# VIP36 localisation to the early secretory pathway

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## SUMMARY

VIP36, an integral membrane protein previously isolated from epithelial MDCK cells, is an intracellular lectin of the secretory pathway. Overexpressed VIP36 had been localised to the Golgi complex, plasma membrane and endocytic structures suggesting post-Golgi trafficking of this molecule (Fiedler et al., 1994). Here we provide evidence that endogenous VIP36 is localised to the Golgi apparatus and the early secretory pathway of MDCK and Vero cells and propose that retention is easily saturated. High resolution confocal microscopy shows partial overlap of VIP36 with Golgi marker proteins. Punctate cytoplasmic structures colocalise with coatomer and ERGIC-53, labeling ER-Golgi intermediate membrane structures. Cycling of VIP36 is suggested by colocalisation with anterograde cargo trapped in pre-Golgi structures and modification of its N-linked carbohydrate by glycosylation enzymes of medial Golgi cisternae. Furthermore, after brefeldin A treatment VIP36 is segregated from resident Golgi proteins and codistributes with ER-Golgi recycling proteins.

Key words: ER-Golgi intermediate structure, Cycling, Brefeldin A, Glycolipid raft

## INTRODUCTION

VIP36 (vesicular integral membrane protein of 36 kDa) was purified from detergent-insoluble complexes of epithelial Madin-Darby canine kidney (MDCK) cells (Fiedler et al., 1994). Our interest in VIP36 derived from the presence of this protein in immunoisolated apical and basolateral trans-Golgi network (TGN)-derived transport vesicles (Wandinger-Ness et al., 1990). The major proteins of the immunoisolated vesicles were also found in CHAPS-insoluble and Triton-insoluble residues allowing their enrichment and subsequent cloning of the corresponding cDNAs (Kurzchalia et al., 1992; Fiedler et al., 1993). Caveolin, VIP17/MAL and annexin XIIIb were identified and these proteins have been shown to play a functional role in apical transport of MDCK cells (Kurzchalia et al., 1992; Cheong et al., 1999; Puertollano et al., 1999; Lafont et al., 1998).

Sequence analysis showed that VIP36 belongs to a family of animal lectins, homologous to leguminous lectins (Fiedler and Simons, 1994). This family includes ERGIC-53, a mannose-specific lectin localised to the ER-Golgi intermediate compartment (Itin et al., 1996). For VIP36 there is evidence for interaction with *N*-acetyl-D-galactosamine (Fiedler and Simons, 1996) and high-mannose type glycans (Yamashita et al., 1998). Overexpressed VIP36 localised to the Golgi apparatus, plasma membrane and endosomes (Fiedler et al., 1994). In view of all these properties, we speculated that VIP36 plays a functional role as a lectin in post-Golgi trafficking.

However, in subsequent work focusing on the localisation of endogenous VIP36, we could not find any evidence for post-Golgi localisation. The observation that VIP36 colocalises partially with coatomer involved in trafficking in the early secretory pathway proved to be a turning point in our studies. We now present evidence that endogenous VIP36 is localised to the Golgi apparatus and to structures cycling between the Golgi complex and the ER. Colocalisation was observed with coatomer and ERGIC-53 which have been implicated in trafficking between the ER and Golgi complex. Furthermore we demonstrated that VIP36 colocalised with anterograde cargo in pre-Golgi structures and was modified by glycosylation enzymes of the Golgi complex.

