Intestinal aminooligopeptidase in diabetic BioBreed rat: altered posttranslational processing and trafficking

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Najjar, Sonia M., Jean-Pierre Broyart, Lydia T. Hampp, and Gary M. Gray. Intestinal aminooligopeptidase in diabetic BioBreed rat: altered posttranslational processing and trafficking. Am J Physiol Gastrointest Liver Physiol 280: G104–G112, 2001.—The structure of aminooligopeptidase (AOP), an intestinal brush-border digestive hydrolase, is abnormal in human diabetes and in the congenitally diabetic BioBreed Wistar (BBd) rat. Its assembly in the BBd rat was examined. After normal initial synthesis and assembly of immature AOP precursor (AOPi) with high-mannose N-linked chains in the endoplasmic reticulum (ER), processing of N-linked glycans in Golgi yielded a smaller than normal mature AOP precursor (AOPm) with persistence of some high-mannose N-linked chains. Deglycosylation analyses suggested that the mass difference could be attributed to a lower mass of N-linked with unaltered O-linked carbohydrates chains that results in a mass change and altered kinetics of its membrane-associated assembly and trafficking. Additionally, we have examined the structure of another enterocyte digestive enzyme, sucrase-α-dextrinase, was also altered in the BBd rat (22), suggesting that altered glycoprotein structure is common in congenital diabetes. The alteration is independent of hyperglycemia or the degree of severity of diabetes and reverts to normal over −3-wk treatment of the diabetic animal with insulin (22, 23). Deglycosylation of brush-border AOP and sucrase-α-dextrinase revealed normal apoprotein products in the BBd rat, suggesting that the mass change of AOP and sucrase-α-dextrinase in congenital diabetes is caused by altered carbohydrate chains (22, 23).

AMINOOLIGOPEPTIDASE (AOP), commonly known as aminopeptidase N, resides in the brush-border surface membrane of epithelial cells of the renal proximal tubules (31, 32) and is most prominent at the intestinal enterocyte surface (15). Analogous aminopeptidases are also expressed variably in other tissues such as splenocytes, hepatocytes, salivary glands, and the genital tract (31). Although the physiological significance of AOP in most organs is uncertain, AOP plays a pivotal role in the small intestine in protein assimilation by acting at the lumen-enterocyte interface to cleave sequentially the NH2-terminal amino acid residues from nutrient oligopeptides having two to six amino acid residues (17). The released amino acids are then transported by the enterocyte.

In vivo pulse-chase experiments with radioactive amino acid precursors in the intact rat showed initial glycosylation of AOP with high-mannose N-linked chains in association with the endoplasmic reticulum (ER) to yield maximal incorporation in an immature AOP precursor (AOPi) after only 15 min of chase (2). Subsequent posttranslational processing of N-linked chains to complex forms and probable addition of O-linked chains occur after its transport to the Golgi compartment, producing a mature AOP precursor (AOPm) by 45 min. AOPm is then transported to the brush-border surface without additional modification.

Since its discovery in 1974, the spontaneously diabetic BioBreed Wistar (BBd) rat has been considered an excellent experimental model for the study of type 1 diabetes mellitus in humans because of many common histopathological and clinical features (20). Previously, we demonstrated (23) an altered structure of newly synthesized and steady-state brush-border AOP in the BBd rat manifesting by a 5-kDa reduction in mass. Similarly, the structure of another enterocyte digestive enzyme, sucrase-α-dextrinase, was also altered in the BBd rat (22), suggesting that altered glycoprotein structure is common in congenital diabetes. The alteration is independent of hyperglycemia or the degree of severity of diabetes and reverts to normal over −3-wk treatment of the diabetic animal with insulin (22, 23). Deglycosylation of brush-border AOP and sucrase-α-dextrinase revealed normal apoprotein products in the BBd rat, suggesting that the mass change of AOP and sucrase-α-dextrinase in congenital diabetes is caused by altered carbohydrate chains (22, 23).

In the present studies, we have examined the intracellular synthesis and assembly of intestinal AOP in the intact diabetic BBd rat. We have identified abnormal posttranslational processing of its N-linked carbohydrate chains that results in a mass change and altered kinetics of its membrane-associated assembly and trafficking through the ER and Golgi compartments. Additionally, we have examined the structure of AOP in human type 2 diabetes. Similar to findings in the congenitally diabetic BBd rat (23), we observed a...
smaller than normal AOP in human subjects. This suggests that altered AOP structure is common in diabetes.

