A Urokinase-type Plasminogen Activator-inhibiting Cyclic Peptide with an Unusual P2 Residue and an Extended Protease Binding Surface Demonstrates New Modalities for Enzyme Inhibition

To find new principles for inhibiting serine proteases, we screened phage-displayed random peptide repertoires with urokinase-type plasminogen activator (uPA) as the target. The most frequent of the isolated phage clones contained the disulfide bridge-constrained sequence CSWGLLENHRMC, which we designated upain-1. When expressed recombinantly with a protein fusion partner, upain-1 inhibited the enzymatic activity of uPA competitively with a temperature and pH-dependent $K_D$, which at 25°C and pH 7.4 was $\sim 500$ nM. At the same conditions, the equilibrium dissociation constant $K_D$ was $\sim 400$ nM. By an inhibitory screen against other serine proteases, including trypsin, upain-1 was found to be highly selective for uPA. The cyclic structure of upain-1 was indispensable for uPA binding. Alanine-scanning mutagenesis identified Arg$^2$ of upain-1 as the P1 residue and indicated an extended binding interaction including the specificity pocket and the 37-, 60-, and 97-loops of uPA and the P$_1$, P$_2$, P$_3$, P$_4$, and the P$_5$ residues of upain-1. Substitution with alanine of the P$_2$ residue, Trp$^3$, converted upain-1 into a distinct, although poor, uPA substrate. Upain-1 represents a new type of uPA inhibitor that achieves selectivity by targeting uPA-specific surface loops. Most likely, the inhibitory activity depends on its cyclical structure and the unusual P$_2$ residue preventing the scissile bond from assuming a tetrahedral geometry and thus from undergoing hydrolysis. Peptide-derived inhibitors such as upain-1 may provide novel mechanistic information about enzyme-inhibitor interactions and alternative methodologies for designing effective protease inhibitors.

Serine proteases of the trypsin family (clan SA) have many physiological and pathophysiological functions. There is therefore extensive interest in generating specific inhibitors to be used for pharmacological interference with their enzymatic activity. Moreover, serine proteases are classical subjects for studies of catalytic and inhibitory mechanisms. Serine protease-catalyzed peptide bond hydrolysis proceeds through a tetrahedral transition state formed by a nucleophilic attack on the carbonyl group of the substrate P$_1$ amino acid by the hydroxyl group of Ser$^{195}$ (using the chymotrypsin template numbering (1)), with His$^{57}$ and Asp$^{102}$ acting as a charge relay system. The protonated His$^{57}$ functions as a general acid to facilitate collapse of the tetrahedral intermediate that is stabilized through interactions at the oxyanion hole and main chain $\beta$-strand-type hydrogen bonds between the P$_1$–P$_2$ and P$_2$–P$_3$ amino acids of the substrate and residues within the polypeptide binding cleft, as well as specific contacts within the S$_1$, S$_2$, S$_3$, S$_4$, $S'_1$, and $S'_2$ pockets, which bind respective side chains of the P$_1$, P$_2$, P$_3$, P$_4$, and P$_5$ residues (for reviews, see Refs. 2 and 3). Substrate specificity is governed by the fit of the P residues into their corresponding S-pockets as well as protease-specific surface loops surrounding the active site. Serine proteases of the trypsin family are biosynthesized as inactive zymogens, with activation occurring by cleavage of the peptide bond between residues 15 and 16 to stabilize the oxyanion hole (4).

One interesting member of the trypsin family is urokinase-type plasminogen activator (uPA), which catalyzes the conversion of the zymogen plasminogen to the active protease plasmin (5). uPA has a catalytic serine protease domain with surface-exposed loops around residues 37, 60, 97, 110, 170, and 185 and an N-terminal extension consisting of a kringle domain and an epidermal growth factor domain, which binds to the cell surface-anchored uPA receptor (uPAR) (6). uPA-catalyzed plasminogen generation participates in the turnover of extracellular matrix proteins in physiological and pathophysiological tissue remodeling (for a review, see Ref. 7). Abnormal expression of uPA is responsible for tissue damage in several pathological conditions, including rheumatoid arthritis, allergic vasculitis, and xeroderma pigmentosum, and in particular, is a key factor for the invasive capacity of malignant tumors (for reviews, see Refs. 8 and 9).

As with other serine proteases, there has been extensive interest in generating specific inhibitors of uPA. The plasminogen activation activity of uPA can be inhibited specifically by polyclonal (10) and monoclonal antibodies (11). Several inhibitory monoclonal antibodies have epitopes encompassing the 37- and 60-loops (12). They preclude access of the activation loop of plasminogen to the active site of uPA but do not inhibit the uPA-catalyzed hydrolysis of low molecular weight substrates comprising three amino acids and a $\alpha$-nitroaniline leaving group, which interacts only with the S$_1$–S$_3$ binding area. Another type of protein

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The abbreviations and trivial names used are: uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; aPC, activated protein C; BSA, bovine serum albumin; DFP, diisopropylfluorophosphate; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; mAb, monoclonal antibody; PAB, $\alpha$-aminobenzamidine; PAL, plasminogen activator inhibitor; PN-1, protease nexin-1; S-2222, benzyl-Ile-Glu-Gly-Arg-$\alpha$-nitroanilide; S-2238, H-o-Phe-piperidine-Arg-$\alpha$-nitroanilide; S-2288, Ile-Pro-Arg-$\alpha$-nitroanilide; S-2302, Pro-Phe-Arg-$\alpha$-nitroanilide; S-2366, pyro-Glu-Pro-Arg-$\alpha$-nitroanilide; S-2403, pyro-Glu-Phe-Lys-$\alpha$-nitroanilide; S-2444, pyro-Glu-Glu-Arg-$\alpha$-nitroanilide; S-2586, methyl-succinyl-Arg-Pro-Tyr-$\alpha$-nitroanilide; S-2765, benzoyloxycarbonyl-Arg-Gly-Arg-$\alpha$-nitroanilide; SPD, serine protease domain; Spectrozyme$^{\text{TM}}$, methansulfonyl-$\alpha$-cyclohexylalanyl-buty1-$\beta$-nitroanilide; tPA, tissue-type plasminogen activator; VLIK-AMC, H-o-Val-Leu-Lys-7-amido-4-methylcoumarin; WT, wild type; mIla, factor VIIa; fxIIa, factor Xa.
protease inhibitor is the dimeric nonspecific serine protease inhibitor ecotin, binding two serine protease molecules per ecotin dimer through interactions with both the protease active site and an exosite. With some success, ecotin has been converted into a high affinity uPA inhibitor by engineering each of the two interaction sites (13). Moreover, several classes of low molecular weight organochemical inhibitors of uPA have been synthesized. The binding modes for many of these were studied by x-ray crystal structure analyses. An important feature of such inhibitors is an Arg analogue inserting into the S1 pocket of uPA. However, the challenge has been to achieve selectivity for uPA over other serine proteases with P1 Arg specificity by exploiting small variations in the subsite geometry of the S1 pocket and its surroundings (14–19).

To isolate uPA inhibitors with a potential for providing mechanistic information, with specificity comparable with that of monoclonal antibodies and protein protease inhibitors and a size eventually allowing for chemical synthesis and modification, we screened phage displayed peptide libraries with uPA as bait. We have isolated a dodecapeptide that binds both to the active site and surface loops of uPA and have shown that it is a highly specific inhibitor of the enzymatic activity of uPA.

**EXPERIMENTAL PROCEDURES**

**uPA**—Two-chain uPA, originating from human urine, was purchased from Wakamoto Pharmaceutical Co., Tokyo, Japan. Protease concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 1.36 ml mg⁻¹ cm⁻¹ and a M₉ value of 54,000. Natural human pro-uPA was purified from serum-free conditioned medium of HT-1080 cells (20). Wild-type (WT) and mutant recombinant human uPA were expressed in and purified from HEK293T cells (12). When the cells were cultured under standard conditions, ~50% of the uPA in the conditioned medium and the purified preparations was in the pro-form and the rest in the active form. Conditioned medium with uPA exclusively in the uncleaved single-chain pro-form was produced by expressing the cleavage-resistant K158/15A uPA variant (we will refer to amino acid residues in uPA by a double numbering system based on numbering from the N terminus of the native protein and on the chymotrypsin template numbering system (1)). uPA was purified from conditioned medium by immunoaffinity chromatography as described previously (20). Low molecular weight uPA (N-terminally truncated at Lys136), the amino-terminal fragment derived from the N terminus of the native protein and on the chymotrypsin preparations was in the pro-form and the rest in the active form. Conditioned medium with uPA exclusively in the uncleaved single-chain pro-form was produced by expressing the cleavage-resistant K158/15A uPA variant (we will refer to amino acid residues in uPA by a double numbering system based on numbering from the N terminus of the native protein and on the chymotrypsin template numbering system (1)). uPA was purified from conditioned medium by immunoaffinity chromatography as described previously (20). Low molecular weight uPA (N-terminally truncated at Lys136), the amino-terminal fragment of uPA (Ser¹–Lys¹³⁶), uPA-PAI-1 complex, low molecular weight uPA-PAI-1 complex, and uPA-protease nexin-1 (PN-1) complex were prepared as described previously (20).

