VIP36 functions as a transport lectin for trafficking certain high mannose type glycoproteins in the secretory pathway. Here we report the crystal structure of VIP36 exoplasmic/luminal domain comprising a carbohydrate recognition domain and a stalk domain. The structures of VIP36 in complex with Ca\(^{2+}\) and mannosyl ligands are also described. The carbohydrate recognition domain is composed of a 17-stranded antiparallel \(\beta\)-sandwich and binds one Ca\(^{2+}\) adjoining the carbohydrate-binding site. The structure reveals that a coordinated Ca\(^{2+}\) ion orients the side chains of Asp\(^{131}\), Asn\(^{166}\), and His\(^{190}\) for carbohydrate binding. This result explains the Ca\(^{2+}\)-dependent carbohydrate binding of this protein. The Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,2-Man, which corresponds to the D1 arm of high mannose type glycan, is recognized by eight residues through extensive hydrogen bonds. The complex structures reveal the structural basis for high mannose type glycoprotein recognition by VIP36 in a Ca\(^{2+}\)-dependent and D1 arm-specific manner.

In eukaryotic cells, post-translational modification of secreted proteins and intracellular protein transport between organelles are ubiquitous features. One of the most studied systems is the N-linked glycosylation pathway in the synthesis of secreted glycoproteins (1–3). The N-linked glycoproteins are subjected to diverse modifications and are transported through the endoplasmic reticulum (ER)\(^{3}\) via the Golgi apparatus to their final destinations inside and outside of the cell. Incorporation of the cargo glycoproteins into the transport vesicles is mediated by transmembrane cargo receptors, which have been identified as intracellular lectins. For example, mannose 6-phosphate receptor (4) functions as a cargo receptor for lysosomal proteins in the trans–Golgi network, whereas ER-Golgi intermediate compartment (ERGIC)-53 (5, 6) and its yeast orthologs Emp46/47p (7) are transport lectins for glycoproteins that are transported out of the ER.

VIP36, vesicular-integral protein of 36 kDa, was originally isolated from Madin–Darby canine kidney cells as a component of detergent-insoluble, glycolipid-enriched complexes containing apical marker (8). Confocal and immunoelectron microscopic experiments have suggested that VIP36 is distributed by either the pre–Golgi secretory pathway (9–11) or post–Golgi pathway (8, 12). Furthermore we showed that VIP36 is involved in intracellular transport, in the secretion of glycoproteins (e.g. clusterin) from polarized Madin–Darby canine kidney cells (13), and in the secretion of \(\alpha\)-amylase from rat parotid glands (14). Taken together, VIP36 appears to play significant roles not only in vesicular transport from the ER to the Golgi complex but also from the trans–Golgi network to the plasma membrane.

The exoplasmic/luminal domain of VIP36 as well as the luminal domain of ERGIC-53 and Emp46/47p share homology with L (leguminous)-type lectins and are thus identified as carbohydrate recognition domains (CRDs). It has been shown that ERGIC-53 interacts with glycoproteins carrying high mannose type glycan by endo-\(\beta\)-N-acetylglucosaminidase H treatment (15–17) and binds glycoproteins in a Ca\(^{2+}\)- and pH-dependent manner (18). We have previously found that VIP36 has high affinity for high mannose type glycans containing Man-\(\alpha\)-1,2-Man residues in Man\(_{-}\)_\(\alpha\)(GlcNAc)\(_{2}\)-Asn peptides (19). Kamiya et al. (20) recently reported in detail the carbohydrate binding properties of VIP36 assayed by frontal affinity chromatography. This work suggested the Ca\(^{2+}\) dependence of carbohydrate binding and the specificity for the D1 arm, Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,2-Man residues, of high mannose type glycans (corresponding to Man(D1)-Man(C)-Man(4); Fig. 1). In addition, using a flow cytometry-based method, it was also demonstrated that...
VIP36 binds glycoproteins carrying high mannose type glycans (21). These observations suggested that VIP36 is involved in the transport of glycoproteins via high mannose type glycans.

Crystal structures of the CRD of rat ERGIC-53 in the absence and presence of Ca$^{2+}$ have been determined, confirming its structural similarity to the L-type lectins (22, 23). In these reports, it was shown that the putative ligand-binding site of ERGIC-53 is similar to the mannose-binding site of the L-type lectins. Very recently, we reported the crystal structures of the CRD of Ca$^{2+}$-independent K$^{-}$-bound Emp46p and the metal-free form of Emp47p (24). To date, however, no structures of mannoses (v, vii, and x) using 1-O-acetyl-2,3,4,6-tetra-O-benzyl-a-D-mannopyranosyl (xii) have been determined. To investigate the structural basis of the mechanism of high mannose type glycoprotein recognition by VIP36, we determined crystal structures of the exoplasmic/luminal domain of VIP36 alone and in complex with Ca$^{2+}$ and mannose, Man-1,2-Man (termed Man$_2$, which corresponds to Man(D1)-Man(C), Man(C)-Man(4), Man(D2)-Man(A), or Man(D3)-Man(B) of Man$_{9}$(GlcNAc)$_{2}$; Fig. 1), and Man-1,2-Man-1,3-Man-1,4-GlcNAc (termed Man$_{5}$GlcNAc, which corresponds to Man(C)-Man(4)-Man(3)-GlcNAc(2); Fig. 1).

