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Polyarginines Are Potent Furin Inhibitors*

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The ubiquitous serine endoprotease furin has been implicated in the activation of bacterial toxins and viral glycoproteins as well as in the metastatic progression of certain tumors. Although high molecular mass bioengineered serpin inhibitors have been well characterized, no small nontoxic nanomolar inhibitors have been reported to date. Here we describe the identification of such inhibitors using positional scanning amidated and acetylated synthetic L- and D-hexapeptide combinatorial libraries. The results indicated that L-Arg or L-Lys in all positions generated the most potent inhibitors. However, further investigation revealed that the peptide terminating groups hindered inhibition. Consequently, a series of non-amidated and acetylated polyarginines was synthesized. The most potent inhibitor identified, nona-L-arginine, had a K_i for furin of 40 nM. The K_i values for the related convertases PACE4 and prohormone convertase-1 (PC1) were 110 nM and 2.5 μM, respectively. Although nona-L-arginine was cleaved by furin, the major products after a 6-h incubation at 37 °C were hexa- and hepta-L-arginines, both of which retained the great majority of their potency and specificity against furin. Hexa-D-arginine was as potent and specific a furin inhibitor as hexa-L-arginine (K_i values of hexa-D-arginine: 106 nM, 580 nM, and 13.2 μM for furin, PACE4, and PC1, respectively). PC2 was not inhibited by any polyarginine tested; indeed, PC2 showed an increase in activity of up to 140% of the control in the presence of L-polyarginines. Data are also presented that show extended subsite recognition by furin and PC2. Whereas N-terminal acetylation was found to reduce the inhibitory potency of the L-hexapeptide LLRVKR against furin 8-fold, C-terminal amidation reduced the potency <2-fold. Conversely, N-terminal acetylation increased the potency against PC2 nearly 3-fold, whereas C-terminal amidation of the same peptide increased the potency by a factor of 1.6. Our data indicate that non-acetylated, poly-D-arginine-derived molecules may represent excellent lead compounds for the development of therapeutically useful furin inhibitors.

A calcium-dependent, membrane-bound serine endoproteinase, furin is considered to be one of the most important enzymes of the seven-member subtilisin-like proprotein/prohormone convertase (PC)[1] family identified to date (for reviews, see Refs. 1–3). Furin has a ubiquitous tissue distribution and cycles between the *trans*-Golgi network, the cell surface, and the endosomes directed by defined sequences within the cytosolic tail (for reviews, see Refs. 4 and 5). Hence, furin is well positioned to process not only intracellular growth factors and serum proteins, but also extracellular matrix proteins and cell-surface receptors. In addition to these important physiological roles, furin is implicated in the cleavage and activation of bacterial toxins and viral coat proteins, where it assists in pathological processes resulting in viral replication and toxin activation (for review, see Ref. 5). Furin has also been implicated in assisting the maturation of membrane type 1 matrix metalloproteinase (6) and stromelysin-3 (7), a process associated with metastatic progression in various tumors (for review, see Ref. 8). Thus, furin is an attractive target for therapeutic agents.

Although the typical furin recognition sequence is R*X*(K/R)R and the minimal recognition sequence is R*XX*R (for review, see Ref. 5), there is increasing evidence that the P6, P1′, and P2′ positions also contribute to effective catalysis (9–12). Like furin substrates, inhibitors of furin also require the occupation of certain subsites by basic residues. The third domain of turkey ovomucoid has been engineered (KPACTLE^{19} → KPRCKRE^{19}) in an attempt to increase its specificity for furin (13); however, the equilibrium constant of 1.1×10^7 M^{-1} was representative of a moderate, rather than a potent, inhibitor. Inhibition of furin in the subnanomolar range was accomplished by bioengineering the reactive-site loop of an α_1-antitrypsin variant, α_1-PDX (α_1-antitrypsin Portland), to contain the minimal furin consensus sequence (LEAIMPS^{359} → LERIMRS^{359}) (14). Kinetic analysis shows that a portion of bound α_1-PDX operates as a tight-binding suicide inhibitor, forming an SDS-stable complex with furin (15); an alternative pathway involves cleavage and release of α_1-PDX (16). The bait region of the general protease inhibitor α_2-macroglobulin was mutated (RVGFYESDVM^{690} → RVRSKRSLVM^{690}) in an attempt to produce a specific furin inhibitor (17). Although no kinetic constants were reported, processing of the endogenous furin substrates von Willebrand factor, human immunodeficiency virus type 1 glycoprotein gp160, and transforming growth factor-β1 was inhibited in COS-1 cells.

More recently, the ovalbumin-type serpin human proteinase inhibitor-8, containing two instances of the minimal furin recognition sequence (VVRNSRCSRM^{343}), has been shown to form SDS-stable complexes with furin with an overall K_i of 53.8 pM (18). However, the inhibition of furin by proteinase inhibitor-8 *in vivo* or, indeed, the colocalization of proteinase inhibitor-8 and furin within the secretory pathway has not yet been dem-

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[1] The abbreviations used are: PC, prohormone convertase; MCA, methylcoumarinamide; HPLC, high pressure liquid chromatography; Ac, acetyl PS-SPCL, positional scanning synthetic peptide combinatorial library.

onstrated. Nevertheless, these studies show that potent furin inhibitors can be synthesized by appropriate protein design.

The only naturally occurring intracellular furin inhibitor described to date found in the constitutive secretory pathway is furin's own propeptide. It is known that the prodomains of many proteases play a role in the activation and regulation of activity of their cognate enzymes (for review, see Ref. 19). Anderson *et al.* (20) observed efficient inhibition of furin by a glutathione *S*-transferase/furin propeptide fusion construct, while Seidah and co-workers (21) showed that furin prosegments expressed intracellularly can act in *trans* to inhibit substrate processing.

The potential therapeutic value of furin inhibitors was recently reinforced by a report showing that exogenous application of α_1-PDX is able to block *in vivo* maturation of pro-gB, the human cytomegalovirus envelope glycoprotein (22). As uptake of α_1-PDX into the cell could not be detected in cell lines lacking the enzyme, it was suggested that α_1-PDX bound to furin at the cell surface. *Pseudomonas* exotoxin A activation has also been prevented by extracellular application of α_1-PDX to A7 melanoma cells (16), as has the processing of human immunodeficiency virus type 1 glycoprotein gp160 in transfected cells (14). These studies collectively demonstrate that limited and selective inhibition of furin (potentially occurring on the extracellular surface rather than the interior of the cell) can result in inhibition of pathological disease processes. However, a furin inhibitor with the ideal characteristics of high potency, stability, and specificity, combined with low toxicity and low molecular mass, has yet to be identified. In this study, we have used positional scanning hexapeptide libraries to identify peptide inhibitors of furin, to describe the specificity requirements of effective inhibitors, and to compare the requirements of different members of the convertase family. The use of combinatorial peptide libraries, when applied to PC1, resulted in the identification of a potent hexapeptide, which was later shown to exist within the sequence of a natural protein inhibitor (23–25).

EXPERIMENTAL PROCEDURES

Materials—- The hexapeptide libraries and synthetic peptides were synthesized at the Torrey Pines Institute for Molecular Studies (San Diego, CA). Two positional scanning hexapeptide libraries were screened for inhibition of furin, one made up of only L-amino acids and the other of only D-amino acids. Each hexapeptide library consisted of 120 peptide mixtures with amino-terminal acetylation and carboxyl-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 amino acids. The undefined positions were occupied by any of the amino acids except cysteine. The positional scanning libraries and the individual compounds were synthesized using simultaneous multiple peptide synthesis methodology as described previously (26–29). The L-polyarginine synthesis was performed by the Louisiana State University Health Sciences Center Core Laboratories; mass spectroscopy was used to verify identity. α_1-PDX was a generous gift of G. Thomas (The Oregon Health Sciences University, Portland, OR). The anti-furin antiserum MON148 was a kind gift of W. Van de Ven (University of Leuven, Leuven, Belgium). Anti-Myc and anti-His antisera were obtained from Invitrogen (Carlsbad, CA). pERTKR-MCA was obtained from Peptides International Inc. (Louisville, KY). *N*-Glycosidase F was obtained from Calbiochem.

