

Selective localization of the polytopic membrane protein prominin in microvilli of epithelial cells – a combination of apical sorting and retention in plasma membrane protrusions

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SUMMARY

Prominin is a recently identified polytopic membrane protein expressed in various epithelial cells, where it is selectively associated with microvilli. When expressed in non-epithelial cells, prominin is enriched in plasma membrane protrusions. This raises the question of whether the selective association of prominin with microvilli in epithelial cells is solely due to its preference for, and stabilization in, plasma membrane protrusions, or is due to both sorting to the apical plasma membrane domain and subsequent enrichment in plasma membrane protrusions. To investigate this question, we have generated stably transfected MDCK cells expressing either full-length or C-terminally truncated forms of mouse prominin. Confocal immunofluorescence and domain-selective cell surface biotinylation experiments on transfected MDCK cells grown on permeable supports demonstrated the virtually exclusive apical localization of prominin at steady state. Pulse-chase experiments in combination with domain-selective cell surface biotinylation showed that newly synthesized prominin was directly targeted to the apical plasma membrane domain. Immunoelectron microscopy

revealed that prominin was confined to microvilli rather than the planar region of the apical plasma membrane. Truncation of the cytoplasmic C-terminal tail of prominin impaired neither its apical cell surface expression nor its selective retention in microvilli. Both the apical-specific localization of prominin and its selective retention in microvilli were maintained when MDCK cells were cultured in low-calcium medium, i.e. in the absence of tight junctions. Taken together, our results show that: (i) prominin contains dual targeting information, for direct delivery to the apical plasma membrane domain and for the enrichment in the microvillar subdomain; and (ii) this dual targeting does not require the cytoplasmic C-terminal tail of prominin and still occurs in the absence of tight junctions. The latter observation suggests that entry into, and retention in, plasma membrane protrusions may play an important role in the establishment and maintenance of the apical-basal polarity of epithelial cells.

Key words: Prominin, Polytopic membrane protein, Apical sorting, Microvillus

INTRODUCTION

A hallmark of epithelial cells is their apical-basal polarity. At the level of the plasma membrane, this is reflected by the existence of distinct, apical and basolateral, domains, which differ from each other in protein and lipid composition. Studies on the biogenesis and maintenance of this plasma membrane polarity in classical epithelia such as kidney, liver and intestine, and in cell lines derived therefrom, led to the identification and characterization of three principal molecular mechanisms, which are not mutually exclusive, that underlie the polarized distribution of plasma membrane proteins in epithelial cells (Rodriguez-Boulant and Nelson, 1989; Nelson, 1992; Simons et al., 1992; Matter and Mellman, 1994; Mays et al., 1995a). First, newly synthesized plasma membrane proteins are sorted

to the appropriate, apical or basolateral, plasma membrane domain. This sorting occurs at two levels: (i) at the trans-Golgi network (TGN), if apical and basolateral proteins are delivered directly to the respective plasma membrane domain, as shown in MDCK cells (Rodriguez-Boulant and Nelson, 1989; Simons et al., 1992); and (ii) at endosomes, if both apical and basolateral proteins are first delivered to one plasma membrane domain followed by transcytosis of one set of proteins via endosomes to the other plasma membrane domain, as shown in enterocytes (Massey et al., 1987), Caco-2 cells (Matter et al., 1990) and hepatocytes (Bartles et al., 1987). Second, plasma membrane proteins are randomly delivered to either cell surface domain but are selectively retained, by interacting with cytoskeletal components (Hammerton et al., 1991; Rotin et al., 1994) and/or the extracellular matrix, in only one domain

whereas they are endocytosed from the other domain. Third, plasma membrane proteins are randomly delivered to either cell surface domain but undergo proteolytic processing in only one domain, resulting in their enrichment in the other domain (Dempsey et al., 1997).

Our group has been studying the apical-basal polarity of neuroepithelial cells, which constitute the inner layer of the neural tube of the vertebrate embryo and give rise to all neurons and macroglial cells of the central nervous system (Huttner and Brand, 1997). In searching for markers to study the apical-basal polarity of these cells, we have recently identified prominin, a novel polytopic membrane protein of as yet unknown function that is selectively localized at the apical surface of neuroepithelial cells and several other embryonic epithelia, and on brush border membranes of the epithelial cells lining the adult kidney proximal tubules (Weigmann et al., 1997). Prominin is a 115 kDa glycoprotein predicted to consist of an extracellular N-terminal domain, five transmembrane domains separating two small cytoplasmic and two large extracellular loops, and a cytoplasmic C-terminal domain (Fig. 1). However, prominin is also expressed in non-epithelial cells: independent work by Buck and colleagues (Miraglia et al., 1997; Yin et al., 1997) has shown that the human AC133 antigen, which appears to be the ortholog of mouse prominin (Corbeil et al., 1998; Miraglia et al., 1998; D. Corbeil et al., unpublished data), is expressed in hematopoietic stem cells.

With regard to the mechanisms underlying the polarized distribution of plasma membrane proteins in epithelial cells, prominin shows two remarkable features. First, within the apical plasma membrane domain, prominin is exclusively associated with microvilli and other plasma membrane protrusions, even if the latter are very small and sparse (Weigmann et al., 1997). This protrusion-specific localization does not depend on an epithelial state of the cell because prominin ectopically expressed in fibroblasts is also selectively found in plasma membrane protrusions (Weigmann et al., 1997). Second, prominin retains an exclusive apical localization in neuroepithelial cells not only at the neural plate stage, when tight junctions are present, but also at the neural tube stage (Weigmann et al., 1997), when tight junctions have disappeared (Aaku-Saraste et al., 1996) and the polarity of certain plasma membrane proteins is down-regulated (Aaku-Saraste et al., 1997).

These observations raise the following questions. Is the apical-specific localization of prominin in epithelial cells solely the consequence of its preference for plasma membrane protrusions? In other words, does the apical-specific localization of prominin simply reflect the greater abundance of plasma membrane protrusions in the apical than basolateral domain, with prominin being delivered randomly to apical and basolateral domains but remaining in the plasma membrane only if incorporated into a protrusion? This mechanism would require only one type of targeting information in the prominin molecule, i.e. to be specifically retained in plasma membrane protrusions. Or is the apical-specific localization of prominin due to both (i) the specific delivery to the apical plasma membrane domain, followed by (ii) its enrichment and retention in plasma membrane protrusions? This mechanism would require a second type of targeting information in prominin in addition to that responsible for its retention in

plasma membrane protrusions, i.e. to be sorted into vesicles destined for the apical plasma membrane.

In the present study, we have investigated these questions. Specifically, we have expressed wild-type prominin and C-terminal deletion mutants in the epithelial cell line MDCK and have analysed (i) the cell surface delivery of these proteins, (ii) their apical vs basolateral distribution, and (iii) their retention in plasma membrane protrusions.

MATERIALS AND METHODS

Materials

Media and reagents for cell culture were purchased from Gibco-BRL. Protein A-Sepharose CL-4B was from Pharmacia Biotech. Streptavidin-agarose beads were from Sigma Chemical Co. TranswellTM-COL collagen-coated filters were obtained from Costar (Cambridge, MA) and Nunc polycarbonate membrane tissue culture inserts from A/S Nunc (Denmark). Goat anti-rat IgG/IgM coupled to dichlorotriazinyl amino fluorescein (DTAF) and affinity pure rabbit anti-rat IgG/IgM were purchased from Jackson ImmunoResearch Laboratories.

