Novel Peptide Inhibitors of Human Kallikrein 2*

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**The abbreviations used are:** PSA, prostate-specific antigen; hK2, human kallikrein 2; mAb, monoclonal antibody; TBS, Tris-buffered saline; ESA, bovine serum albumin; IFMA, immunoﬂuorometric assay; HPLC, high performance liquid chromatography; pNA, p-nitroanilide; TBTU, 2-(1H-benzotriazol-1-yl)-1,3,3-tetramethyluronium tetrafluoroborate.

Human kallikrein 2 (hK2) is a serine protease produced by the secretory epithelial cells in the prostate. Because hK2 activates several factors participating in proteolytic cascades that may mediate metastasis of prostate cancer, modulation of the activity of hK2 is a potential way of preventing tumor growth and metastasis. Furthermore, specific ligands for hK2 are potentially useful for targeting and imaging of prostate cancer and for assay development. We have used enzymatically active recombinant hK2 captured by a monoclonal antibody exposing the active site of the enzyme to screen phage display peptide libraries. Using libraries expressing 10 or 11 amino acids long linear peptides, we identified six different peptides binding to hK2. Three of these were shown to be specific and efficient inhibitors of the enzymatic activity of hK2 toward a peptide substrate. Furthermore, the peptides inhibited the activation of the proform of prostate-specific antigen by hK2. Amino acid substitution analyses revealed that motifs of six amino acids were required for the inhibitory activity. These peptides are potentially useful for treatment and targeting of prostate cancer.

Prostate cancer is the most common cancer of males in industrialized countries. Screening based on determination of serum prostate-specific antigen (PSA)3 can be used to detect early prostate cancer, which when localized to the prostate is potentially curable by radical prostatectomy or radiotherapy (1). However, 20–30% of the cancers detected by screening show extraprostatic spread and will eventually develop into advanced disease, which presently cannot be cured. More aggressive screening strategies are needed to catch these tumors at a curable stage (2), but this will lead to increased over-diagnosis of slowly growing tumors, only part of which need to be cured. It is, therefore, important to identify new therapeutic agents that could slow down the growth of prostate cancer so that clinical disease would not develop during the lifetime of the patient. Advanced disease can be treated by endocrine therapy, but androgen resistance develops within 2–5 years for which there is no effective treatment (3). Thus, novel pharmaceuticals need to be developed for treating both localized and metastatic prostate cancer.

The prostate produces several proteases that are secreted into seminal fluid. Two homologous serine proteases, PSA and hK2, have been shown to be highly prostate-specific (4–6). Even though PSA is a sensitive serum marker of prostate cancer, its expression is actually lower in malignant than in normal prostatic epithelium, and it is even lower in poorly differentiated tumors (7, 8). In contrast, the expression of hK2 is higher in aggressive tumors (9). hK2 has been shown to activate the proforms of PSA and urokinase type plasminogen activator (10) and to inactivate plasminogen activator inhibitor-1 (11), suggesting a possible role in a proteolytic cascade associated with prostate cancer. Thus, hK2 may promote tumor growth, and metastasis and inhibition of hK2 activity may, therefore, slow down tumor spread. Because hK2 is highly prostate-specific, inhibition of its activity is a potential way of preventing prostate cancer growth and metastasis without side effects outside the prostate.

Peptides are a potential alternative to monoclonal antibodies for treatment of cancer (12) and for diagnostic applications (13). Screening of random peptide libraries can be used to develop novel types of enzyme inhibitors, peptide substrates, and ligands binding to the active site of receptors (14). Recently developed peptide ligands binding specifically to PSA show novel types of binding specificity by reacting only with enzymatically active forms of free PSA (15, 16). By using these peptides a novel type of assay designated immunopeptidometric assay has been established (13). Thus, small peptides against prostate-specific proteases are potentially useful ligands for the development of novel diagnostic assays, but perhaps more importantly, they may be used for targeting of prostatic tumors. Because of their small size, peptides are not immunogenic and show faster tissue penetration than antibodies. They are easy to synthesize chemically and can be derivatized in several ways for labeling with isotopes (17) or toxins (18) or for modification of pharmacokinetics (19). Peptides modulating the enzymatic activity of proteases may inhibit the growth of cancer (15, 20). Radioactively labeled peptides are potentially useful for radioimaging, and peptide conjugates with cytotoxic drugs or boron compounds may be used to kill tumor cells (21).

