Involvement of VIP36 in Intracellular Transport and Secretion of Glycoproteins in Polarized Madin-Darby Canine Kidney (MDCK) Cells*

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VIP36, an intracellular lectin that recognizes high mannose-type glycans (Hara-Kuge, S., Ohkura, T., Seko, A., and Yamashita, K. (1999) Glycobiology 9, 833–899), was shown to localize not only to the early secretory pathway but also to the plasma membrane of Madin-Darby canine kidney (MDCK) cells. In the plasma membrane, VIP36 exhibited an apical-predominant distribution, the apical/basolateral ratio being ~2. Like VIP36, plasma membrane glycoproteins recognized by VIP36 were found in the apical and basolateral membranes in the ratio of ~2 to 1. In addition, secretory glycoproteins recognized by VIP36 were secreted ~2-fold more efficiently from the basolateral membrane than from the apical membrane. Thus, the apical/basolateral ratio of the transport of VIP36-recognized glycoproteins was correlated with that of VIP36 in MDCK cells. Upon overproduction of VIP36 in MDCK cells, the apical/basolateral ratios of both VIP36 and VIP36-recognized glycoproteins were changed from ~2 to ~4, and the secretion of VIP36-recognized glycoproteins was greatly stimulated. In contrast to the overproduction of VIP36, that of a mutant version of VIP36, which has no lectin activity, was of no effect on the distribution of glycoproteins to apical and basolateral membranes and inhibited the secretion of VIP36-recognized glycoproteins. Furthermore, the overproduction of VIP36 greatly stimulated the secretion of a major apical secretory glycoprotein of MDCK cells, clusterin, which was found to carry at least one high mannose-type glycan and to be recognized by VIP36. In contrast to the secretion of clusterin, that of a non-glycosylated apical-secretion protein, galectin-3, was not stimulated through the overproduction of VIP36. These results indicated that VIP36 was involved in the transport and sorting of glycoproteins carrying high mannose-type glycan(s).

Newly synthesized secretory and membrane proteins exit from the ER1 in transport vesicles targeted to the Golgi apparatus. Vesicular transport through the Golgi is often accompanied by post-translational modifications, such as glycosylation of cargo proteins until they have reached the trans-Golgi Network. In the trans-Golgi Network, proteins are sorted into vesicles bound for different destinations including the plasma membrane, the endosome/lysosome, and secretory granules. The protein sorting has been one of the most interesting issues in the study of vesicular protein traffic processes, but its molecular mechanisms remain largely unresolved.

It has been recently demonstrated that intracellular lectins play important roles in vesicular transport: for example, mannose-6-phosphate receptor (1) as a receptor recognizing the marker for lysosomal enzymes, calnexin (2, 3) and calreticulin (4) as molecular chaperones, and ERGIC-53 (5) possibly as a transport cargo receptor. ERGIC-53 is an intermediate compartment marker (6), and it is identical to MR60, a mannose-specific membrane lectin (7) with a carbohydrate-binding domain homologous to that of lectins of leguminous plants (8). The N-terminal luminal domain of ERGIC-53, which includes the carbohydrate-binding domain, has an amino acid sequence similar to that of vesicular integral membrane protein of 36 kDa (VIP36) (9). VIP36 has been originally isolated from MDCK cells as a component of detergent-insoluble, glycolipid-enriched complexes containing apical marker proteins (10), and it has been recently shown to exist in early secretory pathway (11). In addition, we have shown that VIP36 has lectin activity recognizing high mannose-type glycans containing over seven mannose residues (12). These observations have implied that VIP36 is an intracellular lectin involved in the intracellular transport of glycoproteins carrying high mannose-type glycan(s). In this study, to address the function of VIP36, we reexamined the localization of VIP36 and investigated the effect of overproduction of VIP36 and a mutant VIP36 defective in lectin activity on the metabolism and transport of VIP36-recognized glycoproteins in MDCK cells.