Recombinant Convertase Preparation—The mouse furin clone was a kind gift from K. Nakayama (Fukuoka University School of Medicine, Fukuoka, Japan). The mouse furin cDNA was truncated N-terminally to the transmembrane domain at His^{711} by polymerase chain reaction. This polymerase chain reaction product was then subcloned into pcDNA3.1(−)-Myc-His (Invitrogen) at the *Nhe*I and *Xba*I restriction sites. Dihydrofolate reductase-negative DG44 Chinese hamster ovary cells (provided by L. Chasin, Columbia University, New York, NY) were transfected using Lipofectin (Life Technologies, Inc.), and colonies were selected at 37 °C and 5% CO_2 in α-minimal essential medium (lacking nucleosides) containing 10% well dialyzed fetal bovine serum (Life Technologies, Inc.). Conditioned medium from colonies was screened using an enzyme assay (see below), and a high expressing clone was selected. Overexpression of furin was achieved by increasing the methotrexate concentration from 5 nM to 50 μM in 5–10-fold steps as described previously (30). Amplified lines were tested for increasing furin expression by enzyme assay. Once the 50 μM methotrexate level had been achieved, cells were split at ratios of 1:6 twice a week. 100 ml of conditioned medium (Opti-MEM (Life Technologies, Inc.) containing 100 μg/ml aprotinin (Miles Laboratories, Kankakee, IL)) was collected from confluent roller bottles every 24 h. The medium was centrifuged at low speed to remove cells, and the supernatant was stored at −80 °C until required.

Purification of Furin—Conditioned medium was thawed, pooled, and diluted 1:3.5 with buffer A (20 mM HEPES, 0.1% Brij 35, and 5 mM $CaCl_2$, pH 7.4) and pumped at 40 ml/min through a Sartorius D100 anion exchange membrane. The membrane was washed with 40 ml of buffer A, followed by 40 ml of buffer A containing 50 mM NaCl and finally by 40 ml of buffer A containing 200 mM NaCl. The fraction eluting with 200 mM NaCl was diluted 1:4 with buffer A and applied to a 1-ml Mono Q HR5/5 anion exchange column (Amersham Pharmacia Biotech) at a flow rate of 1 ml/min. Following a 10-ml wash with buffer A, elution of furin was achieved with a linear increase of 0–500 mM NaCl in buffer A over 30 ml; 2-ml fractions were collected. Fractions containing peak activity were pooled, and 200-μl aliquots were subjected to gel permeation chromatography using a Superose 12 column (Amersham Pharmacia Biotech) and buffer A containing 200 mM NaCl at a flow rate of 0.5 ml/min. Fractions were assayed for activity as described below; protein content was determined following the Bradford method. All purification steps were performed at 4 °C.

Alternatively, the enzyme-containing fraction eluting from the ion exchange membrane with 200 mM NaCl was pumped onto a 1-ml Ni^{2+}-nitrilotriacetic acid Superflow column (QIAGEN Inc.) at 0.3 ml/min; washed with 10 ml of buffer A; and then eluted with a two-step gradient of 0–20 mM imidazole in buffer A over 20 ml, followed by a linear gradient of 20–200 mM imidazole in buffer A. The fractions containing peak enzyme activity were pooled and subjected to ion exchange chromatography with a Mono Q column as detailed above.

PC1 and PC2 were prepared by ion exchange chromatography as described previously (31). PACE4 was partially purified from the overnight conditioned medium of stably transfected HEK-293 cells (a generous gift of R. E. Mains, Johns Hopkins University School of Medicine, Baltimore, MD) (32, 33). Briefly, 100 ml of conditioned medium (Opti-MEM containing 100 μg/ml aprotinin) was loaded onto an Econo-Pac Q column (Bio-Rad) at 4 ml/min, washed with 10 ml of buffer A, and then eluted with a linear gradient of buffer A containing 500 mM NaCl over 50 ml. The active fractions were diluted 1:4 with buffer A prior to loading onto a Mono Q column at 1 ml/min. PACE4 was eluted with a linear gradient of buffer A containing 500 mM NaCl over 10 ml. The resulting active fractions were then pooled and stored at −80 °C until required. The validity and purity of the preparation were verified by Coomassie Blue staining and Western blotting with a polyclonal antiserum (1475) directed against PACE4 (a gift of R. E. Mains).

Enzyme Assays and Hexapeptide Library Screening—The enzyme assays for PC1 and PC2 were performed at pH 5.0 using pERTKR-MCA as described previously (23). The assay for furin was performed using the same substrate at pH 7.0 in 100 mM HEPES, 5 mM $CaCl_2$, and 0.1% Brij 35. All assays were performed at 37 °C in a 96-well fluorometer (Labsystems, Inc.) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The total volume was 50 μl. Unless otherwise stated, the final substrate concentration for all assays was 200 μM. When used, inhibitory peptides were preincubated with enzyme for 30 min at room temperature prior to addition of substrate. All assays were performed in duplicate or triplicate. Inhibition constants were determined using the method previously described (23) and the equation $K_i = K_{i(app)}/(1 + ([S]/K_m))$. The K_m values for PC1, PC2, furin, and PACE4 were 11, 42, 8, and 15 μM, respectively, as determined using a computerized least-squares fitting technique with EnzFitter (Biosoft, Cambridge, United Kingdom).

Digestion of Recombinant Furin with N-Glycosidase F—A 200-μl aliquot of the pooled fractions from Superose chromatography (containing 40 μg of furin) was made up to 4.5% β-mercaptoethanol and 0.45% SDS and boiled for 10 min prior to concentration to 60 μl using a Centricon 10 (Amicon, Inc.). The concentrate was diluted to 400 μl using 50 mM sodium phosphate buffer, pH 7.5, and 0.76% Triton X-100; and 1.8 μg of *N*-glycosidase F was added. The sample was incubated at 37 °C, and 45-μl aliquots were removed at the times indicated and placed in 5 μl of 5× SDS sample buffer prior to boiling for 3 min. The aliquots were subjected to SDS-polyacrylamide gel electrophoresis (8.8%) and visualized by Coomassie Blue staining.

TABLE I
Purification of recombinant furin

Purification step	Total activity[a]	Total protein	Specific activity	Yield	Purification factor
	units	*mg*	*units/mg*	*%*	
Conditioned medium	156	54	2.9	(100)	(1)
Ion exchange 1	84	7.6	11	54	3.8
Ion exchange 2	46	2.4	19	29	6.7
Gel permeation	42	2	21	27	7.2

[a] 1 unit = 1 μmol of aminomethylcoumarin/h.

Cleavage of Nona-L- and Hexa-L-arginines by Furin—Nona-L-arginine (200 μM) or hexa-L-arginine (200 μM) was incubated at 37 °C with or without furin (1.7 μM) in 100 mM HEPES, pH 7, containing 5 mM $CaCl_2$ and 0.2% Brij 35. Aliquots (20 μl) were removed at the indicated times, placed into 480 μl of ice-cold 0.1% trifluoroacetic acid, and immediately frozen prior to HPLC. The aliquots were separated using a 5-μm Beckman ODS column (0.46 × 25 cm) with a linear gradient of 0–15% acetonitrile containing 0.1% trifluoroacetic acid over 40 min at 1 ml/min. Absorbance was monitored at 214 nm. Cleavage products were identified using polyarginine standards. Parallel reactions containing buffer instead of furin were also analyzed.