Plasmid construction

The bacterial expression plasmids pGEX-E2 and pGEX-I3 containing the mouse prominin cDNA from nt 735 to nt 1466 (residues Gln183-Trp426), and nt 2607 to nt 2762 (Lys807-Tyr858), respectively, each fused in-frame to glutathione S-transferase (GST), were constructed by selective PCR amplification of a composite cDNA (derived from clones 3Ab1 and 4B; Weigmann et al., 1997). For pGEX-E2, the oligonucleotides 5'-CGGATCAAAGGATCCCAGAACTG-3' and 5'-AATCAAGGGATCCAGCTACCAGT-3' were used as 5' and 3' primers, respectively; for pGEX-I3 the oligonucleotides 5'-TGTAATCATGGATCCAAGCTGGC-3' and 5'-AAGCAGC-TTCGGATCCAGTTGTCA-3' were used as 5' and 3' primers, respectively. All oligonucleotides created a *Bam*HI restriction site and the E2 3' primer in addition introduced a TAG stop codon. The resulting PCR fragments were digested with *Bam*HI and cloned into the corresponding site of pGEX-2T (Pharmacia).

The eukaryotic expression plasmid pRc/CMV-prominin, containing both the mouse prominin cDNA and a neomycin resistance gene, has been described previously (Weigmann et al., 1997). C-terminally truncated forms of prominin were obtained by PCR mutagenesis using cloned *Pfu* DNA polymerase (Stratagene). The translational stop codons were introduced into the cDNA at positions corresponding to amino acids 833 (mutant Δ 26C) and 812 (mutant Δ 47C). The PCR products were digested with *Cla*I and resulting cDNA fragments (*Cla*I-*Cla*I) containing the mutated region were used to replace the corresponding wild-type cDNA fragment in the pRc/CMV-prominin plasmid.

In all cases, the PCR DNA fragments were sequenced in order to confirm the presence of the mutation and the absence of any reading mistakes of the DNA polymerase.

Antisera against recombinant prominin

GST-fusion proteins containing the E2 (residues Gln183-Trp426), or I3 (Lys807-Tyr858) fragments of prominin (see Fig. 1) were expressed in HB 101 *Escherichia coli* and purified on glutathione Sepharose 4B beads (Pharmacia) according to the batch method as described by the manufacturer's protocol. The E2 fragment was cleaved from the fusion protein bound to the beads by incubation with human thrombin (\approx 25 cleavage units/mg protein) (Sigma) for 2 hours at room temperature. The eluted E2 fragment was then purified on a 15% polyacrylamide gel and an antiserum against this fragment was generated by immunizing a New Zealand white rabbit with an SDS-PAGE gel slice homogenized and mixed (1:1) with complete Freund's

adjuvant. The GST-fusion protein containing I3 fragment was eluted from the glutathione Sepharose 4B beads at room temperature by addition of 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. The fusion protein was then used to generate antisera against the I3 fragment as described above.

Cell culture and transfection

MDCK cells (strain II) were maintained in a humidified incubator at 37°C under a 5% CO₂ atmosphere in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS), 100 i.u./ml penicillin, and 100 µg/ml streptomycin (complete medium). For experiments studying the polarized cell surface delivery, cells were plated at a density of 4.5×10⁵ cells/cm² on permeable membranes (0.4 µm pore size), using either 24 mm TranswellTM-COL chambers or 12 mm Nunc tissue culture inserts. Media were changed every day, and the experiments were performed 3-7 days after seeding to allow the development of a tight monolayer. The integrity of the cell monolayer was assessed by monitoring the diffusion of [¹⁴C]sucrose (Amersham, 615 mCi/mmol) from the apical to the basolateral compartment of the chamber. Filter-grown MDCK cell monolayers with a leakage of >1% (after 1 hour) were discarded.

For some experiments, the cells were plated at confluency on either 24 mm TranswellTM-COL chambers or collagen-coated 60 mm Petri dishes (Collagen R; Serva) in complete medium. After 90 minutes in the incubator to allow for cell attachment, the complete medium was replaced with low-calcium medium (spinner(s) MEM, Gibco) containing 10% FCS dialysed against PBS, and the cultures were incubated for 24 hours (Vega-Salas et al., 1987; Ojakian and Schwimmer, 1988).

MDCK cells were transfected with the prominin eukaryotic expression plasmids (see Plasmid construction) using the calcium phosphate-mediated transfection method for adherent cells in suspension (Sambrook et al., 1989). Cells expressing the neomycin resistance gene were then selected in complete medium containing 600 µg/ml of G418. Two weeks later, G418-resistant colonies were pooled and expanded in the presence of G418. To enhance the expression of the transgene, cells were incubated for 17 hours with 10 mM sodium butyrate (Gorman et al., 1983). All subsequent steps were performed in complete medium lacking butyrate. Under these conditions, 10-30% of the neomycin-resistant cells expressed the recombinant prominin. Transfected cells were then used for northern blot analysis, metabolic labeling, cell surface biotinylation, immunofluorescence, and electron microscopy, or were solubilized in ice-cold solubilization buffer (2% Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 1 µg/ml leupeptin, 10 µg/ml aprotinin), and the Triton extract obtained after centrifugation (10 minutes, 10,000 g) was used for deglycosylation, SDS-PAGE and immunoblotting (see below).

Endoglycosidase digestions and immunoblotting

Triton extracts corresponding to one-fifth of a confluent 100 mm dish were incubated overnight at 37°C in the absence or presence of either 0.8 U peptide-N-glycosidase F (PNGase F) or 10 mU of endo-β-N-acetylglucosaminidase H (endo H) according to the manufacturer's protocols (Boehringer-Mannheim).

Proteins were then analysed by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany; pore size 0.45 µm) using standard procedures. After transfer, nitrocellulose membranes were incubated for 2 hours in blocking buffer (PBS containing 5% low fat milk powder and 0.3% Tween-20). Prominin was then detected using either mAb 13A4 (1 µg/ml) (Weigmann et al., 1997) or one of the two rabbit polyclonal antisera (the αE2 antisera (1/20,000) and αI3 antiserum (1/20,000)) diluted in blocking buffer. In all cases, horseradish peroxidase-conjugated secondary antibodies were used to reveal antigen-antibody complexes and these were visualized with enhanced chemiluminescence developing reagents (ECL system, Amersham Corp.).

Cell surface biotinylation and immunoprecipitation

Unless indicated otherwise, all steps were carried out at 4°C. Just prior to use, the membrane-impermeable sulfo-succinimidyl-6(biotinamido)-hexanoate biotinylation agent (sulfo-NHS-LC-biotin, Pierce) was dissolved in Ca/Mg-PBS (PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂) to a final concentration of 0.2 mM. After repeated washing (five times) with Ca/Mg-PBS, stably transfected MDCK cells (on 100 mm dishes) were incubated for 1 hour in 2 ml of the biotin solution. The cells were washed three times with Ca/Mg-PBS and then incubated for 10 minutes with Ca/Mg-PBS containing 20 mM glycine to quench the residual biotin. Cells were lysed in ice-cold solubilization buffer (see Cell culture and transfection), and the Triton extract was diluted tenfold with Ca/Mg-PBS containing protease inhibitors. Biotinylated cell surface proteins were then adsorbed to streptavidin-agarose beads. After a 2 hours incubation, the beads were washed five times with Ca/Mg-PBS and biotinylated proteins were eluted using Laemmli buffer at 95°C. Eluates were analysed by SDS-PAGE followed by immunoblotting.

