Transmembrane Movement of a Water-soluble Analogue of Mannosylphosphoryldolichol Is Mediated by an Endoplasmic Reticulum Protein

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Abstract. Based on topological studies mannosylphosphoryldolichol (Man-P-Dol) is synthesized on the cytoplasmic face of the RER, but functions as a mannosyl donor in Glc3Man9GlcNAc2-P-P-dolichol biosynthesis after the mannosyl-phosphoryl headgroup diffuses transversely to the luminal compartment. The transport of mannosphosphorylcitronellol (Man-P-Cit), a water-soluble analogue of Man-P-Dol, by microsomal vesicles from mouse liver, has been investigated as a potential experimental approach to determine if a membrane protein(s) mediates the transbilayer movement of Man-P-Dol. For these studies β-[3H]Man-P-Cit was synthesized enzymatically with a partially purified preparation of Man-P-undecaprenol synthase from Micrococcus luteus. The uptake of the radiolabeled water-soluble analogue was found to be (a) time dependent; (b) stereoselective; (c) dependent on an intact permeability barrier; (d) saturable; (e) protease-sensitive; and (f) highest in ER-enriched vesicles relative to Golgi complex-enriched vesicles and intact mitochondria. Consistent with the involvement of a membrane protein, the analogue did not enter synthetic phosphatidylinositol-liposomes. [3H]Man-P-Cit also was not transported by human erythrocytes. These results indicate that the transport of Man-P-Cit by sealed microsomal vesicles from mouse liver is mediated by a membrane protein transport system. It is possible that the same membrane protein(s) participates in the transbilayer movement of Man-P-Dol in the ER.

MANNOSYLPHOSPHORYLDOLICHOL (Man-P-Dol) is a multifunctional mannosid that serves as a mannosyl donor in the assembly of Glc3Man6GlcNAc2-P-P-Dol (oligo-P-P-Dol), the lipid-bound precursor of N-linked oligosaccharides (Hirschberg and Snyder, 1987; Waechter, 1989; Cummings, 1992; Abeijon and Hirschberg, 1992), the biosynthesis of glycosylphosphatidylinositol (GPI) anchors (Menon et al., 1990; Camp et al., 1993; Englund, 1993), and the O-mannosylation of serine/threonine residues in yeast glycoproteins (Tanner and Lehle, 1987; Herscovics and Orlean, 1993). In addition, Man-P-Dol is capable of stimulating the formation of GlcNAc-P-P-Dol in vitro, and may play a regulatory role in the synthesis of the glycolipid intermediate in vivo (Kean, 1982, 1985).

In the current topological model for oligo-P-P-Dol biosynthesis, Man-P-Dol, glucosylphosphoryldolichol (Glc-P-Dol), and Man6GlcNAc2-P-P-Dol, are formed on the cytoplasmic leaflet of the RER by glycosylation reactions in which all of the glycosyl residues are derived directly from the respective sugar nucleotides (Hirschberg and Snyder, 1987; Abeijon and Hirschberg, 1992). Oligo-P-P-Dol synthesis is completed on the luminal monolayer where Man-P-Dol donates four mannosyl units in the conversion of Man6GlcNAc2-P-P-Dol to Man6GlcNAc2-P-P-Dol, and the triglucosyl cap is added by the sequential transfer of three glucosyl units from Glc-P-Dol. There is also evidence that the transfer of mannosyl units from Man-P-Dol to serine/threonine residues in yeast glycoproteins occurs in the luminal compartment of the ER (Sharma et al., 1974; Tanner and Lehle, 1987). In contrast to the orientation of these lipid-mediated mannosylation reactions, there is evidence that Man-P-Dol donates mannosyl units to GPI anchor precursors on the cytoplasmic face of the ER in Trypanosoma brucei (Vidugiriene and Menon, 1994).

