Molecular Cloning and Expression of an Alternative hKLK3 Transcript Coding for a Variant Protein of Prostate-specific Antigen

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Abstract

We report the molecular cloning of a full-length cDNA corresponding to a 2.1-kb hKLK3 mRNA. This mRNA results from the alternative splicing of intron 4, and its accumulation in prostatic LNCaP cells is stimulated by androgen. The cDNA encodes a prepro-prostate-specific antigen (PSA) variant containing 238 amino acids. The new protein, PSA-related protein 1 (PSA-RP1), differs from PSA at the COOH-terminal end and lacks the serine residue that is essential for catalytic activity. Prepro-PSA-RP1 was transiently expressed in COS1 cells fused to the V5 epitope of the paramyxovirus SV5. The recombinant fusion protein was detected in the spent medium by Western blot analysis using anti-V5 and anti-PSA antibodies. This indicates that PSA-RP1 is secreted and has PSA-like antigenic epitopes. A pro-PSA and a pro-PSA-RP1 having a mutated propeptide were overproduced in Escherichia coli fused to glutathione S-transferase. The recombinant PSA and PSA-RP1 were matured in vitro and identified by Western blot with molecular masses of 29 (PSA) and 27 (PSA-RP1) kDa. The data indicate that PSA-RP1, not complexed to serine protease inhibitors, could be present in biological fluids, thus contributing to the free PSA-immunoreactive fraction in serum.

Introduction

The hK family is composed of three related serine proteases encoded by the hKLK1, hKLK2, and hKLK3 genes. The hK1 pancreatic/renal glandular kallikrein is produced from hKLK1, mainly in the kidney, pancreas, and submandibular salivary glands. Both hK2 and hK3 are synthesized primarily in the prostate epithelium from the hKLK2 and hKLK3 genes (1). The major 1.6-kb mRNA from hKLK3 codes for a 261-amino acid prepro form of hK3. The mature, catalytically active form of hK3 is a glycoprotein containing 237 amino acid residues (Mt, 28,000), which has chymotrypsin-like substrate specificity (2). hK3, often called PSA, is responsible for proteolysis of semenogelins I and II (3), which are the major gel-forming proteins produced by the seminal vesicles. hK3/PSA is believed to be involved in semenogelation and expressed it in eukaryotic and prokaryotic cells. We have demonstrated that a secretory protein related to PSA is produced from this alternative hKLK3 mRNA. The new protein, designated PSA-RP1, has PSA-like antigenic epitopes and, thus, could cross-react with anti-PSA antibodies in clinical assays.

Materials and Methods

Cell Culture, Tissue, and RNA Isolation. The expression of hKLK3 mRNAs was measured in LNCaP cells (American Type Culture Collection, Manassas, VA) cultured in RPMI 1640 lacking phenol red and supplemented with 5% charcoal-stripped FCS (Eurobio, Les Ulis, France). Cells were grown in the absence and presence of the synthetic androgen R1881 (0.1 μM; NEN-DuPont, Les Ulis, France). The prostate tissues were obtained by transurethral prostatectomy in a patient with BPH. Total cellular RNA was isolated using the TriReagent method (Euromedex, Souffelweyersheim, France) following the manufacturer’s instructions. Poly(A) RNA was isolated using biotinylated oligo(dT) and streptavidin-coated paramagnetic particles (PolyAtract mRNA isolation system; Promega, Madison, WI).

RACE and DNA Sequencing. The hKLK3 cDNA clones were obtained by 3’ RACE using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). Marathon cDNAs were generated from 1 μg of LNCaP poly(A) RNA according to the manufacturer’s instructions. PCR amplification was done with a hKLK3-specific primer (K3-PCR1, 5’-CCAAAGTTACACCTGACC-3’), based on a sequence just downstream of the transcription initiation site of the hKLK3 gene. The RACE was performed in a Progen thermocycler from Thecne (Cambridge, United Kingdom) using K3-PCR1, the Marathon adaptor primer 1 (AP1), and the Expand Long Template PCR System (Boehringer Mannheim, Mannheim, Germany). The thermocycling protocol was: initial denaturation at 94°C for 2 min; 10 cycles of denaturation at 94°C for 10 s, annealing at 64°C for 30 s, and elongation at 68°C for 3 min and 30 s; 20 cycles of 94°C for 10 s, 64°C for 30 s, and 68°C for an initial duration of 3 min and 30 s and an automatic increment of 20 s at each cycle; and 1 cycle with an elongation step at 68°C for 17 min. Amplified products were cloned into a pGEM-T vector (Promega). DNA constructions were transformed into XL1-Blue MRF’ supercompetent cells (Stratagene, La Jolla, CA). DNA was sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster City, CA).

