Prostate-specific Membrane Antigen Association with Filamin A Modulates Its Internalization and NAALADase Activity

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ABSTRACT

Prostate-specific membrane antigen (PSMA) is an integral membrane protein highly expressed by prostate cancer cells. We reported previously that PSMA undergoes internalization via clathrin-coated pits (Liu et al., Cancer Res., 58: 4055–4060, 1998). In this study we demonstrate that filamin A, an actin cross-linking protein, associates with the cytoplasmic tail of PSMA and that this association of PSMA with filamin is involved in its localization to the recycling endosomal compartment. By ectopically expressing PSMA in filamin-negative and -positive cell lines, we additionally show that filamin binding to PSMA reduces the internalization rate of PSMA and its N-acetylated-α linked-acidic dipeptidase activity. These results suggest that filamin might be an important regulator of PSMA function.

INTRODUCTION

PSMA is a type-II integral membrane protein of Mr 100,000 with a short 19 amino acid cytoplasmic tail, predominantly localized to the epithelial cells of the prostate gland (1, 2). PSMA has been shown to have two enzymatic activities: folate hydrolase (3) and NAALADase (4). In normal prostate epithelial cells, expression of PSMA is very low, and the level increases several fold in high-grade prostate cancers, metastatic diseases, and hormone-refractory prostate carcinoma (5). mAbs raised against the extracellular domain of PSMA have been conjugated with either radioactive ligands or cytotoxins for use as immunotoxins for the specific targeting of prostate cancer cells (6). The importance of PSMA was additionally illustrated by the finding that it is also expressed in endothelial cells of the neovasculature but is absent in normal endothelial cells (7).

PSMA is constitutively internalized via clathrin-coated pits in LNCaP cells (8). In addition, antibody specific for PSMA extracellular domain increased the rate of internalization of PSMA (8). Deletion of the cytoplasmic tail of PSMA resulted in the loss of internalization of PSMA, indicating that the cytoplasmic tail is crucial for its internalization (5). To additionally understand the mechanism of PSMA internalization we used the cytoplasmic tail of PSMA as bait in a yeast two-hybrid screening approach to identify PSMA interacting proteins. In this study we report that the cytoplasmic tail of PSMA associates with filamin A, an actin cross-linking phosphoprotein and that this association modulates the internalization rate of PSMA and its localization to the REC. Our studies also suggest that PSMA-filamin association is involved in the regulation of NAALADase activity of PSMA.

MATERIALS AND METHODS

Antibodies and Reagents. Mouse mAb, J591, against the extracellular domain of PSMA has been described (7). Mouse mAb against filamin A was from Chemicon International Inc. (Temecula, CA). FITC- and Texas Red-labeled, affinity-purified secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), and horseradish peroxidase-conjugated antimouse antibody and streptavidin were from Transduction Laboratories. PC3 cells stably expressing PSMA have been described earlier (9) and were kindly provided by Dr. Warren Heston (Cleveland Clinic Foundation, Cleveland, OH). M2 and A7 melanoma cell lines were generously provided by Dr. Thomas Stossel (Harvard Medical School, Boston, MA).

Cell Culture and Transfections. PC3-PSMA cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, MEM nonessential amino acid solution, and penicillin/streptomycin. The calcium phosphate method was used for all of the transfections (10).

Yeast Two-Hybrid Analysis. Two complementary oligonucleotides, representing the 19 amino acid cytoplasmic tail of PSMA, were synthesized, annealed, and cloned into the bait vector pGBKT7. This bait was used to screen 3 × 10^6 yeast colonies from the Matchmaker Two-Hybrid System 3 of Clontech (Palo Alto, CA).

GST Pull-Down and Immunoprecipitation. A 520-bp DNA fragment corresponding to the NH2-terminal 1–173 amino acids of PSMA was cloned in fusion with GST in the pGEX-4X vector. The GST-PSMA fusion protein overexpressed in Escherichia coli was purified to homogeneity according to the manufacturer’s instructions (Amerham Biosciences, Piscataway, NJ). The DNA representing the 23rd–24th repeats (2466–2646 amino acids) of filamin A was cloned in pET 28-b, a T7-based bacterial expression vector. The GST pull-down assay was carried out in a 50 μl reaction volume [binding buffer: 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 12.5 mM MgCl2, and 10% glycerol] containing 500 ng of affinity purified GST-PSMA, and 5 μl of in vitro transcription and translation reaction containing radioactive filamin A. The binding reaction was carried out at 4°C for 2 h, and the bound filamin was pulled down using GST beads. Purified GST protein incubated with radioactive filamin A was used as a control in the assay. Bound proteins were analyzed by 15% SDS-PAGE followed by autoradiography.

