Processing of MOPC 315 Immunoglobulin A Oligosaccharides: Evidence for Endoplasmic Reticulum and Trans Golgi α1,2-Mannosidase Activity

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ABSTRACT The processing of asparagine-linked oligosaccharides on the α-chains of an immunoglobulin A (IgA) has been investigated using MOPC 315 murine plasmacytoma cells. These cells secrete IgA containing complex-type oligosaccharides that were not sensitive to endo-β-N-acetylglucosaminidase H. In contrast, oligosaccharides present on the intracellular α-chain precursor were of the high mannose-type, remaining sensitive to endo-β-N-acetylglucosaminidase H despite a long intracellular half-life of 2–3 h. The major [3H]mannose-labeled α-chain oligosaccharides identified after a 20-min pulse were Man₉GlcNAc₂ and Man₇GlcNAc₂. Following chase incubations, the major oligosaccharide accumulating intracellularly was Man₆GlcNAc₂, which was shown to contain a single α₁,2-linked mannose residue. Conversion of Man₆GlcNAc₂ to complex-type oligosaccharides occurred at the time of secretion since appreciable amounts of Man₇GlcNAc₂ or further processed structures could not be detected intracellularly. The subcellular locations of the α₁,2-mannosidase activities were studied using carbonyl cyanide m-chlorophenylhydrazone and monensin. Despite inhibiting the secretion of IgA, these inhibitors of protein migration did not effect the initial processing of Man₇GlcNAc₂ to Man₆GlcNAc₂. Furthermore, no large accumulation of Man₇GlcNAc₂ occurred, indicating the presence of two subcellular locations of α₁,2-mannosidase activity involved in oligosaccharide processing in MOPC 315 cells. Thus, the first three α₁,2-linked mannose residues were removed shortly after the α-chain was glycosylated, most likely in rough endoplasmic reticulum, since this processing occurred in the presence of carbonyl cyanide m-chlorophenylhydrazone and monensin. However, the removal of the final α₁,2-linked mannose residue as well as subsequent carbohydrate processing occurred just before IgA secretion, most likely in the trans Golgi complex since processing of Man₇GlcNAc₂ to Man₅GlcNAc₂ was greatly inhibited in the presence of monensin.

A number of membrane, lysosomal, and secretory glycoproteins have been studied to define the subcellular location of the carbohydrate modifications required to form complex-type asparagine-linked oligosaccharide chains. It is known that the biosynthesis of the N-linked complex-type oligosaccharides of proteins involves the transfer of a high molecular weight oligosaccharide containing glucose, mannose, and N-acetylglucosamine from dolichol diphosphate to asparagine residues in the protein (1). Once transferred to protein, oligosaccharides destined to become complex-type are processed extensively. The terminal glucose residues are removed rapidly by specific membrane-bound glucosidases thought to be located in the rough and smooth endoplasmic reticulum (2–4). Subsequently, the four α₁,2-linked mannose residues are removed by one or more specific α₁,2-mannosidases. There is evidence indicating that this initial mannose processing could occur in the Golgi apparatus since enzymes capable of removing these α₁,2-linked mannose residues have been isolated from purified rat liver Golgi membranes (5, 6). The Golgi apparatus is also the major subcellular location of the enzymes responsible for further carbohydrate modifications. These enzymes include the N-acetylglucosaminyltransferase,
which attaches an N-acetylglucosamine residue to a Man$_3$GlcNAc$_2$ species (5, 7, 8), α-mannosidase II, which acts on GlcNAcMan$_5$GlcNAc$_2$ to remove the terminal α1,3-linked and α1,6-linked mannose residues to form GlcNAcMan$_5$GlcNAc$_2$ (5–8), and the glycosyltransferases that are involved in the elongation of the complex oligosaccharides.

Despite the isolation and partial purification of presumed processing α-mannosidases from rat Golgi membranes, others studying different cell types have suggested that the enzymatic removal of mannose residues occurs, at least in part, in the rough endoplasmic reticulum. For example, Tartakoff and Vassalli (9) have shown that trimming of mannose residues from IgM-μ-chain oligosaccharides occurs in mouse plasmablasts even in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP)$^1$, an uncoupler of oxidative phosphorylation known to inhibit intracellular protein migration from the rough endoplasmic reticulum (10). In contrast, Godelaine et al. (11) have suggested that only a single α1,2-linked mannose is removed in the rough endoplasmic reticulum of calf thyroid cells, since they observed the accumulation of thyroglobulin-linked Man$_4$GlcNAc$_2$ in thyroid slices labeled for 1 h and chased in the presence of CCCP. Hakimi and Atkinson (12) also concluded that one α1,2-linked mannose residue is removed from the Sindbis virus proteins B and PE2 in the rough endoplasmic reticulum of chick embryo fibroblasts. An α1,2-mannosidase has recently been partially purified from the rough endoplasmic reticulum of rat liver cells (13).

