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Identification of Inhibitors of Prohormone Convertases 1 and 2 Using a Peptide Combinatorial Library*

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A positional scanning synthetic peptide combinatorial library containing approximately 52 million hexapeptides was used to identify potential inhibitory peptides for recombinant mouse prohormone convertase 1 (PC1) and PC2 and to provide information on the specificity of these enzymes. The library surveys revealed that a P6 Leu, a P4 Arg, a P2 Lys, and a P1 Arg were most inhibitory against PC1, and a P6 Ile and a P4 Arg were most inhibitory against PC2. Using information derived from the library surveys, hexapeptide sets were synthesized and screened for inhibition of PC1 and PC2. The data obtained revealed the preference of both enzymes for a P3 Val. At P5, many substitutions were well tolerated. PC1 and PC2 proved to differ mainly in the selectivity of their S6 subsites. In PC1, this subsite displayed a strong preference toward occupation by Leu; the K_i value for peptide Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 was 28 times lower than that for peptide Ac-Ile-Ile-Arg-Val-Lys-Arg-NH_2. In contrast, PC2 discriminated little between Leu and Ile at P6, as evidenced by the small (1.5-fold) difference in K_i values for these two peptides. Several hexapeptides synthesized as a result of the screen were found to represent potent inhibitors of PC2 (with K_i values in the submicromolar range) and, particularly, of PC1 (with K_i values in the low nanomolar range). The most potent inhibitor, Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2, proved to be the same peptide for both enzymes and inhibited PC1 and PC2 in a competitive, fast-binding manner with K_i values of 3.2 and 360 nM, respectively. The four most potent peptide inhibitors of PC1 and PC2 were also tested against soluble human furin and found to exhibit a different rank order of inhibition; for example, Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 was 440-fold less potent against furin than against PC1, with a K_i of 1400 nM.

Prohormone convertase 1 (PC1)[1] and PC2 are members of the prohormone convertase/kexin subfamily of serine proteinases, which is now thought to be responsible for the proteolytic maturation of a variety of proproteins and prohormones (reviewed in Refs. 1 and 2). Although structurally related to subtilisin in their catalytic domains, these enzymes differ substantially from subtilisin, both in their much greater size and their requirement for basic residues on the NH_2-terminal side of the scissile bond (reviewed in Refs. 1 and 2). Members of this family of enzymes are Ca^{2+}-dependent and, unlike the related family member furin, which is fully active at neutral pH values (3, 4) are most active at acidic pH values (5–10). The yeast homolog of these enzymes, kex2, is a membrane-bound enzyme with a neutral but fairly wide pH optimum (11).

From both *in vivo* and *in vitro* studies, it is evident that the prohormone convertases and furin favor the presence of basic residues at subsites P1, P2, and, in many cases, P4 for efficient catalysis (12–18). The specificity of these enzymes was exploited in the design of several inhibitors for PC1 and furin. These include an irreversible peptidyl chloromethane inhibitor (19), ketomethylene and aminomethyl ketone pseudopeptide analogs (20, 21), and decapeptides or dodecapeptides with the P1′ position occupied by unnatural amino acids (22, 23). Pseudopeptide analogs proved to exhibit K_i values of nanomolar to submicromolar range against furin, and peptides with an unnatural amino acid residue at the P1′ position inhibit furin and PC1 with micromolar K_i values (22, 23). Furin can also be potently inhibited by certain serpins, such as the mutated analog of α_1-antitrypsin known as α_1-antitrypsin Portland (24). This mutant α_1-antitrypsin contains Arg substitutions at positions 358 and 355 (thus generating P1 and P4 Arg residues) and exhibits significantly greater potency for furin over the Pittsburgh variant that lacks a P4 Arg (24). Recent studies have revealed the selectivity of α_1-antitrypsin Portland toward furin as opposed to prohormone convertases (25). The turkey ovomucoid inhibitor has also been used to generate furin inhibitors (26), and serpin proteinase inhibitor 8 has been shown to potently inhibit furin (27).

Little information exists on physiological inhibitors of the prohormone convertases. The COOH-terminal peptide of the neuroendocrine protein 7B2 has been shown to potently inhibit PC2, but not PC1 (9, 28). The COOH-terminal tail of PC1 has been proposed to represent an inhibitor of this enzyme (29); however, this has not yet been directly demonstrated in *in vitro* studies. Recently, in line with the idea that subtilisin propeptides represent potent intramolecular inhibitors (30), the furin propeptide has also been reported to inhibit furin (31). Boudreault *et al.* have also recently demonstrated that the PC1 propeptide (residues 1–98) represents an effective inhibitor of PC1 with a K_i value of 6 nM.[2]

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[1] The abbreviations used are: PC, prohormone convertase; mPC, mouse PC; MCA, methylcoumarinamide; PS-SPCL, positional scanning synthetic peptide combinatorial library; 7B2 CT peptide, human $7B2_{155–185}$; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; AMC, aminomethylcoumarin.

[2] A. Boudreault, D. Gauthier, and C. Lazure, submitted for publication.

In the study reported below, we have used a positional scanning synthetic peptide combinatorial library (PS-SPCL) containing approximately 52 million hexapeptides to identify potential inhibitory peptides for PC1 and PC2 and to provide additional information on the specificity of these two enzymes. PS-SPCLs have proven useful in the identification of antigenic determinants and ligands for various receptors and, more recently, in the identification of enzyme inhibitors (reviewed in Refs. 32 and 33). Deconvolution can be performed either by iterative definition of progressively defined peptide mixtures (34) or by positional scanning of defined libraries and combination of the most potent residues at each position (35). An iterative combinatorial approach was successfully used to identify trypsin inhibitors with micromolar IC_{50} values (36) as well as potent and selective α-glucosidase inhibitors (37). The positional scanning format has also been used to identify chymotrypsin inhibitors (38). In the present study, we show that PC1 and PC2 exhibit rather similar specificity profiles, except for the higher selectivity of the S6 subsite of PC1 compared with that of PC2. We also identify several very potent hexapeptide inhibitors of PC1 (with K_i values in the low nanomolar range) and several potent inhibitors of PC2 (with K_i values in the submicromolar range).

