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Specificity Profiling of Seven Human Tissue Kallikreins Reveals Individual Subsite Preferences*

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Human tissue kallikreins (hKs) form a family of 15 closely related (chymo)trypsin-like serine proteinases. These tissue kallikreins are expressed in a wide range of tissues including the central nervous system, the salivary gland, and endocrine-regulated tissues, such as prostate, breast, or testis, and may have diverse physiological functions. For several tissue kallikreins, a clear correlation has been established between expression and different types of cancer. For example, the prostate-specific antigen (PSA or hK3) serves as tumor marker and is used to monitor therapy response. Using a novel strategy, we have cloned, expressed in *Escherichia coli* or in insect cells, refolded, activated, and purified the seven human tissue kallikreins hK3/PSA, hK4, hK5, hK6, hK7, hK10, and hK11. Moreover, we have determined their extended substrate specificity for the nonprime side using a positional scanning combinatorial library of tetrapeptide substrates. hK3/PSA and hK7 exhibited a chymotrypsin-like specificity preferring large hydrophobic or polar residues at the P1 position. In contrast, hK4, hK5, and less stringent hK6 displayed a trypsin-like specificity with strong preference for P1-Arg, whereas hK10 and hK11 showed an ambivalent specificity, accepting both basic and large aliphatic P1 residues. The extended substrate specificity profiles are in good agreement with known substrate cleavage sites but also in accord with experimentally solved (hK4, hK6, and hK7) or modeled structures. The specificity profiles may lead to a better understanding of human tissue kallikrein functions and assist in identifying their physiological protein substrates as well as in designing more selective inhibitors.

The tissue kallikreins constitute a subgroup of the chymotrypsin-like serine proteinase family S1A of clan PA(S) (1). They are highly homologous to the "true tissue kallikrein" K1, which is encoded in mammals, for example, by the human KLK1 or the mouse Klk1 genes (2). The 15 human KLK genes code for the classical tissue kallikreins hK1, hK2, and the prostate-specific antigen (PSA,[5] hK3) as well as for the 12 more recently discovered so-called new tissue kallikreins, hK4 to hK15, named in chronological order of their characterization (3–7). These genes share common characteristics such as a similar exon/intron organization and encode single-chain pre-pro-proteinases, which consist of a signal peptide, a generally short, 4–9-residue-long pro-peptide (only hK5 harbors a rather long propeptide of 37 residues), and a chymotrypsin- or trypsin-like catalytic domain (Fig. 1) with amino acid sequence identity of about 80% for the classical and 40% among the new tissue kallikreins, respectively (8, 9). According to phylogenetic analyses, hK1, hK2, and hK3 form a subgroup of particularly closely related proteinases, whereas the tissue kallikreins hK4-hK15 diverge into several subgroups, namely hK4/hK5/hK7, hK6/hK13/hK14, hK8/hK10/hK12, and hK9/hK11/hK15 (2, 10). In Table 1, the 15 human tissue kallikreins are listed together with their synonyms, their primary tissue localization, and their data base accession numbers (11).

In healthy humans most tissue kallikreins are expressed in various tissues, such as skin, pancreas, salivary gland, and central nervous system (10) as well as in the endocrine-regulated tissues testis, prostate, and breast, whereas hK2 and hK3 are only expressed in the prostate. For example, hK6 has been found in glandular epithelia of breast, prostate, endometrium, colon, and pancreas and in the central nervous system as well as in milk and cerebrospinal fluid (9). This expression in a wide range of tissues suggests involvement in a variety of physiological and pathological processes (12). Unfortunately, only for a few tissue kallikreins a distinct biological function has yet been established. For example, hK1 plays a role in blood pressure regulation, exerting its biological activity mainly via release of the vasoactive kinin Lys-bradykinin (kallidin) from low molec-

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[5] The abbreviations used are: PSA, prostate-specific antigen; hK, human tissue kallikrein; EK, enterokinase; AMC, 7-amino-4-methylcoumarine; uPA, urokinase-type plasminogen activator; PS-SCL, positional scanning with a synthetic combinatorial peptide library; BPTI, bovine pancreatic trypsin inhibitor.