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK cells, strain II, were donated by Dr. M. Tashiro (National Institute of Infectious Diseases, Tokyo, Japan). MDCK cells were maintained in minimum Eagle’s medium supplemented with 5% fetal bovine serum, and MDCK/VIP36 and MDCK/mVIP36 cells were cultured in the same medium containing 500 μg/ml G418 (Geneticin) (Invitrogen) in plastic dishes. For polarization, cells were seeded at 20,000 cells/cm² in plastic dishes. For polarization, cells were seeded at 20,000 cells/cm² in plastic dishes. For localization, cells were fixed with 3% paraformaldehyde at room temperature for 20 min and incubated with Tris-buffered saline (10 mM Tris hydroxymethylaminomethane, pH 7.4, 150 mM NaCl) containing 0.2% Triton X-100.

VIP36, vesicular integral-membrane protein of 36 kDa; mVIP36, mutant VIP36; PBS, phosphate-buffered saline; sulfo-NHS-biotin, sulfo-N-hydroxysuccinimide-biotin; HRP, horseradish peroxidase.

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‡ The abbreviations used are: ER, endoplasmic reticulum; Endo H, endo-β-N-acetylglucosaminidase H; GST, glutathione S-transferase;
polycarbonate filter, the numbers of cells were 5.0 ± 0.3 x 10^4 cells corresponding to 0.70 ± 0.04 mg of protein in all cases of MDCK, MDCK/VIP36, and MDCK/mVIP36 cells.

Preparation of Antibodies—Anti-VIP36 polyclonal antibody was prepared by immunization of a rabbit with GST-Vip36 as antigen, and anti-clusterin antibody was specific for the glutathione S-transferase (GST) fusion partner were removed by adsorption using a GST protein matrix. Anti-galectin-3 polyclonal antibody was prepared by immunization of a rabbit with recombinant human galectin-3. Anti-clusterin goat polyclonal IgG was purchased from Santa Cruz Biotechnology, Inc.

Immunofluorescence Microscopy—The MDCK cells were immersed in a fixative composed of 1% paraformaldehyde and 0.1% picric acid in 0.01 M PBS for 24 h at 4 °C. After fixation, the tissues were dehydrated with a graded ethanol series at 0 °C, embedded on Lowicryl K4M, and then cured for 3 days at −35 °C. Ultrathin sections were treated with 3% hydrogen peroxide for 10 min and 1% normal goat serum for 1 h and then incubated with anti-VIP36 antibody (1:100) with PBS containing 1% bovine serum albumin for 24 h at 4 °C. After washing, the samples were incubated with purified fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin (1:200) followed by rinsing with PBS and then distilled water. Finally, the sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (Hitachi H-800).

Preparation of Domain-selectively Biotinylated Plasma Membrane Proteins—Cell surface molecules were domain-selectively biotinylated using a membrane-impermeable biotin analog sulfo-NHS-biotin (14) with slight modifications described below. After washing the cells, which were grown to confluence on polycarbonate filters, three times with ice-cold PBS, either the apical or basolateral surface proteins were biotinylated in PBS containing 1 mg/ml sulfo-NHS-biotin for 45 min at 4 °C followed by blocking with 50 mM glycine in PBS. For preparation of domain-selective plasma membrane glycoproteins, the cells were washed three times with ice-cold PBS, and either the apical or basolateral surface proteins were biotinylated. Then the cells were lysed by incubation on ice for 30 min in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.0) containing protease inhibitor mixture. The lysate was centrifuged for 10 min at 3000 rpm, and the supernatant was referred to as the detergent extract. Biotinylated apical or basolateral surface proteins in the supernatant were precipitated by means of A-Sepharose (Amersham Biosciences) as described above, for biotinylated proteins or by autodigestion for metabolic labeled proteins.

Preparation of Domain-selective 35S-Secretory Glycoproteins—For preparation of 35S-labeled secretory proteins, the confluent cells cultured on polycarbonate filters were incubated for 30 min in starvation medium lacking methionine and cysteine and then labeled for 1 h with 250 μCi/ml (9.25 MBq/ml) Expe implants (PerkinElmer Life Sciences).

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 from the apical and basolateral chambers separately and applied to a PD-10 (Amersham Biosciences) column to separate the labeled secretory proteins from free labeled [35S]methionine/cysteine, and concentrated with Centriplus 10 (Amicon Inc.).