## MATERIALS AND METHODS

### Antibodies

Antibodies against VIP36 have been described (Fiedler et al., 1994; Fiedler and Simons, 1996). For all immunofluorescence studies reported here, we used a new antibody raised against the cytoplasmic tail of VIP36 (CFQKRQERNKRFY) coupled to KLH (keyhole limpet hemocyanine). The additional cysteine facilitated affinity purification of the rabbit antiserum on a peptide-agarose column as recommended by the manufacturer (Pierce, Rockford, IL). Polyclonal caveolin antibodies were from Transduction Laboratories (Lexington, KY). Mouse monoclonal antibodies against ERGIC-53 (Schweizer et al., 1988) and giantin (Linstedt and Hauri, 1993) were kindly provided by Hans-Peter Hauri (Dept of Pharmacology, Basel/Switzerland). Other monoclonal antibodies were concentrated from hybridoma supernatant: c-myc (9E10, Evan et al., 1985), VSV-G (P5D4, Kreis 1986) and β′-COP (CM1A10, Palmer et al., 1993). The following
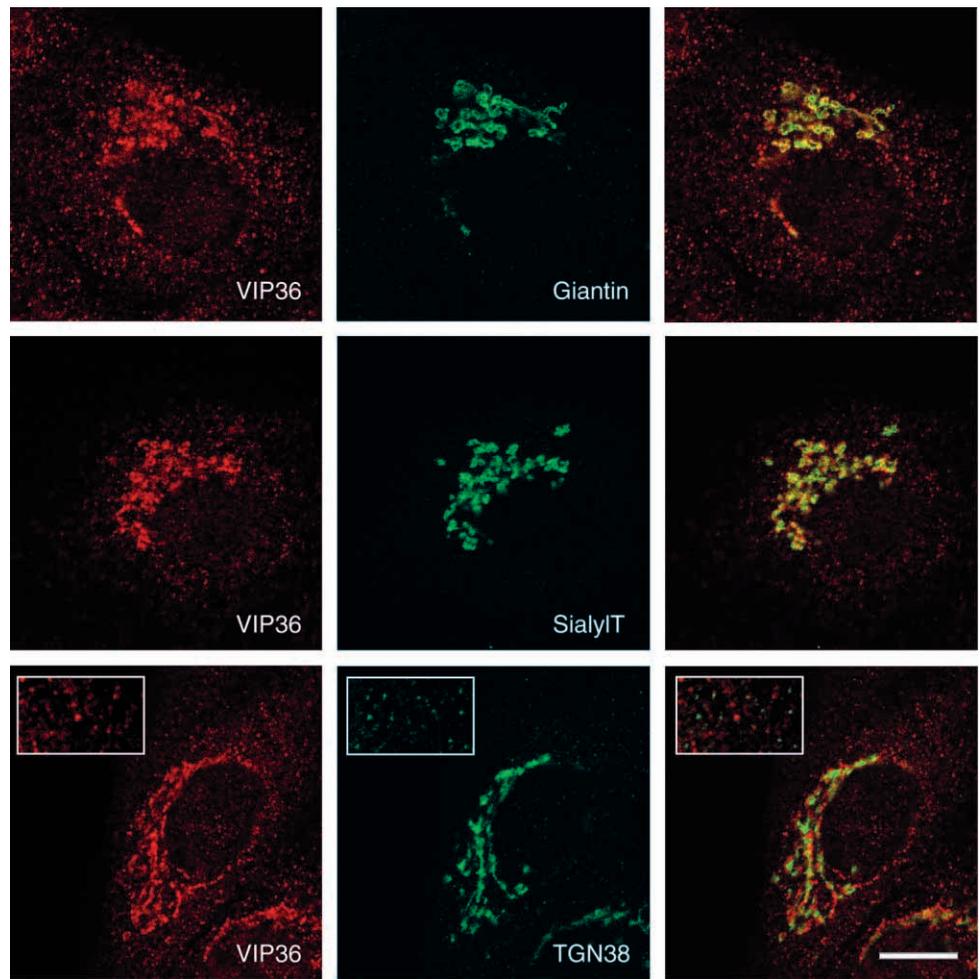
**Fig. 1.** Comparison of VIP36 and Golgi marker proteins in MDCK cells by confocal scanning microscopy. Affinity purified antibodies raised in rabbit against the cytoplasmic tail of VIP36 were used together with mouse monoclonal antibodies against the Golgi marker protein giantin for indirect immunofluorescence of parental MDCK cells. MDCK cells stably transfected with epitope-tagged trans-Golgi/TGN marker proteins α-1,6 sialyltransferase (SialylT) and TGN38 were generated by antibiotic selection and stained with mouse monoclonal antibodies directed against P5D4 or c-myc, respectively. Although VIP36 and the marker proteins are all localised to the perinuclear area of the cell, striking differences in the staining patterns are apparent in the coloured overlays. Images were recorded simultaneously to ensure no pixel shifts were occurring and are single sections. Bar, 10 μm.

secondary antibodies were used: donkey anti-rabbit coupled to Cy3 or FITC, donkey anti-mouse FITC or Cy3 (Dianova, Hamburg, Germany), goat anti-rabbit and goat anti-mouse coupled to Alexa488 (Molecular Probes, Eugene, OR).

### Cell culture and viral infection

MDCKII cells were grown in minimal essential medium (MEM) with 5% FCS. HeLa, Vero, NRK and hybridoma (CM1A10, P5D4) cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FCS under standard tissue culture conditions. Vero cells stably transfected with myc-tagged *N*-acetyl-glucosaminyltransferase I (NAGT I; Yang and Storrie, 1998) were maintained in the presence of 0.2 mg/ml G-418 (Sigma, Deisenhofen/Germany). MDCK cells stably expressing c-myc tagged TGN38 (Miesenböck and Rothman, 1995) or P5D4-tagged α1,6-sialyltransferase (Rabouille et al., 1995) were obtained by electroporation and selection with 0.5 mg/ml G-418.

Vesicular stomatitis virus strain tsO45 was used to infect Vero and MDCK cells for 45 minutes at room temperature in infection medium (MEM without FCS, 20 mM Hepes, pH 7.2, 0.2% BSA). Accumulation of VSV glycoprotein G (VSV-G tsO45) was for 3 hours at 39.5°C in MEM including FCS. Subsequent incubation at 15°C was in the presence of 200 μM cycloheximide and 20 mM Hepes, pH 7.2, for 1.5 hours.

### VIP36 expression plasmids and DNA transfection

VIP36 and N-terminally myc-tagged VIP36 (Fiedler et al., 1994) were cloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). Subconfluent Vero or HeLa cells (10 cm$^2$ Petri dish)

were transfected by calcium-phosphate co-precipitation (Keown et al., 1990) using 3.0 μg plasmid DNA. Cells were processed for immunofluorescence 24 hours after transfection. Brefeldin A (Epicentre Technologies, Madison, WI) was used at a concentration of 5 μg/ml for 30 minutes at 37°C.

### Immunofluorescence and confocal analysis

Subconfluent cells grown on coverslips were fixed with 3% paraformaldehyde for 20 minutes. After quenching aldehyde groups with ammonium chloride, cells were permeabilized with 0.1% saponin. Antibodies were incubated in phosphate-buffered saline (PBS) containing 0.2% fish skin gelatin and 0.1% saponin. Coverslips were mounted in Mowiol and viewed with a Zeiss Axiovert 100TV microscope equipped with a 24-bit RGB CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) or the Leica TCS-NT confocal laser scan microscope (Leica Lasertechnique, Heidelberg/Germany).