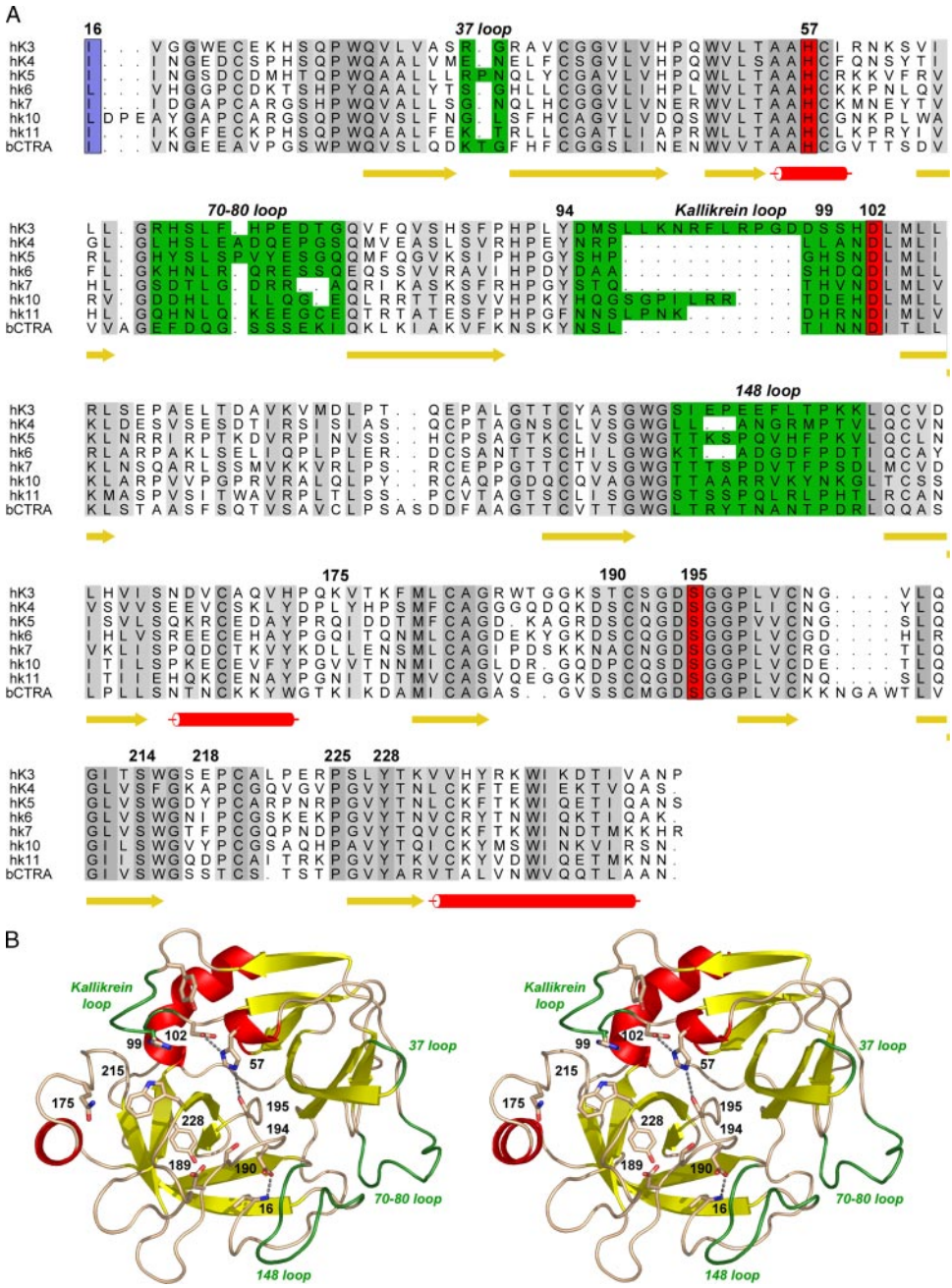

FIGURE 1. **Primary and tertiary structures of tissue kallikreins.** *A*, multiple sequence alignment for human hKs 3, 4, 5, 6, 7, 10, 11, and bovine chymotrypsin, according to the observed or anticipated topological similarities. The degree of conservation is indicated by increasingly *darker shades of gray*, whereas the N-terminal residues are *highlighted* in *blue*, and the catalytic triad residues are in *red*. Further residues that are significant for the discussion of the structure function-relationship of the tissue kallikreins are labeled according to the chymotrypsin numbering. α-Helices and β-sheets are indicated below the sequences. *B*, stereo ribbon plot of hK6 (34), shown in standard orientation, as an example for a typical tissue kallikrein structure. β-Strands and α-helices are shown as *yellow arrows* and *red ribbons*, important loops are colored *green*, and a few side chains addressed in the text are given as *full-length stick models*.

ular weight kininogen, but is also implicated in various other physiological processes (13). hK3/PSA has been shown to rapidly hydrolyze the seminal vesicle proteins semenogelin I and II, resulting in liquefaction of the ejaculated seminal plasma clot (14), whereas hK2 (15, 16) and hK4 (17) apparently activate pro-hK3/PSA *in vitro*, suggesting a physiological regulatory mechanism. hK4 and hK8 seem to be important for tooth development and neural plasticity, like their putative murine or porcine orthologs enamel matrix serine proteinase and neuropsin (18–20), and hK5 and hK7 as well as hK14 are involved in skin desquamation and inflammatory processes (21, 22).

Much more evident is the association of these tissue kallikreins with endocrine-related malignancies. Several tissue kallikreins are differentially expressed in hormone-dependent prostate, ovarian, and breast cancers, rendering them useful diagnostic and prognostic markers for these diseases (11). hK3/PSA is widely used as a tumor marker for prostate cancer screening, diagnosis, and for monitoring therapy response (23). Some of the more recently discovered tissue kallikreins are clear indicators of poor or favorable prognosis (24) for ovarian, breast, prostate, and testicular cancers so that in the future they might become useful clinical biomarkers (25). Data are accumulating that some of these tissue kallikreins are also implicated in other human diseases, such as hK1 in inflammation and hypertension, hK4 in dental diseases (26), hK6 and hK8 in central nervous system inflammatory diseases (27), and hK7 in skin diseases (28). Therefore, these tissue kallikreins might be valuable therapeutic targets, and modulation of their proteolytic activity by selective inhibitors may prevent development or progression of diseases (29).

The tissue kallikreins are synthesized as inactive pre-pro-enzymes, secreted as inactive zymogens, and activated autocatalytically or by other proteinases including tissue kallikreins in novel cascade-like activation pathways (9). The majority of these tissue kallikreins exhibit a preference for basic P1 residues, whereas some tissue kallikreins cleave in a more chymotrypsin-like manner. In all tissue kallikreins, the substrate specificity additionally depends on the amino acids located more distant from the scissile peptide bond (30, 31), that is to say, they recognize their respective substrate via extended substrate-enzyme interactions. Several potential protein substrates have been identified *in vitro*. Further physiological substrates might be derived from the substrate cleavage preference presented in our work.

A full understanding of the functional properties of the tissue

TABLE 1
Human tissue kallikrein members, their main tissue expression, and GenBank™ accession numbers (8, 24, 78)

	Systematic names and synonyms	Tissue localization	GenBank™ accession number	References
hK1	Pancreatic/renal kallikrein	Kidney, pancreas, salivary gland	M25629	79
hK2	Human glandular kallikrein 1	Prostate	M18157	80
hK3	PSA	Prostate	X05332	81
hK4	Prostase, enamel matrix serine protease 1, KLK-L1	Prostate, teeth	AF113141	82
hK5	Human stratum corneum tryptic enzyme, KLK-like 2	Testis, breast, skin	AF168768	3
hK6	Protease M, neurosin, zyme, myelencephalon-specific protease	Brain, pancreas, kidney	U62801	83
hK7	Human stratum corneum chymotryptic enzyme	Pancreas, skin	L33404	84
hK8	Neuropsin, ovasin, brain serine protease 1	Pancreas, brain, skin	AB009849	20
hK9	KLK-like 3	Pancreas	AF135026	4
hK10	Normal epithelial cell-specific 1	Pancreas, breast, ovary colon, intestine, testis, lung	AF024605	85
hK11	Trypsin-like serine protease, hippostasin	Pancreas, skin, prostate, heart, testis, brain	AB013730	66
hK12	KLK-like 5	Pancreas	AF135025	4
hK13	KLK-like 4	Pancreas, esophagus, appendix	AF135024	86
hK14	KLK-like 6	Brain bone marrow, fetal liver	AF283669	35
hK15	Prostinogen	Thyroid gland, prostate	AF242195	33

kallikreins requires knowledge of both their three-dimensional structures and their extended substrate specificity. Crystal structure coordinates of mature human tissue kallikreins are currently available only for hK1 (Protein Data Bank code 1SPJ (32)), hK4,[6] hK6 (1LO6 (34)), and hK7,[7] besides a few porcine (pK1, 2PKA and 2KAI (36, 37), horse, 1GVZ (38)), rat (1TON (39)), and mouse glandular tissue kallikreins (mgK13, 1AO5 (40, 41)). The extended substrate specificity has been assayed for hK3 (42) and hK5 (43) and more extensively and comprehensively for hK4 (44) and hK14 (45). To learn more about the molecular properties of these tissue kallikreins, to gain more insights into the mechanisms whereby these tissue kallikreins contribute to cancer growth, and to offer a basis for rational drug design for developing selective inhibitors, we sought to characterize a number of tissue kallikreins by expressing their active species and determining their non-prime specificity using peptide positional-scanning methods.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—On the basis of the known coding sequences of the human tissue kallikreins (Table 1), appropriate PCR primers were designed and synthesized (Medigenomix GmbH). The full-length tissue kallikrein cDNAs were isolated in our laboratory from ovarian and breast cancer tissue or in the case of PSA from prostate cancer cells. The cDNAs were used as PCR templates for amplification of the DNAs coding for the mature tissue kallikrein domains. All enzymes used for recombinant DNA techniques, PCR, and DNA isolation kits were purchased from New England Biolabs and Qiagen. For hK3, -4, -6, -7, -10, and -11, the chosen constructs coding for a $MRGSH_6GS$ sequence at the N terminus followed by an Asp_4-Lys enterokinase (EK) recognition site as a linker to the respective mature tissue kallikrein were cloned into the bacterial expression vector p*QE30* (Qiagen). *Escherichia coli M15*(*pREP4*) cells (Qiagen) were transformed with the resulting expression vectors. After induction with 1 mM isopropyl-β-thiogalactoside, the constructs were expressed in 2×TY medium, yielding inclusion bodies. The expressed His_6-tagged proteins were purified on a nickel-nitrilotriacetic acid-Sepharose column (Amersham Biosciences), washed by stepwise reducing the pH from 8.0 to 4.5, and eluted with 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris/HCl, pH 4.0. The tissue kallikrein constructs were incubated with 10 mM dithiothreitol overnight at 25 °C, and dialyzed for 12 h against the 100-fold volume of 4 M urea, 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, and 0.005% Tween 20. For example, the refolding of hK4 was performed in 2 M urea, 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5 mM reduced glutathione, 0.5 mM oxidized glutathione (GSH to GSSG ratio 1:10), 2 mM $CaCl_2$, 0.002% NaN_3, 0.005% Tween 20, pH 8.0, in the 100-fold volume of the sample at 4 °C for 40 h and subsequently in the 100-fold sample volume of the same buffer containing 1 M urea at 4 °C for 24 h. Finally, the refolding buffer was exchanged twice with 100 mM NaCl, 50 mM Tris/HCl, 0.002% NaN_3 at pH 8.0 with 24-h incubation periods. The folded proteins were concentrated to the desired volume using Amicon cells with 10-kDa cut-off membranes (Millipore).

