for 2 h at 50 μC_i/ml/disc. Following TCA precipitation of protein and fluorography [Stoll and Krag, 1988], colonies that incorporated low amounts of mannose into protein while staining strongly with Coomassie Blue were picked from the master dish. These colonies were recloned by limiting dilution and then subjected to a secondary screen.

In the secondary screen cells were incubated on a 100 mm dish with 50 μC_i [2-^{3}H]mannose/2.5 ml/dish for 2 h. The incubation was terminated as previously described [Cacan et al., 1992]. Incorporation into oligosaccharide-lipid and protein was determined [Cacan et al., 1992] and compared to that in parental cells. Isolates with reduced incorporation (≤2-fold) into either oligosaccharide-lipid, protein, or both relative to parental cells were subjected to a tertiary screen. In a tertiary screen cells were incubated for 2 h with 15 μC_i [^{35}S]methionine/ml/60 mm dish. Protein was solubilized from the washed dish by incubation with 0.1N NaOH and was precipitated by 10% TCA. Colonies that had ≤50% incorporation of mannose into

glycoprotein but 75–100% incorporation of methionine into protein compared to parental cells were saved for further analysis. For both the secondary and tertiary screens, cells were incubated at 41.5°C for 16 h before labeling. Potential mutants including MI5–4 were isolated at a frequency of about one in 10^3 using this technique.

Metabolic Labeling

Cells were labeled with [2-^{3}H]mannose as described previously [Cacan et al., 1992]. Incorporation into oligosaccharide-lipid and protein was determined as described [Cacan et al., 1992]. Saccharides released from oligosaccharide-lipid by mild acid treatment were separated by gel filtration chromatography as described [Krag, 1979].

Cells were infected with vesicular stomatitis virus and labeled with [^{35}S]methionine for 10 min as described [Stoll et al., 1992]. Labeled cells were solubilized in sample buffer, an equal volume of each sample was applied to an SDS gel, and the labeled proteins were separated by SDS-PAGE and visualized by autoradiography as described previously [Stoll et al., 1992].

Cells were labeled with [^{3}H]mevalonolactone and prenols were extracted, isolated, quantified, and separated as described previously [Rosenwald et al., 1993].

Analysis of Aqueous [2-^{3}H]Mannose-Containing Products

The aqueous phase of the extraction [Cacan et al., 1992] was dried under reduced pressure and resuspended in 1 ml distilled water containing unlabeled standards of GDP-mannose, mannose, and mannose 1-phosphate. The mixture was applied to a 0.5 cm by 4 cm AG1-X8 resin, formate form (Bio-Rad Laboratories, Hercules, CA). Material was eluted in steps using the following: 7.5 ml water, 7.5 ml 0.1 M formic acid (J.T. Baker), 10 ml 1.0 M formic acid, 10 ml of 4 M formic acid, and 10 ml of 4 M formic acid/0.4 M ammonium formate. Fractions of 0.75 ml were collected and aliquots were counted. Standards elute as follows: mannose in the water wash (detected with anthrone) [Radin, 1958]; mannose 1-phosphate in the 4 M formic acid wash (phosphate analysis) [Bartlett, 1959]; and GDP-mannose in the 4 M formic acid/0.4M formate wash (detected using absorbance at 256 nm).

Mannose 1-phosphate was distinguished from mannose 6-phosphate, which also elutes with 4 M formic acid, by mild acid hydrolysis. Fractions eluting with 4 M formic acid were pooled, dried under reduced pressure, and resuspended in 0.1 N HCl. Samples were then heated in a dry heat block at 80°C for 2 h. The products of acid hydrolysis were separated on the AG1–8X resin column as described above.

Hexokinase Assays

Cells were grown at either 34°C or 41.5°C and removed from the culture dishes by incubation in phosphate-buffered saline containing EDTA [Krag, 1979]. Cells were homogenized with 30 strokes of a cold dounce homogenizer in 2 ml 0.02 M Tris/Cl, pH 7.4. A solution (0.1 vol) of 0.03 M $MgCl_2$/0.1 M NaCl was added and mixed with five more strokes of the homogenizer. Unbroken cells and heavy organelles were pelleted in a 20,000*g* centrifugation for 20 min at 4°C. The supernatant fluid was used as the source of enzyme.

Hexokinase activity was assayed using a coupling enzyme, glucose 6-phosphate dehydrogenase, that reduced $NADP^+$ to NADPH during the oxidation of glucose 6-phosphate to 6-phosphogluconic acid [Faik et al., 1989]. The reaction mixture contained the following: 0.1 M Tris-Cl, pH 8.0, 5 mM ATP, 5 mM $MgCl_2$, 10 mM glucose, 0.6 mM $NADP^+$, and two units of glucose 6-phosphate dehydrogenase. When mannose was used as a substrate, the reaction conditions were the same except mannose was substituted for glucose, and two additional coupling enzymes were included, one unit mannose 6-phosphate isomerase and 1.5 units glucose 6-phosphate isomerase. Substrates were at saturating concentrations.

