Demonstration That Golgi Endo-α-D-mannosidase Provides a Glucosidase-independent Pathway for the Formation of Complex N-linked Oligosaccharides of Glycoproteins*

(Received for publication, January 22, 1990)

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Studies on N-linked oligosaccharide processing were undertaken in HepG2 cells and calf thyroid slices to explore the possibility that the recently described Golgi endo-α-D-mannosidase (Lubas, W. A., and Spiro, R. G. (1987) J. Biol. Chem. 262, 3775-3781) is responsible for the frequently noted failure of glucosidase inhibitors to achieve complete cessation of complex carbohydrates unit synthesis. We have found that in the presence of the glucosidase inhibitors, castanospermine (CST) or 1-deoxynojirimycin, there is a substantial production of the glucosylated mannose saccharides (Glc3Man, Glc2Man, and Glc1Man) which are the characteristic products of endomannosidase action. Furthermore, in HepG2 cells, a secretion of these components into the medium could be demonstrated. Characterization of the N-linked polymannose oligosaccharides produced by HepG2 cells in the presence of CST (as well as 1-deoxynojirimycin to prevent processing by α-mannosidase I) indicated the occurrence, in addition to the expected glucosylated species, of substantial amounts of ManαGlcNAc and ManαGlcNAc. Since ManαGlcNAc was almost completely absent and the ManβGlcNAc isomer was shown to be identical with that formed by the in vitro action of endomannosidase on glucosylated polymannose oligosaccharides, we concluded that this enzyme was actively functioning in the intact cells and could provide a pathway for circumventing the glucosidase blockade. Indeed, quantitative studies in HepG2 cells supported this contention as the continued formation of complex carbohydrate units in the presence of CST inhibition could be accounted for by the deglucosylation effect by endomannosidase.

It is now recognized that in most eukaryotic cells N-glycosylation of proteins is accomplished by a co-translational transfer of a triglucosylated polymannose oligosaccharide (Glc3Man,GlcNAc3) from a lipid carrier followed by a complex series of processing reactions to yield mature asparagine-linked carbohydrate units (1-3). A prerequisite to the formation of the complex-type oligosaccharides is the removal of glucose residues, and this modification is generally believed to be accomplished early in the processing scheme through the sequential action of α-glucosidases I and II which are situated in the rough endoplasmic reticulum (3). However, despite this requirement for glucose excision, it has become apparent from studies in a variety of cells that glucosidase inhibitors fail to achieve complete cessation of complex carbohydrate unit synthesis (4-16), and, that furthermore, a glucosidase II-deficient cell line still has the capacity to form these oligosaccharides (17).

These unexpected findings suggested to us that an alternate processing route which can circumvent a glucosidase blockade may exist and that this pathway may be provided by the Golgi endo-α-D-mannosidase recently discovered in this laboratory (18, 19). This enzyme which can achieve deglucosylation by cleaving the bond between the glucose-substituted mannose residue and the remainder of the polymannose unit is not inhibited by the commonly used agents which interfere with glucosidase action.

In the present investigation, we have explored this hypothesis by studying N-linked oligosaccharide processing in HepG2 cells and thyroid slices in the presence of glucosidase inhibitors. In both cell types, an active endo-α-D-mannosidase could be demonstrated by identification of the characteristic products of this enzyme. Furthermore, detailed quantitative studies of the HepG2 cells indicated that this alternate route can account for the continued formation of complex carbohydrate units during glucosidase blockade.

EXPERIMENTAL PROCEDURES

Culture and Radiolabeling of HepG2 Cells—HepG2 cells (20), obtained from ATCC, Rockville, MD, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in an atmosphere of 95% O2, 5% CO2. Radiolabeling was accomplished by incubating cells which had reached 90% confluency on 100-mm dishes (Falcon) with either 100 μCi of [d-1-14C]glucose (315 μCi/μmol) or 100-200 μCi of [2-3H]mannose (23.5 mCi/μmol), obtained from Du Pont-New England Nuclear, in 2 ml of Dulbecco's modified glucose-free Eagle's medium containing 1 mM sodium pyruvate and 2 mM glutamine; the plates were subject to mild agitation on a rocker platform (Bellco, Vineland, NJ) during this incubation. When glycosidase inhibitors were used (CST), a gift from Dr. M. Kang, Merrell Dow Research Institute, Cincinnati, OH, as well as purchased from Genzyme, Boston; DMJ, purchased from Genzyme; and DNJ, a gift from Drs. E. Truscheit and D. Schmidt, Bayer Research Laboratories, Wuppertal, Federal Republic of Germany), the cells were preincubated for 45 min before addition of the radiolabeled sugar. In pulse-chase studies, a

* This work was supported by Grant DK 17477 from the National Institutes of Health. 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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20-min labeling period with [2-<sup>3</sup>H]mannose (200 μCi) was terminated by a wash with medium containing 2 mM mannose and 1 mM pyruvate, and the cells were then subject to incubations in this unlabeled medium for various periods of time.

*Incubation of Thyroid Slices*—Slices (0.5 g) from fresh calf thyroids were incubated at 37 °C in 1.7 ml of a previously defined medium (21) containing 5 mM pyruvate and 120 μCi of d-[U-<sup>14</sup>C]glucose in an atmosphere of 95% O<sub>2</sub>, 5% CO<sub>2</sub>. As with the cells, a 40-min incubation with the addition of the radiolabeled medium was followed by incubations in this unlabeled medium for various periods of time.

