Characterization of the Endomannosidase Pathway for the Processing of N-Linked Oligosaccharides in Glucosidase II-deficient and Parent Mouse Lymphoma Cells*

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Stuart E. H. Moore‡ and Robert G. Spiro§
From the Department of Biological Chemistry and Medicine, Harvard Medical School and the Elliot P. Joslin Research Laboratory, Boston, Massachusetts 02215

Studies on N-linked oligosaccharide processing in the mouse lymphoma glucosidase II-deficient mutant cell line (PHA"2.7) as well as the parent BW5147 cells indicated that the former maintain their capacity to synthesize complex carbohydrate units through the use of the deglucosylation mechanism provided by endomannosidase. The in vivo activity of this enzyme was evident in the mutant cells from their production of substantial amounts of glycosylated mannose saccarides, predominantly Glc2Man; moreover, in the presence of 1-deoxynojirimycin or kifunensine to prevent processing by mannosidase I, N-linked ManαGlcNAc2 was observed entirely in the form of the characteristic isomer in which the terminal mannose of the α1,3-linked branch is missing (isomer A). In contrast, parent lymphoma cells, as well as HepG2 cells in the presence of 1-deoxynojirimycin accumulated ManαGlcNAc2 as the primary deglucosylated N-linked oligosaccharide and contained only about 16% of their ManαGlcNAc2 as isomer A. In the presence of the glucosidase inhibitor castanospermine the mutant released Glc2Man instead of Glc2Man, and the parent cells converted their deglucosylation machinery to the endomannosidase route. Despite the mutant’s capacity to accommodate a large traffic through this pathway no increase in the in vitro determined endomannosidase activity was evident.

The exclusive utilization of endomannosidase by the mutant for the deglucosylation of its predominant N-linked Glc2Man,αGlcNAc2 permitted an exploration of the in vivo site of this enzyme’s action. Pulse-chase studies utilizing sucrose-D2O density gradient centrifugation indicated that the Glc2Man,αGlcNAc2 to ManαGlcNAc2 conversion is a relatively late event that is temporally separated from the endoplasmic reticulum-situated processing of Glc2Man,βGlcNAc2 to Glc2Man,αGlcNAc2 and in contrast to the latter takes place in the Golgi compartment.

On the basis of investigations carried out in a number of laboratories, a general outline for the biosynthesis of complex N-linked carbohydrate units has emerged in recent years in which a cotranslational attachment of a glucosylated polymannose oligosaccharide (Glc4Man3GlcNAc3) is followed by a succession of processing reactions involving ER- and Golgi-situated glycosidases and glycosyltransferases (1, 2). Although this established scheme dictates that removal of the 3 glucose residues, a prerequisite for complex oligosaccharide formation, is accomplished by the stepwise action of glucosidase I and II in the ER, it does not accommodate the frequently reported observation that the formation of these carbohydrate units persists to a substantial extent in the presence of glucosidase inhibitors (3-10). This quandary was resolved with the discovery in our laboratory of an endomannosidase which can effect deglucosylation by cleaving the linkage between the glucose-substituted mannose and the remainder of the polymannose oligosaccharide (11, 12). It has been demonstrated that this enzyme, which is unaffected by agents inhibiting glucosidase action (11, 12), can circumvent glucosidase blockade and thereby make possible the continued formation of complex N-linked oligosaccharides (13).

In the present investigation we have further pursued our exploration of the endomannosidase-initiated processing pathway by focusing our attention on a mutant mouse lymphoma cell line (PHA"2.7) described by Reitman et al. (14) which despite a deficiency in glucosidase II produces N-linked complex oligosaccharides. We have obtained evidence in support of our original proposal relative to these cells (11, 12) that the persistent biosynthesis of complex carbohydrate units in this mutant is made possible by the alternate glucose-removal mechanism provided by the endomannosidase pathway. By comparing and quantitating the relevant processing intermediates of the glucosidase-deficient cells with those of the parent lymphoma, it has been possible to evaluate to what extent this route functions under normal conditions. Furthermore, the mutant cells provided us with a unique opportunity to determine at what stage in the processing sequence in which subcellular compartment the endomannosidase-mediated deglucosylation takes place in the in vivo situation.

**EXPERIMENTAL PROCEDURES**

Culture and Radiolabeling of Cells—The mouse lymphoma glucosidase II-deficient mutant cell line (PHA"2.7) and its parent BW5147 cells were a generous gift of Dr. I. S. Trowbridge (Salk Institute of Biological Studies, San Diego, CA); the latter cells were also pur...

[The abbreviations used are: ER, endoplasmic reticulum; CST, castanospermine; DMJ, 1-deoxynojirimycin; endo H, endo-β-1-N-acetylglucosaminidase; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid. All sugars are presumed to be in the β-configuration; reduced sugars are indicated by the designation H2 following the symbol of the monosaccharide.]

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‡ Present address: Dept. of Biochemistry, University of Dundee, Dundee, United Kingdom.
§ To whom correspondence and reprint requests should be addressed: Elliott P. Joslin Research Laboratory, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215.

