Vesicular integral protein of 36 kDa (VIP36) is an intracellular lectin recognizing high-mannose type glycans and is highly expressed in salivary glands, especially the parotid gland, which secretes α-amylase in large quantities. Here immunoelectron microscopy demonstrated that VIP36 was primarily localized to secretory vesicles in the glandula parotis of the rat, where α-amylase also resided. A secretory vesicle fraction, prepared by Percoll density gradient centrifugation, contained both VIP36 and α-amylase. Moreover, α-amylase that was localized to these secretory vesicles contained high-mannose type glycans. In addition, VIP36 coprecipitated with α-amylase in an endo H treatment-sensitive manner. These results suggest that VIP36 is involved in the secretion of α-amylase in the rat parotid gland.

Key words: α-amylase/high mannose/salivary glands/secretion/VIP36

Introduction

Understanding how newly synthesized glycoproteins are processed through the secretory pathway is of prime importance to our current understanding of biology. Secretory glycoproteins must undergo a complex series of events that results in their correct folding, quality control, and sorting along the secretory pathway (Dodd and Drickamer, 2001; Hauri et al., 2000a). Recently some intracellular lectins have been found to play important roles in these processes. For example, calnexin and calreticulin are known to function as molecular chaperones (Ellgaard and Helenius, 2003; Parodi, 2000), endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC)-53 as a transport cargo receptor (Hauri et al., 2000b; Zhang et al., 2003), and mannose-6-phosphate receptor as a receptor recognizing the marker for lysosomal enzymes (Ghosh et al., 2003).

VIP36 was originally isolated from MDCK cells as a component of detergent-insoluble, glycolipid-enriched complexes containing apical marker proteins and was shown to have a carbohydrate-recognition domain similar to that of ERGIC-53 (Fiedler et al., 1994). In our previous studies, we demonstrated that VIP36 is an intracellular lectin recognizing high-mannose type glycans (Hara-Kuge et al., 1999). Furthermore, we reported that VIP36 is involved in intracellular transport and the secretion of glycoproteins in polarized MDCK cells (Hara-Kuge et al., 2002). However, currently the function(s) of VIP36 in mammalian organs remains unknown. In this study, we demonstrate that VIP36 and α-amylase are colocalized and that they coprecipitate in secretory vesicles of the rat parotid gland, suggesting that VIP36 is involved in α-amylase secretion in this gland.

Results

Colocalization of VIP36 and α-amylase in secretory vesicles of rat parotid gland

Recently, VIP36 was shown to be highly expressed in rat parotid gland (Shimada et al., 2003), which is well known to secrete large quantities of α-amylase. Furthermore, double immunofluorescent staining suggested that VIP36 and α-amylase are both localized in the same compartment of acinar cells (Shimada et al., 2003), implying that VIP36 is involved in the secretion of α-amylase. To determine the precise localization of these proteins, immunoelectron microscopy was performed on rat parotid glands using double-staining methods with polyclonal anti-VIP36 and monoclonal anti-α-amylase antibodies. This indicated that VIP36 was primarily localized to secretory vesicles of the parotid gland, where α-amylase was also expressed (Figure 1). Thus we established that VIP36 and α-amylase were colocalized in secretory vesicles of rat parotid gland.

Isolation of secretory vesicles containing VIP36 and α-amylase

To further investigate VIP36 and α-amylase colocalization in secretory vesicles, we isolated these vesicles from parotid gland homogenates by Percoll density gradient centrifugation (Fujita-Yoshigaki et al., 1996, 1999). Immunoblot analysis of the seven fractions derived from this gradient revealed that both VIP36 and α-amylase resided in the uppermost, lightest fraction (no. 1; Figure 2a and b). α-Amylase but not VIP36 also resided in the lowest and most dense fraction (no. 7; Figure 2a and b). As shown in Figure 3, the distribution of α-amylase enzyme activity in the gradient correlated directly with its expression,
as detected by immunoblotting. Importantly, electron microscopy indicated that both fractions 1 and 7 contained a plethora of condensed vesicles (Figure 4). This suggested that there existed two types of secretory vesicles, one of which contained both VIP36 and α-amylase, and the other that contained α-amylase but not VIP36.

