Molecular Basis of Sugar Recognition by the Human L-type Lectins ERGIC-53, VIPL, and VIP36

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ERGIC-53, VIPL, and VIP36 are related type 1 membrane proteins of the mammalian early secretory pathway. They are classified as L-type lectins because of their luminal carbohydrate recognition domain, which exhibits homology to leguminous lectins. These L-type lectins have different intracellular distributions and dynamics in the endoplasmic reticulum-Golgi system of the secretory pathway and interact with N-glycans of glycoproteins in a Ca\(^{2+}\)-dependent manner, suggesting a role in glycoprotein sorting and trafficking. To understand the function of these lectins, knowledge of their carbohydrate specificity is crucial but only available for VIP36 (Kamiya, Y., Yamaguchi, Y., Takahashi, N., Arata, Y., Kasai, K. I., Ihara, Y., Matsuo, I., Ito, Y., Yamamoto, K., and Kato, K. (2005) J. Biol. Chem. 280, 37178–37182). Here we provide a comprehensive and quantitative analysis of sugar recognition of the carbohydrate recognition domains of ERGIC-53 and VIPL in comparison with VIP36 using a pyridylaminated sugar library in conjunction with frontal affinity chromatography. Frontal affinity chromatography revealed selective interaction of VIPL and VIP36 with the deglucosylated trimannose in the D1 branch of high-mannose-type oligosaccharides but with different pH dependence. ERGIC-53 bound high-mannose-type oligosaccharides with low affinity and broad specificity, not discriminating between monoglucosylated and deglucosylated high-mannose-type oligosaccharides. Based on the sugar-binding properties in conjunction with known features of these proteins, we propose a model for the action of the three lectins in glycoprotein guidance and trafficking. Moreover, structure-based mutagenesis revealed that the sugar-binding properties of these L-type lectins can be switched by single amino acid substitutions.

Growing evidence indicates that N-glycans function as tags recognized by a variety of intracellular lectins that govern the fates of glycoproteins in eukaryotes (1–4). These lectins are postulated to interact specifically with partially trimmed intermediates of high-mannose-type oligosaccharides displayed on the target polypeptide and thereby to control protein translocation, folding, degradation, and trafficking.

The lectins calreticulin and calnexin assist in the folding process of nascent proteins in the endoplasmic reticulum (ER) by capturing their monoglucosylated high-mannose-type oligosaccharides after the action of glucosidase I (1–6). Glucosidase II then removes the remaining glucose residue at the non-reducing terminal of the D1 branch of the substrate glycoproteins, giving rise to the M9 glycoform (the oligosaccharides are designated as shown in Fig. 1) that is no longer capable of interacting with calreticulin and calnexin (1–4, 7). During the folding process, incompletely folded glycoproteins are re-glucosylated by the folding sensor UTP-diphosphate-glucose:glycoprotein glucosyltransferase (1–4). Successful folding of glycoproteins in the ER can be achieved through multiple rounds of the calreticulin/calnexin cycle.

After correct folding, secretory glycoproteins are transported to the Golgi complex via the ER-Golgi intermediate compartment (ERGIC) by vesicular transport. ER export of some proteins is facilitated by cargo receptors. The most completely characterized cargo receptor is the type 1 membrane lectin ERGIC-53, which, together with ERGL, VIP36, and VIPL, constitutes the family of leguminous type (L-type) lectins (8). L-type lectins possess a luminal carbohydrate recognition domain (CRD) that binds to high-mannose-type oligosaccharides in a Ca\(^{2+}\)-dependent manner (9–11). ERGIC-53, the most...
popular marker for the ERGIC, cycles between ER and ERGIC and operates as a cargo receptor in ER export of some glycoproteins, including the lysosomal glycoproteins cathepsin C and cathepsin Z (12–14). Polymerization of IgM is also assisted by ERGIC-53 (15). This lectin forms a complex with MCFD2 (multi-coagulation factor deficiency 2), a 16-kDa protein possessing two EF-hand domains, and thereby recruits blood coagulation proteins, including the lysosomal glycoproteins cathepsin C and cathepsin Z. The polymerization of IgM is also assisted by ERGIC-53 (12–14). Polymerization of IgM is also assisted by ERGIC-53 (15).