In view of these findings, we have examined the processing of immunoglobulin A (IgA) α-chain (heavy chain) oligosaccharides isolated from MOPC 315 plasmacytoma cells. In this study, we present evidence of at least two distinct subcellular locations of α1,2-mannosidase activity involved in the processing of IgA α-chain oligosaccharides. Processing α1,2-mannosidase activity appears to be present in the endoplasmic reticulum and the trans Golgi complex of these cells.

**MATERIALS AND METHODS**

1. Preparation of α-IgA
2. Oligosaccharides
3. Glycopeptides and Oligosaccharides
4. Pulse and chase experiments
5. Incorporation of α-[3H]Leucine into MOPC 315 α-glycopeptides

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$^1$ Abbreviations used in this paper: CCCP, Carbonyl cyanide m-chlorophenylhydrazone; GlcNAcNAc, N-acetylgalactosaminol; HPLC, high performance liquid chromatography; IgA, immunoglobulin A; NP-40, nonident P-40; SaCl, fixed protein A bearing Staphylococcus aureus.

Benzinger (Washington University School of Medicine). 3(30) scintillation fluid and TS-1 were obtained from Research Products International Corp., Mt. Prospect, IL.

**Plasmacytoma Line:** The α- secreting MOPC 315/I cells that were provided by Dr. Richard Lynch (Department of Pathology, University of Iowa). The cells were maintained as suspension cultures in 1 X 15 medium containing 10% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. This cell line has a doubling time of ~24 h at 37°C and was kept at a density of 3 X 10$^5$ to 1 X 10$^6$ cells/ml in RPMI supplemented with 10% fetal calf serum and 2% L-glutamine, 2% sodium bicarbonate, and 2% sodium pyruvate.

**Pulse Labeling of MOPC 315 Cells**

Incorporation of α-[3H]Leucine into MOPC 315 α-glycopeptides

**Preparation and isolation of α-[3H]Mannose-labeled glycopeptides and oligosaccharides:** Glycopeptides were prepared from α-[3H]Mannose-labeled IgA by incubation with the washed SäCi immunoprecipitations with 5 mg of pronase for 48 h in 500 μl of 0.1 M Tris-HCl buffer, pH 8.0 containing 0.001 M CaCl$_2$, 0.1 M mannose, and 0.05 M glucose-6-phosphate. Digestions were carried out at 37°C under toluene. Additional 5-mg amounts of pronase were added to the samples at 12, 24, and 36 h and the reactions were stopped by placing the digestion tubes in boiling water for 2 min. The pronase digests were then subjected to gel filtration on a column (1.5 X 95 cm) of Bio-Gel P-6 equilibrated and eluted with 0.1 M NaH$_2$PO$_4$, pH 7.0.
Glycosidase Digestions: All enzymatic digestions were done at 37°C under an atmosphere of toluene. Digestions with endo-β-N-acetylglucosaminidase H (0.002 U) were done for 24 h in 50 μl of 0.05 M sodium citrate buffer, pH 5.5. Digestions with endo-β-N-acetylglucosaminidase D (0.005 U) were done for 18 h in 50 μl of 0.15 M sodium phosphate buffer, pH 6.5 containing 0.01% bovine serum albumin. Treatments of [3H]mannose-labeled glycopeptides or oligosaccharides with exoglycosidases were done under the following conditions: jack bean α-mannosidase (0.08 U) in 50 μl of 0.05 M sodium citrate buffer, pH 4.5 for 18 h; V. cholerae neuraminidase (0.2 U) in 200 μl of 0.05 M sodium acetate buffer, pH 5.6 containing 0.9% NaCl and 0.004 M CaCl₂ for 48 h; jack bean β-galactosidase (1.3 U) and β-N-acetylglucosaminidase (1.5 U) in 150 μl of 0.05 M sodium citrate buffer, pH 4.5, for 48 h; Aspergillus oryzae α,1,2-mannosidase (0.05 μg) in 30 μl of 0.01 M sodium acetate buffer, pH 5.0 for 24 h.