MATERIALS AND METHODS

Materials. All chemicals and reagents are Baker analyzed reagents except where otherwise indicated.

Experimental animals. Male BioBreed BBd diabetic and normal (Wist) Wistar rats were selectively bred in the Animal Resources Division, Health Protection Branch, Health and Welfare, Ottawa, Canada, and sent to the animal facility at Stanford University 1–10 days after the onset of diabetes. All rats were housed in a room lighted from 6:00 AM to 6:00 PM in cages equipped with filters (Lab Care caging system; Research Equipment, Byran, TX) and fed regular laboratory rat chow ad libitum. The BBd rats developed spontaneous diabetes at 60–90 days of age and weighed 250–350 g. After preliminary experiments established that Wist rats showed no structural change in AOP from 60 to 120 days of age (23), they were age- and weight matched with BBd rats as controls. BBd rats required insulin for survival at the onset of diabetes. They were kept on a daily dose of neutral protamine Hagedorn (NPH) insulin (U-100; Lilly) injected subcutaneously at 4:00 AM. Withdrawal of insulin resulted in an hyperglycemic state (serum glucose level for 24 h) from 100 mg/dl to 300–400 mg/dl. One day before the experiment, rats were fasted overnight and kept solely on drinking water. At 7:00 AM to 6:00 PM in cages equipped with filters (Lab Care caging system; Research Equipment, Byran, TX) and fed regular laboratory rat chow ad libitum. The BBd rats developed spontaneous diabetes at 60–90 days of age and weighed 250–350 g. After preliminary experiments established that Wist rats showed no structural change in AOP from 60 to 120 days of age (23), they were age- and weight matched with BBd rats as controls. BBd rats required insulin for survival at the onset of diabetes. They were kept on a daily dose of neutral protamine Hagedorn (NPH) insulin (U-100; Lilly) injected subcutaneously at 4:00 AM. Withdrawal of insulin resulted in an hyperglycemic state (serum glucose level for 24 h) from 100 mg/dl to 300–400 mg/dl. One day before the experiment, rats were fasted overnight and kept solely on drinking water. At 7:00 AM the following day, rats were either injected intraperitoneally with \[^3H\]leucine or pulse-chased intraluminally with \[^35S\]methionine (see Intraluminal labeling of proximal jejunum of rats). During the experiment whole blood glucose was assayed by a glucose analyzer (Boehringer Mannheim, Indianapolis, IN), and urinary and blood ketone and glucose were checked by the Ketostix and Dipstick methods (Roche Diagnostics, respectively). Serum was kept at −20°C for later determination of \(\beta\)-hydroxybutyrate, a ketoacidosis marker (33), and glucose (Beckman Glucose Analyzer-2).

Human tissues. Jejunal segments (25–60 g) of normal and type 2 diabetic African-American men and women were obtained during surgery or immediately after death from the National Disease Research Interchange (Philadelphia, PA). After removal, tissues were quickly frozen and kept at −70°C for 2–20 mo. The ages of the donors varied between 50 and 80 yr.

Intraluminal labeling of proximal jejunum of rats. A proximal jejunal segment (15- to 20-cm length) prepared in rats anesthetized with pentobarbital sodium as described previously (1) was preperfused for 1 h with 0.9% NaCl (37°C) to remove pancreatic proteases from the lumen. It was then pulsed with 2 mCi of \(^{3}[^{35}S\text{]}\)methionine (1,200 Ci/mmol; Amersham) for 5 min and chased with 1 mM \(^{3}[^{35}S\text{]}\)methionine for 15–180 min. After its removal, the jejunal segment was flushed with 0.9% NaCl and 1 mM dithiothreitol (4°C) and the mucosa was scraped off with a glass microscopic slide and homogenized in 10 mg/l of buffer A (5 mM histidine, pH 7.4 with imidazole, 5 mM EDTA) including protease inhibitors (chymostatin, antipain hydrochloride, leupeptin, and ovomucoid trypsin inhibitor, final concentration 25 \(\mu\)g/ml; Sigma, St. Louis, MO). The homogenate was then subjected to serial differential centrifugation, and the organelles were separated on a final 25–60% sorbitol gradient as described previously (1) with sucrase as a specific marker for brush border and \(\alpha\)-mannosidase II for the Golgi membranes (24). The ER and Golgi membranes comigrated on this gradient and were pooled together as ER-Golgi fractions (ERG) (1).