RESULTS

Overexpression, Purification, and Characterization of Recombinant Mouse Furin—The use of the dihydrofolate reductase-coupled amplification method to overexpress truncated furin resulted in a cell line that secreted ~0.8 μg/ml furin into the culture medium, as estimated by the specific activity of the purified protein. As shown in Table I, an initial ion exchange step, although suffering from a relatively low yield, proved valuable as a rapid method of concentrating the conditioned medium from a large volume while removing phenol red and contaminating protein from the product. Having reduced the volume, it was possible to load the high resolution Mono Q ion exchange column used in the second ion exchange step within a reasonable time frame. As shown in Fig. 1*A*, the majority of the protein eluted as a single peak coincident with the convertase activity. Again, the yield from this step was poor, but an appreciable increase in specific activity was observed (Table I). The fractions with maximum activity were pooled and subjected to gel permeation chromatography as shown in Fig. 1*B*. In this step, virtually all the protein was seen to elute as a single peak, with only minor forms at lower and higher molecular masses; the furin activity was observed to exactly overlie the major absorbance peak. Fractions containing maximum activity were pooled, diluted with glycerol to a final concentration of 10%, and stored at −80 °C until required. Under these conditions, there was no detectable loss of activity over 6 months. Molecular mass standards used to calibrate the gel permeation column indicated a molecular mass for furin of 59 kDa. As shown in Fig. 1*C*, Coomassie Blue staining (*left lane*) and Western blotting (*right lane*) of the gel permeation-purified fractions revealed a single band at 61 kDa. The final specific activity was 21 units/mg of protein, and the overall yield for the purification was 27%, with a purification factor of 7.2.

Initial purifications were attempted using the C-terminally located hexa-His tag as a ligand for affinity chromatography with a metal ion chelation resin (Ni NTA Superflow, Qiagen, Valencia, CA). However, the furin activity was found to elute at very low (≈20 mM) imidazole concentrations, with no increase in specific activity compared with the sample applied. Subsequent Western blotting with both anti-His and anti-Myc antisera showed no immunoreactivity, whereas blotting using the anti-furin antiserum MON148 revealed a strong band at 61 kDa (Fig. 1*C*, *right lane*), indicating that C-terminal truncation of the secreted product had occurred. The metal ion chelation step was subsequently abandoned, and the data presented in this report were obtained using furin purified by ion exchange and gel permeation chromatography as detailed in Table I.

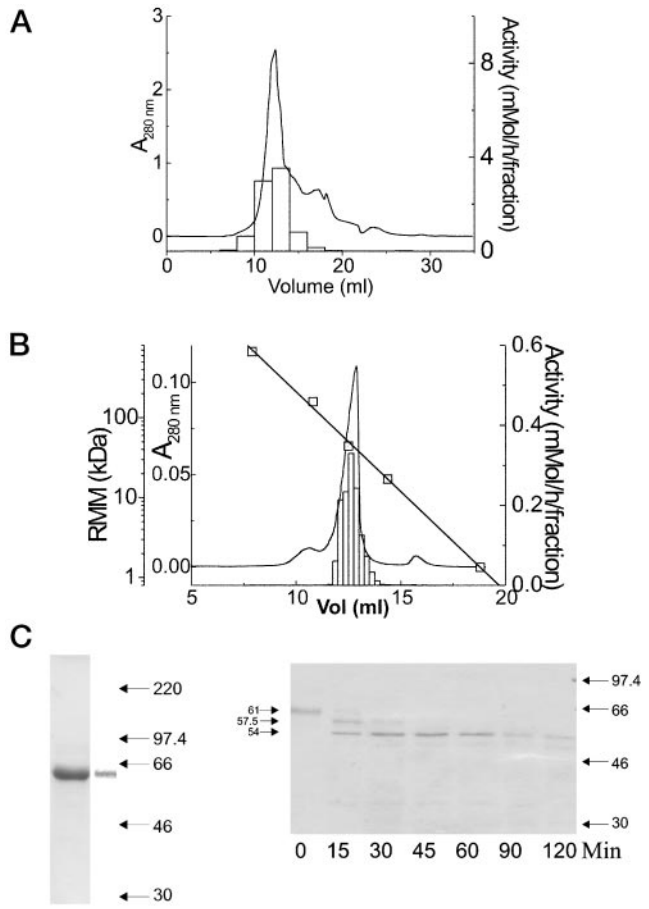

FIG. 1. **Purification of recombinant furin.** *A*, partially purified concentrated recombinant mouse furin from the first ion exchange step was diluted 1:4 with buffer A and pumped through a Mono Q HR5/5 column equilibrated with buffer A. The column was washed with 5 ml of buffer A before elution with a 30-ml gradient of 0–500 mM NaCl in buffer A. The elution volume from the start of the salt gradient is shown. *B*, the fractions containing peak enzyme activity were pooled, and aliquots were applied to a Superose 12 column; a representative chromatogram is shown. *Bars*, activity against pERTKR-MCA; *solid line*, UV absorbance at 280 nm. The gel permeation column was calibrated with the molecular mass standards shown (■): thyroglobulin, 670 kDa; IgG, 150 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; and cyanocobalamin, 1.35 kDa (all obtained from Bio-Rad). *C*, shown are the results from SDS-polyacrylamide gel electrophoresis (8.8%) of the pooled fractions from the Superose 12 purification. *Left lane*, Coomassie Blue stain; *right lane*, Western blotting with MON148. *D*, shown is the effect of *N*-glycosidase F on the mobility of recombinant furin. An aliquot of the pooled fractions from Superose 12 chromatography was reduced with β-mercaptoethanol, denatured by boiling in the presence of SDS, and then diluted into a pH 7.5 buffer containing *N*-glycosidase F. The sample was incubated at 37 °C, and aliquots were removed at the times indicated. *AMC*, aminomethylcoumarin; *RMM*, relative molecular mass.

Treatment of the purified furin with *N*-glycosidase F revealed the presence of two lower molecular mass forms, indicating that two of the three potential sites in the recombinant furin preparation were present and originally glycosylated (Fig. 1*D*). The effect of pH on activity was studied. Although there was a rapid drop in activity below pH 6.5, the enzyme retained >90% of maximum activity at pH 9.0 (data not shown). Calcium concentrations over 1 mM were required for full activity, with no significant difference in activity as calcium concentrations were increased to 50 mM. In accordance with previous results (16), furin was potently inhibited at nanomolar α_1-PDX concentrations, providing further validation of the enzyme preparation (data not shown).