Analysis of Oligosaccharide Alcohols by High Performance Liquid Chromatography (HPLC): Oligosaccharides released by endo-β-N-acetylglucosaminidase H or endo-β-N-acetylglucosaminidase D were reduced in 350 μl of 0.12 M NaBH₄, pH 10 for 16–18 h at room temperature. The reductions were stopped by the addition of 15 μl of glacial acetic acid and 1 ml of methanol was added and methyl borate was removed by repeated methanol additions and evaporation under vacuum. The [3H]mannose-labeled oligosaccharide alchohol standards were desalted on Bio-Gel P-2 or amberlite MB-3 and analyzed by HPLC. Methods used for HPLC were those described by Mellis and Baenziger (17) using a 4 mm x 30 cm MicroPax AX-10 column (Varian Associates, Inc., Palo Alto, CA) and a Varian Liquid Chromatography Model 5000. Columns were run at a flow rate of 1 ml/min. The initial solvent was acetonitrile/H₂O (65:35) and the oligosaccharides were eluted by increasing the H₂O content at 0.5%/min. Fractions were collected in scintillation vials at 0.3-min intervals. Radioactivity was determined after the addition of 0.15 ml of H₂O and 3.8 ml of 3a70. Elution profiles of standards were highly reproducible although they changed with repeated use of a column over a period of several months. Therefore, appropriate oligosaccharide alcohol standards were run on each day on which samples were analyzed.

Paper Chromatography Analyses: Descending paper chromatography was done with Whatman No. 1 paper (Whatman Laboratory Products, Inc., Clifton, NJ). To establish that the radioactivity incorporated into the labeled compounds was mannose, we hydrolyzed portions of various oligosaccharides or glycopeptides or oligosaccharides with exoglycosidases were done under the following conditions: jack bean α-mannosidase (0.08 U) in 50 μl of 0.05 M sodium citrate buffer, pH 4.5 for 18 h; V. cholerae neuraminidase (0.2 U) in 200 μl of 0.05 M sodium acetate buffer, pH 5.6 containing 0.9% NaCl and 0.004 M CaCl₂ for 48 h; jack bean β-galactosidase (1.3 U) and β-N-acetylglucosaminidase (1.5 U) in 150 μl of 0.05 M sodium citrate buffer, pH 4.5, for 48 h; Aspergillus oryzae α,1,2-mannosidase (0.05 μg) in 30 μl of 0.01 M sodium acetate buffer, pH 5.0 for 24 h.

FIGURE 1 Effect of endo-β-N-acetylglucosaminidase H on the electrophoretic migration of intracellular and secreted MOPC 315 α-chains. MOPC 315 cells were incubated with [3H]leucine for 5 h. Portions of NP-40 cell lysates and secretions were incubated with endo-β-N-acetylglucosaminidase H before isolation of [3H]leucine-labeled IgA by immunoprecipitation, as described in Materials and Methods. A 7.5% SDS polyacrylamide slab gel was run in the presence of 2-mercaptoethanol. Light chains are not resolved on 7.5% gels. The labeled low molecular weight material migrating with the tracking dye bromophenol blue (BPB) was not observed in [3H]mannose-labeled samples. After electrophoresis, the gel was prepared for fluorography by the procedure of Bonner and Laskey (16). (A) α-chains isolated from a NP-40 cell lysate; (B) α-chains isolated from a cell lysate after treatment with endo-β-N-acetylglucosaminidase H; (C) secreted α-chains isolated from the labeling medium; (D) secreted α-chains isolated from the labeling medium after treatment with endo-β-N-acetylglucosaminidase H. The arrow indicates the migration of nonglycosylated α-chains synthesized in the presence of tunicamycin.

apparent molecular weights (Fig. 1a). This electrophoretic pattern of murine α-chains has been described previously (18). The heterogeneity appears to be the result of carbohydrate content since subsequent analysis of the treatment with endo-β-N-acetylglucosaminidase H resulted in the appearance of a single band of radioactivity (Fig. 1b). The treated intracellular α-chains have a more rapid electrophoretic migration identical to that of nonglycosylated α-chains synthesized in the presence of tunicamycin. The fact that the entire intracellular pool of α-chains was susceptible to the activity of endo-β-N-acetylglucosaminidase H indicates that oligosaccharide processing is not complete until the time of secretion. This intracellular susceptibility is not complete until the time of secretion. This susceptibility of the intracellular α-chains to endo-β-N-acetylglucosaminidase H is like that described for murine IgM μ-chains (9) and human chorionic gonadotropin (19).

In contrast to intracellular α-chains, the SDS PAGE migra-
tion of secreted IgA α-chains was not affected by treatment with endo-β-N-acetylglucosaminidase H, consistent with the presence of predominantly complex-type oligosaccharide units.