If these models for oligo-P-P-Dol assembly and the lipid-mediated mannosylation of yeast glycoproteins are correct, then newly synthesized Man-P-Dol must diffuse transversely, or "flip-flop," in the ER before functioning...
as an “activated” mannosyl donor. Since the spontaneous flip-flopping of \((\text{GlcNAc})_2\)-P-P-Dol (Hanover and Lenaz, 1978) and spin-labeled analogues of polyisoprenyl compounds (McCloskey and Troy, 1980) in synthetic lipid bilayers occurs at an extremely slow rate, it is possible that intrinsic membrane proteins in the ER facilitate the transbilayer movement of Man-P-Dol and other lipid intermediates.

Although the transverse diffusion of dolichylphosphochitocarboxylic intermediates and newly synthesized membrane phospholipids is a critical process in the assembly of N-linked oligosaccharides and membrane biogenesis, respectively, there is virtually nothing known about the class of membrane proteins generally referred to as “flip-flopases.” Several years ago, Bishop and Bell (1985) synthesized a water-soluble analogue of phosphatidyicholine (PC), diCPC, and characterized its transport into sealed ER vesicles. This approach implicated a membrane protein in the transbilayer movement of PC in the ER.

In this study, we adapted the experimental strategy of Bishop and Bell, and enzymatically synthesized \(\beta\)-mannosylphosphochitocarboxylic (Man-P-Cit), a water-soluble analogue of Man-P-Dol. Man-P-Cit is closely related structurally to Man-P-Dol, and could be considered to have the structure of Man-P-Dol, including the saturated \(\beta\)-iso-protein in the transbilayer movement of PC in the ER.

The possibility that the protein(s) involved in the transport of Man-P-Cit is closely related structurally to Man-P-Dol, and could be considered to have the structure of Man-P-Dol, including the saturated \(\beta\)-iso-protein unit critical to its function as a mannosyl donor (Rush et al., 1993; DeLuca et al., 1994; Dotson et al., 1995) and activator of GlcNAc-P-P-Dol synthesis (Kean et al., 1994). The recognition of Man-P-Cit by the mannosyltransferases catalyzing the transfer of four mannopyruvates from Man-P-Dol into Man\(\beta\)GlcNAc\(\beta\)-P-P-Dol (Rush et al., 1993), suggests that the phosphochitocarboxylic derivative might be a suitable water-soluble analogue for these transport studies.

Using this approach, we have been able to establish that Man-P-Cit is transported into intact microsomal vesicles by a mechanism exhibiting the properties of a protein-mediated system. The possibility that the protein(s) involved in the transport of Man-P-Cit is related to membrane proteins facilitating the transbilayer movement of Man-P-Dol in the ER is discussed.

### Materials and Methods

#### Materials

Phosphorus trichloride oxide was obtained from Alfa Products (Danvers, MA), GDP-mannose, DE-52 cellulose, Con A-Sepharose, monosien, valinomycin, iodomycin, carbonyl cyanide p-trifluoromethoxy-phenoxyhydrozone (FCCP), and S-citronellol were purchased from Sigma Chemical Co. (St. Louis, MO). Econosafe counting cocktail is a product of Research Diagnostics, Inc. (Fitzgerald, NJ). GDP-[\(2^{-3}\text{H}\)]mannose using hexokinase (Sigma Chemical Co.) and a/13-galactosylphosphochitocarboxylic (Gal-P-Cit), and a/13-gluco-sylphosphochitocarboxylic (Glc-P-Cit) were synthesized chemically using a minor modification (Rush et al., 1993) of the method of Warren and Jeanloz (1973, 1978). Chemically synthesized \(\alpha\)-Man-P-Cit was further purified by affinity chromatography on Con A-Sepharose at low pH (Rush and Waechter, 1995). Most (86%) of the Man-P-Cit was retained, but eluted after the addition of 5 mM \(\alpha\)-methylmannoside to the elution buffer. The fractions containing the recovered \(\alpha\)-Man-P-Cit were pooled, purified by chromatography on a DEAE-cellulose column (40 ml), and desalted by chromatography on Bio Gel P-2 (Bio-Rad Laboratories, Richmond, CA).

