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Detection of Weak Sugar Binding Activity of VIP36 using VIP36–streptavidin Complex and Membrane-based Sugar Chains

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High mannose-type glycan–lectin interactions play important roles especially in quality control of glycoproteins. VIP36 is a receptor with homology to plant leguminous lectins in its luminal region. The luminal region of VIP36 with a C-terminal biotinylation-tag (sVIP36) was expressed in *Escherichia coli* and oligomerized with R-phycoerythrin (PE)-labelled streptavidin. Flow cytometric analysis revealed that PE-labelled sVIP36–SA complex (sVIP36–SA) bound to deoxymannojirimycin (DMJ)- and kifunensine (KIF)-treated HeLaS3 cells. The binding of sVIP36–SA to HeLaS3 cells treated with DMJ or KIF was abolished by endo-β-*N*-acetylglucosaminidase H treatment of the cells. Furthermore, the binding of sVIP36–SA to the cells was inhibited by high mannose-type glycans especially $Man_{7–9}$ $GlcNAc_2$, indicating that the binding of sVIP36–SA to cell surfaces was mediated by high mannose-type glycans. Although VIP36 has the lower affinity for ligands than typical homologous plant lectins, we were able to monitor the sugar-binding activity of VIP36 using less than 100 ng of the sVIP36–SA. This method is highly sensitive and suitable for detecting interactions between lectins and sugar chains of low affinity.

Key words: flow cytometry, membrane-based ligand, sugar-binding, VIP36, weak interaction.

Abbreviations: CNX, calnexin; CRT, calreticulin; DMJ, deoxymannojirimycin; DSA, *Datura stramonium* agglutinin; EDEM, ER degradation enhancing α-mannosidase-like protein; Endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; ERGIC-53, ER-Golgi compartment protein of 53 kDa; FCS, fetal calf serum; GNA, *Galanthus nivalis* agglutinin; KIF, kifunensine; MFI, mean fluorescence intensity; PA, pyridylamino; PE, R-phycoerythrin; PE-SA, PE-conjugated streptavidin; PI, propidium iodide; SA, streptavidin; SW, swainsonine; VIP36, vesicular integral protein of 36 kDa.

INTRODUCTION

Intracellular lectins, which are involved in the proper folding of newly synthesized polypeptides, the degradation of misfolded proteins, and the transport and sorting of properly matured proteins use *N*-linked glycans, especially of the high mannose-type, to identify target proteins in the endoplasmic reticulum (ER) (*1, 2*). Monoglucosylated high mannose-type glycans on proteins are recognized by calnexin (CNX) or calreticulin (CRT), which act as chaperones to correctly fold the proteins (*3, 4*). If the glycoproteins are misfolded, it is believed that the ER degradation enhancing α-mannosidase-like protein (EDEM) family proteins bind to the Man_{8B} isoform of high mannose-type glycans on the misfolded proteins (Fig. 1), causing the proteins to be retro-translocated from the ER into the cytosol for degradation by proteasomes (*5–7*). Correctly folded and matured glycoproteins exit the ER and are sorted by cargo receptors. ERGIC-53 and VIP36 have been postulated to belong to a new class of sorting receptors in the secretory pathway (*8, 9*). Both proteins have a type-I transmembrane topology and a stretch of about 200 amino acids in their luminal region that shows around 20% identity to plant leguminous lectins (*10, 11*). To clarify the mechanisms underlying the molecular action of VIP36 and the role of VIP36 in such quality control of glycoproteins, it is essential to identify the sugar-binding specificity of the cargo receptor in detail. Although VIP36 interacts with thyroglobulin, and this interaction is inhibited by higher-molecular-weight high mannnose-type glycans (*12*), its specificity for binding to sugars, including isomeric structures, has not yet been examined.

The carbohydrate-binding specificity of lectins has been studied by traditional hapten inhibition of haemagglutination using various sugars and sugar derivatives as inhibitors. Other approaches for examining the specificities of lectins have also been described, including equilibrium dialysis (*13*), affinity chromatography on immobilized lectin columns (*14*), titration calorimetry

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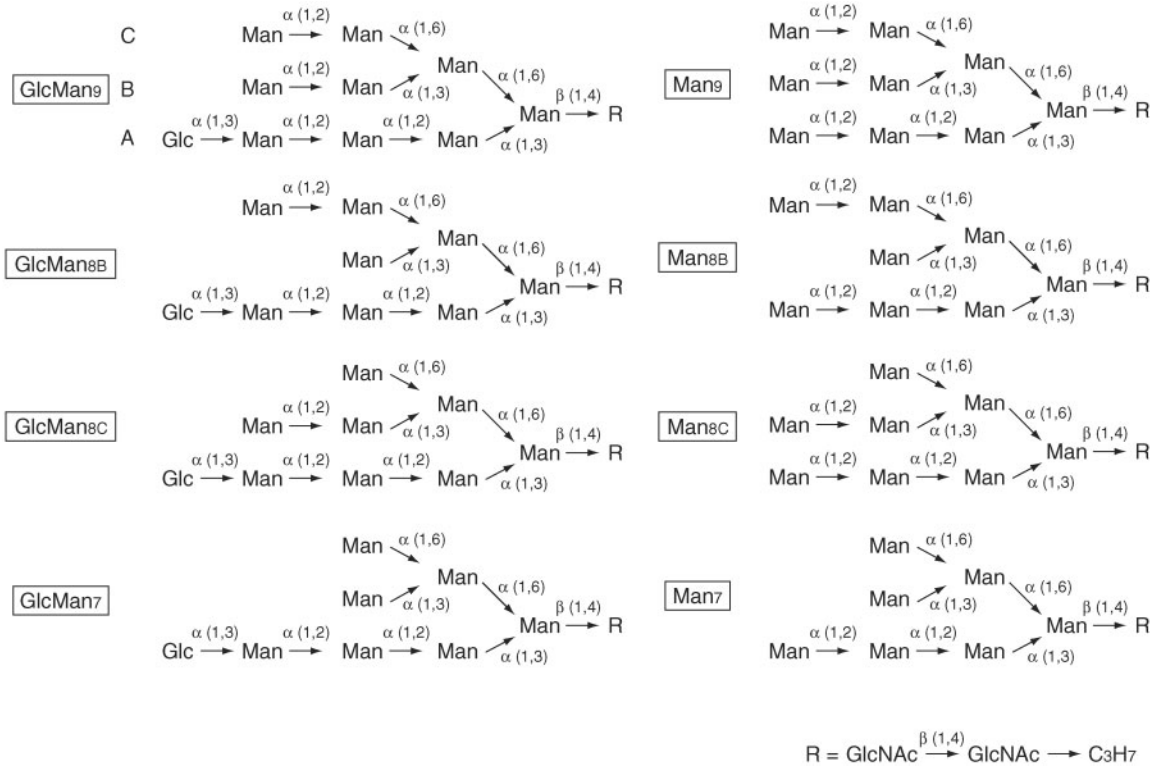