Confocal images of double labeling experiments were obtained simultaneously to exclude any artefacts from sequential acquisition. Both channels were adjusted to ensure that the maximum fluorescence intensity was still in the recording range. Only one focal plane was analysed. Stainings shifted against each other were confirmed by series of z sections and repeated simultaneous scans. Micrographs were arranged with Adobe Photoshop and Illustrator.

### Glycosylation analysis

Maturation of *N*-glycosylated VIP36

MDCK cells (10 cm$^2$ Petri dish) were depleted of endogenous methionine by incubating for 15 minutes at 37°C in methionine-free labeling medium, pulsed with 200 μCi [$^{35}$S]methionine (0.2 mCi/ml
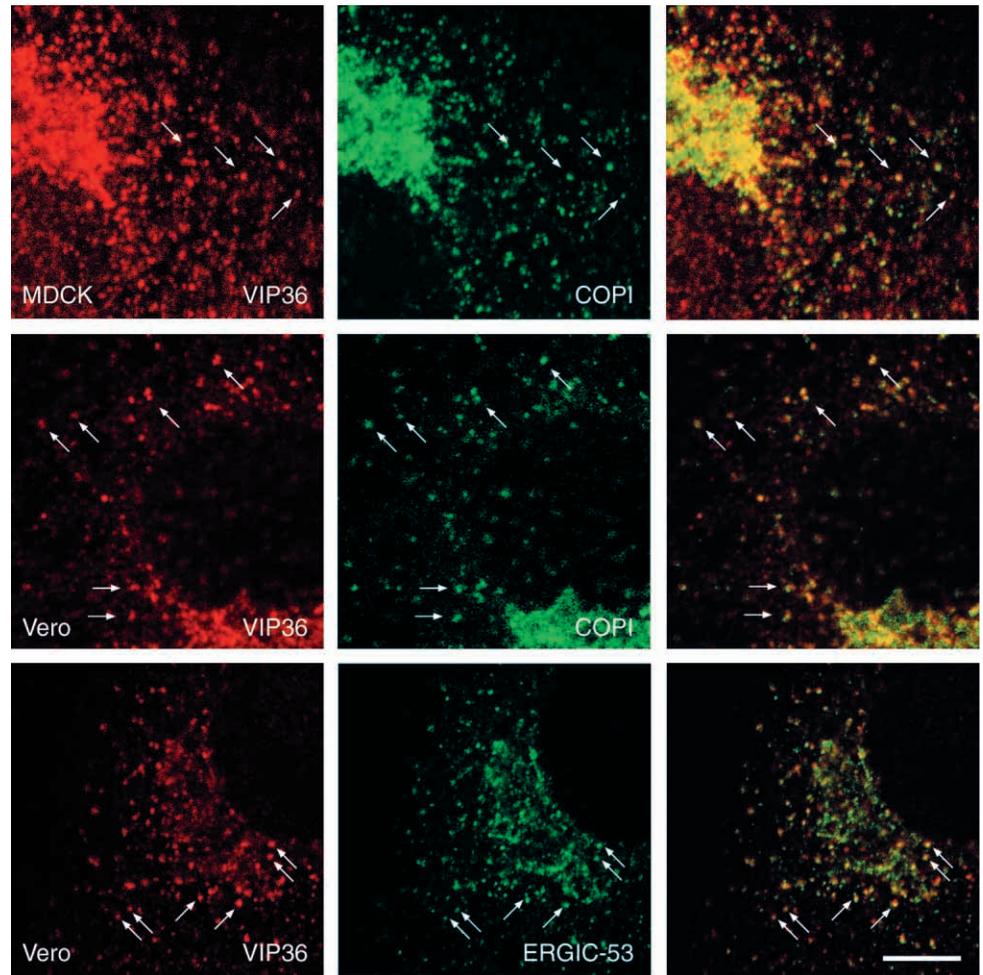
**Fig. 2.** Colocalisation of peripheral cytoplasmic structures of VIP36 with coatomer and ERGIC-53. MDCK and Vero cells were stained with antibodies against VIP36 and β′ COP, a subunit of coatomer (COPI). Arrows indicate overlapping distributions. Antibodies against ERGIC-53 stain ER-Golgi intermediate structures in Vero cells. The overall punctate pattern of VIP36 and ERGIC-53 shows a high degree of colocalisation. Confocal images were recorded simultaneously to ensure no pixel shifts were occurring and are single sections. The fluorescence intensity of the Golgi region in the images comparing COPI and VIP36 is above the recording range to allow for better comparison of the punctate cytoplasmic structures. Bar, 5 μm.

specific activity) for 8 minutes and chased with an excess of unlabeled methionine for 2, 15, 30, 60 and 120 minutes. Lysis was at 4°C using PBS containing 1% w/v NP-40, 0.2% w/v SDS and protein inhibitor cocktail (CLAP). Samples were immunoprecipitated with antibodies and Protein A-Sepharose overnight. After washing with 1% w/v NP-40, 1% w/v SDS in PBS, bound proteins were eluted by boiling for 5 minutes in 150 mM sodium citrate, pH 5.5, 0.6% SDS, 1.5% β-mercaptoethanol. SDS was quenched with NP-40 and incubation with 7.5 mU Endoglycosidase H (EndoH; Boehringer Mannheim/Germany) was for 18 hours at 37°C in the presence of CLAP.

