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VIP17/MAL, a proteolipid in apical transport vesicles

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Abstract **VIP17 is a proteolipid enriched in the CHAPS-insoluble complexes from MDCK cells, and a candidate component of the molecular machinery responsible for the sorting and targeting of proteins to the apical surface. Cloning and sequencing of the cDNA encoding the protein revealed that it is the canine homolog of the human and rat MAL proteins. Analysis by immunofluorescence microscopy of epitope-tagged VIP17/MAL expressed transiently in BHK cells and stably in MDCK cells revealed a perinuclear, vesicular, and plasmalemmal staining. In MDCK cells the distribution was mainly in vesicular structures in the apical cytoplasm. These and other results suggest that VIP17/MAL is an important component in vesicular trafficking cycling between the Golgi complex and the apical plasma membrane.**

Key words: Madin–Darby canine kidney cell; Proteolipid; Apical transport; Myelin biogenesis; Detergent-insoluble complex; Vesicular trafficking

1. Introduction

Epithelial cells generate and maintain a polarized cell architecture to perform vectorial functions in secretion, absorption, and ion transport. The epithelial plasma membrane is segregated into apical and basolateral domains which differ in protein and lipid composition [1,2]. In Madin–Darby canine kidney (MDCK) cells biosynthetic sorting takes place in the trans Golgi network (TGN) [3,4]. Here apical and basolateral transport vesicles are formed to deliver their cargo to the respective membrane domains [5]. To identify putative sorting and targeting machinery, we have immunoisolated the transport vesicles and analyzed their protein composition in 2D gel electrophoresis [6]. Several of these proteins were found to form a detergent-insoluble complex with an apical marker protein, the influenza virus hemagglutinin [7,8]. Among these, the first to be characterized was VIP21/caveolin, a protein also localizing in the Golgi complex and in plasmalemmal caveolae [7–11]. A second component, named VIP36, was a new type I transmembrane protein with a N-terminal domain showing homology to leguminous plant lectins [12].

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Here in this paper we have identified and characterized a third protein, which was referred to as C14 in the 2D gel analysis of apical and basolateral vesicles [6–8]. According to our nomenclature we have named the protein VIP17 [13].

Analysis of the cDNA encoding VIP17 demonstrated that it is the canine homolog of human and rat MAL, a protein previously described with unknown function, which is expressed in T cells, Schwann cells, oligodendrocytes, and also in the kidney [14–16].

2. Materials and methods

2.1. Materials

Monoclonal anti-HA epitope 12CA5 was from Boehringer, Germany; polyclonal anti-caveolin (N-20) from Santa Cruz Biotechnology, Santa Cruz, CA, USA; donkey anti-mouse and anti-rabbit IgG (Rhodamine-conjugated) from Dianova, Hamburg, Germany; pBK-CMV plasmid from Stratagene, CA, USA; 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and the Silver Stain kit from Sigma, MO, USA.

Polymerase chain reaction (PCR) on cDNA was performed using Dynazyme DNA polymerase from Finnzymes Oy, Espoo, Finland. Oligonucleotides and PCR primers were prepared by the oligonucleotide synthesis facility, and sequence reactions on both strands of DNA by the DNA sequence facility, both at the EMBL, Heidelberg, Germany.

2.2. Cell culture

MDCK strain II and BHK21 cells were grown and passaged as previously described [6,7].

