Expression of Human Glandular Kallikrein, hK2, in Mammalian Cells

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ABSTRACT

The human kallikrein family consists of three members, hK1, hK2, and hK3 (prostate-specific antigen (PSA)). PSA is a widely accepted marker for prostate cancer. The mRNAs for both hK2 and PSA are localized predominantly to prostate epithelium and are regulated by androgens. In addition, hK2 has 78% amino acid homology to PSA. Although similarities to PSA make hK2 a potential prostate cancer marker, they also impede biochemical characterization of hK2 in those human tissues and body fluids where PSA is abundant. To study the expression, biosynthesis, and processing of hK2, recombinant hK2 was expressed in the adenovirus-induced Syrian hamster tumor cell line AV12-664 (AV12-hK2). Expression of hK2 was analyzed by Western blots and ELISA using monoclonal antibodies HK1G 464.3 (specific for prohK2 (pK2)) and HK1D 106.4 (specific for pK2 and mature hK2 (hK2)). Western blot and ELISA analyses showed that pK2 was secreted into the media by AV12-hK2 cells on day 1 and was gradually converted to the mature form of hK2 by day 7. N-terminal amino acid sequencing verified the Western blot and ELISA data. This demonstrates for the first time that hK2 is secreted as pK2 and converted to hK2 extracellularly. In addition, hK2 detected in day 4–7 AV12-hK2-spent media was enzymatically active. Recombinant hK2 was also expressed in human prostate carcinoma cell lines, PC3 (PC3-hK2) and DU145 (DU145-hK2), that do not express endogenous hK2 or PSA. Similar to AV12-hK2 cells, both cell lines secreted pK2 that was converted to hK2 extracellularly. pK2 was the major form detected in the spent media of PC3-hK2 cells, even after 7 days, indicating a slow conversion of pK2 to hK2. hK2 was the predominant form detected in the spent media of DU145-hK2 starting on day 1, indicating the rapid conversion of pK2 to hK2. In this study, we demonstrate that hK2 exists in different forms and is secreted as pK2. pK2 is then converted to enzymatically active hK2 extracellularly.

INTRODUCTION

pCa2+ is the most commonly diagnosed malignancy in American males, with an estimated 244,000 new cases and 40,400 deaths expected in 1995, accounting for 36% and 14% of total new cases and deaths of all cancer types, respectively (1). PSA has been widely recognized as a reliable prognostic marker for this disease (2–4). Current PSA tests, however, lack the specificity to distinguish between pCa and BPH, especially when serum PSA value is only slightly elevated (5). hK2 and PSA belong to the family of human glandular kallikreins. The kallikreins are a multigene family of serine proteases. Three kallikreins have been identified in humans, designated hK1, hK2, and hK3 (PSA; Refs. 5–8). Their genes have been mapped to chromosome 19 (9). hK1 pancreatic/renal glandular kallikrein is produced primarily in the kidney, pancreas, and submandibular salivary gland (10). hK2 and PSA share many characteristics. Both are primarily produced by the prostate epithelium (7, 11), are regulated by androgens (12–14), and share 78% sequence homology (6, 15). Because of similarities between hK2 and PSA molecules, it is speculated that measuring serum concentrations of hK2 may enhance or complement the sensitivity of the PSA test for screening, staging, and monitoring of pCa and may help distinguish between pCa and BPH. PSA is a chymotrypsin-like protease (16–19), whereas protein modeling studies suggest that hK2 is a trypsin-like protease (20, 21). This implies that the physiological role of hK2 in prostate is different from PSA. The lack of knowledge regarding the biosynthesis and protein processing of hK2 has impeded the understanding of its physiological role in the normal and cancerous prostate gland.

Significant progress has been made in understanding the biology and biochemistry of PSA. PSA is postulated to liquefy semenogelin and enhance male fertility (16, 17, 22). In contrast, very little is known about hK2 biology. Recently, hK2 was identified in the prostate cytosol and seminal plasma where it was uncomplexed or complexed with PCI (14, 23); however, biological and biochemical properties have not been reported.