**α-Amylase of fraction 1 but not fraction 7 carries high-mannose type glycan(s)**

VIP36 is an intracellular lectin recognizing high-mannose type glycans. Thus it was of interest to determine whether the α-amylase that colocalized with VIP36 carried high-mannose type glycan(s). The immunoblotting membrane used for the detection of α-amylase in the Percoll density gradient fractions was stripped and subjected to lectin staining with concanavalin A (Con A), which has high affinity for high-mannose type glycans. As shown in Figure 2c, a fraction 1 lower band that migrated to exactly the same position on the membrane as that of the protein recognized by anti-α-amylase, was stained by Con A, and the upper bands were also stained by Con A. The biotin-Con A–positive protein may also bind to VIP36 via high-mannose type glycan, although the molecule was not still identified. Furthermore, purified α-amylase, which was derived from parotid gland homogenates by immunoprecipitation with anti-α-amylase, reacted with both Con A and *Galanthus nivalis*, a plant lectin that more specifically recognizes high-mannose type glycans (data not shown). However, in contrast to fraction 1, within fraction 7 the protein recognized by anti-α-amylase antibody did not react with Con A (Figure 2c). When aliquotes of Percoll fractions 1 and 7 were applied on the DSA, AAL, LCA, TJA-I, MAL, and E4-PHA-Sepharose column chromatographies (Kobata and Yamashita, 1993), α-amylase activities flowed through these lectin-Sepharose columns (data not shown). These results indicated that the α-amylase in fraction 1 carried high-mannose type glycan(s) and that in the fraction 7 seems to be nonglycosylated.

To exclude the possibility that the absence of high-mannose type glycan(s) in fraction 7 reflected a specific localization of endo-β-N-acetylglucosaminidase, we determined the activity of this enzyme in fractions 1 and 7. Although endo-β-N-acetylglucosaminidase activity was detected in both fractions, there was no significant difference between fractions 1 and 7 with respect to this activity (Table I).
Association of VIP36 with α-amylase via high-mannose type glycans

VIP36 is an integral membrane protein and α-amylase is a soluble protein. Thus, to examine whether VIP36 and α-amylase associated each other in fraction 1, a detergent phase separation analysis was performed. In this experiment, membrane and soluble proteins were recovered in the detergent and aqueous phases, respectively, and therefore any α-amylase associated with VIP36 was expected to be present in the detergent phase.

As expected, VIP36 was recovered in the detergent phase (Figure 5). In fraction 1, α-amylase was recovered in both the detergent and aqueous phases (Figure 5). In contrast, α-amylase in fraction 7, which was the fraction lacking VIP36, was recovered only in the aqueous phase (Figure 5). These results implied that α-amylase in the detergent phase of fraction 1 was associated with VIP36. To further examine this association, α-amylase in parotid gland homogenates was immunoprecipitated with

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**Table 1. Activity (dpm) of endo-β-N-acetylglucosaminidase in the rat parotid gland**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Substrate</th>
<th>Activity (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>bi</td>
<td>1729</td>
</tr>
<tr>
<td></td>
<td>M9</td>
<td>2618</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>bi</td>
<td>1820</td>
</tr>
<tr>
<td></td>
<td>M9</td>
<td>2863</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>bi</td>
<td>1639</td>
</tr>
<tr>
<td></td>
<td>M9</td>
<td>1477</td>
</tr>
</tbody>
</table>

Fifty milligrams of parotid homogenate was fractionated by Percoll gradient. The secretory vesicles in fractions 1 and 7 were isolated by centrifugation as described in Materials and methods. One-tenth volume of each sample was suspended in 10 mM HEPES buffer (pH 7.4) and used as an enzyme source. Enzyme activity was determined by measuring radioactivities of released glycans from 40,000 dpm of Gal β GlcNAc2 Man3 GlcNAc2 Asn-N-[^14]C Ac (bi) or Man9 GlcNAc2 Asn-N-[^14]C Ac (M9).
Human parotid α-amylase can be divided into two groups: non-glycosylated and glycosylated, both of which are secreted into the mouth cavity. In this study, we showed the presence of two types of α-amylase; one (fraction 1 in Figure 2) contains high-mannose type glycans, and the other (fraction 7 in Figure 2) contains no or very few glycans. We previously showed the involvement of VIP36 in the secretion of clasterin, which has at least one high-mannose type glycan (Hara-Kuge et al., 2002). These facts indicate that α-amylase in fraction 1 binds to VIP36 and is targeted toward the secretion on the apical surface of parotid cells. On the other hand, how is α-amylase in the fraction 7 secreted? Rat nonglycosylated α-amylase should be secreted into the mouth cavity like that of human. One speculation is that α-amylase in fraction 1 may be deglycosylated by intrinsic endo-β-N-acetylgalcosaminidase and be detached from VIP36, and then be accumulated in other type of vesicles; those in fraction 7 are destined to secrete like as those in fraction 1. It is necessary to assess whether nonglycosylated α-amylase contains a GlcNAc residue, which is a reaction product of the action of endo-β-N-acetylgalcosaminidase.

