

Furin-mediated Processing of Pro-C-type Natriuretic Peptide*

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C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family that is involved in a variety of homeostatic processes. Here we characterize the processing essential for the conversion of the precursor, human pro-CNP, to the biologically active hormone. In human embryonic kidney 293 and chondrosarcoma SW 1353 cells, recombinant pro-CNP was converted into a mature peptide intracellularly as detected by Western analysis. Expression of recombinant human corin, a pro-atrial natriuretic peptide convertase, did not enhance the processing of pro-CNP in these cells. The processing of pro-CNP was inhibited in the presence of an inhibitor of the endoprotease furin but was not affected by inhibitors of matrix metalloproteinases and tumor necrosis factor- α convertase. In furin-deficient human colon adenocarcinoma LoVo cells, no conversion of recombinant pro-CNP to CNP was detected. Expression of recombinant human furin in LoVo cells restored the ability of these cells to process pro-CNP. Furthermore, incubation of purified recombinant human furin with LoVo cell lysate containing pro-CNP led to the conversion of the precursor to a mature peptide. The furin-processed CNP was shown to be biologically active in a cell-based cGMP assay. These results demonstrate that furin is a critical enzyme for the processing of human pro-CNP.

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP),¹ brain or B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (1–7). ANP and BNP are produced mainly in cardiomyocytes in the heart and are important in maintaining normal body fluid and sodium homeostasis. CNP is expressed in many tissues and cell types, including the brain, vascular endothelial cells, and chondrocytes (8–13). The dominant receptor for CNP is natriuretic peptide receptor-B, whereas the receptor for ANP and BNP is natriuretic peptide receptor-A. The biological functions of CNP are apparently different from those of ANP and BNP. Studies have shown that CNP inhibits the proliferation of vascular smooth muscle cells in culture (14, 15) and prevents balloon injury-induced coronary artery restenosis in animal models (16–18). Recent studies of CNP-deficient mice indicate that CNP plays an important role in chondrocyte differentiation and bone formation (11).

The natriuretic peptides are synthesized as prepropeptides. The signal peptide is removed to form propeptides, but a further proteolytic cleavage of the propeptide is required to convert the precursor to a biologically active peptide. This activation mechanism is critical in regulation of the activity of the natriuretic peptides, but the enzyme(s) responsible for the conversion remained uncharacterized for many years. Recently, we identified a cardiac serine protease, corin (19), that is a member of the type II transmembrane serine protease family (20, 21). In cell-based functional studies, we showed that corin converted pro-ANP to biologically active ANP in a highly sequence-specific manner (22, 23). Recombinant corin also processed pro-BNP to BNP in cell-based assays (22), indicating that corin is a convertase for pro-ANP and pro-BNP. To date, however, the enzyme responsible for pro-CNP processing has not been fully characterized.

In addition to its abundant expression in cardiomyocytes, the *corin* gene is also expressed in several other tissues, such as the pregnant uterus and developing kidneys and bones (19). In developing bones, corin mRNA was specifically expressed in the prehypertrophic chondrocyte, a subset of chondrocytes important in bone growth and maturation. Interestingly, pro-CNP mRNA is also expressed in the prehypertrophic chondrocyte in developing bones (11). The co-localization of corin and pro-CNP mRNA expression suggested the hypothesis that corin was responsible for the processing of pro-CNP.

In this study, we test this hypothesis by examining the processing of human pro-CNP. Our results showed that in human kidney epithelial 293 cells and chondrosarcoma SW 1353 cells, pro-CNP was processed intracellularly. The processing of pro-CNP was not enhanced in the presence of recombinant corin but was inhibited by an inhibitor of furin, a widely expressed precursor-processing enzyme. We also showed that the processing of pro-CNP did not occur in furin-deficient LoVo cells and that transfection of a plasmid expressing human furin in LoVo cells enabled the cells to process pro-CNP to CNP. Our data indicate that, unlike pro-ANP, pro-CNP is processed intracellularly by the endoprotease, furin, but not by the transmembrane serine protease, corin.