**Miscellaneous Proteases and Inhibitors**—Human activated protein C (aPC) was a gift from Dr. John Fenton, Albany, NY. Human thrombin was a gift from Dr. Inger Schousboe, University of Copenhagen, Denmark. Human plasma kallikrein was a gift from Borean Pharma, Aarhus, Denmark. Human plasmin was purchased from American Diagnostica. Human fXa was a gift from Maxygen, Hørsholm, Denmark. Human thrombin was a gift from Dr. Erkki Koivunen, University of Helsinki, Finland (25). Subsequent to 10 washes with PBS, the bound phase particles were eluted by adding 1 ml of 100 mM glycine adjusted to pH 2.2 with HCl and incubation for 10 min at room temperature. The eluted phase particles were neutralized by addition of 400 μl 0.1 M Tris, pH 9.0, and propagated in E. coli TG-1 cells overnight. After removal of bacterial cells by centrifugation, the secreted phase particles were precipitated by addition of 0.25 volumes of 2.5 M NaCl, 20% polyethylene glycol 6000, followed by incubation for 1 h at 0 °C and centrifugation. The resulting phase pellet was dissolved in PBS with 10% glycerol. Four successive rounds of selection were performed. Alternating antibodies were used for subsequent rounds of selection in order to avoid enrichment of antibody-binding phases.

Expression of the Upain-1 Peptide Sequence and Derivatives thereof. Fused to the N-terminal Domains of the Phage Coat Protein g3p—A DNA fragment encoding the two SfiI cloning sites plus the first two domains of the phage coat protein g3p (D1 and D2, residues 1–219) was amplified from the phage fUSE5 (26) with the PCR primers fUSEfwd (5’-CATGCGCATGGGTGCAGCG-3’) and fUSEbck2 (5’-GATCCGAGCGCCCGCAGCAT-GCAGG-3’) using the PfTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. The generated PCR product was purified with the Qiagick PCR Purification kit (Qiagen, Hilden, Germany) and ligated into the E. coli expression vector pET20b(+) (Merck Novagen, Nottingham, UK) via Ncol and Xhol restriction sites. The resulting vector is referred to as pETD1D2. Using the same approach, but with the upain-1 phage as template for the PCR reaction, a vector was created for expression of the upain-1 peptide sequence fused to D1D2 of
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g3p (MGSADGACSWVRLEHHRMCGAAG-g3p,219-LEHHHHHHH, the upain-1 sequence is underlined). This fusion protein will be referred to as upain-1-D1D2. Expression vectors for derivatives of upain-1 fused to D1D2 were created by ligating suitable oligonucleotide cassettes into SfiI-digested pETD1D2. Likewise, a vector for expression of the peptide-flanking sequences fused to D1D2 (MGSADGACSWVRLEHHRMCGAAG-g3p,219-g3p1–219) was constructed. Individual fusion proteins were expressed from cultures of E. coli BL21(DE3)PlysS (Merck Novagen, Nottingham, UK) containing the relevant plasmids and purified by immobilized metal affinity chromatography as previously described for His5-tagged PAI-1 (27, 28), subjected to size exclusion chromatography on Superdex 75 (Amersham Biosciences) equilibrated in HEPES-buffered saline (HBS, 10 mM HEPES, pH 7.4, 150 mM NaCl), and finally concentrated with Centricron Centrifugal Filter Devices (Millipore Corp., Glostrup, Denmark).

Expression of the Upain-1 Peptide Sequence Fused to the Coiled-coil Domain of Tetranectin—The upain-1 sequence was produced recombinantly in fusion with the trimemerising coiled-coil domain of tetranectin (EPPTQKPKKIVNAKDDVNTKMFEEKSLRDLTQAQVALQQQQQQQQQQQQQQQQQLTVGS) (29). The construct was expressed and purified as follows. Six liters of E. coli BL21 cells were grown to OD600 0.8 before induction of protein expression by addition of bacterial phage ACE6 at a multiplicity of infection of ~5. Bacterial cultures were incubated for 4 h before harvesting the cells by centrifugation at 5000 rpm for 10 min. The bacterial pellet was resuspended in 100 ml of a buffer of 100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, 50 mM dithiothreitol. Phenol (150 ml), adjusted to pH 8.0, was added and the mixture sonicated to extract all protein. The mixture was centrifuged at 8,500 rpm for 30 min to isolate the phenol phase. Protein was precipitated from the phenol phase by addition of 2.5 volumes of ethanol and harvested by centrifugation at 5000 rpm for 10 min. Following resuspension in 6 mM guanidinium chloride, 50 mM Tris-HCl, pH 8.0, the protein solution was gel filtered on a Sephadex G-25 column into 8M urea, 500 mM NaCl, 50 mM Tris-HCl, 5 mM β-mercaptoethanol, pH 8.0, and loaded on a Ni2+ charged nitrilotriacetic acid column. The expressed protein was subjected to a patented cyclic in vitro refolding procedure (30). This construct will be referred to as upain-1-tetranectin.

ELISA for Measuring uPA-Phage Particle Binding—Unless otherwise stated, the buffer used was HBS supplemented with 0.2% BSA. The concentration of uPA variants in conditioned medium was determined by a quantitative sandwich ELISA using the monoclonal antibody mAb 6 for capture and a polyclonal rabbit anti-uPA antibody for detection (12). Activation of pro-forms of uPA variants was achieved by incubation at 37 °C for 16 h at pH 7.4 in the presence of 2 μg/ml plasmin followed by addition of 1 μg/ml bovine pancreatic trypsin inhibitor. For the ELISAs, the antibody to be immobilized on the solid phase (5 μg/ml in 100 mM NaHCO3/Na2CO3, pH 9.6) was coated in the wells of a 96-well Maxisorp plate (Nunc) followed by blocking with 5% BSA in HBS. The wells were incubated with 10 mM uPA for 1 h. Excess uPA was removed by washing before incubation with phage particles (~108 colony-forming units/ml) for 1 h. For competition studies, up to 15 μM peptide-D1D2 fusion proteins were added along with the phage particles. The wells were washed and incubated for 1 h with a 5,000-fold dilution of HRP-conjugated anti-M13 monoclonal antibody directed against the major phage coat protein g8p (Amersham Biosciences). After a final wash, the wells were developed by the addition of 0.5 mg/ml orthophenylenediamine (100 μl) (KemEnTech, Denmark) in 50 mM citric acid, pH 5.0, supplemented with 0.03% H2O2. When suitable color had developed, the reactions were quenched with 50 μl 1 M H2SO4. The A492 of the wells was read in a microplate reader. When testing the binding of upain-1 phage to different uPA variants, the presence of equal amounts of the uPA variants on the solid phase was assured by a parallel ELISA with 1 μg/ml polyclonal rabbit anti-uPA antibody instead of phage particles and with HRP-conjugated swine-anti-rabbit serum (DAKO, Glostrup, Denmark, diluted 2,000-fold) instead of anti-M13 antibody.

Determination of the Mode of Inhibition of uPA by Upain-1—Four nm uPA was incubated with various concentrations of upain-1 peptide (0–400 μM) or upain-1-D1D2 (0–20 μM) in HBS with 0.1% BSA at 37 °C for 15 min prior to the addition of the chromogenic substrate, S-2444. Each inhibitor concentration was combined with a series of S-2444 concentrations in the range 10–2000 μM. The initial reaction velocity was monitored at an absorbance of 405 nm. The following equation is expected to apply for competitive inhibition according to Michaelis-Menten kinetics.

\[
V = \frac{(V_{\text{max}}[S]_0)}{([S]_0 + K_m(1 + [I]_0/K_i))} \quad (\text{Eq. 1})
\]

where \([S]_0\) and \([I]_0\) are the total substrate and inhibitor concentrations, respectively; \(K_m\) is the inhibition constant; \(K_i\) is the Michaelis constant for S-2444 under the assay conditions. In Equation 1, one can define a \(K_m^{\text{app}}\) as

\[
K_m^{\text{app}} = K_m(1 + [I]_0/K_i) \quad (\text{Eq. 2})
\]

The \(K_m^{\text{app}}\) values for S-2444 hydrolysis by uPA were determined by standard Michaelis-Menten kinetics at each inhibitor concentration by a nonlinear fit to the equation

\[
V = \frac{(V_{\text{max}}[S]_0)}{([S]_0 + K_m^{\text{app}})} \quad (\text{Eq. 3})
\]

In the case of competitive inhibition, \(K_m^{\text{app}}\), according to Equation 2, is expected to have a linear relationship to \([I]_0\), whereas \(V_{\text{max}}\) will be independent of \([I]_0\). The \(K_i\) values could thus be estimated as the slope of the line relating \(K_m^{\text{app}}\) to \([I]_0\).