The Man-P-Cit that did not bind to Con A-Sepharose was resistant to jack bean \(\alpha\)-mannosidase digestion (Sigma Chemical Co.), but was cleaved by snail \(\beta\)-mannosidase (Sigma Chemical Co.). Conversely, the purified \(\alpha\)-Man-P-Cit was quantitatively hydrolyzed by incubation with \(\beta\)-mannosidase and completely resistant to treatment with \(\beta\)-mannosidase.

#### Preparation of Microsomes from Mouse and Rat Liver

ER-enriched preparations of mouse or rat liver microsomes were prepared essentially as described by Coleman and Bell (1978). The microsomal vesicles were judged to be >95% intact as assessed by mannose 6-phosphate (Man 6-P) phosphatase latency (see below).

#### Isolation of Human Red Blood Cells

Erythrocytes obtained from a human donor (J. S. R.), were suspended in 5 mM EDTA and sedimented by centrifugation at 1,000 \(g\), 10 min, 4°C. The buffy coat (white cells) was removed by aspiration and the red cells were resuspended in Dulbecco’s PBS and sedimented again. The packed red cells were resuspended in an equal volume of PBS and stored on ice until used for transport assays.

#### Assay of Man-P-Cit and Glucose Transport by Liver Microsomes or Human Erythrocytes

Assay mixtures contained liver microsomes (150–500 \(\mu\)g membrane protein), 40 mM Tris-HCl, pH 7.4, 10 mM MgCl\(\text{2}\), 5 mM EDTA, 0.25 M sucrose, and the indicated concentration of either [\(2^{-3}\text{H}\)]-Man-P-Cit (7–1,000 cpm/\(\mu\)l) or [\(3^{-3}\text{H}\)]glucose (52 cpm/\(\mu\)l) in a total vol of 20 \(\mu\)l. After incubation at 21–24°C for 30 s, transport was stopped by the addition of 0.5 M of ice-cold 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 5 mM CaCl\(\text{2}\), and the amount of labeled Man-P-Cit or glucose transported was determined by a filtration assay (Bishop and Bell, 1985). The diluted assay mixtures were quickly transferred to a chilled filtration manifold equipped with a 0.45-\(\mu\)m HA filter disk (Millipore Corp., Bedford, MA) and suction-filtered. The disks were then washed with 10 ml of ice-cold 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 5 mM CaCl\(\text{2}\). Filtration was usually completed in <30 s. The Millipore filter was transferred to a 20-ml scintillation vial, and the amount of radioactivity retained on the filter determined by scintillation spectrometry in a scintillation spectrometer (Tri-carb Packard Instrument Co., Inc., Meriden, CT). The diluted sample was transferred to a scintillation vial, and the amount of radioactivity retained on the filter determined by scintillation spectrometry in a scintillation spectrometer (Tri-carb Packard Instrument Co., Inc., Meriden, CT).