EXPERIMENTAL PROCEDURES

Materials—Penicillin, streptomycin, L-glutamine, fetal bovine serum, and cell culture medium were purchased from Invitrogen. Human embryonic kidney 293 cells, chondrosarcoma SW 1353 cells, and furin-deficient LoVo human colon adenocarcinoma cells were obtained from the American Type Culture Collection and maintained at the Core Facility at Berlex Biosciences. Anti-V5 antibody was purchased from Invitrogen. Recombinant furin was obtained from New England Biolabs Inc. (Beverly, MA). Furin inhibitor (decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone) and synthetic human CNP were purchased from Bachem Bioscience Inc. (King of Prussia, PA). The matrix metalloproteinase (MMP) inhibitor GM6001 was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Tumor necrosis factor- α convertase (TACE) inhibitor and tumor necrosis factor- α protease inhibitor-1 (TAPI), was purchased from Calbiochem. Transfection

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¹ The abbreviations used are: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; MMP, matrix metalloproteinase; TACE, tumor necrosis factor- α convertase; TAPI, tumor necrosis factor- α protease inhibitor-1.

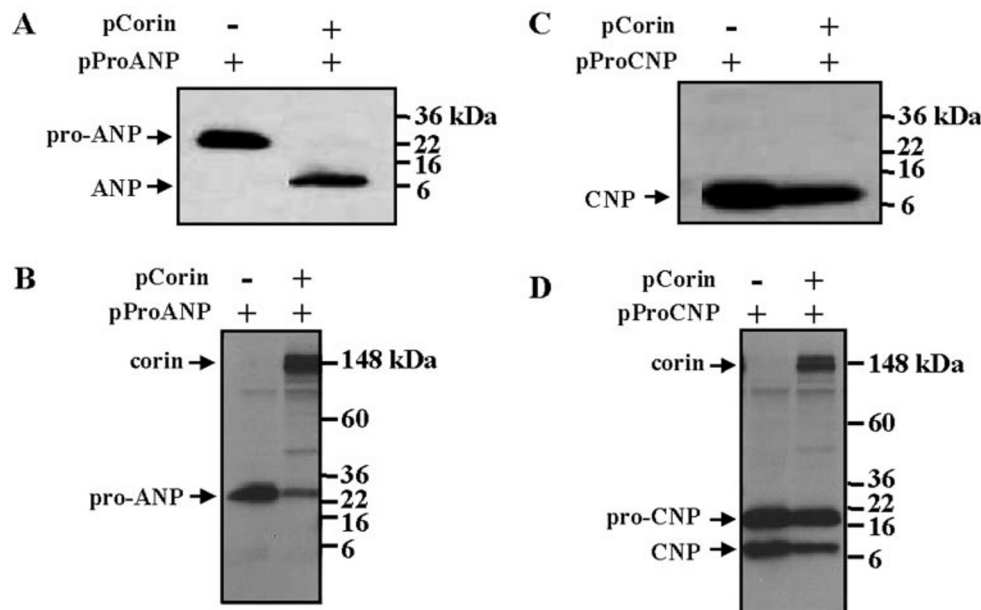
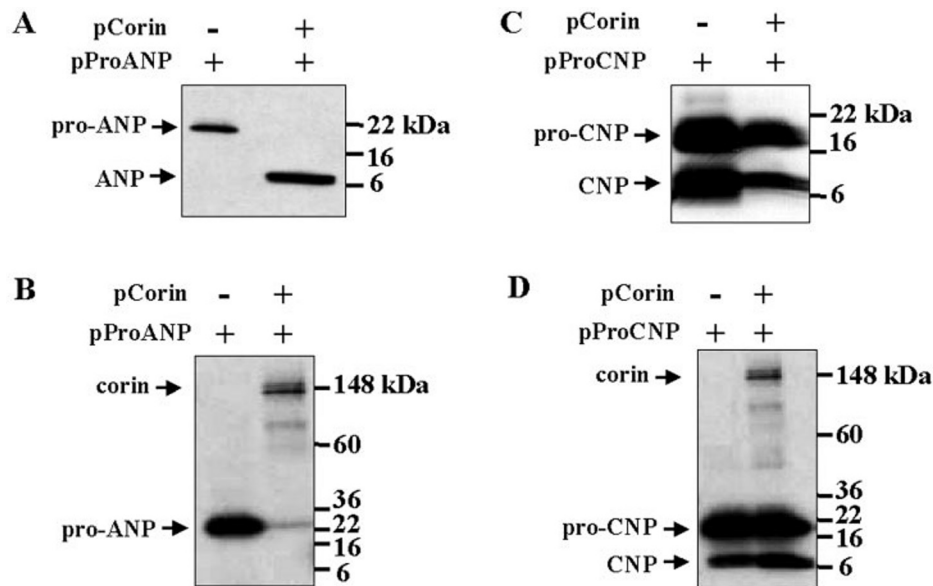


