Molecular Cloning of Sucrase-Isomaltase cDNA in the House Musk Shrew Suncus murinus and Identification of a Mutation Responsible for Isolated Sucrase Deficiency

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Isolated sucrase deficiency has been demonstrated in a line of house musk shrew, Suncus murinus (laboratory name: suncus). This animal belongs to the order Insectivore and is phylogenetically different from ordinarily used laboratory animals. They are believed to have evolved with mainly animal food without sucrose. To study the molecular basis of the sucrase deficiency in suncus, we cloned 6.0-kilobase (kb) sucrase-isomaltase (SI, EC 3.2.1.48–10) cDNA from suncus intestinal cDNA library. The cDNA clone contained a 5442-base pair (bp)-long open reading frame preceded by an in frame termination codon. The deduced 1813-amino acid sequence showed 68.6, 71.2, and 74.7% similarity with those of rat, rabbit, and human, respectively. A cleavage site between isomaltase and sucrase as well as the region surrounding the catalytic sites for sucrase and isomaltase were conserved among the species. Out of 18 potential N-linked glycosylation sites, 5 were common among all 4 species. In the connecting segment which was enriched with O-linked glycosylation sites in the other species, only two sites were present in suncus. Northern blot analysis revealed that the 6.0-kb SI mRNA was expressed in the KAT line with intact sucrase-isomaltase activity. In contrast, 3.0-kb SI mRNA was expressed in suncus of the MI line with isolated sucrase deficiency. The 3.0-kb mRNA cosegregated with sucrase deficiency phenotype as an autosomal recessive trait. Sequence analysis revealed a 2-nucleotide deletion at position 2767–2768, which results in a frame shift and an immature termination codon. The cDNA of the MI line diverged from that of the KAT line at position 2865, having an 18-bp unique sequence followed by a poly(A) tail. The mutant cDNA encodes 922 amino acid residues which preserves the region for isomaltase but lacks that for whole sucrase. While the cells transfected with the plasmids expressing SI in the KAT line showed both sucrase and isomaltase activity, the plasmids expressing MI line cDNA showed only isomaltase activity. Thus it was concluded that the mutation in the SI gene was responsible for isolated sucrase deficiency in the MI line.

Mammals of the order Insectivore have received attention as possible laboratory animals which are phylogenetically different from ordinarily used laboratory animals, such as the mouse, rat, hamster, and rabbit (1, 2). Both primates and rodents apparently originated from Insectivore and then evolved separately (3). In 1976, Oda and Kondo succeeded in rearing and breeding the house musk shrew, Suncus murinus, mammal belonging to the family Soricidae of Insectivore (4). Thereafter, several laboratory lines of suncus which originated from different wild populations in Asian regions have been developed in Nagoya, Japan, and a number of studies have been carried out on the morphology, endocrinology, and physiology of this animal (1). As a result, several lines of suncus were established as a model of sucrase deficiency. It was further established that hereditary sucrase deficiency in suncus was transmitted as an autosomal recessive manner (5, 6).

Sucrase-isomaltase (SI, EC 3.2.1.48–10) is an integral enzyme complex of the brush border membrane of the small intestine in mammals (7). It is synthesized as a single-chain polypeptide which has two catalytic sites (8, 9). Following its insertion into brush border membrane, SI is cleaved by pancreatic proteases to yield two separate enzymes (10). Isomaltase remains anchored to the membrane by its NH2-terminal domain while sucrase is attached to isomaltase by strong ionic interaction (9, 11). The enzyme is responsible for the terminal digestion of dietary sucrose and starch (12).