**Experimental Procedures**

**Protein Expression**—The DNA fragments for residues 1–275 and 1–304, corresponding to the signal sequences and the CRDs of human ERGIC-53 and VIPL, respectively, were cloned into the BamHI and XbaI sites of the pDNA3.1 vector (GE Healthcare) with a C-terminal poly-His tag moiety. HEK293 cells transfected by the calcium phosphate precipitation method were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and nutrient mixture F-12 supplemented with 10% fetal bovine serum, 1% minimum Eagle’s medium 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM sodium pyruvate at 37 °C with 10% CO₂ in humidified air. To enhance the expression levels, 2 mM butyrate was added 2 days before harvesting the medium. The poly-His fusion proteins were purified from the cultured medium with a Ni²⁺-Sepharose high performance column (GE Healthcare). The proteins were further purified using a Superdex 75 gel filtration column (GE Healthcare).

The CRD of human VIP36 encoding amino acid residues 45–296 was mutated using the QuickChange method (Stratagene) with the following primers: D261G, 5′-GCGGGCCACGGCCGGGCTGTCTGACAAAC-3′; and 168KI69, 5′-CCCAATGATGAGAAGACCCTAGGAGGC-3′. The wild-type or mutant VIP36-CRD with a C-terminal poly-His tag moiety was expressed by *Escherichia coli* as described previously (10).

**Frontal Affinity Chromatography**—FAC analyses were carried out according to the literature (23–25). In the previous study, we conducted FAC analyses of VIP36-CRD immobilized on a HiTrap NHS-activated column (GE Healthcare). However, ERGIC-53-CRD with the ERGIC-53-encoding DNA fragment was not copurified from the cultured medium with a Ni²⁺-Sepharose high performance column (GE Healthcare). Whereas VIP36 cycles between the Golgi and the ER, VIPL is a resident of the ER (8, 13, 20, 21). Thus, these three lectins have distinct localizations within the early secretory pathway, pointing to disparate functions despite the structural similarities of their CRDs.

To understand the molecular function of these lectins, it is essential to know the sugar recognition features of their CRDs in detail. In a previous study (10), we elucidated the sugar-binding properties of VIP36 by the frontal affinity chromatography (FAC) method using a pyridylaminated (PA) sugar library. In the present study, we used the FAC method to comprehensively and quantitatively analyze the sugar recognition of ERGIC-53 and VIPL in comparison with that of VIP36. Furthermore, on the basis of the recently reported crystal structure of VIP36 (22), we created rationally designed VIP36-CRD mutants with altered sugar-binding specificity and pH dependence.

Sugar Recognition by Cargo Receptors

The PA oligosaccharide library was constructed as described previously (10). As a new member of this library, Man₁₋₆(Mana₁₋₃)Manα₁₋₆(Glca₁₋₃Manα₁₋₂Manα₁₋₂Manα₁₋₃)Manβ₁₋₄GlcNAcβ₁₋₄GlcNAc-PG (designated as GM7) was produced by digestion of GlcMan₆GlcNAc₂-PG using 30 milliunits of α-1,2-mannosidase (Seikagaku Kogyo Co.) for 1 nmol of the oligosaccharide in 100 μl of 0.1 M citrate-phosphate buffer, pH 5.0, at 37 °C for 24 h and subsequently isolated by high performance liquid chromatography using a TSK gel Amide-80 column (Tosoh, Tokyo, Japan). Consequently, the PA sugar library used in the present study contained 18 kinds of high-mannose-type oligosaccharides.

PA oligosaccharides were dissolved at a concentration of 10 mM in 10 mM HEPES (pH 7.0–8.0) or 10 mM MES (pH 5.5–6.5), both containing 150 mM NaCl and 1 mM CaCl₂, and applied onto the 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, Vᵣ. The dissociation constant, Kᵅ(1/√K₀), of the lectins for Man₆GlcNAc₂-PG (M9) was determined by the concentration-dependent analysis using Equation 1 as described previously (24),

\[
[A]₀ ∙ (Vᵣ - V₀) = Bᵣ - Kᵅ ∙ (Vᵣ - V₀)
\]  

Eqt. 1

where [A]₀, Vᵣ, and Bᵣ are the initial concentration of the PA oligosaccharide, the elution volume of the control sugar, and the total amount of immobilized lectins in the column, respectively. The elution profile was monitored by UV absorption at 300 nm to avoid possible quenching caused by the relatively high concentration of the PA sugar. For the determination of Vᵣ, 50 μM p-nitrophenyl β-D-galactopyranoside was used. Kᵅ was calculated based on the retardation Vᵣ - V₀ measured at concentrations of 5–80 μM M9.