Before activity measurements, the N-terminal His_6 tag was cleaved off at the recognition sequence with enterokinase (Sigma), whereby 0.0016 units of EK cleaved 25 μg recombinant hKs (per 100–125 μl) to 95% completion in 30 h at 37 °C in buffer containing 100 mM NaCl, 50 mM Tris/HCl, pH 8.0, 0.005% Tween 20 in total volumes of 10–15 ml. EK was removed from the reaction mixture with the EK antibody capture kit (Sigma), and the completeness of the activation reaction was evaluated by SDS-PAGE and N-terminal sequencing. The tissue kallikrein samples were further purified by benzamidine-Sepharose (hK4, hK5, hK6, hK10, hK11) and BPTI-Sepharose (Amersham Biosciences) chromatography (hK3 and hK7). After washing the column with 300 mM NaCl, 0.005% Tween 20, and 10 mM Tris/HCl, pH 8.0, the active tissue kallikreins were eluted with 150 mM NaCl, 20 mM *para*-aminobenzamidine, 0.005% Tween 20, and 10 mM Tris/HCl, pH 8.0. The purified tissue kallikreins were concentrated to ~20 mg/ml in Amicon Centricon microconcentrators at 4 °C. This procedure allowed the high yield expression of carbohydrate-free samples in *E. coli*, their rapid isolation, efficient screening of individual refolding conditions, specific activation, and the isolation of seven different mature tissue kallikreins in high yields. Monomeric fractions of these tissue kallikrein species were obtained by final gel

[6] M. Debela, V. Magdolen, V. Grimminger, C. Sommerhoff, A. Messerschmidt, R. Huber, R. Friedrich, W. Bode, and P. Goettig, submitted for publication.
[7] M. Debela, P. Goettig, V. Magdolen, N. Schechter, T. Steiner, W. Bode, and P. Hess, submitted for publication.

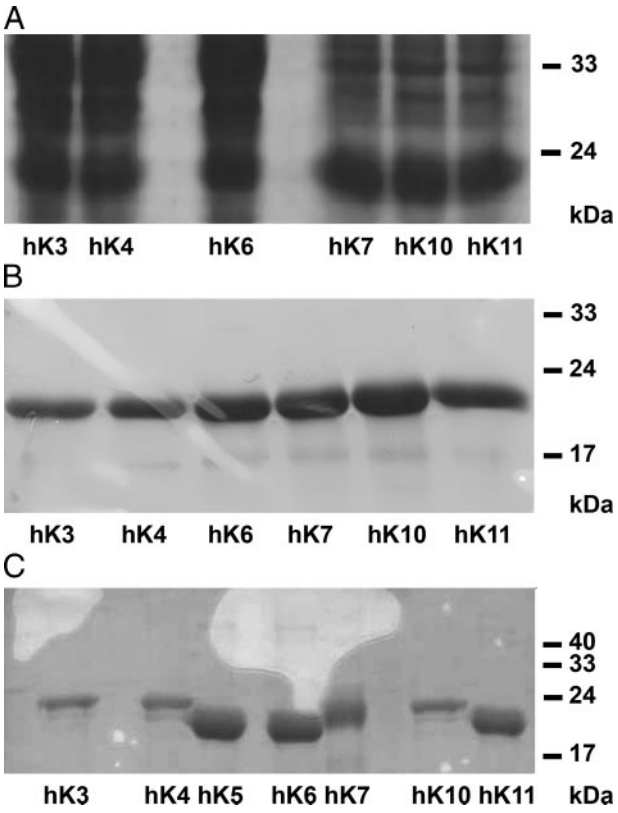

FIGURE 2. **Coomassie-stained SDS gels of recombinant tissue kallikreins during expression and purification.** *A*, protein bands of hKs 3, 4, 6, 7, 10, and 11 from *E. coli* cells with indicated molecular weight markers. *B*, seven recombinant tissue kallikreins carrying synthetic propeptides after affinity chromatography and refolding. *C*, bands of the seven activated proteinases including hK5 after treatment with enterokinase.

filtration. For the screening experiments described below, the stored precursors were activated and isolated immediately before usage. The purified tissue kallikreins were found by SDS-PAGE, ultracentrifugation, and mass spectrometry to be more than 95% pure (Fig. 2).

Recombinant hK5 was expressed in insect cells and purified as previously reported (46). Enzymatically active hK5 was purified by repeated chromatography on heparin-Sepharose, and the active concentration of hK5 was established by titration with standardized soybean trypsin inhibitor to be about 90% that based on UV absorbance at 280 nm.

Enzymatic Activity Assays—The concentrations of the individual tissue kallikrein samples were measured by absorbance at 280 nm and calculated extinction coefficients (47, 48). The enzymatic activity of these tissue kallikrein samples were checked with the fluorogenic 7-amino-4-methylcoumarine substrates (AMCs) succinyl-AAPF-AMC for hK3 and hK7 and benzyloxycarbonyl-LR-AMC and benzyloxycarbonyl-FR-AMC for hK4, hK5, hK6, hK7, hK10, and hK11 in buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.005% Tween 20 and 1% Me_2SO (from the substrate stock solution) at 25 °C by monitoring the initial velocity of the AMC release recorded at excitation and emission wavelengths of 380 and 460 nm with a PerkinElmer Life Sciences LS50B Luminescence spectrofluorimeter. For these enzyme assays final substrate and enzyme concentrations of 250 μM and 10–20 nM were employed.