*Extractions of Cells and Slices*—At the end of the HepG2 cell incubations, the medium was carefully removed, and the plates were washed twice with 2 ml of phosphate-buffered saline; these fractions were pooled and stored at −20 °C for further analyses. Subsequently, 4 ml of an ice-cold mixture of methanol, 0.15 M Tris/HC1, pH 7.4, containing 1.4 M MgCl<sub>2</sub>, (21) was added to the rinsed cells which were then scraped from the plates and combined with a further 2 ml wash with this reagent. Chloroform (6 ml) was then added to achieve a final mixture of chloroform/methanol/water (2:2:1) from which the upper phase containing the soluble oligosaccharides was removed for further study. The interphase material was, after a water wash, further extracted with chloroform/methanol/water (10:1:3) as previously described (21) to yield a depleted protein pellet.

At the termination of the thyroid slice incubations, comparable upper phase oligosaccharide and interphase delipidated protein fractions were prepared after separating the tissue from the medium and disrupting the former with a Polytron homogenizer (Brinkmann Instruments) as previously reported (21).

*Preparation of Free Oligosaccharides from Medium and Cells*—For the characterization of free oligosaccharides, organic solvents were evaporated in vacuo at 40 °C from the upper phase of the lipid extracts while the media, after addition of bovine serum albumin (1.25 mg), were deproteinized with a final concentration of 10% ice-cold trichloroacetic acid subsequent to which the latter was removed by repeated extractions with peroxide-free ether. After such treatment, the upper phase and medium samples, in aqueous solution, were passed through coupled columns of Dowex 50-X2, 200-400 mesh (H<sup>+</sup> form) and Dowex 1-X2, 200-400 mesh (acetate form) to yield neutral salt-free fractions which in turn were applied to columns (3 × 10 mm) of charcoal-Celite (Daroce or Bio-Celite 555, 1:1 by weight) (22). After extensive washing with water to remove monosaccharides, these columns were eluted with 30% ethanol (9 ml) to yield an oligosaccharide fraction which was resolved by thin layer chromatography.

*Preparation of Glycopeptides and Endo H Digestion*—The delipidated protein pellets from the HepG2 cells or thyroid slices, as well as the ether-extracted trichloroacetic acid precipitates from the media of the cells, were digested at 37 °C with Pronase (Calbiochem) under the conditions previously specified (23). Upon termination of the digestion, the glycopeptides were absorbed on columns of Dowex 1-X2 (acetate form) overlaid with Dowex 50-X2 (H<sup>+</sup> form), eluted with 2 M pyridine acetate, pH 5.0, and lyophilized. Polyanomannose units were released by digestion of the glycopeptides with 4 milliunits of endo H (ICN Biochemicals Inc. or Genzyme) as previously described. The reaction mixture was desalted on a column (1.5 × 34 cm) of Bio-Gel P-2 equilibrated with 0.1 M ammonium acetate, pH 4.0, and eluted with the same solvent at a flow rate of 0.4 ml/min. The column was monitored with a Model 171 radioisotope detector (Beckman Instruments) using pyridylamine elution mixture in which Bovine Trp III (Beckman Instruments) was mixed with the effluent in a 6:1 ratio. Data were recorded on a SP 4270 recording integrator interfaced with a Workstation Integrator (Spectra-Physics Inc., San Jose, CA). Prior to HPLC analysis, each sample was mixed with a [2-<sup>3</sup>H]mannose-labeled GlcMan<sub>3</sub>GlcNAc pyridylamine derivative to serve as an internal standard.

Thin layer chromatography of monosaccharides through pentasaccharides was carried out on plastic sheets precoated with cellulose (0.1 mm thickness, Merck) for 6 h in pyridine/ethanol/water/acetic acid, 5:5:3:1 (Solvent System A), while resolution of larger oligosaccharides (GlcMan<sub>4</sub>GlcNAcH<sub>2</sub> to Man<sub>4</sub>GlcNAc) was carried out on plastic sheets precoated with Silica Gel 60 (0.2-mm thickness, Merck) for 26 h in 1-propanol/acetic acid/water 3:3:2 (Solvent System B); for the resolution of the pyridylaminol derivatives of the oligosaccharides, chromatography was carried out for 36 h in this system. For the separation of heparosides from hexitols, chromatography was undertaken for 20 h on cellulose coated plates in nitromethane/acetic acid/water saturated with boric acid, 8:1:1 (Solvent System C). The chromatography in all systems was carried out with a wick of Whatman No. 5MM paper clamped to the top of the thin layer plates, and the components were detected by fluorography.

Quantitation was achieved by scraping the areas corresponding to the radioactive spots into vials to which 0.4 ml of water was added followed by 4.6 ml of a 1% solution of trichloroacetic fluid (Ultrafluor, New England Nuclear, Inc.). For preparative purposes, resolved oligosaccharides were eluted from the plates with water, and the resultant eluates, after extraction with peroxide-free ether to remove scintillant, were passed through small coupled columns of Dowex 50 (H<sup>+</sup>) and Dowex 1-X2 (acetate) coupled columns.