For immunoprecipitation, PC3-PSMA were rinsed with ice-cold PBS and lysed in 1 ml of lysis buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton-X-100, 40 mM N-octylglucoside, 0.2 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 50 μg/ml DNase, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of antipain, leupeptin, and pepstatin] on ice for 30 min. The lysates were subjected to immunoprecipitation using mAb J591 and immunoblotted with antibo.
for filamin. Total PSMA was immunoprecipitated with mAb prepared from PC3 cells expressing PSMA, and of PSMA and filamin A in vivo. GST (D) alone was used as a control (Ref. 8). Obtained partial cDNA clones identical to the COOH-terminal region of filamin A, filamin B, or C cDNA clones on medium lack- substitutions. Among all of the positive clones sequenced, 50% represented conserved regions of the three isoforms of filamin that interacted with the cytoplasmic domain of PSMA. Among all of the positive clones sequenced, 50% represented two distinct COOH-terminal regions, corresponding to the 22–24th and 23–24th repeats of filamin A. Filamin has three isoforms: filamin A (also called ABP 280, filament α, filament B) and was used to pull-down the COOH-terminal region of filamin B and filamin C (Ref. 12). We also obtained partial cDNA clones identical to the COOH-terminal region of filamin B and filamin C (Fig. 1B). The amino acid sequence alignment of the regions of the three isoforms of filamin that interacted with the cytoplasmic tail of PSMA encompassed their 22–24th repeat as shown in Fig. 1A. There was 70% identity at the amino acid level among the different filamin isoforms in this region.

PSMA Binds to Filamin in Vitro and in Vivo. In vitro GST pull-down assay was carried out to determine whether PSMA interacted directly with filamin A. As shown in Fig. 1C the interaction between PSMA and filamin was readily detected. Coimmunoprecipitation analysis was performed to determine whether filamin and PSMA associate in vivo. PC3-PSMA cells were lysed in an immunoprecipitation buffer, and PSMA was immunoprecipitated by mAb J591. Coimmunoprecipitating filamin was detected by antifilamin antibody. In agreement with the two-hybrid and bacterial fusion protein analyses, filamin was coimmunoprecipitated in vivo from prostate epithelial cells (Fig. 1D).

Colocalization of PSMA and Filamin in PC3-PSMA Cells. Confoocal microscopy revealed that PSMA colocalized with filamin at the plasma membrane region (Fig. 2, A–C) indicating that PSMA and filamin associate at the plasma membrane. In addition to its plasma membrane localization, PSMA also exhibited a distinct spot-like staining at the perinuclear region (Fig. 2B, arrowhead), a staining pattern similar to that of the recycling endosome (13). Therefore, to examine whether PSMA is localized to the recycling endosome, we tested whether the PSMA codistributes with a recycling endosomal marker (internalized transferrin). The internalized transferrin clearly colocalized with PSMA in the perinuclear region (Fig. 2, D–F), indicating that PSMA is localized to the REC in PC3-PSMA cells.
Next, we tested whether PSMA-filamin association is involved in the localization of PSMA to the REC. Because PC3 cells contain filamin, we used M2 cells, a cell line derived from human malignant melanoma, which is negative for filamin expression, and A7 cells, a subline generated by stable transfection of M2 with filamin cDNA. These cells do not express endogenous PSMA, and, therefore, PSMA was ectopically expressed in these cell lines. Strikingly, in filamin-negative M2 cells, PSMA showed vesicular staining throughout the cytoplasm (Fig. 2G), whereas in A7 cells, a distinct spot-like staining (Fig. 2H), as seen in PC3-PSMA cells (compare Fig. 2, B and H) was observed. These results indicate that filamin is involved in the localization of PSMA to the REC.

Decreased Internalization of PSMA in Filamin-positive Cells. Localization of PSMA to the REC in filamin-positive A7 cells indicated that PSMA filamin association might affect internalization of PSMA. Therefore, we tested the rate of internalization of PSMA in M2 and A7 cells using a cell surface biotinylation assay. M2 cells showed ~2-fold increase in the internalization of PSMA (Fig. 3A and B). Strikingly, at 60 min, 100% of the total surface PSMA was internalized in M2 cells. In A7 cells, at 60 min there was a slight decrease in the internalized PSMA as compared with the 30-min time point, which is possibly because of degradation. These results indicate that filamin might be involved in the modulation of internalization rate of PSMA.

Increased NAALADase Activity of PSMA in Filamin-deficient Cells. We then tested whether PSMA association with filamin is involved in the regulation of its NAALADase activity using PSMA expressing M2 and A7 cells. NAALADase activity was determined using [3H]NAAG as the substrate. The activity was normalized to the amount of cell surface PSMA determined by cell surface biotinylation assay. The NAALADase activity was 1.5-fold higher in M2 cells compared with A7 cells (Fig. 4) indicating that the filamin association decreases the enzymatic activity of PSMA.

