Identification, Expression, and Characterization of a cDNA Encoding Human Endoplasmic Reticulum Mannosidase I, the Enzyme That Catalyzes the First Mannose Trimming Step in Mammalian Asn-linked Oligosaccharide Biosynthesis*

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We have isolated a full-length cDNA clone encoding a human α1,2-mannosidase that catalyzes the first mannos trimming step in the processing of mammalian Asn-linked oligosaccharides. This enzyme has been proposed to regulate the timing of quality control glycoprotein degradation in the endoplasmic reticulum (ER) of eukaryotic cells. Human expressed sequence tag clones were identified by sequence similarity to mammalian and yeast oligosaccharide-processing mannosidases, and the full-length coding region of the putative mannosidase homolog was isolated by a combination of 5′-rapid amplification of cDNA ends and direct polymerase chain reaction from human placental cDNA. The open reading frame predicted a 663-amino acid type II transmembrane polypeptide with a short cytoplasmic tail (47 amino acids), a single transmembrane domain (22 amino acids), and a large COOH-terminal catalytic domain (594 amino acids). Northern blots detected a transcript of ~2.8 kilobase pairs that was ubiquitously expressed in human tissues. Expression of an epitope-tagged full-length form of the human mannosidase homolog in normal rat kidney cells resulted in an ER pattern of localization. When a recombinant protein, consisting of protein A fused to the COOH-terminal luminal domain of the human mannosidase homolog, was expressed in COS cells, the fusion protein was found to cleave only a single α1,2-mannose residue from Man₉GlcNAc₂ to produce a unique Man₉GlcNAc₁ isomer (Man9B). The mannos cleavage reaction required divalent cations as indicated by inhibition with EDTA or EGTA and reversal of the inhibition by the addition of Ca²⁺. The enzyme was also sensitive to inhibition by deoxymannojirimycin and kifunensine, but not swainsonine. The results on the localization, substrate specificity, and inhibitor profiles indicate that the cDNA reported here encodes an enzyme previously designated ER mannosidase I. Enzyme reactions using a combination of human ER mannosidase I and recombinant Golgi mannosidase IA indicated that that these two enzymes are complementary in their cleavage of Man₉GlcNAc₂ oligosaccharides to Man₈GlcNAc₂.

The maturation of Asn-linked oligosaccharides in mammalian cells is initiated in the endoplasmic reticulum (ER) through the cleavage of three glucose residues and as many as two mannose residues soon after the Glc₃Man₉GlcNAc₂ oligosaccharide is transferred to the nascent polypeptide chain (1, 2). For glycoproteins that are destined for secretion or transport to other intracellular compartments, additional mannos trimming occurs in the Golgi complex through the action of members of a multigene family of mannosidases that remove the remaining α1,2-mannose residues to yield a Man₈GlcNAc₂ structure (2). Further maturation by the action of GlcNAc transferase I, Golgi mannosidase II, the collection of branching GlcNAc transferases, and additional glycosyltransferases results in the array of complex oligosaccharides that are found on cellular and secreted glycoproteins (1).

The initial stages of mannose trimming in the ER were originally examined by the metabolic radiolabeling of oligosaccharides on newly synthesized glycoproteins in the presence of mannosidase processing inhibitors or ionophores, such as carbonyl cyanide m-chlorophenylhydrazone, that blocked the transport of proteins from the ER (3–11). The oligosaccharide structures were then examined on glycoproteins that were either secreted from the cell or retained within the ER either as ER-resident proteins or as a result of the carbonyl cyanide m-chlorophenylhydrazone blockade. These studies revealed the presence of two processing mannosidase activities in the ER. The first activity, termed ER mannosidase I (12), was originally identified by the partial accumulation of oligosaccharides containing a unique Man₉GlcNAc₂ isomer structure (Man9B; Fig. 1) in mammalian cells in the presence of low concentrations of dMNJ (5). Subsequent in vitro assays using ER membrane preparations identified a catalytic activity that could generate the Man9B structure from Man₉GlcNAc₂, and this activity was shown to be sensitive to inhibition by dMNJ, kifunensine, and EDTA but not swainsonine or 1,4-dideoxy-1,4-imino-D-mannose; 1-deoxymannojirimycin; pNP-α-Man, p-nitrophenyl-α-D-mannoside; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s minimal medium; FCS, fetal calf serum; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; bp, base pair(s); NRK, normal rat kidney; MES, 4-morpholineethanesulfonic acid; ORF, open reading frame; PA, pyridylamine.

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The abbreviations used are: ER, endoplasmic reticulum; dMNJ, 1-deoxymannojirimycin; pNP-α-Man, p-nitrophenyl-α-D-mannoside; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s minimal medium; FCS, fetal calf serum; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; bp, base pair(s); NRK, normal rat kidney; MES, 4-morpholineethanesulfonic acid; ORF, open reading frame; PA, pyridylamine.
many catalytic characteristics in common with the enzyme identified in mammalian tissues.

In contrast to ER α-mannosidase I, a second ER-resident α-mannosidase activity, termed ER mannosidase II, has been identified in mammalian cells and has been shown to cleave Man9GlcNAc2 to a distinct Man8GlcNAc2 isomer (Man8C; Fig. 1) and potentially smaller structures (12, 13). This enzyme was sensitive to inhibition by dMNJ and 1,4-dideoxy-1,4-imino-D-mannitol and to partial inhibition by swainsonine but not kifunensine or EDTA. An α-mannosidase activity that is immunologically and biochemically related to ER mannosidase II has also been found in the cytosol (12, 27), but the cytosolic form of the enzyme was considerably larger (27–29). The similarity in characteristics between the ER and cytosolic forms of the enzyme has led to the hypothesis that ER α-mannosidase II was derived from a post-translational translocation of the cytosolic form by a process that involves a proteolytic cleavage event (12).

A potential role for ER mannosidase I in the quality control degradation of misfolded glycoproteins in the ER has been recently reported in both S. cerevisiae and mammalian cells. In S. cerevisiae, the formation of the Man8B product of the ER processing mannosidase was shown to be essential for the rapid degradation of a misfolded form of carboxypeptidase Y in the ER (30, 31). Expression of mutant carboxypeptidase Y under conditions that blocked the formation of the isomer B structure, either through gene disruptions in the processing α-glucosidases or the ER processing mannosidase or through defects in the synthesis of the lipid linked precursor structure, led to an accumulation of the misfolded protein in the ER. A role for an ER processing mannosidase has also been described in mammalian cells by an examination of the degradation of a misfolded variant form of α1-antitrypsin (32) and the T cell receptor subunit CD3-δ (33). The turnover of these glycoproteins in the ER is accomplished by translocation into the cytoplasm and proteolysis by the cytosolic proteosome. Treatment of the cells with dMNJ blocked the quality control degradation of these proteins, indicating a requirement for a dMNJ-sensitive ER mannosidase prior to proteolysis. Treatment of cells with kifunensine, a selective inhibitor of ER mannosidase I but not ER mannosidase II, also blocked degradation of α1-antitrypsin, implicating ER mannosidase I in targeting proteins for degradation (32).