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chased from ATCC, Rockville, MD. The suspended cells were grown in Dulbecco’s modified Eagle’s medium containing glucose (4.5 g/liter), fetal calf serum (10% Gibco), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in an atmosphere of 95% O2, 5% CO2, and 2% air in a humidified incubator (14). The cells were harvested by centrifugation (140 × g for 5 min) and washed twice with glucose- and serum-free Dulbecco’s modified Eagle’s medium containing 5 mM sodium pyruvate, and radiolabeling was then accomplished by incubating 2.5 × 106 cells in 2 ml of this medium with either 180 μCi of [2-3H]mannose (50 μCi/μmol) or 170 μCi of D-[U-14C]glucose (30 μCi/μmol), Du Pont-New England Nuclear) while being subjected to mild agitation on a rocker platform (Bellco, Vineland, NJ). When the glycosidase inhibitors CST (a gift from Dr. M. Kang, Merrell Dow Research Institute, Cincinnati, OH) and D MJ (purchased from Genzyme) were used, the cells were preincubated with these agents (2 mM each) for 30 min before addition of the radiolabeled sugar. In the incubations used for subcellular fractionation, 1 × 106 cells were pulse labeled for 20 min with 330 μCi of [2-3H]mannose in 1 ml of the glucose-free Dulbecco’s modified Eagle’s medium containing 20 mM sodium pyruvate, and the chase was then initiated by the direct addition of 2 ml of the medium used for cell growth in which 37.5 mM mannose was included throughout these pulse-chase studies as well as in a 30-min preincubution, kifunensine (kindly provided by M. Shihara of the Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) at a concentration of 0.1 mM was present. HepG2 cells (ATCC, Rockville, MD) were cultured and radiolabeled in a manner previously described (13): 100 μCi of D-[U-14C]glucose (315 μCi/μmol) were included in incubations of 100-mm dishes.

Subcellular Fractionation—After pulse-chase radiolabeling of PHA2/2.7 cells, they were separated from the medium and were washed with ice-cold 50 mM HEPES, buffer, pH 7.4, containing 0.15 M NaCl. The cell pellets were suspended in 10 mM HEPES, pH 7.4, 0.25 M sucrose and disrupted by multiple passages of a tight fitting Dounce homogenizer until greater than 90% of the cells had been broken. Postnuclear supernatants (1 ml), prepared by centrifugation of the homogenates at 450 × g for 15 min, were layered on top of 10-50% sucrose gradients as described (13). Centrifugation was carried out at 4°C for 3 h at 164,000 × g in Beckman SW 41 Ti rotor. The gradients were then separated into 0.9-ml fractions with an ISCO gradient collector; sucrose concentrations were determined with an ABBE-3L refractometer (Bellco, Vineland, NJ) at a concentration of 0.1 mM was present.

Extraction of Radiolabeled Cells and Density Gradient Fractions—After radiolabeling, the lymphoma cells were separated from the medium by centrifugation (140 × g for 5 min) and then extracted in a mixture (12 ml) of chloroform/methanol/buffer (3:2:1) as previously described. The extracts, after agitation with an upper phase containing gulosaccharides and an interphase fraction; the latter, after a water wash, was further extracted with chloroform/methanol/water (10:3:10) to yield an oligosaccharide-lipid fraction and a delipidated protein pellet (17).

Fractions from the sucrose density gradients were treated in the same manner as the liver samples, except that all volumes were halved and that carrier protein (2 mg of thyroid membranes) was added prior to the organic solvents.

Preparation of Free Oligosaccharides—After deproteinization of the media with trichloroacetic acid and removal of organic solvents from the upper phase of the cell extracts, oligosaccharides from these two sources were obtained by charcoal-Celite chromatography (30% ethanol eluate) subsequent to removal of salt by passage through Dowex 50 (H+ form) and Dowex 1 (acetate form) as previously described (13).

Isolation of Glycopeptides and Endo H Digestion—Glycopeptides were prepared in a manner previously reported (13) after Pronase digestion of the delipidated protein from the cell extracts and the sucrose density fractions as well as the ether-extracted trichloroacetic acid precipitates from the media. Since more than 90% of the radiolabeled oligosaccharides were found to be associated with medium and cellular glycopeptides were pooled for most of the analyses. Polymannosidase oligosaccharides were released from the glycopeptides by endo H digestion (4 million units, Genzyme); they were desalted and separated from the endo H-resistant glycopeptides by chromatography on columns of Dowex 50 and Dowex 1 and further resolved by high voltage electrophoresis (15).

Endomannosidase and Glucosidase II Assays—In preparation for the enzyme assays the cells (3–6 × 109) were washed with phosphate-buffered saline, suspended in 4 volumes of 100 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM dithiothreitol (14), and disrupted at 4°C with 3 × 5-s bursts of a Branson sonifier (setting No. 1). The resulting homogenates were centrifuged (100,000 × g for 60 min) into a clear supernatant and a membrane pellet; the latter was washed twice with sucrose-glucose medium and separated by centrifugation at a protein concentration of 10 mg/ml. Both the mutant and parental cell line contained 50–60 μg of protein/106 cells, and in both cases about 55% of the total protein was present in the membrane fraction.

For the glucosidase II and endomannosidase assays, 14C-labeled Glc,Man,GlcNAc (10,000 dpm), prepared in a manner previously described (11), was used as substrate in a 100-μl volume. For the glucosidase assay the reaction mixture also contained 100 mM sodium phosphate, pH 7.0, 0.25% Triton X-100, and 0–50 μg of protein while the endomannosidase activity was measured in 100 mM NaMES of 50 mM EDTA 100 mM 2-aminopyridine, pH 7.0, 0.25% Triton X-100, and 0–500 μg of protein; as previously indicated for the latter assay (11), an incubation at 2°C preceded the addition of substrate. Both assays were conducted for 45 min at 37°C at which time the incubation mixtures were deproteinized and desalted by the procedure previously described (11). The products of the enzyme reactions, namely glucose or the disaccharide GlcαGlcMan, were separated by thin layer chromatography in Solvent System A and quantitated by scintillation counting after fluorographic visualization and elution from the plates (11).

