Substrate Specificity of Human Kallikrein 6
SALT AND GLYCOSAMINOGLYCAN ACTIVATION EFFECTS

Received for publication, September 14, 2005, and in revised form, November 30, 2005 Published, JBC Papers in Press, December 1, 2005, DOI 10.1074/jbc.MS100962000

Pedro Francisco Angelo, Aurelio Resende Lima, Fabiana M. Alves, Sachiko I. Blaber, Isobel A. Scarisbrick, Michael Blaber, Luiz Juliano, and Maria Aparecida Juliano

From the Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Três de Maio 100, 04044-20 São Paulo, Brazil, the Department of Biomedical Sciences, Florida State University College of Medicine, Tallahassee, Florida 32306-4300, and the Departments of Neurology, Physical Medicine, and Rehabilitation, Mayo Medical and Graduate School, Mayo Clinic, Rochester, Minnesota 55905

Human kallikrein 6 (hK6) is abundantly expressed in the central nervous system and is implicated in demyelinating disease. This study provided biochemical data about the substrate specificity and activation of hK6 by glycosaminoglycans and by kosmotropic salts, which followed the Hofmeister series. The screening of fluorescence resonance energy transfer (FRET) peptide families derived from Abz-KLRSSQ-EDDnp in the finding that Abz-AFRFSQ-EDDnp (where Abz is ortho-aminobenzoic acid and EDDnp is N-[2,4-dinitrophenyl]ethylenediamine) is the best synthetic substrate described so far for hK6 ($k_{cat}/K_m = 38.667 \text{ s}^{-1} \text{mM}^{-1}$). It is noteworthy that the AFRES sequence was found as a motif in the amino-terminal domain of seven human ionotropic glutamate receptor subunits. We also examined the hK6 hydrolytic activity on FRET peptides derived from human myelin basic protein, precursor of the $\alpha_3$-antichymotrypsin, plasminogen, and maturation and inactivation cleavage sites of hK6, which were described earlier as natural substrates for hK6. The best substrates were derived from myelin basic protein. The hK6 maturation cleavage site was poorly hydrolyzed, and no evidence was found to support a two-step self-activation process reported previously. Finally, we assayed FRET peptides derived from sequences that span the cleavage sites for activation of protease-activated receptors (PAR) 1–4, and only the substrate with the PAR 2 sequence was hydrolyzed. These results further supported the hypothesis that hK6 expressed in the central nervous system is involved in normal myelin turnover/demyelination processes, but it is unlikely to self-activate. This report also suggested the possible modulation of ionotropic glutamate receptors and activation of PAR 2 by hK6.

Human kallikreins (hK) are a multigene family of 15 secreted serine-type proteases aligned in tandem on chromosome 19q13.4 (1–4) (the hKallikrein genes are abbreviated as hKLK, mKLK, and rKLK, and the proteins as hK, mK, and rK depending on whether they are from human, mouse, or rat, respectively). Similarly, the mKLK and rKLK kallikrein gene families are composed of a large number of closely related members (5–9) that possibly arose from gene duplication events. The members of mouse, rat, and human kallikreins have a high degree of amino acid identity but present different substrate specificity, particularly toward oligopeptides (10–17). The natural substrates and the substrate specificities of the kallikreins are known only for a few of them. hK1 is the best characterized kallikrein, it is a glycoprotein expressed most abundantly in pancreas, salivary gland, kidney, and urine (18), releases Lys-bradykinin by limited proteolysis from high and low molecular weight kininogens by cleavage at the Met$^{342}$-Lys$^{343}$ and Arg$^{389}$-Ser$^{390}$ bonds (19), and has both trypsin- and chymotrypsin-like activities (20–23). hK2 is present in the seminal plasma, hydrolyzes certain components of the semen coagulum (24), and cleaves substrates with restrictive trypsin-like specificity (25). In contrast, hK3 (prostate-specific antigen) has only chymotrypsin-like activity and is expressed in the glandular epithelial cells of the prostate gland. hK3 rapidly hydrolyzes both semenogelin I and semenogelin II in vivo and synthetic substrates derived from these proteins (26) and was reported to be strongly activated by Na$_2$SO$_4$ (27) and by heparin (28). hK14 is mainly expressed in breast, skin, and prostate, and similar to hK1, hK14 also accepts either basic or aromatic amino acids within its $S_1$ subsite (29).

hK6 was cloned from different tissues and was identified by various names, including protease M from breast tissue (30), neurosin from a colon carcinoma cell line and oligodendrocytes (31, 32), or zyme from brain tissue (33). The rodent orthologue of hK6 was identified (34) and shown to be abundantly expressed in the central nervous system (CNS) and was termed myelencephalon-specific protease (MSP) (35, 36). hK6 was demonstrated to play an important role in the progression of inflammatory diseases of the CNS and in its demyelination processes (37, 38). hK6 is reduced in brain extracts of Alzheimer disease patients (39, 40) and increased in serum of patients with ovarian cancer (41, 42). hK6 exists mainly as a proenzyme in milk and cerebrospinal fluid, and a fraction of hK6 was described to be partially complexed with $\alpha_1$-antichymotrypsin in milk and ascites fluid of ovarian cancer patients (43).

Some of the enzymatic properties of rat MSP (44) and of hK6 (45) were described, including the crystal structure of recombinant hK6 (46) and of pro-hK6 (47). Recombinant rat MSP and hK6 have restricted specificity for basic amino acids at the $P_1$ position of the substrates with large preference for Arg (44, 45); however, all the reported peptidyl-$p$-nitroanilide and peptidyl-7-amino-4-methylcoumarin substrates were poorly hydrolyzed by MSP and hK6 compared with other arginyl hydroxylases (44–46) such as hK1 (20–23).

The identification of substrate specificity and activators for hK6 is a
relevant goal to understand the biological roles of this enzyme, to identify potentially susceptible substrates, and to elucidate functional information. We examine the modulation of salts and glycosaminoglycans (GAGs) on hydrolytic activity of hK6 and their effects on the pH profile activity of this enzyme. The rationale for investigation of the effects of GAGs on hK6 activity was based on the co-localization of both molecules in the CNS. GAGs act in neurogenesis, plasticity, and protection of the CNS (48–51), and hyaluronan accumulates in demyelinated lesions (52). Furthermore, hK6 is described to be involved in degenerative, demyelinating, and inflammatory diseases of CNS, such as multiple sclerosis (38). We also present a detailed substrate specificity analysis of hK6 that was performed by using two strategies; in the first strategy, the S$_x$ to S$_y$' subsite specificities are examined by taking as reference the fluorescence resonance energy transfer (FRET) peptide Abz-KLRSSKQ-EDDnp (Abz is ortho-aminobenzoic acid and EDDnp is N-[2,4-dinitrophenyl]ethylenediamine), which was designed based on previously reported subsite requirements for hK1 substrates (20–23). A series of analogues of this peptide were synthesized with modification by all amino acids, except Gln, and assayed as substrates. For comparison, we also determined the kinetic parameters for hydrolysis by hK1 of the FRET peptides derived from Abz-KLRSSKQ-EDDnp. In the second strategy, we examined the hK6 hydrolytic activity on FRET peptides derived from proteins described previously to be hydrolyzed by hK6, including human myelin basic protein (MBP) (46), the precursor of the Aβ amyloid peptide (45), the reactive center loop of α$_i$-antichymotrypsin (a serpin that was reported to be complexed with hK6) (43), plasminogen (53), and the maturation and inactivation cleavage sites of hK6 (45, 46, 53).