In the present study we have identified novel hK2-binding peptides, of which some were efficient and specific inhibitors of hK2. The motifs required for inhibitory activity were characterized by amino acid substitution analysis, which also led to the identification of peptides with improved inhibitory capacity.

**EXPERIMENTAL PROCEDURES**

**Materials**—The monoclonal anti-PSA antibody (mAb) 5E4 (22) and recombinant wild-type hK2 (23) were produced as described. PSA was purified from human seminal plasma as described (24). ProPSA was isolated from LNCP4 cell culture media (16). A polyclonal antisera to
Novel Peptide Inhibitors of Human Kallikrein 2

M13 bacteriophage coat proteins (ab6188) was purchased from Abcam (Cambridge, UK). The proteinases bovine trypsin and α-chymotrypsin were obtained from Athens Research and Technology (Athens, GA). Human plasmin, thrombin, and plasma kallikrein were from Sigma. Recombinant human trypsin types 1, 2, 3, and C were produced in-house. The chromogenic substrates S-2586, S-2222 S-2238, S-2403, and S-2302 for chymotrypsin and PSA, trypsin, thrombin, plasmin, and kallikrein, respectively, were from Chromogenix Instrumentation Laboratory (Milano, Italy).

Phage Display Library Screening—The phage display libraries were constructed in FUSE-5-phage as previously described (25). We screened libraries with the structures CX-C, CX-C, Xα1, Xα1, CX-CX-CX-C, where C stands for cysteine, and X is any of the 20 randomly occurring natural amino acids. Screening was performed according to Koivunen et al. (26).

We screened each peptide library against hK2, which was captured by mAb 5E4 coated onto microtiter wells (15). mAb 5E4 binds PSA and hK2 in an equivmolar manner and reacts with an epitope, which is remote from the active site of these enzymes (22, 27). Twenty-four ng of hK2 was incubated in antibody-coated wells for 30 min at 37 °C in 100 μl of 0.05 M Tris buffer, pH 7.7, containing 0.154 M NaCl and 8 mM NaN₃ (TBS) followed by washing to remove unbound hK2. An aliquot of each phage library containing 10⁹-10¹ⁱ transducing units was added into the microtiter wells in 100 μl of TBS containing 10 g/liter bovine serum albumin (BSA). Incubation took place at 4 °C with gentle shaking for 16–24 h in the first round and for 1 h for the following three rounds. The wells were washed 10 times with TBS containing 0.5% Tween 20. To elute bound phages 100 μl of 0.1 M glycine-HCl, pH 2.2, containing 1 g/liter BSA was added. The eluted phage suspension was neutralized with 1 M Tris buffer, pH 9.0, amplified by infection of kanamycin-resistant Escherichia coli K91 cells and purified by polyethylene glycol precipitation (15). After four rounds of selection and amplification, single-stranded DNA from individual phage clones was prepared. The sequences encoding peptides were determined after amplification by PCR (28). The relevant part of the viral DNA was sequenced with an ABI 310 Genetic analyzer and the Dye Terminator Cycle Sequencing Core kit (PE Applied Biosystems, Foster City, CA) using as a primer 5'-TAA TAC GAC TCA CTA TAG GGC AAG CTG ATA AAC CGA-3'.

Immunoﬂuorometric Assay (IFMA)—The phage IFMA was performed essentially as described (15). The solid phase antibody (5E4) was coated onto microtiter wells at a concentration of 5 mg/liter in TBS for 16 h at 22 °C, the solution was discarded, and the wells were saturated 10 g/liter BSA in TBS for 3 h at 22 °C. In the assay 50 ng of hK2 in 200 μl of assay buffer (TBS, pH 7.7, containing 2.3 g/liter bovine serum albumin and 0.15 g/liter bovine globulin) was captured onto wells coated with mAb 5E4. After washing, 2.5 μl of peptide (10⁻⁵⁻¹⁰⁻⁶) transducing units was added into wells in which hK2 had been captured by mAb 5E4. After incubation for 30 min, phage (10⁻¹²–10⁻¹¹ units) was added, and after 1 h incubation the wells were washed, and bound phage were detected as described above for phage IFMA.