Positional Scanning with a Synthetic Combinatorial Peptide Library (PS-SCL)—The diverse PS-SCL with the general molecular structure acetyl-P4-P3-P2-P1–7-amino-4-carbamoylmethylcoumarin consists of 20 P1-, 20 P2-, 20 P3-, and 20 P4-libraries in which the corresponding P1, P2, P3, or P4 position is fixed and occupied by one of the 20 canonical amino acids (omitting cysteine and including norleucine), whereas the three remaining positions contain an equimolar mixture of these amino acids (Fig. 3) (49). Therefore, each of the P1–P4 libraries comprised 20 sublibraries containing a mixture of 8000 different fluorogenic tetrapeptide substrates. The final enzyme concentration of the activated tissue kallikreins was 5–15 nM, and the final concentration for each of the 8000 compounds per well was 31.25 nM in a 100 μl total reaction volume. Triplicate samples were assayed at 25 °C with a buffer containing 150 mM NaCl, 50 mM Tris/HCl, pH 8.0, 0.005% Tween 20, and 1% Me_2SO (from the substrate stock solutions). All library assays were performed in 96-well Microfluor-1 Black "U" bottom plates (Dynex Technologies, Chantilly, VA) on a SpectraMax Gemini fluorescence spectrometer (Molecular Devices) with excitation, emission, and cut-off wavelengths of 380, 460, and 435 nm, respectively for 10 min.

RESULTS AND DISCUSSION

Expression and Purification Strategy—In vivo, the human tissue kallikreins are secreted into the endoplasmic reticulum as inactive precursors, which are converted to the 224–237-amino acid residue active forms by removal of their generally short activation peptides, mostly by means of proteinases with trypsin-like specificity. To produce six of the seven active tissue kallikrein species studied in this paper, we prepared chimeric constructs coding for the respective catalytic domain preceded by a His_6 tag linked to an accessible EK cleavage site. These constructs in combination with standardized procedures allowed the expression in *E. coli*, efficient refolding and isolation, activation, and high yield production of six carbohydrate-free mature tissue kallikreins. Because of problems with the bacterial preparation, hK5 was expressed in insect cells (46). For all positional scanning experiments, the stored precursors were activated and purified immediately before usage. In general, the isolated tissue kallikreins were more than 95% pure, as shown by SDS-PAGE (Fig. 2), and their identity was determined by N-terminal sequencing and mass spectrometry, whereas their activity was qualitatively checked with appropriate AMC substrates.

Extended Substrate Specificity by Positional Scanning—For the determination of the extended substrate specificity of the selected human tissue kallikreins, positional-scanning with fluorogenic peptide substrates from a synthetic combinatorial library (PS-SCL screening (49–51)) was used. Fig. 3 shows the results of the positional scanning of the P1-P4 positions with a so-called diverse tetrapeptide library for the seven different tissue kallikreins tested. In the following these cleavage preferences are discussed in light of hitherto known cleavage sites and of the experimentally solved structures (hK4,[6] hK6 (32), and hK7[7]) or modeled tissue kallikreins with substrates. For modeling the following has been assumed (compare with the modeled hK6 hexapeptide in Fig. 4). (i) The all-L-poly-α-amino acid tetrapep-

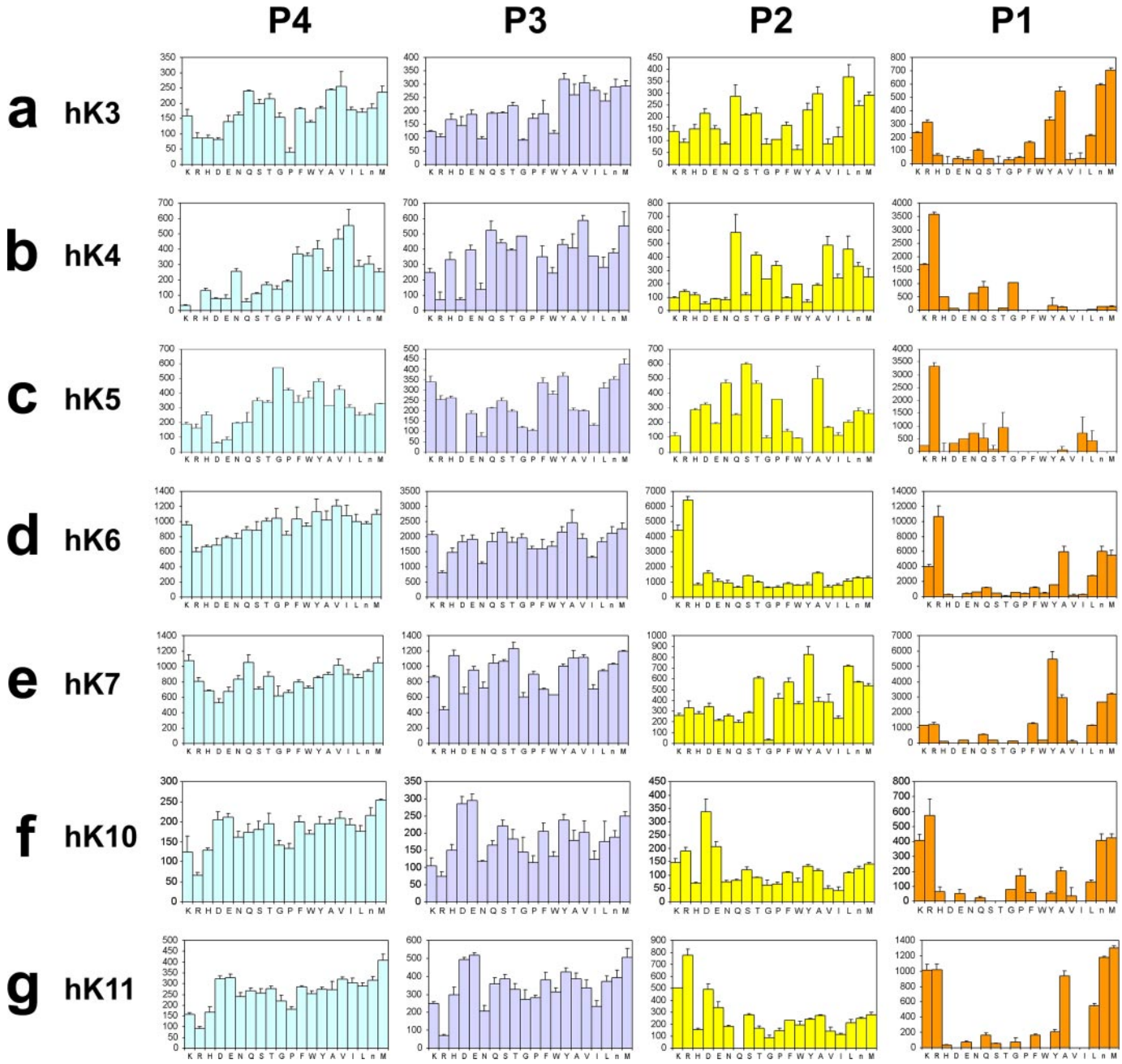

FIGURE 3. **Extended substrate specificity of seven human tissue kallikreins.** Results from P1, P2, P3, and P4 diverse positional scanning libraries are shown for recombinant mature hK3/PSA (*a*), hK4 (*b*), hK5 (*c*), hK6 (*d*), hK7 (*e*), hK10 (*f*), and hK11 (*g*). The *y* axis represents the substrate cleavage rates in picomolar concentrations of fluorophore released per second. The *x* axis indicates the amino acid held constant at each position, designated by the one-letter code (with *n* representing norleucine).