2.3. cDNA cloning and sequence analysis

CHAPS pellets from dog kidney and 2D gel electrophoresis were carried out according to Fiedler et al. [8]. The spots corresponding to C14 [6–8] were excised from Coomassie blue stained gels. Amino acid sequence analysis on tryptic fragments was performed as described [7]. Two sequences were obtained KQYHENISAVVF and PAAASGGSSLPSGF. These were used to screen the Swissprot database with MPsearch [17] (accessible by e-mail under Blitz@EMBL-Heidelberg.de) and to prepare the degenerated oligonucleotides for PCR amplification of cDNA from MDCK cells. Cytoplasmic RNA was isolated from MDCK cells as described [18], and converted into cDNA by priming with oligo-dT and reverse transcriptase. The degenerated primers 5′-G(TC)(TA)(GC)ICTICC(ACGT)AG(TC)GGITT-3′ (sense) and 5′-IAT(AG)TT(CT)TC(AG)TG(AG)TA(CT)TG(CT)TT-3′ (antisense) were synthesized and used to amplify the cDNA by PCR [12]. Based on the sequence obtained from the PCR fragment, new oligonucleotides directed either upstream or downstream on the cDNA were synthesized. The downstream primer was used in combination with a oligo-dT primer to obtain the 3′-end sequence of the VIP17 cDNA as described [19]. The 5′-end of the VIP17 cDNA was obtained by using the upstream primer in an anchor ligation based PCR [20]. Finally we amplified a cDNA fragment corresponding to the coding region of the VIP17/MAL cDNA and cloned it into pBAT-4 (*Bam*HI-blunted, *Nco*I), which is a T7-based *Escherichia coli* expression vector (Peränen, unpublished). DNA sequencing was performed on both strands on cDNAs obtained from separate PCR, using the dideoxynucleotide chain termination method [21].

Sequence analysis was carried out with the Wisconsin University GCG software package (Madison, WI, USA) [22]. EMBL/Genbank and Swissprot databases were searched for homology to VIP17.

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2.4. Extraction and purification of proteolipids

MDCK cells (about 80% confluent) were scraped in ice-cold phosphate buffer (NaCl 135 mM, KCl 2.7 mM, Na_2HPO_4 6.5 mM, KH_2PO_4 1.5 mM) with a rubber policeman from 4 × 530 cm^2 dishes, washed with ice-cold SHE buffer (sucrose 250 mM, HEPES/NaOH 10 mM, EGTA 2 mM, pH 7.4) and resuspended in the same buffer with a cocktail of protease inhibitors (chymostatin, leupeptin, antipain, pepstatin, 10 μg/ml each). Cells were disrupted by sonication (Branson tip sonicator; 30 s, 0.5 Hz, strength 3) and centrifuged for 30 s at 10,000 × *g*. The pellet was washed once and the supernatants pooled (PNS, 3 ml, 10 mg/ml).

For chloroform/methanol extraction the PNS was stirred 50 min at 4°C with 15 volumes of chloroform/methanol 2:1 and filtrated through a Whatman filter paper. Then 0.2 volumes of water were added and mixed 30 min at 4°C. After phase separation at 4°C for 3 hours, the water phase was removed and the organic phase dried under a stream of nitrogen.

The floated membrane fraction was prepared according to Fiedler et al. [8] from 12 × 150 cm^2 dishes by flotation of the PNS brought to 2 M sucrose and overlayed with 7.5 ml of 1.2 M and 3 ml of 0.8 M sucrose in 10 mM HEPES/NaOH (pH 7.4) and 2 mM EGTA, and centrifuged at 4°C for 20 h at 38,000 rpm in a SW40 rotor (Beckman, CA, USA). The membrane fraction was recovered from the 0.8/1.2 M sucrose interface (20 ml, 0.5 mg/ml). The CHAPS pellet, prepared according to Fiedler et al. [8] was solubilized in 0.1% SDS, 0.192 M glycine, 25 mM Tris pH 8.3, and extracted into chloroform-methanol. The extracted material from the PNS or the CHAPS pellet was dissolved in 500 μl of chloroform/methanol/acetic acid/HCl(10 mM) 2:1:0.03:0.03 and loaded onto a 65 cm × 1 cm LH-20 column (Pharmacia) and eluted with the same eluent at 800 μl/min (600 μl/fraction). For SDS-PAGE 1/20th and 1/6th of the PNS and CHAPS pellet extracts were loaded, while 1/5th and 1/3rd, respectively for 2D gel electrophoresis.

2.5. SDS-PAGE and 2D gel electrophoresis

SDS-PAGE on 15% gels and resolution of proteins in two dimensions by isoelectric focusing and SDS-PAGE (15% gels) was performed as described [5,6,23]. The proteolipid fractions, dried under nitrogen stream, were resuspended by vortexing directly into sample buffer [24] or 2D gel sample buffer. Detection of proteins was by silver stain.

