Molecular Cloning and Expression of Rat Liver Endo-α-mannosidase, an N-Linked Oligosaccharide Processing Enzyme*

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A clone containing the open reading frame of endo-α-mannosidase, an enzyme involved in early N-linked oligosaccharide processing, has been isolated from a rat liver Agt11 cDNA library. This was accomplished by a strategy that involved purification of the endomannosidase from rat liver Golgi by ligand affinity chromatography (Hiraizumi, S., Spohr, U., and Spiro, R. G. (1994) J. Biol. Chem. 269, 4697–4700) and preparative electrophoresis, followed by sequence determinations of tryptic peptides. Using degenerate primers based on these sequences, the polymerase chain reaction with rat liver cDNA as a template yielded a 470-base pair product suitable for library screening as well as Northern blot hybridization. EcoRI digestion of the purified λ DNA released a 5.4-kilobase fragment that was amplified in Bluescript II SK(−) vector. Sequence analysis indicated that the deduced open reading frame of the endomannosidase extended from nucleotides 89 to 1441, encoding a protein of 451 amino acids and corresponding to a molecular mass of 52 kDa. Data base searches revealed no homology with any other known protein. When a molecular mass of 52 kDa. Data base searches revealed a protein of 451 amino acids and corresponding to a

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Bluescript II SK(−) yielded a 5.4-kilobase fragment that was amplified in Bluescript II SK(−) vector. Sequence analysis indicated that the deduced open reading frame of the endomannosidase extended from nucleotides 89 to 1441, encoding a protein of 451 amino acids and corresponding to a molecular mass of 52 kDa. Data base searches revealed no homology with any other known protein. When a vector coding for this protein fused to an NH2-terminal peptide containing a polyhistidine region was introduced into Escherichia coli, high levels of the enzyme were expressed upon induction with isopropyl-β-D-thiogalactoside. Purification of the endomannosidase to electrophoretic homogeneity from E. coli lysates was accomplished by Ni2+-chelate and Glcα1→3Man-O-(CH2)8CONH-Affi-Gel ligand chromatographies. Polyclonal antibodies raised against this protein reacted with Golgi endomannosidase. By both immunoblotting and silver staining, the purified protein carrying this activity could be isolated to electrophoretic purity. This permitted the generation of antibodies which reacted with the rat liver Golgi enzyme and provided a tool for future explorations of its biological function and subcellular distribution.

EXPERIMENTAL PROCEDURES

Isolation of Endomannosidase and Sequencing of Tryptic Peptides—

Endomannosidase was isolated from rat liver Golgi membranes by affinity chromatography on Glc-Man-Affi-Gel as described previously (6). The purified enzyme preparation was submitted to 12% polyacrylamide gel electrophoresis in SDS and then electropholotted onto a polyvinylidene difluoride membrane (Bio-Rad) for 6 h (60 V) at 4 °C in 10 mM CAPS, pH 10.6, buffer in a manner previously described (7). After visualization of the two protein bands by a brief exposure to 0.1% Ponceau S in 1% acetic acid, the 56-kDa component was excised, washed with water, and sent frozen to the Harvard University Microchemistry Facility. Under the direction of William S. Lane, solid phase trypsin digestion was carried out, followed by reverse phase-high performance liquid chromatography of the resulting peptides. Several of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) AF023657.

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1 The abbreviations used are: Glc-Man-Affi-Gel, Glcα1→3Man-O-(CH2)8CONH-Affi-Gel; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PCR, polymerase chain reaction; IPTG, isopropyl 1-thiogalactopyranoside; MES, 2-(N-morpholino)ethanesulfonic acid; CST, castanospermine; DMJ, 1-deoxymannojirimycin; bp, base pair(s); kb, kilobase pair(s).

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the latter were then selected for amino acid sequencing by automated Edman degradation (8).