FIG. 1. Processing of pro-ANP and pro-CNP in transfected 293 cells. Transfection experiments were performed in human 293 cells using plasmids expressing human pro-ANP (*pProANP*) (A and B) or pro-CNP (*pProCNP*) (C and D) with or without a plasmid expressing human corin (*pCorin*), as described under "Experimental Procedures." Recombinant pro-ANP, pro-CNP and their derivatives in the conditioned media (A and C) and cell lysates (B and D) were analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody. The Western analysis also detected in the cell lysate the expression of recombinant human corin as two bands possibly caused by differences in glycosylation.

FIG. 2. Processing of pro-ANP and pro-CNP in transfected SW 1353 cells. Transfection experiments were performed in human chondrosarcoma SW 1353 cells using plasmids expressing human pro-ANP (*pProANP*) (A and B) or pro-CNP (*pProCNP*) (C and D) with or without a plasmid expressing human corin (*pCorin*), as described under "Experimental Procedures." Recombinant pro-ANP, pro-CNP, and their derivatives in the conditioned media (A and C) and cell lysates (B and D) were analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody. The Western analysis also detected in the cell lysate the expression of recombinant human corin, as indicated.



reagent LipofectAMINE 2000 was purchased from Invitrogen. All other chemical reagents were obtained from Sigma.

Cell Culture—Human 293 cells were cultured in α -minimum essential medium (α -MEM) (Invitrogen) supplemented with 10% fetal bovine serum and 1% L-glutamine. Chondrosarcoma SW 1353 cells were grown in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% L-glutamine. LoVo cells were cultured in Kaighn's modification of Ham's F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% L-glutamine. Rat aortic smooth muscle cells (Cambrex Bioscience Walkersville, Inc.) were grown in a smooth muscle cell growth medium (Cambrex Bioscience). All cells were cultured at 37 °C in humidified incubators with 5% CO₂/95% air.

Expression Vectors—Plasmid vectors expressing human pro-ANP (*pcDNAproANP*) and corin (*pcDNACorin*) were described previously (22). Recombinant pro-ANP and corin expressed by these vectors contain a viral V5 and a His₆ tag at their carboxyl termini, which facilitates the detection of the proteins. A plasmid expressing human furin was provided by E. Tuley and J. E. Sadler (Washington University, St. Louis, MO) (24). The full-length human pro-CNP cDNA was cloned by

an overlap PCR method (25) using the following four oligonucleotide primers: CNP1S, 5'-TGCCGCCCGTGTGCGCCCTCGAC-3'; CNP2A, 5'-ACATCCCAGGCCGCTCATGGAGCC-3'; CNP3A, 5'-GGGTTTCGCGGACCTTCGGCGCGCGCCCGGGCTTG-3'; and CNP4S, 5'-GTCCCGCGAACCCCGCCGCGCAGAGGAG-3' (26). Briefly, two separate PCR products were amplified from human genomic DNA using primers CNP1S and CNP3A or CNP2A and CNP4S. The two PCR products were gel-purified and mixed. A second PCR was performed using primers CNP1A and CNP4S. The final PCR product was cloned into the expression plasmid *pcDNA3.1/V5-His-TOPO* (Invitrogen). The sequence of the insert and its orientation were verified by automated DNA sequencing. The presence of the viral V5 tag at the carboxyl terminus of pro-CNP allows the detection of the recombinant protein by Western blotting using an anti-V5 antibody (Invitrogen).

Transfection and Western Analysis—Transient transfection was performed in 293, chondrosarcoma SW 1353, or LoVo cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Conditioned medium was collected 13 to 24 h after transfection and subjected to centrifugation at 15,000 rpm to remove cell debris. Cells were lysed in a buffer containing 100 mM Tris-HCl, pH 7.5, and

FIG. 3. Inhibition of pro-CNP processing by small molecule inhibitors. Transfection experiments were performed in human 293 cells using a plasmid expressing human pro-CNP (*pProCNP*). The transfected cells were grown in the presence of increasing concentrations of a furin inhibitor (*Dec-RVKR-CMK*), or a high concentration (20 μ M) of an MMP inhibitor (*GM6001*) or a TACE inhibitor (*TAPI*). Recombinant pro-CNP and its derivatives in the conditioned media (*top*) and cell lysates (*bottom*) were analyzed by an anti-V5 antibody.