To study the biochemical and biological properties of hK2, the protein needs to be overexpressed in a heterologous system. This approach provides a reliable source for hK2 that is devoid of PSA. We have previously reported expression of recombinant pphK2 in Escherichia coli that proved to be an invaluable immunogen (24). However, to study biosynthesis, protein processing, secretion, and activity, it is imperative to express hK2 in mammalian cells. Lovgren et al. (25) reported transient, low-level expression of hK2 in BHK-21 cells using the Semiliki Forest virus system to study cross-reactivity of antibodies used in immunoassays for free PSA and PSA. However, this system did not provide a stable source of hK2 which is necessary for studying the biosynthetic processing of the protein. In studies presented here, hK2 was cloned and stably expressed in Syrian hamster tumor cells, AV12, and human prostate carcinoma cell lines PC3 and DU145. We demonstrate for the first time that hK2 is secreted into the spent media by mammalian cells as pK2 and converted to enzymatically active hK2 extracellularly. These observations establish the biosynthetic processing of hK2 and suggest that pK2 may exist in biological fluids and could be a useful marker for prostate cancer and/or BPH.

MATERIALS AND METHODS

Expression Vectors. A 0.8-kb DNA fragment coding for entire pphK2 was PCR amplified using primers A (5'-ATATGGATCCATATGTCAGCATGGACCTGGUCTCTCCA), primer B (5'-ATATGGATCCTCAGGGGTFFGGCTGCGATGGT), and plasmid pVL1393 containing pphK2 cDNA (gift from Dr. Charles Young, Mayo Clinic, Rochester, MN) as the template. The PCR products were cloned into the TA cloning vector (Invitrogen Corp., San Diego, CA) to obtain TA-hK2 and sequenced to ensure the fidelity of PCR.

Fig. 1 shows the hK2 expression vectors used in the study. pGTD-hK2 was constructed by cloning the 0.8-kb hK2 insert from the TA-hK2 clone into the BclI site of pGTD (26) with PCI (14, 21). This implies that the physiological role of hK2 in prostate is different from PSA. The lack of knowledge regarding the biosynthesis and protein processing of hK2 has impeded the understanding of its physiological role in the normal and cancerous prostate gland.

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pGTD-hK2

Amp

GBMT

hK2

Bcl 1

Bcl 1

SVe
dhfr

pLNS-hK2

LTR

NEO

SV40

hK2

LTR

Hind III

Cla 1

Hind III

Cla 1

W

pLNC-hK2

LTR

NEO

CMV

hK2

LTR

Hind III

Hpa I

Fig. 1. Schematic representation of hK2 expression vectors. Arrows, transcrioption start sites.

In all of the mammalian expression vectors, the orientation of the insert was confirmed by DNA sequencing.

Cell Lines and Transfection. AV12-664 (ATCC CRL 9595) and DU145 cells were cultured in DMEM (high glucose) containing 10% fetal clone (HyClone, Logan, UT). PC3 cells were cultured in minimal Eagle’s medium containing 10% fetal clone. AV12-664, DU145, and PC3 cell lines were transfected, respectively, with pGTD-hK2, pLNS-hK2, and pLNC-hK2 expression vectors using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. Transfected AV12-664 cells (AV12-hK2) were selected in 200 µm methotrexate (Sigma Chemical Company, St. Louis, MO). Transfected DU145 (DU145-hK2) and PC3 (PC3-hK2) cells were selected in 400 µg/ml G418 (Life Technologies, Inc., St. Louis, MO). Transfected AV12-664 cells were cultured in DMEM (high glucose) containing 10% fetal calf serum and 10% fetal calf serum (Hyclone, Logan, UT). PC3 cells were cultured in minimal Eagle’s medium containing 10% fetal calf serum (Hyclone, Logan, UT). Transfected cells were selected in 400 µg/ml G418 (Life Technologies, Inc., St. Louis, MO). Transfected DU145 (DU145-hK2) and PC3 (PC3-hK2) cells were selected in 400 µg/ml G418 (Life Technologies, Inc., St. Louis, MO). Transfected AV12-664 cells were cultured in DMEM (high glucose) containing 10% fetal calf serum and 10% fetal calf serum (Hyclone, Logan, UT). PC3 cells were cultured in minimal Eagle’s medium containing 10% fetal calf serum (Hyclone, Logan, UT). Transfected cells were selected in 400 µg/ml G418 (Life Technologies, Inc., St. Louis, MO). Transfected DU145 (DU145-hK2) and PC3 (PC3-hK2) cells were selected in 400 µg/ml G418 (Life Technologies, Inc., St. Louis, MO).