Fig. 1. **High mannose-type glycan derivatives used in this study.** Structures of the synthesized high mannose-type glycans used in the inhibition assay are shown. The reducing end of each glycan is modified by a propyl group. The three branches of the glycan are designated the A, B and C arms (*2*).

using a microcalorimeter (*15*) and current surface plasmon resonance (*16*). However, the interactions between proteins of the ER quality control machinery and their substrates, as well as between cargo receptors and their cargo, are often of a weak and transient nature and are therefore difficult to study. In particular, associations with high mannose-type glycans appear to be very weak ($K_a = 1.16 \sim 0.07 \times 10^4\,M^{-1}$ in the case of VIP36) (*17*) compared with those of typical plant lectins ($K_a = 10^7$ to $10^5\,M^{-1}$).

In the present study, we propose an alternative method for detecting carbohydrate-mediated interactions of VIP36 using a soluble VIP36–SA complex and cell surface sugar chains as ligands. This method provides a specific and sensitive assay to monitor the weak interactions between sugar chains and animal lectins, including intracellular lectins.

MATERIALS AND METHODS

Materials—The synthesized high mannose-type glycans used in this study are shown in Fig. 1 (*18, 19*). The biotin-labelled lectins *Galanthus nivalis* agglutinin (GNA) and *Datura stramonium* agglutinin (DSA) were purchased from EY Laboratories (San Mateo, CA), and monosaccharides were purchased from Wako (Osaka, Japan).

Construction of Plasmids for Soluble VIP36 and its Mutant—A DNA encoding human VIP36 was PCR-amplified from HL60 cDNA. To express the soluble VIP36 protein (sVIP36) corresponding to the luminal part of VIP36, residues 45 to 322 were amplified by PCR using the primers 5′-GGGAATTTC*CATATG*GATATAA CTGACGGCAACAG-3′ and 5′-*ATGATGATGATGATGA TG*CCGCCACCCCGTCAGGG-3′, which have an *Nde* I site and a 6x His-tag sequence, respectively (shown with italic font). The amplified DNA was digested with *Nde* I and then cloned into a pET3c vector that had been modified to include an enzymatic biotinylation signal at the C-terminus (*20*). VIP36 containing a D131N mutation was generated by introducing a point mutation with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the primers 5′-CATG GAAACGGCATCGCCTTGTGGTAC-3′ and 5′-GATGCCG TTTCCATGGAAGGTTCTTCTTC-3′.

Preparation of Biotinylated Recombinant Soluble VIP36–SA Complex—sVIP36 and its mutant D131N were expressed in the BL21(DE3) pLysS strain of *Escherichia coli*, and each protein was obtained from harvested *E. coli* cell lysates. These proteins were purified by sequential Ni-affinity and gel filtration chromatographies on columns of HiTrap Chelating HP and Superdex 75 10/300 GL (Amersham Pharmacia, Piscataway, NJ), respectively. Biotinylation of these proteins was performed with the biotin ligase BirA (Avidity, Denver, CO) as previously described (*21*). Biotinylation was validated with a gel shift assay in polyacrylamide gels using streptavidin (SA) as follows. One microgram of sVIP36 was mixed with SA at a molar

ratio of 1:2 at 4°C for 1 h in 10 μl of 10 mM sodium phosphate, pH 7.4, containing 137 mM NaCl and 2.68 mM KCl [PBS(−)]. The sample was added to 2 μl of 6× solubilizing buffer (375 mM Tris-HCl, pH 6.8, containing 12% SDS, 0.6% bromophenol blue and 60% glycerol) and electrophoresed in 12.5% polyacrylamide gels without boiling. Complexes of sVIP36 and SA were analysed by gel filtration chromatography on a column of Superdex 200 HR 10/30 (10 mm × 300 mm) (Amersham Pharmacia) equilibrated with PBS(−) and elution was performed with the same buffer. For preparing the R-phycoerythrin (PE)-labelled sVIP36–SA complex (sVIP36–SA), biotinylated sVIP36 proteins were incubated with PE-conjugated streptavidin (PE-SA) (BD Biosystems, San Jose, CA) at a molar ratio of 4:1 at 4°C for 1 h. A PE-labelled D131N–SA complex (D131N–SA) was also prepared according to the method previously described.