The relative affinity of each oligosaccharide was calculated under conditions where [A]₀ is negligibly small compared with Kᵅ using Equation 2.

\[
Vᵣ - V₀ = Bᵣ/Kᵅ
\]  

Eqt. 2

To determine Vᵣ, (GlcNAcβ₁₋₆(GlcNAcβ₁₋₄)(GlcNAcβ₁₋₂)Manα₁₋₆(Glca₁₋₃Manα₁₋₂Galβ₁₋₄GlcNAcβ₁₋₄Manα₁₋₃)Manβ₁₋₄GlcNAcβ₁₋₄GlcNAc-PG and Neu5Acα₂₋₃Galβ₁₋₃GalNAcβ₁₋₄( Neu5Acα₂₋₈Neu5Acα₂₋₃ Galβ₁₋₄Glc-PG were used as control sugars for the analyses of VIP36-CRD and the other CRDs, respectively. Kᵅ values are the mean ± S.D. of three independent experiments.
RESULTS AND DISCUSSION

Sugar-binding Properties—Fig. 1B shows typical elution profiles of a series of PA oligosaccharides after application to an immobilized VIPL-CRD column at pH 7.4. From the retardation volume, relative affinities of the individual PA oligosaccharides could be estimated. In a similar way, the FAC data were collected for ERGIC-53, VIP36, and the VIP36 mutants (D261G and 168K169). Immobilization and elution conditions were the same as those used for VIPL-CRD. VIP36-D261G became capable of binding to GM9 (as indicated by the arrow), whereas VIP36–168K169 retained the sugar-binding specificity pattern of the wild type.

Molecular Basis of Carbohydrate-binding Specificity—We attempted to identify the amino acid residues accounting for the differences in the sugar-binding properties of the lectins on the basis of the recently reported crystal structures of the VIP36-CRD in comparison with that of the rat ERGIC-53-CRD (22, 26). The structure of the VIP36-CRD with mannopyranosyl ligands indicated that the D1 branch is accommodated in a pocket neighboring the Ca\(^{2+}\)-binding site with canonical interactions of Man-C with Asp-131 and Asn-166, which are widely conserved among L-type lectins (supplemental Fig. S2) (22).
This explains why the existence of Man-C is a prerequisite for the preferential binding to all three lectins. In VIP36-CRD, Man-D1 makes contacts with Ser-96 and Asp-261, putatively leading to steric hindrance between Glu-98 and the non-reducing terminal glucose residue. Although Ser-96 is conserved in ERGIC-53 and VIPL, Asp-261 is substituted with glycine in ERGIC-53 but not VIPL. Inspection of the crystal structures indicates that this amino acid substitution resulted in the lack of the protrusive side chain, rendering the putative sugar-binding pocket of ERGIC-53 significantly shallower (supplemental Fig. S2D). We speculated that abolishing the structural constraint by the Asp-261 side chain may enable conformational rearrangement of the D1 outer branch, avoiding the steric crash of the glucose residue in VIP36-CRD. To test this, we produced a D261G-mutated VIP36-CRD and examined its sugar-binding specificity. The FAC data in Fig. 1C indicate that the mutant VIP36-CRD can now bind the monoglucosylated high-mannose-type oligosaccharides. These findings suggest that the high specificity of VIP36 and VIPL for the deglucosylated D1 branch is governed by the single aspartate residue in their sugar-binding pockets.

The pH dependence of sugar binding is a unique property of the L-type lectins. Like ERGIC-53 (8), VIPL effectively binds oligosaccharides under neutral conditions but not at lower pH, whereas VIP36 exhibits a bell-shaped pH dependence of binding to M9 with a lower pH optimum (10). Previous mutagenesis studies have suggested that ionization of the histidine residue located in the loop participating in the Ca$^{2+}$ coordination, i.e. His-178 of human ERGIC-53 (His-186 in rat), is responsible for the decrease in their sugar binding below pH 7.0 (9). The corresponding histidine (His-188) is conserved in VIPL-CRD and therefore is likely to be responsible for the decrease in affinity of this lectin upon lowering the pH. The bell-shaped pH dependence of VIP36 cannot be attributed to a single titratable group. Inspection of the crystal structure of VIP36 suggests that all titratable groups in and around the sugar-binding site are conserved in VIPL. It is noteworthy, however, that there exists an extra lysine residue (Lys-166) near the putative sugar-binding site of VIPL-CRD (supplemental Fig. S1). This inspired us to design a mutant VIP36-CRD in which a lysine residue is inserted into the corresponding position, i.e., between Glu-168 and Thr-169. FAC analysis demonstrated that this mutant, designated as 168K169, retained the sugar-binding selectivity of the wild type (Fig. 1C) but no longer exhibited the bell-shaped pH dependence: rather, it showed a steady increase in affinity for M9 with increasing pH, very much like VIPL (Fig. 2). The three-dimensional model of VIP36-CRD complexed with Man$_8$GlcNAc$_2$Asn based on the crystal structure indicates that the Glu$_{168}$-Thr$_{169}$ segment is not in direct contact with the ligand (supplemental Fig. S2) (22). This suggests that the insertion of the lysine indirectly influences the affinity at higher pH, accounting for the differences in the pH dependence of sugar binding between VIP36 and VIPL.

