A human cDNA encoding a 70.9-kDa type II membrane protein with sequence similarity to class I α,1,2-mannosidases was isolated. The enzymatic properties of the novel α,1,2-mannosidase IC were studied by expressing its catalytic domain in Pichia pastoris as a secreted glycoprotein. α,1,2-Mannosidase IC sequentially hydrolyzes the α,1,2-linked mannose residues of [3H]mannose-labeled Man₉GlcNAc to form [3H]Man₅GlcNAc and a small amount of [3H]Man₄GlcNAc. The enzyme requires calcium for activity and is inhibited by both 1-deoxymannojirimycin and kifunensine.

The order of mannose removal was determined by separating oligosaccharide isomers formed from pyridylaminated Man₉GlcNAc by high performance liquid chromatography. The terminal α,1,2-linked mannose residue from the middle branch is the last mannose removed by the enzyme. This residue is the mannose cleaved from Man₅GlcNAc by the endoplasmic reticulum α,1,2-mannosidas I or Man₅GlcNAc isomer B. The order of mannose hydrolysis from either pyridylaminated Man₅GlcNAc or Man₄GlcNAc isomer B differs from that previously reported for mammalian Golgi α,1,2-mannosidases IA and IB. The full-length α,1,2-mannosidase IC was localized to the Golgi of MDGBK and MDCK cells by indirect immunofluorescence. Northern blot analysis showed tissue-specific expression of a major transcript of 3.8 kilobase pairs.

The expression pattern is different from that of human Golgi α,1,2-mannosidases IA and IB. Therefore, the human genome contains at least three differentially regulated Golgi α,1,2-mannosidase genes encoding enzymes with similar, but not identical specificities.

α,1,2-Mannosidases play an essential role in the maturation of N-glycans to hybrid and complex structures in mammalian cells (for reviews, see Refs. 1–3). They remove the four α,1,2-linked mannose residues from Man₅GlcNAc, following cleavage of glucose from Glc₃Man₉GlcNAc. Thus, α,1,2-mannosidases provide the Man₅GlcNAc substrate required for GlcNAc transferase I that initiates formation of complex and hybrid N-glycans. They belong to class I α-mannosidases (familly 47 of glycosyl hydrolase classification (Ref. 4)) that have been conserved through eukaryotic evolution. The α,1,2-mannosidases are type II transmembrane proteins with amino acid similarity throughout their large C-terminal catalytic domains. They are inverting calcium-dependent glycosyl hydrolases that are inhibited by 1-deoxymannojirimycin and kifunensine. However, they have different N-terminal regions and intracellular localizations. A class I α,1,2-mannosidase localized to the ER of mammalian cells has been cloned (5, 6). It has the same properties as the yeast ER α,1,2-mannosidase, the structure of which has recently been determined by x-ray crystallography (7). The ER α,1,2-mannosidase removes a single specific mannose residue from Man₅GlcNAc to form Man₄GlcNAc isomer B, which lacks the terminal α,1,2-mannose from the middle branch of the oligosaccharide. Two class I Golgi α,1,2-mannosidases, IA and IB, that are about 65% identical in amino acid sequence have also been cloned from mammalian cells (8–11).

These Golgi enzymes remove the four α,1,2-linked mannose residues from Man₅GlcNAc to yield Man₄GlcNAc. Their specificity is complementary to that of the ER α,1,2-mannosidase since the mannose residue cleaved by the ER enzyme is the last residue removed by the two Golgi α,1,2-mannosidases (12). The major difference between Golgi α,1,2-mannosidase IA and IB is their tissue- and cell-specific expression as shown by Northern blot analysis of human and murine tissues (9–11), and by immunolocalization in cells of the rat testis (13). In addition, there is some difference in their specificity with Man₅GlcNAc as substrate (12).