Plasmid Construction and Isolation of Clones Showing Stable Overproduction—Expression of the GST fusion protein GST-Vip36 in Escherichia coli and confirmation of its functionality have been described previously (12). Site-directed mutagenesis was performed by a two-step polymerase chain reaction method (15) using DNA of GST-Vip36 as the template and sets of appropriate primers that overlap in the region where the mutation was introduced. The cDNA of mutated GST-Vip36(D131N) was cloned into pGEX-2TK (Amersham Biosciences).

The full-length cDNA encoding VIP36 (D131N) was also prepared by a two-step polymerase chain reaction method using VIP36 cDNA as the template and subcloned into pSVKneo (16). A full-length cDNA encoding mutant VIP36 (D131N) was also prepared for preparation of clusterin, metabolic-labeled clusterin in medium was immunoprecipitated for 1 h at 4 °C with anti-clusterin antibody and Protein A-Sepharose. The bound materials were eluted with 0.4 N acetic acid, neutralized with pyridine, and dried. N-glycans were released from the clusterin treatment with 5 units of N-glycanase (Roche Diagnostic GmbH) at 37 °C for 18 h.

Purified [3H]glucosamine-labeled glycoproteins were subjected to hydrazinolysis at 100 °C for 8 h to release 3-H-glycans from glycoproteins (17). After N-acetylation and reduction with NaBH₄, neutral oligosaccharides were separated by high voltage paper electrophoresis with pyridine-acetic acid buffer, pH 5.4 (pyridine:acetic acid:water, 3:1.35:7) at a potential of 1000 V/cm for 1 h and applied to a C₂₄ Sepharose column (1 ml) equilibrated with PBS. Oligosaccharides were eluted with 5 mM a-methyl-β-glucoside (elution for biantennary glycans) and then 0.2 M α-methyl-β-mannoside (elution for high mannosetype glycans) in PBS (18). The fractions of high manno-type glycans were further fractioned by Bio-Gel P-4 (extra fine, 2 × 100 cm) column chromatography (19). The peak fractions from Bio-Gel P-4 chromatography were digested with 1 unit of a-mannosidase (Aspergillus niger) (20) in 0.1 M acetate buffer (pH 4.5) in a total volume of 50 μl at 37 °C for 18 h and then fractionated by Bio-Gel P-4 chromatography to determine the manno-type glycans structure.

RESULTS

VIP36 Is Localized Not Only to the Early Secretory Pathway but Also to Plasma Membrane—We previously determined that VIP36 binds to high manno-type glycans bearing Man 1-2 residues (12). To elucidate the functional role of VIP36, we first analyzed the localization of VIP36 biochemically using an immunopurified polyclonal anti-VIP36 antibody raised against GST-Vip36 (12). Using immunogold electron microscopy, we confirmed for the first time that endogenous VIP36 is predominantly localized to the early secretory pathway (Fig. 1a). Fig. 1a supports the results of Füllekrug et al. (11) and Dahn et al. (21), who recently showed that endogenous VIP36 localizes to the early secretory pathway, cycling between ER-
Fig. 1. Distribution of VIP36 in MDCK cells. Immunoelectron micrograph of VIP36 in a MDCK cell showed VIP36 localized on ER and Golgi complex (a) and on plasma membrane (b). Nuc, nucleus. Immunoelectron micrograph without primary anti-VIP36 antibody (c) is shown as control. Bar = 1 μm. Immunofluorescence of VIP36 in a MDCK cells with (d) or without (e) treatment with Triton X-100 showed that VIP36 is distributed from ER to plasma membrane throughout the cell. Immunofluorescence micrograph without primary anti-VIP36 antibody (f) is shown as control corresponding to the phase contrast micrograph (g). The length of one side = 40 μm.
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Fig. 2. Localization of VIP36 in plasma membrane. The plasma membrane proteins of MDCK cells were labeled with (lanes 1 and 2) or without (lanes 3 and 4) membrane-impermeable biotin reagent, and the cells were then lysed with Nonidet P-40 lysis buffer. The cell lysate was incubated with monomeric avidin-Sepharose to purify plasma membrane. The avidin-bound proteins (lane 1, plasma membrane, and lane 3, background) and avidin-unbound proteins (lane 2, proteins without plasma membrane, and lane 4, total proteins) were subjected to immunoblot using anti-VIP36 antibody. When the chemiluminescence of the VIP36 bands was measured using Image Gauge, the chemiluminescence level of the VIP36 band in the plasma membrane (lane 1) was 15%. Results are the means of three experiments. (Standard deviations were less than 5.)