Preparation of Primers and PCR Treatment of Rat Liver cDNA—Degenerate primers (9) based on the amino acid sequences for three peptides from the affinity purified endomannosidase were synthesized and purified (Bio-Rad). Certified DNase-free DEPC-treated water (Gibco-BRL) and water purified with rat liver cDNA as a template (CLONTECH, oligo(dT) primed mRNA from 10–12-week-old Sprague-Dawley rats). The reactions were performed in 100 μl of 20 mM Tris chloride, pH 8.3, buffer containing 0.2 mM mixed deoxynucleotides, 2 mM MgCl₂, 300 μmol of each primer, 1 ng of cDNA, and 2.5 units of Taq DNA polymerase (U. S. Biochemical Corp.) with a Techne Thermal Cycler. Cycles were carried out as follows: 3 min at 72 °C, 45 s at 94 °C, and 2 min at 46 °C; these were repeated 35 times with an 8-min extension at 72 °C following the final cycle. Products were ligated into a pCR-II vector (TA cloning kit, Invitrogen Corp.) with a Techne Thermal Cycler. Cycles were carried out as follows:

1. **Screening of cDNA Library**—A 1 μg cDNA library prepared from adult male Sprague-Dawley rat liver by oligo(dT) and random priming was obtained from CLONTECH (5–Stretch Plus) and screened with the PCR-generated probe EM1 after radiolabeling with [α-32P]dCTP (NEN Life Science Products) using the Megaprime Labeling Kit (Amersham Corp.). Host cells (strain Y1090r, CLONTECH) and phage were grown on 150-mm plates; nitrocellulose filters were prehybridized in 50% formamide in 5 × Denhardt’s solution containing 5 × SSPE, 0.1% SDS, and 100 μg/ml salmon sperm DNA and hybridized in the same solution for 20 h at 42 °C. Filters were washed at room temperature three times in 2 × SSC containing 0.5% SDS, followed by washes in 0.2 SSC, 0.1% SDS for 1 h at 50 °C, 1 h at 55 °C, and 30 min at 60 °C. A total of four rounds of screening were performed before selecting a single positive plaque. After amplification of the clone, purification of the λ DNA was accomplished with Wizard Lambda columns (Promega).

Subcloning of Endomannosidase Insert—Purified λ DNA containing the endomannosidase sequences was digested with EcoRI (Life Technologies, Inc.) followed by electrophoresis on low-melt agarose gel (Bio-Rad). Recovery of the EM1 from the gel was accomplished by digestion with β-agarase (Calbiochem) at 45 °C followed by precipitation of the DNA with ethanol. The size of the electrophoresed DNA fragments was assayed with 123-bp and 1-kb ladders (Life Technologies, Inc.).

Expression of Endomannosidase in E. coli—To produce the E. coli endomannosidase as a fusion protein containing its NH₂-terminal region a polyhistidine tag suitable for nickel-affinity purification, as well as an enterokinase susceptible cleavage sequence, the pTrcHisB vector (Perkin-Elmer), which contains the trp-lac promoter (11), was chosen and appropriate PCR primers designed. The 5′ primer contained the nucleotide sequence of EM2 (Fig. 1) from positions 78–96 (CCAGAAAAACATGGGAGC), which included the first in-frame ATG; additionally, the sequence GGATC was added to the 5′ end to permit digestion with BamHI (Fig. 1). The antisense primer (ACAGTAGCAACGAGACACAT) was complementary to positions 1540 to 1521 of EM2. The template for PCR treatment was the pBluescript II plasmid pEM2 (160 ng) containing the 5.4-kb fragment; the reaction volume was 100 μl and contained 50 mM Tris chloride, pH 9.2, 16 mM NaSO₄, 1.75 mM MgCl₂, 0.2 mM each dNTP, 1 μM each primer, and 2.5 units of Taq polymerase (Perkin-Elmer). All of the 27 cycles involved 45 s at 94 °C, 1 min at 52 °C, and 2 min at 72 °C. The PCR product was cloned into the pBluescript vector of the TA Cloning System (Invitrogen) and its identity confirmed by sequencing.

After release of the insert by digestion of the TA plasmid with BamHI and EcoRI, ligation was carried with the similarly cleaved pTrcHisB vector to produce pTrcHisEM (Fig. 1) which was used to transform the competent E. coli strain TOP10F² and JM109 (Invitrogen) using the heat-shock procedure (10). Similar transformations of the E. coli with the pTrcHisB vector itself were also performed to serve as controls. These transformed cells were streaked on ampicillin-containing plates, and colonies were selected for growth in SOB medium containing 50 μg/ml ampicillin.

The kinetics of expression were determined from a time course after initiation of induction. Cells were grown in SOB medium containing 100 μg/ml ampicillin at 37 °C to an absorbance of 0.6 at 600 nm. After addition of IPTG to a concentration of 1 mM, the cells were shaken vigorously at 27 or 37 °C, and samples were taken at various times. For determination of endomannosidase activity, cell pellets suspended in 20 mM phosphate, pH 7.8, with 500 mM NaCl were submitted to 4 × 10-s bursts of a Branson sonifier (setting 1) followed by four cycles of freeze-thawing; subsequent to centrifugation (4,000 × g for 30 min) aliquots of the supernatants were assayed for enzyme activity.

