Differing effects of microtubule depolymerizing and stabilizing chemotherapeutic agents on t-SNARE–mediated apical targeting of prostate-specific membrane antigen

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Abstract

Prostate-specific membrane antigen (PSMA) is a protein up-regulated in the vast majority of prostate cancers. Antibodies to PSMA have proved highly specific for prostate cancer cells, and the therapeutic potential of such antibodies is currently being assessed in clinical trials. We have previously shown that PSMA at the cell surface of polarized epithelial cells is predominantly expressed at the apical plasma membrane and that microtubule depolymerization abolishes apical PSMA targeting. In the current report, we implicate a functional role for a target membrane soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptor, syntaxin 3, in the microtubule-dependent apical targeting of PSMA. PSMA and syntaxin 3 are similarly localized to the apical plasma membrane of the prostatic epithelium and Madin-Darby canine kidney cells. Introduction of a point mutation into syntaxin 3 abolishes its polarized distribution and causes PSMA to be targeted in a nonpolarized fashion. Additionally, treatment of polarized Madin-Darby canine kidney cells with vinblastine, a microtubule depolymerizing chemotherapeutic agent, causes both syntaxin 3 and PSMA to redistribute in a nonpolarized fashion. However, following treatment with the microtubule stabilizing chemotherapeutic agent Taxotere, both syntaxin 3 and PSMA continue to localize in a polarized manner at the apical plasma membrane. Thus, microtubule depolymerizing and stabilizing chemotherapeutic drugs might exact similar cytotoxic effects but have disparate effects on protein targeting. This phenomenon might have important clinical implication, especially related to antibody-mediated immunotherapy, and could potentially be exploited for therapeutic benefit. [Mol Cancer Ther 2006;5(10):2468–73]

Introduction

Prostate-specific membrane antigen (PSMA) is a potentially important clinical biomarker for the detection, management, imaging, and treatment of prostate cancer (1). PSMA is a type II transmembrane protein that is largely restricted to cells of the prostatic epithelium (2, 3). Elevated expression of PSMA is a hallmark of prostatic adenocarcinoma, and levels of PSMA are directly proportional to disease grade and stage (4). Clinical strategies have attempted to exploit the integral membrane association of PSMA and its correlation with advancing malignancy by using this protein as a tumor-associated antigenic target for monoclonal antibodies (mAb; refs. 5, 6). Antibodies to PSMA have received Food and Drug Administration approval for the detection and imaging of metastatic prostate cancer in soft tissues (5, 7, 8), and antibodies conjugated to radionuclides and cytotoxic drugs are currently in clinical trials for use in mAb-mediated immunotherapy (6, 9–12). These mAbs have shown the ability to deliver therapeutic agents specifically to the site of prostate cancer cells while limiting toxicity experienced by surrounding benign tissue. Although these antibodies have proved efficient at killing prostate tumor cells *in vitro* and in xenograft studies, the practical application to therapy has yet to be fully developed.

For such therapeutic strategies to be effective, the mAbs must reach the antigen on the malignant cell. In the case of solid tumors, accessibility of the antigen is restricted by a number of biological impediments and is contingent on the intracellular trafficking of the target antigen (13). We have previously shown that newly synthesized PSMA is targeted to the apical plasma membrane in polarized epithelial cells and identified signals responsible for sorting at the trans-Golgi network (14). After sorting at the trans-Golgi network, post-Golgi intermediate vesicles containing apical or basolateral cargo must traverse the cytoplasm and fuse with the appropriate plasma membrane domain. The

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microtubule cytoskeleton is particularly important for polarized targeting of apical cargo. Microtubule depolymerization or disruption of dynein function results in aberrant delivery of several apical proteins, including PSMA, to the basolateral surface (14–16); however, the role of microtubules in apical sorting of PSMA is not understood.

To elucidate the role of microtubules in apical targeting, we investigated the effects of microtubule disruption on the localization of soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptors (SNARE) at the plasma membrane. SNAREs are a class of membrane anchored proteins that contain a variable NH_2-terminal domain, a highly conserved coiled-coil domain, and a COOH-terminal α-helical membrane anchor. Following initial membrane contact between carrier vesicles and the target membrane, which is mediated largely by GTPases and tethering proteins, the SNAREs mediate the process of docking and membrane fusion (17, 18). In addition to mediating the physical fusion of the membranes, SNAREs also confer specificity for protein cargo delivery. Vesicular membrane SNAREs interact specifically with cognate target membrane SNAREs, which are assembled at specific intracellular locations (19).

