Metformin-stimulated Mannose Transport in Dermal Fibroblasts*

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The biguanide drug metformin stimulates AMP-activated protein kinase, a master regulator of cellular energy metabolism, and has antihyperglycemic activity due to attenuation of gluconeogenesis in hepatocytes and 2-fold stimulation of glucose transport by skeletal muscle. Here we identify a metformin-stimulated α-mannose transport (MSMT) activity in dermal fibroblasts. MSMT increased mannose uptake 1.8-fold and had greater affinity for mannose than basal mannose transport activity. It was attributed to robust stimulation of a transporter expressed weakly in untreated cells. MSMT was not explained by greater glucose transporter activity because metformin unexpectedly decreased transport of 2-deoxy-D-glucose and 3-O-methyl-D-glucose by fibroblasts. Effective inhibitors of MSMT retained specificity for the 3-, 4-, and 6-OH groups of the mannose ring but not the 2-OH group. Thus, MSMT could be strongly inhibited by glucose and 2-deoxy-D-glucose even though the latter was not a good transport substrate. MSMT was significant because in the presence of 2.5 μM mannose, metformin corrected experimentally induced deficiencies in the synthesis of glucose-mannosylGlcNAc2-P-P-dolichol and N-linked glycosylation. MSMT was also identified in congenital disorder of glycosylation types Ia and Ib fibroblasts, and metformin acted synergistically with 100 μM mannose to correct lipid-linked oligosaccharide synthesis and N-glycosylation in the Ia cells. In conclusion, metformin activates a novel fibroblast mannose-selective transport system. This suggests that AMP-activated protein kinase may be a regulator of mannose metabolism and implies a therapy for congenital disorders of glycosylation-Ia.

* The work was supported by National Institutes of Health Grant GM38545 and the Robert Welch Foundation Grant I-1168. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: LLO, lipid-linked oligosaccharide; AMPK, AMP-activated protein kinase; CDG-I, congenital disorder of glycosylation type I; 2DG, 2-deoxyglucose; 3mG, 3-O-methylglucose; MSMT, metformin-stimulated mannose transport; TN, tunicamycin; HPLC, high pressure liquid chromatography.

d-Mannose is a component of many eukaryotic glycoconjugates, such as asparagine (N)-linked glycans and their precursor lipid-linked oligosaccharides (LLO), glycosylphosphatidylinositol, glycosylphosphatidylinositol-anchored proteins, mannos-containing serine/threonine O-linked glycans, and C-mannosyltryptophan. By metabolic conversion, extracellularly derived l-glucose can be a major source of d-mannosyl residues. This is illustrated by the human genetic disease congenital disorder of glycosylation (CDG) type Ib, which is characterized by defective phosphomannose isomerase and deficient conversion of the glucose metabolite Fru-6-P to Man-6-P (2). Man-6-P is converted to Man-1-P by phosphomannomutase, which generates GDP-mannose. GDP-mannose is used to form mannose-P-dolichol, and both are required for synthesis of complete LLOs. Thus, CDG-Ib cells have diminished LLO production and incomplete N-linked glycosylation (2).

Extracellularly derived mannose itself is also a significant physiological precursor of mannosyl residues. It is well known that [3H]mannose can be incorporated into glycoproteins when added to eukaryotic cell cultures. In dermal fibroblasts, this process is selective over glucose (3). Furthermore, [3H]mannose is incorporated into glycoproteins when injected into mice (4), and a mannose-selective transporter has been identified in human dermal fibroblasts (5). Extracellular mannose can counteract abrogated LLO synthesis caused by culturing normal fibroblasts in glucose-deficient medium (6), and it can correct the LLO defects in cultured CDG-Ia (phosphomannomutase-deficient) and CDG-Ib cells (2, 7, 8). Paradoxically, disease in CDG-Ia patients is not corrected by dietary mannose therapy, and CDG-Ib transferrin remains underglycosylated (9). CDG-Ib is successfully treated with dietary mannose, but excess quantities of mannose can cause side effects such as bloating and kidney damage (10). Thus, in both cases mannose therapy might be more effective if its uptake by cells could be improved.

Given the importance of mannose transport activity in normal physiology, as well as the potential for treatment of glycosylation disorders by mannose supplementation, we sought to identify agents that would enhance mannose transport. Metformin is a biguanide drug used to treat type II diabetes and has antihyperglycemic properties (11, 12) due to its ability to stimulate glucose transport activity in skeletal muscle about 2-fold (13–15) and decrease hepatic gluconeogenesis (16). Metformin acts by stimulating AMP-activated protein kinase (AMPK), a master regulator of energy homeostasis in liver, skeletal muscle, adipocytes, and pancreatic islets (14, 17). AMPK is normally activated when cellular AMP concentrations rise relative to ATP, such as during vigorous exercise, and triggers a set of responses that help restore the AMP/ATP ratio to normal. Although an activator, metformin does not appear to interact with AMPK directly (18).

