Identification of a Novel Complex between Human Kallikrein 2 and Protease Inhibitor-6 in Prostate Cancer Tissue


HPLC, high-performance liquid chromatography; ACT, antichymotrypsin; PI-6, protease inhibitor-6; PCa, prostate cancer; mAb, monoclonal antibody; PAI, plasminogen activator inhibitor

Abstract

Human kallikrein (hK) 2 is an arginine-selective serine protease expressed predominantly in the prostate that has an 80% sequence identity with prostate-specific antigen. Expression of hK2 is elevated in the tumor epithelium compared to benign prostate tissue. We have purified, sequenced, and identified a novel hK2 complex in prostate tissue consisting of hK2 and a serine protease inhibitor known as protease inhibitor-6 (PI-6). This 64-kDa SDS-PAGE stable complex is elevated in the tumor and is approximately 10% of total hK2. No comparable complex of prostate-specific antigen was detected. PI-6, also known as cytoplasmic antiprotease, has been characterized as an intracellular inhibitor of trypsin and chymotrypsin-like proteases, which has high homology to plasminogen activator inhibitor 1 and 2. The physiological role of PI-6 in the prostate and its relationship to hK2 and prostate cancer are under investigation.

Introduction

Three members of the hK2 family have been identified thus far, designated hK1, hK2, and hK3. All are serine proteases with a high sequence identity. Two of these kallikreins, hK2 and hK3, are found almost exclusively in the prostate (reviewed in Ref. 1). hK3, known more commonly as PSA, is a widely used serum marker for PCa. More recently, hK2 has become the focus of investigations into its possible role as a PCA marker as well as its possible roles in PCA biology. hK2 levels are elevated in the serum of patients with PCa, but hK2 shows a different serum profile than PSA (2). Immunohistochemical studies using hK2-specific mAbs have shown hK2 to be more highly expressed in prostate carcinoma than in normal tissues (3). This is the inverse of PSA, which tends to be expressed at lower levels in more poorly differentiated cancer epithelium than in normal tissues. hK2 has been shown to activate urokinase-type plasminogen activator (4, 5). We recently reported that hK2 rapidly complexes with PAI-1 and inactivates a 7-fold molar excess of PAI-1 during complex formation (6). The search for this complex in prostate tissues revealed significant levels of a hK2 complex with a molecular mass similar to that of in vitro-prepared hK2-PAI-1. Purification and NH2-terminal sequencing of this complex revealed that this was a complex with PI-6, a serine protease inhibitor with a similar mass and high homology to PAI-1 and PAI-2 (7). PI-6 is a member of the ovalbumin family of serine protease inhibitors (8). PI-6 has been reported in platelets and tissues such as the kidney, heart, and skeletal muscle, where it is expressed in epithelial and endothelial cells (9). In all cases thus far, PI-6 appears to be localized cytoplasmically (10). The finding of prostatic PI-6 in complex with hK2 raises a number of questions about the regulation of hK2 and the role that PI-6 may play in the prostate and PCa. The hK2-PI-6 complex may represent a novel prostate-specific marker for PCa.

Materials and Methods

Materials. PAI-1 was obtained from Oncogene Research Products (San Diego, CA). ACT was obtained from Athens Research (Athens, GA). Purified recombinant hK2 was expressed in the Syrian hamster carcinoma cell line AV12 and immunopurified using the murine hK2-specific mAb HK1G586.1 as described previously (11, 12). HK1G586.1 has been shown to have negligible cross-reactivity with PSA (2, 13). PFDF1D215.2 is a mAb developed toward PSA fragments that is also cross-reactive with hK2 and hK2 fragments. PSM773 is a mAb that is specific for PSA and does not cross-react with hK2.

Extraction and SDS-PAGE of Prostate Tissues. Prostate tissues were frozen in liquid nitrogen, pulverized, and then homogenized in PBS buffer containing the complete mixture of protease inhibitors (Boehringer Mannheim, Indianapolis, IN). SDS-PAGE was performed on a Novex mini-gel with a 4–20% gradient. In vitro complexes of hK2 with ACT and PI-1 were prepared by incubating hK2 with excess inhibitor as described previously (6). Western blots were probed with a primary antibody at 5 μg/ml and with goat antimonospecific horseradish peroxidase as the secondary antibody (1:50,000; Jackson Immunoresearch Laboratories, Inc., West Grove, PA). The immunoreactive signals were detected by Supersignal Ultra (Pierce, Rockford, IL) according to the manufacturer’s instructions.

