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The Metabolic Origins of Mannose in Glycoproteins*◆

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Background: Metabolic origins of mannose in glycoproteins have not been studied.
Results: Glucose is the major source of *N*-glycan mannose, but exogenous mannose provides up to 50%. Man salvaged from glycoprotein turnover, glycogen, and gluconeogenesis is insignificant.
Conclusion: Exogenous mannose is incorporated into *N*-glycans much more efficiently than that derived from glucose.
Significance: This study provides quantitation of metabolic origins of Man in *N*-glycans.

Mannose in *N*-glycans is derived from glucose through phosphomannose isomerase (MPI, Fru-6-P ↔ Man-6-P) whose deficiency causes a congenital disorder of glycosylation (CDG)-Ib (MPI-CDG). Mannose supplements improve patients' symptoms because exogenous mannose can also directly contribute to *N*-glycan synthesis through Man-6-P. However, the quantitative contributions of these and other potential pathways to glycosylation are still unknown. We developed a sensitive GC-MS-based method using [1,2-^{13}C]glucose and [4-^{13}C]mannose to measure their contribution to *N*-glycans synthesized under physiological conditions (5 mM glucose and 50 μM mannose). Mannose directly provides ~10–45% of the mannose found in *N*-glycans, showing up to a 100-fold preference for mannose over exogenous glucose based on their exogenous concentrations. Normal human fibroblasts normally derive 25–30% of their mannose directly from exogenous mannose, whereas MPI-deficient CDG fibroblasts with reduced glucose flux secure 80% of their mannose directly. Thus, both MPI activity and exogenous mannose concentration determine the metabolic flux into the *N*-glycosylation pathway. Using various stable isotopes, we found that gluconeogenesis, glycogen, and mannose salvaged from glycoprotein degradation do not contribute mannose to *N*-glycans in fibroblasts under physiological conditions. This quantitative assessment of mannose contribution and its metabolic fate provides information that can help bolster therapeutic strategies for treating glycosylation disorders with exogenous mannose.

Both glucose and mannose can contribute mannose to *N*-glycan synthesis through a common precursor, Man-6-P,[2] which is converted to Man-1-P via phosphomannomutase (PMM2) and on to GDP-mannose. For over 35 years, biosynthetic studies of glycoproteins have relied on the labeling specificity of [2-^{3}H]mannose. Intracellular conversion to [2-^{3}H]Man-6-P then leads to two mutually exclusive fates: 1) glycan synthesis or 2) MPI-mediated catabolism to Fru-6-P with release of ^{3}HOH (1). Up to 98% of the label from [2-^{3}H]mannose entering cells is catabolized (see Scheme 1). When radiolabeled [^{3}H]glucose and [^{14}C]mannose are provided at physiological concentrations of 5 mM and 50 μM, respectively, 80–98% of mannose in *N*-glycans appears to be derived from glucose. A major factor determining the fate of [2-^{3}H]mannose is the PMM2:MPI ratio because both compete for Man-6-P. Reducing MPI activity either by RNAi knockdown (1) or by genetic mutation (1) increases the proportion of [2-^{3}H]mannose in *N*-glycans because [2-^{3}H]Man-6-P catabolism is reduced.

Other studies in cells (2), mice (3), and humans (4, 5) all show that surprisingly small increases in exogenous mannose dramatically improve deficient glycosylation (2–5). Patients carrying hypomorphic, life-threatening mutations in *MPI* suffer from coagulopathy, protein-losing enteropathy, hypoglycemia, and liver dysfunction, but most of these abnormalities reverse when patients are given ~0.6 g of mannose/kg/day as dietary supplements, raising their plasma mannose concentration by 3–4-fold (4). Mannose also reverses insufficient glycosylation in patient-derived cells (2). Mannose rescues embryonic lethality of mice carrying mutations in *Pmm2* that die by mid-gestation, but when dams are given 0.9% mannose in their drinking water, pups are born and thrive (3). However, patients with *PMM2* mutations do not seem to respond to mannose therapy (6, 7). These observations prompted us to quantitatively assess the contributions of mannose to mannose (Man^M) and glucose to mannose (Man^G) in glycosylation as well as their catabolic fate. In this study, we describe and apply new methods to metabolically label cells with stable isotopes (8) and determine the relative and quantitative contributions of mannose (Man^M) to *N*-glycans. We found that the contribution of exogenous mannose was previously underestimated. We also combined stable monosaccharide isotope labeling with deuterium oxide (D_2O) and found that other potential sources of mannose including mannose salvaged from degraded glycoproteins (Man^S), glycogen (Man^{GL}), and gluconeogenesis do not make significant contributes to *N*-glycosylation.

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[2] The abbreviations used are: Man-6-P, mannose 6-phosphate; Fru-6-P, fructose 6-phosphate; Glc-6-P, glucose 6-phosphate; [UL-^{13}C], [Universal label-$^{13}C_6$]; PMM2, phosphomannomutase; MPI, Man-6-phosphate isomerase; MEF, mouse embryonic fibroblast; TMS, trimethylsilyl.

EXPERIMENTAL PROCEDURES

Radiolabels and Stable Isotopes—[2-^{3}H]Mannose and [2-^{3}H]2-deoxyglucose were obtained from PerkinElmer. Monosaccharides with stable isotopes, [1,2-^{13}C], [6,6-^{2}H], and [6-^{13}C]glucose; [1,2-^{13}C], [2-^{13}C], and [4-^{13}C]mannose; and [2-^{13}C]glycerol and [3-^{13}C]pyruvate were purchased from Omicron Biochemicals. Deuterium water was purchased from Cambridge Isotope Laboratories, Inc.

Cell Lines—Normal fibroblasts were obtained from American Type Culture Collection and Coriell Cell Repositories. All the cell lines were grown in DMEM with 1 g/liter of glucose containing 2 mM glutamine, 10% fetal bovine serum (FBS), and penicillin-streptomycin. DMEM and FBS were obtained from Mediatech and Thermo Scientific HyClone, respectively. Dialyzed FBS was obtained from Invitrogen. Glutamine and antibiotics were purchased from Omega Scientific Inc.

Reagents—*N,O*-bis[Trimethylsilyl]tri-fluoroacetamide was purchased from Thermo Scientific. All other reagents and enzymes were purchased from Sigma-Aldrich.

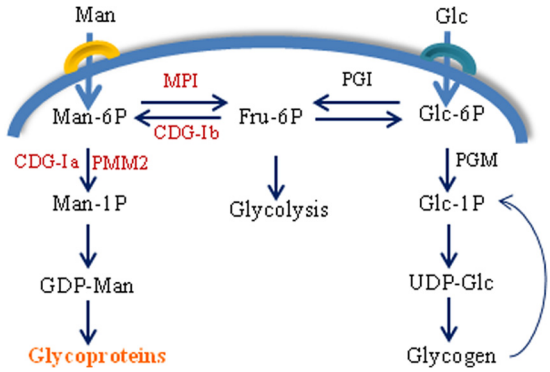
General Procedure for Stable Isotope Labeling and Sample Preparation—Cells were grown in a 60-mm dish to 75% confluency and labeled in DMEM glucose-free medium reconstituted with stable isotopes of glucose and mannose (5 mM and 50 μM, respectively) plus 10% dialyzed FBS for 24 h unless otherwise noted.

For glycan analysis, cells were washed with PBS and extracted with chloroform methanol (2:1), deionized water, and chloroform-methanol-water (10:10:3) (9). The cell pellet was dried and resuspended in PBS, 1% SDS, and *N*-glycans were released by *N*-glycosidase F digestion. Purified glycans were hydrolyzed by 2 M trifluoroacetic acid at 100 °C and dried.

For glycogen analysis, cells were extracted with 85% EtOH, and the resulting pellets were dissolved in 0.1 M NaOH, 0.1% sodium lauryl sulfate and digested with amyloglucosidase (10) Hydrolysates from *N*-glycan and digests from glycogen were derivatized as described in (11), dried, resuspended in chloroform, and analyzed by GC-MS.

For metabolite analysis from glycolysis, an aliquot of 50 μl of medium was extracted with 250 μl of 50% methanol and 150 μl of chloroform. The aqueous layer was separated and dried. The resulting solid was derivatized with 50 μl of ethoxylamine hydrochloride in pyridine (20 mg/ml) at 80 °C for 20 min followed by the addition of 50 μl of *N-tert*-butyl-dimethylsilyl-*N*-methyl-trifluoroacetamide and heated at 80 °C for another 1 h. Derivatized samples were centrifuged, and supernatant was directly injected for GC/MS analysis (12).