For chromatographic standards, metabolically radiolabeled Glc<sub>4</sub>Man<sub>4</sub>GlcNAc and Man<sub>4</sub>GlcNAc were prepared from thyroid oligosaccharide-lipids and glycoproteins, respectively, as previously de-
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RESULTS

Identification of Endomannosidase-generated Mono-, Di-, and Triglycosylated Mannose in HepG2 Cells and Thyroid Slices during Glucosidase Blockade—After incubation of HepG2 cells with [14C]glucose in the presence of glucosidase inhibitors, thin layer chromatographic examination of the free intracellular and medium oligosaccharides revealed the presence of radiolabeled components which co-migrated with the characteristic di-, tri-, and tetrasaccharide products (Glc,Man, Glc2Man, and Glc3Man) of in vitro endo-α-D-mannosidase action (Fig. 1). Although these glucosylated mannose oligosaccharides were not evident in control incubations, the presence of a glucosidase inhibitor, either DNJ or CST, resulted in their formation albeit in distinctively different ratios; the predominance of the tetrasaccharide in the presence of CST is consistent with its known effectiveness as a glucosidase I inhibitor (29, 30).

While the components observed in the media were limited to the Glc,3Man species, the cellular fraction contained a number of larger oligosaccharides (Fig. 1) which were identified as belonging to the previously reported (24) polymannose-GlcNAc2 series; in the presence of glucosidase inhibitors, they remained close to the origin due to their larger size. The components in the cellular fraction of control incubations (Fig. 1, lane 1) which migrated slightly ahead and behind Glc,Man were identified as Man, GlcNAc and Man, GlcNAc, respectively, on the basis of their conversion to Manβ1 → 4GlcNAc by jack bean α-mannosidase treatment. Since these GlcNAc-terminating oligosaccharides were not observed in the media (Fig. 1), the presence of Glc,Man components in this fraction cannot be attributed to a simple leakage from damaged cells. Indeed, pulse-chase studies suggested that the HepG2 cells actively secrete Glc,Man oligosaccharides into the medium. As illustrated for Glc,Man, this tetrasaccharide progressively accumulates in the medium after a period in which the concentration inside the cell reaches a plateau level (Fig. 2).

To confirm that the di-, tri-, and tetrasaccharide species which are produced by HepG2 cells in the presence of the glucosidase inhibitors are in fact identical with the Glc,Man, Glc2Man, and Glc3Man products of in vitro endomannosidase action (19), they were isolated by preparative thin layer chromatography. The purified components (Fig. 3) migrated to the same position on rechromatography as the in vitro products (Rm = 0.76, 0.61, and 0.28, respectively) and were found to be resistant to jack bean α-mannosidase treatment (data not shown). Furthermore, after acid hydrolysis of the native and NaBH4-reduced oligosaccharides, only glucose and mannose or glucose and mannitol, respectively, could be detected in ratios consistent with their proposed structures (Table I).

In order to determine if other cell types also display in vitro endomannosidase activity, the free oligosaccharides produced by calf thyroid slices during [14C]glucose labeling were examined by thin layer chromatography (Fig. 4). Although the chromatographic pattern from these cells was complicated by the presence of prominent radiolabeled components belonging to the maltooligosaccharide series (28), it was apparent that in the presence of DNJ or CST components migrating to the position of Glc,Man and Glc2Man, respectively, were produced (Fig. 4). Treatment of the thyroid oligosaccharide fractions with amyloglucosidase to degrade the maltose oligomers left the presumptive Glc,Man and Glc2Man components intact and presented a chromatographic picture comparable to that observed in the HepG2 cells (cf. Figs. 1 and 4); since the glucosylated mannose oligosaccharides were found to be resistant to amyloglucosidase action, the decrease of the spot at the Glc,Man position in the CST-inhibited sample after treatment with this enzyme (cf. lanes 6 and 7) is due to the removal of a maltooligosaccharide. In thyroid as in HepG2 cells, the glucosylated mannose oligosaccharides were not evident in
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**FIG. 3.** Thin layer chromatography of purified radiolabeled di-, tri-, and tetrasaccharides from HepG2 cell incubations. The components were isolated by preparative thin layer chromatography from the medium after [14C]glucose labeling. The disaccharide and trisaccharide were obtained from incubations carried out in the presence of DNJ while the tetrasaccharide was isolated from CST-inhibited cells (see Fig. 1). Chromatography was performed for 6 h in Solvent System A on a cellulose-coated plate; 2000 dpm was applied to each lane, and the components were visualized by fluorography. The migration of standard mannose is indicated by the arrow.

**TABLE I**

Composition of glucosylated mannose saccharides isolated from HepG2 cell incubations

| Saccharide | Reduction | Ratio of Glc to Man or ManH₂ | Observed | Expected |
|------------|-----------|-----------------------------|----------|----------|
| Disaccharide | -         | 1.0                         | 1        | 1        |
| Disaccharide | +         | 1.0                         | 1        | 1        |
| Trisaccharide | -         | 2.0                         | 2        | 2        |
| Trisaccharide | +         | 2.1                         | 2        | 2        |
| Tetrasaccharide | -       | 2.4                         | 3        | 3        |
| Tetrasaccharide | +         | 3.4                         | 3        | 3        |

* Isolated by preparative thin layer chromatography after labeling of HepG2 cells with [14C]glucose; the di- and trisaccharides were obtained from incubations performed in the presence of DNJ, while the tetrasaccharide was isolated from CST-inhibited incubations (see Fig. 1).

* (+) indicates that the saccharide was reduced with NaBH₄ prior to acid hydrolysis.

