Human tissue kallikrein 9: Production of recombinant proteins, antibody generation and ELISA development

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ABSTRACT

Human tissue kallikreins (genes, *KLK*s; proteins, hKs) are a subgroup of hormonally regulated serine proteases. Two kallikreins, namely, hK2 and hK3 (PSA), are currently used as serological biomarkers of prostate cancer. Human tissue kallikrein 9 (*KLK9*) is a newly identified member of the kallikrein gene family. Recent reports indicate that *KLK9* mRNA is differentially expressed in ovarian and breast cancer.

Here, we report for the first time, production of recombinant hK9 in prokaryotic and mammalian cells, generation of polyclonal and monoclonal anti-hK9 antibodies, and development of an hK9-specific enzyme-linked immunosorbent assay (ELISA). Total testis tissue mRNA was reverse-transcribed to cDNA, amplified by PCR and was then cloned into pET/200 TOPO plasmid vector for protein production in *E. coli*. Full-length KLK9 cDNA was also cloned in pcDNA3.1 vector and was expressed in CHO, as well as (HEK)-293, cells. CHO-derived hK9 (pro-hK9 having no tag) was purified using FPLC and reversed-phase HPLC and was used as immunogen to generate polyclonal and monoclonal antibodies.

An immunoassay with no cross-reactivity with other members of the kallikrein gene family was developed. hK9-ELISA could detect the hK9 generated in *E. coli* as well as CHO derived and HEK-293 derived hK9. Our preliminary results indicate that hK9 is present in a variety of tissues including liver, muscle, testis and seminal vesicle as well as in biological fluids such as human breast milk, amniotic fluid, and seminal plasma. The reagents generated here will help define the physiological role of this kallikrein and its involvement in human disease.

INTRODUCTION

Human tissue kallikreins gene family is a group of 15 closely related genes, located on chromosome 19 (q13.4) in tandem, which encode for secreted serine proteases with various physiological functions. Pancreatic/renal kallikrein (hK1), human glandular kallikrein 2 (hK2) and prostate-specific antigen (hK3, PSA) are amongst the first tissue kallikreins identified. In addition to these "classical" tissue *KLK*s, in recent years, 12 additional tissue kallikrein genes have been identified in the same locus (Borgono and Diamandis, 2004; Clements *et al*., 2004).

Human tissue *KLK* gene family has attracted significant attention mainly due to their association with various malignancies and their potential applicability as novel biomarkers (Obiezu and Diamandis, 2005). KLK2, KLK3, and KLK11 are established or candidate serologic biomarkers in early diagnosis and monitoring of prostate cancer. In addition to prostate cancer, many of the newly identified kallikreins are differentially expressed in ovarian (Ni *et al*., 2004), breast (Fritzsche *et al*., 2006), testicular (Luo *et al*., 2003), pancreatic (Yousef *et al*., 2004), cervical (Santin *et al*., 2004), and colorectal (Ogawa *et al*., 2005) cancer. No rich natural source for many of the newly identified kallikreins is currently available; consequently the production of recombinant tissue kallikreins for antibody generation and functional studies in recent years is highly pursued.

Human tissue kallikrein 9 (*KLK9*) was originally identified in our laboratory in the year 2000. The full sequence of the gene (GenBank accession # AF135026) and its precise chromosomal localization have been characterized (Yousef and Diamandis, 2000). Similar to other kallikreins, hK9, is predicted to be synthesized as a preproenzyme which is processed into a mature form (229 amino acids). hK9 harbors a signal peptide of 19 amino acids and a 3-amino acid pro-segment. *KLK9* mRNA, by RT-PCR analysis, is found in a wide variety of tissues. Recent reports indicate that *KLK9* mRNA is differentially expressed in ovarian and breast cancer.

Among the fifteen members of the *KLK* gene family, *KLK9* is the only kallikrein for which production of recombinant protein has not been reported to date. In this study we describe the production of recombinant hK9 and generation of antibodies against this kallikrein.