In the present work, the characterization of Golgi human α,1,2-mannosidase IC, a novel member of the mammalian class I α,1,2-mannosidases is reported. This enzyme displays a distinct pattern of tissue-specific expression and trims Man₅GlcNAc to Man₄GlcNAc, forming different high-mannose oligosaccharide intermediates from those previously observed for mammalian Golgi α,1,2-mannosidases IA and IB.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by BioCorp (Montréal, Canada). The C-terminal peptide was synthesized and conjugated to keyhole limpet hemocyanin by the Sheldon Biotechnology Centre (McGill University, Montréal, Canada) [3H]mannose-labeled Man₅GlcNAc was prepared from rat liver and Man₅GlcNAc from soybean agglutinin as described previously (14, 15). Man₅GlcNAc-PA oligosaccharides were purchased from Takara Shuzo Co. (Otsu, Japan). Kifunensine, 1-deoxymannojirimycin, and swainsonine were obtained from the newcastle, coventry, and astra companies.

The abbreviations used are: ER, endoplasmic reticulum; BSA, bovine serum albumin; ORF, open reading frame; HPLC, high performance liquid chromatography; PA, pyridylamino; PAGE, polyacrylamide gel electrophoresis; Endo H, endo-β-N-acetylg glucosaminidase H; PIPES, 1,4-piperazinediethanesulfonic acid; contig, group of overlapping clones; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus Tween 20; kb, kilobase pair(s); EST, expressed sequence tag; PCR, polymerase chain reaction; MES, 4-morpholineethanesulfonic acid; MDCK, Madin-Darby canine kidney; MDBK, Madin-Darby bovine kidney.

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from Toronto Research Chemicals, Inc. (Downview, Canada).

Isolation of Human α1,2-Mannosidase IC cDNA—ESTs encoding α1,2-mannosidase IC were identified by querying the NCBI dbEST data base with the yeast α1,2-mannosidase amino acid sequence (16) using the BLASTn algorithm. Most of the α1,2-mannosidase IC ESTs identified in UniGene were then resolved by HPLC on a Micro-Pak-SP C18 column (4.6 × 150 mm, Varian), eluted isocratically at 1 ml/min with 100 mM acetic acid containing 0.05% n-butyly alcohol adjusted to pH 4 with triethylamine. The isomers were monitored at an excitation of 320 nm and an emission of 400 nm. The identity of the products was determined by comparing their elution to that of standard Man9–5GlcNAc2-PA, Man8GlcNAc2-PA, and anadisorption peptides (Amersham Pharmacia Biotech) were sequenced. Primers within the 5′ region of the consensus sequence (5′-CTCTGAGCTTGCGCCAGAGGAG-3′; 5′-CTCTGTTGCGGAGAGAAGGAG-3′) were used to screen a fetal brain cDNA library for the presence of the catalytic domain (amino acids 165–630) was affinity-purified (17) using a column prepared by coupling the peptide to cyanogen bromide-activated Sepharose 4B according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The ORF sequence was amplified by PCR using a sense primer containing a HindIII site (5′-AAAAGTGCCACACCTGGCTATGAGAAGTTG-3′) and an antisense primer with a XbaI site following the stop codon (5′-AAATCTAGATCAGTGTCGCCCCAGCAAGAGT-3′). The amplicon was inserted into the HindIII/ApaI sites of pPICZaA (Invitrogen) in frame with the α-factor signal sequence yielding the expression construct pZAHMHCIC93. The expression construct (10 μg) was linearized with PmeI and electroporated into P. pastoris strain GS115 (his4) (Invitrogen), and transformants were grown as described previously (11). Clones expressing recombinant α1,2-mannosidase were identified by assays with [3H]Man9–5GlcNAc (18).

