Kallikrein 4 is a Predominantly Nuclear Protein and Is Overexpressed in Prostate Cancer

Zhijun Xi, Tove Irene Klokck, Kemal Korkmaz, Piotr Kurys, Cem Elbi, Bjørn Risberg, Håvard Danielsen, Massimo Loda, and Fahri Saatcioglu

1 Department of Biology, University of Oslo, Oslo, Norway; 2 Laboratory of Receptor Biology and Gene Expression, National Cancer Institute, Bethesda, Maryland; 3 Department of Pathology, Norwegian Radium Hospital, Oslo, Norway; 4 Division of Genomic Medicine, University of Sheffield, United Kingdom; and 5 Department of Pathology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Kallikreins (KLKs) are highly conserved serine proteases that play key roles in a variety of physiological and pathological processes. KLKs are secreted proteins that have extracellular substrates and function. For example, prostate-specific antigen (or KLK3) is a secreted protein that is widely used as a diagnostic marker for prostate cancer. KLK4 is a recently identified member of the kallikrein family that is regulated by androgens and is highly specific to prostate for expression. Here, we show that the gene product of KLK4, hK4, is the first member of the KLK family that is intracellularly localized. We provide strong evidence that the previously assigned first exon that was predicted to code for a signal peptide that would target hK4 for secretion is not part of the physiologically relevant form of KLK4 mRNA. In addition to detailed mapping of the KLK4 mRNA 5' end by RT-PCR, this conclusion is supported by predominantly nuclear localization of the hK4 protein in the cell, documented by both immunofluorescence and cell fractionation experiments. Furthermore, in addition to androgens, hK4 expression is regulated by estrogen and progesterone in prostate cancer cells. Finally, in situ hybridization on normal and hyperplastic prostate samples in tissue microarrays indicate that KLK4 is predominantly expressed in the basal cells of the normal prostate gland and overexpressed in prostate cancer. These data suggest that KLK4 has a unique structure and function compared with other members of the KLK family and may have a role in the biology and characterization of prostate cancer.

INTRODUCTION

Androgens play a critical role in the development and maintenance of the normal prostate as well as in the development and progression of prostate cancer (1). However, the molecular mechanisms responsible for the effects of androgens on prostate biology and pathophysiology are largely unknown. Recent studies have begun to identify androgen-regulated and prostate-enriched genes and the proteins that they encode, which started to define the molecular details of androgen action in the prostate and in prostate carcinogenesis. The best known of these genes is KLK3, also called prostate-specific antigen (PSA), that is a widely used clinical tumor marker for detection and monitoring of prostate cancer progression (2). Another gene that is androgen regulated and highly enriched to prostate for expression is KLK2, which may also have utility as a prostate cancer marker in conjunction with PSA (3). KLK4 is a more recently cloned member of the family (also known as prostate, KLK4-L1, PRSS17, and ARM1), is also a highly prostate-enriched gene, and is androgen regulated (4–7). KLK4 was mapped to chromosome 19q13-4 in humans, a region that contains the other KLK family members (4, 5, 8).

The gene structure of the kallikrein family is highly conserved among the different members, both in size and organization (9). Of significance, the KLK genes have five coding exons, the first one of which encodes, or is predicted to encode, the initiation of the presignal peptide (9). On this basis, and the fact that all kallikrein (KLK) proteins characterized to date have extracellular functions, KLKs are considered to be secreted proteins. KLK4 was also assumed to have 5 exons based on sequence similarity with other KLKs identified to date based on computer-aided analysis and sequence alignment (4, 5, 7). Our previous findings, however, suggested that KLK4 mRNA does not include sequences derived from exon 1 (10). This is an important point to clarify, because the predicted first exon is suggested to code for a signal peptide that would target the protein encoded for secretion, whereas the absence of this exon will result in intracellular localization and function for hK4. This is in contrast to the extracellular location and function of the other kallikreins that have been characterized to date (9, 11).