2.6. Epitope tagging and transfection

A construct encoding VIP17/MAL with the hemagglutinin (HA) 12CA5 epitope [25,26] at the N terminus was created by PCR (overlap extension) using pBAT-VIP17 as a template. A 63 bp oligonucleotide encoding a *Nco*I restriction site at the 5′-end and the amino acids **MGYPYDVPDYA**SGMAPAAA (epitope in bold), and a 3′ specific CCCAAGCTTTATGAAGACTTCCATCTG oligonucleotide were used according to Ho et al. [27]. The amplification product (*Nco*I, *Bam*HI-blunted) was then cloned into pBAT4 (*Nco*I, *Hin*dIII-blunted) creating pBAT-V17HA. Expression of the tagged protein in BHK cells with the T7 RNA polymerase-recombinant vaccinia virus [28] was performed as described [7,29]. Stable MDCK cell clones expressing the tagged protein were created by electrotransfection of cells (according to [30]) with a plasmid (pCMV-V17HA) created by inserting the fragment coding for the tagged protein cut out from pBAT-V17HA (*Not*I, *Nco*I-blunted), into the pBK-CMV vector (*Not*I, *Nhe*I-blunted).

2.7. Conventional and confocal immunofluorescence

Immunofluorescence was performed according to Fiedler et al. [31] omitting the denaturation step with guanidine.

3. Results

3.1. cDNA cloning and sequence analysis

VIP17 protein was purified from Coomassie blue stained 2D gels of the CHAPS pellet from total membrane fraction of dog kidney according to Fiedler et al. [8] and, after trypsin digestion, the fragments were subjected to amino acid sequence analysis. The complete nucleotide sequence was obtained as described in section 2.

An EMBL/GenBank database search (FASTA program, GCG software package) revealed homology with the MAL protein described in T-lymphocytes [14, 32] and the rMAL/MVP17 protein found in rat myelin [15,16]. Fig. 1 shows the alignment of the three proteins, with identical aminoacid residues represented by dots and the consensus (Pretty program, GCG software package) in capital letters. The 88% and 87% identity with the MAL and rMAL proteins respectively suggests VIP17 to be the canine homolog of the MAL protein. We call the protein VIP17/MAL.

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            1                                                  50
VIP17/MAL   MAPAAASGGS sLPSGFSVFT TFPDLLFIFE FIFGGLVWIL IASSLVPiPL
  hMAL      ......t... T. .....T .L..... ..... V......w..
  rMAL      ......S... T.....v .F..... ..... I......m..

            51                                                 100
VIP17/MAL   VQGWVMFVSV FCFMATTaLL VLYIIGAHGG EnSWVTLDAA YHCIAALFYL
  hMAL      .......... ...v...t.i IL....A... .T..V..... ...t......
  rMAL      .......... ...L...s.M VM....t... .T..I..... ...V......

            101                                                150
VIP17/MAL   SASVLEALAT IgMQEGYTYk qYHENIsAVV FSYVATLLYV VHAVFSLIRW
  hMAL      .......... .T.QD.F..R H.....A... .S.I..... V.........
  rMAL      .......... .T.fD.F..R H.....A... .a.V..... I.........