### Deoxymannojirimycin treatment

MDCK cells (25 cm$^2$ Petri dish) were preincubated with 1 mM deoxymannojirimycin (DMM; Oxford GlycoSystems/UK) for 90 minutes at 37°C. Cells were pulse-labeled with 400 μCi [$^{35}$S]methionine (0.4 mCi/ml specific activity) for 30 minutes in the presence of DMM. Labeling medium was removed and the following chase was for 4 hours in the continued presence of DMM. The procedure so far just ensured that there was labeled VIP36 which had not been modified by α1,2-mannosidase I and was therefore EndoH sensitive. Cells were washed three times with PBS to wash out DMM and incubated in standard tissue culture medium for 0, 2, 4 and 10 hours to evaluate modification by α1,2-mannosidase I and later Golgi enzymes. Lysis, immunoprecipitation and EndoH treatment were as described above.

### Analysis of detergent-insoluble glycolipid-enriched complexes

The previous methodology for analyzing detergent-insolubility using CHAPS or Triton X-114 (Fiedler et al., 1993, 1994) has been changed

and optimized for finding out whether a protein is in detergent-insoluble glycolipid-enriched complexes (DIGs) or not (Scheiffele et al., 1999; J. Benting and K. Simons, unpublished). Using the modified method we did not find significant amounts of VIP36 in DIGs. In short, filter-grown MDCK cells were scraped in PBS and extracted with 800 μl lysis buffer (1% v/v Tx-100, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA) for 15 minutes on ice. After passing three times through a 22 G needle, 1.14 ml 60% w/v Optiprep (Nycomed, Oslo/Norway) were added to a final concentration of 35%, overlayered with 2 ml 30% w/v Optiprep in lysis buffer and finally with 400 μl lysis buffer only. After centrifugation in a Beckmann SW60 rotor for 4 hours at 40,000 rpm, five fractions were collected. Proteins were incubated with 1% NP-40 at room temperature, precipitated with TCA, washed with 80% acetone and analysed by SDS-PAGE and western blotting.

## RESULTS

### Comparison between endogenous VIP36 and Golgi marker proteins

We previously demonstrated by indirect immunofluorescence of MDCK cells that antibodies against VIP36 stained a perinuclear region presumably corresponding to the Golgi apparatus. In addition, many punctate structures throughout the cytoplasm were observed (Fiedler et al., 1994). In an effort to investigate the localisation of endogenous VIP36 in more

detail, we compared VIP36 to Golgi marker proteins by high resolution confocal scanning microscopy.

Giantin is a cytoplasmically oriented Golgi membrane protein (Linstedt and Hauri, 1993). At low resolution and with a standard microscopy set-up, both giantin and VIP36 overlap in the perinuclear region of MDCK cells (not shown). However, simultaneous recording of thin optical sections revealed differences in the distribution of these two proteins (Fig. 1). The pattern for giantin is more compact and ribbon-like whereas VIP36 staining has a granular appearance in the Golgi region and additionally consists of numerous small punctate structures in the cytoplasm.

Few other antibodies against Golgi marker proteins are available for canine kidney derived tissue culture cells. Stable expression of epitope-tagged Golgi proteins has been used successfully in the past to circumvent this problem in other cell lines (Rabouille et al., 1995). MDCK cells stably expressing α1,6-sialyltransferase or TGN38 were generated by antibiotic selection. Transfer of sialic acid is one of the final steps in the maturation of N-glycans and is thought to occur in the trans-Golgi/TGN (Roth et al., 1985). The staining pattern of sialyltransferase showed some overlap with VIP36 in the perinuclear Golgi region, but cytoplasmic punctate structures stained exclusively for VIP36 (Fig. 1). TGN38 is mainly localised to the TGN at steady state but is also cycling to plasma membrane and endosomes (Reaves et al., 1993). Little coincident staining could be observed between TGN38 and VIP36. The small punctate structures presumably corresponding to TGN38 recycling structures also did not overlap significantly with VIP36 (Fig. 1, inset).

## Localisation of VIP36 to COPI-coated membranes of ER-Golgi intermediate structures

The immunofluorescence pattern of VIP36 suggested that this protein was trafficking between the Golgi apparatus and other compartments. Coatomer or COPI is an heptameric cytosolic protein complex apparently involved in bi-directional transport between the ER and the Golgi apparatus (Letourneur et al., 1994; Aridor et al., 1995; Scales et al., 1997) and intra-Golgi transport (Orci et al., 1997; Nickel et al., 1998). Careful analysis by confocal scanning microscopy of subconfluent MDCK cells led to the surprising finding that some of the cytoplasmic punctate structures of VIP36 colocalised with coatomer (Fig. 2, arrows). Colocalisation between coatomer and VIP36 was more obvious in the fibroblast-like monkey kidney derived Vero cells (Fig. 2, arrows). NRK and HeLa cells gave essentially the same result (not shown). ERGIC-53 is a membrane protein localised to the early secretory pathway and has been used extensively as a marker protein for the ER-Golgi intermediate compartment (Schweizer et al., 1990). Significant but not complete overlap is observed between VIP36 and ERGIC-53 in Vero cells (Fig. 2). The Golgi region of Vero cells showed less prominent staining for VIP36 as compared to MDCK cells (Fig. 2). In many Vero cells, perinuclear staining of VIP36 consisted of numerous punctate rather than continuous structures. It is not clear if this reflects differences in cell shape or cycling preferences which might be cell type specific.