**Analysis of Intracellular [3H]Mannose-labeled α-Chain Glycopeptides**

Further experiments with endo-β-N-acetylglucosaminidase H were performed to confirm that the changes in electrophoretic migration of the intact intracellular α-chains were in fact the result of the removal of high mannose-type oligosaccharide units. Fig. 2 shows the Bio-Gel P-6 elution profile of [3H]mannose pulse-labeled α-chain glycopeptides immunoprecipitated immediately at the conclusion of the pulse and after 5½ h of chase incubation. Two major peaks of radioactivity were observed at both time points (Fig. 2, a and c), each of which was susceptible to endo-β-N-acetylglucosaminidase H treatment as indicated by the change in gel filtration elution shown in Fig. 2, b and d. Thus, glycopeptides derived from pulse-labeled α-chains chased for 5½ h remained susceptible to endo-β-N-acetylglucosaminidase H, a result consistent with the data shown in Fig. 1. This demonstrates that the carbohydrate processing of intracellular α-chains is not complete even after prolonged periods of time and that the final processing events must occur rapidly at the time of secretion, since no intracellular α-chain glycopeptides resistant to endo-β-N-acetylglucosaminidase H could be identified. The two major glycopeptide peaks identified by gel filtration contain oligosaccharides of similar size as determined by gel filtration (Fig. 2, b and d) and HPLC (Fig. 3) of the endo-β-N-acetylglucosaminidase H released structures.

**Characterization of Endo-β-N-Acetylglucosaminidase H Released α-Chain Oligosaccharides**

Fig. 3 shows the elution pattern of pulse-labeled intracellular α-chain oligosaccharide alcohols. The two major glycopeptide peaks isolated by gel filtration (Fig. 2 a) had identical oligosaccharide structures that eluted from the HPLC column in fractions identical to the known standards Man9GlcNAc, Man9GlcNAc, Man9GlcNAc, Man9GlcNAc, and Man9GlcNAc, respectively. The arrow labeled G indicates the elution of GlcMan9GlcNAc.
that glucose-containing oligosaccharides are transferred to α-chains and is consistent with previous findings that the terminal glucose residues are rapidly removed following transfer of the oligosaccharides from lipid to protein (1). Further characterization of the 20-min pulse-labeled α-chain oligosaccharides was obtained by measuring the susceptibility of these structures to the exoglycosidase jack bean α-mannosidase. Fig. 4 shows the analysis by paper chromatography in solvent B of a jack bean α-mannosidase digestion revealing a large peak of radioactivity that co-migrated with free mannose and a smaller peak of radioactivity with a migration similar to that of Man₆GlcitolNAc. A very small peak of radioactivity was observed near the origin of the chromatogram shown in Fig. 4. This α-mannosidase-resistant material was tentatively identified as Glc₆Man₆GlcitolNAc by comparison to the migration of known standards and most likely was derived from the small shoulder of glucosylated oligosaccharides detected by HPLC (Fig. 3).

Fig. 3 also shows the presence of pulse-labeled α-chain oligosaccharides containing less than nine mannose residues. A sizable portion of the HPLC-characterized oligosaccharides obtained at the conclusion of a 20-min pulse eluted in a position similar to that of the standard Man₉GlcitolNAc. The proportion of Man₉GlcitolNAc relative to Man₆GlcitolNAc varied from 38 to 70% in several 15- to 20-min pulse-labeling experiments (data not shown). In addition, lesser amounts of labeled structures judged by HPLC to be Man₆GlcitolNAc and Man₇GlcitolNAc were also identified. We cannot exclude the possibility that small amounts of these structures are transferred directly to α-chains. However, the presence of the much higher percentage of glucosylated structures identified after a shorter 3-min pulse indicates that most of these oligosaccharides are derived from a larger glucosylated precursor. Thus, the removal of some α₁,₂-linked mannose residues begins soon after glycosylation of α-chains despite the relatively long intracellular half-life of this glycoprotein.

The intracellular oligosaccharides present after 1, 3, and 5½ h of chase incubation of pulse-labeled α-chains are shown in Fig. 5. By 1 h, the predominant peak of radioactivity identified by HPLC was Man₆GlcitolNAc, although >50% of the radioactivity was still present in peaks identified as Man₆GlcitolNAc and Man₇GlcitolNAc. No glucosylated oligosaccharides were identified by HPLC, and no jack bean α-mannosidase-resistant structures were observed by paper chromatography analyses (data not shown). After 3 h of chase incubation, at a time when ~50% of the pulse-labeled IgA has been secreted, there was a relative increase of Man₇GlcNAc on the remaining intracellular α-chains. At 5½ h, the major oligosaccharide alcohol identified by HPLC was Man₆GlcitolNAc. Only small amounts of Man₇GlcitolNAc, Man₈GlcitolNAc, and Man₉GlcitolNAc could be identified. No further processing of the Man₇GlcNAc could be detected even after this long chase incubation. No pools of smaller oligosaccharides were found, suggesting that the subsequent processing of Man₇GlcNAc must be closely coupled to secretion of the glycoprotein.