EXPERIMENTAL PROCEDURES

Materials—A human 7B2 CT peptide consisting of residues 1–18 (terminating in Lys-Lys) was synthesized by Louisiana State University Medical Center Core Laboratories. pGlu-Arg-Thr-Lys-Arg-MCA was purchased from Peptides International, Inc. (Louisville, KY). The PS-SPCL consisted of 120 hexapeptide mixtures with NH_2-terminal acetylation and COOH-terminal amidation divided into six groups corresponding to each position within the hexapeptide. For each position, 20 mixtures were surveyed, each of which was defined by 1 of the 20 natural L-amino acids. The undefined positions were occupied by any of the L-amino acids except cysteine. The PS-SPCL and the inhibitory peptides were synthesized at the Torrey Pines Institute for Molecular Studies (San Diego, CA) using simultaneous multiple peptide synthesis methodology as described previously (32, 35).

Production of Recombinant mPC1—Purified recombinant mPC1 was obtained from the conditioned medium of methotrexate-amplified Chinese hamster ovary/mPC1 cells using fast performance liquid chromatography as described previously (7), with some modifications. Briefly, a Protein-Pak™ Q 8HR column (5 × 50 mm; Waters, Milford, MA) was used in the purification procedure. PC1 was eluted from the column using a 0–100% linear gradient of Buffer B (1 M sodium acetate, 20 mM BisTris, pH 6.5, and 0.1% Brij 35) in Buffer A (20 mM BisTris, pH 6.5, and 0.1% Brij 35) for 120 min. This PC1 preparation consisted of an equal mixture of three PC1 forms (87, 74, and 66 kDa), as judged by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining.

Production of Recombinant mPC2—Purified recombinant mPC2 was obtained as described in Lamango *et al.* (10) from the conditioned medium of methotrexate-amplified Chinese hamster ovary/mPC2 cells stably supertransfected with cDNAs encoding 21-kDa rat 7B2 (39).

Hexapeptide Library Screen—Each hexapeptide mixture (final concentration, 1 or 0.5 mg/ml) was preincubated with preactivated PC1 (200 ng) or PC2 (65 ng) in 100 mM sodium acetate buffer, pH 5.5, or pH 5.0 containing 5 mM $CaCl_2$ and 0.4% *n*-octyl glucoside for 30 min at room temperature, followed by the addition of substrate (pGlu-Arg-Thr-Lys-Arg-MCA; final concentration, 200 μM). The total volume of each reaction mixture was 50 μl. The rate of hydrolysis relative to control samples lacking inhibitors was then determined by measuring the fluorescence of the released AMC over a 3-h (in the case of PC1) or 1-h (in the case of PC2) incubation period at 37 °C, during which time hydrolysis of the substrate proceeded linearly in the absence of inhibitor.

Analysis of Inhibition of PC1 and PC2 by Synthetic Peptides—Based on the information obtained from the hexapeptide library screening, various hexapeptides (NH_2-terminally acetylated and COOH-terminally amidated) were synthesized to further study the subsite binding preferences of potential PC1 and PC2 inhibitors. Initial screening of the peptides for PC1 or PC2 inhibition was performed in an identical fashion to the screening of the PS-SPCL, except that a final peptide concentration of 0.1 μg/ml (against PC1) or 10 μg/ml (against PC2) was used. The apparent inhibition constants ($K_{i(app)}$) for peptides showing strong inhibition at these concentrations were then determined as described by Salvesen and Nagase (40) by measuring the rate of substrate hydrolysis in the presence of varying inhibitory peptide concentrations. The K_i values were calculated using the K_m values of 11 and 18 μM for PC1 and PC2, respectively, and the relationship $K_i = K_{i(app)}/(1 + [S]/K_m)$. Against PC1, concentrations between 0 and 1 μg/ml were used for peptides 3, 6, 9, and 12; concentrations between 0 and 3 μg/ml were used for peptide 5; and concentrations between 0 and 10 μg/ml were used for peptides 4, 10, 32, and 38. Against PC2, concentrations between 0 and 100 μg/ml were used for peptides 4, 24, 25, 30, 31, 35, and 36; concentrations between 0 and 40 μg/ml were used for peptides 5, 12, and 32; and concentrations between 0 and 20 μg/ml were used for peptides 3, 6, and 9 (see Tables II–IV).

Progress curves of mPC1 or mPC2 activity in the presence of Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 or 7B2 CT peptide 1–18 were recorded with a Perkin-Elmer model 650–40 fluorescence spectrophotometer using an excitation and an emission wavelength of 370 (slit width, 5 nm) and 460 nm (slit width, 3 nm), respectively. Assay mixtures (total volume, 400 μl) contained 100 mM sodium acetate buffer, pH 5.5 or 5.0, 5 mM $CaCl_2$, 0.4% *n*-octyl glucoside, varying amounts of Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 (0, 30, 61, and 121 nM in the case of PC1 and 0, 1.2, 6, and 12 μM in the case of PC2) or 7B2 CT peptide 1–18 (0, 4, 10, and 25 nM), 200 μM substrate, and preactivated PC1 (1.6 μg) or PC2 (180 ng). Reactions were initiated by adding the enzyme and conducted at 37 °C.

