Human prostate-specific glandular kallikrein is expressed as an active and an inactive protein

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A polymorphism in the human prostate-specific glandular kallikrein (hKLK2) gene was described by direct sequencing (by PCR) of genomic DNAs isolated from prostatic cancer tissue, benign prostatic hyperplasia tissue, and blood leukocyte specimens. Results showed two forms of human prostate-specific glandular kallikrein protein (hK2), a consequence of a change from C to T at base 792 in the hK2 coding region. Producing the two forms as recombinant proteins in insect cells demonstrated that Arg226-hK2 (CC genotype) is an active protein and Trp 226-hK2 (TT genotype) is inactive. Polymorphism studies of 36 patients with prostatic diseases identified only 1 with the TT genotype. The same kind of polymorphism was not detected in the human prostate-specific antigen (hKLK3) gene. Arg 226-hK2 possessed only trypsin-like enzyme activity, whereas recombinant human prostate-specific antigen (hPSA) had only chymotrypsin-like activity. Monoclonal and polyclonal antibodies raised against hPSA purified from seminal plasma detected both active and inactive hK2. Thus, because inactive as well as stable hK2 protein may be present, a lack of trypsin-like activity in hPSA standards is not enough to confirm that the materials are free of hK2 contamination.

INDEXING TERMS: prostate-specific antigen • prostatic cancer • recombinant protein • allelic frequency • polymorphism • enzyme activity • purification of assay components

The human glandular kallikrein gene family comprises three different genes: prostate-specific antigen (hKLK3), prostate-specific glandular kallikrein (hKLK2), and pancreatic/renal kallikrein (hKLK1) genes [1]. All three kallikrein genes are located on chromosome 19, and hKLK3 and hKLK2 are aligned 12 kb apart [2–4]. The similarity of the coding region of the hKLK1 gene and that of the hKLK3 and hKLK2 genes is 74% and 75%, respectively. Moreover, the coding regions of hKLK3 and hKLK2 are 85% identical, and 91% of their promoter regions are the same [5, 6]. hKLK1 encodes tissue kallikrein, shows kininogenase activity, and is expressed in kidneys, pancreas, and salivary glands [7–9]. In situ hybridization has shown that hKLK3 and hKLK2 are expressed only in prostatic epithelial cells [10–14]. As tested in LNCaP cells, the presence of androgens has a clear up-regulation effect on the amounts of hKLK2 and hKLK3 mRNA [15].

Recently, the prostate-specificity of hKLK2 and hKLK3 gene expression has been questioned. The expression of all three human kallikrein genes in human endometrium has been detected by reverse transcription-PCR [16]. Human prostate-specific antigen (hPSA) has been detected in the milk of lactating women by hPSA immunoassay [17]. In addition, 30–40% of breast tumors and steroid hormone-stimulated normal breast tissue produce low amounts of hPSA [18–21].

The nucleotide sequence of the five coding exons of hKLK2 predicts the production of a 261-amino acid preproprotein that includes a signal peptide of 17 amino acids and an activation peptide of 7 amino acids, similar to the structure of hPSA [22]. Human prostate-specific glandular kallikrein (hK2) possess the catalytic triad (His11–Asp96–Ser189) typical of serine proteases; moreover, the presence of Asp183 in hK2 predicts a trypsin-like activity towards substrate, although the function of hK2 is unknown. In contrast, hPSA has a serine residue at this position, which apparently confers chymotrypsin-like activity [23, 24]. The function of hPSA is to cleave the

1 Nonstandard abbreviations: hKLK3, human prostate-specific antigen gene; hKLK2, human prostate-specific glandular kallikrein gene; hKLK1, human pancreatic/renal kallikrein gene; hK2, human prostate-specific glandular kallikrein; hPSA, human prostate-specific antigen; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and pNA, p-nitroanilide.
semenogelin clot; it also digests insulin-like growth factor (IGF)-binding protein-3 [25, 26].

Serum concentrations of hPSA are increased in patients with cancerous and benign hyperplastic prostatic tissues, and this protein is widely used as a tumor marker for detecting and monitoring prostatic cancer [27, 28]. Because of the high homology between hKLK3 and hKLK2, it may be difficult to purify the hK2 and the hPSA proteins separately [29]. Furthermore, the close similarity of these proteins could cause cross-reactivity between hK2 and hPSA as antigens [30]. The hK2 protein remains to be adequately purified, and the purity of hPSA is not always unambiguous. Problems of antigen purity could lead to complications in hPSA assays that are based on polyclonal or monoclonal antibodies raised against hPSA purified from seminal fluid. Here we report our production and characterization of two polymorphic forms of hK2 protein that are detectable by the monoclonal and polyclonal antibodies raised against hPSA purified from seminal plasma [28].