The absorbance change produced by the reduction of $NADP^+$ was followed at 340 nm on a Varian multicell spectrophotometer. A data point was taken every 30 sec, the signal averaging time was 2 sec, and the reaction time was 15 min. For certain experiments, the cell extract was warmed for 10 min prior to the addition of substrates. The linear rate of absorbance change versus time was calculated using the slope from 7 to 15 min. An extinction coefficient for $NADP^+$ of 6250 $M^{-1}cm^{-1}$ was used to convert the absorbance change to pmol product formed.

Determination of GDP-Mannose Pool

GDP-mannose was extracted from cells, separated from other metabolites by lectin affinity

chromatography and ion exchange chromatography, and quantitated spectrophotometrically. Six 100 mm dishes of cells were used for each measurement. Cells from two dishes were harvested and counted to determine cell number. The cells in four dishes were lightly labeled with [2-^{3}H]mannose (125 μC_i) for 1 h to allow detection of mannose-containing compounds during the work-up.

Labeling medium was removed by aspiration and 0.7 ml ice-cold methanol was added per dish to kill the cells and extract the sugar nucleotide. Cells were removed from the dish by scraping and dishes were rinsed with additional methanol. The methanol contained a trace amount of GDP-[^{14}C]mannose as an internal standard.

The methanol suspension was centrifuged 10 min at 1,400*g* to remove cellular debris, and the supernatant solution was transferred to a 100 ml pear flask. The sediment was washed twice; first with 1 ml ice-cold methanol and then with 1 ml ice-cold 70% ethanol containing 15 mM KH_2PO_4, pH 4.5. The washes were combined with the methanol extract and dried on a rotary evaporator. The residue was dissolved in 1 ml of buffer A (15 mM KH_2PO_4, pH 4.5, 1 mM Mg acetate, 1 mM Ca acetate,1 mM Mn acetate). The slight precipitate was removed by centrifugation at top speed in a microfuge, and the soluble material was loaded onto a 4 ml concanavalinA sepharose column [Rush and Waechter, 1995] equilibrated with buffer A. The column was eluted stepwise with 4 ml of buffer A, 12 ml of 15 mM KH_2PO_4, pH 6.0, and 25 ml 15 mM KH_2PO_4, pH 7.5. Fractions of 1 ml were collected. Fractions containing GDP-mannose (8–10 ml), which eluted at pH 7.5 were pooled, dried on a rotary evaporator, and resuspended in 1 ml water. The solution was centrifuged through an 0.2µ filter (Amicon, Micropure) and a 0.5 ml sample was injected onto a Dionex Carbopac PA1 column [Gibson et al., 1994] on a Waters 660E HPLC. GDP-mannose was eluted with a linear gradient in which the concentration of KH_2PO_4, pH 4.5 was increased from 0.25 to 0.35 M over a period of 60 min at a flow rate of 1 ml/min. The sugar nucleotide was detected by absorbance at 253 nm. Under these conditions, GDP-mannose emerged as a single sharp peak at about 25 min. The amount of GDP-mannose was calculated from the area of the absorbance peak. The area was determined to be linear for GDP-mannose standards over a range of 100–1,000 pmoles injected. Overall, recovery of internal standard was 70–80%.

RESULTS

Isolation of MI5–4

We developed a new method for isolating mutants in CHO cells by combining a colony screening procedure with a mannose suicide enrichment procedure (see Materials and Methods). Although this procedure could isolate mutants in the N-linked glycosylation pathway, it was not specific for that pathway. Any mutant whose lesion resulted in a lower incorporation of labeled mannose from the medium into cellular glycoproteins would be isolated by this procedure.

One mutant isolated by this new method was MI5–4. Preliminary labeling experiments during the secondary and tertiary screens indicated that MI5–4 cells had 70–75% reduction in incorporation of [2-^{3}H]mannose into oligosaccharide-lipid and protein compared to parental cells but maintained 80% of parental levels of incorporation of [^{35}S]methionine into protein (data not shown). An analysis of [^{3}H]mevalonolactone-labeled cells indicated that MI5–4 synthesized dolichol rather than polyprenol, and therefore was not a Lec9 CHO mutant [Rosenwald et al., 1993; data not shown].

Mannose-Labeling Phenotype of MI5–4

The amount of mannose incorporated into protein (Fig. 1A) and oligosaccharide-lipid intermediates (Fig. 1B) at 34°C and 41.5°C for parental and MI5–4 cells is shown in Figure 1.