tide substrates bind in an extended manner antiparallel to the segment Ser-214–218 according to canonical binding (52). (ii) The S1 pockets are lined by segment Pro-225—Tyr-228 (internal, back with respect to the standard orientation, as used in Fig. 4), the overall chymotrypsin-like entrance frame segment Ser-214—Cys-220 (forming the front ceiling), segment 189–Ser-195 (floor), and disulfide bridge Cys-191—Cys-220 (lower closure). (iii) The S2 subsites are based on the side-chain moiety of residue 215 (in most cases an indole) and framed by the flat side of the His-57 imidazole moiety and the 99 side chain. In hK3 the S2 pocket will be, as in hK1 and hK2, additionally bordered by the extended 99 or "kallikrein" loop; however, in the new hKs, the S2 subsites are relatively open, allowing P2 side chains to bend and to extend toward the bulk solvent. (iv) The S3 subsites are located on the enzyme surface based on the tissue kallikrein-characteristic 218/192 "balcony." (v) The S4 subsites, based on the 215 (mostly Trp) side chain, extend alongside the S2 subsites, further framed by the side chain of residue 174/175.

hK3; Prostate-specific Antigen—As shown in Fig. 3*a*, hK3/PSA slightly prefers Met over Nle and Ala at the P1 position of the fluorogenic substrates but also tolerates Leu, Tyr, Phe, and the basic residues Arg and Lys, whereas Asp and Thr are not accepted at all. In the S2 subsite Leu is slightly preferred over Ala, Gln, Met, and Tyr. The S3 subsite shows some preference

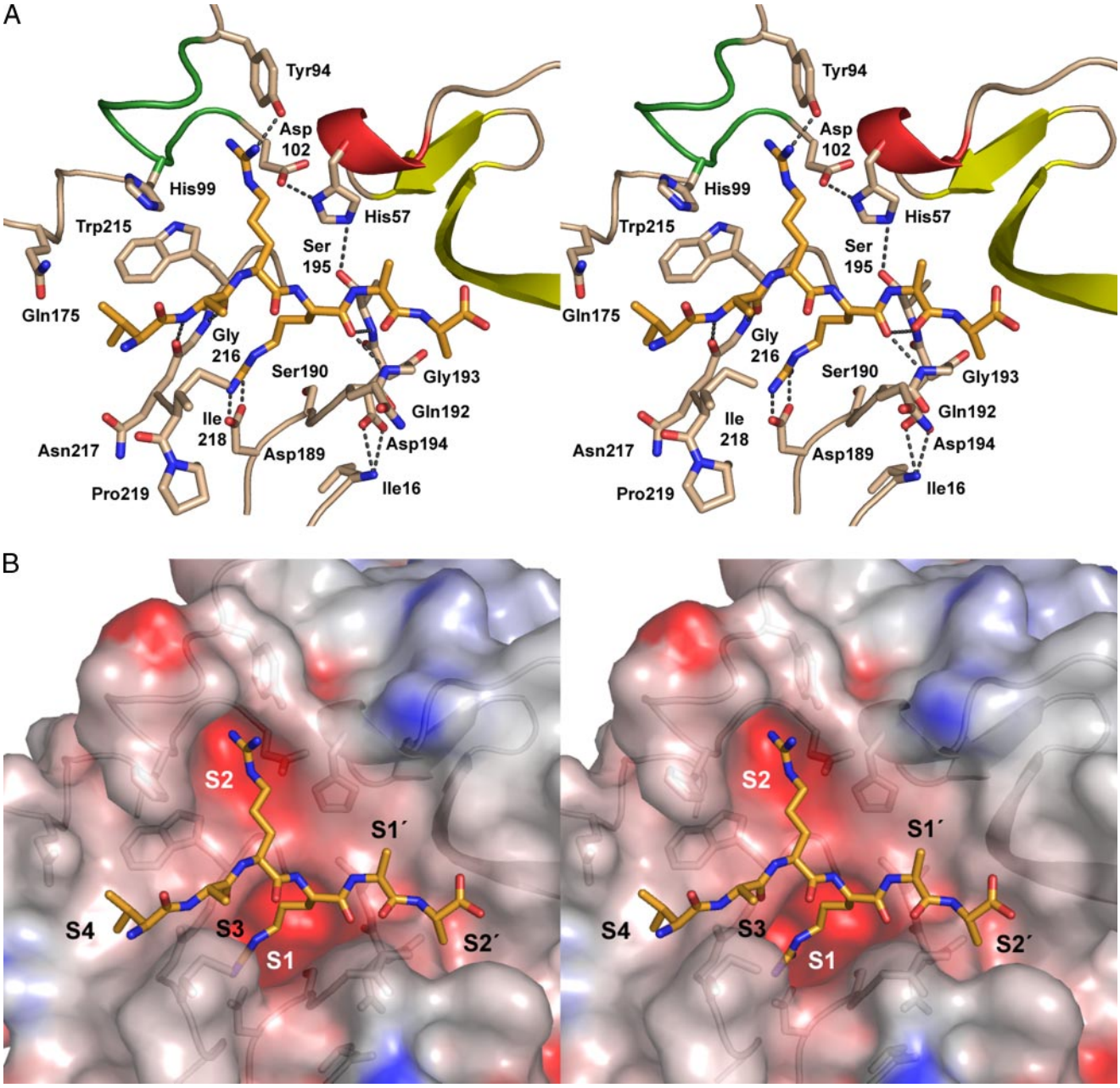

FIGURE 4. **Peptide substrate binding by hK6 (34) in standard orientation.** *A*, stereo stick model of the active-site cleft of hK6, displayed together with a modeled hexapeptide substrate (P4-Val-P3-Ala-P2-Arg-P1-Arg-↓-P1′-Ala-P2′-Ala). *B*, active-site cleft of hK6 in stereo, represented as *solid surface-colored* according to the electrostatic surface potential (*intense blue* and *red* corresponding to extreme positive and negative values, respectively), depicted with the modeled hexapeptide (VARRAA), which is bound to subsites S4 to S2′.

for medium-sized hydrophobic residues, whereas all medium-sized hydrophobic as well as polar and uncharged residues are tolerated at P4.

The dual P1 specificity of hK3 is also reflected by the known cleavage sites in natural hK3 protein substrates (53), which seem to be preferentially cleaved after medium-sized hydrophobic (Tyr, Leu, Val, Phe) or basic residues (His, Lys, Arg). This finding is largely in accordance with our P1 profiling pattern, albeit hK3 prefers to cleave the seminal clot-forming proteins semenogelins I and II after P1-Tyr and Gln, mostly after Tyr-Gln and Gln-Tyr tandems (54). The efficient inhibition by aprotinin/BPTI (bovine pancreatic trypsin inhibitor) (with a P1-Lys) and leupeptin (Leu-Leu-Arg-OH) reflects the readiness of hK3 to accommodate Lys and Arg side chains in its S1 pocket. The documented conversion of single- to two-chain urokinase-type plasminogen activator (uPA), with the much less suitable P4-P1 target sequence Pro-Arg-Phe-Lys (compare with Fig. 3*a*), indicates, however, that most likely other favorable exosite interactions determine the cleavage site in addition (42, 55).

