Vesicular-integral membrane protein, VIP36, recognizes high-mannose type glycans containing \(\alpha 1\rightarrow 2\) mannosyl residues in MDCK cells

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The 36 kDa vesicular-integral membrane protein, VIP36, has been originally isolated from MDCK cells as a component of glycolipid-enriched detergent-insoluble complexes containing apical marker proteins, and its luminal domain shows homology to leguminous plant lectins and ERGIC-53. As the first step to identify the functional role of VIP36, the carbohydrate binding specificity of VIP36 was investigated using a fusion protein of glutathione-S-transferase and luminal domain of VIP36 (Vip36). It was found that VIP36 recognizes high-mannose type glycans containing \(\alpha 1\rightarrow 2\) Man residues and \(\alpha\)-amino substituted asparagine. The binding of Vip36 to high-mannose type glycans was independent of Ca\(^{2+}\) and the optimal condition was pH 6.0 at 37°C. The concentration at which half inhibition of the binding by Man\(_7\alpha\)-GlcnAc2\(\cdot\)NacAsn occurred was 1.0 \(\times\) \(10^{-9}\) M. The association constant between Man\(_2\alpha\)-GlcnAc2 in porcine thyroglobulin and immobilized Vip36 was 2.1 \(\times\) \(10^{8}\) M\(^{-1}\) as determined by means of a biosensor based on surface plasmon resonance. These results indicate that VIP36 functions as an intracellular lectin recognizing glycoproteins which possess high-mannose type glycans, \((\text{Man} \alpha 1\rightarrow 2)\text{Man}\alpha 4\text{Man}\text{GlcnAc2}.

Key words: high-mannose/lectin/MDCK cells/vesicular transport/VIP36

Introduction

Polarized epithelial cells have distinct apical and basolateral membrane domains, each of which contains specific proteins and lipid components (Rodriguez-Boulan and Powell, 1992). In polarized MDCK cells, not only proteins and phospholipids but also various glycoconjugates are sorted into transport vesicles in the trans-Golgi network (TGN), delivered to the apical or basolateral cell surface (Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990; Matter and Mellman, 1994), and function in the specialized domains.

It has been recently demonstrated that lectin-like proteins such as mannose-6-phosphate receptor (Dahms et al., 1989), calnexin (Ou et al., 1993; Ware et al., 1995), and calreticulin (Nauseef et al., 1995) play important roles in intracellular vesicular transport. Accordingly, we focused on an intracellular lectin which might be related to trafficking mechanism of glycoproteins. VIP36 (vesicular-integral membrane protein of 36 kDa) was isolated as a component of glycolipid-enriched detergent-insoluble complexes containing the apical marker protein and is present in carrier vesicles (Fiedler et al., 1994). The exoplasmic/luminal domain of VIP36 is homologous to the N-terminal luminal domain of ERGIC (ER-Golgi intermediate compartment)-53 (Fiedler and Simons, 1994), which is an intermediate compartment marker (Schweizer et al., 1988) and identical to MR60, a mannos-binding protein (Arar et al., 1995). Although the biological function of VIP36 is unknown as in the case of ERGIC-53, both proteins show a significant similarity to leguminous plant lectins, suggesting that they might be involved in the delivery of saccharide-bearing molecules in the secretary pathway (Fiedler and Simons, 1994). Fiedler and Simons (1996) previously reported that VIP36 might weakly recognize N-acetylgalactosamine (GalNAc) residue and does not interact with \(^{3}\text{H}\)mannose-labeled glycopeptides. However, since we obtained the contradictory results in preliminary experiments, we reinvestigated the precise carbohydrate binding specificity of VIP36.

Here we show that recombinant VIP36 expressed as a fusion protein with glutathione-S-transferase (GST), GST/Vip36, binds to secretion proteins and postnuclear supernatant (PNS) proteins in MDCK cells under the condition of pH 6.0 at 37°C. This binding is independent of Ca\(^{2+}\) and is specifically inhibited by several nanomoles of glycopeptides with high-mannose type sugar chains containing \(\alpha 1\rightarrow 2\)Man residues and \(\alpha\)-amino substituted asparagine. The binding constant between GST/Vip36 and thyroglobulin containing high-mannose type sugar chains can be measured by means of a biosensor based on surface plasmon resonance. Our results strongly suggest that VIP36 functions as an intracellular lectin recognizing high-mannose type sugar chains in MDCK cells.

