N-glycosylation and microtubule integrity are involved in apical targeting of prostate-specific membrane antigen: implications for immunotherapy

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Abstract
Prostate-specific membrane antigen (PSMA) is an important biomarker expressed in prostate cancer cells with levels proportional to tumor grade. The membrane association and correlation with disease stage portend a promising role for PSMA as an antigenic target for antibody-based therapies. Successful application of such modalities necessitates a detailed knowledge of the subcellular localization and trafficking of target antigen. In this study, we show that PSMA is expressed predominantly in the apical plasma membrane in epithelial cells of the prostate gland and in well-differentiated Madin-Darby canine kidney cells. We show that PSMA is targeted directly to the apical surface and that sorting into appropriate post-Golgi vesicles is dependent upon N-glycosylation of the protein. Integrity of the microtubule cytoskeleton is also essential for delivery and retention of PSMA at the apical plasma membrane domain, as destabilization of microtubules with nocodazole or commonly used chemotherapeutic Vinca alkaloids resulted in the basolateral expression of PSMA and increased the uptake of anti-PSMA antibody from the basolateral domain. These results may have important relevance to PSMA-based immunotherapy and imaging strategies, as prostate cancer cells can maintain a well-differentiated morphology even after metastasis to distal sites. In contrast to antigens on the basolateral surface, apical antigens are separated from the circulation by tight junctions that restrict transport of molecules across the epithelium. Thus, antigens expressed on the apical plasma membrane are not exposed to intravenously administered agents. The ability to reverse the polarity of PSMA from apical to basolateral could have significant implications for the use of PSMA as a therapeutic target.

Introduction
Prostate-specific membrane antigen (PSMA) is a 100-kDa transmembrane glycoprotein with a highly restricted profile of tissue expression. In addition to the benign prostatic epithelium, PSMA is expressed in tumor-associated neovasculature and at increased levels in most cases of prostate cancer, with the greatest levels associated with high-grade tumors, metastases, and androgen-independent disease (1–4). Overexpression of PSMA is a good prognostic indicator of disease outcome and is more highly expressed in poorly differentiated tumors than the better-known marker, prostate-specific antigen (4).

In contrast to other prostate-specific proteins, such as prostate-specific antigen or prostatic acid phosphatase, PSMA is a transmembrane protein expressed on the surface of the prostatic epithelium. As an integral membrane protein, PSMA offers a potentially valuable antigenic target for therapeutic and in vivo imaging strategies involving monoclonal antibodies (mAb; ref. 5). The capacity for antibodies to recognize tumor cells with high specificity and affinity has long been appreciated, and the successful clinical application of mAbs depends upon the subcellular localization and trafficking of antigen within the target cell (6).

Like all epithelial tissues, the prostatic epithelium is comprised of highly polarized cells with biochemically distinct apical and basolateral plasma membrane surfaces (7). These plasma membrane domains maintain an asymmetrical distribution of proteins and lipids, are physically separated by tight junctions that promote cell-cell contact, restrict the flow of fluid through intercellular spaces, and prevent the lateral diffusion of membrane components (8, 9). Thus, apical and basolateral plasma membrane domains are exposed to disparate extracellular environments. Whereas the basolateral plasma membrane is relatively accessible to the underlying vasculature, tight junctions prevent molecules within the circulation from reaching the apical surface (6).

The establishment of plasma membrane asymmetry requires vectorial targeting of newly synthesized proteins to either the apical or basolateral surfaces (10). Proteins targeted for a particular plasma membrane domain may arrive at their destinations via alternate routes. Whereas...
many proteins are targeted directly from the trans-Golgi network to their appropriate plasma membrane domain, others are first delivered to the opposing membrane surface before undergoing transcytosis to their ultimate destination (11). The particular transport pathway by which a given protein reaches the plasma membrane is dependent upon the individual protein and governed by interactions between the cellular sorting machinery and signals encoded within that protein. Wherein the signals for basolateral targeting generally involve short amino acid–based motifs, often containing critical tyrosine or leucine residues (12, 13), targeting to the apical surface seems mediated by a far more heterogeneous array of divergent signals, including N- or O-linked oligosaccharides (14, 15), PDZ-interacting domains (16), membrane anchors (17), and amino acid sequences encoded within membrane spanning (18) or cytoplasmic domains (19). This diversity of sorting signals underscores the complexity of apical targeting and implies the existence of multiple pathways for apical targeting.

Like the plasma membrane, cytoskeletal elements also display a nonuniform distribution in polarized epithelial cells. As cells establish polarity, microtubules emanating from the microtubule organizing centers are rearranged to form longitudinal arrays with their minus ends facing the apical surface (20). This polarized arrangement of microtubules seems critical for targeting a number of apical proteins, because microtubule depolymerization or disruption of dynein function results in aberrant targeting of several apical proteins to the basolateral surface (21, 22) but does not seem to have a significant effect on targeting of basolateral proteins (21, 23).