RESULTS

Isolation and Characterization of Human α1,2-Mannosidase IC—Human ESTs encoding α1,2-mannosidase IC were identified by querying the EST Data base as described under “Experimental Procedures.” The 1.3-kb consensus sequence obtained upon aligning the ESTs encoded the C-terminal region of the catalytic domain (216 amino acids) including two of the highly conserved class 1 α1,2-mannosidase amino acid sequence motifs, 991 base pairs of flanking 3′-untranslated region and a poly(A) tail. Thereafter, a cDNA clone encoding the entire catalytic domain (1.1 kb) as well as the 3′-untranslated region (1 kb) was identified by a PCR screen of a fetal brain cDNA library using primers within the 5′ region of the consensus sequence. In addition, a partially sequenced EST clone...
identified in the UniGene data base was completely sequenced (2.9 kb) and shown to encode the entire ORF. The α<sub>1</sub>,2-mannosidase IC cDNA (2.9 kb) is predicted to encode a 70.9-kDa type II membrane protein with a short cytoplasmic tail of about 22 amino acid residues, a transmembrane domain of 22 residues and a large C-terminal domain (Fig. 1). The C-terminal domain contains a proline-rich “stem” region (amino acids 45–164) not required for enzyme activity, followed by the catalytic domain (amino acids 165–630). The latter encodes class I α<sub>1</sub>,2-mannosidase signature motifs (see the Carbohydrate-Active Enzymes server, available via the world wide web) and the nine invariant acidic amino acids and cysteine residues shown to be essential for the activity of the yeast class I α<sub>1</sub>,2-mannosidase (23, 24). Three potential N-glycosylation sites are located within the catalytic domain.

α<sub>1</sub>,2-Mannosidase is about 54% identical to the human (X74837, AF027156), murine (U04299, U03458), and porcine (Y12503) α<sub>1</sub>,2-mannosidases IA and IB, and 38% identical to the human ER α<sub>1</sub>,2-mannosidase (AF145732, AF148509) amino acid sequences (5, 6, 8–11, 25).

Expression of Recombinant α<sub>1</sub>,2-Mannosidase IC in P. pastoris—The catalytic domain starting at amino acid 165 was cloned in the P. pastoris expression vector pPICZαA in frame with the α-factor signal sequence. α<sub>1</sub>,2-Mannosidase activity was detected in the medium 2 days following induction with methanol of yeast cells transformed with the resulting construct pZαAHMIC493. No activity was found in the medium of cells transformed with the empty vector pPICZαA. The secreted recombinant α<sub>1</sub>,2-mannosidase consists of a 55-kDa and a heterogeneous 67-kDa form. Treatment with Endo H gives rise to a single band of the expected size of 52 kDa (Fig. 3). These results indicate that one glycoform only acquires core N-glycans, whereas the other contains outer chains with an average of about 16 residues per core structure, assuming all three sites are equally glycosylated.

Properties of Recombinant α<sub>1</sub>,2-Mannosidase IC—The enzymatic properties of recombinant α<sub>1</sub>,2-mannosidase IC were analyzed using [3H]mannose-labeled Man<sub>9</sub>GlcNAc as substrate. The enzyme has a pH optimum of about 5.9 and requires the addition of calcium for maximum activity. Inhibition of the enzyme by preincubating with 50 μM EDTA is reversed by the addition of 10 μM Ca<sup>2+</sup>, but not by 10 μM Mg<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>, Zn<sup>2+</sup>, or Fe<sup>2+</sup>. The α<sub>1</sub>,2-mannosidase IC activity is inhibited by the class I α-mannosidase inhibitors 1-deoxymannojirimycin

FIG. 1. Nucleotide and deduced amino acid sequence of human α<sub>1</sub>,2-mannosidase IC. Numbers at the right in normal font refer to the nucleotide sequence and those in bold to the deduced amino acid sequence. The conserved class I α<sub>1</sub>,2-mannosidase motifs are indicated in bold and underlined, and invariant acidic amino acid residues and cysteines are circled. The putative transmembrane domain is denoted in bold and underlined by a dotted line. The starting amino acid residue of the recombinant enzyme expressed in P. pastoris is indicated by a diamond, and asterisk marks potential N-glycan sites.
IC50 = 250 μM) and kifunensine (IC50 = 0.5 μM), but not by the class II α-mannosidase inhibitor swainsonine (Table I). Therefore, α1,2-mannosidase IC has all the properties ascribed to class I α1,2-mannosidases.