RNA Analysis. The size fractionation and Northern blotting of RNAs have been described previously (7). RNAs were prehybridized for 30 min at 68°C with the QuikHyb Hybridization solution (Stratagene) and hybridized overnight in this buffer at the same temperature. Washing was performed twice at 68°C for 30 min in 2 x SSC-0.1% SDS and twice for 20 min in 0.1 x SSC-0.1% SDS. The membrane was scanned using the Instant Imager (Packard Instrument Co., Meriden, CT) to estimate the extent of hybridization and then exposed to Kodak AR X-ray film at -70°C using intensifying screens for 1–7 days. A 32P-labeled probe was generated from a fragment of the cDNA by

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3 The abbreviations used are: hK, human kallikrein; PSA, prostate-specific antigen; PSA-RP1, PSA-related protein 1; BPH, benign prostatic hyperplasia; RACE, rapid amplification of cDNA ends; GST, glutathione S-transferase.

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primer extension. This fragment was obtained by PCR using the forward primer 5'-TGGGTCACTGTGGCAGCGTAAT-3' and reverse primer 5'-ACAGATACGGTTGGTCCAGTGGTT-3'. Labeling was carried out at 37°C for 2 h using 100 ng of denatured DNA, 30 pmol of reverse primer, and 2 units of Klenow fragment.

**Expression Vectors and Cell Transfection.** A 0.75-kb DNA fragment coding for the entire PSA-RP1 was PCR amplified using the Expand Long Template PCR System, the forward primer K3-PCR1, the reverse primer B (5'-GACACTTCTCCTCCAGGGCAC-3'), and the NH070707 clone as template. The PCR product was cloned into the pDNA3.1/V5-His-TOPO vector using the Eukaryotic TOPO TA Cloning Kit (Invitrogen, San Diego, CA). This vector permits the synthesis of the recombinant protein as a COOH-terminal fusion to the V5 epitope of the paramyxovirus SV5 and to a polyhistidine tag. A control vector containing the entire sequence encoding PSA was constructed using a similar approach. COS-1 cells were transfected with the expression vectors using Lipofectamine (Life Technologies SARL, Cergy Pontoise, France) according to the manufacturer's instructions. Cells and spent media were recovered 72 h after transfection. The recombinant proteins were purified using Talon Metal Affinity Resin (Clontech) according to the manufacturer's instructions.

**Construction of the GST/proPSA-RP1 Fusion Protein.** The Ser23 in the propeptide of PSA and PSA-RP1 was replaced by a proline residue using two oligonucleotides (5'-TGAGAAAAGAGGCAGGGATGAGGGGAGCTTG-3' and 5'-AACCCAGCCAGGATGAGGGGAGCTTG-3') as an adaptor segment. The adaptor sequence was designed to have a 5'-XhoI cohesive end and a 3'-BamHI cohesive end, and it contained the sequence coding the entire propeptide with the desired mutation. A plasmid containing the complete hK3/PSA cDNA (NH01040797) was used as template in a PCR performed with the K3-PCR1 primer, the K3-5700 reverse primer (5'-TGGGTCACTGTGGCAGCGTAAT-3') and the Expand Long Template PCR System. The K3-5700 primer matched nucleotides 118–140 downstream from the stop codon of the PSA cDNA and contained a mutation creating an EcoRI restriction site. The PCR product was cleaved with BamHI and EcoRI, ligated to the adaptor, inserted into plasmid pBluescript II SK+ (Stratagene) at XhoI and EcoRI sites, and propagated in Escherichia coli XL1 Blue (Stratagene). The insert was then excised and ligated into vector pGEX-5X-3 (Pharmacia Biotech Europe, Orsay, France) at NotI and SmaI sites. The resulting recombinant plasmid (SO04060398) contained the mutated prohK3/PSA cDNA sequence in the correct frame with GST as confirmed by sequencing. GST-proPSA-RP1 expression vector was constructed from the hKLK3 cDNA encoding hK3/PSA was isolated from the 3' RACE library. Comparison of the novel cDNA sequence (Fig. 1) with the hKLK3 genomic sequence revealed that the corresponding mRNA resulted from the use of an alternative acceptor site located in intron 4, upstream from the used site for the mRNA encoding hK3/PSA. The insert was ligated into vector pGEX-5X-3 (Pharmacia Biotech Europe, Orsay, France) at NotI and SmaI sites. The resulting recombinant plasmid (SO04060398) contained the mutated prohK3/PSA cDNA sequence in the correct frame with GST as confirmed by sequencing. GST-proPSA-RP1 expression vector was constructed from the plasmid SO04060398 by replacing the Saci-I K3–5700 fragment of hK3/PSA with the Saci-I K3-REV2 fragment of PSA-RP1. This fragment was generated by a Saci cleavage of a blunt-ended PCR product obtained using the primers K3-PCR1 and K3-REV2 (5'-ACAGATACGGTTGGTCCAGTGGTT-3'). The plasmid (SO20140598) containing the GST-proPSA-RP1 construct and the plasmid SO04060398 were used to transform E. coli BL21 (Pharmacia Biotech).