In an effort to further characterize the mammalian form of ER mannosidase I and examine its role in oligosaccharide maturation and glycoprotein quality control degradation, we have isolated a full-length cDNA clone encoding a homolog of the S. cerevisiae ER processing mannosidase and members of the multigene family of mammalian Golgi processing mannosidases (Class I mannosidases (2), also known as the Swiss-Prot glycosylhydrolase family 47 (34–36)). A recombinant form of the enzyme was shown to cleave only a single mannoside residue from Man9GlcNAc2 to form Man8B, and it displayed the anticipated response to inhibitors that would be predicted for ER mannosidase I, based on in vitro assays of the enzyme from membrane extracts from mammalian tissues (12). Stable transfection of an epitope-tagged form of the enzyme into normal rat kidney (NRK) cells resulted in a co-localization with ER-resident proteins. Northern blots indicated that transcripts encoding the enzyme were ubiquitously expressed in human tissues. These data indicate that the cDNA encodes human ER mannosidase I and will provide the basis for further studies on the localization of the enzyme and the role of the enzyme in glycoprotein maturation and catabolism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA), Roche Molecular Biochemicals, or Promega (Madison, WI). 1-Deoxynojirimycin was from Genzyme (Cambridge, MA). Kifunensine was from Toronto Research Chemicals, Inc. (Downview, Ontario, Canada). The pCDNA 3.1/Myc-His vector, the PCR II vector, and the mouse monoclonal anti-Myc antibody were from Invitrogen (Carlsbad, CA). Polyclonal (rabbit) anti-cathepsin antibody was from Affinity Bioreagents, Inc. (Golden, CO). Polyclonal (rabbit) anti-p58 antibody was a gift from Dr. J. Saraste (University of Bergen, Norway). Cy5-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG were from Rockland (Gilbertsville, PA). Expand Long PCR reagent kit was from Roche Molecular Biochemicals. Plasmid purification columns were purchased from Qiagen (Valencia, CA). IgG-Sepharose was from Amersham Pharmacia Biotech. COS-7 and NRK cells were from ATCC (Rockville, MD). All other reagents were at least reagent grade and obtained from standard suppliers.

**DNA Sequencing and Primer Preparation**—DNA sequences were determined using Taq polymerase in the dideoxy dye terminator reaction (37) and analyzed on an Applied Biosystems 373A DNA Sequencer (Molecular Genetic Facility, University of Georgia) following the standard protocol as described by the manufacturer. Primers for PCR and DNA sequencing were synthesized by the Molecular Genetics Instrumentation Facility, University of Georgia.

**Expressed Sequence Tag (EST) Identification and Computer Analyses**—The human EST data bank was searched using World Wide Web-based BLAST search engines (National Center for Biotechnology Information), while the S. cerevisiae and Caenorhabditis elegans data banks were searched using their respective search engines. ESTs were purchased from Genome Systems, Inc. (St. Louis, MO). Multiple sequence alignments were performed using Pileup and Boxshade subroutines

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2 The search engines for the S. cerevisiae and C. elegans data banks can be found on the World Wide Web.
ER Mannosidase I Cloning and Characterization

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from the Genetics Computer Group (Madison, WI). Phylogenetic analysis
was performed using the ClustalW 1.6 program (38).

Isolation of the 5' End of the cDNA by 5' RACE—The 5' end of the cDNA encoding the human mannosidase homolog was isolated by two consecutive rounds of 5' RACE (39). The template for the 5' RACE reaction was cDNA prepared from the placenta source as described above. The cDNA-specific primer pairs for the first round of 5' RACE were a pair of EST-specific nested primers designed based on the 5' end of the sequence of the human EST R55729. Two rounds of PCR were performed for each round of 5' RACE. The first amplification employed 5 µl of ligated cDNA and a general primer pair corresponding to the complement of the sequence at base pair positions 1066–1102 (Fig. 3). The conditions for the secondary round of PCR were identical to the first round. The resulting 613-bp 5' RACE product was isolated by agarose gel electrophoresis and purified from the gel using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), subcloned into the pCR II cloning vector (Invitrogen), and sequenced. Comparison with the S. cerevisiae ER processing mannosidase and C. elegans putative open reading frames CELT03G11.4 and ZC410.3 indicated that the full 5' end of the cDNA had not yet been obtained. Therefore, a second round of 5' RACE was performed using an antisense cDNA-specific primer pair corresponding to positions 502–532 and 483–512 (Fig. 3), matched with the AP-1 and AP-2 adaptor primers in amplifications as described above. A 610-bp amplifier was obtained containing a 5' sequence that indicated that the complete coding region had been obtained. To confirm that the 5' end of the cDNA was complete, a third round of 5' RACE was performed using an antisense cDNA-specific primer pair corresponding to positions 10–40 and 1–30 (Fig. 3) matched with the AP-1 and AP-2 primers as described above. The 5' end of the amplifier that was obtained was identical to the product of the second round 5' RACE reaction, indicating that the full-length cDNA had been obtained.

Expression and Isolation of the Fusion Protein Containing the Human Mannosidase Homolog Linked to Protein A—The fusion protein of the human mannosidase homolog linked to protein A was expressed by transient transfection of the pPROTA-ERMan plasmid in COS-7 cells. COS-7 cells were grown in Dulbecco's modified Eagle's minimal medium supplemented with 0.1 µg/ml penicillin, 0.1 µg/ml streptomycin, and 8% fetal calf serum to confluence. The resulting construct was excised by digestion with EcoRI and subcloned into the vector, pcDNA3.1/Myc-His (Invitrogen), that had been isolated following digestion with the same enzyme.

To generate an in-frame fusion with the vector-encoded COOH-terminal Myc epitope tag, the human mannosidase homolog in the pPROTA plasmid was altered by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and primers to positions 1971–2009 to change the stop codon, UAG, to a lys codon, AAG. The resulting construct (ERMan/ppcDNA3.1 Myc-His) encodes a polypeptide containing an extra 50 amino acids added to the COOH-terminal end of the coding region including the vector-encoded Myc epitope sequence and a His peptide.