Immunoprecipitation and electrophoresis. ER-Golgi and brush-border fractions were solubilized in 0.5% Triton X-100 and assayed for AOP activity as described previously (15). AOP was then specifically immunoprecipitated with monoclonal anti-AOP antiserum (1), washed, heated at 100°C for 5 min in 200 \(\mu\)l of SDS buffer (63 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10 mM dithiothreitol, and 1 mM EDTA), and analyzed by 7% SDS-electrophoresis and autoradiography (Kodak X-OMAT films at −70°C) as described previously (2). The ratio of the distance from the origin to the AOP band relative to the distance from the origin to the gel front (Rf) was measured independently by four observers, and the molecular mass was then estimated by plots of log Rf vs. molecular mass of Bio-Rad standards. In some experiments, the AOP immune pellet was treated with enzymatic probes before electrophoresis or the bands were excised from the dried gels and the radioactivity quantified after solubilization in Solulyte-Lipofluor-water (1:10:0.2). In other experiments, the autoradiogram was scanned on an imaging densitometer (Eagle Eye II, Stratagene) and the proteins were quantitated with the NIH Image (version 1.60) Macintosh software program.

Treatment by endo-\(\beta\)-N-acetylglucosaminidase H. The AOP immunoprecipitate (see Immunoprecipitation and electrophoresis) was taken up and boiled in 100 \(\mu\)l of 150 mM sodium citrate (pH 5.5), 2% SDS for 2 min, and 1 \(\mu\)l of endo-\(\beta\)-N-acetylglucosaminidase H (Endo H; Boehringer Mannheim) per 75 \(\mu\)l of AOP was then added to the supernatant (15,000 g, 5 min) as described previously (10). Briefly, Endo H (10 \(\mu\)U) was added to the recovered supernatant, and the digestion mixture was placed at 37°C for 1 h with a thin overlay of toluene. Control samples were treated identically except that incubation was in buffer without Endo H. After addition of albumin (15 \(\mu\)l of 10 mg/ml) to enhance recovery of the protein pellet, the Endo H-treated samples were precipitated with 10% trichloroacetic acid, washed with cold acetone (−20°C), and analyzed by SDS-PAGE.

Treatment of AOP by N-glycosidase F. The AOP immunoprecipitate was washed and taken up in 100 \(\mu\)l of 0.2 M sodium phosphate buffer (pH 8.6), 2% SDS, 2% mercaptoethanol at 100°C for 2 min. The supernatant (15,000 g, 5 min) was then collected and treated with N-glycosidase F (PNGase F; Boehringer Mannheim) as described previously (10). Briefly, the SDS supernatant was brought up to 900 \(\mu\)l with the sodium phosphate buffer, 1% NP-40, and 0.5% mercaptoethanol. Five units of PNGase F in 100 \(\mu\)l of the same buffer were then added, and the mixture was covered with a thin overlay of toluene at 37°C for 18 h. The reaction was stopped by placing the tubes on ice. Albumin (150 \(\mu\)g) was added as the protein carrier, and trichloroacetic acid (final concentration 10%) was added to precipitate AOP protein. The mixture was then rotated on a wheel at 4°C for 1–2 h. The protein pellet (15,000 g, 5 min) was washed with acetone (−20°C), taken up in 200 \(\mu\)l of SDS buffer, and applied to 7% SDS-acrylamide gel electrophoresis as described above.

