Prostate-Specific Membrane Antigen and Its Truncated Form PSM′

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BACKGROUND. Prostate specific membrane antigen (PSMA) is a type II transmembrane protein overexpressed in prostate cancer as well as in the neovasculature of several non-prostatic solid tumors. In addition to full-length PSMA, several splice variants exist in prostatic tissue. Notably, the N-terminally truncated PSMA variant, termed PSM′, is prevalent in healthy prostate, and the ratio of PSMA/PSM′ mRNA has been shown to correlate with cancer progression. The widely accepted hypothesis is that the PSM′ protein is a translation product arising from the alternatively spliced PSM′ mRNA.
METHODS. Differential ultracentrifugation, cell surface biotinylation, Western blotting, and enzyme activity measurement were used to study the origin and localization of the PSMA/PSM′ variants in prostatic (LNCaP; lymph-node carcinoma of the prostate) and non-prostatic (HEK293) cell lines. These experiments were further complemented by analysis of the *N*-glycosylation patterns of the PSMA/PSM′ proteins and by site-directed mutagenesis.
RESULTS. We identified PSM′ protein expression in both the LNCaP cell line and a non-cancerous HEK293 human cell line transfected with a plasmid encoding full-length PSMA. Differential centrifugation revealed that PSM′ is localized predominantly to the cytosol of both these cell lines and is proteolytically active. Furthermore, the PSM′ protein is *N*-glycosylated by a mixture of high-mannose and complex type oligosaccharides and therefore trafficked beyond the *cis*-Golgi compartment.
CONCLUSIONS. Our data suggest that the PSM′ protein is likely not generated by alternative splicing of the PSMA gene but by different mechanism, probably via an endoproteolytic cleavage of the full-length PSMA. *Prostate 69: 471–479, 2009.* © 2008 Wiley-Liss, Inc.

KEY WORDS: **prostate specific membrane antigen; glutamate carboxypeptidase II; prostate cancer**

Abbreviations: Endo H_f, recombinant protein fusion of Endoglycosidase H and maltose binding protein; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK 1-750, human embryonic kidney cell line transfected with plasmid encoding PSMA (amino acids 1-750); Hsp, heat shock protein; IMDM, Iscove's modified Dulbecco's medium; IP, immunoprecipitation; LNCaP, lymph-node carcinoma of the prostate cell line; LAMP, lysosome associated membrane protein; mAb, monoclonal antibody; NAAG, N-acetyl-L-aspartyl-L-glutamate; NA-HRP, neutravidin-horseradish peroxidase; PBS, phosphate buffered saline; PNGase F, peptide N-glycosidase F; PSM′, N-terminally truncated form of PSMA; PSMA, prostate specific membrane antigen.

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INTRODUCTION

Prostate-specific membrane antigen (PSMA) is a 100 kDa type II membrane glycoprotein expressed in all types of prostatic tissues, including normal epithelial cells, benign prostatic hyperplasia, prostatic intraepithelial neoplasia, and cancerous tissue [1,2]. It is recognized as a novel prostate cancer marker; moreover, it is studied intensively as a possible therapeutic target [1,3]. In the prostate tissues, several alternatively spliced variants of PSMA have been identified, namely PSM′, PSM-C, PSM-D, and PSM-E [4–6].

In PSM′, bases 114–379 of the genomic PSMA sequence are absent due to alternative splicing [4,5]. PSM-C has the same splice donor site as PSM′ (nucleotide 114) but an alternative acceptor site is located in intron one of PSMA (the mRNA contains an additional 133 nucleotides in comparison to PSM′). PSM-D also has the same splice donor site as PSM′ and a unique acceptor site within intron one. It includes a novel exon [4]. PSM-E mRNA contains a unique exon (97 nucleotides), which is inserted between nucleotides 379 and 380 of PSMA cDNA. Furthermore, PSM-E lacks a 93-nucleotide region, nucleotides 2,232–2,324 of PSMA [6]. The protein products that may result from translation of these mRNA splice variants are summarized in Figure 1.

It has been shown that PSM′ mRNA is predominantly expressed in normal prostate. The ratio of PSMA/PSM′ mRNA levels can be used as a tumor index, which increases during prostate cancer (with increasing Gleason score) and can therefore be a useful indicator for diagnosis of prostate cancer and tumor progression [4,5]. PSM-C expression remains unchanged in normal prostate, primary prostate tumor, and metastasis; PSM-D is mainly expressed in metastasis [4]; and PSM-E mRNA expression levels vary significantly in normal prostate, benign prostatic hyperplasia, and prostate cancer [6].

