Human Small Intestinal Maltase-glucoamylase cDNA Cloning

HOMOLOGY TO SUCRASE-ISOMALTASE*

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It has been hypothesized that human mucosal glucoamylase (EC 3.2.1.20 and 3.2.1.3) activity serves as an alternate pathway for starch digestion when luminal α-amylase activity is reduced because of immaturity or malnutrition and that maltase-glucoamylase plays a unique role in the digestion of malted dietary oligosaccharides used in food manufacturing. As a first step toward the testing of this hypothesis, we have cloned human small intestinal maltase-glucoamylase cDNA to permit study of the individual catalytic and binding sites for maltose and starch enzyme hydrolysis activities in subsequent expression experiments. Human maltase-glucoamylase was purified by immunosolubilization and partially sequenced. Maltase-glucoamylase cDNA was amplified from human intestinal RNA using degenerate gene-specific primers with the reverse transcription-polymerase chain reaction. The 6,513-base pair cDNA contains an open reading frame that encodes a 1,857-amino acid protein (molecular mass 209,702 Da). Maltase-glucoamylase has two catalytic sites identical to those of sucrase-isomaltase, but the proteins are only 59% homologous. Both are members of glycosyl hydrolase family 31, which has a variety of substrate specificities. Our findings suggest that divergences in the carbohydrate binding sequences must determine the substrate specificities for the four different enzyme activities that share a conserved catalytic site.

Starches are a mixture of two structurally different polysaccharides: amylase, a linear [4-O-α-D-glucopyranosyl-β-D-glucose]ₙ polymer, and amylpectin, with additional 6-O-α-D-glucopyranosyl-β-D-glucose links (about 4% of total), which result in a branched configuration. Dietary starches are a mixture of approximately 25% amylase in amylpectin, a fact of nutritional significance because of the multienzyme complexity of the mammalian starch digestion pathway. α-amylase (EC 3.2.1.1) is the endoenzyme found in mature human salivary and pancreatic secretions that produces linear maltose oligosaccharides by hydrolysis of α₁→4 linkages of amylose (2, 3). α-amylase bypasses the α₁ → 6 linkages of amylopectin and produces branched isomaltose oligosaccharides. The starch-derives oligosaccharides are not fermentable by yeast without further processing by β-amylase (EC 3.1.1.2), which hydrolyzes the nonreducing ends at 1→4 and 1→6 linkages (2). In mammals, hydrolysis of the nonreducing ends is carried out by small intestinal mucosal brush border-anchored sucrase-isomaltase (SIM)1 (EC 3.2.1.48 and 3.2.1.10) and maltase-glucoamylase (MGA) (EC 3.2.1.20 and 3.2.1.3) complexes (1). Enzyme substrate specificities of SIM overlap with those of MGA. In vivo, SIM accounts for 80% of maltase (1,4-α-D-glucanohydrolase) activity, all sucrase (β-D-glucanomannan-β-D-fructohydrolase) activity, and almost all isomaltase (1,6-α-D-glucanohydrolase) activity (1). MGA accounts for all glucoamylase exoenzyme (1,4-α-D-glucanohydrolase) activity for amylose and amylopectin substrates, 1% of isomaltase activity, and 20% of maltase activity (1, 4). Some have hypothesized that human mucosal glucoamylase exoenzyme activity is an alternate pathway for starch digestion when luminal α-amylase endoenzyme activity is reduced because of immaturity and malnutrition and that MGA plays a unique role in the digestion of malted dietary oligosaccharides used in food and beverage manufacturing (5–8). The objective of this study was the cloning and sequencing of the human small intestinal MGA cDNA to allow subsequent testing of this hypothesis by analysis of the individual catalytic and binding sites for maltose and starch by expression and mutation experiments. In this paper, we describe the cloning of cDNA for MGA from human intestinal RNA. The identity of the isolated cDNA clone was confirmed by expression of a recombinant protein in Escherichia coli transformed by the cDNA coding for the N-terminal domain.

MGA activities associated with proteins of the same size as the intestinal enzyme have been reported from human kidney and granulocytes (9, 10). A clinical deficiency of intestinal glucoamylase has been reported that consisted of chronic diarrhea responding to a starch elimination diet in children with normal mucosal morphology and low starch hydrolyzing activity (11). A genetic form of glucoamylase deficiency has been reported in mice (12). In rats and rabbits, SIM activity is undetectable until weaning but MGA is present from birth (1). In pigs and humans, both SIM and MGA activities are present from birth. In malnourished human infants, SIM and MGA activities are reduced (13, 14), but SIM and MGA activities are increased in malnourished rats (15). There have been charac-

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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) AF016833.