**EXPERIMENTAL PROCEDURES**

**Synthesis of Man-1,2-Man-1,2-Man-1,2-Man-1,3-Man, and Man-1,2-Man-1,6-Man**—Couplings of phenyl 3,4,6-tri-O-benzyl-a-D-mannopyranosyl (i), phe- nyl 2,3,4,6-tri-O-benzyl-a-D-thiomannopyranoside (ii), and phe- nyl 2,3,4-tri-O-benzyl-a-D-thiomanopyranoside (iii) having hydroxyl groups at the C-2, C-3, and C-6 positions (25) and 1,2-di-O-acetyl-3,4,6-tri-O-benzyl-a-D-mannopyranoside (iv) (26) were performed under conditions well established for a-mannosidation (trimethylsilyl trifluoromethanesulfonate/CH$_{2}$Cl$_{2}$) to give phenyl 2-O-acetyl-3,4,6-tri-O-benzyl-a-D- mannopyranosyl-(1→2)-3,4,6-tri-O-benzyl-a-D-thio- manopyranoside (v), phenyl 2-O-acetyl-3,4,6-tri-O-benzyl-a-D-thiomanopyranosyl-(1→2)-3,4,6-tri-O-benzyl-a-D-thiomanopyranoside (vi), and phenyl 2-O-acetyl-3,4,6-tri-O-benzyl-a-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-a-D-thiomanopyranoside (vii) (27), respectively. Subsequent deacetylation of the mannobioses (v, vi, and vii) gave phenyl 3,4,6-tri-O-benzyl-a-D-mannopyranosyl-(1→2)-3,4,6-tri-O-benzyl-a-D-thiomanopyranoside (viii), phenyl 3,4,6-tri-O-benzyl-a-D-mannopyranosyl-(1→3)-2,3,4,6-tri-O-benzyl-a-D-thiomanopyranoside (ix), and phenyl 3,4,6-tri-O-benzyl-a-D-mannopyranosyl-(1→6)-2,3,4,6-tri-O-benzyl-a-D-thiomanopyranoside (x) (27), respectively. Introduction of the non-reducing end of the mannose residue to the mannobioses (vii, ix, and x) using 1-O-acetyl-2,3,4,6-tetra-O-benzyl-a-D-mannopyranosyl (xi) (28) was performed using the same a-mannosidation method to give phenyl 2,3,4,6-tetra-O-benzyl-a-D-mannopyranosyl-(1→2)-3,4,6-tri-O-benzyl-a-D-mannopyranosyl-(1→2)-3,4,6-tri-O-benzyl-a-D-thiomanopyranoside (xii), phenyl 2,3,4,6-tetra-O-benzyl-a-D-mannopyranosyl-(1→2)-3,4,6-tri-O-benzyl-a-D-thiomanopyranoside (xiii), and phenyl 2,3,4,6-tetra-O-benzyl-a-D-mannopyranosyl-(1→2)-3,4,6-tri-O-benzyl-a-D-thiomanopyranoside (xiv), respectively. Finally complete deprotection of synthesized mannobiosides derivatives (xii, xiii, and xiv) afforded a-D-mannopyranosyl-(1→2)-a-D-mannopyranosyl-(1→2)-a-D-mannopyranosyl-(1→2)-a-D-mannopyranosyl-(1→3)-a-D-mannopyranoside, and a-D-mannopyranosyl-(1→2)-a-D-mannopyranosyl-(1→6)-a-D-mannopyranoside, respectively. These mannobioses were isolated on a COSMOSIL Sugar-D column (Nacalai Tesque) using an isocratic solvent composed of 65% MeCN and 35% H$_{2}$O. MS spectra of these compounds were in good agreement with those reported for closely related compounds (29, 30).