Although KLK4 is highly expressed in the prostate, its expression in normal and malignant prostate tissue has not yet been rigorously characterized. Furthermore, expression of hK4 in prostate cancer cell lines or tissue samples remains poorly documented. In this study, we provide strong evidence that the predicted first exon is not part of the physiologically relevant form of KLK4. In addition to detailed mapping of the KLK4 mRNA 5' end by RT-PCR, this conclusion is supported by predominantly nuclear localization of the hK4 protein in the cell, indicated by both immunofluorescence and cell fractionation experiments. We also show that in addition to androgens, hK4 expression is regulated by estradiol and progesterone in prostate cancer cells. Finally, in situ mRNA hybridization on normal and hyperplastic prostate samples in tissue microarrays indicates that KLK4 is predominantly expressed in the basal cells of the normal prostate gland and overexpressed in prostate cancer. These data suggest that KLK4 has a unique structure and function compared with other members of the kallikrein family and may have a role in prostate carcinogenesis.

MATERIALS AND METHODS

Cell Culture. The human prostate cell lines LNCaP, DU145, and PC-3 were obtained from the American Type Culture Collection (Rockville, MD). The LNCaP cells were routinely maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 5 mg/ml penicillin/streptomycin, and 200 nm l-glutamine. The passage number of LNCaP cells was 12–18. The African green monkey kidney cell line COS-7, also from American Type Culture Collection, was grown in high-glucose DMEM with 10% fetal bovine serum, penicillin/streptomycin and l-glutamine as the human prostate cell lines. Cells were treated as described previously (10).

Semiquantitative RT-PCR and Primers. The total RNA of LNCaP cells was extracted using TRizol (Invitrogen) and used for first-strand cDNA synthesis with the Superscript II system (Invitrogen). For the RT-PCR, Advantage Polymerase Mix (Clontech) was applied according to the manufacturer’s recommendations. Primers were designed to amplify the KLK4 from different 5’ ends. A common reverse primer and six different forward primers (primers’ sequences available on request) were designed to have similar
melting temperatures, (Tm) and GC content. The integrity and concentration of all primers were confirmed on a 1% denaturing polyacrylamide gel.

Plasmids and Transfection. The cloning of KLK4 has been described previously (10). The pcDNA4-hismax-TOPO vector (Invitrogen) was used to generate Xpress-tagged KLK4. The 3HA-tagged KLK4 was constructed by inserting the Neol-BamHI fragment of pZEROKLK4 into the Sreα-3HA vector (12). The KLK4-PR with putative first exon was amplified from LNCaP race library by PCR (forward primer 5′-ACGGATCCATGGCCACACGAG-GAAATCCCT-3′, reverse primer 5′-TAAACTGGCCTGAGCCTTTTGCTC-3′, 40 cycles) and cloned into PCR II TOPO vector. The pSG5-KLK4-PR was constructed by inserting the BamHI-BamHI fragment from pCR II-LKLK4-PR into pSG5 vector. All constructs have been confirmed by sequencing. FuGene 6 (Roche) was used for transfection into COS-7 cells according to the manufacturer’s recommendations.

Production of Anti-hK4 Antibody. An NH₂-terminal hK4 peptide (QI-INGEDCPSHIQPWM) was used as an antigen to raise the anti-hK4 antisera (Medprobe). The antisera was affinity purified before use.