Dec-RVKR-CMK	-	1	5	20	-	-	μ M
GM6001	-	-	-	-	20	-	μ M
TAPI	-	-	-	-	-	20	μ M

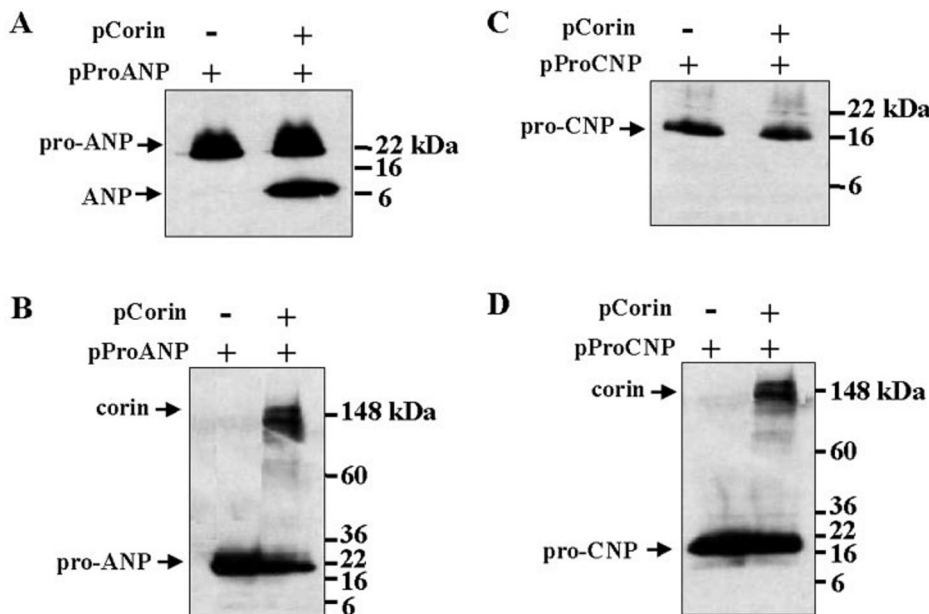
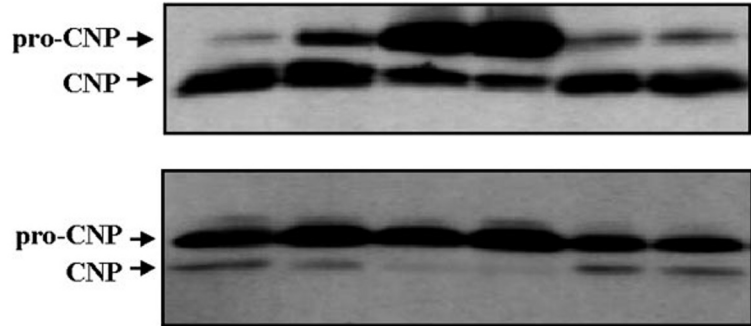


FIG. 4. Processing of pro-ANP and pro-CNP in transfected LoVo cells. Transfection experiments were performed in human colon adenocarcinoma LoVo cells using plasmids expressing human pro-ANP (*pProANP*) (A and B) or pro-CNP (*pProCNP*) (C and D) with or without a plasmid expressing human corin (*pCorin*), as described under "Experimental Procedures." Recombinant pro-ANP, pro-CNP, and their derivatives in the conditioned media (A and C) and cell lysates (B and D) were analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody. The Western analysis also detected in the cell lysate the expression of recombinant human corin, as indicated.

0.6% Triton X-100. To analyze pro-ANP and pro-CNP processing, recombinant pro-ANP and pro-CNP and their derivatives in the conditioned media were immunoprecipitated by an anti-V5 antibody (Invitrogen). Protein samples from the conditioned media or cell lysates were separated by SDS/PAGE and analyzed by Western blotting using a horseradish peroxidase-conjugated anti-V5 antibody (Invitrogen).