NH2-terminal Sequence Analysis. NH2-terminal analysis was performed on a PE-Applied Biosystems Model 492 amino acid sequencer. Proteins to be sequenced directly from blots were prepared by electroblotting onto a PVDF membrane, visualized with Coomassie Brilliant Blue G-250, excised, and applied to the sequencer. Samples for internal sequencing were reduced with 2 mM DTT and alkylated with iodoacetamide before SDS-PAGE. Coomassie Brilliant Blue G-250-stained bands were excised from the gel, cut into small pieces, washed twice with 50% acetonitrile and 0.2 M sodium bicarbonate for 30 min, and dried completely in a speedvac. Dried gel slices were then reconstituted with 50 μl of 0.2 M sodium bicarbonate containing 1 μg of sequencing-grade trypsin (Promega, Madison WI). Samples were digested for 20 h at 37°C. Peptides were extracted with 50% acetonitrile and 0.1% trifluoroacetic acid, concentrated to 20 μl, and applied directly to the PE-Applied Biosystems Model 173A capillary HPLC microblotter system. Peptide peaks were cut from the PVDF membrane and applied to the sequencer.

Results

HK2-PI-6 in Prostate Tissues. Tumor and benign prostate tissues were extracted, and the supernatant solutions were subjected to SDS-PAGE under nonreducing conditions. Fig. 1, Lanes 1 and 2, shows the Western blot of benign tissue and tumor, respectively, probed with the hK2-specific mAb hK1G586.1. The band at 33 kDa is free hK2, and the 64-kDa band shows the hK2 complex. The level of the hK2 complex is higher per milligram of total protein in the tumor extract.

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1 To whom requests for reprints should be addressed, at Hybritech Inc., P. O. Box 269006, San Diego, CA 92136-9006. Phone: (858) 621-3264; Fax: (858) 621-4610; E-mail: smikolajczyk@beckman.com.

2 The abbreviations used are: hK, human kallikrein; PSA, prostate-specific antigen; PI-6, protease inhibitor-6; PCa, prostate cancer; mAb, monoclonal antibody; PAI, plasminogen activator inhibitor; PCI, protein C inhibitor; PVDF, polyvinylidene difluoride; HPLC, high-performance liquid chromatography; ACT, α1-antichymotrypsin.

Received 4/30/99; accepted 7/2/99.

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A total of 10 matched samples were tested, with 7 samples showing the trend seen in Fig. 1. Lanes 3 and 4 were identical to Lanes 1 and 2, except that these lanes were probed with PSM773 to detect PSA and PSA complexes. Faint bands of higher molecular mass PSA complex were detectable with extended exposure times. Immunoassay measurements of the extracts gave values of approximately 10 μg PSA/mg total protein and 0.3 μg hK2/mg total protein in both the tumor and benign samples. Thus, a significant percentage of the hK2 is found as a complex in prostate tissue, whereas the much higher levels of PSA are predominantly seen in the free, noncomplex form. Fig. 1 further indicates that the 64-kDa hK2 complex is the major kallikrein complex found in prostate tissue extracts.

**Amino Acid Sequence of hK2 Purified from Prostate Tissues.** To identify the inhibitor bound to hK2, approximately 50 g of uncharacterized prostatectomy tissue were extracted. hK2 and the hK2 complex were purified from the extract with an immunoaffinity column containing bound HK1G586.1. Fig. 2 shows the Western blot profile of the purified hK2 forms eluted from the affinity column, together with hK2 complex standards prepared in vitro (Lanes 3–5). Lanes 1 and 2 are identical samples of the purified molecular forms of hK2 obtained from prostate tissue. Lane 1 was probed with HK1G586.1, which recognizes an epitope on the NH2-terminal half of the hK2, whereas Lane 2 was probed with PF1D215.2, which recognizes an epitope on the COOH-terminal half of hK2. In both lanes, the 64-kDa hK2 complex band was detected along with the 33-kDa intact hK2. In Lane 1, a 22-kDa clipped fragment of hK2 was also detected, corresponding to hK2 fragments 1–145. In Lane 2, PF1D215.2 detected the 10-kDa fragment corresponding to hK2 fragment 146–237 but also detected a major band at ~50 kDa. These results suggested that under reducing conditions, the hK2 complex was present as two forms: (a) an inhibitor in complex with intact hK2 (64 kDa); and (b) an inhibitor covalently attached to the clipped hK2 146–237 fragment (50 kDa). Under nonreducing conditions, as in Fig. 1, only the 64-kDa complex was detected with both mAbs (data not shown). The anti-PSA mAb PSM773 showed no reactivity on these bands (data not shown). Lanes 3–5 show hK2 complexes with PAI-1, PCI, and ACT, respectively. Lane 6 is purified hK2. The mobilities of these hK2 complexes with PAI-1, PCI, and ACT are different due to the molecular mass of the inhibitors, which is 43, 58 and 68 kDa, respectively. Thus, the inhibitor in complex with intact hK2 has a mobility similar to that of hK2-PAI-1.