#### Preparation of Microsomal Fractions and Mitochondria from Rat Liver

Light (Golgi complex–enriched) and heavy (ER-enriched) microsomes, recovered at the interface between the 0.25-M and 1.3-M sucrose fractions. All fractions were collected from the 1.1 M/1.3 M sucrose (5 ml), 0.85 M sucrose (5 ml), and 1.3 M sucrose (5 ml). All fractions contained 5 mM Hepes, pH 7.0, 0.25 M sucrose by 10 strokes with a motor-driven homogenizer (Bellco Biotechnology, Vineland, NJ) at 1,000 rpm. Unbroken cells and other debris were removed by centrifugation at 1,000 \(g\), 10 min. The supernatant was sedimented at 25,000 \(g\), 10 min. The 25,000-\(g\) supernatant was used for the preparation of light and heavy microsomes, whereas the 25,000-\(g\) pellet contained the mitochondrial fraction. Highly purified mitochondria were prepared from the 25,000-\(g\) pellet exactly as described by Fleischer and Kervina (1974). In the 25,000-\(g\) supernatant (30 ml) was layered onto 10 ml of 5 M Hepes, pH (7.0), 1.3 M sucrose, and centrifuged at 26,000 \(rpm\), 2 h in a Sorvall rotor AH629 (Dupont Instruments, Wilmington, DE). The heavy microsomes in the 1.3 M sucrose layer and the pellet were used for the preparation of ER-enriched membrane fractions as described below. The light microsomes, recovered at the interface between the 0.25-M and 1.3-M sucrose layers, were removed by aspiration, adjusted to 1.1 M sucrose with 5 mM Hepes, pH 7.0, and layered (25 ml tube) on a four-step sucrose gradient containing 0.25 M sucrose (5 ml), 0.85 M sucrose (5 ml), and 1.3 M sucrose (5 ml). All fractions contained 5 mM Hepes, pH 7.0, in addition to sucrose. The gradients again were centrifuged at 26,000 \(g\) for 2 h in a Sorvall AH629 rotor. Light microsomes were collected from the 1.1 M/1.3 M sucrose interface, diluted with ice-cold 5 mM Hepes (pH 7.0), 2.5 M sucrose, and sedimented. Light microsomes were resuspended in a membrane protein concentration of ~10 mg/ml and stored on ice until analysis.
The 1.3-M sucrose layer and the pellet from the initial sucrose gradient were resuspended, diluted with 5 mM Hepes, pH 7.0, to a sucrose concentration of 0.85 M, and layered (15 ml/tube) on a four-step sucrose gradient containing 0.25 M sucrose (5 ml), 1.1 M sucrose (10 ml), and 1.3 M sucrose (10 ml). Each of the sucrose layers contained 5 mM Hepes, pH 7.0, in addition to sucrose. The gradients were centrifuged at 26,000 g for 2 h in a Sorvall AH629 rotor. The pellet containing the ER-enriched microsomes was resuspended in 5 mM Hepes, pH 7.0, 0.25 M sucrose to a membrane protein concentration of ~20 mg/ml, and stored on ice.

**Distribution of Marker Enzyme Activities in Rat Liver Subcellular Fractions.** Cross-contamination of the subcellular fractions from rat liver was assessed by measuring the established marker enzyme activities: ER/glucose 6-P phosphatase (Rush and Waechter, 1992); Golgi/galactosyltransferase (Fleischer and Smigel, 1978) and mitochondria/succinate dehydrogenase (Pennington, 1961).

**Assessment of the Integrity of ER-enriched and Golgi-enriched Vesicles and Mitochondria.** The integrity of liver ER microsomes was determined by measuring Man 6-P phosphatase latency using [3H]Man 6-P as substrate (Rush and Waechter, 1992). The integrity of rat liver Golgi vesicles was estimated by measuring the latency of rat liver galactosyltransferase activity to proteolytic digestion with trypsin. Rat liver Golgi vesicles (2 mg/ml) were incubated at room temperature in 5 mM Tris-HCl (pH, 7.4), 0.25 M sucrose, 10 mM MgCl₂, and trypsin (1 mg/ml) in a total vol of 0.01 ml. After incubation for 30 min, the fractions were assayed for galactosyltransferase activity as described by Fleischer and Smigel (1978). The Golgi complex–enriched vesicles were at least 90% intact based on the latency trypsin sensitivity of galactosyltransferase activity. The intactness of rat liver mitochondria was estimated by measuring the sensitivity of succinate-dehydrogenase activity to proteolytic digestion with trypsin. Rat liver Golgi vesicles in unfractured rat liver mitochondria was inhibited >85% by antinmycin A.

**Preparation of Synthetic Liposomes.** Synthetic liposomes were prepared using a lipid mixture of egg PC, 54 μmol dicytrophosphate, and 27 μmol cholesterol. The organic solvent (15 ml) was removed by rotary evaporation under reduced pressure in a 100-ml round-bottom flask. Liposomes were formed by sonication for 2 h with 10 ml of 0.25 M sucrose, 10 mM Tris-HCl, (pH 7.4), in a bath ultrasonic cleaner (5200; Branson Ultrasonics Corp., Danbury, CT). The liposomes were dialyzed overnight with 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4. Large aggregates were removed by centrifugation at 1,000 g for 10 min.