**Preparation of Man$_{5}$GlcNAc and Man$_{5}$GlcNAc$_{2}$-Asn—**

Man$_{5}$GlcNAc was prepared from urine of a mannosidosis patient as described previously (31). Briefly 10 ml of urine containing 10 mg of creatinine was concentrated to 1 ml and centrifuged for 20 min at 3,000 rpm. The supernatant was subjected to Bio-Gel P-4 (200–400 mesh) column chromatography (2.6 × 97 cm). The column was eluted with water containing 0.002% phenylmercuric nitrate, and the hexose content in each fraction was analyzed with phenol-sulfuric acid reagent. Fractions between Man$_{5}$GlcNAc and Man$_{5}$GlcNAc were pooled, sequentially subjected to Bio-Gel P-4 (under 400 mesh) column chromatography (2 × 100 cm) at 55 °C, and eluted with distilled water by monitoring with a refractometer. The fraction corresponding to Man$_{5}$GlcNAc was collected, and the struc-
Crystallization and X-ray Data Collection—Initial crystallization conditions were screened using the Large Scale Protein Crystallization and Monitoring System (PXS) (33). The crystallographic parameters of VIP36 are shown in Table 1. The recombinant VIP36 was expressed in Escherichia coli BL21(DE3). Cells were harvested after induction with 0.1 mM isopropyl β-D-thiogalactoside (Wako) for 15 h at 20 °C and lysed by sonication in phosphate buffered saline buffer containing 2 mM CaCl2. The cell lysate was loaded on a glutathione-Sepharose 4B column (GE Healthcare). The cleaved proteins were passed through a glutathione-Sepharose 4B column to remove GST protein and further purified by a benzamidine-Sepharose 4FF column (GE Healthcare) to remove the thrombin protease. The purified protein was dialyzed against 10 mM MES (pH 6.5) and 2 mM CaCl2.

Crystallographic and NMR System (CNS) (36) and REFMAC5 (37). The crystallographic parameters of VIP36 were solved by the molecular replacement method using the program MOLREP (35) with the Ca2+-bound ERGIC-53 (Protein Data Bank code 1R1Z) (23) as a search model. The refinement procedures were carried out with Crystallography and NMR System (CNS) (36) and REFMAC5 (37). Model fitting to the electron density maps was performed manually using Coot (38). The stereochemical quality of the final models was assessed by PROCHECK (39). Final refinement statistics are summarized in Table 1. Figures were prepared using GRASP (40), LIGPLOT, (41), and PyMOL (42).

Computer-aided Model Building—The model of VIP36-Man8(GlcNAc)2-Asn complex was built using coordinates of well ordered high mannos type glycans on glycoprotein crystal structures (human pancreatic α-amylase (Protein Data Bank code 1BSI), Erythrina corallodendron lectin (Protein Data Bank code 1LTE), and exo-(1,3)-β-glucanase (Protein Data Bank code 1H4P)) and mannosyl ligand-bound VIP36 structures. The corresponding glycan residues were superimposed on each other, and appropriate coordinates were used as follows: Asn-GlcNAc(1), Protein Data Bank code 1BSI; GlcNAc(2), Protein Data Bank code 1LTE; Man(3)-Man(4)-Man(C), ManαGlcNAc-bound VIP36; Man(D1), Manβ-bound VIP36; Manβ-bound crystal with the above model and human salivary α-amylase (Protein Data Bank code 1SMD) structures, the complex model of VIP36-rat salivary α-amylase carrying Manα(GlcNAc)2 was built. The salivary α-amylase was docked onto the VIP36-Manα(GlcNAc)2-Asn complex model through superimposition with an N-glycosylation site (Asn461) of the salivary α-amylase and the asparagine residue of the high mannos type glycan bound with VIP36.

Structure Determination and Refinement—The crystal structure of VIP36 was solved by the molecular replacement method using the program MOLREP (35) with the Ca2+-bound ERGIC-53 (Protein Data Bank code 1R1Z) (23) as a search model. The refinement procedures were carried out with Crystallography and NMR System (CNS) (36) and REFMAC5 (37). Model fitting to the electron density maps was performed manually using Coot (38). The stereochemical quality of the final models was assessed by PROCHECK (39). Final refinement statistics are summarized in Table 1. Figures were prepared using GRASP (40), LIGPLOT, (41), and PyMOL (42).

Surface Plasmon Resonance (SPR) Analysis—SPR measurements were carried out at 25 °C using a Biacore 2000 (Biacore) equipped with a CM5 sensor chip. GST-VIP36 (residues 51–322) was purified by affinity chromatography using glutathione-Sepharose 4B and benzamidine-Sepharose 4FF columns. The purified protein was immobilized on the flow cell using the amine coupling method. Various concentrations of mannotrioses (Man-α-1,2-Man-α-1,3-Man, Man-α-1,2-Man-α-1,3-Man, and Manα-1,2-Manα-1,6-Man) and Manα(GlcNAc)2-Asn in sample buffer (50 mM HEPES (pH 6.0) and 1 mM CaCl2) were injected over the flow cells at a flow rate of 20 μl/min using HBS-P buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.005% surfactant P20) as the running buffer.