Gas Chromatography/Mass Spectrometry—Sugar analysis was done using a 15 m × 0.25 mm × 0.25-μm SHRXI-5ms column (Shimadzu, Kyoto, Japan) on a QP2010 Plus gas chromatograph mass spectrometer (GC-MS) (Shimadzu, Kyoto, Japan) with *m/z* range 140–500 and ~1.5-kV detector sensitivity. GC-MS ion fragment intensities were obtained using GC/MS Solution version 2.50 SU3 from Shimadzu Corp. For each fragment, the intensities were corrected for the natural abundance of each element using matrix-based probabilistic methods as described (13–15). The $^{13}C/(^{12}C + {}^{13}C)$ or $^{2}H/(^{1}H + {}^{2}H)$ ratios were used to calculate isotopic labeling proportion.



SCHEME. 1 **Mannose and glucose metabolic pathway.**

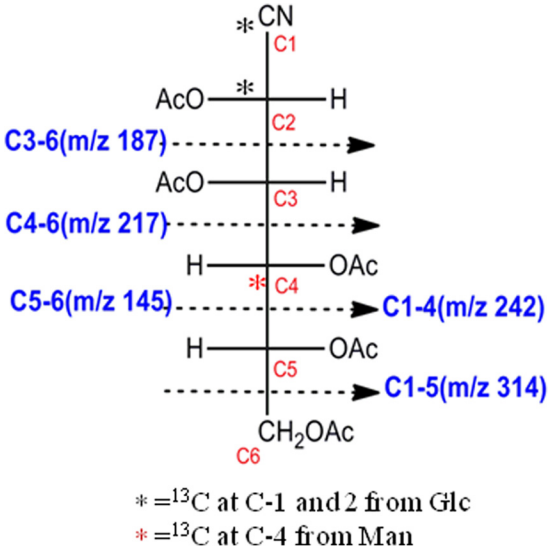


FIGURE 1. **Example of mass distribution calculation.** A fragment of C1-5 gives a molecular mass of 314, which is an isotopomer without stable isotope and defined as *m*0. If mannose is originated from glucose, two additional mass units will be observed (*m*2) because glucose contains two ^{13}C at C-1 and 2. The percentage of contribution from glucose was calculated as $m2/(m0 + m2)$. In a similar manner, the percentage of contribution from mannose was calculated as $m1/(m0 + m1)$. Natural abundance heavy isotope was corrected using matrix based probabilistic methods.

Choice of Isotopes to Determine the Origin of Man in N-Glycans—The following fragmentation series were used to identify the origin of mannose in *N*-glycan: *m/z* 145 and 187 for [6,6-^{2}H]glucose, *m/z* 242 and 314 for [1,2-^{13}C]glucose/mannose, [2-^{13}C]mannose, *m/z* 187 and 217 for [6-^{13}C]glucose, and *m/z* 217 and 314 for [4-^{13}C]mannose. (Fig. 1). For each experiment, cells labeled without stable isotopes were included to calculate a background value to subtract from samples with stable isotopes. Error bars show the range between fragments to calculate the abundance of each sugar.

Total N-Glycan and Glycogen—The amount of mannose derived from *N*-glycans was analyzed by GC-MS and calculated as mannosyl units/mg of protein based on the mannose standard curve. Glucose derived from glycogen was also analyzed by GC-MS and calculated as the difference from untreated samples. A glucose standard curve was used to calculate glycogen, and its total amount was expressed as glycosyl unit per mg protein (16).

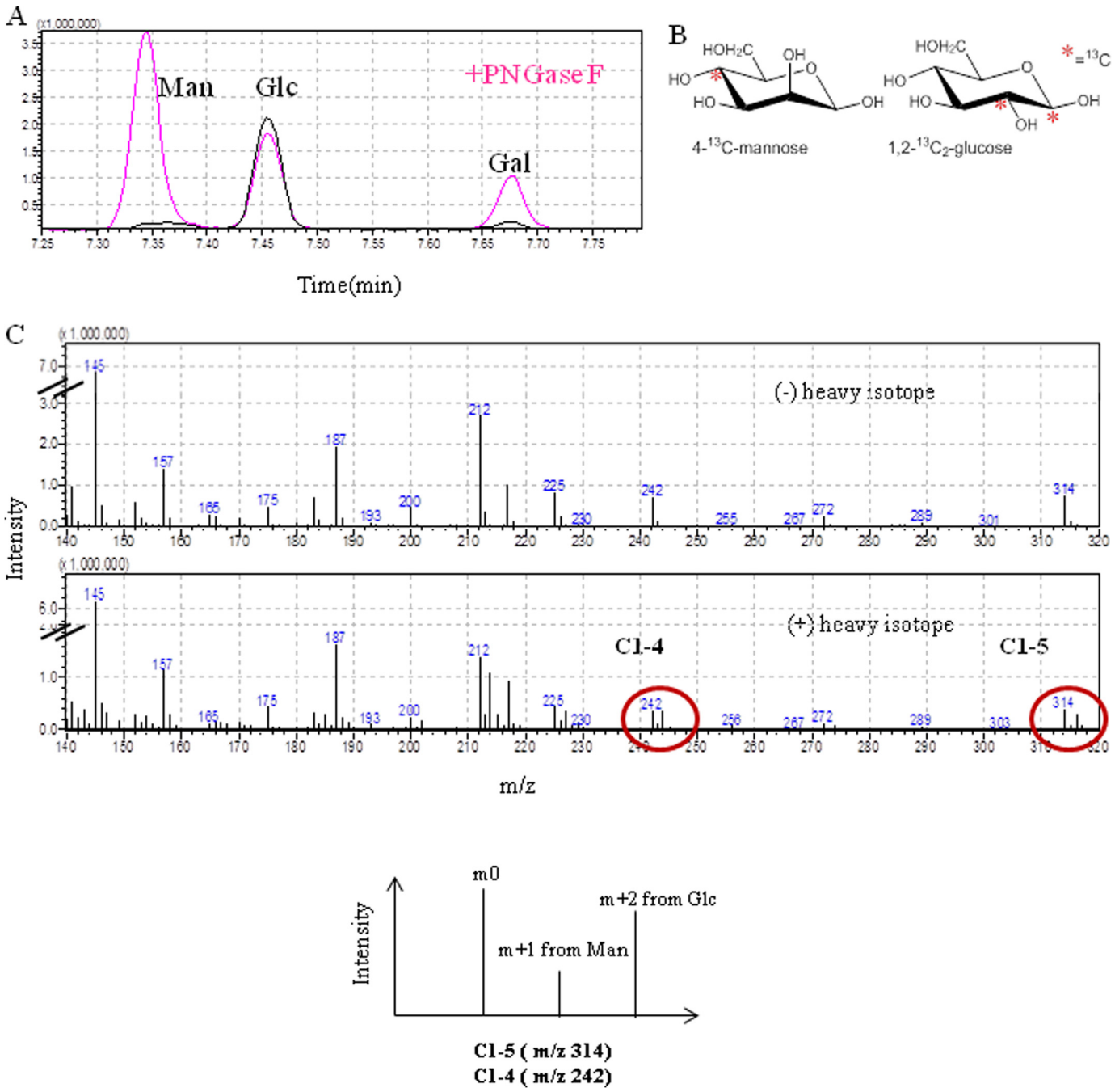


FIGURE 2. **GC and MS chromatogram of mannose.** *A*, GC chromatogram of before (*black line*) and after *N*-glycosidase F (*PNGase F*) digestion (*pink line*). (Glucose is a routine contaminant.) *B*, location of heavy isotopic labels in glucose and mannose. *C*, MS fragments of mannose. *Upper panel*, MS fragment without heavy isotope; *lower panel*, MS fragment with heavy isotope.

Gluconeogenesis to N-Glycosylation—Control fibroblasts were labeled with 5 mM [6,6-^{2}H]glucose and 1 mM [3-^{13}C]pyruvate or [2-^{13}C]glycerol for 24 h. Contribution from [6,6-^{2}H]-glucose was calculated as an average of $m147/(m145 + m147)$ and $m219/(m217 + m219)$.

The contribution from [3-^{13}C]pyruvate was calculated as an average of $m218/(m217 + m218)$ and $m315/(m314 + m315)$, and the contribution from [2-^{13}C]glycerol was calculated as an average of $m218/(m217 + m218)$ and $m316/(m314 + m316)$ because they would be in [1,6-^{13}C]glucose from [3-^{13}C]pyruvate, and [2,5-^{13}C]glucose from [2-^{13}C]glycerol, respectively, assuming that final product mannose comes from one labeled and one unlabeled precursor.

Preparation for Stable Isotopes with D_2O Labeling or Chase with D_2O—DMEM glucose-free medium with dialyzed serum was lyophilized and reconstituted in deuterium water prior to labeling. Stable isotopes and other additives were also dissolved in deuterium water prior to use.