**Analytical Methods**

Protein concentrations were determined by the method of Rodriguez-Vico et al. (1989) using a protein assay reagent (BCA; Pierce, Rockford, IL). Lipid phosphorus was determined by the method of Bartlett (1959).

**Results**

**Man-P-Cit Uptake by Sealed Mouse Liver Microsomal Vesicles Is Time-dependent and Stereoselective**

To develop a model system for studying the properties of membrane proteins that might be involved in the transmembrane movement of Man-P-Dol in the ER, a water-soluble analogue of Man-P-Dol (Man-P-Cit) was synthesized. Man-P-Cit (Man-P-Dol₀) was chosen for the transport studies described below because of its close structural relationship to Man-P-Dol (Fig. 1). The water-soluble analogue contains the same ω-terminal isoprenyl unit and a saturated α-isoprenyl unit characteristic of dolichols, but lacks the long, hydrophobic, intervening polyisoprenyl chain. Aqueous solutions of Man-P-Cit can be made at concentrations at least as high as 50 mM.

When sealed mouse liver microsomal vesicles (vesicular integrity was >98%) were incubated with 0.2 mM β-[3H]-Man-P-Cit, there was time-dependent uptake of the radiolabeled analogue (Fig. 2). The distribution of β-Man-P-Cit between the extramicrosomal and luminal compartments approached equilibrium within 2 min, with ~4% of the Man-P-Cit internalized.

Although Man-P-Cit previously was shown to function as a mannosyl donor for Man₆GlcNAc₂-P-P-Dol synthesis in vitro with brain microsomes (Rush et al., 1993), <0.4% of the label associated with the sealed liver microsomes was due to the transfer of [3H]mannosyl units into lipid intermediates or N-linked glycoproteins during the short duration and the relatively low temperature of the transport assay.

Under identical assay conditions, the uptake system exhibited a stereoselective preference for β-Man-P-Cit over α-Man-P-Cit (Fig. 2). The α-stereoisomer of the water-soluble analogue was taken up by rat liver microsomes, but at a slower rate, and the affinity of the uptake system for
Properties of Man-P-Cit Transport System

Man-P-Cit uptake was characterized further by examining several properties of the transport system. First, the requirement of an intact permeability barrier was demonstrated by showing that the uptake of the water-soluble analogue decreased closely in parallel with the loss of latency of Man-6-P phosphatase activity when increasing concentrations of Triton X-100 were added (Fig. 3). Approximately 50% of the transport activity and the latent Man-6-P phosphatase activity were lost when mouse liver microsomes were incubated with 0.1% (wt/vol) Triton X-100. Also consistent with the entry of Man-P-Cit into microsomal vesicles and the amount of radiolabeled Man-P-Cit transported into microsomal vesicles during a 30-s incubation at 23°C were determined as described in Materials and Methods.

β-Man-P-Cit ($K_i = 0.66$ mM) was significantly higher than for α-Man-P-Cit ($K_i = 7.1$ mM).

Possible Involvement of a Membrane Protein in Man-P-Cit Transport

To determine if a membrane protein(s) is involved in the transport of Man-P-Cit, the effect of trypsin treatment on transport activity was evaluated. As seen in Fig. 6, preincubation at room temperature for 4 h did not affect the uptake of Man-P-Cit. However, when the microsomal vesicles were treated with trypsin (1 mg/ml), there was a time-dependent loss of Man-P-Cit transport activity. Consistent with the trypsin sensitivity being due to proteolytic cleavage of a membrane protein, the loss of transport activity caused by incubation with trypsin was prevented by the presence of trypsin inhibitor from soybean.

The possible involvement of a membrane protein in Man-P-Cit uptake was investigated further by testing the
ability of the water-soluble analogue to enter synthetic PC-liposomes (Fig. 7). In this experiment, radiolabeled Man-P-Cit was mixed with liver microsomes or PC liposomes. After an incubation of 10 min at 23°C, the microsomal or liposomal vesicles were separated from untransported [3H]Man-P-Cit by exclusion chromatography on a Sephacryl S-200 column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). In Fig. 7 A it can be seen that while sealed microsomes containing a substantial amount of [3H]Man-P-Cit were recovered in the void volume, virtually no labeled Man-P-Cit entered the synthetic PC liposomes (Fig. 7 B). However, if the synthetic liposomes were sonicated in the presence of Man-P-Cit before gel-filtration, [3H]Man-P-Cit was trapped in the liposomes and was recovered in the void volume (Fig. 7 C). Thus, the diffusion of [3H]Man-P-Cit through the lipid bilayer of PC vesicles occurs at a substantially slower rate than transport into microsomal vesicles.