Determination of the Inhibition Constants (\(K_i\)) for the Inhibition of uPA and uPA Variants by Upain-1 Peptide and Upain-1-D1D2—For routine determination of inhibition constants \(K_i\) for the inhibition of purified uPA under equilibrium inhibition conditions, a fixed concentration of uPA (4.0 nM) was preincubated in a 200-μl volume of HBS with 0.1% BSA at 37 °C, at pH values of 6.0, 7.4, and 8.1, with various concentrations of upain-1 peptide (0–400 μM) or upain-1-D1D2 (0–20 μM) for 15 min prior to the addition of the chromogenic substrate, S-2444 (46.9 μM). The initial reaction velocities were monitored at an absorbance of 405 nm. Apparent equilibrium inhibition constants \(K_i^{\text{app}}\) were subsequently determined from the nonlinear regression analyses of plots of \(V_i/V_o\) versus \([I]_0\) using Equation 4 (31).

\[
\frac{V_i}{V_o} = 1 - \left(\frac{[E]_0 + [I]_0 + K_i^{\text{app}}}{\sqrt{(([E]_0 + [I]_0 + K_i^{\text{app}})^2 - 4([E]_0 [I]_0)}}\right) \quad (\text{Eq. 4})
\]

where \(V_i\) and \(V_o\) are the reaction velocities in the presence and absence of inhibitor, respectively, and \([E]_0\) and \([I]_0\) are the total protease and inhibitor concentrations, respectively. \(K_i^{\text{app}}\) represents the apparent inhibition constant in the presence of chromogenic substrate. The true \(K_i\) was subsequently determined by correcting for the competitive effect of the substrate, S, using the relationship

\[
K_i = K_i^{\text{app}}/(1 + [S]_0/K_m) \quad (\text{Eq. 5})
\]

Experimental determinations of the \(K_i\) values for inhibition of the uPA variants by upain-1-D1D2 were accomplished essentially as described above except for the following modifications: 20 μl of conditioned HEK293T cell medium was used for each uPA variant instead of the purified protease, the tested upain-1 concentrations ranged from 0
to 150 μM, and the concentration of S-2444 used was increased to 100 μM. In these experiments, the exact concentration of uPA was not readily known, and therefore these data were analyzed by the simplified expression of Equation 6 (21),

\[
V/V_0 = \frac{1}{1 + [I]/K_{\text{app}}}
\]  
(Eq. 6)

where \( V \) and \( V_0 \) are the reaction velocities in the presence and absence of inhibitor, respectively, \([I]_0\) is the total inhibitor concentration, and \( K_{\text{app}} \) is the apparent inhibition constant with the final value of \( K_i \) being determined as described above using the experimentally determined \( K_{\text{app}} \) values for WT uPA and uPA variants in cell media (see TABLE FOUR). Equation 6 assumes that \( [I]/K_i < 0.1 - 0.01 \) and \([I]_{\text{free}} \approx [I]_{\text{total}} \) a condition that was observed considering that the assay typically contained a final concentration of uPA or uPA variant ranging from 2 to 20 nM.

**Determination of the Inhibition Constants (K) for the Inhibition of Serine Proteases by Upain-1-D1D2—Inhibition constants (K) for the inhibition of related clan SA purified serine proteases were determined under equilibrium inhibition conditions in HBS with 0.1% BSA at 37 °C, except for assays with fVIIa, which were carried out in TBS (Tris-buffered saline, 50 mM Tris, pH 8.4, 100 mM NaCl) with 0.1% BSA. The assays were performed essentially as described above for uPA. The following protease-substrate combinations were tested: aPC (8.5 nM) and S-2366 (300 μM); FXa (0.5 nM) and S-2765 (100 μM); fVIIa (10 nM) and Spectrozyme fVIIa (500 μM); plasma kallikrein (4.0 nM) and S-2230 (300 μM); plasmin (2.0 nM) and S-2403 (125 μM); thrombin (0.5 nM) and S-2238 (50 μM); tPA (2.0 nM) and S-2288 (300 μM); β-trypsin (2.0 nM) and S-2222 (50 μM); murine uPA (4.0 nM) and S-2444 (750 μM). Protease and substrate concentrations were chosen to obtain an optimal [substrate]/[Km] ratio that minimized the magnitude of the correction factor. As above, the \( K_m \) values for these proteases were determined by standard Michaelis-Menten kinetics and used in the calculation of the reported \( K_i \) values (TABLE FIVE).

**Determination of the Equilibrium Binding Constant (Kd) for Upain-1-D1D2 Binding to uPA and the Relative Affinity of Upain-1-D1D2 Variants for uPA—**The equilibrium binding constant (Kd) for the interaction of upain-1-D1D2 with uPA and the S356/195A-uPA variant were determined by a modification of a previously described method based on the competitive displacement of the fluorescent probe p-aminobenzamidine (PAB) from the active site of uPA (22). Fluorescence emission spectra were recorded at 25 °C on a Shimadzu 5301PC spectrofluorophotometer in a 2 × 10-5 mM semi-micro quartz cuvette and in a buffer of 30 mM HEPES, pH 7.4, 0.135 mM NaCl and 0.1% polyethylene glycol 8000. Excitation was at 335 nm, and the emission was scanned from 350 to 400 nm using excitation and emission band passes of 5 and 10 nm, respectively. Equilibrium binding reactions were performed over a range of uPA concentrations (0 μM–100 μM) with 0.4 μM uPA or 0.8 μM S356/195A-uPA in the presence of 20 or 100 μM PAB, respectively. Emission spectra were collected using an integration of 1–2 s over a 1.0-nm step resolution, and the individual data points for equilibrium binding isotherms were subsequently determined from the integrated fluorescence peaks after correction for dilution and inner filter effects and subtraction of the appropriate control titrations in the absence of uPA or S356/195A-uPA. Integrated fluorescence data were normalized as \( \Delta F_{\text{obs}}/\Delta F_{\text{max}} \) where \( \Delta F_{\text{obs}} \) is the observed integrated fluorescence at each uPA titration point and \( \Delta F_{\text{max}} \) represents the total displacement of specific PAB binding to uPA following the addition of a 2-fold (uPA) or 3-fold (S356/195A uPA) molar excess of recombinant wild-type PAI-1. Equilibrium binding isotherms were analyzed by Equation 7, describing competitive binding under the assumption that the stoichiometry of probe and inhibitor binding to the protease are both 1:1 (22),

\[
\Delta F_{\text{obs}} = \frac{\Delta F_{\text{max}}}{2[E_0]} \times \left( K_{\text{d}} \frac{[PAB]}{[K_{\text{d}, PAB}]} + [I]_0 + [E_0] \right) - \left( K_{\text{d}} \frac{[PAB]}{[K_{\text{d}, PAB}]} + [I]_0 - [E_0] \right)^2
\]

\[
- 4[E_0]K_{\text{d}} \left( 1 + \frac{[PAB]}{[K_{\text{d}, PAB}]} \right)^{1/2}
\]

(Eq. 7)

where \( \Delta F_{\text{max}} \) is the maximal fluorescence change, \([E]_0\), \([I]_0\), and \([PAB]_0\) are the total proteinase, inhibitor, and p-aminobenzamidine concentrations, respectively. \( K_i \) is the dissociation constant for the inhibitor, and \( K_{\text{d}, PAB} \) is the experimentally determined dissociation constant for PAB. The value of \( K_{\text{d}, PAB} \) used in the nonlinear regression analysis of Equation 7 was determined from titrations of uPA or S356/195A-uPA with PAB, by monitoring the change in fluorescence at the emission peak of 365 nm, which yielded the maximal difference between bound and free probe. Following correction for the dilution effects, the background fluorescence of the PAB probe, and inner filter effects, PAB binding isotherms were evaluated by Equation 8 (22),

\[
\Delta F_{\text{obs}} = \frac{\Delta F_{\text{max}}}{2[E_0]} \times (\frac{[E]_0 + [PAB]_0 + K_{\text{d}, PAB}}{\sqrt{[E]_0 + [PAB]_0 + K_{\text{d}, PAB}^2}} - 4[E_0]K_{\text{d}, PAB})
\]

(Eq. 8)

where \( \Delta F_{\text{max}} \) is the maximal fluorescence change, \([E]_0\), \([I]_0\), and \([PAB]_0\) are the total proteinase and p-aminobenzamidine concentrations, respectively, and \( K_{\text{d}, PAB} \) is the dissociation constant of the ligand.