Within epithelial cells, target membrane SNAREs are expressed in a highly polarized fashion, with syntaxins 3 and 4 localized to the apical and basolateral plasma membrane of Madin-Darby canine kidney (MDCK) cells, respectively (20). This asymmetrical localization provides specificity for vesicle fusion and likely promotes the polarized epithelial phenotype (21, 22). In this study, we show that syntaxin 3 and PSMA are both expressed on the apical plasma membrane of the prostatic epithelium *in situ* and in cultured Madin-Darby canine kidney (MDCK) cells. We show that alteration of syntaxin 3 localization, either by microtubule depolymerization or by a specific point mutation, also resulted in aberrant targeting of PSMA to the basolateral plasma membrane. In these cases, mAbs against PSMA were efficiently bound and internalized from the basolateral surface. These observations could have significant clinical implications, as PSMA at the basolateral surface would be more accessible to therapeutic antibodies in cases of well-differentiated carcinoma. Although *Vinca* alkaloids and Taxotere both inhibit microtubule dynamics, the stabilization of microtubules with the drug Taxotere did not cause syntaxin 3 or PSMA to redistribute in a nonpolarized fashion. Rather, these antigens remained localized specifically at the apical plasma membrane, indicating that this effect is specific for microtubule depolymerization.

Materials and Methods

DNA Constructs

The cDNA encoding full-length PSMA (generously provided by Dr. Warren Heston, Cleveland Clinic Foundation, Cleveland, OH) was cloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA). The cDNA encoding rat syntaxin 3 was cloned into the pCB7 expression vector as previously described (20). Wild-type human syntaxin 3 and a point mutant ($syn3_{E34A}$) disrupting the apical targeting signal were cloned into the vector pcDNA4-TO-myc-his as previously described (23).

Cell Culture and Transfections

MDCK cells (clone II) were cultured in DMEM 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% CO_2.

To generate stable cell lines, MDCK cells were transfected using the calcium phosphate method previously described (24) and clones were selected in the presence of 500 μg/mL geneticin (G418; Life Technologies, Inc., Rockville, MD). Immunofluorescence and immunoblotting were used to confirm expression. Transient transfection was done with FuGENE6 reagent (Roche, Indianapolis, IN) according to the instructions of the manufacturer.

MDCK cell lines expressing PSMA (MDCK-PSMA) were treated overnight in media containing 10 mmol/L sodium butyrate to increase PSMA expression. Cells were treated for 3 hours with 4 μg/mL nocodazole (Sigma, St. Louis, MO) in DMEM or with 2 μmol/L vinblastine (Sigma) to depolymerize microtubules or with 25 nmol/L Taxotere (Aventis, Bridgewater, NJ) to stabilize microtubules.

Antibodies

The mAb J591 directed against an extracellular epitope of PSMA has previously been described (25). The rabbit polyclonal antibody against syntaxin 3 has previously been described (26). Alexa Fluor 488– and Texas red–conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR) and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively.

Immunofluorescence

Human prostate tissue was fixed in paraformaldehyde, embedded in paraffin, sectioned, subjected to antigen retrieval by pressure-cooking and immunofluorescence microscopy as previously described (27). MDCK cells were grown on glass coverslips and fixed in cold methanol at −20°C for 30 minutes. Following fixation, specimens were placed in humidified chambers and washed with PBS containing 1 mmol/L $CaCl_2$ and $MgCl_2$ and 0.5% bovine serum albumin (PBS-CM-BSA). Specimens were incubated for 1 hour in the presence of primary antibody, washed with PBS-CM-BSA, incubated for 30 minutes in secondary antibody, washed with PBS-CM-BSA, and rinsed with distilled water. Specimens were mounted in Vectashield (Vector, Burlingame, CA) and the coverslips were sealed.

Cell-Surface PSMA Staining

MDCK-PSMA cells were grown on 0.4-μm pore size polycarbonate transwell filters (Corning, Corning, NY) and the transepithelial electrical resistance was determined with an EVOM Epithelial Voltohmeter (World Precision Instruments, Sarasota, FL). Values were normalized for filter area after subtracting the background resistance of a filter without cells. Transepithelial electrical resistance

values of >200 Ω/cm^2 were indicative of tight junction formation in MDCK cells. Medium was removed and chilled DMEM containing 5.0 μg/mL J591 was added to the indicated chamber (apical or basolateral). Cells were incubated on ice for 30 minutes, rinsed with cold PBS-CM-BSA, fixed in cold methanol, and incubated with secondary antibody as described above.