**Specificity of Man-P-Cit Transport**

Initial studies indicated that Man-P-Cit transport was stereoselective (Fig. 2). To evaluate further the specificity of Man-P-Cit uptake by sealed microsomes, competition experiments were performed with a variety of structurally related compounds. As shown in Table I, none of the sugar derivatives tested had a significant effect on the transport of Man-P-Cit. This list includes three hexose monophosphates (Man 1-P, Man 6-P, Glc 6-P), Cit-P, and three other glycosylphosphorylcitronellols (Gal-P-Cit, Glc-P-Cit, and α-Man-P-Cit). The failure of a large excess of Glc 6-P to compete for uptake of Man-P-Cit is good evidence that the water-soluble analogue does not enter nonspecifically via the Glc 6-P transporter.

**Man-P-Cit Uptake System Is Enriched in ER Vesicles**

If the transport of Man-P-Cit were mediated by a protein involved in the transbilayer movement of Man-P-Dol, it would be expected to be enriched in the ER (Hirschberg and Snider, 1987; Waechter, 1989; Abeijon and Hirschberg, 1992; Cummings, 1992). In a limited survey, the distribution of Man-P-Cit transport activity in rat liver subcellular fractions containing ER-enriched vesicles, Golgi complex–enriched vesicles, or intact mitochondria were examined (Fig. 8). Although all three subcellular fractions transport Man-P-Cit to some extent, the specific activity for Man-P-Cit transport was highest in sealed vesicles from the ER-enriched fraction (Fig. 8 A). Based on the relative amounts of galactosyltransferase (Fig. 8 C) and succinate dehydrogenase activity (Fig. 8 D), the ER-enriched fraction contained only minor contamination with either Golgi or mitochondrial vesicles. Since the mitochondrial and Golgi fractions contained significant levels of Man 6-P phosphatase activity (Fig. 8 B), the relatively low Man-P-Cit transport activity in these fractions is probably due to the presence of contaminating ER vesicles. Because of the difficulty in establishing the intactness of vesicle preparations, other subcellular compartments were not surveyed in this study.

Man-P-Cit was not transported by human red blood cells (Fig. 9 A), providing adducing proof that the water-soluble analogue does not adsorb to the lipid bilayer of...
cell membranes. Under identical conditions human red blood cells transport a substantial amount of glucose (Fig. 9 B), as anticipated due to the presence of the facilitative glucose transporter (Bell et al., 1993).

Although it is still uncertain whether the Man-P-Cit uptake system is involved in the transbilayer movement of Man-P-Dol, it is encouraging that Man-P-Cit transport is enriched in the ER, the subcellular location where Man-P-Dol is synthesized and functions as a mannosyl donor.

Discussion

Considering the evidence that the “unassisted” transbilayer movement of glycerophospholipids (Kornberg and McConnell, 1971; Rothman and Dawidowicz, 1975) and polyisoprenylphosphosaccharides (Hanover and Lennarz, 1978; McCloskey and Troy, 1980) in artificial lipid bilayers is relatively slow compared to the cellular processes they are involved in, it seems likely that membrane proteins facilitate the transverse diffusion or flip-flopping of the polar head groups of these lipid molecules. The class of membrane proteins referred to as flippases may be essential for the transbilayer movement of a number of membrane phospholipids (Bishop and Bell, 1985; Zachowski and Devaux, 1990; Schroit and Zwaal, 1991; Zachowski, 1993; Trotter and Voelker, 1994), lipid-linked components of cell walls and envelopes in bacteria (Baddiley, 1973; Weppner and Heuhaus, 1978; Mulford and Osborn, 1983; Raetz, 1987), and other membrane proteins such as the Man-P-Cit transport protein.