Metabolic Detour with D_2O as Tracer—Contributions from Man via Fru-6-P to Man of *N*-glycans were assessed as follows. Control fibroblasts, $Mpi^{-/-}$ mouse embryonic fibroblast (MEF), and wild type MEF were labeled with 5 mM glucose and 100

or 200 μM [1,2-^{13}C]Man in D_2O/DMEM medium described above for 24 h. Species of [1,2-^{13}C-2D]Man in *N*-glycan were calculated with an average of *m*245/(*m*242 + *m*245) and *m*317/(*m*314 + *m*317).

Sugar Phosphate Analysis—Contribution from Man and Glc to sugar phosphates (Man-6-P, Glc-6-P, and Fru-6-P) were assessed as follows. Wild type MEFs grown in 15-cm dishes were labeled with 5 mM [UL-^{13}C]Glc and 50 μM Man or 5 mM Glc and 50 or 500 μM [UL-^{13}C]Man for 6 h. Cells were harvested, extracted twice with 0.1 M AcOH with sonication, and centrifuged for 5 min at 15,000 × *g*. The supernatants were combined, filtered through a membrane filter (0.22 μm), and lyophilized. The resulting solid was derivatized with 50 μl of hydroxylamine hydrochloride in pyridine (20 mg/ml) at 80 °C for 20 min followed by the addition of 50 μl of *N,O*-bis [trimethylsilyl]trifluoroacetamide and heated at 80 °C for another 1 h. Derivatized samples were centrifuged, and the supernatant was directly injected for GC/MS analysis. First, we assigned characteristic MS fragments for sugar phosphate derivatives after phosphorylation of [1,2-^{13}C], [UL-^{13}C]Man; [1,2-^{13}C], [UL-^{13}C]Glc; and [UL-^{13}C]Fru by hexokinase and compared their fragment masses with standards for Man-6-P/Glc-6-P/Fru-6-P. Fragmentation series of *m/z* 459 and *m/z* 471 were used to identify which labeled species were precursors of Fru-6-P and Man6-P/Glc6-P, respectively. The contribution of [UL-^{13}C]Man/Glc to Man-6-P and Glc-6-P was calculated with the *m*475/(*m*471 + *m*475) ratio. The contribution of [UL-^{13}C]Man/Glc to Fru-6-P was calculated with the *m*462/(*m*459 + *m*462) ratio (see Fig. 6*B*).

Other Sources of Man in N-Glycans—To assess whether either glycogen or degradation of *N*-glycans serves as a source of Man, cells were first labeled with [1,2-^{13}C]Glc/[^{12}C]Man or [1,2-^{13}C]Man/[^{12}C]Glc for 48–72 h to enrich labeled glycogen or Man in *N*-glycans, respectively. Subsequently, cells were washed with medium without stable isotopes and chased in D_2O/DMEM medium for 12–24 h in the presence or absence of [^{12}C]Glc/[^{12}C]Man. For the analysis, the same mass fragments were used to calculate the origin of glycogen as [1,2-^{13}C-2D]Glc or degradation of *N*-glycans as [1,2-^{13}C-2D]Man with average of *m*245/(*m*242 + *m*245) and *m*317/(*m*314 + *m*317).

PMM2 and MPI Enzyme Assays—A standard coupled assay to estimate MPI and PMM2 activities was previously described (1).

Rate of Sugar Uptake from the Medium—Rates of sugar uptake were calculated based on the remaining amount of each sugar in the culture medium at time 0–24 h. Cells were grown in 5 mM glucose and 50 μM mannose, and an aliquot of 50 μl of medium was extracted with 250 μl of 50% methanol and 150 μl of chloroform. The aqueous layer was separated and dried. The resulting solid was derivatized in the same manner as that described under "Sugar Phosphate Analysis" (17). For quantitation of glucose and mannose, the total ion count peak area of the respective *Z*- and *E*-forms of the sugars, or specific mass ions, was used for calculation based on standard curves.

RESULTS

Choice of Mannose and Glucose Isotopes—Exogenous mannose and glucose can both contribute to mannose in *N*-glycans, as shown in Scheme 1. We supplied cells with mannose and glucose containing stable isotopes (^{2}H or ^{13}C) at different positions in the sugar backbone. Monosaccharides incorporated into cellular *N*-glycans were released by *N*-glycosidase F digestion and hydrolyzed. The released monosaccharides were converted to aldonitrile acetate derivatives for analysis by GC-MS. Mannose and glucose were baseline-separable by GC (Fig. 2*A*), and each produced similar mass fragments. By varying the locations of the heavy isotope on each precursor sugar (Fig. 2*B*), we could distinguish their origin in *N*-glycans as being from Glc or Man. (Fig. 2*C*) (11). We tested combinations of [1,2-^{13}C], [6-^{13}C]-, and [6,6-^{2}H]glucose and [1,2-^{13}C]-, [2-^{13}C]-, and [4-^{13}C]mannose as precursors. Most of the variously labeled glucose or mannose species (Man^G or Man^M, respectively) were incorporated into mannose in *N*-glycans at similar rates, suggesting little isotope effect. The single exception was [6,6-^{2}H]-glucose, which showed a 26% reduction in incorporation into *N*-glycans, presumably due to the presence of two bulky deuterium labels at the 6-position, which reduced either the rate of glucose transport or the subsequent metabolism (data not shown). We chose [1,2-^{13}C]glucose and [4-^{13}C]mannose for further studies because they gave the clearest signals using the

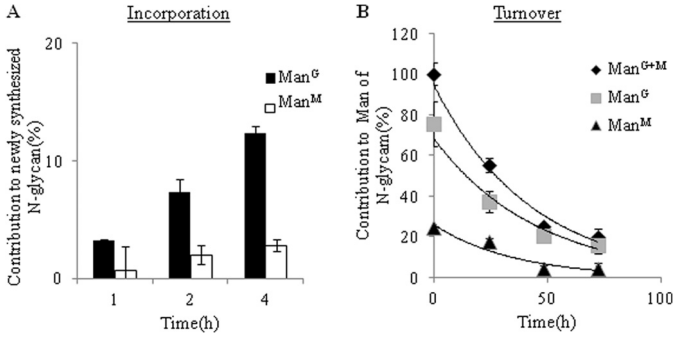


FIGURE 3. **Labeling and turnover of labeled *N*-glycans.** *A*, incorporation of mannose into *N*-glycans is shown as Man^G, indicating contribution from glucose to mannose, and as Man^M, indicating contribution of mannose to mannose. Control fibroblasts were labeled with 5 mM [1,2-^{13}C]Glc and 50 μM [4-^{13}C]Man for 1, 2 or 4 h and followed by glycan analysis. *B*, turnover rates of mannose in *N*-glycans. Control fibroblasts were labeled with 5 mM [1,2-^{13}C]Glc and 50 μM [4-^{13}C]Man for 72 h and chased in unlabeled medium. Total ^{13}C-labeled from glucose and mannose (Man^{G+M}) was defined as 100% at the beginning of the chase. Contribution from Glc (Man^G) and Man (Man^M) was calculated as Man^G/Man^{G+M} and Man^M/Man^{G+M}, respectively.

TABLE 1
Mannose and glucose uptake and incorporation into *N*-glycans and glycogen

Cell type	Sugar uptake[a] (nmole/mg/h)		N-Glycan (nmole/mg/h)		Glycogen (nmole/mg/h)	
	Man	Glc	Man^M	Man^G	Man	Glc
Fibroblast	12.7	1500	0.22	0.40	0	16.7
Hep3B	9.4	1237	0.06	0.27		
A549	15.6	1633	0.12	0.13		
293T	10.9	1262	0.08	0.38		
U87	21.8	2182				

[a] Rate of sugar uptake was calculated as described under "Experimental Procedures." Man^M and Man^G were calculated by proportions of ^{13}C-labeled mannose derived from [1,2-^{13}C]Glc and [4-^{13}C] Man and total amount of mannose (Man^G + Man^M) in *N*-glycans. Glucose and mannose contributions to glycogen were also calculated the same way.