Enzyme Assays—Oligosaccharide substrates were obtained, and enzyme assays were performed essentially as described previously (42). Briefly, 10–20 µl of the washed IgG-Sepharose bead suspension with bound fusion protein was used in a total assay volume of 50 µl containing 100 mM MES (pH 7.0) and pyridylamine-tagged Man,GlcNAc as the oligosaccharide substrate. Incubations with added cations contained the indicated concentrations of chloride (Ca, Mg, Mn, Co, Br, I, Y, Zn) salts. The incubation conditions for the C18 column were described previously (42). Enzyme assays using the disaccharide substrate, Man1,2Man,GlcNAc, were performed in potassium phosphate buffer (pH 6.5), using 2 mM substrate (42). Assays using the p-nitrophenyl-a-mannopyranoside (pNP-a-Man) substrate were performed as described previously (43) with the exception that the buffer was 100 mM MES (pH 7.0).

Northern Blot Analysis—Northern blots containing poly(A) RNA from various human tissues were purchased from CLONTECH Laboratories. The blots were prehybridized, hybridized, and washed as described (44) using a 900-bp HindIII–PstI restriction fragment from the mannosidase homolog coding region as a radiolabeled probe. The blots were subsequently hybridized with a radiolabeled human β-actin control probe (CLONTECH) to act as an RNA load control for the blots.

Generation of Stably Transfected NRK Cells and Immunofluorescence Microscopy—NRK cells were grown on eight-well coverslips or 100-mm dishes in DMEM/10% FCS for 10 days. The cultures were maintained in fresh DMEM/10% FCS and grown at 37 °C for 36 h. The growth medium was collected, 160 µl of a 50% suspension of IgG-Sepharose beads were added per 10 ml of culture medium, and the suspension was incubated at 4 °C overnight with constant shaking. The beads were collected by centrifugation at 1700 x g for 15 min, washed three times with resuspension and centrifugation in 15 ml of PBS, and used directly in enzyme assays.

To generate a construct encoding the full-length human mannosidase, a nested pair of PCRs was used to generate a 907-bp PCR amplimer containing the front end of the coding region from the human placenta cDNA source as described above. The cDNA-specific primer pairs for the first round of PCR corresponded to positions 927–957 (Fig. 3). The second round of PCR employed a cDNA-specific primer pair corresponding to base pair positions 71 to 1–30 and amplified the sequence at base pair positions 805–836 (Fig. 3). The amplifier fragment was isolated, subcloned, and sequenced to confirm that no errors were introduced into the cDNA sequence as a result of the PCR. To generate the full-length construct in the pCR II vector, the 907-bp amplifier corresponding to the front end of the coding region was excised from pCR II by digestion with NsiI, which cleaves in the vector polylinker and in the insert at a unique site corresponding to base pair position 668 (Fig. 3). The pCR II vector containing the 1476-bp amplimer corresponding to the back end of the coding region was digested with NsiI, and the small NsiI fragment that was excised was replaced with the NsiI fragment from the front end of the coding region to result in a construct containing the full-length coding region corresponding to base pair positions 1–805. This construct was excised by digestion with EcoRI and subcloned into the vector, pcDNA3.1/Myc-His (Invitrogen), that had been isolated following digestion with the same enzyme.

To generate an in-frame fusion with the vector-encoded COOH-terminal Myc epitope tag, the human mannosidase homolog in the pPROTA plasmid was altered by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and primers to positions 1971–2009 to change the stop codon, UAG, to a lys codon, AAG. The resulting construct (ERMan/ppcDNA3.1 Myc-His) encodes a polypeptide containing an extra 50 amino acids added to the COOH-terminal end of the coding region including the vector-encoded Myc epitope sequence and a His peptide.

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isolated and screened for expression of the Myc fusion protein by indirect immunofluorescence using the mouse anti-Myc monoclonal antibody (Invitrogen).

Stably transfected NRK cells were grown on eight-well chamber slides prior to washing with PBS and fixation with 3.5% formaldehyde in 100 mM potassium phosphate (pH 7.0) for 15 min at 37 °C. Following fixation, the cells were washed with PBS and permeabilized by incubation with 0.2% saponin and 10% fetal calf serum in PBS (buffer A) for 15 min at 37 °C. Primary and secondary antibodies were diluted in buffer A at the following dilutions: anti-p58 polyclonal antibody (gift of Dr. J. Saraste, University of Bergen, Norway (45, 46)) and anti-calreticulin polyclonal antibody (Affinity Bioreagents, Golden, CO), 1:100 dilution; anti-Myc monoclonal antibody (Invitrogen), anti-Golgi mannosidase II polyclonal antibody (47), fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Rockland), and Cy5-conjugated goat anti-mouse IgG (Rockland), 1:1000 dilution. Primary antibody incubations were for 1 h at room temperature followed by several washes with PBS. Secondary antibody incubations were also for 1 h at room temperature followed by several washes with PBS. The slides were then mounted in Permafluor mounting medium (Lipshaw-Immunon, Pittsburgh, PA), examined, and photographed with a Bio-Rad MRC-600 laser scanning confocal microscope.

RESULTS

Identification of Putative C. elegans Open Reading Frames and Human ESTs Encoding Homologs of Processing Mannosidases—We anticipated that the mammalian ER mannosidase I sequence would be homologous to the mammalian Golgi processing α-mannosidases IA (42) and IB (48) and the yeast ER mannosidase I (49) based on two lines of evidence. In vitro assay data have indicated that the mammalian ER mannosidase I was inhibited by dMNJ and kifunensine but not swainsonine or 1,4-dideoxy-1,4-imino-D-mannitol, and the enzyme required Ca²⁺ for catalytic activity (12). These characteristics are in common with the previously described Class I mannosidases (Swiss-Prot glycosylhydrolase family 47) (2, 34–36), including mammalian Golgi mannosidase IA and IB and yeast ER mannosidase I. In addition, the yeast enzyme catalyzes the same enzymatic reaction predicted for the mammalian ER mannosidase I, cleaving Man₆GlcNAc₂ to the same Man₆GlcNAc₂ B isomer (26), indicating that the yeast enzyme may be an ortholog of the human enzyme. In an attempt to identify clones encoding the human ER mannosidase I, we decided to first make a sequence search of the C. elegans genome data base for mannosidase homologs and follow this search with a search of the human EST sequence data base using the yeast mannosidase, the mammalian Golgi mannosidases, and the putative homologs from the C. elegans data base as query sequences. Sequence similarity searches using the cloned Class I (glycosylhydrolase family 47) mannosidases (2, 34–36) as query sequences identified four predicted open reading frames in the C. elegans genome that had translations with sequence similarity to the processing mannosidases (C. elegans predicted open reading frame (ORF) designations: CELT03G11.4, ZE410.3, C52E4.5, and CED2030.1). The two former ORFs were found to have a higher similarity to the yeast ER processing mannosidase (49), and the latter two were found to be more similar to the mammalian Golgi processing mannosidases IA and IB (48, 50) (Fig. 4).