**Specificity of Human α1,2-Mannosidase IC**—The enzyme was incubated with [3H]mannose-labeled Man9GlcNAc, and the products obtained at different times were resolved by HPLC. The recombinant enzyme catalyzed the stepwise removal of mannose from Man9GlcNAc to form Man6GlcNAc and a small amount of Man5GlcNAc (Fig. 4). Man9GlcNAc incubated for 48 h with medium of yeast transformed with the vector alone was not hydrolyzed.

To determine the order of mannose removal, the enzyme was incubated with Man9GlcNAc2-PA. The Man8–6 GlcNAc2 intermediates were first fractionated according to size by HPLC (data not shown). Each oligosaccharide fraction was then further fractionated into its component isomers by HPLC on a reverse phase column. Hydrolysis of Man9GlcNAc2 yields primarily a single Man8GlcNAc2 isomer (about 90%), equivalent amounts of two Man7GlcNAc2 isomers, and a single Man6GlcNAc2 isomer that were identified by comparison with elution of standard oligosaccharides-PA (Fig. 5, A–C). These results indicate that the terminal α1,2-linked mannose residue on the middle arm is the last to be removed. Since Man9GlcNAc2 isomer B is formed by human ER α1,2-mannosidase I, Man9GlcNAc, PA isomer B was also incubated with α1,2-mannosidase IC. In this case the enzyme first cleaves the terminal mannose on the α1,3-branch of the substrate, yielding essentially a single Man7GlcNAc2 isomer (about 85%). Thereafter, equivalent amounts of two Man7GlcNAc2 isomers (Fig. 5, D and E) were formed by the hydrolysis of either of the two remaining α1,2-linked mannose residues. Thus, the order of mannose removal from Man9GlcNAc2-PA was identical to the order observed for the Man9GlcNAc2-PA.

**Immunolocalization of α1,2-Mannosidase IC in Transfected MDBK and MDCK Cells**—The full-length α1,2-mannosidase IC expression. [α-32P]dATP-labeled α1,2-mannosidase EST clone W19722 was hybridized to Northern blots containing 2 mg of poly(A+) RNA isolated from human tissues. The blots were exposed to x-ray film for 5 days. Molecular size markers are indicated beside each blot.

**TABLE I**

| Inhibitor           | Activitya % of control |
|---------------------|------------------------|
| 1-Deoxymannojirimycin |                        |
| 10 μM               | 100                    |
| 50 μM               | 82                     |
| 250 μM              | 50                     |
| 500 μM              | 31                     |
| Kifunensine         |                        |
| 0.1 μM              | 82                     |
| 0.5 μM              | 51                     |
| 1.0 μM              | 28                     |
| 10.0 μM             | 2                      |
| Swainsonine         |                        |
| 10 μM               | 100                    |
| 100 μM              | 100                    |
| 500 μM              | 100                    |

a Activity is expressed as the percentage of [3H]mannose released from [3H]Man9GlcNAc in the absence of inhibitors (100 cpm). Assays were performed as described under “Experimental Procedures” except the enzyme was preincubated on ice for 30 min with the inhibitor and following addition of 20,000 cpm [3H]Man9GlcNAc and 1 mM Man6GlcNAc incubated for 3 h at 37 °C.

**FIG. 4.** Time course of α1,2-mannosidase IC hydrolysis of Man9GlcNAc. [3H]Man9GlcNAc was incubated with medium (10-fold concentrated) from P. pastoris transformed with pZaAHMIC493 at 2 days after induction. The products, Man9GlcNAc (●), Man8GlcNAc (○), Man7GlcNAc (■), Man6GlcNAc (□), and Man5GlcNAc (▲), were resolved by HPLC on an Aminosphereisorb column as described under “Experimental Procedures.” The results are expressed as a percentage of the total radioactivity recovered at each time point.