* Measured by scintillation counting after acid hydrolysis and thin layer chromatographic separation of the released hexoses and hexitols as described under "Experimental Procedures"; the values were corrected for the experimentally determined difference in specific activity of the glucose and mannose residues (Glc/Man = 1.2). No glucitol was detected in any of the reduced samples.

the incubation conducted in the absence of the inhibitors.

**Characterization of N-linked Polymannose Saccharide Units Present on HepG2 Cell Glycoproteins during Glycosidase Blockade**—In order to establish that endomannosidase-initiated processing does indeed occur in the HepG2 cells, we undertook the task of characterizing the N-linked polymannose oligosaccharides produced during glucosidase blockade and determining if the characteristic Man₉GlcNAc₂ variant (isomer A) produced by the action of this endoglycosidase (18, 19) is a predominant component. Since in the intact cell, as in isolated Golgi membranes (18), peptide-linked Man₉GlcNAc₂ could be rapidly further processed by the action of mannosidase I, we added an inhibitor of this enzyme, DMJ (31), in addition to the CST, to the HepG2 cell incubations.

**FIG. 4.** Thin layer chromatographic examination of free oligosaccharides present in thyroid slices after radiolabeling with [14C]glucose. Following a 160-min incubation with 120 μCi of radiolabeled glucose in the absence (CONTROL) or presence of glucosidase inhibitors (DNJ, 5 mM; CST, 2.2 mM), the oligosaccharides were isolated as described under "Experimental Procedures" and applied to a cellulose-coated plate with (+) or without (−) prior amyloglucosidase treatment. Chromatography was carried out in Solvent System A for 6 h, and the components were visualized by fluorography. The abbreviations for the glucosylated mannose standard are the same as in Fig. 1.

**FIG. 5.** Comparison of the N-linked polymannose units synthesized by HepG2 cells in the absence and presence of glycosidase inhibitors. After incubation of the cells with [14C]glucose (100 μCi) in the presence of CST (2.2 mM) plus DMJ (2 mM) or absence of inhibitor (CONTROL), the reduced endo H-released oligosaccharides from cellular (C) and medium (M) glycoproteins were resolved by chromatography on a silica gel-coated plate in Solvent System B for 26 h. Aliquots representing 3% and 5%, respectively, of the cell and medium fractions were applied to the plate, and the components were detected by fluorography. The migration of radiolabeled standard oligosaccharides is indicated by the following abbreviations: G₃M₉, Glc₃Man₉GlcNAc₂H₉; M₉, Man₉GlcNAc₂H₉; M₆, Man₆GlcNAc₂H₆; M₅, Man₅GlcNAc₂H₅. The letters a-f designate components from the glycosidase-inhibited incubations which were eluted for further structural characterization.

Chromatographic examination of the radiolabeled oligosaccharides released from cell and medium glycoproteins by endo H revealed a very different pattern for control and glycosidase-inhibited incubations (Fig. 5). In the absence of the inhibitors, Man₉GlcNAc and Man₉GlcNAc were the predominant cellular species, while the saccharide units of the medium glycoproteins were largely endo H-resistant presumably because they had been processed to complex-type structures. In contrast, large amounts of endo H-susceptible oligosaccha-
rides were present in cellular as well as medium glycoproteins formed in the presence of CST and DMJ and even a cursory examination indicated a substantial complement of slow-moving glycosylated oligosaccharides as well as prominent Man\(_4\)GlcNAc and Man\(_3\)GlcNAc components and yet very little of the Man\(_5\)GlcNAc (Fig. 5).

After isolation by preparative thin layer chromatography, the oligosaccharides (a–f) from the inhibited cells were characterized by the products which they yielded after extensive jack bean \(\alpha\)-mannosidase digestion. In this procedure, tri-, di-, and monoglucosylated oligosaccharides yield Glc\(_3\)Man\(_4\)GlcNAc, Glc\(_3\)Man\(_3\)GlcNAc, and Glc\(_3\)Man\(_2\)GlcNAc, respectively, while those which have no glucose substitution are degraded to Man\(_3\)GlcNAc, Man\(_2\)GlcNAc, and Glc\(_3\)Man\(_2\)GlcNAc, respectively, while components e and f consisted almost entirely of Man\(_2\)GlcNAc and Man\(_3\)GlcNAc, respectively. Treatment of components a–e with rat liver Golgi endomannosidase confirmed these identifications indicating that indeed a and b were primarily triglucosylated, c and d monoglucosylated, and e unglucosylated (Fig. 6).

A quantitation of the various endo H-released oligosaccharides indicated that even in the glucosidase-inhibited cells a substantial portion was present in the nonglucosylated form as Man\(_2\)GlcNAc and Man\(_3\)GlcNAc (Table II). However, the almost complete absence of Man\(_3\)GlcNAc stood in marked contrast to the situation observed in control incubations and was consistent with an endomannosidase-mediated deglucosylation by the HepG2 cells during glucosidase inhibition (Table II).

The effectiveness of CST as an inhibitor of glucosidase II is evident from the virtual absence of Man\(_3\)GlcNAc; however, the presence of some monoglcsylated oligosaccharides suggests that the action of this agent on glucosidase I, although superior to DNJ (see Fig. 1), was not complete.

**Characterization of Man\(_3\)GlcNAc and Man\(_3\)GlcNAc\(_2\) Isomers**

![Fig. 6. Characterization by in vitro endomannosidase digestion of oligosaccharides from HepG2 glycoproteins synthesized in the presence of CST and DMJ.](image)

The radioactive material evident at the origins primarily represents the polymannose-GlcNAc product of endomannosidase action. The abbreviations for the Glc\(_3\)Man standards are the same as in Fig. 1.