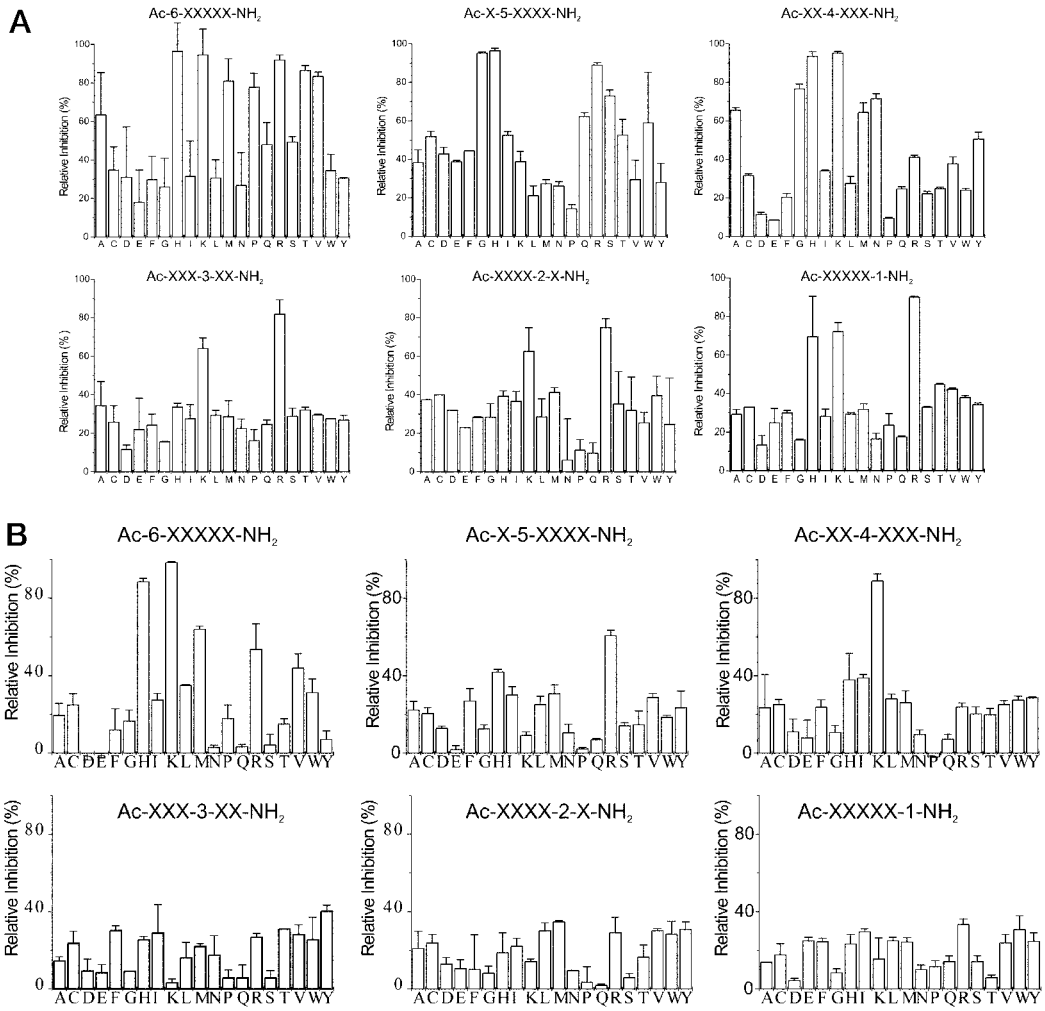

FIG. 2. **Inhibition of furin by an L-hexapeptide positional scanning-synthetic peptide combinatorial library.** Each peptide mixture was preincubated with furin in assay buffer for 30 min prior to addition of substrate (final concentration of 100 μM). The rate of hydrolysis of pERTKR-MCA was followed for 1 h. Inhibition is given as the percentage decrease in activity in the presence of the peptide mixture relative to that of controls. *A*, 1 mg/ml peptide concentration; *B*, 0.5 mg/ml peptide concentration.

L-Hexapeptide Library Scan—In an attempt to identify residues important for inhibition of furin, we screened a positional scanning L-hexapeptide library (amino-terminally acetylated and carboxyl-terminally amidated) using the substrate pERTKR-MCA. In total, the library was screened nine times at inhibitor concentrations of 1.0 and 0.5 mg/ml and at substrate concentrations of 200 and 100 μM. The concentrations of inhibitor and substrate were found to exert an influence on the relative inhibition observed. Screening at the lower substrate concentration resulted in better discrimination between residues at all inhibitor concentrations. In addition, at the lower substrate concentration, better discrimination was shown for positions P1, P2, and P3 at 1 mg/ml inhibitor (Fig. 2*A*), whereas better discrimination for positions P4, P5, and P6 was seen at 0.5 mg/ml inhibitor (Fig. 2*B*).

From Fig. 2*A* (using an inhibitor concentration of 1 mg/ml), it can be seen that at position P1, Arg, Lys, and His exerted greater than average inhibition, whereas at positions P2 and P3, Arg and Lys, but not His, were the preferred residues. At positions P4 and P5 and especially position P6, many residues showed greater than average inhibition, but no clear distinction could be made on the basis of size, hydrophobicity, or charge. In contrast, at the lower inhibitor concentration (Fig. 2*B*), although there were no clearly preferred residues at positions P1, P2, and P3, there was good discrimination at positions P4, P5, and P6. On the basis of the screens shown in Fig. 4, we selected Arg in positions P1, P2, and P3; Lys in position P4; His or Arg in position P5; and His, Met, Lys, or Arg in position P6 for assembly into discrete peptide sequences.

D-Hexapeptide Library Scan—The positional scanning acetylated and amidated D-hexapeptide library was screened a total of nine times at either 0.5 or 1.0 mg/ml inhibitor and either 50 or 100 μM substrate; in all cases, the results were similar. A representative screen is shown in Fig. 3. Whereas D-Arg was one of the preferred residues in all positions, the remainder of the inhibitory residues were hydrophobic. Interestingly, D-Lys effected greater than average inhibition only in position P6, but still showed less inhibition than the most effective residue, D-Trp. In positions P3, P4, and P5, D-Arg was marginally the most potent residue when all results were averaged. In position P2, D-Arg and D-Ile consistently produced relatively high inhibition, whereas the same was true for D-Arg and D-Leu in position P1. In all cases, relative inhibition values were consistently lower than those of the L-hexapeptide library, indicating a preference of furin for L-residues. Nevertheless, a series of D-hexapeptides was synthesized based upon the results described above, whereby position P1 was either D-Arg or D-Leu; position P2 was either D-Arg or D-Ile; positions P3, P4, and P5 were always D-Arg; and position P6 was always D-Trp.

Inhibition of Furin and PC2 by Synthetic Peptides—The amidated and acetylated D- and L-hexapeptides based on the results of the D- and L-hexapeptide library screens were tested

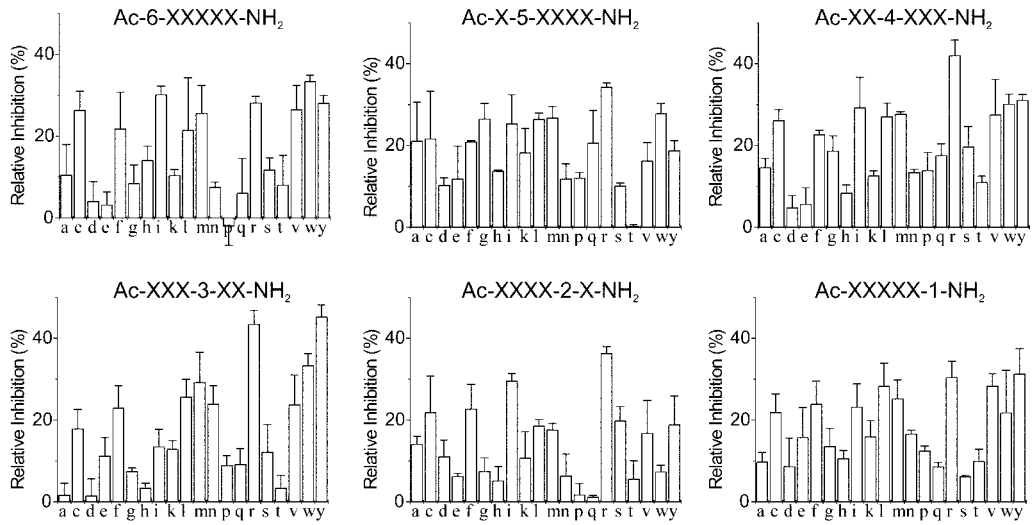

FIG. 3. **Inhibition of furin by a D-hexapeptide positional scanning-synthetic peptide combinatorial library.** Each peptide mixture (final concentration of 1 mg/ml) was preincubated with furin in assay buffer for 30 min prior to addition of substrate (final concentration of 100 μM). The rate of hydrolysis of pERTKR-MCA was followed for 1 h. Inhibition is given as the percentage decrease in activity in the presence of the peptide mixture relative to that of controls.