**Effect of CCCP and Monensin on the Processing of IgA α-Chain Oligosaccharides**

The above experiments are consistent with the presence of glycoprotein processing α₁,₂-mannosidase activity in two different subcellular locations since the removal of the final α₁,₂-linked mannose residue was closely associated with late processing enzymes such as N-acetylgalactosaminyl transferase I, α-mannosidase II, and other glycosyltransferases thought to be located in the trans Golgi complex (20, 21). CCCP and monensin were used, therefore, in an attempt to define further the subcellular sites of α₁,₂-mannosidase activity. CCCP has been shown to block the intracellular migration of immunoglobulins at the level of the rough endoplasmic reticulum.
whereas monensin blocks within the Golgi apparatus (10).
Initially, pulse-chase experiments were performed to
determine whether these agents blocked the secretion of the MOPC 315 IgA. As shown in Fig. 6, the presence of either CCCP or monensin in the chase medium inhibited the secretion of 315 IgA during the 5 h after pulse labeling with [3H]leucine. This inhibition of secretion was not the result of increased intracellular degradation, as determined by immunoprecipitation of the labeled IgA in NP-40 cell lysates at each time point (data not shown). A small amount of [3H]leucine-labeled IgA was secreted in the presence of monensin (25% of control at 5 h), whereas secretion in the presence of CCCP was inhibited by >85% at all time points analyzed. Similar results have been reported previously for MOPC 315 IgA secretion (10).
Analyses of the oligosaccharides obtained from α-chains that were pulse labeled with [3H]mannose for 20 min and then blocked intracellularly with CCCP or monensin are shown in Fig. 7. Despite the presence of these inhibitors of secretion, no gross alterations of intracellular carbohydrate processing could be detected. The major oligosaccharide present after 3 h of chase eluted with the Man₅GlcitolNAc standard regardless of whether the intracellular block occurred at the level of the rough endoplasmic reticulum (CCCP) or within the Golgi apparatus (monensin). Thus, the removal of the first three α₁,₂-linked mannose residues occurs in the endoplasmic reticulum, since the time course for their removal is not affected by a CCCP-induced block of protein migration. In addition, the fact that the major oligosaccharide that accumulated in monensin-treated cells also has six mannose residues indicates that the final α₁,₂-linked mannose residue is removed distal to the monensin block, and therefore in the trans Golgi complex. A small peak of radioactivity was identified in both CCCP- and monensin-treated cells that eluted with the standard Man₅GlcitolNAc. However, no large accumulation of Man₆GlcitolNAc or smaller structures was ever observed. The major peak of radioactivity was identified as Man₅GlcitolNAc even with chase periods of 5–6 h (data not shown).

Characterization of the Oligosaccharides With Six Mannose Residues

Although the above data are compatible with two subcellular locations of α₁,₂-mannosidase activity involved in the processing of IgA secreted from MOPC 315 plasmacytoma

![Figure 7](https://example.com/fig7.png)

**Figure 7** HPLC analyses of endo-β-N-acetylglycosaminidase H-released oligosaccharides from pulse-labeled α-chains chased in nonradioactive medium containing either CCCP or monensin. MOPC 315 cells were labeled for 20 min with [3H]mannose and chased in nonradioactive medium containing either CCCP (50 μM) or monensin (10 μM). At the indicated times, IgA was immunoprecipitated from NP-40 cell lysates. The labeled oligosaccharides were obtained and prepared prior to HPLC analyses as described in Materials and Methods and the legend to Fig. 3. A, α-chain oligosaccharide alcohols from cells chased in the presence of CCCP for 1 h; B, α-chain oligosaccharide alcohols from cells chased in the presence of CCCP for 3 h; C, α-chain oligosaccharide alcohols from cells chased in the presence of monensin for 1 h; D, α-chain oligosaccharide alcohols from cells chased in the presence of monensin for 3 h. The arrows indicate the elution of Man₅GlcitolNAc, Man₆GlcitolNAc, Man₇GlcitolNAc, Man₈GlcitolNAc, and Man₉GlcitolNAc standards.

This conclusion can only be made if the structures identified as Man₅GlcitolNAc contain an α₁,₂-linked mannose residue and if the mature, secreted oligosaccharides have been further processed to complex type structures. Purified Aspergillus satoi α₁,₂-mannosidase therefore was used to characterize the Man₅GlcitolNAc structures. This α-mannosidase releases only terminal α₁,₂-linked mannose residues from carbohydrate structures (22, 23). As seen in Fig. 8a, HPLC analysis shows that the major intracellular [3H]mannose-labeled oligosaccharide alcohol structure from pulse-labeled α-chains chased for 5½ h was Man₅GlcitolNAc. Treatment of this sample with Aspergillus satoi α₁,₂-mannosidase before HPLC resulted in the formation of two different peaks of radioactivity, shown in Fig. 8b. The major peak had an elution profile that corresponded to that of the standard Man₅GlcitolNAc, whereas the smaller peak eluted much earlier in a position that corresponded to mannose. The relative sizes of the radioactive peaks are consistent with the release of a single α₁,₂-linked mannose residue. Thus, the structure identified as Man₅GlcitolNAc does contain an α₁,₂-linked mannose residue which, if further processing occurs, is removed at the time of secretion.