Whereas numerous investigations have attempted to address the relevance of PSMA as a diagnostic marker and a therapeutic target, most of these studies have been done using highly transformed carcinoma cell lines such as LNCaP cells and PC3 and have largely neglected the significance of epithelial polarity. Because a well-differentiated polarized epithelial cell culture model for prostate is not available, we established the Madin-Darby canine kidney (MDCK) cell culture model for studying prostate restricted proteins. We showed that LNCaP cells and PC3 and have largely neglected targeting of dynein function results in aberrant targeting of several apical proteins to the basolateral surface (21, 22) but does not seem to have a significant effect on targeting of basolateral proteins (21, 23).

In this study, we investigated the mechanisms and cellular machinery involved in apical targeting of PSMA. We employed a series of biochemical and morphologic assays to elucidate the targeting pathway of PSMA and ascertain the role of N-glycosylation and microtubules in the delivery of PSMA to the apical surface. We show that N-glycosylation is necessary for proper targeting of PSMA into apically targeted vesicles, whereas integrity of the microtubules is necessary to deliver and retain PSMA at the apical plasma membrane domain. We exploited this intrinsic role of microtubules in the apical delivery of PSMA for the clinical benefit by using Vinca alkaloids to destabilize microtubules and show that these alkaloids redirect PSMA to the basolateral plasma membrane, which is accessible to the circulating therapeutic antibodies.

Materials and Methods

Cell Culture

MDCK cells (clone II) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum, 2 mmol/L l-glutamine, 25 units/mL penicillin, 25 µg/mL streptomycin, and 100 µmol/L nonessential amino acids. Cells were grown at 37°C in a humidified incubator with 5% CO2. Cells were treated for 10 hours with 10 mmol/L sodium butyrate to enhance PSMA expression. For experiments involving inhibition of N-glycosylation or microtubule depolymerization, cells were treated for 3 hours with 5 µg/mL tunicamycin (Sigma, St. Louis, MO) or 4 µg/mL nucodazole (Sigma), or 2 µmol/L vinblastine, vincristine, or vinorelbine (Sigma) in DMEM at 37°C before the indicated experiment unless otherwise noted.

DNA Constructs and Transfection

The cDNA encoding full-length PSMA (provided by Dr. Warren Heston, Department of Cancer Biology, The Lerner Research Institute at the Cleveland Clinic Foundation, Cleveland, OH) was cloned into the pcDNA3 expression vector from Invitrogen (Carlsbad, CA). The sPSMA construct was generated by RT-PCR and amplification of codons 53 to 751 of PSMA using total RNA extracted from LNCaP cells as a template. The 5’ and 3’ primers were used to introduce SfiI and AplI restriction sites, respectively. This cDNA was cloned into the pSecTag2A vector (Invitrogen) in fusion with an NH2-terminal sequence encoding the cleavable murine immunoglobulin κ chain leader sequence for protein secretion. To create the green fluorescent protein (GFP)–tagged PSMA-A103-750 construct, a 309-bp DNA fragment encoding the cytoplasmic, transmembrane, and a 60-amino-acid region of the extracellular domain of PSMA was generated by reverse transcription-PCR using total RNA isolated from LNCaP cells. The PCR product was digested with XhoI and BamHI and cloned into the pEGFP-N3 expression vector (Clontech, Palo Alto, CA). The cDNA encoding the β-subunit of the canine sodium pump (Na,K-ATPase; provided by Dr. Robert Farley, Department of Biochemistry and Molecular Biology, Keck School of Medicine, University of Southern California, Los Angeles, CA) was PCR amplified and inserted into pEGFP-N3 to create a GFP fusion at the COOH terminus (Na,K-β-GFP).

MDCK cells were transfected using calcium phosphate, as previously described (25). Stable clones were selected in 500 µg/mL genetin (G418, Life Technologies) for pcDNA3 vectors or 300 µg/mL Zeocin (Invitrogen) for pSecTag2 vector and expression verified by immunofluorescence and immunoblot.

Antibodies

The mAb J591 against an extracellular epitope of PSMA has been described (26). The mAb 7E11 against an...
intracellular epitope of PSMA was prepared from hybridoma 7E11 (American Type Culture Collection, Rockville, MD). Mouse mAbs raised against Na,K-ATPase α1 (M7-PB-E9) and β1-subunit (M17-FS-F11) have been described (27, 28). Rabbit anti-mouse and mAb against α-tubulin were purchased from Sigma. Horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G was purchased from Transduction Laboratories (Lexington, KY). FITC and CY3 conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

**Immunofluorescence and Confocal Microscopy**

Tissue sections and MDCK cells were fixed in cold methanol at −20°C for 30 minutes. Following fixation, specimens were placed in humidified chambers, washed with PBS containing 0.1 mmol/L CaCl₂ and 1 mmol/L MgCl₂ and 0.5% bovine serum albumin (PBS-CM-BSA), and subject to SDS-PAGE and immunoblot analysis as described (30).