Incorporation into both protein and oligosaccharide-lipid was decreased in MI5–4 cells compared to parental cells, especially at 41.5°C, where a three- to four-fold reduction was seen. MI5–4 was isolated in our screen because it incorporated less tritiated mannose into protein at 41.5°C.

Although the amount of incorporation of [^{3}H]mannose into oligosaccharide-lipid was decreased, the labeled oligosaccharide appeared to have the same structure in MI5–4 and parental cells, as judged by elution on a gel filtration column (Fig. 2). The majority of radioactivity in both cell lines eluted excluded of the $GlcNAc_6$ standard, consistent with it being $Glc_3Man_9GlcNAc_2$ [Krag, 1979]. Thus, MI5–4 cells appeared to label reduced amounts of the normal oligosaccharide-lipid intermediate.

We next analyzed the incorporation of mannose into GDP-mannose and mannose phos-

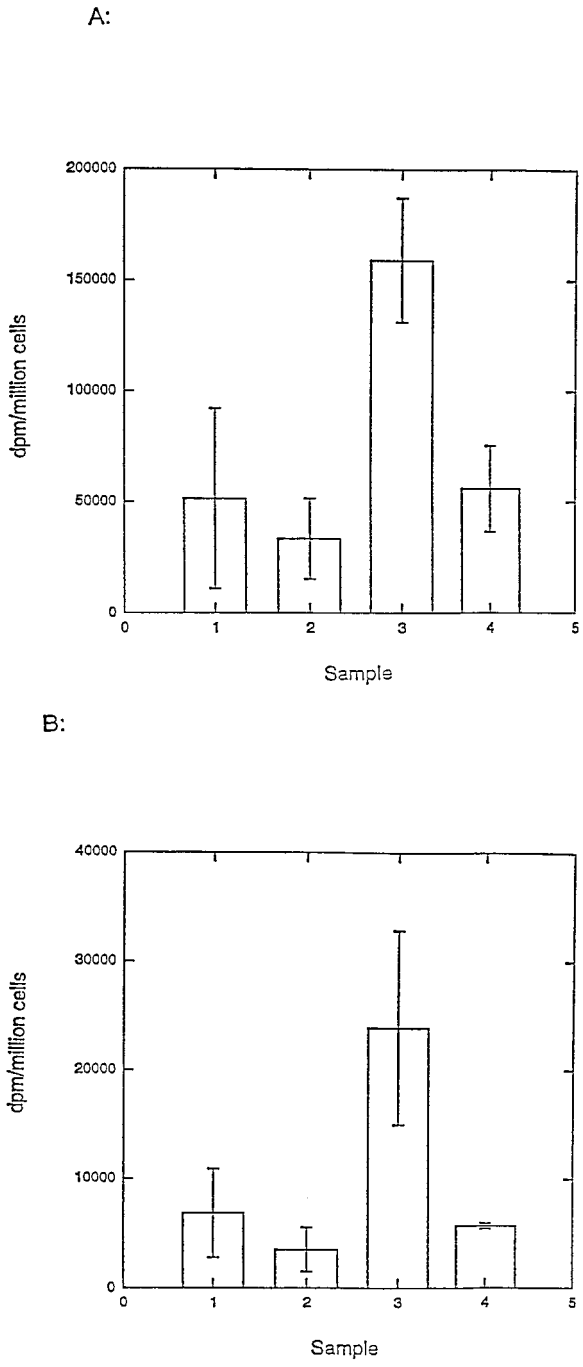

Fig. 1. Incorporation of D-[2-^{3}H]mannose into oligosaccharide-lipid and protein in parental and MI5–4 cells at 34°C and 41.5°C. Cells were grown at 34°C for 2 days, then incubated overnight at either 34°C or 41.5°C. Cells were labeled with tritiated mannose for 1 h as described in Materials and Methods. Oligosaccharide-lipid and protein were analyzed for incorporation of radioactivity as described in Materials and Methods. Incorporation was normalized per million cells present on each dish. **A**: Sample 1: K1–2 glycoprotein at 34°C; sample 2: MI5–4 glycoprotein at 34°C; sample 3: K1–2 glycoprotein at 41.5°C; sample 4: MI5–4 glycoprotein at 41.5°C. **B**: Sample 1: K1–2 oligosaccharide-lipid at 34°C; sample 2: MI5–4 oligosaccharide at 34°C; sample 3: K1–2 oligosaccharide-lipid at 41.5°C; sample 4: MI5–4 oligosaccharide-lipid at 41.5°C.

phate. As indicated in Table 1, incorporation into mannose phosphate and GDP-mannose was reduced in MI5–4 compared to parental cells. Again, the defect was greater at 40.5°C than at 34°C.