For domain-selective cell surface biotinylation (Le Bivic et al., 1989), 7-day-old monolayers on duplicate Transwell filters were washed three times with Ca/Mg-PBS and then biotinylated (see above) from either the apical (1.5 ml) or basolateral (2.6 ml) chamber compartment. The compartment not receiving sulfo-NHS-LC-biotin was filled with Ca/Mg-PBS. After quenching, cells were lysed in ice-cold solubilization buffer and Triton extracts were then divided into two equal aliquots. One was subjected to streptavidin-agarose adsorption and biotinylated proteins were analysed by immunoblotting as described above. The other was first subjected to immunoprecipitation. Briefly, Triton extracts were diluted four-fold in immunoprecipitation buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.8, 10 µg/ml aprotinin, 2 µg/ml leupeptin and 1 mM PMSF), and αI3 antiserum was added (1:100 dilution). The samples were incubated overnight at 4°C end-over-end. The immune complexes were collected with Protein A-Sepharose beads and analysed by SDS-PAGE followed by blotting with horseradish peroxidase-conjugated streptavidin (see Immunoblotting, except that the horseradish peroxidase-conjugated streptavidin was diluted in PBS).

Pulse-chase studies

Stably transfected MDCK cells on 60 mm dishes were rinsed once with met/cys-free medium (methionine- and cysteine-free DMEM supplemented with 1% FCS dialysed against PBS), and incubated for 30 minutes at 37°C in this medium. The cells were then pulse-labeled for 20 minutes at 37°C with fresh met/cys-free medium containing 500 µCi/ml [³⁵S]Pro-mix (Amersham, >1000 Ci/mmol). After the pulse, cells were chased for the indicated times in fresh met/cys-free DMEM supplemented with 5% FCS and unlabeled methionine and cysteine at a concentration of 0.15 mg/ml each. At the end of the chase period, the cells were washed three times with ice-cold PBS containing 1 mM PMSF and then lysed in ice-cold solubilization buffer (see Cell culture and transfection). Prominin was then immunoprecipitated from Triton extracts with mAb 13A4, and the immune complexes were collected using Protein A-Sepharose, which had been preincubated with rabbit anti-rat antibody, followed by SDS-PAGE and phosphoimaging.

In order to study the cell surface delivery, Transwell filter-grown MDCK cells were radiolabeled for 20 minutes with [³⁵S]Pro-mix (see above), with the labeling medium added to the basolateral compartment since uptake of amino acids has previously been found to be more efficient from the basolateral domain (Balcarova-Ständer et al., 1984). After the pulse, cells were chased for the indicated times, chilled in cold Ca/Mg-PBS and cell-surface proteins were labeled using the domain-specific biotinylation protocol described above. The cells were then lysed, and prominin molecules were first immunoprecipitated with αI3 antiserum (see Cell surface biotinylation and immunoprecipitation). To separate the biotinylated from the non-

biotinylated prominin molecules in the immunoprecipitates, the Protein A-Sepharose beads were boiled in 20 μ l of 10% SDS for 5 minutes, diluted with 1 ml of 0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 7.5, and centrifuged (1 minute, 10,000 g). The supernatants were then incubated with streptavidin-agarose beads as described in Cell surface biotinylation and immunoprecipitation. The beads were washed five times with PBS and biotinylated proteins were eluted using Laemmli buffer at 95°C. Eluates were subjected to SDS-PAGE and analysed using a Fuji phosphorimager.

Immunofluorescence and confocal microscopy

Cell surface immunofluorescence was performed as described previously (Pimplikar and Huttner, 1992). Prominin-transfected MDCK cells grown on glass coverslips were washed with Ca/Mg-PBS, first at room temperature and then on ice, and double-surface-labeled for 30 minutes at 4°C by addition of the mAb 13A4 (10 μ g/ml) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated wheat germ agglutinin (WGA) (1:300, Sigma) in immunofluorescence buffer (Ca/Mg-PBS containing 0.2% gelatin). Unbound antibodies and free WGA were removed by five washes with ice-cold immunofluorescence buffer. Fixative, 3% paraformaldehyde in PBS, was added to the cells on ice, and the coverslips were placed at room temperature for 30 minutes. The fixative was removed by three washes with Ca/Mg-PBS, and the residual formaldehyde was quenched for 30 minutes with 0.1 M glycine in PBS. The cells were then incubated for 30 minutes at room temperature with DTAF-conjugated anti-rat IgM/IgG (1:150). The coverslips were rinsed sequentially with immunofluorescence buffer, PBS and distilled water, and mounted in Mowiol 4.88 (Calbiochem-Behring GmbH).

In immunofluorescence experiments on filters, 5-day-old, Nunc filter-grown monolayers of prominin-transfected MDCK cells were washed with PBS and then fixed with 2% paraformaldehyde/0.1% glutaraldehyde in PBS for 15 minutes at room temperature. Quenching was performed by three 5-minute washes with a 1 mg/ml NaBH₄ solution. The cells were permeabilized and blocked with 0.2% saponin/0.2% gelatin in PBS (blocking solution) for 10 minutes. The cells were then incubated sequentially for 30 minutes each with mAb 13A4 and DTAF-labeled secondary antibody diluted in blocking solution. Both primary and secondary antibodies were added to both sides of the filter. Nuclei were labeled with propidium iodide (0.3 μ g/ml) during the incubation with the secondary antibody. The filters were washed in PBS, cut out, dipped in water and mounted essentially as described by Reinsch and Karsenti (1994) except that we used a mounting solution consisting of 90% glycerol, 0.1% (w/v) paraphenylenediamine in PBS, pH 9.0. The cells were observed with a Leica TCS^{4D} confocal laser scanning microscope. The confocal microscope settings were such that the photomultipliers were within their linear range. The images shown were prepared from the confocal data files using Adobe photoshop software and printed using a Kodak XLS 8600 PS printer.

Immunoelectron microscopy

Stably transfected MDCK monolayers cultured on collagen-coated 60 mm Petri dishes (Collagen R; Serva) or Transwell-filters were washed with 250 mM HEPES-NaOH, pH 7.4, and fixed with 8% paraformaldehyde/0.1% glutaraldehyde in HEPES buffer overnight at 4°C. The cells were then infiltrated with 15% polyvinylpyrrolidone, 1.95 M sucrose in PBS (Tokuyasu, 1989) and ultrathin cryosections (Griffiths, 1993) were cut at -100°C with a Reichert FCS cryoultramicrotome (Leica). Cryosections were stretched using 2.3 M sucrose and mounted on Formvar/carbon-coated grids. Sections were blocked with 10% FCS and 20 mM glycine in PBS for 10 minutes, and sequentially incubated with the mAb 13A4 (500 μ g/ml), rabbit anti-rat antibody (1:30), and Protein A-gold (9 nm or 14 nm), each in blocking solution containing only 5% FCS. The sections were treated with 0.3% uranyl acetate/1.8% methyl cellulose, and air dried. The

immunogold-labeled sections were examined in an electron microscope (EM 10, Zeiss).