**Determination of the Number of Mannose Residues in Secreted IgA α-Chain Oligosaccharides**

The exact structures of the fully processed IgA carbohydrate chains are not known, but the secreted protein appears to...
have mainly complex-type oligosaccharides as determined by resistance to endo-β-N-acetylgalactosaminidase H (Fig. 1). Typically, processing of complex-type oligosaccharides results in core structures containing only three mannose residues linked to a chitobiose unit (24). To determine the number of mannose residues present in the fully processed secreted molecule, we isolated and characterized [3H]mannose-labeled MOPC 315 IgA from the medium. Fig. 9a shows the gel filtration profile of the glycopeptides derived from secreted IgA. In contrast to their intracellular precursors, these structures have a higher apparent molecular weight, eluting near the void volume of the P-6 column. Treatment of these glycopeptides with endo-β-N-acetylgalactosaminidase H released only a small amount of radioactivity (~10%, data not shown). The exact origin of these high-mannose structures is unknown. Nevertheless, most of the [3H]mannose-labeled, secreted IgA glycopeptides were resistant to endo-β-N-acetylgalactosaminidase H.

Fig. 9b shows the P-6 gel filtration profile of these glycopeptides following sequential treatment with neuraminidase, β-galactosidase, and endo-β-N-acetylgalactosaminidase. Two new peaks of radioactivity with lower apparent molecular weights could be identified suggesting that the secreted α-chains contain terminal monosaccharides other than mannose. Following exoglycosidase treatment, the oligosaccharides could be released from peptides by treatment with endo-β-N-acetylgalactosaminidase D as judged by gel filtration on the P-6 column (Fig. 9c). This endoglycosidase will cleave the chitobiose unit of complex-type oligosaccharides once the outer chain sugars are removed from the core structure Manα1→3(Manα1→6)-Manβ1→4GlcNAcβ1→4GlcNAc→Asn. The α-mannose residue linked to the innermost β-linked mannose residue by a 1–3 linkage must be unsubstituted for enzymatic activity (25, 26). Fig. 10 shows the HPLC analysis of the oligosaccharide alcohol derived from the major peak of endo-β-N-acetylgalactosaminidase D-released radioactivity. A large peak of radioactivity could be identified that had an elution profile identical to that of the standard Man6GlcNAc2, indicating that complete processing to typical complex-type core structures does occur in the majority of IgA-associated oligosaccharides. Thus, the final α,1,2-linked mannose residue is released from the intracellular Man6GlcNAc2 α-chain oligosaccharides as well as further processing to complex-type structures. As shown, these events occur rapidly at the time of secretion, since no large pools of partially processed oligosaccharides could be identified.

Characterization of Oligosaccharides on IgA α-Chains Secreted in the Presence of Monensin

As shown in Fig. 6, monensin inhibited the secretion of IgA from MOPC 315 cells. However, 25% of the [3H]leucine pulse-labeled IgA was secreted after 5 h of chase. This incubation time was used to obtain sufficient amounts of secreted [3H]mannose-labeled IgA so that the oligosaccharides could be characterized. A higher percentage (~40%) of [3H]mannose-labeled α-chain glycopeptides and oligosaccharides were analyzed by gel filtration on a Bio-Gel P-6 column as described in Fig. 2. (A) the immunoprecipitated IgA containing [3H]mannose-labeled α-chains was treated with pronase for 48 h and applied to the P-6 column; (B) the major peak of radioactivity, indicated by the bar in A, was concentrated and treated sequentially with neuraminidase, β-galactosidase, and β-N-acetylgalactosaminidase. A portion of the final digestion was applied directly to the P-6 column; (C) following sequential treatment with exoglycosidases, the [3H]mannose-labeled glycopeptides were de-salted and treated with endo-β-N-acetylgalactosaminidase D before applying to the P-6 column. The major peak of radioactivity was pooled as indicated by the bar, concentrated, reduced, and analyzed by HPLC. V10 and V100 indicate the elution positions of bovine serum albumin and mannose, respectively, determined in a separate experiment.
nose-labeled IgA was secreted in the presence of monensin relative to $[^{3}H]$leucine-labeled IgA. As shown below, this is a result of incomplete oligosaccharide processing. Fig. 11a shows a Bio-Gel P-6 gel filtration profile of the glycopeptides obtained after extensive pronase digestion. The elution profile was considerably different from that of IgA glycopeptides isolated from control cell secretions depicted in Fig. 9a. In fact, the gel filtration elution profile was similar to that of glycopeptides derived from intracellular IgA (Fig. 2c). Furthermore, a large proportion of the glycopeptides derived from IgA secreted in the presence of monensin were susceptible to endo-$\beta$-$N$-acetylglucosaminidase H treatment, as determined by gel filtration (Fig. 11b). Analysis by HPLC of the endo-$\beta$-$N$-acetylglucosaminidase H-released oligosaccharides revealed that they were similar in size to intracellular oligosaccharides present after several hours of chase incubation. Fig. 12 shows that the major oligosaccharide had an elution identical to that of the known standard Man$_5$GlcNAc$_2$. Smaller amounts of Man$_6$GlcNAc$_2$, Man$_7$GlcNAc$_2$, and Man$_8$GlcNAc$_2$ were also identified. Free mannose and Man$_3$GlcNAc$_2$ were identified when these oligosaccharides were treated with jack bean $\alpha$-mannosidase and analyzed by paper chromatography in solvent A, further showing that only mannose and GlcNAc were present (data not shown).