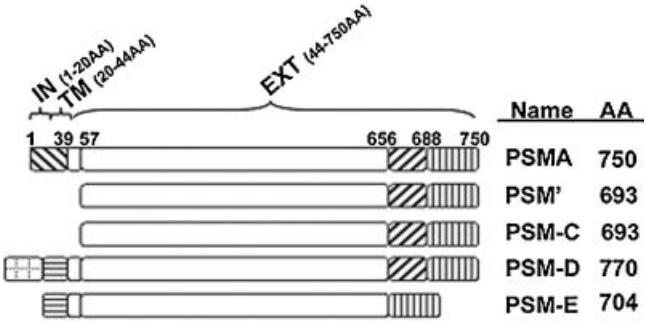

Fig. 1. Proteins and hypothetical proteins translated from different splice variants of PSMA mRNA. Patterned boxes illustrate the differences in amino acid sequence between the translated protein variants. AA, amino acids; IN, intracellular domain; TM, transmembrane domain; EXT, extracellular domain.

PSM′ is the most frequently described alternatively spliced variant of PSMA. Its cDNA lacks nucleotides at the 5′ end [4,5], which encode the intracellular and transmembrane domain in PSMA (Fig. 1). PSM′ was identified in LNCaP cells as a 95 kDa cytosolic protein with the amino acid sequence starting at Ala60 of the full-length PSMA sequence [7,8]. The cytosolic fractions of LNCaP cells and prostate tissues have been analyzed, and PSM′ has been reported to have an equivalent enzymatic activity to PSMA [8–10].

All studies performed on PSM′ have assumed that the protein arises from alternatively spliced mRNA. However, at the same time, data have been presented that PSMA is *N*-glycosylated [11–13] and that *N*-glycosylation is indispensable for its enzymatic activity. This seems contradictory in the case of PSM′, since cytosolic proteins are generally not *N*-glycosylated. Therefore, in this report we set out to analyze the glycosylation, intracellular localization, and possible origin of PSM′.

MATERIALS AND METHODS

Expression Plasmids

pcDNA4/PSMA. The DNA fragment encoding PSMA (amino acids 1–750) in the plasmid pMTNA 1–750 [14] was excised by digestion with *Kpn*I and *Xho*I restriction endonucleases (New England Biolabs, Ipswich, MA). The 2,258-bp fragment was ligated into the pcDNA4/V5-HisA vector (Invitrogen, San Diego, CA) via *Kpn*I/*Xho*I sites to generate the expression plasmid pcDNA4/PSMA.

pcDNA4/PSMA L4A/L5A. The pcDNA4/PSMA plasmid was used as a template, and mutations in the internalization signal (two leucines in the intracellular domain of PSMA were changed into alanines) were introduced by two complementary oligonucleotide primers harboring the desired mutation. The following primers were used: FNAL4A/L5A 5′-CAAAGATGTGGAATGCCGCTCACGAAACCGACTCGG-3′ and RNAL4A/L5A 5′-CCGAGTCGGTTTCGTGAGCGGCATTCCACATCTTTGG-3′. The presence of individual mutations was verified by dideoxynucleotide-terminated sequencing.

Transfection and Stable Cell Line Generation

Stable cell lines expressing individual proteins were generated by transfection with the expression plasmids using Lipofectamine 2000 (Invitrogen). Selection of transfected cells was achieved by culturing in IMDM (Sigma, St Louis, MO) medium supplemented with 10% fetal bovine serum (Invitrogen) and 300 μg/ml of

Zeocine (Invitrogen). Plates were inspected for colonies 3 weeks post-transfection, and healthy colonies were picked using cloning cylinders. The zeocine-resistant clones were analyzed for expression of the desired protein.

Antibodies

The following antibodies recognizing cell markers were used: anti-human CD107a (LAMP-1) (1:250, BioLegend, San Diego); GAPDH antibody (1:10,000, Acris, Hiddenhausen, Germany); Hsp70 (3A3) (1:250, Santa Cruz Biotechnology, Santa Cruz).

Monoclonal Antibodies Against PSMA/PSM′

Three different monoclonal antibodies (mAbs) designated GCP-04 [12], 7E11 [15], and GCP-05 were used in this study. GCP-04 mAb recognizes an extracellular epitope (amino acids 100–104 [16]) of denatured PSMA or PSM′. The epitope of 7E11 mAb was mapped to the N-terminus of the protein, and 7E11 mAb recognizes both native and denatured PSMA but not PSM′. The epitope of GCP-05 mAb is located in the extracellular part of the protein (amino acids 44–750), and GCP-05 mAb recognizes only native PSMA/PSM′. Hybridomas secreting GCP-05 mAb were prepared by standard methods from mice (F1 hybrids of BALB/c and B10.A strains) immunized with recombinant human PSMA as previously described [11].