Structural and Analytical Procedures—Oligosaccharides released by each assay were purified and resolved by thin layer chromatography were coupled to 2-amino-4pyridine (Aldrich Chemical Co.) by the procedure of Hase et al. (18). Reduction of oligosaccharides was carried out with NaBH4, as previously described (11) while acetylation was performed by the procedure of Hase et al. (18) and subsequently purified on columns of Bio-Gel P-2 (13). Quantitation of radiolabeled neutral sugars was carried out after hydrolysis (1 n HCl, 5 h at 100°C under nitrogen). After passage of the hydrolysates through coupled columns of Dowex 50 (H+ form) and Dowex 1 (acetate form) (20), the components were separated by thin layer chromatography, and the radioactivity was determined by scintillation counting after elution. When oligosaccharides from the mutant were radiolabeled with [14C]glucose the relative specific activities of their glycose and mannose residues was determined in a hydrosate of purified Glc,Man,GlcNAc prepared by endo H digestion of the glycopeptides by the procedure of Bradford (21) using bovine serum albumin as a standard.

Chromatographic Procedures—Affinity chromatography of glycopeptides on concanavalin A-Sepharose (Pharmacia LKB Biotechnol. Inc.) was performed as previously described (13) to yield complex multiantennary (unbound), complex biantennary (10 mM methyl-a-D-mannoside eluate), and polymannose (500 mM methyl-a-D-mannoside eluate) fractions.

Reverse-phase HPLC of desalted pyridylamin-derivatives of polymannose oligosaccharides was performed by a modification of the procedure of Hase et al. (22). Samples (∼10,000 dpm 14C-label) were applied to a LiChrospher 100 RP-18 column (4.6 × 250 mm, 5 μm, Merck) and eluted with the same buffer at a flow rate of 0.4 ml/min. Monitoring of the column effluent was performed with a model 171 radioisotope detector (Beckman Instruments) as previously described (13). To each sample a pyridylamine derivative of [2-3H]mannose labeled Glc,Man,GlcNAc was added for endo H digestion of the glycopeptides by the procedure of Hase et al. (21) using bovine serum albumin as a standard. Thin layer chromatographic resolution of monosaccharides and small oligosaccharides was achieved on plastic sheets precoated with cellulose (0.1-mm thickness, Merck) for 6 h in pyridine/ethyl acetate/water/acetic acid, 5:5:3:1 (Solvent System A). Separation of larger oligosaccharides on plates was accomplished with Silica Gel-Gel (30 μm thickness, Merck) for 24 h in 1-propanol/acetate acid/water, 3:3:2 (Solvent System B). For the separation of hexoses from heptitols, chromatography was undertaken for 20 h on cellulose-coated plates in nitromethane/acetate acid/ethanol/water saturated with Silica Gel-60 (0.2 mm thickness, Merck) for 30 min in 1-propanol/acetate acid/water, 6:3:2 (Solvent System C). The internal standard for all systems was carried out with a wick of Whatman 3MM paper clamped to the top of the thin layer plates. The components were detected by fluorography and quantitated by scintillation counting after elution with water as previously described (13). For preparative
purposes, the oligosaccharide eluates were extracted with peroxide-free ether to remove scintillants and passed through small coupled columns of Dowex 50 (H⁺) and Dowex 1 (acetate). The preparation of radiolabeled oligosaccharide standards has been previously reported (11-13).

Radioactivity Measurements—Liquid scintillation counting was carried out in Ultrafluor with a Beckman LS 7500 instrument. Components on thin layer plates were detected by fluorography at -70°C with X-Omatic AR film (Eastman) after spraying with a scintillation mixture containing a-methylnaphthalene (23).

RESULTS

Evaluation of N-linked Complex Oligosaccharide Synthesis by Parent and Mutant Mouse Lymphoma Cells—Fractionation by concanavalin A-Sepharose chromatography of glycopeptides prepared after [2-³H]mannose labeling indicated that parent lymphoma cells continued to synthesize complex carbohydrate units, although in somewhat reduced amounts, in the presence of the glucosidase inhibitor, CST (24) (Table I). Complex oligosaccharide formation was also carried out by the glucosidase II-deficient mutant as previously reported (14), but this was of a lower level than in the parent cells and was unaffected by the presence of CST (Table I). These results indicate that lymphoma cells can utilize a glucosidase-independent processing pathway for the formation of N-linked complex oligosaccharides.

Identification of Endomannosidase-generated Saccharides—After incubation with [2-³H]mannose, thin layer chromatographic examination of the combined intracellular and medium-free oligosaccharides from CST-treated parent lymphoma cells revealed components which migrated to the positions of the characteristic di-, tri-, and tetrasaccharide products of endomannosidase action (12) with the predominance of Glc3Man; these components were not present in the absence of the glucosidase inhibitor (cf. lanes 1 and 3, Fig. 1). In mutant cell incubations carried out without CST, a prominent trisaccharide was evident which comigrated with Glc3Man (lane 2, Fig. 1), but in the presence of the inhibitor the amount of this component was greatly reduced with the concurrent appearance of an intense spot which moved to the position of Glc4Man (lane 4, Fig. 1). The slower moving components observed in all the lanes (Fig. 1) belong to the previously observed polymannose-GlcNAc₁₂ series (25); in the glucosidase-deficient mutant and the CST-treated parent cells, these oligosaccharides remained closer to the origin due to their larger size.

| Cell type | Distribution of radioactivity | Complex | Biant | Polymannose | Total radioactivity |
|-----------|-------------------------------|---------|-------|--------------|-------------------|
| Parent    | CST                          | Multiant|       |              |                   |
|           | %                             | dpm x 10⁶|
| Parent    | - 34                           | 13      | 58    | 3.3          |
| Parent    | + 25                           | 14      | 61    | 3.1          |
| Mutant    | - 15                           | 9       | 76    | 2.2          |
| Mutant    | + 16                           | 9       | 75    | 2.5          |

* Parent and mutant cells (2 x 10⁶) were incubated with (+) or without (-) CST (2 nm) for 3 h as described under "Experimental Procedures," and an aliquot (8%) of the pooled cellular and medium glycopeptides was fractionated on the lectin columns.