When this collection of mannosidase coding regions were used as query sequences in BLAST searches against the human EST data base, more than 30 overlapping human EST sequences were identified that had a high degree of sequence similarity to the yeast and putative C. elegans ER mannosidase I sequences. Several of these EST clones were obtained from EST repositories and fully sequenced. The longest of the EST clones was ~1800 bp in length (EST R55729 in Fig. 2), contained all of the sequences of the shorter EST clones, and terminated at the 3′-end with a poly(A) tail. This clone was apparently missing the 5′-end of the coding region based on a comparison of the translation of the EST clone with the translations of the yeast and putative C. elegans mannosidases genes.

5′-RACE Extension of the Human EST Sequence—Since the human EST clone, R55729, did not contain the entire coding region for the putative human mannosidase homolog, we isolated sequences extending further upstream by employing a 5′-RACE approach using a ligation-anchored human placenta cDNA template. A nested pair of primers were designed to the 5′-end of the R55729 EST sequence, and two sequential rounds of PCR were carried out using two EST-specific nested primers in conjunction with adaptor primers complementary to the anchor primer sequence on the human placenta cDNA as described under “Experimental Procedures.” A 613-bp amplification product of the 5′-RACE reaction was subcloned and sequenced (Fig. 2). Comparison of the translation of the resulting sequence with the peptide sequences of the yeast and putative C. elegans mannosidases indicated that the RACE sequence was still incomplete. An additional round of 5′-RACE was performed as described above using the 5′ sequence of the first round RACE product to design the cDNA-specific nested primers. The second round of 5′-RACE generated a 610-bp amplifier, which was also subcloned and sequenced (Fig. 2). The translation of this sequence was consistent with a full-length
Coding region based on a comparison with the S. cerevisiae, C. elegans, and mammalian mannosidase sequences and the presence of a putative initiating ATG, followed by a hydrophobic sequence that could act as a potential transmembrane domain. To confirm that the complete 5'-end of the coding region was obtained, a third round of 5'-RACE product was performed using the sequence derived from the second round 5'-RACE product. The product of the third round of 5'-RACE terminated at the same position as the second round 5'-RACE product (Fig. 2), confirming that the 5'-end of the transcript had been obtained.

Characteristics of the cDNA Sequence and Comparison with the Sequence of Other Processing Mannosidases—The composite sequence obtained by assembling the products of the 5'-RACE amplimers with the EST sequences resulted in a transcript of 2679 bp (excluding the poly(A) tail) that consisted of a 98-bp 5'-untranslated region, a 1989-bp coding region, and 592 bp of 3'-untranslated region followed by a poly(A) tail (Fig. 3). Although the putative 5'-untranslated region does not contain an upstream in-frame stop codon, three lines of evidence suggest that the indicated ATG is the correct initiation site. First, the 5'-RACE data terminated at the same position in two independent 5'-RACE reactions, and the indicated ATG is the first potential initiation site in the sequence. Second, the sequence surrounding this ATG conforms to the consensus sequence for eukaryotic translation initiation sequences with a purine at position -3, the most critical residue for initiation (51). Third, all of the Class I mannosidases (2) are type II transmembrane proteins with short cytoplasmic tails and single transmembrane domains, consistent with the data for the translation of the human mannosidase homolog coding region downstream from the proposed translation start site.

The 3'-end of several of the EST clones, including EST R55729, contained a poly(A) tail at an identical position, and the poly(A) tail was preceded by a rarely used GAUAAA polyadenylation signal (single underline in Fig. 3, top) (52). The open reading frame encodes a 663-amino acid (M ± 76,002) protein that predicts a single type II transmembrane domain from amino acid residues 48–69 as indicated by hydropathy analysis (Fig. 3, bottom), and no consensus Asn-linked glycosylation sites were identified.

Comparison of the translation of the cDNA with other known mannosidase sequences indicated a similarity to Class I mannosidases (glycosylhydrolase family 47) (2, 34–36) and a higher degree of sequence similarity to a subset of these gene products, including the two putative C. elegans “ER mannosidase I” homolog sequences and the ER processing mannosidase from S. cerevisiae (Fig. 4, bottom). A direct sequence comparison with the latter three sequences (Fig. 4, top) demonstrated that the sequence similarity is restricted to the COOH-terminal domain, ranging from 21 to 26 amino acids, the cytoplasmic tails vary in length from 2 amino acids (yeast ER processing mannosidase) to 42 and 47 amino acids (C. elegans CELT03G11.4 and the human mannosidase homolog, respectively). Even more variable is the length of the putative “stem domain,” a region that has been previously described for Golgi glycosyltransferases as
a potential spacer region between the catalytic domain and the transmembrane domain that may provide flexibility to the catalytic domain within the lumen of the ER and Golgi (53). While the yeast processing mannosidase and the putative *C. elegans* CELT03G11.4 gene product contain a relatively short putative “stem domain,” the “stem domains” of the human mannosidase homolog and the putative *C. elegans* ZE410.3 gene product are considerably larger (Table I and Fig. 4, top) and contain an unusually high content of proline residues. The significance of a lack of conservation in the sequence and the length of these NH2-terminal domains is unclear.

**Fig. 4.** Multiple sequence alignment of protein sequences corresponding to a subset of the class I mannosidases and sequence relationship to other processing mannosidases. The upper panel shows an optimized multiple sequence alignment using the Pileup and Boxshade subroutines as described under “Experimental Procedures.” Protein sequences included in the comparison are the translation of the human mannosidase homolog (Human_ER_ManI, from Fig. 3), the *S. cerevisiae* ER processing mannosidase (Yeast_ER_ManI), and the two putative *C. elegans* mannosidase ORFs that are indicated in the shaded area of the bottom panel (C_e_CELT03G11 and C_e_ZE410). Sequences shown with white text on a black background are identical in at least two of the aligned proteins. Sequences that are black on gray are conserved in amino acid character. Dots and dashes indicate gaps and spaces introduced to optimize the sequence alignment. The bottom panel indicates a phylogenetic tree of the known Class I mannosidases (Swiss-Prot glycosylhydrolase family 47) with species indicated in italics and the GenBank™ accession numbers shown in parenthesis. The unrooted tree was generated with the ClustalW 1.6 software. The shaded cluster of sequences in the bottom panel correspond to the putative subgroup of ER mannosidase I-like enzymes.