**Apical Targeting of PSMA**

Apical Targeting of PSMA was studied using an apical targeting assay as described (27, 28). Rabbit anti-mouse and mAb against α-tubulin were purchased from Sigma. Horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G was purchased from Transduction Laboratories (Lexington, KY). FITC and CY3 conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

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**Immunoblotting and Image Analysis**

Immunoblot analysis was performed using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Densitometric analysis and quantification of bands was done using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Metabolic Labeling**

Confluent monolayers of MDCK-PSMA cells on transwell filters were rinsed twice and incubated for 30 minutes in starving media (cystine/methionine-free DMEM; CellGro, Herndon, VA) supplemented with 0.2% BSA. Cells were either pulsed for 20 minutes in labeling medium (starving medium containing 250 μCi/mL Trans-[35S]-label; ICN, Costa Mesa, CA) or labeled for 4 hours in the presence or absence of 10 μg/mL of tunicamycin (Sigma). Chase was done by rinsing filters thrice with starving medium and incubating in DMEM (10% fetal bovine serum) containing 50 μg/mL of cycloheximide (Sigma).

**Targeting Assays**

For the antibody internalization targeting assay, MDCK cells were metabolically labeled and chased in DMEM containing 5 μg/mL of mAb J591 added to either the apical or basolateral chamber. Following incubation, cells were rinsed thoroughly with cold PBS-CM. Filters were rinsed and incubated for >4 hours in lysis buffer at 4°C. Immunocomplexes were precipitated using rabbit antimouse–coated protein A agarose beads. Beads were rinsed and subject to SDS-PAGE. Gels were fixed in a solution of 20% methanol and 10% acetic acid, dried, enhanced with salicylic acid, and exposed to film. The relative amount of PSMA for each was calculated as a percentage of the total amount of labeled PSMA precipitated throughout the course of the experiment, as quantified by densitometry.

**Polarized Secretion Assay**

MDCK cells expressing sPSMA (MDCK-sPSMA) were grown on transwell filters. Following the establishment of

**SDS-PAGE and Immunoblot Analysis**

Samples were separated on 10% SDS polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated in 5% nonfat milk for 1 hour and immunoblotted with primary antibodies (1:1,000) for 2 hours in milk. Membranes were washed thrice with PBS with 0.3% Tween 20 (PBST), incubated with horseradish peroxidase–conjugated secondary antibody (1:4,000) in milk. After washing thrice in PBST and once in PBS, bound antibody was detected by peroxidase-catalyzed Enhanced Chemiluminescence-Plus (Amersham, Buckinghamshire, United Kingdom). Densitometric analysis and quantification of bands was done using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).
tight junctions, as assessed by transepithelial electrical resistance, cells were rinsed thrice with fresh DMEM and 2.0 mL of fresh medium were added to both the apical and basolateral chambers. Cells were incubated for 8 to 10 hours at 37°C, at which point the conditioned medium was collected and sPSMA immunoprecipitated using immobilized J591 bound to protein A coated agarose beads. Samples were washed and subjected to SDS-PAGE, immunoblot analysis, and densitometry as described above.

Post-Golgi Analysis Assays
MDCK-PSMA cells transiently transfected to express Na,K-β-GFP were grown on glass coverslips and treated with nocodazole or taxol. Cells were incubated for 6 hours at 20°C to accumulate newly synthesized protein in the Golgi and trans-Golgi network, subsequently transferred to 37°C for 30 minutes, and subjected to immunofluorescence analysis as described above. Laser scanning confocal microscopy was done using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Inc., Thornwood, NY). Samples were excited with Argon and Helium/Neon lasers and single channel images were generated and analyzed using the Zeiss LSM 510 Meta imaging system (Carl Zeiss) by recording light emitted between 505 and 543 nm for GFP and above 560 nm for Cy3.

Domain-Specific Internalization Assay
MDCK-PSMA cells were grown on 0.04-μm pore size polycarbonate transwell filters (Corning, Corning, NY) and grown to confluence as measured by transepithelial electrical resistance using an EVOM Epithelial Voltohmeter. Cells were treated with 2 μmol/L of vinblastine, vincristine, or vinorelbine at 37°C for 3 hours and subsequently incubated at 37°C for 30 minutes in the presence of the indicated drug and 5 μg/mL of J591 added to either the apical or basolateral chamber. Cells were rinsed in PBS-CM, fixed, and subjected to immunofluorescence analysis with FITC-conjugated secondary antibody. Single-channel digital microscopic images were collected with an Olympus AX70 upright microscope using identical exposure variables and analyzed with SPOT imaging software, version 4.0.4 (Diagnostic Instruments, Inc., Sterling Heights, MI).