† The percentage distribution of radioactivity into three fractions namely complex multiantennary (multiant), complex biantennary (biant), and polymannose (polymannose) is tabulated.

‡ Radioactivity present in the total glycopeptides from the incubation; more than 90% of the radiolabeled glycopeptides synthesized by the two cell lines were associated with the cells.

The identity of the trisaccharide produced by the lymphoma mutant was confirmed following isolation of this component by preparative thin layer chromatography from [³H]glucose-labeled cells. While acid hydrolysis of the native trisaccharide yielded only glucose and mannose, the latter was released as mannitol when the NaBH₄-reduced oligosaccharide was analyzed (data not shown); these observations as well as the ratio of glucose to mannose of 1.8 (after correction for differences in specific radioactivities) are consistent with a GlcMan₃ structure (12).

Characterization of N-Linked Polymannose Oligosaccharides—Chromatographic examination of the endo H-released oligosaccharides from parent lymphoma cells revealed Man₃GlcNAc and Man₃GlcNAc as the predominant components (lane 1, Fig. 2). In the mutant cells three oligosaccharide species (designated a-c) were evident (lane 2, Fig. 2), which on the basis of their chromatographic migration and the products released by in vitro endomannosidase treatment (Fig. 3) were identified as Glc₃Man, Glc₄Man, and Glc₅Man, respectively, intermixed with small amounts of monoglycosylated components (Glc₃Man, Glc₄Man, and Glc₅Man, respectively).

To inhibit further processing of deglucosylated polymannose units which would be formed in the mutant cells by endomannosidase action, DMJ, a mannosidase I inhibitor (24), was added to the incubations. In the presence of this agent, oligosaccharides appeared among the endo H-released components of the mutant cells which migrated to the position of Man₃GlcNAc and Man₃GlcNAc (f and g in lane 4, Fig. 2.) and which were distinguished from glucosylated species by their resistance to in vitro digestion with endomannosidase (Fig. 3). However, addition of DMJ to parent cell incubations...
ponents were detected by fluorography. The migrations of radiola-
beled standard oligosaccharides are indicated by the following
abbreviations: Glc3Man9GlcNAc; Man9GlcNAc; Man8GlcNAc; Man7GlcNAc; Man6GlcNAc; Man5GlcNAc; Man4GlcNAc; Man3GlcNAc; Man2GlcNAc; Man1GlcNAc; ManGlcNAc; MansGlcNAc; MangGlcNAc; Glc2Man7GlcNAc; Glc2Man6GlcNAc; Glc2Man5GlcNAc; Glc2Man4GlcNAc; Glc2Man3GlcNAc; Glc2Man2GlcNAc; Glc2ManGlcNAc; Glc2ManGlcNAc; Glc2ManGlcNAc; Glc2ManGlcNAc; Glc2ManGlcNAc; Glc2ManGlcNAc. The letters a-g designate components from the mutant
cells which were taken for further study after preparative thin layer
chro-matography. The radioactive material evident at the origins
primarily represents the polymannose-GlcNAc product of endoman-
nosidase action.

resulted in a pronounced accumulation of Man9GlcNAc (lane
3, Fig. 2), a component, which consistent with a glucosidase II deficiency, was not present in the mutant cell line. Indeed
a quantitation of the various endo H-released oligosaccharides formed by the mutant in the presence of DMJ indicated that a substantial portion (~35%) was present as nonglucosylated
species among which, in marked contrast to the parent cells,
Man9GlcNAc was almost completely absent (Table II); the latter component was also missing when CST was added to
DMJ-treated mutant cells (data not shown).

Characterization of Man9GlcNAc Isomers—Since the action of
domannosidase on glucosylated Man9GlcNAc leads to the forma-
tion of a characteristic Man9GlcNAc variant (isomer A), in which the terminal mannoside of the α1,3-linked branch is missing (11), an examination of this oligosaccharide should provide information in regard to the processing routes employed by the parent and mutant lymphoma cells. While HPLC analysis of the Man9GlcNAc from the parent cells indicated that isomer B, in which the terminal mannoside from the middle branch of the polymannose unit is missing (11, 13) was almost exclusively present, essentially only isomer A was detected in the mutant (Fig. 4, Table III). Addition of DMJ
to the parent cell incubations led to a substantial increase in the proportion of isomer A (Fig. 4, Table III) indicating that this component is more rapidly processed in vivo by mannosidase action.

