Pancreatic α-Amylase Controls Glucose Assimilation by Duodenal Retrieval through N-Glycan-specific Binding, Endocytosis, and Degradation*

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Background: Secreted pancreatic α-amylase binds to N-glycans of the duodenal brush-border membrane (BBM) and inhibits glucose uptake by SGLT1 at high doses.

Results: Enterocytes endocytosed and degraded the α-amylase in lysosomes about 30 min after BBM binding.

Conclusion: N-Glycan recognition and subsequent internalization of α-amylase suppressed and then gradually allowed duodenal glucose absorption.

Significance: A newly revealed mechanism regulates postprandial intestinal glucose uptake.

α-Amylase, a major pancreatic protein and starch hydrolase, is essential for energy acquisition. Mammalian pancreatic α-amylase binds specifically to glycoprotein N-glycans in the brush-border membrane to activate starch digestion, whereas it significantly inhibits glucose uptake by Na+/glucose cotransporter 1 (SGLT1) at high concentrations (Asanuma-Date, K., Hirano, Y., Le, N., Sano, K., Kawasaki, N., Hashii, N., Hiruta, Y., Nakayama, K., Umemura, M., Ishikawa, K., Sakagami, H., and Ogawa, H. (2012) Functional regulation of sugar assimilation by N-glycan-specific interaction of pancreatic α-amylase with glycoproteins of duodenal brush border membrane. J. Biol. Chem. 287, 23104–23118). However, how the inhibition is stopped was unknown. Here, we show a new mechanism for the regulation of intestinal glucose absorption. Immunohistochemistry revealed that α-amylase in the duodenum of non-fasted, but not fasted, pigs was internalized from the pancreatic fluid and immunostained. We demonstrated that after N-glycan binding, pancreatic α-amylase underwent internalization into lysosomes in a process that was inhibited by α-mannoside. The internalized α-amylase was degraded, showing low enzymatic activity and molecular weight at the basolateral membrane. In a human intestinal Caco-2 cell line, Alexa Fluor 488-labeled pancreatic α-amylase bound to the cytomembrane was transported to lysosomes through the endocytic pathway and then disappeared, suggesting degradation. Our findings indicate that N-glycan recognition by α-amylase protects enterocytes against a sudden increase in glucose concentration and restores glucose uptake by gradual internalization, which homeostatically controls the postprandial blood glucose level. The internalization of α-amylase may also enhance the supply of amino acids required for the high turnover of small intestine epithelial cells. This study provides novel and significant insights into the control of blood sugar during the absorption stage in the intestine.

Endocytosis is the process used by cells to absorb proteins and nutrients that are too large to pass through the plasma membrane. In enterocytes, endocytosis facilitates intestinal nutrient absorption, mucosal renewal, immune defense, hormone response, and membrane conservation (1–3).

α-Amylase (EC 3.2.1.1) catalyzes the initial step of starch hydrolysis for glucose production and is thus a key enzyme in energy acquisition. As such, α-amylase is a target molecule for the treatment of type 2 diabetes mellitus; its inhibitors and its relationship with the disease have been extensively investigated (4, 5). Pancreatic α-amylase is synthesized by pancreatic acinar cells and secreted into the duodenum as a major component of pancreatic fluid (6). In the duodenum, α-amylase digests starch to maltose or maltotriosaccharides, which are subsequently hydrolyzed by brush-border membrane (BBM)2 enzymes, such as sucrase-isomaltase (7). The final glucose product is then carried into the enterocytes by Na+/glucose cotransporter 1 (SGLT1) at the BBM (8). The small intestine BBM is heavily glycosylated (9).