Since the partial colocalisation between VIP36 and coatomer/ERGIC-53 was quite an unexpected finding, several control experiments were performed. Vero and HeLa cells transiently transfected with epitope-tagged or wild-type VIP36 were analysed using limiting amounts of antibodies against the cytoplasmic tail. Under these conditions, transfected cells were easily recognised and the data demonstrated that the punctate cytoplasmic structures were genuine, persisted in the presence of the protein synthesis inhibitor cycloheximide, and co-stained with coatomer (not shown). In addition, as shown before (Fiedler et al., 1994), peptide competition abolished VIP36 staining. VIP36 antibodies microinjected into Vero cells gave the same overall pattern as on fixed cells, indicating that the native carboxyterminus of VIP36 is recognized (not shown).

## Colocalisation of VIP36 with anterograde cargo in pre-Golgi structures

A characteristic feature of proteins cycling in the early secretory pathway is their colocalisation with viral membrane proteins accumulating at 15°C in heterogeneous membranes between ER and cis Golgi (Saraste and Kuismanen, 1984; Schweizer et al., 1990). Strain tsO45 of vesicular stomatitis virus (VSV) contains a temperature sensitive mutant of the membrane glycoprotein VSV-G, which misfolds and is retained in the ER at the restrictive temperature (39.5°C). At 15°C VSV-G accumulates in punctate structures probably representing ER exit sites and vesiculo-tubular structures on their way from ER to the Golgi complex, but is not processed by Golgi-localised glycosylation enzymes. Double immunofluorescence of VIP36 and VSV-G at the restrictive temperature shows an ER pattern for VSV-G, whereas VIP36 is localised to the perinuclear Golgi region and cytoplasmic punctate structures. As mentioned above, the perinuclear staining of VIP36 is less prominent in Vero cells (Figs 2, 3) as compared to MDCK cells (Figs 1, 2). In addition, some infected cells expressing high amounts of VSV-G showed even less than usual Golgi staining but this phenomenon was not observed consistently. After incubation at 15°C in the presence of the protein synthesis inhibitor cycloheximide, many of the cytoplasmic structures positive for VIP36 are strikingly coincident with VSV-G protein arrested on its way from ER to Golgi (Fig. 3). The overlap was not complete which can be explained by the nature of the 15°C temperature block which will slow down one pathway while allowing others to function. However, the differences generated would not be sufficient to transport all VIP36 molecules to anterograde pre-Golgi transport structures. The colocalisation between VSV-G and VIP36 was also observed in MDCK cells (not shown), but was slightly less obvious since under our conditions VSV-G left the ER less efficiently than in Vero cells.

## VIP36 is modified by glycosylation enzymes of the medial Golgi

VIP36 is N-glycosylated and resistant to digestion with endoglycosidase H (EndoH) at steady state (Fiedler and Simons, 1996). A kinetic analysis of the maturation of the N-linked carbohydrate showed that more than half of the newly synthesized VIP36 was already EndoH resistant after 60 minutes (Fig. 4A). Acquisition of EndoH resistance requires the action of glycosylation enzymes localised to medial Golgi cisternae.

Continued cycling of VIP36 is supported by studies employing DMM inhibiting α1,2-mannosidase activity in the

**Fig. 3.** VIP36 colocalisation with anterograde cargo in the 15°C intermediate compartment. Vero cells were infected with vesicular stomatitis virus tsO45 expressing a glycoprotein (VSV-G tsO45) with temperature-sensitive folding properties. At the non-permissive temperature (39.5°C) VSV-G accumulates in the ER. Upon incubation at 15°C, VSV-G is rearranged and properly folded and can leave the ER. However fusion with Golgi membranes is temperature sensitive and does not occur efficiently at 15°C. VSV-G accumulates in pre-Golgi structures which overlap extensively although not completely with the VIP36 staining pattern. This suggests that VIP36 can be transported retrograde from the perinuclear area to ER-Golgi transport intermediates. Bar, 10 µm.
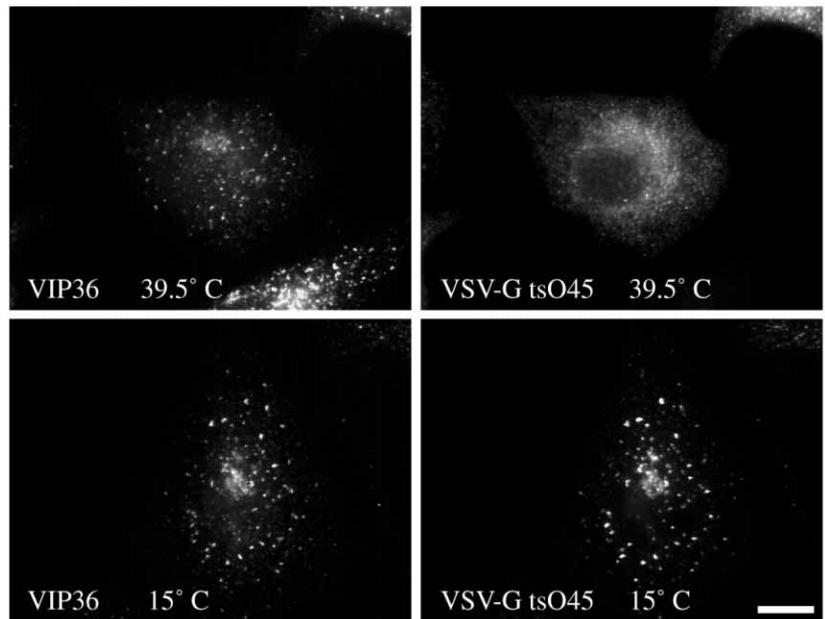
Golgi complex (and further processing of the N-linked carbohydrate). In the experiment shown in Fig. 4B, newly synthesized VIP36 was chased for 4 hours without or with DMM (lanes 1-3). Without DMM, VIP36 is now completely EndoH resistant. In the presence of DMM however, removal of mannose residues in the Golgi is inhibited and the carbohydrate stays EndoH sensitive. After washout of DMM (lanes 4-6), VIP36 acquires EndoH resistance indicative of modification by medial Golgi enzymes. This modification takes longer than that shown for newly synthesized VIP36 in Fig. 4A. One possible explanation is that the re-activation of $\alpha1,2$-mannosidase I after washout of DMM is not efficient. The point we want to make here is that although we do not know exactly where VIP36 is localized 4 hours after synthesis (most likely in ER-Golgi intermediate structures and across the whole Golgi stack), it continues cycling and is evidently passing through medial Golgi cisternae. We take this as an indication that VIP36 is not transported just once to the medial Golgi following its synthesis (a single 'maturation journey') but is continuously cycling within the Golgi.