Table 1. Man-P-Cit Transport into Sealed RER Vesicles from Mouse Liver Is Not Affected by a Variety of Structurally Related Compounds

<table>
<thead>
<tr>
<th>Additions</th>
<th>[3H]Man-P-Cit transported Percentage of control</th>
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<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Man 1-P</td>
<td>91</td>
</tr>
<tr>
<td>GDP-Man</td>
<td>116</td>
</tr>
<tr>
<td>Man 6-P</td>
<td>138</td>
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<tr>
<td>Glc 6-P</td>
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</tr>
<tr>
<td>Glc-P-Cit</td>
<td>101</td>
</tr>
<tr>
<td>Gal-P-Cit</td>
<td>96</td>
</tr>
<tr>
<td>Cit-P</td>
<td>88</td>
</tr>
<tr>
<td>o-Man-P-Cit</td>
<td>89</td>
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Man-P-Cit transport assay mixtures contained 40 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 5 mM EDTA, 0.25 M sucrose, mouse liver microsomal vesicles (200 µg membrane protein), 0.01 mM [3H]Man-P-Cit, and the indicated additions at a final concentration of 1 mM in a total vol of 20 µl. After incubation at 23°C for 30 s, Man-P-Cit transport was assayed as described in Materials and Methods. Vesicular integrity was not affected by any of the added compounds as determined by Man 6-P phosphatase latency.

Figure 7. Man-P-Cit does not enter synthetic liposomes. Man-P-Cit transport assays contained 40 mM Tris-Cl, pH 7.4, 2 mM MgCl₂, 5 mM EDTA, 0.25 M sucrose, 4 µM [2-3H]Man-P-Cit (771 cpm/pmol), and either mouse liver microsomes (280 µg membrane protein) (A) or synthetic liposomes prepared as described in Materials and Methods (B and C) in a total vol of 0.04 ml. After incubation for 1 min at room temperature, transport reaction mixtures were diluted with 0.2 ml ice-cold 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, and chromatographed on a Sephacryl S-200 column (16 ml) equilibrated in 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose at 4°C. 0.75 ml fractions were collected and analyzed for either radioactivity (○) or lipid-phosphorus (■). In the analysis depicted in C, synthetic liposomes were formed by sonication (20 min in a bath sonicator) in the presence of 4 µM [2-3H]Man-P-Cit (771 cpm/pmol) before chromatographic analysis.

Figure 8. Subcellular distribution of Man-P-Cit transport activity in rat liver fractions. ER-enriched vesicles (●), Golgi-enriched vesicles (○), and mitochondria (▲) were assayed for Man-P-Cit transport (A), Man 6-P phosphatase activity (B), galactosyltransferase activity (C), or succinate dehydrogenase activity (D) at various protein concentrations. The procedures for the isolation of the subcellular fractions and evaluation of the integrity of the various organelles are described in Materials and Methods.

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Man-P-Cit transport assay mixtures (A) contained 40 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 5 mM EDTA, 0.25 M sucrose, 0.5 mM [³²H]Man-P-Cit (32 cpm/pmol), and the indicated amount of either rat liver microsomes (●) or human red blood cells (○) in a total vol of 0.02 ml. Glucose transport assay mixtures (B) were identical to Man-P-Cit transport assay mixtures except that Man-P-Cit was replaced with 0.5 mM [¹⁴C]glucose (52 cpm/pmol). After incubation at 21°C for 1 min, the amounts of radio-labeled Man-P-Cit and glucose transported were assayed as described in Materials and Methods. ●—●, ER enriched; ○—○, Golgi complex–enriched; Δ—Δ, mitochondria.