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1 The abbreviations used are: SIM, sucrase-isomaltase; MGA, maltase-glucoamylase; IPTG, isopropyl β-D-thiogalactopyranoside; RT, reverse transcription; PCR, polymerase chain reaction; mAB, monoclonal antibody; bp, base pair; EST, Expressed Sequence Tag.
terizations of MGA activity, synthesis, and processing in chickens, mice, rats, rabbits, and pigs (12, 16–24). There were two mature MGA protein subunits in all of these species. Studies of pig MGA peptide sequence demonstrated that it is anchored to the membrane by the N terminus (16–18). Rat studies found that maltase activity was associated with the membrane, and gliucoamylase was associated with the luminal ends (1, 19).

**EXPERIMENTAL PROCEDURES**

**Materials**—Human small intestine from organ donors was used for the preparation of antibodies and isolation, and characterization of MGA (25–27). The usage of this tissue was approved by the committees of all involved hospitals and institutions (25–27). All chemicals and biologicals used for protein isolation and characterization were purchased from Sigma unless otherwise noted (27). All molecular reagent and kit suppliers are indicated and were used according to manufacturer’s instructions.

**Electrophoresis and Immunoblotting Procedures**—The procedures used for SDS-polyacrylamide gel electrophoresis and immunoblots were previously reported (27, 28). Molecular mass standards were run with all gels and ranged from 43 to 202 kDa (27).

**Antibody Reagents**—Monoclonal mouse HBB 2/143/17 (25) and HMA (26) anti-human maltase-glucoamylase antibodies have been previously described (31, 32). These primers were synthesized by Microsynth (Windisch, Switzerland) from areas of the sequenced MGA peptides with lowest homology to other eukaryotic species (human, monkey, rat, mouse, dog, cow, rabbit, chicken, yeast). The Geno probe contained 4 μg of EcoR I-digested genomic DNA cut with EcoR I, HindIII, BamHI, PstI, and BglII. The probe was prepared by PCR from the MGA-P1 template with primers encompassing 1669–2049 in the intestinal MGA sequence. The amplifier was randomized with [32P]dCTP. The hybridization (CLONTECH) was at 68°C, the membrane was washed at 50°C, and the image was developed.

**Expression in E. coli**—A full-length construct was assembled by cutting clones K3, 13-13, and T46 with Hae III and Sac I. The DNA fragments were ligated and transformed into E. coli BL21(DE3) E. coli (Novagen). Expression was induced by 1 mm IPTG, and proteins were harvested by osmotic shock and sonication. The induced and uninduced proteins were separated on a 6% gel and stained with Coomassie Blue or immunoblotted with HMA mAbs.

**Computer Analysis of Sequences**—The software from the Genetics Computer Group, Inc. (26) was used, and the Baylor College of Medicine molecular biology computation resource. Sequence analysis was performed with the GCG programs (32). GenBankTM data were searched using the BLAST Service (33) or FastA and TFASTA (34) programs. Primer design used the PRIMER (35) program. The PROSITE analysis accessed the patterns section (36) and Blocks data base (37). Chromosomal assignment was made by searching the Expresssed Sequence Tag (EST) data base (38).

**RESULTS**

**MGA Peptide Sequences**—The HBB 2/143/17-immunoisolated proteins had the predicted 335- and 285-kDa forms of MGA when separated on SDS gels (25–27). In experiments with a heavier P2 immunoisolate, an additional band of about 210 kDa, similar in size to those previously reported in higher resolution human explant (27) and immunoblot (26) experiments, was visualized. All HBB 2/143/17-immunoisolated MGA bands reacted with individual HMA-5–7 mAbs on immunoblots. The 335- and 285-kDa MGA bands were separately excised from blots, and N-terminal amino acids were sequenced. The human MGA N-terminal sequence had 82% homology with hog MGA (18) and 52% with human SIM (29, 30). The initiator methionine was missing from the peptide sequences of the human MGA and human, rabbit, and porcine SIM N termini (16–18, 29, 30). Five identical bands were visualized on electrophoretic separation after independent CNBr hydrolysis of the 335- and 285-kDa forms. The polypeptide fragment bands were individually excised and sequenced. Band 2 could not be successfully sequenced. Band 4 was only sequenced from the