**FIG. 3.** Recombinant α1,2-mannosidase IC expressed in P. pastoris. Ten microliters of medium (concentrated 10-fold), treated with or without Endo H, was subjected to 10% SDS-PAGE (reducing) and visualized by Western blotting. Lanes 1 and 2, GS115 transformed with pZaAHMIC493; lane 3, GS115 transformed with pPICZα A, at 48 h after induction. Molecular mass markers are indicated on the right.
was expressed in MDBK and MDCK cells to determine its subcellular localization by indirect immunofluorescence. Punctate perinuclear Golgi staining was detected in cells 24–48 h after transfection with both the hemagglutinin tagged (pMH-HMICT) and native (pMHMMICS) α1,2-mannosidase IC (Fig. 6). The staining pattern shows that α1,2-mannosidase IC is in the Golgi since it co-localizes with endogenous Golgi β1,4-galactosyltransferase. No immunofluorescence was observed with pre-immune serum, secondary antibodies alone, or cells transfected with the pMH vector.

Genomic Organization and Chromosomal Localization—The α1,2-mannosidase IC gene contains 12 exons encoded by GenBank clones AL031280 and AL020996. These clones overlap by 2.3 kb within the intronic region between exons 2 and 3. The gene is localized on chromosome 1p35.1–36.13 and spans 167 kb of genomic sequence between the markers D1S2843 and D1S417 on Gene Map 98 (26). The intron and exon boundaries of the coding region are identical to those found in the human α1,2-mannosidase IA gene, which spans 188 kb on chromosome 1p13 (UniGene Hs.2750). The reported genomic organization of the α1,2-mannosidase IB (11) localized on human chromosome 1p13 differs from α1,2-mannosidase IA and IC at a few positions within the ORF (Fig. 7), particularly at the N terminus, which is encoded by two exons.

DISCUSSION

The present results demonstrate the existence of a previously unsuspected third mammalian Golgi α1,2-mannosidase derived from a distinct gene. This enzyme is capable of trimming high mannose oligosaccharides to Man₉GlcNAc₂ during N-glycan biosynthesis. Human α1,2-mannosidase IC requires calcium for activity and is inhibited by 1-deoxymannojirimycin and kifunensine; thus, it possesses the characteristic properties of class I α1,2-mannosidases. The amino acid sequence of the catalytic domain is similar to previously described mammalian Golgi α1,2-mannosidases IA and IB, but the cytoplasmic tail and stem region sequence differ. Furthermore, α1,2-mannosidase IC displays a distinct tissue-specific expression pattern and order of α1,2-linked mannose removal (Fig. 8).

Human Golgi α1,2-mannosidases IA, IB, and IC are encoded by independent genes on chromosomes 6q22, 1p13, and 1p35–36, respectively. Gene duplication occurring late in evolution probably gave rise to the mammalian Golgi α1,2-mannosidase gene family (27) since the positions of the intron and exon boundaries within the gene are very similar. However, in both humans and mice these genes are independently regulated, thus giving rise to distinct patterns of expression (9–11, 13).

α1,2-Mannosidase IC readily hydrolyzes three of the four α1,2-linked mannose residues of Man₉GlcNAc₂ and slowly cleaves the remaining terminal α1,2-linked mannose residue on the middle branch (Fig. 8, upper section). The enzyme produces the same Man₉GlcNAc₂ isomer as α1,2-mannosidase IA.

**Fig. 5.** Oligosaccharide intermediates formed by α1,2-mannosidase IC. The Man₉GlcNAc₂-PA isomers formed from Man₉GlcNAc₂-PA (A–C) and Man₉GlcNAc₂-PA (D and E) were resolved by HPLC on a C18 column as described under "Experimental Procedures." The structures of the substrates are shown above the profiles. Arrows indicate the elution position of standards whose structures are shown above the arrows. O, α1,2-linked mannose residues; □, α1,3- and α1,6-linked mannose residues; □, GlcNAc₂-PA.