Peptide Synthesis—Peptides hK2p01–hK2p06 (Table 1) were synthesized with an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Kentucky) with Fmoc (N-(9-fluorenylethoxycarbonyl) strategy and TBTU/disopropylethanolamine as coupling reagents. All peptides were synthesized on Gly-Wang resin (Advanced ChemTech). The side-chain protecting groups used in synthesis were t-butyloxycarbonyl for Trp and Lys, tert-buty1 for Ser and Thr, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonil for Arg, and trityl for Gln. For Cys both acetamidomethyl and trityl protection groups were used. The peptides were purified by HPLC (Shimadzu, Kyoto, Japan) on a C₁₈ reverse phase column with acetonitrile as eluent (0.1% trifluoroacetic acid in H₂O, 0–60% acetonitrile gradient for 60 min). Alain replacement experiments were done for peptides hK2p01–hK2p03 by changing each of the amino acids to alanine individually.

Peptides containing cysteines (hK2p04, -p05, and -p06) were cyclized by the iodination method (30). Lyophilized peptides were dissolved in 50% acetic acid at a concentration of 2 g/liter. 1 M HCl (0.1 ml/mg of peptide) was added followed immediately by 0.1 M iodine solution in 50% acetic acid (molar excess of the iodine/acetamidomethyl group). The solution was stirred vigorously at room temperature for 40 min. The reaction was stopped with 0.1 M sodium thiosulfate. After filtering through a 0.45-μm disposable polypropylene filter (Puradisc, Whatman), the peptides were purified by HPLC as described above. The structure of each peptide was verified by mass spectrometry on a Biflex III matrix-assisted laser desorption ionization time-of-flight (Bruker, Germany), and the purity was determined by analytical HPLC with a 240 × 1.4-mm C₁₈ column eluted with 0–60% acetonitrile in 0.1% trifluoroacetic acid for 30 min.

Effect of the Peptides on the Enzymatic Activity of hK2—The effect of the peptides on the enzymatic activity of hK2 was studied by using the chromogenic substrate S-2302 (H-D-Pro-Phe-Arg-pNA (H means open primary amine, and D means right-handed (steroisomer)). hK2 (0.17 μM) was incubated with a 100–1000-fold molar excess of synthetic peptide in 20 μl Tris buffer, pH 8.0, containing 0.1% BSA for 30 min at room temperature. After the addition of the substrate to a final concentration of 0.2 μM, the absorbance at 405 nm was monitored at 5–10–min intervals for 1 h with a Victor 1420 Multilabel fluorometer. Student’s t test was used to evaluate the significance of the hK2 inhibition by the peptides. For this, 12 replicates were analyzed.

The inhibitory capacity of the peptides was compared also by determining IC₅₀ for hK2 inhibition, determined based on the velocity of S-2302 cleavage at 200 μM concentration by 0.17 μM hK2 in the presence of 0–400 μM peptide. In addition, the Kᵢ value for the most potent inhibitory peptide was determined by a Lineweaver-Burk plot after determining the velocity of the cleavage of 0–400 μM S-2302 substrate by 0.2 μM hK2 in the presence of 4 μM of peptide.

The specificity of the peptides was analyzed by studying their effect on the activity of other serine proteases including α-chymotrypsin, bovine trypsin, human trypsin 1, 2, 3, and C, urokinase, thrombin, plasmin, plasma kallikrein, and PSA. The assay was performed as described for hK2, but the substrate for PSA and α-chymotrypsin was S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA), for urokinase it was S-2444 (pyroGlu-Gly-Gly-Arg-pNA), for thrombin it was S-2238 (H-D-Phe-pipolic acid-Arg-pNA), for plasmin it was S-2403 (pyroGlu-Phe-Lys-pNA), for plasma kallikrein it was S-2302 (H-D-Phe-Pro-Arg-pNA), and for the various trypsin isoenzymes it was S-2222 (CO-Ile-Glu-(OR)-Gly-Arg-pNA).
Cleavage of the Peptides by Trypsin—Cleavage of the peptides by trypsin was studied by incubating 0.1 mM synthetic peptides with 3.9 nM bovine trypsin in 20 mM Tris-buffer, pH 8.0, containing 0.1% BSA for 30 min at room temperature. The reaction mixture was applied on a Nova-Pack C\textsubscript{18} column (3.9 × 75 mm, Waters, MA) that was connected to an ÄKTA purifier system (Amersham Biosciences) and eluted with 0–70% acetonitrile in 0.1% trifluoroacetic acid for 30 min.