### BFA-induced vesicular structures contain VIP36 and ERGIC-53, but not resident Golgi glycosylation enzymes

The fungal metabolite brefeldin A leads to the redistribution of coatomer from Golgi membranes to the cytosol and subsequently to the fusion of Golgi membranes with the ER, forming an ER-Golgi hybrid compartment (reviewed by Klausner et al., 1992). Resident Golgi proteins change their morphology to an ER-like pattern (Fig. 5). However, certain cycling membrane proteins of the early secretory pathway, like ERGIC-53, KDEL-receptor and p24 family proteins, accumulate instead in tubulo-vesicular clusters scattered in the cytoplasm (Lippincott-Schwartz et al., 1990; Tang et al., 1993; Füllekrug et al., 1999). These structures are morphologically and biochemically distinct from the ER-Golgi hybrid compartment. Analysis of VIP36 demonstrated that the staining pattern after BFA treatment does not

colocalise with resident Golgi proteins in the ER, but shows a high degree of coincident staining with ERGIC-53 (Fig. 5). Vero cells were used for these experiments since the Golgi apparatus of MDCK cells is not susceptible to brefeldin A (Hunziker et al., 1991).
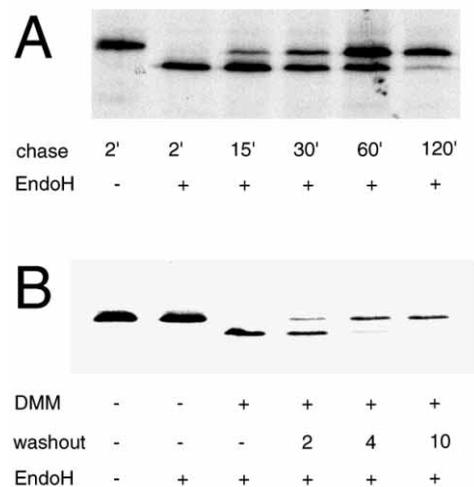


**Fig. 4.** Glycosylation analysis of VIP36. (A) Maturation of *N*-glycosylated VIP36. MDCK cells were labeled with [$^{35}$S]methionine for 8 minutes according to Materials and Methods and chased for various times. Immunoprecipitation and digestion with endoglycosidase H (EndoH) shows that the carbohydrate attached to VIP36 is getting progressively EndoH resistant. This indicates that VIP36 is reaching medial Golgi cisternae containing glycosylation enzymes conferring EndoH resistance. (B) Glycosylation of VIP36 after treatment with the $\alpha1,2$-mannosidase I inhibitor DMM. MDCK cells were pulse-labeled with [$^{35}$S]methionine and chased in the presence of DMM for 4 hours as described in Materials and Methods. Directly after this procedure, VIP36 is completely EndoH sensitive (lane 3). Subsequent incubation in DMM-free medium (lanes 4-6) and analysis by immunoprecipitation, EndoH treatment and gel electrophoresis shows that VIP36 acquires an EndoH resistant carbohydrate.
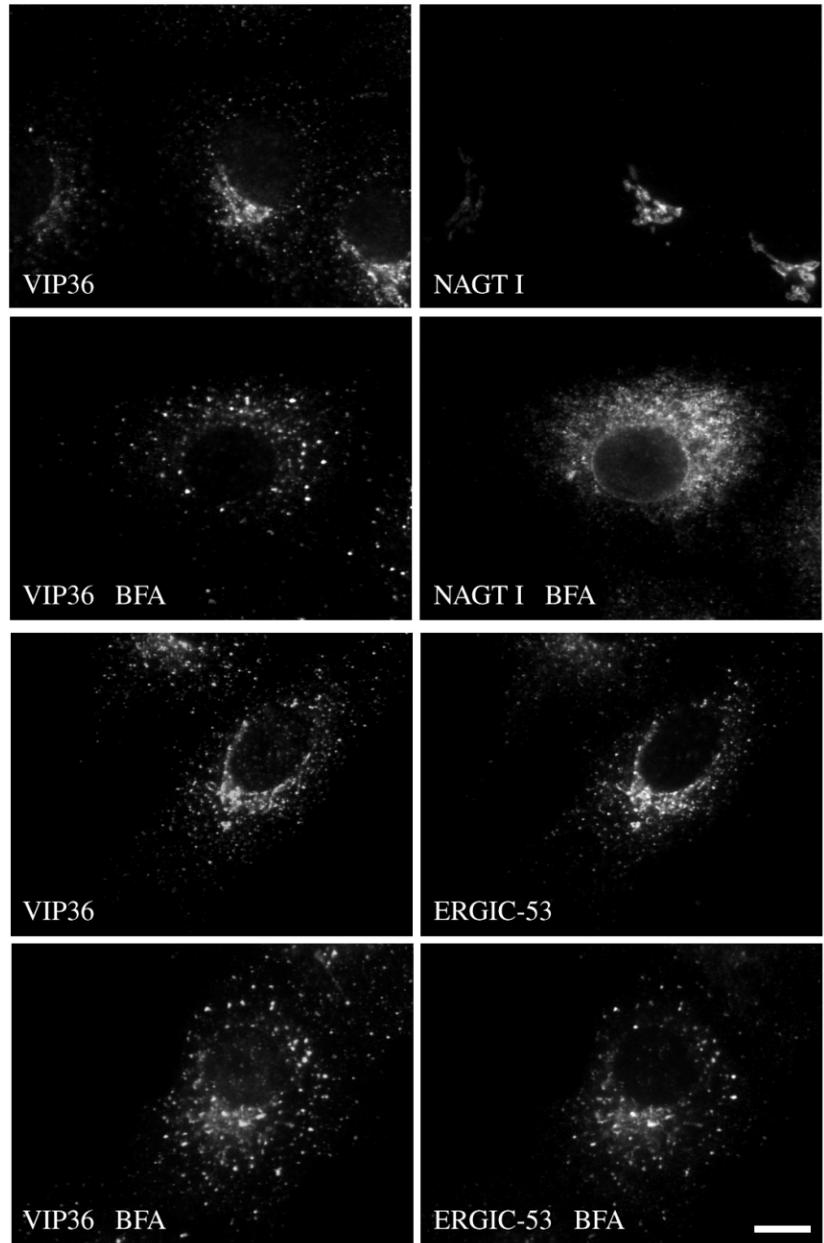
**Fig. 5.** Accumulation of VIP36 in brefeldin A-induced structures containing ER-Golgi recycling membrane proteins. Vero cells stably transfected with the epitope-tagged resident Golgi glycosylation enzyme N-acetyl-glucosaminyltransferase I (NAGT I) were treated with brefeldin A for 30 minutes. VIP36 stains dotty structures throughout the cytoplasm, colocalising with ERGIC-53. These brefeldin A-induced structures are different from the ER-Golgi hybrid compartment marked here by NAGT I. Bar, 10 μm.