Effect of Small Molecule Inhibitors—Human 293 cells were transfected with the pro-CNP expressing plasmid and grown in Opti-MEM medium for 3 h. Small molecule inhibitors, including a furin inhibitor (decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone) (27), an MMP inhibitor (GM6001) (28), and a TACE inhibitor (TAPI-1) (29) were added separately to the medium and incubated for 24 h. The conditioned medium containing recombinant pro-CNP was collected. Cell lysate was prepared as described above. Pro-CNP and its derivatives were analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody.

Cleavage of Pro-CNP by Purified Recombinant Furin—LoVo cells were transfected with a pro-CNP-expressing plasmid or the control vector pcDNA and grown in Opti-MEM medium for 24 h. The cells ($\sim 10^6$) were washed once with phosphate-buffered saline and lysed in 1 ml of 100 mM HEPES, 1% Triton X-100, 1 mM 2-mercaptoethanol, and 1 mM calcium chloride (30). Purified recombinant human furin was added to the cell lysate, and the mixture was incubated at 30 $^{\circ}$ C for 2 h. Cleavage of pro-CNP was analyzed by Western blotting using an anti-V5 antibody.

cGMP Assay—To examine the activity of recombinant CNP, a cGMP assay was performed using a Biotrak enzyme immunoassay kit (Amersham Biosciences), as described previously (23). Briefly, rat aortic smooth muscle cells were grown in 96-well plates in a smooth-muscle cell growth medium (Cambrex Bioscience). Confluent cells were washed

once with phosphate-buffered saline. The LoVo cell lysate (180 μ l), which contained recombinant pro-CNP and was treated with purified furin, was added to each well and incubated at 37 $^{\circ}$ C for 10 min. In this experiment, synthetic human CNP was used as a positive control. The cells were lysed by addition of a lysis buffer (20 μ l/well) containing 2% dodecyltrimethylammonium and 50 mM sodium acetate, pH 5.8. The intracellular cGMP concentration in CNP-stimulated aortic smooth muscle cells was then determined with the Biotrak enzyme immunoassay kit. Each experimental condition was assayed in triplicate.

RESULTS

Processing of Pro-ANP and Pro-CNP in 293 Cells—To examine whether corin is involved in the processing of pro-CNP, transfection experiments were performed in 293 cells. As reported in our previous studies and shown here as a control, corin was required for the processing of human pro-ANP to ANP. Western analysis of the conditioned medium showed the conversion of recombinant pro-ANP to ANP when the cells were co-transfected with plasmids expressing human corin and pro-ANP (Fig. 1A). Expression of recombinant human corin in the transfected cells was confirmed by Western analysis of the cell lysates (Fig. 1B). Western blotting also showed that only pro-ANP, but not ANP, was present in the cell lysates, indicating that pro-ANP was processed extracellularly by corin. In contrast, processed CNP was detected in the conditioned medium from cells transfected with the pro-CNP expressing plas-

mid alone or together with the corin expressing plasmid (Fig. 1C). Western analysis of the cell lysates showed that pro-CNP was processed similarly in the absence or presence of recombinant corin (Fig. 1D), indicating that pro-CNP was processed intracellularly by a corin-independent mechanism.

Processing of Pro-ANP and Pro-CNP in Chondrosarcoma SW 1353 Cells—It is possible that the processing of pro-CNP observed in kidney-derived 293 cells might not reflect conditions in chondrocytes because two cell types will have different proteomes. Both corin and pro-CNP are expressed in chondrocytes; in these cells, corin might be the pro-CNP convertase. To test this, we next performed experiments in SW 1353 cells that were derived from a human chondrosarcoma. As shown in Fig. 2A and B, co-transfection of plasmids expressing human pro-ANP and corin led to the conversion of pro-ANP to ANP. Corin, however, was not required to process pro-CNP. Western analysis detected both pro-CNP and CNP in the conditioned medium and cell lysate from the cells transfected with the pro-CNP-expressing plasmid alone or together with the corin-expressing plasmid (Fig. 2, C and D). Expression of corin did not enhance the processing of pro-CNP. The results showed that the processing of pro-CNP in SW 1353 cells again occurred intracellularly but was less efficient than that in 293 cells. This data is consistent with the observation that corin is not required for the processing of pro-CNP.