A congenital deficiency of sucrase, either isolated or combined with isomaltase deficiency, can cause malabsorption syndrome in humans (13–15). When subjects with sucrase deficiency take carbohydrate, they manifest abdominal distention and osmotic-fermentative diarrhea with liquid feces containing sucrose and glucose (14). To study the pathophysiology of sucrase-(isomaltase) deficiency or sucrase intolerance, a hereditary deficiency in SI in animal models is useful. However, there are no animal models which manifest congenital SI deficiency (CSID) or sucrase intolerance except for the suncus. To elucidate the molecular mechanism for CSID in suncus would contribute to our understanding of an autosomal recessive CSID in human. We thus cloned suncus SI cDNA from two lines of suncus, KAT line, expressing both sucrase and isomaltase, and MI line, expressing only isomaltase but not sucrase. We identified a mutation that resulted in deletion of the region coding for sucrase in an MI line. Transient transfection of the mutant cDNA revealed that peptide encoded by the mutant cDNA had isomaltase activity, but not sucrase activity in accordance with the phenotype of the animals.

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‡ The abbreviations used are: SI, sucrase-isomaltase; pro-SI, precursor sucrase-isomaltase; CSID, congenital sucrase-isomaltase deficiency; UTR, untranslated region; bp, base pair(s); kb, kilobase(s).
**Sucrase-Isomaltase cDNA in Suncus**

**EXPERIMENTAL PROCEDURES**

**Animals**—Two lines of suncus were employed in the present study. The KAT line has both sucrase and isomaltase activities in the small intestine which originated from a wild population in Katmandu. The MI line, which has isomaltase activity but not sucrase activity, was established among hybrid lines derived from wild populations in Nagasaki, Okinawa, Tarama of Japan and west Java of Indonesia. All these lines have been described in the previous reports (5–6). Suncus of MI line develop diarrhoea and weight loss when they are given water containing sucrose, while those of KAT line or KAT/MI heterozygote do not (5–6). They were kept on a solid diet of 5p pellets (Nippai Co. Ltd., Chita, Japan) used as food for aquafarming rainbow trout. The pellet contains high proteins (36–40%) but not sucrose. The enzyme activities in the animals used in this study were determined by the semi-quantitative assay method (16–18). These two suncus lines were bred at Research Institute of Environmental Medicine, Nagoya University.

**RNA Extraction from the Small Intestine**—Two-month-old suncus were killed by cardiac exsanguination under ether anesthesia. Proximal jejunal segments were removed and washed with cold 0.9% NaCl. Total RNAs were extracted by the acid guanidium thiocyanate-phenol-chloroform-extraction method (19). Poly(A) RNAs from each KAT and MI line were prepared with Oligotex®-DNA-300 Super (Takara, Tokyo, Japan) according to the instructions of the manufacturer.

**Library Construction and Screening**—The intestinal cDNA libraries were prepared by using Zap-cDNA® Gigapack® III Gold Cloning Kit (Stratagene) and 5 μg of poly(A) RNA from each KAT and MI line. The Uni-ZAP XR cDNA library of KAT line yielded 1.5 × 10⁶ independent clones and that of MI line yielded 1 × 10⁶. After plating, approximately 2 × 10⁵ clones were transferred onto GeneScreen Plus membranes (NEN Life Science Products). After denaturation in alkali followed by neutralization, the membranes were incubated in a hybridization buffer containing 20% formamide, 900 mM NaCl, 50 mM sodium phosphate (pH 7.4), 1% SDS, 5 × Denhardt’s solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), and denatured sheep serum DNA (0.1 mg/ml) for 4 h at 42 °C. An EcoRI fragment of GC ring sperm DNA (0.1 mg/ml) for 2° C. A EcoRI fragment of GC was labeled with [α-³²P]dATP (Boehringer Mannheim) and then used as a probe. After hybridization at 42 °C for 20 h, the membranes were then washed in 2 × SSC and 0.1% SDS at 65 °C (2 × 5 min), 1 × SSC and 1% SDS at 50 °C (2 × 30 min), and 0.1 × SSC at 20 °C (2 × 10 min). The positive plaques were isolated based on the image from computer-assisted imaging analysis using a BAS-2000 Fuji Bioimage Analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan).

**Plasmid Construction, DNA Sequence Analysis**—The positive λ clones were converted to pBluescript according to the instruction of the manufacturer. The sequence of the cDNA insert was determined by using ABI 373A DNA sequencer as described previously (20). Sequence searchings were carried out using the BLAST Local Alignment Search Tool program (21). The homology of nucleotide and amino acid sequences of SI among human, rabbit, rat, and suncus were determined with Genetyx: Homology 1.0.1 (Software Development, Tokyo, Japan). The suncus SI amino acid sequence was aligned with the other three species by FASTA program (22).