Materials and Methods
Cloning hK2 cDNAs. The hK2 coding sequence was amplified by PCR from the human prostatic cancer tissue cDNA library (Clontech, Palo Alto, CA). For PCR, the N-terminal oligomer, 5'-TCTGCAGGGGTTGGC-3', contained Smal and BglII restriction sites, whereas the C-terminal oligomer, 5'-CGCTCTAGATGCGGATCTTC-3', contained an XbaI restriction site in addition to the hK2 coding sequences. (Nucleotides that differ between hK2 and hPSA are shown in bold type, whereas underlining indicates identical nucleotides in the C-terminal oligomers.) The hK2 PCR product was cloned into PCRII-vector (Invitrogen, San Diego, CA), and both strands of the insert were sequenced [31]. This hK2 cDNA contained T nucleotide at position 792, which leads to the amino acid change from originally reported Arg226 [22] to Trp226 and suggests possible polymorphism in the hKLK2 gene.

Another hK2 cDNA was generated by using PCR with the N-terminal oligomer described above and a longer C-terminal oligomer, 5'-CCGGACCTCTAAGGGTTCGGCGGATCTTC-3', contained a XbaI restriction site in addition to the hK2 coding sequences. (Nucleotides that differ between hK2 and hPSA are shown in bold type, whereas underlining indicates identical nucleotides in the C-terminal oligomers.) This hK2 cDNA contained T nucleotide at position 792 (surrounded by asterisks), which codes forArg226 in hK2 protein. The sequence of this cDNA was also confirmed from both directions.

Detections of the Arg226/Trp226 polymorphism. Genomic DNA was isolated from prostatic tissue obtained by prostatectomy, biopsy, or transurethral resection and from blood leukocytes [32]. Female and young male blood leukocyte DNAs were used as a control material. The 5’ oligomer (5’-TTCTCAGGTGTCTCTCTCC-3’) and the biotin-labeled 3’ oligomer (5’-GTGGGACAGGGGCACTCA-3’) were used to amplify the hKLK2 gene region from nucleotide 4942 in intron 4 to nucleotide 5144 in exon 5. The PCR products were sequenced [33] by using the fluorescein amidite-labeled oligomer 5’-ATCATGGGG-CCCT-GAGCC-3’, which corresponds to the region of nucleotides 5023 to 5041 in exon 5. The products were electrophoresed under standard conditions with the Automated Laser Fluorescent DNA Sequencer (Pharmacia LKB Biotechnology, Uppsala, Sweden), as recommended by the manufacturer.

Construction of recombinant plasmid pVL1392 transfer vectors and generation of recombinant hK2 baculoviruses. The hK2 cDNAs were subcloned into the BglIII/XbaI site of the pVL1392 transfer vectors (Invitrogen). The pVL1392 transfer vectors containing either of the hK2 cDNA fragments, confirmed by sequencing, were cotransfected with linearized wild-type BaculoGold-DNA (Pharmingen, San Diego, CA) into Spodoptera frugiperda (Sf9) insect cells (CRL1711; ATCC, Rockville, MD) [34].

Production and purification of recombinant Arg226-hK2 and Trp226-hK2. The recombinant hK2 proteins were produced on a pilot scale with 2-L (Biosat MD) and 30-L (Biosat UD30) bioreactors (B. Braun Biotech International, Melsungen, Germany) [35]. The harvested medium from the recombinant virus infection was concentrated with a Pellicon cassette system (10-kDa cutoff; Millipore, Bedford, MA) and dialyzed into 50 mmol/L sodium acetate buffer (pH 5.5). The concentrate was loaded onto a 10 × 10 cm cation-exchange column (S-Sepharose HP; Pharmacia), the column was washed, and the recombinant hK2 protein was eluted from the column with a linear NaCl gradient (0–200 mmol/L). The fractions that were immunoreactive with polyclonal hPSA antibody were concentrated (with a PELICON concentrator from Amicon, Beverly, MA) for gel-filtration chromatography through a 2.6 × 80 cm column of Superdex 75 (Pharmacia), from which they were eluted with 50 mmol/L sodium acetate (pH 5.8) containing 150 mmol/L NaCl. The hK2-containing fractions were pooled and dialyzed in 50 mmol/L sodium acetate (pH 5.8) for cation-exchange chromatography through a 0.5 × 5.0 cm Mono-S HR column (Pharmacia). The recombinant hK2 protein was eluted from the column with a linear NaCl gradient (0–200 mmol/L). The S-Sepharose and the Superdex 75 columns were connected to a BioPilot automated chromatography system; the Mono-S column was used with the FPLC system (all from Pharmacia).