Western Blotting

Proteins were resolved by 10% SDS–PAGE and electroblotted onto a nitrocellulose membrane. The membrane was probed with GCP-04 mAb (1 mg/ml, 1:5,000 dilution [12]) overnight at room temperature, followed by incubation with a goat anti-mouse horseradish peroxidase conjugated secondary antibody (1:25,000; Pierce, Rockford, IL) for 1 hr. The blot was developed using SuperSignal West Dura Chemiluminiscence Substrate (Pierce).

Sequential Immunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 2% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0) with protease inhibitor cocktail (Complete Mini, EDTA-free; Roche, Indianapolis, IN) added for 30 min at 4°C. The cell lysate was pre-cleared with Protein G Sepharose (GE Healthcare Bio-Science, Uppsala, Sweden) and incubated with 7E11 mAb (3 μg) for 2 hr at 4°C. The 7E11 mAb-GCPII complex was incubated with Protein G Sepharose (GE Healthcare Bio-Science) for 1 hr at 4°C. The flow-through fraction was mixed with the GCP-05 mAb (3 μg) overnight at 4°C. The GCP-05 mAb-GCPII complex was further incubated with Protein G Sepharose (GE Healthcare Bio-Science) for 4 hr. Both resins were washed with RIPA buffer, and proteins were eluted with 8 M guanidinium hydrochloride. The eluted proteins were precipitated by addition of 5% trichloroacetic acid and centrifuged. The pellet was dissolved in 1× SDS–PAGE sample buffer.

Endoglycosidase Digestion

Immunoprecipitated proteins and cell lysates were treated with 100 U of PNGase F or 400 U of Endo H_f (New England Biolabs) overnight at 37°C in a total volume of 20 μl.

Differential Centrifugation

LNCaP and HEK 1–750 cells were harvested in 20 mM Tris, 130 mM NaCl, pH 7.4, with protease inhibitor cocktail (Roche) added, frozen and thawed three times, and homogenized with 50 up and down strokes in a glass homogenizer. The homogenate was centrifuged at 600*g* for 5 min (the pellet contained whole cells, large debris, and nuclei). The supernatant was then centrifuged at 3,000*g* for 20 min to spin down mostly mitochondria (and some lysosomes). Another centrifugation was performed at 10,000*g* for 20 min (pelleting lysosomes, peroxisomes), followed by centrifugation at 100,000*g* for 1 hr (pelleting the microsomal fraction) and a final centrifugation at 300,000*g* for 3 hr (pelleting macromolecules/free ribosomes). The remaining supernatant consisted of the soluble cytoplasmic fraction.

Radioenzymatic Assay

Each fraction from differential centrifugation was diluted by reaction buffer (50 mM Tris–HCl, 20 mM NaCl, pH 7.4) to a final volume of 180 μl and preincubated at 37°C for 3 min. The reaction was started by addition of 20 μl of radioactive NAAG (1 μM, 1850 Bq/20 μl, tritium label on the glutamate, PerkinElmer, Boston, MA). After 20 min, the reaction was stopped by the addition of 200 μl chilled 200 mM sodium phosphate (pH 7.4, Lachema, Brno, Czech Republic). Two hundred microliters of the mixture was separated in a glass column on AG 1-X8 Resin (BioRad, Munich, Germany), the cleaved glutamate was eluted with 2 ml of 1 M formic acid, mixed with scintillation cocktail (Rotiszint ECO Plus, Roth, Karlsruhe, Germany) and measured in a liquid scintillator (LS 6500, Beckman, Fullerton, CA).

Cell Surface Biotinylation

LNCaP cells were grown to 90% confluency (in 100 mm dishes), the medium was removed, and the

cells were washed with ice-cold PBS. Cells were treated with 0.5 mg water-soluble Sulfo-NHS-SS-Biotin (Pierce) dissolved in PBS for 30 min at 4°C and then washed with PBS. Biotinylation was stopped with 10 mM glycine (in PBS), and cells were transferred back to IMDM complete medium and incubated at 37°C for 5 hr to allow internalization of membrane proteins. The incubation was stopped by transferring the dishes to 4°C; biotins on cell surface proteins were stripped in a reducing solution (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10% fetal bovine serum in PBS) for 30 min at 4°C. Cells were washed thrice in PBS. PSMA/PSM′ proteins were immunoprecipitated, resolved by non-reducing SDS–PAGE and analyzed by Western blot using GCP-04 mAb (1:5,000) or a Neutravidin-horseradish peroxidase conjugate (1:2,500; Pierce) specific for biotin.