**Southern Blot Analysis**—Species specificity and genomic context were evaluated with the Zoo blot and Geno blot (CLONTECH). The Zoo blot containing 4 μg of EcoR I-digested genomic DNA cut from nine eukaryotic species (human, monkey, rat, mouse, dog, cow, rabbit, chicken, yeast). The Geno probe contained 4 μg of human genomic DNA cut with EcoR I, HindIII, BamHI, PstI, and BglII. The probe was prepared by PCR from the MGA-P1 template with primers encompassing 1669–2049 in the intestinal MGA sequence. The amplifier was randomized with [32P]dCTP. The hybridization (CLONTECH) was at 68°C, the membrane was washed under high stringency conditions at 50°C, and the image was developed (Fig. 4, B and C).

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been listed as GenBankTM accession number AF016833. Sequences were recognized (Fig. 2). The nucleotide sequence has


tional internal peptides and a WIDMNE catalytic site se-


reading frame of the consensus was continued, and two addi-


tions, each of which included one catalytic site, were ligated


(MGA-P1a, N-terminal domain; MGA-P1b, C-terminal do-


mains), were present. The amplimer pattern suggested that MGA


salivary or pancreatic RNA, although control actin amplimers


were not detected from kidney total RNA. No MGA amplimers were detected from


the uninduced bacterial nor the induced MGA-P1b pro-


tin the amino acid sequence in Fig. 2. Sequenced Not Sequenced


335-kDa digest. The N terminus and internal peptide sequences from the 335-kDa band were identical to those from the 258-kDa band. The locations of these peptide sequences are underlined in the amino acid sequence in Fig. 2.


Clones and Sequences—Four overlapping clones were obtained from the degenerate primer amplifications, and two additional internal peptides and a WIDMNE catalytic site sequences were recognized (Fig. 2). The nucleotide sequence has been listed as GenBank™ accession number AF016833.


Chromosomal Location—A search of the EST chromosomal data bank with the MGA nucleotide sequence revealed that the 3' end of the untranscribed cDNA had 100% identity with a 147-base cDNA EST GS1365 (38). There were no other EST identified.


Expression of Cloned cDNA in E. coli—Because of the length of the cDNA, 2,584- and 2,687-bp fragments of the cDNA (MGA-P1a, N-terminal domain; MGA-P1b, C-terminal domain), each of which included one catalytic site, were ligated into expression vectors and induced in E. coli. Proteins with the expected molecular mass of about 100 kDa were induced by IPTG in these E. coli cells from both constructs (Fig. 3A). When the bacterial proteins were immunoblotted to confirm expression MGA protein identity, the induced 100-kDa band from MGA-P1a was stained by three HMA mAb (Fig. 3B), but neither the uninduced bacterial nor the induced MGA-P1b proteins were stained.


Tissue Distribution—The same amplimer patterns were visualized after RT/PCR from small intestine, granulocyte, or kidney total RNA. No MGA amplifiers were detected from salivary or pancreatic RNA, although control actin amplimers were present. The amplifier pattern suggested that MGA mRNA was expressed in small intestine, granulocyte, and kidney but not in salivary gland or pancreas (Fig. 4A). A Southern blot from this gel revealed that all the MGA amplimers from small intestine, granulocyte, and kidney were stained. The names and locations of the sequencing clones are diagramed (5). The full-length clone MGA-P1 was assembled from clones 13–13, K3, and T46. The bars in black indicate the areas sequenced from the clones.


DISCUSSION

Two major studies have addressed isolation and synthesis of human MGA proteins (27, 39). The first study isolated human MGA by chromatography after treatment with papain and found a single band of about 312 kDa with 32–38% glycosylation. This isolated protein had substrate specificities for maltose and whole starch (39). The second study utilized in vitro organ culture followed by immunosolation (27). Electrophoresis of the precipitate revealed a major band of about 335 kDa (27). In metabolically labeled explants, a polypeptide of about 285 kDa was visualized at 30 min, and the 335-kDa form was visualized at 60 min. The 335-kDa isofrom was found to be a complex glycosylated form of the high mannose 285-kDa form. Treatment of both 335- and 285-kDa forms with trifluoroethanesulfonic acid resulted in a single unglycosylated protein (described as ~210 kDa in this paper) and revealed that the 335-kDa form was heavily glycosylated. Because of reports of two ~100 kDa MGA proteins in rats and pigs (18, 22), the human explants were treated with trypsin, but MGA was not cleaved (27).