**Investigation of Substrate Behavior of Upain-1—**To analyze for substrate behavior of upain-1-D1D2 fusion protein or its alanine-substituted variants against uPA, they were incubated in PBS at 37 °C for 16 h at a final concentration of 4 μM in the presence of 0, 10, or 100 nM uPA. The reaction products were boiled in sample buffer with 10 mM dithiothreitol, separated by SDS-PAGE (12% acrylamide), and visualized by Coomassie Blue staining. The time course of upain-1-D1D2 W3A cleavage at 37 °C during incubation with equimolar amounts of uPA (2 μM) was monitored by SDS-PAGE analysis as described above. For determination of the cleavage site, 2.5 μM upain-1-D1D2 W3A was treated with 400 nM uPA at 37 °C for 16 h in PBS, precipitated with trichloroacetic acid, and N-terminally sequenced by Edman degradation on an Applied Biosystems 477A sequencer. To analyze for substrate behavior of the upain-1-D1D2 fusion protein or its alanine-substituted variants against other serine proteases, they were incubated at a final concentration of 2.5 μM in the presence of 50 mM tPA, aPC, thrombin, plasma kallikrein, fVIIa, FXa, β-trypsin, or plasmin, respectively, in PBS at 37 °C for 30 min followed by SDS-PAGE analysis as described above to detect any cleavage of the upain-1 sequence by these proteases. Similar assays with incubation times up to 4 h and 5 or 50 μM β-trypsin or plasmin, respectively, were performed with upain-1-D1D2, upain-1-D1D2 R4A, or upain-1-D1D2 R10A.

**Plasminogen Activation Assays—** uPA (4 nM) was preincubated with 1.5 mM S-2403 for 15 min at 37 °C in PBS with 0.01% Tween 20. The plasminogen activation reaction was initiated by addition of plasminogen to a final concentration of 110 nM. The A405 was recorded at regular intervals for time periods between 30 and 60 min. When evaluating the
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effect of inhibitors in the assay, these were added during the preincubation period in variable concentrations as indicated for each single experiment.

For assaying uPA-catalyzed plasminogen activation activity with uPA bound to uPAR at cell surfaces, U937 cells were cultured as described previously (32), washed extensively, and resuspended in TBS with 0.1% BSA at a density of 10⁷ cells/ml. The cells were then preincubated for 1 h at 37°C with 50 µM uPA. The cells were subsequently washed three times with TBS with 0.1% BSA to remove unbound uPA. They were then aliquoted into the wells of nontransparent 96-well microtiter plates for fluorescence readings (Nunc) at 37°C in a total volume of 200 µl with upain-1-D1D2 and other compounds in final concentrations as indicated for each single experiment. After 30 min, the plasminogen activation reaction was stopped by the addition of anti-uPA mAb 5 in a final concentration of 100 nM. The relative amounts of plasmin generated in the wells were estimated by the addition of VFK-AMC to a final concentration of 200 µM. The fluorescence of the wells was monitored at 1-min intervals in a Spectromax Gemini fluorescence plate reader (Molecular Devices) using an excitation wavelength of 390 nm and an emission wavelength of 480 nm.

RESULTS

Selection of a uPA Binding Peptide—For isolating uPA binding peptides, we used a combination of four different phage-displayed random peptide libraries in the formats X₇, CX₇-X₇, CX₇-X₇, and CX₇-CX₇, where X denotes random natural amino acids. The cysteine residues shown are oxidized, resulting in constrained circular peptides in the CX₇-CX₇ formats, whereas the CX₇-CX₇-X₇-CX₇ exhibits a more complicated and presumably variable disulfide bond pattern depending on the other residues. The theoretical diversity of the combined libraries is >10¹⁰ unique peptide inserts (25). As a bait, we used uPA immobilized on the monoclonal anti-uPA antibodies mAb 6, mAb 12, or mAb 390, respectively, all having epitopes in the kringle domain of uPA (12). Alternating antibodies were used for different rounds of selection to avoid enrichment of antibody-binding phages. Four rounds of selection were performed. Among 28 individual phages showing uPA-binding in ELISA, 19 displayed a peptide with the sequence CSWRGLENHRMC. This sequence was chosen for further analysis and will be referred to as upain-1.

The Selected Peptide Sequence Is a Competitive Inhibitor of uPA—A peptide corresponding to the selected sequence, with oxidized cysteines, was synthesized chemically. The upain-1 sequence was also expressed in fusion with the two N-terminal domains (D1 and D2) of the g3p phage coat protein and in fusion with the coiled coil domain of the trimERIC protein tetranectin. These fusion proteins will be referred to as upain-1-D1D2 and upain-1-tetranectin.

The upain-1 peptide, upain-1-D1D2, and upain-1-tetranectin inhibited the peptidolytic activity of uPA against S-2444, whereas the fusion partners D1D2 and tetranectin, without the upain-1 sequence, showed no or only weak inhibitory activity (see also below). By determining the rate of S-2444 hydrolysis at several substrate and several inhibitor concentrations, an apparent Km (Km app) could be determined at each inhibitor concentration. The Km app increased linearly with the inhibitor concentration, whereas Vmax changed only negligibly in the case of upain-1-D1D2 (TABLE ONE and Fig. 1), upain-1-tetranectin, and the upain-1 peptide (data not shown), clearly in agreement with the expected inhibition mode of a competitive inhibitor. We therefore concluded that upain-1 competitively inhibits uPA.

In our analysis of inhibition kinetics, we routinely used plots of V/V₀ versus inhibitor concentration at a single substrate concentration near the Km value and analysis by nonlinear regression curve-fitting according to Equation 4. The Km values for the inhibition of uPA by upain-1 peptide and upain-1-D1D2 under equilibrium conditions and at a physiological temperature of 37 °C were determined for pH values of 6.0, 7.4, and 8.1 (Fig. 2 and TABLE TWO). The Km values for inhibition of uPA by the upain-1-D1D2 construct were significantly lower (14–36-fold) than the corresponding values for the chemically synthesized peptide, suggesting that presentation of the peptide sequence in a protein scaffold considerably increased the affinity for the uPA target. Despite having significantly different affinities, both the synthetic peptide as well as upain-1-D1D2 showed Kᵢ values that decreased markedly with pH (TABLE TWO). D1D2 without the peptide insert had only negligible inhibitory activity (Kᵢ ~ 300 µM) (see below). As expected, the Kᵢ also depended on temperature, being ~4-fold lower at 25°C than at 37°C for upain-1-D1D2 (TABLE TWO). We did not perform an extensive kinetic analysis of the inhibition of uPA by upain-1-tetranectin. However, it also inhibited uPA with a pH-dependent Kᵢ value, which at pH 7.4 and 37 °C was 3.93 ± 0.95 µM (n = 3). Analysis of the synthetic upain-1 peptide by reverse phase high pressure liquid chromatography and upain-1-D1D2 by SDS-PAGE after incubation with uPA demon-

| Table One | Km app and Vmax values for the hydrolysis of S-2444 by uPA in the presence of upain-1-D1D2 |
|-----------|-----------------------------------------------|
|           | km app (M) | Vmax (µM) |
| 0.0       | 86.9 ± 11.3 | 5.85 ± 0.69 x 10⁻⁸ |
| 1.0       | 105.8 ± 12.3 | 5.64 ± 0.74 x 10⁻⁸ |
| 2.0       | 134.7 ± 21.4 | 5.48 ± 0.59 x 10⁻⁸ |
| 4.0       | 163.3 ± 29.1 | 5.39 ± 0.63 x 10⁻⁸ |
| 6.0       | 200.6 ± 40.6 | 5.23 ± 0.50 x 10⁻⁸ |
| 8.0       | 230.4 ± 49.9 | 5.19 ± 0.46 x 10⁻⁸ |
| 10.0      | 275.9 ± 57.4 | 5.02 ± 0.36 x 10⁻⁸ |
| 20.0      | 474.0 ± 103.6 | 4.67 ± 0.19 x 10⁻⁸ |

FIGURE 1. Upain-1-D1D2 is a competitive inhibitor of uPA activity. Reactions following the uPA mediated hydrolysis of varying concentrations of S-2444 in the presence of increasing concentrations of upain-1-D1D2 were monitored at 405 nm in a microtiter plate reader. The apparent Km values (Km app) were determined by nonlinear regression analysis of substrate dose curves by Equation 1. Km app values were plotted subsequently against the inhibitor concentration. The dashed line represents a linear regression fit of the data where the slope = Km/Kᵢ (Equation 2), resulting in a calculated Kᵢ of 4.6 ± 0.1 µM (± S.E. of the fit). The bars indicate S.D. for 4–6 independent determinations.
A Cyclic Peptide Inhibiting uPA

Determination of the Equilibrium Binding Constant (K_D) for the Binding of Upain-1-D1D2 to uPA by p-Aminobenzamidine Displacement—As a complementary approach to determine the affinity of upain-1-D1D2 for uPA, we designed an experiment that followed the competitive displacement of PAB, a fluorescent probe and arginine analogue, which binds to the S_1 pocket of Arg-specific serine proteases. Displacement experiments carried out at 25 °C and pH 7.4 resulted in a dose-dependent quench of the fluorescence enhancement associated with probe binding (Fig. 3B) and yielded the equilibrium binding isothem presented in Fig. 3A. Further analysis of the binding isothem by Equation 7 gave a fitted K_D value of 429 nM, an equilibrium binding constant very close to the K_D of ~600 nM determined for upain-1-D1D2 at pH 7.4 and 25 °C (TABLE TWO). This finding is rather consistent with the expectations for a competitive inhibitor where K_D will be equivalent to the value k_{on}/k_{off} and will approximate the dissociation constant K_D.