Golgi intermediate structures and the Golgi complex. To examine whether or not endogenous VIP36 is localized not only to the early secretory pathway but also to the plasma membrane, a section with plasma membrane (Fig. 1b) was examined, and several immunogold particles were observed on plasma membrane. Furthermore, immunofluorescence staining of MKD cells was performed with or without Triton X-100 treatment. When immunofluorescence staining was performed after Triton X-100 treatment, which enabled the antibody to permeate through plasma membrane, VIP36 seemed to exist broadly in MDCK cells (Fig. 1d). Even without Triton X-100 treatment, the antibody reacted with the preparation, and staining of plasma membranes was observed (Fig. 1e), suggesting that VIP36 existed on the cell surface as shown before (10).

In addition, when plasma membrane proteins of intact MDCK cells were biotinylated by a membrane-impermeable sulfo-NHS-biotin, plasma membrane proteins were isolated using a monomeric avidin-Sepharose by using intact cells. As shown in Fig. 2, the amount of VIP36 biotinylated by using intact cells was estimated to be ~15% of the total cellular VIP36. These results indicated that endogenous VIP36 was localized not only to the early secretory pathway but also to the plasma membrane of MDCK cells.

VIP36 in the Apical and Basolateral Domains of the Plasma Membrane of MDCK Cells in the Ratio of ~2:1—To examine whether there is a correlation between the distribution of VIP36 and VIP36-recognized glycoproteins in the polarized plasma membrane, we determined also the distribution of the VIP36-recognized glycoproteins in the polarized plasma membrane of MDCK cells. The apical and basolateral plasma membrane proteins were separately biotinylated, detergent extracts were prepared, and these preparations were incubated with a recombinant fusion protein, GST-Vip36 (12), which consisted of GST and a VIP36-derived peptide, or with GST alone. The membrane proteins bound to GST-Vip36 and GST were collected using glutathione-Sepharose beads, fractionated by SDS-PAGE, and then transferred to a nitrocellulose membrane followed by detection of biotinylated proteins using streptavidin-HRP and chemiluminescence reagent. As shown in Fig. 4, the apical membrane contained more glycoproteins bound to GST-Vip36 than the basolateral membrane. When the chemiluminescence of each lane was measured using Image Gauge (Fuji Photo Film), the amount of VIP36-binging glycoproteins in the apical membrane was estimated to be ~2-fold more than that in the basolateral membrane, although the total amount of biotinylated proteins derived from the apical membrane was similar to that of biotinylated proteins derived from the basolateral membrane (Table 1). In addition, the amount of VIP36-binding glycoproteins decreased markedly upon treatment of the apical and basolateral proteins with Endo H (Fig. 4 and Table 1). This confirmed that VIP36 recognized high mannose-type glycans and indicated that our assay system for measuring VIP36-binding glycoproteins worked well.