Rat parotid endo-β-N-acetylgalcosaminidase acts on both high-mannose type glycans and complex-type glycans (as summarized in Table I), whereas human parotid endo-β-N-acetylgalcosaminidase has specific activity for complex-type glycans (Ito et al., 1993). Glycan structures of human glycosylated α-amylase are complex type (Yamashita et al., 1980, 1981), and are recognized by human endo-β-N-acetylgalcosaminidase. On the contrary, in this study, it is clear that rat parotid α-amylase contains high-mannose type glycans. At this time, it is unlikely that VIP36 is involved in the intracellular transport and secretion of human glycosylated-α-amylase. Thus the mechanism of parotid α-amylase secretion could differ substantially between human and rat.

Recently, a VIP36-like protein, VIPL, that displays molecular similarities to VIP36, was identified (Neve et al., 2003; Nufer et al., 2003). The polyclonal anti-VIP36 antibody, which we raised against the luminal domain of VIP36 (Hara-Kuge et al., 1999) and used in this study, also might recognize VIPL, reflecting their very close amino acid homology. Because it has been reported that VIPL localizes to the ER and may affect the recycling of ERGIC-53 (Neve et al., 2003; Nufer et al., 2003), the protein we identified in secretory vesicles using this anti-VIP36 antibody should be VIP36.

Furthermore, there appeared to be two types of secretory vesicle that contained α-amylase, one residing in the lightest Percoll fraction and one in the densest fraction. Both types of vesicle might function in secreting glycoproteins, because α-amylase in each vesicle was enzymatically active. Although the α-amylase of fraction 1 seems to be regulated by VIP36, other functional differences between fraction 1 and fraction 7 secretory vesicles may emerge in the near future.

Discussion

Previously, we demonstrated that VIP36 is an intracellular lectin recognizing high-mannose type glycans (Hara-Kuge et al., 1999) and is involved in secretion of glycoproteins in MDCK cells (Hara-Kuge et al., 2002). However, it had not been determined where VIP36 functions or with which secretory glycoprotein it associates in mammalian tissues. Recently we reported that VIP36 is highly expressed in secretory vesicles of the rat parotid gland, which also contain α-amylase (Shimada et al., 2003). Here we further demonstrated that VIP36 colocalizes with α-amylase in secretory vesicles of the rat parotid gland by double labeling at an immunoelectron microscopic level. Moreover, we succeeded in isolating a subset of secretory vesicles in which VIP36 binds to α-amylase in an endo H–sensitive manner. Although the involvement of VIP36 in α-amylase secretion may have been expected by virtue of its lectin activity, this study provides the first demonstration of this function in a mammalian organ.

Reagents and antibodies

Endo H and biotin–Con A were purchased from New England Biolabs (Beverly, MA) and Funakoshi (Tokyo, Japan). Bovine pancreas α-amylase was purchased from Sigma Chemical Co. (St. Louis, MO), and V8 protease was obtained from Nakarai Tesque (Kyoto, Japan). The polyclonal anti-VIP36 antibody, which we raised against the luminal domain of VIP36 (Hara-Kuge et al., 1999) and used in this study, also might recognize VIPL, reflecting their very close amino acid homology. Because it has been reported that VIPL localizes to the ER and may affect the recycling of ERGIC-53 (Neve et al., 2003; Nufer et al., 2003), the protein we identified in secretory vesicles using this anti-VIP36 antibody should be VIP36.

Fig. 6. Binding of VIP36 to α-amylase in the parotid gland homogenate. α-Amylase was subjected to immunoprecipitation with an anti-α-amylase antibody and detected by Con A staining (a). The precipitated proteins were analyzed by immunoblotting using anti-VIP36 (b). Endo H was added before (lane 3) or after (lane 4) immunoprecipitation.

Materials and methods

Reagents and antibodies

Endo H and biotin–Con A were purchased from New England Biolabs (Beverly, MA) and Funakoshi (Tokyo,

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Japan), respectively. The EnzChek α-amylase assay kit was purchased from Molecular Probes (Eugene, OR).

Biotin–**Galanthus nivalis** lectin (Sigma-Aldrich, St. Louis, MO) was prepared by biotinylation using an enhanced chemiluminescence protein biotinylation module (Amersham Bioscience, Little Chalfont, U.K.), according to the manufacturer’s instructions.

Anti-VIP36 antibody was prepared as described previously (Hara-Kuge et al., 2002). Anti-α-amylase goat polyclonal IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-α-amylase monoclonal IgG (for immunoelectron microscopy) from QED Bioscience (San Diego, CA).