Characterization of recombinant Arg226-hK2 and Trp226-hK2. The purity and characterization of recombinant hK2 proteins were evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and nonreduc-
ing PAGE. Both types of electrophoresis were carried out on a PhastSystem (Pharmacia) with PhastGel gradient media 10–15 for SDS-PAGE and 8–25 for nonreducing PAGE, and the products were silver-stained [36–40]. For Western blotting, the proteins were transferred onto nitrocellulose membranes with PhastTransfer (Pharmacia) [41]. Rabbit polyclonal antibody raised against hPSA purified from seminal fluid [28] was used with a ProtoBlot AP system (Promega, Madison, WI) to detect the expressed protein. In both electrophoresis procedures, the recombinant hK2 proteins were always compared with commercial hPSA purified from seminal fluid (Calbiochem, La Jolla, CA).

To determine the nature of the isolated recombinant hK2, we assessed its ability to hydrolyze Pro-Phe-Arg-pnitroanilide (pNA) (which would demonstrate trypsin-like activity) and Arg-Pro-Tyr-pNA (for chymotrypsin-like activity), both from Chromogenix (Möln达尔, Sweden), at a final concentration of 1 mmol/L. The reactions, performed at 37 °C, were initiated by adding 50 μL of the chromogenic substrate to 200 μL of 50 mmol/L sodium phosphate buffer (pH 7.8) containing 100 mmol/L NaCl and hK2. After 1 h, the reaction was stopped by adding 800 μL of 0.6 mol/L acetic acid, and the absorbance of the reaction mixture was measured at 405 nm [42]. The reaction rate, nanomoles of pNA formed per minute, was calculated from the calibration curve for pNA. The same reactions were also carried out with recombinant hPSA [43].

**Results**

**DETECTION OF THE ARG<sup>226</sup>/TRP<sup>226</sup> POLYMORPHISM**

The coding region of the hK2 cDNA was amplified from the human prostatic cancer tissue cDNA library by PCR. The hK2 cDNA sequence from this prostatic cancer tissue library had one base different from that reported earlier [22]. The difference at base 792 (C vs T) results in production of different amino acids at residue 226, Arg<sup>226</sup> and Trp<sup>226</sup>, respectively. Sequenced DNA samples from both tissue and leukocyte specimens from 36 patients with prostatic diseases revealed a polymorphism at this base position (Table 1 and Fig. 1). In this limited specimen material, 13 of 24 patients with prostatic cancer were heterozygotes (CT), 10 were homozygotes (CC), and 1 was a homozygote (TT). Genotypes of sequenced specimens from the other groups are summarized in Table 1, as are the frequencies of the Arg<sup>226</sup> and Trp<sup>226</sup> alleles. When the respective region of the hKLK3 gene was sequenced from the same genomic DNA specimens, only CC homozygotes were detected at base position 792.

**Figure 1. Detection of polymorphism at base 792 in hKLK2 gene.**

Genomic sequences from three DNA specimens showing homozygous CC (top chart), heterozygous CT (middle chart), and homozygous TT forms (bottom chart).

**Table 1. Genotypes at nucleotide position 792 in hK2 and frequency of Arg<sup>226</sup> and Trp<sup>226</sup> alleles.**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>No. of specimens</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Allele frequency, no. (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostatic cancer</td>
<td>24</td>
<td>10</td>
<td>13</td>
<td>1</td>
<td>33 (69)</td>
<td>15 (31)</td>
</tr>
<tr>
<td>Benign prostatic hyperplasia</td>
<td>12</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>16 (67)</td>
<td>8 (33)</td>
</tr>
<tr>
<td>Female blood leukocyte</td>
<td>10</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>21 (66)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11 (34)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Young male blood leukocyte</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> CC, homozygous Arg<sup>226</sup>-hK2; CT, heterozygous; TT, homozygous Trp<sup>226</sup>-hK2.

<sup>a</sup> In n = 16 controls (female and young male leukocytes combined).
which moved farther than the broad band for Trp226-hK2. When digested with N- and O-glycosidase enzymes, neither hK2 form showed a detectable change in molecular mass (data not shown).