We also examined the amount of apical and basolateral secretory glycoproteins that was capable of binding to VIP36. The apical and basolateral secretory proteins of MDCK cells were metabolically labeled with [35S]methionine/cysteine, and we examined the mobility and intensity of glycoproteins in the polarized plasma membrane of MDCK cells. Next we examined whether VIP36 existed in the apical or basolateral membrane, or in both plasma membrane domains, of polarized MDCK cells. To discriminate between apical and basolateral VIP36, the apical and basolateral membrane proteins of MDCK cells were metabolically labeled with [35S]methionine/cysteine and then selectively biotinylated by the addition of membrane-impermeable sulfo-NHS-biotin in the apical or basolateral chamber. After extraction with detergent, proteins from apically and basolaterally biotinylated MDCK cells, preparations of which had the same total radioactive activity, were subjected to immunoprecipitation using anti-VIP36 antibody. The immunoprecipitated proteins were fractionated by SDS-PAGE and then transferred to a nitrocellulose membrane followed by detection of biotinylated VIP36 using streptavidin-HRP and chemiluminescence reagent. As shown in Fig. 3 (lanes 1 and 2), bands of VIP36 were found in both preparations from apically and basolaterally biotinylated MDCK cells. The amount of VIP36 in both bands was ~2-fold more than that in the plasma membrane of MDCK cells. Therefore, we prepared a bacterially produced mutant GST-Vip36 in which the Asp residue corresponding to Asp-131 of VIP36 was replaced with Asn (Fig. 5), and we examined the binding activity to glycoproteins. Unlike the original GST-Vip36, the mutant GST-Vip36 was incapable of binding to [35S]-labeled MDCK cells at all (Fig. 5B). This indicated...
VIP36 Is Involved in the Intracellular Transport of Clusterin—As described above, the results obtained from the experiments involving bulk membrane and secretory glycoproteins suggested that VIP36 was involved in the transport and sorting of glycoproteins. To confirm the involvement of VIP36 in the transport of glycoproteins, next we performed experiments focusing on a major apical secretory glycoprotein in MDCK cells. Clusterin is an 80-kDa glycoprotein, and it is released as a disulfide-linked heterodimeric complex (after intracellular proteolytic maturation) from the apical surface of the polarized MDCK cells (22). Clusterin contains seven potential N-glycosylation sites, three in the 35-kDa subunit and four in the 45-kDa subunit, and all N-glycosylation sites are used (23). The following results indicated that clusterin carried at least one high mannose-type glycan recognized by VIP36. After immunoprecipitation of \[^{3}H\]\(\text{glucosamine}\)-labeled clusterin, the \[^{3}H\]\(\text{N-glycans}\) were recovered from clusterin precipitated by Protein A beads as described under “Experimental Procedures.” As shown in Table III, 92.5% of the total radioactivity of \[^{3}H\]\(\text{N-glycans}\) was recovered as complex type glycans, consistent with the previous results (22). However, the radioactivity, which amounted to 7.5% of the total radioactivity, was recovered in a high mannose-type glycan fraction. Since a high mannose-type glycan contains only 2 mol of N-acetylglucosamine residues, whereas most of complex type glycans abundant in MDCK cells contain 5 mol of N-acetylglucosamine, this result indicated that over 15% of total N-glycans of clusterin was of high mannose-type glycans. Thus, one of the N-glycans of clusterin was most likely of high mannose-type glycan.

To examine whether the high mannose-type glycan of clusterin, Man\(_{n}\)GlcNAc\(_{2}\), is recognized by VIP36, the detergent extract from MDCK cells was subjected to immunoprecipitation with anti-VIP36 antibody followed by immunoblot with anti-clusterin antibody. As shown in Fig. 6a, clusterin was bound to VIP36. These results involving MDCK/VIP36 and MDCK/mVIP36 suggested that VIP36 was involved in the transport and sorting of glycoproteins bearing high mannose-type glycans.

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That the mutation changing Asp-131 to Asn (D131N) in VIP36 resulted in loss of lectin activity.