**Immunoelectron microscopy**

Young male Wistar rats weighing 80–100 g were anesthetized with pentobarbital and fixed by perfusing 5% acrolein in 0.15 M phosphate buffer (PB), pH 7.4, though the aorta at 37°C. The parotid glands were removed and cut into small pieces, which were washed well with PB and immersed in a fixative composed of 2% paraformaldehyde, 0.125% glutaraldehyde, and 0.1% picric acid in PB overnight at 4°C. The tissues were postfixed in 1% osmium tetroxide and 7% sucrose in PB for 2 h at 4°C; dehydrated with a graded ethanol series at 0°C; embedded in Lowicryl K4M; and cured under UV light for 3 days at –35°C, as described previously (Shimada et al., 2003). Ultrathin sections were treated with 3% hydrogen peroxide at room temperature for 10 min, washed three times with water, and then treated with 0.1% ammonium chloride for 10 min. After washing well with water, the ultrathin sections were incubated with 10% normal goat serum in phosphate buffered saline (PBS) for 1 h and then incubated with anti-VIP36 immunoglobulin adequately diluted with 100 mM PBS, pH 7.4, containing 1% bovine serum albumin for 24 h at 4°C. After rinsing well with PBS, the sections were incubated for 1 h with 10 nm colloidal gold-labeled anti-rabbit IgG (1:200) at room temperature, followed by rinsing with PBS and then water. For double-labeling analysis, the reverse side of the sections labeled with anti-VIP36 antibody was incubated with anti-α-amylase antibody as described. The sections were rinsed well and then incubated for 1 h with 15 nm colloidal gold-label anti-mouse IgG (1:200) at room temperature, followed by rinsing with PBS and then water. Finally, the sections were examined under an electron microscope (Hitachi H-7500).

**Isolation of secretory vesicles**

Secretory granules of rat parotid glands were isolated by Percoll density gradient centrifugation as described by Fujita-Yoshigaki et al. (1996, 1999). Briefly, parotid glands were minced in ice-cold homogenizing buffer composed of 250 mM sucrose, 20 mM HEPES (pH 7.2), 1 mM ethylene glycol bis(2-aminoethyl ether)-tetra acetic acid, 0.1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride and dispersed by eight strokes in a glass homogenizer. The homogenate was centrifuged at 1,500 × g, for 10 min. The pellet was suspended in the homogenizing buffer containing 40% Percoll (Amersham Bioscience) and centrifuged at 20,000 × g for 20 min. When the sample was collected in seven fractions, secretory granules were recovered in both the lightest and the most dense fraction.

**Immunoblotting and immunoprecipitation**

Rat parotid gland homogenates (25 µg protein) or aliquots (50 µl) of each Percoll fraction (diluted with 10 volumes of homogenizing buffer and centrifuged at 1500 × g for 10 min) were separated on 12% or 15% polyacrylamide gels and transferred to nitrocellulose membranes. Proteins were detected by incubation with anti-VIP36 antibody (1:200), anti-α-amylase antibody (1:2000) or biotin-conjugated lectin (1:1000) followed by a horseradish peroxidase–conjugated secondary antibody or horseradish peroxidase–conjugated streptavidin and then visualized by enhanced chemiluminescence (Amersham Bioscience), according to the manufacturer’s instructions.

For immunoprecipitation, the homogenate was incubated for 1 h at 4°C with the relevant antibody. Protein A–Sepharose was added to the incubation mixture, and incubation was continued for 1 h at 4°C. After washing the Sepharose beads with homogenizing buffer, the proteins bound to the beads were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting.

**Endo H assay**

The homogenates and isolated secretory vesicles were suspended in 10 mM HEPES/NaOH (pH 7.2) to a concentration of 0.26 µg/µl protein and used as an enzyme source. The enzyme was assayed using 1.9 × 10³ dpm/µg each of biantennary glycan (Gal₃. GlcNAc₂. Man₃. GlcNAc₂. Asn-N-[¹⁴C]Ac) or high mannose-type glycan (Man₉. GlcNAc₂. Asn-N-[¹⁴C]Ac) as substrate. The substrate (40,000 dpm) was mixed with enzyme solution in 50 mM HEPES/NaOH (pH 7.2), 0.1% (w/v) Triton X-100, and 10 mM CaCl₂. The reaction mixture was incubated at 37°C overnight and subjected to paper chromatography (pyridine:ethyl acetate:acetic acid:H₂O 5:5:1:3). The enzyme activity was measured by counting radioactivities of segregated glycans.

**Phase partitioning with Triton X-114**

The procedure used was as described previously with minor modifications (Brusca and Radolf, 1994). The isolated secretory vesicles were suspended in 1 ml ice-cold TNE/TX114 (25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM ethylenediamine tetra-acetic acid, 1% Triton X-114, 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein U/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) and incubated on ice for 30 min. The incubation mixture was warmed to 37°C to solubilize membrane proteins. After cooling the Triton X-114 extract, residual insoluble material was removed by centrifugation. The resulting supernatants were partitioned into detergent and aqueous phases by temperature-induced phase separation. The detergent phases were reextracted with 10 volumes of TNE.

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Abbreviations

Con A, concanavalin A; endo H, endo-β-N-acetylglucosaminidase H; ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment; PB, phosphate buffer; PBS, phosphate-buffered saline.

References