Internalization and Immunofluorescence

Cells were grown on cover slips in IMDM (Sigma) medium supplemented with 10% fetal bovine serum (Invitrogen) until 70% confluency. Primary mouse monoclonal antibody GCP-05 (5 μg) was added to the culture medium and cells were incubated in the presence of antibody at 37°C for 20 min. Cells were transferred to ice cold PBS, fixed by 2% formaldehyde (in PBS) for 10 min and permeabilized by 0.1% saponin (Sigma). Non-specific interactions were blocked by 10% FBS (Invitrogen) in PBS for 20 min and secondary antibody was added (anti mouse FITC, 1:200, Jackson ImmunoResearch Laboratiries, Baltimore Pike). The cells were washed in PBS after 1 hr incubation and fluorescence was studied by Olympus IX81 (Olympus C&S s.r.o., Praha, Czech Republic).

RESULTS

N-Glycosylation of PSM′

The expression of PSM′ (on the protein level) was studied in two different cell lines: LNCaP cells endogenously expressing PSMA and HEK293 cells transfected with a plasmid encoding full-length PSMA (amino acids 1–750; HEK 1–750). Two protein species of approximately 100 and 96 kDa were detected in the lysates of both cell lines, albeit in different ratios, using Western blot analysis. The lower molecular weight protein was the minor form in both cell lines studied (Fig. 2A).

The lower molecular weight protein (approx. 96 kDa) was identified as an N-terminally truncated PSMA (known as PSM′) using sequential immunoprecipitation by two specific antibodies, 7E11 and GCP-05, which recognize epitopes at the N-terminus and in the extracellular region of PSMA, respectively (Fig. 2B).

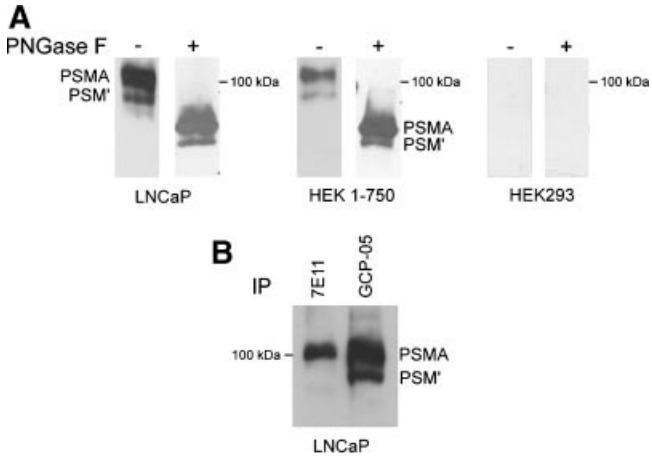

Fig. 2. Expression of PSMA/PSM′ proteins in two cell lines. Panel **A**: Lysates from LNCaP, HEK293 and HEK 1–750 cells (untreated or deglycosylated using PNGase F) were subjected to SDS–PAGE, electroblotted onto a nitrocellulose membrane, and probed using the GCP-04 mAb. Panel **B**: Sequential immunoprecipitation of PSMA from LNCaP cells with 7E11 mAb (against the N-terminus), followed by immunoprecipitation with GCP-05 mAb (against the extracellular domain). Immunoprecipitated proteins were separated by SDS–PAGE, electroblotted onto a nitrocellulose membrane, and probed with GCP-04 mAb.

Note that GCP-05 mAb pulls down both forms of the protein in this experiment. The identity of PSM′ was further confirmed by N-terminal sequencing and mass spectroscopy of the protein immunoprecipitated from LNCaP and HEK 1–750 cells. The N-terminal sequence was determined to be Ala-Phe-Leu-Asp-Glu, which corresponds to residues 60–64 of PSMA (data not shown).

The nature and extent of *N*-glycosylation of PSMA/PSM′ in LNCaP and HEK 1–750 cells was studied using PNGase F and endoglycosidase H_f (Endo H_f), two *N*-glycosidases with differing specificities (Fig. 3). While PNGase F removes N-linked oligosaccharides indiscriminately, Endo H_f cleaves within the chitobiose core of high-mannose and some hybrid-oligosaccharides from N-linked glycoproteins. Our data show that both PSMA and PSM′ are sensitive to PNGase F treatment and therefore both forms are *N*-glycosylated (Figs. 2A and 3). Furthermore, Endo H_f treatment also results in a partial shift of sample mobility (Fig. 3A,B). These data suggest that the proteins are posttranslationally modified by a mixture of high-mannose (or hybrid) and complex type oligosaccharides and are therefore trafficked beyond the *cis*-Golgi compartment.