We previously showed that mammalian pancreatic α-amylase binds to N-linked oligosaccharides of glycoproteins in a pH-dependent manner; it binds to the high-mannose type at neutral pH and to both complex and high-mannose types at acidic pH (10). The carbohydrate-binding activity of pancreatic α-amylase, which is common to pigs and humans (11), allows it to interact with N-glycans on the duodenal BBM. This increases
glucose production up to 240% through the cooperation of sucrase-isomaltase and α-amylase at a wide range of concentrations; however, glucose uptake by SGLT1 is inhibited at high α-amylase concentrations in the intestine (11). Therefore, we hypothesized that this process may be an innate regulatory mechanism in the intestinal lumen to suppress the sudden elevation of glucose absorption and maintain blood glucose homeostasis. This hypothesis raises an important point regarding intestinal glucose recovery, which is that the behavior of pancreatic enzymes after reaching the duodenum has not yet been clarified. To our knowledge, the only report concerning the fate of these enzymes stated that *Bacillus species* α-amylase reaches the blood circulation via the enterocytes under *in vivo* conditions in rats (12). However, the mechanism and biological significance of internalization of pancreatic α-amylase are yet unknown.

In the present study, we investigated the intestinal endocytosis of pancreatic α-amylase using pig duodenum tissue and human intestine Caco-2 cells. We found that pancreatic α-amylase was internalized into the duodenal mucosa after binding to N-glycans on the duodenal BBM. We further showed that internalized pancreatic α-amylase was transported to lysosomes through the endocytic pathway and subsequently degraded. These findings significantly advance our knowledge of mammalian regulation of sugar absorption after eating.

**Materials and Methods**

**Reagents and Antibodies—α-Amylase** from pig pancreas was purchased from Elastin Products Co., Inc. (Owenswill, MO). Human pancreatic α-amylase was expressed in yeast and purified as described previously (11). Rabbit anti-α-amylase IgGs to human pancreatic α-amylase (K50894R) were purchased from Biomeda Corp. (Foster City, CA). Horseradish peroxidase (HRP)-rabbit anti-α-amylase IgGs to *Bacillus amyloliquefaciens* α-amylase (ab34578), mouse anti-early endosome antigen 1 (EEA1) IgGs (ab70521), mouse anti-lysosome-associated membrane protein 1 (LAMP1) (H4A3) IgGs (ab25630), and rabbit anti-SGLT1 IgGs (ab14686) were purchased from Abcam (Cambridge, MA). Alexa Fluor 488- and 594-labeled secondary antibodies were obtained from Invitrogen. HRP-conjugated secondary antibodies were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). HRP-conjugated avidin-biotin complex was purchased from Sigma. Alexa Fluor 488- and 594-labeled protein labeling kit (Invitrogen) according to the manufacturer’s instructions. Alexa Fluor 488-α-amylase were dialyzed against 0.1 M borate buffer (pH 8.0) to remove the sugars and then against PBS.

**Preparation of Alexa Fluor 488-α-Amylase** from pig pancreas in PBS (1 or 0.5 mg/ml, respectively) containing 0.2 m methyl α-mannopyranoside and 0.2 m methyl α-galactopyranoside as protecting sugars was labeled with an Alexa Fluor 488-N-hydroxysuccinimidyl ester dye using the Alexa Fluor 488 protein labeling kit (Invitrogen) according to the manufacturer’s instructions. Alexa Fluor 488-α-amylases were then washed with cold PBS (pH 7.2) and cut into 1-cm cross-sections. The fresh duodenum sections were soaked in 10 μM pig pancreatic α-amylase contained in 1 mM phenylmethylsulfonyl fluoride in PBS (pH 7.2) and incubated at 4°C or 37°C for 0–60 min with gentle agitation. After incubation, the tissues were fixed in 4% paraformaldehyde/PBS, and paraffin-embedded sections were prepared (11).

**Internalization Assays with Tissue**—The duodena from fasting pigs were washed with cold PBS (pH 7.2) and cut into 1-cm cross-sections. The fresh duodenum sections were soaked in 10 μM pig pancreatic α-amylase contained in 1 mM phenylmethylsulfonyl fluoride in PBS (pH 7.2) and incubated at 4°C or 37°C for 0–60 min with gentle agitation. After incubation, the tissues were fixed in 4% paraformaldehyde/PBS, and paraffin-embedded sections were prepared (11).

