Constitutive and Antibody-induced Internalization of Prostate-specific Membrane Antigen

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Abstract

Prostate-specific membrane antigen (PSMA) is a cell surface glycoprotein expressed predominantly by prostate cancer cells. We have characterized four monoclonal antibodies that bind to the extracellular domain of PSMA (Liu et al., Cancer Res., 57: 3629–3634, 1997). Here we report that viable LNCaP cells internalize these antibodies. Laser scanning confocal microscopy reveals that the internalized antibodies accumulate in endosomes, and immunoelectron microscopy reveals that endocytosis of the PSMA-antibody complex occurs via clathrin-coated pits. In addition, a quantitative cell surface biotinylation assay demonstrates that PSMA is constitutively endocytosed in LNCaP cells and that anti-PSMA antibodies increase the rate of internalization of PSMA. These studies suggest that PSMA might function as a receptor mediating the internalization of a putative ligand. The availability of prostate-specific internalizing antibodies should aid the development of novel therapeutic methods to target the delivery of toxins, drugs, or short-range isotopes specifically to the interior of prostate cancer cells.

Introduction

PSMA is the single most well-established highly restricted prostate epithelial cell membrane antigen (1–8). In contrast to other highly restricted prostate-related antigens such as prostate-specific antigen and prostatic acid phosphatase, which are secretory proteins, PSMA is an integral cell membrane protein. The PSMA gene has been cloned, sequenced (9), and mapped to chromosome 11q14 (10). One of the reasons for significant interest in PSMA is that it is ideal for in vivo prostate-specific targeting strategies. In addition to its prostate specificity (1–8), PSMA is expressed by a very high proportion of PCAs characterized four monoclonal antibodies that bind to the extracellular domain and define two distinct epitopes of PSMA (PSMAa and PSMAB; Ref. 11). Because these mAbs are capable of binding viable PSMA-expressing cells, we have begun to use them in an effort to further understand the function of PSMA.

Materials and Methods

Antibodies and Reagents. mAbs J591, J415, and J533 (all IgG1) and E99 (IgG3) to PSMAa, and mAb 156 (IgG1; negative control) to inhibit were generated as described previously (9). Purified mAb 7E11.C5 was a generous gift from Dr. Gerald P. Murphy (Pacific Northwest Research Foundation, Seattle, WA). Secondary antibody reagents conjugated with FITC and Texas Red were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Antibody Uptake. LNCaP cells (15 x 104) were plated on glass coverslips in 35-mm dishes and grown for 2-3 days before initiating the experiments. For the internalization assay, the cells were washed and further processed for IF and confocal microscopy as described below. For immunoelectron microscopic detection of antibody uptake, the above-mentioned procedure was followed, except that the cells were grown directly on 35-mm culture dishes.

IF and Laser Scanning Confocal Microscopy. After primary mAb incubation, FITC-conjugated goat antimouse IgG [Jackson ImmunoResearch Laboratories; 1:100 in 1% BSA in PBS (pH 7.4)] was incubated for 30 min and washed extensively in 1% BSA in PBS. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA).

The internalization of FITC-conjugated transferrin and antibodies against PSMA was examined using a Photobios 1000 laser scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA) as described previously (16). To detect FITC- and Texas Red-labeled reagents simultaneously, samples were excited at 514 nm with an argon laser; the light emitted between 525 and 540 nm was recorded for FITC, and the light emitted above 630 nm was recorded for Texas Red. Serial optical sections of the monolayer were recorded at 0.4 μm intervals. A total of 30–40 horizontal (X-Y) confocal sections were obtained for each cell type and used to generate three-dimensional images using the Image Space software program (version 3.01; Molecular Dynamics) on an Iris Indigo Workstation (Silicon Graphics, Mountain View, CA).

PSMA has been shown to have peptidase (12) and folate hydrolase (13) activity. Although it shares some homology with rat brain N-acetylated α-linked acidic dipeptidase (12) and the transferrin receptor (9), PSMA does not share the latter’s internalization signal (9). The function of PSMA with respect to PCA and vascular endothelial cell biology and the direct correlation between its expression and increasing PCA aggressiveness remain intriguing and unclear.