Subcellular Localization of PSM′

Differential centrifugation analysis revealed that PSMA is localized in mitochondrial, lysosomal, and

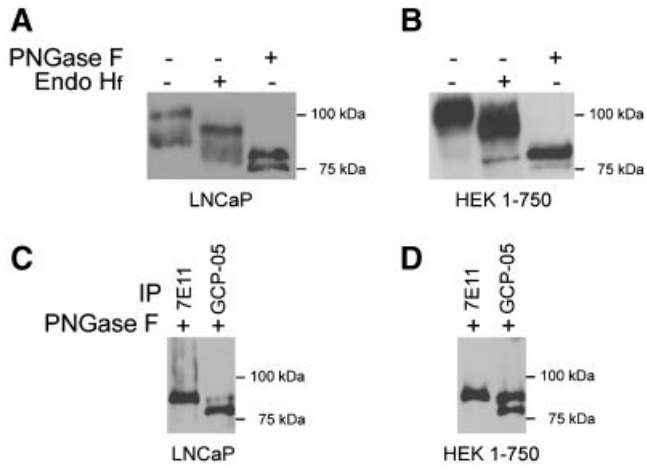

Fig. 3. Effect of endoglycosidases on PSMA/PSM′ proteins. Lysates from LNCaP (Panel **A**) and HEK 1–750 cells (Panel **B**) were either untreated or deglycosylated using PNGase F or Endo H_f. Protein samples were resolved by SDS–PAGE, electroblotted onto a nitrocellulose membrane, and probed with GCP-04 mAb. Panels **C**,**D**: PSMA/PSM′ from LNCaP (Panel C) and HEK 1–750 (Panel D) lysates were sequentially immunoprecipitated using monoclonal antibodies 7E11 and GCP-05, and proteins were subsequently deglycosylated with PNGase F. Samples were resolved by SDS–PAGE, electroblotted onto a nitrocellulose membrane, and probed with GCP-04 mAb.

microsomal fractions of the mammalian cells, while PSM′ is predominant in the macromolecule fraction (sometimes referred to as free ribosomes) and especially in the cytosol of both LNCaP and HEK 1–750 cells (Fig. 4, panels A,B,E). The identity of individual fractions was confirmed by immunochemical detection of specific protein markers (lysosome associated membrane protein-1, glyceraldehyde-3-phosphate dehydrogenase, heat-shock protein 70). The fractions containing the highest amounts of PSM′ were treated with PNGase F, and a lower molecular weight protein product corresponding to deglycosylated PSM′ was observed (Fig. 4, panel B). Individual fractions obtained by differential centrifugation were subsequently analyzed for proteolytic activity using *N*-acetyl-L-aspartyl-L-glutamate (NAAG), a natural substrate for PSMA in the brain [10]. The activity measurements confirmed that PSM′ is also able to cleave NAAG (Fig. 4, panel C,D).

PSMA Trafficking

To test the hypothesis that PSM′ is a product of posttranslational processing of PSMA, cell surface biotinylation experiments were performed with LNCaP cells. The biotinylated cell surface PSMA protein was internalized into the cells, and lysates prepared from these cells were inspected for the presence of PSMA/PSM′ proteins by sequential immunoprecipitation and Western blotting (Fig. 5). Detection with GCP-04 mAb (specific for PSMA/PSM′; Fig. 5A) revealed the presence of PSMA (immunoprecipitated with 7E11 mAb) and PSM′ (immunoprecipitated with GCP-05 mAb). (Note that in this particular experiment, GCP-05 mAb used sequentially after 7E11 mAb pulls down PSM′ alone, as opposed to the experiment shown in Fig. 2B. We speculate that the results of sequential immunoprecipitations in individual experiments reflect the amounts and ratios of the PSMA/PSM′ proteins present in the cells, which might not be constant.) On the other hand, detection with NA-HRP (specific for any biotinylated protein) shows only PSMA, suggesting that no PSM′ was biotinylated and hence present on the cell membrane (Fig. 5B). This observation challenges the hypothesis that the truncated form PSM′ arises from processing of PSMA during endosomal trafficking. Identical results were obtained when a rabbit polyclonal antibody was used for the immunoprecipitation of cell lysates in the biotinylation experiments (data not shown).

A band of higher molecular weight was observed in experiments in which a non-reducing SDS sample buffer was used (Fig. 5, upper bands). Our experiments suggest that both PSMA and PSM′ form homodimers in non-reducing conditions. Cross-linking experiments with the purified extracellular part of PSMA in reducing and non-reducing conditions also revealed a band of higher molecular weight (data not shown), confirming that the enzyme is a homodimer as suggested by Schülke et al. [17].