The protease-activated receptors (PARs) constitute a family of G-protein-coupled receptors that undergo a unique mechanism of activation via proteolytic cleavage of the amino terminus, subsequently exposing a tethered peptide ligand (54). PARs 1–4 are activated upon hydrolysis after specific Arg or Lys residues, are widely expressed in the CNS (55), and are activated in several diseases (56) (particularly PAR 2, which is involved in inflammation by a neurogenic mechanism (57)). These properties of PARs motivated us to include in this study FRET peptides derived from sequences that span the cleavage sites for activation of these receptors because hK6 has preference for Arg at the P$_1$ site of substrates and hK6 is involved in inflammatory diseases as mentioned above.

**MATERIALS AND METHODS**

**Recombinant Kallikrein, hK6 and hK1**—The mature hK6 was expressed and purified from a baculovirus/insect cell line system as described previously (46). hK1 was expressed and purified as described previously (58). Molar concentrations of active hK6 and hK1 were described previously (46). hK1 was expressed and purified as described above. The enzymes were preincubated in the assay buffer for 3 min before the addition of substrate. The enzymes were preincubated in the assay buffer for 3 min before the addition of substrate. Fluorescence changes were monitored continuously at $\lambda_{ex} =$ 320 nm and $\lambda_{em} =$ 420 nm. The enzyme concentrations for initial rate determinations were chosen at a level intended to hydrolyze less than 5% of the amount of added substrate over the time course of data collection. The slope of the generated fluorescence signal was converted into micromoles of substrate hydrolyzed per min based on a calibration curve obtained from the complete hydrolysis of each peptide. The substrate concentration for determination of the kinetic parameters was between two $K_s$ values higher and lower than the obtained value. The kinetic parameters $K_m$ and $k_{cat}$ were calculated by nonlinear regression using the Grafit program (62). For peptides hydrolyzed at more than one site, or for substrate exhibiting inhibition, the apparent $k_{cat}/K_m$ values were also determined under pseudo-first order conditions (where [S] $\ll$ $K_m$) and performed under two different substrate concentrations. Errors were less than 5% for each of the obtained kinetic parameters.

**RESULTS**

**Effects of Salts and Glycosaminoglycans on hK6 Activity**—The FRET peptide Abz-KLRSSKQ-EDDnp, which was designed based on the substrate specificities of hK1 (20–23), was taken as the initial substrate with which to search for the best conditions required for hK6 activity. We initially observed that this peptide was a better substrate for hK6 in the pH range 7–9.0 than any other commercially available substrate. The relative hydrolytic activity of hK6 on the peptide Abz-KLRSSKQ-EDDnp at pH 7.5 and 9.0 in the presence of 1 M sodium citrate, sodium sulfate, and sodium acetate and 5 mM NaCl are shown in Table 1. hK6 exhibited the greatest activity with sodium citrate and sodium sulfate, pH 7.5, whereas these same salts inhibited the enzyme at pH 9.0. The sodium citrate activation of hK6 is concentration-dependent and is more effective at pH 7.5 than pH 9.0 (Fig. 1). The enzyme activity on an Econosil C-18 column. The molecular weight and purity of synthesized peptides (94% or higher) were checked by amino acid analysis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry, using a ToFSpec-E from Micromass, Manchester, UK. Stock solutions of the peptides were prepared in N,N-dimethylformamide, and the concentrations were measured spectrophotometrically using the molar extinction coefficient of 17,300 M$^{-1}$ cm$^{-1}$ at 365 nm.

**Glycosaminoglycans**—We have used a size-defined (10 kDa) bovine lung heparin (The Upjohn Co.), prepared by using size exclusion column approach (61); heparan sulfate (16,000 Da) from bovine lung was a generous gift from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy) (61); dermatan sulfate (12,000 Da) and chondroitin sulfate (25,000 Da) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan).

**Kinetic Measurements**—The FRET peptides were assayed in a Shimadzu RF-1501 spectrofluorometer at 37°C. The assays were performed in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 2 mM sodium citrate or 20 mM Tris-HCl, pH 9.0, containing 1 mM EDTA. The enzymes were preincubated in the assay buffer for 3 min before the addition of substrate. Fluorescence changes were monitored continuously at $\lambda_{ex} =$ 320 nm and $\lambda_{em} =$ 420 nm. The enzyme concentrations for initial rate determinations were chosen at a level intended to hydrolyze less than 5% of the amount of added substrate over the time course of data collection. The slope of the generated fluorescence signal was converted into micromoles of substrate hydrolyzed per min based on a calibration curve obtained from the complete hydrolysis of each peptide. The substrate concentration for determination of the kinetic parameters was between two $K_s$ values higher and lower than the obtained value. The kinetic parameters $K_m$ and $k_{cat}$ were calculated by nonlinear regression using the Grafit program (62). For peptides hydrolyzed at more than one site, or for substrate exhibiting inhibition, the apparent $k_{cat}/K_m$ values were also determined under pseudo-first order conditions (where [S] $\ll$ $K_m$) and performed under two different substrate concentrations. Errors were less than 5% for each of the obtained kinetic parameters.
reduced in 1 m sodium acetate and 5 mM NaCl. The chloride salts of K⁺, NH₄⁺, Mg²⁺, and Ca²⁺ inhibited hK6 at pH 7.5 or 9.0. The monovalent cations at 5 μM concentration inhibited 20–50% of the enzyme activity, whereas Mg²⁺ and Ca²⁺ completely inhibited the enzyme at the same concentration.

Chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin activated hK6 in a concentration-dependent manner, but with distinctly different profiles at pH 7.5 and 9.0 (Fig. 2). At pH 9.0 all the glycosaminoglycans activated hK6, with a maximum stimulation occurring at a concentration of 1–2 μM for heparan and chondroitin sulfate and 10–20 μM for heparin and dermatan sulfate, respectively. At higher concentrations, all the GAGs reduced their activation or even inhibited the hK6 activity. At pH 7.5, all the GAGs activated hK6 with higher relative efficiency than either a similar comparison at pH 9.0 or in the absence of GAGs. Inhibition was not detected at pH 7.5 even at higher GAG concentrations, although with heparin a maximum activity of hK6 was reached at 20 μM and then decreased at higher concentrations. NaCl inhibited the activation of hK6 by heparin and by 2 M sodium citrate as shown in Fig. 3, indicating that chloride ions compete with heparin for their interactions with hK6. The decrease of the sodium citrate activation by chloride ions could be a result of perturbing the kosmotropic (water maker structure) properties of citrate (63–65). On the other hand, as shown in Fig. 4, heparin was able to increase the hK6 activity in the presence of 2 M sodium citrate, indicating that the activation by these two compounds is additive. The pH-profile activity of hK6 is modified in the presence of sodium citrate or heparin, as shown in Fig. 5. The optimum activity is displaced to pH 8 by citrate, whereas in the presence of 10 μM heparin the hK6 activity has little variation in the pH range 7–9. This result is in accordance with that obtained with the dose

---

**FIGURE 1.** Activation of hK6 with sodium citrate at pH 7.5 (○) and pH 9.0 (●). The reactions were performed in 20 mM Tris-HCl, 1 mM EDTA with sodium citrate, pH 7.5 or pH 9.0, using as substrate Abz-KLRSSKQ-EDDnp ([S] = 5.0 μM), and the concentration of enzyme was 0.5 nM at 37 °C.