Internalization Assays

MDCK cells were grown to confluence on 0.4-μm pore size polycarbonate transwell filters as determined by transepithelial electrical resistance. Cells were treated with the indicated drugs 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 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).

Microscopy

Single-channel digital microscopic images were collected with an Olympus AX70 upright microscope and analyzed with SPOT imaging software, version 4.0.4. Laser scanning confocal microscopy was done with 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 Pascal imaging system (Carl Zeiss) by recording light emitted between 505 and 543 nm for Alexa Fluor 488 and above 560 nm for Texas red.

Results

Syntaxin 3 Is Localized to the Apical Plasma Membrane

Syntaxin 3 has previously been shown to localize to the apical plasma membrane in a variety of polarized epithelial cell types (20, 28–30). Here the localization of syntaxin 3 was evaluated in the human prostatic epithelium. *In situ* immunofluorescence done on human prostate tissue sections revealed that syntaxin 3 was restricted to the apical plasma membrane facing the lumen of the gland whereas the basolateral marker E-cadherin was clearly restricted to regions of intercellular contact and the underlying basolateral plasma membrane (Fig. 1A). Immunofluorescence and confocal microscopy done in polarized MDCK cells revealed a similar pattern of localization, with E-cadherin and ectopically expressed rat syntaxin 3 localized to the basolateral and apical plasma membranes, respectively (Fig. 1B). Antibodies to rat syntaxin 3 did not recognize endogenous syntaxin 3 in the MDCK cells.

Functional Syntaxin 3 Is Essential for Apical Targeting of PSMA

A stable cell line of MDCK cells expressing PSMA (MDCK-PSMA) was transfected with either wild-type or a mutant form of syntaxin 3 (23). This syntaxin 3 mutant contained a point mutation that changed a glutamic acid residue at position 34 into an alanine ($syn3_{E34A}$).

MDCK-PSMA cells were transiently transfected with either wild-type syntaxin 3 or $syn3_{E34A}$ and were grown to confluency on transwell filters. Immunofluorescence analysis revealed that wild-type syntaxin 3 localized exclusively to the apical surface of MDCK cells (Fig. 2A). In contrast, the mutant $syn3_{E34A}$ was localized in a nonpolarized fashion at both apical and basolateral plasma membrane surfaces (Fig. 2B). PSMA on the cell surface has previously been shown to localize almost exclusively to the apical plasma membrane in polarized MDCK and prostatic epithelial cells (31). Polarized monolayers of MDCK-PSMA cells grown on transwell filters were subjected to staining at the basolateral surface with antibodies to the extracellular domain of PSMA. These domain-specific cell-surface staining assays revealed that expression of wild-type syntaxin 3 did not increase the amount of PSMA at the basolateral plasma membrane (Fig. 2C). However, surface staining of PSMA at the basolateral membrane was apparent in MDCK-PSMA cells expressing $syn3_{E34A}$ (Fig. 2D).