We introduced the D131N mutation into a full-length VIP36 cDNA clone carried by a mammalian expression vector and then introduced the wild type VIP36 cDNA and the mutant VIP36 cDNA into MDCK cells by transfection. Stable transfectants obtained (designated as MDCK/VIP36 and MDCK/mVIP36, respectively) which overproduced the wild type of VIP36 or the D131N-mutant VIP36 at a similar level, were purified and subjected to biochemical characterization. When the distribution of VIP36 (or mutant VIP36) in MDCK, MDCK/VIP36, and MDCK/mVIP36 cells was analyzed, we unexpectedly found that overproduction of VIP36 enhanced the apical distribution of VIP36; the apical/basolateral ratios of VIP36 in MDCK/VIP36 and MDCK/mVIP36 were 3.5 and 3.6, respectively, whereas it was 1.9 in MDCK cells (Fig. 3). This finding led us to examine whether or not the enhancement of apical distribution of VIP36 had an effect on the distributions of VIP36-recognized glycoproteins in polarized plasma membrane. The apical/basolateral ratios of membrane and secretory proteins that were capable of binding to VIP36 were 4.6 and 5.8, respectively, in the case of MDCK/VIP36 cells and 2.4 and 1.9, respectively, in the case of MDCK cells (Table II). Thus, in MDCK/VIP36 cells, the apical distribution of both VIP36 and VIP36-recognized glycoproteins was enhanced coincidentally. In contrast to MDCK/VIP36 cells, the MDCK/mVIP36 cells exhibited normal apical/basolateral ratios of VIP36-recognized membrane glycoproteins and secretory glycoproteins, similar to those exhibited by MDCK cells (Table II). Furthermore, overproduction of VIP36, but not that of the mutant, led to a striking increase in the apical secretion of glycoproteins that bound to VIP36 (Table II). In contrast, overproduction of the mutant VIP36 inhibited the secretion of glycoproteins that bound to VIP36. These results involving MDCK/VIP36 and MDCK/mVIP36 suggested that VIP36 was involved in the transport and sorting of glycoproteins bearing high mannose-type glycan(s).

VIP36 Is Involved in the Intracellular Transport of Clusterin—As described above, the results obtained from the experiments involving bulk membrane and secretory glycoproteins suggested that VIP36 was involved in the transport and sorting of glycoproteins. To confirm the involvement of VIP36 in the transport of glycoproteins, next we performed experiments focusing on a major apical secretory glycoprotein in MDCK cells. Clusterin is an 80-kDa glycoprotein, and it is released as a disulfide-linked heterodimeric complex (after intracellular proteolytic maturation) from the apical surface of the polarized MDCK cells (22). Clusterin contains seven potential N-glycosylation sites, three in the 35-kDa subunit and four in the 45-kDa subunit, and all N-glycosylation sites are used (23). The following results indicated that clusterin carried at least one high mannose-type glycan recognized by VIP36. After immunoprecipitation of \[^{3}H\]\(\text{glucosamine}\)-labeled clusterin, the \[^{3}H\]\(\text{N-glycans}\) were recovered from clusterin precipitated by Protein A beads as described under “Experimental Procedures.” As shown in Table III, 92.5% of the total radioactivity of \[^{3}H\]\(\text{N-glycans}\) was recovered as complex type glycans, consistent with the previous results (22). However, the radioactivity, which amounted to 7.5% of the total radioactivity, was recovered in a high mannose-type glycan fraction. Since a high mannose-type glycan contains only 2 mol of N-acetylglucosamine residues, whereas most of complex type glycans abundant in MDCK cells contain 5 mol of N-acetylglucosamine, this result indicated that over 15% of total N-glycans of clusterin was of high mannose-type glycans. Thus, one of the N-glycans of clusterin was most likely of high mannose-type glycan.

To examine whether the high mannose-type glycan of clusterin, Man\(_{n}\)GlcNAc\(_{2}\), is recognized by VIP36, the detergent extract from MDCK cells was subjected to immunoprecipitation with anti-VIP36 antibody followed by immunoblot with anti-clusterin antibody. As shown in Fig. 6a, clusterin was
TABLE I

|                        | Apical | Basolateral |
|------------------------|--------|-------------|
| Total biotinylated plasma membrane proteins | 3.27   | 4.00        |
| Biotinylated plasma membrane glycoproteins bound to VIP36 | 1.02   | 0.43        |
| Biotinylated plasma membrane glycoproteins bound to VIP36 after Endo H treatment | 0.14   | 0.09        |

Values are the averages of duplicate assays with a variation of <8% between duplicates. The data are for one of three experiments with reproducible results.