RESULTS

Crystallization and Overall Structure of Exoplasmic/Luminal Domain of VIP36—The exoplasmic/luminal domain (residues 51–301) of VIP36, corresponding to the CRD and part of the short stalk domain, was crystallized. Despite extensive crystallization screening, diffraction quality crystals of the CRD (residues 51–278) alone could not be produced. The crystal structure of the exoplasmic/luminal domain of Ca2+-bound VIP36 was solved by the molecular replacement method using the Ca2+-bound ERGIC-53 CRD (Protein Data Bank code...
1R1Z) (23) as a search model. The crystal belongs to space group C2 with two molecules (A and B) per asymmetric unit. Both VIP36 molecules are related by ~2-fold symmetry, forming a pseudo-homodimer. However, gel filtration data demonstrate that this protein is monomeric in solution (supplemental Fig. 1).

To obtain crystals of mannose-bound VIP36, the Ca\(^{2+}\)-bound crystals were soaked in a solution containing mannose in molar excess. The Man\(_{2}\)-bound form was obtained by co-crystallization with longer oligomannoses, Man\(_{1,3}\)-Man, Man\(_{3}\)GlcNAc, and Man\(_{3}\)GlcNAc\(_{2}\)-Asn. Diffraction quality crystals were obtained from co-crystallization with the Man\(_{2}\)GlcNAc alone. The crystal belongs to space group P2\(_{1}\)2\(_{1}\)2\(_{1}\) with five molecules (A, B, C, D, and E) per asymmetric unit. The structure has the Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,3-Man moiety in molecules A and B, whereas the GlcNAc moiety is disordered. In contrast, only one mannose residue is ordered in molecule C, and all the carbohydrate residues are disordered in molecules D and E. The dimer interfaces of the P2\(_{1}\)2\(_{1}\)2\(_{1}\) crystal form are different from that of the C2 crystal form, suggesting that VIP36 is monomeric.

The CRD of VIP36 has an overall globular shape composed of a \(\beta\)-sandwich of two antiparallel \(\beta\)-sheets and is composed of 17 \(\beta\)-strands and three 3\(_{10}\) helices, each with a single turn (Fig. 2A). The \(\beta\)-sandwich comprises a seven-stranded (\(\beta2\)-\(\beta5\)-\(\beta14\)-\(\beta7\)-\(\beta8\)-\(\beta9\)-\(\beta10\)) concave \(\beta\)-sheet and a six-stranded (\(\beta1\)a,\(\beta1\)-\(\beta5\)-\(\beta6\)-\(\beta11\)-\(\beta12\)-\(\beta13a\)) convex \(\beta\)-sheet in a variation of the jelly roll fold. The \(\beta\)-strands are numbered according to the secondary structure numbering scheme of ERGIC-53 (22). A \(\beta\)-hairpin (strands \(\beta3\) and \(\beta4\)) is inserted between \(\beta2\) and \(\beta5\). Residues Cys\(_{202}\) (strand \(\beta10\)) and Cys\(_{239}\) (strand \(\beta13a\)) form a disulfide bond. The stalk domain is composed of a \(\beta\)-strand (\(\beta16\)), a short \(\alpha\)-helix (H4), and one turn of a 3\(_{10}\) helix (H5). The \(\beta16\) forms a \(\beta\)-sheet with \(\beta13b\) on the face of the protein opposite to the ligand-binding site between the concave and convex \(\beta\)-sheets. The stalk domain (residues...
289–301) contributes to an extensive crystal contact (Fig. 2A and supplemental Fig. 2) that explains the successful crystallization of this construct.

**Ca**\(^{2+}\) binding Site and Its Structural Changes in VIP36 CRD—The **F**\(_{o}\) − **F**\(_{c}\) electron density map of VIP36 shows one prominent peak corresponding to a Ca\(^{2+}\) ion coordinated between two loops, which are termed Loop 1 (between \(\beta 8\) and \(\beta 9\)) and Loop 2 (between \(\beta 9\) and \(\beta 10\)). The side-chain oxygen atom of Asp\(^{162}\) (O\(-\)1 of O\(-\)6-2), Asn\(^{166}\) (O\(-\)1-1), and Asp\(^{193}\) (O\(-\)2-2); main-chain carbonyl oxygen atoms of Tyr\(^{164}\) (O); and two water molecules termed W1 and W2 (O) are coordinated to the Ca\(^{2+}\) with distances of 2.4–2.5 Å (Fig. 3).