Until recently, the only available mAb to PSMA was 7E11.C5 (14), which targets an epitope located within the short cytoplasmic tail of the molecule (15, 16). As a result, 7E11.C5 did not bind viable cells (11, 14, 16). We recently reported the development of four IgG1 mAbs that react with the external domain and define two distinct epitopes of PSMA (PSMAa and PSMAB; Ref. 11). Because these mAbs are capable of binding viable PSMA-expressing cells, we have begun to use them in an effort to further understand the function of PSMA.
IEM. After antibody incubation at 37°C for 2 h, the cells were washed in PBS in BSA, fixed for 20 min in cold methanol, and hydrated in PBS in BSA. Cells were then incubated for 1 h with 15-nm gold beads conjugated with goat antimouse IgG (Amersham Life Science, Inc., Arlington Heights, IL). After washing, the cells were fixed in 2.5% glutaraldehyde for 15 min, scraped gently, pelleted, and processed for IEM as described previously (11, 17). Electron micrographs were taken with a Joel 100 CX electron microscope.

Cell Surface Biotinylation Assay for Endocytosis. Biotinylation assays were performed as described by Bretscher and Lutter (18). Briefly, LNCaP cells (60 x 10⁴) were grown on polylysine (3%)-coated 60-mm dishes. Cells were washed in precooled PBS containing 1 mM each of calcium chloride and magnesium chloride. To biotinylate the cell surface proteins, cells were treated with the water-soluble, membrane-impermeable, cleavable biotin analogue sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-biotin; Pierce Chemical Co., Rockford, IL; 0.5 mg/ml) at 4°C for 20 min and then washed in RPMI-BSA. Two control dishes were kept on ice, whereas the other dishes were incubated at 37°C. The incubation was stopped at various times by transferring cells back to 4°C. After washing in 10% FCS in PBS, the cells were incubated twice for 20 min in reducing solution [310 mg of glutathione-free acid (Sigma, St. Louis, MO) dissolved in 17 ml of H₂O; 1 ml of 1.5 M NaCl, 0.12 ml of 50% NaOH, and 2 ml of serum were added just before use] to remove the residual cell surface exposed biotin. One control dish was reduced, and the second dish was not reduced, thereby serving as 0 and 100% biotinylation references, respectively. After washing, free sulfhydryl groups were quenched in iodoacetamide (5 mg/ml; Sigma) in BSA in PBS for 15 min. Cells were lysed, and PSMA was immunoprecipitated as described previously (11). Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions. The gels were transferred to nitrocellulose membranes, and the blots were probed with 125I-streptavidin (Amersham), autoradiographed, and quantified using a densitometer (Molecular Dynamics).

Results

Staining of Viable Cells and Internalization of mAbs. IF analysis of viable LNCaP cells incubated with mAbs J591, J533, E99, and J415 at 4°C showed distinct plasma membrane staining (data not shown), whereas mAb 7E11.C5 revealed no plasma membrane staining. Incubation of cells at 37°C with mAb J591 revealed labeling of both plasma membrane and intracellular vesicles (Fig. 1). After a 5-min incubation at 37°C, the labeling was detected primarily on the plasma membrane (Fig. 1A). At 20 min, distinct staining of intracellular vesicles was apparent (Fig. 1B), and at 180 min, intense labeling was observed in the juxtanuclear region, with sparse labeling throughout the cytoplasm (Fig. 1C). mAbs J415, J533, and E99 gave identical results (data not shown). mAb 7E11.C5 showed neither cell surface nor intracellular staining (data not shown) in these viable cells. These results indicate that mAbs to PSMAex are internalized by viable PCA cells.

Endosomal Localization of Internalized Antibodies. To test whether internalized antibodies accumulate in endosomes, a simultaneous uptake of mAbs and FITC-labeled transferrin (an endosomal marker) was carried out. Laser scanning confocal microscopy revealed that internalized J591 (Fig. 2A) and transferrin (Fig. 2C) codistributed to a large extent (Fig. 2E), indicating that the internalized PSMA-antibody complex accumulates in endosomes. Control experiments with 7E11.C5 confirmed that this antibody is not internalized (Fig. 2, B, D, and F).

IEM. IEM of nonpermeabilized LNCaP cells at 4°C revealed mAb J591 binding to the extracellular side of the plasma membrane (11). IEM of viable LNCaP cells incubated with mAb J591 at 37°C for 10 min showed an accumulation of gold particles in clathrin-coated pits (Fig. 3, A and B) and in vesicles close to the plasma membrane (Fig. 3C). After a 2-h incubation at 37°C, vesicles containing gold beads were found in a juxtanuclear location (Fig. 3D). These findings indicate that mAb J591 internalization occurs via clathrin-coated pits in LNCaP cells.