In this study we investigated the effects of metformin on mannose transport and protein glycosylation. Because these processes are not well characterized in skeletal muscle, experiments were performed instead with dermal fibroblasts from both normal and CDG type I patients. A metformin-stimulated mannose transport (MSMT) activity was identified. The stimulation of mannose transport, although only roughly 2-fold, is comparable with the 2-fold effect of metformin on glucose transport by skeletal muscle. This was not due to the doubling of activity of a single transporter but rather an increase in the activity of a novel transporter that has little contribution in untreated cells. Unexpectedly, metformin decreased glucose transport activity in fibroblasts, a distinction with MSMT. Fi-
nally, we show that the effects of metformin can contribute a major fraction of the hexose necessary to assemble lipid-linked oligosaccharides and that metformin has the potential to counteract the glycosylation defect in CDG-Ia cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Metformin (D5035), phloretin (P7912), and phlorizin (P3449) were from Sigma. Non-radioactive sugars were obtained from the sources listed in Table I. Cell culture media were from Invitrogen, and sera were from Atlanta Biologicals. [2-3H]Mannose (10–20 Ci/ mmol) was from Amersham Biosciences, and both 2-deoxy-[G-3H]D-glucose (10 Ci/mmole) and 3-O-methyl-[G-3H]D-glucose (60 Ci/ mmole) were from American Radiolabeled Chemicals.

**Culture of Normal and CDG Type I Dermal Fibroblasts**—Dermal fibroblasts were cultured as described earlier (6, 19) in RPMI 1640 medium with 10% fetal bovine serum. Cells were generally grown to 80–90% of confluence for experiments. As described earlier (19), the fibroblasts used were normal CRL-1904 (American Type Culture Collection), CDG-Ia (20), and CDG-Ib (21).

**Analysis of [3H]Mannose-labeled LLOs and N-Linked Glycans by HPLC**—Unless indicated otherwise, cultures were incubated for 20 min in RPMI 1640 medium with 0.5 mM glucose containing 10% dialyzed fetal bovine serum and 40 μCi/ml (2.5 μCi) [3H]mannose. [3H]Labeled LLOs were extracted with organic solvent, fractionated by HPLC, and detected as described previously (6, 19). The proteinaceous pellet remaining after organic extraction was digested with N-glycanase (Calbiochem catalogue number 382185) as directed by the manufacturer, and the released N-glycans were analyzed by HPLC as described (22). In some experiments the amounts of [3H]mannose were varied, or 0.1 mM unlabeled D-mannose was included during the 20-min labeling period. As described earlier (6), the peak heights of [3H]mannose-labeled glycans detected by HPLC were normalized to their mannose contents to determine their percentages in glycan pools.

**Analysis of [3H]Mannose Incorporated into Glycoconjugate Fractions**—Cells were incubated with [3H]mannose for 10 min, and the total [3H] incorporated into each of four fractions was determined as follows. Cells from 35-mm wells of 6-well plates were washed three times with ice-cold phosphate-buffered saline and scraped into methanol. After drying under nitrogen, the cellular residue was extracted with 10 ml of chloroform/methanol (2:1). The supernatant recovered by centrifugation was dried under nitrogen, resuspended with 4 ml of chloroform/methanol (2:1), mixed with 0.8 ml of water, and centrifuged to yield two layers. The lower organic layer was mixed with 2 ml of chloroform/methanol/water (3:48:47) and centrifuged. The resulting lower phase containing sugar-P-dolichols was saved, and the upper aqueous phase was combined with that from the first step for later use. The residue remaining after initial chloroform/methanol (2:1) extraction was further extracted with 10 ml of water, which (after recovery by centrifugation) was combined with the aqueous phases obtained earlier to yield free mannose plus water-soluble metabolites. The residue was then extracted with 10 ml of chloroform/methanol/water (10:10:3) to obtain LLOs. The three solvent extracts were evaporated to dryness. The proteinaceous residue that remained was dissolved in 1% SDS with boiling. The distribution of [3H]mannose in the four fractions was determined by liquid scintillation spectrometry.

**Hexose Uptake Measurements**—0.5 or 1.0 × 10⁵ cells were seeded per 35-mm well of a multwell plate and grown to 80–50 or 80–90% of confluence (yielding ~0.04 or 0.08 mg of cell-associated protein), respectively. No systematic differences in the outcomes of experiments were noted between the two conditions. After treatment in the absence or presence of metformin, fibroblasts were typically incubated for 10 min at 37 °C with 0.5 ml of RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum, and the indicated concentrations of [2-3H]mannose, [G-3H]2-deoxy-o-glucose, or 3-O-methyl-[G-3H]D-glucose were essentially as described (6). Prior to use, [3H]labeled sugars were subjected to two rounds of drying under nitrogen and dissolution in water to eliminate [3H]water formed by isotopic exchange during storage. 0.5 or 1 mM unlabeled glucose (as indicated), which had small effects on basal and metformin-stimulated mannose transport (see “Results”), was provided as a source of energy during the assay. After incubation, plates were placed on ice and washed three times with ice-cold phosphate-buffered saline. Cells were lysed with 1% SDS, and total cell-associated tritium was measured by liquid scintillation spectrometry. For [3H]mannose, essentially all of the tritium remaining in the medium or associating with cell pellets failed to evaporate under a stream of nitrogen, indicating that formation of [3H]water by phosphomannose isomerase was inconsequential.