Upon Ca\(^{2+}\) binding, significant conformational changes occur around Loops 1 and 2 (Fig. 3). Large movements of the Ca\(^{2+}\) -coordinating and neighboring atoms are observed for the O\(-\)2 of Asp\(^{131}\), O\(-\)1 of Asn\(^{166}\), and N\(-\)1 of His\(^{190}\). The and mannose, the mannose is located in a pocket neighboring the Ca\(^{2+}\)-binding site on the concave \(\beta\)-sheet and has well defined electron density (Fig. 5A). The mannose-binding site (also called the “primary binding site” hereafter) comprises \(\beta 7\) and Loops 1, 2, and 3. A number of specific contacts can be seen between the protein and the ligand. The mannose is bound by Asp\(^{131}\) (O\(-\)6-1 and O\(-\)6-2), Asn\(^{166}\) (N\(-\)3-2), His\(^{190}\) (N\(-\)2), Gly\(^{260}\) (N), Asp\(^{261}\) (N), and Leu\(^{262}\) (N) through hydrogen bonds in the presence of 1 mM Ca\(^{2+}\) was 70 \(\mu\)M. On the other hand, no interaction was observed in the presence of 5 mM EDTA.

**Structure of VIP36 in Complex with Ca**\(^{2+}\) and Mannose—In the structure of VIP36 in complex with Ca\(^{2+}\) and mannose, the mannose is located in a pocket neighboring the Ca\(^{2+}\)-binding site on the concave \(\beta\)-sheet and has well defined electron density (Fig. 5A). The mannose-binding site (also called the “primary binding site” hereafter) comprises \(\beta 7\) and Loops 1, 2, and 3. A number of specific contacts can be seen between the protein and the ligand. The mannose is bound by Asp\(^{131}\) (O\(-\)6-1 and O\(-\)6-2), Asn\(^{166}\) (N\(-\)3-2), His\(^{190}\) (N\(-\)2), Gly\(^{260}\) (N), Asp\(^{261}\) (N), and Leu\(^{262}\) (N) through hydrogen bonds in the presence of 1 mM Ca\(^{2+}\) was 70 \(\mu\)M. On the other hand, no interaction was observed in the presence of 5 mM EDTA.

Specific Binding of VIP36 to D1 Arm of High Mannose Type Glycans—Previous studies have suggested that VIP36 CRD recognizes D1 arm, Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,2-Man residues of high mannose type glycans (19, 20). In fact, we observed using SPR analysis that VIP36 has higher affinity for Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,2-Man oligosaccharide (corresponding to Man(D1)-Man(C)-Man(4) of the D1 arm) than for Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,3-Man (Fig. 4). No interaction was observed between VIP36 and Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,6-Man. In addition, we observed that Ca\(^{2+}\) ion is required for the interaction between VIP36 and Man\(_n\)(GlcNAc)\(_2\)-Asn (supplemental Fig. 3). The calculated dissociation constant between VIP36 and Man\(_n\)(GlcNAc)\(_2\)-Asn in the presence of 1 mM Ca\(^{2+}\) was 70 \(\mu\)M. On the other hand, no interaction was observed in the presence of 5 mM EDTA.

The bound Ca\(^{2+}\) ions are coordinated between slow B, and neighboring atoms are observed for the O\(-\)2 of Asp\(^{131}\), O\(-\)1 of Asn\(^{166}\), and N\(-\)1 of His\(^{190}\). The distances are 1.7, 1.6, and 4.0 Å, respectively. The O\(-\)6-2 of Asp\(^{131}\) and N\(-\)1 atom of His\(^{190}\) form hydrogen bonds with Ca\(^{2+}\) -coordinating water molecules W1 and W2, respectively, whereas the O\(-\)1 of Asn\(^{166}\) is directly coordinated with Ca\(^{2+}\). As we will describe below, these residues bind the carbohydrate moiety in the presence of Ca\(^{2+}\) suggesting a mechanism for the Ca\(^{2+}\)-dependent carbohydrate binding of VIP36.
respectively (Fig. 5E). In contrast, the hydrogen bond between the 6-OH group and Asp261 (O–H1) is not observed in molecule B (supplemental Fig. 4A). The binding site of the reducing-end mannose residue of \( \text{Man}_3 \) is almost the same as the primary binding site (Fig. 5, D and E).

Structure of VIP36 in Complex with \( \text{Ca}^{2+} \) and \( \text{Man}_3 \text{GlcNAc} \)—In the structure of VIP36 with \( \text{Man}_3 \text{GlcNAc} \), the \( \text{Man}_3 \) moiety is ordered, whereas the GlcNAc moiety is disordered. The \( \text{Man}_1 \text{ alpha}-1,2-\text{Man}_1 \text{ alpha}-1,3-\text{Man} \) residues have defined electron density (Fig. 5C). The binding site of the non-reducing mannose residue of \( \text{Man}_3 \text{GlcNAc} \) is almost the same as the primary binding site (Fig. 5, D and F). The 6-OH group of the \( \alpha_1-2 \) and \( \alpha_1-3 \)-linked mannose residue makes hydrogen bonds with Tyr164 (O–H1) and Asn166 (O) (Fig. 5F). The \( \alpha_1-3 \) and \( \beta_1-4 \)-linked mannose residue is recognized by Asp261 (O–H1) through a hydrogen bond, whereas the hydrogen bond is not observed in molecule B (supplemental Fig. 4B).