Internalization of PSMA. A cell surface protein biotinylation assay was developed to test whether PSMA is internalized in the absence of antibody, or whether antibody binding induces PSMA internalization. In this assay, a cleavable biotin analogue (NHS-SS-biotin) was used to label proteins exposed on the surface at 4°C. The return of surface biotinylated cells to a temperature of 37°C allows the internalization of the appropriate cell surface proteins with their biotin tag. The NHS-SS-biotin label is removed from noninternalized cell surface proteins through cleavage of the disulfide linkage with glutathione (18), whereas internalized biotinylated proteins are protected from this cleavage. The appearance of biotin-labeled protein that is resistant to glutathione reduction was taken as an indicator of inter-
Fig. 2. Confocal microscope analysis of the internalization mAb J591. LNCaP cells were incubated with mAb J591 and FITC-conjugated transferrin (A, C, and E) or mAb 7E11.C5. (B, D, and F) for 2 h and processed for IF as described in “Materials and Methods.” mAbs J591 (A) and 7E11.C5. (B) were detected with a Texas Red-conjugated secondary antibody. FITC-conjugated transferrin uptake is shown (C and D). Images in A and C were merged to obtain the image in E (mAb J591 and FITC-conjugated transferrin colocalization, yellow). Images in B and D were merged to obtain the image in F, in which only transferrin uptake is seen because 7E11.C5. neither binds nor internalizes.

As shown in Fig. 4A, biotinylated PSMA is sensitive to glutathione reduction after labeling cells at 4°C (Fig. 4A, compare Lanes 1 and 2). However, with progressively longer incubation periods at 37°C, an increasing proportion of the labeled PSMA becomes increasingly resistant to reduction (Fig. 4A, Lanes 3–6). Quantitation of the blots revealed that 60% of the total cell surface PSMA was internalized (Fig. 4C) within 2 h.

When the biotinylated cells were incubated with 1 μg/ml mAb J591 at 37°C, a 3-fold increase in the rate of internalization of PSMA was observed (compare Fig. 4B, Lanes 3 and 4 and Fig. 4D). A higher concentration of mAb J591 did not show any further increase in the internalization of PSMA (Fig. 4B, Lanes 5–7; Fig. 4D). Similar results were obtained with monovalent Fab fragments (data not shown).

Discussion

The prostate-restricted nature of PSMA, coupled with the direct association between the level of PSMA expression and increasingly aggressive disease (4), implies a potentially important role for PSMA in PCA biology. The importance of understanding the function of PSMA is further stimulated by its expression in vascular endothelium specifically supplying cancers but not in normal, resting endothelium (7, 11). In the past, investigating PSMA function has been compromised because the sole antibody to PSMA reacted with a cytoplasmic epitope of the molecule and therefore bound only to cells that were permeabilized or dead (11, 14, 16). Our recent development of mAbs to the extracellular domain of PSMA and their demonstrated ability to
bind viable cells (11) have provided a means to study the function of PSMA.

In this study, we demonstrate by a combination of microscopical and biochemical techniques that PSMA and mAbs to PSMA<sub>ext</sub> are internalized by LNCaP cells. Confocal microscopy and IEM reveal that PSMA-mAb complexes are endocytosed via clathrin-coated pits (Figs. 2 and 3). A quantitative cell surface biotinylation assay demonstrates that PSMA is constitutively internalized in the absence of antibody binding. At 20 min, 15% of the total biotinylated surface PSMA is internalized (Fig. 4C). The proportion of surface PSMA internalized increases to 60% at 60 min and remains fairly constant at that level for 240 min thereafter, when the assay was terminated. The stability of the labeled PSMA for a period of over 6 h (data not shown) indicates that PSMA degradation during this period is minimal. Internalization of only 60% of the total labeled surface PSMA may be explained by the recycling of internalized, biotinylated PSMA back to the cell surface, where it would be reduced and rendered undetectable in this assay.

Constitutive internalization of PSMA may reflect the recycling of a structural protein through a plasma membrane location or may be mediated by the binding of a ligand. Whereas the finding that anti-PSMA antibody significantly increases the rate of internalization of PSMA is consistent with the latter ligand receptor-type function, it does not necessarily indicate that PSMA has a transport function. In the presence of mAb to PSMA<sub>ext</sub>, the rate of internalization of PSMA increased up to 3-fold in a dose-dependent manner, reaching a maximum rate at an antibody concentration of 1–2 μg/ml (Fig. 4). A similar increase in the internalization rate has been shown for epidermal growth factor and its ligand (19).