Characterization of endo H-susceptible oligosaccharides
synthesized by parent and mutant mouse lymphoma cells in
the absence and presence of a mannosidase inhibitor. After
incubation of parent (Pt) and mutant (Mt) cells with [3H]glucose
(170 μCi) for 8 h in the presence of DMJ (2 mM) or the absence of inhibitor (CONTROL), the endo H-released oligosaccharides from pooled cellular and medium glycoproteins were chromatographed on a silica gel-coated plate in Solvent System B for 24 h. Equal aliquots (5%) from each incubation were applied to the plate, and the com-
ponents were detected by fluorography. The migrations of radiola-
beled standard oligosaccharides are indicated by the following
abbreviations: G2M5, Glc3Man9GlcNAc; M5, Man9GlcNAc; M6, Man8GlcNAc; M7, Man7GlcNAc; M8, Man6GlcNAc; M9, Man5GlcNAc; M10, Man4GlcNAc; M11, Man3GlcNAc; M12, Man2GlcNAc; M13, ManGlcNAc. The letters a-g designate components from the mutant
cells which were taken for further study after preparative thin layer
chromatography.

FIG. 2. Comparison of endo H-susceptible oligosaccharides
synthesized by parent and mutant mouse lymphoma cells in
the absence and presence of a mannosidase inhibitor. After
incubation of parent (Pt) and mutant (Mt) cells with [3H]glucose
(170 μCi) for 8 h in the presence of DMJ (2 mM) or the absence of inhibitor (CONTROL), the endo H-released oligosaccharides from pooled cellular and medium glycoproteins were separated by cellulose-coated plate for 6 h in Solvent System A. The abbrevia-
tions for the Glcl-zMan standards shown to the left of the chro-
mogram are the same as in Fig. 1. The oligosaccharide composition of each component as determined from the endomannosidase pro-
ducts is indicated above each lane, and the system of abbreviations is
primarily the same as in Fig. 2. The radioactive material evident at the origins
primarily represents the polymannose-GlcNAc product of endoman-
nosidase action.

FIG. 3. Characterization of mutant polymannose oligosac-
charides by in vitro endomannosidase digestion. Endo H-re-
leased components a-g (5000 dpm) obtained from CONTROL and
DMJ-treated mutant lymphoma cells (see Fig. 2) were digested with
Golgi rat liver endomannosidase under conditions described under
"Experimental Procedures" and then submitted to chromatography
on a cellulose-coated plate for 6 h in Solvent System A. The abbrevia-
tions for the Glc1-zMan standards shown to the left of the chro-
mogram are the same as in Fig. 1. The oligosaccharide composition of each component as determined from the endomannosidase pro-
ucts is indicated above each lane, and the system of abbreviations is
primarily the same as in Fig. 2. The radioactive material evident at the origins
primarily represents the polymannose-GlcNAc product of endomannosidase action.

TABLE II

| Oligosaccharides | Mutant | Parent |
|------------------|--------|--------|
| Glc3Man9GlcNAc   | 58     | —      |
| Glc3Man8GlcNAc   | 7      | 8      |
| Man9GlcNAc       | 1      | 62     |
| Man8GlcNAc       | 17     | 18     |
| Man7GlcNAc       | 13     | 6      |
| Man6GlcNAc       | 3      | 4      |
| Man5GlcNAc       | 1      | 2      |
| (Total dpm × 10⁻³) | (409) | (444) |

a The cells were incubated with [3H]glucose in the presence of DMJ as described in Fig. 3. The endo H-released oligosaccharides from pooled cellular and medium glycoprotein were separated by preparative thin layer chromatography in Solvent System B.

b The molar distribution of oligosaccharides was determined by
scintillation counting of the eluted components. The extent of glu-
cosylation of the slower components was determined by endomannosidase digestion (Fig. 4); under the conditions of digestion employed
monoglucosylated species were completely hydrolyzed to yield Glc2Man while diglucosylated oligosaccharides were cleaved to the
extent of 47% to yield Glc3Man. The values for glucosylated oligosaccharides were furthermore corrected for the experimentally deter-
mined difference in specific activity of the glucose and mannoside
resides (Glc/Man = 1.3) as described under "Experimental Procedures."

c Sum of diglucosylated components; primarily Glc3Man9GlcNAc
in mutant while below detection (—) in parent cells.

d Sum of monoglucosylated components; primarily Glc3Man9GlcNAc in mutant as well as parent cells.

e Values in parentheses indicate total radioactivity in the endo H-
released oligosaccharides.
idase I than the B isomer which would also account for the absence of ManαGlcNAc in the mutant when this agent was not present (Fig. 2). Indeed when the mannosidase inhibitor was added to HepG2 cells, this increase in the proportion of isomer A was also evident especially in the secreted glycoproteins (Table III).

Acetolysis studies on the ManαGlcNAc from mutant and parent cells were in agreement with the HPLC analyses. While the reduced oligosaccharide from mutant cells, yielded only products characteristic of isomer A, namely mannobiase, mannotriose, and ManαGlcNACh2, the fragments obtained from the ManαGlcNACh2 of parent cells reflected the presence of both isomers B and A with the latter becoming more prominent when DMJ was added to the incubations (Fig. 5).

Quantitation of the Endomannosidase Processing Pathway in Mutant and CST-treated Parent Lymphoma Cells—Upon incubation with [2-3H]mannose, a close correlation was observed in both types of cells between the sum of the distinctive endomannosidase-derived di-, tri-, and tetrasaccharides and the total molar equivalents of deglucosylated N-linked oligosaccharides among which complex carbohydrate units predominated (Table IV). From these data it becomes evident that endomannosidase can provide a route in lymphoma cells by which a glucosidase deficiency or blockade can be circumvented.