            151
VIP17/MAL   KSS
  hMAL      ...
  rMAL      ...
```

Fig. 1. Alignment of canine VIP17/MAL with the human (hMAL) and rat (rMAL) MAL proteins. Dots represent identity, capital letters the consensus according to the Pretty program of the GCG software package. Accession numbers: VIP17/MAL, X92505; MAL, M15800; rMAL, X82557; MVP17, U31367.

3.2. VIP17/MAL is a proteolipid

The proteolipid fraction from MDCK cell post-nuclear supernatant was isolated by a standard chloroform/methanol extraction procedure for lipids. The extracted material was delipidated by LH-20 gel filtration [33]. Three peaks were obtained (Fig. 2a). The first peak contained proteolipids. Phospholipids were found in the second peak, whereas cholesterol (as well as other small lipidic molecules) was in the third peak (analysis by thin layer chromatography, data not shown). An SDS-PAGE analysis (Fig. 2b, lane 1) of the first peak fraction revealed the presence of several proteins. The prominent band around 17 kDa was identified as VIP17/MAL according to the 2D gel analysis showed in Fig. 2c. The isoelectric point and the apparent molecular weight were identical to those of VIP17 in immunoisolated TGN-derived vesicles [6–8]. This is in agreement with studies demonstrating that the MAL protein from T-lymphocytes also is soluble in chloroform/methanol [32].

A proteolipid fraction from the CHAPS pellet was also prepared. The chloroform/methanol extracted material contained little lipid. The LH-20 column elution profile almost totally lacked the second and third peaks, and the low content of lipids was confirmed by thin layer chromatographic analysis which showed the expected enrichment in sphingolipids and cholesterol (data not shown and see Fiedler et al. [8]). Lane 2 in Fig. 2b and Fig. 2d show the SDS-PAGE and 2D gel patterns of proteolipids present in the CHAPS pellet. Only VIP17/MAL and another unidentified proteolipid were detected.

3.3. Cellular localization of VIP17/MAL in BHK and MDCK cells

VIP17/MAL carrying the HA epitope recognized by the 12CA5 monoclonal antibody was transiently expressed in BHK cells using the T7 RNA polymerase recombinant vaccinia virus expression system [7,28,29]. After 2.5 hours of expression the tagged VIP17/MAL protein was localized to the perinuclear