Activity measurements showed that Arg226-hK2 had trypsin-like but not chymotrypsin-like activity, its specific activity (per milligram) being 320.6 nmol/min when measured with the Pro-Phe-Arg-pNA synthetic peptide. Trp226-hK2, however, had no detectable trypsin- or chymotrypsin-like activity. The recombinant hPSA had chymotrypsin-like but not trypsin-like activity [43].

Discussion
We have identified polymorphism in the hKLK2 gene. A nucleotide change from C to T at a base 792 in hK2 cDNA leads to two different hK2 proteins, active Arg226-hK2 and inactive Trp226-hK2. The frequency of the Arg226-allele is about twice that of the Trp226-allele among 36 patients with prostatic diseases. The same kind of polymorphism was not detectable in the hKLK3 gene.

To obtain hK2 free of hPSA contamination, we produced the hK2 as a recombinant protein, using a baculovirus expression vector system in insect cells. The inactive recombinant Trp226-hK2 was stable, but the active recombinant Arg226-hK2 protein seemed to be labile, which might account for its low production yields. hK2 (CC genotype) has been previously expressed as an inactive prepropolypeptide in an Escherichia coli system [30]. SDS-PAGE run under reducing conditions showed that the molecular masses for both of the expressed, nonglycosylated recombinant hK2 proteins was 33 kDa. The molecular mass of natural, glycosylated hK2 proteins is probably closer to that of hPSA, 34 kDa.

The amino acid change from Arg226 to Trp226 affects the net charge of the protein and could account for the difference in mobility of the hK2 forms in nonreducing PAGE. Arg226-hK2 showed two bands, possibly from oligomerization of the protein. Both forms of hK2 were less heterogeneous in nonreducing PAGE than was commercial hPSA purified from seminal fluid. The Arg226-hK2 had only trypsin-like activity when synthetic peptide substrates were used, differing, as expected, from the chymotrypsin-like activity of hPSA. The heterogeneity seen in commercial hPSA purified from seminal fluid possibly results from endoproteolytic cleavage of the protein into two or four polypeptide chains that are held together by disulfide bridges [24]. The recent results [29] obtained by mass spectroscopy indicate molecular mass differences between hPSA isoforms. Some of these differences are probably the results of differences in glycosylation or the sialic acid content of hPSA [44].

Protease inhibitors and internal peptide bond cleavages are known mechanisms for inactivating hPSA. For example, of the hPSA isolated from seminal fluid, 30% does not have any catalytic activity because of the internal peptide bond cleavage between Lys145 and Lys146 [45]. Possible mechanisms for inactivation of hK2 are not known. The Arg226 in hK2 is located in an area that is highly similar between the hK2 and hPSA proteins, but the inactivating mutation to Trp226 was not found in hPSA.

The existence of inactive and stabile hK2 enzyme makes questionable the purity of currently used hPSA standards. Previously, aprotinin–Sepharose affinity chromatography, based on the trypsin-like activity of hK2, has been used to remove contaminating hK2 from PSA samples [14]. The lack of trypsin-like activity and confirmation of the N-terminal sequence of hPSA as described in a recently published method for rapid hPSA purification [46] are not sufficient to guarantee the absence of inactive hK2 contamination, because the first 16 N-terminal amino acids of hK2 and hPSA are identical [23, 24]. Moreover, aprotinin affinity chromatography was not able to remove the trypsin-like activity of hPSA purified from seminal fluid [47]. Unresolved is whether hPSA was contaminated with Arg226-hK2 or whether Trp226-hK2 can be self-activated. Recombinant protein technology is probably the only sure way to get pure hPSA for standardizing hPSA assays [43].

Fig. 2. Silver-stained polyacrylamide gel electrophoreses and immunoblot analyses of recombinant hK2 proteins.
Top panels: Pure recombinant Trp226-hK2 protein (lane 1) and commercial hPSA (lane 2) were silver-stained in native (A) and reduced SDS-PAGE (B) analyses. Lower panels: Rabbit polyclonal antibody raised against hPSA purified from seminal fluid was used to detect recombinant Arg226-hK2 (lane 1), recombinant Trp226-hK2 (lane 2), and commercial hPSA (lane 3) on immunoblotted native PAGE (C) and reduced SDS-PAGE (D).
In closing, we note that the purified recombinant hK2 proteins have been used as antigens for production of monoclonal antibodies to be used in further studies of the function and expression of active and inactive hK2 proteins and of the relations of these proteins to prostatic diseases. We also plan to screen the commercially available hPSA assays for possible cross-reactivity with active and inactive hK2 proteins.

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