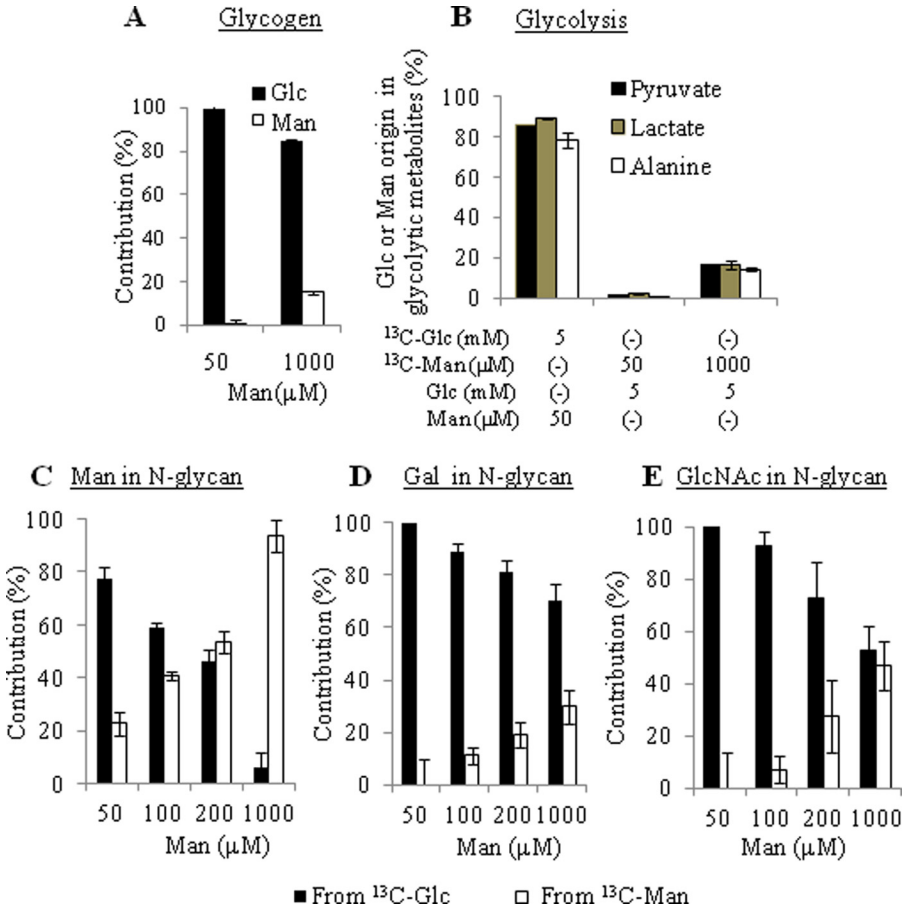


FIGURE 4. **Fate of mannose, including incorporation into glycogen, glycolysis, and other monosaccharides.** *A*, glycogen was analyzed by labeling control human fibroblasts with 5 mM [1,2-^{13}C]Glc and 50 μM or 1 mM [4-^{13}C]Man for 24 h, respectively. *B*, glycolytic metabolite analysis was carried out by labeling control human fibroblasts with 5 mM [1,2-^{13}C]Glc and 50 μM Man or 5 mM Glc and 1 mM [1,2-^{13}C]Man for 24 h, and culture medium was processed as described under "Experimental Procedures." C–E, origin of mannose (*C*), galactose (*D*), or GlcNAc (*E*) in *N*-glycans was analyzed with increasing concentrations of exogenous [4-^{13}C]Man with 5 mM [1,2-^{13}C]Glc. Contributions from ^{13}C-Glc (G) and ^{13}C-Man (M) origin were calculated as G/(G+M), M/(G+M), respectively, except for glycolysis (*B*), where the contribution of ^{13}C-Glc or ^{13}C-Man was adjusted to twice the percentage of incorporation into these metabolites because one glucose/mannose unit produces two pyruvate/lactate/alanine molecules.

mass isotopomer patterns of *m/z* 314 and *m/z* 242 aldonitrile-sugar fragments (+2 or +1 mass unit labeling, respectively). Exchanging the location of the labels between the two precursors had no effect on their relative proportions in *N*-glycans.

Samples containing as little as 5% incorporated heavy isotope gave reliable values above natural abundance background for each isotope after 2 h of labeling (Fig. 3*A*). Fibroblasts were routinely labeled for 16–24 h without deleterious effects to the cells. Chasing heavy isotope-labeled cells with unlabeled medium showed that *N*-glycan mannose (content = 24 nmol/mg of protein) has a $t_{1/2}$ of 24 h (Fig. 3*B*).

Fate and Distribution of Mannose—Under physiological concentrations (5 mM glucose, 50 μM mannose), several studies have shown that only a small amount of exogenous [2-^{3}H]mannose is incorporated into glycans (~2%), whereas most is released as ^{3}HOH (98%) (1). Using stable isotopes and unlabeled sugars, we found that fibroblasts take up exogenous mannose at a rate of 9.4–22 nmol/mg/h protein and glucose at 1500–2200 nmol/mg/h protein (Table 1). Among five different cell lines tested, glucose and mannose contribution to *N*-glycans ranged from 0.1–0.4 and 0.1–0.2 nmol/mg/h, respectively, showing that, relative to their uptake rates, mannose is incorporated into *N*-glycans (1–2%) much more efficiently than glucose (0.01–0.03%). Under these labeling conditions, conversion of [4-^{13}C]mannose to other monosaccharides found in *N*-glycans or to glycogen is undetectable (Table 1). To assess the contribution of mannose (to glucose) in glycogen and glycolysis, control fibroblasts were labeled with either 5 mM [1,2-^{13}C]Glc/50 μM Man (shown as ^{13}C-Glc) or 5 mM Glc/1 mM [1,2-^{13}C]Man (shown as ^{13}C-Man) for 24 h, respectively. Under the first condition, ^{13}C-Man is undetectable in glycogen or in glycolytic products. At a 20-fold higher concentration, mannose is clearly metabolized identically to glucose because it shows a similar distribution in glycogen and in pyruvate, lactate, and alanine in the medium (Fig. 4, *A* and *B*). For assessing the mannose contribution to other sugars in *N*-glycans, control fibroblasts were labeled with 5 mM [1,2-^{13}C]Glc and various concentrations of [4-^{13}C]Man, ranging from 50 to 1,000 μM for 24 h. Sugars (mannose, galactose, and *N*-acetylglucosamine) in *N*-glycans were analyzed by GC/MS as their corresponding aldonitrile derivatives. At 50 μM, mannose contribution to Man of *N*-glycans (Man^{M}) was about 25–30%; however, increasing mannose to 1 mM shows that it can completely replace glucose-derived Man (Man^{G}) in *N*-glycans (Fig. 4*C*). Mannose can also