Production and Partial Purification of Furin—Partially purified furin was obtained from the medium of the baculovirus-infected Hi5 cells. Hi5 cells (20×10^6) in serum-free EXCELL 401 medium were transfected with furin-baculovirus stock virus (16). After 74 h, the medium was centrifuged at 1,000 × *g* for 15 min to remove cells and dialyzed against several changes of Buffer A (10 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, and 0.1 mM *o*-phenanthroline) as described previously (16). The dialysate was centrifuged at 100,000 × *g* for 30 min, and the supernatant was applied onto a 25-ml (1.6 × 12.5 cm) fast-flow Q-Sepharose column (Amersham Pharmacia Biotech) equilibrated with Buffer A. The column was washed with 200 ml of Buffer A and eluted with a gradient of 0–80% Buffer B (Buffer A with 300 mM NaCl and 5% glycerol) for 80 min.

Determination of K_i Values against Furin—In a total volume of 50 μl, preactivated furin (corresponding to 250–300 pmol/h AMC released from pGlu-Arg-Thr-Lys-Arg-MCA) was preincubated with varying amounts (ranging from 0 to 0.1 mg/ml) of different synthetic peptides in 100 mM HEPES, pH 7.5, 0.5% Triton X-100, and 1 mM $CaCl_2$ for 30 min at room temperature. The reaction was initiated by adding pGlu-Arg-Thr-Lys-Arg-MCA to a final concentration of 60 μM; incubations were then conducted for 1 h at 37 °C. The K_i values were determined in the same fashion as described for PC1 and PC2, using a K_m value of 3 μM for this substrate.

RESULTS

Screening of the PS-SPCL—To gain insight into which residues might be important for inhibition of PC1 and PC2, the PS-SPCL with acetylated NH_2 and amidated COOH termini was screened for competitive inhibition of PC1 or PC2 activity. With PC2, the mixtures were screened a total of 11 times at several different peptide concentrations. For PC2, but not for PC1, some variability was observed between screenings, possibly due to differences in enzyme preparations, library handling, and/or unknown factors. With PC1, the mixtures were screened a total of three times at final peptide concentrations of 1 and 0.5 mg/ml, and the results of the three screenings were consistent.

A representative screen with PC1 is shown in Fig. 1, using a final library concentration of 1 mg/ml. At positions P1, P2, P4, and P6, a clear preference emerged for Arg, Lys, Arg, and Leu, respectively. At the P3 position, a slight preference for Val was found. At the P5 position, no amino acid residue had remarkable inhibitory potency, and no clear consensus emerged. Surprisingly, some peptides appear to have activating effects. The reasons for this are not clear; one possible explanation is that activating peptides may bind to PC1 not at the active site but at an allosteric site, thereby increasing enzyme activity. However, further work must be done to support this idea.

Results of the hexapeptide library screenings against PC2 are shown in Table I. At position P1, Arg consistently exhibited