Purification of Endomannosidase Fusion Protein—Large scale (250–500 ml) preparations of JM109 or Top10F² cells containing the pTrcHisEM vector were grown in SOB medium containing 100 μg/ml ampicillin as above and induced with 1 mM IPTG. After centrifugation to recover the cells, extraction medium (20 mM phosphate, pH 7.8 containing 500 mM NaCl, 2 μg/ml leupeptin, 10 units/ml aprotinin, and 1 mM benzamidine) was added and the mixture shaken for 10 min at 4 °C for the cell culture which represented approximately 6 mg of protein. The suspended cells were then disrupted at 4 °C in 5-ml portions with 4 × 10-s bursts of a Branson sonifier (setting 1) and subjected to four cycles of freeze-thawing (ethanol/dry ice followed by 37 °C water); this was followed by a 15-min room temperature digestion with DNase (10 μg/ml) in the presence of 1 mM magnesium acetate. After centrifugation (20,000 × g for 20 min) the lysates contained approximately 30% of the total cellular protein and 80% of the endomannosidase activity. For purification of the polyhistidine-tagged fusion protein, nickel-affinity chromatography was carried out at a room temperature on a column (1 × 13 cm) of Ni-NTA resin (Qiagen), equilibrated with the extraction medium. The lysate from 250 to 500 ml of cell culture, after concentration (Centriprep 30, Amicon) to 12 ml, was applied to the column in 4-ml aliquots, each of which was allowed to equilibrate for 20 min. The column was then washed with extraction medium and subsequently was eluted with this medium containing 20 mM imidazole. The chromatography was carried out at room temperature, and aliquots were taken for endomannosidase assay and electrophoretic examination. The tubes containing the enzyme were pooled for further purification by Glc-Man-Aff-Gel chromatography.

After concentration, the Ni-NTA column enzyme pool was applied to Glc-Man-Aff-Gel at 2 °C in the presence of 0.1% Triton X-100, 0.2 mM Cysteine, and protease inhibitors as described previously (6). After a wash with the buffer containing 1 mM NaCl, the enzyme was eluted with 0.1 mM glycine HCl buffer, pH 3.0 (purified by filtration using a Centricon-30 membrane), containing 0.1% Triton and 1 mM NaCl (6) while 4-mL frac-
tions were collected; these acidic fractions were immediately neutralized by the addition of solid NaMES. For evaluation of the NH2-terminal region of the fusion protein the purified endomannosidase was digested with 1 unit of recombinant enterokinase (Novagen) at 25 °C for 16 h prior to examination by SDS-polyacrylamide gel electrophoresis.

**Protein Purification** — Antiserum against peptide 2 (Table I), synthesized by the Joslin Diabetes Center Peptide Laboratory employing the Applied Biosystem Model 430A synthesizer and subsequently coupled to keyhole limpet hemocyanin through an NH2-terminal cysteine (12), was prepared in a New Zealand White rabbit with a program of multiple intradermal injections. Polyclonal antibodies of highly purified endomannosidase from transfected JM109 E. coli lysates were prepared in rabbits by intradermal injection of the enzyme (204 μg of protein) followed by two booster doses of 68 μg each of this protein.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting** — Electrophoretic analysis of E. coli lysates and column fractions was performed by the procedure of Laemmli (13) on 10% gels (1.5 mm thick); protein bands were visualized by silver staining (14).

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**Endomannosidase Assays** — For enzyme analysis of tissues from male rats (200 g, CD strain, Taconic, Inc.), homogenization was carried out in 4 volumes of 0.1 M MES buffer, pH 6.5, with a Polytron for three 10-s bursts at setting 5. Postnuclear supernatants (800 × g for 10 min) of the homogenates were then centrifuged for 60 min at 100,000 × g to obtain membrane pellets which, after a wash with the homogenizing buffer, were suspended in the same buffer at a protein concentration of about 18 mg/ml. Endomannosidase activity of the postnuclear membranes of the rat tissues as well as of E. coli lysates and column fractions was determined by incubations with 3H-labeled Glc2Man4GlcNAc substrate (10,000 dpm) in the presence of CST (1 μM) and DMJ (2 μM) in 60 μl of 0.1 M NaMES buffer, pH 6.5, containing 0.2% Triton X-100 at 37 °C for 2 h in a manner similar to that previously described (1). The released disaccharide (Glc1–3Man) was separated from the oligosaccharide substrate by thin layer chromatography of the desalted and deproteinized samples on plastic sheets precoated with cellulose (0.1-mm thickness, Merck) in pyridine/ethyl acetate/water/acetic acid, 5:5:3:1. The radioactive components were detected by fluorography and quantitated after elution with water as previously reported (1). One unit of endomannosidase activity is defined as the amount of enzyme that catalyzes the release of 1,000 dpm of Glc1–3Man per h.