**Protein Analysis.** The proteins were characterized by SDS-PAGE on a 15% gel under reducing conditions. PSA from seminal plasma was used to immunize a rabbit using the standard procedure. Immunodetection by Western blotting was performed with the Amersham ECL Western blotting system (Amersham Life Sciences, Les Ulis, France) using a rabbit PSA antiserum or an anti-V5 monoclonal antibody (Invitrogen). The second antibody was either peroxidase-conjugated rabbit antiserum immunoglobulin or peroxidase-conjugated pig antirabbit immunoglobulins (Dako S.A., Trappes, France).

**Results**

A complete cDNA (clone NH070707) that was different from the cDNA encoding hK3/PSA was isolated from the 3' RACE library. Comparison of the novel cDNA sequence (Fig. 1) with the hKLK3 genomic sequence revealed that the corresponding mRNA resulted from the differential use of two polyadenylation splice sites. Results from the differential use of two polyadenylation splice sites. We investigated the relative abundance of the two alternative mRNAs (NH070707 and PA 525) in prostate cells by hybridizing total RNA from tissue or LNCaP cells to a specific probe. Two RNA species of ~2 kb (2.1 and 1.9 kb) were detected in adenomatous

![Fig. 1. Complete nucleotide and deduced amino acid sequences of the NH070707 cDNA. This cDNA obtained by 3' RACE-PCR using the forward K3-PCR1 primer consists of 1902 nucleotides encoding 238 amino acids. The positions of the BsmAI and Saci sites and the positions of the reverse primer B and the K3-REV2 primer used to construct the expression vectors are indicated. The putative poly(A) signal is in boldface type. The signal peptide (---) and the propeptide of PSA-RP1 are underlined, whereas the amino acid sequence different from PSA is double underlined.](image-url)
RNA induction was monitored by treating LNCaP cells with 0.1 nM of with a glyceraldehyde-3-phosphate dehydrogenase-specific probe. Cells. The intensity of the hybridization signals was determined as described in “Materials and Methods” and corrected for procedural errors following a rehybridization of the blot with a glyceraldehyde-3-phosphate dehydrogenase-specific probe. Columns, means of two experiments.

prostates (Fig. 2A). Because most mRNAs have a 200–250-residue poly(A) tail, these species probably correspond to the NH070707 (2.1-kb mRNA) and the PA525 cDNAs (1.9-kb mRNA). The relative abundance of the two species varied greatly from specimen to specimen; the 1.9-kb mRNA accounted for 10–60% of the transcripts detected. Using an exon 2 oligonucleotide probe (6), we estimated the level of expression of the 2.1-kb mRNA to be from 6 to 13% of that observed for the 1.6-kb mRNA encoding PSA. Only the 2.1-kb species was detected in LNCaP cells cultured in medium containing androgen (Fig. 2A). The time course of 2.1-kb mRNA steady-state RNA induction was monitored by treating LNCaP cells with 0.1 nM of the nonmetabolizable synthetic androgen R1881 for increasing periods of time. The amount of the 2.1-kb mRNA increased continuously after hormone stimulation (Fig. 2B) to reach 19-fold above control levels without R1881, after 11 days. The 1.9-kb mRNA species was undetectable at all times, whereas the 2.1-kb mRNA species accounted for ~5% of the hKLK3 transcripts at the end of the culture time.

The full-length cDNA encodes a 238-amino acid protein (Fig. 1). Comparison of the postulated-protein sequence and the prepro-hKLK3/PSA sequence revealed that only the COOH-terminal parts of the proteins differed. They should have the first 210 amino acids in common. The structural prediction suggests that the novel protein PSA-RP1 may be synthesized as a prepro protein because the signal peptide (amino acids 1–17) and the prosegment (residues 18–24) of hKLK3/PSA are unchanged. In contrast, the serine residue at position 189 in mature hKLK3/PSA is not found in PSA-RP1. This residue belongs to the catalytic triad of the hKLK3 serine protease, and it is essential for the proteolytic activity of this enzyme.