Effect of the Peptides on the Activation of proPSA by hK2—hK2 is known to activate proPSA (31). To determine whether the peptides are able to inhibit this activity, the hK2 binding peptides, hK2p01, hK2p02, and hK2p03, were incubated with hK2 for 50 min at room temperature in TBS buffer containing 1 g/liter BSA before the addition of proPSA. Concentrations of peptides, hK2, and proPSA were 0–100 μM, 7.5 nM, and 150 nM, respectively, in a final volume of 30 μl. After incubation for 2 h, 10 μl of 2.4 μM fluorogenic PSA substrate (4-morpholinecarbonyl-HSSKLQ-amidomethylcoumarin (JPT custom peptide (32)) was added. Fluorescence (excitation at 355 nm and emission at 460 nm) was measured after 17.5 h of incubation in a Victor 1420.

Similarity Searches—BLAST searches (www.ncbi.nlm.nih.gov/BLAST) (33) using the Swiss-Prot data base were performed to find if the hK2 binding sequences display sequence similarity with previously identified human peptides or proteins.

RESULTS

Isolation and Structure of the Peptides—Screening with six different phage display peptide libraries revealed phages reacting with hK2 in libraries with the structures X\textsubscript{10} and X\textsubscript{11}. Among 24 isolates from the X\textsubscript{10} library, four different hK2-binding peptides were identified, designated hK2p01–hK2p04, of which the most prevalent one was hK2p02 (AARRPPFPAPST). Three of the peptides were linear (hK2p01–hK2p03), whereas hK2p04 contained two Cys residues, suggesting that it forms a cyclic structure through a disulfide bridge. Among 10 isolates from the X\textsubscript{11} library, we identified two peptide sequences (FRTCLRFRQGCWVIT and CYRMPTCMQRD) containing two Cys residues, suggesting that they also are cyclic. Common motifs can be found among the peptides, i.e. peptides hK2p02 and hK2p03 both contain the motif AARRFP and when the three cyclic peptides, hK2p04, hK2p05, and hK2p06, are aligned according to the location of the disulfide bridges, they all have an Arg residue as the third residue after the first Cys residue. Furthermore, in peptides hK2p05 and hK2p06, the second Cys residue is followed by a Met residue. The Arg residue, which is present in all peptides, is known to be located in the P1 position of hK2 substrates (34).

Inhibition of Phage Binding with Synthetic Peptides—The relative binding specificity of the five peptides was studied by inhibition experiments using phage-displayed and synthetic peptides. Synthetic peptides hK2p01, -02, and -03, unlike hK2p04 and -06, inhibited the binding of the phage carrying the corresponding peptide as part of pIII protein showing that the peptides were active without the protein fusion part (Fig. 1). Phage hK2p05 showed weak binding in phage IFMA and was not further studied. In cross-inhibition experiments, synthetic peptides hK2p01–hK2p03 efficiently inhibited the binding of all five phages studied, indicating that they bind to the same region or close to each other on hK2 (Fig. 1).

Effect of the Peptides on Enzymatic Activity of hK2—Peptides hK2p01, hK2p02, and hK2p03 caused a significant (p < 0.001) reduction in the enzymatic activity of hK2 (Fig. 2, Table 1), but their inhibitory activities were different (for all p < 0.001), with hK2p01 the most effective. When