RESULTS

Altered N-linked chains in brush-border AOP. Wist and BBd rats were injected intraperitoneally with 4 mCi of \(^{3}[^{3}H\text{]}\)leucine and killed 4 h later. Brush-border AOP was solubilized, immunoprecipitated, and incubated with either Endo H or PNGase F or in buffer alone without either enzymatic probe before electrophoresis (Fig. 1). Untreated AOP from BBd migrated as a 135-kDa structure, 5 kDa smaller than normal (140
traluminally into a jejunal segment for 5 min and
Fig. 1. Acute alteration of N-linked carbohydrate chains of brush-
tissue) of brush-border AOP in the BBd rat (Fig. 1)
AOP (100 mU) was immunoprecipitated from Triton-solubilized jejunal brush-bor-
membranes and treated with either endo-β-N-acetylglu-
cosaminidase (Endo H) or glycopeptidase F (PNGase F) before elec-
trophoresis and autoradiography. Lanes from the same gel were
aligned to allow direct comparison. Masses were determined as
described in MATERIALS AND METHODS. Note the faster migration of the
AOP for BBd (Mr, 135 kDa) than Wist (Mr, 140 kDa) rats and its
partial Endo H sensitivity (BBd Endo H+ lane; arrowheads denote
doublet). The residual mass of diabetic AOP after PNGase F was not
changed from normal (PNGase F+ lane). The decreased density of
bands in the Endo H and PNGase F lanes was caused by aggregative
loss of a portion of the sample during glycosidase treatment.
kDa) (Fig. 1; Rf of 0.388 in Wist vs. 0.407 in BBd). AOP
from normal rats displayed no significant mass change when treated with Endo H (Fig. 1), indicating essentially
complete processing of N-linked carbohydrates to
complex structures during maturation of the precursor
in the Golgi membranes. In contrast, Endo H treat-
ment of AOP from the brush-border membranes (BBM)
of BBd rats produced a heterogeneous band ranging
between 130 and 135 kDa (Fig. 1), suggesting persis-
tence of some Endo H-sensitive high-mannose glycans
or hybrid N-linked chains in brush-border AOP of
the BBd rat. Removal of N-linked carbohydrates by
PNGase F produced a single broad band that migrated
as a similar structure of ~120 kDa in both Wist and
BBd rats (Fig. 1; Rf of 0.455 in Wist and 0.459 in BBd
rats), suggesting that there was no additional post-
translational structural alteration of AOP in the BBd
rat such as modification of its O-linked chains in the
Golgi compartment.
Normal protein synthesis and glycosylation with
high-mannose N-linked carbohydrates of AOP in the
BBd rat. The finding of altered N-linked glycosylation
(with no accompanying alteration in O-linked structures)
of brush-border AOP in the BBd rat (Fig. 1) prompted the analysis of the intracellular synthesis and
processing of the AOP precursor. For this purpose,
rats were administered 2 mCi of [35S]methionine in-
traluminally into a jejunal segment for 5 min and
chased with 1 mM methionine for 30 and 60 min
(normal) and 60 and 90 min (diabetic) to achieve max-
imal incorporation into the ERG (see MATERIALS AND
METHODS). AOP (40–55 mU) from solubilized ERG was
recovered, treated with Endo H, and analyzed by elec-
trophoresis and autoradiography (Fig. 2). The AOPi
was indistinguishable in normal and diabetic BBd rats
[Fig. 2A, Endo H− lane at 30 min (normal) vs. Fig. 2B,
Endo H− lane at 60 min (diabetic)]. Similarly, removal
of high-mannose N-linked chains by Endo H produced
AOP for BBd and Wist rats (125 kDa), and removal of N-linked
chains by PNGase F yielded an apoprotein product [AOPi(e)]
that displayed an identical pattern for both rat groups [Fig.
2A, Endo H+ lane at 30 min (normal) vs. Fig. 2B,
Endo H+ lane at 60 min (diabetic)]. The Rf of AOPi and
AOPi(e) were indistinguishable in normal and diabetic
rats and amounted to 0.422–0.425 and 0.451–0.454,
respectively, when AOP extracted from ER-Golgi frac-
tions after 15 min of chase was applied on the same gel
(data not shown). This suggested that protein synthesis
and initial cotranslational assembly of high-mannose
carbohydrates proceeded normally in the BBd rat.
Moreover, Endo H had only an equivocal effect on the
migration of AOPi in the Wist rat (Fig. 2A; Rf of Endo
H− vs. Endo H+ after 30 and 60 min of chase), reflect-
ning complete maturation of N-linked chains to the
complex type in the Wist rat. In contrast, there was an
appreciable reduction in mass produced by Endo H
treatment of AOPm in the diabetic rat [AOPm(e), Fig.
2B]. These data suggest that, in contrast to the normal
species that attained almost complete maturation of its
N-linked chains, AOPm retained some high-mannose
or hybrid N-linked chains in the ERG of the BBd rat.
Because AOP at the BBM retained its Endo H sensi-
tivity (Fig. 1), it appears that complete maturation of the
N-linked chains is not necessary for proper assem-
ly at the surface membrane.
Altered intracellular maturation of N-linked carbo-
hydrate chains of AOP in the BBd rat. Wist and BBd
rats were pulse-chased for 15, 60, and 150 min as
described in MATERIALS AND METHODS. [35S]methionine-
labeled AOP was then isolated from ERG and BBM
fractions, treated with PNGase F and analyzed by SDS-electrophoresis and autoradiography (Fig. 3).
AOPi at 15 min appeared initially in the ERG as a
major band in both normal and diabetic rats (Fig. 3)
and was subsequently converted to a larger mature
AOPi species that was eventually transported to the
BBM in both diabetic and normal animals (Fig. 3).
Removal of N-linked chains of AOPi at 15 min of chase
by PNGase F yielded an apoprotein product [AOPi(e)]
indistinguishable in the normal and diabetic rat (Fig.
3), supporting our observation of normal protein syn-
thesis and initial carbohydrate assembly in the ER of
the diabetic rat (Fig. 2). Determining the molecular
masses of AOPi and AOPm by log-linear plots of Rf vs.
mass before and after removal of N-linked high-man-
noise and complex N-linked chains by PNGase F (Fig.
3), as represented in Table 1, revealed that the change
in AOPm mass is caused by alteration of N-linked
chains during maturation. For instance, there was no
detectable difference in the mass of AOPi for the BBd
and Wist rats (125 kDa), and removal of N-linked
chains by PNGase F produced an apparently identical apoprotein product of ~110 kDa in each case (Table 1). Thus the mass of high-mannose N-linked chains of AOP_i appeared to be indistinguishable in the BB_d and Wist rats and amounted to ~15 kDa (Table 1). However, the conversion of AOP_i to AOP_m involved the addition of ~15 kDa for the Wist rat (Table 1; 125 → 140 kDa) and only 10 kDa of mass for the BB_d rat (Table 1; 125 → 135 kDa) during the maturation of the precursor in the Golgi membranes. Removal of N-linked carbohydrates by PNGase F from AOP_m yielded a similar residual mass (120 kDa) in both Wist and BB_d rats [AOP_m(pf); Fig. 3 and Table 1]. Because PNGase F removes both high-mannose and complex N-linked chains, the decrease in the apparent molecular mass of AOP_m after PNGase F treatment from 140 to 120 kDa in the Wist rat and from 135 to 120 kDa in the BB_d rat accounts for a total mass of ~20 kDa for N-linked chains in normal and ~15 kDa in BB_d rats (Table 1). The apparent mass of O-linked chains, added during the conversion of AOP_i to AOP_m in the Golgi compartment, can be estimated from the difference between AOP_m(pf) (N-linked chains removed; putative O-linked chains attached) and AOP_i(pf) (the completely deglycosylated apoprotein). Notably, this mass difference in the molecular mass of AOP_i(pf) and AOP_m(pf) is ~10 kDa in both Wist and BB_d rats; hence the mass of their O-linked chains (possibly also including other post-translationally added structures) appears to be indistinguishable from normal in congenital diabetes (Table 1). These data confirm those of Fig. 1.