![Diagram of hexose transport](https://example.com/diagram.png)
RESULTS AND DISCUSSION

Model for Metformin-stimulated Mannose Transport (MSMT)—

For clarity, the reader is referred to Fig. 1, which presents a model for three transport activities indicated by this study as follows: (i) an activity responsible for most 3-O-methyl-D-glucose (3mG) transport and a minor portion of the 2-deoxy-D-glucose (2dG) transport under basal conditions, which is relatively resistant to phlorizin and is diminished by about half with metformin treatment; (ii) an activity responsible for most of the 2dG transport and mannose transport under basal conditions that is not strongly affected by metformin but is relatively sensitive to phlorizin; and (iii) an activity that is stimulated by metformin, selective for mannose, and relatively resistant to phlorizin (MSMT). Although this model is consistent with the data presented below, other models may also be plausible.

Metformin Increases Uptake of D-Mannose—As shown in Fig. 2, metformin treatment increased uptake of mannose by dermal fibroblasts. Assays were based upon the total amount of cell-associated tritium derived from [3H]mannose. Results are reported as transport by untreated cells (basal), transport by cells after treatment with metformin (metformin-treated), or the difference (metformin-stimulated mannose transport, MSMT). For a 10-min incubation with 2.5 mM mannose, the magnitude of the increase (1.8-fold, average of 23 determinations, Fig. 2, panel a) was comparable with the 2-fold effect of metformin on glucose transport in skeletal muscle (13, 15). This enhancement was evident at multiple mannose concentrations (panel b), dependence of mannose uptake upon time in the transport assay. Panel c, inhibition of [3H]mannose transport with unlabeled mannose. Panel d, dependence of basal and metformin-stimulated mannose transport upon [3H]mannose concentration; the specific activity was 8.4 mCi/mmol.

Fig. 2. Identification of metformin-stimulated mannose transport activity. Transport assays for [3H]mannose were performed as described under “Experimental Procedures.” Assays were for 10 (panels a–c) or 20 min (panels d–f) at 37 °C with 0.6 μM (panel d), 2.5 μM (panels a, c, and e), or variable [3H]mannose (panels b and f) in the presence of 0.5 mM (panels a–d) or 1.0 mM glucose (panels e and f). Panel a, mannose transport was measured without (white bar) or with (black bar) 24-h treatment of cells with 2 mM metformin. The increase after metformin treatment was determined to be 1.8 ± 0.03-fold (23 total measurements over 9 experiments). For panels b–f, open circles indicate untreated cells (basal activity); closed circles indicate metformin-treated cells; and open triangles indicate the difference (MSMT). Error bars are ± S.E. of triplicate experiments; in some cases error bars are smaller than the graph points. Panel b, [3H]mannose uptake by untreated and metformin-treated cells. Panel c, dependence of mannose uptake upon time in the transport assay. Panel d, dependence of mannose uptake upon treatment time with metformin. Panel e, inhibition of [3H]mannose transport with unlabeled mannose. Panel f, dependence of basal and metformin-stimulated mannose transport upon [3H]mannose concentration; the specific activity was 8.4 mCi/mmol.
and MSMT is seen more clearly by directly measuring transport with different concentrations of [3H]mannose of a constant specific activity (panel f). MSMT became saturated with ~1 mM mannose, although the dependence of basal mannose transport upon concentration was linear with as much as 30 mM mannose (panel f and data not shown).

Thus, two fibroblast mannose transport activities were discernible: a high capacity/low affinity basal mannose transport activity in untreated fibroblasts, and an MSMT activity with lower capacity but higher affinity (EC50 value ~0.5 mM, Fig. 2, panel f). Detailed kinetic comparisons could not be performed, because calculation of MSMT was associated with unacceptable experimental error at mannose concentrations above 2 mM. At first glance, the stimulation of mannose transport shown in Fig. 2 may appear modest and not representative of a substantial biochemical effect. However, as shown below, this increase is best explained by a robust stimulation of a transporter that is expressed weakly in untreated cells, rather than the doubling of an existing transporter.

**Basal and Metformin-stimulated Mannose Transport Involve Distinct Transporters**—To assess whether basal transport and MSMT were due to distinct transporters, inhibition by phloretin and phlorizin was evaluated. The effects of phloretin on basal and MSMT were indistinguishable (Fig. 3, panel a), with greater than 80% inhibition by 100 μM phloretin. In contrast, MSMT was resistant to inhibition by 0.5 mM phlorizin, although basal mannose transport was inhibited by about half, consistent with two distinct transport activities. Both were strongly inhibited with 2.5 mM phlorizin (panel b).

To evaluate the possible roles of glucose transporters in MSMT, uptake of 0.6 and 100 μM 2dG and 3mG, both commonly used substrates for glucose transporters, were compared with mannose (Fig. 4). In surprising contrast to the enhancing effects of metformin on glucose transport by skeletal muscle (13, 15) and mannose transport by fibroblasts (Fig. 2; Fig. 4, panels a and d), transport measured with both glucose derivatives was decreased by metformin treatment (Fig. 4, panels b, c, e, and f). Thus, it is unlikely that MSMT is due to the activity of a conventional glucose transporter. Instead, the model presented in Fig. 1 is applicable. 2dG is equivalent to 2-deoxy-D-mannose, and as will be shown below, basal mannose transport tolerated epimerization at the 2-position. Thus, it is possible that the same transporter (transporter 2 in Fig. 1) was responsible for basal transport of both mannose and 2dG. However, because neither basal mannose transport nor MSMT tolerated substitutions at the 3-posi-

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**FIG. 3.** Inhibition of basal and MSMT activities by phloretin and phlorizin. **Panel a**, inhibition of basal activity (circles) and MSMT (triangles) by phloretin. Assays were done in the presence of 0.5% ethanol by adding various amounts of ethanol or a 100 mM phloretin/ethanol solution. **Panel b**, as for panel a but with a 500 mM phlorizin/ethanol solution. Error bars are ± S.E. of triplicate experiments; in some cases error bars are smaller than the graph points.