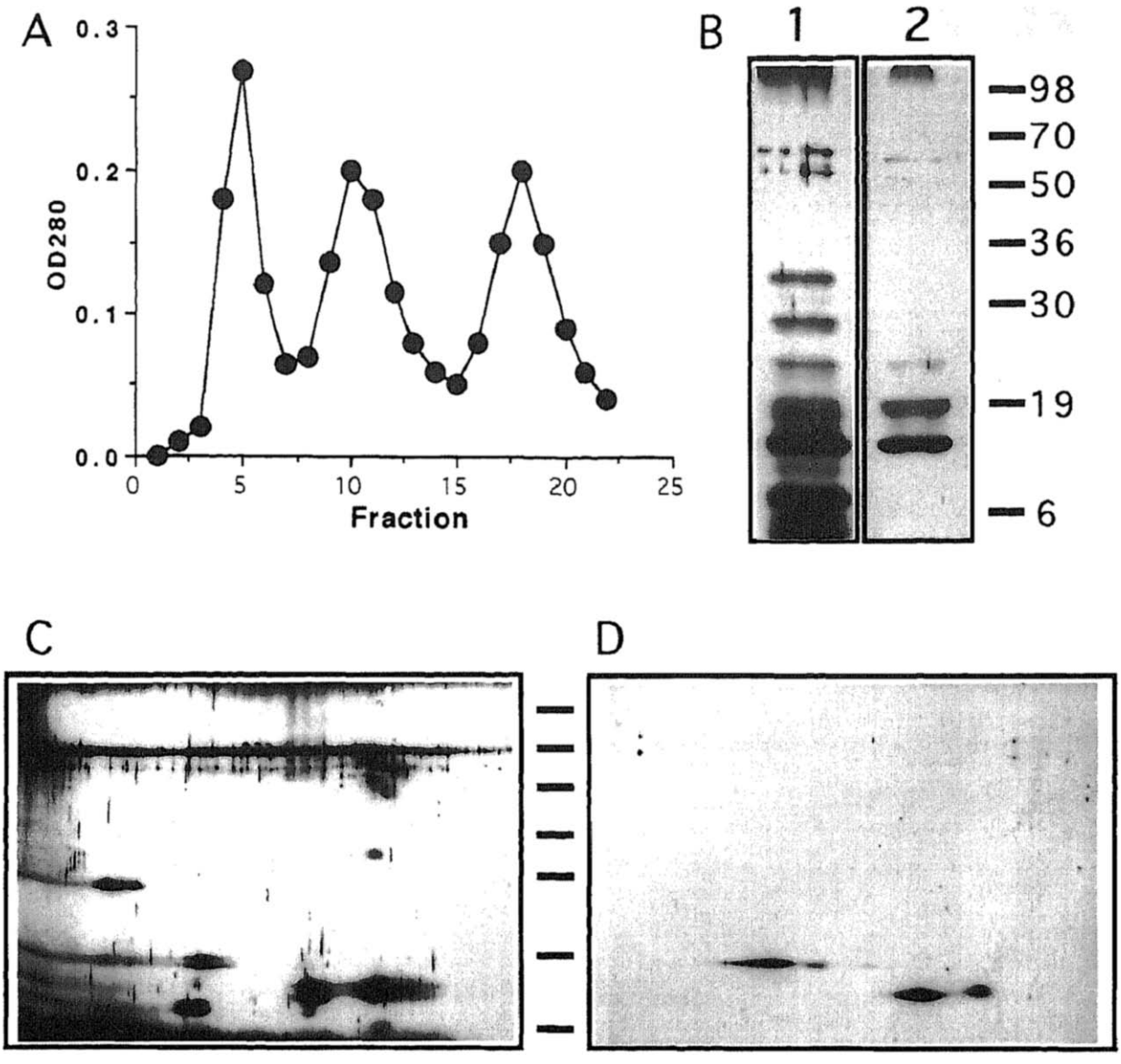

Fig. 2. Proteolipid purification from the PNS and the CHAPS pellet. (A) Elution profile (measured by optical density at 280 nm) of the chloroform/methanol extract from the PNS, chromatographed on the LH-20 column. (B) SDS-PAGE comparison of the proteolipid pattern from the PNS (lane 1) and the CHAPS pellet (lane 2). (C,D) 2D gel analysis of the proteolipids extracted from the PNS (C) or the CHAPS pellet (d). Basic side is on the right. The same molecular weight markers (horizontal bars) are used as in (B).

area and to punctate structures throughout the cell (Fig. 3a). After a chase with cycloheximide (4 hours of expression followed by 90 min cycloheximide treatment) the perinuclear staining decreased and surface staining was seen, whereas the punctate-vesicular pattern was unchanged (Fig. 3b). No signal was detected from non permeabilized cells suggesting that the N terminus is on the cytoplasmic side (not shown).

The tagged protein was also stably expressed in MDCK cells. No difference in the gross morphology could be observed between the expressing clone and the parental line. The VIP17/MAL expressing MDCK cells exhibited normal transepithelial resistance and the distribution of the apical marker 114 kDa protein and the basolateral marker 58 kDa protein was polarized in the filter-grown cells (not shown) [34]. Immunofluorescence analysis of the tagged protein in the confocal microscope revealed a punctate-vesicular and plasmalemmal pattern in agreement with the results obtained in BHK cells. In addition, a preferential localization towards the apical side could be observed in the comparison with the VIP21/caveolin staining (Fig. 4).

4. Discussion

VIP21/caveolin, VIP36 and VIP17/MAL were all identified as components of a detergent-insoluble complex, which forms when the newly synthesized influenza virus hemagglutinin reaches the TGN in MDCK cells [7]. This high molecular weight complex is then incorporated into vesicles routed to the apical membrane. The discovery that these same proteins could be isolated by an extremely simple procedure relying on their