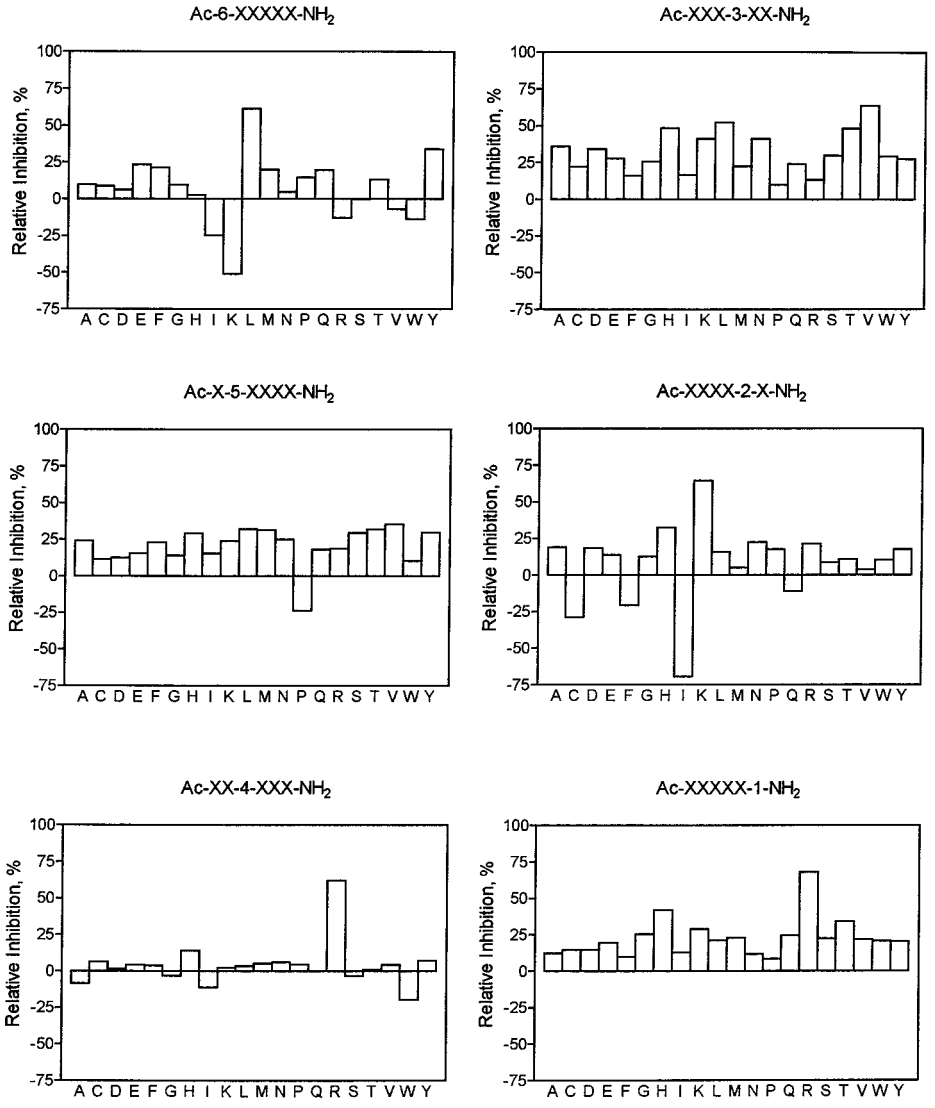

FIG. 1. **Inhibition of PC1 activity by a hexapeptide PS-SPCL.** Each peptide mixture (final concentration, 1 mg/ml) was preincubated with the enzyme in assay medium for 30 min at room temperature before the addition of substrate. The rate of hydrolysis of pGlu-Arg-Thr-Lys-Arg-MCA was followed for 3 h. Inhibition is given as the percentage decrease in activity in the presence of the various peptide mixtures relative to that of controls.

TABLE I
SPCL survey for PC2

Results are from eight independent experiments.

Test no.	Inhibitory amino acid residues					
	P6	P5	P4	P3	P2	P1
1	MEHIK	SHT	R	HLK	KH	DEH
2	E	IY	R	None	None	HR
3	EIP	GHIKR	KMR	Many	EFKM	HR
4	EI	GST	YN	FLMVY	EFKPY	None
5	E	I	MR	FMV	K	R
6	EIRK	HIKPR	R	ACFGLV	DFK	EKR
7	KEFHW	PHI	R	KI	nd[a]	nd[a]
8	EFH	None	R	FHKN	HKR	HK
Consensus	EI	IK	RM	FV	K	R

[a] nd, not done.

profound inhibition; in other screenings, Lys His, and Glu were also frequently found to be inhibitory. At P2, many amino acids were inhibitory. Lys was among the top three inhibitory amino acids in most screenings, consistent with the known cleavage site preference for Lys-Arg pairs within prohormones. At the P3 position, no clear consensus emerged during any of the screenings, except a slight preference for Phe, Val, Leu, and occasionally Lys. At the P4 position, as in the case with PC1, a clear preference for Arg emerged; in five of eight screenings of the library, Arg was by far the most inhibitory amino acid. Met, Lys, and Tyr were also occasionally inhibitory at this position. At the P5 position, Ile and Lys were slightly preferred in half of the screenings; other inhibitory amino acids included His, Ser, and Pro. At the P6 position, Glu was preferred in six screen-

TABLE II
Inhibition of PC1 by various hexapeptides tested at 100 ng/ml
Inhibition was quantified as the percentage decrease in activity relative to the control without peptides. The values represent the means ± S.D. ($n = 2$).

No.	Peptide (P6 P5 P4 P3 P2 P1)	Relative inhibition (%)
1	Ac-Leu-Lys-Arg-His-Lys-Arg-NH_2	11 ± 3
2	Ac-Leu-Lys-Arg-Thr-Lys-Arg-NH_2	19 ± 7
3	Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2	59 ± 1
4	Ac-Leu-Leu-Arg-His-Lys-Arg-NH_2	18 ± 1
5	Ac-Leu-Leu-Arg-Thr-Lys-Arg-NH_2	34 ± 5
6	Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2	75 ± 1
7	Ac-Leu-Met-Arg-His-Lys-Arg-NH_2	10 ± 8
8	Ac-Leu-Met-Arg-Thr-Lys-Arg-NH_2	25 ± 1
9	Ac-Leu-Met-Arg-Val-Lys-Arg-NH_2	61 ± 1
10	Ac-Leu-Tyr-Arg-His-Lys-Arg-NH_2	5 ± 2
11	Ac-Leu-Tyr-Arg-Thr-Lys-Arg-NH_2	22 ± 2
12	Ac-Leu-Tyr-Arg-Val-Lys-Arg-NH_2	51 ± 2

ings; other inhibitory amino acids at this position included Ile, Lys, and His. Based on the results of early screenings, several consensus peptides were synthesized. P5/P6 Arg/Lys was used, based primarily on the known presence of this basic pair at PC cleavage sites within prohormones.

Inhibition of PC1 by Synthetic Peptides—Twelve PC1-targeted peptides were synthesized by selecting the best-inhibiting amino acids at the P1–P6 positions. Each peptide had Leu, Arg, Lys, and Arg at positions P6, P4, P2, and P1, respectively. Position P3 was occupied by either Val, Thr, or His, and position P5 was occupied by either Leu, Met, Tyr, or Lys.

Synthetic peptides were initially tested for their inhibitory potencies against PC1 at a final concentration of 0.1 μg/ml. These results are shown in Table II. K_i values were then determined for selected peptides (Table III). As is evident in Tables I and II, a distinct binding preference can be observed at the P3 subsite. The inhibition potency declined in the order Val, Thr, and His. Hexapeptides with Val at this position proved to be very potent inhibitors of PC1, with K_i values in the low nanomolar range. The most potent peptide, Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2, exhibited a K_i value of 3.2 nM. Replacement of Val with Thr resulted in a 5-fold increase in the K_i value (peptides 6 and 5), and the presence of His rather than Val at this position produced an even larger increase of 20–30-fold (peptides 6 and 4; peptides 12 and 10).

Substitutions at position P5 were better tolerated than those at the P3 subsite. Replacement of Leu (which proved to be more preferable at this subsite) with Met, Lys, or Tyr resulted in only a 2–3.6-fold increase in the K_i values (peptides 6, 3, 9, and 12; peptides 4 and 10). Interestingly, a double substitution of Leu by Ile at positions P5 and P6 (peptide Ac-Ile-Ile-Arg-Val-Lys-Arg-NH_2, synthesized based upon the results of the SPCL screens against PC2, see below) resulted in a 28-fold increase in its K_i value against PC1.