**FIG. 4.** Effects of phlorizin and metformin on transport of mannose, 2-deoxyglucose, and 3-O-methylglucose. Transport by untreated or metformin-treated cells is represented by white or black bars, respectively. Cells were incubated for 20 min with either 0.6 (panels a–c) or 100 μM (panels d–f) [3H]-labeled sugar in the presence of 0.5% ethanol or 0.5% ethanol plus 0.5 mM phlorizin as indicated. Panels a and d, mannose; panels b and e, 2dG; panels c and f, 3mG. Error bars are ± S.E. of triplicate experiments.
Inhibition of basal and metformin-stimulated mannose transport by saccharides

Transport assays with normal fibroblasts were carried out for 20 min as described under “Experimental Procedures” with the listed inhibitors, and the percent inhibition was determined ± S.E. ND (not detectable) indicates that inhibition of transport was less than 10%. n indicates the number of determinations. Values for para-nitrophenyl (pNP) and methylumbelliferyl (MLMB) derivatives were determined after subtraction of any effects of the vehicle Me2SO. These data were obtained separately from any of the experiments shown in figures.

### Table I

| Inhibitor      | n | Glucose in assay medium | Inhibitor concentration | [3H]Mannose | Inhibition of basal mannose transport | Inhibition of MSMT |
|----------------|---|-------------------------|-------------------------|-------------|--------------------------------------|--------------------|
| Simple sugars  |   |                         |                         |             | %                                    |                    |
| d-Allose†      | 3 | 1                       | 5                       | 2.9         | ND                                   | ND                 |
| d-Allo†        | 3 | 0.5                     | 5                       | 0.6         | ND                                   | ND                 |
| l-Fucose†      | 3 | 1                       | 5                       | 2.9         | ND                                   | ND                 |
| d-Galactose†   | 3 | 1                       | 5                       | 2.9         | ND                                   | ND                 |
| d-Glucose‡     | 3 | 0.5                     | 5                       | 59.0 ± 0.8  | 90.9 ± 0.9                           |                    |
|                | 3 | 0.5                     | 5                       | 64.5 ± 0.6  | 95.3 ± 2.0                           |                    |
|                | 3 | 0.5                     | 11                      | 76.8 ± 0.7  | 92.3 ± 1.6                           |                    |
| d-Mannose§     | 6 | 1                       | 5                       | 36.4 ± 6.3  | 90.3 ± 3.1                           |                    |
| d-Talose§      | 3 | 1                       | 5                       | 27.6 ± 1.5  | ND                                   |                    |
| d-Xylose§      | 3 | 1                       | 5                       | 32.5 ± 1.5  | 12.4 ± 4.2                           |                    |
| Glucose derivatives | | | | | | |
| 2-Deoxy-Glcα   | 3 | 0.5                     | 2                       | 63.8 ± 3.8  | 75.1 ± 8.7                           |                    |
|                | 3 | 0.5                     | 5                       | 68.1 ± 0.5  | 91.4 ± 2.2                           |                    |
|                | 3 | 1                       | 5                       | 39.5 ± 1.4  | 48.3 ± 2.5                           |                    |
| 3-O-Me-Glcα    | 3 | 1                       | 5                       | 28.5 ± 3.2  | ND                                   |                    |
| GlcNAcα        | 3 | 0.5                     | 5                       | 37.0 ± 2.1  | ND                                   |                    |
| Glycosides     |   |                         |                         |             | %                                    |                    |
| α-Me-Glcα      | 3 | 1                       | 5                       | 2.9         | ND                                   | ND                 |
| α-Me-Manα      | 3 | 1                       | 5                       | 2.9         | ND                                   | ND                 |
| β-Me-Glcα      | 3 | 1                       | 5                       | 2.9         | ND                                   | ND                 |
| pNP-α-Glcα     | 3 | 1                       | 5                       | 25.8 ± 0.6  | ND                                   |                    |
| MLMB-α-Manα    | 3 | 1                       | 5                       | 25.4 ± 0.9  | 27.5 ± 1.8                           |                    |
| MLMB-α-Glcα    | 3 | 1                       | 5                       | 20.1 ± 0.8  | ND                                   |                    |
| a-Glc-1-Pα     | 3 | 1                       | 5                       | 2.9         | ND                                   | ND                 |
| a-GlcNAc-1-Pα  | 3 | 1                       | 5                       | 2.9         | ND                                   | ND                 |
| Maltoseα       | 3 | 1                       | 5                       | 2.9         | ND                                   | ND                 |

† Provided by Sigma.
‡ Provided by Aldrich.
§ Provided by Fluka.
α Provided by Fisher.