Target Cells with Modified Glycosylation—HeLaS3 cells were obtained from the Cell Resource Center for Biochemical Research (Tohoku University, Miyagi, Japan), and maintained in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine and 50 μM 2-mercaptoethanol under 5% CO_2 at 37°C. To modify the cell surface glycans, cells were cultured for 24 h in the presence of 1 mM deoxymannojirimycin (DMJ) (Sigma), 2 μg/ml kifunensine (KIF) (Calbiochem, San Diego, CA), or 10 μg/ml swainsonine (SW) (Calbiochem). Endo-β-*N*-acetylglucosaminidase H (Endo H, 5.0×10^3 U; New England Biolabs, Ipswich, MA) was added to 2×10^6 DMJ-treated HeLaS3 cells suspended in 500 μl of HEPES-buffered saline, pH 7.4, containing 0.1% bovine serum albumin, 1 mM $CaCl_2$, and 0.1% NaN_3 (HBSB). After incubation at 37°C for 1 h, the cells were washed twice with HBSB.

Flow Cytometry—HeLaS3 cells were washed with PBS(−) containing 1 mM EDTA and were suspended in HBSB at a concentration of 2×10^7/ml. Ten microlitres of cell suspension was incubated with the indicated concentration of PE-labelled sVIP36–SA at 25°C for 30 min in a 96-well plate (Millipore, Bedford, MA). After washes with HBSB, cells were suspended in 200 μl of HBSB containing 1 μg/ml propidium iodide (PI). For GNA or DSA binding experiments, 10 μg/ml of each biotynylated lectin was complexed with PE-SA at a molar ratio of 4:1 and then incubated with the cells at 25°C for 30 min. The fluorescence of stained cells was measured using a FACS Caliber and CellQuest software (BD Biosystems). The fluorescence at 575 nm associated with PE on the surface of the cells was recorded and converted to a mean fluorescence intensity (MFI). In total, 10,000 events of live cells gated by forward and side scattering and exclusion of PI were acquired for analysis.

When we examined the binding of the sVIP36–SA to the cells in the absence of calcium, 1 mM EDTA was added instead of 1 mM $CaCl_2$ in every step. For the monosaccharide inhibition assay, the sVIP36–SA was pre-incubated with the indicated concentration of monosaccharide at 25°C for 1 h before the addition of the cells. For the inhibition experiments with several high mannose-type glycans, 10 μg/ml of sVIP36–SA was mixed with 5 mg/ml of the various high mannose-type glycans at 25°C for 1 h before the incubation with the cells. sVIP36 was added to the cells as a monomer to examine the effect of oligomerization on the binding of sVIP36 to the cells. After incubation with the sVIP36 monomer at 25°C for 30 min, the cells were washed with HBSB and then stained with 10 μg/ml PE-SA at 25°C for 30 min.

Preparation of Biotinylated Cell Surface Proteins—Cells were cultured in the presence or absence of 1 mM DMJ for 24 h. After two washes with PBS, pH 8.0, containing 0.1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ [PBS(+)], 1.5×10^7 HeLaS3 cells were resuspended in 1.5 ml of PBS(+) containing 0.11% (v/v) of 10 mg/ml NHS-sulfo-biotin (Dojindo, Tokyo, Japan). The cells were then incubated at 4°C for 1 h. Serum-free RPMI medium was added to the cell suspension to quench the biotinylation reaction. The cells were washed with PBS(+) and lysed by adding lysis buffer (100 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, and 1 μg/ml leupeptin). Biotinylated proteins were precipitated with SA-agarose (Invitrogen, Carlsbad, CA), and then the precipitate was dried in a desiccator.

Preparation of Pyridylamino (PA)-Derivatized Oligosaccharides and HPLC Analysis—The *N*-linked oligosaccharides were liberated from the biotinylated glycoproteins by hydrazinolysis at 100°C for 2 h. After reacetylation, the sample was loaded onto 500 μl of Dowex 50W × 8 (H^+ form, 100–200 mesh, Muromachi Technos Co., Japan) packed into a 1 ml syringe. The column was washed with 1.5 ml of water, and the effluent and wash fractions were collected and lyophilized. The sample was dissolved with 1 ml of water, loaded onto a Carbograph tube (150 mg, Alltech), washed with 2 ml of water, and then eluted with 25% acetonitrile containing 0.05% trifluoroacetic acid (*22*). *N*-glycans were labelled with 2-aminopyridine according to the method of Kondo *et al*. (*23*). PA-oligosaccharides were desialylated with neuraminidase from *Arthrobacter ureafaciens* (Marukin Chuyu, Kyoto, Japan), then isolated with reverse- and normal-phase HPLC as reported previously by Tomiya *et al*. (*24*). Briefly, the neutral PA-sugars were separated on a Shim-Pak CLC-ODS column (6.0 mm × 150 mm). Elution was performed at a flow rate of 1 ml/min at 55°C using two solvents, A (10 mM sodium phosphate buffer, pH 4.2) and B (0.5% 1-buthanol in A). The column was equilibrated with a mixture of solvents A and B, 80:20 by volume. After the injection of a sample, the ratio of solvent B to A was increased with a linear gradient to 50:50 over 60 min. Each peak was identified by fluorescence monitoring (excitation, 320 nm; emission, 400 nm), collected, and lyophilized. Each lyophilized sample was dissolved in 20 μl of solvent C (described further) and then subjected to normal-phase HPLC with a TSK-Gel Amide-80 column (4.6 mm × 250 mm). Elution was performed at a flow rate of 1 ml/min at 40°C using two solvents, C and D. Solvent C was composed of 3% acetic acid in water with triethylamine, pH 7.3, and acetonitrile, 35:65 by volume. Solvent D was composed of 3% acetic acid in water with triethylamine, pH 7.3, and acetonitrile,

