Sugar-binding Properties of VIP36, an Intracellular Animal Lectin Operating as a Cargo Receptor*

Received for publication, May 26, 2005, and in revised form, August 25, 2005 Published, JBC Papers in Press, August 29, 2005, DOI 10.1074/jbc.M505757200

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The vesicular integral protein of 36 kDa (VIP36) is an intracellular animal lectin that acts as a putative cargo receptor, which recycles between the Golgi and the endoplasmic reticulum. Although it is known that VIP36 interacts with glycoproteins carrying high mannose-type oligosaccharides, detailed analyses of the sugar-binding specificity that discriminates isomeric oligosaccharide structures have not yet been performed. In the present study, we have analyzed, using the frontal affinity chromatography (FAC) method, the sugar-binding properties of a recombinant carbohydrate recognition domain of VIP36 (VIP36-CRD). For this purpose, a pyridylaminated sugar library, consisting of 21 kinds of oligosaccharides, including isomeric structures, was prepared and subjected to FAC analyses. The FAC data have shown that glucosylation and trimming of the D1 mannosyl branch interfere with the binding of VIP36-CRD. VIP36-CRD exhibits a bell-shaped pH dependence of sugar binding with an optimal pH value of ~6.5. By inspection of the specificity and optimal pH value of the sugar binding of VIP36 and its subcellular localization, together with the organellar pH, we suggest that VIP36 binds glycoproteins that retain the intact D1 mannosyl branch in the cis-Golgi network and recycles to the endoplasmic reticulum where, due to higher pH, it releases its cargos, thereby contributing to the quality control of glycoproteins.

N-linked oligosaccharides contribute to the folding, transport, and degradation of glycoproteins via interactions with a variety of intracellular lectins (1–4). The processing of the N-linked oligosaccharides is initiated in the endoplasmic reticulum (ER) by the removal of the glucose residues from the Glc3Man9GlcNAc2 oligosaccharide structure. Mannose trimming is subsequently initiated in the ER and continues in the Golgi complex by a series of mannosidases prior to the branching and extension of the oligosaccharides by Golgi glycosyltransferases (5–7). Calnexin and calreticulin, which are ER chaperones with lectin activities, specifically bind monoglucosylated high mannose-type oligosaccharides, i.e. Glc1Man9GlcNAc2, expressed on unfolded proteins and assist their folding (8–10). Degradation of glycoproteins is governed by the trimming of the middle branch of their carbohydrate moieties by ER mannosidase I and by association with ER degradation-enhancing α-mannosidase-like protein (EDEM) (11–13).

Secretory and membrane glycoproteins from ER-resident proteins that are correctly folded and sorted leave the ER and are exported to the Golgi complex via the ER-Golgi intermediate compartment (ERGIC). ERGIC-53, which is a 53-kDa type I transmembrane protein used as a popular marker for the ERGIC, constitutively cycles between the ER and the ERGIC, serving as a cargo receptor for some glycoproteins (14). The crystal structure of the carbohydrate recognition domain (CRD) of p58, the rat homologue of human ERGIC-53, revealed a striking structural similarity to Ca2+-dependent leguminous lectins and calnexin (15, 16).

Cargo glycoproteins are further sorted in the Golgi, where the vesicular integral protein of 36 kDa (VIP36), the type I membrane glycoprotein with a CRD similar to that of ERGIC-53, is thought to act as a cargo receptor for quality control (17, 18). Although VIP36 is highly localized in the cis-Golgi network, the carbohydrate moieties of VIP36 are modified by Golgi enzymes, suggesting that this glycoprotein recycles between the Golgi and the ER (19).

To gain deeper insight into the mechanisms underlying the molecular actions of VIP36 and ERGIC-53, it is essential to reveal the sugar-binding properties of these cargo receptors in detail. Although both of the cargo receptors interact with glycoproteins carrying high mannose-type oligosaccharides (20–23), detailed analyses of the sugar-binding specificity that discriminates isomeric oligosaccharide structures have not yet been performed. Although ERGIC-53 has been shown to bind glycoproteins in a Ca2+- and pH-dependent manner (24), a Ca2+ requirement for the sugar binding of VIP36 remains controversial (25, 26).