Mutagenesis of Residues in the Upain-1 Sequence—To evaluate the importance of individual residues in upain-1 for binding to uPA, each residue, except the cysteines, was independently substituted by Ala on the upain-1-D1D2 background. A linear variant with the N- and C-terminal cysteine residues replaced by serine residues was also constructed. The effect of these substitutions on the binding was evaluated initially by the ability of the upain-1-D1D2 variants to compete for uPA-1 phage particle binding to immobilized uPA in an ELISA. The analysis demonstrated that substitution of all residues except Ser, Leu, Arg, and Met resulted in a strongly reduced binding (TABLE THREE). Furthermore, the cysteine residues at positions 1 and 12, and therefore the cyclical nature of the inserted peptide sequence, were found to be necessary for binding.

The effects of alanine substitutions in upain-1-D1D2 on the binding to uPA were further investigated by an assay in which the displacement of PAB was compared at a 15 μM concentration of upain-1-D1D2 WT or upain-1-D1D2 variant, a concentration in which ~85% of the PAB is displaced by upain-1-D1D2 WT (TABLE THREE). A profile of binding activity was obtained similar to that demonstrated by the phage ELISA. Nevertheless, the increased resolution of the PAB displacement assay indicated that alanine substitutions of Trp, His, and Leu, and to a lesser extent of Gly, had less effect than the other alanine substitutions, suggesting less importance of these residues for the binding (TABLE THREE).

To see whether the alanine substitutions reduced the binding by converting upain-1 into a substrate, we analyzed the condition of the upain-1-D1D2 mutants by SDS-PAGE after a prolonged incubation with uPA. We found that only the W3A substitution in the upain-1-D1D2 sequence rendered upain-1-D1D2 a substrate for uPA. N-terminal sequencing of the uPA-cleaved upain-1-D1D2 W3A variant identified the Arg4–Gly5 peptide bond as the scissile bond (Fig. 4A). Hydrolysis was not inhibited by the pancreatic trypsin inhibitor, indicating that the cleavage was performed directly by uPA and not by contaminating trypsin or plasmin (data not shown). Although we did not determine the precise kinetic parameters for this reaction, the hydrolysis was relatively slow, with a working concentration of more than 100 nM uPA required to cleave 4 μM upain-1-D1D2 W3A fusion protein within 16 h at 37 °C (Fig. 4B). When the concentration of uPA was increased to an equimolar concentration (2 μM each), the turnover was only partially complete within 60 min at 37 °C (Fig. 4C). Thus, under the reaction conditions employed in

| pH tested | Temperature °C | K_m μM | Upain-1 peptide μM | Upain-1-D1D2 μM |
|-----------|----------------|--------|-------------------|-----------------|
| 6.0       | 37             | 175.4 ± 0.1 | 6.7 ± 0.3         | 0.29 ± 0.01     |
| 7.4       | 37             | 84.9 ± 0.1  | 29.9 ± 0.8        | 2.2 ± 0.1       |
| 8.1       | 37             | 59.1 ± 0.1  | 169.1 ± 6.1       | 4.8 ± 0.1       |
| 7.4       | 25             | 42.0 ± 0.1  | ND                | 0.59 ± 0.02     |
A Cyclic Peptide Inhibiting uPA

FIGURE 3. Equilibrium binding of upain-1-D1D2 to uPA as determined by displacement of p-aminobenzamidine. The equilibrium binding constant (K_D) for upain-1-D1D2 binding to 0.4 μM uPA was determined from the competitive displacement of PAB (20 μM). A, equilibrium titrations using increasing concentrations of upain-1-D1D2 (0–15 μM) produced a saturating binding isotherm with individual data points representing the means ± S.E. of the integrated fluorescence peaks for three independent titrations normalized to ΔF_{max}/ΔF_{max}. Individual data points represent the means of 3–4 experimental determinations ± S.E. The solid line denotes the nonlinear regression fit of the data to Equation 7, giving a K_D value of 428.9 ± 53.7 nM (± S.E. of the fit). B, representative titration of 0.4 μM uPA with increasing concentrations of upain-1-D1D2 illustrates a dose-dependent quench of PAB fluorescence. Fluorescence spectra were acquired using an excitation wavelength at 335 nm and collected over an emission range of 350 to 400 nm. The representative experiment has the following titration points depicted: 0 μM upain-1-D1D2 (black trace), 0.5 μM upain-1-D1D2 (red trace), 1.0 μM upain-1-D1D2 (green trace), 2.0 μM upain-1-D1D2 (yellow trace), 6.0 μM upain-1-D1D2 (blue trace), 15 μM upain-1-D1D2 (magenta trace), and PAB-1 control for the total displacement of PAB, i.e., ΔF_{max} (dashed line).

TABLE THREE

| Upain-1-D1D2* | IC_{50} | PAB displacement | % of control |
|---------------|---------|------------------|--------------|
| CSWRGLENHRMC  | 3.4 ± 2.7 | 100              |              |
| CAWRGLENHRMC  | 3.2 ± 2.2 | 109.4 ± 19.4     |              |
| CSARGLENHRMC  | >15      | 25.4 ± 4.9       |              |
| CSWAGLENHRMC  | >15      | 6.9 ± 2.4        |              |
| CSWRALENHRMC  | >15      | 11.4 ± 4.9       |              |
| CSWRGAENHRMC  | 3.7 ± 2.9 | 106.4 ± 8.8      |              |
| CSWRGLANHRMC  | >15      | 6.3 ± 8.0        |              |
| CSWRGLEANHRMC| >15      | 2.9 ± 2.5        |              |
| CSWRGLENARMC | >15      | 33.0 ± 0.6       |              |
| CSWRGLENAMC  | 4.1 ± 2.8 | 99.7 ± 7.7       |              |
| CSWRGLENHRAC | 3.2 ± 2.7 | 98.1 ± 7.6       |              |
| SWRGENHSM     | >15      | 10.0 ± 5.1       |              |
| D1D2 without peptide | >15 | ND               |              |

* This is a modified sequence. The binding affinities were estimated by the ability of upain-1-D1D2, harboring the indicated mutations (bolded and underlined), to compete with the binding of upain-1 phage particles to uPA in a phage ELISA.

b The IC_{50} values represent the upain-1-D1D2 concentrations causing half-maximal inhibition of phage particle binding. Data are reported as the mean ± S.D. of up to three independent determinations. IC_{50} values listed as >15 μM indicate that no measurable competition was observed at concentrations up to the highest tested (15 μM).

c Relative binding affinities were determined by the competitive displacement of PAB from the active site of uPA and are calculated from ΔF_{max}/ΔF_{max} data. ND indicates that PAB displacement was not determined with the “empty” D1D2 scaffold.

both the phage ELISA and the PAB displacement experiments, where low nM concentrations of uPA were incubated with upain-1-D1D2 W3A at 25 °C for at most 60 min, there was not likely to be any significant cleavage of upain-1-D1D2 W3A. Therefore, we may conclude that any cleavage found during the extended incubations at 37 °C would most likely be irrelevant to the observed effects of upain-1-D1D2 W3A binding in the equilibrium and ELISA experiments and that the results are therefore expected to report the reduced binding of the intact upain-1-D1D2 W3A and not that of an apparently reduced affinity because of hydrolysis of the inhibitor.

Mapping the Binding Site for Upain-1 on uPA with Monoclonal Antibodies—To characterize the binding site for the upain-1 peptide sequence on uPA, we first tested the effect on the binding of upain-1 phage particles to uPA by competition with different monoclonal anti-uPA antibodies having previously identified epitopes (12). uPA was immobilized on the antibodies in microtiter plates. When coating with
anti-uPA mAb 5, mAb 16, mAb 394, and mAb 3689, each of which has overlapping but distinct epitopes within the 37- and the 60-loop regions of the serine protease domain (SPD), we observed no detectable binding of upain-1-phages. In contrast, substantial binding was observed with the anti-kringle antibodies mAb 12, mAb 6, and mAb 390 (12) (data not shown).