**Tissue Distribution of mRNA Transcripts for the Human Mannosidase Homolog—** Transcript levels of the human mannosidase homolog were determined by Northern blot analysis using a 900-bp restriction fragment from the coding region as a radiolabeled probe. A major transcript of \(~2.8\) kilobase pairs
ER Mannosidase I Cloning and Characterization

Comparison of the relative lengths of the cytoplasmic tails, transmembrane domains, putative "stem" domains, and catalytic domains of the "ER mannosidase I" family of proteins

| Species (gene or putative ORF designation) | Cytoplasmic tail | Transmembrane domain | Putative "stem" domain | Catalytic domain |
|------------------------------------------|-----------------|----------------------|-----------------------|-----------------|
| C. elegans (CELT03G11.4)                 | 42              | 21                   | 28                    | 440             |
| C. elegans (ZEU410.3)                    | 9               | 26                   | 54                    | 443             |
| Human mannosidase homolog                | 47              | 22                   | 151                   | 443             |
| S. cerevisiae ER Man I (P32908)          | 2               | 23                   | 20                    | 504             |

a The length of the cytoplasmic tail was defined as the number of amino acids between the NH₂ terminus and the last charged amino acid prior to the transmembrane domain.

b The length of the transmembrane domain was defined as the number of amino acids between the first noncharged amino acid flanking the transmembrane domain (defined by Kyte-Doolittle hydrophathy analysis (75)) and the next downstream charged amino acid. In each instance, the last charged amino acid on the cytoplasmic side of the transmembrane domain was Lys or Arg, and the first charged amino acid on the luminal side was Glu or Asp.

c The length of the putative "stem domain" was arbitrarily defined as the number of amino acids between the end of the transmembrane domain and the first entirely conserved amino acid in the "catalytic domains" of the aligned sequences. This amino acid corresponds to Phe²²¹ in the human mannosidase homolog but is at different positions in the other proteins (refer to Fig. 4, top).

d The length of the luminal catalytic domain was arbitrarily defined as the distance from the first fully conserved amino acid in the aligned sequences (Phe²⁰¹) in the human mannosidase homolog to the COOH terminus.

This work. (see Fig. 3).

Fig. 5. Tissue distribution of mRNA transcripts for the human mannosidase homolog. A Northern blot of human tissue poly(A⁺) RNAs was hybridized with a radiolabeled probe corresponding to a 900-bp restriction fragment within the human mannosidase homolog coding region as described under "Experimental Procedures." The blot was rehybridized with β-actin cDNA as a control (lower panel). Lanes on the blot represent the RNA isolated from the tissues indicated at the top. The locations of the size standards (in kilobases) are indicated on the left. The arrow on the right indicates the transcript corresponding to the human ER mannosidase I transcript at ~2.8 kilobase pairs, in close agreement with the size predicted for the polyadenylated transcript equivalent to the full-length cDNA clone.

was found in all tissues (Fig. 5), consistent with the size of the transcript predicted from the length of the full-length cDNA including the poly(A) tail. The transcript was equally abundant in most tissues, consistent with the expected role for the enzyme in glycoprotein processing in all cells, but colon, kidney, lung and peripheral blood leukocytes had slightly lower transcript levels.

Recombinant Protein Expression in COS-7 Cells and Characterization of the Enzyme Activity of the Fusion Protein—To demonstrate a catalytic activity associated with the putative human mannosidase cDNA expression product, we generated a construct encoding the COOH-terminal end of the coding region for the human mannosidase homolog (corresponding to amino acids 178–663) fused in frame and downstream from the coding region for protein A. This construct contains an NH₂-terminal signal sequence that would target the fusion protein for translocation into the ER lumen (41). Constructs of this type have previously been used to generate secreted forms of the catalytic domains of ER and Golgi glycoprotein processing enzymes in mammalian cells (48, 54–60), where they can be recovered from the culture media by binding to IgG-Sepharose. When the medium from COS cells transfected with this expression construct was incubated with IgG-Sepharose beads, the fusion protein bound to the beads was tested for mannosidase activity using a variety of substrates. Incubation of the beads containing the immobilized recombinant fusion protein with a pyridylamine-tagged Man₉GlcNAc₂ substrate resulted in the release of a single mannose residue (Fig. 6, left panels). This cleavage was linear with time and concentration of culture media (data not shown), and prolonged incubation resulted in no further cleavage of the substrate. No substrate cleavage was detected when assays were performed using medium from control transfections with a vector without an insert (data not shown). The Man₉GlcNAc₂ product of the reaction was identified as the Man₈B isomer (Fig. 7B), using C18-HPLC, by comparison with the elution positions of standards that were previously identified by NMR (42). When the recombinant fusion protein bound to the IgG-Sepharose beads was tested with either npNP-α-Man or the disaccharide substrate, Man₁₉, 2Manα-O-CH₃, no substrate cleavage was detected. As positive controls, a recombinant form of the human lysosomal α-mannosidase (43) was shown to cleave the npNP-α-Man substrate, and a recombinant form of murine Golgi mannosidase IA (42) hydrolyzed the disaccharide substrate (data not shown).

The catalytic characteristics of the recombinant enzyme were determined using Man₉GlcNAc₂-PA as the substrate. The enzyme was active between pH 6.0 and 8.0 with an optimum at pH ~7.0. Kifunensine, dMNJ, EDTA and EGTA, a specific chelator of Ca²⁺ (61), inhibited the mannosidase activity (Figs. 8 and 9), but swainsonine did not show inhibition at a concentration of 1 mM (not shown). The enzyme inhibition by EDTA or EGTA could be reversed by the addition of Ca²⁺ (Fig. 9) and to a lesser extent by Fe³⁺ and Mn²⁺. Other cations tested were unable to reverse the EDTA inhibition and were inhibitory in the absence of prior incubation with EDTA (Fig. 9), suggesting that they compete with Ca²⁺ for binding to the enzyme. Experiments testing the effects of Ca²⁺ on the recovery of enzyme activity revealed that the enzyme bound to the IgG-Sepharose beads had been partially stripped of bound Ca²⁺ during washing with buffer (data not shown). Enzyme assays without added Ca²⁺ gave variable but lower activity than assays in the presence of added Ca²⁺. The increase in mannosidase activity as a result of the addition of exogenous Ca²⁺ could be used as a measurement of Ca²⁺ association and allowed the determination of an approximate Kd for Ca²⁺ of ~8 μM (data not shown), similar to the affinity constants previously determined for the yeast ER processing mannosidase (25) and rabbit liver Golgi mannosidase IA (62).