### TABLE 1

| Code    | Display library | Peptide sequence       | No. of isolates | IC\textsubscript{50} (μM) |
|---------|-----------------|------------------------|----------------|---------------------------|
| hK2p01  | X\textsubscript{10} | SFKVFVWAAG              | 1              | 3.4                       |
| hK2p02  | X\textsubscript{10} | AARRPPFPAPST            | 19             | 30.5                      |
| hK2p03  | X\textsubscript{10} | PARRPPFPAPST            | 1              | 162.0                     |
| hK2p04  | X\textsubscript{10} | CFRQGCWVIT              | 3              |                           |
| hK2p05  | X\textsubscript{11} | FRTCLRFRQGCWVIT         | 6              |                           |
| hK2p06  | X\textsubscript{11} | CYRMPTCMQRD             | 4              |                           |
Novel Peptide Inhibitors of Human Kallikrein 2

The enzymatic activity of PSA was measured with a specific fluorogenic substrate in the presence or absence of hK2 and hK2-inhibiting peptides (data not shown). The enzymatic activity of PSA was measured with a specific fluorogenic substrate in the presence or absence of hK2 and hK2-inhibiting peptides (n = 8, mean ± S.E.). Using 0.17 μM hK2, inhibition was observed with a 100-fold molar excess of peptides, and with a 1000-fold excess inhibition it was 70–80%. Peptides hK2p04 and hK2p06 did not affect the enzymatic activity of hK2 (data not shown). None of the peptides showed inhibition of the other serine proteinases studied. Fractionation of the reaction mixture containing trypsin and the hK2-binding peptides by C18 chromatography showed that the peptides were not degraded by bovine trypsin (data not shown).

Effect of the Peptides on the Activation of ProPSA by hK2—Incubation of proPSA with hK2 increased PSA activity (Fig. 3), and this activation was inhibited in a dose-dependent manner by the peptides hK2p01, hK2p02, and hK2p03, indicating that they inhibit the activity of hK2 toward proPSA. IC50 values were 2.6 ± 0.1 μM for hK2p01, 6.5 ± 0.3 μM for hK2p02, and 18.6 ± 1.0 μM for hK2p03 (mean ± S.E., n = 8, p < 0.001 between all, Student’s t-test). proPSA or hK2 alone had no enzymatic activity toward the PSA-specific fluorogenic substrate.

Amino Acid Substitution and Deletion Analyses—To define the motifs required for inhibitory activity of the peptides, amino acid substitution and deletion analyses were performed for peptides hK2p01–hK2p03. In the deletion analysis, each peptide was shortened one amino acid at a time either from the NH2 or COOH terminus (Table 2). In peptide hK2p01 (SRFKVWWAAG) Ala replacement of Arg-2, Phe-3, Lys-4, Val-5, Trp-6, and Trp-7 strongly reduced inhibition of hK2, and maximal inhibition is achieved with the ARFKVW- motif, which is present in both hK2p02 and hK2p03, respectively, without significant reduction of activity. Thus, the ARRPXP motif, which is present in both hK2p02 and hK2p03, is essential for hK2 inhibition.

Sequence Similarity by BLAST Searches—In the Swiss-Prot data base 13 proteins containing the ARRP motif (8) were identified. Sequence similarity searches were performed for the hK2-inhibiting peptides. Inhibition was observed with a 100-fold molar excess of hK2p01, and 18.6 μM for hK2p03 (mean ± S.E., n = 8, p < 0.001 between all, Student’s t-test). proPSA or hK2 alone had no enzymatic activity toward the PSA-specific fluorogenic substrate.

Amino Acid Substitution and Deletion Analyses—To define the motifs required for inhibitory activity of the peptides, amino acid substitution and deletion analyses were performed for peptides hK2p01–hK2p03. In the deletion analysis, each peptide was shortened one amino acid at a time either from the NH2 or COOH terminus (Table 2). In peptide hK2p01 (SRFKVWWAAG) Ala replacement of Arg-2, Phe-3, Lys-4, Val-5, Trp-6, and Trp-7 strongly reduced inhibition of hK2, suggesting that these residues are key mediators of the activity. Deletion of Ser-1 led to partial loss of activity, whereas replacement of it with Ala increased the inhibitory activity of the peptide, and IC50 decreased from 3.4 to 2.2 μM. This peptide showed the strongest inhibition with a Ks value of 1.41 μM. As determined by Lineweaver-Burk plot analysis, the peptide is a competitive inhibitor. Thus, some mutations in the NH2-terminus of amino acid are tolerated, but it is important that a spacer amino acid is included before Arg-2. Deletion of the NH2-terminus SR motif abolished the activity, confirming the essential role of the second residue. Deletion analysis further showed that the AAG motif in the COOH terminus of hK2p01 is not required for inhibition. The RFKXWW motif is required for inhibition of hK2, and maximal inhibition is achieved with the ARFKVWWWAG peptide.