Immunohistochemical Studies
Formalin-fixed, paraffin-embedded tissue samples from patients with metastatic prostate cancer were obtained from the tissue procurement core laboratory at the University of California at Los Angeles. Metastatic prostate–derived specimens included four lesions isolated from lymph nodes and two isolated from bone marrow. Serial 5-μm sections were deparaffinized to water and subjected to antigen retrieval for 10 minutes at room temperature in 0.05% trypsin or microwaved in citrate buffer. Following antigen retrieval, specimens were incubated in 1% hydrogen peroxide for 10 minutes, blocked with 4% fetal bovine serum in PBS for 1 hour, and incubated with mAb 7E11 (1:50) overnight at 4°C. Samples were subsequently washed and incubated at room temperature with biotinylated goat anti-mouse secondary antibody (Vector) for 1 hour. Samples were rinsed and subjected to A and B reagent. Immunoreactivity was visualized by incubation with diaminobenzidine in the presence of hydrogen peroxide. Sections were counterstained with hematoxylin and mounted for microscopic analysis. Control experiments were also done by incubating tissues with an irrelevant mouse monoclonal immunoglobulin G.

Results
PSMA Is Expressed on the Apical Plasma Membrane of Polarized Epithelial Cells
We have previously shown that the MDCK cell line is a suitable model system to study polarized sorting of prostate-restricted transmembrane and secretory proteins (24). Immunofluorescence analysis of tissue sections revealed prominent PSMA localization at the apical plasma membrane of prostatic epithelial cells, with staining at the luminal interface of the gland (Fig. 1A), recapitulating in situ observations (24). Surface immunofluorescence analysis done on confluent monolayers of MDCK cells expressing PSMA (MDCK-PSMA) revealed a similar pattern of expression, with PSMA staining localized primarily to the apical membrane in these cells (Fig. 1B). A selective cell surface biotinylation assay was done to provide a quantitative analysis of relative surface PSMA levels. Results of this assay showed that 70% to 79% of PSMA at the cell surface was localized to the apical plasma membrane (Fig. 1C) thus confirming previous results (24).

PSMA Is Targeted Directly to the Apical Plasma Membrane
Apical proteins may be targeted directly from the trans-Golgi network or may be delivered first to the basolateral plasma membrane before undergoing transcytosis to the apical surface. To distinguish between these pathways, an antibody internalization-based targeting assay was employed. Confluent monolayers of MDCK-PSMA cells grown on transwell filters were metabolically labeled with [35S]-cysteine/methionine for a brief pulse and chased in the presence of mAb J591 added to either the apical or basolateral chamber. The J591 mAb recognizes an extracellular epitope on PSMA and is readily internalized by cells expressing PSMA (26). During the period of antibody incubation, PSMA at a particular plasma membrane surface will bind extracellular antibody, even if this localization is transient. Immune complexes are then precipitated from cell lysates and subjected to SDS-PAGE and autoradiography. If PSMA were to undergo direct targeting to the apical plasma membrane, a greater amount of metabolically labeled PSMA would be recovered when antibody is added to the apical chamber relative to the basolateral. At each time point subsequent to release, the level of metabolically labeled PSMA immunoprecipitated was between 1.7- and 2.0-fold greater when antibody was added to the apical rather than the basolateral chamber (Fig. 2A and B) thus indicating that whereas some PSMA may initially be delivered to the basolateral surface, the majority is targeted directly to the apical plasma membrane.

These results were supported by a selective cell surface biotinylation-based targeting assay. Confluent monolayers of MDCK-PSMA cells were metabolically pulsed and chased
in normal culture medium before opposing membrane surfaces were labeled with biotin. Autoradiography showed that PSMA is seen predominantly on the apical plasma membrane with a smaller fraction localized to the basolateral surface throughout the course of the experiment (Fig. 2C). The fact that PSMA is primarily observed at the apical plasma membrane even at the initial time point of 30 minutes further suggests that the majority of newly synthesized PSMA is targeted to the apical surface.

**Extracellular Domain of PSMA Contains Information for Apical Targeting**

Although signals for apical targeting may be localized throughout the length of a given transmembrane protein, such signals most commonly reside within the extracellular domain. To assess the significance of this domain in apical targeting, a GFP-tagged form of PSMA was created in which the majority of the extracellular domain was removed (PSMA-D103-750). Cell surface biotinylation assays showed that this protein was localized in a nonpolarized fashion (Fig. 3A). Immunoblot analysis done on the same membranes revealed that 90% to 95% of the α-subunit of the sodium pump (Na,K-ATPase α-sub) was localized at the basolateral surface of these cells (Fig. 3B), demonstrating that the uniform plasma membrane distribution of PSMA is not merely attributable to a general loss of epithelial polarity.