**FIGURE 2.** Activation of hK6 with glycosaminoglycans (chondroitin sulfate, dermatan sulfate, heparan sulfate) at pH 7.5 (○) and pH 9.0 (●). The activation was performed in 20 mM Tris-HCl, 1 mM EDTA with sodium citrate, pH 7.5 or pH 9.0, using as substrate Abz-KLRSSKQ-EDDnp ([S] = 5.0 μM), and the concentration of enzyme was 0.5 nM at 37 °C. The bars represent the S.E. of at least three determinations.

**FIGURE 3.** Effect of sodium chloride on human kallikrein 6 (hK6) activated by sodium citrate (○) and heparin (●). All the reactions were done in 20 mM Tris-HCl, 1 mM EDTA, 2 M sodium citrate, pH 7.5, or 20 mM Tris-HCl, 1 mM EDTA, 10 μM heparin, pH 9.0, using as substrate Abz-KLRSSKQ-EDDnp ([S] = 5.0 μM), and the concentration of hK6 was 0.5 nM at 37 °C.
hK6 Activity Modulation and Substrate Specificity

**FRET Peptides Derived from Abz-KLRSSKQ-EDDnp**—The peptide Abz-KLRSSKQ-EDDnp was taken as reference, and a series of five related FRET peptides were synthesized and assayed as substrates for hK6. Assays were performed in 50 mM Tris, pH 7.5, in the presence of 2 M sodium citrate, a condition in which we observed the maximum activity of hK6, as well as pH 9.0, in the absence of this salt. The specificity of the S1 subsite was explored with the peptide series Abz-KLRSSKQ-EDDnp (where X indicates Arg, Lys, Phe, Tyr, Met, Leu, Val, Trp, Asp, Glu, Gly, His, Ile, Asn, Pro, Gln, Ser, and Thr). Only the peptide Abz-KLRSSKQ-EDDnp was hydrolyzed at pH 7.5 in the presence 2 M sodium citrate, and at pH 9.0 without salt, with the kinetic parameters as follows: pH 7.5, \( k_{cat} = 14.2 \text{ s}^{-1}, K_m = 2.8 \mu M, k_{cat}/K_m = 5071 \text{ s}^{-1} \text{ M}^{-1} \); pH 9.0, \( k_{cat} = 2.3 \text{ s}^{-1}, K_m = 4.0 \mu M, k_{cat}/K_m = 575 \text{ s}^{-1} \text{ M}^{-1} \). All other peptides (including X indicating Lys) were resistant in both conditions up to 50 mM of hK6; therefore, these results show that the S1 subsite of hK6 has a very restricted specificity for Arg. In contrast, the activity of hK1 on the same peptide series (Abz-KLRSKQ-EDDnp) hydrolyzed the peptides with X indicating Arg, Lys, Phe, Tyr, and Met at the peptide bonds Arg–Ser (\( k_{cat}/K_m = 2467 \text{ s}^{-1} \text{ M}^{-1} \)), Lys–Ser (\( k_{cat}/K_m = 519 \text{ s}^{-1} \text{ M}^{-1} \)).

The Best Designed Substrate for hK6 and Its Relation to the ATD of the Ionotropic Glutamate Receptor (iGluR) Subunits—The peptide Abz-AFRFSKQ-EDDnp was synthesized containing the selected amino acids that produced the best substrates in each one of the five FRET peptide series described above. hK6 hydrolyzed this peptide at pH 7.5 with 2 M sodium citrate with the parameters as follows: \( k_{cat} = 16 \text{ s}^{-1}, K_m = 0.8 \mu M, k_{cat}/K_m = 20,000 \text{ s}^{-1} \text{ M}^{-1} \), which resulted in the best substrate yet among all the tested FRET peptides. A shorter homologue of Abz-
**hK6 Activity Modulation and Substrate Specificity**

**TABLE 2**

Kinetic parameters for the hydrolysis of the FRET peptide series Abz-XLRSSKQ-EDDnp and Abz-KXRSSKQ-EDDnp by recombinant hK6 for the characterization of its $S_1$ and $S_2$ substrate specificities.

The following conditions for substrate hydrolysis were used: for pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, and 2 mM sodium citrate; for pH 9.0, 50 mM Tris-HCl, 1 mM EDTA (values in parentheses). All the reactions were done at 37 °C, with [substrate] = 0.1–10 μM and [hK6] = 7.5–15.0 nM.

| X       | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|----------|-------|-----------|----------------|
| Arg      | 5.7 (1.7) | 4.5 (0.6) | 790 (353) |
| Lys      | 8.0 (1.5) | 3.0 (0.4) | 375 (267) |
| His      | 6.3 (4.9) | 29.6 (1.5) | 4698 (265) |
| Phe      | 4.2 (6.3) | 5.7 (2.5) | 1357 (362) |
| Tyr      | 8.2 (3.2) | 0.9 (0.3) | 110 (94) |
| Met      | 3.2 (1.9) | 4.1 (0.8) | 1281 (421) |
| Pro      | 6.3 (1.9) | 7.8 (0.7) | 1238 (368) |
| Leu      | 2.9 (4.0) | 14.2 (2.3) | 4897 (575) |
| Ile      | 4.0 (2.4) | 7.5 (0.9) | 1875 (375) |
| Val      | 4.6 (2.4) | 10.0 (0.9) | 2174 (375) |
| Ala      | 5.4 (2.6) | 7.9 (0.7) | 1463 (269) |
| Gly      | 7.7 (1.4) | 2.8 (0.4) | 364 (286) |
| Ser      | 6.4 (1.3) | 3.9 (0.4) | 609 (308) |
| Gln      | 5.7 (2.4) | 12.7 (0.8) | 2228 (333) |
| Thr      | 8.0 (2.8) | 8.1 (1.5) | 1012 (625) |
| Ser      | 4.1 (3.9) | 5.4 (1.6) | 1317 (440) |
| Glu      | 6.1 (3.6) | 6.5 (0.3) | 1066 (83) |

| $k_{cat}/K_m$ |
|---------------|
| 3.3 (5.3)      |
| 2.9 (4.0)      |
| 5.5 (14.7)     |
| 5.4 (12.4)     |

AUSRFSKQ-EDDnp was also synthesized (removing Lys at the carboxy-terminal site), and the resulting peptide AUSRFSQ-EDDnp was an even more efficient substrate ($k_{cat} = 11.6 s^{-1}$, $K_m = 0.3 \mu M$, $k_{cat}/K_m = 38,667 s^{-1} \mu M^{-1}$). This result suggests that Lys at the P$_2$ position has some influence on the susceptibility of the substrate hydrolysis by hK6, which was confirmed by the kinetic parameters of hydrolysis at pH 7.5 with 2 M sodium citrate of the peptides Abz-KLRSAQ-EDDnp ($k_{cat} = 11.2 s^{-1}$, $K_m = 1.6 \mu M$, $k_{cat}/K_m = 7,000 s^{-1} \mu M^{-1}$), Abz-KLRSQQ-EDDnp ($k_{cat} = 8.1 s^{-1}$, $K_m = 0.8 \mu M$, $k_{cat}/K_m = 10,125 s^{-1} \mu M^{-1}$), and Abz-KLRSQKQ-EDDnp ($k_{cat} = 37.8 s^{-1}$, $K_m = 1.4 \mu M$, $k_{cat}/K_m = 27,000 s^{-1} \mu M^{-1}$).