Initially, we produced the pro-form of hK9 using an *E .coli* expression system. Purified *E. coli*-derived hK9 was used as an immunogen to generate polyclonal antibodies. We then cloned the full length hK9 mRNA (pre-pro form) in pcDNA3.1. The construct was used for protein expression in Chinese Hamster Ovary (CHO) cells and a stable cell line secreting pro-hK9 with no tag was generated. The identity of the purified mammalian hK9 was confirmed by mass spectroscopy. Using Western blotting, the hK9 rabbit antiserum specifically recognized the mammalian hK9 with no cross-reactivity from other kallikreins. We further used the CHO-derived hK9 (with no tag) to generate additional monoclonal and polyclonal antibodies against this kallikrein. These antibodies are currently being utilized to generate the first hK9-specific enzyme-linked immunosorbent assay (ELISA).

METHODOLOGY

Total testis tissue mRNA was reverse-transcribed to cDNA. Polymerase chain reaction using oligonucleotide primers specific to the pro-form human KLK9 was conducted. The PCR product was cloned into pET/200 TOPO plasmid vector and the construct was used to transform Top10 *E. coli* strain BL21(DE3) for protein production. rhK9 was purified to homogeneity using nickel-nitrilotriacetic metal affinity chromatography followed by reversed-phase high performance liquid chromatography (HPLC).

Highly purified rhK9 was used as immunogen for antibody production. hK9 rabbit anti-serum displayed no cross-reactivity with other kallikreins and could specifically recognize *E. coli* and CHO-derived hK9 in Western blots. Full-length *KLK9* cDNA was cloned in pcDNA3.1 vector, and the construct was used to transfect Chinese hamster ovary (CHO) cells as well as human embryonic kidney (HEK)-293 cells.

Stable cell lines secreting pro-hK9 with no tag were generated. CHO-derived hK9 was purified using FPLC, followed by reversed-phase HPLC. Following confirmation of protein identity by tandem mass spectroscopy, hK9 was used as immunogen to generate additional anti-hK9 polyclonal and monoclonal antibodies in rabbit and mice. Utilizing these antibodies, an immunoassay with sensitivity of 0.5 ng/ml was developed.

RESULTS

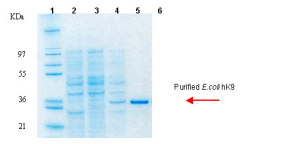

Figure 1. Detection of hK9 fusion protein in bacterial cell pellets and during purification steps, as detected by SDS-PAGE (Coomassie stain). Lane 1) Mark-12 molecular mass marker; Lane 2) BL21 cell pellet [no insert]; Lane 3) BL21 cell pellet [containing hK9 insert] in the absence of IPTG, at 4 hrs; Lane 4) BL21 cell pellet [containing hK9 insert] 4 hours post IPTG stimulation; Lane 5) Purified hK9 after NI-NTA chromatography.

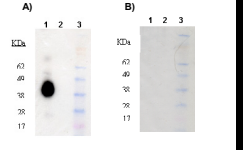

Figure 4. Detection of CHO-derived hK9 as detected by Western blotting using hK9 rabbit serum (A) or pre-immune control rabbit serum (B) as the primary antibody. Lane 1) Purified CHO-derived hK9; Lane 2) Blank; Lane 3) "See blue plus2" molecular weigh marker.

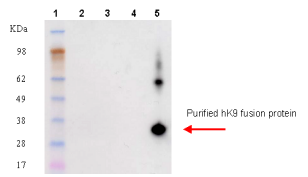

Figure 2. hK9 fusion protein as detected by Western blotting using 10,000-fold dilution of the hK9 rabbit serum. Lane 1) "See blue plus2" molecular mass marker; Lane 2) BL21 cell pellet [no insert]; Lane 3) BSA; Lane 4) Blank; Lane 5) Purified hK9 fusion protein .