To evaluate the targeting potential offered by the luminal domain, a secreted form of PSMA (sPSMA) lacking the cytoplasmic and transmembrane domains was created. The sPSMA protein was secreted from MDCK cells as a ~100-kDa glycoprotein that was recognized by the mAb J591 and that migrated with a molecular mass of ~80 kDa following treatment with tunicamycin or N-glycosidase (data not shown). A stable MDCK cell line expressing sPSMA (MDCK-sPSMA) was grown to confluence on transwell filters, and the conditioned medium was collected from the apical and basolateral chambers. As shown in Fig. 3C, sPSMA was secreted almost exclusively from the apical plasma membrane.

**Figure 1.** PSMA is expressed on the apical surface of polarized epithelial cells. A, immunofluorescence analysis of prostate tissue sections reveals that PSMA is predominantly localized to the apical plasma membrane surface of the prostatic epithelium, lining the lumen of the gland. B, XY and XZ confocal sections of surface staining of MDCK-PSMA cells reveal PSMA localized to the apical plasma membrane. C, results from three independent cell surface biotinylation assays confirm predominant apical localization and demonstrate that 70% to 79% of surface PSMA is localized to the apical plasma membrane; bars, SD. Apical (A), basolateral (BL). Bar, 10 μm (A and B).

**Figure 2.** PSMA is targeted directly to the apical plasma membrane. MDCK-PSMA cells on transwell filters were briefly pulsed with [35S]-cystine/methionine and chased in the presence of mAb J591 added to either the apical or basolateral chamber. A, the amount of newly synthesized PSMA precipitated was significantly greater when antibody was added to the apical chamber (A) compared with the basolateral (BL). Densitometric analysis of three independent antibody internalization targeting assays reveals increased apical targeting at each time point. B, data indicate that 1.7 to 2.0 times more PSMA is precipitated when J591 is added to the apical chamber (A) compared with the basolateral (BL); bars, SD. C, representative data from two independent biotinylation based targeting assays also show greater levels of newly synthesized PSMA at the apical plasma membrane relative to the basolateral throughout the course of the experiment.
membrane, further implicating the existence of a targeting signal encoded within the extracellular domain of PSMA.

**Apical Targeting of PSMA Requires N-Glycosylation**

The extracellular domain of PSMA is highly glycosylated, with ∼25% of the mass of PSMA attributable to N-linked carbohydrates (32). Given the significance of oligosaccharide moieties in apical targeting, we investigated the role of N-glycosylation in trafficking of PSMA (14, 33).

Confluent monolayers of MDCK-PSMA cells were metabolically labeled in the presence or absence of tunicamycin. This drug prevents N-glycosylation in the endoplasmic reticulum and has been used extensively to assess the role of glycosylation in protein trafficking (34, 35). Selective biotinylation of the apical or basolateral plasma membrane revealed that whereas the majority of surface PSMA is normally localized to the apical plasma membrane, inhibition of N-glycosylation abolished the polarized expression of PSMA and resulted in equivalent levels at both plasma membrane surfaces (Fig. 4A).

Inhibition of N-glycosylation also resulted in a dramatic alteration in PSMA localization within post-Golgi transport vesicles. Incubation of MDCK-PSMA cells at 20°C was used to inhibit post-Golgi transit and accumulate proteins within the trans-Golgi network (36). As seen in Fig. 4B, both PSMA and a GFP-tagged version of the basolaterally targeted Na,K-ATPase β-subunit (Na,K-β-GFP) were localized to the trans-Golgi network following incubation at 20°C. These cells were subsequently transferred to 37°C, allowing proteins to exit from the trans-Golgi network. In the absence of tunicamycin, PSMA and Na,K-ATPase localized to distinct post-Golgi vesicles in regions proximal to the trans-Golgi network, with only ∼8% (7 of 87) of red and green vesicles overlapping. However, the level of colocalization of vesicles containing these markers increased to ∼43% (38 of 88) when cells are incubated with tunicamycin (Fig. 4C), indicating a role for N-glycosylation PSMA sorting into distinct post-Golgi vesicles.

**Microtubules Are Necessary for Apical Targeting of PSMA**

The integrity of the microtubule cytoskeleton is essential for the targeted delivery of many apical proteins in polarized epithelial cells (37, 38). To address the significance of microtubules in PSMA targeting, MDCK-PSMA cells were treated with the microtubule-depolymerizing agent, nocodazole. As shown in Fig. 5, nocodazole treatment resulted in a dramatic redistribution of PSMA. Surface immunofluorescence revealed increased PSMA expression at the basolateral plasma membrane relative to untreated cells (Fig. 5A). These data were also confirmed by cell surface biotinylation experiments, which show a homogeneous distribution of PSMA at both plasma membrane domains following nocodazole treatment (Fig. 5B). Polarity of the basolateral marker Na,K-ATPase was unaffected by nocodazole treatment, confirming the conservation of tight junction integrity and epithelial polarity in these cells (Fig. 5C).