For the immunofluorescence technique, tissue sections were deparaffinized, rehydrated, and heated in 10 mM citrate buffer (pH 6.0) at 95 °C for 10 min. The sections were blocked with 5% bovine serum albumin and 0.1% fish gelatin in PBS containing 0.1% Tween 20 (PBS-T) for 30 min. Incubation with primary antibodies (anti-α-amylase (K50894R), HRP-anti-α-amylase (ab34578), anti-LAMP1 (diluted to 1:150), or anti-SGLT1 (diluted to 1:50)) was carried out overnight at 4 °C. Sections were then washed with PBS-T and incubated with one of three secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG (H+L), Alexa Fluor 594 goat anti-rabbit IgG (H+L), or Alexa Fluor 594 goat anti-mouse (H+L) (diluted to 1:200)) for 1 h at room temperature. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics GmbH, Mannheim, Germany). Finally, coverslips were mounted using 50% glycerol. Fluorescence was examined by confocal scanning microscopy LSM710 (Carl Zeiss, Inc.). Quantification of total fluorescence was performed with LSM Software ZEN 2009 (Carl Zeiss).

**Internalization Assays with Caco-2 Cells**—Internalization assays were performed according to previously published protocols (13). Caco-2 cells were grown on polyester Transwell permeable supports (0.4-μm pores, 12-mm diameter) at 2.6 10^5 cells/cm² and cultured for 20–27 days. Every 2–3 days, the culture medium was renewed. Then cells were starved in a serum-free medium for 18 h and washed two times with Dulbecco’s phosphate-buffered saline (DPBS) with Ca²⁺ and Mg²⁺. The cells were incubated with 20 μg/ml Alexa Fluor 488-α-amylase in a serum-free medium at 4 °C for 30 min. After...
incubation, cells were extensively washed with DPBS with Ca\(^{2+}\) and Mg\(^{2+}\), placed in prewarmed complete medium, and incubated at 37 °C for the indicated periods. For TF labeling, cells were chased at 37 °C for the indicated periods. For Tf labeling, cells were chased at 37 °C for the indicated periods with 10 μM pancreatic α-amylase solution containing 1 mg/ml phenylmethylsulfonyl fluoride for 10 or 30 min; subsequently, they were fixed and paraffin-embedded. The prepared sections were incubated with rabbit anti-α-amylase IgGs (K50894R) and visualized by Alexa Fluor 488-goat anti-rabbit IgGs (green fluorescence) and then examined by confocal microscopy. Bars, 20 μm. B, fresh 1-cm cross-sections of duodena from fasted pigs were incubated at 37 °C with 10 μM pancreatic α-amylase solution containing 1 mg/ml phenylmethylsulfonyl fluoride for 10 or 30 min; subsequently, they were fixed and paraffin-embedded. The prepared sections were incubated with rabbit anti-α-amylase IgGs (K50894R) and visualized by Alexa Fluor 488-goat anti-rabbit IgGs (green fluorescence) and then examined by confocal microscopy. Bars, 20 μm.

**Preparation of BBM from Duodenum—**BBM was isolated from pig duodenal mucosa by Ca\(^{2+}\) precipitation methods as described previously (11, 16). Alkaline phosphatase, which is chiefly located in BBM, was concentrated 18-fold. The protein concentration was determined by a BCA protein assay (Thermo Fisher Scientific) using bovine serum albumin as the standard. The fractions other than BBM in duodenal mucosa were called basolateral membrane (BLM) fraction, whose alkaline phosphatase activity was 5.2% that of the purified BBM fraction, even lower than that of crude BBM homogenate.

**Enzyme Activity of α-Amylase in BBM and BLM—**The enzyme activity of α-amylase was measured according to the method of Bernfeld (17) on a small scale as described previously (11). BBM and BLM fractions from duodenal mucosa (each 0.56 mg/ml protein) and α-amylase from pig pancreas (0.56 μg/ml) as the standard (50 μl) were preincubated at 37 °C for 15 min; 100 μl of prewarmed 1% starch in 20 mM phosphate buffer, pH 6.9, was added; and the mixture was reacted at 37 °C for 30 min. To stop the reaction, cold 3,5-dinitrosalicylic acid (100 μl) was added, and the mixture was heated at 98 °C for 5 min to develop
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The color. The reaction mixture was cooled on ice. Aliquots (50 µl) of the reaction mixture were diluted with 200 µl of water in a 96-well microtiter plate and measured at 540 nm. Maltose was used as a standard for reducing sugar. BBM, BLM, and α-amylase that had been heated at 98 °C for 20 min were used as blanks.