**Other Procedures** — Protein was determined by the dye-binding technique (17) with bovine serum albumin as a standard; for analysis of E. coli cell fractions, solubilization was accomplished by heating in 0.05 N NaOH at 100 °C for 5 min.

To visualize radioactive components after thin layer chromatography, the plates were sprayed with a mixture containing 2-methylnaphthalene (18) and exposed to X-Omat AR film (Kodak) at −80 °C. Staining of gels was accomplished by autoradiography as described previously (16).

### Table I

**Sequence of trypsin peptides from rat liver Golgi endomannosidase**

| Peptidea | Sequenceb | Position in proteinc |
|----------|-----------|----------------------|
| P-1      | YGNHPAFYR | 234–242              |
| P-2      | TWWNLTPSSQXVR | 266–280        |
| P-3      | YYEVLG(S)AALQTG(S)LI(L)IT | 370–389 |

a Peptides are designated by the order of their HPLC elution which coincided with their relative position in the peptide chain.
b The amino acid symbols given in parentheses represented probable amino acids; the nucleotide sequence confirmed the presence of all except the (L) at position 387 which proved to be serine.
c See Fig. 2 for the numbering of the amino acid sequence.

### Table II

**Primers utilized for PCR and sequencing**

| Degenerate primers based on peptide sequencesd | Primer sequence |
|-----------------------------------------------|----------------|
| PR-1S                                         | TAY GKI AAY CAY CCI GCN TTY TA |
| PR-1AS                                        | TA RAA NCC NNG RTT RIT |
| PR-2S                                         | ACI TGG GCC AAY YTI YTI ACI CCI WSI WSI CAR III GTI CGI |
| PR-2AS                                        | TG ISW ICC ISW IGG IGI IAR IAR RTT IGGCCA IGA |
| PR-3S                                         | TAY TAY GAR GII GTI CTN III GCI GCN CTI CAR ACN CA |
| PR-3AS                                        | GT IAT IAG IAT III NNG YTG IGT YAG NCC |
| Modified peptide-based primersf              |                 |
| PRM-1S                                        | TAT GKS AAC CAT CYY GCC TCC TA |
| PRM-2AS                                       | ACA TGG GCC AAT CTA TTA ACA CCC TGA TCA CT CA |
| PRM-SS                                        | CCC GCS AAC ACT CTG AGA TCC TGA GSS TGT TAA TAG ATT |
| EM clone sequence-based primersg              |                 |
| C1 (S 3–21)                                   | GGT TTT GGT GAG GCC ATT C |
| C2 (S 235–252)                                | CCT CCA AAG AGA TGA TCG |
| C3 (S 285–304)                                | AAG GGG CTT CTG TGA CTG TG |
| C4 (AS 398–397)                               | GGT GTC TCC CAT ACC AAC TG |
| C5 (AS 514–495)                               | ACT GGA GCC AAT GTC ATC TG |
| C6 (S 739–758)                                | TCG AGA TGA CCA AAA CAT GC |
| C7 (AS 1314–1295)                             | GCA GTC CTT TGG GGG ACA GC |
| C8 (S 1322–1341)                              | GTA TAC CTG GAT TAC GGC CC |
| C9 (S 1446–1445)                              | CAG CTG CTT GCT TCA TAA TG |
| C10 (S 1682–1701)                             | GAA ATC TTA ATG GAG TTG CC |
| C11 (S 2015–2034)                             | CTG TTA GCC ATG TGT TG |
| Primers for PCR generation of TrcHis inserth  |                 |
| IN-1 (S 575–96)                               | G CAT CCC AGG AAA AAT ATG GGA GC |
| IN-2 (AS 1545–1520)                           | ACA GTA GCA ACC ACA CAT TG |

d Degenerate sense (S) and antisense (AS) primers (PR) based on sequences obtained for peptides 1, 2, and 3 (Table I). The abbreviations used for nucleotides are: K for G or T; R for A or G; Y for T or C; W for A or T; S for C or G; N for A, C, G, or T; I, inosine.