In Vitro Assessment of Endomannosidase Activity in Mutant and Parent Cells—In order to determine if the mutant cell line compensated for its glucosidase II deficiency by increasing its expression of endomannosidase, enzyme analyses were carried out on its soluble and membrane fractions and compared to those from parent cells. These studies clearly indicated that while glucosidase II activity was, as anticipated (14), essentially absent in the mutant, the level of endomannosidase was similar to that observed in the parent cells (Fig. 6). Furthermore, it became evident that endomannosidase was predominantly associated with the membrane fraction in contrast to glucosidase II which was recovered to a substantial extent (~50%) in the soluble portion of the parent cells (Fig. 6); the total in vitro glucosidase II activity toward the GlcManαGlcNAc substrate was calculated to be about 50 times as great as that of endomannosidase.

Subcellular Localization of Endomannosidase Action in the In Vivo State—The glucosidase II-deficient mutant cells, which apparently achieve deglucosylation of their N-linked oligosaccharides exclusively by the action of endomannosidase, provided an attractive system for exploring the subcellular locale in which this enzyme functions since all the ManαGlcNAc produced is in the form of the A isomer. When pulse-chase incubations were carried out with the mutant in the presence of the recently described potent mannosidase I inhibitor, kifunensine, (26) to prevent further processing of
Glycoprotein Processing by Endomannosidase in Lymphoma Cells

**FIG. 5** Characterization of the acetolysis products obtained from Man₆GlcNAc formed by parent and mutant lymphoma cells. After radiolabeling of parent (Pt) and mutant (Mt) cells in the presence (+) or absence (−) of DMJ as described in Fig. 2, the thin layer chromatographically purified and NaBH₄-reduced endo H-released Man₆GlcNAc component was submitted to acetolysis as described under “Experimental Procedures.” The acetolysis products (3000 dpm) were resolved by chromatography on a cellulose-coated plate for 6 h in Solvent System A, and the components were visualized by fluorography. The migration of standards is indicated by the following abbreviations: M, mannose; M₃, mannotriose; M₄GN, Man₆GlcNAcH₂; M₅GN, Man₇GlcNAcH₂. A fragmentation scheme of the Man₆GlcNAc isomer consistent with the acetolysis products is shown above the chromatograms of the samples from the mutant cells treated with DMJ (lane 3) and parent cells incubated without this agent (lane 1). The acetolysis products from the DMJ-treated parent cells (lane 2) indicate a mixture of the two depicted Man₆GlcNAc isomers.

deglucosylated N-linked carbohydrate units, thin layer chromatographic examination of the endo H-released oligosaccharides clearly revealed that the conversion of Glc₃Man₃GlcNAc to Glc₃Man₇GlcNAc is essentially complete at a time when the formation of Man₆GlcNAc is just beginning (Fig. 7). Resolution of the postnuclear supranatants of the radiolabeled cells on the sucrose-D₂O gradient described by Lodish et al. (16) indicated that Man₆GlcNAc-containing glycoproteins, which become evident only after a 90-min chase, are primarily located in two peaks which correspond in density to Golgi vesicles (16), in contrast to the oligosaccharide-lipids which become evident only after a 90-min chase, are primarily situated in the heavier ER membranes (Fig. 8). Due to the presence of the mannosidase inhibitor, the N-linked carbohydrate units remained endo H susceptible throughout the pulse-chase study, and thin layer chromatographic examination of the oligosaccharides released by this enzyme indicated that the ratio of Man₆GlcNAc to its Glc₃Man₇GlcNAc parent type.

**TABLE IV** Evaluation of the amounts of endomannosidase-derived saccharide and deglucosylated polymannose and complex N-linked oligosaccharide formed in mutant and CST-treated parent lymphoma cells during incubation with [2-³H]mannose

| Oligosaccharide       | Cell type* | Mutant | Parent + CST |
|-----------------------|------------|--------|--------------|
|                       | dpm × 10⁻¹⁴|
| Endomannosidase derived|           |        |              |
| Glc₃Man               |            | 4      | 35.0 (35.0)  |
| Glc₃Man               | 19.2 (19.2)| 3.8 (3.8)|            |
| Glc₃Man               | 6.4 (6.4)  | 5.1 (5.1)|            |
| Total                 | (25.6)     | 43.9   |              |
| Deglucosylated N-linked|           |        |              |
| Man₆GlcNAc            | 7.4 (1.0)  | 11.2 (1.6)|            |
| Man₆GlcNAc            | 9.3 (1.6)  | 7.6 (1.3)|            |
| Man₆GlcNAc            | 3.4 (0.7)  | 4.3 (0.9)|            |
| Complex              | 50.0 (16.7)| 110.4 (36.8)|            |
| Total                | (20.0)     | (40.6) |              |

* Lymphoma cells (2 × 10⁷) were incubated with 180 μCi of [2-³H]mannose for 3 h as described under “Experimental Procedures”; CST (2 mM) was included in the parent cell incubations.

† The values represent the radioactivity present in the component from the combined cells and medium; figures in parentheses represent the molar equivalent radioactivity which was calculated by dividing the dpm in the oligosaccharide by the number of mannose residues which it contains.

‡ The tri-, di-, and monoglucosylated mannosaccharides were assayed by scintillation counting after elution from thin layer chromatograms (see Fig. 1).

§, −, indicated that component could not be detected.