against furin and PC2. For both series, the K_i values for furin were all in the low micromolar range; these peptides were 10–100-fold more potent inhibitors of furin than of PC2 (Table II). The potency increased against furin as the sequence became more basic, an observation that did not hold for PC2. Examination of the K_i values of the L-hexapeptides for furin revealed that Arg was preferred to His in position P5 and that the inhibitory potency of these peptides against furin increased as position P6 was changed in the order His, Met, Lys, Arg. The same order of inhibitory potency was not seen against PC2; in this instance, His was preferred to Arg in position P5. Although the combination of a P5 Arg and a P6 Met was severely unfavorable as a PC2 inhibitor, the influence of the P5 residue appeared to outweigh that of the P6 residue against furin.

The D-hexapeptide inhibitors were also assayed against furin and PC2 (Table II). D-Arg was preferred to D-Ile in position P2, and D-Arg was preferred to D-Leu in position P1 against furin. However, the presence of a basic residue at position P1 or P2 was sufficient to produce a relatively potent furin inhibitor, despite the presence of a hydrophobic residue at position P2 or P1. Conversely, when D-Arg was present at position P2, substituting D-Leu for D-Arg in position P1 produced a more potent PC2 inhibitor. Thus, like the L-peptide inhibitors, increasing basicity resulted in the generation of a more potent furin inhibitor, but not of a more potent PC2 inhibitor.

Mechanism of Inhibition—Lineweaver-Burk plots of the most potent D-and L-hexapeptides identified from the library screens are shown in Fig. 4. These demonstrate strictly competition-type inhibition for both the L- and D-peptides. No deviation was seen from classical Michaelis-Menten-type kinetics, indicative of tight-binding or suicide inhibitors such as α_1-PDX and the chloromethyl derivatives, both of which function by forming an irreversible complex with the enzyme (15, 34).

Effect of N-terminal Acetyl and C-terminal Amide Groups on Inhibition—As there have been several reports of the substrate specificity of furin extending beyond P1–P4 recognition (9–12), we were interested in examining the effect of the terminal acetyl and amide modifications on inhibitory potency. Thus, in addition to the peptides synthesized as a result of the combinatorial library screens described here, various forms of an amidated and acetylated L-hexapeptide previously identified as a nanomolar inhibitor of PC1 (23) were tested against furin and PC2; the results are shown in Table III. Interestingly, removing the terminal amide and acetyl groups of the hexapeptide increased its inhibitory potency against furin 8-fold. It appeared that the relative lack of inhibitory potency of the unacetylated and unamidated peptide against furin was almost solely due to the N-terminal acetyl group. In contrast, when the same peptides were tested against PC2, the terminating groups appeared to assist in inhibition. The K_i of the acetylated and amidated peptide was nearly 4-fold smaller for PC2 than the K_i of the unmodified peptide. Comparing peptides, it can be seen that removing the N-terminal acetyl group resulted in the loss of inhibitory potency against PC2, implying that PC2 has sequence recognition ability that extends beyond the P6 side chain.

TABLE II
Inhibition constants of various L- and D-hexapeptides for furin and PC2

The rate of hydrolysis of pERTKR-MCA was determined in the presence of various concentrations of the different peptides as described under "Experimental Procedures." The results obtained were then used to compute the K_i values of the peptides. Each value represents the mean ± S.D. determined from three independent experiments.

	K_i	
	Furin	PC2
	μM	
L-Peptides		
Ac-HHKRRR-NH_2	13.2 ± 1.6	235 ± 16
Ac-MHKRRR-NH_2	10.3 ± 1.4	216 ± 13
Ac-KHKRRR-NH_2	5.2 ± 0.9	280 ± 29
Ac-RHKRRR-NH_2	3.4 ± 0.6	152 ± 30
Ac-HRKRRR-NH_2	2.1 ± 0.5	309 ± 29
Ac-MRKRRR-NH_2	2.3 ± 0.5	1,500 ± 300
Ac-KRKRRR-NH_2	1.6 ± 0.5	391 ± 60
Ac-RRKRRR-NH_2	1.3 ± 0.9	461 ± 75
D-Peptides		
Ac-wrrril-NH_2	22.7 ± 4.3	601 ± 200
Ac-wrrrir-NH_2	7.0 ± 0.9	399 ± 75
Ac-wrrrrl-NH_2	5.3 ± 1.0	203 ± 20
Ac-wrrrrr-NH_2	2.4 ± 0.8	334 ± 37

Inhibition of Furin, PACE4, PC1, and PC2 by Polyarginine Peptides—A series of L-polyarginine peptides with chain lengths of 4–9 residues and no terminal modifications was synthesized and tested for inhibitory potency against furin, PC1, PC2, and PACE4. Table IV shows that the K_i of the L-polyarginine peptides for furin increased from 40 nM to 6 μM as the chain length decreased from 9 to 4 residues. Whereas the

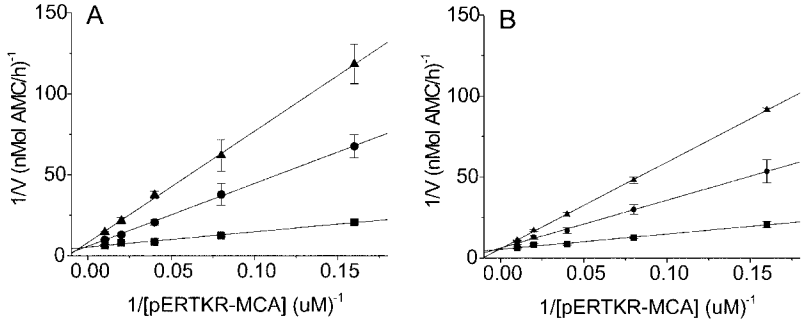

FIG. 4. **Lineweaver-Burk plots of the most potent L- and D-hexapeptides identified from the library screens.** Furin (30 nM) was preincubated in 100 mM HEPES, 5 mM $CaCl_2$, and 0.1% Brij 35, pH 7.0, with 0 (■), 20 (●), or 40 (▲) μM Ac-RRKRRR-NH_2 (*A*) or Ac-wrrrrr-NH_2 (*B*) prior to addition of substrate at the final concentrations indicated. *AMC*, aminomethylcoumarin.

TABLE III
Effect of N-terminal acetylation and C-terminal amidation on the inhibition of furin and PC2

The rate of hydrolysis of pERTKR-MCA was determined in the presence of various concentrations of the different peptides as described under "Experimental Procedures." The results obtained were then used to compute the K_i values of the peptides. Each value represents the mean ± S.D. determined from three independent experiments.