**Functional Implications**—In the present study, we have established the sugar-binding specificities and pH dependence of VIPL, ERGIC-53, and VIP36. Like VIP36, VIPL specifically interacts with the deglucosylated trimannose in the D1 branch, whereas ERGIC-53 binds high-mannose-type oligosaccharides with broad specificity. Unexpectedly, in our FAC analysis, ERGIC-53 did not discriminate between the monoglucosylated and deglucosylated oligosaccharides, although a previous study indicated that this lectin exhibits impaired binding to glycoprotein cargo after treatment of cells with the glucosidase inhibitor castanospermine (12). One possible explanation for this apparent discrepancy is that castanospermine treatment gives rise to di- and/or triglucosylated oligosaccharides, which may not fit into the sugar-binding site of ERGIC-53-CRD.

How can the sugar-binding properties established in the current study be translated into function? We propose the following working model for the function of the three lectins. VIPL binds deglucosylated high-mannose-type glycopolypeptides immediately after their exit from the calreticulin/calnexin cycle given its ER localization and its high selectivity for binding the D1 branch, provided glucose is removed. Thereby, VIPL may protect the folded glycoproteins from demannosylation by ER mannosidases and interaction with the EDEM (ER degradation-enhancing α-mannosidase-like protein) family of mannose-binding proteins. Demannosylation and binding to EDEMs is a key step in ER-associated degradation of newly synthesized glycoproteins (27). Hence, interaction with VIPL may prevent the degradation of folded proteins.

Because VIPL is a resident protein of the ER, its interaction with secretory glycoproteins must be transient, but how is cargo released from VIPL for ER export? A known mechanism of cargo release involves a drop in pH, as exemplified by transport receptors cycling between the plasma membrane and endosomes. There is no indication, however, of pH differences within the ER. Overexpression of VIPL is known to redistribute ERGIC-53 to the ER, suggesting interaction and functional interplay between these two lectins (8, 21). One possibility is that the VIPL-bound glycoproteins are handed over to ERGIC-53. At first glance, such a process seems difficult to imagine because the glycan affinity of the ERGIC-53-CRD is lower than that of the VIPL-CRD. In vivo, however, ERGIC-53 forms homohexamers (28), which may considerably increase its gly-
can affinity. Moreover, the binding of cargo glycoproteins to ERGIC-53 can be enhanced by protein-protein interactions, as exemplified by cathepsin Z, which binds not only through a high-mannose-type glycan but also by a β-hairpin loop nearby (13). In addition, ERGIC-53 forms a complex with MCFD2, providing an additional possibility for protein-protein interactions as known for coagulation factors V and VIII. The cooperative binding to glycan and polypeptide appears to be the basis for the selective cargo receptor function of ERGIC-53. In the absence of protein-protein interaction sites, ERGIC-53 may facilitate glycoprotein transport with only low efficiency via its absence of protein-protein interaction sites, ERGIC-53 may for the selective cargo receptor function of ERGIC-53. In the native binding to glycan and polypeptide appears to be the basis providing an additional possibility for protein-protein interaction (13). In addition, ERGIC-53 forms a complex with MCFD2, exemplified by cathepsin Z, which binds not only through a high-mannose-type glycan but also by a β-hairpin loop nearby (13). It is conceivable that the up-regulation of ERGIC-53 under ER stress enhances the export capacity of glycoprotein cargo (27, 28). The broad specificity of ERGIC-53 for larger high-mannose-type glycans may be advantageous for efficient removal of proteins from the ER in this case of emergency.

The current data on VIP36 confirm our previous conclusions regarding the function of VIP36 (10, 29). Because of a slightly acidic pH optimum corresponding to the luminal pH of the Golgi and selectivity for the D1 branch, VIP36 may retrieve misfolded glycoproteins back to the ER that have inadvertently escaped quality control in the ER. VIP36 is therefore postulated to act in post-ER quality control.

In conclusion, the L-type lectins VIPL, ERGIC-53, and VIP36 share structural similarities in their CRDs but exhibit distinct sugar-binding specificities and affinities, which can be finely tuned by sensing pH. The combination of sugar specificity and pH dependence is proposed to provide the molecular basis for different functions of the three lectins in transport and quality control of glycoproteins. Our findings also reveal that the carbohydrate specificities of these lectins can be switched by single amino acid substitutions.

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