A mutant GST/Vip36: Leu His Gly Asn Gly Ile Ala

**Fig. 5.** The mutation changing Asp-131 to Asn (D131N) in VIP36 results in loss of lectin activity. One hundred µl of the binding buffer containing the indicated amount of GST-Vip36 or mutated D131N GST-Vip36 and 4–5 × 10^6 dpm of [35S]-detergent extract was incubated for 60 min at 37 °C. The bound proteins were precipitated by the addition of glutathione-Sepharose beads, and the amount of radioactivity was determined by scintillation counting. (C), GST-Vip36; (●), mutated D131N GST-Vip36.

Inconsistent with our finding that a significant fraction of VIP36 is localized to the plasma membrane, Füllekrug et al. (11) have reported that, unlike overexpressed VIP36, endogenous VIP36 is not detected on the plasma membrane of MDCK cells. This inconsistency might be attributed to the difference in the antibody used for the detection and/or to the alteration of property of MDCK cells during cell passages. VIP36 is localized to the early secretory pathway, cycling between ER-Golgi intermediate structures and the Golgi complex (21), and it is localized also to the plasma membrane in a significant amount, at least in the case of our MDCK cells. Therefore, it is likely that, at the late Golgi or trans-Golgi Network, a large fraction of VIP36 is retrieved to ER-Golgi intermediate structures, and a small but significant fraction of VIP36 is exported out to the plasma membrane.

It is uncertain whether VIP36 cycles between the plasma membrane and the Golgi complex or endosomes. VIP36 has no obvious internalization signal in its short cytoplasmic tail. However, there is the possibility that VIP36 is associated with plasma membrane protein(s) carrying cytoplasmic internalization signal and is therefore co-internalized with such proteins. It is also possible that VIP36 has an unknown internalization signal on the apical plasma membrane.

In 30 min of chase time, a significant amount of [35S]clusterin was secreted by MDCK/VIP36 cells (see Fig. 8, lane 3) but not by MDCK cells (see Fig. 8, lane 7), implying that the rate of secretion of clusterin from MDCK/VIP36 cells was faster than that in MDCK cells (Fig. 7, a and c, and Fig. 8). On the other hand, overproduction of mutant VIP36 inhibited the secretion of clusterin, with the result that the rate of secretion of clusterin from MDCK/mVIP36 cells was slower than that in MDCK cells (Fig. 7, a and c). These results indicated that the overproduction of VIP36 in MDCK cells led to an increase in the rate of clusterin transport. In contrast to the secretion of glycosylated clusterin, the secretion of galectin-3, which is a non-glycosylated apical secretory protein in MDCK cells (24), was not affected by the overproduction of VIP36 (Fig. 7, b and d). Thus, VIP36 seemed to be involved in the transport of glycoproteins such as clusterin.
signal. To clarify the functional role of VIP36, we are currently examining whether VIP36 is internalized at the cell surface or not.

In the polarized plasma membrane of MDCK cells, both VIP36 and VIP36-recognized membrane proteins exhibited an apical-predominant distribution, their apical/basolateral ratios being 2. In addition, secretory glycoproteins recognized by VIP36 were secreted 2-fold more efficiently from the apical membrane than from the basolateral membrane. Thus, the apical/basolateral ratio of the transport of VIP36-recognized glycoproteins was correlated with that of VIP36 in MDCK cells. When VIP36 was overproduced in MDCK cells, the apical/basolateral ratio of plasma membrane VIP36 was changed from 1.9 to 3.5. Furthermore, the overproduction of VIP36 induced a marked enhancement of the apical distribution of VIP36-recognized glycoproteins and strikingly stimulated the apical secretion of VIP36-recognized glycoprotein.

| TABLE II |
| Plasma membrane and secretory glycoproteins that bound to VIP36 in polarized MDCK, MDCK/VIP36, and MDCK/mVIP36 cells |

For preparation of domain selectively plasma membrane, either the apical or basolateral surface proteins were biotinylated and purified by means of an avidin column as described under “Experimental Procedures.” For preparation of [35S]-labeled secretory proteins, the cells cultured on polycarbonate filters were labeled for 1 h with 250 μCi/ml Expre35S and incubated for another 2 h after washing the cells, and the medium was collected from the apical and basolateral chambers as described under “Experimental Procedures.” The domain selectively plasma membrane and secretory proteins were subjected to binding assay to GST/Vip36.