When glucose is listed as an inhibitor, the concentration value includes the glucose in the assay medium.

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**Fig. 5. Glucose as an inhibitor of mannose transport.** Basal (circles) and metformin-stimulated (triangles) transport of 2.5 μM [3H]mannose was determined in the presence of 0.25–1 mM glucose (panel a) or 0.5–11 mM glucose (panel b). For panel a, 100% was based upon transport with 0.25 mM glucose. For panel b, 100% was based upon transport with 0.5 mM glucose. Error bars are ± S.E. of triplicate experiments; in some cases error bars are smaller than the graph points.

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**Fig. 6. Inhibition of MSMT by tunicamycin.** Six fibroblast cultures were exposed to vehicle (0.1% ethanol; “−”) or 5 μM tunicamycin (TN) for 1 h, 100 nM thapsigargin (TG) for 30 min, 2 mM dithiothreitol (DTT) for 20 min, or 200 μM diamide (DIA) for 20 min. Five hours after beginning the experiment, triplicate cultures were incubated with either 0 or 2 mM metformin for an additional 24 h, at which point transport of 0.6 mM [3H]mannose was measured in the presence of 0.5 mM glucose. Basal and MSMT are expressed as percentages (±S.E.) of that measured with ethanol.

Comparisons of the effects of 0.5 mM phlorizin on transport of 0.6 and 100 μM mannose, 2dG, and 3mG helped to distinguish further the transport activities (Fig. 4) and support the model shown in Fig. 1. 0.5 mM phlorizin was without effect on transport of 3mG, in the absence or presence of metformin (Fig. 4, panels c and f), or on MSMT (Fig. 3, panel b). However, 0.5 mM
phlorizin partially inhibited basal mannose transport. Thus, the apparent decrease of total mannose transport by phlorizin in metformin-treated cells (black bars in Fig. 4, panels a and d) can be accounted for by the decrease in basal transport. Like basal mannose transport, basal 2dG transport was partially inhibited by 0.5 mM phlorizin (white bars in panels b and e), suggesting that both sugars were transported by the same phlorizin-sensitive transporter under basal conditions (transporter 2 in Fig. 1).

Because metformin diminished 2dG transport (Fig. 4, panels b and e), it is possible that the same metformin-inhibitable transporter responsible for 3mG uptake (transporter 1 in Fig. 1), might also be responsible for transport of a portion of 2dG. However, in the presence of 0.5 mM phlorizin, metformin had little or no effect on 2dG transport. This result suggests that the inhibition of basal 2dG transport activity by phlorizin was counter-acted by a phlorizin-resistant 2dG transport activity stimulated by metformin. The latter activity could be due to

**Table II**

| Cell type                     | Fold effect of metformin on mannose uptake (±S.E.) | n  |
|-------------------------------|---------------------------------------------------|----|
| Mouse embryonic fibroblast    | 2.5 ± 0.1                                          | 3  |
| Human dermal fibroblast       | 2.0 ± 0.04                                         | 18 |
| HeLa                          | 1.8 ± 0.1                                         | 6  |
| Madin-Darby bovine kidney     | 1.8 ± 0.2                                          | 9  |
| RAW 264.7                     | 1.2 ± 0.04                                         | 6  |
| COS-1                         | 1.1 ± 0.04                                         | 6  |
| CHO-K1                        | 0.7 ± 0.03                                         | 6  |

**FIG. 7.** Metformin corrects experimentally induced defects in LLO synthesis and N-linked glycosylation with normal fibroblasts. Cultures were treated in the absence (panels a, c, and e) or presence (panels b, d, and f) of 2 mM metformin for 24 h and then incubated in 0.5 mM glucose medium containing 40 μCi/ml [3H]mannose for 20 min in the absence (panels a, b, e, and f) or presence (panels c and d) of 0.1 mM mannose. [3H]-LLOs (panels a–d) or N-[3H]glycans (panels e and f) were recovered and analyzed by HPLC. The positions of glycans standards are indicated (M₃, M₅, Man₃GlcNAc₂; M₇, Man₆GlcNAc₂; G₇M₇, Glc₃Man₆GlcNAc₂). Two N-glycans, designated x and y, were found to be high mannose oligosaccharides as judged by sensitivity to endoglycosidase H and sizing by HPLC. Compared with a [3H]mannose-labeled fibroblast glycoprotein sample treated with N-glycanase, which released x, y, and glycans with fewer than six mannose residues (panel g), only x and y were released with endoglycosidase H (panel h). Note the slightly reduced elution times due to removal of a GlcNAc residue. After endoglycosidase H (Endo H) treatment, further digestion with N-glycanase recovered glycans with fewer than six mannose residues (panel i). The glycan eluting at 49 min in panel i is likely to be Glc₃Man₆GlcNAc₂. HPLC elution times for x and y in panel h were consistent with Man₆GlcNAc₂ and Glc₃Man₆GlcNAc₂, respectively, although in some experiments there appeared to be some digestion by endoplasmic reticulum mannosidase to the corresponding eight-mannose glycans. After digestion with jack bean α-mannosidase, a mixture of x and y yielded free mannose plus glycan products with retention times consistent with Glc₃Man₆GlcNAc₂ and Glc₃Man₆GlcNAc₂ (data not shown).
2dG uptake by transporter 3 (Fig. 1). Consistent with this, MSMT was moderately inhibited by 2dG (Table I). If so, each of the three transporters suggested in Fig. 1 would contribute to uptake of 2dG.