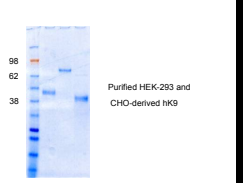

Figure 5. Purified HEK-293 and CHO-derived hK9 as detected by SDS-PAGE. Lane 1) "See blue plus2" molecular weigh marker; Lane 2) Purified HEK 293-derived hK9 lower band; Lane 3) Purified HEK 293-derived hK9 upper band; Lane 4) Purified CHO-derived hK9. All bands have been confirmed by Mass spectrometry. The difference in molecular size between HEK-293 and CHO-derived hK9 is possibly due to variation in degree of glycosylation in these cell lines.

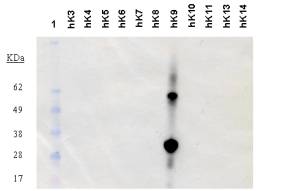

Figure 3. Western blot to test for cross-reactivity of hK9 rabbit antiserum with other members of kallikrein family available in house. 100 ng of each protein was loaded into wells of a 12 well polyacrylamide gel. Note: No detectable cross-reactivity from other kallikreins.

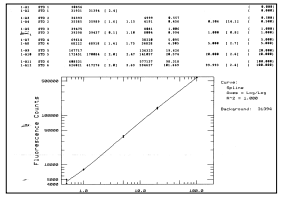

Figure 6. Calibration curve of rhK9 double sandwich ELISA. Using this ELISA a sensitivity of 0.5 µg/L (0.5 ng/ml) was obtained.

DISCUSSION

Two of the classical kallikreins, hK2 and hK3, currently have utility in cancer diagnostics. With the identification of additional human kallikrein genes, the potential value of this hormonally regulated family of serine proteases as biomarkers of cancer has been the focus of intense research. Numerous reports indicate that the mRNA of many of the newly identified kallikreins is differentially expressed in hormone dependent malignancies.

In order to study the expression of kallikreins at the protein level, the production of recombinant forms of these proteases (for functional studies as well as for antibody generation) has been highly pursued. Generation of recombinant kallikreins such as hK6, hK10, and hK11 in recent years and consequent generation of antibodies against these kallikreins not only has provided new avenues in characterizing their structure and function but also has resulted in generation of highly sensitive serological assays. These assays confirm the potential utility of these kallikreins as cancer biomarkers at the protein level.

Various recent clinical studies suggest that *KLK9* mRNA is differentially expressed in ovarian and breast cancer. Due to lack of a sensitive method for the detection of this protein (hK9), the prognostic and diagnostic significance of this kallikrein in cancer, at the protein level, remains elusive.

In this study, for the first time, we report production of recombinant hK9 as a fusion protein using an *E. coli* protein system. Polyclonl antibodies against this recombinant protein were used to generate anti-hK9 antibodies, thereby facilitating the detection and purification of recombinant hK9 with no tag in a mammalian protein expression system. Purified mammalian rhK9 was further used for generation of additional monoclonal and polyclonal antibodies which could be utilized in the generation of the first hK9-specific Enzyme-Linked Immunosorbent Assay (ELISA) capable of recognizing native hK9 in biological samples.

hK9-ELISA showed no cross-reactivity with other members of the kallikrein gene family. hK9-ELISA was able to detect the mammalian form of hK9 (CHO derived and HEK-293 derived hK9) as well as hK9 generated in *E. coli*. A sensitivity of 0.5 ng/ml is currently achieved.

Analysis of a panel of human tissue cytosolic extracts indicated that hK9 is present in a variety of tissues including liver, muscle, testis and seminal vesicle. These results are in agreement with the detection of other newly identified tissue kallikreins in various tissues, as well as *KLK9* mRNA expression which is also not tissue specific and is expressed in a wide array of tissues as detected by RT-PCR. hK9 was also detected in biological fluids such as human breast milk, amniotic fluid, and seminal plasma confirming that hK9 is a secreted protein as predicted from its amino-acid sequence.

CONCLUSION

The production of the different forms of hK9 reported in this study provide the research tools required to characterize the structure and function of this kallikrein. Utilization of hK9 specific polyclonal and monoclonal antibodies, as well as development of hK9-specific ELISA, may soon determine the potential utility of this kallikrein in cancer diagnosis.

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