To complement the cell surface biotinylation experiment, we analyzed the internalization of a PSMA variant with a mutation in the internalization signal. PSMA internalization is regulated by the MXXXL signal, and a mutation in this signal results in impaired internalization of PSMA into the cell [18]. We therefore transfected HEK293 cells with plasmid pcDNA4/PSMA L4A/L5A containing a specific mutation in the internalization signal (L4A/L5A) and analyzed the cells by immunochemistry and the Western blot (Fig. 6). The presence of PSM′ in the lysates of the cells transfected by the (L4A/L5A) mutant suggests that a defect in the internalization signal does not prevent the formation of PSM′.

DISCUSSION

In this study we investigated the N-terminally truncated form of PSMA at the protein level. PSM′ expression was observed in LNCaP cells, which is in agreement with previously reported data [7]. Surprisingly, it was also detected in HEK293 cells transfected with a plasmid encoding the full-length form, PSMA.

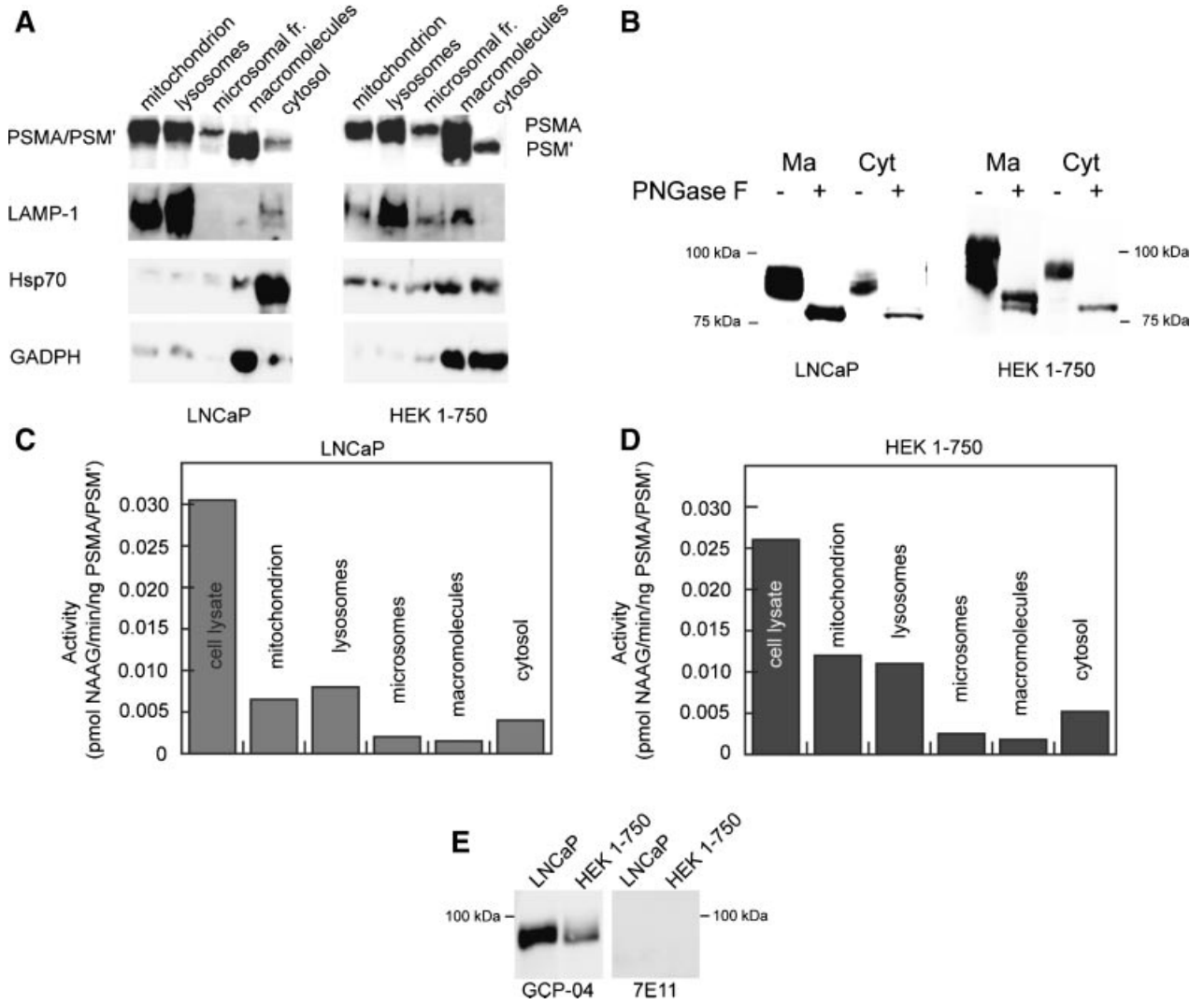

Fig. 4. PSM′ localization to cytosol. Lysates from LNCaP and HEK 1–750 cells were subjected to differential centrifugation and individual fractions were inspected for the presence of PSMA/PSM′ proteins (Western blotting using GCP-04 mAb, Panels **A**,**B**,**E**, and NAAG-hydrolyzing activity, Panels **C**,**D**). Panel A: Immunodetection of PSMA/PSM′ in different cellular fractions together with lysosomal (LAMP-1) and cytosolic (Hsp70, GAPDH) markers. Panel B: Immunodetection of untreated or PNGase F treated macromolecule (Ma) and cytosolic (Cyt) fractions. Panels C,D: Individual fractions from the differential centrifugation of LNCaP (Panel C) and HEK 1–750 (Panel D) lysates were assayed for NAAG-hydrolyzing activity using a radioenzymatic assay. The band intensities corresponding to amount of PSMA/PSM′ in individual fractions were recorded using a charge-coupled device camera and followed by analysis using ImageQuant software (version v2003, Amersham Biosciences, Uppsala, Sweden). Panel E: Immunodetection of PSMA/PSM′ in cytosol of LNCaP and HEK 1–750 cells by GCP-04 mAb and 7E11 mAb.

This observation suggests that PSM′ could be generated by a mechanism that is distinct from the generally accepted notion of an alternative splicing of the PSMA gene.