Whereas tunicamycin and nocodazole treatment both resulted in a loss of PSMA polarity, the localization of PSMA within post-Golgi vesicles after treatment with these drugs was distinctly different. Following release from a 20°C block, PSMA and Na,K-β-GFP did not show an increased colocalization in the presence of nocodazole, with only ∼8% (4 of 52) of red and green post-Golgi vesicles (Fig. 5D). These results indicate that targeting of PSMA into distinct post-Golgi vesicles was unaffected by microtubule depolymerization.

Whereas microtubule depolymerization does not affect the sorting of PSMA into post-Golgi vesicles, the delivery of these vesicles to the plasma membrane fails to occur in a polarized manner. Confluent monolayers of MDCK-PSMA cells on transwell filters were pulsed and chased in the presence of extracellular mAb J591. In the absence of nocodazole, ∼1.9-fold more radiolabeled PSMA was precipitated when J591 was added to the apical chamber compared with the basolateral, consistent with our earlier findings (Fig. 5E). However, in the presence of nocodazole, equivalent levels of radiolabeled PSMA were precipitated regardless of the chamber to which J591 was added, thus showing that newly synthesized PSMA was delivered in a nonpolarized fashion (Fig. 5E). These results suggest that microtubule integrity is necessary for proper delivery and retention of PSMA at the plasma membrane domain.
Vinca Alkaloids Promote mAb J591 Uptake from the Basolateral Plasma Membrane

Because nocodazole treatment reversed the polarity of PSMA, we investigated the effect of commonly used chemotherapeutic agents that inhibit microtubule assembly. The Vinca alkaloids are a class of drugs applied to the treatment of a number of malignant diseases, including prostate cancer. Treatment of MDCK-PSMA cells with vinblastine, vincristine, or vinorelbine was sufficient to induce extensive depolymerization of the microtubule cytoskeleton (Fig. 6A-D). Confluent monolayers of MDCK-PSMA cells were subjected to J591 internalization assays to determine how Vinca alkaloid treatment influences PSMA localization. Whereas polarized monolayers of untreated MDCK-PSMA cells readily internalized mAb J591 added to the apical chamber (Fig. 6E), very little antibody was internalized from the basolateral surface (Fig. 6I). Following treatment with Vinca alkaloids, J591 was also taken up from the apical surface, albeit at decreased levels relative to untreated cells (Fig. 6F-H); however, these cells exhibited a dramatic increase in J591 internalization from the basolateral surface (Fig. 6J-L).

Polarized Morphology of Prostate Tumor Cells

We next investigated whether our observations using a polarized cultured cell line might have a practical significance in the context of prostate cancer cells, in situ. Histologic assessment of a metastatic lesion from lymph node shows diffused prostate tumor infiltration replacing the lymph node parenchyma (Fig. 7A). The enlarged tumor cells contain large and prominent nuclei and mitotic figures are readily observed. The prostate cancer cells form sheets with several areas of glandular differentiation. These glandular structures have clearly identifiable luminal spaces occasionally containing pink secretions. The tumor cells surrounding the luminal spaces show similar morphology to that seen in well-differentiated primary adenocarcinoma of the prostate with distinct plasma membrane organization (Fig. 7A and B). Immunohistochemical analysis revealed that these cells express PSMA and that this antigen is restricted to the apical surface facing the lumen (Fig. 7C and D). This staining was clearly distinct from that of the endothelial cell marker CD34 and CD31. Antibodies to these antigens stained small vessels but not the glandular structures thus excluding the possibility that these PSMA expressing structures are actually blood vessels (Fig. 7E and F; data not shown). These results indicate that prostatic cancer in some patients retains a well-differentiated morphology, even following metastasis to distal sites.

Discussion

With expression largely restricted to the cells of the prostatic epithelium and protein levels proportional to tumor grade, PSMA has emerged as a potentially important biomarker for the management and therapy of prostate cancer. In this report, we have investigated the trafficking of PSMA in polarized epithelial cells and showed N-glycosylation and microtubule requirements for apical targeting.