DISCUSSION

Recent investigations have elucidated the steps involved in the formation of complex-type $N$-linked asparagine oligosaccharides (1). Following the transfer of Glc$_3$Man$_9$GlcNAc$_2$ from dolichol diphosphate to proteins, the glucose residues are rapidly removed by glucosidases located in the rough and smooth endoplasmic reticulum. This investigation has focused on the subsequent removal of the four $\alpha 1,2$-linked mannose residues, which is the next major stage of processing involved in the formation of complex-type $N$-linked oligosaccharides. Enzymes capable of specifically cleaving $\alpha 1,2$-linked mannose residues from high mannose substrates have been partially purified from mammalian tissues (5, 6, 13, 27). Since several of these have been isolated from rat Golgi membrane preparations, it has been speculated that the Golgi complex is the subcellular site for this processing step (5, 6). However, others have presented evidence to indicate that the rough
endoplasmic reticulum is a major site of α1,2-mannosidase activity (9, 11-13).

Our data indicate that there is α1,2-mannosidase activity in the rough endoplasmic reticulum of MOPC 315 cells. The pulse-chase experiments show that, despite the long intracellular half-life of IgA α-chains, processing of the first three α1,2-linked mannose residues occurs soon after the high-mannose oligosaccharide is transferred to this protein. The use of CCCP, an inhibitor of protein migration in the rough endoplasmic reticulum, provided further evidence to define the subcellular location of processing α1,2-mannosidase activity. The time course for the removal of the first three α1,2-linked mannose residues was not affected by a CCCP-induced block, consistent with mannosidase activity in the rough endoplasmic reticulum.

The extent of endoplasmic reticulum α1,2-mannosidase processing of α-chains in MOPC 315 cells is somewhat different from that observed for other proteins in other cell types. Tartakoff and Vassalli (9) suggested that the mannose trimming of newly synthesized μ-chains in murine plasmablasts occurred in the rough endoplasmic reticulum. However, structural analyses were not performed to document the exact extent of processing. Godaillen et al. (11) have reported processing α1,2-mannosidase activity in both the endoplasmic reticulum and Golgi apparatus of calf thyroid cells. They found that a single α1,2-linked mannose residue was removed from thyroglobulin in the endoplasmic reticulum. Likewise, Hakimi and Atkinson (12) concluded that one mannose residue was removed from Sindbis virus proteins B and PE2 in the rough endoplasmic reticulum of chick embryo fibroblasts. Our evidence that α1,2-mannosidase activity is in the rough endoplasmic reticulum is consistent with those described above. However, three α1,2-linked mannose residues of newly synthesized MOPC 315 α-chain oligosaccharides are removed in the endoplasmic reticulum. Compared with the other studies, this difference may be a result of the particular glycoprotein studied since our investigation does not exclude the presence of α1,2-mannosidase activity in the early or cis Golgi apparatus of MOPC 315 plasmacytoma cells. Indeed, the extent of α1,2-mannosidase processing in any subcellular location may depend on the individual protein. It may be that the relatively slow secretory rate of the α-chains lengthens the time of their exposure to endoplasmic reticulum α1,2-mannosidase, resulting in the formation of ManαGlcNAc2 oligosaccharides.