It is well established that many ligands and their transmembrane receptors are internalized via clathrin-coated pits (receptor-mediated endocytosis) (20). The formation of antigen-antibody complexes on the cell surface often results in internalization through a pathway closely resembling the receptor-mediated endocytosis of peptide hormones, growth factors, and other natural ligands (21). Based on our findings, we hypothesize that PSMA may have a transport function of an as yet unidentified ligand. The baseline internalization rate of PSMA may indicate that PSMA may internalize in the absence of...
whereas in PCA, the membrane form predominates by 10-100-fold variant without a significant membrane-expressed component, example, at the mRNA level, normal and benign hyperplastic prostate epithelia predominantly express the cytosolic PSM′ splice becomes increasingly PCA-specific via different mechanisms. For internalize. Therefore, the highly restricted expression of PSMA Tyr-X-Arg-Phe and dileucine motifs (25) and presumably does not present in the cytoplasmic tail of PSMA. Experiments are under way to confirm the dileucine internalization motif of PSMA.

Interestingly, whereas PSMA is 85% homologous to a rat brain neuropeptidase (24), this homology is located primarily at the NH2-termini. Furthermore, rat brain neuropeptidase lacks both the COOH-termini and declines to less than 50% homology at the phosphatase receptor, asialoglycoprotein receptor, polymeric immunoglobulin receptor, and others, are all tetrapeptides (Tyr-X-Arg-Phe) having an aromatic residue in the fourth position of the sequence (23). The cytoplasmic tail of PSMA lacks a sequence similar to the Tyr-X-Arg-Phe motif (9). Another signal is the dileucine motif, for which the only known requirement is the presence of two consecutive leucines or a leucine-isoleucine pair. The dileucine motif has been shown to mediate internalization and targeting to endosomes and lysosomes (24). A dileucine motif is present in the cytoplasmic tail of PSMA. Experiments are under way to confirm the dileucine internalization motif of PSMA. Interestingly, whereas PSMA is 85% homologous to a rat brain neuropeptidase (24), this homology is located primarily at the COOH-termini and declines to less than 50% homology at the NH2-termini. Furthermore, rat brain neuropeptidase lacks both the Tyr-X-Arg-Phe and dileucine motifs (25) and presumably does not internalize. Therefore, the highly restricted expression of PSMA becomes increasingly PCA-specific via different mechanisms. For example, at the mRNA level, normal and benign hyperplastic prostate epithelia predominantly express the cytosolic PSM′ splice variant without a significant membrane-expressed component, whereas in PCA, the membrane form predominates by 10-100-fold (26). Another form of functional specificity is demonstrated in rat brain astrocytes (25); although there is expression of a homologous neuropeptidase, this neuropeptidase is presumably not internalized as is PSMA in PCA cells.

The property of mAbs to PSMAext to be internalized in PCA cells adds another dimension to their in vivo therapeutic potential. In addition to selective-specific binding to the PCA cell surface, the mAb or fragment would be internalized into the targeted cells, providing direct access to the neoplastic cell machinery. As such, this property opens up options such as the use of toxin or drug conjugates. Similarly, the juxtanuclear location of the internalized vesicles should increase the potency of mAb-α particle conjugates by improving the incident angle of the isotope and the target DNA.

Lastly, although mAb may function as a surrogate ligand, the question remains as to the identity of the putative natural ligand of PSMA. Troyer et al. (16) noted a M, 40,000 band that coimmunoprecipitated with PSMA that they identified as S-glutamic oxalacetic transaminase. We have not been able to demonstrate the binding of S-glutamic oxalacetic transaminase to PSMA (data not shown). Fur incorporation remains as to the identity of the putative natural ligand of PSMA. Troyer et al. (16) noted a M, 40,000 band that coimmunoprecipitated with PSMA that they identified as S-glutamic oxalacetic transaminase. We have not been able to demonstrate the binding of S-glutamic oxalacetic transaminase to PSMA (data not shown). Further study will be required to define the putative natural ligand, which, in turn, may shed additional light on the role of PSMA in cancer biology and tumor angiogenesis. The natural ligand, if similarly restricted in its tissue receptor binding profile, may substitute for the mAb in a targeted therapy approach.

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References


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