Comparison of the Activity of the Human Mannosidase Homolog with the Activity of Murine Golgi Mannosidase IA—To determine whether the human mannosidase homolog had an activity that was complementary with Golgi mannosidase IA, we performed a digestion time course of Man₉GlcNAc₂-PA with the human mannosidase homolog alone, murine Golgi mannosidase IA alone, or the two recombinant enzymes mixed together in approximately equal proportions based on their rate...
of cleavage of Man₉GlcNAc₂-PA (Fig. 6). The human mannosidase homolog progressively cleaved Man₉GlcNAc₂-PA to the Man₈B isomer (Fig. 6, left panels), whereas the recombinant murine mannosidase IA partially cleaved this substrate to Man₆GlcNAc₂-PA and several larger intermediates over the same 60-min time course (Fig. 6, middle panels). In contrast, a mixture of similar quantities of the two enzymes resulted in a rapid and efficient cleavage of the Man₉GlcNAc₂-PA substrate to Man₅GlcNAc₂-PA in 60 min (Fig. 6, right panels). These data indicate that the recombinant product of the human mannosidase homolog cDNA encodes an enzyme activity that is complementary to the activity of Golgi mannosidase IA and that the combination of the two enzymes provides an efficient cleavage route for the processing of Man₉GlcNAc₂ oligosaccharides to the Man₅GlcNAc₂ structures that are necessary for further oligosaccharide maturation.

**Relative Inhibition by dMNJ of the Human Mannosidase Homolog in Comparison with Golgi Mannosidase IA**—Since both the recombinant human mannosidase homolog and murine Golgi mannosidase IA are sensitive to inhibition by dMNJ (Fig. 8 and Ref. 42), we tested the relative sensitivity of the two enzymes to inhibition by dMNJ in a reaction where both enzymes were present (Table II). As a measurement of the relative contributions of the two enzymes in the cleavage reaction, we isolated the Man₈GlcNAc₂ intermediates from an enzymatic time course and determined the ratios of the Man₈GlcNAc₂ isomers. The human mannosidase homolog was shown to exclusively produce the Man₈B isomer (Fig. 7B), while the recombinant murine Golgi mannosidase IA produces predominantly Man₈A with small amounts of the Man₈C isomer during the progress of digestion to smaller structures (Fig. 7C and Ref. 42). The combination of the two enzymes produced a mixture of the Man₈GlcNAc₂ isomers A, B, and C with a ratio of Man₈B to Man₈A + Man₈C of ~0.4. This isomer ratio indicates that the ratio of the enzyme activity of the human mannosidase homolog to Golgi mannosidase IA was ~0.4:1 at each of the time points tested (Table II). In contrast, when identical samples were incubated in the presence of 5 μM dMNJ, the cleavage beyond Man₈GlcNAc₂ was significantly reduced, and the ratio of the Man₈GlcNAc₂ isomers was shifted toward the formation of the Man₈B structure (Man₈B/Man₈A + Man₈C) = 2.14. These data indicate that there was a significantly greater inhibition of the murine Golgi mannosidase IA at this inhibitor concentration than the human mannosidase homolog.
Fig. 8. Enzymatic properties of the human mannosidase homolog. Enzyme assays of the human mannosidase homolog were carried out using the recombinant fusion protein bound to IgG-Sepharose beads and the Man$_9$GlcNAc$_2$-PA substrate. A, pH profile with enzyme activity expressed as a percentage of maximal activity; $B$, kifunensine inhibition of the catalytic activity expressed as a percentage of control activity in the absence of inhibitors; $C$, dMNJ inhibition of the catalytic activity expressed as a percentage of control activity in the absence of inhibitors; $D$, EGTA inhibition expressed as a percentage of control activity in the absence of inhibitors; $E$, recovery of mannosidase activity by the addition of CaCl$_2$ after prior incubation in the presence of 200 $\mu$M EGTA.

**Immunolocalization of an Epitope-tagged Form of the Human Mannosidase Homolog in Transfected Cells**—The subcellular localization of the human mannosidase homolog was determined by the stable transfection of a construct encoding a Myc-tagged form of the full-length protein (ERMannI pcDNA3.1 Myc-His) into NRK cells followed by detection of the Myc epitope tag by indirect immunofluorescence. Double staining of the cells was accomplished by the use of a mouse monoclonal anti-Myc antibody, detected with a Cy5-tagged secondary antibody, and rabbit polyclonal antibodies to compartment-specific marker proteins, detected with fluorescein isothiocyanate-tagged anti-rabbit IgG. The immunofluorescence pattern of the Myc-tagged fusion protein was broadly distributed throughout the cytoplasm in a reticular pattern (Fig. 10, A, C, and E). Untransfected cells or cells transfected with vector alone showed no detectable fluorescence. Co-localization of the anti-Myc immunofluorescence pattern with the antibody to the ER marker protein, calreticulin, was detected in double-labeled cells (Fig. 10, B, D, and F), although minor differences in intensity and staining pattern were seen in regions of some cells. A similar partial co-localization of the Myc tag immunofluorescence pattern was seen with an antibody to the intermediate compartment protein, p58/ERGIC 53 (45, 46) (data not shown), which also has a reticular appearance in our transfected NRK cell line. A lack of co-localization was seen with an antibody to the Golgi marker protein, $\alpha$-mannosidase II (data not shown). These data indicate that the Myc-tagged form of the enzyme is localized in the ER and possibly in intermediate compartment structures but not in the Golgi of transfected cells.

**DISCUSSION**

The extent of $\alpha$1,2-mannose trimming of Asn-linked oligosaccharides in the early secretory pathway varies in eukaryotic organisms from the removal of a single mannose residue in $S$. cerevisiae (15) to the removal of all four $\alpha$1,2-mannose residues in metazoan organisms (2). In all eukaryotic organisms examined, with the possible exception of the fission yeast $Schizosaccharomyces pombe$ (64), mannose trimming is initiated in the ER by the removal of a single mannose residue from Man$_9$GlcNAc$_2$ to produce the Man88B structure. Further trimming of $\alpha$1,2-mannose residues in animal and plant systems occurs through the action of multiple mannosidases in the ER and Golgi (2). Primary sequence similarity between the $S$. cerevisiae processing mannosidase and the mammalian Golgi processing $\alpha$1,2-mannosidases, along with their common requirement for Ca$^{2+}$ for catalytic activity, their sensitivity to inhibition by dMNJ and kifunensine, and their common reaction mechanism (20, 42), has led to the classification of these enzymes as Class I mannosidases (2). This classification contrasts them with the more heterogeneous collection of processing and catalytic mannosidases, termed Class II mannosidases (2), in the ER, Golgi, lysosomes, and cytosol, that do not require Ca$^{2+}$ for catalytic activity, their sensitivity to inhibition by dMNJ and kifunensine, and have a different mechanism of action (65). A similar separation of the mannosidases into two distinct families was made during the classification of glycosylhydrolases based on sequence similarities (Class I = Swiss-Prot glycosylhydrolase family 47; Class II = Swiss-Prot glycosylhydrolase family 38) (34–36).