Phloretin, phlorizin, and some of the inhibitors listed in Table I required the vehicles ethanol and Me2SO. Curiously, basal mannose transport activity was more sensitive to inhibition by 5% ethanol (66.8 ± 1.5% inhibition, n = 3) and 0.2 mM Me2SO (26.7 ± 1.6% inhibition, n = 9) than was MSMT (less than 10% inhibition by each), suggesting that MSMT may be more resistant to disruptive effects of these solvents.

Specificity of Metformin-stimulated Mannose Transport—Table I lists the effects of 5 mM concentrations of saccharides on mannose transport and reveals additional differences between basal and MSMT. Up to 1 mM glucose inhibited only a minor fraction of the total mannose transport (Fig. 5), so these assays were done in the presence of 0.5 or 1 mM glucose to provide a source of energy. Low glucose concentrations were used in mannose uptake assays with fibroblasts in a previous study (5).

5 mM mannose and 5 mM glucose inhibited both the basal and MSMT activities (Table I), and consistent with the results of Figs. 2 and 5, basal mannose transport was somewhat less sensitive in each case. Thus, inhibitors of basal transport or MSMT could have axial or equatorial conformations for the 2-hydroxyl of hexose ring. Consequently, 5 mM 2dG, which lacks the 2-hydroxyl, also inhibited both activities (transporters 2 and 3 in Fig. 1) even though it did not appear to be a good substrate for the latter. GlcNAc was not an inhibitor, indicating that a bulky group at the 2-position was not tolerated.

Neither d-altrose (the 3-epimer of mannose) nor d-allose (the 3-epimer of glucose) were effective inhibitors of either basal or MSMT activity, indicating that the 3-equatorial conformation is strongly preferred. 3mG caused mild inhibition of basal, but not MSMT activity, suggesting that bulky groups at the 3-position were not tolerated by MSMT. D-Talose (the 4-epimer of mannose) was a better inhibitor of basal mannose transport than of MSMT. This indicated that MSMT had a preference for equatorial conformations of the 4-hydroxyl, consistent with the
observation that d-galactose (the 4-epimer of glucose) was unable to inhibit MSMT. D-Xylose, the 6-deoxyhexose analogue of glucose, inhibited neither basal nor MSMT activity indicating a requirement for the 6-deoxyhexose group. None of the other hexoses or hexosamines tested, whether in α or β linkage, significantly inhibited either basal or MSMT, indicating that neither activity tolerated modification of the 1-position. Thus, as expected the transporters recognized free sugars but not glycosides. However, basal and MSMT were somewhat inhibited by 5 mM concentrations of para-nitrophenyl and methylumbelliferyl glycosides, suggesting a possible interaction with the aglycone.

In summary, MSMT (transporter 3 in Fig. 1) can be distinguished from basal mannose transport (transporter 2 in Fig. 1) by the greater sensitivity of MSMT for inhibition by mannose and glucose, less tolerance for bulky groups at the 3-position (this property also distinguishes MSMT from transporter 1 in Fig. 1), less tolerance for epimerization of the 4-position, greater resistance to phlorizin, and relative resistance to disruptive effects of ethanol and Me$_2$SO. Presumably, the basal transport of mannose observed in these studies is due to the combined actions of the high affinity ($K_{\text{uptake}} = 35 \mu M$) and low affinity ($K_{\text{uptake}} = 850 \mu M$) mannose transport activities reported previously in human dermal fibroblasts (5). Apparently, the basal uptake data presented here (Fig. 2 and data not shown) did not have sufficient resolution to distinguish these two mannose transport activities. Interestingly, both the basal activity reported here and the high affinity mannose transporter reported earlier (5) retained about half of their activities in 0.5 mM phlorizin (Fig. 3) and physiological, i.e., 5 mM, glucose (Fig. 5). In contrast, MSMT was insensitive to 0.5 mM phlorizin, yet completely inhibited by physiological glucose, indicative of a specialized function (see below).

Additional evidence distinguishing the two transport activities is shown in Fig. 6. MSMT was inhibited by about half by pretreatment of cells with tunicamycin (TN), an inhibitor of LLO synthesis. Basal transport was unaffected. TN also causes endoplasmic reticulum stress, but other endoplasmic reticulum stress inducers such as azetidine-5-carboxylic acid, thapsigargin, and diithiothreitol (23), as well as the cytoplasmic stress inducer diame (23) did not affect MSMT. Thus, the effect of TN on MSMT was not due to a stress response. Presumably, an N-glycan is involved in the function of one or more components of the MSMT system.

Metformin enhanced mannose uptake by some, but not all, mammalian cell types. As listed in Table II, little or no enhanced uptake was detected in RAW 264.7 (macrophage), COS-1 (kidney), and CHO-K1 (ovary) cells. In fact, metformin consistently decreased mannose uptake by CHO-K1 cells. These cells might be useful for expression of candidate genes for transporter 3. In contrast, metformin increased mannose uptake in mouse embryonic fibroblasts, Madin-Darby bovine kidney cells, and HeLa (adenocarcinoma) cells. However, experiments analogous to those shown in Figs. 2–6 will be necessary to determine whether the effects of metformin are due to increased activity of an existing transporter or expression of an additional transporter similar to MSMT in dermal fibroblasts.