**FIG. 6** Comparison of in vitro soluble and particulate endomannosidase and glucosidase II activities in parent and mutant lymphoma cell lines. The enzymes were assayed with ¹⁴C-labeled Glc₃Man₆GlcNAc and increasing amounts of protein from the membrane (O, O) and supernatant (Δ, Δ) fractions of parent (O, Δ) and mutant (○, △) lymphoma cells as described under “Experimental Procedures.” The substrate included in each incubation (10,000 dpm) can yield a maximum of 2233 dpm as disaccharide (Glc1Man₃Man) and 1262 dpm as glucose in the endo-mannosidase and glucosidase assays, respectively.
which causes it to accumulate in the parent cell line, is out in Solvent System B for 24 h, and the components were visualized. Chromatography was carried out in Solvent System B for 24 h, and the components were visualized by fluorography. The system of abbreviations for the oligosaccharide standards are the same as in Fig. 2.

cursor was highest in the lighter membrane fractions of the gradient; a plot of this ratio clearly defined the two Golgi peaks and indicated that no substantial endomannosidase-mediated deglucosylation took place in the ER compartment (Fig. 9).

**DISCUSSION**

From the results presented in this paper, it is apparent that the glucosidase II-deficient mouse lymphoma cell line mutant (PHA*²,7) described by Reitman et al. (14) employs the endomannosidase-initiated processing pathway to maintain its capacity to synthesize complex N-linked oligosaccharides. Indeed the observations made in this study and in a previous investigation with glucosidase inhibitors in HepG2 cells (13) support the proposal made upon discovery of endomannosidase, that the unique deglucosylation mechanism provided by this enzyme can make possible circumvention of a glucosidase

The in vivo operation of endomannosidase in the mutant was most clearly demonstrated by the observation that the characteristic products of this enzyme, namely Glc3Man and N-linked Man₅GlcNAc isomer A, were produced by these cells. The finding that the addition of CST to mutant incubations resulted in the formation of Glc₃Man instead of Glc₃Man indicated that these cells have the capacity to attach as well as remove the outer α1→2-linked glucose residue in a normal manner; indeed in the parent lymphoma cell line, as in HepG2 cells (13), triglucosylmannose is the primary product released in the presence of CST. On the other hand, the failure of the mutant to form Man₆GlcNAc, even in the presence of DMJ which causes it to accumulate in the parent cell line, is consistent with the in vitro demonstrable absence of glucosidase II activity determined with Glc₃Man₅GlcNAc as substrate. The occurrence of small amounts of Glc₃Man and monoglucosylated polymannose units in the mutant, however, suggests the continued presence of some glucosidase function; such residual activity may preferentially degrade the diglucosylated oligosaccharides as it has been reported that at least in vitro glucosidase II has higher activity toward Glc₃Man₅GlcNAc than Glc₃Man₆GlcNAc (27).

Substantial quantities of deglucosylated N-linked polymannose oligosaccharides were produced by the mutant, but because of their rapid further processing by mannosidase I they only become evident when an inhibitor of this enzyme was added to the incubations. The major deglucosylated oligosaccharide species was Man₅GlcNAc (isomer A) although also present were Man₆GlcNAc and Man₇GlcNAc which are presumed to have originated by endomannosidase action on Glc₃Man₅GlcNAc and Glc₃Man₆GlcNAc, respectively. The apparently exclusive utilization of the endomannosidase pathway by the mutant cells for the deglucosylation of the N-linked Glc₃Man₅GlcNAc facilitated our exploration of the in vivo locale of this enzyme’s action. The pulse-chase studies carried out with the mutant cells clearly indicated that the Glc₃Man₅GlcNAc to Man₅GlcNAc conversion is a rather late event which is temporally separated from the ER-situated processing of Glc₃Man₅GlcNAc to Glc₃Man₆GlcNAc, and, in contrast to the latter, takes place in the Golgi compartment. These observations are consistent with previously reported in vitro studies which demonstrated that Golgi membranes have...
two values was plotted for each fraction. The radioactivity recovered from each density gradient fraction of 90-min chased tides prepared from each density gradient fraction of 90-min chased mutant cells (see Fig. 8) were treated with endo H, and the liberated oligosaccharides (50,000 dpm) were resolved by thin layer chromatography on a silica gel-coated plate in Solvent System B. After elution the radioactivity in the Man,GlcNAc and Glc,Man,GlcNAc components was determined by scintillation counting, and the ratio of these two values was plotted for each fraction. The radioactivity recovered from the gradient in Man,GlcNAc and Glc,Man,GlcNAc was 2.73 × 10^5 dpm and 19.9 × 10^5 dpm, respectively.

Although it is apparent from the present investigation and our previous study (13) that endomannosidase can provide glucosidase-deficient or inhibited cells with an alternate processing route, it is not yet clear to what extent this enzyme functions under physiological conditions; in normal cells the appearance of the characteristic glucosylated mannose saccharides (Glc,Man) cannot be used as an index of endomannosidase-deficient or inhibited cells with an alternate processing route. Our analyses of the Man,GlcNAc from parent lymphoma cells revealed that about 16% is present in the form of isomer A and a similar observation was made in HepG2 cells. However, the production of this Man,GlcNAc variant was not evident unless DMJ was added to the incubations indicating that the A isomer is preferentially processed by mannosidase I. Even if all of the Man,GlcNAc and Man,GlcNAc which are formed by the parent lymphoma cells in the presence of DMJ are presumed to the incubations indicating that the A isomer is preferentially processed by mannosidase I, it is unlikely that this relatively modest involvement of endomannosidase in the deglucosylation process of normal cells is due to the low total cellular activity of this enzyme when compared to glucosidase II, as we have shown that mutant lymphoma cells as well as CST-treated HepG2 cells (13) can accommodate a large traffic through this alternate route. Indeed in the glucosidase II-deficient lymphoma cells the increase in the operation of the endomannosidase pathway did not even require a higher level of this enzyme as determined by in vitro assay. The limiting factor to endomannosidase function may be the number of proteins with glucosylated polymannose oligosaccharides which reach the Golgi complex where this enzyme is located. Although in glucosidase-inhibited or deficient cells, all of the glycoproteins which enter this organelle still retain their glucose residues, under normal circumstances extensive removal of these sugars would already have taken place by the ER-situated glucosidase I and II, and only a small portion of the total molecules would still be in the glucosylated form. It is possible that under physiological conditions endomannosidase acts on carbohydrate units with truncated mannone branches for which it is known to have a particularly high affinity (12). Since such partially processed oligosaccharides (e.g. Glc,Man,GlcNAc and Glc,Man,GlcNAc) are poor substrates for cleavage by glucosidase II (28, 29) they would be more likely to reach the Golgi complex.