In order to further pinpoint the defect, we determined the proportion of [^{3}H]mannose 6-phosphate and [^{3}H]mannose 1-phosphate in the mannose phosphate fraction from the AG1–8X column. Both mannose 1-phosphate and mannose 6-phosphate elute from the ion exchange column in 4 M formic acid. We utilized the acid lability of the phosphoglycosidic bond in mannose 1-phosphate compared to the phosphodiester bond in mannose 6-phosphate to distinguish the two compounds in the mixture of labeled mannose phosphate. Material eluting with 4 M formic acid from the AG1-X8 column (Table 1) was concentrated to dryness and then treated with mild acid for 2 h (see Materials and Methods). The products of this mild acid treatment were rechromatographed on a AG1–8X formate column. As seen in Table II, the ratio of mannose, the product of acid hydrolysis of mannose 1-phosphate, to mannose 6-phosphate was identical in MI5–4 and K1-2, indicating that incorporation of label into both mannose 1-phosphate and mannose 6-phosphate was reduced in MI5–4 cells. This result suggests that phosphomannomutase is active in MI5–4 cells.

Measurement of Hexokinase Activity in MI5–4 Cells

Incorporation of tritiated mannose into mannose 6-phosphate could be lower in the mutant for two reasons. First, mannose transport could be defective or second, the level of hexokinase activity could be reduced. We assayed crude cytosolic preparations of parental and MI5–4 cells for hexokinase activity. As seen in Figure 3, the activity of K1–2 extracts using glucose as the substrate was 74 pmol/min/μg, while that of MI5–4 was 8 pmol/min/μg. In 20 assays, the range of activity observed was from 56 to 92 pmol/min/μg for K1–2 extracts and 5.5 to 10 pmol/min/μg for MI5–4 extracts. The concentration of glucose used in the reaction (10 mM glucose) was saturating for both cell lines (Fig. 4). Hexokinase activity with mannose as substrate was 43 pmol/min/μg in extracts from parental cells and 5 pmol/min/μg in extracts from MI5–4. In 10 assays, using mannose as substrate, activities ranged from 35 to 47 pmol/min/μg for K1–2 extracts and from 4 to 6 pmol/min/μg for MI5–4 extracts.