Results

\(\text{The recombinant GST/Vip36 bound } \text{[35S]-labeled proteins from MDCK cells}\)

VIP36 is isolated from TGN-derived vesicles of MDCK cells and its luminal domain shows homology to leguminous plant lectins and ERGIC-53 (Fiedler and Simons, 1994; Fiedler et al., 1994). As the first step to identify the functional role of VIP36, the carbohydrate binding specificity of VIP36 was studied. The luminal region of VIP36, Vip36, was expressed as a fusion protein with GST in \(E.\text{coli}\), and the GST/Vip36 was purified by means of a glutathione Sepharose 4B column. First, we investigated whether the GST/Vip36 binds \(^{35}\text{S}\)-labeled secretory proteins or \(^{38}\text{S}\)-labeled PNS prepared from metabolically labeled MDCK cells under the conditions described in Materials and methods. The amount of binding observed using GST itself in the presence of glutathione-coupled Sepharose 4B, which was less than 10% of the amount using GST/Vip36, was taken as background. As shown in Figure 1, GST/Vip36 bound \(^{35}\text{S}\)-labeled PNS...
Effects of temperature on specific binding of GST/Vip36 to post-nuclear supernatant (PNS) (a), and effects of pH on specific binding to secretory proteins (b) and PNS (c) prepared from metabolically [35S]-labeled MDCK cells. [35S]-labeled PNS and secretory proteins were prepared as described in Materials and methods and incubated for 1 h at 37°C in binding buffer (pH 6.0) containing 0.8 µM GST/Vip36, 4 × 10^6 d.p.m. [35S]-labeled protein and 5 mM EDTA or the indicated cation at 1 mM.

**Fig. 1.** Effects of temperature on specific binding of GST/Vip36 to post-nuclear supernatant (PNS) (a), and effects of pH on specific binding to secretory proteins (b) and PNS (c) prepared from metabolically [35S]-labeled MDCK cells. [35S]-labeled PNS and secretory proteins were prepared as described in Materials and methods and incubated for 1 h at 37°C in binding buffer (pH 6.0) containing 0.8 µM GST/Vip36, 4 × 10^6 d.p.m. [35S]-labeled protein and 5 mM EDTA or the indicated cation at 1 mM.

dose-dependently at 37°C (Figure 1a, solid circles) but the binding activity was markedly reduced at 4°C (Figure 1a, open circles). The maximal binding activity was observed at pH 6.0 rather than under neutral pH conditions in the case of both secretory proteins (Figure 1b) and PNS (Figure 1c). The binding activity was not inhibited by 0.2 M α-methylmannoside, 0.2 M GalNAc, or 1 mM trypsin-digested transferrin containing bi-antennary sugar chains (×, solid stars, solid squares, respectively, in Figure 1b,c), but was markedly inhibited upon addition of 1 mM trypsin-digested porcine thyroglobulin containing high-mannose type and bi- to tetra-antennary sugar chains (open squares in Figure 1b,c), suggesting that the binding between Vip36 and PNS/secretory (glyco)proteins occurs via some specific glycopeptide moieties. When the cell homogenate was fractionated into membrane and cytosolic proteins, the binding activity was recovered in the membrane fraction (Figure 2). These results indicate that the Vip36-binding PNS proteins are mainly membrane-derived glycoproteins. Furthermore, it was demonstrated that the binding activity of Vip36 was not affected by EDTA or metal ions including Ca²⁺, Mg²⁺, and Mn²⁺, although Vip36 contains a Ca²⁺ binding domain as well as sugar binding domains (Fiedler et al., 1994). The divalent cation-independent character of Vip36 is different from ERGIC-53 which also contains both types of domains and which binds sugar residues in a Ca²⁺-dependent manner (Itin et al., 1996). From the results, the binding character of Vip36 in MDCK cells was further analyzed using PNS at 37°C, pH 6.0.