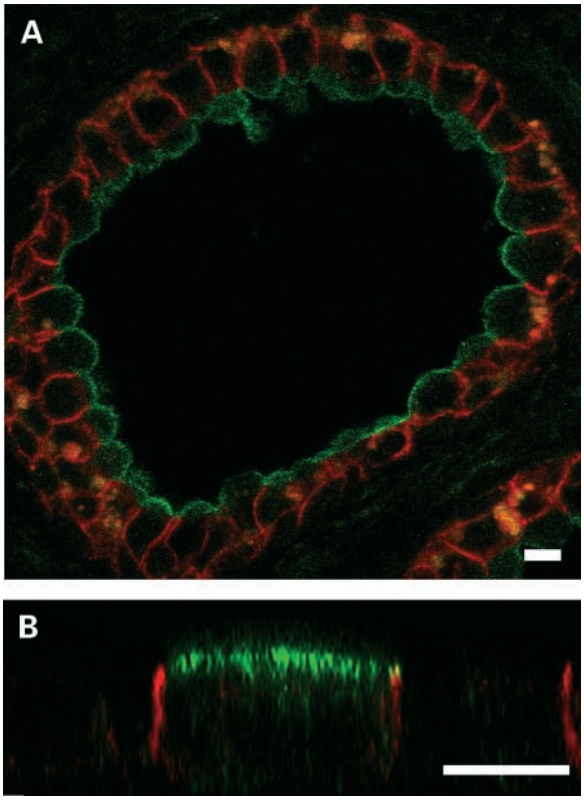

Figure 1. Syntaxin 3 is localized to the apical plasma membrane. *In situ* immunofluorescence reveals that syntaxin 3 (*green*) is localized to the apical plasma membrane of prostatic epithelial cells whereas E-cadherin (*red*) is localized to the basolateral membrane (**A**). Immunofluorescence analysis was done on polarized MDCK cells transiently transfected with syntaxin 3 cDNA. A vertical confocal section of a single polarized cell expressing ectopic syntaxin 3 reveals that this antigen (*green*) is localized to the apical plasma membrane and E-cadherin (*red*) is localized to the basolateral (**B**). Bar, 10 μm.

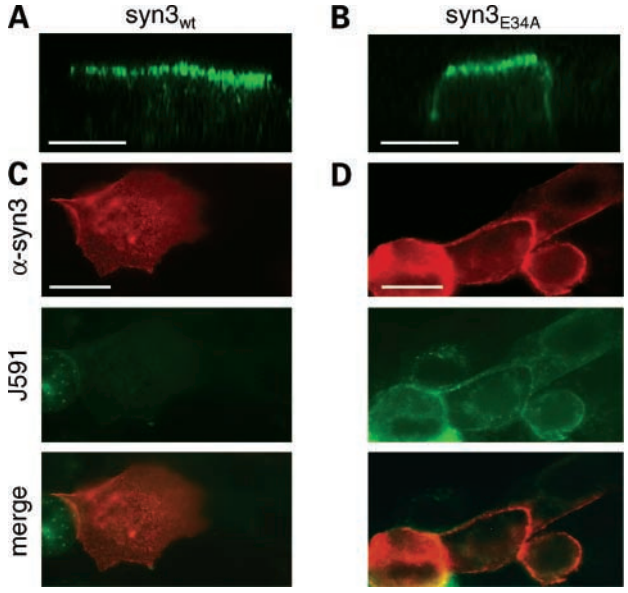

Figure 2. A mutation that affects syntaxin 3 polarity also affects the polarity of PSMA. Confocal vertical sections reveal that wild-type syntaxin 3 (*syn3*$_{WT}$) is localized to the apical plasma membrane in MDCK-PSMA cells (**A**). In contrast, a mutant form of syntaxin 3 (syn3$_{E34A}$), containing a point mutation that changes a glutamic acid residue to an alanine at position 34, is distributed in a nonpolarized fashion in MDCK-PSMA cells (**B**). Cell-surface PSMA labeling of polarized MDCK-PSMA cells transfected with wild-type syntaxin 3 does not reveal appreciable levels of PSMA at the basolateral surface (**B**). However, expression of syn3$_{E34A}$ resulted in PSMA cell-surface labeling at the basolateral surface of polarized MDCK-PSMA cells (**D**). Bar, 10 μm.

Microtubule Depolymerization, but not Stabilization, Alters the Polarity of Syntaxin 3

Apical localization of PSMA has previously been shown to require intact microtubules (14). To investigate the significance of microtubules on syntaxin 3 localization, MDCK cells transiently expressing rat syntaxin 3 were grown on glass coverslips and treated with either the microtubule depolymerizing drug nocodazole or the microtubule stabilizing drug Taxotere. Immunofluorescence analysis with antibodies against α-tubulin or rat syntaxin 3 revealed that untreated MDCK cells had intact microtubules and syntaxin 3 localized to the apical plasma membrane (Fig. 3A). Treatment with nocodazole resulted in extensive disassembly of microtubules and increased the localization of syntaxin 3 at the basolateral surface and in the cytoplasm (Fig. 3B), consistent with previous results (32, 33). Treatment with Taxotere, which binds to and stabilizes microtubules, resulted in the formation of dense bundles of microtubules. Syntaxin 3 in these cells continued to localize at the apical plasma membrane (Fig. 3C).