Delayed maturation of AOP_i in the ERG in vivo. As noted above, the 15-min chase experiments suggested normal synthesis and assembly of the AOP_i precursor in the ER of the diabetic animal (Fig. 3B vs. Fig. 3A). AOP_i eventually undergoes conversion to AOP_m in both normal and diabetic rats, as indicated by the 150-min chase experiment (Fig. 3). Thus it appears that the kinetics of AOP_i appearance were indistinguishable from normal in the BB_d rat (Figs. 2 and 3). However, there appeared to be a possible variation in the processing rate of AOP_i among the normal and diabetic rat groups. For instance, as indicated in Figs. 2A and 3A, conversion of AOP_i to AOP_m began in the Wist rat by 15 min and was almost complete by 60 min. In the BB_d rat, however, AOP_m was essentially undetectable by 30 min of chase (not shown), and an appreciable amount of AOP_i was still relatively detected at 60 min of chase (Fig. 2B). Moreover, Fig. 3 indicates that AOP_i persisted in the ERG fractions of the diabetic rat even after 150 min of chase, at which point it essentially disappeared from the ERG fractions of the normal animal. This suggests a longer half-life of AOP_i in the diabetic animal. Moreover, scanning of the autoradio-
gram in Fig. 3 and calculating the AOP\textsubscript{m}to-AOP\textsubscript{i} ratio as an index of maturation revealed a lower maturation level of AOP precursor in the diabetic rat compared with its normal counterpart (AOP\textsubscript{m}:AOP\textsubscript{i} in diabetic vs. normal: 0.00 vs. 0.16 at 15 min, 8.38 vs. 12.21 at 60 min, and 8.43 vs. 49.34 at 150 min of chase). Thus it appears that the conversion of AOP\textsubscript{i} to AOP\textsubscript{m} in ER-Golgi membranes is delayed in the BB\textsubscript{d} animal.