**Fig. 2.** Effects of divalent cations on GST/Vip36 binding. Total membrane and cytosolic proteins were prepared as described under Materials and methods. The binding assay was carried out by incubation for 1 h at 37°C in binding buffer (pH 6.0) containing 0.8 µM GST/Vip36, 4 × 10^6 d.p.m. [35S]-labeled protein and 5 mM EDTA or the indicated cation at 1 mM.

**GST/Vip36 bound glycoproteins**

Vip36 binds secretory proteins as well as membrane-bound proteins in MDCK cells as shown in Figure 1 and Figure 2. In order to examine whether Vip36 binds glycoproteins via the specific glycan portions, various glycoproteins for which the sugar chain structures had been determined as shown in Table I were used for the binding assays except for batroxobin. Because of its thrombin activity, asialo-batroxobin glycopeptides were used for the binding assay. As shown in Figure 3, the binding of Vip36 to PNS was markedly inhibited by porcine thyroglobulin (Figure 3, open circles) or variant surface glycoproteins (VSG) of Trypanosoma brucei (Figure 3, solid circles) in a dose-dependent manner. Both of these glycoproteins have high-mannose type sugar chains containing mostly Man₇,9GlcNAc₂ (Ito et al., 1977; Zamze et al., 1991). Although ribonuclease B and ovalbumin also contain one high-mannose type sugar chain per molecule, which consists of rather smaller-sized sugar chains, Man₇GlcNAc₂ >>

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**Notation:**
- α-methylmannoside
- GalNAc
- trypsin-digested transferrin
- porcine thyroglobulin
- EDTA
- Ca²⁺, Mg²⁺, Mn²⁺
- Man₇,9GlcNAc₂
- Man₇GlcNAc₂

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**Materials and methods**

- [35S]-labeled PNS and secretory proteins
- 0.8 µM GST/Vip36
- 5 mM EDTA
- pH 6.0
- 37°C
- Binding buffer

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**Results**

- Binding activity at 4°C reduced compared to 37°C
- Maximal binding activity at pH 6.0
- Not inhibited by α-methylmannoside, GalNAc, trypsin-digested transferrin
- Markedly inhibited by trypsin-digested porcine thyroglobulin

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**References**

- Fiedler et al., 1994
- Itin et al., 1996
- Ito et al., 1977
- Zamze et al., 1991
VIP36 recognizes high-mannose type glycans (Tai et al., 1975; Liang et al., 1980), the inhibitory effects of ribonuclease B and ovalbumin were not significant (Figure 3, inverted solid triangles and open triangles, respectively). These results suggested that VIP36 recognizes larger-sized high-mannose type sugar chains. Furthermore, placental alkaline phosphatase containing GPI (glycosylphosphatidylinositol) anchor (Redman et al., 1994) and bi-antennary complex type sugar chains (T. Endo et al., 1988) did not inhibit the binding (Figure 3, inverted open triangles). Transferrin (Figure 1b,c, solid squares), hCG (human chorionic gonadotropin) (Figure 3, solid triangles) containing four mucin type sugar chains/mol and α2→3 sialylated bi-antennary sugar chains (Y. Endo et al., 1979), and asialo-batroxobin glycopeptides (Figure 3, solid squares) containing β1→4 N-acetylgalactosaminylated bi-antennary sugar chains (Tanaka et al., 1992) did not show any effects on the binding activity. From these results, it seems that the inhibitory effects of VSG and thyroglobulin might be due to the high-mannose type sugar chains, not the glycan portion of the GPI anchor, any complex type sugar chain or mucin type sugar chain, although there remains the possibility that the number of carbohydrate units per glycoprotein may also affect the inhibitory efficiency.

Binding of GST/Vip36 was inhibited by high-mannose type glycopeptides containing α1→2Man residues

The results described so far suggested that Vip36 binds some high-mannose type sugar chains of secretory glycoproteins and membrane glycoproteins. In order to determine the further precise carbohydrate binding specificity of Vip36, various high-mannose type glycoconjugates were prepared and added to the binding assays. As summarized in Table II, upon testing of a series of high-mannose type glycopeptides, only Man₆₋₉GlcNAc₂·Nac·Asn inhibited the binding of Vip36 effectively and, in contrast, Man₅·GlcNAc₂·Nac·Asn did not inhibit the binding, consistent with the effect of glycoproteins on the binding activity as shown in Figure 3. Because the concentration required for 50% inhibition by Man₆₋₉GlcNAc₂·Nac·Asn (1×10⁻⁷ M) was two orders higher than those in the cases of Man₇₋₉GlcNAc₂·Nac·Asn (1×10⁻⁹ M), it is suggested that the presence of the two α1→2Man residues in high-mannose type sugar chains is important for strong carbohydrate recognition by VIP36.