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**Table II**

| Oligosaccharide \* | Control | CST + DMJ |
|-------------------|---------|-----------|
|                   | Cell    | Medium    | Cell    | Medium    |
|                   | molar % | molar %   | molar % | molar %   |
| Glc\(_3\)Man\(_5\)GlcNAc | 1     | 25        | 21      | 21        |
| Glc\(_3\)Man\(_4\)GlcNAc | 1     | 5         | 2       | 2         |
| Glc\(_3\)Man\(_3\)GlcNAc | 1     | 4         | 2       | 2         |
| Glc\(_3\)Man\(_2\)GlcNAc | 7     | 12        | 3       | 3         |
| Glc\(_3\)Man\(_2\)GlcNAc | 1     | 8         | 3       | 3         |
| Man\(_5\)GlcNAc    | 21     | 17        | 2       | 3         |
| Man\(_4\)GlcNAc    | 48     | 20        | 25      | 24        |
| Man\(_3\)GlcNAc    | 14     | 46        | 12      | 23        |
| Man\(_2\)GlcNAc    | 10     | 17        | 4       | 8         |
| (Total dpm \(\times 10^{10}\)) | (770) | (107)     | (1672)  | (914)     |

\*After incubation of HepG2 cells with \[^{14}C\]glucose under conditions described in Fig. 5, the oligosaccharides released from cellular and medium glycoproteins by endo H were resolved by preparative thin layer chromatography as described in "Experimental Procedures." The individual components (illustrated in Fig. 5, a–f) were eluted from a preparative chromatogram and characterized by their migration before and after treatment with jack bean \(\alpha\)-mannosidase as discussed in text.

\*The molar distribution of oligosaccharides was established by scintillation counting of the eluted components. When \(\alpha\)-mannosidase digestion revealed the presence of more than one species, their molar ratio was ascertained from the core oligosaccharides obtained after \(\alpha\)-mannosidase treatment. The values for glucosylated oligosaccharides were corrected for the experimentally determined difference in specific activity of the glucose and mannose residues (Glc/Man = 1.2).

\*Values in parentheses indicate the total radioactivity of the endo H-released oligosaccharides.

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**Effect of glycosidase inhibitors on the nature and molar distribution of endo-H-susceptible N-linked carbohydrate synthesized by HepG2 cells**

Examination of the Man\(_3\)GlcNAc obtained from glycoproteins synthesized by HepG2 cells under control and exoglycosidase-inhibited (CST + DMJ) conditions indicated a distinct difference in the elution position of the predominant isomer (Fig. 7). In the uninhibited cells, Man\(_3\)GlcNAc co-eluted with the major isomer from thyroid (component B) which is known to have the terminal mannose of the middle branch of the polymannose unit missing (18). The Man\(_3\)GlcNAc from the cells incubated in the presence of CST and DMJ co-eluted almost exclusively with component A from thyroid (Fig. 7) and in a position identical to the product of in vitro action of rat liver Golgi endomannosidase on Glc\(_3\)Man\(_5\)GlcNAc substrate (chromatograph not shown) in which the terminal mannose of the \(\alpha\,\beta\)-linked branch of the polymannose saccharide is absent (18).

To further establish the identity of the Man\(_3\)GlcNAc isomers, acetylation studies were undertaken. When this procedure, which selectively cleaves 1,6-glycosidic bonds, was applied to the reduced Man\(_3\)GlcNAc from cellular and medium glycoproteins, the oligosaccharide from control HepG2 cells was fragmented into manno- and Man\(_3\)GlcNAc\(_2\) while
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FIG. 7. Reverse-phase HPLC separation of Man₉GlcNAc₅ isomers as their pyridylamino derivatives. After incubation of thyroid slices and HepG2 cells with [¹⁴C]glucose under the conditions specified in Figs. 4 and 5, respectively, the endo H-released oligosaccharides were coupled to 2-aminopyridine. Subsequent to isolation by preparative thin layer chromatography, the Man₉GlcNAc-pyridylamino component (10,000 dpm) was analyzed on a Microsorb C₅ column as described under “Experimental Procedures”; radioactivity was determined by scintillation counting with a flow-through detector. The position of elution of the three isomers of Man₉GlcNAc are designated by the letters A, B, and C, and these were identified as having a mannose residue missing in the α₁,3-linked, middle, and α₁,6-linked chain of the polymannose unit, respectively. The pyridylamino derivative of [³H]mannose-labeled GlcMan₉GlcNAc which was used as an internal standard emerged from the column at 82 min.

Fig. 8. Identification of the acetylation products derived from Man₉GlcNAc synthesized by HepG2 cells in the presence and absence of glycosidase inhibitors. After incubation of the cells with [¹³C]glucose under the conditions specified in Fig. 5, the endo H-released Man₉GlcNAc from cell (C) and medium (M) glycoproteins was isolated by preparative thin layer chromatography and, after NaBH₄ reduction, was submitted to acetylation as described under “Experimental Procedures.” The acetylation products were resolved by chromatography on a cellulose-coated plate for 6 h in Solvent System A, and the components were detected by fluorography. The migration of standards are indicated (M, mannose; M*, mannobiose; M₁, mannotriose; M₂GN, Man₃GlcNAc; M₃GN, Man₅GlcNAc). A scheme showing acetylation cleavages on the Man₉GlcNAc isomer consistent with the observed fragments is shown above the chromatograms from the control and inhibited samples; the symbols M and GN are used for mannose and N-acetylglucosamine, respectively.