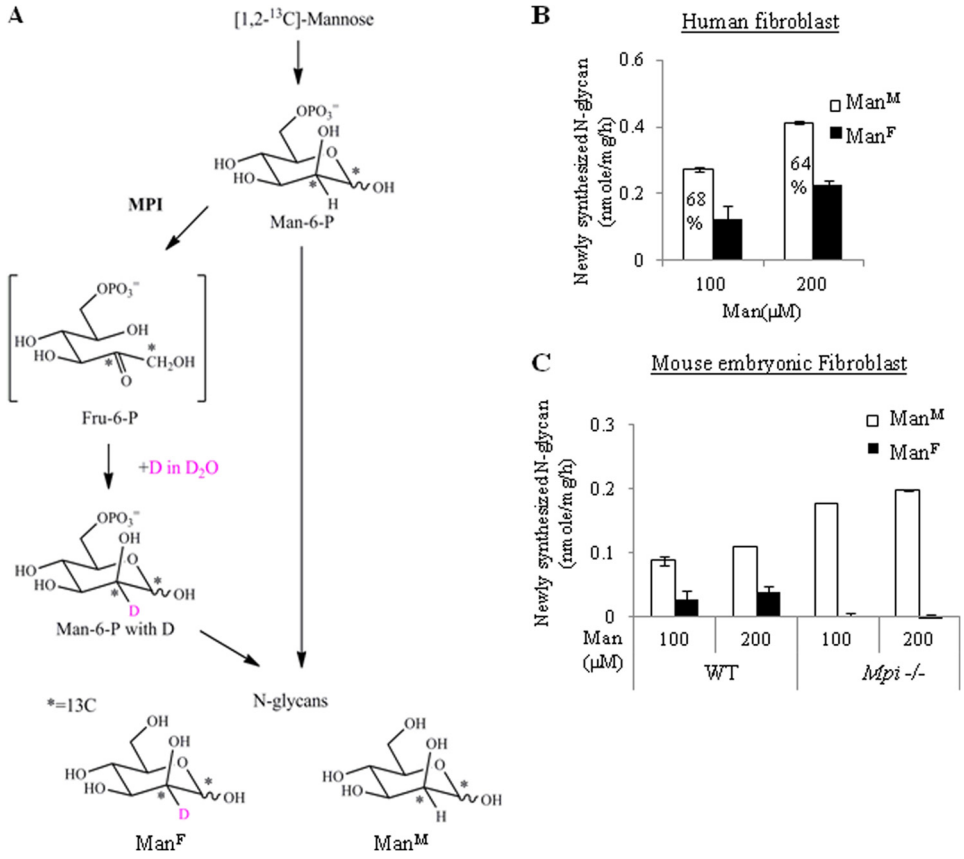


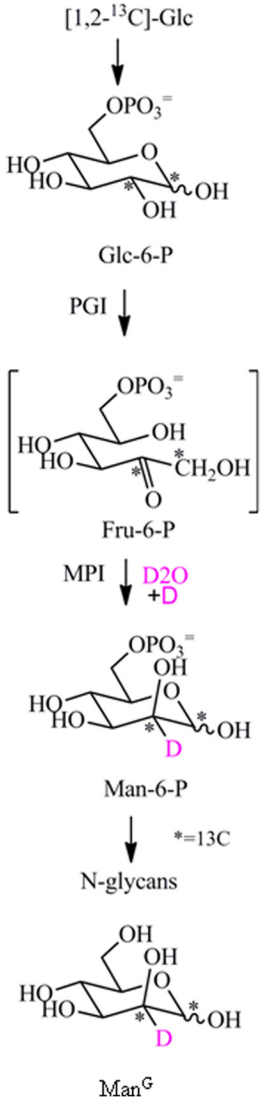
FIGURE 5. **Metabolic detour by MPI.** *A*, mechanism for incorporation of deuterium into [1,2-^{13}C]Man-6-P through Fru-6-P (Man^{F}) catalyzed by MPI. *B*, control human fibroblasts were labeled with 100 or 200 μM [1,2-^{13}C]Man and 5 mM Glc in the presence of D_2O for 12 h prior to glycan analysis to quantify the contribution of Man^{M} and Man^{F} into *N*-glycans. *C*, the same experiments were done with *Mpi* WT MEF and *Mpi* KO MEF.

contribute to galactose and *N*-acetylglucosamine in *N*-glycans. It is undetectable at 50 μM; however, at 1 mM, 30% of galactose and 50% of *N*-acetylglucosamine were derived from Man (Fig. 4, *D* and *E*). Mannose presumably competes for glucose transport at 1 mM because it reduces the uptake of 2-deoxy-[2-^{3}H]-glucose by 40% (data not shown).

Mannose and Glucose Take "Metabolic Detours" within the Cell—Scores of previous studies using [2-^{3}H]mannose indicated two mutually exclusive fates: conversion to Fru-6-P (and loss of ^{3}HOH) and catabolism via glycolysis or, alternatively, conversion to [2-^{3}H]Man-1-P and incorporation into glycans. Stable isotope labeling suggests that the route is more complex. The MPI epimerase reaction requires loss of a hydrogen atom at C-2 of the substrate followed by its replacement with a hydrogen atom from water, or deuterium in the presence of D_2O. To test the assumption that conversion to Fru-6-P is irreversible, we labeled fibroblast cells with [1,2-^{13}C]mannose in the presence of D_2O. Unexpectedly, this introduced deuterium was incorporated into about one-third of the mannose-derived molecules found in *N*-glycans (Fig. 5*B*). This shows that a substantial amount of [1,2-^{13}C]Man-6-P is not directly incorporated into the glycosylation pathway, but is first converted to [1,2-^{13}C]Fru-6-P and then reconverted to [1,2-^{13}C-2-D]Man-6-P (Man^{F}) before incorporation into *N*-glycans. (Fig. 5*A*). This reaction is totally dependent on MPI because the deuterium-labeled product (Man^{F}) is not produced in *Mpi*-null mouse embryonic fibroblasts (Fig. 5*C*). This suggests that previous determinations of the proportion of [2-^{3}H]mannose incorporated into glycans are probably substantially underestimated because loss of ^{3}HOH from Man-6-P was assumed to indicate irreversible commitment to further catabolism.

To test whether similar metabolic detours occur in the utilization of glucose, we incubated cells with [1,2-^{13}C]glucose in the presence of D_2O. We first confirmed that all glucose incorporated into glycans as mannose (Man^{G}) trafficked through an MPI-dependent pathway as [1,2-^{13}C-2-D]Man-6-P (Scheme 2), as evidenced by the extra mass labeling of mannose in glycans (data not shown). Next, we assayed incorporation into glycogen. Although glucose is primarily used as an energy source, a substantial amount is incorporated into glycogen (content = 404 $\pm$ 37 nmol/mg of protein) through a pathway that requires intermediates Glc-6-P, Glc-1-P, and UDP-Glc (Scheme 1). Labeling cells with [1,2-^{13}C]glucose in the presence of D_2O showed that 80% of glucose incorporated into glycogen is first converted into [1,2-^{13}C]Fru-6-P via phosphoglucose isomerase (Glc-6-P $\leftrightarrow$ Fru-6-P) and then back to [1,2-^{13}C-2-D]Glc-6-P prior to incorporation into glycogen (data not shown). Together these results suggest that Fru-6-P may serve as a checkpoint for exogenous mannose directed toward glycosylation and glucose directed toward glycogen because this intermediate is not on the "direct" biosynthetic pathway for either product.

Multiple Metabolic Pools of Fru-6-P—To investigate the hypothesis of Fru-6-P as metabolic checkpoint for glycosyla-

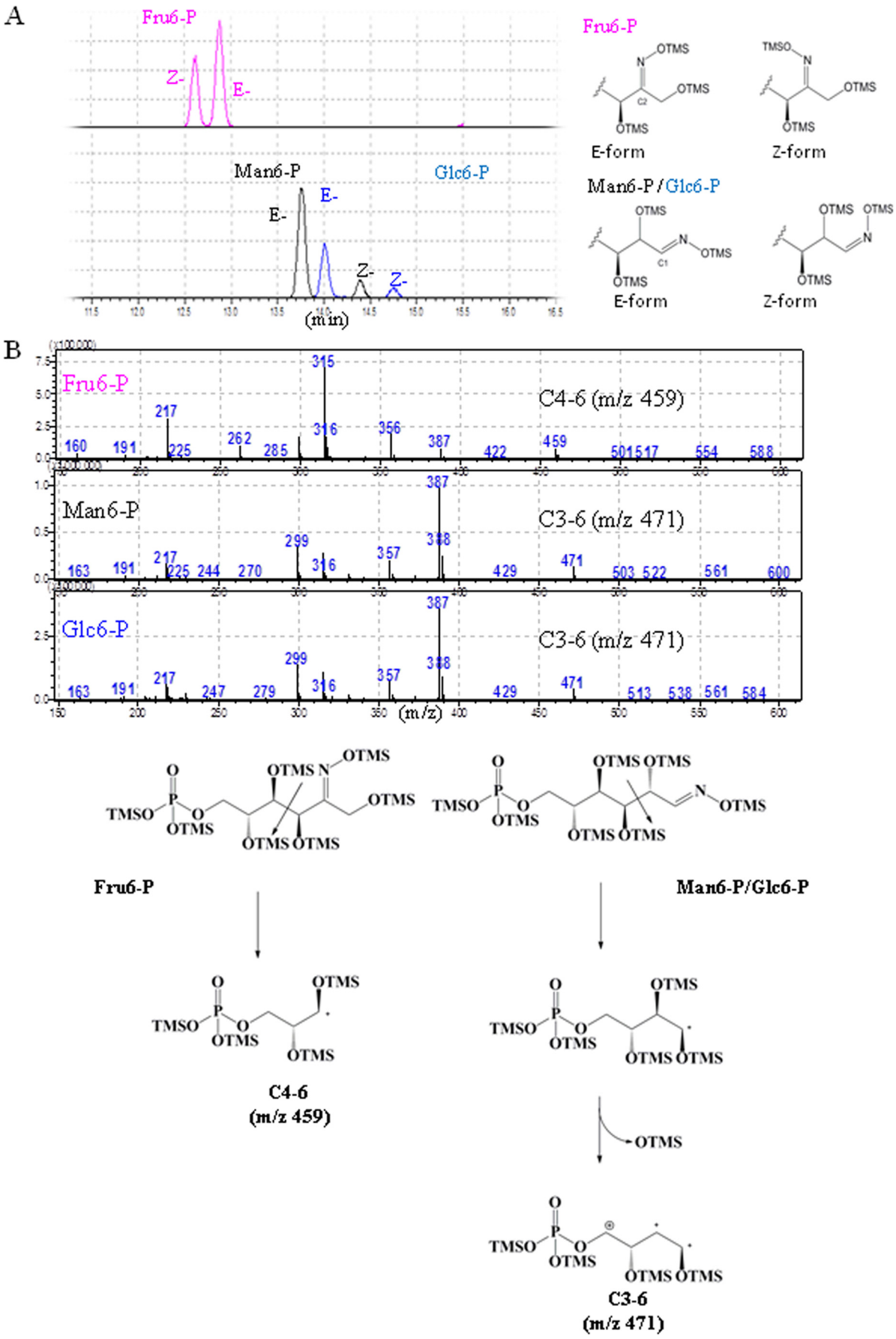


SCHEME. 2 **Deuterium incorporation in mannose of *N*-glycans from glucose via MPI-dependent pathway.**

tion, cells were labeled with either [UL-^{13}C]glucose or [UL-^{13}C]mannose for 6 h, harvested, extracted, and dried. Dried samples were derivatized as oxime-TMS and subjected to GC-MS analysis. Man-6-P, Glc-6-P, and Fru-6-P are well separated on GC as their corresponding derivatives (Fig. 6*A*). The origin of sugar phosphate was determined by characteristic fragments of *m/z* 459 (for Fru-6-P) or *m/z* 471 (for Man-6-P/Glc-6-P) and their enrichment from [UL-^{13}C]sugar (+3 or +4 mass unit labeling, respectively) (Fig. 6*B*). As expected, contribution from glucose and mannose to Man-6-P (Man^{G}-6-P, Man^{M}-6-P) was proportional to Man^{G} and Man^{M} found in *N*-glycans (Fig. 7). However, ^{13}C-mannose was not detected in Fru-6-P, although the D_2O experiment showed that a substantial amount of Man-6-P is converted to Fru-6-P prior to *N*-glycosylation (Fig. 5*B*). Increasing the concentration of mannose to 500 μM still did not show detectable contribution to the total cellular Fru-6-P pool (Fig. 7). We conclude that the Fru-6-P derived from exogenous mannose does not equilibrate with the total cellular pool of Fru-6-P, which is overwhelmingly derived from glucose.