50:50 by volume. The column was equilibrated with 100% solvent C. After the injection of a sample, the ratio of solvent D to C was increased with a linear gradient from 0% to 100% solvent D over 50 min. Each peak was identified by fluorescence monitoring (excitation, 300 nm; emission, 360 nm), collected, lyophilized, and analysed with MALDI-TOF-MS (Voyager DE-STR; Applied Biosystems, Foster City, CA), using 10 mg/ml 2,5-dihydroxybenzoic acid containing 5 mM NaCl as a matrix.

RESULTS AND DISCUSSION

Preparation of Soluble VIP36–SA Complex—First, we prepared a recombinant soluble VIP36 protein (sVIP36) corresponding to the luminal domain of wild-type VIP36 and a sugar-binding-deficient mutant of sVIP36 (D131N) (*25*) and expressed them in *E. coli*. sVIP36 and the D131N mutant both had a C-terminal enzymatic biotinylation signal and were biotinylated with the biotin ligase BirA to allow tetramerization with SA, which possesses four binding sites for biotin. SDS-polyacrylamide gel electrophoresis without boiling could validate biotinylation of sVIP36 and the band corresponding to sVIP36 monomer was disappeared after the addition of SA. The data indicated that almost all of the sVIP36 and D131N was biotinylated (data not shown). To clarify the content of several kinds of oligomeric forms of sVIP36–SA complex in the probe, we performed gel filtration chromatography of the complex of sVIP36 and SA mixed at ratios of 1:1, 2:1, 4:1 and 10:1. As shown in Fig. 2A and B, each of oligomeric form of sVIP36–SA complex was separated successfully. A mixture of sVIP36 and SA mixed at molar ratio 10:1 was eluted in two peaks corresponding to molecular weight 198 and 34 kDa, respectively (Fig. 2A). Based on the relative molecular weight of the peaks, these are thought to be sVIP36 tetramer complexed with SA and sVIP36 monomer, respectively. To rule out the inhibition of monomeric sVIP36 for the binding of the PE-labelled probe, we used the complex of sVIP36 and SA mixed at a ratio of 4:1 (sVIP36–SA) and it contained sVIP36 tetramer, trimer and dimmer, each complexed with SA at a ratio of about 2:4:1 and smaller amount of sVIP36 (Fig. 2B). Soluble VIP36 tetramer complexed with PE–SA can be purified by gel filtration. However, a part of sVIP36 is released from SA during storage of sVIP36 tetramers, and it may influence the binding. So we used the sVIP36–SA complex without purification. This procedure is easy, reproducible, and suitable for the limited amount of samples.

Preparation of Membrane-based Glycans—The second strategy, preparation of membrane-based high mannose-type glycans, was accomplished by the treatment of HeLaS3 cells with several α-mannosidase inhibitors, such as DMJ, KIF and SW. DMJ inhibits ER α-mannosidase I and II, and causes accumulation of the $Man_9GlcNAc_2$ isomer (*2, 26*). KIF strongly inhibits ER α-mannosidase I but not ER α-mannosidase II, and causes accumulation of $Man_9GlcNAc_2$ and Man_{8C} isoform (*27*). SW, which inhibits Golgi α-mannosidase II, causes hybrid-type glycans to be expressed on the cell surface (*28*).

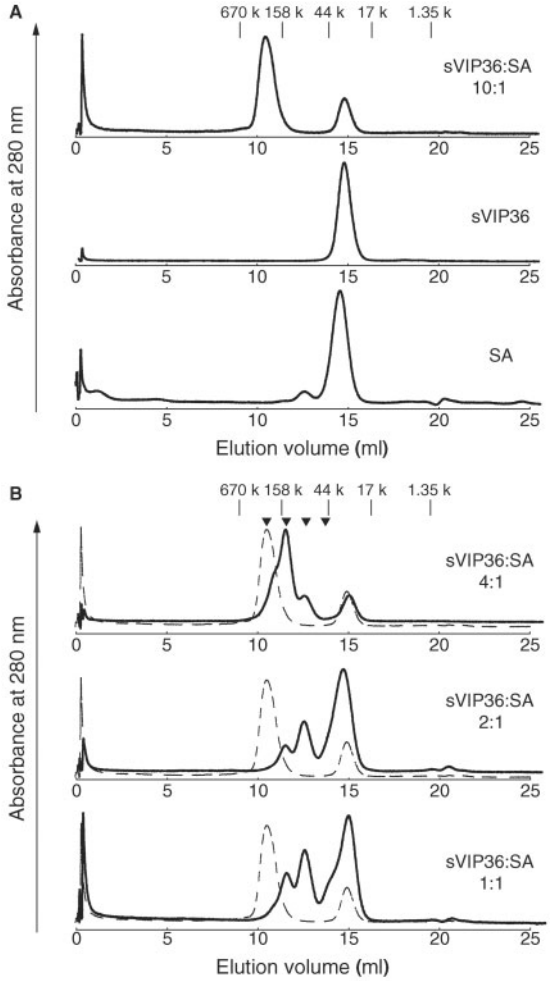

Fig. 2. **Analysis of sVIP36–SA complex by gel filtration chromatography.** Biotinylated sVIP36 was incubated with SA at indicated molar ratios at 25°C for 30 min and then analysed by gel filtration as described in Materials and Methods. Eluting position of molecular weight standards are shown by vertical bars on top. (A) Elution profile of sVIP36 and SA mixed at a ratio 10:1, and those of sVIP36 and SA, respectively. (B) Elution profiles of sVIP36 and SA mixed at a ratio 4:1, 2:1 and 1:1, respectively (solid line). Broken line indicates the elution profile of sVIP36 and SA mixed at molar ratio of 10:1. Arrowheads indicate the positions of sVIP36 tetramer-SA (198 kDa), sVIP36 trimer-SA (163 kDa), sVIP36 dimer-SA (128 kDa), and sVIP36 monomer-SA (94 kDa), respectively.