Peptide	K_i	
	Furin	PC2
	μM	
LLRVKR-NH_2	0.8 ± 0.1	2.3 ± 0.2
Ac-LLRVKR	3.5 ± 0.2	1.3 ± 0.6
Ac-LLRVKR-NH_2	3.4 ± 0.1	1.0 ± 0.08
LLRVKR	0.42 ± 0.02	3.7 ± 0.17

TABLE IV
Inhibition constants of various polyarginine peptides for furin, PACE4, and PC1

The rate of hydrolysis of pERTKR-MCA was determined in the presence of various concentrations of the different peptides as described under "Experimental Procedures." The results obtained were then used to compute the K_i values of the peptides. Each value represents the mean ± S.D. determined from three independent experiments.

	K_i		
	Furin	PACE4	PC1
	μM		
Tetra-L-arginine	6.4 ± 0.9	>200	>200
Penta-L-arginine	0.99 ± 0.08	0.98 ± 0.120	14 ± 6.1
Hexa-L-arginine	0.114 ± 0.006	0.52 ± 0.045	3.9 ± 0.62
Hepta-L-arginine	0.068 ± 0.001	0.24 ± 0.045	5.2 ± 1.2
Octa-L-arginine	0.061 ± 0.001	0.15 ± 0.060	5.1 ± 2.0
Nona-L-arginine	0.042 ± 0.003	0.11 ± 0.013	12 ± 2.5
Hexa-D-arginine	0.106 ± 0.010	0.58 ± 0.040	13 ± 0.25

K_i values of the nona-, octa-, hepta-, and hexamers ranged from 42 to 114 nM, there was an ~10-fold increase in the K_i between the hexamer and heptamer and a 5-fold increase between the heptamer and tetramer.

The K_i values for PACE4 also increased as the chain length of the inhibitor decreased; but unlike furin, the K_i of the pentameric polyarginine was approximately twice that of the hexamer, and no sharp change was seen as the chain length was reduced below the $n = 6$ level. The minimum K_i observed was 110 nM; the tetramer was not found to inhibit even at millimolar concentrations. In contrast, the polyarginine peptides were only moderate inhibitors of PC1, with a minimum K_i of 4 μM. Interestingly, the K_i of the nonamer was significantly greater than those of the hexa-, hepta-, and octamers. Similarly to PACE4 (but not to furin), PC1 was not inhibited by tetra-L-arginine at micromolar concentrations. Overall, it appears that the binding pockets of furin and PACE4 are more closely related to each other than to that of PC1, but that furin has a unique dependence on the S6 binding pocket.

In contrast to the other convertases studied here, PC2 activity was consistently stimulated by the polyarginine peptides. The stimulatory effect was noticeable with all polyarginines tested, starting at concentrations as low as 0.1 nM and increasing with concentration up to ~10 μM peptide, whereupon a relative decrease in activity was observed (Fig. 5). No effect could be confidently correlated with the peptide length, except at low nanomolar peptide inhibitor concentrations, where the tetra- and penta-L-arginines appeared to produce a smaller stimulatory effect than the longer peptides. The data shown in Fig. 5 represent the mean value of all six poly-L-arginines tested.

In addition, hexa-D-arginine was synthesized and tested for inhibitory potency against furin, PACE4, PC1, and PC2. The K_i values for furin and PACE4, as shown in Table IV, were remarkably similar to those of hexa-L-arginine, whereas a 3-fold increase in K_i for PC1 was observed, indicating better discrimination. When tested against PC2, no stimulatory or inhibitory effect was observed (data not shown).

Mechanism of Inhibition—Lineweaver-Burk plots of hexa-L-, nona-L-, and hexa-D-arginines are shown in Fig. 6. Like the acetylated and amidated hexapeptides shown in Fig. 4, these compounds demonstrated strictly competition-type inhibition. Note that the concentrations of polyarginine used to generate the data shown in Fig. 6 were 40-fold less than the concentrations of amidated and acetylated hexapeptides used in Fig. 4.

Cleavage of Nona-L- and Hexa-L-arginines by Furin—Cleavage of nona-L-arginine was first observed 40 min after the reaction commenced, with the appearance of hexa- and hepta-L-arginines (Fig. 7*C*). It was not until 4 h had elapsed that penta-L-arginine was observed, by which time no nona-L-arginine remained. The heptapeptide was still present after 6 h of digestion (Fig. 7*D*); but after 24 h had elapsed, only the penta- and hexapeptides were present (Fig. 7*E*). Tetra-L-arginine was not observed at any time. Cleavage of hexa-L-arginine proceeded much less rapidly than that of nona-L-arginine; indeed, no cleavage was seen after 6 h of incubation with furin (data not shown). After 24 h, partial digestion of hexa-L-arginine had occurred, producing penta-L-arginine (Fig. 7*G*); again, the complete absence of a tetra-L-arginine product was noteworthy. Controls (in which buffer replaced furin) are shown in Fig. 7 (*F* and *H*) after a 24-h incubation at 37 °C. These data show that L-polyarginine is preferentially oriented into the catalytic pocket of furin such that side chains interact with the S1–S6 binding pockets. When the experiment was repeated with hexa-D-arginine and furin or with nona-L-arginine and PC2, no cleavage was observed after 24 h of incubation (data not shown).

DISCUSSION

In this study, we have purified and partially characterized a recombinant truncated mouse furin from the conditioned medium of Chinese hamster ovary cells. Our purified furin preparation was homogeneous, with apparent molecular masses of 61 kDa by SDS-polyacrylamide gel electrophoresis and 59 kDa by gel permeation chromatography. The enzyme was shown to be C-terminally processed, as the C-terminally located tags

could not be detected by Western blotting, giving a molecular mass of ~60 kDa. Treatment with *N*-glycosidase F suggested that this furin was glycosylated at two of the three potential sites. A truncated furin preparation has previously been shown to be C-terminally processed, with a similar 5-kDa shift in apparent mobility on SDS-polyacrylamide gel electrophoresis following *N*-glycosidase F treatment (35); however, to the best of our knowledge, this is the first time the number of glycosylation sites has been shown. The specific activity of the purified enzyme against pERTKR-MCA of 21 μmol of aminomethylcoumarin/h was similar to that reported by Bravo *et al.* (36), who obtained a maximum specific activity of 30 μmol of aminomethylcoumarin/h using *t*-butoxycarbonyl-RVRR-MCA. The overall yield of 27% we obtained was considerably lower than the 49% obtained by Bravo *et al.* (36), but probably reflects the use of two (rather than one) ion exchange steps in our protocol.

Surprisingly, the enzyme suffered only a slight loss of activity at pH 9.0; this contrasts with results obtained by Hatsuzawa *et al.* (37), who observed only 30% of full activity at pH 9 using *t*-butoxycarbonyl-RVRR-MCA as the substrate against unpurified conditioned medium. However, Thomas and coworkers (38), also using *t*-butoxycarbonyl-RVRR-MCA, observed >60% of full activity at pH 9.0 using purified vaccinia virus-expressed truncated human furin. Using unpurified medium collected from Sf9 cells transfected by baculovirus techniques, Cieplik *et al.* (39), using *t*-butoxycarbonyl-RTKR-MCA, showed that bovine furin had a sharp pH optimum at pH 7.0, whereas *Spodoptera frugiperda* furin had a much broader optimum centered around pH 8.0. Thus, the pH dependence of furin appears to depend on the source, substrate, and degree of purification. Reassuringly, the purified enzyme used in this study was potently inhibited by the furin-specific serpin α_1-PDX at concentrations identical to those previously reported (18). Thus, this new recombinant furin compares well with those previously published.