We have searched the sequence AUSRFS in the human Protein Data Bank using the BLAST program (www.ncbi.nlm.nih.gov/BLAST), and this sequence was found in the ATD of ionotropic glutamate receptor kainate 3 (iGluR-7) subunit. Based on this information, we compared the amino acid sequences of the ATD of kainate, AMPA, and N-methyl-D-aspartic acid iGluR subunits, and the AUSRFS motif or similar sequences were found in iGluR-1 through iGluR-7 subunits as shown in Table 4. The subunits KA1 and KA2 of kainate iGluR and the subunits NR1, NR2A through NR2D, NR3A, and NR3B do not present either AUSRFS motif or similar sequences. FRET peptides containing the variations of the AUSRFS motif found in iGluR-1 through iGluR-7 subunits (AURFKA, AFKFA, AFRVG, and AFRLA) were synthesized, and the kinetic parameters of their hydrolysis by hK6 were determined (Table 5). AUSRFAFQ-EDDnp was hydrolyzed with a high $k_{cat}/K_m$ value as observed for the hydrolysis of Abz-AFRFAQ-EDDnp. However, the peptides derived from AMPA-2 and AMPA-4, which are AUSRFAVRGQ-EDDnp and AUSRFAFAQ-EDDnp, respectively, were still hydrolyzed but with lower $k_{cat}/K_m$ values, and the peptide derived from kainite iGluR (AUSRFAFAQ-EDDnp, i.e., P$_3$ indicates Lys) was resistant to hydrolysis by hK6 (Table 5).

It is noteworthy that two or three amino acid residues upstream of the motif AUSRFS there are one or two acidic Glu residues, which are also present in similar places relative to the auto-activation cleavage site of pro-hK6 and in the precursor of the Aβ amyloid peptide (see below). To investigate the effect of these acidic Glu residues, the following peptides were synthesized and assayed with hK6: AUSRFAFAQ-EDDnp, AUSRFAFAQ-EDDnp, and AUSRFAFAQ-EDDnp, which correspond to Glu$_{25}$-Ser$_{32}$, iGlu kainate-3; Ala$_{19}$-Ala$_{27}$, iGlu AMPA-3; and Ala$_{27}$-Ala$_{35}$, iGlu kainate-1 receptors, respectively. The peptides

**TABLE 3**

Kinetic parameters for the hydrolysis of the FRET peptide series Abz-KLRSSQKQ-EDDnp and Abz-KLRSSKQ-EDDnp by recombinant hK6 for the characterization of its $S_1$ and $S_2$ substrate specificities.

The following conditions for substrate hydrolysis were used: for pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, and 2 mM sodium citrate; for pH 9.0, 50 mM Tris-HCl, 1 mM EDTA (values in parentheses). All the reactions were done at 37 °C, with [substrate] = 0.1 to 10 μM and [hK6] = 7.5–15.0 nM.

| X       | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|----------|-------|-----------|----------------|
| Arg      | 2.6 (2.0) | 6.0 (0.4) | 2308 (200) |
| His      | 1.0 (1.7) | 4.5 (0.4) | 4500 (235) |
| Phe      | 1.0 (1.6) | 12.0 (0.6) | 12000 (375) |
| Pro      | 0.8 (2.0) | 2.6 (0.6) | 3250 (300) |
| Leu      | 0.25 (1.6) | 0.9 (0.2) | 3600 (125) |
| Val      | 0.6 (1.6) | 1.6 (0.2) | 2667 (125) |
| Ala      | 1.2 (2.6) | 4.6 (0.7) | 3833 (269) |
| Gly      | 4.8 (1.2) | 4.6 (0.2) | 958 (167) |
| Asn      | 3.2 (3.3) | 8.8 (0.8) | 2750 (242) |
| Gln      | 2.5 (1.9) | 4.6 (0.2) | 1840 (105) |
| Ser      | 2.9 (4.0) | 14.2 (2.3) | 4897 (575) |
| Glu      | 3.8 (4.7) | 2.8 (0.1) | 737 (21) |

| $k_{cat}/K_m$ |
|---------------|
| 1.0 (1.3)      |
| 8.3 (1.9)      |
| 10.1 (2.0)     |
| 3.6 (2.2)      |
| 2.4 (3.6)      |
| 3.3 (5.8)      |
| 6.9 (11.9)     |
| 6.3 (2.6)      |
| 10.1 (1.5)     |
| 2.9 (4.0)      |
| 6.6 (11.2)     |

*No hydrolysis of the peptide Abz-GLRSSQKQ-EDDnp was detected at pH 9.0.*
**TABLE 4**

**ATD sequences of the first 45 amino acids of kainate and AMPA iGluR subunits**

The alignment of the sequences was done forcing the superposition of the AFRFS sequence indicated in boldface and capital letters. The first amino acid in each sequence corresponds to the first amino acid of iGluR in its mature form. The nomenclature is as follows: kainate iGluR is stimulated by kainic acid ((2S,3S,4R)-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid) and AMPA iGluR is stimulated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid).

| Accession no. | ATD-iGlu | Sequence (amino-terminal amino acids) |
|---------------|----------|---------------------------------------|
| Q13003        | GluR-7   | 1mphvirigfeyadgpnqvmn ValehAFRFSanimrtlipn |
| Q13002        | GluR-6   | 1kthfjergyeyegpmqesAFRFAvtnimrtlipmtt |
| P39086        | GluR-5   | 1qtapqvirigfetvenpevnneAFLFACvtnimrtlipmt |
| P48058        | GluR-4   | 1gafpssvjqiggfimrtqeyAFRLAlifhntnapaseapfnvs |
| P42263        | GluR-3   | 1gfpnisiqfgfimrtqehsAFRFAvynqntteegphlny |
| P42262        | GluR-2   | 1vssissiqiggfpragdxyAFRFGvqfisfrtpd |
| P42261        | GluR-1   | 1anfnniejqiggfplnqseohaAFRFAbsqleppklpdiv |
Abz-EHSAFRFAQ-EDDnp and Abz-EHSARFAQ-EDDnp were hydrolyzed with k_{cat}/K_{m} values 5–7 times lower than the corresponding short homologues Abz-AFRFAQ-EDDnp and Abz-AFRFAQ-EDDnp exclusively because of an increase of K_{m} values. On the other hand, Abz-EELAFKFAQ-EDDnp was one of the rare peptides hydrolyzed by hK6 (albeit at a very low level) at the carboxyl-terminal side of Lys, whereas its short homologue, Abz-AFRFAQ-EDDnp, was resistant (Table 5).

The large differences in the kinetic parameters for the hydrolysis of Abz-AFRFAQ-EDDnp and Abz-AFRFAQ-EDDnp in the presence of citrate at pH 7.5 and in the absence of this salt at pH 9.0 is noteworthy (Table 5). In particular, the presence of citrate increased the k_{cat}/K_{m} value for hydrolysis of Abz-AFRFAQ-EDDnp by almost 3 orders of magnitude. The presence of the acidic Glu residues in the amino-terminal extension of these two peptides (Abz-EHSAFRFAQ-EDDnp and Abz-EHSARFAQ-EDDnp) did not change the effect of citrate as evaluated only by the k_{cat}/K_{m} parameter.