RESULTS

Expression and biosynthesis of prominin in MDCK cells

Prominin was expressed in the MDCK epithelial cell line by stable transfection with the pRc/CMV-prominin plasmid. Expression of mouse prominin was monitored using SDS-PAGE and immunoblotting with the rat monoclonal antibody (mAb) 13A4. The apparent molecular mass of mouse prominin expressed in MDCK cells was found to be different from prominin derived from mouse kidney, which consisted of a single band with an average molecular mass of 115 kDa (Fig. 2A, lane 1). In prominin-transfected MDCK cells, two distinct sets of immunoreactive bands with apparent molecular mass of 120-125 kDa (referred to as 123 kDa band) and 102-106 kDa (referred to as 104 kDa band) were detected (Fig. 2A, lane 4). The same results were obtained with two rabbit polyclonal antisera, designated α 13 and α E2 (see Fig. 1 and Materials and Methods) (data not shown). Densitometric scanning revealed that the 104 kDa band represented about 10-20% of the total amount of immunoreactive material. No prominin immunoreactivity was detected when MDCK cells were transfected with the expression vector without insert (data not shown; see Fig. 6B, lane 2), which suggests that this kidney-derived cell line is devoid of endogenous prominin. Consistent with the immunoblotting data, no prominin mRNA was found in untransfected MDCK cells by northern blot analysis (data not shown).

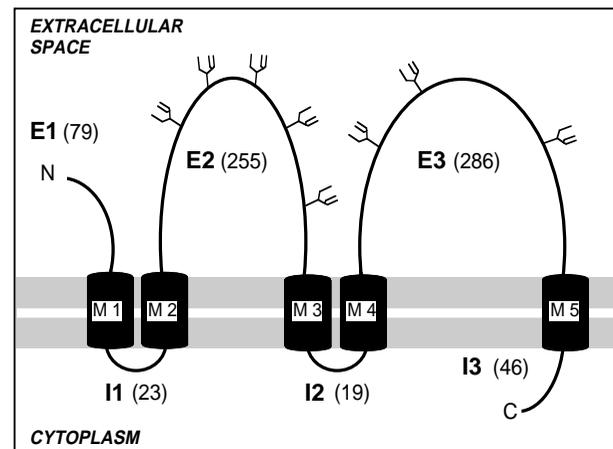


Fig. 1. Topological model of prominin. The N-terminal 19 amino acid residues of mouse prominin are assumed to constitute a cleaved signal peptide rather than an uncleaved signal anchor and are not shown. M1-M5, putative transmembrane segments. The numbers of amino acid residues in the three extracellular domains (E1-E3) and the three intracellular, cytoplasmic domains (I1-I3) are given in parentheses. The two large extracellular loops (domains E2 and E3) contain all eight potential N-glycosylation sites. The extracellular location of the E2 and E3 domains is shown by the presence of N-linked glycans (Weigmann et al., 1997) and by cell surface immunofluorescence (not shown), the cytoplasmic localization of the I3 domain has been shown previously (Weigmann et al., 1997).

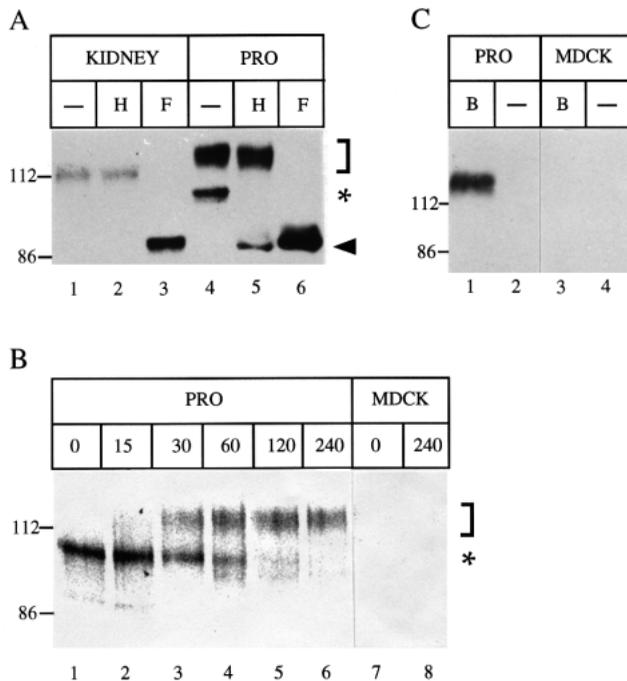


Fig. 2. Biosynthesis of mouse prominin in transfected MDCK cells. (A) Lysates of prominin-transfected MDCK cells (PRO) and, for comparison, adult mouse kidney membranes were incubated in the absence (-) or presence of 10 mU endo H (H) or 1 U PNGase F (F) and analysed by immunoblotting with mAb 13A4. Bracket, endo H-resistant, PNGase F-sensitive form of prominin; asterisk, endo H- and PNGase F-sensitive form; arrowhead, product after N-deglycosylation. (B) MDCK cells stably transfected with the mouse prominin expression vector (PRO) or vector DNA without insert (MDCK) were pulse-labeled for 20 minutes with [³⁵S]methionine/cysteine and chased for the indicated times (minutes). After immunoprecipitation of prominin with mAb 13A4, labeled proteins were analysed by SDS-PAGE and phosphoimaging. Bracket, 123 kDa prominin band; asterisk, 104 kDa prominin band. (C) MDCK cells stably transfected with the mouse prominin expression vector (PRO) or vector DNA without insert (MDCK) were incubated for 1 hour at 4°C without (-) or with (B) sulfo-NHS-LC-biotin, solubilised, and biotinylated proteins adsorbed to streptavidin-agarose followed by immunoblotting of the adsorbed material using the α I3 antiserum.

Treatment with PNGase F converted the 123 kDa and 104 kDa bands of recombinant prominin found in transfected MDCK cells into the same 94 kDa product (Fig. 2A, lane 6, arrowhead) as that obtained in the case of mouse kidney prominin (Fig. 2A, lane 3), indicating that the molecular mass difference between mouse prominin expressed in MDCK cells and mouse kidney prominin resulted from differential N-glycosylation. The significance, if any, of this differential N-glycosylation is unknown. The 123 kDa band found in transfected MDCK cells, like the kidney protein, was largely resistant to endo H digestion (Fig. 2A, lanes 2 and 5, bracket), suggesting that it had passed through the Golgi apparatus. In contrast, the 104 kDa band found in transfected MDCK cells (Fig. 2A, lane 4, asterisk) was converted to the 94 kDa form upon endo H treatment (Fig. 2A, lane 5, arrowhead), consistent with the 104 kDa band representing the high-mannose form of prominin

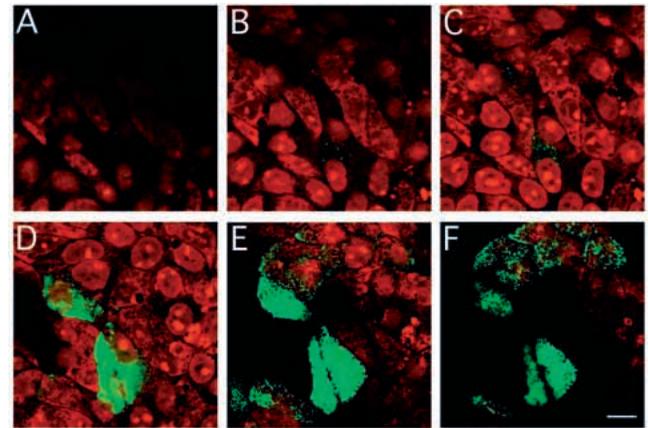


Fig. 3. Localization of mouse prominin in polarized MDCK cells by confocal immunofluorescence microscopy. Prominin-transfected MDCK cells were cultured to confluency on Nunc filters and then stained with mAb 13A4 followed by DTAF-conjugated anti-rat antibody (green). Nuclei were labeled with propidium iodide (red). (A-F) A series of single optical *xy*-plane sections from the basal (A) to the apical (F) side of the cells. Bar, 10 μ m.

localized in the endoplasmic reticulum or an early Golgi compartment.