The cDNAs were excised from pBluescript by digestion with NotI and Apal and inserted into the same sites in an expression vector, pCMV (Invitrogen, San Diego, CA) to construct p-KSI expressing SI cDNA of KAT line and p-MSI expressing that of MI line. Twenty five SI cDNA derived from KAT line and p-KSI expressing SI cDNA of MI line, which has isomaltase activity but not sucrase activity, was established among hybrid lines derived from wild populations in Nagasaki, Okinawa, Tarama of Japan and west Java of Indonesia. All these lines have been described in the previous reports (5–6). Suncus of MI line develop diarrhoea and weight loss when they are given water containing sucrose, while those of KAT line or KAT/MI heterozygote do not (5–6). They were kept on a solid diet of 5p pellets (Nippai Co. Ltd., Chita, Japan) used as food for aquafarming rainbow trout. The pellet contains high proteins (36–40%) but not sucrose. The enzyme activities in the animals used in this study were determined by the semi-quantitative assay method (16–18). These two suncus lines were bred at Research Institute of Environmental Medicine, Nagoya University.

**RESULTS**

Isolation of SI cDNA Clones of Suncus KAT Line—The Uni-ZAP XR cDNA library constructed from the intestinal mRNA of suncus KAT line with intact SI activity was screened with rat SI cDNA. Screening of 2 × 10⁷ phage plaques resulted in the identification of approximately 400 positive clones, indicating that SI mRNA comprised 0.2% of the intestinal mRNAs. KSI (SI cDNA derived from KAT line). One of the clones that contained the longest insert was selected for sequencing.

**Nucleotide Sequence of KSI and Deduced Amino Acids Sequence of SI**—Sequencing of KSI insert revealed a single open reading frame of 5442 bp. The ATG start codon was found 54 bp downstream from the 5’ end of the cDNA. In-frame termination codon was present 39 bp upstream of the translation start codon. The 3’-untranslated region (UTR) was 395 bp followed by a poly(A) tail. The coding sequence showed 78.8, 77.3, and 71.8% homology with human (25), rabbit (26), and rat (27), respectively. The comparative analysis of available sequences for the 5’-UTR showed approximately 50% sequence homology with other species including human, rabbit, and rat. The 3’-UTR showed around 70% sequence homology among these species. The amino acid sequence of KAT line showed 74.7, 71.2, and 68.6% overall similarity with human (25), rabbit (26), and rat (27), respectively.

The suncus SI amino acid sequence was alignment with the other species in Fig. 1. Stretchs of 16 and 14 amino acids surrounding the suncus sucrase and isomaltase active sites, respectively (26), were identical among the species (Fig. 1, boxes). A cleavage site between isomaltase and sucrase was present at the same position as the other species (arrowhead in Fig. 1). As indicated by N in boldface type, 17 potential N-glycosylation sites were present in SI of KAT line. Out of eight N-glycosylation sites conserved among rat, rabbit, and human, only five N-glycosylation sites were conserved in suncus (Fig. 1, indicated by #). The NH2 terminus of isomaltase containing the putative transmembrane domain (Fig. 1, indicated by a continuous line) showed 85% sequence identity among the four species. However, the region called stretch, putative connecting segment following the transmembrane domain with potential O-glycosylation sites (Fig. 1, dashed line), was much shorter in suncus when compared with the other species.

**Northern Blot Analysis**—Northern blot analysis of the intestinal RNA using KSI insert as a probe showed that a 6.0- and a 3.0-kb mRNA was expressed in KAT line and MI line, respectively (see Fig. 2). The size of mRNA in KAT line was similar to that in the previous reports (25–27). To examine whether the short mRNA is limited to the sucrase deficiency phenotype, we crossed an animal of KAT line with an animal of MI line. The resulting male heterozygote was further crossed with the mother (Fig. 2). As expected, the heterozygote expressed both 6.0- and 3.0-kb mRNAs. Out of four pups obtained from the backcross, three showed sucrase deficiency, and only 3.0 kb of mRNA could be detected in these animals. It was thus confirmed that lack of the 6.0-kb SI mRNA expression is re-
sponsible for the isolated sucrase deficiency in MI line.