e Optimized primers (PRM) for peptides based on sequences determined for PCR product EM1. The abbreviations used for nucleotides are the same as in footnote a.

f Sense and antisense primers (C) based on sense (S) and antisense (AS) sequences determined for 5.4-kb clone isolated from Agt11 rat liver library. Numbers refer to the position of nucleotides relative to the start of the 5.4-kb clone (Fig. 1). For sequencing this clone, primers representing lambda regions were also synthesized: 5′- (antisense): GAC TCC TGG AGC CCG; 3′- (antisense): GGT ACG GAC CGC CC.

g In order to prepare an appropriate PCR product with a BamHI site for insertion into the vector TrcHisB, a sense primer containing the 5′ region of the 5.4-kb clone beginning 11 nucleotides prior to the ATG start of the EM open reading frame plus an additional restriction site-specific sequence (underlined). The antisense primer was based on the sequence starting 76 nucleotides beyond the TAA stop codon. The EcoRI recognition sequence for the 3′ end of this insert was derived from the TA vector into which the PCR product was amplified.
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When degenerate tide Sequence and Open Reading Frame—

rected to the 56-kDa band which we assumed to carry the

performed in Monofluor (National Diagnostics) in a Beckman LS7500

yses for the endomannosidase fusion protein (Fig. 1), endoman-

Cloning and Expression of Rat Liver Endomannosidase—As previously reported, the affinity purified endomanno-

results in the release of characteristic GlcNAc-I→3Man disaccharide from Glc3Man3GlcNAC substrate, although control cells containing the TrcHisB vector did not demonstrate such activity (cf. lanes 1 and 2; Fig. 4, left panel). However, when rat liver Golgi was added to the latter, the full activity known to be present in these membranes (1, 6) was evident (cf. lanes 2 and 3; Fig. 4). Like the purified enzyme from rat liver Golgi (6), the E. coli-expressed endomannosidase was unaffected by the exoglycosi-

3DMJ, an analogue of the re-

The time-dependent expression of the endomannosidase in the transfected cells after addition of IPTG was evident from the increasing level of endomannosidase activity as seen from the release of the Glc3Man disaccharide (Fig. 5).

A major portion of the enzyme activity (~80%) was solubili-

cloned by application of the combined sonication and freeze-

seen from incubations with the Glc1Man9GlcNAc sub-

terminal methionine (23–25). No consensus sequences for N-linked glycosylation were found in this protein, which is consistent with the observation that its electrophoretic mobility was not affected by digestion with N-glycanase.2

Fig. 1. Strategy for cloning and expression of endomannosidase. PCR treatment of rat liver cDNA using primers PR 1-8 and PR 3-AS (Table II) yielded a 470-bp product, EM1, that was found to contain sequences coding for peptides 1–3 (Table I). Subsequent screening of a Agt11 rat liver cDNA library with radiolabeled EM1 resulted in the isolation of one clone from which the 5.4-kb fragment EM2 was released by EcoRI digestion. The open reading frame of endomannosidase is indicated by the dashed-lined box; the asterisk indicates the end of the determined sequence. The BamHI-EcoRI insert (containing nucleotides 78–1540) produced to use the TrcHisEM vector for expression in E. coli was generated by PCR treatment of the 5.4-kb DNA using the primers shown in Table II; the corresponding endomannosidase sequences are indicated by double underlining in Fig. 2. The fusion protein produced after E. coli transfection begins at the ATG indicated in the vector and contains the sequence of six histidines (His)6) suitable for nickel-column purification, as well as an enterokinase-cleavable site (EK); this additional NH2-terminal peptide represents a 3-kDa segment. The pTrcHisEM vector, which includes the trc promoter and is inducible by addition of IPTG, is not drawn to scale, since the vector is 4.4 kb in comparison to the insert which is only 1.5 kb.

470-bp DNA (designated EM1) contained the sequences for the three endomannosidase peptides (Fig. 1).