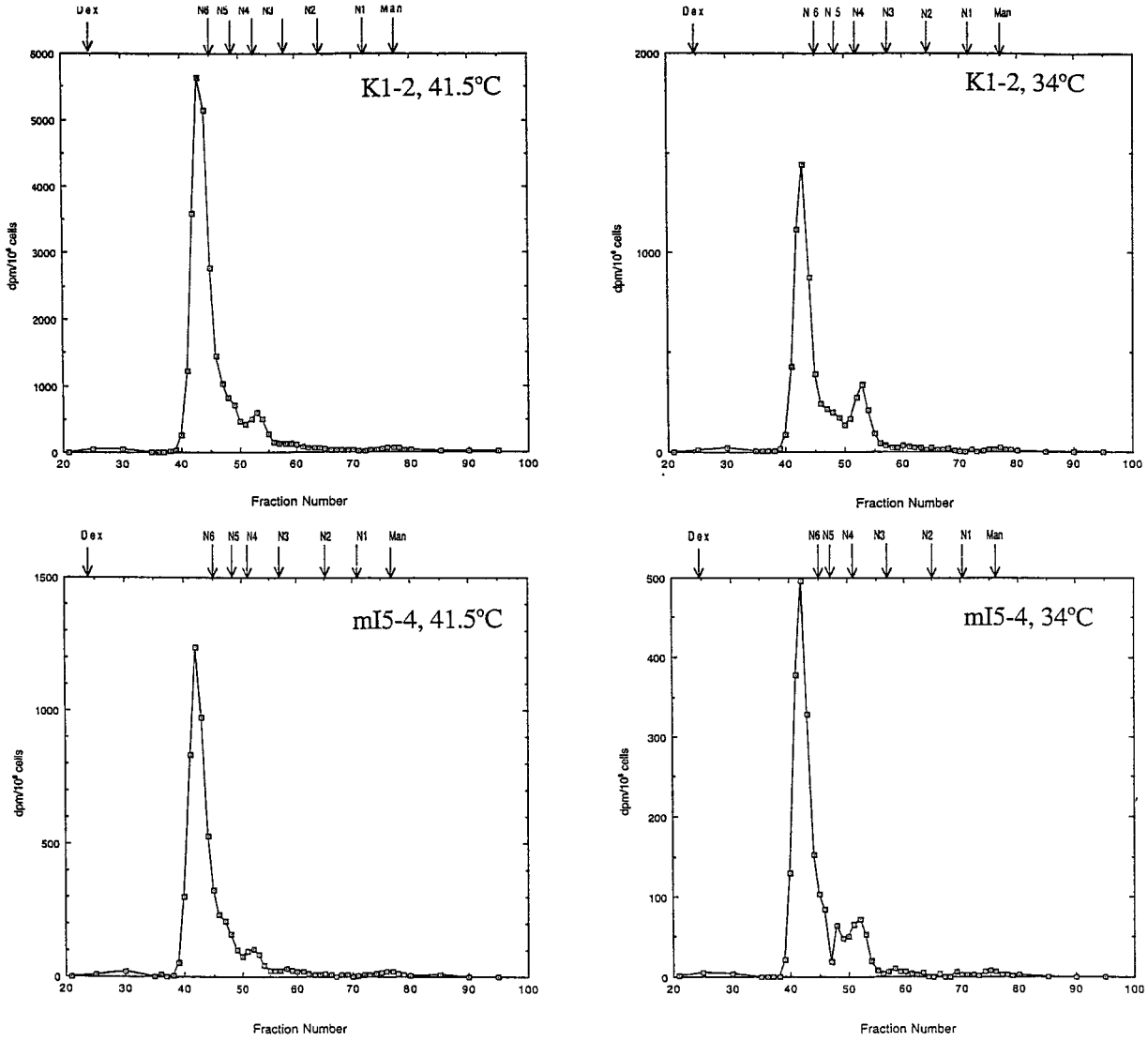

Fig. 2. Profile of saccharides released from oligosaccharide-lipid extracted from K1–2 and MI5–4. Cells were grown at 34°C for 2 days, incubated overnight at either 34°C or 41.5°C, then incubated with [2-^{3}H]mannose for 1 h. Labeled oligosaccharide-lipids were extracted. Labeled saccharides were released by mild acid hydrolysis and analyzed by gel filtration chromatography as described in Materials and Methods. Fractions were collected and the radioactivity recovered in each fraction was normalized to cell number. Internal standards [Krag, 1979] were as follows: Dex, dextran; Man, mannose; N1–N6, oligomers of GlcNAc.

In some experiments, known amounts of protein from each cell line were mixed, and the activity was found to be additive, when either glucose or mannose was used as a substate (data not shown). This indicates that an inhibitor of hexokinase was not present in the mutant extracts. The activity of hexokinase did not appear to be more heat-labile in MI5–4 cells than in parental cells (Fig. 5). Finally, MI5–4 cells grown at either 34°C or 39.5°C had reduced levels of hexokinase (Table 3). Thus, it appears that MI5–4 has about seven-fold less hexokinase activity than parental cells regardless of growth conditions or assay conditions.

N-Linked Glycosylation of G Protein of VSV-Infected MI5–4 Cells

One indicator of the efficiency of N-linked glycosylation in CHO cells is the glycosylation pattern of G protein produced by cells infected with vesicular stomatitis virus (VSV). CHO cells infected with VSV cease to produce cellular proteins, and only produce five viral proteins, one of which, G protein, is an N-linked glycopro-

TABLE I. Incorporation of Labeled Mannose Into Water-Soluble Products[a]

Cell	Temperature	Mannose phosphate dpm/10^6 cells	GDP-mannose dpm/10^6 cells
K1-2	34°C	4.0×10^3	7.8×10^3
K1-2	40.5°C	8.7×10^3	14.0×10^3
MI5-5	34°C	3.1×10^3	3.9×10^3
MI5-4	40.5°C	2.9×10^3	3.5×10^3

[a]Each cell line was grown at 34°C and then incubated at 34°C or 40.5°C overnight prior to labeling with [2-^{3}H]mannose for 1 h and extraction as described in Materials and Methods. The aqueous fraction was applied to an AG1-X8 (formate form) anion exchange column and eluted as described in Methods. The elution positions of unlabeled internal standards were determined. Data in this table are from one of eight comparable experiments. Data from all eight experiments were not pooled because the absolute incorporation varied two-fold.

TABLE II. Determination of the Ratio of Labeled Mannose 6-Phosphate to Mannose 1-Phosphate in K1-2 and MI5-4 Cells[a]

Cell	Temperature	Mannose dpm/10^6 cells (percentage)	Mannose 6-phosphate dpm/10^6 cells (percentage)
K1-2	34°C	2,698 (37)	3,723 (51)
K1-2	40.5°C	4,588 (34)	7,895 (59)
MI5-4	34°C	1,029 (26)	2,144 (56)
MI5-4	40.5°C	1,062 (32)	1,896 (57)

[a]Labeled material eluting from the AG1-8X column with 4 M formic acid (mannose phosphate) was concentrated to dryness under reduced pressure and then incubated with 0.1 N HCl for 2 hr at 80°C. The hydrolysis products were then separated by an AG1-8X column as described in Materials and Methods. Fractions were collected and counted. The radioactivity that eluted in the neutral wash (mannose) was separated from that eluted with 4 M formic acid (mannose 6-phosphate). The eluted radioactivity in each fraction was divided by the total radioactivity loaded onto the column and the percentages are given in the parentheses.

tein [Morrison and Lodish, 1975]. Normally in CHO cells, G protein is N-glycosylated at two sites [Stanley, 1982] and di-, mono-, and nonglycosylated forms can easily be separated by SDS-PAGE [Rosenwald et al., 1989].