DISCUSSION

Many lectins, such as mannose-binding proteins and the asialoglycoprotein receptors, achieve higher affinity and selectivity through oligomerization of their CRDs (43). For instance, ERGIC-53 and Emp46/47p are known to form oligomeric complexes through the putative coiled-coil region in the stalk domain (15, 44, 45). On the other hand, it has been shown that

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**FIGURE 4.** Specific binding of GST-VIP36 to the D1 arm (Man-\( \alpha_1,2-\text{Man}_1,2-\text{Man} \)) revealed by SPR analysis. Three different mannotrioses were added over flow cells at the indicated concentrations. Specific binding of mannotrioses was obtained by subtracting the resonance unit (RU) value of the GST immobilized sensor chip from the values of GST-VIP36 immobilized sensor chips. The plots were obtained by subtracting the values measured using the sample buffer without carbohydrates. The dose binding curves were obtained from the resonance unit value at 200 s. Solid circle, Man-\( \alpha_1,2-\text{Man}_1,2-\text{Man} \); open circle, Man-\( \alpha_1,2-\text{Man}_1,2-\text{Man} \); solid triangle, Man-\( \alpha_1,2-\text{Man}_1,2-\text{Man} \).

**FIGURE 5.** Carbohydrate ligand-binding site of VIP36. 2F, –F, electron density map of mannose of the Man-bound form (A), Man-\( \alpha_1,2-\text{Man} \) of the Man-\( \alpha_1,2-\text{Man} \) bound form (B), and Man-\( \alpha_1,2-\text{Man}_1,3-\text{Man} \) of the Man-\( \alpha_1,2-\text{Man} \) bound form (C) contoured at 1.2\( \sigma \). Secondary structures are shown as in Fig. 2A. D, structure of mannose and \( \text{Ca}^{2+} \)-binding site of VIP36 (molecule A). E, structure of Man-\( \alpha_1,2-\text{Man}_1,2-\text{Man} \) and \( \text{Ca}^{2+} \)-binding site of VIP36 (molecule A). F, structure of Man-\( \alpha_1,2-\text{Man}_1,3-\text{Man} \) and \( \text{Ca}^{2+} \)-binding site of VIP36 (molecule A). The bound carbohydrate residues are shown as green stick models. Residues of VIP36 binding to the ligands are shown in ball-and-stick models.
Structure of VIP36-Mannosyl Ligand Complex

FIGURE 6. A, overall structure of ConA monomer. A purple ribbon model of ConA (molecule A) is shown. The bound Mn\(^{2+}\) (S1) and Ca\(^{2+}\) (S2) are shown as large gray and black spheres, respectively. B, comparison between VIP36 and ConA metal-binding site structures. The VIP36 (molecule A) and ConA structures are colored yellow and purple, respectively. Residues of VIP36 and ConA are labeled in black and purple, respectively. The Ca\(^{2+}\) in VIP36 is shown as a large pink sphere. Because the position of Ca\(^{2+}\) at the S2 site in ConA is almost the same as in VIP36, it is not shown. Water molecules found in the S1 site of ConA are shown as small white spheres and are labeled W3 and W4. C, overall structure of ERGIC-53 CRD. A cyan ribbon model of ERGIC-53 (molecule A) is shown. The bound Ca\(^{2+}\) is shown as large magenta spheres (M1 and M2). D, comparison between VIP36 (yellow) and ERGIC-53 (cyan) Ca\(^{2+}\) binding site structures. Because the position of Ca\(^{2+}\) at the M2 site in ERGIC-53 is almost the same as in VIP36, it is not shown. Water molecules found in the M1 site of ERGIC-53 are shown as small orange spheres and are labeled W5 and W6. Residues involved in the metal binding are shown as ball-and-stick models.

no disulfide-linked or stable non-covalent oligomers of recombinant exoplasmic/luminal domain (residues 45–322) or endogenous VIP36 could be detected by cross-linking or sedimentation analysis (46). Furthermore we confirmed that the exoplasmic/luminal domain of VIP36 (residues 51–301 and 51–322) is monomeric in physiological solution by gel filtration analyses (supplemental Fig. 1). Indeed the stalk domain (residues 279–322) of VIP36 is 95–162 residues shorter than those of ERGIC-53, Emp46p, and Emp47p. The portion of the stalk domain of VIP36 included in our construct (residues 279–301) does not form coiled-coil structure (Fig. 2A and supplemental Fig. 2). The short stalk domain and the absence of coiled-coil domain suggest that VIP36 may likely function as a monomer.