To investigate the kinetics of maturation of mouse prominin expressed in MDCK cells, pulse-chase experiments with [³⁵S]methionine/cysteine were performed. Cells were metabolically labeled for 20 minutes, collected at various times, and prominin immunoprecipitated from Triton X-100 extracts. At 0 minutes of chase, a single band of 104 kDa was detectable (Fig. 2B, lane 1, asterisk). This band was converted to the 123 kDa band (Fig. 2B, lanes 3-6, bracket) with a $t_{1/2}$ of 1 hour, indicating that the 104 kDa band represented the immature form of prominin. Cell-surface biotinylation experiments provided direct evidence that the 123 kDa form, but not the 104 kDa form, is located at the cell surface. After cell surface labeling with the membrane impermeant sulfo-NHS-LC-biotin followed by streptavidin-agarose precipitation and immunoblotting with α I3 antiserum, only the 123 kDa band, but not the 104 kDa band, of prominin was recovered (Fig. 2C, lane 1).

Prominin is specifically associated with apical microvilli, even in the absence of tight junctions

To determine the localization of prominin with regard to the apical versus basolateral plasma membrane domain of MDCK cells, indirect immunofluorescence followed by confocal laser scanning microscopy was performed with confluent monolayers of transfected MDCK cells grown on permeable filters. To facilitate the analysis, the nuclei were visualized by propidium iodide staining. A gallery of confocal sections from the basal (Fig. 3A) to the apical (Fig. 3F) side revealed that prominin immunostaining (green) was predominantly located at the apical domain.

The previous observation (Weigmann et al., 1997) that prominin is specifically associated with microvilli and related plasma membrane protrusions present at the apical surface of neuroepithelial cells *in vivo* prompted us to investigate the ultrastructural localization of prominin in the transfected MDCK

cells (Fig. 4). Immunogold EM revealed that the prominin immunoreactivity was preferentially associated with microvillar membranes; no significant labeling could be detected in the neighbouring planar areas of the apical plasma membrane (Fig. 4A). The lateral membrane was devoid of labeling (Fig. 4B). These data show that the signal specifying the enrichment of mouse prominin in microvillar protrusions of the apical plasma membrane is operational in canine cells. Therefore, this transfected epithelial cell line constitutes a useful model system for studying the mechanism underlying this enrichment.

To investigate whether the enrichment of prominin in microvilli required the integrity of cell-to-cell contacts, prominin-transfected MDCK cells were cultured in low-calcium medium, which is known to prevent the formation of intercellular junctions (Gonzalez-Mariscal et al., 1985). Under these conditions, the cells lost their characteristic cuboidal structure and adopted a dome-like shape (data not shown; see Vega-Salas et al. (1987)). Interestingly, despite these dramatic morphological alterations, prominin remained selectively associated with microvilli (Fig. 4C,D) and other, small plasma membrane protrusions (Fig. 4D, inset), and was absent from the planar areas of the plasma membrane (Fig. 4C,D).

Prominin is directly delivered to the apical domain

To quantitate the distribution of prominin between the apical and basolateral plasma membrane, domain-selective cell surface biotinylation was performed. Confluent prominin-transfected MDCK cells grown on Transwell filters were biotinylated with sulfo-NHS-LC-biotin on either the apical or the basolateral surface. Triton extracts were incubated with either the α I3 antiserum to immunoprecipitate the entire pool of prominin, or with streptavidin-agarose to selectively precipitate the biotinylated proteins. Cell surface labeled-proteins were then quantified by probing the immunoprecipitates with HRP-conjugated streptavidin ($n=3$), or the streptavidin-agarose precipitates with α I3 antiserum ($n=2$) (Fig. 5A). Using either method, we found that, in agreement with the microscopy observations, in steady state more than 98% of the biotinylated prominin was present at the apical side.

The observations described so far established the basis for investigating whether the enrichment of prominin in apical microvilli is solely due to its preference for, and stabilization in, these plasma membrane protrusions, or is due to both sorting to the apical plasma membrane domain and subsequent enrichment in these membrane protrusions. We addressed this

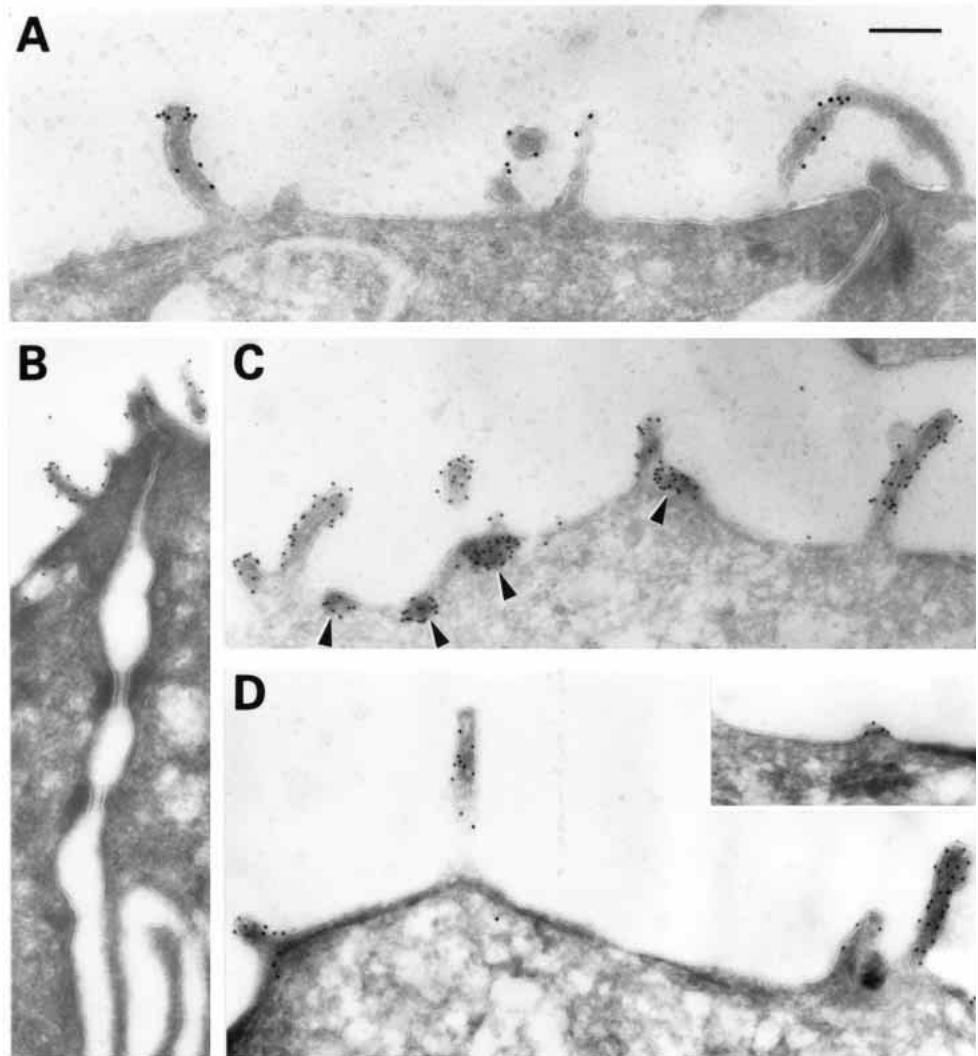


Fig. 4. Immunogold electron microscopy showing the localization of mouse prominin at the plasma membrane of transfected MDCK cells. Ultrathin cryosections were stained with mAb 13A4 followed by rabbit anti-rat IgG/IgM and 9 nm Protein A-gold. (A) Prominin immunoreactivity is confined to microvilli present at the apical cell surface of MDCK cells growing on Transwell filters. (B) No gold particles are observed at the lateral membrane. (C,D) When MDCK cells were cultured on either collagen-treated Petri dishes (C) or Transwell filters (D) in low-calcium medium, prominin immunoreactivity is restricted to microvilli and other, small plasma membrane protrusions (inset in D). Arrowheads indicate 'flaccid' microvilli, i.e. whose projection is not parallel to the plane of sectioning. Bar, 230 nm.