Effects of Small Molecule Inhibitors on Processing of Pro-CNP—The observation that pro-CNP was processed intracellularly suggested that the propeptide may be processed by one of the subtilisin-like proteases such as furin, which is known for its role in the processing of many precursor proteins (31–35). To test this hypothesis, we examined the effect of a furin inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (27), on the processing of pro-CNP. Transfection experiments were performed in 293 cells using the pro-CNP expressing plasmid, after which the cells were incubated with the furin inhibitor. Western analysis showed an increase of pro-CNP and a decrease of mature CNP in the conditioned medium from the cells treated with increasing concentrations of the furin inhibitor (Fig. 3, top). Consistent with this result, mature CNP in the cell lysate was decreased in the presence of increasing concentrations of the furin inhibitor (Fig. 3, bottom). In contrast, the processing of pro-CNP was not inhibited when the transfected cells were cultured in the presence of either a broad-spectrum MMP inhibitor (GM6001) or a TACE inhibitor (TAPI) (Fig. 3). These results suggest that furin, but not MMPs or TACE, is involved in the processing of pro-CNP.

Processing of Pro-CNP in LoVo Cells—It is possible that the effect of the inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone on pro-CNP processing was not mediated through its inhibition of furin because the compound is also known to inhibit other processing enzymes. To examine the importance of furin in the processing of pro-CNP, we performed experiments using furin-deficient LoVo cells that were derived from a lymph node metastasis of a human colon adenocarcinoma and contain compound mutations in the *furin* gene (36). As shown in Fig. 4, C and D, pro-CNP, but not CNP, was detected in the conditioned medium and cell lysates from cells transfected with the pro-CNP expressing plasmid alone or together with the corin-expressing plasmid. In controls, the conversion of pro-ANP to ANP was detected in LoVo cells co-transfected with pro-ANP- and corin-expressing plasmids (Fig. 4, A and B), indicating that furin is not required for the corin-mediated processing of pro-ANP. The results demonstrate that furin deficiency prevented the processing of pro-CNP in LoVo cells.

Co-transfection of Plasmids Expressing Furin and Pro-CNP in LoVo Cells—We next tested whether expression of recombi-

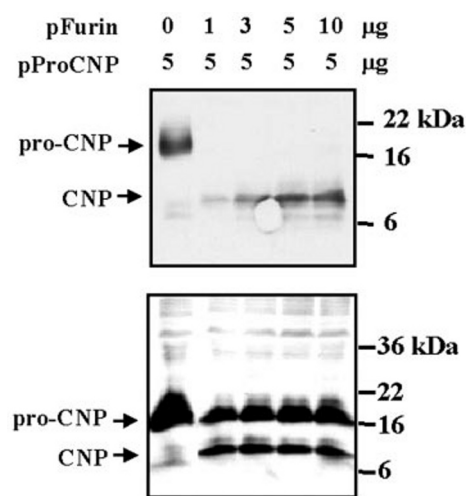


FIG. 5. Co-transfection of plasmids expressing furin and pro-CNP in LoVo cells. LoVo cells were transfected with a pro-CNP expressing plasmid together with increasing amounts of a plasmid expressing human furin, as described under "Experimental Procedures." Recombinant human pro-CNP and its derivatives in the conditioned media (top) and cell lysates (bottom) were analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody. In addition to specific bands representing pro-CNP and CNP (indicated by arrows), several weak nonspecific bands are also present.

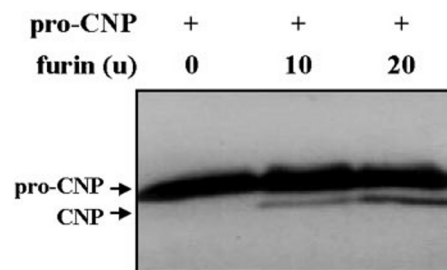


FIG. 6. Cleavage of pro-CNP by purified recombinant furin. Cell lysates from LoVo cells transfected with a plasmid expressing human pro-CNP (pro-CNP) were prepared and incubated with purified recombinant human furin (10 or 20 units) at 30 °C for 2 h. Recombinant pro-CNP and its derivatives were analyzed by SDS-PAGE and Western analysis using an anti-V5 antibody.

nant furin could restore the processing of pro-CNP in LoVo cells. Transfection experiments were performed in LoVo cells using plasmids expressing pro-CNP and human furin. As shown in Fig. 5, Western analysis detected both pro-CNP and CNP in cell lysates from LoVo cells co-transfected with plasmids expressing pro-CNP and furin. Consistently, increasing levels of processed CNP were found in the conditioned medium from LoVo cells transfected with the pro-CNP expressing plasmid and increasing amounts of the furin expressing plasmid (Fig. 5).