In the present study, we analyzed the sugar-binding properties of a recombinant CRD domain of VIP36 (VIP36-CRD) by the frontal affinity chromatography (FAC) method, because this method is suitable for analyzing weak interactions (27–29). For FAC analyses, we prepared a pyridylaminated (PA) sugar library consisting of 21 kinds of oligosaccharides, including isomeric structures. On inspection of the FAC data, the mannose residues predominantly involved in association with VIP36-CRD had been identified in the high mannose-type oligosacchar-
ride structures. The sugar branch specificity of VIP36 thus revealed a basis for understanding the molecular function of this cargo receptor in transport of glycoproteins.

**EXPERIMENTAL PROCEDURES**

**PA Sugar Library—**000.1 (the code numbers and structures of PA oligosaccharides are recorded in the GALAXY web site at www.glycoanalysis.info/ENG/index.html (30) and/or described in the literature (31)), M2.1, M2.2, M4.7, M5.1, M6.1, M6.10, M7.1, M7.2, M8.1, M8.2, and M8.4 were purchased from Takara Bio, Inc. Other high mannose-type oligosaccharides were derived from IgY. IgY was isolated from egg yolk using the Eggcellent™ chicken IgY purification kit (Pierce) and then subjected to MALDI-TOF-MS analyses, contained two digestion products, separated on Amide and octadecyl silica columns at 37 °C overnight. Oligosaccharides were released with PNGase F (New England Biolabs) in 50 mM Tris-HCl, pH 8.0, at 37 °C overnight. Oligosaccharides were digested with 2 milliunits of M8.1 and M8.2, respectively, which had already been recorded in the GALAXY web site at www.glycoanalysis.info/ENG/index.html (30) and/or described in the literature (31). M9.1 and M9.2 were successfully isolated from IgY as PA derivatives. To prepare the analogues of the monoglucosylated high mannose-type oligosaccharides, 1 nmol of GlcMan\(_3\)GlcNAc\(_2\)-PA (M9.2) was digested with 2 milliunits of \(\alpha\)-mannosidase from jack bean (Seikagaku Kogyo Co.) in 40 \(\mu\)l of 0.1 M acetate buffer, pH 5.0, containing 10 mM \(\text{ZnSO}_4\), at 37 °C for 4 h. The digestion products, separated on Amide and octadecyl silica columns and then subjected to MALDI-TOF-MS analyses, contained two isoforms of GlcMan\(_3\)GlcNAc\(_2\)-PA, which had not been recorded in the GALAXY data base and are designated as M8.3 and M8.5 (31). Glucosidase II was purified from rat liver microsomes as described by Trombetta et al. (34). Glucosidase II treatments of M8.3 and M8.5 gave rise to M8.1 and M8.2, respectively, which had already been recorded in GALAXY, indicating that the structures of M8.3 and M8.5 are \(\text{Man}_1-2\text{Man}_1-6(\text{Man}_1-3)\text{Man}_1-6(\text{GlcNAc}_1-3\text{Man}_1-2\text{Man}_1-2\text{Man}_1-3)\text{Man}_1-4\text{GlcNAc}_1\text{B1-4GlcNAc}_1\text{PA}\) and \(\text{Man}_1-2\text{Man}_1-3\text{Man}_1-6(\text{GlcNAc}_1-3\text{Man}_1-2\text{Man}_1-3)\text{Man}_1-4\text{GlcNAc}_1\text{B1-4GlcNAc}_1\text{PA}\), respectively. Extensive \(\alpha\)-mannosidase digestion of GlcMan\(_3\)GlcNAc\(_2\)-PA, using 1 unit of the enzyme for 1 nmol of the oligosaccharide in 40 \(\mu\)l of 0.1 M acetate buffer, pH 5.0, containing 10 mM \(\text{ZnSO}_4\), at 37 °C for 27 h, resulted in production of GlcMan\(_3\)GlcNAc\(_2\)-PA (designated as M5.5 (31)), which was confirmed by a MALDI-TOF-MS analysis. Hence, the PA sugar library used in the present study consists of 21 kinds of oligosaccharides, including high mannose-type, hybrid-type, and complex-type oligosaccharides.