K1–2 and MI5–4 cells were infected with VSV and then labeled with [^{35}S]methionine as described in Methods in the presence or absence of tunicamycin, which inhibits N-linked glycosylation [Elbein, 1987]. As can be seen in Figure 6, di- and nonglycosylated (lanes with tunicamycin treatment) G protein were easily distinguished. Importantly, the glycosylation

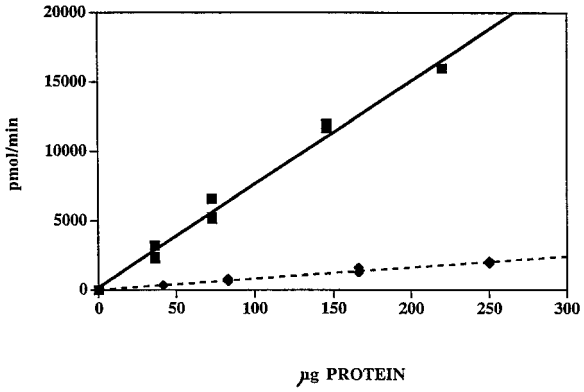

Fig. 3. Hexokinase activity of cytosolic extracts from K1–2 and MI5–4 cells. Crude cytosolic extracts were prepared from K1–2 cells and MI5–4 cells grown at 34°C and were assayed for hexokinase activity using 10 mM glucose as described in Materials and Methods. K1–2 (▩) and MI5–4 (◆).

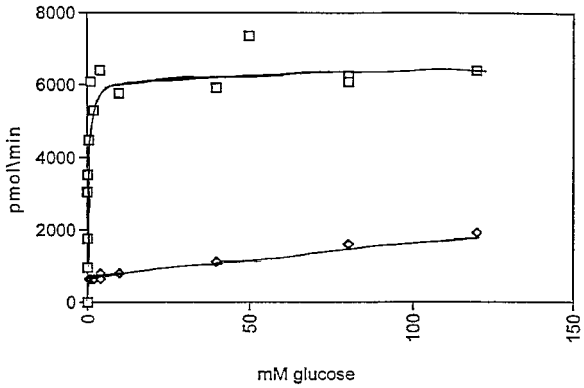

Fig. 4. Glucose concentration curve for hexokinase activity in cytosolic extracts of K1–2 and MI5–4 cells. Crude cytosolic extracts were prepared from K1–2 cells and MI5–4 cells grown at 34°C and were assayed for hexokinase activity. In this experiment, 73 µg of protein from K1–2 and 83 µg from MI5–4 were assayed using various amounts of glucose as substrate. The hexokinase specific activity was 74 pmol/min/µg in K1–2 extracts (□—□) and 8 pmol/min/µg in MI5–4 extracts (△—△).

patterns of G protein from MI5–4 and K1–2 cells were indistinguishable. The great majority of G protein in both cases was diglycosylated. Therefore, despite the reduction in hexokinase activity, MI5–4 cells produce a normally N-glycosylated viral protein.

Determination of the Size of the GDP-Mannose Pool in MI5–4 Cells

Although the reduced incorporation of [^{3}H]mannose into GDP-mannose and glycoproteins suggested a defect in glycosylation, the normal glycosylation patterns of VSV G protein in MI5–4 cells contradicted this suggestion. To

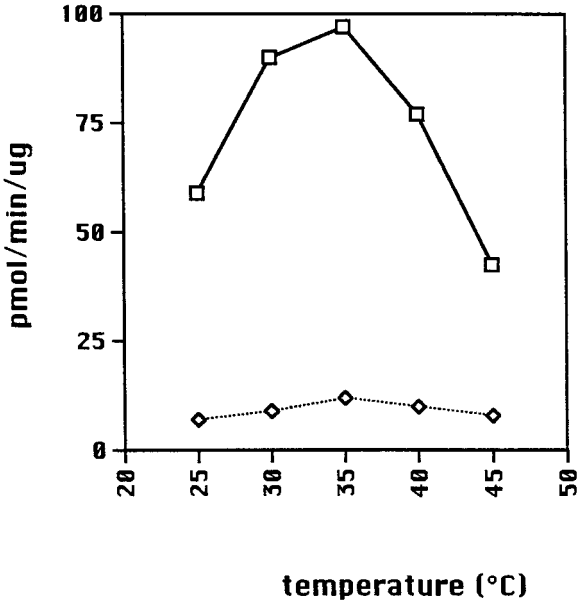

Fig. 5. Hexokinase activity as a function of assay temperature. The specific activity of hexokinase at various reaction temperatures in cytosolic extracts of K1–2 (□—□) and MI5–4 (△—△) cells grown at 34°C was determined as described in Materials and Methods with 10 mM glucose as substrate. Cytosolic extracts were preincubated at the assay temperature for 10 min before the addition of the rest of the reaction components. Points are an average of two determinations.