In peptides hK2p02 (AARRPFPAAPS) and hK2p03 (PARRPFVPVTAG), substitution of Arg-3, Arg-4, Pro-5, and Pro-7 with Ala strongly reduced the inhibitory activity, whereas substitution of Pro-1 or Val-8 in hK2p03 slightly increased activity (Table 2). Deletion of Pro from the NH2 terminus of hK2p02 led to partial reduction in activity, whereas deletion of PA abolished activity. The APS and VTA motifs could be deleted from hK2p02 and hK2p03, respectively, without significant reduction of activity. Thus, the ARRPXP motif, which is present in both hK2p02 and hK2p03, is essential for hK2 inhibition.

### Table 2

| Sequences (NH2-terminus) | IC50 (μM) | % Inhibition |
|--------------------------|-----------|--------------|
| hK2p01 derivatives       |           |              |
| SRFKVWWAAG               | 1207.35   | 79           |
| ARFKVWWAAG               | 1191.35   | 94           |
| SRAFKVWWAAG              | 1122.24   | 27           |
| SRAPVWWAAG               | 1131.25   | 0            |
| SRFAVWWAAG               | 1150.26   | 0            |
| SRAPKWWAAG               | 1179.30   | 39           |
| SRAFPWWAAG               | 1092.22   | 10           |
| SRAPFWWAAG               | 1092.22   | 30           |
| SRFKVWWAAG               | 1207.35   | 78           |
| RPKVWWAAG                | 1120.27   | 22           |
| RPKVWWAAG                | 964.08    | 0            |
| RAKVWWAAG                | 589.60    | 0            |
| SRFKVWWAAG               | 1136.27   | 80           |
| SRAFKVWWAAG              | 1065.19   | 79           |
| SRAFKVWWAAG              | 878.98    | 8            |
| SRAFKVWWAAG              | 692.77    | 7            |
| SRAFVGG                  | 593.64    | 0            |
| hK2p02 derivatives       |           |              |
| GAARRPPFPAPG             | 1183.34   | 77           |
| GAARRPPFPAPG             | 1098.23   | 0            |
| GAARRPPFPAPG             | 1098.23   | 0            |
| GAARRPPFPAPG             | 1157.30   | 0            |
| GAARRPPFPAPG             | 1107.24   | 75           |
| GAARRPPFPAPG             | 1157.30   | 24           |
| GAARRPPFPAPG             | 1157.30   | 69           |
| GAARRPPFPAPG             | 1167.43   | 78           |
| RRPPAP                   | 595.70    | 0            |
| RRPPF                    | 671.80    | 0            |
| RRPPA                    | 555.60    | 0            |
| RRPPQ                    | 631.70    | 0            |
| GRARPAPG                 | 709.74    | 0            |
| GRARPFPQ                 | 785.84    | 0            |
| AARRPPAP                 | 794.88    | 36           |
| AARRAPPAG                | 865.96    | 51           |
| AARRAPPFG                | 870.98    | 28           |
| AARRAPPFG                | 942.06    | 37           |
| hK2p03 derivatives       |           |              |
| GPARRPFVPVTAG            | 1225.42   | 49           |
| GPARRPFVPVTAG            | 1199.38   | 61           |
| GPARRPFVPVTAG            | 1140.31   | 0            |
| GPARRPFVPVTAG            | 1140.31   | 0            |
| GPARRPFVPVTAG            | 1199.38   | 0            |
| GPARRPFVPVTAG            | 1149.32   | 49           |
| GPARRPFVPVTAG            | 1199.38   | 17           |
| GPARRPFVPVTAG            | 1197.36   | 58           |
| GPARRPFVPVTAG            | 1195.39   | 45           |
| GPARRPFVPVTAG            | 1154.28   | 48           |
| GPARRPFVPVTAG            | 1053.17   | 37           |
| GPARRPFVPVTAG            | 954.04    | 33           |
| GPARRPFVPVTAG            | 856.92    | 0            |
| GPARRPFVPVTAG            | 709.74    | 4            |
| GPARRPG                  | 612.62    | 8            |
| GPARG                    | 456.43    | 0            |
| GPARRPFVPVTAG            | 1225.36   | 58           |
| GPARRPFVPVTAG            | 1128.24   | 27           |
| GRRPPFPVTAG              | 1057.16   | 2            |
| GRRPPFPVTAG              | 900.97    | 0            |
| GPPFPVTAG                | 744.78    | 0            |
| GPPFPVTAG                | 647.66    | 0            |
| GPTAG                    | 500.48    | 0            |
| GVTAG                    | 403.36    | 0            |
peptides needed for hK2 inhibition, were found. These proteins included serine/threonine protein kinase p21-activated kinase 4 (PAK4) (GenBank™ accession code O96013), cytokine receptor-like factor 1 precursor (GenBank™ accession code O75462), and substance P part of protachykinin 1 precursor (GenBank™ accession code P20366). The rest of the proteins containing the motif were integral membrane proteins, cytoplasmic and nuclear proteins. There were no exact matches with the other hK2 inhibitory sequence RFFXWW.