Figure 4. N-glycosylation is required for targeting PSMA into the proper post-Golgi vesicles. Cell surface biotinylation reveals that PSMA is distributed in a nonpolarized fashion following treatment with tunicamycin. Apical (A), basolateral (BL). A, following incubation at 20°C, PSMA (red) and Na,K-β-sub-GFP (green) are accumulated in the trans-Golgi network. B, after release from the 20°C block, PSMA (red) and Na,K-β-sub-GFP (green) localize to distinct post-Golgi vesicles, with limited colocalization. In the presence of tunicamycin, a significant increase in colocalization is observed. C, the circles in the merged image were artificially added using Photoshop to highlight the location of individual post-Golgi vesicles containing Na,K-ATPase (green), PSMA (red), or both (yellow). Bar, 5 μm (B and C).
Currently, no well-differentiated cell lines of prostatic origin exist that maintain epithelial polarity under culture conditions. The commonly used prostate-derived cell lines, such as LNCaP, DU145, and PC3, are all highly transformed cells that lack epithelial junctions. Therefore, we have used polarized MDCK cells to investigate PSMA trafficking. Previous efforts have shown similar patterns of localization for several prostatic antigens, including PSMA, prostate-specific antigen, and PSCA, indicating that the components of the protein trafficking machinery are conserved between cell types (24). Thus, information about protein targeting in MDCK cells can effectively be applied to prostatic epithelial cells, in situ.

Our results indicate that the PSMA is targeted directly from the trans-Golgi network to the apical plasma membrane and that the signal for apical sorting resides in the luminal domain of PSMA. This apical sorting signal is dependent upon the presence of N-linked oligosaccharides, which are essential for proper targeting of PSMA into post-Golgi vesicles devoid of basolateral cargo. Unfortunately, attempts to express mutant forms of PSMA in which the various N-glycosylation sites had been mutated were largely unsuccessful at generating cell lines with sufficient expression at the plasma membrane. Thus, we were unable to define the specific N-glycosylation sites required for apical targeting.

Like N-glycosylation, intact microtubules are also required for proper targeting of PSMA to the apical plasma membrane. Whereas treatment with either tunicamycin or nocodazole both resulted in nonpolarized delivery of PSMA in MDCK cells, only tunicamycin treatment was associated with aberrant localization of PSMA to post-Golgi vesicles, regardless of nocodazole treatment. The circles in the merged image were artificially added using Photoshop to highlight the location of individual post-Golgi vesicles containing Na,K-ATPase (green), PSMA (red), or both (yellow). E, representative data from two independent antibody internalization targeting assays reveal that polarized delivery of PSMA is abolished following 3 h of nocodazole treatment. Bar, 5 μm (A and D).

Figure 5. Intact microtubules are required for proper delivery of PSMA-containing vesicles to the apical plasma membrane. Immunofluorescence analysis of MDCK-PSMA cells (-Noco) reveals that PSMA is expressed on the apical membrane. A, following microtubule depolymerization (+Noco), XY and XZ confocal sections reveal PSMA staining throughout the plasma membrane and at cell borders. B, cell surface biotinylation assays confirm that PSMA is distributed throughout the plasma membrane. C, Na,K-ATPase β subunit is still restricted to the basolateral plasma membrane following nocodazole treatment, indicating that such treatment does not result in a general loss of polarity. D, following release from a 20°C block, Na,K-ATPase (green) and PSMA (red) are localized to distinct post-Golgi vesicles, regardless of nocodazole treatment. The circles in the merged image were artificially added using Photoshop to highlight the location of individual post-Golgi vesicles containing Na,K-ATPase (green), PSMA (red), or both (yellow). 

- Noco + Noco

D Na,k-β-GFP PSMA Merge

E PSMA

- Noco +Noco

- Noco + Noco
cancer immunotherapy. PSMA-specific mAbs conjugated to radionuclides or cytotoxic drugs have already proven effective at reducing the size of spheroids in cell culture and tumor xenografts in mouse models (39–41). However, these studies have used highly transformed cells lacking plasma membrane polarity and may not accurately reflect the more complex situation that exists in vivo.

To gain access to antigens on the surface of malignant cells in vivo, therapeutic mAbs must traverse a gamut of formidable obstacles. With few barriers to impede mAb binding, hematologic malignancies are well suited to this form of therapy (42). In comparison, successful treatment of solid tumors with mAbs has proven considerably more elusive. Studies using radiolabeled mAbs show that only 0.01% to 0.1% of the original injected dose will ever reach the antigen within a solid tumor mass, per gram of tissue (43, 44). Following i.v. injection and diffusion throughout the vascular space, therapeutic antibodies must traverse the microvascular endothelium and contend with stromal and interstitial barriers associated with a sizeable tumor mass (45, 46). After navigating these formidable impediments, a mAb may still be confronted by an additional set of epithelial barriers that may severely restrict accessibility of antigens to circulating antibodies.

Whereas over 90% of all cancers are carcinomas derived from epithelial tissues, the significance of these epithelial barriers is often disregarded in the treatment of malignant disease.