Other Sources of Mannose for N-Glycans—Previous studies suggest that glycogen can serve as a reserve source of mannose for glycosylation (18, 19). To test this concept, we preincubated control fibroblasts with [1,2-^{13}C]glucose for 2 days to label >80% of glycogen. These cells were then incubated for 24 h in D_2O and various concentrations of unlabeled glucose. Mannose derived directly from [1,2-^{13}C]glucose (Man^{G}) in *N*-glycans will increase by +2 mass units, but mannose in glycans derived from glycogen (Man^{GL}) during the D_2O chase will have an additional mass unit (deuterium) at the C-2 position because all of it must transit through Fru-6-P via MPI (Scheme 2). However, no detectable (<5%) glycogen-derived label (Man^{GL}) was seen under standard culture conditions (5 mM glucose) or when glucose was reduced to either 1.0 mM or 0.5 mM (Fig. 8*A*). Under these conditions of limiting glucose, all cellular glycogen was depleted during the 24-h D_2O chase. As endoplasmic reticulum stress is known to increase Man-6-P from glycogen, we tested an endoplasmic reticulum stress inducer, DTT; however, it did not increase incorporation of glycogen-derived mannose into *N*-glycans (Fig. 8*A*). Cells cultured in the complete absence of glucose and mannose for 12 h had very little metabolic activity, and only under these severe conditions, a small amount of glycogen-derived mannose was seen in *N*-glycans (Fig. 8*A*). These results make it unlikely that glycogen routinely provides mannose for glycan synthesis, even when glucose is limiting. Similar experiments using two different hepatoma cell lines, HepG2 and Hep3B, led to similar conclusions (data not shown). We also considered the possibility that gluconeogenesis provides mannose for *N*-glycans, but found no evidence that any label (<5%) was derived from either labeled pyruvate or labeled glycerol under our standard labeling conditions (Fig. 8*B*). Increasing the concentration of labeled pyruvate or glycerol to 2 mM and extending labeling time to 48 h also did not show any contribution of gluconeogenesis-derived mannose to *N*-glycans either (data not shown).

Reutilization of Salvaged Mannose—Previous studies showed that mannose released from *N*-glycan processing is rapidly transported from the endoplasmic reticulum/Golgi back into the medium (9, 20). However, the fate of mannose derived from glycoprotein degradation is unknown. To address this question, we labeled fibroblasts for 72 h with 0.5 mM [1,2-^{13}C]mannose to replace ~60% of the mannose in glycoproteins with Man^{M}. Following a brief washout in unlabeled medium, cells were incubated for 12 h in D_2O with ^{12}C-monosaccharides. This is sufficient time to turnover ~25% of cellular *N*-glycans (Fig. 3). Based on the results presented above, reincorporation of mannose into newly synthesized glycoproteins would require Man-6-P, and ~50% of those molecules would be converted to Fru-6-P and acquire an additional deuterium at C-2. Analysis of *N*-glycans showed that no salvaged mannose (Man^{S}) with deuterium was found (<5%) under physiological condition (5 mM glucose, 50 μM mannose). In the complete absence of glucose and mannose during the incubation with D_2O, we did detect a small contribution of mannose from salvaged *N*-glycans (Man^{S}). However, under normal conditions, we conclude that the mannose salvaged from *N*-glycans (Man^{S}) does not make a significant contribution to newly synthesized *N*-glycans (Fig. 8*C*).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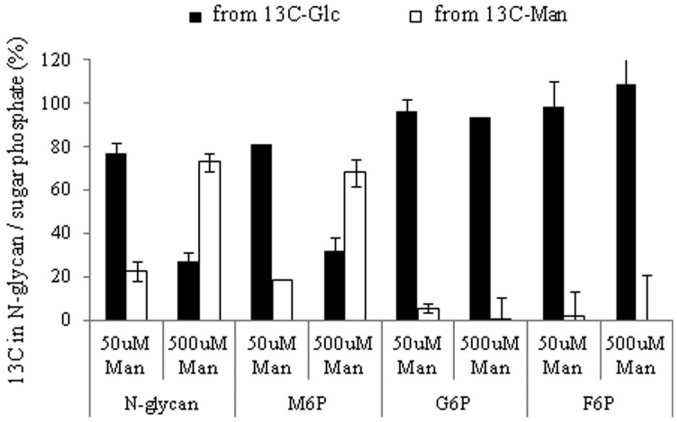

FIGURE 7. **Sugar phosphate analysis.** Contributions from Man and Glc to sugar phosphates (Man-6-P, Glc-6-P, and Fru-6-P) were assessed by labeling wild type MEF with 5 mM [UL-^{13}C]Glc and 50 μM Man or 5 mM Glc and 50 or 500 μM [UL-^{13}C]Man for 6 h prior to GC-MS analysis described under "Experimental Procedures." For comparison, *N*-glycans were also analyzed under the same labeling conditions except that incubation was done for 24 h.

Direct Mannose Contribution in Various Cell Lines—Cancer cell lines are known to possess extremely active sugar metabolism. To evaluate whether they have a unique mannose flux, a wide variety of tumor cell lines were labeled with [4-^{13}C]mannose and [1,2-^{13}C]glucose to determine the contribution from each monosaccharide (Fig. 9*A*). Mannose contribution varied from ~10% to ~45% at 50 μM mannose and reached up to 75% at 200 μM mannose. Direct mannose contribution generally correlates with the PMM2:MPI ratio, *i.e.* more mannose is delivered to glycosylation when the ratio is high (Fig. 9*B*). However, some exceptions suggest that other factors, such as a putative mannose-preferential transporter, may also influence the contribution of Man^{M}.

DISCUSSION

Small amounts of dietary mannose rescue glycosylation-deficient cells (2), mice (3), and humans (4, 5), suggesting its efficient use for glycosylation. Radiolabel tracer studies using [2-^{3}H]mannose indicate that the great majority (98%) is catabolized and only a small portion appears in *N*-glycans. The low glycan labeling efficiency seems at odds with the remarkable physiological benefits. Which perspective is accurate? [2-^{3}H]Mannose labeling has shortcomings because it does not indicate: 1) the quantity of mannose incorporated; 2) the relative contributions of glucose and mannose to Man-6-P; or 3) the fate of exogenous mannose.

To address these issues, we developed stable isotope-labeled methods using glucose and mannose at physiological concentrations, 5 mM and 50 μM, respectively. We detected the origin of mannose of *N*-glycans based on mass fragment analysis using GC-MS. This efficient, nontoxic, and inexpensive method provides both the actual and the relative amounts derived from each monosaccharide.

We showed that the amount of mannose directly incorporated into *N*-glycans is ~50% greater than previously estimated.