Microtubule Depolymerization, but not Stabilization, Results in Uptake of PSMA-Specific Antibodies from the Basolateral Surface

The apical plasma membrane in polarized epithelial cells is inaccessible to i.v. administered agents within the circulation. Therefore, we next wanted to investigate the influence of commonly used microtubule disrupting chemotherapeutic agents on PSMA polarity and domain-specific antibody uptake. MDCK-PSMA cells were grown on transwell filters and antibody was added to either the apical or basolateral chamber. These cells expressed only endogenous syntaxin 3 and thus eliminated the possibility that any effect on PSMA distribution could be ascribed to overexpression of syntaxin 3. Untreated MDCK-PSMA cells with intact microtubules internalized J591 exclusively from the apical surface (Fig. 4A). We next treated cells with the microtubule depolymerizing agent vinblastine. Vinblastine is a member of a class of chemotherapeutic drugs known as the *Vinca* alkaloids. The use of these drugs has been investigated for chemotherapy of a variety of malignancies, including prostate cancer (34, 35). Like nocodazole treatment, incubation with vinblastine resulted in microtubule depolymerization. Similar to previous observations, vinblastine-treated cells internalized a significant amount of mAb J591 from the basolateral surface (Fig. 4B). In contrast, treatment with Taxotere did not increase PSMA uptake from the basolateral surface. Although the overall efficiency of mAb uptake seemed to be slightly reduced relative to untreated cells, PSMA internalization was still highly polarized and, like in control cells, occurred almost exclusively from the apical plasma membrane (Fig. 4C).

Discussion

Results from this study show that the targeting of PSMA is contingent on the localization of the target membrane SNARE syntaxin 3. Normally, syntaxin 3 is localized to

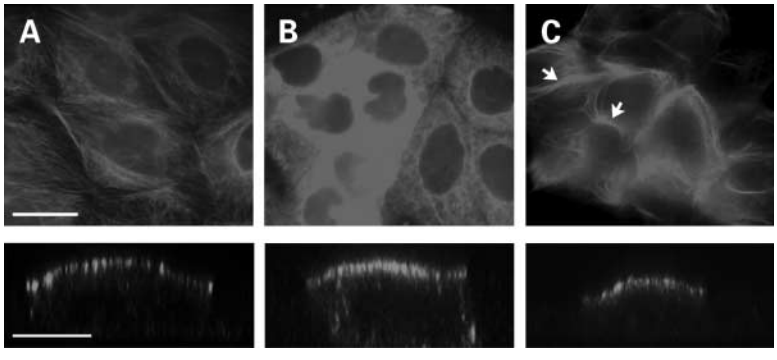

Figure 3. Microtubule depolymerization, but not stabilization, results in a nonpolarized distribution of syntaxin 3. Untreated MDCK cells have intact microtubules and localize syntaxin 3 to the apical surface (**A**). Treatment with nocodazole resulted in microtubule depolymerization, with diffuse α-tubulin staining dispersed throughout the cytoplasm. These cells also exhibit a nonpolarized distribution of syntaxin 3 (**B**). Taxotere treatment resulted in formation of thick bundles of microtubules (*arrows*) but did not affect apical localization of syntaxin 3 (**C**). Bar, 10 μm.

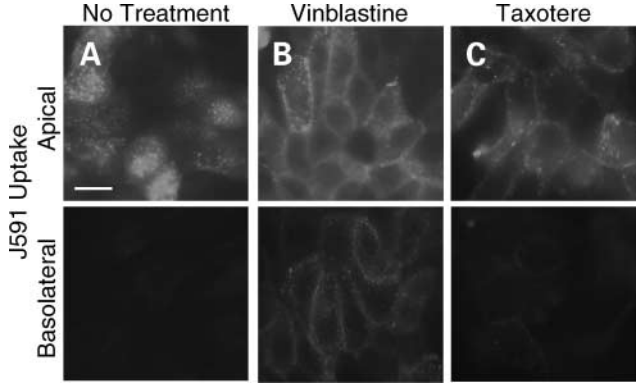

Figure 4. Microtubule depolymerization, but not stabilization, results in a nonpolarized plasma membrane distribution of PSMA. Untreated MDCK-PSMA cells have intact microtubules and internalize J591 exclusively from the apical surface (**A**). Treatment with the *Vinca* alkaloid vinblastine resulted in microtubule depolymerization. Following vinblastine treatment, MDCK-PSMA cells internalized a significant level of mAb J591 from the basolateral surface (**B**). Taxotere treatment resulted in formation of dense bundles of microtubules; these cells also internalized mAb J591 exclusively from the apical surface (**C**). Bar, 10 μm.

the apical plasma membrane in prostatic epithelial cells and in cultured MDCK cells. Introduction of point mutations causes syntaxin 3 to distribute in a nonpolarized fashion and also results in aberrant targeting of PSMA to both plasma membrane surfaces. The localization of syntaxin 3 requires intact microtubules as treatment with the drug nocodazole causes syntaxin 3 to redistribute, increasing localization at the basolateral plasma membrane and within the cytoplasm. Concomitant with the change in syntaxin 3 localization, PSMA is targeted to the cell surface in a nonpolarized fashion. Following treatment with nocodazole, PSMA in MDCK cells becomes accessible to antibodies at the basolateral plasma membrane. This may be a potentially significant observation from a clinical standpoint.