Southern Blot Analysis—To characterize the structure of the SI gene in suncus, genomic DNA from KAT line and MI line were digested with EcoRI, HindIII, and SacI and probed with KSI insert (Fig. 3). No gross difference between KAT (1/1) and MI (–/–) lines could be detected, indicating that a point mutation, a small deletion/insertion or a large alteration in intron region, is responsible for the generation of 3-kb mRNA.

Isolation and Characterization of SI cDNA Clones of MI Line with Isolated Sucrase Deficiency—To characterize the 3-kb mRNA in the sucrase-deficient line, we screened a cDNA library constructed from the intestinal poly(A) RNA of MI line using KSI insert as a probe. Screening of 2 \(3 \times 10^5\) phage plaques resulted in the identification of approximately 200 positive clones, indicating that SI mRNA comprised 0.1% of the intestinal mRNAs. One of the clones, MSI (SI cDNA derived from MI line) contained the longest insert of 3.0 kb. The size was compatible with that of the mRNA detected by the Northern blot analysis (Fig. 2).

Sequencing of the MSI insert revealed that it contained an open reading frame of 2769 bp, a 5′-UTR of 39 bp, and a 3′-UTR of 93 bp followed by a poly(A) tract. The coding sequence was identical to that of KSI from the initiation codon to 2766 nucleotides, where two nucleotides were deleted. This deletion of two nucleotides resulted in a frameshift and appearance of an immature stop codon. It was deduced that MI-sucrase-isomaltase mRNA encoded an enzyme with 922 amino acid residues which lacked COOH-terminal, 69 amino acids of isomaltase and the entire region of sucrase. An arrow in Fig. 1 shows the COOH-terminal end of the enzyme. The alignment of the 3′ sequence of MSI with that of KSI is depicted as Fig. 4. Downstream of the 2-nucleotide deletion, three base substitutions were present in the following 78 bp. Then the cDNA of MI line completely diverged from that of KAT line, having an 18-bp...
unique sequence followed by a poly(A) tail. The sequence AG-TAA at the position 2845 in MI line was considered to serve as a poly(A) signal as described by Guntaka (28).

**Transfection and Enzyme Assay**—To study the function of SI encoded by cDNAs in KAT line and MI line, we carried out the expression study using COS-7 cells and measured glucose production from sucrose or isomaltose. The cells transfected with p-KSI expressing SI of KAT line could digest sucrose to glucose after incubation of 120 min. In contrast, no significant glucose production could be detected in cells transfected with p-MSI expressing SI of MI line as in the nontransfected control cells after 120 min of incubation (Fig. 5A). Transfection efficiency monitored by β-galactosidase activity was not significantly different between cells transfected with p-KSI and those transfected with pMSI (data not shown). After incubating the cells with isomaltose for 60 and 120 min, glucose production could be detected in both cells transfected with p-KSI and p-MSI (Fig. 5B). Thus transient transfection study demonstrated that this mutant clone, MSI, retained isomaltase activity but not sucrase. It is concluded that 3-kb deleted mRNA with the immature termination codon is responsible for the isolated sucrase deficiency in MI line.