Evaluation of Complex Carbohydrate Unit Formation by HepG2 Cells during Glucosidase Blockade and Quantitation of the Endomannosidase Processing Pathway—Since analyses of the N-linked polymannose carbohydrate units produced in HepG2 cells indicated that the block in glucose removal could be circumvented by endomannosidase action, we undertook to determine if further processing into complex oligosaccharides could take place during glucosidase inhibition. When glycopeptides prepared after [2⁻³H]mannose labeling were fractionated by a concanavalin A chromatographic procedure (35), it became evident that indeed the synthesis of complex carbohydrate units continues in CST-inhibited cells, although in reduced amounts (Fig. 9). In the presence of the glucosidase inhibitor, an anticipated increase in the polymannose units of cellular glycoproteins was observed while there was about a 50% reduction in the complex multiantennary oligosaccharides; the concanavalin A fraction of the cellular glycoproteins in which the complex biantennary oligosaccharides are known to elute did not show a decrease presumably because of an enhanced formation of hybrid units (Fig. 9). While in the presence of CST, the radioactivity of glycopeptides from the medium was reduced to about one-half of the control levels, concanavalin A chromatography indicated that these secreted glycoproteins, unlike the cellular proteins, had the normal ratio of multiantennary, biantennary, and polymannose oligosaccharides (Fig. 10).
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In order to assess whether endomannosidase action could account for the persistent formation of complex carbohydrate units by HepG2 cells in the presence of CST, we determined the sum of the distinctive products released by this enzyme (Glc, Man, Glc, Man, and Glc, Man) during an incubation with [2-\(3^H\)]mannose and compared it, on a molar basis, to the endo H-resistant oligosaccharides formed during that time taking into account the presence of 3 mannose residues per unit and correcting for radiolabeling of fucose substituents. Such a calculation could be made with reasonable precision as in the presence of CST, without an additional mannosidase inhibitor, no N-linked unglucosylated polymannose oligosaccharides (Man\(_n\)-GlcNAc) could be detected (data not shown) presumably due to their rapid processing into complex carbohydrate units. The close correlation evident from these calculations between complex carbohydrate unit production and the appearance of mono-, di-, and triglucosylated mannose (Table III) indicated that endomannosidase does indeed provide the route by which the glucosidase blockade is circumvented.

**DISCUSSION**

It is apparent from the studies reported in this paper that the recently described Golgi endo-\(\alpha\)-D-mannosidase (18, 19) does function in vivo and can initiate a processing route which permits the formation of complex N-linked carbohydrate units in the presence of a glucosidase blockade. Indeed, our findings indicate that the frequently noted failure of glucosidase inhibitors to stop complex oligosaccharide synthesis (4-16) in a variety of cells can be attributed to the alternate glucose-removal mechanism which the endomannosidase provides; furthermore, the continued formation of those oligosaccharides observed in glucosidase-deficient cells (17) could be explained on this basis.

The identification of the characteristic glucosylated mannose residues (designated as "endomannosidase-derived") in the endo H-resistant glycopeptides was in close agreement to that recovered in fractions 1 and 2 from concanavalin A column fractionation.

**TABLE III**

Quantitation of endomannosidase-derived saccharides and complex N-linked oligosaccharides formed by HepG2 cells during incubation with [2-\(3^H\)]mannose in the presence of CST

| Component* | Radioactivity* | Relative molar amount* |
|------------|---------------|----------------------|
| Endomannosidase derived: | | |
| Glc3Man | 26 | 0.58 |
| Glc2Man | 7.1 | 0.16 |
| Glc,Man | 16 | 0.35 |
| Total | (49.1) | (1.09) |
| Complex oligosaccharides | 135* | 1.00 |

* Following incubation of HepG2 cells with [2-\(3^H\)]mannose (100 \(\mu Ci\) for 3 h in the presence of CST, the tri-, di-, and monoglucosylated mannose saccharides (designated as "endomannosidase-derived") were purified from cells and medium and assayed after elution from thin layer chromatography by scintillation counting as described under "Experimental Procedures." Treatment of pronase-digested cellular and medium glycoproteins with endo H yielded glycopeptides containing the "complex oligosaccharides." The radioactivity recovered in these endo H-resistant glycopeptides was in close agreement to that recovered in fractions 1 and 2 from concanavalin A column fractionation.

* The values represent the sum of the radioactivity present in the component isolated from cells and medium.

* The molar ratios were calculated from the radioactivity on the basis that each complex oligosaccharide contains 3 mannose residues, while the endomannosidase-derived saccharides each have a single mannose residue.

* Values represent radioactivity in mannose residues as determined after acid hydrolysis of the glycopeptides and separation of this monosaccharide from radiolabeled fucose by thin layer chromatography.
nose products (Glc₃Man, Glc₂Man, and GlcMan) of endo-
mannosidase (19) in both HepG2 cells and thyroid slices in
the presence of glucosidase inhibitors provided the major
evidence for the in vivo action of this enzyme. These compo-

tents, which were resistant to α-mannosidase and anyoglu-
cosidase treatment, were found intracellularly as well as in
the medium. Indeed, pulse-chase studies in HepG2 cells in-
dicated that the Glc₃Man saccharides are actively secreted
in contrast to the large assortment of polymannose-GlcNAcz-
oligosaccharides (24) which remain associated with the cells.
The mechanism by which the glucosylated mannose products
of endomannosidase action reach the exterior of the cell is
not known and is currently under investigation.