**Protein Expression**—The DNA fragment encoding amino acids residues 45–296 of human VIP36, which was designated VIP36-CRD, was cloned into the pET-3c plasmid vector with a C-terminal poly(H) tag moiety. The protein was expressed in *Escherichia coli* BL21 (DE3) pLysS strain (Stratagene) in LB medium. Production of recombinant proteins was induced by the addition of 0.08 mM isopropyl-\(\beta\)-D-thiogalactopyranoside. The poly(H) fusion protein was purified from cell lysates with M8.4 was purchased from Takara Bio, Inc. Other high mannose-type oligosaccharides are recorded in the GALAXY web site at www.glycoanalysis.info/ENG/index.html (30) and/or described in the literature (31), and the horizontal axes indicate time (in minutes).

![Elution profile of a series of PA oligosaccharides after application to an immobilized VIP36-CRD column.](image)

**FIGURE 1.** Elution profile of a series of PA oligosaccharides after application to an immobilized VIP36-CRD column. Each elution pattern was superimposed on that of the pyridylamine-labeled heptasaccharide. The horizontal axis indicates concentration of 10 mM in 10 mM Tris-HCl (pH 8.0), 10 mM HEPES (pH 7.0–7.5), or 10 mM MES (pH 5.5–6.5) containing 1 mM \(\text{CaCl}_2\) or 5 mM EDTA and applied onto the VIP36-CRD column at a flow rate of 0.25 ml/min at 20 °C. The elution profile was monitored by the fluorescence intensity at 400 nm (excitation at 320 nm). The retardation compared with the control oligosaccharide was computed using the difference of each elution volume, \(V_f - V_0\).

The dissociation constant, \(K_d\) of VIP36-CRD for Man\(_9\)GlcNAc\(_2\)-PA (M9.1) was determined using equation 1 (28),

\[
[A]_0(V_f - V_0) = B_f - K_d(V_f - V_0)
\]

where \([A]_0\), \(V_f\), and \(B_f\) are initial concentration of the PA oligosaccharide, the elution volume of the control sugar, and the total amount of immobilized VIP36-CRD in the column, respectively. The elution profile was monitored by UV light absorption at 300 nm to avoid possible quenching caused by the relatively high concentration of the PA sugar. For the determination of \(V_0\), 50 \(\mu\)M \(p\)-nitrophenyl-\(\beta\)-D-galactopyranoside was used. \(K_d\) was calculated based on the retardation \(V_f - V_0\) at concentrations of 150, 100, 50, 25, and 10 mM M9.1.

The relative affinity of each oligosaccharide was calculated under conditions where \([A]_0\) is negligibly small compared with \(K_d\), using Equation 2.

\[
V_f - V_0 = B_f/K_d
\]

In this case, the heptasaccharide Neu5Ac\(_2\)-3Gal\(_\beta\)-3GalNAc\(_\beta\)-4( Neu5Ac\(_2\)-8 Neu5Ac\(_2\)-3)Gal\(_\beta\)-4Glc-PA was used as a control sugar to determine \(V_0\). \(K_d\) values are mean ± S.D. of three independent experiments.

**RESULTS**

**Characterization of Sugar-binding Specificity of VIP36 by the FAC Analysis—**In this study, we constructed a PA sugar library for the FAC analyses to determine the sugar-binding specificity of VIP36-CRD. Fig. 1 shows typical elution profiles of the PA oligosaccharides overlaid with...
that of the control sugar. Based on the retardation volume $V_f - V_0$, we could estimate relative affinities of the individual PA oligosaccharides for VIP36-CRD. The $K_a$ of VIP36-CRD for M9.1 was determined as $0.97 \pm 0.02 \times 10^4$ M$^{-1}$ on the basis of dependence of retardation on the ligand concentration. The obtained data were treated according to Equation 1 (Fig. 2). The value of $K_a$ was calculated from the slope, which corresponds to $-K_a$. The $B_t$ value, which was obtained from the intercept on the ordinate, was $2.1 \times 10^{-8}$ mol for the immobilized VIP36-CRD column. Inspection of these data allows us to calculate $K_a$ values for all other PA oligosaccharides using Equation 2 (TABLE ONE). The PA sugars with higher affinities for VIP36-CRD are all high mannose-type possessing the Man$_{1-2}$Man$_{1-2}$Man branch (D1 branch), i.e. M9.1, M8.1, M8.2, and M7.1. Glucosylation or trimming of the D1 arm resulted in significant reduction in affinity for VIP36-CRD. The complex- and hybrid-type oligosaccharides were shown to exhibit lower affinities, which is consistent with the previous analysis (26).