Recently, the crystal structure of horse prostate kallikrein has been offered as a model for hK3 (38). Because of significant differences in the substrate binding sites, however, this equine tissue kallikrein structure is certainly not an appropriate model for hK3; the bulky hydrophobic Ile-190 that replaces the smaller Thr residue of hK3 and the unusually exposed Val-227 side chain completely block the bottom of the S1 pocket, and

the tissue kallikrein characteristic Pro-219 is replaced by a Glu residue. In addition, the molecule contained in the horse prostate kallikrein crystals exhibits a quite deformed, inaccessible substrate binding site with a closed S1 pocket and a kinked 214–220 segment, which would not be competent for productive substrate binding without large rearrangements.

Probably, the substrate recognition architecture of active hK3 can be better inferred from a superposition of hK1/pK1 and mgK13 with the recently determined crystal structure of the chymotrypsin-like hK7.[7] Accordingly, the S1 pocket of hK3 should be, because of to the flanking polar Thr-190 and Ser-226 side chains and the small polar Ser-189 residue, accessible and quite deep but relatively narrow at its bottom. Thus, shape and polar lining would be in good accordance with the observed preference of hK3 (Fig. 3*a*) for medium-sized polar as well as hydrophobic P1 side chains. Further in line with our hK3 profiling, an indole moiety of a P1-Trp residue should not fit well into this S1 pocket based on a comparison with the structure of a P1-Trp-containing bovine γ-chymotrypsin (56). The extended 99/kallikrein loop of hK3 comprises 11 residues more than chymotrypsin and presumably hangs over the active-site cleft as in hK1 and mgK13. This loop should leave some space for medium-sized P2 residues, in conformance with the observed hydrolytic activity of active hK3 and its inhibition by aprotinin. Because of the bulk solvent exposure across the Ser-192—Glu-218 balcony, various P3 side chains should be accepted by hK3, whereas the S4 subsite, flanked by the Gln-174 side chain and possibly limited by the overhanging 99 loop, should prefer medium-sized polar/hydrophobic side chains.

hK4; Prostase—Our data demonstrate that hK4 clearly prefers P1-Arg over Lys side chains in its specificity pocket (Fig. 3*b*). Besides these two basic residues also Gly, Gln, and Asn are tolerated as P1 residues. Although our data for the P2, P3, and P4 specificity of hK4 were in contrast to another study (Matsumura *et al.* (44)) obtained with "P1-diverse" instead of "P1-Arg-fixed" libraries, our results were similar; at S2, hK4 slightly prefers Gln over Val, Leu, Thr, and Pro (*i.e.* medium-sized polar and hydrophobic residues), whereas large aromatic and basic side chains are least welcome. With respect to S3, hK4 seems to be quite promiscuous, accepting in particular Gln, Val, Met, and (in contrast to the measurements of Matsumura and coworkers (44)) Gly; peptide substrates with Pro, Arg, and Asp are hardly turned over. The S4 subsite of hK4 prefers bulky hydrophobic side chains, such as of Ile, Val, Tyr, Phe, and Trp, whereas the charged residues of Arg, Lys, Asp, and Glu are scarcely accepted.

The preference of hK4 for P1-Arg over P1-Lys and P1-Tyr is also reflected by its higher specificity for tripeptidyl-*p*-nitroanilides with P1-Arg compared with P1-Lys and P1-Tyr tripeptidyl-*p*-nitroanilides (17). *In vitro* hK4 has been shown to cleave the proform of hK3/PSA at the ILSR ↓ IV activation site much faster than hK2 and to rapidly degrade prostatic acid phosphatase (an androgen-mediated regulator of prostate epithelial cell growth) after VYIR but also to efficiently convert single-chain into active two-chain uPA by cleaving after PRFK (17) as well as after the uPA receptor sequence TYSR (57). These data indicate that hK4 might be the physiological activator or inactivator of these cancer-involved enzymes.

Overall, these substrate preferences of hK4 are in good conformance with the active-site architecture of our current hK4 crystal structures.[6] First, the large S1 pocket, with an Asp-189 at its bottom, is particularly well suited to accommodate a P1-Arg side chain, whereas the adjacent small and polar Ser-190 also makes P1-Lys side chains acceptable. Second, the uniquely notched S2 groove can easily accept medium-sized hydrophobic and polar P2 side chains. Third, the wide S3 gap between the side chains of Asn-192 and Ala-218 is ready to tolerate a multitude of P3 side chains. Finally, the hydrophobic S4 cleft, based on the Phe-215 phenyl side chain, is bordered by Leu-175 and Leu-99 and can be perfectly filled by medium-sized hydrophobic P4 side chains.

hK5; Human Stratum Corneum Tryptic Enzyme—As shown in Fig. 3*c*, the S1 pocket of hK5 is very specific for P1-Arg side chains. P1-Thr, -Asn, -Gln, and -Ile are tolerated, whereas the residual amino acids, in particular with large aromatic side chains and Lys, are hardly accepted. In S2, P2-Ser is slightly preferred over Asn, Thr, and Ala, whereas among all other residues only Arg and Tyr are rejected. Most amino acids, in particular Met, Phe, and Tyr but not Asp, are accepted in S3, whereas the S4 subsite has an unusual preference for Gly followed by Tyr, Val, and Pro.

As the synonym human stratum corneum trypsin-like enzyme (3) indicates, hK5 is known to exhibit a trypsin-like activity, with a strong preference for P1-Arg over -Lys but not accepting chymotrypsin-specific substrates with a Phe or Tyr at P1 (43). A comparison of some fluorogenic tripeptidyl substrates revealed a preference for Ala and Lys at P2 and for Leu and Gln at P3. The best substrates found were the AMC of *t*-butoxycarbonyl-Val-Pro-Arg and *t*-butoxycarbonyl-Phe-Ser-Arg. The specificity of hK5 for cleavage of Arg-Xaa bonds is also reflected by its rapid interaction with the P1-Arg residue-containing serpin inhibitors α2-antiplasmin and antithrombin III compared with the α1-proteinase inhibitor and α1-antichymotrypsin (43). hK5 has been found to activate pro-hK7 at a low rate (58); therefore, it seems to be able also to cleave after P1-Lys.

According to their sequences (Fig. 1*A*) and specificities, hK5 and hK4 presumably have very similar three-dimensional structures. Based upon the presence of residues Asp-189, Ser-190, Gly-216, and Gly-226, both S1 specificity pockets are well designed to accept P1-Arg side chains. The even more exclusive P1-Arg preference of hK5 (Fig. 3*c*) might be due to the replacement of the hK4 residue Ala-218 by Tyr-218, which in hK5 could provide an additional hydrophobic entrance barrier toward the S1 pocket (see Fig. 4). Additionally, the His-99 residue of hK5, which replaces Leu-99 of hK4, would render the S2 cleft more polar. The minor preference for more hydrophobic P3 residues might be caused by the Tyr-218 "wall" at the S1 entrance, and the Gln-175 side chain will probably confer a higher polarity to the S4 subsite, in agreement with a weak tendency of hK5 for more polar P4 side chains.

hK6; Neurosin—As shown in Fig. 3*d*, hK6 exhibits a strong preference for P1-Arg over Lys, which is accepted nearly equally as well as Ala, Met, and Nle. With respect to the S2 subsite, hK6 is most selective among the seven tissue kallikreins tested, preferring P2-Arg over Lys, far ahead of all other amino

acid residues. In strong contrast, hK6 is not very selective concerning P3 and P4 residues. This high hK6 specificity for P1-Arg agrees well with the reported efficient usage of fluorescent *XX*R-AMC substrates in enzymatic hK6 assays (59) and with the cleavage after Arg residues of the myelencephalon-specific protease, the hK6 homolog from rat (60).