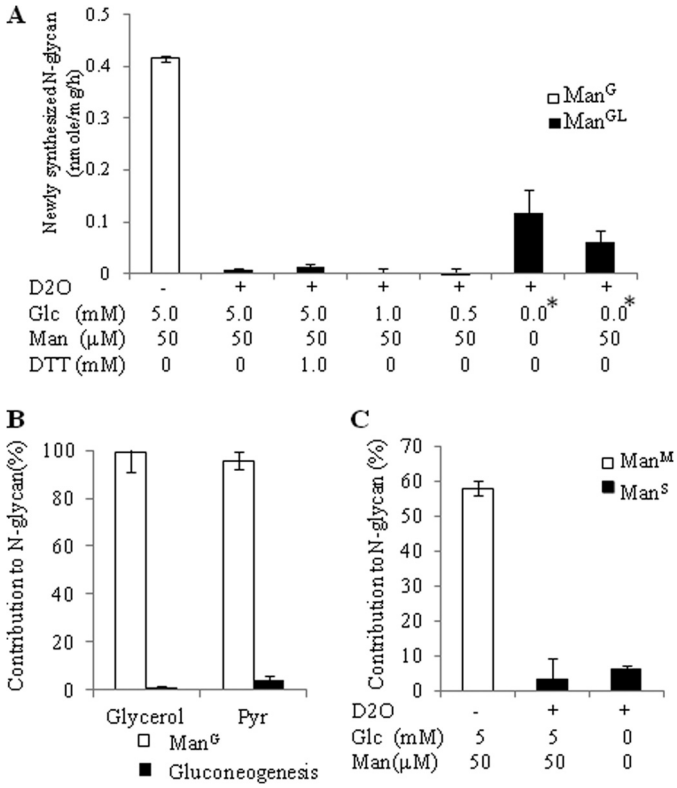

FIGURE 8. **Other sources of mannose for *N*-glycans.** *A*, contribution of glycogen to *N*-glycans. Control fibroblasts were labeled for 48 h with 5 mM [1,2-^{13}C]Glc and chased in D_2O for 24 h with 0.5–5.0 mM Glc except for 12 h with 0 mM as indicated by (*) in the presence or absence of 1 mM DTT, and glycogen-derived mannose of *N*-glycans (Man^{GL}) was analyzed. *White bar* shows glucose contribution to mannose in *N*-glycans (Man^{G}) without chase in D_2O for 24 h. *B*, contribution of gluconeogenesis to *N*-glycans was assessed by labeling control fibroblasts with 1 mM [2-^{13}C]glycerol or [3-^{13}C]pyruvate and 5 mM [6,6-^{2}H]glucose for 24 h. Contribution from [2-D]Glc (Man^{G}) and gluconeogenesis was calculated as the percentage of total incorporation of stable isotope into *N*-glycans. *C*, reutilization of mannose from glycan salvage was assessed by labeling control fibroblasts with 500 μM [1,2-^{13}C]Man and 5 mM [^{12}C]Glc for 72 h and chasing in D_2O for 12 h with or without 5 mM Glc and 50 μM Man. *White bar* shows percentage of labeled *N*-glycans from ^{13}C-Man (Man^{M}) before D_2O chase. *Black bar* represents salvaged mannose from *N*-glycans (Man^{S}) during D_2O chase.

Although conversion of [2-^{3}H]Man-6-P to Fru-6-P through a *cis*-enediol intermediate indeed releases ^{3}HOH from ^{3}H at C-2 in the medium, the reverse reaction regenerates Man-6-P, or 2-(D)-Man-6-P if the reaction is performed in D_2O.

Stable isotopes also allowed us to determine unambiguously whether mannose in *N*-glycans is derived from other sources. We found no evidence that gluconeogenic precursors (pyruvate or glycerol) contribute to *N*-glycan mannose under physiological conditions. Previous studies suggested that endoplasmic reticulum stress (21) or reduced exogenous glucose (22) could recruit glycogen for glycosylation, but we could not find any evidence for this. The one exception was when cells were completely deprived of all exogenous glucose and mannose for 12 h. We also showed that mannose salvaged from *N*-glycan degradation did not make a significant contribution to mannose in *N*-glycans under normal conditions.

FIGURE 6. **GC and MS chromatogram of sugar phosphates.** *A*, GC chromatogram corresponding to Fru-6-P, Man-6-P, and Glc-6-P as TMS derivatives (*E*- and *Z*-form). Peaks corresponding to *E*-forms were used for characterization. *B*, MS fragments of TMS-derivatized sugar phosphates. Characteristic fragments are highlighted as C4-6 (*m/z* 459) for Fru and C3-6 (*m/z* 471) for Glc and Man.

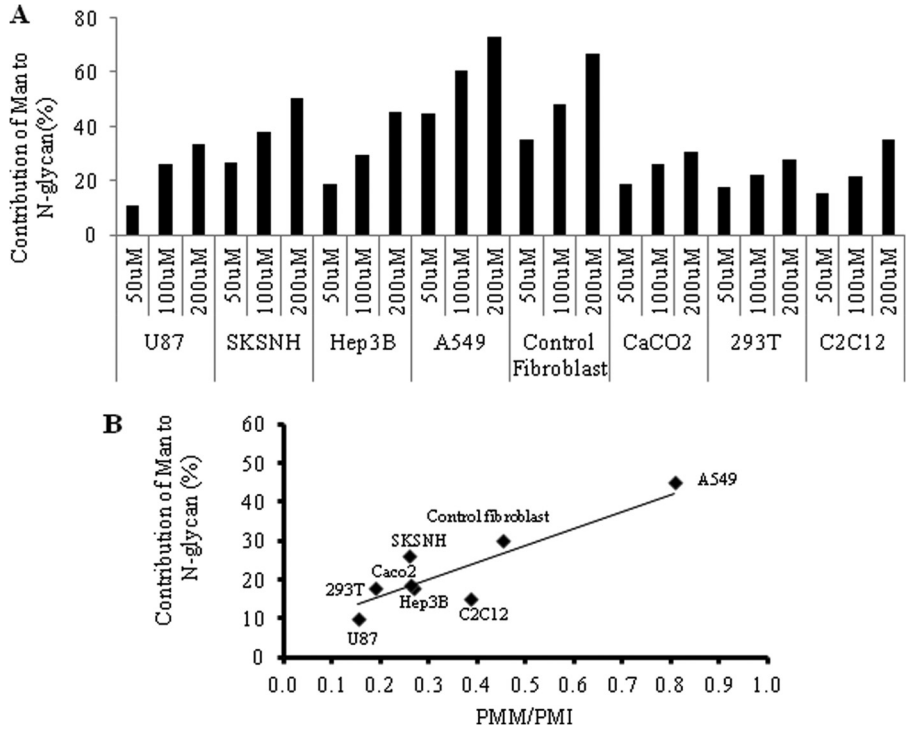

FIGURE 9. **Direct mannose contribution to glycans in various cell lines.** *A*, various cell lines were labeled with 5 mM [1,2-^{13}C]Glc and 50–200 μM [4-^{13}C]Man for 24 h, and the proportion of ^{13}C-Man contributing to total mannose of *N*-glycans was plotted. *B*, correlation between Man contribution to *N*-glycans and ratio of PMM2/MPI enzyme specific activity of cell lines tested in *A* was displayed.

Stable isotopes can be used to track mannose under non-physiological conditions as well. Increasing mannose concentration to 1 mM shows that it is metabolized identically to glucose, with similar contributions to glycogen, and to pyruvate, lactate, and alanine in the medium. In the complete absence of glucose, mannose salvaged from *N*-glycans or glycogen-derived glucose shows small contributions to *N*-glycans.

The direct contribution of mannose to *N*-glycans varies among different cell lines. The ratio of PMM2:MPI activities is an important determining factor; however, other undefined factors may also be important because some cell lines show large increases in direct mannose incorporation into glycans when exogenous mannose is increased (Fig. 9*A*, C2C12, U87, Hep3B), whereas others (293T, CaCo2) show only modest increases. Other factors such as levels and activities of the GDP-Man-pyrophosphorylase, dolichol-phosphate-mannose (Dol-P-Man) synthase, and mannosyltransferases are also known contributors, but their roles need to be re defined in light of the new approach and direct mannose metabolism.

A much higher proportion of transported mannose is used for *N*-glycosylation (~1.8%) when compared with glucose (0.026%), and a lingering question is how a 100-fold lower concentration of exogenous mannose *versus* glucose can contribute nearly 50% of the mannose to *N*-glycans. One explanation is that mannose employs a high affinity mannose-preferential or -specific hexose transporter that directs it to glycosylation. Studies using [2-^{3}H]Man labeling suggested the existence of such a GLUT-like mannose transporter (23), but no specific transporter was identified. A kidney-localized sodium-glucose linked transporter (SGLT)-type mannose-specific transporter was described (24) that may reabsorb mannose.

Another possibility is that distinct metabolic pools of Fru-6-P exist for glycosylation, glycogenesis, and glycolysis. Our observation that Man^{M}-6-P and Glc-6-P are both transiently converted to Fru-6-P before appearing in glycoproteins and glycogen, respectively, is puzzling because Fru-6-P would seem to be an "unnecessary" intermediate.

It is unclear whether this detour process has physiological significance or results from an incidental mixing of Glc-6-P/Man-6-P metabolizing enzymes. To investigate the hypothesis that different pools of Fru-6-P exist for glycosylation, sugar phosphates were analyzed with stable isotopes to determine their origin. The ratio of Man^{M}-6-P and Man^{G}-6-P in the Man-6-P pool was proportional to Man^{M} and Man^{G} found in *N*-glycans. However, mannose contribution to Fru-6-P neither at physiological concentration (50 μM) nor at a 10-fold higher concentration (500 μM) was detected. This suggests the presence of a separate pool of Fru-6-P that does not equilibrate with the total cellular pool.