When tested in cell culture using HeLaS3 cells, complex-type glycans were decreased from 36.2% to 2.7% by treatment of 1 mM DMJ for 24 h as demonstrated by reverse- and normal-phase HPLC and MALDI-TOF-MS analysis (Fig. 3 and Table 1). In contrast, the amount of high mannose-type glycans, especially of $Man_{7-9}GlcNAc_2$, was increased from 30.7% to 69.6% by DMJ treatment on the HeLaS3 cells (Table 1). Then we performed the binding assay of sVIP36–SA against such DMJ-, KIF- and SW-treated HeLaS3 cells, because we expected that ligands on the cell surface would be clustered due to the lateral mobility and therefore would be easily accessible to multivalent lectins.

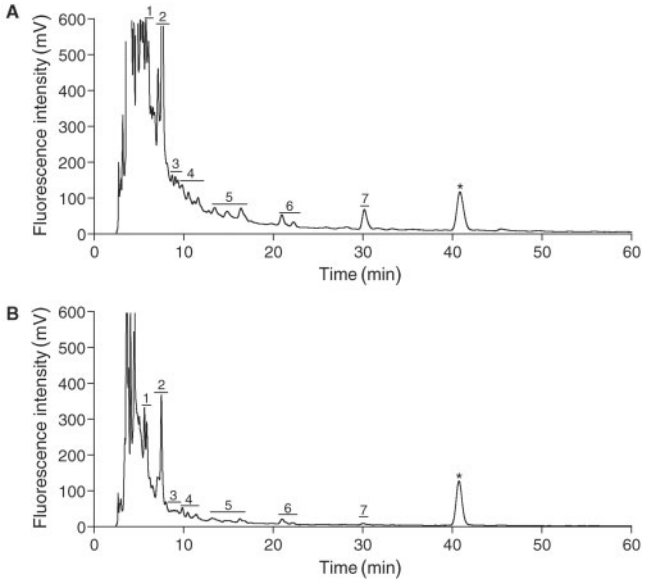

Fig. 3. **Analysis of PA-*N*-glycans by reverse-phase HPLC.** *N*-glycans from HeLaS3 cells cultured in the absence (A) or presence (B) of DMJ were prepared as described in the section 'Materials and Methods'. Released *N*-glycans were PA-derivatized, digested with sialidase, and then subjected to reverse-phase HPLC using an ODS column. Each fraction from 1 to 7 was separated with normal-phase HPLC using an Amide-80 column. The isolated PA-*N*-glycans were analysed with MALDI-TOF-MS. The composition of the major *N*-glycans can be found in Table 1. An asterisk indicates that the peak is a contaminant that was not detected in normal phase-HPLC.

PE-labelled sVIP36–SA Bound to HeLaS3 Cells Treated with DMJ or KIF—To use sVIP36 for flow cytometric analysis, we oligomerized sVIP36 with PE-labelled SA. Then we performed a binding assay using PE-labelled sVIP36 oligomers and HeLaS3 cells treated with DMJ, KIF or SW. First, to confirm the enhanced expression of high mannose-type glycans on the cell surface following inhibitor treatment, we stained the cells with two plant lectins (Fig. 4A). The binding of GNA, a high mannose-type selective lectin, to the HeLaS3 cells was increased by treatment with DMJ or KIF. This agreed with the data obtained from an HPLC analysis (Fig. 3 and Table 1). SW treatment also increased the binding of GNA to HeLaS3 cells because GNA can bind to the terminal Manα1,3Man sequence of hybrid-type sugar chains (*29*), whose expression is enhanced by SW. The binding of DSA, which is specific for GlcNAcβ2(GlcNAcβ6 or 4)Man containing *N*-glycans (*30*), to the cells showed a staining pattern opposite to that of GNA. The binding of DSA to the cells was decreased by DMJ and KIF treatment, because of lower expression of complex- and hybrid-type glycans on the cell surface (Fig. 4A). The binding of DSA to the SW-treated cells decreased slightly, indicating that the expression of tri- and tetraantennary complex-type glycans on the cell surface also decreased.

Second, we monitored the binding of the sVIP36–SA to HeLaS3 cells after DMJ, KIF or SW treatment. The sVIP36–SA bound to cells treated with DMJ or KIF in the presence of 1 mM Ca^{2+}, but failed to bind to intact cells or cells treated with SW (Fig. 4A). In the presence of 0.1 mM Ca^{2+}, the sVIP36–SA binding was observed to the same extent (data not shown). In contrast, 1 mM EDTA inhibited the binding of sVIP36–SA to cells treated with DMJ and KIF (Fig. 4A) and further addition of calcium at the final concentration of 1 mM recovered the binding (data not shown), indicating that this binding was Ca^{2+}-dependent and reversible. This is consistent with a previous report that VIP36 is a Ca^{2+}-dependent lectin (*17*). We also prepared a D131N mutant of sVIP36, because Asp131 is conserved and has been reported to be essential for both the sugar-binding and Ca^{2+} coordination of leguminous lectins (*31*). As shown in Fig. 4B, the D131N-SA did not bind to the cells treated with DMJ, although sVIP36–SA bound to the cells in a dose-dependent manner. This finding suggests that the binding of sVIP36 to the cells is mediated by a sugar-binding site homologous to those of leguminous lectins.