According to the crystal structure of hK6 (Bernett *et al.* (34)), the S1 pocket is very similar to those of hK4 and bovine trypsin (61), with the Asp-189 side chain at the bottom favoring basic P1 residues, the two Gly residues at positions 216 and 226 keeping the pocket open, the Ser-190 side chain making Lys side chains acceptable, and Gln-192 allowing appropriate fixation of peptide substrates by hydrogen bonds. The low acceptance of hydrophobic P1 side chains may be caused by the shielding of the S1 pocket from bulk water by the Ile-218 side chain. Also, the S2 pocket resembles that of trypsin, except for the bordering Leu-99 side chain that is replaced by the His-99 imidazole group. This latter side chain would basically limit the size of the S2 pocket if it does not give way to incoming substrates, as observed for the equivalent Tyr-99 side chain of pK1 in complex with BPTI (37). Therefore, there is no straightforward explanation for the strong preference of hK6 for P2-Arg and -Lys residues. The low specificity of hK6 for P3 and P4 side chains is in accordance with the solvent-exposed S3 site and the quite open, partially polar S4 groove.

hK7; Human Stratum Corneum Chymotryptic Enzyme—Interestingly, the most favored P1 residue of hK7 is Tyr followed by Ala, Met, and Nle, whereas Phe is ranked rather low, and Trp seems to be totally excluded (Fig. 3*e*). At S2, the most preferred residue is again Tyr, well ahead of the medium-sized hydrophobic residues Leu, Nle, Thr, Met, and Phe, whereas Gly is almost not tolerated. At S3 and S4, nearly all residues are accepted, with a slight preference for hydrophobic side chains. In accordance with this profile, MeO-succinyl-Arg-Pro-Tyr-tripeptidyl-*p*-nitroanilide has been identified as an efficient hK7 substrate. By contrast, no turnover for succinyl-Ala-Ala-Pro-Phe-tripeptidyl-*p*-nitroanilide was reported, although hK7 cleaved the insulin B chain after Asn-Gln-His-Leu, Glu-Ala-Leu-Tyr, Gly-Phe-Phe-Tyr, and Arg-Gly-Phe-Phe (62); another study demonstrated a slow turnover of substrates with Phe in P1 position (46).

Recently, the three-dimensional crystal structure of hK7 has been determined in our laboratory and will be published elsewhere.[7] Its overall architecture resembles hK1/pK1, hK4, and hK6, but its S1 pocket differs from these tissue kallikreins with trypsin-like specificity in that the polar Asn-189 replaces the negatively charged Asp at the bottom and the hydrophobic Ala-190 substitutes the polar Ser-190 in its middle part and in that the entrance to this pocket is framed by the side chains of Phe-218 and Asn-192. The relatively wide but not extremely deep S1 pocket of hK7 is well suited for accommodation of medium-sized to large side chains with polar tips, which explains the preference for Tyr at P1 as well as the acceptance for Met and Ala. Upon superposition with the P1-Trp-containing bovine γ-chymotrypsin structure (56), some unfavorable contacts are predicted for the indole side chain with the bottom of the S1 pocket, in accordance with the low acceptance of P1-Trp by hK7. Its S2 groove is mainly limited in size by the His-99 imidazole side chain, allowing accommodation only for small hydrophobic P2 residues, unless the His-99 side chain swings over toward the S4 pocket, leaving space for larger P2 residues. P3 side chains resting on the Phe-218 phenyl group could extend into the bulk water, whereas P4 side chains would point into the triangular hydrophobic S4 cleft, confined by the Leu-175 isobutyl side chain.

hK10; Normal Epithelial Cell-specific 1—As shown in Fig. 3*f*, hK10 prefers Arg residues at the P1 position over Lys, Met, and Nle. Albeit Asp is a more suitable P2 residue than Glu, Arg, and Lys, the S2 pocket appears to be rather unspecific for all other residues. At S3, hK10 displays some preference for the acidic residues Glu and Asp and tolerates large hydrophobic or polar residues such as Met, Tyr, Phe, and Ser but mostly rejects Lys and Arg. The S4 subsite seems to be particularly unspecific, since it accepts all hydrophobic, polar, and negatively charged side chains, with Arg being the least welcome residue.

Most likely, the still unknown three-dimensional structure of hK10 will differ considerably from all other tissue kallikreins and trypsin-like proteinases with respect to the three residue longer N terminus of our proteolytically active recombinant hK10 with the sequence ^{16}LDPE. Usually, the catalytic chains of almost all other mammalian trypsin-like serine proteinases start with combinations of ^{16}(I/L/V)(V/I)*XG*, concomitantly forming an internally buried salt bridge between the α-ammonium group and Asp-194 and triggering the formation of a functional active site. Also quite unique is residue Ala-193, which replaces the highly conserved Gly residue that contributes to the formation of the oxyanion hole. Any 193 side chain would clash to some extent with the P3′ main chain of a canonically binding extended peptide substrate, thereby affecting the efficient cleavage of C-terminal elongated substrates (63). This effect has been exemplified and discussed for the snake venom serine proteinase TSV plasminogen activator possessing a Phe-193 (64) and for human brain trypsin containing an Arg-193 (65).

Based on the assumed location of Asp-189 at the bottom of the S1 pocket, hK10 should prefer P1-Arg and -Lys residues, in agreement with the profiling results (Fig. 3*f*). The hK10 residues Pro-190 and Ala-226, replacing the more common Ser-190 and Gly-226, should render the S1 pocket smaller and more hydrophobic, probably accounting for the acceptance of aliphatic P1 residues such as Met. The sequence alignment (Fig. 1) suggests that hK10 possesses a kallikrein/99 loop that is significantly longer than in the other "new" tissue kallikreins but still four residues shorter than in hK1, hK2, and hK3, precluding a reasonable prediction of its S2 subsite. One may speculate that almost all P3 side chains could be accepted by hK10 due to the phenolic Tyr-218 basis. Similarly, no distinct preference for P4 side chains is suggested by our profiling data (Fig. 3*f*).

hK11; Hippostasin—As depicted in Fig. 3*g*, the substrate profile of hK11 looks similar to that of hK10. However, in hK11 P1-Met and -Nle are the most favored residues ahead of Arg, Lys, and Ala. At S2, P2-Arg is clearly preferred over Lys and Asp followed by Glu and all other amino acids except Gln. At P3 and P4, most amino acids, in particular Met and acidic residues, are accepted, whereas basic residues are least welcome.