α-Amino substituted asparagine residue is necessary for binding of GST/Vip36

Next, in order to determine the aglycon specificity, the inhibitory effects of high-mannose type sugar chains with various reducing terminal side structures were examined. As shown in Table II and Figure 4, Man₆₋₉GlcNAc₂·Nac·Asn inhibited Vip36 binding to PNS and, in contrast, Man₅·GlcNAc₂·Nac·Asn did not show any inhibitory effects. Accordingly, the carbohydrate binding specificity of VIP36 is considered to be as shown in Figure 5.

**Table I.** Structures and number of sugar chains of glycoproteins used in this study

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>N-Glycans/mol</th>
<th>Structure of glycan</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroglobulin</td>
<td>ND*</td>
<td>Bi- to tetra-antennary High-mannose type</td>
<td>Kondo et al., 1977</td>
</tr>
<tr>
<td>VSG from Trypanosoma</td>
<td>5</td>
<td>Bi-antennary High-mannose type</td>
<td>Ito et al., 1977</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>1</td>
<td>Bi-antennary GPI anchor</td>
<td>Endo et al., 1988</td>
</tr>
<tr>
<td>Ribonuclease B</td>
<td>1</td>
<td>High-mannose type GPI anchor</td>
<td>Ferguson et al., 1988</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>1</td>
<td>High-mannose type Hybrid type</td>
<td>Liang et al., 1980</td>
</tr>
<tr>
<td>Transferrin</td>
<td>2</td>
<td>α2→6Sialylated bi-antennary</td>
<td>Tai et al., 1975</td>
</tr>
<tr>
<td>hCG</td>
<td>4</td>
<td>α2→3Sialylated mono- and bi-antennary mucin type</td>
<td>Spik et al., 1975</td>
</tr>
<tr>
<td>Asialo-batroxobin</td>
<td>2</td>
<td>β1→4N-Acetyl-galactosaminylated bi-antennary</td>
<td>Endo et al., 1979</td>
</tr>
</tbody>
</table>

*Not determined.
Fig. 4. Effects of high-mannose type sugar chains with various reducing terminal side structures on specific binding of GST/Vip36 to PNS. The experimental conditions were the same as those in the Figure 3 caption, except that various kinds of high-mannose type sugar chains were added at indicated concentrations. Open circles, M₉GlcNAc₂·N-ac·Asn; solid circles, M₉GlcNAc·Asn; open triangles, M₉GlcNAc-GlcNAc; solid triangles, M₉GlcNAc.

Fig. 5. The carbohydrate binding specificity of GST/Vip36.

These results suggest that the lectin activity of VIP36 requires not only α₁→2Man residues in high-mannose type sugar chains, but also α-amino substituted asparagine residues as aglycon specificity.

The association constant between GST/Vip36 and porcine thyroglobulin was $2.1 \times 10^8$ M⁻¹

Using a biosensor based on surface plasmon resonance, we analyzed the association constant between GST/Vip36 and glycoproteins. Transferrin or thyroglobulin, at 500 nM in each instance, was introduced onto the sensor chip with immobilized GST/Vip36 or GST. No specific affinity was observed between transferrin and GST/Vip36, whereas a specific interaction between thyroglobulin and GST/Vip36 was evident (Figure 6). After subtracting the background value (the amount of interaction between thyroglobulin and GST), the $K_D$ value was calculated to be $4.7 \times 10^{-9}$ M and the $K_A$ value was $2.1 \times 10^8$ M⁻¹. Porcine thyroglobulin contains at least one Man₈₋₉·GlcNAc₂ per molecule. Since half inhibition of the interaction between GST/Vip36 and PNS was seen with $1 \times 10^{-9}$ M of Man₈₋₉·GlcNAc₂·N-ac·Asn (Table II), the $K_P$ value between thyroglobulin and GST/Vip36 obtained from the biosensor analysis may reflect an interaction via carbohydrate.