To confirm the identities of the hK2 bands and to identify the inhibitor bound to hK2, the purified prostatic hK2 was blotted onto a PVDF membrane and subjected to NH2-terminal terminal sequencing. In Fig. 3, approximately 10 μg of hK2 were loaded in the lane. Bands 1 and 2, the 10- and 22-kDa bands, were confirmed as hK2 fragments 1–145 and 146–237, respectively. Band 1 began with the sequence SLQXVSLHL, and band 2 began with the hK2 NH2 terminus sequence IVGGWEXEK. The sequence of the 33-kDa band was consistent with intact hK2.

However, band 4 (the 50-kDa hK2 complex band seen in Fig. 2, Lane 2) contained only the sequence SLQXVSLHL, corresponding to the 10-kDa hK2 fragment. The absence of a second sequence indicated that the NH2 terminus of the inhibitor was blocked. The reactive serine in hK2 that forms the covalent bond with serpins is residue 189, which is COOH-terminal to the 145 clip. This is consistent with Fig. 2, Lane 2, in which PF1D215.2 was shown to recognize both the 10-kDa hK2 146–237 fragment and the 50-kDa complex consisting of the hK2 146–237 fragment covalently attached to the inhibitor.

Band 5, corresponding to the faint 64-kDa band in Fig. 2, contained only the hK2 NH2 terminus sequence, consistent with intact hK2. The absence of a second sequence corresponding to the inhibitor again suggested that the inhibitor was blocked. The stronger band just above band 5 was determined to be human serum albumin by sequencing. Band 6 had two sequences, intact hK2 and ACT, which indicates that this is intact hK2 bound to ACT.
The sequences in Fig. 3 indicate that the vast majority of the free hK2 is clipped at arginine 145 and that about 80% of the hK2 complex consists of inhibitor bound to the 10-kDa fragment of hK2. The relative percentage of hK2 in each band was calculated, based on the total pmol of all bands measured during sequence analysis. Band 1 contained 48 pmol of the 10-kDa hK2 fragment, which represented 73% of the total hK2 sequenced on this blot. The intact hK2 band (33 kDa) contained 13% of the total hK2. The hK2 attached to the blocked inhibitor (the 50- and 64-kDa bands) contained 12% of the total hK2. About 2% of the hK2 was present as a complex with ACT.

Identification of PI-6. To identify the blocked inhibitor, it was necessary to digest the complex with trypsin and sequence the internal peptide fragments. The 50-kDa hK2 complex band was used for digestion because the majority of the inhibitor was bound to the 10-kDa hK2 fragment. The tryptic fragments were separated by the capillary C18 reversed-phase HPLC microblotter and automatically blotted onto a PVDF membrane. The chromatogram showing the resolution of the tryptic digest of the hK2 complex is seen in Fig. 4. Each of the indicated peaks was sequenced. A database search of the internal sequences revealed that the other protein present in the hK2 complex was a serine protease inhibitor designated PI-6. A total of 21 peptide fragments of PI-6 were positively identified, comprising more than 70% of the total PI-6 protein mass. As expected, no peptide fragments of PI-6 blocked the NH2 terminus, or the fragments after the PI-6 residue 341 reactive site were detected. hK2 fragments were identified in peaks 2, 5, 15, 18, and 19. This represents five of the seven possible tryptic fragments of the hK2 146–237 fragment. No unidentified sequences were detected.