**FRET Peptides Derived from Proteins Described as Substrates for hK6**

All the peptides in this study were assayed with hK6 in 50 mM Tris, 1 mM EDTA, 2 mM sodium citrate, pH 7.5, and 37 °C. **Myelin Basic Protein**—We selected from the sequence of MBP 18 peptide fragments of 7 amino acids in which Arg or Lys is located in the middle of them and the corresponding FRET peptides were synthesized. All the susceptible peptides were hydrolyzed on the carboxyl-terminal side of Arg, and only the peptides 1, 10, and 12 (Table 6) were resistant to hK6. These three peptides have Lys as the only basic amino acid available for cleavage, which confirmed the very restricted S1 specificity for Arg as described above. In addition, the large number of Arg residues along the MBP protein sequence and the observed susceptibility to hydrolysis by hK6 of the peptides derived from MBP containing this basic amino acid is in accordance to the early described high fragmen-

tation of MBP by hK6 (46). The peptides 2, 9, 15, and 16 (Table 6) were hydrolyzed with the higher k_{cat}/K_{m} values, and they present as a common feature the presence of amino acids with a small side chain, particularly peptide 16, which contains Gly, Gly, and Ala at positions P_{5}, P_{3}, and P_{1}' respectively. These amino acids do not result in peptides that are hydrolyzed by hK6 with high efficiency compared with the best substrates derived from Abz-KLRSSQK-EDDnp described above; however, the peptides 2, 9, 15, and 16 are flexible sequences, and they could be the preferential sites of cleavage inside MBP. The peptides 7 and 13 were digested with low k_{cat}/K_{m} values, and this could be related to the presence of Pro at the P_{2} and P_{1}' positions in these substrates; similarly Pro at these same positions in the substrates derived from Abz-KLRSSQK-EDDnp were also poorly hydrolyzed. Substrate inhibition of hK6 was observed with the peptides 5, 15, and 18, and the k_{cat}/K_{m} values for their hydrolysis were obtained in pseudo-first order condition.

**Precursor of the AB Amyloid Peptide**—Three peptides from this protein were selected based on the earlier reported cleavage sites on this protein by hK6 (45), and the corresponding FRET peptides were synthesized (Table 7). By using 15 nM hK6, only peptide 20 was hydrolyzed at the Arg–His bond, whereas peptides 19 and 21 that contain Lys as basic residue were resistant to hydrolysis. Although peptide 20 is a poor substrate to hK6 compared with other peptides described above, this result indicates that amyloid precursor protein would be predominantly hydrolyzed by hK6 inside the Aβ amyloid peptide because the Arg–His bond is available at the amino terminus (as its fifth peptide bond). In addition, peptides 19 and 21 were extended to the amino-terminal side with the same sequence of Aβ amyloid peptide precursor to introduce the glutamic acid residues (peptides 19-A and 21-A in Table 7). These peptides were hydrolyzed but with very low k_{cat}/K_{m} values.

**a1-Antichymotrypsin**—Three FRET peptides derived from the reactive center loop of a1-antichymotrypsin were synthesized and assayed as substrate for hK6, because this serpin was described to inhibit (45) and to be covalently associated with hK6 (43). Peptide 22 contains residues P_{1} to P_{1}' of the reactive center loop of a1-antichymotrypsin that is cleaved by chymotrypsin and chymotrypsin-like proteases at the Leu–Ser bond with formation of a stable complex. This peptide was resistant to hK6, which is in accordance to the restricted requirement of the enzyme for Arg, but remains the question as to how a1-antichymotrypsin interacts and establishes a covalent complex with hK6. Two other peptides (peptides 23 and 24) were synthesized but now with sequences of the reactive center loop of a1-antichymotrypsin that contain the Arg, which are downstream to the Leu–Ser bond (see the structures in the Table 7 and in its legend). Peptide 23 was hydrolyzed with very low k_{cat}/K_{m} value, and peptide 24 was a better substrate, but inhibition of its hydrolysis was detected at a concentration above 5 μM.

**Plasminogen**—hK6 was described to cleave plasminogen in the three peptide bonds Lys^{66}–Lys^{77}, Lys^{97}–Val^{106}, and Ser^{460}–Val^{465} (53). We synthesized two FRET peptides containing the sequences 91-ValFEKVKY^{100} (Abz-VLFEKVKYQ-EDDnp) and 464-GTEAVSV^{466} (Abz-GTENASVPO-EDDnp) from human plasminogen that included in the middle of them the reported cleavage sites. However, both peptides at 1 μM concentration were resistant to hydrolysis of 50 nM hK6 for 30 min.

**Maturation and Inactivation Cleavage Sites of hK6**—The inactive hK6 proprotein is converted to an active peptidase upon cleavage at the Lys^{13}–Leu^{16} peptide bond. The ability of hK6 itself to perform this step is a matter of some debate (45–47, 53), and hK6 in one report has been characterized as exhibiting a dual specificity for cleavage after both Gin^{15}–Asn^{14} and Lys^{55}–Leu^{16} peptide bonds (53). We synthesized the
Kinetic parameters for the hydrolysis of the FRET peptide derived from human MBP

The following conditions for substrate hydrolysis were used: for pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, and 2 mM sodium citrate. The number in parentheses for each FRET peptide corresponds to the numbered and underlined basic amino acids in the sequence of MBP (see below) that were fixed in the middle of each FRET peptide. ↓ indicates the cleaved peptide bond. The $k_{cat}/K_m$ values for peptides 5, 15, and 18 were determined in pseudo-first order condition due to substrate inhibition. No hydrolysis was detected up to 50 nM hK6. The amino acid sequence of MBP is as follows: ASQKRPSQ-EDDnp (see below) that were fixed in the middle of each FRET peptide.

1. Abz-ASQKRPSQ-EDDnp
2. Abz-BPSQR ↓ HATQ-EDDnp
3. Abz-DHAR ↓ HFGQ-EDDnp
4. Abz-FLPR ↓ HRIQ-EDDnp
5. Abz-LPR ↓ HR ↓ DTGQ-EDDnp
6. Abz-SIGR ↓ FFQG-EDDnp
7. Abz-GGDR ↓ GSGQ-EDDnp
8. Abz-AKPR ↓ GSGQ-EDDnp
9. Abz-APKR ↓ GSGQ-EDDnp
10. Abz-APKR ↓ GSGQ-EDDnp
11. Abz-SHGR ↓ QGGR-EDDnp
12. Abz-SLPQKSHGQ-EDDnp
13. Abz-TrpTR ↓ TPQQ-EDDnp
14. Abz-AKQGR ↓ GSQG-EDDnp
15. Abz-SLSR ↓ FSQG-EDDnp
16. Abz-LIGGR ↓ DSRQ-EDDnp
17. Abz-LGGR ↓ DSRQ-EDDnp
18. Abz-RDSR ↓ GSQG-EDDnp

**Peptides**

| Peptides | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (s⁻¹ mM⁻¹) |
|-----------|------------|-----------------|--------------------------|
| 1) Abz-ASQKRPSQ-EDDnp | 4.6 | 4.9 | 1065 |
| 2) Abz-BPSQR ↓ HATQ-EDDnp | 8.4 | 1.8 | 214 |
| 3) Abz-DHAR ↓ HFGQ-EDDnp | 4.3 | 2.5 | 592 |
| 5) Abz-LPR ↓ HR ↓ DTGQ-EDDnp | 1.0 | 0.1 | 100 |
| 7) Abz-GGDR ↓ GSGQ-EDDnp | 2.6 | 0.8 | 308 |
| 9) Abz-APKR ↓ GSGQ-EDDnp | 1.9 | 4.0 | 2105 |
| 10) Abz-SLPQKSHGQ-EDDnp | No hydrolysis | No hydrolysis | No hydrolysis |
| 11) Abz-SHGR ↓ QGGR-EDDnp | No hydrolysis | No hydrolysis | No hydrolysis |
| 12) Abz-SLPQKSHGQ-EDDnp | No hydrolysis | No hydrolysis | No hydrolysis |
| 13) Abz-TrpTR ↓ TPQQ-EDDnp | 3.5 | 0.3 | 87 |
| 14) Abz-AKQGR ↓ GSQG-EDDnp | 2.4 | 0.5 | 195 |
| 15) Abz-SLSR ↓ FSQG-EDDnp | No hydrolysis | No hydrolysis | No hydrolysis |
| 16) Abz-LIGGR ↓ DSRQ-EDDnp | No hydrolysis | No hydrolysis | No hydrolysis |
| 17) Abz-LGGR ↓ DSRQ-EDDnp | No hydrolysis | No hydrolysis | No hydrolysis |
| 18) Abz-RDSR ↓ GSQG-EDDnp | No hydrolysis | No hydrolysis | No hydrolysis |