Sandwich ELISA—Rabbit anti-α-amylase IgGs (K50894R) (1 µg/ml, 100 µl) were immobilized in wells of a microtiter plate (Immuno 1B, Dynex) in TBS containing 5 mM CaCl₂ (TBS-Ca) overnight at 4 °C. All other procedures were performed at room temperature using TBS-Ca as the dilution buffer. After washing three times, the wells were blocked with 3% bovine serum albumin for 2 h. The solubilized BBM or BLM with 1% Triton X-100 (each 100 µg/ml, 100 µl) or α-amylase from pig pancreas (0–10 µg/ml, 100 µl) was added to each well, followed by incubation for 2 h. The wells were then washed and incubated with HRP-

FIGURE 2. Binding and internalization of pancreatic α-amylase were inhibited by polysaccharides. Fresh 1-cm cross-sections of duodena from fasted pigs were incubated with 10 µM pancreatic α-amylase in PBS, which had been preincubated with 0.1 M polysaccharide (α-D-mannan, β-D-galactan, or colominic acid) at 4 °C overnight, at 4 or 37 °C for 10 or 30 min as indicated. The duodenal sections were fixed and paraffin-embedded. The prepared sections were incubated with rabbit anti-α-amylase IgGs (K50894R) as the primary antibody and visualized by Alexa Fluor 488-goat anti-rabbit IgGs (green fluorescence) and then examined by confocal microscopy. The controls were incubated without the primary antibody and stained in the same way. Nuclei were counterstained with DAPI. A, confocal image after incubation with pancreatic α-amylase for 30 min. A typical result of three independent experiments is shown. Bars, 20 µm. B and C, quantification of α-amylase localization observed as green fluorescence in A at 4 °C (B) or at 37 °C (C). Error bars, S.D.

FIGURE 3. Internalized pancreatic α-amylase decreased in Caco-2 cells. A, time course. Caco-2 cells were incubated with 20 µg/ml Alexa Fluor 488-α-amylase (green fluorescence) at 4 °C for 30 min and washed to remove unbound α-amylase. After the cells were chased at 37 °C for 0–60 min, they were fixed and examined by confocal microscopy. Nuclei were counterstained with DAPI. Bars, 10 µm. B, quantification of green fluorescence in A. The mean intensity (%) is calculated the value at 0 min as 100%. The experiments were repeated three times. Error bars, S.D.
rabbit anti-α-amylase IgGs (ab34578) (10 μg/ml, 100 μl) for 2 h. After the wells were washed three times, color was developed by adding 0.4 mg/ml o-phenylenediamine, 0.008% H2O2 in 0.1 M citrate-phosphate buffer, pH 5.0 (200 μl), and then 50 μl of 2.5 M H2SO4 was added to stop the reaction. Absorbance was measured with a microplate reader (Vient, DS Pharma Biomedical) at 490 nm.

Western Blotting—The BBM and BLM solubilized with 1% Triton X-100 and α-amylase from pig pancreas were subjected to SDS-PAGE using 15% polyacrylamide gels under reducing conditions. SDS-polyacrylamide gels were transferred to polyvinylidene difluoride membranes and immunostained using HRP-rabbit anti-α-amylase (ab34578) or rabbit α-amylase IgGs (K50894R) and HRP-conjugated secondary antibodies. Membranes were subsequently reacted with 3,3′-diaminobenzidine, tetrahydrochloride (0.2 mg/ml) and 0.006% H2O2 in TBS.

Statistical Analysis—All data were presented as the mean of five different fields (4,000 μm²) ± S.D. Statistical significance was evaluated with Student’s t test using Microsoft Excel.