Of interest was the time course for the removal of the final α1,2-linked mannose residue from the IgA α-chains. Processing of the oligosaccharides containing six mannose residues to complex-type structures occurred rapidly at the time of secretion, since no oligosaccharides containing five or less mannose residues could be isolated from cells during long chase incubations. Thus, this α1,2-mannosidase activity is associated with the subsequent processing enzymes known to be located in the trans Golgi apparatus (20, 21). The results obtained from the experiments using monensin are consistent with the presence of α1,2-mannosidase in this subcellular location. Recent studies have indicated that the site of action of monensin is between the cis and trans Golgi elements (20, 21, 28), although evidence for a block beyond the Golgi apparatus has also been proposed (29). In the presence of monensin, the major α-chain oligosaccharide isolated from the cells and medium contained six mannose residues. Therefore, α1,2-mannosidase activity that can remove the final α1,2-linked mannose residue is located beyond the monensin-induced block. We cannot exclude the possibility that some α1,2-mannosidase activity responsible for the removal of the final α1,2-linked mannose exists before the monensin block since very small, but measurable, amounts of Man6GlcNAc2 were isolated from cells treated with either monensin or CCCP (Fig. 7) and from the medium of cells treated with monensin (Fig. 12). Nevertheless, appreciable amounts of Man6GlcNAc2 α-chain oligosaccharides were not identified after long incubations in the presence of these inhibitors. This, plus the pulse-chase experiments depicting an association of the removal of the final α1,2-linked mannose residue with enzymes responsible for subsequent carbohydrate processing, provides strong evidence for the presence of α1,2-mannosidase activity in the trans Golgi apparatus. Processing α1,2-mannosidase in this Golgi subcompartment has not been characterized extensively. In fact, there have been conflicting reports as to whether α1,2-mannosidase activity exists in the cis or trans Golgi apparatus (30-32). These studies have involved subcellular fractionation of various cell types. However, the documentation of α1,2-mannosidase activity in various subcellular fractions may well require specific substrates. For example, we have recently determined that the structure of the intracellular IgA oligosaccharides containing six mannose residues is a single species with the α1,2-linked mannose residue present on the mannose-linked α1,3 to the innermost β-linked core mannose residue (data not shown). This suggests that the purported MOPC 315 trans Golgi α1,2-mannosidase is specific. If so, it would be necessary to have a Manα-substrate containing a single specific α1,2-linked mannose residue in order to detect enzymatic activity in the trans Golgi apparatus of these cells. Such an oligosaccharide used as a substrate would be ideal for detecting the trans Golgi apparatus α1,2-mannosidase activity but may well be a poor substrate for rough endoplasmic reticulum or cis Golgi α1,2-mannosidases. Likewise, a substrate containing eight mannose residues might be a poor substrate for a trans Golgi α1,2-mannosidase. We are currently investigating this hypothesis using a number of well characterized [3H]mannose-labeled substrates.

It is not known whether a trans Golgi apparatus α1,2-mannosidase that specifically removes the last α1,2-linked mannose residue from Man6GlcNAc2 is present in all cell types. Preliminary data have indicated the presence of such an enzyme in MOPC 460 cells, another murine IgA-secreting plasmacytoma cell line. Likewise, Rudden et al. (19) using cultured human choriocarcinoma cells have shown that pulse-labeled α-subunits of human chorionic gonadotropin have a relatively long intracellular half-life and contain oligosaccharides that remain sensitive to endo-β-N-acetylglucosaminidase H until secreted. Furthermore, a major α-subunit oligosaccharide species accumulating intracellularly appeared to be Man6GlcNAc2, a result similar to our findings with the α-chain of MOPC 315 IgA. Both human chorionic gonadotropin and IgA are secretory proteins known to be secreted continuously without appreciable intracellular storage. Whether this pattern of α1,2-linked mannose processing is unique to secreted glycoproteins remains to be verified.

The removal of the final α1,2-linked mannose residue and subsequent carbohydrate modifications do not appear to be absolute requirements for the secretion of IgA since even in the presence of monensin, some α-chains with partially processed high mannose-type oligosaccharides are secreted. Recently, it has also been shown that monensin treatment of
human fibroblasts in culture results in the secretion of fibronectin with abnormal carbohydrate chains (33). Our study clearly demonstrates that the initial carbohydrate-processing events are not affected by monensin, as the glucose residues are rapidly removed and the time course for the removal of the first three α1,2-linked mannose residues is unchanged. However, α-chains secreted in the presence of monensin have carbohydrate structures similar to those accumulating intracellularly. Whether monensin directly inhibits the processing enzymes in the trans Golgi apparatus or in some way causes the partially processed α-chains to bypass this site leading to secretion by another subcellular route remains to be elucidated.

We conclude that the processing of MOPC 315 IgA α-chain oligosaccharides involves the activity of α1,2-mannosidase in at least two subcellular locations. This may represent the presence of several enzyme proteins as have been described in rat liver (5, 6). To date, we do not have direct evidence for the presence of multiple α1,2-mannosidase enzymes in MOPC 315 cells. It is possible that a single enzyme protein exists within the endoplasmic reticulum and Golgi apparatus. Various changes in the subcellular environment might affect substrate-enzyme interactions, resulting in preferential hydrolysis of mannose residues from a specific oligosaccharide structure. Although we believe that this is unlikely, direct evidence must await isolation and characterization of the proteins responsible for the α1,2-mannosidase activities demonstrated in this study.

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