**Km**

The following conditions for substrate hydrolysis were used: for pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, and 2 mM sodium citrate. The number in parentheses for each FRET peptide corresponds to the numbered and underlined amino acids in the sequences of the precursor of Aβ amyloid peptide and the reactive center loop of α-antichymotrypsin (see below) that were fixed in the middle of each FRET peptide. ↓ indicates the cleaved peptide bond. The $k_{cat}/K_m$ value for the peptide 24 was determined in pseudo-first order condition due to substrate inhibition. No hydrolysis was detected up to 50 nM hK6. The amino acid sequence of MBP is as follows: ASQKR1PSQR2HATASTMDHAR3 HGFLPR4HR5DTGILDSIGR6FFGGDR7GAPKR8GSGKD-

**kinetic parameters for the hydrolysis of the FRET peptide derived from human MBP**

The following conditions for substrate hydrolysis were used: for pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, and 2 mM sodium citrate. The number in parentheses for each FRET peptide corresponds to the numbered and underlined amino acids in the sequences of the precursor of Aβ amyloid peptide and the reactive center loop of α-antichymotrypsin (see below) that were fixed in the middle of each FRET peptide. ↓ indicates the cleaved peptide bond. The $k_{cat}/K_m$ value for the peptide 24 was determined in pseudo-first order condition due to substrate inhibition. No hydrolysis was detected up to 50 nM hK6. The amino acid sequence of MBP is as follows: ASQKR1PSQR2HATASTMDHAR3 HGFLPR4HR5DTGILDSIGR6FFGGDR7GAPKR8GSGKD-

**kinetic parameters for the hydrolysis of the FRET peptide derived from human MBP**

The following conditions for substrate hydrolysis were used: for pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, and 2 mM sodium citrate. The number in parentheses for each FRET peptide corresponds to the numbered and underlined amino acids in the sequences of the precursor of Aβ amyloid peptide and the reactive center loop of α-antichymotrypsin (see below) that were fixed in the middle of each FRET peptide. ↓ indicates the cleaved peptide bond. The $k_{cat}/K_m$ value for the peptide 24 was determined in pseudo-first order condition due to substrate inhibition. No hydrolysis was detected up to 50 nM hK6. The amino acid sequence of MBP is as follows: ASQKR1PSQR2HATASTMDHAR3 HGFLPR4HR5DTGILDSIGR6FFGGDR7GAPKR8GSGKD-

**kinetic parameters for the hydrolysis of the FRET peptide derived from human MBP**

The following conditions for substrate hydrolysis were used: for pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, and 2 mM sodium citrate. The number in parentheses for each FRET peptide corresponds to the numbered and underlined amino acids in the sequences of the precursor of Aβ amyloid peptide and the reactive center loop of α-antichymotrypsin (see below) that were fixed in the middle of each FRET peptide. ↓ indicates the cleaved peptide bond. The $k_{cat}/K_m$ value for the peptide 24 was determined in pseudo-first order condition due to substrate inhibition. No hydrolysis was detected up to 50 nM hK6. The amino acid sequence of MBP is as follows: ASQKR1PSQR2HATASTMDHAR3 HGFLPR4HR5DTGILDSIGR6FFGGDR7GAPKR8GSGKD-

**kinetic parameters for the hydrolysis of the FRET peptide derived from human MBP**

The following conditions for substrate hydrolysis were used: for pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, and 2 mM sodium citrate. The number in parentheses for each FRET peptide corresponds to the numbered and underlined amino acids in the sequences of the precursor of Aβ amyloid peptide and the reactive center loop of α-antichymotrypsin (see below) that were fixed in the middle of each FRET peptide. ↓ indicates the cleaved peptide bond. The $k_{cat}/K_m$ value for the peptide 24 was determined in pseudo-first order condition due to substrate inhibition. No hydrolysis was detected up to 50 nM hK6. The amino acid sequence of MBP is as follows: ASQKR1PSQR2HATASTMDHAR3 HGFLPR4HR5DTGILDSIGR6FFGGDR7GAPKR8GSGKD-

**kinetic parameters for the hydrolysis of the FRET peptide derived from human MBP**

The following conditions for substrate hydrolysis were used: for pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, and 2 mM sodium citrate. The number in parentheses for each FRET peptide corresponds to the numbered and underlined amino acids in the sequences of the precursor of Aβ amyloid peptide and the reactive center loop of α-antichymotrypsin (see below) that were fixed in the middle of each FRET peptide. ↓ indicates the cleaved peptide bond. The $k_{cat}/K_m$ value for the peptide 24 was determined in pseudo-first order condition due to substrate inhibition. No hydrolysis was detected up to 50 nM hK6. The amino acid sequence of MBP is as follows: ASQKR1PSQR2HATASTMDHAR3 HGFLPR4HR5DTGILDSIGR6FFGGDR7GAPKR8GSGKD-
DISCUSSION

The anion-dependent activation of hK6 followed the Hofmeister series, citrate > sulfate > acetate > chloride only at pH 7.5, which contrasts with the inhibition effect of all assayed cations in the chloride form. The effect of citrate seems to be restricted to a local rather than global effect in increasing the hK6 peptidase activity because if any conformational modification of hK6 occurred because of a kosmotropic effect of citrate, it was not large enough to be detected by CD spectroscopy, gel filtration chromatography, or anilinonaphthalenesulfonic acid binding to the enzyme (results not shown). These results are not in accord with an earlier reported 100-fold increase of hK3 activity produced by kosmotropic salts that was also accompanied by changes of hK3 structure detectable by CD spectroscopy and gel filtration chromatography (27). It is noteworthy that the effects of salts on the hydrolytic activity reported for hK3 (27) and those reported here for hK6 occur at high ionic strength. This condition obtained by 2 M sodium citrate for hK6 activation was perturbed by further addition of an increased amount of NaCl (Fig. 3), and then the structure of water is probably taking a significant role in enzyme activity. This dependence on the buffer composition of the activity of tissue kallikreins still requires a more detailed study because the function of these enzymes seems to have a strict dependence on the milieu composition that can be the way the organism controls their functions.

The modulation of hK6 activity observed with GAGs and its dependence upon pH are indications of the hK6-GAG interaction. In addition, the hydrolysis of the peptide Abz-AFRFSQ-EDDnp at pH 7.5 was also activated in the presence of 10 μM heparin (data not shown), although this substrate differs in two positive charged amino acids from Abz-KLRSSKQ-EDDnp. Particularly noteworthy are the k\textsubscript{cat} values at pH 7.5 without this anion, and displaced the optimum pH of the enzyme (Fig. 2). The activation by GAGs seems not to be a general property of the kallikrein family of proteases because no effect was observed with the four GAGs on hK1 activity (results not shown), although hK3, but not hK2, was described to be activated by heparin (28). The activation of hK6 by GAGs is particularly noteworthy because this enzyme is highly expressed in the CNS, and GAGs are extracellular matrix components associated with CNS remodeling, development, and disease (for reviews see Refs. 49 and 66). Consequently, further studies of the modulation mechanism of hK6 activity by GAGs on potentially natural substrates is worthwhile from a physiological and pathological point of view, particularly because the extent of the activation effects of GAGs on hK6 also varied with the substrate sequence.