Discussion

We have identified a novel prostatic complex between hK2 and the cytoplasmic serine protease inhibitor PI-6. This is the first evidence of a tissue-specific complex of hK2 and has implications for the regulation of hK2 activity and function in the prostate. It is equally compelling that the inhibitor is identified as PI-6, a cytoplasmic inhibitor that has not been reported in association with a cancer marker. The reasons for the interaction between hK2 and PI-6 are less clear. hK2 has been shown to be secreted by mammalian cells and is thought to be an extracellular protease (12). The reaction between hK2 and PI-6 could therefore result from abnormal intracellular hK2 activation as part of a defective oncolytic pathway or from the leakage of PI-6 from damaged or necrotic neoplastic tissue. In the latter case, hK2-PI6 may exist in the interstitial spaces of cancerous tissue.

![Fig. 3. SDS-PAGE and NH2-terminal sequence of a blot of purified hK2 from prostate tissues. The purified hK2 was reduced and alkylated before SDS-PAGE. The sample contained 10 μg of total hK2. The blot was stained with Coomassie Brilliant Blue G-250, and the bands were sequenced through nine cycles. This is the same sample probed by Western blot in Fig. 2, Lanes 1 and 2. The hK2 complex bands at 50 and 64 kDa showed only hK2 sequences, indicating that the inhibitor was NH2-terminally blocked.](image)

![Fig. 4. Reversed-phase C-18 HPLC profile of the peptides generated after trypsin digestion of the 50-kDa hK2 complex band, as seen in Fig. 3. NH2-terminal sequencing of the peaks revealed 21 peptide sequences identical to the serpin PI-6. Peaks 2, 5, 15, 18, and 19 contained five of the seven tryptic fragments from the COOH-terminal half of hK2, residues 145–237. Aside from four tryptic fragments, no other sequences were detected. The position of the two internal dye markers is indicated by D.](image)
Whereas it is difficult to rule out the formation of hK2-PI-6 during the extraction procedure, protease inhibitors were added to minimize this possibility. In addition, the large percentage of clipped free hK2 and clipped hK2 attached to PI-6 suggests prior in vivo complex formation because clipped hK2 has been shown to be enzymatically inactive (14).

Two physiological forms of the hK2 complex have been reported: (a) a complex with PCI in seminal plasma (15); and (b) a complex with ACT in the serum of PCa patients (13). Thus, the major difference between the hK2 in tissues and seminal plasma is that the tissues contain the hK2-PI-6 complex, whereas seminal plasma contains hK2-PCI. No hK2-PCI complex was detected in tissue in our work. Similarly, no hK2-PI-6 was detected in pooled seminal plasma (data not shown), indicating that this inhibitor is not part of the normal pathway of PSA and hK2 secretion into the glandular lumen of the prostate. It is possible that seminal plasma levels of hK2-PI-6 could become detectable in patients with prostate disease.

As a potential serum marker, hK2-PI-6 may have a physiological significance that differs from complexes with ACT. PI-6 is intracellular; therefore, any hK2-PI-6 would most likely be of prostatic origin. By contrast, hK2-ACT could result from hK2 contact with the high levels of ACT in the serum.

PI-6 inhibits trypsin, thrombin, urokinase-type plasminogen activator, and factor 10a (7) and has also been shown to possess dual inhibitory activity toward chymotrypsin (16). However, in the current study, the chymotrypsin-like PSA did not show evidence of complex formation with PI-6 in tissue extracts. PI-6 has been shown to be localized cytoplasmically; therefore, its most likely physiological role would be expected to be intracellular protection against internal proteolytic damage by both trypsin and chymotrypsin-like proteases. However, no specific role or endogenous protease complex with PI-6 has been reported in any tissue thus far.

The complex between hK2 and PI-6 is an intriguing finding that raises a number of questions about both hK2 and PI-6. Is the level of PI-6 itself variable in tumor tissue compared to benign tissue? Is this inhibitor correlated with neoplastic development? It remains to be determined whether PI-6 or hK2-PI-6 is positively or negatively correlated with factors such as stage, metastatic potential, and patient outcome. PI-6 antibodies are currently in development and, together with our panel of hK2-specific mAbs, may help answer these questions.

References
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