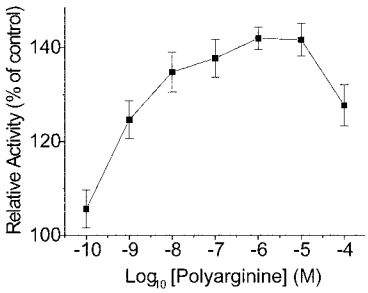

FIG. 5. **Effect of poly-L-arginine peptides on the activity of PC2.** The activity of PC2 against pERTKR-MCA was determined in the presence or absence of varying concentrations of tetra-L-, penta-L-, hexa-L-, hepta-L-, octa-L-, and nona-L-arginines, each polyarginine being assessed individually. For each peptide concentration, the data shown are an average of all polyarginines studied (a total of 18 assays per peptide concentration) and are expressed as a percentage of that observed in the absence of polyarginine peptides. The *error bars* represent the S.D.

Basic Residues in All Positions Favor Inhibition of Furin, but Not of PC1 and PC2—Unlike the results of the L-hexapeptide combinatorial library screens against PC1 and PC2 carried out in our laboratory (23), furin revealed a preference for Arg and Lys in all six positions, with Arg being the more inhibitory of the 2 residues in all positions except P4. In addition, some preference was also shown for His in positions P1, P4, P5, and P6. In contrast, our previous work with PC1 (23) showed a strong preference for Arg in positions P1 and P4, Lys in position P2, and Leu in position P6, whereas residues at positions P3 and P5 could be interchanged with relatively little effect on inhibition. Screens against PC2 showed that Arg in positions P1 and P4 consistently gave the highest inhibition, whereas in the other four positions, no clear consensus was seen. Thus, it appears that the binding pocket of furin has, unlike PC1 and PC2, a requirement for basic residues that stretch from the S1 to S6 subsites. Zhong *et al.* (21) showed that peptides based on the prodomain sequences of both furin and PC7 could act as potent inhibitors of either enzyme. The furin propeptide could be reduced to a 10-residue sequence (QQVAKRRTKR), with K_i values of 40 nM for furin and ~500 nM for PC7. When the C-terminal residue was changed to a non-basic alanine, inhibitory potency was abolished. A decapeptide fragment (EQRLLKRAKR) of the propeptide of PC7 showed K_i values of 80 nM for furin and 6 nM for PC7. It should be noted, however, that differences in the furin preparation and in the method used to calculate the K_i values preclude an accurate comparison of our values with those of Zhong *et al.* (21). Nonetheless, these results show that the inhibitory potency of peptides against furin is correlated with the concentration of positive charges and indicate that this might be a selective property of furin.

D-Residues Can Be Used to Construct Relatively Potent Inhibitory Peptides—Although the D-hexapeptide library screen showed less inhibition of furin than the L-hexapeptide library, the K_i values of the synthetic D-peptides were surprisingly similar to those of the L-peptides (Table II), indicating a similar mechanism of inhibition. As D-peptides should be more resistant to hydrolysis than L-peptides *in vivo*, this finding provides lead compounds for the development of stable inhibitors for therapeutic purposes.

Furin and PC2 Are Sensitive to Groups Distal to the P1 and P6 Residues—We have shown above that furin is sensitive to groups C-terminal to the P1 side chain, with a doubling of the K_i with C-terminal amidation of hexapeptides. Lazure *et al.* (11) reported that although substrates based upon the sequence of parathyroid hormone (*o*-aminobenzoyl-KSVKKRSVSEYS) were cleaved by furin, substitution of Ser for Val in the P1′ position in combination with substitution of Val for Arg in

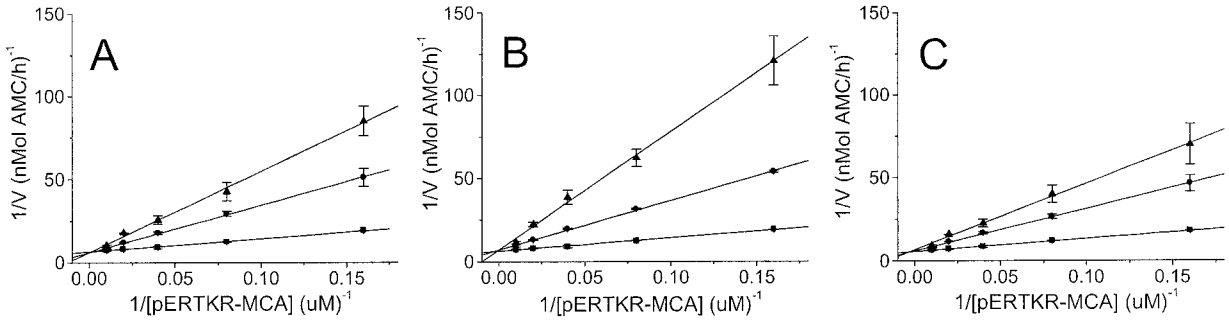

FIG. 6. **Lineweaver-Burk plots of hexa-L-, nona-L-, and hexa-D-arginines.** Furin (30 nM) was preincubated in 100 mM HEPES, 5 mM $CaCl_2$, and 0.1% Brij 35, pH 7.0, with 0 (■), 0.5 (●), or 1 (▲) μM hexa-L-arginine (*A*), nona-L-arginine (*B*), or hexa-D-arginine (*C*) prior to addition of substrate at the final concentrations indicated. *AMC*, aminomethylcoumarin.

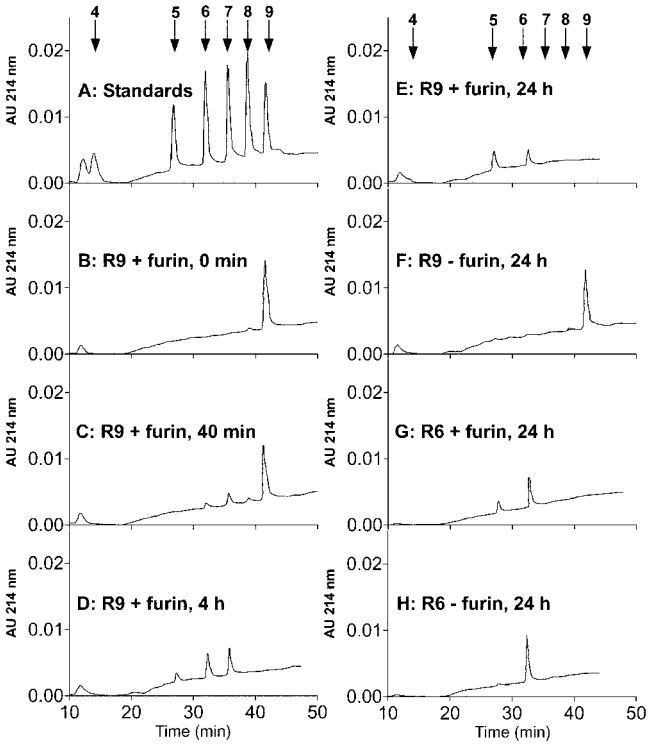

FIG. 7. **Cleavage of nona-L- and hexa-L-arginines by furin.** Furin (*B–E* and *G*) or buffer (*F* and *H*) was incubated with nona-L-arginine (*B–F*) or hexa-L-arginine (*G* and *H*) at 37 °C for 0 min (*B*), 40 min (*C*), 6 h (*D*), or 24 h (*E–H*) prior to separation by HPLC as described under "Experimental Procedures." In *A*, the separation of a standard mixture of polyarginines is shown. The number of residues/poly-L-arginine is indicated by the *arrows* in the *upper panels*. *AU*, absorbance units.

the P2′ position produced a furin inhibitor with a K_i of 4.6 μM. Additionally it has been shown that substrates containing P′ residues are more efficiently cleaved by furin than peptidyl methylcoumarinamide substrates (11, 40, 41), and Berman *et al.* (42) reported that a prorenin-related internally quenched fluorescence PC1 substrate (*o*-aminobenzoyl-RMARLTL-ethylenediamine-2,4-dinitrophenyl) was not cleaved by furin, but that a single substitution (Leu for Ala) in the P1′ position produced a relatively efficient substrate. We have also shown that furin is very sensitive to groups distal to the P6 side chain: N-terminal acetylation of an L-hexapeptide increased its K_i by a factor of 8. This is in agreement with the data of Fuller and co-workers (12), who showed substrate inhibition with hexapeptide (but not tetrapeptide) substrates. In the same study, a comparison of furin with the related convertase Kex2 revealed that although the residue at the P1 position had a large effect on catalysis, the P4 and P6 residues were especially important for furin. Furthermore, favorable residues at positions P2 and P6 were able to compensate for less than optimal residues at positions P1 and P4. Our data indicate that the effect of acetylation and amidation on the inhibition of PC2 was the opposite of that seen for furin, with both modifications increasing the inhibitory potency. However, like furin, P6 acetylation of PC2 inhibitors had the largest single effect on inhibition, demonstrating that, like furin, the binding pocket of PC2 extends beyond the P6 residue.