These findings are not inconsistent with reports on the efficient cleavage of the hK1 characteristic substrates PFR-AMC

and benzyloxycarbonyl-FR-AMC and the rejection of other synthetic substrates with P1-Tyr, -Ala, or -Asp (66, 67) by the three isoforms of hK11 from brain and prostate (68). According to its sequence, the architecture of the S1 pocket of hK11, with an Asp-189 and a Ser-190 at its bottom and Gly-216, Gly-226, and Gln-192 at its sides, should be similar to that of hK6. This model is in accordance with the turnover of P1-Arg and -Lys residues but does not explain the observed strong acceptance of P1-Met, -Nle, and -Ala (Fig. 3*f*). Furthermore, hK11 exhibits a kallikrein/99 loop of medium length that might slightly hang over the active-site cleft. As in the known structure of hK6, His-99 might border the S2 subsite, leaving a relatively small S2 niche, which cannot easily explain the experimentally observed preference of hK11 for P2-Arg residues. Again, all P3 side chains could relatively freely extend away from the enzyme surface, and P4 side chains should be accommodated in a relatively polar S4 groove bordered by Asn-175.

Conclusions—In this study, seven different tissue kallikreins have been expressed as mature recombinant proteinases, and their extended substrate specificity has been determined using a positional scanning combinatorial tetrapeptide substrate library. As expected, two of these tissue kallikreins (hK3/PSA and hK7) exhibit a chymotrypsin-like specificity for medium sized to large hydrophobic or polar P1 residues (Table 2). Intriguingly, PSA features an uncommon P1-specificity for Met and Nle and possibly for the second sulfur-containing natural amino acid, Cys, which was not tested in these assays. Also, hk7 is distinguished from chymotrypsin by a clear preference for Tyr over Phe in P1, which is paralleled by Tyr as the most favored residue in P2. Three of the other tissue kallikreins (hK4, hK5, and hK6) display a trypsin-like specificity, with a strong preference for P1-Arg (hK5 > hK4 > hK6), whereas two (hK10 and hK11) possess a bivalent specificity, accepting both basic as well as large aliphatic P1 residues (Table 2). With the exception of the latter two, the existing three-dimensional structures (hK4, hK6, and hK7) or derived models make all these S1 specificities comprehensible. In contrast, only part of the observed preferences for P2, P3, and P4 residues can be predicted due to lack of structural information such as the architecture of the S2 groove with the partially bordering kallikrein/99 loop but also because the intuitive estimation of the activity-assisting effects of the specific docking in shallow surface grooves is often difficult.

Therefore, these positional scanning data are complementary to structural data and may help in the search for physiologically relevant substrates. Currently, the best studied natural substrates of hK3/PSA are the semenogelins I and II with favorable cleavage sequences (P4-P2′) of the type SSIY ↓ SQ (54, 69, 70). Although these particular residues are not the most preferred ones in each position of our profiling pattern, the whole sequences fit to the observed specificity of recombinant hK3 (Fig. 3*a*). Moreover, two insulin-like growth factor binding proteins have been identified as potential substrates for hK3 (71). hK4 may be a regulator of tumor-associated substrates because it cleaves peptides from the uPA and the uPA receptor, such as VQYR ↓ SG (57), which is consistent with our specificity profiling except for the P2 residue Tyr (Fig. 3*b*). Apparently, hK5 efficiently activates itself at the cleavage site SSSR ↓ II as well as hK7 and hK14 in a proteolytic cascade of the stratum corneum that serves for skin desquamation (72). Other efficient substrate sequences for hK5, such as the N-terminal propeptide of hK3 ILSR ↓ IVG, which closely resembles our scanning library result (Fig. 3*c*), have been reported recently (73). Variations of the motif AFR ↓ FS from diverse subunits of the human ionotropic glutamate receptors have been found to be suitable substrates for hK6 (74). Compared with our PS-SCL results, P1 and P3 coincide, whereas P2 differs to some extent (Fig. 3*d*). Human β-glucocerebrosidase and acidic sphingomyelinase from the stratum corneum seem to be physiological substrates of hK7 (75). Intriguingly, these lipid-processing enzymes contain altogether four potential hK7 recognition sequences for P4-P1 that agree very well with our specificity profiling data (NLYY, LQYY, IKYY, NLLY; see Fig. 3*e*). No defined natural substrates or corresponding synthetic substrates of hK10 have been described up to now. Recently, a role of hK11 in an enzymatic cascade for semen liquefaction together with hK2, hK3, and hK4 has been proposed whereby only tryptic activity was reported (76), in contrast to our finding of a mixed specificity (Fig. 3*g*).

TABLE 2
P1 preference of human tissue kallikreins determined with combinatorial fluorogenic substrates compared with P1 residues in physiological substrate cleavage sites (78)

Name	Tissue kallikrein PS-SCL	Cleavage sites in physiological substrates
hK3	Met > Nle = Ala > Tyr = Arg	Leu, Phe, Tyr > Gln
hK4	Arg ≫ Lys	Arg, Lys
hK5	Arg	Arg > Lys
hK6	Arg ≫ Ala = Nle = Met > Lys	Arg > Lys
hK7	Tyr ≫ Ala = Met > Nle	Tyr
hK10	Arg > Lys = Met = Nle	Unknown
hK11	Met > Nle > Arg = Lys = Ala > Leu	Arg

Because most or all of these tissue kallikrein substrates are proteins, they could additionally contact the corresponding tissue kallikrein by surface regions outside the active site. We have previously found distinct negatively and positively charged surface patches in hK4[6] and hK7,[7] respectively, which could serve as specific exosites. In many cases such exosite contacts influence or even determine the efficiency of cleavage, which often accounts for the frequently observed phenomenon of physiological protein substrates with apparently improper cleavage sequences.

The profiling pattern of hK4 is similar to that recently published with a similar positional scanning procedure (44), and the scanning profile obtained for hK3/PSA coincides well with specificity results for selected chromogenic and fluorogenic peptide substrates (42). It should be taken in mind that the amino acid preferences at the four positions from our diverse library do not rule out special neighboring effects between the four side chains, which certainly exist in particular between P2 and P4. Such effects can only be discovered by analyzing synthetic substrates with systematically varied P1-P4 sequences. For example, a recent study on hK6 specificity using fluorescent resonance energy transfer peptide families (74) shows clear differences in the P2 position compared with our results, whereas there is a remarkable congruence for P3 (Fig. 3*d*). In the future our data should, therefore, be refined by similar strategies to identify optimal substrates.

Besides, the availability of large amounts of homogeneous, carbohydrate-free hKs will certainly support further research on the role and function of the tissue kallikreins, *e.g.* by allowing for further three-dimensional structure determination and screening for selective inhibitors. Nevertheless, the mostly minor structural and functional differences of the tissue kallikreins may be important for comprehending their special physiological function. This work should contribute to a better understanding of these differences and elucidate the respective structure-function relationships. The very recent discovery of the LEKTI domains as potent inhibitors for hK5 and hK7 with inhibition constants in the nanomolar range (22, 46, 77) will certainly stimulate research on the physiological regulation and function of the hKs. Eventually, the detailed specificity patterns of the hKs selected in this study could facilitate the design of therapeutic substances for medical applications.

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