Antibodies. Murine mAbs HK1D 106.4 (against a peptide corresponding to 17–71 aa of hK2), HK1G 464.3 and HK1G 586.1 (against phK2

A hindering of sites. 

Purification of hK2. mAb HK1D 586.1 was coupled with GammaBind Plus Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) according to the published procedure (29). Tissue culture petri dishes containing various clones grown to approximately 60–70% cell confluency were washed with HBSS (Life Technologies, Inc.) and serum-free HH4 medium (JRH Biosciences, Lenexa, KS) was added. Spent media was concentrated (YM-10 membrane; Amicon Inc., Beverly, MA) and incubated with the above resin at 4°C for 48 h with gentle agitation. The resin was spun down and washed three times with PBS and bound proteins were eluted with 1% SDS. The eluent was subjected to SDS-PAGE on a 4–20% polyacrylamide gel and electroblotted to a polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was stained with Coomassie blue, and the band corresponding to the appropriate molecular weight was excised and subjected to protein sequencing using an Applied Biosystems model 477A protein sequencer. phK2

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Western Blot and ELISA Analyses. Western blot analysis was performed as described in "Purification of hK2" from resistant clones were collected at specified days, concentrated 10-fold using Centricon 10 (Amicon, Inc.), and subjected to SDS-PAGE on a 4–20% gel (Bio-Rad, Inc., Melville, NY). After electrophoresis, proteins were electroblotted onto nitrocellulose membranes. Primary anti-

bodies (1:1000 dilution of ascites) and secondary antibodies (goat anti-mouse horseradish peroxidase, 1:500; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) were used to probe the blots. The immunoreactive signals were detected using the enhanced chemiluminescence (Amersham, Buckinghamshire, England) system according to the manufacturer’s instructions.

To measure hK2 by ELISA, microtiters plates (Becton Dickinson Labware, Lincoln Park, NJ) were coated with 50 µl of spent media overnight at 37°C. The wells were washed with PBS + 0.1% Tween 20, blocked with 2% BSA in PBS, and incubated for 1 h at 37°C with 50 µl of HK1D 106.4 or HK1G 464.3. The wells were washed again with PBS + 0.1% Tween 20 and incubated for 1 h at 37°C with 50 µg of goat anti-mouse IgG Fcγ antibodies coupled to horseradish peroxidase (1:500; Jackson Immunoresearch Laboratories). The wells were washed with PBS + 0.1% Tween 20 again, incubated with α-phenylenediamine dihydrochloride (Sigma) for 15 min, and quenched with 4 N sulfuric acid. The colorometric reaction was measured at A405 with an ELISA reader (model EL310; Biotek Instruments, Inc., Burlington, VT). All samples were assayed in triplicate. Spent media from AV12 cells transfected with vector alone were used as negative control. Purified phK2