**MSMT Can Enhance LLO Synthesis**—Primary dermal fibroblasts in typical RPMI 1640 medium (11 mM glucose) make normal LLOs bearing Glc$_3$Man$_9$GlcNAc$_2$ as determined by fluorophore-assisted carbohydrate electrophoresis (24). However, in our hands when primary dermal fibroblasts are labeled for 20 min with 2.5 $\mu M$ [2-^3$H$]mannose in RPMI 1640 medium with 0.5 mM glucose and dialyzed serum, a dramatic shift in the structures of LLOs occurs, from mostly Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol to LLO intermediates (Man$_{n-5}$GlcNAc$_2$-P-P-dolichol) as the predominant species. This shift to Man$_{n-5}$GlcNAc$_2$-P-P-dolichol is prevented if the 0.5 mM glucose medium is supplemented with 0.1 mM mannose (6). Thus, the mannose restores optimal conditions for LLO synthesis. This model system for LLO synthesis permitted us to evaluate the impact of metformin treatment under conditions where hexose precursors were limited, or under optimal conditions, in the same cell type.

As shown in Fig. 7, metformin enhanced extension of LLO intermediates in normal fibroblasts when hexose precursors were limiting (panels a and b), and increased the fraction of highly mannosylated glycans (originating from Glc$_3$Man$_9$GlcNAc$_2$-P-P-dolichol, see panels g–i) transferred to protein (panels e and f). However, in the presence of 0.1 mM mannose, metformin did not affect LLO synthesis (panels c and d) or N-linked glycans (data not shown). To determine whether increased mannose uptake accounted for all of the action of metformin on LLO extension, mannose uptake and LLO profiles were compared directly for cells incubated with 40 $\mu$Ci/ml (2.5 $\mu M$) [3$H$]mannose, 40 $\mu$Ci/ml [3$H$]mannose plus metformin, or 80 $\mu$Ci/ml [3$H$]mannose. As shown in Fig. 8, panel a, compared with 40 $\mu$Ci/ml [3$H$]mannose, treating cells with metformin had an effect on net uptake that was similar to the effect of changing to 80 $\mu$Ci/ml [3$H$]mannose. After normalization of peak heights to mannose contents (see “Experimental Procedures”), quantification of LLO profiles (panels b–d) showed that Glc$_3$Man$_g$GlcNAc$_2$-P-P-dolichol represented, respectively, 1.3, 7.9, and 3.2% of the LLO pool under these conditions. Therefore, a substantial part of the effect of metformin on LLO extension can be explained by its enhancement of mannose uptake, but a second mechanism also appears to contribute. Given the many downstream effects of AMPK activation (14), the nature of the second mechanism remains elusive.

As further evidence that metformin treatment enhances LLO extension, incorporation of [3$H$]mannose into relevant glycoconjugate fractions was determined. Because LLO extension is limited by the chemical amount (2.5 $\mu M$) of the [3$H$]mannose added to cultures when 0.5 mM glucose medium is used (Fig. 7) (6), greater uptake of [3$H$]mannose should also result in more [3$H$]mannose incorporated into LLO. As shown in Fig. 9, metformin increased the [3$H$]mannose incorporated in the LLO and protein fractions, as well as the water fraction containing mannose and aqueous metabolites such as mannosyl phos-
Metformin-stimulated Mannose Transport

Fig. 11. Metformin acts as an adjuvant for phenotypic correction of CDG-Ia cells by mannose supplementation. CDG-Ia cells were treated in the absence (panels a, c, e, and g) or presence (panels b, d, f, and h) of 2 mM metformin for 24 h. LLOs (panels a–d) and N-linked glycans (panels e–h) were analyzed by HPLC after labeling with 2.5 μM [3H]mannose alone (panels a, e, and f) or in the presence of 0.1 mM unlabeled mannose (panels c, d, g, and h). Chromatograms are labeled with standards as described for Fig. 6.

The greater incorporation of [3H]mannose into LLO and protein due to metformin treatment is consistent with the greater number of mannose residues per mol of LLO and N-glycan (Fig. 7). In contrast, [3H]mannose incorporated into the mannos-P-dolichol fraction (chloroform-methanol 2:1) was not affected, probably because the mannose content is limited to 1 mol/mol lipid.

MSMT in Congenital Disorder of Glycosylation Type Ia and Ib Cells—LLO synthesis is abrogated in type I CDG (9, 25). Type Ia (phosphomannomutase-deficient) and Ib (phosphomannose isomerase-deficient) CDG each result in diminished GDP-mannose production (8). However, the effects on the LLO pools differ. Type Ia cells accumulate LLO intermediates, resulting in a pattern with predominantly Manα2-Manα2-GlcNAc2-P-P-dolichol. Thus, a key hallmark of the CDG-Ia phenotype is a relatively high percentage of undermannosylated glycans found on newly synthesized glycoproteins. Because undermannosylated LLOs are poor donor substrates for oligosaccharyltransferase, CDG-Ia glycoproteins also tend to be underglycosylated. By comparison, type Ib cells appear to contain fewer LLO molecules, but they are extended efficiently to Glcα3-Manα2-GlcNAc2-P-P-dolichol. As mentioned earlier, CDG-Ib patients can be treated effectively with dietary mannose (2), but curiously, therapy with dietary mannose is not effective for CDG-Ia patients (9).