Cleavage of Pro-CNP by Recombinant Furin—We also examined the effect of purified recombinant human furin on pro-CNP processing. Recombinant pro-CNP was expressed in LoVo cells and cell lysate was prepared and incubated with increasing concentrations of purified recombinant human furin. The processing of pro-CNP was analyzed by Western blotting. As shown in Fig. 6, in the presence of increasing concentrations of recombinant furin, increasing amounts of pro-CNP were converted to CNP, demonstrating that purified furin is a pro-CNP convertase.

The Activity of Recombinant CNP—The biological function of CNP is mediated through its receptor that has guanylyl cyclase activity. Binding of CNP to its receptor stimulates the guanylyl cyclase activity, leading to generation of intracellular cGMP.

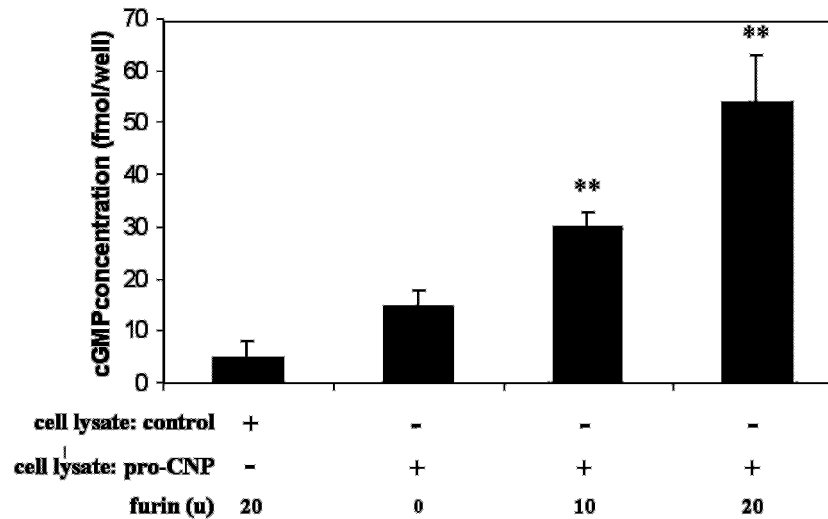


FIG. 7. **Stimulation of intracellular cGMP production.** Cell lysates from LoVo cells transfected with a control vector (*control*) or a plasmid expressing human pro-CNP (*pro-CNP*) were incubated with purified recombinant human furin (10 or 20 units) at 30 °C for 2 h and added to wells in a 96-well plate that had rat aortic smooth muscle cells cultured in it. The cell culture plates were incubated at 37 °C for 10 min. The cells were lysed by addition of a buffer containing 2% dodecyl trimethylammonium and 50 mM sodium acetate, pH 5.8. The intracellular concentration of cGMP in rat aortic smooth muscle cells was determined using the Biotrak enzyme immunoassay kit, as described under "Experimental Procedures." The data are presented as mean $\pm$ S.D. from three independent experiments. **, $p < 0.01$ versus samples containing pro-CNP but no furin by Student's t test.

To determine whether the furin-processed recombinant CNP is biologically active, an aortic smooth muscle cell-based cGMP assay was performed (37). As shown in Fig. 7, low levels of cGMP-stimulating activity were detected in the cell lysate from LoVo cells transfected with a control plasmid or the pro-CNP expressing plasmid alone. The cGMP-stimulating activity increased 2- and 3.7-fold, respectively, when 10 and 20 units of purified recombinant furin were added to the cell lysate containing pro-CNP (Fig. 7). Calculated amounts of recombinant CNP processed by 10 and 20 units of purified furin were 22.5 and 46 ng/ 10^6 cells, respectively. These results are consistent with the data showing that purified recombinant furin cleaves pro-CNP and demonstrate that furin-cleaved recombinant CNP is biologically active.