**Ca$^{2+}$ and pH Dependence of Sugar Binding of VIP36-CRD**—Next, we analyzed the Ca$^{2+}$ and pH dependence of the sugar-binding affinity of VIP36-CRD by the FAC method. Fig. 3 shows the elution profiles of M9.1 on the VIP36-CRD affinity column in the presence of 1 mM CaCl$_2$ or 5 mM EDTA. No significant retardation of elution of M9.1 was observed in the presence of EDTA, indicating that Ca$^{2+}$ is required for sugar binding of VIP36-CRD.

Both ERGIC-53 and VIP36 possess two Ca$^{2+}$-binding sites in their CRDs. The x-ray crystallographic study of the CRD of p58/ERGIC-53 has revealed that one Ca$^{2+}$-binding site consists of Asp-160, Phe-162, Asn-164, and Asp-189, and the other is composed of Asp-163, Asp-165, Asn-169, Asn-170, and Asp-189 (16). The amino acid residues, forming the former Ca$^{2+}$-binding site are all conserved in VIP36-CRD. There exists one conserved histidine residue (His-178 and His-190 in ERGIC-53 and VIP36, respectively) in close proximity in this Ca$^{2+}$-binding site. A neutral mutation of His-178 inactivated the lectin function of ERGIC-53 (24).

We assessed the effect of a mutation of His-190 on the sugar binding of VIP36-CRD by the FAC method. A His$\rightarrow$Tyr substitution at position 190 resulted in the disappearance of the retardation of the elution of M9.1, indicating that His-190 plays an essential role in the sugar binding of VIP36-CRD (data not shown).

Finally, we analyzed the pH dependence of the sugar binding of VIP36-CRD. Fig. 4 shows the $K_a$ values under varying pH conditions of VIP36-CRD for M9.1, which exhibits a bell-shaped pH dependence with a maximum near pH 6.5.

**DISCUSSION**

VIP36 has been reported to recognize high mannose-type oligosaccharides containing Man$_{1-2}$Man residues (26). In this study, we attempted to estimate the contribution to VIP36 binding of the individual mannosyl branches of high mannose-type oligosaccharides by use of
Man5GlcNAc2 structure, which also has a lower affinity for VIP36-CRD. The D1 arm is trimmed by glucosidase II and reglucosylation by UDP-glucose:glycoprotein glucosyltransferase coupled with the correct folding of some glycoproteins in the ER, which is assisted by calnexin and/or calreticulin (1, 36). The FAC data has shown that glucosylation and trimming of the D1 mannosyl branch high mannose-type oligosaccharides. Principally, the FAC data has shown that glucosylation and trimming of the D1 mannosyl branch high mannose-type oligosaccharides assessed in the present study were in the range 0.07–1.16 M⁻¹ (TABLE ONE). Such a low affinity binding is widely observed for lectin-carbohydrate interaction systems, which often adopt affinity enhancement by multivalent interaction (35). It is conceivable that VIP36 clusters on the luminal membrane and thereby achieve affinity enhancement due to multiple interactions with its cargos.

We have revealed that VIP36 preferentially binds the D1 branch of high mannose-type oligosaccharides. Principally, the FAC data has shown that glucosylation and trimming of the D1 mannosyl branch interfere with the binding of VIP36-CRD. A cycle of glucose trimming by glucosidase II and reglucosylation by UDP-glucose:glycoprotein glucosyltransferase coupled with the correct folding of some glycoproteins in the ER, which is assisted by calnexin and/or calreticulin (1, 36). The D1 arm is trimmed by cis-Golg mannosidase I, giving rise to the Man6GlcNAc2 structure, which also has a lower affinity for VIP36-CRD (6, 38). These data indicate that VIP36 exhibits optimal binding affinities for the glycoproteins that leave the calnexin/calreticulin cycle in the ER and yet do not undergo trimming of the D1 mannosyl branch in the cis-Golg (Fig. 5). The present FAC data also suggest that the affinity for cargos of VIP36 is nominally affected by mannosidase trimming by ER mannosidase I and II, which gives rise to Man8 and Man7 structures (5).