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RESULTS

The cDNA for hK2 was cloned into pGT-d vector under the GBMT promoter. This promoter is activated by the Ela tumor antigen. AV12-664 cells are derived from an adenovirus type 12-induced tumor in Syrian hamster and constitutively express Ela tumor antigen. Thus, when pGT-d vector is transfected into the AV12 cells, the GBMT promoter is activated by the Ela antigen, leading to the overexpression of the protein cloned under this promoter. This host vector system has been used for expression of other serine proteases such as human protein C and tissue plasminogen activator (26). To study the expression of hK2 in mammalian cells, AV12 cells were transfected with pGTD-hK2 expression vector. Cells were selected in 200 µm methotrexate for 2 to 3 weeks, and single-cell clones were analyzed for hK2 expression using an ELISA. Spent media from five randomly selected hK2 positive clones were analyzed with Western blot. A hK2-immunoreactive band migrating at ~34 kDa was detected in all of the clones (data not shown). AV12-hK2#27 was selected for further characterization and purification.

To determine the identity of the protein that was secreted at day 1, spent media from AV12-hK2#27 was collected, concentrated, and affinity purified using HK1D 586.1 resin. N-terminal sequencing analysis of the purified protein revealed a sequence: VPLIQSRIVGEPH. No competing sequence was evident from the profile of aa released sequentially by the Edman degradation procedure. This sequence corresponds with the sequence predicted for the NH2 terminus of phK2. A similar purification protocol was used to purify the protein from day 7 spent media of AV12-hK2#27 cells. N-terminal sequencing analysis of the purified protein revealed a sequence, IVGG-WEXEK, that corresponds with the sequence predicted for the NH2 terminus of hK2.

To study the biosynthesis of hK2 in mammalian cells, spent media from AV12-hK2#27 was collected for 8 consecutive days and evaluated for the presence of hK2. The samples were concentrated and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with either HK1D 106.4 or HK1G 464.3 mAbs (Fig. 2). As also shown in Fig. 2, HK1D 106.4 recognizes both phK2 and hK2, whereas HK1G 464.3 recognizes only phK2 since its epitope lies in ~7 to +7 region of hK2 protein (data not shown).
Expression of hK2 (~34 kDa) peaked by day 3 and plateaued thereafter as detected by HK1D 106.4 mAb (Fig. 2). Two other immuno-reactive bands migrating at ~70 kDa and ~90 kDa were also detected from day 4 onward. On the other hand, when the same samples were blotted and probed with HK1G 464.3, a gradual reduction in the level of phK2 expression was detected starting at day 4 (Fig. 2). By day 8, very low levels of phK2 were found in the spent media. This result shows that the pro form of hK2 is being secreted into the media by AVI2-hK2#27 and is gradually converted to mature form of hK2 extracellularly. We did not observe ~70- and ~90-kDa bands with HK1G 464.3 mAb, indicating that these bands are either homooligomers of hK2 or hK2 covalently complexed with yet unknown protein(s). Although the identity of these bands is not known at this time, they serve as markers for the presence of hK2 in the spent media. Purified phK2A217V (phK2 with Ala to Val mutation at aa 217) was used as the positive control in this experiment. This protein is stable to purification and can be converted from phK2A217V to hK2A217V by trypsin digestion.3

To study the relationship between extracellular conversion of phK2 to hK2 and viability of AV12-hK2#27 cells in culture, cell viability was determined by trypan blue exclusion and correlated with hK2 expression. Expression of phK2 and hK2 in the spent media was measured with an ELISA using both HK1D 106.4 and HK1G 464.3 mAbs. As shown in Fig. 3 (inset), the number of viable cells peaked at 3.8 × 10^7 in culture by day 3 and gradually decreased thereafter. By day 8, the number of viable cells was reduced to <1.0 × 10^7. The expression of both phK2 (measured by HK1G 464.3) and hK2 (measured by HK1D 106.4) also peaked by day 3. However, the expression of phK2 declined thereafter, whereas the expression of hK2 plateaued after day 4. This result indicates that phK2 is secreted by AV12-hK2#27 and a fraction of it is converted extracellularly to hK2 by day 4. Moreover, it shows that conversion of phK2 to hK2 correlates with a decrease in cell viability, suggesting that intracellular proteases released by the dying cells are responsible for this conversion. Expression of phK2 was highest when cells were most viable (day 3). A decrease in phK2 paralleled a decline in cell viability, suggesting that phK2 is secreted by these cells, as opposed to being released following cell death and lysis. In addition, the rise in hK2 corresponded to the drop in phK2, indicating that the pro form of hK2 was being converted to the mature form of hK2 over time. The reason for ~50% decline in total hK2 concentration from day 3 to day 4 is not understood at present. It is possible that the sudden release of unstable proteases from dying cells degrade hK2 in the culture media.