To analyze further the apparent delayed conversion of AOP\textsubscript{i} to AOP\textsubscript{m} in the BB\textsubscript{d} rat, we pulse-chased rats for 30–180 min with methionine (see MATERIALS AND METHODS). AOP was then isolated from ERG and analyzed by SDS-PAGE and autoradiography. AOP\textsubscript{i} and AOP\textsubscript{m} were excised from the dried gels (Fig. 4), and the radioactivity ratio of AOP\textsubscript{m} to AOP\textsubscript{i} was calculated (Fig. 4C). Because AOP\textsubscript{i} is converted stoichiometrically to AOP\textsubscript{m} in intact normal rats (2), we expressed the conversion of AOP\textsubscript{i} to AOP\textsubscript{m} by estimating the 50% conversion (AOP\textsubscript{m}:to-AOP\textsubscript{i} radioactivity ratio = 1:1) as an internal control to correct for any variation in label incorporation among animals. Notably, there was a lower AOP\textsubscript{m}:AOP\textsubscript{i} in BB\textsubscript{d} rats than in Wist rats at all periods of chase (Fig. 4C). The 1:1 ratio of AOP\textsubscript{m} to AOP\textsubscript{i} occurred at ~30 min of chase in the Wist rat but was not achieved until 60–90 min in the diabetic BB\textsubscript{d} rat (Fig. 4). As maturation proceeded, AOP\textsubscript{m} became the predominant species for both normal and diabetic rats, but even at 180 min of chase, the AOP\textsubscript{m}:AOP\textsubscript{i} for the diabetic animal achieved one-half the value of that of the normal animal (3.3 vs. 6.8, Fig. 4C). Statistical analysis by the t-test revealed that these differences between the animal groups were significant ($P < 0.05$).

Also, a plot of the maturational AOP\textsubscript{m}:AOP\textsubscript{i} vs. the chase time produced linear regression curves (not shown, $R^2 = 0.936$ for Wist rats and 0.829 for BB\textsubscript{d} rats) with significantly different slopes (0.019 for Wist rats and 0.004 for BB\textsubscript{d} rats; $P < 0.01$); this verified the delayed maturation of AOP in the diabetic animal. The reduced AOP\textsubscript{m}:AOP\textsubscript{i} at each time period in BB\textsubscript{d} rats is consistent with the relative persistence of AOP\textsubscript{i}, caused by a slower processing of the high mannose, a delay in exit from the ER, or both in the BB\textsubscript{d} rat.
The data presented above suggested that maximal assembly of AOPm in Golgi is achieved at 60 min in Wist rats and 120–150 min in BBd rats (Figs. 3 and 4). Because the time required for maximal maturation to AOPm in the ER-Golgi is prolonged in the diabetic rat and involves assembly of altered N-linked carbohydrate chains, it is reasonable to predict that the subsequent exit of AOPm from the Golgi might be altered in diabetes. To test this hypothesis, [35S]methionine-labeled AOP from Triton-solubilized ER-Golgi was immunoprecipitated and analyzed on SDS-PAGE and AOPm was excised from the gel and counted. Total radioactivity incorporated in AOP was determined from the total yield of ER-Golgi specific enzyme marker (α-mannosidase II). Logarithmic-linear plots of decay (decline) after maximum incorporation in ER-Golgi as a function of chase time were used to determine the half-life ($t_{1/2}$, in min) of AOPm in the Golgi fractions. On the basis of two repeated paired experiments carried out 30 days apart, the rate constant ($K_i$, in min$^{-1}$) of the exit of AOPm from Golgi was found to be nearly 2.5-fold greater in BBd rats than in Wist rats (0.077 min$^{-1}$ on day 1 and 0.073 min$^{-1}$ on day 2 in BBd vs. 0.029 min$^{-1}$ on both days in Wist), indicating an ~2.5-fold shorter residence time of AOPm in the Golgi of BBd rats than in Wist rats ($t_{1/2}$ in Golgi: 9.0–9.5 min in BBd vs. ~24 min in Wist). Because of the unavailability of a sufficient number of spontaneously diabetic rats, we did not carry out more than two sets of this experiment. However, because the results of these two sets agreed within 10%, we believe that our observations fairly suggest that the residence time of newly synthesized AOPm in Golgi is shorter in the diabetic animal than in its normal counterpart.