The Binding of sVIP36–SA to DMJ-treated Cells is Dependent on High Mannose-type Glycans—To verify that the binding of sVIP36–SA to DMJ-treated cells depends on high mannose-type glycans, we examined the binding of sVIP36–SA to DMJ-treated cells after a 1 h incubation with Endo H at 37°C. The binding of GNA and sVIP36–SA to the cells treated with Endo H was decreased (Fig. 5A). The binding of sVIP36–SA was highly sensitive to Endo H compared to that of GNA. On the other hand, the binding of DSA was almost the same. This was because that DSA bound to complex-type glycans, which were not digested with Endo H.

Next, we performed the inhibition of the binding using various monosaccharides. The binding of sVIP36–SA was specifically inhibited by mannose in a dose-dependent manner (Fig. 5B). These data support that the sVIP36–SA bound to the mannose-containing glycans generated by DMJ treatment. Furthermore, we carried out an inhibition assay with a panel of high mannose-type glycans. As shown in Fig. 6, Man_9, Man_{8B}, Man_{8C} and Man_7 inhibited the binding of sVIP36–SA to the DMJ-treated cells more effectively than Glc_1Man_9, Glc_1Man_{8B}, Glc_1Man_{8C} and Glc_1Man_7. Kamiya *et al*. (*17*) reported that VIP36 recognizes the A arm (Fig. 1) of deglucosylated high mannose-type glycans, and our data are consistent with their observation.

Oligomerization of sVIP36 is Necessary for Strong Binding—Finally, we estimated the contribution of oligomerization of sVIP36 to the binding of sVIP36 to the cells. For this purpose, we examined the binding of monomeric sVIP36 to DMJ-treated cells under the same conditions. Cells treated with DMJ were allowed to bind to monomeric sVIP36, followed by staining with PE-SA. As shown in Fig. 4C, no binding of monomeric sVIP36 was observed, indicating that oligomerization of sVIP36 by PE-SA is necessary to monitor the binding of sVIP36 to the cells. When DMJ-treated HeLaS3 cells was stained with sVIP36 and PE-SA complex mixed at a molar ratio 4:1, fluorescence intensity was 4 times larger than that stained with sVIP36 and PE–SA complex mixed at a ratio 1:1 (data not shown). In case of leguminous *Griffonia simplicifolia* isolectins, the association constants of monovalent, divanent, trivalent, and tetravalent isolectins for human type A erythrocytes

Table 1. **Composition of the major PA-*N*-glycans of biotinylated cell surface glycoproteins fractionated by HPLC and analysed by MALDI-TOF-MS.**

Fractions from ODS[a]	Peak from amide	*m/z*[b]			Composition	Relative quantity[c] (%)	
		Observed (control, DMJ)		Theoretical		Control	DMJ
1	1-a	1984.54	1984.74	1984.80	$Man_9GlcNAc_2$-PA	9.5	23.9
	1-b	1822.57	1822.62	1822.66	$Man_8GlcNAc_2$-PA	13.7	30.3
	1-c	1660.81	1660.63	1660.51	$Man_7GlcNAc_2$-PA	7.5	15.4
2	2-a	1497.93	–	1498.37	$Man_6GlcNAc_2$-PA	8.9	–
3	3-a	1336.97	–	1336.23	$Man_5GlcNAc_2$-PA	2.8	–
5	5-a	1742.77	–	1742.62	$Hex_2HexNAc_2Man_3GlcNAc_2$-PA	7.8	–
7	7-a	2619.93	–	2619.44	$Hex_4HexNAc_4dHex_1Man_3GlcNAc_2$-PA	24.8	–
	7-b	1889.42	1888.58	1888.76	$Hex_2HexNAc_2dHex_1Man_3GlcNAc_2$-PA	3.6	2.7

Monosaccharides were denoted as follows: Man, mannose; GlcNAc, N-acetylglucosamine; Hex, Hexose; HexNAc, N-acetylhexosamine; dHex, deoxyhexose.

[a]PA-derivatized *N*-glycans were isolated with ODS column, and then separated with Amide-80 column. Each PA-*N*-glycan was analyzed with MALDI-TOF-MS. Fractions 4 and 6 in Fig. 3 were not assigned because they contained only minor peaks (<2%/each peak) which could not be identified by MALDI-TOF-MS.

[b]*m/z* of each peak was observed as $[M + Na]^+$.

[c]Relative quantity was calculated based on Amide-80 elution profile. The sum of PA-oligosaccharides on this table is 78.6% (control) and 72.3% (DMJ), respectively.