**DISCUSSION**

We isolated several peptides binding to hK2 using phage display peptide libraries. Most of the peptides specifically inhibited the enzymatic activity of hK2. We have previously identified specific PSA-binding peptides by using the same methodology (15), but contrary to the hK2-inhibiting peptides the PSA-binding peptides enhance the enzyme activity of PSA. Despite the high sequence similarity (79%) between hK2 and PSA, there was no similarity between the PSA- and hK2-binding peptides.

A critical factor for the screening of the libraries for both PSA- and hK2-binding peptides was the capture of the target enzyme with a mAb, which presents the active site of these enzymes efficiently to the potential ligands. When PSA or hK2 were directly absorbed to the solid phase, no specific peptides were obtained, suggesting that absorption to plastic destroys or hides the active site of these enzymes.

Six different peptides were isolated, and some recurring motifs were identified in their sequences. Three of the peptides contained two Cys residues, which can form a disulfide bridge leading to formation of a peptide with a cyclic structure. All cyclic peptides contained a motif of the three linear peptides, two were highly similar, containing the ARRP motif. Even though we identified three hK2-binding peptides with cyclic structures from X10 and X11 libraries, we could not isolate any such peptides from the libraries expressing peptides constrained into a cyclic form by engineered Cys residues. It is noteworthy that the cyclic peptides identified had no effect on hK2 activity, and except hK2p06, all showed only weak binding to hK2. The constrained libraries contained peptides having 3, 7, 8, or 11 residues between the Cys residues, but in the peptides selected from non-constrained libraries the loops had 4–5 amino acids between the Cys residues. Thus, it is possible that a cyclic peptide reacting with hK2 requires a small loop with a size of 4–5 amino acid residues. Another possible reason for the lack of hK2-binding peptides in the constrained libraries is that the linear peptide libraries were more diverse than the cyclic peptide libraries. A common factor for all of the six peptides isolated was the presence of one or two Arg residues. This is not surprising since hK2 is a trypsin-like serine proteinase cleaving its substrates after arginine residues (35, 36). However, the method used does not facilitate identification of substrates because it requires strong binding between the phage and hK2 rather than cleavage by hK2.

Because the Arg residue is a potential cleavage site also for trypsin, we analyzed whether trypsin cleaves the peptides. Although no cleavage was observed under these conditions, our preliminary analyses suggest that the peptides are not very stable against proteolytic degradation. However, by various modifications the stability can be markedly improved without loss of activity. The presence of an Arg residue in all six peptides identified suggests that it is an important mediator of the binding, which was confirmed by amino acid substitution analyses. Based on substitution and deletion analyses, the optimal motifs for inhibition of hK2 are RFFXWW or ARRPXP. Stronger inhibition was observed if certain spacer residues were included around the motifs, e.g. the strongest inhibitor identified among all peptides evaluated was the ARFKVWW derivative of hK2p01. Lineweaver-Burk analyses showed that this peptide is a competitive inhibitor of hK2, suggesting that it binds to the active site. The enzymatic activity of hK2 was inhibited in a dose-dependent manner by 17–170 μM concentrations of peptide. These concentrations are similar to those required for PSA-binding peptides to enhance the enzymatic activity of PSA (15) or for other peptides selected from phage display libraries used to inhibit other proteases (37) and ligand-receptor binding (28). The hK2-inhibiting peptides did not show any significant effect on the activity of PSA, chymotrypsin, and various trypsin isoenzymes, showing that the peptides act specifically on hK2. The three peptides (hK2p04–hK2p06) containing two Cys residues showed no inhibitory effect on hK2.