The hypothetical PSM′ protein arising from alternative splicing (or from alternative translation) would not contain the cytosolic and transmembrane domain and would therefore lack the signal sequence necessary to drive the newly synthesized protein into the endoplasmic reticulum and through the glycosylation process. Previous reports on PSM′ did not include analysis of glycosylation [7]. Our experiments using endoglycosidases with different specificities show that PSM′ is *N*-glycosylated and contains a mixture of high mannose or hybrid and complex types of oligosaccharides. Even though *O*-glycosylation of PSMA has been reported [19] it was not analyzed in this study. We focused solely on *N*-glycosylation, which is known to be indispensable for the enzymatic activity of PSMA [11–13].

The observation of complex type oligosaccharides in PSM′ indicates that the truncated protein is trafficked

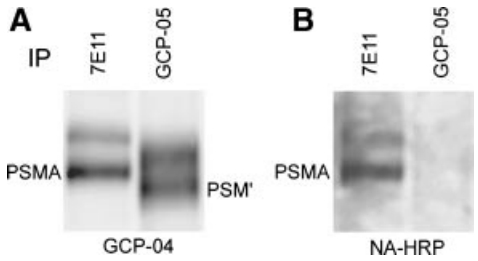

Fig. 5. Cell surface biotinylation. Cell surface proteins were biotinylated with Sulfo-NHS-SS-Biotin and then incubated 5 hr at 37°C to allow internalization of plasma membrane molecules. Biotin labels were stripped from proteins remaining at the cell surface, cells were lysed, and cell lysates were subjected to sequential immunoprecipitation using monoclonal antibodies 7E11 (recognizing the N-terminus) and GCP-05 (recognizing the extracellular domain). Proteins were resolved by non-reducing SDS–PAGE and electroblotted onto a nitrocellulose membrane. Panel **A**: The immunoblot probed with GCP-04 mAb. Panel **B**: The immunoblot probed with NeutrAvidin conjugated to horseradish peroxidase, specific for biotin.

not only into the ER but also to the Golgi apparatus. This process usually requires the presence of a signal peptide, but the signal sequence is deleted from mRNA encoding PSM′. In fact, we have previously shown that insect cells transfected with cDNA encoding PSM′ expressed a cytosolic protein that was not glycosylated and that was devoid of NAAG-hydrolyzing activity [11]. Furthermore, mammalian cells transfected with cDNA encoding PSM′ also express inactive, non-glycosylated protein (data not shown). The above-mentioned results are difficult to reconcile with the