Results

α-Amylase Is Found in Duodena of Non-fasted but Not Fasted Pigs—α-Amylase in the duodenal mucosa of fasted or non-fasted pigs was labeled with two specific primary antibodies, HRP-anti-α-amylase (ab34578) and anti-α-amylase (K50894R). Low-level staining of α-amylase was observed in the villi of fasted pig duodena (Fig. 1A, a, c, and e). In contrast, clear and significant staining with both antibodies was observed in the villi of the duodena of non-fasted pigs (Fig. 1A, b, d, and f), and strong staining with anti-α-amylase (K50894R) was present in the perinuclear region (Fig. 1A, f). Omitting the primary antibody resulted in the absence of staining (Control; Fig. 1A, g and h).

Exogenous Pancreatic α-Amylase Is Internalized in Duodenum—α-Amylase in pancreatic fluid is secreted into the duodenum, where it digests starch. Thus, the α-amylase detected in the enterocytes of duodena of non-fasted pigs may have been secreted by the pancreas and then endocytosed by the duodenum. To test this possibility, duodenum sections from fasted pigs were incubated with exogenous pancreatic α-amylase, and immunostaining was performed. As shown in Fig. 1B, the α-amylase signal was not detected at 0 min but was observed after 10 min of incubation and was mainly associated with the BBM of epithelial cells. At 30 min, the signal was enhanced and expanded to the entire villus. These results suggest that the positive immunostaining presented in Fig. 1A, b, d, and f, shows α-amylase that has been internalized from the pancreatic fluid rather than endogenous α-amylase.

Carbohydrate-specific Binding Internalizes Pancreatic α-Amylase—We previously reported that human and pig pancreatic α-amylases specifically bind to the N-glycans of glyco-
proteins (10, 11) and that the binding is inhibited by certain sugars of pancreatic α-amylases, such as α-mannan (11). To examine whether the carbohydrate-specific binding of pancreatic α-amylase to the BBM is responsible for internalization into the enterocytes, the effects of various polysaccharides on the binding and internalization of exogenous pancreatic α-amylase at 4 °C and 37 °C were compared. In the absence of polysaccharides, α-amylase mainly localized to the BBM of enterocytes at 4 °C, whereas it was dispersed throughout the enterocytes at 37 °C (Fig. 2A). Only α-D-mannan markedly inhibited the binding of α-amylase at both 4 and 37 °C, whereas β-D-galactan and D-colominic acid, which are the polysaccharides of β-D-galactose and N-acetyl-D-neuraminic acid, respectively, inhibited binding only weakly compared with α-D-mannan. Quantification of the staining intensity showed that, compared with the α-amylase signal in the absence of a polysaccharide at 30 min, which was set to 100%, α-D-mannan inhibited α-amylase entry into the enterocytes to 31.3% at 4 °C and 26.6% at 37 °C (Fig. 2,
B and C). These results indicate that pancreatic α-amylase is internalized by the duodenal tissue after initial carbohydrate-specific binding to N-glycans on the mucosal epithelium.

Pancreatic α-Amylase Is Internalized and Degraded in Caco-2 Cells—To study the intestinal internalization of pancreatic α-amylase at the cellular level, the human intestinal epitel-
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A. Duodenum