PSMA offers an intriguing antigenic target for mAb-mediated immunotherapy of prostate cancer. Unfortunately, one of the persistent problems facing immunotherapy for the treatment of solid tumors involves the relatively low fraction of the original i.v. administered dose of antibody that will ever reach the target antigen. Studies have previously suggested that only ~0.01% to 0.1% of the original i.v. administered dose of antibody will reach target antigens within a solid tumor per gram of tumor tissue (36). This situation can primarily be ascribed to the substantial biological impediments that prevent the antibody from reaching the cell surface. Tight junctions are among the most frequently overlooked barriers that may have a major effect on mAb-mediated immunotherapy (13). The loss of tight junctions is often presumed to be a universal feature of advancing invasiveness that accompanies the transition to a malignant phenotype. However, carcinomas represent a highly heterogeneous population of cells with varying morphologic characteristics. In addition, individual cells may display a high degree of plasticity, enabling them to disassemble and reform epithelial junctions and assume different morphologic characteristics in response to genetic, epigenetic, or environmental changes. Thus, it is not uncommon to find advanced metastatic and invasive tumors with well-differentiated epithelial characteristics, intact epithelial junctions, and plasma membrane polarity.

In these cells, the apical surface, which normally faces the external environment or luminal contents of the gland, would not be accessible to i.v. administered mAbs due to the gate and fence functions of the tight junctions. Only the basolateral surface would be accessible to the underlying stroma and vasculature (13). Thus, antibodies to PSMA would be relatively ineffective for the treatment of these morphologically differentiated carcinoma cells, especially in comparison with mAbs such as Herceptin, which targets the basolateral antigen Her-2 (37).

The microtubule requirement for polarized localization of syntaxin 3 is consistent with previous results (32, 33) and offers an interesting potential mechanism to improve the accessibility of apical antigens to i.v. agents in polarized cells. Microtubule depolymerizing drugs, such as *Vinca* alkaloids, nocodazole, and colchicine, are commonly used in chemotherapy and work by binding to tubulin monomers and preventing their assembly. These agents specifically target dividing cells by disrupting the mitotic spindle and activating the spindle checkpoint. The results presented in this study suggest that, in addition to this primary cytotoxic effect, these drugs would also redirect syntaxin 3. This would result in increased basolateral targeting of some apical tumor-associated antigens, such as PSMA, making them better targets for immunotherapy in well-differentiated carcinoma cells. However, such treatments would also potentially redirect apical antigens in benign tissues. Altered cell polarity and polarized protein trafficking contribute to a variety of pathologic conditions, and temporary alteration of polarized trafficking in response to microtubule depolymerization therapeutics may potentially result in inflammation, tissue damage, or even organ failure (38, 39).

The effect on syntaxin 3 localization seems to be specific for microtubule depolymerization and not for stabilization. Like the microtubule depolymerizing drugs, microtubule stabilizing drugs, such as Taxotere and Taxol-related analogues, also bind tubulin and target dividing cells by interfering with spindle assembly, preventing cell division, and activating the spindle checkpoint. However, these drugs promote the formation of highly stable microtubules that are resistant to depolymerization. Recently, syntaxin-containing vesicles were shown to contain an adaptor protein that provided a link to the microtubule cytoskeleton. This particular adaptor, known as syntabulin, is bound to conventional kinesin I heavy chain (40). Such microtubule-associated motor proteins interact and traverse along stabilized microtubules, both *in vitro* and *in vivo*, thus explaining why syntaxin 3 and PSMA were not directed in a nonpolarized fashion in Taxotere-treated cells. Therefore, whereas both microtubule depolymerizing and stabilizing drugs would activate cell cycle checkpoints and initiate the

onset of cell death programs in actively proliferating cells, only microtubule depolymerizing drugs would have the added effect of altering the polarized distribution of certain apical antigens, including PSMA. A comprehensive understanding of how these drugs affect protein trafficking in polarized epithelial cells can be used to adapt specialized treatment regimens, improve efficacy of synergistic therapeutic modalities, or limit specific types of peripheral damage to benign tissues.

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