## VIP36 is not associated with detergent-insoluble glycolipid-enriched complexes (DIGs)

We previously showed that detergent-insoluble complexes from MDCK cells are enriched in glycosphingolipids, apical cargo and a subset of membrane proteins also found in TGN to plasma membrane carrier vesicles (Kurzchalia et al., 1992; Fiedler et al., 1993). VIP36 was found in both apical and basolateral vesicle preparations as well as in detergent-insoluble complexes prepared by treatment with either CHAPS or Triton X-114. One of the biochemical characteristics of the Triton-insoluble complexes or glycolipid rafts is their low density due to high lipid content. Proteins contained within glycolipid rafts will not be solubilized by treatment with the non-ionic detergent Triton X-100 on ice. When placed at the bottom of a density gradient, Triton-insoluble complexes will separate from solubilized proteins and float upwards during centrifugation (Brown and Rose, 1992). We have now modified

the original method that we previously used to find out whether a protein is in DIGs or not (see Materials and Methods). Using this method no significant amounts of VIP36 were found in fractions of lower density corresponding to glycolipid rafts (Fig. 6). We conclude that VIP36 is not associated with DIGs and therefore probably not with glycolipid rafts and is unlikely to play a role in the formation of these structures.

## DISCUSSION

In this study we present evidence that endogenous VIP36 is localised to the early secretory pathway, cycling between ER-Golgi intermediate structures and the Golgi complex. However, in our previous work we localised *overexpressed* VIP36 not only to the Golgi complex but also to the plasma membrane and endosomal structures (Fiedler et al., 1994). Although we
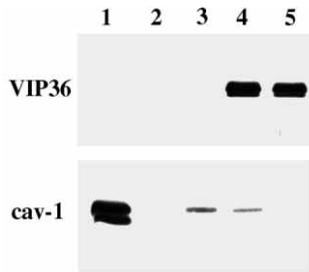
**Fig. 6.** Flotation gradient of glycolipid rafts. MDCK cells were treated with the non-ionic detergent Triton X-100 on ice, and the lysate placed at the bottom (fractions 4 and 5) of an Optiprep density step gradient. After high-speed centrifugation, fractions were collected from top to bottom and analysed by SDS-PAGE and western blotting. VIP36 is not contained in floating glycolipid rafts (fractions 1, 2) marked here by caveolin-1.

could not find any *endogenous* VIP36 in endosomal structures or on the plasma membrane, it is impossible to exclude the possibility that small amounts below the detection limit of our studies could be localised there.

We therefore have to conclude that it is the overexpression of VIP36 which leads to post-Golgi transport of this protein. This is not without precedence, since the closest related mammalian protein to VIP36, ERGIC-53, also appears on the cell surface after overexpression (Kappeler et al., 1994). Following expression at the cell surface, both ERGIC-53 and VIP36 are re-internalised. A putative endocytosis signal in the cytoplasmic tails of these two proteins has been characterised (Itin et al., 1995). In the light of recent findings that the same 'endocytosis' signal interacts with a component of the COPII coat involved in budding from the ER (Kappeler et al., 1997), it seems more appropriate to state that export from the ER and endocytosis from the plasma membrane are related to each other, and some sorting determinants are apparently shared. The KDEL-receptor and mammalian Rer1, two other membrane proteins cycling between ER and Golgi localise to the ER when overexpressed and do not leak to the cell surface (Hsu et al., 1992; Füllekrug et al., 1997). This suggests that there are different mechanisms for the cycling of membrane proteins between the ER and the Golgi complex, and that the retention/retrieval mechanism for ERGIC-53 and VIP36 is easily saturated.