Although often overlooked, epithelial barriers may exert a profound effect on the efficacy of mAb therapy. The tight junctions would severely restrict the accessibility of antibodies to antigens at the apical plasma membrane. For example, the carcinoembryonic antigen, which is expressed at similar levels in both benign and malignant cells of the colonic epithelium, is restricted to the apical surface of normal tissues and well-differentiated tumors (47). However, loss of tight junction integrity in poorly differentiated tumors results in nonpolarized expression of carcinoembryonic antigen throughout the plasma membrane (47, 48). The altered localization of carcinoembryonic antigen allows accessibility of this antigen to the underlying vasculature and would explain why immunoscintigraphic studies using i.v. injected mAbs to carcinoembryonic antigen are able to specifically label primary and metastatic tumors but not normal or well-differentiated tissues (49, 50).

Because PSMA is normally targeted directly to the apical plasma membrane, therapeutic antibodies to PSMA would only bind to highly transformed and poorly

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**Figure 6.** Vinca alkaloid treatment enhances J591 uptake from the basolateral surface. A, immunofluorescence analysis of α-tubulin reveals intact microtubules in untreated control cells. Extensive microtubule depolymerization is observed following treatment with the Vinca alkaloids vinblastine (B), vincristine (C), and vinorelbine (D). E, immunofluorescence analysis following a 30-min incubation with mAb J591 (5 μg/mL) added to the apical (A) chamber reveals intense staining for untreated control cells. F–H, although staining is relatively less intense than in control cells, immunofluorescence staining is also observed following treatment with Vinca alkaloids when J591 was added to the apical chamber. I, very minimal immunofluorescence staining is observed in control cells following incubation with J591 added to the basolateral (BL) chamber. J–L, treatment with Vinca alkaloids results in a dramatic increase in J591 internalized from the basolateral chamber. Bar, 10 μm.
Immunohistochemical analysis of CD31 shows identical staining to cells surrounding lymphovascular lumens stain positively for CD34.

Figure 7. Metastatic prostate cancer cells within the lymph node maintain a well-differentiated epithelial morphology. A, histologic examination of a pelvic lymph node replaced by metastatic prostate adenocarcinoma reveals high tumor cell density and extensive tumor infiltration. Small areas where tumor cells are foaming acinar/glandular-appearing structures (arrows). B, on the high-magnification microscopic picture, the boxed region reveals a structure reminiscent of well-differentiated epithelial tissue, with a distinct lumenal space. C and D, immunohistochemical analysis of this area with antibodies to PSMA reveals polarized expression of PSMA on the apical plasma membrane at the lumenal surface of these glandular structures. In addition, some PSMA staining was visible in the cytoplasm of PSMA on the apical plasma membrane at the lumenal surface of these glandular structures. In addition, some PSMA staining was visible in the cytoplasm of primary and metastatic prostate tumors, even with Gleason scores up to 9 (24). Furthermore, our data show that PSMA is distributed in a highly asymmetrical fashion on the plasma membrane of some metastatic prostate cancer cells. Whereas metastatic cells are generally considered highly transformed and nonpolarized, our novel finding suggests that even within occult metastatic lesions, populations of prostatic carcinoma cells maintain a well-differentiated epithelial morphology. These results indicate that cells within high-grade primary and metastatic prostate tumors are capable of maintaining a well-differentiated morphology.

Therefore, whereas PSMA-based immunotherapy would be most effective at combating highly transformed prostate cancer cells, such an approach would offer little tangible benefit for treatment of well-differentiated tumor cells. Thus, therapeutic strategies designed to reduce the obstructive influence of epithelial barriers could substantially improve the efficacy of mAb based immunotherapy. Analogous strategies to mitigate endothelial barriers by enhancing vascular permeability or intraepithelial transport have improved mAb uptake into tumor tissues (43, 51, 52). Whereas such an approach to increase the permeability of epithelial junctions may not always be feasible, those designed to alter the polarized protein trafficking may hold significant promise for the treatment of disease. Therefore, the microtubule requirement for proper PSMA localization to the apical plasma membrane could have important implications for therapy.

We have shown that treatment of polarized epithelial cells with microtubule-targeting chemotherapeutic Vinca alkaloids resulted in increased binding and endocytosis of PSMA-specific antibodies from the basolateral surface in an in vitro system. To our knowledge, this is the first study to suggest that commonly used chemotherapeutic agents can be exploited to target intrinsic protein trafficking machinery as a means to reverse the apical polarity of an antigen to the basolateral plasma membrane. Although this has yet to be proven using an in vivo system, it seems that a combined therapeutic strategy to target both microtubules and an antigenic target, like PSMA, could have a synergistic effect on overall patient outcome.

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References

Apical Targeting of PSMA


N-glycosylation and microtubule integrity are involved in apical targeting of prostate-specific membrane antigen: implications for immunotherapy

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