Protein Extraction and Western Analysis. The whole cell extract was used by resuspending the cells in 200 μl of lysis buffer (20 mM HEPES (pH 7.4), 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM NaVO₃, 0.5 mM PMSF, 1 mM leupeptin, and 5 μg/ml aprotinin). The suspension was rotated at 4°C for 2 h, followed by centrifugation at 15,000 × g for 30 min. The supernatant was collected and stored at −80°C. For the preparation of the cytosolic and nuclear extracts, cells were first lysed in buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor mixture]. The suspension was kept on ice for 15 min, followed by centrifugation at 15,000 × g for 3 min, and addition of Igepal to 0.6%. The supernatant was collected and stored at −80°C. For the nuclear extract, the cell pellet was washed twice with buffer A and resuspended in buffer C [20 mM HEPES (pH 7.9), 0.4 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor mixture]. After rotating at 4°C for 20 min, the nuclear protein extract was obtained by collecting the supernatant after centrifugation at 15,000 × g for 5 min. To obtain the protein extracts in the culture medium, the culture medium was collected after R1881 induction and was dialysed against 1 mM Tris (pH 7.6) for 24 h at 4°C with three changes of the dialysis buffer, followed by concentration with Amicon Ultra-15 centrifugal filter unit (Millipore). All protein concentrations were determined by the Bio-Rad protein assay. Protein extracts were electrophoresed on 15% SDS-PAGE gel. The proteins were then transferred to a polyvinylidene difluoride membrane (BIO-RAD), which was blocked with 5% milk for 2 h, followed by incubation with the primary antibody at 4°C overnight. The enhanced chemiluminescence kit (Amersham Pharmacia) was used for detection according to the manufacturer’s recommendations. The primary antibodies that have been used were hK4 rabbit polyclonal antibody (1:600 dilution), STAMP1 rabbit polyclonal antibody (1:8000 dilution), and rehydrated by serial dilution of ethanol, fixed with fresh 4% paraformaldehyde for 10 min, and digested with proteinase K (20 μg/ml) for 10 min at 37°C. After another fixation with 4% paraformaldehyde, sections were washed with 0.5 × SSC and rehydrated (1% SDS, 4 × SSC, 0.5 mg/ml RNA, 10% dextran sulfate, 50% formamide, 25 μg/ml salmon sperm DNA) at 56°C for 30 min. Lastly, digoxin/digoxigenin-labeled riboprobe (1:50) was applied and hybridized at 55°C overnight. The DIG (Digoxin/Digoxigenin) Detection Kit (Roche) was used according to the manufacturer’s recommendations. The stained slides were scanned with the “PathScope” (Fairfield Imaging Ltd., Kent, United Kingdom) and scored by an experienced pathologist using the “Path-Array” TMA software (Fairfield Imaging Ltd.). Each slide was scanned with a pixel resolution of 0.16 μm/pixel and scored at 400–800× magnifications. Digital images of each TMA core were manually scored and subsequently grouped and displayed according to staining intensity and morphology. Each tissue cylinder was evaluated for degree of cytoplasmic staining intensity (0, negative; 1, weak positive; 2, moderate positive; 3, strong positive), and the average of the five scores (one TMA cylinder) was used as a single value for each specimen. If high-grade prostatic intraepithelial neoplasia was present, this was also noted and scored for degree of in situ hybridization staining.

Statistical Analysis. Differences between the groups were evaluated using SigmaStat 2.03 (SPSS) software, followed by Mann-Whitney rank-sum test, with P < 0.05 being considered as significant.

RESULTS

The Predicted First Exon in KLK4 Is Not Physiologically Significant. To thoroughly assess the presence or absence of the predicted exon 1 in KLK4, we have performed a detailed semi-quantitative PCR study using a common reverse primer that spans the junction between exons 3 and 4, and six different forward primers, three from each of exons 1 and 2 (Fig. 1A). All primer pairs worked at similar efficiency in the PCR of a cloned KLK4 cDNA (Fig. 1B). Total RNA was extracted from LNCaP cells that were treated with R1881 (10⁻⁸ m) for 24 h. The cDNA was made from the RNA, used in PCR with the different primers as described above, and the reaction products were resolved and visualized in an agarose gel.

As shown in Fig. 1C, when F5 and F6 primers were used, corresponding to exon 2, faint bands were observed similar to those obtained with a control plasmid containing the predicted KLK4 (KLK4-PR) fragment that includes exon 1. After five additional cycles, it was still only the reactions containing F5 and F6 primers that resulted in specific amplification. At 10 additional cycles (cycle 30), in addition to F5 and F6, F4 primer also resulted in a PCR product, but it was significantly weaker than those obtained with F5 and F6. It was at 35 cycles that the primers corresponding to the putative first exon (F1, F2, and F3) gave rise to weakly visible bands. The primer intensities were confirmed on an acrylamide gel, the melting temperatures of the primers were similar, and all primers functioned similarly on a cloned KLK4 cDNA with the predicted exon 1 (Fig. 1B), indicating that the differences observed are not because of primer-specific/PCR-related effects. Thus, there were approximately 15 cycles between the appearances of amplification corresponding to the second exon compared with the putative first exon. This shows that there is a difference of at least 3 orders of magnitude in the steady-state accumulation of the KLK4 mRNA species that contain the second exon, as compared with those that have the predicted exon 1. Similar results were obtained by quantitative RT-PCR analysis (data not shown). These data suggest that the putative first exon of KLK4, if present, is at extremely insignificant low levels compared with downstream sequences.