**Fig. 6.** Localization of α1,2-mannosidase IC in MDBK cells. MDBK cells were transiently transfected with either hemagglutinin tagged (A–D) or native (E and F) α1,2-mannosidase IC. The cells were fixed and stained with monoclonal HA11 hemagglutinin antibody (A and C), and polyclonal β1,4-galactosyltransferase antibodies (B) or polyclonal α1,2-mannosidase IC antibodies (D and E). The HA11 (A and C) and polyclonal (B, D, and E) antibodies were detected with CY2- and rhodamine-conjugated antibodies, respectively. Phase contrast of the cell in panel E is presented in panel F.

**Fig. 7.** Organization of human Golgi α1,2-mannosidase genes. α1,2-Mannosidase IA is encoded by GenBank genomic clones AL078600 (exons 1–3) and AL022722 (exons 4–12). The intron and exon boundaries within the coding region are identical to those of the α1,2-mannosidase IC gene (AL031280 (exons 1 and 2) and AL020996 (exons 3–12)). The α1,2-mannosidase IB gene organization (11) differs at the indicated positions. Coding region exons are indicated by numbered boxes, introns are denoted by dotted lines, and solid lines represent the 5′- and 3′-untranslated regions. Numbers above the boxes correspond to the position of the 3′ nucleotide in the exons relative to the first nucleotide of the ORF.
These results demonstrate that purified rat Golgi a,2-mannosidase of purified porcine those previously reported for Golgi insect (29) and fungal (30) GlcNAc2. The human Golgi formed by Golgi order observed for Man 9GlcNAc2. However, this order differs and ER arrow 1,2-mannosidase indicate the active site of class I from that observed with murine Man9GlcNAc2 last. Hydrolysis of Man 8GlcNAc2 isomer B by oligosaccharide substrate (7). Furthermore, mutation of one of the Man 7GlcNAc2 isomers is also formed by (12) and then forms equivalent amounts of two Man5GlcNAc2 isomers. One of the Man5GlcNAc2 isomers is also formed by recombinant murine a,2-mannosidases IA and IB (12), and purified rat Golgi a,2-mannosidase (28), whereas the other isomer (not produced by IA or IB) is formed by recombinant insect (29) and fungal (30) a,2-mannosidases, and is an inferred intermediate of purified porcine a,2-mannosidase (31). The human Golgi a,2-mannosidase activities are complementary to the human ER a,2-mannosidase (5, 6) since they hydrolyze the terminal a,1,2-linked mannose of the middle arm of Man5GlcNAc2 last. Hydrolysis of Man5GlcNAc2 isomer B by a,1,2-mannosidase IC proceeds readily to Man3GlcNAc2 (Fig. 8, lower section). The mannose residues are removed in the same order observed for Man6GlcNAc2. However, this order differs from that observed with murine a,1,2-mannosidases IA and IB (12). These results demonstrate that a,1,2-mannosidase IC has a unique specificity that differs from that of mammalian Golgi a,2-mannosidases IA and IB.

Recent x-ray crystallographic studies of the yeast ER a,1,2-mannosidase indicate the active site of class I a,2-mannosidases is located within an ⟨αα⟩2 barrel with many non-conserved amino acids interacting with different parts of the oligosaccharide substrate (7). Furthermore, mutation of one of these amino acids was demonstrated to change the specificity of the yeast ER a,1,2-mannosidase (22). Therefore, it is likely that the variations in the order of mannose removal by the various class I a,1,2-mannosidases is largely determined by the differences in non-conserved amino acids interacting with the oligosaccharide substrate within the barrel. The relative expression of mammalian Golgi a,1,2-mannosidases with slightly different specificities can thus provide different high mannose oligosaccharide isomers with possible variation in recognition functions.

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