hK2 has previously been shown to activate the zymogen forms of PSA (31). In our study inhibition of hK2 with peptides hK2p01, hK2p02, and hK2p03 resulted in reduced activation of proPSA, showing that they are also capable of inhibiting hK2 activity toward a natural protein substrate. Although the IC50 values of inhibition of proPSA activation were somewhat different than those acquired using small peptide substrate, the order of the effectiveness of the peptides was the same with both substrates. If hK2-inhibiting peptides would be used for treatment of prostate cancer, e.g. via inhibition of proteolytic cascades essential for cancer growth and metastasis, it might be beneficial that the peptides also inhibit PSA activation by hK2. PSA has been suggested to cleave fibronectin and laminin, leading to extracellular matrix degradation, which might promote prostate cancer growth and metastasis (38). However, it is noteworthy that hK2 is not the only PSA activator in prostate, and PSA has also been suggested to inhibit the growth of prostate cancer by releasing angiostatin from plasminogen (39, 40).

To our knowledge, this is the first report describing development of specific peptide inhibitors of hK2. Specific peptide substrates have been developed previously for hK2 by substrate phage display (34), and based on these, hK2-specific inhibitors have been developed by site-specific mutations of the bait region of the serpin α1-antitrypsin (41). Comparison of the sequences of the substrate peptides with those of our inhibitory peptides does not show any significant sequence similarity except that Arg was present in all the peptides isolated in both studies. However, the specific hK2 inhibitors designated rACTT2a and rACTT3b have similar inhibitory activities as hK2p01 and hK2p02, respectively. A search of the peptide sequences against Swiss-Prot data base revealed that the essential motif, ARRPXP, present in peptides hK2p02 and hK2p03, is found in serine/threonine protein kinase PAK4, which is highly expressed in testis, colon, and in the prostate. This cytoplasmic protein has been shown to activate the c-Jun NH2-terminal kinase (JNK) pathway and to be involved in actin cytoskeleton reorganization and filopodia formation (42). The motif is also found in the signal sequence of human cytokine receptor-like factor 1, and therefore, it probably does not affect the activity of hK2. Protachykinin 1 also contains the sequence ARRPXP. Previous immunohistochemical studies show presence of substance P in the human prostate (43). However, its effect on regulation of hK2 has not been studied.

Active PSA and hK2 show 79% amino acid sequence identity, and it has been difficult to develop specific antibody-based assays for hK2. The hK2-binding peptides present a possible solution to this problem. Synthetic peptides have been used in combination with an antibody to develop an immunopeptidometric assay, as we have previously reported for PSA (13). Detection of hK2 is important, as hK2 has been shown to...
be useful for prostate cancer diagnosis (44, 45). Measurement of active hK2 may further improve the diagnostic accuracy, as is the case with various forms of PSA (46, 47).

There is evidence that hK2 expression is up-regulated in aggressive prostate cancer, and hK2 has been shown to activate several factors mediating cancer spread (48). The peptides developed represent a new type of ligand, which inhibits the activity of hK2. They are, therefore, potentially useful agents for inhibiting growth and spread of prostate cancer. Thus, these peptides may be useful in prostate cancer treatment as such or in combination with other ligands modulating the activity of factors mediating tumor growth. Furthermore, they are potentially useful as radioactively labeled derivatives for tumor imaging and, if conjugated with cytotoxic drugs, for targeted killing of tumor cells.

In conclusion, we have identified two different types of hK2-binding peptides; linear peptides, which inhibit the activity of hK2, and cyclic gated with cytotoxic drugs, for targeted killing of tumor cells.

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