It is known that leguminous lectins coordinate Mn\(^{2+}\) and Ca\(^{2+}\) ions, termed S1 and S2, respectively, in their β-sandwich structures (Fig. 6, A and B) (47). The S1 ion stabilizes the S2-binding site, and the S2 ion fixes the positions of the amino acids that interact with the oligosaccharide ligands. In this study, we showed that VIP36 has a single Ca\(^{2+}\) in the CRD structures and that the Ca\(^{2+}\) fixes the positions of Asp\(^{131}\), Asn\(^{166}\), and His\(^{190}\) that interact with carbohydrate ligands in the primary binding site (Figs. 3 and 5). Specifically significant conformational changes upon Ca\(^{2+}\) binding take place around the Ca\(^{2+}\) and primary carbohydrate-binding site of VIP36 (Fig. 3). Similar but more pronounced structural changes of the corresponding site upon metal binding were also observed in concanavalin A (ConA) (48) and ERGIC-53 (23). The Ca\(^{2+}\) of ConA induces large conformational changes to stabilize the correct geometry of the Ca\(^{2+}\)-binding site that comprise the trans to cis isomerization of the Ala\(^{200}\)-Asp\(^{208}\) peptide bond accompanied by the formation of the carbohydrate-binding site (48). In VIP36 structures, such isomerization changes were not observed. We also observed that Ca\(^{2+}\) is required for interaction between VIP36 and Man\(_{n}\\(\text{GlcNAc})_{2}\\)-Asn by SPR experiments (supplemental Fig. 3). These results explain the Ca\(^{2+}\)-dependent carbohydrate binding of VIP36.

It was shown that ERGIC-53 contains two Ca\(^{2+}\) ions termed M1 and M2 (Fig. 6, C and D) and that the M1 ion does not lie at the same site as the S1 ion, although M2 is located at the corresponding S2 site (23). The Ca\(^{2+}\) of VIP36 corresponds to the M2 site of ERGIC-53. When the VIP36 and ERGIC-53 structures are superimposed, the M2 metal ions overlay with a separation that is less than 0.2 Å. Although the Ca\(^{2+}\) of VIP36 is equivalent to the M2 site of ERGIC-53, the electron density maps of VIP36 show no peak that could be assigned as a metal ion either at the corresponding M1 site or at any other sites within the structure. In the Ca\(^{2+}\)-binding site (M2 and S2), the Ca\(^{2+}\)-coordinating residues are structurally very well conserved except for Asp\(^{193}\) in VIP36 (Fig. 6, B and D). The corresponding Asp\(^{193}\) (ConA) and Asp\(^{189}\) (ERGIC-53) residues are coordinated by the two metal ions. In VIP36, the Oδ-1 of Asp\(^{193}\) forms a hydrogen bond with the main-chain nitrogen atom of Asp\(^{167}\) to stabilize the Ca\(^{2+}\)-binding site. As a result, only one Ca\(^{2+}\) ion fixes the ligand binding residues in VIP36. Our crystallographic studies also suggest that VIP36 did not bind other divalent cations, neither Mn\(^{2+}\) nor Mg\(^{2+}\) (data not shown). Loop 1 of VIP36 is two residues longer than that of ERGIC-53. Likewise the residues coordinating the M1 ion in ERGIC-53 (Asp\(^{163}\), Asp\(^{165}\),
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Next the carbohydrate-binding site of VIP36 was compared with that of ConA in complex with Man−α1,3-(Man−α1,6-)Man (corresponding to Man(3)-(Man(4′))-Man(4)) (49). Although the carbohydrate binding specificity of VIP36 and ConA is essentially different, the structural conservation of ligand-binding sites between them is observed in not only the Man(C)-binding site but also the Man(D1)- and Man(4)-binding sites of VIP36 (Fig. 7C). In the Man(C)-binding site of VIP36, the ligand binding residues are structurally very well conserved except for His190 in VIP36 (Fig. 7C). The corresponding main-chain nitrogen atom of Arg228 in ConA is bound to only the 3-OH of the mannose residue. In contrast, Ne-2 of His190 in VIP36 is bound to the 3-OH and 4-OH groups and acts simultaneously as hydrogen bond donor and acceptor (Fig. 7B). In the Man(4)-binding site, although the carbohydrate binding loop conformation of VIP36 is largely different than that of ConA, the side-chain position of Tyr164 is very similar to that of Tyr12 in ConA.