Abnormal jejunal AOP in type 2 diabetes. Jejunal AOP was immunoprecipitated from detergent-solubilized BBM of normal and diabetic female subjects and analyzed by 7% acrylamide electrophoresis (Fig. 5). AOP from diabetic subjects migrated as a 5-kDa smaller structure than normal (135 vs. 140 kDa). Similar observations were made in male subjects (data not shown).

### DISCUSSION

Similar to our previous studies in the congenitally diabetic BBd rat (23), we have observed in the current studies that the brush-border pool of AOP in humans with type 2 diabetes is smaller (~5 kDa) than normal. Thus it appears that abnormal AOP structure is common in diabetes.

Furthermore, chemical removal of all carbohydrate chains by trifluoromethanesulfonic acid suggested that the reduced mass of the intact AOP glycoprotein in the

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#### Table 1. Comparison of N- and O-linked chains of AOP precursor

The ratios of origin-to-aminoaligopeptidase (AOP) band distance to origin-to gel front distance ($R_f$) of immature and mature AOP precursors (AOPi and AOPm, respectively) and glycopeptidase F-sensitive AOP and AOPm were measured to the center of the bands in Fig. 3A and B, and the molecular masses were estimated independently from log-linear plots by 4 independent observers. The mass attributed to N-linked chains (CHOn) and the mass of O-linked chains (CHOo) were calculated as described in RESULTS.

Fig. 4. Kinetics of intracellular synthesis and assembly of AOP. Jejunal loops of normal (A) and diabetic BBd (B) Wistar rats were pulse-chased for 30, 60, 90, 120, and 180 min. [35S]methionine-labeled AOP from Triton-solubilized ERG was immunoprecipitated and analyzed by electrophoresis and autoradiography. C: [35S]methionine-labeled AOP and AOPm bands were excised from gels shown in A and B, and the ratio of AOPm to AOP, was calculated as a measure of AOP maturation.
BB_d rat appears to involve structural changes in its carbohydrate structure in diabetes (23). Because this alteration was also present in the newly synthesized AOP within hours after administration of a radiolabeled precursor, structural alterations either during intracellular synthesis and assembly or shortly after insertion into the BBM were suggested. Nonenzymatic glycosylation (21) that occurs slowly over days to weeks as a function of the elevated extracellular glucose concentrations in diabetes seemed an unlikely mechanism because of the rapid nature of the mass change (only 3 days after the onset of diabetes) and the lack of correlation with plasma glucose levels (23).

Change in AOP_m mass is caused by alteration of its N-linked chains. In this report, we have examined the intracellular processing of the AOP precursor in intact rats as it is synthesized and assembled in association with intracellular membranes and then inserted into the brush-border surface membrane of the enterocyte. The apoprotein molecular mass, estimated after deglycosylation of AOP_1, with Endo H, was the same in BB_d and Wist rats. This suggests that protein synthesis and cotranslational assembly of high-mannose N-linked chains of AOP_1 proceeded rapidly and normally in the ER-Golgi of the BB_d rat. Although AOPi in the BB_d rat initially appeared rapidly (15 min) and displayed a normal mass on SDS gels, its subsequent conversion to AOP_m (1:1 labeling ratio of AOPi:AOP_m) was delayed by approximately two- to threefold (Fig. 4C; 60–90 min in BB_d vs. 30 min in Wist). The retardation of AOP maturation in the BB_d rat may involve several mechanisms. Among these are