Upon screening a total of 12 150-mm plates representing 1.8 × 107 phage plaques with a radiolabeled EM1 probe, one positive clone was found, and this was carried through three additional rounds of screening before selecting a single positive plaque. After amplification of the clone, EcoRI digestion released a 5.4-kb insert, which was designated EM2 (Fig. 1). This DNA was ligated into pBluescript II (pEM2) for amplification and sequencing. Additional primers for DNA sequencing (Table II) were synthesized based on the nucleotide sequences obtained for both EM1 and EM2.

Although the EcoRI-released DNA segment EM2 was 5.4 kb in length, the automated DNA sequencing procedure provided reliable data only through nucleotide 2552 (Fig. 2); beyond this were several regions of poly(dt) that interfered with the sequence analysis. An untranslated 5’ region (1–88 bp) preceded the first ATG of the open reading frame; this codon had an A at position −3 and a G at location +4, consistent with a Kozak consensus sequence for translation (22). At the 3’ end, a substantially larger untranslated segment was found, with an additional 4-kb segment occurring after the TAA stop codon at 1442–1444 bp. The deduced open reading frame coded for a protein of 451 amino acids with an M, value of 51,762 (Fig. 2). The three tryptic peptides (Table I) are contained in the open reading frame, and as anticipated each one is preceded by a Lys residue (Fig. 2). Polar and hydrophobic amino acids constituted 33 and 27 residues per 100 total residues, respectively. The hydropathy plot (Fig. 3) for the endomannosidase open reading frame indicated only a few hydrophobic regions, the most prominent occupying residues 25–36 and 375–390. The sequence (GALMAT), represented by residues 2–7, is consistent with N-myristoylation which would occur after removal of the NH2-terminal methionine (23–25). No consensus sequences for N-linked glycosylation were found in this protein, which is consistent with the observation that its electrophoretic mobility was not affected by digestion with N-glycanase.2

Expression of Endomannosidase Activity in E. coli—After transfection of TopF10 E. coli with the TrcHisEM vector coding for the endomannosidase fusion protein (Fig. 1), endomannosidase assays conducted on cell lysates resulted in the release of the characteristic GlcNAc-I→3Man disaccharide from Glc2Man3GlcNAC substrate, although control cells containing the TrcHisB vector did not demonstrate such activity (cf. lanes 1 and 2; Fig. 4, left panel). However, when rat liver Golgi was added to the latter, the full activity known to be present in these membranes (1, 6) was evident (cf. lanes 2 and 3; Fig. 4). Like the purified enzyme from rat liver Golgi (6), the E. coli-expressed endomannosidase was unaffected by the exoglycosidase inhibitors CST and DMJ but was completely inhibited (Fig. 4, right panel) by Glc1→3DMJ, an analogue of the released disaccharide (26). The E. coli lysates did not demonstrate α-mannosidase or α-glucosidase processing activities as was evident from incubations with the Glc3Man3GlcNAC substrate in the absence of any inhibitor (Fig. 4, right panel).
Transfected JM109 E. coli cells were consistently observed to produce a larger amount of IPTG-induced enzyme (approximately three times that of the TOP10F cells), and accordingly this strain was favored for high yield preparative purposes.

Purification of Endomannosidase Fusion Protein—The presence of the polyhistidine region in the fusion protein permitted binding of the endomannosidase from E. coli lysates on the Ni-NTA resin (Fig. 6). Although some of the enzyme was only weakly bound, emerging in the buffer wash, 20 mM imidazole was required to achieve elution of most of the endomannosidase activity (Fig. 6). Since complete enzyme recovery was affected with 20 mM imidazole, this fractionation procedure could be abbreviated for preparative purposes by eluting the column with this histidine analogue as soon as the predominant protein peak had emerged.

Further purification of the endomannosidase could be achieved by applying the Ni-NTA enzyme pool to Glc-Man-Affi-Gel from which it could be selectively eluted with pH 3.0 glycine buffer (Fig. 6). Indeed, polyacrylamide gel electrophoresis revealed only a single protein band (47 kDa) in the first fraction (G-1) emerging after application of the pH 3.0 buffer, and this coincided with enzyme peak which accounted for about 90% of the endomannosidase activity loaded onto the column (Fig. 7); other proteins present in Ni-NTA pool were not bound to the Glc-Man-Affi-Gel (Fig. 7). The endomannosidase identity of the component revealed by silver staining in fraction G-1 was further substantiated by the observation that antiserum directed against peptide 2 of the enzyme reacted with a single co-migrating band (Fig. 7). Furthermore, the molecular mass of this component was reduced by about 3 kDa after digestion with FIG. 2.