Cloned cDNA—We have isolated cDNA for human MGA that codes for a single open reading frame of 6,513 nucleotides and includes 58 nucleotide 5'- and 885 nucleotide 3'-untranslated regions. There is a single polyadenylation signal AATAAA at nucleotide 6,404. There is a 174 nucleotide sequence in the N- and C-terminal domains.

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with a calculated molecular mass of 209,702 Da (Fig. 2). This protein is similar in molecular mass to the smallest band visualized on overloaded lanes and previously identified as a unglycosylated stalk in SIM (O). The induced proteins were of the expected size, ~100 kDa (Fig. 3A), and the N-terminal-expressed bacterial protein selectively reacted with mAbs used to characterize the isolated MGA protein. The MGA-P1b (C-terminal domain)-induced protein was unstained (not shown). A

Recombinant Expression—N-terminal and C-terminal halves (each including one catalytic site) of MGA cDNA were expressed in E. coli. The induced proteins were of the expected size, ~100 kDa (Fig. 3A), and the N-terminal-expressed bacterial protein selectively reacted with mAbs used to characterize the human MGA starting material (Fig. 3B). The expression and peptide sequence data support the conclusion that we have cloned the authentic human intestinal MGA cDNA.

N-terminal Domains—In the cytoplasmic tail domain, MGA has 26 amino acids with 5 lysines. The N-terminal domain has a hydrophobic segment, a putative type II membrane anchor, with 16 branched chains in this 21-amino acid sequence. The anchoring domain is followed by threonine- and serine-rich residues with a calculated molecular mass of 209,702 Da (Fig. 2). This protein is similar in molecular mass to the smallest band visualized on overloaded lanes and previously identified as a unglycosylated MGA (27). There was 59% sequence identity between human MGA and SIM proteins.

Disulfide Bonds—MGA has 24 cysteines; it is reported that all the cysteines of MGA are joined in disulfide linkages (29, 39). Of these cysteines, 11 are in homologous regions in the N-terminal and C-terminal domains (Fig. 2). These have been described as trefoil-type domains (37). There are three additional conserved cysteines that bracket the N-terminal and two that follow the C-terminal signature 1 WIDMNE sites. There are also paired, conserved cysteines within each of the signature 2 sequences (discussed below).

Glycosylation—MGA has 19 potential N-glycosylation sites. There is a total of 253 sites of potential O-glycosylation in MGA. It is reported that mature MGA has a total of 28 carbohydrate residues with a total oligosaccharide molecular mass of 50,400 kDa (41). Carbohydrates were found to make up about a third of the molecular mass of mature MGA, and no sialic acid could be detected (27, 39).
Fig. 5. Comparison of internal homologies of MGA and SIM. The internal alignments were constructed with the Pileup program (31) with settings of gap weight 3 and gap length 0.1. The SIM and MGA C termini were aligned by consensus at Pileup position 90, well ahead of the SIM trypsin site at Pileup 177. The numbering is based upon a Pileup length of about 1,000 amino acids. Only the Pileup portion from amino acid 451 to 750 is shown. This alignment finds conserved amino acid sequences in 33% of the four entire enzyme domains and 31% in the portion shown. The two glycosyl hydrolase family 31 signature sequences are in bold. Three loops that are unique to the C terminus of MGA and/or SIM are noted by brackets.

Dimer Formation—All isolated bands were recognized by eight different mAbs specific for hMGA (25–27). The CNBr fragment patterns and the peptide sequences were identical from the 335- and 285-kDa proteins. The complete removal of all glycosylation from either the 335- or 285-kDa form resulted in a single protein band of ~210 kDa (27), which was consistent with the molecular mass as determined by sedimentation equilibrium (39, 40, 42) and cloning. These observations suggest the presence of a single protein structure in the 335- and 285-kDa forms of human MGA. Only differences in type of glycosylation and molecular mass distinguished the human 335- from 285-kDa bands (27). Parallel glycosylation differences were found in uncleaved MGA bands immunoisolated from pig or rat intestine (17, 19). It has been reported that the largest band in the rat (similar to human 335 kDa) is a dimer formed by noncovalent adhesion between complex glycosylation sites (37): 562–567 and 698–730, which are duplicated (bold) in Figs. 2 and 5).

Enzyme Family—MGA has two glycosyl hydrolase family 31 signature sequences (37): 562–567 and 698–730, which are duplicated (bold in Figs. 2 and 5).

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