To demonstrate that hK2 detected in the spent media of AV12-hK2#27 is enzymatically active, a spectrophotometric assay was developed using the commercially available chromogenic substrate, H-D-Pro-Phe-Arg-pNA (S-2302). Data shown in Fig. 3 indicate no hK2 activity in the spent media of AV12-hK2#27 cells at day 1 or day 3. However, hK2 activity was detected in the spent media at day 4 and increased until day 7. Since phK2 is enzymatically inactive, the above results support our previous observations and again indicate that phK2 is secreted by the AV12-hK2#27 cells and is gradually converted to...
hK2 starting from day 3. Day 7 spent media of AV12 or AV12-pGTD cells showed no detectable activity in this assay, indicating that hK2-like proteases are not secreted by AV12 cells (data not shown).

Moreover, in the process of hK2 purification (see above), removal of hK2 from day 7 spent media of AV12-hK2 cells by passage through the HK1G 586.1 immunoaffinity column also removed ~90% of the enzymatic activity detected in the spent media. These results indicate that the enzymatic activity in the spent media of AV12-hK2 cells as detected in our assay was specific to hK2 and not due to other proteases released from dying cells.

To ascertain whether phK2 is converted to hK2 extracellularly, an equal volume of day 1 spent media of AV12-hK2 cells was incubated with that of either AV12 (day 7 spent media) or AV12-pGTD (day 7 spent media) for 48 h. The mixtures were then concentrated, electrophoresed, and electroblotted as described previously. The blot was probed with HK1D 106.4 and HK1G 464.3 mAbs (Fig. 4). As is clearly visible in Lanes 5 and 6, the ~70-kDa hK2 immunoreactive band was present in AV12-hK2 supernatants upon incubation with either spent media of AV12 or AV12-pGTD cells. When day 1 spent media of AV12-hK2 was incubated alone or with plain media, the ~70-kDa band was not present. Appearance of an ~70-kDa immunoreactive band indicates that a fraction of phK2 was converted to hK2. This suggests that intracellular proteases released into the media by the lysed AV12 cells may be responsible for the initial conversion of phK2 to hK2.

Three other independently isolated AV12-hK2 clones were examined with Western blot (using HK1D 106.4 and HK1G 464.3 mAbs) for expression of both phK2 and hK2. Results showed that the patterns of phK2 and hK2 expression were the same in all clones, indicating that the above results were not limited to a single AV12-hK2 clone (data not shown).

To study the expression of hK2 in human prostate carcinoma cell lines, pLNC-hK2 (hK2 under the control of the cytomegalovirus promoter) was transfected into PC3 cells, and pLNS-hK2 (hK2 under the control of the SV40 promoter) was transfected into DU145 cells (Fig. 1).