B. Caco-2 Cells

C

FIGURE 9. SGLT1 was internalized and returned to cell surface in an α-amylase-independent manner. A, duodenum. Fresh 1-cm cross-sections of duodenal from fasted pigs were incubated at 37 °C with 10 μM pancreatic α-amylase solution for 0 – 60 min, fixed, and paraffin-embedded. The prepared serial sections were incubated with rabbit anti-α-amylase IgGs (K50894R) or rabbit anti-SGLT1 IgGs (ab14686) and visualized by Alexa Fluor 488–goat anti-rabbit IgGs (green fluorescence) or Alexa Fluor 594–goat anti-rabbit IgGs (red fluorescence), respectively. The stained sections were observed by confocal microscopy. Bars, 20 μm. B, Caco-2 cells. The cells were incubated with 20 μg/ml Alexa Fluor 488–α-amylase (green fluorescence) at 4 °C for 30 min and washed to remove unbound α-amylase. After the cells were chased at 37 °C for 0 – 60 min, they were fixed. The cells were immunostained with anti-SGLT1 IgGs and Alexa Fluor 488–α-amylase and traced its internalization into Caco-2 cells. Alexa Fluor 488–α-amylase was allowed to bind maximally to the cells by incubating at 4 °C for 30 min, and then the unbound α-amylase was removed by washing, and the bound α-amylase was chased at 37 °C. Pancreatic α-amylase that bound to the cell membrane at 4 °C was internalized within 10 min at 37 °C, and the signal disappeared within 60 min (Fig. 3, A and B). Similarly, in Caco-2 cells cultured for 7 days, α-amylase from human and pig pancreas that bound to the cell membrane at 0 min was internalized in 5–10 min and had disappeared at 60 min (data not shown). These results suggest that internalized pancreatic α-amylase is degraded.

Pancreatic α-Amylase Is Transported to Lysosomes through Early Endosomes in Caco-2 Cells—To investigate the internalization pathway of pancreatic α-amylase, the colocalization of internalized Alexa Fluor 488–α-amylase with endocytic marker proteins was examined. When internalized, α-amylase was initially colocalized with EEA1, an early endosome marker, after 10 min of incubation at 37 °C (Fig. 4, A and C). After incubation at 37 °C for more than 30 min, the majority of internalized α-amylase was colocalized with LAMP1, a lysosome marker (Fig. 4, B and E). Less colocalization was observed with the recycling endosomal marker Tf at 30 and 60 min at 37 °C (Fig. 4D). Together, these results suggest that the majority of the internalized α-amylase is transported via the endosomal-lysosomal degradation pathway and that a small amount may enter the recycling pathway. Human α-amylase was also colocalized with LAMP1 (data not shown). Furthermore, the effects of a lysosome inhibitor, chloroquine, were examined. As shown in Fig. 5, A and B, the colocalization of α-amylase with LAMP1 was inhibited by chloroquine treatment, which raises the pH of the lysosomes, and the internalized α-amylase tended to colocalize with EEA1. The reduction of fluorescence intensity of α-amylase, which indicates the intracellular degradation of α-amylase, was inhibited with chloroquine, not only at 30 min (Fig. 5C) but also from 10 to 60 min (Fig. 6), suggesting a decrease of the lysosomal degradation of α-amylase. These results indicate that α-amylase is degraded in lysosomes via the endocytic pathway.

Pancreatic α-Amylase Is Transported to Lysosomes and Degraded in Duodenal Tissue—We next investigated the degradation of pancreatic α-amylase in duodenal tissue. In duodenal tissue from non-fasted pigs, pancreatic α-amylase was
SGLT1 Was Internalized and Returned to Cell Surface—Localization of SGLT1 in duodenum and Caco-2 cells during endocytosis of pancreatic α-amylase was investigated. As shown in Fig. 9A, immunoreaction with α-amylase had gradually increased in the duodenum tissue by 60 min after the start of incubation with α-amylase. On the other hand, the immunoreaction with SGLT1 in the duodenum was observed at the BBM at 0 min, gradually moved to the basolateral region by 30 min, and then returned to the BBM at 60 min. In Caco-2 cells, SGLT1 was detected at both cell membranes and intracellular areas in permeabilized cells (Fig. 9B). The immunoreaction of SGLT1 was observed at the cell surface in non-permeabilized cells at 0 min, it was greatly reduced at 30 min, and it had returned to the cell surface at 60 min, whereas pancreatic α-amylase was internalized and degraded by 60 min. α-Amylase colocalized with SGLT1 at 0 min, and the colocalization decreased at 10–60 min (Fig. 9, B and C), which suggested that the internalization pathway of α-amylase and that of SGLT1 are different. The SGLT1 recycling was found to occur with PBS or medium alone at 37 °C in the same manner as that with α-amylase in both duodenum and Caco-2 cells (Fig. 10), which indicates SGLT1 trafficking; in other words, internalization of SGLT1 by 30 min and its return to the cell surface at 60 min is carried out in an α-amylase-independent manner in both Caco-2 cells and duodenum. Chloroquine had no effects on SGLT1 trafficking, whereas it inhibited the decrease of α-amylase in Caco-2 cells, as shown in Fig. 6. The results represented the endocytosis of SGLT1, which occurred in an α-amylase-independent manner.