The type of inhibition of PC1 exhibited by synthetic peptides was tested by performing incubations with various concentrations of inhibitors and substrate. As shown in the Lineweaver-Burk plot (Fig. 2), inhibition of PC1 by the best synthetic peptide (Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2) was competitive. Progress curves of PC1 activity demonstrated that the interaction of PC1 with the peptide inhibitor followed fast binding kinetics (Fig. 3), because the shape of the curves in the presence of varying concentrations of the inhibitor remained the same as in the absence of inhibitor.

Inhibition of PC2 by Synthetic Peptides—Seventeen peptides were synthesized based upon the results of the SPCL screenings, and the assumption of a P2 Lys and a P1 Arg. An additional peptide was synthesized that was based on the PC2-binding site of the 7B2 CT peptide. These peptides were tested for their inhibitory potencies at a final concentration of 10 μg/ml (Table IV), and K_i values were then determined for the most potent peptides (Table III). K_i values against PC2 were also determined for certain peptides originally synthesized as PC1-targeted inhibitors.

The results obtained revealed a preference for Arg over Met at the P4 subsite, as evidenced by the data in both Tables III and IV. Replacement of Arg with Met resulted in a 16-fold increase in the K_i value (peptides 32 and 30). As with PC1, many substitutions at the P5 subsite were well tolerated (peptides 6, 3, 9, and 12; peptides 31 and 35). Only the introduction of Pro at this position resulted in a dramatic 20-fold increase in the K_i value (peptides 32 and 37). At P3, a preference for Val over Phe emerged. The K_i values increased by two to six times after the substitution of Phe for Val (peptides 32 and 31; peptides 36 and 35). Replacement of Val with Thr produced a 2-fold increase in the K_i value (peptides 6 and 5). The occupancy of subsite P6 with Ile resulted in better inhibition than Glu. The peptide Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2, which was found to be the most potent inhibitor against PC1, also proved to exhibit the lowest K_i value against PC2. This was surprising because Leu was not found among the top inhibitory amino acid residues in the PS-SPCL screenings against PC2 (see Fig. 2).

Interestingly, the synthetic peptide Ac-Asn-Val-Val-Ala-Lys-Lys-NH_2, whose sequence was based upon the PC2-binding site of the only known natural PC2 inhibitor, the 7B2 CT peptide, was inactive as an inhibitor of PC2 (Table IV).

As with PC1, the interaction of inhibitory peptides with PC2 followed fast binding kinetics (Fig. 4).

Cleavage of Peptide Inhibitors by PC1 and PC2—We examined the possibility of internal cleavage of peptide inhibitors by enzymes using two synthetic peptides: Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 and Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2, with the latter peptide being the most likely candidate for being internally cleaved with PC1 or PC2, because it contains an internal dibasic site. Each of these peptides was incubated with PC2 at 37 °C for different time periods; the final concentration chosen, 10 μg/ml, does not result in complete inhibition of PC2. After the incubation, the reaction mixtures were analyzed by reverse-phase high performance liquid chromatography. We found that a 5-h or even a 24-h incubation of PC2 with either of the two inhibitory peptides did not result in a decrease of the size of the peptide peak as compared with the zero time incubation mixture (data not shown). This suggests that the peptides are not internally cleaved by PC2 upon incubation for 24 h. The peptide Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2 was incubated with PC1 at a final concentration of 1 μg/ml at 37 °C for 15 h; the reaction mixture was then analyzed by mass spectroscopy. Again, no cleavage peptides were observed, with the mass of the peptide remaining constant (data not shown). Because the primary structures of the other synthetic peptides used in this paper are similar to those of Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 and Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2, our data suggest a probable lack of internal cleavage by PC2 or PC1.

Inhibition of Furin with Synthetic Peptides—The various convertases exhibit overlapping substrate specificities; we therefore tested the inhibitory potency of four synthetic peptides against furin, another member of the prohormone convertase/kexin subfamily. Interestingly, three of the peptides tested were three to five times less potent against furin than against PC2, but the potency of Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2 was over three times higher against furin than against PC2 (Table III). The rank order of the peptides thus differed for the various enzymes, *i.e.* the lowest K_i value against furin was displayed by the peptide Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2, not by Ac-Leu-

TABLE III
Inhibition constants for various hexapeptides against PC1, PC2, and furin

No.	Peptide P6 P5 P4 P3 P2 P1	K_i (nM)[a] PC1	PC2	Furin
6	Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2	3.2 ± 1.0	360 ± 50	1400 ± 230
9	Ac-Leu-Met-Arg-Val-Lys-Arg-NH_2	4.9 ± 1.0	530 ± 70	1900 ± 100
3	Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2	5.7 ± 1.5	620 ± 150	190 ± 20
12	Ac-Leu-Tyr-Arg-Val-Lys-Arg-NH_2	6.5 ± 2.3	720 ± 160	3400 ± 300
5	Ac-Leu-Leu-Arg-Thr-Lys-Arg-NH_2	16 ± 1	860 ± 200	ND[b]
4	Ac-Leu-Leu-Arg-His-Lys-Arg-NH_2	60 ± 14	3100 ± 300	ND
10	Ac-Leu-Tyr-Arg-His-Lys-Arg-NH_2	220 ± 23	ND	ND
32	Ac-Ile-Ile-Arg-Val-Lys-Arg-NH_2	92 ± 17	530 ± 120	ND
31	Ac-Ile-Ile-Arg-Phe-Lys-Arg-NH_2	ND	3400 ± 1300	ND
36	Ac-Ile-Lys-Arg-Val-Lys-Arg-NH_2	200 ± 18	3700 ± 200	ND
35	Ac-Ile-Lys-Arg-Phe-Lys-Arg-NH_2	ND	8400 ± 600	ND
30	Ac-Ile-Ile-Met-Val-Lys-Arg-NH_2	ND	8600 ± 1300	ND
37	Ac-Ile-Pro-Arg-Val-Lys-Arg-NH_2	ND	10400 ± 1800	ND
24	Ac-Glu-Ile-Arg-Val-Lys-Arg-NH_2	ND	12600 ± 1400	ND
27	Ac-Glu-Lys-Arg-Phe-Lys-Arg-NH_2	ND	>25 μM	ND
25	Ac-Glu-Lys-Met-Phe-Lys-Arg-NH_2	ND	>45 μM	ND