DISCUSSION

In this study, we show that the first 19 NH2-terminal amino acids of PSMA associate with the 23rd-24th repeat of filamin A. This interaction between PSMA and filamin A found in the yeast two-hybrid analysis was confirmed using in vitro pull-down assays using GST-PSMA, and in vivo coimmunoprecipitation and colocalization experiments. We confirmed that PSMA is localized to the REC in filamin-positive cells. We also provided biochemical evidence that in the absence of filamin association, PSMA is internalized faster and exhibits increased NAALADase activity.

Filamin A is a dimeric actin cross-linking phosphoprotein located in the cortical cytoplasm adjacent to the plasma membrane. Parallel filamin dimers cross-link actin and facilitate the orthogonal branching of actin filaments, and serve as a docking site for various cell surface receptors and certain intracellular proteins involved in signal trans-
PSMA ASSOCIATION WITH FILAMIN

In this study, we have shown that PSMA association with filamin is necessary for its localization to the REC. In filamin-negative M2 cells, the internalized PSMA accumulated in vesicles, which were highly diffused throughout the cytoplasm. This diffused staining pattern changed into a bright spot like perinuclear staining (REC) in cells transfected with filamin A cDNA (A7 cells). REC is a collection of 50–70 nm diameter tubules containing recycling receptors and some ligands that are known to undergo recycling, and is generally found to localize near the microtubule-organizing center (14). Our results are consistent with a view that PSMA filamin association is involved in the transport of PSMA from the cell surface to the REC. However, we do not rule out the possibility that filamin might also be involved in the organization and maintenance of REC in mammalian cells. Additional experiments are necessary to unravel the role of filamin in targeting PSMA to REC and its possible involvement in the regulation of REC structure.

LNCaP cells, which express endogenous PSMA and filamin, did not show localization of PSMA to the REC, and PSMA did not coimmunoprecipitate with filamin.6 It is possible that PSMA association with filamin is modulated by intracellular signaling mechanisms. The PSMA cytoplasmic tail has three putative phosphorylation sites. It is known that filamin A undergoes phosphorylation in vivo, and this regulates its interaction with the actin cytoskeleton (15). Whether the phosphorylation status of filamin or PSMA is different in LNCaP compared with PC3 cells and whether this modulates PSMA localization to the REC remains to be studied.

PSMA association with filamin A decreased its rate of internalization by ~50%. This is in agreement with an earlier observation that endocytosis of furin, an endoprotease, was reduced 42% upon binding with filamin A (16). This may occur by the stabilization of these molecules on the membrane by linking to the actin cytoskeleton via filamin A. Dissociation from filamin might facilitate the binding of adaptor proteins required for endocytosis. Liu et al. (16) suggested the existence of a competition between filamin A and adaptors binding to the same site of furin, thereby regulating the rate of internalization of furin. Our observations that mutation of specific cytoplasmic tail amino acid residues of PSMA resulted in the loss of its internalization5 and that these mutants interacted more strongly with filamin A6 support the above possibility.

In our study, we have shown that filamin binding reduced the NAALADase activity of PSMA by 64%. It is possible that the interaction between filamin A and the cytoplasmic tail of PSMA changes the conformation of the extracellular domain of PSMA, resulting in reduced substrate (NAAG) binding. The observation that the cytoplasmic tail of a protein regulates the conformation of its extracellular domain has been reported previously for calreticulin, a M60,000 intracellular calcium-binding protein. The cytoplasmic tail of calreticulin interacts with a highly conserved GFFKR motif present in all of the α-subunits of integrin cytoplasmic domains, and promotes integrin to maintain a high affinity state for ligand binding (17).

The significance of the internalization of PSMA in prostate epithelial cells is not known. Antibody-induced endocytosis, high similarity at amino acid level between transferrin receptor and PSMA, and the presence of a domain similar to the transferrin receptor dimerization domain at the extracellular region of PSMA strongly suggest that PSMA could be a membrane receptor for an unknown ligand. On binding to ligand, the PSMA-ligand complex could undergo internalization. At present, there is no clue whatsoever about the nature of the ligand. If PSMA transduces a cell growth advantage signal, then its proper down-regulation by endocytosis would be important to prevent transformation of normal cells into cancerous cells. This is observed in the case of epidermal growth factor receptor. An epidermal growth factor receptor mutant unable to undergo endocytosis has been identified in human cancers (18). Endocytosis of PSMA might also be important for its role in the metastasis of prostate cancer. In this case, an increased internalization of PSMA could promote the invasive property of prostate cancer cells. It is reported that cytoplasmic tail-dependent internalization of membrane-type I matrix metalloproteinase was important for its invasion-promoting activity (19). We are currently carrying out experiments to delineate the potential importance of endocytosis of PSMA in prostate cancer.

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