While this paper was in preparation, a study by Fujimoto and Kornfeld (30) was published which demonstrated that the glucosidase II-deficient mouse lymphoma cell line produces Glc,Man and Glc,Man in a sufficient amount to account for its complex N-linked oligosaccharides formation. While our study is in agreement with these findings, it addresses itself to a substantial number of additional aspects of the glucosidase-independent pathway in lymphoma cells. Our characterization of the deglucosylated N-oligosaccharides has shown that the mutant cells, in contrast to the parent, produces exclusively the distinctive endomannosidase-generated isomer A of Man,GlcNAc. This finding has permitted us to determine the subcellular locale in which this enzyme acts and, furthermore, from a measurement of the Man,GlcNAc isomers in normal cells, we have obtained some insight into the extent to which endomannosidase functions under physiological conditions.

REFERENCES
1. Spiro, R. G., and Spiro, M. J. (1982) Philos. Trans. R. Soc. Lond. Biol. Sci. 300, 117-127
2. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631-664
3. Pan, Y. T., Hori, H., Saul, R., Sanford, B. A., Molyneux, R. J., and Elbein, A. D. (1983) Biochemistry 22, 3975-3984
4. Gross, V., Andus, T., Tran-Thi, T.-A., Schwartz, R. T., Decker, K., and Heinrich, P. C. (1985) J. Biol. Chem. 258, 12203-12209
5. Lodish, H. F., and Kong, N. (1984) J. Cell Biol. 98, 1720-1729
6. Parent, J. B., Yeo, T.-K., Yeo, K.-T., and Olden, K. (1986) Mol. Cell. Biochem. 72, 21-33
7. Foddy, L., and Hughes, R. C. (1988) Eur. J. Biochem. 175, 291-299
8. Duronio, V., Jacobs, S., Romero, P. A., and Herscovics, A. (1988) J. Biol. Chem. 263, 5436-5445
9. Stannard, B. S., Gesundheit, N., Ronin, C., Burnside, J., and Weintraub, B. C. (1988) J. Biol. Chem. 263, 8309-8317
10. Matter, K., McDowell, W., Schwartz, R. T., and Hauri, H.-P. (1988) J. Biol. Chem. 264, 13131-13139
11. Lubas, W. A., and Spiro, R. G. (1987) J. Biol. Chem. 262, 3775-3781
12. Lubas, W. A., and Spiro, R. G. (1988) J. Biol. Chem. 263, 3990-3998
13. Moore, S. E. H., and Spiro, R. G. (1996) J. Biol. Chem. 265, 13194-13112
14. Reitman, M. L., Trowbridge, I. S., and Kornfeld, S. (1982) J. Biol. Chem. 257, 10357-10363
15. Trowbridge, I. S., Hyman, R., Ferson, T., and Mazaukas, C. (1978) Eur. J. Immunol. 8, 715-723
16. Lodish, H. F., Kong, N., Hirani, S., and Rasmussen, J. (1987) J. Biol. Chem. 262, 221-230
17. Spiro, M. J., Spiro, R. G., and Bhoyroo, V. D. (1976) J. Biol. Chem. 251, 6400-6408
18. Hase, S., Iwaki, T., and Ikennaka, T. (1984) J. Biochem. (Tokyo) 95, 197-203
19. Varki, A., and Kornfeld, S. (1980) J. Biol. Chem. 258, 2808-2818
20. Spiro, R. G. (1966) Methods Enzymol. 8, 26–52
21. Bradford, M. (1976) Anal. Biochem. 72, 248–254
22. Hase, S., Natsuka, S., Oku, H., and Ikenaka, T. (1987) Anal. Biochem. 167, 321–326
23. Spiro, M. J., and Spiro, R. G. (1985) J. Biol. Chem. 260, 5808–5815
24. Elbein, A. D. (1987) Annu. Rev. Biochem. 56, 497–534
25. Anumula, K. R., and Spiro, R. G. (1983) J. Biol. Chem. 258, 15274–15282
26. Elbein, A. D., Tropea, J. E., Mitchell, M., and Kaushal, G. P. (1990) J. Biol. Chem. 265, 15599–15605
27. Grinna, L. S., and Robbins, P. W. (1979) J. Biol. Chem. 254, 8814–8818
28. Spiro, R. G., Spiro, M. J., and Bhooryoo, V. D. (1979) J. Biol. Chem. 254, 7659–7667
29. Grinna, L. S., and Robbins, P. W. (1980) J. Biol. Chem. 255, 2255–2258
30. Fujimoto, K., and Kornfeld, R. (1991) J. Biol. Chem. 266, 3571–3578
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S E Moore and R G Spiro

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