Table II. Inhibition of GST/Vip36 binding to the postnuclear supernatant (PNS) derived from MDCK cells by various high-mannose type glycoconjugates

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration for 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man₅·GlcNAc₂</td>
<td>---*</td>
</tr>
<tr>
<td>Man₅·GlcNAcβ₁→Asn</td>
<td>---</td>
</tr>
<tr>
<td>Man₅·GlcNAcβ₁→N-ac·Asn</td>
<td>---</td>
</tr>
<tr>
<td>Manα₁→2Man₅·GlcNAc₂</td>
<td>---</td>
</tr>
<tr>
<td>Manα₁→2Man₅·GlcNAcβ₁→Asn</td>
<td>---</td>
</tr>
<tr>
<td>Manα₁→2Man₅·GlcNAcβ₁→N-ac·Asn</td>
<td>$1 \times 10^{-7}$M</td>
</tr>
<tr>
<td>(Manα₁→2)₂Man₅·GlcNAc₂</td>
<td>---</td>
</tr>
<tr>
<td>(Manα₁→2)₂Man₅·GlcNAcβ₁→Asn</td>
<td>---</td>
</tr>
<tr>
<td>(Manα₁→2)₂Man₅·GlcNAcβ₁→N-ac·Asn</td>
<td>$1 \times 10^{-9}$M</td>
</tr>
<tr>
<td>(Manα₁→2)₃Man₅·GlcNAc₂</td>
<td>---</td>
</tr>
<tr>
<td>(Manα₁→2)₃Man₅·GlcNAcβ₁→Asn</td>
<td>---</td>
</tr>
<tr>
<td>(Manα₁→2)₃Man₅·GlcNAcβ₁→N-ac·Asn</td>
<td>$1 \times 10^{-9}$M</td>
</tr>
<tr>
<td>(Manα₁→2)₄Man₅·GlcNAc₂</td>
<td>---</td>
</tr>
<tr>
<td>(Manα₁→2)₄Man₅·GlcNAcβ₁→Asn</td>
<td>---</td>
</tr>
<tr>
<td>(Manα₁→2)₄Man₅·GlcNAcβ₁→N-ac·Asn</td>
<td>$1 \times 10^{-9}$M</td>
</tr>
</tbody>
</table>

* No inhibition up to 1 mM.

Furthermore, it was investigated whether both [³⁵S]-labeled PNS and [³⁵S]-labeled secretory glycoproteins bound to Vip36 via high-mannose type N-glycans. The respective [³⁵S]-labeled proteins bound to Vip36 were developed to SDS–PAGE and visualized with autoradiography. Although many newly synthesized proteins were [³⁵S]-labeled (Figure 7, lanes 1 and 6), Vip36 bound to several [³⁵S]-labeled PNS proteins and secretory proteins (Figure 7, lanes 3 and 8). Their binding was inhibited in
VIP36 recognizes high-mannose type glycans

VIP36 interacts with β-GalNAc residues, the binding strength should be very weak. Furthermore, Fiedler and Simons used \[^{3}H\]mannose-labeled glycopeptide, which were prepared by the digestion of high concentrations of pronase for binding assay. Because it is well known that high concentration of pronase hydrolyzes high-mannose type glycans of glycoproteins to (Man\(_{3}\)β1→4GlcNAc\(_{1}\)→4GlcNAc-Asn, thus prepared \[^{3}H\]mannose-labeled glycopeptides should not bind VIP36, as identified in this paper. However, there remains the possibility that the carbohydrate binding specificity of GST/Vip36 might be affected by the presence of GST.

ERGIC-53 recycling between ER and cis-Golgi is one of the candidates possessing lectin activity which may function in relation to intracellular transport. ERGIC-53 is identical to the mannose-binding protein MR60 (Arar et al., 1995) and shows high homology to VIP36 in the regions of the carbohydrate binding sites and Ca\(_{2+}\) binding site; however, these two proteins are quite different from each other in terms of lectin character. ERGIC-53 recognizes a mannose in a Ca\(_{2+}\)-dependent manner (Itin et al., 1996), while VIP36 neither recognizes monosaccharides nor requires any divalent cations for binding to high-mannose type sugar chains. Because VIP36 was shown to bind Ca\(_{2+}\) as reported by Fiedler and Simons (1996), there remains the possibility that VIP36 binds other components in a Ca\(_{2+}\)-dependent manner. This point has to be resolved by analyzing point mutants of VIP36 in the calcium binding site in subsequent studies.