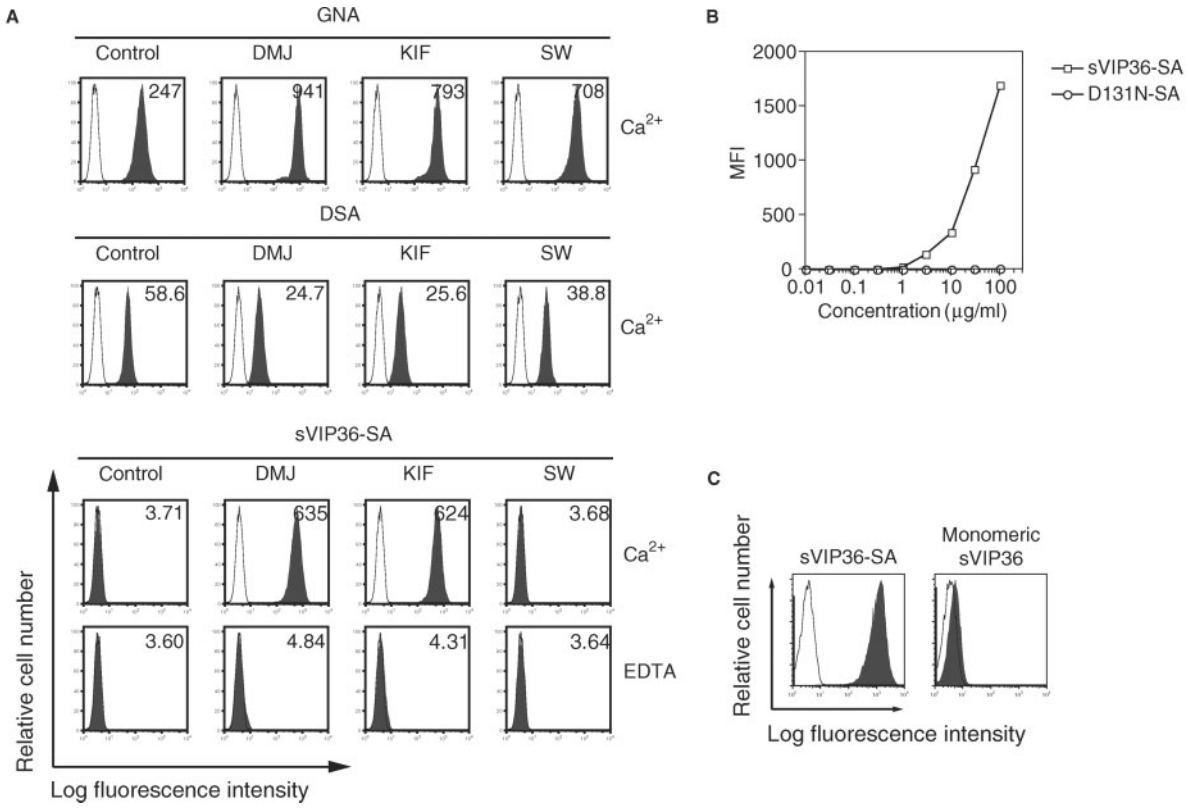

Fig. 4. **Binding of sVIP36–SA to the cells treated with glycosylation inhibitors.** (A) HeLaS3 cells were cultured in the presence of 1 mM DMJ, 2 μg/ml KIF, or 10 μg/ml SW for 24 h. Then the cells were incubated with 10 μg/ml PE-labelled GNA, DSA or sVIP36–SA as indicated (filled histograms) or PE-SA as the control (thin lines). The value in each panel indicates the MFI of PE-labelled lectins. Data are representative of three independent experiments with similar results. (B) HeLaS3 cells treated with 1 mM DMJ for 24 h were incubated with the indicated concentration of sVIP36–SA (open squares) or D131N-SA (open circles) and analysed by flow cytometry. Data are representative of three independent experiments with similar results. (C) sVIP36 was added to DMJ-treated HeLaS3 cells at 25°C for 30 min and then mixed with PE-SA. The same cells were mixed with sVIP36–SA. Filled histograms indicate the staining of above probes and thin lines indicate the staining of PE-SA only. Data are representative of three independent experiments with similar results.

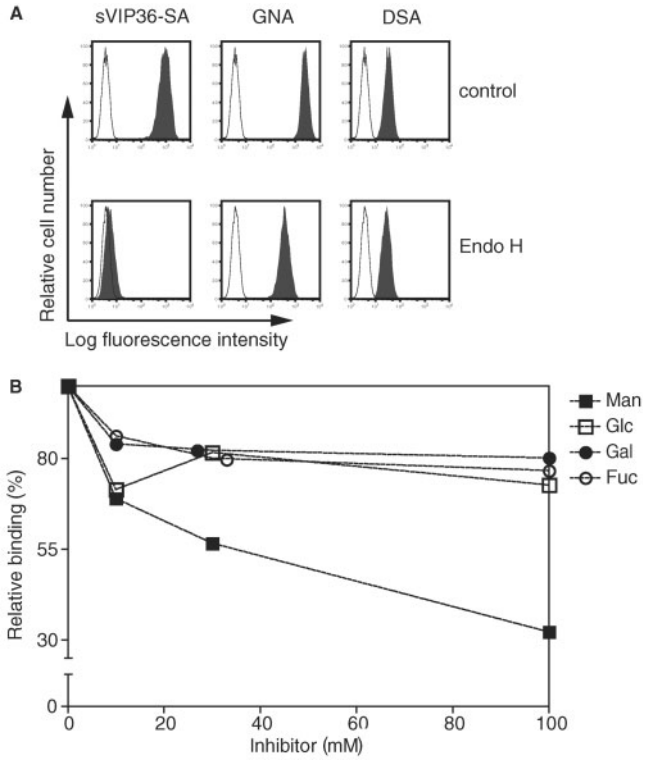

Fig. 5. **The binding of sVIP36–SA to DMJ-treated cells is mediated by high mannose-type glycans on the cell surface.** (A) HeLaS3 cells cultured in the presence of 1 mM DMJ for 24 h were incubated with or without 1.0×10^4U/ml Endo H at 37°C for 1 h. The cells were then stained with PE-labelled lectins (filled histograms) or PE-SA (thin lines). Data are representative of three independent experiments with similar results. (B) sVIP36–SA (10 μg/ml) and the indicated concentrations of various monosaccharide were mixed and kept at 25°C for 1 h before the addition of DMJ-treated HeLaS3 cell. The binding of sVIP36–SA in the presence of each inhibitor is shown relative to that in the absence of inhibitor, adjusted to 100%. The data shown are means of three independent experiments with similar tendencies. The inhibition by GlcNAc and GalNAc is similar to Glc and Gal, respectively (data not shown).