We are aware of the difficulties associated with drawing precise conclusions about the localisation of a protein by light microscopy. A coincident staining, especially in the perinuclear Golgi region cannot be taken as unequivocal evidence for colocalisation. Regarding the cytoplasmic punctate structures however we think it is unlikely that the observed overlap is being generated by two different structures which happen to be close to each other. There are several reasons why even overlapping punctate structures do not appear simply as bright yellow dots in the overlay images: first, only structures binding fluorochrome-coupled antibodies to the same extent will appear yellow. If intensities are mismatched, orange or light green colour hues will be the consequence. Second, since primary antibodies have to be recognised by fluorochrome-coupled secondary antibodies, steric hindrance and the distances involved might generate a slight shift between the signals. In addition to all this, vesiculo-tubular

structures on their way from ER to Golgi could be polarised, with some proteins at the leading edge, whereas others would be closer to a possible retrieval site. These important questions can only be investigated by the resolution of electron microscopy. Unfortunately, our antibody against the cytoplasmic tail of VIP36 used for all immunofluorescence experiments in this study does not work in immunoelectron microscopy.

The N-glycan structure of VIP36 suggests transport to the medial cisternae of the Golgi apparatus. This behaviour is similar for a distantly related yeast homologue termed Emp47p. The N-linked carbohydrate of Emp47p is modified by enzymes of the medial or later Golgi, but Emp47p can also be relocated to the ER (Schröder et al., 1995). Furthermore the N-glycan of gp27, a member of the p24 protein family of putative cargo/coat receptors, is modified with similar kinetics as VIP36 but localises at steady state to the cis-Golgi network and not to medial cisternae of the Golgi apparatus (Füllekrug et al., 1999). Trafficking of VIP36 in the Golgi is more extensive than that observed for a glycosylated variant of ERGIC-53 measured by the rate of acquisition of Endo H-resistant N-glycans (Itin et al., 1995). This is in line with the presence of a strong ER retrieval signal in the cytoplasmic tail of ERGIC-53 (Tisdale et al., 1997), which is lacking for VIP36.

Our efforts to demonstrate localisation and trafficking of endogenous VIP36 beyond the Golgi apparatus were unsuccessful. Using a recently developed system for TGN to plasma membrane transport (Toomre et al., 1999), no overlap between VIP36 and transport containers on their way to the plasma membrane could be documented (D. Toomre and J. Füllekrug, unpublished). Antibody internalisation was confirmed for cells overexpressing VIP36 (Fiedler et al., 1994), but could not be detected in MDCK cells with normal levels of VIP36 (P. Scheiffele, unpublished). The drugs chloroquin, bafilomycin and concanamycin A all inhibit endosomal acidification, and lead to accumulation of proteins cycling between plasma membrane and TGN in large endosomal vacuoles. No VIP36 was found in these structures (U. Lahtinen and J. Füllekrug, unpublished). We confirmed that overexpression of wt and epitope-tagged VIP36 led to the appearance of VIP36 at the plasma membrane, as reported previously (Fiedler et al., 1994).

In a detailed study applying nanoelectrospray tandem mass spectrometry to identify components of trans-Golgi network-derived transport vesicles and detergent-insoluble complexes of MDCK cells, two membrane proteins termed p23 and gp25l2 belonging to the p24 protein family were detected (Shevchenko et al., 1997). These proteins are localised at steady state to the cis-Golgi network (Dominguez et al., 1998; Rojo et al., 1997) and colocalise with anterograde cargo blocked at 15°C (J. Füllekrug and T. Nilsson, unpublished). It is puzzling that p23, gp25l2 and VIP36 are enriched after immunoisolation of apical and basolateral vesicles. We speculate that part of the Golgi to ER transport structures might be uncoated (and therefore 'sticky') and would have densities similar to TGN-derived vesicles. Furthermore, the 20°C temperature block for accumulation of secretory proteins in the trans-Golgi network is not absolute and viral proteins can also be present in structures proximal to the stack. A contamination of our TGN-derived preparations by vesicular intermediates derived from the Golgi apparatus and earlier is likely.

If VIP36 is not involved in post-Golgi trafficking what function might this protein have? The closest related mammalian protein is ERGIC-53, and database searches indicate that in *Drosophila melanogaster* and *Caenorhabditis elegans* there are also only two lectins of this class. Since VIP36 and ERGIC-53 are both localised to the early secretory pathway, they might differ in their lectin specificity. The benefit of having lectins could be that sorting of secretory glycoproteins into COPII budding profiles for export out of the ER would be more efficient. Alternatively, they could form a 'team' with a certain redundancy, and be involved in quality control, similar to calnexin and calreticulin in the ER. Human individuals deficient for ERGIC-53 have reduced levels of heavily glycosylated coagulation factors in the blood plasma and suffer from a bleeding disorder, but do not show any other obvious symptoms (Nichols et al., 1998). This implies that embryonic development and secretion of other glycoproteins are not seriously affected. Only future work will clarify how this family of lectins performs their function in the secretory pathway.

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