COS1 cells were transfected with a pcDNA3/PSA-RP1/V5-His expression vector to determine whether PSA-RP1 can be produced and secreted from mammalian cells. A parallel experiment was performed using a pcDNA3/PSA/V5-His construct. The spent media were collected 72 h after transfection, and the fusion proteins were purified by affinity chromatography. The recovered proteins were electrophoresed, electroblotted, and probed with an anti-V5 monoclonal antibody (Fig. 3). Like PSA/V5-His, PSA-RP1/V5-His was detected in spent media, indicating that mammalian cells can express and secrete PSA-RP1. We inferred from the great structural similarity of PSA-RP1 and PSA that PSA-RP1 could cross-react with antibodies raised against PSA. This was confirmed by the immunodetection of PSA-RP1/V5-His using an anti-PSA polyclonal antibody (Fig. 3).

To further characterize the variant protein, we produced pro-PSA and pro-PSA-RP1 as fusion proteins with GST in E. coli. We created a cleavage site for thrombin (10) at the end of the prosegment of pro-PSA and pro-PSA-RP1 to facilitate their recovery in a mature form. This was done by replacing the Ser23 (Fig. 1) with a proline residue. The recombinant fusion proteins were purified by affinity chromatography on glutathione-Sepharose; ~6 mg of purified protein were obtained from 1 liter of culture. The recovered fusion proteins contained two major bands (molecular masses of 55 and 47 kDa) that reacted positively with the hKLK3/PSA polyclonal antiserum by Western blotting (Fig. 4). The upper band probably corresponds to the complete fusion protein, whereas the lower band could be a truncated form due to a premature arrest of translation. The fusion proteins were incubated with thrombin and analyzed by reduced SDS-PAGE and Western blotting. The recombinant mature PSA was ~2 kDa smaller than the mature hKLK3/PSA purified from seminal plasma (Fig. 4). The absence of glycosylation in the recombinant product in contrast to the presence of one carbohydrate chain in hKLK3/PSA from seminal plasma contributes to this mass difference. A new major band (27 kDa) representing the backbone of mature PSA-RP1 under the conditions of the gels was observed in the recombinant product. This band was not present in mature PSA-RP1/V5-His but was present in the mature PSA-RP1/V5-His expressed in COS1 cells transfected with a pcDNA3/PSA-RP1/V5-His construct. This band was found to correspond to a truncated form of PSA-RP1 that was not detected in the mature PSA-RP1/V5-His expressed in COS1 cells transfected with a pcDNA3/PSA-RP1/V5-His construct.

Fig. 2. Alternative hKLK3 mRNAs in BPH specimens and in LNCaP cells. Twenty µg of total RNA from different prostate tissues or from LNCaP cells cultured with 0.1 nM R1881 were analyzed by Northern blot using a specific probe. A, autoradiogram showing amounts of alternative mRNAs in tissue and in LNCaP cells (induction with R1881 for 11 days). B, effect of R1881 on the steady-state level of the 2.1 alternative mRNA in LNCaP cells. The intensity of the hybridization signals was determined as described in “Materials and Methods” and corrected for procedural errors following a rehybridization of the blot with a glyceraldehyde-3-phosphate dehydrogenase-specific probe. Columns, means of two experiments.

Fig. 3. Immunodetection of PSA and PSA-RP1 fusion proteins in the spent media of COS1 cells. Prepro-PSA (Lanes 2) and prepro-PSA-RP1 (Lanes 3) were transiently expressed in COS1 cells as a COOH-terminal fusion to V5 epitope of the paramevirus SV5 and polyhistidine tag. They were purified by affinity chromatography, subjected to SDS-PAGE under reducing conditions on a 15% gel, and analyzed by Western blot using an anti-V5 monoclonal antibody (left) and an anti-PSA immunoserum (right). Lanes 1, PSA purified from seminal fluid. Numbers on the left, molecular mass markers.
signal peptide, and a proregion, it is reasonable to assume that PSA-activation as does the hK3/PSA mRNA, have the same 5'
observations suggest that hK3/PSA is activated after secretion, probably by the inactive zymogen to a catalytically active mature form. Recent
species in LNCaP cells.

mRNA by androgens is primarily due to a transcriptional induction. The increase in hK3/PSA changes are similar to those previously observed for the major 1.6-kb mRNA species encoding hK3/PSA (12). The increase in hK3/PSA