Regulation of hK4 Expression in LNCaP Cells. To confirm that KLK4 codes for a protein and to study its expression and distribution pattern in cells that normally express it, we raised an antipeptide antibody against the predicted NH₂ terminus of hK4 corresponding to an epitope expected to be encoded by exon 2. The antisera was affinity purified and used in Western analysis to detect either...
KLK4-PR that was ectopically expressed in COS-7 cells or endogenous hK4 in LNCaP cells. Whereas there was no specific band in untransfected COS-7 cells, a single band of expected size (30 kDa) was observed in cells transfected with the KLK4-PR expression plasmid (data not shown). In contrast, hK4 was detected at low levels in untreated LNCaP cells that significantly increased after R1881 treatment to reach 20-fold higher levels at 48 h compared with untreated cells (Fig. 2A), consistent with androgen regulation of KLK4 mRNA accumulation (6, 10). hK4 that is detected in LNCaP cell extracts had an apparent molecular mass of 45 kDa, significantly higher than the calculated molecular mass of 28 kDa, suggesting that hK4 is posttranslationally modified in LNCaP cells.

To confirm that the bands detected in Western analysis were specific to hK4, we performed a competition experiment with the peptide that was originally used to raise the hK4 antiserum. In the presence of the specific peptide, the putative hK4 band in the extracts from COS-7 cells that ectopically expressed KLK4-PR, as well as the endogenous hK4 in LNCaP cells, were drastically diminished (data not shown). This indicates that the bands observed in Western analysis are specific to hK4.

Previous studies have shown that KLK4 mRNA expression can be regulated by estradiol and progesterone, in addition to testosterone (7, 10, 14). To assess whether this regulation is also at the protein level, we have used Western analysis. LNCaP cells were either left untreated or treated with R1881 (10^{-8} M), estradiol (10^{-8} M), or progesterone (10^{-8} M) for 48 h. Cell extracts were prepared and used in Western analysis with the hK4 antiserum. As shown in Fig. 2B, R1881 treatment resulted in approximately 20-fold increase in hK4 accumulation. In contrast, estradiol and progesterone treatment resulted in approximately 2.5-fold increase of hK4 levels. These data show that in addition to R1881, hK4 is also regulated, albeit more weakly, by estradiol and progesterone in LNCaP cells. Two other prostate cancer cell lines, DU145 and PC-3, showed no expression of hK4, consistent with earlier findings by Northern analysis (10).

hK4 Is Predominantly Localized to the Nucleus. We have previously used the green fluorescent protein to tag KLK4 and its variants to determine their intracellular localization (10). Because the large green fluorescent protein tag could influence the cellular localization of proteins, we have used two additional small tags, Xpress (15) and hemagglutinin (HA; Ref. 16), to study the cellular distribution of hK4. COS-7 cells were transiently transfected with expression vectors encoding KLK4, tagged either with the Xpress or the HA epitopes, and the localization of the expressed fusion proteins was examined by immunofluorescence microscopy.
As shown in Fig. 3, A and B, with both tags, hK4 was predominantly localized to the nucleus. Although some cytoplasmic staining was seen at variable levels, this never approached the levels of hK4 observed in the nuclei (data not shown). We also examined the localization of intracellular hK4 in LNCaP cells. LNCaP cells were grown in the presence of R1881 for 48 h; cells were fixed and observed by immunofluorescence microscopy using the hK4 antiserum. As shown in Fig. 3C, endogenous hK4 was predominantly localized to the nucleus of LNCaP cells. Taken together, these data show that hK4 is an intracellular protein that is principally localized to the nucleus. This is consistent with the essential absence of the putative exon 1 in KLK4 mRNA species in LNCaP cells.