When the carbohydrate-binding site of VIP36 was compared with the corresponding site of ERGIC-53 in complex with Ca2+, the Manα binding residues of VIP36 are largely identical to the corresponding residues of ERGIC-53 (Fig. 7D and supplemental Fig. 2). The structural conservation suggests that ERGIC-53 also binds the D1 arm moiety of high mannose type glycoproteins, which is consistent with the function of ERGIC-53 as a transport lectin for high mannose type glycoproteins. These contain Manα2Manα2GlcnAc with an intact D1 arm and are transported from the ER by the ER quality control mechanism (2, 3). However, there are some structural differences in the β5, β7, and Loops 1 and 3 regions: (i) in β5 the side-chain orientation of Ser196 of ERGIC-53 is dissimilar to that of VIP36, (ii) the side-chain orientation of Asp129 of ERGIC-53 is different than that of the corresponding Asp131 of VIP36, (iii) Phe162 of ERGIC-53 is replaced by Tyr164 in VIP36, and (iv) the Loop 3 of ERGIC-53 is positioned further away from the ligand when compared with VIP36, and Asp261 of VIP36 is replaced by Gly260 in ERGIC-53. Most significantly, Tyr164 and Asp261 in VIP36 are better suited than Phe162 and Gly260 in ERGIC-53 for binding to the Manα. In ERGIC-53, the

Asn169, Asn170, and Asp189; shown in green in supplemental Fig. 2) are poorly conserved in VIP36. Taken together, we conclude that VIP36 binds only one Ca2+ ion and that the single Ca2+ fixes the positions of residues involved in carbohydrate ligand binding.

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Structure of VIP36-Mannosyl Ligand Complex

A

FIGURE 8. Model for binding between VIP36 and high mannose type glycan (Man$_8$(GlcNAc)$_2$-Asn). A, the high mannose type glycan is indicated by a stick model. In the oligosaccharide, the part determined in this study is colored in green. The modeled D2 and D3 arms and N-linked chitobiose moiety of the high mannose type glycan are shown in purple. The types of glycosidic linkages are also indicated. The individual carbohydrate residues of Man$_8$(GlcNAc)$_2$-Asn are shown as in Fig. 1. Residues involved in the ligand binding are shown as ball-and-stick models. B, model for binding between VIP36 and salivary α-amylase carrying Man$_8$(GlcNAc)$_2$, in rat secretory vesicles.

presumptive weak interactions between ERGIC-53 and carbohydrate ligands might be compensated by the oligomerization of the CRDs.

To demonstrate that the crystal structure strictly represents the complex formed in solution, we simulated a complex model of Man$_8$(GlcNAc)$_2$-Asn and VIP36 (Fig. 8A). In this model, there are no significant steric clashes between high mannose type $N$-glycan and VIP36. The monomeric VIP36 seems to accommodate the glycan along an extended ligand-binding site. Kamiya et al. (20) have suggested that VIP36 recognizes the D1 arm and showed that mannose trimming and monoglucosylation of the D1 arm resulted in significant reduction in affinity for VIP36 CRD using frontal affinity chromatography analysis. When a glucose residue is modeled into the VIP36 for VIP36 CRD using frontal affinity chromatography analysis. It has been shown that VIP36 recycles between the ER and the Golgi complex (9–11). To date, however, there is no obvious evidence that VIP36 is involved in retrograde transport of glycoproteins from the Golgi complex to the ER. On the other hand, we revealed that VIP36 localizes in the trans-Golgi network (12) and is involved in secretion of high mannose type glycoproteins clusterin and α-amylase (13, 14). It has been generally known that the D1 arm is trimmed by cis-Golgi mannose I to form Man$_n$(GlcNAc)$_2$ in the cis-Golgi. The carbohydrate structure has a lower affinity for VIP36 (19, 20). In this study, we have shown that VIP36 specifically binds the Man-$\alpha$-1,2-Man-$\alpha$-1,2-Man residues of the D1 arm of high mannose type glycan. Taken together, VIP36 might be involved in anterograde transport of certain glycoproteins carrying high mannose type glycan with the D1 arm from the ERGIC via the Golgi complex to the plasma membrane by protecting the D1 arm against trimming by cis-Golgi manniosidase I. Although it is not known whether or not high mannose type glycan of rat salivary α-amylase has the D1 arm, a possible model for binding between VIP36 and salivary α-amylase carrying high mannose type glycan (Man$_8$(GlcNAc)$_2$) in rat parotid acinar cells is shown (Fig. 8B).

In summary, we determined the first complex structure of the exoplasmic/luminal domain of the transport lectin VIP36 and Ca$^{2+}$ and Man, Man$_2$, and Man$_3$GlcNAc, which are part of the D1 arm of high mannose type glycans. Our results provide structural insights into the mechanism of recognition of high mannose type glycoproteins by VIP36 in a Ca$^{2+}$-dependent and D1 arm-specific manner. Further biochemical analysis such as subcellular localization of VIP36 on a wide variety of cells and identification of its cargo glycoproteins together with the detailed carbohydrate structures will provide further insight into the mechanism of high mannose type glycoprotein transport by VIP36.

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