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FIG. 3. Hydrophobicity profile of the deduced amino acid sequence of rat liver endomannosidase. The plot was carried out according to the method of Kyte and Doolittle (21); values were calculated by the Protean program of DNASTAR, Inc., using a 9-amino acid frame. The dotted line indicates the average hydrophobicity for the protein. The most hydrophobic region of the protein extends from residues 25 to 36 and has an average hydrophobicity of 1.6.

Transfected JM109 E. coli cells were consistently observed to produce a larger amount of IPTG-induced enzyme (approximately three times that of the TOP10F cells), and accordingly this strain was favored for high yield preparative purposes.
with enterokinase (Fig. 7) as would be anticipated from the nature of the fusion protein. The average yield of purified endomannosidase from JM109 E. coli was 495 μg per liter of cultured cells, and its specific activity was 68 units/μg protein, which represented an approximately 280-fold purification over the cell lysate. This enzyme preparation was utilized for the preparation of polyclonal antibodies in rabbits.

Proteolytic Cleavage of Endomannosidase by E. coli—The polyclonal antibody against the Glc-Man-Alf-Gel purified enzyme also detected only a single component with a molecular mass of approximately 47 kDa (Fig. 8). This antibody provided us with the opportunity to explore the basis for the discrepancy in the size of the purified E. coli endomannosidase (47 kDa) and that anticipated from the fusion protein which would include the deduced open reading frame (52 kDa) plus its 3-kDa NHemail terminal addition. Immunoblots of the E. coli lysates clearly showed that the expected 55-kDa protein was indeed present at early times subsequent to induction but was degraded to a 47-kDa component (Fig. 8); this proteolysis of the endomannosidase was observed in both TOP10F cells and at 44 h of the vector control. The components were detected by fluorography.

**FIG. 4.** Thin layer chromatographic demonstration of endomannosidase expression in E. coli and evaluation of the effect of glycosidase inhibitors. Incubations of E. coli lysates were carried out with 3H-labeled Glc,Man,GlcNAc (10,000 dpm) for 2 h at 37 °C, and the desalted-deproteinized samples were then chromatographed on cellulose-coated plates for 22 h as described under "Experimental Procedures." The left panel represents a chromatograph of incubations of lysates (4 μg of protein) of E. coli TOP10F cells transfected with either the pTrcHisEM vector (lane 1) or the pTrHisB control vector (lane 2). The latter was also incubated after the addition of rat liver Golgi membranes (20 μg of protein) to exclude the presence of interfering material in the E. coli (lane 3). In the right panel the E. coli pTrHisEM-transfected lysate was incubated in the presence (+) or absence (−) of castanospermine (CST), 1-deoxymannojirimycin (DMJ), and Glc1→3DMJ (GDMJ). The radioactive components were visualized by fluorography for the migrations of the released Glc1→3Man (G1M1) as well as that of glucose (G) and mannose (M) standards are indicated to the left of the chromatograms. The radioactive material at the origins represents the split as well as the unsplit polymannose-GlcNAc substrate.

**FIG. 5.** Induction of endomannosidase expression in E. coli as a function of time. E. coli TOP10F cells transfected with either the pTrcHisEM vector (+) or the pTrHisB control vector (−) were induced with IPTG (1 mM) at 27 °C for various periods following which their lysates were analyzed for endomannosidase activity. The Glc1→3Man (G1M1) product of endomannosidase was resolved by thin layer chromatography as in Fig. 4 after incubation of equal volumes of the E. coli lysates (0.5 μl) with 3H-labeled Glc,Man,GlcNAc (10,000 dpm) under conditions described under "Experimental Procedures." The assays were carried out at various induction times of the pTrcHisEM transfected cells and at 44 h of the vector control. The components were detected by fluorography.

**Tissue Distribution of Endomannosidase Activity and mRNA Level—**Endomannosidase was found to be present in all rat tissues examined (Fig. 9), with liver, in which this enzyme was first detected (1), having the highest specific activity. Hybridization of Northern blots with the radiolabeled probe EM1, which contains sequences for the three isolated trypsin peptides (P-1, P-2, and P-3) demonstrated an mRNA band at 4.9 kb in all of these tissues. Although this message is substantially larger than expected from the size of the protein, it is consistent with the 5.4-kb cDNA of the clone. The mRNA content exhibited a correlation with enzyme activity; the highest levels of message as well as enzyme activity were observed in liver and lung.