Although biochemical evidence for an ER mannosidase I-like activity in mammalian cells was originally described over 16 years ago (3, 6) and the proposed contribution of the enzyme to the maturation of Asn-linked oligosaccharides has been de-
The effect of dMNJ on the relative activity of human ER mannosidase I and murine Golgi mannosidase IA in a mixed enzyme reaction

The relative sensitivity of recombinant human ER mannosidase I and murine Golgi mannosidase IA to inhibition by dMNJ was determined in an enzyme reaction containing both enzymes at an approximate ratio of 0.4:1 (human mannosidase homolog to Golgi mannosidase IA). Three reactions were prepared with the Man$_9$GlcNAc$_2$-PA substrate and incubated for the times indicated in the presence or absence of dMNJ. The Man$_8$GlcNAc$_2$-PA peak was isolated by NH$_2$-HPLC, as indicated by the arrow on the profiles, and the relative ratios of the Man$_8$GlcNAc$_2$ isomers were determined by C$_{18}$-HPLC as described under “Experimental Procedures.” The sizes of the individual oligosaccharide peaks on the NH$_2$-HPLC column are shown at the bottom of the table (M5, Man$_8$GlcNAc$_2$-PA; M6, Man$_8$GlcNAc$_2$-PA; M7, Man$_8$GlcNAc$_2$-PA; M8, Man$_8$GlcNAc$_2$-PA; M9, Man$_8$GlcNAc$_2$-PA). As indicated in Fig. 6, the presence of Man$_8$GlcNAc$_2$ isomer B is indicative of the human mannosidase homolog activity, and the presence of Man$_8$GlcNAc$_2$ isomers A and C are indicative of Golgi mannosidase IA activity. The ratio of the isomers (Man8B/(Man8A + Man8C)) would represent the relative activity of the human mannosidase homolog versus Golgi Man IA in the reaction. The dMNJ appeared to have a greater effect on inhibition of Golgi Man IA as indicated by the increase in the ratio in response to the inhibitor.

| Time of Digestion | dMNJ conc. | NH$_2$-HPLC Column Profile | Percent distribution of the Man$_8$GlcNAc$_2$ isomer structures |
|-------------------|------------|---------------------------|-------------------------------------------------------------|
| 5 min 0           | 27.3       | 63                        | 9.8  | 0.375 |
| 20 min 0          | 29.6       | 61.9                      | 8.5  | 0.42  |
| 20 min 5 μM       | 68.2       | 27.7                      | 4.1  | 2.14  |

scribed in reviews (1, 2, 66), very little is known about the enzyme other than biochemical characteristics determined through enzyme assays in crude membrane extracts (12). Previous attempts to clone the enzyme have resulted in the isolation of cDNAs encoding either the rat cytosolic/ER mannosidase II (12, 28) or mouse Golgi mannosidase IB (48).

We anticipated that the mammalian ER mannosidase I would be a Class I mannosidase based on two lines of evidence. First, in vitro assay data indicated that the mammalian ER mannosidase I was inhibited by dMNJ and kifunensine but not swainsonine, and the enzyme required Ca$^{2+}$ for catalytic activity (12). Second, the only known Class I mannosidase in S. cerevisiae is an ER processing mannosidase that catalyzes the equivalent enzymatic reaction as the mammalian ER mannosidase I, cleaving Man$_9$GlcNAc$_2$ to the same Man$_8$B isomer (26), indicating that the yeast enzyme may be an ortholog of the human enzyme.

In an attempt to identify clones encoding ER mannosidase I, we searched both the C. elegans genome data base and the human EST sequence data base using the known Class I mannosidases as query sequences. These searches identified four putative Class I mannosidase homologs in C. elegans, two of which had a greater similarity to the yeast ER processing mannosidase, as well as a collection of overlapping human EST sequences. The EST sequences were present in EST libraries from a number of human tissue sources, consistent with our Northern blot data (Fig. 5) that demonstrated a ubiquitous expression pattern for the transcripts in human tissues. The data base searches also identified 186-bp genomic sequence isolated by exon amplification (67, 68) that had a 100% identity with the human mannosidase homolog in base pair positions 623–809 (Fig. 3) (GenBank™ accession no. T12605, data not shown). The exon sequence was amplified from human chromosome 9-specific cosmids, indicating that the gene encoding the human mannosidase homolog is likely to be present on this chromosome.

The partial human EST sequences were extended by repetitive rounds of 5'-RACE, and the full-length coding region was isolated by direct PCR from human placental cDNA. Comparison of the sequence translation with the other Class I mannosidases indicated that the COOH-terminal ~440 amino acids of the protein were most similar to the S. cerevisiae and the putative C. elegans ER mannosidase I sequences and less similar to the other Class I mannosidases. The translation of the coding region also predicted a type II transmembrane topology, a characteristic common in other Class I mannosidases (2), but the primary sequence of the first 220 amino acids, including the transmembrane domain, had no significant sequence similarity to the other mannosidase sequences. The high content of Pro residues in the putative “stem domain” and the cytoplasmic tail led to the identification of a sequence similarity to proline-rich proteins within these regions (data not shown). The significance of the proline-rich regions, including a continuous stretch of seven Pro residues in the NH$_2$-terminal cytoplasmic tail, is uncertain, but the stem domains of several Golgi glycosyltransferases and mannosidases also have a high proline content (44, 50, 53, 69), suggesting that they may contribute to the flexibility of this region.

Expression of an epitope-tagged form of the mannosidase homolog in transfected NRK cells demonstrated an apparent co-localization of the fusion protein with the ER marker protein, calreticulin, and lack of co-localization with the Golgi marker, mannosidase II. The co-localization with the ER marker is strongly suggestive of an ER localization for the enzyme, but further confirmation of this subcellular localization will require an antibody to the human mannosidase homolog allowing a direct detection of the polypeptide in untransfected cells and tissues.

Expression of the COOH-terminal 435 amino acids of the mannosidase homolog as a fusion with protein A demonstrated that this region, in common with the other Class I mannosidases, contains the catalytic domain of the enzyme and that the
The immunofluorescence signal with the immunofluorescence pattern of the anti-rabbit IgG secondary antibody. The coincidence of the anti-Myc calreticulin followed by a fluorescein isothiocyanate-conjugated goat secondary antibody (B) and the detection of the anti-rabbit IgG secondary antibody (E), employed a rabbit polyclonal antibody to calreticulin followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG secondary antibody. The coincidence of the anti-Myc immunofluorescence signal with the immunofluorescence pattern of the ER marker is strongly suggestive of an ER localization of the enzyme.