PC3-hK2 and DU145-hK2 cells were selected in G418 (400 μg/ml)-containing media for 2 to 3 weeks, and resistant clones were analyzed for hK2 expression using Western blotting. Spent media of PC3-hK2 were collected at day 7, concentrated, subjected to SDS-PAGE, and electroblotted onto nitrocellulose membranes. Blots were probed with HK1D 106.4 and HK1G 464.3 mAbs (Fig. 5). Both HK1D 106.4 and HK1G 464.3 mAbs detected a major band of ~34 kDa in the spent media of PC3-hK2 cells, indicating that phK2 is present in the spent media of this cell line even after 7 days. To confirm these observations, the ~34-kDa immunoreactive band was partially purified from day 7 spent media of PC3-hK2 cells by affinity purification. N-terminal analysis revealed a sequence of VPLIQS-RIVGWEXEK, verifying it to be phK2. Spent media of DU145-hK2 was collected at day 1 and analyzed as described above (Fig. 5). HK1D 106.4 detected a stronger band of ~34 kDa compared to HK1G 464.3. This result indicated that predominantly hK2 is present in DU145-hK2 spent media even at day 1. Western blot analysis of PC3-hK2 and DU145-hK2 cell lysates revealed negligible levels of either phK2 or hK2 (data not shown). A time course experiment similar to the one described for AV12-hK2 (Fig. 2) was also conducted for PC3-hK2 and DU145-hK2 cells. The Western blot patterns did not change significantly from day 1 to day 7, indicating that predominantly phK2 was present in PC3-hK2 spent media even after 7 days, whereas predominantly hK2 was present in DU145-hK2 spent media starting from day 1 (data not shown).

DISCUSSION

Serine proteases are produced as propeptides inside the cell. After passage through the secretory pathway, the signal peptide (pre) is cleaved, yielding the pro form of the protein. Although propeptides are generally cleaved inside the cell (e.g., tissue plasminogen activator, protein C, and tumor necrosis factor), there are exceptions (e.g., renin, trypsin, and chymotrypsin) which are secreted as proproteins and cleaved extracellularly (reviewed in Refs. 30–32). The following four lines of evidence presented in this article demonstrate that hK2 is expressed as prohK2 in mammalian cells and is converted to the mature form extracellularly: (a) presence of phK2 at day 1 and hK2 at day 7 as determined by Western blot and enzymatic analyses of spent media of AV12-hK2 cells; (b) purification and sequencing of...
phK2 from day 1 spent media and hK2 from day 7 spent media of AV12-hK2#27 cells; (c) detection of gradually increasing hK2 activity in spent media of AV12-hK2#27 cells from day 4 onward; and (d) similar expression patterns of recombinant hK2 in Syrian hamster tumor cells (AV12) and human prostate carcinoma cell lines, PC3 and DU145. The evidence that phK2 is secreted in the spent media of two human prostate carcinoma cell lines (DU145 and PC3) indicates that phK2 may be present in biological fluids and thus may be a relevant marker for screening, staging, and monitoring progression of the prostate diseases.

phK2 is converted to hK2 by protease cleavage and release of a short propeptide (7 aa) ending in arginine. The presence of phK2 in the day 1 spent media of AV12-hK2#27 cells at first indicated that AV12 cells may not have the enzymatic machinery to convert phK2 to hK2. However, when the cells were left in culture for longer periods of time, hK2 was detected. The appearance of hK2 correlated with a decrease in viability of the AV12-hK2#27 cells. This indicated that although proteases responsible for conversion of phK2 to hK2 are present in the cells, they may be compartmentalized away from phK2. Upon cell lysis, such proteases become accessible and may be responsible for the initial conversion of phK2 to hK2. This observation was further supported by the conversion of phK2 in day 1 spent media of AV12-hK2#27 cells to hK2 upon coinubcation with day 7 spent media of either AV12 or AV12-pGT-D cells (Fig. 4). Based on reactivity with the HK1G 464.3 mAb, it seems that a major fraction of phK2 was still left in these samples (data not shown), suggesting that proteases responsible for initial conversion of phK2 to hK2 are not stable in the extracellular environment under the experimental conditions used here. It is interesting to note that day 7 spent media of AV12 or AV12-pGT-D cells had no detectable activity on chromogenic substrate (data not shown), indicating that AV12 cells lack any hK2-like proteases. It is speculated that the process of phK2 to hK2 conversion is initiated by as yet unknown non-hK2 like proteases that appear to be either unstable or present at low levels or both. The process of conversion from phK2 to hK2 is an important step in understanding the regulation of hK2 and its role in prostate development. Thus, more research is necessary to better characterize this process and to identify the protease(s) responsible for hK2 activation.