In relation to the vectorial transport in the epithelial cells, Wandler-Ness et al. (1990) isolated distinct transport vesicles mediating the delivery of plasma membrane proteins to the apical and basolateral domains of MDCK cells. The apical membrane has an unusually high glycosphingolipid content, leading to the hypothesis that glycolipids and apical proteins are co-sorted in the TGN into the vesicular carriers that are formed by glycosphingolipid self-association into microdomains or “rafts” onto which apically destined proteins attach. Because VIP36 is one of the membrane proteins present in TGN-derived transport vesicles isolated from the MDCK cells and its coding cDNA sequence is highly homologous to that of legume lectin (Fiedler and Simons, 1994; Fiedler et al., 1994), the lectin activity of VIP36 might be somewhat related to the sorting machinery in TGN; although we do not have any evidence at this time.

\(\alpha\)-Amino substituted Asn-linked high-mannose type glycans are recognized in a Ca\(_{2+}\)-independent manner by VIP36, and VIP36 binds sugar chains most effectively at pH 6.0, which is very close to the pH condition in TGN. Such characters may be useful to resolve the functional role of VIP36. It is the subsequent interest whether VIP36 functions to sort glycoproteins having high-mannose type glycans in TGN and to transport them to the apical surface in MDCK cells. There have already been some reports concerning the roles of N-linked glycans in intracellular transport. Scheiffele et al. (1995) showed that growth hormone, which is nonglycosylated and secreted from both sides in MDCK cells, is secreted from the apical side when N-glycosylated. Gut et al. (1998) demonstrated that N-linked glycans are recognized in apical sorting of membrane proteins.

In addition to VIP36, VIP21 (Kurzchalia et al., 1992) which is identical to caveolin (Glenney, 1992), and annexin XIIIb (Fiedler et al., 1995) were identified as vesicular integral membrane proteins. The functional roles of each factor and the correlation among such factors including VIP36 remain unclear and further studies are needed. These studies might lead to elucidation of the roles of glycoconjugates in the mechanisms of sorting and vesicle formation in intracellular transport.

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**Discussion**

This paper clearly demonstrated that VIP36 recognizes high-mannose type glycans containing two \(\alpha\)→2mannose residues and \(\alpha\)-amino-substituted asparagine, and the glycon specificity is unique as follows: VIP36 does not bind (Man\(_{3}\)β1→2)\(_{1-4}\)Man\(_{5}\)GlcNAc\(_{2}\)Asn, or (Man\(_{3}\)β1→2)\(_{1-4}\)Man\(_{5}\)GlcNAc\(_{2}\) but binds (Man\(_{3}\)β1→2)\(_{1-4}\)Man\(_{5}\)GlcNAc\(_{2}\)N-acetylAsn. The binding character indicates that the positively charged primary amino residue of asparagine disturbs the lectin activity of VIP36, which requires not only carbohydrate moieties but also \(\alpha\)-amino substituted asparagine. Such a binding character of VIP36 might allow it to discriminate between glycoproteins to be transported and luminal degradation products such as free oligosaccharides and asparagine-linked oligosaccharides. Although the importance of reducing terminal side structures in interactions with lectins has been reported for several lectins including lentil lectin (Kornfeld et al., 1981), milk lectin (Kornfeld et al., 1981), and E\(_{4}\)-PHA (Kobata and Yamashita, 1989), the carbohydrate binding specificity of VIP36 including \(\alpha\)-amino substituted asparagine is demonstrated for the first time in this paper.