**DISCUSSION**

The present study in which we have cloned, determined the open reading frame, and expressed rat liver endomannosidase has made it possible for this enzyme to take its place among the oligosaccharide processing hydrolases for which such information is currently available (27). Judging from nucleic acid and protein data base searches that we have carried out, it would appear that the deduced amino sequence of the endomannosidase codes for a unique protein that stands in contrast to the processing exomannosidases that have been grouped into two distinct classes on the basis of protein sequence homologies (27). The absence of a molecular relation with the other trimming glycosidases is not unexpected in view of the fact the endomannosidase is quite distinct in its catalytic properties, substrate specificities as well as response to inhibitors and divalent ions (1, 2, 26). Furthermore, it has recently become apparent that the endomannosidase, in contrast to the α-gluco- and α-mannoside processing hydrolases, is a recent evolutionary addition to the enzymatic machinery involved in N-linked oligosaccharide processing (28). Of possible future interest was our search finding that nucleotides 868–1165 of the endomannosidase had an 87% identity with nucleotides 2–299 of a yet uncharacterized Homo sapiens cDNA clone (H80483, clone 239648 5').
Our ability to express the endomannosidase in two strains of *E. coli* was particularly fortunate as not unexpectedly this enzyme is naturally absent in these cells. Indeed, even in the unfractionated lysate of JM109 cells the specific activity of the endomannosidase was about 10-fold higher than in rat liver Golgi membranes. Moreover, the purified *E. coli*-expressed endomannosidase had a substantially higher activity than the enzyme obtained from liver (6), and this can most likely be primarily attributed to the fact that the molecular chaperone, calreticulin, was not present in the bacterial preparation. The high purity of our *E. coli*-derived endomannosidase is to a large extent due to the high purity of our cloning and expression system, and the high purity of the enzyme obtained from liver (6). This can be more specifically attributed to the fact that the molecular chaperone, calreticulin, was not present in the bacterial preparation.
extent the result of the selective Glc-Man-Affi-Gel adsorption step, although the introduction of a polyhistidine tag onto the enzyme made possible an initial fractionation on a nickel resin.

The finding that the open reading frame of the endomannosidase represents a molecular mass (52 kDa) somewhat smaller than that of the rat liver enzyme as determined by SDS-polyacrylamide gel electrophoresis is most likely attributable to eukaryotic posttranslational biosynthetic events. Although N-glycosylation consensus sequences are not evident in the E. coli-expressed endomannosidase, an observation that is consistent with the lack of effect which N-glycosidase has on the rat liver enzyme, the possibility that O-linked oligosaccharides or other modifications may be present on the peptide chain of the latter protein has not been excluded. Indeed, the presence of a sequence suitable for myristoylation in the NH2-terminal region of the enzyme would indicate that a posttranslational addition of this fatty acid could occur.

Even taking into account the possibility that the E. coli and rat liver endomannosidase differ from each other by posttranslational modification of the latter, the fusion protein (47 kDa), which as anticipated could be reduced in size by about 3 kDa by enterokinase excision of its NH2-terminal polyhistidine tag, appeared to be smaller than expected from the open reading frame. It became apparent that this discrepancy can be attributed to some trimming of the COOH-terminal end of the enzyme’s peptide chain by E. coli proteases (29, 30), since immunoblots of the lysates demonstrated a time-dependent degradation from 55 to 47 kDa in molecular mass. It is apparent that neither the active site of the endomannosidase nor the Glc-Man-Affi-Gel binding region is present in this cleaved 8-kDa COOH-terminal peptide.

The substantial yield of endomannosidase that could be isolated from the JM109 E. coli strain made possible the production of a high titer polyclonal antibody against the enzyme which reacted strongly with the 56-kDa component from rat liver Golgi. These antibodies promise to be useful in conducting further explorations of the biological function and subcellular localization of endomannosidase. Although previous studies have shown that the endomannosidase is associated with Golgi membranes (1) and functions in vivo prior to O-mannosidase I in the processing sequence (31), the morphological situation of the enzyme, whether in cis-Golgi or in the endoplasmic reticulum-Golgi intermediate compartment, has not yet been determined either in liver or any of the various other tissues in which the enzyme was found to occur. The antibodies furthermore will be helpful in examining the postulated interrelationship (7) between endomannosidase and calreticulin in assisting proteins to fold or oligomerize in a post-endoplasmic reticulum cellular compartment.

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