[a] The rate of hydrolysis of pGlu-Arg-Thr-Lys-Arg-MCA was determined in the presence of various concentrations of the different peptides (each in duplicate or triplicate) as described under "Experimental Procedures." The results obtained were then used to compute the K_i values for the different peptides. Each value is the mean ± S.D., determined from two to four independent experiments.
[b] ND, not done.

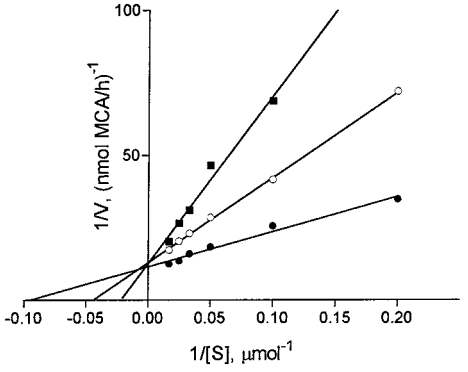

FIG. 2. **Lineweaver-Burk analysis of inhibition of PC1 cleavage by Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2.** PC1 (50 nM) was preincubated in 100 mM sodium acetate, pH 5.5, 5 mM $CaCl_2$, and 0.4% *n*-octyl glucoside with 0 (●), 18 (○), and 30 nM (■) Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 before the addition of substrate at the final concentrations indicated. The rate of hydrolysis was followed for 3 h.

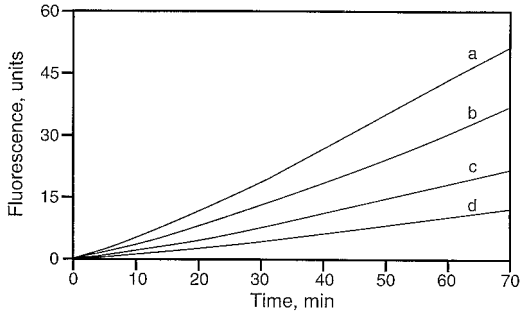

FIG. 3. **Progress curves of PC1 activity in the presence of Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2.** PC1 (50 nM) was reacted with 0 (*a*), 30 (*b*), 61 (*c*), and 121 nM (*d*) peptide Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 in 100 mM sodium acetate, pH 5.5, 5 mM $CaCl_2$, and 0.4% *n*-octyl glucoside at 37 °C in the presence of 200 μM pGlu-Arg-Thr-Lys-Arg-MCA. The reaction was initiated by the addition of enzyme.

Leu-Arg-Val-Lys-Arg-NH_2 (as was the case with PC1 and PC2).

7B2 CT-Peptide 1–18 Is a Slow Binding Inhibitor of PC2—The 31-residue 7B2 CT peptide is very potent natural inhibitor of PC2, with an IC_{50} value of 57 nM (9). The NH_2-terminal portion of this peptide, encompassing the first 18 amino acid residues (7B2 CT peptide 1–18), is responsible for the potent inhibition of PC2 (28). As described above, the interaction of synthetic peptides with both PC1 and PC2 follows fast binding kinetics (Figs. 3 and 4). However, several recently described potent inhibitors of PC1 and furin have been shown to exhibit slow binding inhibition kinetics (27).[2] Therefore, we found it of interest to determine what type of inhibitor the 7B2 CT peptide 1–18 represents.

TABLE IV
Inhibition of PC2 by various hexapeptides tested at 10 μg/ml
Inhibition was quantified as the percentage decrease in activity relative to the control without peptides. The values represent the means ± S.D. of triplicate determinations.

No.	Peptide P6 P5 P4 P3 P2 P1	Relative inhibition (%)
21	Ac-Glu-Ile-Met-Phe-Lys-Arg-NH_2	3.9 ± 1.0
22	Ac-Glu-Ile-Met-Val-Lys-Arg-NH_2	7.9 ± 1.2
23	Ac-Glu-Ile-Arg-Phe-Lys-Arg-NH_2	12.2 ± 2.3
24	Ac-Glu-Ile-Arg-Val-Lys-Arg-NH_2	15.1 ± 1.4
25	Ac-Glu-Lys-Met-Phe-Lys-Arg-NH_2	11.0 ± 0.7
26	Ac-Glu-Lys-Met-Val-Lys-Arg-NH_2	9.4 ± 1.7
27	Ac-Glu-Lys-Arg-Phe-Lys-Arg-NH_2	13.6 ± 1.2
28	Ac-Glu-Lys-Arg-Val-Lys-Arg-NH_2	6.5 ± 1.6
29	Ac-Ile-Ile-Met-Phe-Lys-Arg-NH_2	10.3 ± 2.1
30	Ac-Ile-Ile-Met-Val-Lys-Arg-NH_2	19.8 ± 2.3
31	Ac-Ile-Ile-Arg-Phe-Lys-Arg-NH_2	15.9 ± 2.3
32	Ac-Ile-Ile-Arg-Val-Lys-Arg-NH_2	54.4 ± 0.3
33	Ac-Ile-Lys-Met-Phe-Lys-Arg-NH_2	1.3 ± 1.7
34	Ac-Ile-Lys-Met-Val-Lys-Arg-NH_2	6.2 ± 0.9
35	Ac-Ile-Lys-Arg-Phe-Lys-Arg-NH_2	13.8 ± 1.2
36	Ac-Ile-Lys-Arg-Val-Lys-Arg-NH_2	27.0 ± 2.1
37	Ac-Ile-Pro-Arg-Val-Lys-Arg-NH_2	11.6 ± 4.8
38	Ac-Asn-Val-Val-Ala-Lys-Lys-NH_2	0

Fig. 5 shows progress curves of PC2 activity recorded in the presence of various concentrations of 7B2 CT-peptide 1–18. Based upon these curves, which show a lag to assumption of inhibition, we conclude that the inhibition of PC2 by this peptide follows slow binding kinetics.