BB_d rat may involve several mechanisms. Among these are
1) altered activity of glycosidases, mannosidases, and transferases responsible for the modification to complex N-linked chains, 2) changes in the concentration of glycosyl substrates, 3) alternative routing by possibly bypassing the Golgi apparatus, and 4) prolonged residence time in ER. Whether the apparent alterations in N-linked carbohydrate composition of AOP in congenital diabetes are a consequence of changes in glycosyl substrates or altered processing enzymes (glycosidases or glycotransferases) necessary for the processing of AOP carbohydrates awaits detailed structural analysis of these carbohydrate chains and of the enzymes and substrates required for N- and O-linked glycan assembly. Whether some AOP, bypass the Golgi compartment in diabetes on their exit from the ER is at the moment unknown, but this is possible based on the significant role of N-linked carbohydrates in the proper compartmentalization and intracellular routing of glycoproteins (8). Moreover, a default pathway for the transport of membrane proteins from ER to Golgi has been described (25). For instance, some proteins may return to the ER after exiting the Golgi by a yet-unknown mechanism. How much of these proteins may shuttle back to the Golgi is not clear. Prolonged residence time in ER of aquaporin-2 has been reported to occur in nephrogenic diabetes insipidus (30). Moreover, site-directed mutagenesis of some N-glycosylation sites in the α-chain of the insulin receptor led to accumulation of the receptor precursor in ER (4, 6). Thus accumulation of AOP precursor in ER might occur in congenital diabetes, perhaps among other factors, as a result of inefficient removal of the three nonreducing terminal glucose residues by α-glucosidase I or II, a step that may be a prerequisite for glycoprotein transport from the ER to Golgi (26). Because the ER and Golgi fractions were of necessity pooled together (ER-Golgi) in our experiments, we cannot distinguish between prolonged residence in the ER and a reduced rate of vesicular mediated transport of the AOP, from the ER to the Golgi compartment. Similar to our observations, Koh et al. (18) reported delayed processing of the insulin proreceptor to mature insulin receptor in hepatocytes derived from STZ-diabetic rats. Thus coordinate changes in the kinetics of intracellular assembly of membrane glycoproteins may constitute a common phenomenon in diabetes.

Maturation and exit through trans-Golgi has been identified as a rate-limiting step in the transport of intestinal hydrolases to the surface membrane of Caco-2 human colon adenocarcinoma cells (29). Although AOP, does eventually manage to be transported to the Golgi of the BB₄d rat, approximately one-third of its N-linked chains never become complex (Figs. 1 and 2B). This may be caused by its shorter residence time in the Golgi (~2.5-fold shorter than normal) and by its rapid exit from this compartment before maturation is completed. The difference in residence time in Golgi between the rat groups may be even greater than it appears, because the slower approach to a maximum of AOPₘ in the Golgi in BB₄d rats produced a relatively prolonged peak, which could mask the true rate of log-linear decline in radioactivity in AOPₘ. Thus the loss of AOPₘ from Golgi may even be more rapid than indicated by the apparent half-life. Incomplete maturation of AOP may be attributed to its shorter residence time in the Golgi of congenitally diabetic rats. The mechanism for this shorter residence time in the Golgi deserves further study.

The discrete changes in AOP structure are the result of modifications in the intracellular trafficking of the precursor followed by transfer to the apical surface membrane of a slightly reduced protein harboring a decrease in the mass of N-linked glycans and retaining some of its immature N-linked carbohydrate chains. This culminates in altered apparent mass of AOP at the apical surface membrane (Fig. 1 and Ref. 23). We (23) previously reported normal AOP activity in this congenital diabetic rat model of type 1 diabetes. Moreover, it appears that the glycosylation defect in AOP is not correlated with significant functional alterations of this peptidase in congenital diabetes. Despite the putative importance of carbohydrate chains for orderly intracellular assembly, molecular folding, protection from intracellular degradation, and transfer to the cell surface, the exact role for N-linked carbohydrate side chains of AOP has not been as well studied as other glycoproteins with structural changes in diabetes. For instance, altered insulin signaling and function has been attributed to abnormal carbohydrate assembly of the insulin receptor (4, 6). Thus it seems likely that the modified membrane glycoprotein structure and assembly is a common effect of the metabolic changes in diabetes. Because only few glycoproteins have been examined, additional information about the structural alterations of other membrane glycoproteins and their functional correlation may enhance our understanding of the underlying metabolic mechanisms leading to altered glycosylation in diabetes.

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