Discussion

We have isolated and characterized a full-length cDNA (NH070707) corresponding to a variant of the mRNA coding for hK3/PSA, the most important marker available today for diagnosing and monitoring patients with prostate cancer. Two RNA species (2.1 and 1.9 kb) were detected in adenomatous prostates using a specific probe elaborated from the variant cDNA. Only the 2.1-kb mRNA contained the AAUAAA motif, which is essential for 3' end formation of polyadenylated RNA. Because reports indicate that base changes in the AAUAAA sequence result in low yields of 3' ends (11), the absence of a recognizable AAUAAA element (no sequence differing by less than two nucleotides from the consensus) in the 1.9-kb mRNA suggests that its poly(A) site is weaker than that of the 2.1-kb mRNA. The distribution of the two alternative mRNAs in the prostate tissue samples varied considerably, suggesting that the inherent strength of the poly(A) signals is not the sole factor controlling the steady-state levels of these mRNAs. This is consistent with the lack of accumulated 1.9-kb mRNA in the LNCaP cells, although no mutation affects the sequences surrounding its poly(A) signal. Further investigations are required to determine whether our observations are due to a factor or factors acting on the processing or stability of the variant hKLK3 mRNAs.

Our studies on the LNCaP cell line showed that androgens increase the steady-state level of the 2.1-kb alternative mRNA in a time-dependent fashion. Both the amplitude and the time course of the changes are similar to those previously observed for the major 1.6-kb mRNA species encoding hK3/PSA (12). The increase in hK3/PSA mRNA by androgens is primarily due to a transcriptional induction. Posttranscriptional mechanisms also seem to be involved in the response because androgens stabilize the hK3/PSA mRNA (13). Our observations suggest that androgens have little or no differential effect on the processing or/and stability of the 1.6- and 2.1-kb mRNA species in LNCaP cells.

The serine proteases undergo cleavage of a pro region that converts the inactive zymogen to a catalytically active mature form. Recent reports suggest that hK3/PSA is activated after secretion, probably by the related kallikrein hK2 (8, 14). Because the alternative mRNAs have the same 5' sequences required for translation, secretion, and activation as does the hK3/PSA mRNA, i.e., a 5' leader sequence, a signal peptide, and a proregion, it is reasonable to assume that PSA-RP1 is synthesized in prostate cells, enters the secretory pathway, and successively undergoes cleavage of the signal peptide and of the pro region. This is supported by our data on the polysomal location of the 2.1-kb mRNA and the secretion of recombinant PSA-RP1/V5-His into the spent medium of transfected COS cells. In situ hybridization experiments showed that hKLK3 mRNAs are found exclusively in the secretory epithelial cells of the prostate (15). Thus, hK3/PSA and PSA-RP1 are probably both located in the cellular and extracellular compartments of the prostate.

hK3/PSA is mainly secreted into the lumen of the prostate ducts, but small amounts of PSA enter the blood. There are many forms of hK3/PSA in this compartment and in seminal fluid. These include complexes of PSA with protease inhibitors, such as α1-antichymotrypsin, protein C inhibitor, and α2-macroglobulin (2, 16). Because PSA-RP1 lacks the serine essential for the catalytic activity, it cannot interact with these inhibitors to form complexes. The extensive molar excess of α2-macroglobulin and α1-antichymotrypsin in serum (100–1000-fold) makes it most likely that any enzymatically active hK3/PSA is efficiently inactivated by these inhibitors. However, 10–30% of the PSA immunoreactivity in serum is due to uncomplexed molecules (free fraction; Refs. 17 and 18). Most investigators have suspected that this free fraction is hK3/PSA zymogen or PSA inactivated as a result of internal peptide bond cleavage. Recent attempts to isolate and characterize the uncomplexed molecules have given divergent results (19, 20), so the identity of the free forms remains questionable. PSA-RP1 has several features that are compatible with its distribution in the free immunoreactive fraction: PSA-RP1 is not a serine protease, and thus, it may remain uncomplexed in the serum; the apparent molecular mass of the mature recombinant PSA-RP1 is similar to the molecular mass of free recombinant PSA; and both proteins have common antigenic determinants, as demonstrated by Western blot analysis.

There are several independent studies using different immunological reagents and patient materials that have shown that the free:total PSA ratio is significantly lower in patients with prostatic carcinoma than in those with BPH (21). Measurement of the different serological forms of PSA improves the discrimination between benign and malignant prostate diseases. This discovery of PSA-RP1 now challenges the concept of “free PSA.” Because PSA-RP1 and PSA have the same NH2-terminal sequence, including the internal cleavage site, these proteins cannot be distinguished by NH2-terminal sequencing. Thus, a detailed biochemical and immunological characterization of PSA-RP1 is needed to clarify the molecular nature of the various PSA-immunoreactive forms in the serum. Antibodies that are specific for PSA-RP1 that do not cross-react with PSA could be most useful for showing whether the measurement of PSA-RP1 in serum can be used to improve the distinction between prostate cancer and BPH.

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