Discussion

This study demonstrated that mammalian pancreatic α-amylase is internalized by binding to carbohydrates on the BBM and transported to lysosomes through the endocytic pathway in both pig duodenal tissue and human Caco-2 cells. To our knowledge, this is the first study to show that a pancreatic enzyme undergoes lysosomal degradation in the intestine. α-Amylase was detected in duodenal enterocytes (Fig. 1), suggesting that α-amylase secreted by the pancreas is internalized into enterocytes. The internalization was initiated by the specific binding of α-amylase to carbohydrates (mannose-containing N-glycans) on the BBM of enterocytes (Fig. 2) (10). Several lines of evidence indicate that the internalized α-amylase is degraded in lysosomes. The internalized pancreatic α-amylase disappeared almost entirely within 60 min (Fig. 3A), and the α-amylase was present in lysosomes, as shown by colocalization with LAMP1 (Figs. 4, 5, and 7). In the BLM, but not the BBM, starch-hydrolyzing activity decreased, and low molecular weight immunoreactive bands were detected (Fig. 8).

A typical and well known function of pancreatic α-amylase is the digestion of starch for energy acquisition. We previously showed that pancreatic α-amylase binds specifically to N-glycans (10). When pancreatic α-amylase interacts with duodenal BBM possessing many glycoproteins, including sucrase-isomaltase and SGLT1, glucose production by α-amylase and sucrase-isomaltase is enhanced, whereas glucose uptake of SGLT1 is strongly inhibited by a high dose of α-amylase (11). Here, we observed that pancreatic α-amylase bound to N-gly-
cans on the cell membrane at 4 °C and was removed from the cell membrane by endocytosis, an energy-dependent mechanism (19), at 37 °C (Figs. 2 and 3). These results suggest that the inhibition of SGLT1 is abolished as α-amylase is removed from the BBM, and glucose uptake by SGLT1 remaining in the BBM is thereby restored.

Based on the observations in this study, we propose a novel biological mechanism that minimizes the effects of sudden changes in starch digestion efficiency and intestinal glucose concentration on the blood sugar concentration. This occurs through the carbohydrate-specific interaction of pancreatic α-amylase with BBM, as summarized in Fig. 11.

In this study, SGLT1 was demonstrated to be internalized and then returned to the cell surface. Kipp et al. (20) reported that SGLT1 in Caco-2 cells resides in early intracellular compartments and the apical plasma membrane and suggested that most intracellular SGLT1 is not en route from biosynthesis but represents an intracellular reserve pool by eliminating SGLT1 synthesis and determining the half-life of SGLT1 (2.5 days). Subsequently, Khoursandi et al. (21) reported that the intracellular compartments containing SGLT1 are accessible by endocytosis during incubation of the cells for 30 min at 37 °C. Incubation of Caco-2 cells with mastoparan, a drug that enhances apical endocytosis, shifted a large amount of SGLT1 from the apical membrane to intracellular sites and significantly reduced sodium-dependent glucose uptake (60%) (21). These papers showed that SGLT1 is internalized from the apical surface to the endosomes of Caco-2 cells by incubation of the cells for 30 min at 37 °C and that most intracellular SGLT1 remains in the intracellular reserve pools. Their observations support the results of Figs. 9 and 10.