Fiedler and Simons (1996) previously reported that the interaction between MDCK cells and VIP36 is inhibited by high concentrations of GalNAc, and 0.1% of \[^{3}H\]galactose-labeled glycopeptides in MDCK cells binds to VIP36-agarose column and eluted by 0.1 M GalNAc; by contrast, \[^{3}H\]mannose-labeled glycopeptides pass through the column. However, we obtained the different results by using \[^{35}S\]labeled membrane/secretory proteins in MDCK cells for VIP36-binding assay. The binding activity between GST/Vip36 and \[^{35}S\]labeled membrane/secretory proteins in MDCK cells was not inhibited by 0.2 M GalNAc or 1 mM batroxobin glycopeptides containing \(\beta\)-GalNAc residues on the nonreducing terminal (Figures 1, 3). Accordingly, even if...
Materials and methods

Materials and chemicals

MDCK cells, strain II were donated by Dr. M. Tashiro (National Institute of Infectious Diseases, Tokyo, Japan) and maintained in MEM supplemented with 5% FBS in plastic dishes. Expre[35S]-labeled proteins were purchased from DuPont NEN (Boston, MA), and endo-β-N-acetylgalactosaminidase H (Endo H) from Seikagaku Kogyo Co., LTD. (Tokyo, Japan). Man-6-phosphate, transferrin, hCG, thyroglobulin, and ribonuclease B were purchased from Sigma Chemical Co. Human placental alkaline phosphatase was kindly provided by Dr. Y. Ikehara (Fukuoka University, School of Medicine, Japan). Batroxobin was from American Diagnostical Inc. (Greenwich, CT). VSG of Trypanosoma brucei were prepared as described previously (Fukushima et al., 1997). Man$_{5-9}$GlcNAc were obtained from Man$_{5-9}$GlcNAc$_2$ by Endo H digestion. Man$_{5-6}$GlcNAc$_2$ and Man$_{5-9}$GlcNAc$_2$ were prepared by hydrazinolysis of ovalbumin (Tai et al., 1975) and porcine thyroglobulin (Ito et al., 1977), respectively.

Metabolic labeling and cell fractionation

Cells at confluence in 100 mm dishes were incubated for 16 h in medium containing 1.5 mg/l l-methionine, 3.1 mg/l l-cysteine·2HCl, and 25 µCi/ml Expre[35S]-labeled cells were washed twice in PBS containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$, and lysed by incubation on ice for 30 min in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris/HCl, pH 7.0) containing 1 mM PMSF, 100 kallikrein U/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin (protease inhibitor cocktail). The lysate was centrifuged for 10 min at 3000 r.p.m., and the supernatant, after centrifugation in 150 mM NaCl, and 1 mM PMSF, and the dialysates were concentrated with an Amicon Centricon-30.

Assay of binding of GST/Vip36 to [35S]-labeled proteins of MDCK cells

The binding of GST/Vip36 to [35S]-labeled proteins was assayed in a solution containing 0.8 µM GST/Vip36 or GST, 4 × 10$^6$ d.p.m. [35S]-labeled proteins derived from MDCK cells, 25 mM MES and 150 mM NaCl, pH 6.0, in a total volume of 100 µl, with incubation for 60 min at 37°C. After incubation, 50 µl of a 50% slurry of glutathione Sepharose 4B equilibrated with the same binding buffer above was added to each assay sample and mixed gently for 30 min. The Sepharose beads were then washed four times with binding buffer by centrifugation at 5000 r.p.m. for 10 s at 4°C. Scintillation fluid containing Triton X-100 was added, the samples were mixed and centrifuged. Then the radioactivity of the supernatant was determined. The radioactivity obtained in control samples with GST was taken as background. In another case, the samples were subjected to SDS–PAGE and the proteins bound to GST/Vip36 were visualized by autoradiography.

Preparation of high-mannose type aspartyloligosaccharides from porcine thyroglobulin