Having separate pools does not necessarily mean that they reside in different intracellular locations. For example, separate sugar phosphate pools could be generated by the anomeric selectivity of Glc-6-P/Man-6-P metabolizing enzymes. For Glc-6-P, PGM1 (Glc-6-P ↔ Glc-1-P) highly prefers the α-anomer leading to glycogen synthesis, but the competing enzyme, phosphoglucose isomerase, prefers the β-anomer to supply Fru-6-P for glycolysis. The ratio of α- and β-Glc-6-P and rate of interconversion can influence the metabolic fate (25). For Man-6-P, PMM2 is α-anomer-specific to form Man-1-P for glycosylation, whereas the competing enzyme, MPI, uses β-Man-6-P to form Fru-6-P for glycolysis. Moreover, α-Man-6-P is a weak inhibitor of MPI (26). Thus, the much higher efficiency of man-

nose use in glycosylation might simply result from α- and β-Man-6-P and the anomeric preferences of MPI and PMM2.

In summary, the significant findings of this study employing stable isotopes combined with D_2O were: 1) the direct contribution of mannose to glycoprotein synthesis has been underestimated by at least 1.5-fold; 2) metabolic origin of mannose into *N*-glycosylation and fate of mannose is unambiguously identified; and 3) PMM2:MPI ratio plays a dominant, but not exclusive role in determining the contribution of mannose to *N*-glycans.

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REFERENCES

1. Sharma, V., Ichikawa, M., He, P., Scott, D. A., Bravo, Y., Dahl, R., Ng, B. G., Cosford, N. D., and Freeze, H. H. (2011) Phosphomannose isomerase inhibitors improve *N*-glycosylation in selected phosphomannomutase-deficient fibroblasts. *J. Biol. Chem.* **286,** 39431–39438
2. Panneerselvam, K., and Freeze, H. H. (1996) Mannose corrects altered *N*-glycosylation in carbohydrate-deficient glycoprotein syndrome fibroblasts. *J. Clin. Invest.* **97,** 1478–1487
3. Schneider, A., Thiel, C., Rindermann, J., DeRossi, C., Popovici, D., Hoffmann, G. F., Gröne, H. J., and Körner, C. (2012) Successful prenatal mannose treatment for congenital disorder of glycosylation-Ia in mice. *Nat. Med.* **18,** 71–73
4. Niehues, R., Hasilik, M., Alton, G., Körner, C., Schiebe-Sukumar, M., Koch, H. G., Zimmer, K. P., Wu, R., Harms, E., Reiter, K., von Figura, K., Freeze, H. H., Harms, H. K., and Marquardt, T. (1998) Carbohydrate-deficient glycoprotein syndrome type Ib. Phosphomannose isomerase deficiency and mannose therapy. *J. Clin. Invest.* **101,** 1414–1420
5. Harms, H. K., Zimmer, K. P., Kurnik, K., Bertele-Harms, R. M., Weidinger, S., and Reiter, K. (2002) Oral mannose therapy persistently corrects the severe clinical symptoms and biochemical abnormalities of phosphomannose isomerase deficiency. *Acta Paediatr.* **91,** 1065–1072
6. Kjaergaard, S., Kristiansson, B., Stibler, H., Freeze, H. H., Schwartz, M., Martinsson, T., and Skovby, F. (1998) Failure of short-term mannose therapy of patients with carbohydrate-deficient glycoprotein syndrome type 1A. *Acta Paediatr.* **87,** 884–888
7. Mayatepek, E., and Kohlmüller, D. (1998) Mannose supplementation in carbohydrate-deficient glycoprotein syndrome type I and phosphomannomutase deficiency. *Eur. J. Pediatr.* **157,** 605–606
8. Nakajima, K., Ito, E., Ohtsubo, K., Shirato, K., Takamiya, R., Kitazume, S., Angata, T., and Taniguchi, N. (2013) Mass isotopomer analysis of metabolically labeled nucleotide sugars and *N*- and *O*-glycans for tracing nucleotide sugar metabolisms. *Mol. Cell. Proteomics* **12,** 2468–2480
9. Sharma, V., and Freeze, H. H. (2011) Mannose efflux from the cells: a potential source of mannose in blood. *J. Biol. Chem.* **286,** 10193–10200
10. Passonneau, J. V., and Lauderdale, V. R. (1974) A comparison of three methods of glycogen measurement in tissues. *Anal. Biochem.* **60,** 405–412
11. Price, N. P. (2004) Acylic sugar derivatives for GC/MS analysis of 13C-enrichment during carbohydrate metabolism. *Anal. Chem.* **76,** 6566–6574
12. Scott, D. A., Richardson, A. D., Filipp, F. V., Knutzen, C. A., Chiang, G. G., Ronai, Z. A., Osterman, A. L., and Smith, J. W. (2011) Comparative metabolic flux profiling of melanoma cell lines: beyond the Warburg effect. *J. Biol. Chem.* **286,** 42626–42634
13. Nanchen, A., Fuhrer, T., and Sauer, U. (2007) Determination of metabolic flux ratios from ^{13}C-experiments and gas chromatography-mass spectrometry data: protocol and principles. *Methods Mol. Biol.* **358,** 177–197
14. van Winden, W. A., Wittmann, C., Heinzle, E., and Heijnen, J. J. (2002) Correcting mass isotopomer distributions for naturally occurring isotopes. *Biotechnol. Bioeng.* **80,** 477–479
15. Portnoy, V. A., Scott, D. A., Lewis, N. E., Tarasova, Y., Osterman, A. L., and Palsson, B. Ø. (2010) Deletion of genes encoding cytochrome oxidases and quinol monooxygenase blocks the aerobic-anaerobic shift in *Escherichia coli* K-12 MG1655. *Appl. Environ. Microbiol.* **76,** 6529–6540
16. Sickmann, H. M., Schousboe, A., Fosgerau, K., and Waagepetersen, H. S. (2005) Compartmentation of lactate originating from glycogen and glucose in cultured astrocytes. *Neurochem. Res.* **30,** 1295–1304
17. Halket, J. M., and Zaikin, V. G. (2003) Derivatization in mass spectrometry-1. Silylation. *Eur. J. Mass Spectrom. (Chichester, Eng.)* **9,** 1–21
18. Lehrman, M. A. (2006) Stimulation of *N*-linked glycosylation and lipid-linked oligosaccharide synthesis by stress responses in metazoan cells. *Crit. Rev. Biochem. Mol. Biol.* **41,** 51–75
19. Shang, J., and Lehrman, M. A. (2004) Activation of glycogen phosphorylase with 5-aminoimidazole-4-carboxamide riboside (AICAR). Assessment of glycogen as a precursor of mannosyl residues in glycoconjugates. *J. Biol. Chem.* **279,** 12076–12080
20. Moore, S. E., Bauvy, C., and Codogno, P. (1995) Endoplasmic reticulum-to-cytosol transport of free polymannose oligosaccharides in permeabilized HepG2 cells. *EMBO J.* **14,** 6034–6042
21. Gill, A., Gao, N., and Lehrman, M. A. (2002) Rapid activation of glycogen phosphorylase by the endoplasmic reticulum unfolded protein response. *J. Biol. Chem.* **277,** 44747–44753
22. McMahon, R. J., and Frost, S. C. (1996) Glycogen: a carbohydrate source for GLUT-1 glycosylation during glucose deprivation of 3T3-L1 adipocytes. *Am. J. Physiol.* **270,** E640–645
23. Panneerselvam, K., Etchison, J. R., and Freeze, H. H. (1997) Human fibroblasts prefer mannose over glucose as a source of mannose for *N*-glycosylation: evidence for the functional importance of transported mannose. *J. Biol. Chem.* **272,** 23123–23129
24. Grempler, R., Augustin, R., Froehner, S., Hildebrandt, T., Simon, E., Mark, M., and Eickelmann, P. (2012) Functional characterisation of human SGLT-5 as a novel kidney-specific sodium-dependent sugar transporter. *FEBS Lett.* **586,** 248–253
25. Malaisse, W. J., Zhang, Y., and Sener, A. (2004) Enzyme-to-enzyme channeling in the early steps of glycolysis in rat pancreatic islets. *Endocrine* **24,** 105–109
26. Rose, I. A., O'Connell, E. L., and Schray, K. J. (1973) Mannose 6-phosphate: anomeric form used by phosphomannose isomerase and its 1-epimerization by phosphoglucose isomerase. *J. Biol. Chem.* **248,** 2232–2234