To further assess the cellular localization of hK4, we biochemically examined the distribution of hK4 in different cellular compartments. LNCaP cells were grown in the presence or absence of R1881 for 48 h, and protein extracts were prepared from the whole cell, the cytosol, the nucleus, or the culture medium, which were then subjected to Western analysis with various antisera. As shown in Fig. 4, hK4 was found in the nuclear and whole cell but not in the cytosolic extracts, and its expression significantly increased in response to R1881. The proper fractionation of the cellular compartments was confirmed by Western analysis of the same extracts with antisera against a cytosolic protein, STAMP1 (13), or an androgen-regulated nuclear protein, NKK3.1 (17–19), which displayed distribution in the expected compartments. In addition, PSA (KLK3) was also used as a control; as expected, it was present in the cytosol, as well as in the culture medium, but not in the nucleus (20). These data confirm the immunofluorescence microscopy results presented above documenting that hK4 is a predominantly nuclear protein.

The KLK4 Is Principally Expressed in the Basal Cells of the Normal Prostate Gland and Overexpressed in Prostate Cancer.

Having analyzed hK4 expression by Western and immunofluorescence analysis, we next studied its expression in human prostate tissues by mRNA in situ analysis. To that end, we used a tissue microarray that contained samples representing various stages of prostate cancer progression, in addition to normal prostate glands. KLK4 was localized predominantly to the basal cells of the normal prostate epithelium, with lower levels also present in luminal cells, but not in the stroma (Fig. 5). KLK4 was also expressed in the tumor glands where the levels of expression were significantly higher compared with that found in the normal epithelium or prostatic intraepithelial neoplasia (Table 1). KLK4 expression was not significantly different, however, between the normal epithelium and prostatic intraepithelial neoplasia, or among the three grades of tumor glands. These data indicate that KLK4 expression is increased in prostate cancer.

**DISCUSSION**

The data we present here establish that the physiologically relevant form of KLK4 does not have sequences that would encode a signal peptide; as a result, the protein it encodes is intracellular, primarily localized to the nucleus. This suggests that hK4 has a dramatically different function compared with other members of the KLK family.

The RT-PCR strategy that we have used to assess the presence of exon 1 indicates a difference of at least 3 orders of magnitude in the levels of transcripts that contain, or are deficient in, exon 1. Similar data were obtained by quantitative RT-PCR (data not shown). These results extend our previous findings (10) and are also consistent with the sequences in the expressed sequence tag database indicating that there is no expressed sequence tag corresponding to the first exon of KLK4 of 16 independent entries (data not shown). In contrast, KLK1, KLK2, and KLK3 are represented by at least six expressed sequence tags that include the corresponding first exon encoding a signal peptide (data not shown) consistent with the secreted proteins that they encode.

These data are also consistent with the finding that hK4 protein is primarily localized to the nucleus documented by immunofluorescence microscopy analysis. Two different tagged versions of hK4, as well as the endogenous hK4, are principally localized to the nucleus. The hK4 distribution in the nucleus shows a punctate pattern reminiscent of the promyelocytic leukemia protein bodies that have been suggested to be involved in a variety of important processes, such as transcription, RNA stability and transport, and DNA replication (21). In addition, immunohistochemistry of clinical specimens indicate that hK4 is predominantly found in the nuclei of prostate cancer cells (data not shown). Additional work is required to assess whether hK4 is associated with any specific known structure within the nucleus and their ascribed functions.
Cell fractionation experiments also clearly show that hK4 is mainly localized to the nucleus. Whereas PSA (hK3) is present in the cytosol and is secreted to the culture medium, as already known (20), hK4 is only found in the nuclear fraction of prostate cancer cells. A nonsecretory role for hK4 is also supported by in situ analysis of prostate tissue, indicating that KLK4 is expressed in the basal cell compartment of the prostate epithelium, which does not have any known secretory functions. PSA and hK2, in contrast, which are secreted proteins, are expressed in the luminal cells of the prostate epithelium (3). Taken together, these data strongly suggest that hK4 is a distinct member of the kallikrein family and may serve a unique physiological function that is different from other kallikreins characterized to date.