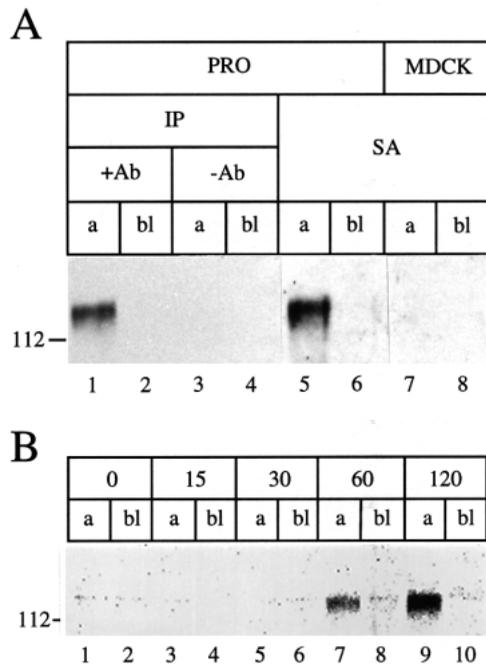


Fig. 5. Mouse prominin is directly delivered to the apical plasma membrane of transfected MDCK cells. (A) Steady state distribution between the apical and basolateral plasma membrane. Transwell filter-grown MDCK cells stably transfected with either an expression vector containing mouse prominin cDNA (PRO) or vector DNA without insert (MDCK) were biotinylated from either the apical (a) or basolateral (bl) surface. Cells were lysed, and one half of the lysates was subjected to immunoprecipitation (IP) using either α I3 antiserum (+Ab) or, as a control, no antibody (-Ab), and the immunoprecipitates were analysed by blotting with horseradish peroxidase-conjugated streptavidin. The other half of the cell lysate was adsorbed to streptavidin-agarose (SA) followed by immunoblotting of the adsorbed material using the α I3 antiserum. (B) Cell surface delivery of newly synthesized prominin. Transwell filter-grown prominin-transfected MDCK cells were pulse-labeled for 20 minutes with [35 S]methionine/cysteine. At the indicated times of chase (minutes), proteins at the cell surface were labeled with sulfo-NHS-LC-biotin from either the apical (a) or basolateral (bl) side. The cells were lysed, prominin was immunoprecipitated with α I3 antiserum, the immunoprecipitates were solubilised, and biotinylated prominin was adsorbed to streptavidin-agarose and analysed by SDS-PAGE and phosphoimaging.

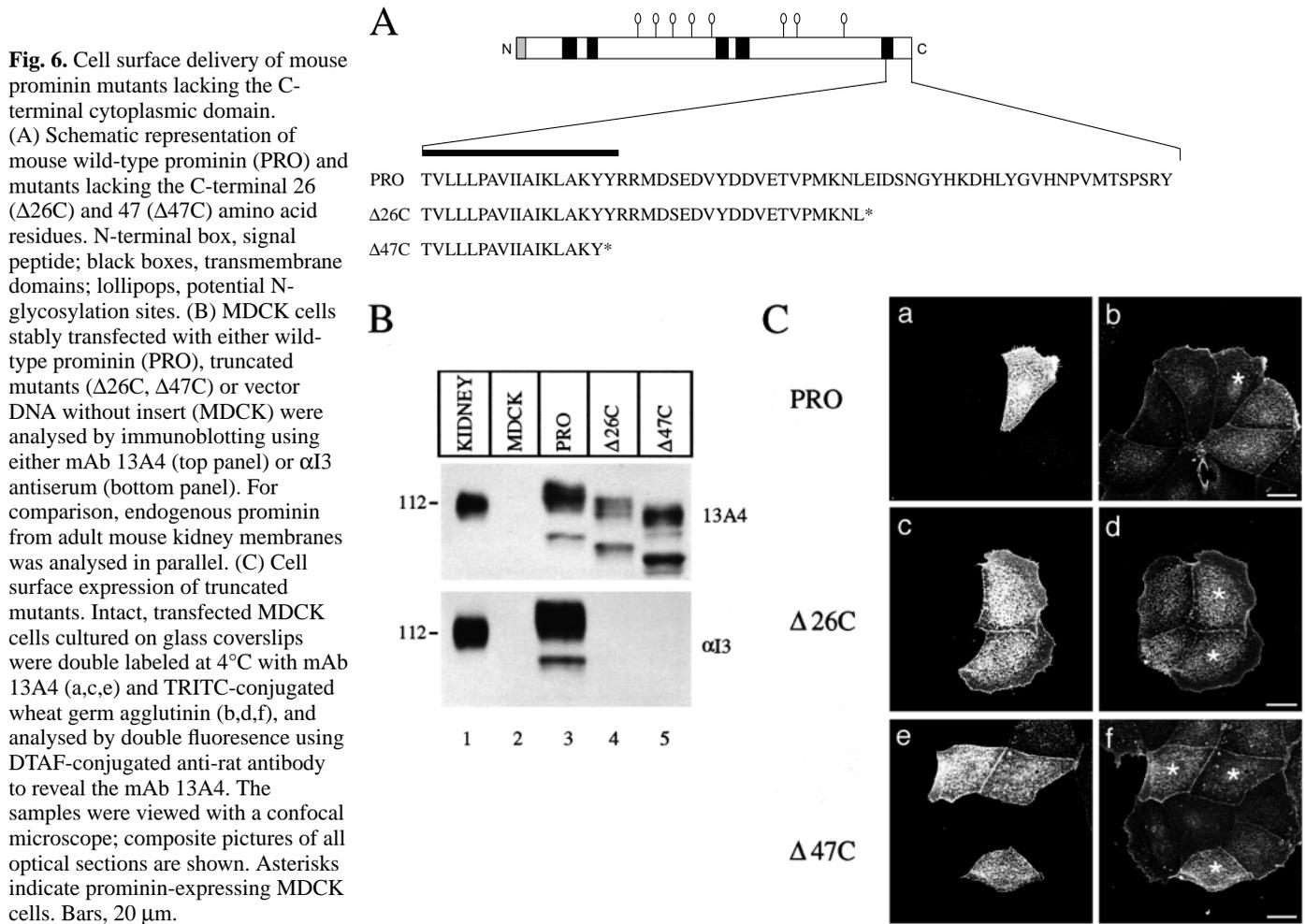
issue by analysing the cell surface appearance of newly synthesized prominin. Confluent monolayers of transfected MDCK cells grown on filters were pulse-labeled for 20 minutes with [35 S]methionine/cysteine and, after various times of chase, were selectively biotinylated with sulfo-NHS-LC-biotin on either their apical or basolateral surface. After immunoprecipitation with α I3 antiserum (or α E2 antiserum, data not shown), biotinylated prominin was isolated using streptavidin-agarose beads and analysed by SDS-PAGE followed by phosphoimaging. Prominin appeared at the apical surface after 60 minutes of chase (Fig. 5B, lanes a); no transient peak of prominin could be detected on the basolateral surface after shorter chase periods (Fig. 5B, lanes bl). These data suggest that prominin was directly sorted to the apical domain, without delivery to the basolateral domain followed by transcytosis to the apical membrane.