Figure 9. Man-P-Cit is not transported by human red blood cells. Man-P-Cit transport assay mixtures (A) contained 40 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 5 mM EDTA, 0.25 M sucrose, 0.5 mM [³²H]Man-P-Cit (32 cpm/pmol), and the indicated amount of either rat liver microsomes (●) or human red blood cells (○) in a total vol of 0.02 ml. Glucose transport assay mixtures (B) were identical to Man-P-Cit transport assay mixtures except that Man-P-Cit was replaced with 0.5 mM [¹⁴C]glucose (52 cpm/pmol). After incubation at 21°C for 1 min, the amounts of radio-labeled Man-P-Cit and glucose transported were assayed as described in Materials and Methods. ●—●, ER enriched; ○—○, Golgi complex–enriched; Δ—Δ, mitochondria.

The properties of the Man-P-Cit transport process meet several criteria for a protein-mediated uptake system. The Man-P-Cit uptake system in sealed mouse microsomes exhibits a stereoselective preference for β-Man-P-Cit over α-Man-P-Cit, is saturable and protease sensitive. Based on competition studies, the Man-P-Cit transporter exhibits a high degree of recognition for Man-P-Cit relative to several closely related sugar derivatives, including Gal-P-Cit and Glc-P-Cit. All of these properties support the conclusion that Man-P-Cit is transported specifically into microsomal vesicles by a protein-mediated system. It is also unlikely that the mannologue is nonspecifically transported by a sugar nucleotide uptake system since there is apparently no transporter for GDP-Man in the ER (Abeijon and Hirschberg, 1992).

Several experimental observations indicate that the water-soluble analogue is transported into the luminal compartment of sealed microsomes, and does not simply adsorb to the outer leaflet of the lipid bilayer. First, Man-P-Cit does not become associated with synthetic PC liposomes, and is not transported by human erythrocytes. Man-P-Cit transport activity is highest in ER-enriched vesicles relative to Golgi complex–enriched vesicles and mitochondrial fractions. In addition, as noted above, the transport of the water-soluble analogue by liver microsomes is stereoselective, saturable, protease-sensitive, and requires an intact permeability barrier. The calculation of an intravesicular volume from transport data, similar to other reported values (Bishop and Bell, 1985; Carey et al., 1980), also supports the conclusion that Man-P-Cit is transported into the luminal compartment of the microsomal vesicles, and is not bound just by intercalation of the C10-isoprenyl unit into the outer leaflet of the bilayer. The observation that Man₉GlcNAc₂-P-P-Dol is labeled enzymatically when sealed brain microsomes are incubated with [³²H]Man-P-Cit (Waechter and Rush, 1993), provides additional convincing evidence that the water-soluble analogue enters the luminal compartment of the vesicles.

Although the Man-P-Cit transport system satisfies many of the criteria for being a protein-mediated system, it is still speculative that the protein(s) involved in the transport of Man-P-Cit functions as a Man-P-Dol flippase. Despite these promising initial results, additional genetic and biochemical evidence is required to establish definitively that the protein(s) mediating Man-P-Cit transport, is indeed involved in the transbilayer movement of Man-P-Dol. Ultimately, it would be desirable to devise methods for the purification and reconstitution of the putative flippase, and to isolate a mutant cell line with a defective Man-P-Dol flippase which was also defective in Man-P-Cit transport. So far, efforts to select such a mutant have been unsuccessful because of the technical problems of preparing and screening populations of sealed ER vesicles from cultured mammalian cells.

In view of the proposal that Man-P-Dol synthase also mediates the transbilayer movement of the mannolipid (Haselbeck and Tanner, 1984), it will be of interest to examine Man-P-Cit uptake in ER vesicles from the CHO mutant Lec15 which lacks Man-P-Dol synthase activity (Stoll et al., 1982). Man-P-Cit transport studies are also warranted with the CHO mutant, Lec35, which may have a defective flippase since it synthesizes Man-P-Dol, and contains the mannosyltransferases required to convert Man₉GlcNAc₂-P-P-Dol to Man₉GlcNAc₂-P-P-Dol, but the Man-P-Dol–mediated reactions do not occur in vivo (Camp et al., 1993). Current studies are aimed at establishing biochemical correlates by identifying factors that effectively inhibit or stimulate both Man-P-Cit uptake and the transverse diffu-