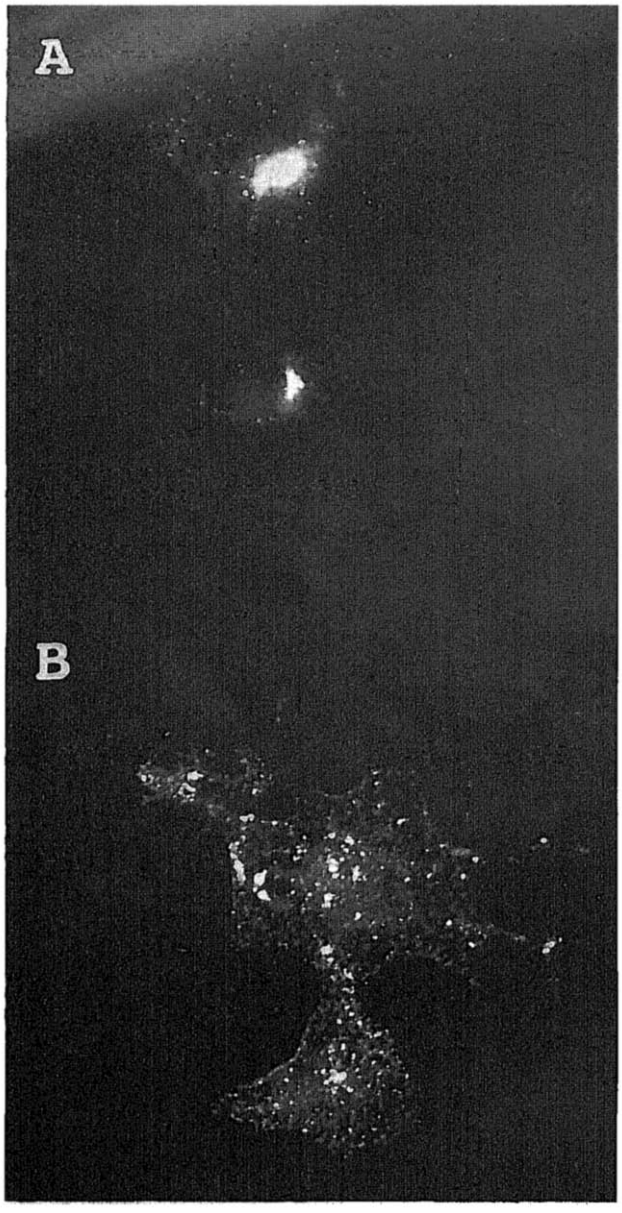

Fig. 3. Localization of tagged VIP17/MAL in transfected BHK cells grown on coverslips after 2.5 hours of expression (A), or 4 hours of expression followed by 90 min chase with cycloheximide (B). Monoclonal anti-HA (12CA5 epitope) was used at a concentration of 1 μg/ml.

CHAPS-insolubility at 4°C, enabled us to characterize these major proteins of the CHAPS complex [7,8,12]. Because detergent-insolubility is such a non-specific purification criterion, it is reassuring that all of these three proteins are indeed localized in the post-Golgi trafficking routes to the cell surface. The intracellular localization of VIP17/MAL was analyzed expressing the epitope-tagged protein both in BHK cells and MDCK cells. The analysis showed a very similar cellular distribution to that previously reported for VIP36 [12]. The VIP17/MAL protein is seen in a perinuclear location probably corresponding to the Golgi complex, in cytoplasmic vesicles, and on the cell surface. During treatment with cycloheximide to block protein synthesis, the perinuclear labelling of VIP17/MAL decreased in BHK cells, presumably because its steady-state localization is more towards post-Golgi compartments, as was seen in stably transfected MDCK cells.

VIP21/caveolin, VIP36 and VIP17/MAL are not only present in apical vesicles, but are also in basolateral vesicles [6]. The reason for this is not yet clear, but we favor the possibility that these proteins also play a role in basolateral to apical transcytosis. VIP21/caveolin is also known to form caveolae on the basolateral surface [9].

Cloning and sequencing the cDNA encoding VIP17 revealed that it is the canine homolog of the human MAL protein which is expressed in the late stages of T-cells maturation [14,32]. Recently a rat myelin protein has also been shown to be a homolog of MAL and VIP17 [15,16]. MAL belongs to the group of proteins called proteolipids [35,36] based on their solubility in organic solvents and on their high content of hydrophobic amino acids [32]. We showed here that VIP17/MAL can be also purified by chloroform/methanol extraction.