To determine whether the above observations in phage ELISA were due to the possible steric hindrance of binding bulky phage particles in the presence of the antibodies, we tested whether upain-1-peptide is also capable of inhibiting the peptidolytic activity of uPA in the presence of these antibodies. The anti-SPD antibodies mAb 5, mAb 16, and mAb 394 did not by themselves inhibit the peptidolytic activity of uPA (12), but mAb 3689 caused a slight increase of the $K_i$ for S-2444 hydrolysis from 85 to 136 $\mu$M. These observations suggest that the active site cleft is still accessible by small molecule tripeptide-$\rho$-nitroanilino substrates in the presence of bound monoclonal antibodies. With an excess of mAb 5, mAb 16, or mAb 394, there was no inhibition of uPA by upain-1-peptide at concentrations up to 200 $\mu$M. Assuming a detection limit of 5% inhibition and using Equations 5 and 6, this observation implies that the $K_i$ in the presence of these antibodies is more than 1300 $\mu$M, as compared with a value of $\sim 30$ $\mu$M in the absence of competing mAb (TABLE TWO). mAb 3689 also caused an increase of the $K_i$ value for inhibition by upain-1-peptide, although to only 202.1 $\pm$ 5.3 $\mu$M ($n = 3$); so although this mAb was not as effective at blocking the binding of upain-1-peptide as the other tested anti-SPD antibodies, the $K_i$ value was still increased by more than 6-fold. Together, these observations are in clear agreement with an overlap between the epitopes for the anti-SPD antibodies and the binding site for upain-1.

Mapping the Binding Site for Upain-1 on uPA by Screening Different uPA Conformations—To further identify the binding site of upain-1 on uPA, we investigated the binding of upain-1 to pro-uPA and uPA in complex with serpin inhibitors or small molecule compounds by phage particle ELISA. There was no measurable binding of phage particles to pro-uPA (Fig. 5), the amino-terminal fragment of uPA, uPA-Conformations

Mapping the Binding Site for Upain-1 on uPA by Alanine-scanning Mutagenesis—To describe the binding interface of upain-1 in greater detail, we performed a comprehensive phage particle ELISA using con-
Effects of alanine substitutions in uPA on the $K_{i}$ and $K_{m}$ values for hydrolysis of S-2444 and inhibition by upain-1-D1D2

Kinetic parameters were determined in HBS with 0.1% BSA at 37 °C with conditioned media from HEK293T cells transfected with the corresponding cDNAs. Besides the variants shown in the table, the following variants were tested but were found to have $K_{i}$ values differing by less than 2-fold from uPA WT: Y177/34A, H180/37A, K211/61A, K212/62A, E213/65A, D214/63A, I216/65A, Y218/67A, R221/70A, R223/72A, N225/74A, N227/76A, K233/82A, E235/84A, Y237/86A, I240/89A, K243/92A, R262/109A, K264/110bA, E265/110cA, R267/110eA, K348/187A, Q353/192A. The activities of H204/57A, D350/189A, S356/195A, and W376/215A were undetectable, such that neither $K_{i}$ nor $K_{m}$ could be determined. The reported inhibition constants were determined from the combined data of 3–6 independent experiments and global evaluation by Equation 4 (‘Experimental Procedures’). Data are reported as the fitted value ± S.E. after correction for the competitive effect of the chromogenic substrate.

| uPA variant | $K_{m}$ (μM) | $K_{i}$ (μM) | ΔΔ$G^\circ$ (kcal/mol) |
|-------------|--------------|--------------|----------------------|
| Wild type   | 63 ± 20      | 2.6 ± 0.1    | −0.46                |
| R178/35A    | 115 ± 1      | 5.5 ± 0.2   | −0.53                |
| R179/36A    | 106 ± 1      | 6.1 ± 0.6   | −0.53                |
| R181/37A    | 100 ± 1      | 6.1 ± 0.2   | −0.53                |
| Y187/40A    | 103 ± 1      | 4.8 ± 0.1   | −0.38                |
| F206/59A    | 116 ± 5      | 9.5 ± 0.5   | −0.80                |
| D208/60A    | 66 ± 8       | 117.5 ± 5.1 | −2.35                |
| Y209/60bA   | 84 ± 10      | 8.2 ± 0.2   | −0.71                |
| Y215/64A    | 82 ± 12      | 61.4 ± 2.7  | −1.95                |
| Y245/94A    | 59 ± 9       | 28.2 ± 1.1  | −1.47                |
| H252/99A    | 427 ± 58     | 21.3 ± 0.5  | −1.30                |
| Y308/151A   | 252 ± 34     | 23.2 ± 0.5  | −1.35                |
| W347/186A   | 860 ± 36     | 29.9 ± 0.1  | −1.51                |

* Modified residue.
* Significantly different from WT according to Student’s $t$ test ($p < 0.0001$).

A complemented media from a panel of uPA alanine variants recombinantly expressed by HEK293T cells. Principally, the alanine substitutions focused on charged, aliphatic, and aromatic residues present on the surface of the protease in and around the active site and the 37-loop and 60-loop regions. The most significant reductions in upain-1 phage binding (>5-fold) were found with the following Ala substitutions: H204/57A, D208/60aA, Y215/64A, Y245/94A, H252/99A, Y308/151A, W347/186A, D350/189A, S356/195A, and W376/215A were undetectable, such that neither $K_{i}$ nor $K_{m}$ could be determined. The reported inhibition constants were determined from the combined data of 3–6 independent experiments and global evaluation by Equation 4 (‘Experimental Procedures’). Data are reported as the fitted value ± S.E. after correction for the competitive effect of the chromogenic substrate.

**FIGURE 6. Equilibrium binding of upain-1-D1D2 to S195A-uPA as determined by displacement of $p$-aminobenzamidine.** The equilibrium binding constant ($K_{i}$) for upain-1-D1D2 binding to 0.8 μM S195A-uPA was determined from the competitive displacement of PAB (100 μM). Equilibrium titrations using increasing concentrations of upain-1-D1D2 (0–80 μM) produced a binding isotherm with individual data points representing the mean ± S.E. of the integrated fluorescence peaks for three independent titrations normalized to Δ$F_{\text{max}}$/Δ$F_{\text{max}}$. The solid line denotes the nonlinear regression fit of the data to Equation 7 (see “Experimental Procedures”), giving a $K_{i}$ value of 17.0 ± 5.6 μM (±S.E. of the fit).
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TABLE FIVE

Reactivity of upain-1-D1D2 toward serine proteases related to uPA

Kinetic parameters were determined in HBS with 0.1% BSA at pH 7.4 and at 37 °C except for fVIIa, which was determined in TBS at pH 8.1. The reported inhibition constants were determined from the combined data of three independent experiments and global evaluation by Equation 4 ("Experimental Procedures"). Data are reported as the fitted value ± S.E. after correction for the competitive effect of the chromogenic substrate.

| Protease      | Substrate | $K_{m}$ | $K_{i}$ |
|---------------|-----------|---------|---------|
|               |           | $\mu M$ | $\mu M$ |         |
|               | Upain-1-D1D2 |         |         | Empty-D1D2 |
| uPA           | S-2444    | 849 ± 0.1 | 2.1 ± 0.1 | 291.8 ± 11.1 |
| Murine uPA    | S-2444    | 1187 ± 0.3 | 1169 ± 93 | ND |
| tPA           | S-2288    | 330.6 ± 0.1 | 397.2 ± 29.8 | 338.6 ± 17.9 |
| Plasmin       | S-2403    | 105.5 ± 0.9 | NIb | ND |
| Thrombin      | S-2238    | 17.3 ± 0.9 | NI | ND |
| $\beta$-Trypsin| S-2222   | 60.0 ± 0.2 | 109.9 ± 3.9f | 455.9 ± 31.4 |
| aPC           | S-2366    | 711.3 ± 0.4 | NI | ND |
| fVIIa         | Spectrozyme$^ {\text{viiia}}$ | >500$^d$ | NI | ND |
| fXa           | S-2765    | 100.7 ± 0.5 | 406.1 ± 10.2 | ND |
| Plasma kallikrein | S-2302  | 299.9 ± 0.3 | 692.5 ± 23.2 | ND |

$^a$ND, inhibition constants were not determined.
$^b$NI, no inhibition of protease activity up to the highest tested upain-1-D1D2 concentration (150 $\mu M$), and $K_{i}$ values could not be determined.
$^c$Determined with the cleavage-resistant upain-1-D1D2 R10A variant.
$^d$Saturating levels of substrate could not be reached in the absence of tissue factor, and thus 500 $\mu M$ represents a lower limit for the $K_{m}$ value under the tested conditions.