Apical microvilli-specific localization of prominin is independent of its cytoplasmic C-terminal domain

To evaluate the role of prominin's C-terminal domain, which has been shown to be localized in the cytoplasm (Weigmann et al., 1997), in its enrichment in microvilli of the apical plasma membrane of transfected MDCK cells, we constructed two deletion mutants of mouse prominin (Fig. 6A). In the first one (Δ 26C), 26 amino acid residues were deleted by introduction of a translational stop codon (TGA) at position 833. In the second mutant (Δ 47C), the entire C-terminal cytoplasmic domain was deleted by introducing a stop codon (TAA) at position 812. Truncated prominin mutants were stably transfected in MDCK cells and analysed by immunoblotting and immunofluorescence. Immunoblotting with mAb 13A4 revealed two distinct sets of immunoreactive bands for both mutants (Fig. 6B, top panel, lanes 4 and 5), as observed for wild-type prominin (Fig. 6B, top panel, lane 3). The reduced molecular mass of the Δ 26C and Δ 47C mutants was consistent with a deletion of 26 and 47 residues, respectively. The proportion of the endoplasmic reticulum/early Golgi form of prominin was greater for the Δ 26C (20-35%) and Δ 47C (40-50%) mutants than for the wild-type (10-20%). Probing parallel immunoblots with the α I3 antiserum showed that both mutants did not contain the C-terminal domain (Fig. 6B, bottom panel, lanes 4 and 5). The latter implies that the I3 epitope is located on the C-terminal 26 amino acid residues of prominin.

To determine whether the deletion mutants of prominin reach the plasma membrane, cell surface immunofluorescence with mAb 13A4 was performed. Immunofluorescence staining was observed for both mutants, suggesting that they were delivered to the cell surface of MDCK cells (Fig. 6C, c and e). The subcellular localization of the truncated prominin mutants was then determined by confocal microscopy and cell surface biotinylation. Vertical xz confocal images of transfected MDCK cells grown on permeable filters expressing either the Δ 26C or Δ 47C mutants revealed significant intracellular immunoreactivity (Fig. 7A, arrows) in addition to cell surface immunoreactivity, in particular for the Δ 47C mutant, consistent with the increased proportion of the endoplasmic reticulum/early Golgi form (Fig. 6B). With respect to the cell surface distribution, both mutants were mainly located at the apical plasma membrane (Fig. 7A), indicating that the C-terminal cytoplasmic domain of prominin does not contain essential information for apical delivery. This conclusion was further supported by the domain-selective cell surface biotinylation experiments (Fig. 7B). Densitometric scanning of the immunoreactive prominin recovered after streptavidin-agarose precipitation indicated that $94 \pm 4.5\%$ ($n=3$) and $87 \pm 4\%$ ($n=3$) of biotinylated Δ 26C and Δ 47C prominin mutants were found in the apical plasma membrane, respectively.

While the apical localization of prominin was not disrupted upon removal of its C-terminal domain, the cytoplasmic tail could be required for the enrichment of prominin in microvilli. To address this question, we examined the subcellular distribution of both truncated forms of prominin by immunogold EM in transfected MDCK cells (Fig. 8). Like wild-type prominin, both deletion mutants were concentrated in microvillar membranes (Fig. 8A, Δ 26C, Fig. 8B, Δ 47C). This implies that the C-terminal domain of prominin does not contain information essential for enrichment in microvilli.



DISCUSSION

The data presented here show that prominin contains dual targeting information: (i) for direct delivery to the apical plasma membrane domain; and (ii) for the selective retention in the microvillar rather than planar subdomain of the plasma membrane.

Direct delivery of prominin from the TGN to the apical plasma membrane

Our results show that the apical-specific plasma membrane localization of prominin is due to direct apical delivery of the newly synthesized protein from an intracellular compartment, probably the TGN, as reported for other apical membrane proteins expressed in MDCK cells (Matlin and Simons, 1984; Le Bivic et al., 1990; Jalal et al., 1992). This implies that the molecular mechanism underlying prominin's apical localization involves sorting in the TGN rather than selective retention in microvilli or selective proteolysis upon random cell surface appearance. Prominin thus differs from other polytopic plasma membrane proteins whose polarized cell surface distribution in epithelial cells is thought to be due to selective retention, such as the Na^+K^+ -ATPase (Hammerton et al., 1991; Mays et al., 1995a,b) and the α_{2B} -adrenergic receptor (Wozniak and Limbird, 1996). In these cases, after a nonvectorial delivery, only the basolateral pool was specifically

retained via interactions with the submembrane cytoskeleton, whereas the apical pool of these molecules was removed from the plasma membrane by endocytosis.

Why does a plasma membrane protein which is expressed not only in epithelial but also in non-epithelial cells (i.e. hematopoietic stem cells) contain an apical sorting signal? With regard to its expression in non-epithelial cells, it should be noted that such cells, e.g. fibroblasts, may deliver proteins from the TGN to distinct plasma membrane subdomains via distinct, apical-like and basolateral-like, vesicles (Yoshimori et al., 1996; M \ddot{u} sch et al., 1996; Per \ddot{a} nen et al., 1996). Perhaps this is of relevance for the cell surface expression of prominin in hematopoietic stem cells. With regard to prominin's expression in epithelial cells, it should be noted that plasma membrane protrusions exist not only on the apical surface but may also exist on the basolateral surface (Schulz et al., 1988). We find it likely that the presence of an apical sorting signal in prominin serves to prevent its appearance in the 'wrong', i.e. basolateral-type, of plasma membrane protrusions.

The molecular nature of prominin's apical sorting signal is unknown, and candidate structures are difficult to predict given the scarce knowledge about such signals in polytopic membrane proteins (Gottardi and Caplan, 1993; Turner et al., 1996; Perego et al., 1997). However, our data obtained for the truncated forms of prominin demonstrate that its cytoplasmic C-terminal tail is not essential for its apical localization. While

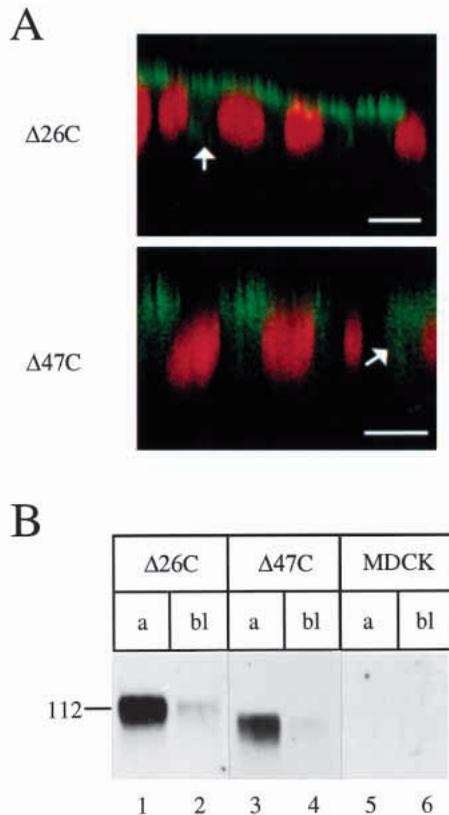


Fig. 7. Mouse prominin C-terminal deletion mutants are delivered to the apical plasma membrane of transfected MDCK cells.