TABLE III. Effect of Growth Temperature and Assay Temperature on Hexokinase Activity of K1-2 and MI5-4 Cell Extracts[a]

Cell	Growth temperature °C	Assay temperature °C	Specific activity (pmol/min/μg)
K1-2	34	30	59
MI5-4	34	30	8
K1-2	34	40	50
MI5-4	34	40	8
K1-2	39.5	30	50
MI5-4	39.5	30	5
K1-2	39.5	40	45
MI5-4	39.5	40	7

[a]K1-2 and MI5-4 cells were grown at 34°C for 2 days, and then either kept at 34°C or shifted to 39.5°C overnight. Cytosolic extract was prepared and assayed for hexokinase activity using glucose as substrate as described in Materials and Methods. The cytosolic extract was preincubated at the assay temperature for 10 min before beginning the assay.

resolve this discrepancy, the absolute level of GDP-mannose in K1–2 cells and MI5–4 cells was measured and used to calculate the specific activity of GDP-[^{3}H]mannose in each cell line. As shown by the data in Table 4, at both temperatures MI5–4 has 70% of parental levels of

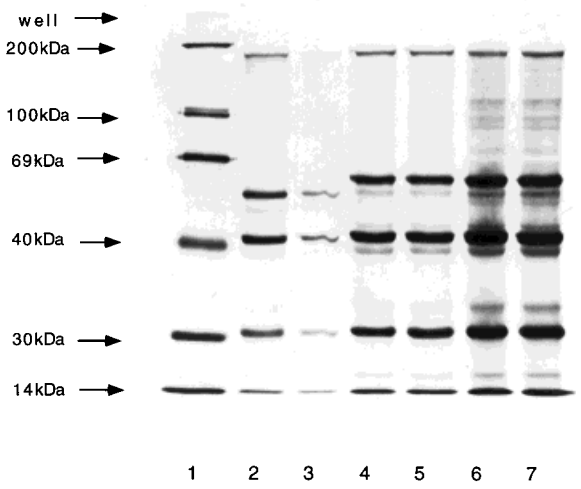

Fig. 6. Biosynthesis of [^{35}S]labeled G protein in VSV-infected parental and MI5–4 cells. Cells were incubated for 2 days at 34°C and then shifted overnight to 39.5°C. Five h postinfection, the cells were incubated in the presence of [^{35}S]methionine to label viral proteins (see Materials and Methods). In some cases, 1 μg/ml tunicamycin was added during the labeling in order to inhibit N-linked glycosylation [Elbein, 1987]. The cells were lysed in sample buffer, and the samples analyzed by SDS-PAGE as described in Materials and Methods. **Lane 1:** [^{14}C]Methylated proteins as standards; **Lane 2:** infected K1–2 cells treated with tunicamycin; **Lane 3:** infected MI5–4 cells treated with tunicamycin; **Lane 4 and 5:** infected MI5–4 cells; **Lane 6 and 7:** infected K1–2 cells. One-third of each sample was loaded on each lane.

GDP-mannose. Thus, the size of the GDP-mannnose pool of MI5–4 cells is near normal but MI5–4 cells have a lower specific activity of the GDP-mannose pool than K1–2 cells (Table 4) due to reduced hexokinase activity. Mannose from the medium is underutilized by MI5–4 cells compared to parental cells. Nonetheless, the pool size of GDP-mannose is near normal in MI5–4 cells, leading to normal N-glycosylation protein (Fig. 6).

Phenotypes of MI5–4 Cells

Although N-glycosylation of protein is normal in hexokinase-deficient MI5–4 cells, there are other observable biological consequences of the defect. First, MI5–4 cells are temperature-sensitive for growth, in a plating-efficiency assay (data not shown).