High-mannose type oligosaccharides, Man$_{7-9}$GlcNAc$_2$, were prepared from 3 g of porcine thyroglobulin as described in a previous paper (Ito et al., 1997). Each oligosaccharide was converted to aspartyloligosaccharides by treatment with Arthrobacter protophormiae endo-β-N-acetylgalactosaminidase (Endo A) (Fan et al., 1995). Aspartyl-β-N-acetylgalactosamine (Asn-GlcNAc) was prepared from Fmoc-conjugated Asn-GlcNAc (kindly provided by Dr. T. Inazu, Noguchi Institute, Tokyo, Japan) by treatment with 20% piperidine. Five hundred microliters of reaction mixture containing 0.1 M Asn-GlcNAc, 30% acetone, each oligosaccharide at 1 mM, and 40 mM of Endo A (kindly provided by Dr. K. Takegawa, Faculty of Agriculture, Kagawa University, Japan) in 50 mM ammonium acetate (pH 6.0) was incubated at 37°C for 15 min and boiled to stop the reaction. After Sephadex G-25 gel filtration to remove excess Asn-GlcNAc, oligosaccharide fractions were applied to a AG 50W-X2 cation exchange column (0.7 × 5.2 cm, H$^+$ form; Bio-Rad). Unreacted oligosaccharides passed through the column, whereas aspartyl oligosaccharides bound to the column and were eluted with 50 mM CaCl$_2$. Fractions were immediately neutralized and desalted. The yield of aspartyl oligosaccharides in the endo A reaction was 40%. Man$_5$GlcNAc$_2$-Asn and Man$_6$GlcNAc$_2$-Asn were prepared from ovalbumin as described in a previous paper (Tai et al., 1975).

Preparation of [35S]-labeled secretory proteins

The confluent cells cultured in plastic dishes were incubated for 30 min in starvation medium lacking methionine and cysteine, and labeled for 2 h with 250 µCi/ml Expre[35S]. After removing the labeling medium, the cells were washed three times with normal culture medium and incubated for another 2 h in the culture medium. The medium was collected and applied to a PD-10 (Pharmacia Biotech) column in each instance to separate the labeled glycoproteins from free [35S]methionine.

Preparation of GST/Vip36

Total RNA was extracted from MDCK cells using ISOGEN (Nippon Gene, Tokyo, Japan) and cDNA synthesis was carried out using Ready-to-Go T-Primed First-Strand Kit (Pharmacia Biotech). The target cDNA encoding the extracellular domain of VIP36 (Vip36, amino acids 76–304) was amplified by PCR performed by 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The primers used were 5'-GGGGATCCGATA- TACGTACGGT-3' as sense primer and 5'-GGGGAATTC- CCCCATCTCCGGAAGTTC-3' as antisense primer. After the sequence of the amplified fragment was confirmed the identical one shown by Fiedler et al. (1994), the fragment was inserted into downstream of GST using the EcoRI and BamHI sites in pGEX-2TK (Pharmacia Biotech). Vip36 was expressed as a fusion protein, i.e., fused with GST in E.coli and GST/Vip36 was purified by affinity chromatography using glutathione Sepharose 4B according to the manufacturer’s instructions. GST was expressed and purified by the same method. The purified GST/Vip36 and GST were dialyzed against 25 mM MES, pH 6.0, 150 mM NaCl, and 1 mM PMSF, and the dialysates were concentrated with an Amicon Centricon-30.
Solid phase binding assay based on surface plasmon resonance

The affinity between VIP36 and human transferrin or porcine thyroglobulin was measured using a GST/Vip36- or GST-immobilized assay system in a BIACore 2000 instrument (BIACORE AB, Uppsala, Sweden). GST/Vip36 or GST was covalently immobilized on the sensor surface by amine coupling. The amount of GST/Vip36 immobilized was 12,759.0 RU and the interaction was monitored at 37°C as the change in the surface plasmon resonance response. The apparent rate constants for binding were determined by the methods of Fägerstam et al. (1992).

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Abbreviations

TGN, trans-Golgi network; Man, mannose; GcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Asn, l-asparagine; PNS, post nuclear supernatant; Endo H, endo-β-N-acetylglucosaminidase H; HCG, human choric gonadotropin; VSG, variant surface glycoproteins; GPI, glycosylphosphatidylinositol; Endo A, endo-β-N-acetylglucosaminidase A; Asn-GlcNAc, asparaty-β-N-acetylglucosaminyl; TBS, Tris-buffered saline (25 mM Tris base, 137 mM NaCl, 2.7 mM KCl, pH 7.4); ERGIC, ER-Golgi intermediate compartment; VIP36, vesicular integral membrane protein of 36kDa; GST, glutathione-S-transferase; NAc-Asn, Nα-acetyl-L-asparagine.

References