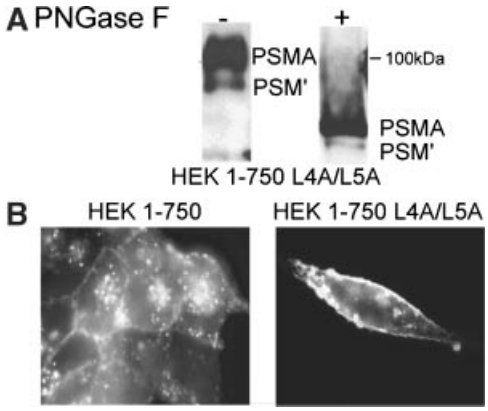

Fig. 6. Internalization in HEK 1–750 L4A/L5A. Panel **A**: Lysates from HEK 1–750 and HEK 1–750 L4A/L5A cells (untreated or deglycosylated using PNGase F) were resolved by SDS–PAGE, electroblotted onto a nitrocellulose membrane, and probed using the GCP-04 mAb (1:5,000). Panel **B**: Immunofluorescence was performed using GCP-05 mAb on HEK 1–750 and HEK 1–750 L4A/L5A cells. Antibody GCP-05 was added to cell culture and internalization was performed at 37°C for 20 min. Cells were permeabilized by saponin and secondary antibody was added (anti-mouse FITC, 1:200).

notion of PSM′ being a product of alternative translation or alternative splicing of the PSMA gene. Our data therefore suggest that the truncated form might rather be generated via post-translational endoproteolytic cleavage of full-length PSMA. A similar hypothesis was made, for example, for adaptor protein FE65, which is now known to be converted into an N-terminally truncated fragment through endoproteolysis [20].

Recently, Castelletti et al. reported the existence of two PSMA protein species of differing molecular weight in COS-1 and MDCK cell lines transfected with the full-length PSMA. The authors concluded that these species represent full-length PSMA protein modified by either mannose-rich (lower molecular weight) or complex types (higher molecular weight) of oligosaccharides [19,21]. However, the PSM′ protein (i.e., the lower molecular weight immunoreactive band identified in our study) is clearly distinct from the mannose-rich variant described earlier. It is interesting to note that Castelletti et al. did not observe any PSM′ protein in their studies and, conversely, we did not observe the mannose-rich PSMA in the study presented here. It is likely that these differences stem from differences in the experimental setups used in the two studies. For example, the relatively short metabolic labeling time (up to 6 hr) employed by Castelletti et al. might not be sufficient to generate detectable amounts of the PSM′ protein, while our 'steady state' approach favors higher abundance of the fully mature form of PSMA (i.e., bearing complex oligosaccharides) over its mannose-rich precursor.

The subcellular localization experiments show that PSMA is localized to mitochondria and lysosomes, which has been reported previously [22,23]. The localization of PSM′ to the cytosol as well as its NAAG-hydrolyzing activity have been reported several times [7–10]. However, since the *N*-glycosylation of PSMA is indispensable for the hydrolytic activity of the enzyme [11], it is difficult to imagine that the cytosolic protein not secreted via the ER would be proteolytically active. In this report we confirm that PSM′ is indeed localized to the cytosol and possesses hydrolytic activity. It is very rare to find N-linked glycoproteins existing in cytosol, yet there are some exceptions, such as a Na^+-pump alpha subunit with N-linked carbohydrates facing the cytoplasm [24]. Furthermore, Sato et al. [25] have identified 14 glycoproteins containing high mannose and/or hybrid type *N*-glycans in the cytosolic fraction during aging in the brain. PSM′ could be another such exception.

Even if we accept that PSM′ arises from the post-translational endoproteolytic cleavage of full-length PSMA, the question of where this process takes place remains unanswered. PSMA possesses a known

internalization signal [18] and can undergo constitutive and antibody-induced internalization into the cell [26], but our biotinylation experiments do not provide evidence that PSM′ originates from PSMA degradation during endocytosis and internalization. Moreover, the N-terminally truncated PSM′ protein is also observed in the HEK293 cell line expressing the PSMA mutant with impaired endocytosis/internalization. At the same time, however, we cannot fully exclude the possibility that the formation of detectable amounts of PSM′ is a very slow process that would require considerably longer experimental times than those that are achievable using biotinylation and/or pulse-chase experimental setups.

CONCLUSIONS

We provide evidence that a truncated form of PSMA protein, referred in the literature to as PSM′, is a proteolytically active *N*-glycoprotein localized predominantly to the cell cytosol. The mechanism by which this truncated protein is formed is unclear; we speculate that it might be generated by the endoproteolysis of the full-length PSMA along the secretory pathway, followed by translocation into the cytosol by an unknown mechanism.

A better understanding of PSMA processing and the origin of PSM′ might provide insight into *N*-glycosylation and trafficking of cytosolic proteins in general. Furthermore, a more detailed knowledge of PSMA biology could be beneficial for design and implementation of therapeutics targeting the PSMA system.

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