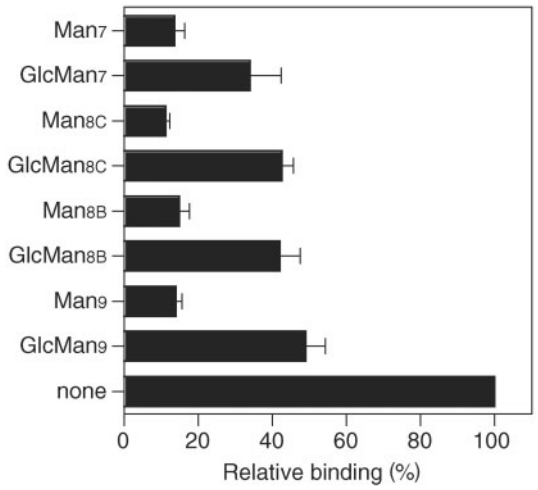

Fig. 6. **Inhibition of the binding of sVIP36–SA to DMJ-treated cells by high mannose-type glycans.** sVIP36–SA (10 μg/ml) and various high mannose-type glycans (5 mg/ml) were mixed and kept at 25°C for 1 h before the addition of HeLaS3 cells treated with 1 mM DMJ for 24 h. The binding of sVIP36–SA in the presence of each inhibitor is shown relative to that in the absence of inhibitor (none) adjusted to 100%. The data shown are means of two independent experiments with similar tendencies.

are 7.5×10^5, 2.9×10^6, 1.4×10^7 and $1.2 \times 10^7\,M^{-1}$, respectively (*32*). Further several reports indicated that lectins bind with several orders of magnitude greater affinity to cells than to the haptenic sugars. These reports are in good agreement with the observation showing that oligomerization of sVIP36 would increase its avidity, making it easy to monitor the binding of sVIP36 to ligands.

These data indicate that binding of VIP36 to high mannose-type glycan ligands can successfully be visualized *in vitro*. Stalk region of VIP36 may function to keep away from steric hindrance of each lectin domain and sugar-binding activity of sVIP36–SA complex was able to monitor successfully. A number of proteins that recognize high mannose-type glycans are beginning to emerge. For example, the molecular chaperones CNX and CRT bind to monoglucosylated high mannose-type glycans to fold glycoproteins correctly (*3, 4*); EDEM, a key molecule in the degradation of misfolded glycoproteins, acts through interactions with high mannose-type glycans (*5–7*); and the cargo receptors ERGIC-53 and VIP36 bind to high mannose-type glycans to transport glycoproteins (*33, 34*). These interactions between lectin and glycans are reported to be weak and transient, probably because the receptors have to both catch and release the ligands during transport within cells. Therefore, it is desirable to establish a method with high sensitivity and reliability to monitor the interaction between these lectins and high mannose-type glycans. Although methods for the detection of lectin–glycan interactions have recently become available, only a few have succeeded in monitoring the interaction of intracellular lectins with high mannose-type glycans. Frontal affinity chromatography has some benefits, including the simultaneous screening of many samples and the ability to determine the dissociation constant. However, a large amount of lectin is required when the affinity of the lectin to the glycan is low (*17*); therefore, this method depends on lectins for exploratory experiments examining the sugar-binding activity of putative lectin proteins. Our current method was able to monitor the lectin activity of sVIP36 with a small amount of sample (0.1 μg), and is especially suitable for first-step experiments. Fukui *et al*. (*35*) developed microarrays of oligosaccharides in the form of neoglycolipids displayed on a nitrocellulose membrane to detect the weak binding of lectin to glycan and succeeded in detecting the weak sugar-binding activity of the cation-independent mannose 6-phosphate receptor (*36*). One advantage of their method is that the neoglycolipids on the membrane could be multivalent ligands. Our method using membrane-based glycans also has this advantage. Intracellular lectins mainly function in the ER to

recognize high mannose-type glycans, so DMJ or KIF treatment of the cells is suitable for the expression of ligands on the cell surface. For lectins recognizing another complex-type glycans, several lectin-resistant CHO and BHK cells are available (*37, 38*). Furthermore, the avidity of the lectin is another merit of our method, because sVIP36 monomers did not bind to DMJ-treated HeLaS3 cells but higher-affinity oligomers did.

In summary, we treated cells with *N*-glycosylation inhibitors to generate membrane-based high mannose-type glycans, and then tested the binding of sVIP36–SA to the cells. The binding of sVIP36–SA to the cells was specifically mediated by high mannose-type glycans, especially Man_9, Man_8, and Man_7. These data are in good agreement with a previous report using frontal affinity chromatography. Our membrane-based high mannose-type glycan assay is suitable for small samples and is an extremely powerful tool for detecting the lectin activity. Our strategy is useful for understanding weak interactions between the lectins and glycans in biological phenomena and is applicable to the identification of novel lectins of unknown classes.

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