The kinetic parameters of hydrolysis of the series of FRET peptides derived from Abz-KLRSSKQ-EDDnp (Tables 2 and 3) show that citrate essentially increased the catalytic process of hK6 because the k\textsubscript{cat} values at pH 7.5 in the presence of 2 M citrate are ~10-fold higher than at pH 9.0 without this anion, and displaced the optimum pH of the enzyme toward lower values (Fig. 5). In contrast, when comparing the k\textsubscript{cat} values at pH 7.5 in the presence of citrate with those obtained at pH 9.0, the relative values depend on the amino acid sequence of the substrates derived from Abz-KLRSSKQ-EDDnp. Particularly noteworthy are the lower k\textsubscript{cat} values at pH 9.0 for the hydrolysis of the series with variations at P\textsubscript{2} position (Abz-KXRSSKQ-EDDnp), except for the reference peptide that contained Leu.

The FRET peptide Abz-AFRFSQ-EDDnp is the best substrate described so far for hK6, and it is composed of amino acids that resulted in the best substrate in each one of the five series of substrates derived from Abz-KLRSSKQ-EDDnp used to screen the S\textsubscript{0} to S\textsubscript{2} subsites of hK6. The AFRFS sequence was found in the ATD of the ionotropic glutamate receptor kainate 3 (GluR-7) subunit, and similar sequences were also found in GluR-1 to GluR-7 subunits as shown in Table 4.
hK6 Activity Modulation and Substrate Specificity

hK6 in this biological compartment is controversial in Alzheimer disease patients, because it was described by Mitsui et al. (40) to be decreased and by Diamandis et al. (41) to be increased. In addition, two synthetic peptides derived from the precursor of Aβ amyloid peptide were assayed as substrates for hK6 (45), and three cleavage sites were qualitatively reported after 24 h of incubation at 37 °C. Two cleavage sites were observed inside the Aβ amyloid peptide and the third site at the Lys–Met bond that is one amino acid immediately upstream of the Aβ amyloid peptide. The peptide sequence of the precursor of the Aβ amyloid peptide is shown in Table 7 legend. Only the FRET peptide Abz-DAEFRHDSQ-EDDnp that is derived from the amino-terminal segment of Aβ amyloid peptide was hydrolyzed by hK6 at the Arg–His bond under the conditions evaluated, whereas Abz-ISEVKMDAQ/H9251 and Abz-VHHQKLVFQ-EDDnp were resistant (Table 7). These two peptides correspond to the sequence upstream and inside, respectively, of the Aβ amyloid peptide and have Lys as the only basic residue. As mentioned above, a consistent result in the present study is the selectivity of hK6 for Arg over Lys at the P1 position. Therefore, our results support the hypothesis that hK6 could play a role in the hydrolysis of the Aβ amyloid peptide at the amino-terminal Arg residue, impairing its accumulation, and the deficiency of hK6 could facilitate the formation of the plaques in the neurons in the Alzheimer disease.

An α1-antichymotrypsin-hK6 complex was reported in milk and ascites fluids (43). Three peptides derived from the reactive center loop of α1-antichymotrypsin were synthesized (peptides 22–24 in Table 7), and only those with Arg were hydrolyzed. Peptide 22 (Abz-KITLSSLVQ-EDDnp) that contains the cleavage site of chymotrypsin (Leu–Ser bond) during the serpin–enzyme complex formation was resistant to hK6. Therefore, the α1-antichymotrypsin-hK6 complex seems not to be established as chymotrypsin does, or alternatively, hK6 could exceptionally hydrolyze the Leu–Ser bond but only in the entire reactive center loop of α1-antichymotrypsin.

The FRET peptide derived from PAR 2 was the only PAR susceptible to hK6 cleavage and is hydrolyzed with a high kcat value compared with all the studied substrates of this paper. Similar to hK6, the highest densities of PAR 2 were observed in the hippocampus, cortex, amygdala, thalamus, hypothalamus, and striatum (55), and in the rat hippocampus PAR 2 is associated with neurodegeneration (74). In addition, PAR 2 has also been associated with hyperalgesia (75), and agonists of PAR 2 induce inflammation by a neurogenic mechanism (57). Therefore, the high susceptibility of the PAR 2-derived FRET peptide to hK6 highlights the necessity to examine the involvement of this kallikrein in neuronal processes in which PAR 2 is activated.

In conclusion, this study provides biochemical data focused on the substrate specificity in the presence of kosmotropic salts or GAGs. We succeeded in identifying efficient substrates for hK6 and correlating them with two potential and noteworthy natural substrates, namely ionotropic glutamate receptor subunits and PAR 2. These results further support the original hypothesis that the MSP/KLK6 gene codes for an Arg-specific trypsin-like degradative protease that is expressed in the brain (34). Because the earlier studies imply excess MSP/hK6 activity in the development of immune-mediated demyelination in both animal models of multiple sclerosis and in human multiple sclerosis lesions (37, 44), the availability of an atomic model of mature hK6 (46) and the enzyme specificity data (this work) may prove useful in the design of specific and potentially therapeutic inhibitors for hK6.

REFERENCES

1. Yousef, G. M., Luo, L. Y., Scherer, S. W., Sotiropoulou, G., and Diamandis, E. P. (1999) Genomics 62, 251–259
2. Borgoño, C. A., and Diamandis, E. P. (2004) Nat. Rev. Cancer 4, 876–890
3. Harvey, T. J., Hooper, J. D., Myers, S. A., Stephenson, S. A., Ashworth, L. K., and Clements, J. A. (2000) J. Biol. Chem. 275, 37397–37406
4. Yousef, G. M., and Diamandis, E. P. (2001) Endocr. Rev. 22, 184–204
5. Evans, B. A., Drinkwater, C. C., and Richards, R. I. (1983) J. Biol. Chem. 262, 8027–8034
6. Mason, A. J., Evans, B. A., Cox, D. R., Shine, J., and Richards, R. I. (1983) Nature 303, 300–307
7. Wines, D. R., Brady, J. M., Pritchett, D. B., Roberts, J. L., and MacDonald, R. J. (1989) J. Biol. Chem. 264, 7653–7662
8. Wines, D. R., Brady, J. M., Southard, E. M., and MacDonald, R. J. (1999) J. Med. Eval. 32, 476–492
9. Gerald, W. L., Chao, J., and Chao, L. (1986) Biochim. Biophys. Acta 866, 1–14
10. Blaber, M., Isackson, P. J., Marsters, J. C., Jr., Burnier, P. J., and Bradshaw, R. A. (1989) Biochemistry 28, 7813–7819
11. Hosoi, K., Tsunashawa, S., Kurihara, K., Aoyama, H., Uehe, T., Murai, T., and Sakaiya, F. (1994) J. Biochem. (Tokyo) 115, 137–143
12. Bothwell, M. A., Wilson, W. H., and Shooter, E. M. (1979) J. Biol. Chem. 254, 7287–7294
13. Kim, W. S., Nakayama, K., Nakagawa, T., Kawamura, Y., Haraguchi, K., and Murakami, K. (1991) J. Biol. Chem. 266, 19283–19287
14. Jichlinski, P., Gygi, C. M., Leisinger, H. J., Diamandis, E. P., and Deperthes, D. (2005) J. Mol. Evol. 62, 225–231
15. Yousef, G. M., and Diamandis, E. P. (2001) J. Comp. Neurol. 434, 347–361
16. Del Nery, E., Chagas, J. R., Juliano, M. A., Prado, E. S., and Juliano, L. (1995) Biochem. J. 308, 233–238
17. Scarisbrick, I. A., Towner, M. D., and Isackson, P. J. (1997) J. Neurosci. Res. 47, 219–230
18. Blaber, S. I., Ciric, B., Christophi, G. P., Bennett, M. J., Blaber, M., Rodriguez, M., and Scarsbrick, I. A. (2004) FASEB J. 18, 920–927
19. Zarghooni, M., Soosaipillai, A., Grass, L., Scorilas, A., Mirzaiini, N., and Diamandis, E. P. (2002) Clin. Biochem. 35, 225–231
20. Mitsui, S., Okui, A., Uemura, H., Mizuno, T., Yamada, T., Yamamura, Y., and Yamaguchi, N. (2002) Ann. N. Y. Acad. Sci. 977, 216–223