Characterization of the N-linked polymannose oligosaccha-
rides formed in HepG2 cells during glucosidase blockade
provided persuasive additional evidence that the endomann-
osidase is actively functioning. Incubation of the cells with
CST, with the further addition of DMJ to prevent processing
by mannosidase I of endomannosidase-generated saccharide
units, resulted in the appearance of substantial amounts of
N-linked Man₃GlcNAcz which, in contrast to control incuba-
tions, occurred almost exclusively as the isomer in which the
terminal mannose on the α1,3-linked branch is missing
(isomer A). Also of importance was the observation that in
the exoglycosidase-inhibited cells Man₄GlcNAcz was almost
completely absent although this component was present in
substantial amounts in control cells and was, as anticipated
(36), even more abundant when DMJ alone was used as a
inhibitor (data not shown). The presence of substantial
amounts of Man₄GlcNAcz in CST-inhibited cells despite the
almost complete absence of Man₃GlcNAcz indicated that the
endomannosidase-catalyzed conversion of Glc₃Man₃Glc-
NAcz to Man₄GlcNAcz must represent a major deglucosyla-
tion pathway which stands in contrast to the sequential
removal of glucose residues by glucosidases I and II followed
by mannose excision through the action of exo α-D mannos-

ides (3).

The occurrence of N-linked Man₄GlcNAcz and Man₅Glc-
NAcz oligosaccharides in the CST + DMJ-treated HepG2
cells is quite consistent with an endomannosidase-mediated
deglucosylation route as in vitro studies have shown that this
enzyme acts optimally on oligosaccharides with truncated
mannose branches (19). Glucosylated Man₅GlcNAcz and
Man₆GlcNAcz, which have been noted as minor N-linked
processing intermediates under physiological conditions (37-
39), could, at the elevated levels occurring during glucosidase
blockade, provide the substrate for the formation of substan-
tial quantities of Man₄GlcNAcz and Man₅GlcNAcz, respec-
tively, by endomannosidase action. Indeed, we have observed
that Glc₃Man is released in substantially greater amounts
(1.6-fold) in CST-treated HepG2 cells than in those exposed
to CST plus DMJ, and this is consistent with an enhanced in vivo
action by endomannosidase on mannose-trimmed car-
bohydrate units.

Our studies with the HepG2 cells indicated that, despite the
dramatically effective glucosidase inhibition brought about by
CST, complex carbohydrate unit formation was inhibited by no more than 50%. This observation is in accord
with the findings of numerous investigations (4-16) which
indicated that only a partial blockade of complex oligosaccha-
ride synthesis could be imposed by glucosidase inhibitors with
an effectiveness ranging from 30% to 90%. Our measurements
of radioactivity in the characteristic glucosylated mannose
saccharides (Glc₃Man) produced by CST-inhibited HepG2
cells during [3H]mannose labeling clearly indicated that the
endomannosidase activity was sufficient to account for all of
the complex oligosaccharides formed during the glucosidase
blockade. The possibility that the glucosidase inhibition could
be circumvented by a direct transfer of Man₅GlcNAcz from a
dolichol intermediate to protein as has been suggested from
studies with F9 teratocarcinoma cells (35) would not explain
the large amount of Man₅GlcNAcz observed in the HepG2
cells and is furthermore made unlikely by the observation
that endo H-released Man₅GlcNAcz in our system is distinct
from the isomer which is associated with the lipid intermedi-
ate (34).

Although our studies with glucosidase-inhibited cells pro-
vide a clear indication of the potential capacity of the endomannosidase-initiated processing route, we are unable to de-
dtermine at this time to what extent this alternate mechanism
for deglucosylation operates under physiological conditions.
Our inability to detect the characteristic Glc₃Man saccha-
rades in control HepG2 incubations does not necessarily in-
dicate a lack of endomannosidase activity as these compo-
nents might undergo degradation in the absence of a glucosi-
dase inhibitor. While analyses of the N-linked polymannose
oligosaccharides from uninhibited cells indicated that only
relatively small amounts of the Man₅GlcNAcz isomer A was
present in thyroid and essentially none in HepG2 cells, the
interpretation of these findings in relation to endomannosi-
dase activity has again to be qualified as this isomer once
formed may undergo rapid further processing.

Judging from the substrate specificity of the endomanno-
sidase (19), it is likely that, under normal circumstances, its
primary in vivo action is on monoglucosylated N-linked pol-
ymannose oligosaccharides. Since it has been generally ob-
served that excision of the outer 2 glucose residues occurs
much more rapidly than that of the mannose-linked glucose
(37,39,40), it may indeed be anticipated that oligosaccharides
containing this latter substituent are the most likely to be
exported to the Golgi complex where the endomannosidase
is known to be located (18). From our studies with glucosidase-
inhibited cells, it is however apparent that even proteins with
triglucosylated polymannose units can leave the rough endo-
plasmic reticulum and enter the Golgi cisternae to present
themselves as substrate for the endomannosidase. The action
of this enzyme apparently makes possible the secretion of
proteins with a normal ratio of complex to polymannose N-
linked oligosaccharides as long as further processing by man-
nosidase I can proceed; however, if the latter enzyme is also
inhibited, the HepG2 cells do export glycoproteins which
contain almost exclusively the polymannose carbohydrate
units.