Recently, it was reported that when the blood glucose level is high in type 2 diabetes mellitus, serum amylase activity is significantly low (5). Not only type 2 but also type 1 diabetes mel-
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litus patients have been reported to show a high prevalence of exocrine pancreatic insufficiency (22, 23). Compared with diabetes mellitus patients, healthy individuals have a higher α-amylase level in the serum and a lower blood glucose level. These phenomena may be explained by the fact that the inhibition of SGLT1 cannot be achieved by a low concentration of α-amylase, due to the exocrine dysfunction in diabetes, which results in a high blood glucose level. This suggests that modulation of sugar assimilation via SGLT1 activity by altering the concentration of pancreatic α-amylase in the intestine might be important for stabilizing blood glucose levels. Recovery of exocrine secretion might therefore be a therapeutic strategy for post-prandial hyperglycemia in diabetes mellitus patients.

Related to the discovery that endocytosis of pancreatic α-amylase occurs via binding to N-glycans on the duodenal BBM, we previously identified several glycoproteins from the duodenal BBM as glycoglycans for pancreatic α-amylase (11). A large number of the glycoglycans identified are known to be internalized via the endocytic pathway. For example, a glycoprotein complex of Tf and Tf receptor is transported to early and recycling endosomes (24); the cation-dependent mannose 6-phosphate receptor is involved in the intracellular targeting of lysosomal enzyme (25, 26), and aminopeptidase N and dipeptidylpeptidase IV are internalized in a clathrin-dependent manner, which is the same as that of Tf (27). These reports support our finding that pancreatic α-amylase bound to membrane glycoprotein ligands in the BBM is transported to lysosomes through the endocytic pathway.

Pancreatic α-amylase has a pH-dependent N-glycan-specific binding activity. It specifically binds to high-mannose type oligosaccharides at neutral pH and both complex and high-mannose type oligosaccharides at acidic pH (10). In this study, we found that the endocytosis pathway of pancreatic α-amylase starts at the duodenal BBM (pH 6.5–7.5) via early endosomes (pH 6), late endosomes (pH 5–6), and finally acidified lysosomes (pH 4–5) (28–30). Pancreatic α-amylase may therefore transit the glycoligands in each endosome on the pathway from high-mannose type N-glycans in the BBM (11) to complex ones, such as those present in LAMPs in acidic lysosomes. This study showed that most pancreatic α-amylase undergoes lysosome degradation. We propose two biologically significant consequences of internalization of pancreatic α-amylase through the intestinal mucosa. The most important biological effect of the release of SGLT1 inhibition is to promote blood glucose homeostasis, as described above (Fig. 11). One further possibility is that pancreatic α-amylase serves as a source of amino acids for the proliferation of small intestine epithelial cells. It is known that epithelial cells require a large supply of amino acids to maintain their turnover rate, which is the most rapid of any tissue in the body (31–33). Amino acids are produced by constant proteolysis, which breaks down both dietary and endogenous proteins for reuse in protein synthesis (34). One of the major sites of intracellular proteolysis is the endosome-lysosome system (35), which was found to incorporate pancreatic α-amylase in this study. Pancreatic α-amylase is the main component of pancreatic fluid, which flows into the duodenum in large quantities at high protein concentrations (6, 36) and must contribute significantly as a source of amino acids for duodenal enterocytes.

In summary, this study reveals a new aspect of the intestinal fate of pancreatic α-amylase. Exocrine pancreatic α-amylase is retrieved by duodenal enterocytes through recognition of N-glycans on the BBM, thus releasing its inhibition of glucose absorption by SGLT1 at the BBM, and is subsequently degraded in lysosomes. We elucidated a link between the N-glycan-specific binding activity and the intracellular behaviors of α-amylase, which performs two previously unidentified functions: stabilizing blood glucose levels and supplying amino acids to enterocytes. These findings uncover a new homeostatic mechanism performed by exocrine enzymes and intestinal glycans.

Author Contributions—K. D. and H. O. conceived the project and designed the experiments. K. D. prepared the samples and performed all experiments. A. S. gave advice on studying endocytosis and protein trafficking in the cell. K. I. gave special advice about blood glucose level and diabetes mellitus. K. D. and H. O. wrote the manuscript. All authors participated in the redaction of the manuscript and approved the final manuscript.

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