FEbruary 10, 2006•vOluMe 281•NuMer 6

JOURNAL OF BIOLOGICAL CHEMISTRY 3125

1572-322X/2006/281(6)/216-325$20.00/0

© 2006 the American Society for Biochemistry and Molecular Biology, Inc.
41. Diamandis, E. P., Yusef, G. M., Soosaipillai, A. R., and Buting, P. (2000) Clin. Biochem. 33, 579–583
42. Ni, X., Zhang, W., Huang, K. C., Wang, Y., Ng, S. K., Mok, S. C., Berkowitz, R. S., and Ng, S. W. (2004) Br. J. Cancer 91, 725–731
43. Hutchinson, S., Luo, L. Y., Yusef, G. M., Soosaipillai, A., and Diamandis, E. P. (2003) Clin. Chem. 49, 746–751
44. Blaber, S. I., Scarisbrick, I. A., Bernett, M. J., Dhanarajan, P., Seavy, M. A., Jin, Y., Schwartz, M. A., Rodriguez, M., and Blaber, M. (2002) Biochemistry 41, 1165–1173
45. Magklara, A., Mellati, A. A., Wasney, G. A., Little, S. P., Sotiropoulou, G., Becker, G. W., and Diamandis, E. P. (2003) Biochem. Biophys. Res. Commun. 307, 948–955
46. Bentner, M. J., Blaber, S. I., Scarisbrick, I. A., Dhanarajan, P., Thompson, S. M., and Blaber, M. (2002) J. Biol. Chem. 277, 24562–24570
47. Gomis-Ruth, F. X., Bayes, A., Sotiropoulou, G., Pampalakis, G., Tsetsenis, T., Villegas, V., Aviles, F. X., and Coll, M. (2002) J. Biol. Chem. 277, 27273–27281
48. Bovolenta, P., and Fernaud-Espinosa, I. (2000) Prog. Neurobiol. 61, 113–132
49. Bandtlow, C. E., and Zimmermann, D. R. (2000) Physiol. Rev. 80, 1267–1290
50. Sugahara, K., Mikami, T., Uyama, T., Mizuguchi, S., Nomura, K., and Kitagawa, H. (2003) Curr. Opin. Struct. Biol. 13, 612–620
51. Rhodes, K. E., and Fascetti, J. W. (2004) J. Anat. 204, 33–48
52. Back, S. A., Tseoby, T. M., Chen, H., Wallingford, N., Craig, A., Struve, J., Luo, N. L., Banine, F., Liu, Y., Chang, A., Trapp, B. D., Bebo, B. F., Rao, M. S., and Sherman, L. S. (2005) Nat. Med., 11, 966–972
53. Bayes, A., Tsetsenis, T., Ventura, S., Vendrell, J., Aviles, F. X., and Sotiropoulou, G. (2004) Biol. Chem. 385, 517–524
54. Vu, T. K., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068
55. Striggow, F., Riek-Burchardt, M., Kiesel, A., Schmidt, W., Henrich-Noack, P., Breder, J., Krug, M., Reymann, K. G., and Reiser, G. (2003) Eur. J. Neurosci. 14, 595–608
56. Ossovskaya, V. S., and Bunnett, N. W. (2004) Physiol. Rev. 84, 579–621
57. Steinhoff, M., Vergnolle, N., Young, S. H., Tognetto, M., Amadesi, S., Ennes, H. S., Trevisani, M., Hollenberg, M. D., Wallace, J. L., Caughey, G. H., Mitchell, S. E., Williams, L. M., Geppetti, P., Mayer, E. A., and Bunnett, N. W. (2000) Nat. Med. 6, 151–158
58. Laxmikanth, G., Blaber, S. I., Bentner, M. J., Scarisbrick, I. A., Juliano, M. A., and Blaber, M. (2005) Proteins 58, 802–814
59. Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., and Elmore, D. T. (1973) Biochem. J. 131, 107–117
60. Pirin, I., Cezari, M. H. C., Nakaie, C. R., Boshcov, P., Ito, A. S., Juliano, M. A., and Juliano, L. (1994) J. Neurosci. 14, 29939–3003
61. Dietrich, C. P., Tersario, I. L. S., Toma, L., Moraes, C. T., Porcionatto, M. A., Oliveira, F. W., and Nader, H. B. (1998) Cell. Mol. Biol. 44, 417–429
62. Leatherbarrow, R. J. (1992) Graft, Version 3.0, Erithacus Software, Staines, UK
63. Timasheff, S. N. (1993) Annu. Rev. Biophys. Biomol. Struct. 22, 67–97
64. Cacace, M. G., Landau, E. M., and Ramasden, J. J. (1997) Q. Rev. Biophys. 30, 241–277
65. Ilesaraw, I., Durr, E., Thomas, R. M., and Booshard, H. R. (1998) Biochemistry 37, 7539–7550
66. Rauch, U. (2004) Cell. Mol. Life Sci. 61, 2001–2045
67. Madden, D. R. (2002) Nat. Rev. Neurosci. 3, 91–101
68. Levens, J. (2003) Nat. Rev. Neurosci. 4, 481–495
69. Wollmuth, L. P., and Sobolevsky, A. I. (2004) Trends Neurosci. 27, 321–328
70. Oswald, R. E. (2004) Adv. Protein Chem. 68, 313–349
71. Choi, D. W. (1992) J. Neurobiol. 23, 1261–1276
72. McDonald, J. W., Althomsons, S. P., Hyrc, K. L., Choi, D. W., and Goldberg, M. P. (1998) Nat. Med. 4, 291–297
73. Ghosh, M. C., Grass, L., Soosaipillai, A., Sotiropoulou, G., and Diamandis, E. P. (2004) Tumor Biol. 25, 193–199
74. Smith-Swintosky, V. L., Cheo-Iaaacs, C. T., D’Andrea, M. R., Santulli, R. J., Darrow, A. L., and Andrade-Gordon, P. (1997) J. Neurochem. 69, 1890–1896
75. Vergnolle, N., Bunnett, N. W., Sharkey, K. A., Brussee, V., Compton, S. J., Grady, E. F., Cirino, G., Gerard, N., Basbaum, A. I., Andrade-Gordon, P., Hollenberg, M. D., and Wallace, J. L. (2001) Nat. Med. 7, 821–826