It is apparent that the function of endomannosidase under
physiological conditions requires further definition, and, as
part of such a study, our laboratory is currently evaluating
the selectivity of this enzyme for specific glycoproteins and
N-glycosylation sites.

REFERENCES
1. Hubbard, S. C., and Ivatt, R. J. (1981) Annu. Rev. Biochem. 50,
555-583
2. Spiro, R. G., and Spiro, M. J. (1982) Philos. Trans. R. Soc. Lond.
B Biol. Sci. 300, 117-127
3. Kasai, K., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54,
631-664
4. Pan, Y. T., Horii, H., Saul, R., Sanford, B. A., Molyneux, R. J.,
and Elbein, A. D. (1983) Biochemistry 22, 3975-3984
5. Gross, V., Andus, T., Tran-Thi, T. A., Schwarz, R. T., Decker,
K., and Heinrich, P. C. (1985) J. Biol. Chem. 258, 12203-
12209
6. Lodish, H. F., and Kong, N. (1984) J. Cell Biol. 98, 1720-1729
7. Romero, P. A., Saunier, B., and Herscovics, A. (1985) Biochem.
J. 226, 733-740
8. Sasaki, V. W., Ordovas, J. M., Elbein, A. D., and Berninger, R.
In Vivo Processing of Glycoproteins by Endo-α-D-mannosidase

W. (1985) Biochem. J. 232, 759-766

10. Gross, V., Tran-Thi, T.-A., Schwarz, R. T., Elbein, A. D., Decker, K., and Heinrich, P. C. (1986) Biochem. J. 236, 853-860

11. Parent, J. B., Yeo, T.-K., Yeo, K.-T., and Olden, K. (1986) Mol. Cell. Biochem. 72, 21-33

12. Hughes, R. C., Foddy, L., and Bause, E. (1987) Biochem. J. 247, 537-545

13. Foddy, L., and Hughes, R. C. (1988) Eur. J. Biochem. 175, 291-299

14. Duronio, V., Jacobs, S., Romero, P. A., and Herscovics, A. (1988) J. Biol. Chem. 263, 5438-5445

15. Stannard, B. S., Gesundheit, N., Ronin, C., Burnside, J., and Weintraub, B. C. (1988) J. Biol. Chem. 263, 8309-8317

16. Matter, K., McDowell, W., Schwartz, R. T., and Hauri, H. P. (1989) J. Biol. Chem. 264, 13131-13139

17. Reitman, M. L., Trowbridge, I. S., and Kornfeld, S. (1982) J. Biol. Chem. 257, 10357-10363

18. Lubas, W. A., and Spiro, R. G. (1987) J. Biol. Chem. 262, 3775-3781

19. Lubas, W. A., and Spiro, R. G. (1988) J. Biol. Chem. 263, 3990-3998

20. Knowles, B. B., Howe, C. C., and Aden, D. P. (1980) Science 209, 497-499

21. Spiro, M. J., Spiro, R. G., and Bhoyroo, V. D. (1976) J. Biol. Chem. 251, 6400-6408

22. Spiro, R. G. (1966) Methods Enzymol. 8, 26-52

23. Spiro, M. J., Spiro, R. G., and Bhoyroo, V. D. (1979) J. Biol. Chem. 254, 7666-7674

24. Anumula, K. R., and Spiro, R. G. (1983) J. Biol. Chem. 258, 15274-15282

25. Varki, A., and Kornfeld, S. (1983) J. Biol. Chem. 258, 2808-2818

26. Hase, S., Ibuki, T., and Ikenaka, T. (1984) J. Biochem. (Tokyo) 95, 197-203

27. Hase, S., Natsuka, S., Oku, H., and Ikenaka, T. (1987) Anal. Biochem. 167, 321-326

28. Spiro, M. J., and Spiro, R. G. (1985) J. Biol. Chem. 260, 5808-5815

29. Palamarczyk, G., and Elbein, A. D. (1985) Biochem. J. 227, 795-804

30. Kaushal, G. P., Pan, Y. T., Tropea, J. E., Mitchell, M., Liu, P., and Elbein, A. D. (1988) J. Biol. Chem. 263, 17278-17283

31. Bischoff, J., and Kornfeld, R. (1984) Biochem. Biophys. Res. Commun. 125, 324-331

32. Byrd, J. C., Tarentino, A. L., Maley, F., Atkinson, P. H., and Trimble, R. B. (1982) J. Biol. Chem. 257, 14557-14566

33. Romero, P. A., and Herscovics, A. (1986) J. Biol. Chem. 261, 15936-15940

34. Chapman, A., Li, E., and Kornfeld, S. (1979) J. Biol. Chem. 254, 10243-10249

35. Kornfeld, K., Reitman, M. L., and Kornfeld, R. (1981) J. Biol. Chem. 256, 6633-6640

36. Bischoff, J., Liacum, L., and Kornfeld, R. (1986) J. Biol. Chem. 261, 4766-4774

37. Godelaine, D., Spiro, M. J., and Spiro, R. G. (1981) J. Biol. Chem. 256, 10161-10168

38. Atkinson, P. H., and Lee, J. T. (1984) J. Cell Biol. 98, 2245-2249

39. Kornfeld, S., Li, E., and Tabas, I. (1978) J. Biol. Chem. 253, 7771-7778

40. Hubbard, S. C., and Robbins, P. W. (1979) J. Biol. Chem. 254, 4569-4576