The NH₂-terminal 220 amino acids are not required for catalytic activity. The enzyme cleaved a single residue from Man₉GlcNAc₂ to produce the Man₈B isomer with no further digestion of the substrate even after a prolonged incubation. The recombinant enzyme was inhibited by dMNJ and kifunensine but not swainsonine and could not cleave pNP-α-Man. The requirement for divalent cations in the enzyme reaction was demonstrated by the strong inhibition with either EDTA or EGTA and the recovery of the enzyme activity by the addition of Ca²⁺. These inhibition profiles, along with requirement for divalent cations and the inability to cleave pNP-α-Man, are hallmarks of Class I mannosidases. In combination with the specificity for producing the Man₈B isomer from Man₉GlcNAc₂, the catalytic characteristics indicate that the human cDNA encodes an enzyme activity previously described for ER mannosidase I (12, 13). The immunolocalization of the Myc-tagged form of the enzyme in the ER of NRK cells and the Northern blots demonstrating a ubiquitous transcript expression pattern are also consistent with this conclusion.

Although we have not been able to detect any other sequences in the human EST data base that have similarity to this subgroup of Class I mannosidases, the present data do not allow us to conclude that the cDNA that we have cloned encodes the only source of ER mannosidase I-like activity in mammalian cells. We have previously used immunodepletion of Golgi mannosidase IA activity from Golgi membrane extracts to determine the role of this enzyme in glycoprotein maturation (42). A similar set of immunodepletion studies with an antiserum specific for ER mannosidase I will allow us to confirm the role of this enzyme in glycoprotein maturation in the ER. We have recently initiated the expression of the human ER mannosidase I cDNA in P. pastoris,³ as we have for other processing and catabolic mannosidases (42, 43, 70, 71), in order to generate sufficient quantities of purified recombinant enzyme necessary for antibody production. Once we have generated a specific antiserum to the recombinant expression product, we will be able to use it for both the immunodepletion studies and further immunolocalization studies on the endogenous enzyme in mammalian cells.

The accumulation of glycoproteins containing the Man₈B structure in cultured cells incubated with low concentrations of dMNJ was among the first evidence suggesting the presence of a unique mannosidase activity in the ER (5). These data demonstrated that ER mannosidase I was more resistant to inhibition by dMNJ than the Golgi enzymes, mannosidase IA and IB (42). Subsequent work indicated that the ER might also contain a distinctive dMNJ-sensitive mannosidase activity that would produce the Man₈B isomer from Man₉GlcNAc₂ (5, 10). Our data on the recombinant human ER mannosidase I have shown that the enzyme is sensitive to inhibition by dMNJ. In addition, our enzyme mixing experiments (Table II) have shown that the ER enzyme was relatively less sensitive to inhibition than Golgi mannosidase IA. These data indicate that treatment of cells with less than fully inhibitory concentrations of dMNJ would result in the production of oligosaccharides with the Man₈B isomer structure. Higher dMNJ concentrations would be predicted to result in full ER mannosidase I inhibition and accumulation of Man₉GlcNAc₂ structures. It is noteworthy that the concentration of dMNJ used previously to demonstrate a dMNJ-resistant ER mannosidase activity in cultured cells (150 μM dMNJ (5)) would not be expected to fully inhibit ER mannosidase I. In contrast, when higher dMNJ concentrations (0.4–1 mM) were employed, a partial (5) or complete (10) inhibition of cleavage from Man₉GlcNAc₂ to Man₈B was observed in cultured cells. An additional complexity arises from the fact that the early enzymology and biosynthetic labeling studies (3–5, 10, 27) predated the biochemical identification of ER mannosidase II (12–14), an enzyme that has been shown to produce the Man₈C isomer and potentially smaller structures from Man₉GlcNAc₂ in the ER of mammalian cells (12). This latter enzyme is also sensitive to inhibition by dMNJ but not kifunensine. As a result of the action of these two enzymes, several factors could contribute to the variable oligosaccharide structures observed in dMNJ-treated cells (5, 10). Although the absolute enzyme activity levels of ER mannosidase I and ER mannosidase II in different cell types are not known, the relative ratios of the two enzymes have been previously shown to vary widely in different cell types (13). Moreover, the capacity of different cell types to transport dMNJ into the lumen of the ER is unknown and may also vary between cell types. Finally, the residence time of glycoprotein substrates in the ER is known to be quite variable (72) and may influence the degree of processing by ER mannosidases. Conclusions about the dMNJ-sensitivity of ER mannosidase I based on an examination of the effect of dMNJ treatment of cultured cells could, therefore, be misleading, and this enzyme could be responsible for both of the activities that have previously been termed dMNJ-sensitive or dMNJ-resistant ER mannosidases.

It is interesting to note that the substrate specificity of ER mannosidase I was found to be complementary to the substrate specificity of Golgi mannosidases IA (Fig. 6). The former enzyme cleaves a single α1,2-mannose from Man₉GlcNAc₂ to produce Man₈B, while the latter enzyme will cleave the other three α1,2-mannose residues on Man₉GlcNAc₂ but recognizes the central branch mannosyl substrate for ER mannosidase I.

³ K. Karaveg, D. S. Gonzalez, and K. W. Moremen, unpublished data.
with at least 10-fold lower efficiency (42). Enzyme mixing experiments confirmed the complementarity of ER mannosidase I activity with Golgi mannosidase IA to result in the rapid and efficient cleavage of Man9GlcNAc2 to Man8GlcNAc2. The differences in substrate recognition by the two enzymes are striking considering their similarity in sequence. Recent crystallization of recombinant Golgi mannosidase IA (73) and the S. cerevisiae processing mannosidase (17), a presumed ortholog of the human ER mannosidase described here, will hopefully lead to structure determination of these two enzymes and should be instrumental in determining the differences in their substrate recognition.

ER mannosidase I-like activity, cleaving Man9GlcNAc into the Man8B structure, is the last step in oligosaccharide processing that is fully conserved from yeast to mammals. In S. cerevisiae, further extension of the oligosaccharide by mannan addition leads to the formation of mannans (15, 74). In mammals, oligosaccharides are further processed by mannose trimming and further extension into complex type structures (1). As the last conserved step in eukaryotic oligosaccharide processing, ER mannosidase I presumably accomplishes a critical role in oligosaccharide maturation. In S. cerevisiae, the enzyme does not appear to be essential for growth (49) or extension of mannan structures (23), but the enzyme may contribute to the timing step in the quality control degradation of glycoproteins in the ER (30, 31). A similar role for ER mannosidase activity has been implicated in mammalian glycoprotein turnover in the ER (32, 33). The availability of the mammalian cDNA encoding ER mannosidase I should allow a more direct testing of the hypothesis that the conserved role of the enzyme in eukaryotes is to target malfolded glycoproteins for quality control degradation.

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