Hexa-L-arginine Is a Potent Inhibitor of Furin, but Stimulates PC2 Activity—In previous work, peptides corresponding to known substrate cleavage sites have been used as a starting point for the synthesis of peptide inhibitors of furin. A series of deca- and dodecapeptides based upon a partial sequence of the junction between the propeptide and catalytic domains of PC1 was tested for inhibition of PC1 and furin (43, 44). These peptides contained a variety of unnatural amino acids in the P1′ position. Interestingly, the compounds were found to be slightly better inhibitors of furin than of PC1, with K_i values of the dodecapeptides ranging from 0.8 to 10 μM for furin compared with 1.0–170 μM for PC1 (44). The K_i values of the decapeptides ranged from 1.0 to 8.6 μM for PC1 and from 0.8 to 2.2 μM for furin (43). A sequence containing a ketomethylene–arginyl pseudopeptide bond (again based on the PC1 propeptide) was also found to have a K_i in the low micromolar range for both furin and PC1 (40). This observation fits with data demonstrating that the propeptides of PC1 and furin are interchangeable (32). Although the K_i of the 10-residue propeptide fragment identified by Zhong *et al.* (21) was essentially the same as that of nona-L-arginine, if cleavage at the P3–P2 bond were to occur, as with nona-L-arginine, the resulting fragment (QQVAKRRT) would have little inhibitory ability due to the lack of a basic residue at position P1. In contrast, cleavage of nona-L-arginine results in peptide K_i values in the low nanomolar range.

A comparison of inhibition of the convertases PC1, PC2, furin, and PACE4 with polyarginine derivatives revealed striking differences. Whereas furin and PACE4 were both inhibited to approximately the same extent by all polyarginines tested except tetra-L-arginine, PC1 was much less sensitive to the peptides than furin, and PC2 was consistently stimulated. From these results, it appears that the binding pocket of PACE4 is relatively similar to that of furin, in agreement with Tsuji *et al.* (45), who reported that α_1-PDX could form an SDS-stable acyl intermediate with PACE4.

It has been observed previously that PC2 is fundamentally different from the other members of the convertase family, being the only member requiring the presence of the neuroendocrine protein 7B2 for full activity, activating late in the secretory pathway, and possessing an Asp rather than an Asn in the oxyanion hole (for review, see Ref. 1). The stimulation of PC2 by L-polyarginines that we observed was not due to more rapid activation of the recombinant pro-PC2, as maximum activity was attained within 30 min of reduction of pH from 7.4 to 5.0 irrespective of the presence of polyarginine and remained constant for 90 min thereafter (data not shown). Thus, either a greater proportion of the enzyme preparation became activated via interactions at an allosteric site, or the polyarginine peptides somehow directly assisted substrate turnover. The exact nature of the activation phenomenon will be an interesting subject for future study.

The Furin Catalytic Pocket: Differences from PC1 and PC2—As the polyarginines tested contained the furin cleavage consensus sequence, it was assumed that cleavage would occur. However, although nona-L-arginine could indeed be cleaved by furin, the two primary products were the hexa- and heptamers; the penta-L-arginine product observed after 4 h (Fig. 7*E*) was most likely due to further cleavage of the heptamer, as incubation of the hexamer with furin produced only the pentamer after 24 h. These results demonstrate that furin cannot cleave hexa-L-arginine at the P2 position, an important finding for the development of useful inhibitors *in vivo* and *ex vivo*. It is also interesting that furin showed an absolute preference for substrates with 5 or 6 residues N-terminal to the cleavage site over substrates with only 2, 3, or 4 residues N-terminal to the cleavage site. This implies that the S6 binding pocket of furin is as important to specificity as the sites closer to the catalytic triad.

Taken together, our results imply that furin subsites are all consistently negatively charged, as opposed to PC1 and PC2, whose S3 and S6 subsites presumably utilize hydrophobic and/or steric interactions. The similar specificity of the pol-

yarginines for PACE4 and furin agrees with the observation that these two convertases are more closely related to each other, both structurally and spatially, than to either PC1 or PC2 (46, 47).

Polyarginines as Therapeutically Useful Furin Inhibitors—Although the observation that hexa-D-arginine is a potent and relatively specific furin inhibitor forms the basis for therapeutic design, there may be problems associated with the use of highly charged peptide-based molecules as proteinase inhibitors *in vivo*, such as the ability of the molecule to cross the cell membrane unaided. However, one of the defining features of furin is the ability to cycle between the *trans*-Golgi network, the cell surface, and the endosomes (4, 48–50). Thomas and co-workers (22) showed that α_1-PDX could be internalized by cells producing furin, but not by furin-deficient cells. It remains to be determined whether polyarginines can also be internalized by cell-surface exposed furin, but given their small size and solubility compared with α_1-PDX, there is good reason to believe that this process could be efficient.

Compared with other low molecular mass convertase inhibitors that have been reported, polyarginines are likely to exhibit relatively little toxicity. Angliker (51) tested a series of ketomethylene and aminomethyl ketone peptides against furin and found the best inhibitor to be decanoyl-RVKR-CH_2-AVG-NH_2, with a K_i of 3.4 nM, whereas the ketomethylene series had K_i values in the low micromolar range. Another low molecular mass inhibitor, the octapeptidyl chloromethane derivative Ac-YEKERSKR-CH_2Cl, was found to be a low nanomolar irreversible inhibitor of both PC1 and furin (10). However, *in vivo*, ketones and chloromethane derivatives tend to exhibit unacceptable toxicity; hence, their use has largely been confined to probing enzyme-structure relationships. In contrast, many studies have been reported using polyarginines *in vivo* without any apparent cytotoxicity, from studies of mucin release in goblet cells to the activation of phospholipase D (52, 53). Interestingly, poly-L-arginine has been used to mimic the cationic major basic protein, a protein implicated in the airway hyperresponsiveness seen in asthma patients (54, 55). The major basic protein is found in the granules of eosinophils, where matrix metalloproteinases play a crucial role in mediating tissue turnover and matrix remodeling (56, 57). As furin has been implicated in the activation of the matrix metalloproteinases (6, 7), it is possible to speculate that the as yet undefined role of the major basic protein involves inhibition of furin-mediated processes. Although questions remain to be answered concerning the ability of D-polyarginines to inhibit furin-mediated processes both on the cell surface and within the *trans*-Golgi network/biosynthetic and endocytic pathways, the observation that hexa-D-arginine is an effective and relatively specific furin inhibitor provides a starting point for the development of modified derivatives capable of successfully inhibiting furin-mediated pathogenic processes *in vivo*.

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