The hK2 detected in the spent media of AV12-hK2#27 from day 4 onward was enzymatically active on a chromogenic substrate (Fig. 3). The increased activity correlated with a decrease in phK2 concentration and increase in hK2 concentration in the spent media. Day 1 and day 3 spent media that contained mainly phK2 had no detectable activity. Based on molecular modeling studies, hK2 is predicted to have a trypsin-like specificity (20, 21). The enzymatic activity of hK2 is specific for selected arginines and is different from the substrate specificity of trypsin.3 Thus, it may be possible to generate hK2-specific substrates that could be used in activity-based diagnostic assays to detect hK2 in bodily fluids.

hK2 is also secreted as phK2 from PC3-hK2 and DU145-hK2 cells; however, the process of extracellular conversion from phK2 to hK2 appears to be more efficient for DU145 cells than for PC3 cells (Fig. 5). The reason for this observation is not known at present. It may be because DU145 cells secrete more varieties or quantities of proteases in the media compared to PC3 cells. Differences in expression of urokinase and plasminogen activator inhibitor 1 have been observed between PC3 and DU145 cells (33). Furthermore, we have observed that PC3 cells survive for more than 2 weeks in serum-free media, whereas DU145 cells lose viability rapidly without serum, thereby liberating proteases more rapidly into the media.

We detected two hK2 immunoreactive bands (~70 and 90 kDa) in the spent media of AV12-hK2#27 cells. These proteins could be homooligomers of mature hK2 or complexes of mature hK2 with other proteins. hK2 has been shown to complex with ACT3 and PCI (23). In an attempt to identify ~70- and 90-kDa bands, samples of the spent media were probed with anti-ACT or anti-PCI antibodies on Western blots. The results indicated that ~70- and 90-kDa bands were not complexes of hK2 with these ACT or PCI (data not shown). Although these bands are represented as a minor fraction in the spent media of AV12-hK2#27 cells, they nonetheless remain a useful marker for the presence of hK2.

A considerable amount of work has been done to elucidate the physiological role of PSA. PSA is a chymotrypsin-like enzyme and the native substrates for PSA are semenogelins. One function for PSA in seminal fluid is to dissolve the seminal clot, thereby increasing the sperm motility (16). Enzymatic activity of PSA may also be related to proliferation, migration, and metastasis of prostatic cancer cells. For example, PSA has been shown to hydrolyze interleukin 2, fibrinogen, gelatin, insulin, A and B, myoglobin, and ovalbumin in vitro (34). PSA can proteolytically modulate activities of transforming growth factor β and cell surface receptors. Recently, PSA has been reported to cleave the IGF-binding protein 3, resulting in increased IGF activity, a function which may be important for the regulation of epithelial tissue growth in the prostate (35). There is no report in the literature indicating the possible physiological role of hK2. As predicted by molecular modeling studies, we have shown hK2 to have trypsin-like activity, suggesting hK2 has a different physiological role than PSA. The reagents developed in this study will be useful in deciphering the role of hK2 in the normal prostate and in prostate cancer.

The above results collectively suggest that hK2 is expressed as the pro form in mammalian cells and is converted to the enzymatically active mature form extracellularly by as of yet an unknown mechanism. These results also suggest that prohK2 may be present in the biological fluids and therefore could be a useful prognostic marker for the prostate diseases. The cell lines described in this report constitute a valuable source of phK2 and enzymatically active hK2 to study their biological roles in greater detail.

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