Proteases that prefer a basic P$_{1}$ residue, using upain-1-D1D2 in competitive chromogenic substrate assays, each one with the corresponding optimal substrate for the respective protease (TABLE FIVE). We observed no detectable inhibition of aPC, plasmin, thrombin, and fVIIa by upain-1-D1D2 at upper limit concentrations ranging from 150 to 350 $\mu M$. If one then assumes a lower limit for detection of ~5% inhibition, we estimate that the $K_{i}$ values for inhibition of these proteases will at the very least exceed 500 $\mu M$. For tPA, $\beta$-trypsin, plasma kallikrein, fXa, and mouse-uPA, the calculated $K_{i}$ values ranged from 217 to 1170 $\mu M$. These values, however, were also in the same range as the $K_{i}$ values determined in control experiments for the nonspecific inhibition of uPA and several related proteases by the D1D2 scaffold without the inserted upain-1 peptide sequence (TABLE FIVE).

To further characterize the interaction of upain-1 with serine proteases other than uPA, we investigated whether any of the tested proteases were able to cleave the upain-1 sequence. The upain-1-D1D2 construct (2.5 $\mu M$) was incubated with individual serine proteases (50 nM) at 37 °C for 30 min, and the reaction products were analyzed by SDS-PAGE. Under these conditions, upain-1-D1D2 was not cleaved by tPA, aPC, thrombin, plasma kallikrein, fVIIa, or fXa. In contrast, 5 nM $\beta$-trypsin or 50 nM plasmin cleaved 100% and ~50% of 2.5 $\mu M$ upain-1-D1D2, respectively (Fig. 7 and data not shown). The cleavage reactions catalyzed by both trypsin and plasmin were delayed by an R4A amino acid substitution and fully avoided by an R10A substitution (Fig. 7 and data not shown). Although Arg$^4$ appears to affect the rate of degradation, this observation showed that the Arg$^{10}$-Met$^{11}$ bond is the scissile bond for trypsin and plasmin. Because of the rapid hydrolysis of the Arg$^{10}$-Met$^{11}$ bond by trypsin, we analyzed upain-1 inhibition of trypsin with upain-1-D1D2 R10A and found that the mutant had a $K_{i}$ value 2-fold lower than the WT (TABLE FIVE). The most ready explanation of this observation is that upain-1-D1D2 was cleaved by trypsin during the assay, reducing the effective inhibitor concentration. Importantly, our finding that upain-1-D1D2 does not inhibit plasmin (TABLE FIVE) cannot be explained as a consequence of plasmin-mediated cleavage of upain-1, because under the experimental conditions used (2 nM plasmin and up to 100 $\mu M$ upain-1), only a negligible fraction of the upain-1 was cleaved (data not shown). We conclude that upain-1-D1D2 has at least a 50-fold lower affinity for these tested proteases than for uPA.
Upain-1 Inhibits Plasminogen Activation Activity of uPA—In a plasminogen activation assay coupled with a plasmin substrate, the plasminogen activation activity of uPA could be inhibited by upain-1 peptide, upain-1-tetranectin, and upain-1-D1D2 (data not shown).

To test the effect of upain-1-D1D2 on cell surface-associated, uPA-catalyzed plasminogen activation, U937 cells were saturated with uPA and incubated with plasminogen in the absence and presence of upain-1-D1D2. The relative amount of plasmin generated during the incubation was then estimated by the use of a fluorogenic plasmin substrate. Twenty μM upain-1-D1D2 inhibited plasmin generation strongly and to the same extent as saturating concentrations of anti-uPA mAb 5 (Fig. 8). We therefore conclude that upain-1 is able to inhibit uPAR-bound uPA on the surface of intact cells in cell cultures.

DISCUSSION

We have described here the isolation of a human uPA-binding peptide sequence from a set of phage-displayed libraries of disulfide bridge-constrained peptides. Libraries with disulfide bridge-constrained peptides were previously used to isolate an activity-enhancing, prostate-specific antigen-binding peptide sequence (36, 37) and an inhibitory chymotrypsin-binding peptide sequence with a micromolar Ki value (38). The peptide sequence described here is a competitive inhibitor of the enzymatic activity of uPA, inhibiting its plasminogen activation activity as well as its peptidolytic activity. The upain-1 sequence is also an inhibitor of uPA bound to uPAR at the surface of intact cells in cell cultures. In equilibrium binding experiments, the upain-1-D1D2 fusion protein bound to uPA with a K<sub>D</sub> value in the high nM range. Our results are noteworthy for the following reasons: (i) the isolated peptide sequence was a more effective inhibitor when displayed on a protein scaffold than when assayed as a chemically synthesized peptide; (ii) the protease specificity of the inhibitor for uPA, as compared with other related serine proteases, is comparable with the best of the currently developed organochemical uPA inhibitors; and (iii) analysis of the binding mode for upain-1 has the potential for providing novel information about modalities of protease inhibition.

The Ki values for inhibition of uPA by upain-1-D1D2 and upain-1-tetranectin were 10–30-fold lower than the Ki value for inhibition of uPA by a chemically synthesized upain-1 peptide. The difference does not seem to be because of differences in the primary structures, as Edman degradation of upain-1-D1D2 showed no evidence for posttranslational modifications. At the moment, we cannot exclude the possibility that the difference in Ki is because of subtle differences in the stereochemistry of the sequence as found in the two backgrounds. However, it is relevant to note that the observed Ki values for upain-1-D1D2 and upain-1-tetranectin are almost identical. This observation implies that the lower Ki values of the fusion proteins do not result from specific interactions between a specific protein partner and uPA. Moreover, the observation made here is not unique. Our findings resemble previously reported results with several specific peptide substrates for uPA and tPA demonstrating significant differences in Ki values when used as chemically synthesized peptides or in fusion with a protein partner. For instance, the Ki values for cleavage by tPA of a peptide sequence selected from a phage-displayed peptide library was 950-fold lower when introduced as an N-terminal extension to Trypanosoma brucei ornithine decarboxylase as compared with the same sequence as a peptide (39). Also, both tPA and uPA hydrolyzed target sequences engineered into loops in staphylococcal nuclease with up to 200-fold lower Ki values relative to cleavage of analogous sequences within 15-residue synthetic peptides (40). The explanation offered by the authors of these two papers (39, 40) is that nonspecific interactions between the protease and the proteinaceous fusion partner are important in determining the interaction affinity. The observations described in the present work may therefore be used as a basis for further elucidation of this rather general phenomenon.

Although a determination of the exact binding site for upain-1 will clearly require the analysis of the three-dimensional structure of the upain-1-uPA complex, certain conclusions and predictions can be drawn from the site-directed mutagenesis analysis. Of the two Arg residues in the upain-1 sequence, only Arg<sup>4</sup> was essential for binding. Upain-1-D1D2 R10A displaced the upain-1 phage in ELISA, whereas upain-1-D1D2 R4A did not. Likewise, upain-1-D1D2 R10A displaced PAB from the specificity pocket of uPA as efficiently as upain-1-D1D2 WT, whereas upain-1-D1D2 R4A was unable to compete for PAB binding to uPA. Moreover, the Asp<sup>350/189</sup> residue of uPA, in the S<sub>1</sub>p pocket, is essential for upain-1 binding. From these observations, we conclude that Arg<sup>4</sup> corresponds to the P<sub>1</sub> residue of protease substrates, inserting into the S<sub>1</sub>p pocket and forming a productive salt bridge with Asp<sup>350/189</sup>. Our findings that Ala substitutions of His<sup>204/57</sup>, Tyr<sup>308/151</sup>, Trp<sup>347/186</sup>, Ser<sup>356/195</sup>, and Trp<sup>376/215</sup> reduced or abolished
upain-1 phage binding, that upain-1 phage does not bind to pro-uPA or uPA-serpin complexes, and that S356/195A displays a 40-fold increase in the $K_D$ value for upain-1 binding. The orientation in agreement with the notion that upain-1 binding occurs in or near the active site and requires an active conformation of the catalytic triad. As the Arg$^5$ residue functions as the P$_1$ residue in the inhibitor, one would then expect the CSW sequence of upain-1 to be oriented toward the S$_2$–S$_3$ binding area and the GLE sequence of upain-1 to be oriented toward the S$_1$–S$_3$ binding area (Fig. 9). As the S$_1$–S$_3$ pockets are in close proximity to the epitopes for the monoclonal anti-SPD antibodies, which span the 37- and the 60-loops (12), it is in agreement with the observation that the antibodies interfere with the ability of upain-1 to interact with uPA but not with the activity of uPA against the peptidolytic substrate that only binds to uPA binding area (Fig. 9). As the S$_1$–S$_3$ pockets are in close proximity to the epitopes for the monoclonal anti-SPD antibodies, which span the 37- and the 60-loops (12), it is in agreement with the observation that the antibodies interfere with the ability of upain-1 to interact with uPA but not with the activity of uPA against the peptidolytic substrate that only binds to uPA.