Metformin increased mannose uptake in CDG-Ia (Fig. 10, panel a) and CDG-Ib (panel b) cells. The potential effects of metformin on LLO synthesis in CDG-Ib cells were difficult to assess because extension was already efficient, but the abnormal LLO pool in CDG-Ia cells was more amenable to analysis. Although proper LLO synthesis in normal cells incubated with 0.5 mM glucose was achieved by adding 0.1 mM mannose (Fig. 7), 0.1 mM mannose supplementation had only a modest effect on glycosylation in CDG-Ia cells (Fig. 11, panels a, c, e, and g).

By itself, metformin did not affect CDG-Ia LLO synthesis (Fig. 11, panels a and b) or the types of oligosaccharides transferred to protein (panels e and f). However, metformin acted synergistically with mannose. By combining 2 mM metformin treatment with 0.1 mM mannose supplementation, extension of LLO intermediates to Glcα3-Manα2-GlcNAc2-P-P-dolichol increased from 5 (panel c) to 19% (panel d) of the LLO pool. The fraction of highly mannosylated glycans on protein increased from 46 (panel g) to 69% (panel h), and the resulting HPLC profiles of N-glycans resembled those from normal cells labeled for the same period under optimal conditions with 0.1 mM mannose (data not shown). Similar data for CDG-Ia cells were observed with 0.5 and 5.0 mM metformin.

As indicated by Fig. 8, these results can be attributed to the dual effects of metformin on fibroblast LLO synthesis, one of which is due to increased mannose uptake. The CDG-Ia phenotype may be tissue-dependent (26), and it is difficult to predict how the results obtained here with dermal fibroblasts apply to cells of other tissues. However, in CDG-Ia patients, metformin may be a useful adjuvant for dietary mannose therapy, which by itself is ineffective. Similarly, metformin may allow CDG-Ib patients to be treated with lower quantities of mannose.

Implications for AMPK as a Regulator of Mannose Metabolism and Protein Glycosylation in Dermal Fibroblasts—The only known target of metformin is AMPK (18), and its role in mannose transport has not been reported. In skeletal muscle, AMPK activation results in fusion of intracellular vesicles containing glucose transporters with the cell surface (14). However, because conventional glucose transport activity was not increased in metformin-treated fibroblasts, this is not a likely cause of the enhanced mannose uptake. Taken together, these data support the possibility that a population of transporters in skin fibroblasts with a selectivity for mannose, distinct from those already described (5), may respond to metformin treatment.

Increased mannose uptake due to activation of AMPK may also explain earlier paradoxical results (27) in which the LLO extension defect in CDG-Ia cells was corrected by 12 h of glucose deprivation. This result was surprising, because withdrawal of a source of hexose precursors for LLO extension would be expected to make the problem worse. It is important to understand the mechanism of this effect because, as discussed above, there is currently no effective treatment for CDG-Ia. Extended glucose deprivation would be expected to activate AMPK. As shown with metformin, mannose uptake should then be increased. Because mannose is a much more efficient precursor for LLO synthesis than glucose (3, 4), even a...
small increase of mannose transport might be effective for correction of the CDG-Ia defect.

Possible Biological Function of MSMT—Two aspects of this study suggest that the function of the transport process stimulated by metformin may be specialized. First, MSMT is completely inhibited by physiological concentrations of glucose in the blood (typically 5 mM). Second, with physiological glucose, LLO synthesis in fibroblasts is highly efficient and does not require additional mannose (24). In this regard, the biological role of the dermal fibroblast should be considered. Typically quiescent in healthy skin, fibroblasts aid in the repair of damaged skin by replicating to form granulation tissue and by producing extracellular components. In the absence of a well formed dermal microvasculature, the supply of precursors for extracellular components such as glucose and mannose may be variable. Indeed, direct microprobe measurements of glucose concentrations at sites of skin inflammation were four times lower than blood glucose concentrations (28). It is therefore plausible that signals within the granulation tissue, perhaps associated with compromised access to glucose, activate AMPK to stimulate processes such as mannose transport. Activation of AMPK may promote uptake of mannose as an alternative energy source to compensate for the lack of glucose, as well as an alternative for glucose as a precursor for glycan synthesis. MSMT has an EC_{50} for mannose of ~500 μM (Fig. 2) and was easily detected with both 0.6 and 100 μM mannose (Fig. 4). As shown in Fig. 8, the potential contribution of MSMT to glycan synthesis was significant with 2.5 μM mannose. Because blood contains 20–50 μM mannose (4), even a small fraction of mannose originating from the circulation would be effective.

Acknowledgment—We thank Biswanath Pramanik for assistance with cell culture.

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J. Biol. Chem. 2004, 279:9703-9712.
doi: 10.1074/jbc.M310837200 originally published online December 17, 2003

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