| Plasma membrane glycoproteins | Secretory glycoproteins |
|-----------------------------|-------------------------|
| apical (a) | basolateral (b) | a/b* | apical (a) | basolateral (b) | a/b* |
| × 10⁻⁶ AU | × 10⁻⁴ dpm |
| MDCK | 1.02 | 0.43 | 2.4 | 1.88 | 0.99 | 1.9 |
| MDCK/VIP36 | 1.43 | 0.31 | 4.6 | 7.68 | 1.32 | 5.8 |
| MDCK/mVIP36 | 0.79 | 0.40 | 2.0 | 0.74 | 0.32 | 2.3 |

* Ratios are the average of triple assays (p < 0.02).

| TABLE III |
| The N-glycan structures of clusterin |

Clusterin that was metabolic-labeled with [3H]glucosamine was immunoprecipitated with anti-clusterin antibody, and [3H]-labeled N-glycans were released by N-glycanase.

| Structure | Recovered radioactivity | % molar ratio |
|-----------|------------------------|--------------|
| dpm       | %                      |
| High mannose-type glycans | 1,850 | 7.5 |
| Complex type glycans        | 9,650 | 39.2 |
| Neutral glycans               | 11,320 | 46.0 |
| Sialylated glycans            | 1,780 | 7.3 |
| Sulfated glycans              |               |               |

FIG. 6. Binding of VIP36 to clusterin. a, detergent extracts from MDCK cells immunoprecipitated with anti-VIP36 antibody. The precipitated proteins were separated by SDS-PAGE followed by transfer to nitrocellulose membrane. Clusterin was detected by immunoblot with anti-clusterin antibody. Clusterin was not detected when the detergent extract was treated with Endo H (lane 2). b, detergent extracts from MDCK cells immunoprecipitated with clusterin antibody. The precipitated proteins with (lane 1) or without (lane 2) treatment with Endo H were separated by SDS-PAGE followed by transfer to nitrocellulose membrane. VIP36 was detected by immunoblot with anti-VIP36 antibody.

FIG. 7. Transport of newly synthesized clusterin and galectin-3 in MDCK, MDCK/VIP36, and MDCK/mVIP36 cells. The polarized MDCK, MDCK/VIP36, and MDCK/mVIP36 cells were pulse-labeled with [35S]methionine/cysteine for 20 min and chased for the indicated periods. The cell lysate and apical medium were subjected to immunoprecipitation with anti-clusterin antibody and anti-galectin-3 antibody. The radioactive counts of clusterin (a) and galectin-3 (b) or the percentage ratios against the radioactive counts at the end of pulse-labeled of clusterin (c) and galectin-3 (d) are shown. ● and ○, MDCK; ■ and □, MDCK/VIP36; ▲ and △, MDCK/mVIP36; ●, ■, and ▲, cell; ○, □, and △, medium.
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The polarized MDCK/VIP36 (lanes 3, 4, 7, and 8) cells were pulse-labeled with [35S]methionine/cysteine for 20 min and chased for the indicated periods. Clusterin was immunoprecipitated using cell lysate (lanes 1, 2, 5, and 6) or apical medium (lanes 3, 4, 7, and 8). The intracellular lectins calnexin and calreticulin are chaperones, functioning in quality control of newly synthesized glycoproteins in the ER (2, 3, and 4). Another intracellular lectin, ERGIC-53, may function as a receptor for cargo glycoprotein in the ER-to-ERGIC transport vesicle (5). In this study, VIMP was shown to be involved in the transport and sorting of glycoproteins and localized to the early secretion pathway and to the plasma membrane. Interestingly, all intracellular lectins identified so far recognize high mannose-type glycans, although the recognized mannose residues in high mannose-type glycans are different among them. These observations concerning the localization and binding specificity of intracellular lectins raise the possibility that intracellular lectins cooperate in the ER-to-plasma membrane transport of glycoproteins carrying high mannose-type glycans.

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