DISCUSSION

We have used a hexapeptide combinatorial library to reveal amino acid residues that are most inhibitory at the different subsites of PC1 and PC2. Based upon the results of hexapeptide library screenings, we synthesized several peptides that contained the most inhibitory residues at each position and tested these peptides for their inhibitory potency against both enzymes.

The data obtained reveal that the specificities of the S5, S4, and S3 subsites of PC1 generally appear to resemble those of PC2. Both with PC1 and PC2, a P4 Arg residue exhibits the

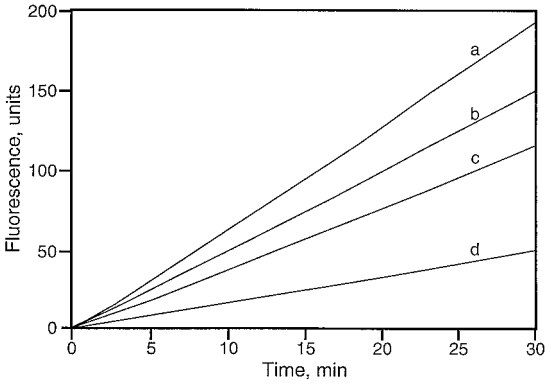

FIG. 4. **Progress curves of PC2 activity in the presence of Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2.** PC2 (7 nM) was incubated with 0 (*a*), 1.2 (*b*), 6 (*c*), and 12 μM (*d*) Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 in 100 mM sodium acetate, pH 5.0, 5 mM $CaCl_2$, and 0.4% *n*-octyl glucoside at 37 °C in the presence of 200 μM pGlu-Arg-Thr-Lys-Arg-MCA. The reaction was initiated by the addition of enzyme.

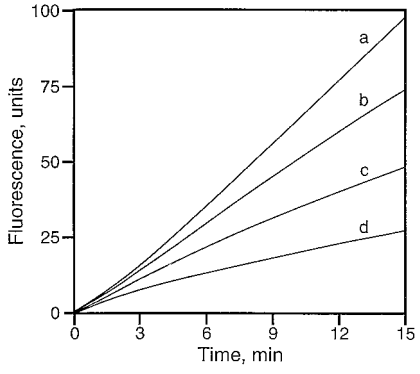

FIG. 5. **Progress curves of PC2 activity in the presence of 7B2 CT peptide 1–18.** PC2 (7 nM) was incubated with 0 (*a*), 4 (*b*), 10 (*c*), and 25 nM (*d*) 7B2 CT peptide 1–18 in 100 mM sodium acetate, pH 5.0, 5 mM $CaCl_2$, and 0.4% *n*-octyl glucoside at 37 °C in the presence of 200 μM pGlu-Arg-Thr-Lys-Arg-MCA. The reaction was initiated by the addition of enzyme.

most inhibition. Previous studies with recombinant PC1 have demonstrated that a P4 Arg is also favored by both enzymes for effective substrate hydrolysis (7, 17, 19). At P5, no clear consensus emerged in the PS-SPCL screenings against either PC1 or PC2; in agreement, data obtained with peptide inhibitors showed that many substitutions, except for Pro, were well tolerated at P5 for both enzymes. However, peptides with a Leu residue at P5 proved to be slightly more potent inhibitors of both PC1 and PC2 as compared with those having either a Lys, Thr, or His residue at this position. Interestingly, the specificity pattern displayed by PC2 at the P5 subsite appears to differ in the case of inhibition and catalysis. Recent studies in our laboratory on the substrate specificity of PC2 using internally quenched substrates have shown that PC2 favors an Arg residue at this position for effective catalysis (41).

At the P3 position, PC1 exhibited a clear preference for Val; substitution of Val by Thr resulted in a 5-fold increase in the K_i value, and substitution by His resulted in a dramatic 20–30-fold increase. In the case of PC2, Val was preferred over Phe (2–6-fold difference in the K_i values) and slightly preferred over Thr (2-fold difference) at P3. However, the rank order of potency for the various substitutions at the P3 position was similar for the two enzymes. Taken together, our data suggest that the S3 subsites of PC1 and PC2 are more similar than they are different.

Despite these similarities, the specificities of PC1 and PC2 do not appear to be identical. In particular, the S6 subsite of PC1 appears to be more selective than that of PC2. The library screens against PC1 revealed Leu to be the most inhibitory residue at position P6. Peptide Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 exhibited the lowest K_i value (3.2 nM); this value was 28 times lower than that observed using the Ile-substituted peptide Ac-Ile-Ile-Arg-Val-Lys-Arg-NH_2. In contrast, PC2 discriminated little between Leu and Ile, as evidenced by the small (1.5-fold) difference in K_i values for these two peptides. Given the relative lack of importance of P5 substitutions discussed above, the decreased potency of the Ile-Ile-containing peptide may be due mainly to the Leu→Ile substitution at the P6 subsite. Leu and Ile residues differ only in the geometry of their side chains; based upon the potent inhibition of the Leu-containing peptide, we suggest that good geometrical complementarity exists between the S6 subsite of PC1 and the corresponding Leu side chain of the peptide inhibitor.