(A) Confocal immunofluorescence microscopy of the $\Delta 26C$ and $\Delta 47C$ mutants. Stably transfected MDCK cells cultured on Nunc filters were stained with mAb 13A4 followed by DTAF-conjugated anti-rat antibody (green). Nuclei were labeled with propidium iodide (red). Single optical xz -plane sections show the apical localization of the $\Delta 26C$ (top panel) and $\Delta 47C$ (bottom panel) mutants. Arrows indicate intracellular immunoreactivity. Bars, 10 μm . (B) Steady state distribution of truncated mutants of prominin between the apical and basolateral plasma membrane. Transwell filter-grown MDCK cells stably transfected with the $\Delta 26C$ and $\Delta 47C$ mutants or vector DNA without insert (MDCK) were biotinylated from either the apical (a) or basolateral (bl) surface. Cell lysates were adsorbed to streptavidin-agarose followed by immunoblotting of the adsorbed material using the $\alpha E2$ antiserum.

a similar conclusion has been drawn for another apical polytopic membrane protein, γ -aminobutyric acid transporter (Perego et al., 1997), the cytoplasmic tail of rhodopsin has recently been shown to contain an apical sorting signal (Chuang and Sung, 1998). In the case of prominin, it is therefore likely that apical sorting information resides in the luminal and/or transmembrane domains of prominin, as shown for single-pass transmembrane proteins (Corbeil et al., 1992; Vogel et al., 1992; Kundu et al., 1996; Huang et al., 1997; Lin et al., 1998). Concerning the luminal domain, recent reports have documented a role of N- and O-glycans as apical sorting signals (Scheiffele et al., 1995; Yeaman et al., 1997). Unfortunately, our attempts to address the potential role of N-glycans in the apical delivery of prominin were hampered by the observation that, as in the case of other membrane proteins (Gut et al., 1998), cell surface delivery was strongly reduced

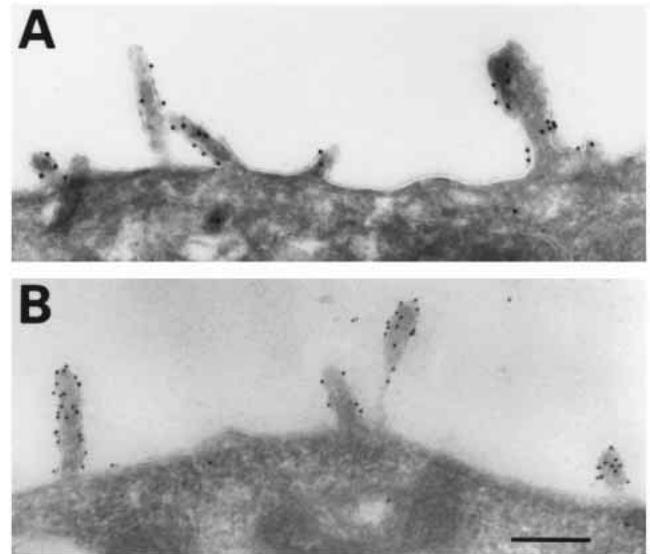


Fig. 8. The enrichment of prominin in microvilli of the apical plasma membrane does not require its C-terminal domain. Ultrathin cryosections of Transwell filter-grown MDCK cells expressing the $\Delta 26C$ mutant (A) or the $\Delta 47C$ mutant (B) of prominin were stained with mAb 13A4 followed by rabbit anti-rat IgG/IgM and 14 nm (A) or 9 nm (B) Protein A-gold. Bar, 300 nm.

upon inhibition of N-glycosylation by tunicamycin (data not shown). Likewise, another deletion mutant of prominin lacking the N-terminal luminal domain (E1) and the first transmembrane domain (M1) (see Fig. 1) did not provide further information about an apical sorting signal because it was not transported to the cell surface. This deletion mutant: (i) did not show any cell surface fluorescence; (ii) exhibited a punctate intracellular immunostaining distributed throughout the cytoplasm; and (iii) carried N-linked glycans which were completely sensitive to digestion by Endo H (data not shown).

Selective retention of prominin in microvilli

Once sorted to the apical plasma membrane domain, prominin is selectively concentrated in microvilli and other, small plasma membrane protrusions. Two modes of interaction, 'vertical' and 'lateral', could contribute to this enrichment. First, prominin may enter plasma membrane protrusions because of a 'vertical' interaction with the submembrane cytoskeleton, which is known to function as an organizer in microvilli, filopodia and microspikes (Costa de Beauregard et al., 1995; Sheetz et al., 1992; Bray and Chapman, 1985). Consistent with this proposal, we have observed by double confocal immunofluorescence microscopy of cytochalasin D-treated MDCK cells that prominin was redistributed in large patches which colocalized with actin aggregates (unpublished data). In this context, it will be interesting to investigate whether prominin interacts with a member of the ezrin-radixin-moesin family of proteins, which are known to serve as general plasma membrane protrusion-actin filament linkers (Bretscher, 1983; Algrain et al., 1993).

Second, prominin may interact 'laterally' with other integral membrane constituents specifically enriched in plasma membrane protrusions, as recently suggested for the localization of integrins in leukocyte microvilli (Abitorabi et

al., 1997). Extrapolating from the concept that lipid rafts play a key role in apical sorting (Brown and Rose, 1992; Fiedler et al., 1993), the formation of prominin-containing specialized lipid microdomains may be an important factor in the 'lateral' mode of interaction leading to prominin's retention in microvilli. Interestingly, not only the 104 kDa (endoplasmic reticulum/early Golgi) form, but also the 123 kDa (TGN/plasma membrane) form of prominin is solubilized by 0.2% Triton X-100 in the cold, whereas, in line with previous observations by others (Zurzolo et al., 1994; Sargiacomo et al., 1993), VIP21/caveolin is not (D. Corbeil, K. Röper and W. B. Huttner, unpublished observations). This suggests that prominin does not interact 'laterally' with the previously characterized cholesterol-sphingolipid raft implicated in apical sorting (Simons and Ikonen, 1997), and also implies that any 'vertical' interaction with the cytoskeleton, if it exists, would be disrupted by Triton X-100.

Maintenance of both the apical-specific localization of prominin and its enrichment in plasma membrane protrusions in epithelial cells in the absence of tight junctions

Remarkably, the apical-specific localization of prominin was observed not only when MDCK cells were cultured in normal medium but also in low-calcium medium. Under these experimental conditions, these epithelial cells lose their tight junctions and other cell-cell interactions (Vega-Salas et al., 1987; McNeill and Nelson, 1992). Hence, functional tight junctions, which are known to prevent the lateral movement between the apical and basolateral plasma membrane domain of transmembrane proteins and lipids of the extracellular membrane leaflet (Dragsten et al., 1981; van Meer and Simons, 1986), are not required for prominin's apical-specific localization. We find it likely that its retention in plasma membrane protrusions prevented the lateral movement of prominin into the basolateral plasma membrane domain of MDCK cells in the absence of tight junctions. Presumably the same holds true for neuroepithelial cells at the neural tube stage, which have lost functional tight junctions (Aaku-Saraste et al., 1996) and in which prominin retains its apical-specific localization, being concentrated in plasma membrane protrusions (Weigmann et al., 1997). Entry into, and retention in, plasma membrane protrusions may therefore play an important role in the establishment and maintenance of apical-basal polarity of the epithelial cells.

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