VIP17/MAL is expressed in white and grey matter oligodendrocytes, in myelinating Schwann cells and in the kidney. Amazingly, rat MAL is not expressed in the thymus [15,16]. Thus, the VIP17/MAL has a very specific tissue expression. Although its function in myelin is not known, it is important to point out that VIP17/MAL is expressed at the time when myelin sheets are being formed [15]. Kim et al. demonstrated that VIP17/MAL is a major component of CHAPS-insoluble complexes in oligodendrocytes starting to produce myelin. VIP21/caveolin and VIP36 were not identified in these complexes [16]. Since myelin is enriched in glycolipids, particularly galactosylceramide and sulfatide [37], it is tempting to speculate that VIP17/MAL has a function in glycolipid transport to the cell surface, i.e. to the myelin sheets in oligodendrocytes and Schwann cells and to the apical surface in kidney cells. This conforms with our working hypothesis for apical membrane biogenesis, involving glycolipid–cholesterol rafts as sorting platform that load cargo in the TGN destined to the apical membrane [13]. Recent results in our laboratory suggest that the apical traffic route uses a mechanism for docking and fusion different from that employing the Rab/NSF/SNAP/SNARE machinery [38]. We, therefore, expect to unravel a new mode of vesicular transport depending on known and unknown VIPs and annexins [31]. Glycolipid rafting may indeed be involved not only in apical and myelin biogenesis, but also in the transport of newly synthesized proteins to the axolemma in neurons [39], as well as in endocytosis and transcytosis involving surface caveolae [40]. Only further work will demonstrate whether this hypothesis is correct or not.

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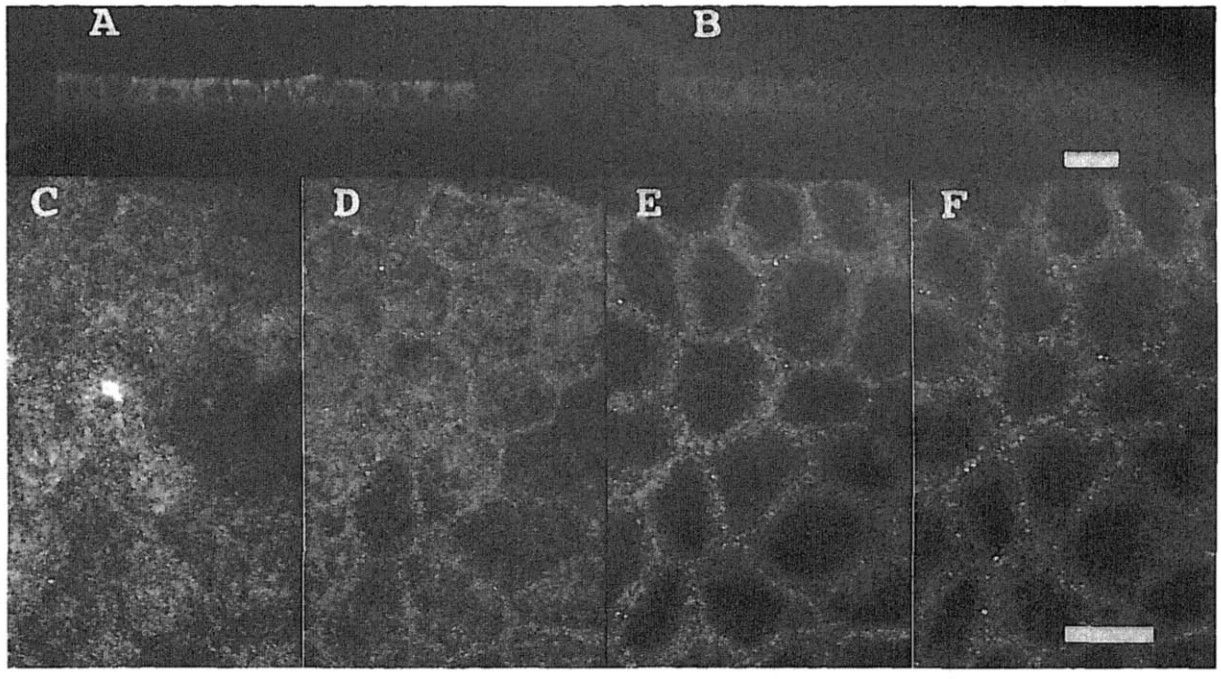

Fig. 4. Localization of epitope-tagged VIP17/MAL and of VIP21/caveolin in filter-grown MDCK cell by confocal microscopy immunofluorescence. (A,B) X–Z views of cells labelled with monoclonal anti-HA (12CA5 epitope) 1 μg/ml (A) or with polyclonal anti-VIP21/caveolin (N-20) 1:500 (B). Bar = 20 μm. (C–F) X–Y serial sections of 1.8 μm from the apical to the basolateral plane of cells labelled with monoclonal anti-HA 1:150. Bar = 10 μm

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