Sugar binding of ERGIC-53 has shown to be Ca²⁺-dependent (24, 39). Appenzeller et al. (22) have reported that the efficiency of cross-linking of ERGIC-53 and its cargo was reduced in Lec1 cells treated with castanospermine, indicating that sugar binding of ERGIC-53 is affected by untrimmed glucose residues. Taken together, our data suggest that ERGIC-53 and VIP36 share common sugar-binding specificities. However, there exist significant differences in the pH dependence of sugar binding between them. ERGIC-53 efficiently binds immobilized mannoside at pH 7.4 but not at slightly lower pH, whereas VIP36 exhibits a bell-shaped pH dependence of binding to M9.1, with optimal pH values of ~6.5. Similar pH dependence has been reported for the binding of VIP36 to secretory and postnuclear supernatant proteins (26).

In ERGIC-53, on the basis of the mutagenesis data, ionization of His-178, which is located in the α-helix participating in the Ca²⁺ coordination, has been suggested to lead to the loss of Ca²⁺ in the sugar-binding pocket, thereby resulting in a reduction of its lectin activity upon acidification (24). Although the data presented here suggest that ionization of His-190 of VIP36, which corresponds to His-178 of ERGIC-53, is similarly responsible for the decrease in its sugar-binding affinity to below pH 6.5, the bell-shaped pH dependence cannot be attributed to a single titratable group. There exist several titratable groups, e.g., His-129, in the proximity of the putative sugar-binding site of VIP36-CRD, which was predicted based on the crystal structure of p58-CRD (15, 16). These amino acid residues are candidates for pH sensing. Further structural and mutational analyses of VIP36-CRD will help to identify the amino acid residue(s) responsible for the pH dependence of sugar binding.

There is progressive acidification from the ER through Golgi to the trans-Golg network because of H⁺ pumping by v-ATPase (41). The typical pH values of the ER, cis-Golg, and trans-Golg network have been reported to be 7.2, 6.4, and 5.4, respectively (41, 42). It has been reported that ERGIC-53 binds to the glycoprotein cargo in the ER and dissociates before reaching the cis-Golg (22), which is consistent with its pH dependence of sugar binding (24). By inspection of the specificity and optimal pH of the sugar binding of VIP36 and its subcellular localization, together with the organellar pH, we suggest that VIP36 binds glycoproteins retaining the intact D1 mannosyl branch in the cis-Golg network and recycles to the ER, where it releases the cargos due to higher pH. It is possible that VIP36 catches and retrieves glycoproteins that have escaped the trimming of the D1 branch, and glycoprotein carrying the correctly trimmed high mannose-type oligosaccharides or

![Image](image-url)
Sugar-binding Properties of VIP36

more mature hybrid- or complex-type oligosaccharides can no longer interact with VIP36 and move in the secretory pathway.

It has been shown that imperfectly folded or partially assembled proteins that exit the ER fail to escape the early secretory system entirely, because they are retrogradely transported from post-ER compartments to the ER (43, 44). This retrieval mechanism is mediated at least partially by interactions between proteins possessing a C-terminal Lys-Asp-Glu-Leu (KDEL) sequence, such as immunoglobulin light chain-heavy binding protein (BiP) and the KDEL receptor (40). A cross-linking experiment has demonstrated that VIP36 interacts with BiP in HEK293 cells (37).

One intriguing possibility is that VIP36 contributes to the retrograde transport of some misfolded or partially assembled glycoproteins in cooperation with BiP and the KDEL receptor, protecting the D1 branch of the cargos, which is subjected to quality control in the ER afterward, against the attack of cis-Golgi mannosidase I.

In summary, our study provides direct evidence that VIP36 binds the D1 mannosyl branch of its target glycoproteins, which is tightly associated with quality control at the ER.

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