When proposing an inhibition mechanism for upain-1, one has to take into account not only the evidence for its binding mode but also the observation that it inhibits plasminogen activation as well as hydrolysis of S-2444. Despite overlapping binding sites, upain-1 thus inhibits uPA by a mechanism different from that of the monoclonal antibodies, which inhibit only plasminogen activation but not S-2444 hydrolysis. The binding mode proposed above is based on analogy to the binding modes of substrates, but why is upain-1 an inhibitor and not a substrate?

An attractive hypothesis for the inhibition mechanism of upain-1 is that the binding to uPA occurs in a way that does not permit proper stabilization of the transition state and therefore does not permit hydrolysis of the potentially scissile bond to proceed. Several findings have suggested that interactions between substrate and enzyme remote from the active site may be important for catalysis by properly aligning the substrate in the active site of the protease. Small changes around the scissile bond, which result from the changes in geometry during the transformation from planar substrate to tetrahedral transition state, can propagate into significant displacements down the peptide chain, and remote interactions can thereby contribute to stabilizing or destabilizing the transition state (for a review, see Ref. 3). Such interactions may therefore also be involved in determining whether a compound binding to a serine protease will be a substrate or an inhibitor. X-ray crystal structure analysis of serine proteases in complex with standard mechanism inhibitors of the Kazal, Kunitz, and related families have shown that, in general, the carbonyl group of the P$_1$ residue always projects into the oxyanion hole where it forms two hydrogen bonds with Gly$^{164}$N and Ser$^{196}$N, whereas the scissile bond is intact. In some complexes, there is a slight out of plane deformation of the carbonyl oxygen. The reactive center loop of these inhibitors forms the same main chain hydrogen bonds in the S$_1$–S$_3$ and S$_1$–S$_4$ binding areas as the substrates (for a review, see Ref. 41). The circular structure of upain-1 and its presumed extended binding contacts may have the same function as the constrained loop in standard mechanism inhibitors. Compared with other serine proteases, the size of the S$_1$ binding area of uPA is small, explaining the preference of uPA for substrates with small aliphatic residues in the P$_1$ position (1). Compared with the substrates, the special feature about upain-1 is the tryptophan residue in the P$_1$ position, which is expected to be too bulky to allow proper alignment into the S$_1$ binding area. In keeping with this expectation, we propose that the Trp$^3$ residue may function to prevent proper alignment of the upain-1 sequence in the active site of uPA in a way that allows peptide bond hydrolysis to proceed. This proposal is in excellent agreement with the fact that the W3A substitution converts upain-1 from an inhibitor to a distinct, although slowly hydrolyzed, substrate.

On the basis of the measured $K_I$ values, upain-1-D1D2 shows a
52-fold selectivity for uPA over trypsin, a 193-fold selectivity over fXa, a 189-fold selectivity over tPA, a 330-fold selectivity over plasma kallikrein, and a more than 250-fold selectivity over plasmin, thrombin, fVIIa, and aPC. We have thus documented an excellent selectivity for uPA over a broad range of serine proteases with the same P1 specificity. Upain-1 also showed a 557-fold selectivity for human uPA over murine uPA. By site-directed mutagenesis, we showed that surface-exposed, protease-specific loops contribute strongly to the binding affinity of upain-1. Among the residues found to contribute to upain-1 binding, the most important ones are Asp208/60a, Tyr215/64, and Tyr245/94. The variation of the residues in these positions among the other proteases suggests that Asp208/60a is the predominant determinant of specificity, as this Asp is absolutely specific for human uPA (TABLE SIX). It is interesting to note that a 50-fold selectivity for uPA as compared with β-trypsin was also achieved with 6-[N-(4-aminomethyl)phenyl]carbamyl]-2-naphtalene carboxamidine, the aminomethyl group of which was shown to form a hydrogen bond to Asp208/60b of uPA (19). Like upain-1, this inhibitor showed a high selectivity for human uPA over murine uPA (42). Among other organochemical uPA inhibitors, a 30-fold selectivity for uPA over plasmin, thrombin, fXa, tPA, and/or trypsin was also achieved with a series of (4-aminomethyl)phenylguanidine derivatives targeting the S1 and the S1′ subsites, displaying K_i values for uPA in the micromolar range (15). In other reported studies, selectivity ranging from more than 100-fold up to 2000-fold for uPA inhibition over that of plasmin, trypsin, thrombin, tPA, and fXa has been described for a series of 6-halo-5-amidinoindole and 6-halo-5-amidino-benzimidazole inhibitors with K_i values in the nanomolar range (14, 43–45). In contrast to our results here, measurements of selectivity over plasma kallikrein were reported for only a few of these inhibitors (14), and selectivity over aPC was not investigated. Furthermore, it has not been demonstrated convincingly that a good selectivity may be achieved concurrently over trypsin, plasmin, and plasma kallikrein. Thus, in terms of documented selectivity, upain-1 is comparable with or superior to the most selective of the previously published organochemical uPA inhibitors.

We have demonstrated here that upain-1 is an effective inhibitor of plasminogen activation in cell cultures. In fact, the upain-1-D1D2 fusion protein may be a uniquely interesting tool for studying the biological functions of uPA in cell culture models systems by transfecting upain-1-D1D2 cDNA into various cells having uPA-dependent functions. Still, the stability of peptide-based protease inhibitors in biological settings may be a concern, as they may be substrates for non-target proteases. We have demonstrated that the Arg10-Met11 bond of the original upain-1 sequence is a good substrate for trypsin but that the substrate behavior may be prevented by substituting Arg10 with alanine without affecting the affinity to uPA. The fact that a reasonable affinity, with a K_D in the high nM range, was achieved in only a single phage display selection step from a library with a size allowing only 0.01% of all possible CX_10,C sequences suggests that the uPA-upain-1 affinity almost certainly can be improved by affinity maturation of the upain-1 peptide sequence, whether used as a synthetic peptide or as a fusion protein. Obviously, a potential drug development based on the upain-1 sequence should utilize chemically synthesized peptides, and the K_i for the upain-1 peptide is not very impressive (29.9 μM). On the other hand, the use of synthetic peptides offers unique possibilities for affinity maturation by the use of non-natural amino acids. Also, we recently began employing techniques for replacing peptide bonds by a hydroxyethylene isoster at the scissile bond of the upain-1 sequence (46). Thus, replacing the P1–P1′ bond by a non-scissile bond may give additional freedom for affinity maturation, without the risk of converting the sequence into a uPA-cleavable peptide. Also, the introduction of non-scissile bonds elsewhere in upain-1 may add to the biological stability.

In conclusion, we have isolated a disulfide bond-constrained dodecapeptide that is a competitive inhibitor of uPA through binding interactions at the active site cleft but that acquires specificity by targeting uPA-specific loops surrounding the active site. Biochemical insight into the inhibitory mechanism of upain-1 has provided a rational explanation for the inhibitory properties of this peptide. Consequently, we propose that this compound and derivatives thereof will be valuable tools for future investigations of the mechanisms of serine protease inhibition and catalysis.

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**TABLE SIX**

| Proteinase   | 35 | 36 | 37 | 40 | 59 | 60a | 60b | 64 | 94 | 99 | 151 | 186 |
|--------------|----|----|----|----|----|-----|-----|----|----|----|-----|-----|
| uPA          | R  | R  | R  | R  | Y  | F   | D   | Y  | Y  | Y  | H   | Y   | W   |
| Murine uPA   | Q  | K  | N  | K  | F  | Q   | L   | Y  | Y  | Y  | Y   | W   |
| tPA          | A  | K  | H  | R  | F  | E   | R   | L  | F  | Y  | Y   | S   |
| Plasmin      | T  | R  | L  | K  | S  | Y   | R   | G  | G  |    |     |     |
| Thrombin     | R  | K  | S  | L  | K  | N   | L   | Y  | L  | Q  | D   |     |
| Human trypsin| S  | H  | Y  |    |    |     |     |    |    |    |     |     |
| Bovine trypsin|    |    |     |     |    |     |     |    |    |    |     |     |
| aPC          | D  | S  | K  | L  | M  | D   | E   | L  | Y  | T  | R   | D   |
| fVIIa        | V  | N  | F  | K  | I   | L   | Y   | T  | T  | G   |     |     |
| fXa          | N  | E  | L  | Q  | A   | F   | F   | Y  | Q  | K   |     |     |
| Plasma kallikrein| V | K  | L  | T  | F  | G   | L   | W  | Y  | G   | I   |     |

Amino acid residue variation among related serine proteases in the positions critical for binding of upain-1 to uPA.