**DISCUSSION**

The cloning of KSI, sucrase isomaltase cDNA in KAT line suncus with both enzyme activities provided some unique features in suncus, as well as conserved features among human, rat, rabbit, and suncus (25–27). Deduced amino acid sequence of KAT line suncus cDNA revealed 17 potential N-glycosylation sites, 7 in isomaltase site, and 10 in sucrase site. In human (25), rat (26), and rabbit (27), eight N-glycosylation sites are conserved (Fig. 1, #), and only five of these were conserved in suncus. The five N-glycosylation sites, one in the isomaltase region and the other four in the sucrase region (Fig. 1, asterisk), conserved among the four species are considered to be important for the formation of the tertiary structure of SI.
region called connecting segments contains more than ten O-glycosylation sites in the other species, and it has been speculated that the glycosylated segment may protect itself from pancreatic enzymes (26). In KAT line suncus sucrase isomaltase, however, the connecting segment was very short and contains only two serine/threonine residues that may be glycosylated. Together with the fact that the SI activity of KAT line suncus was not much lower than that of rabbit (6), it was suggested that the glycosylations of this segment are not playing a critical role for the expression and the activity of SI. On the other hand, stretches of 16 and 14 amino acid residues of the catalytic site of sucrase and isomaltase, respectively, were all conserved among human, rat, rabbit, and suncus, suggesting an important role of these residues in substrate recognition.

Mammals of the order Insectivore including suncus are phylogenetically very different from rat and rabbit (1, 2) and are believed to conserve features of the common ancestor for primates and rodents. It is of note that the highest homology against suncus SI, both at nucleic acid and protein level, was observed in human, but not in rabbit or rat. If this is the case for other genes in suncus, suncus will emerge as a better animal model for human diseases (4).

In the present study, the function of suncus sucrase isomaltase expressed in heterologous cells was also examined by transient transfection. The enzyme complex is cleaved into two subunits by pancreatic enzymes in vivo. In contrast, this cleavage was considered not to take place in the transfection study. It has been reported that pro-SI on intestinal epithelia in pancreasectomized pig is enzymatically fully active (29). Thus, the present observation supports the conclusion that the catalytic sites for the specific substrates are already exposed to the outside of globular molecule in single-chain pro-SI complex.

The molecular basis of isolated sucrase deficiency in MI line suncus was also elucidated in the present study. SI mRNA which has a smaller size was detected in MI line suncus. Mating experiments confirmed that sucrase deficiency inherited in an autosomal recessive manner is linked to this abnormal mRNA. Cloning of MI line cDNA revealed 2-nucleotide deletion, resulting in a frameshift and appearance of an immature termination codon followed by unique sequence and an aberrant polyadenylation. Sucrase deficiency in MI line is attributable to the 2-nucleotide deletion. On the other hand, the precise mechanism for generation of the short mRNA from the genome remains to be elucidated. The genomic region across the 2-base pair deletion could not be amplified by polymerase chain reaction using several pairs of primers either in KAT line or MI line, suggesting the presence of a large intron near the deleted two nucleotides.

The cDNA derived from MI line encodes a 922-amino acids...
protein that lacks COOH-terminal 69 amino acids of isomaltase and the entire region of sucrase. A transient transfection study demonstrated that the protein encoded by the mutant cDNA retained isomaltase activity but not sucrase, confirming that the mutation is responsible for isolated sucrase deficiency in MI line suncus. It is of note that the sucrase subunit as well as COOH-terminal part of the isomaltase subunit are not required for proper folding and membrane-targeted transport of the molecule. SI gene is believed to have evolved by gene duplication from an ancestral, single subunit gene (26). The present results indicate that a single subunit isomaltase similar to the hypothetical ancestral gene is functional, providing some insights to the molecular evolution of SI.

The human congenital SI deficiency (CSID) has been considered as an example of a disease due to transport incompetent molecules (30, 31). In most cases, isomaltase deficiency is combined with sucrase deficiency. Synthesis and transport of SI molecule has been extensively studied using peroral biopsy specimen (32, 33), and the abnormality of the synthesis/transport has been classified into several types (15). Recently, single amino acid substitution was identified in sucrase subunit in a specimen (32, 33), and the abnormality of the synthesis/transport may be also shown in other species belonging to the order. These species would be similar to the hypothetical ancestral gene is functional, providing some insights to the molecular evolution of SI.

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The mutation in suncus did not occur in the laboratory lines, and the mutant gene already had existed in the wild population (5). Thus, there would be no positive selection of animals with a normal SI activity, and the abnormal gene would remain in the population like human cases (37). The Insectivore, including suncus, has probably evolved with mainly animal food (17), and several types of sucrase deficiency may be also shown in other species belonging to the order. These species would be useful for the animals models of CSID.

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