An interesting feature of the endogenous hK4 in LNCaP cells is that its apparent molecular weight of 45 kDa on SDS-PAGE is significantly greater than the predicted molecular weight of 28 kDa. This was also recently observed in ovarian carcinoma cells in which hK4 is expressed (14, 22). One possible modification that gives rise to a difference in predicted and apparent molecular weights is glycosylation, which is known to be used for other kallikreins (23). For example, PSA has a predicted molecular weight of 26 kDa, but migrates as a 33 kDa protein on SDS-PAGE because of glycosylation (24). Recent data on the porcine KLK4, which is 77% identical with hK4, indicate that it is a glycoprotein, and glycosylation results in a molecular weight shift of approximately 10 kDa on SDS-PAGE (25). Further studies are required to assess possible glycosylation status of hK4.

The regulation of KLK4 mRNA accumulation by multiple hormones has been documented previously (5, 7, 10, 14). hK4 protein accumulation in prostate cancer cells increases in response to androgen, and to a lesser extent by estrogen and progesterone treatment, suggesting that the major point of regulation for KLK4 expression is transcriptional. Because the endogenous androgen receptor in LNCaP cells contains a point mutation that increases its ligand repertoire, including estrogens and progesterone (26), it is possible that the

Fig. 4. Biochemical evidence that hK4 is a nuclear protein. LNCaP cells were either left untreated or treated with R1881 (10^-8 m) for 48 h. Western analysis was performed with kallikrein 4 (KLK4) antiserum on cytoplasmic (Cyt.) or nuclear (Nuc.) extracts or on extracts made from concentrated culture medium (Med.) of the same cells as well as the whole cell extract (WCE). The membrane was stripped and used in Western analysis with antiserum for STAMP1 and NKX3.1, two proteins known to reside in the cytoplasmic and nuclear compartments, respectively. Western analysis with prostate-specific antigen (PSA) is also presented that is primarily a secreted protein. Note that the protein loaded in each well was adjusted to correspond to the same number of cells. The experiment was repeated three times with identical results.

Fig. 5. KLK4 expression in normal and malignant prostate. An antisense riboprobe of KLK4 was used for in situ hybridization on a tissue microarray that contains both normal and tumor glands from human prostates. The sense probe did not result in any specific staining (data not shown). The KLK4 mRNA is predominantly detected in the basal cell layer of the normal prostate epithelium (left). The expression pattern is unordered and more intensive in the tumors (right). Two different magnifications are presented.
effects of estrogen and progesterone are mediated by this mutated androgen receptor. Our studies with tamoxifen and RU486, which act as antagonists for the estrogen and progesterone receptors, respectively, indicate that hK4 accumulation induced by estrogen and progesterone can be, at least in part, inhibited by these antagonists (data not shown). In addition, flutamide, an inhibitor of androgen receptor, does not affect estrogen induction of KLK4 in LNCaP cells (data not shown). These data suggest that the effects of estrogen and progesterone on KLK4 expression are at least in part mediated by their respective receptors, which are known to be present and functional in LNCaP cells (27). It will now be important to characterize the KLK4 promoter to identify the hormone-response elements that are involved in KLK4 expression and study their regulation.

An intriguing finding of KLK4 expression in the normal prostate gland is its highly specific localization to the basal cell compartment. The basal cells are thought to be the precursors for the terminally differentiated luminal cells (28) and are therefore considered to have a possible stem cell function in the genesis of the prostate epithelium (29). In addition, luminal cells do not express estrogen or progesterone receptors. Because KLK4 expression is increased in prostate tumors compared with normal cells, it may be a useful marker for prostate cancer and a tool for better understanding of normal prostate physiology and prostate carcinogenesis. Additional work is required to definitively test this hypothesis.

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