Surprisingly, we found that the most potent inhibitor of PC1, the peptide Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2, also proved to be the most potent inhibitor of PC2. However, the K_i value of this peptide for PC2 was 110 times higher than its K_i value for PC1, confirming differences in the active sites between these two enzymes. Whereas a 110-fold difference between the two K_i values is not ideal for effective discrimination between the two enzymes, it may be possible to use this peptide as a basis to develop related peptides that can more effectively distinguish between the two enzymes.

Interestingly, the most PC-inhibitory peptide, Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2, was not the most potent inhibitor of furin among the several peptides tested. Instead, the most potent peptide was Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2, supporting the idea that furin (in contrast to PC1 and PC2) exhibits a preference for a Lys or a positively charged residue at the P5 subsite for effective inhibition.

Four hexapeptide inhibitors identified in the present study (Ac-Leu-Met-Arg-Val-Lys-Arg-NH_2, Ac-Leu-Tyr-Arg-Val-Lys-Arg-NH_2, Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2, and Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2) are among the most potent PC1 inhibitors identified thus far. Indeed, their K_i values of 3.2–6.5 nM are much better than those obtained for the best P1′-substituted decapeptide PC1 analogs (approximately 1 μM; Refs. 22 and 23) and for an isostere-containing peptidyl inhibitor (K_i = 7.2 μM; Ref. 21) and are in the same range as the value for the PC1 propeptide 1–98 (K_i = 6 nM).[2] Interestingly, the hexapeptides we identified do not share significant sequence homology with the PC1 propeptide, outside of the conservation of basic residues at P1, P2, and P4. Likewise, little homology exists at the P3, P5, and P6 subsites between these peptides and proteinase inhibitor 8, the highly potent inhibitor of furin (27). In the furin inhibitor α_1-antitrypsin Portland (24), the P6 and P3 subsites are occupied with Leu and Val, respectively, as in our peptides; however, these residues are not conserved between rat and human α_1-antitrypsins (42).

Interestingly, the naturally occurring PC2 inhibitor, the 7B2 CT peptide, which inhibits PC2 (but not PC1) at high nanomolar concentrations (9), lacks a P4 basic residue but contains the P5 and P6 hydrophobic aliphatic residues observed in effective PC2 inhibitors generated by the combinatorial screen. Indeed, we have mutagenized 7B2 to show that the addition of a P4 Arg destroyed its inhibitory potency, indicating the absolute requirement for a Val (or a similarly hydrophobic amino acid) at this position for effective inhibition (but probably not for catalysis; Ref. 43). Whereas a peptide consisting of the first 18 residues of the human 7B2 CT peptide represents a highly potent inhibitor for PC2 (28), we found that an acetylated, amidated hexapeptide based upon this sequence and including the inhibitory Lys-Lys pair (*i.e.* residues 13–18) was completely inactive against PC2, thus explaining why this peptide was not

identified during the library screen. Clearly, the potency of the 7B2 CT peptide must be attributable to residue interactions outside the hexapeptide region. This supposition is borne out by the recent finding of an almost completely conserved heptapeptide NH_2-terminal to the hexapeptide sequence (P17–P11 with respect to the scissile bond) in two invertebrate 7B2 sequences (44). Residue interactions outside the immediate context of the scissile bond may also contribute to the great potency of PC1 propeptide 1–98 against PC1, as is apparently the case with subtilisin and its propeptide (45). Taken together, these data support the potential for the binding by PC enzymes of prohormone substrates and inhibitors at multiple sites, *i.e.* both within and outside the immediate context of the scissile bond.

We further explored the mechanism of inhibition by the various inhibitors by examining the kinetics of interaction of prohormone convertases 1 and 2 with the hexapeptide inhibitors. Our data show that the mechanism of inhibition with the hexapeptide inhibitors differs from that observed with larger inhibitors such as PC1 propeptide 1–98 and 7B2 CT peptide 1–18. The interaction of PC1 with the PC1 propeptide obeys typical slow binding kinetics.[2] A similar type of inhibition was observed for the interaction of PI8 with furin (27). In the present study, we have shown that 7B2 CT peptide 1–18 also interacts with PC2 with slow binding kinetics, implicating a multiple-step interaction. However, the inhibition of PC1 and PC2 with the peptide inhibitors identified in this study exhibited fast binding kinetics. These data suggest that the mechanism of binding of PC1 and PC2 with short peptide inhibitors may involve a more limited number of interactions compared with that obtained with longer peptide and protein inhibitors. However, the fact that the peptide inhibitors identified in this study were almost equally potent to the longer proteins implies that the design of effective inhibitors for these enzymes need not involve the consideration of a multiple-step process.

In conclusion, we have demonstrated using a hexapeptide combinatorial library that PC1 and PC2 exhibit a specificity profile for inhibitors that is similar but not identical to each other. The hexapeptides Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2, Ac-Leu-Met-Arg-Val-Lys-Arg-NH_2, Ac-Leu-Tyr-Arg-Val-Lys-Arg-NH_2, and Ac-Leu-Lys-Arg-Val-Lys-Arg-NH_2 were found to represent very potent competitive inhibitors of PC1 (K_i values, 3.2–6.5 nM). These peptides, as well as Ac-Ile-Ile-Arg-Val-Lys-Arg-NH_2, also proved to be potent inhibitors of PC2 (K_i values, 360–720 nM). These hexapeptides could potentially be useful for the titration of prohormone convertase activity, for distinguishing between the activity of the two enzymes in tissue extracts, and as a basis for the successful design of synthetic inhibitors for use as therapeutic agents.

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