Second, MI5–4 cells grown in regular medium supplemented with 10 mM glucose and 4 mM glutamine had a slower growth rate than K1–2 cells (38 h doubling time versus 28 h, respectively). CHO cells, as other mammalian cells, use glutamine as an energy source [Reit-

TABLE IV. Incorporation of [^{3}H]Mannose Into the GDP-Mannose Pool[a]

Cells	Growth temperature	Radioactivity in GDP[^{3}H]Man per 10^6 cells dpm $\times 10^{-3}$	pmoles GDP-Man per 10^6 cells	Specific activity of GDP-man (dpm $\times$ pmol^{-1})
K1-2	34°C	4.47	49.8	90
MI5-4	34°C	2.08	34.6	60
K1-2	41.5°C	20.5	105	195
MI5-4	41.5°C	6.79	75.6	90
K1-2	41.5°C	13.1	112	117
MI5-4	41.5°C	3.21	75.2	43

[a]K1-2 and MI5-4 cells were grown to near confluence at 34°C and then either kept at 34°C or switched to 41.5°C overnight prior to labeling for 1 h with [^{3}H]mannose. Cells were harvested with cold methanol containing [^{14}C]GDP-mannose as an internal standard (see Materials and Methods). GDP-mannose was isolated from the methanol extract as described in Materials and Methods and was detected on HPLC by absorbance at 253 nm. Levels of GDP-mannose were quantitated by comparison to GDP-mannose standards and corrected for the minor contribution of the GDP[^{14}C]mannose internal standard. Fractions were collected during HPLC and radioactivity measured to determine recovery of the [^{14}C]labeled standard and the total [^{3}H] incorporation into GDP-mannose. The values at 41.5°C were determined in two identical experiments. Although the levels of [^{3}H]incorporation varied between experiments, the absolute amounts of GDP-mannose were comparable.

zer et al., 1979]. K1–2 cells had the same growth rate in medium with 10 mM glucose/4 mM glutamine, 10 mM glucose/0.3 mM glutamine, and 0.5 mM glucose/4 mM glutamine (28 h). The growth rate of K1–2 cells was not compromised until cells were grown in 0.5 mM glucose/0.3 mM glutamine (43 h). On the other hand, MI5–4 cells doubled slowly (45 h) in medium with 10 mM glucose/0.3 mM glutamine, 0.5 mM glucose/4 mM glutamine, and 0.5 mM glucose/0.3 mM glutamine. This data is consistent with CHO cells being able to use glutamine as an energy source, but also depending on glucose, and thus hexokinase activity, for optimal growth; this observation has been seen for other cultured cells [Morgan and Faik, 1981].

DISCUSSION

This paper reports on a new method to isolate mutants in CHO cells. This method combines two procedures used previously, namely, a colony screen for mannose incorporation [Stoll and Krag, 1988] with a suicide enrichment technique [Hirschberg et al., 1981]. Although we plan to use this procedure to isolate glycosylation mutants, the results in this paper illustrate clearly that this procedure is not specific for mutants with lesions in the N-linked glycosylation pathway. This paper reports on a mutant MI5–4 isolated by this procedure that appears normal in glycosylation, but was deficient in hexokinase activity.

CHO cells deficient in glycolysis had previously been isolated by Morgan and Faik [1981] and were found to be deficient in hexokinase, phosphoglycerate kinase, and glucose phosphate isomerase [Faik et al., 1989]. These cells were used to study the transport and accumulation of 2-deoxy-D-glucose. N-linked glycosylation was not examined in this mutant.

MI5–4 is apparently defective only in hexokinase activity. This defect lowers the utilization of sugars transported into the cell from the medium. This defect appears to compromise growth rate, even in the presence of high glutamine concentrations. Glutamine is known to be a carbon source for oxidative phosphorylation in cultured cells [Reitzer et al., 1979]. Importantly, however, optimal growth rate of CHO cells seems to require utilization of glucose from the medium.

Interestingly, MI5–4 cells, despite a lowered amount of hexokinase activity, were able to maintain near normal amounts of GDP-mannose (Table 4) and thus glycosylate N-linked glycoproteins normally (Fig. 6). This is somewhat surprising in light of recent studies by Alton et al. [1998] showing that under normal conditions cultured human hepatoma cells derive most mannose for glycoprotein biosynthesis from extracellular mannose. Our results with MI5–4 cells suggest that cells can maintain near normal levels of GDP-mannose without the contribution of extracellular mannose. However, MI5–4 cells do display a lowered growth rate and a temperature-sensitive growth phenotype.

Carbohydrate-deficient Glycoprotein Syndrome (CDGS) is a heterogeneous genetic disease of humans, characterized by underglycosylation of proteins. Recently, two groups have found that CDGS cells have defects in enzymes early in the synthesis of GDP-mannose, and in

some of these cells, the defects could be reversed by growth in high amounts of mannose [Powell et al., 1994; Krasnewich et al., 1995; Panneerselvam and Freeze, 1996]. Another group has presented evidence that in some CDGS cells, the synthesis of dolichol is reduced [Ohkura et al., 1997]. Thus, CDGS seems to be a disease with diverse genotypes. Although MI5–4 cells are also defective in an early step in GDP-mannose synthesis, based on the phenotype of MI5–4, we would predict that a hexokinase deficiency would not lead to CDGS.

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