DISCUSSION

In this study, we characterized the processing of human pro-CNP in cell-based experiments, and we present evidence that pro-CNP is processed by furin but not corin. In transfected epithelial 293 and chondrosarcoma SW 1353 cells, we found that recombinant human pro-CNP was processed intracellularly, in contrast to pro-ANP, which was processed extracellularly. Expression of recombinant human corin did not enhance the processing of pro-CNP in these cells, whereas, in control experiments, recombinant corin cleaved pro-ANP to ANP under similar experimental conditions. Thus, corin is unlikely to play a role in processing pro-CNP despite the fact that both corin and pro-CNP mRNA expression was detected in prehypertrophic chondrocytes in developing bones (11, 19). The functional significance of corin expression in chondrocytes remains to be determined.

The observation that pro-CNP was processed intracellularly suggests that the propeptide may be processed by furin, a prohormone convertase that is predominantly localized in the *trans*-Golgi network and involved in processing a variety of constitutively secreted precursor proteins (31–35). Consistent with this hypothesis, addition of a small molecule compound that blocks furin activity to the culture medium inhibited the processing of recombinant pro-CNP in transfected 293 cells. In contrast, potent inhibitors of MMPs (GM6001) and TACE (TAPI) had little effect on pro-CNP processing in the trans-

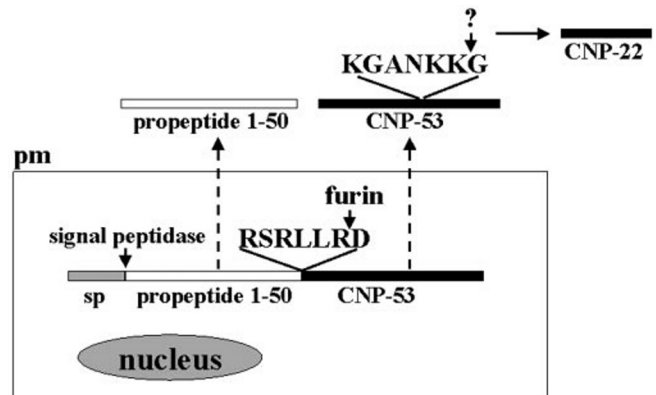


FIG. 8. **A proposed model for the processing of human pro-CNP.** Human pro-CNP is synthesized as a prepropeptide of 126 amino acids (26). After the signal peptide (*sp*) is removed by the signal peptidase, pro-CNP is cleaved by furin at the peptide bond R50-D51 to produce the propeptide 1–50 and CNP-53, both of which are secreted outside the cell. CNP-53 is subsequently cleaved at K80-G81 by an unknown extracellular enzyme to generate CNP-22. Nucleus and plasma membrane (*pm*) are indicated.

fected cells, indicating that MMPs and TACE are unlikely to play an important role in pro-CNP processing. The importance of furin in pro-CNP processing was supported by additional studies using LoVo cells, which are deficient in furin (36). Western analysis showed that there was no detectable conversion of recombinant pro-CNP to CNP in these cells. Transfection of a plasmid expressing human furin in LoVo cells restored the ability of these cells to process pro-CNP. Finally, incubation of purified recombinant human furin with LoVo cell lysate containing recombinant pro-CNP led to conversion of the precursor to the mature peptide. We also showed that the furin-processed CNP was biologically active in a cell-based cGMP assay. Together, these data provide strong evidence that furin is critical in the processing of pro-CNP. Thus, the molecular mechanisms responsible for the processing of pro-CNP and pro-ANP are different despite the fact that these peptides have high sequence homology.

The identification of furin as an intracellular processing

enzyme for pro-CNP provides an insight into the post-translational modification of the peptide. CNP was first isolated as a 22-amino acid peptide (CNP-22) from porcine brain (38). Subsequent studies have shown that a larger 53-amino acid peptide (CNP-53) exists as the major form of CNP in porcine, human, and ovine brain tissues (39–41) and in cultured human endothelial cells (13). Human CNP-53 and CNP-22 are carboxyl-terminal fragments generated by proteolytic cleavages at pro-CNP sequences Arg⁴⁵-Ser⁴⁶-Arg⁴⁷-Leu⁴⁸-Leu⁴⁹-Arg⁵⁰↓-Asp⁵¹ and Lys⁷⁶-Gly⁷⁷-Ala⁷⁸-Asn⁷⁹-Lys⁸⁰-Lys⁸⁰↓-Gly⁸¹, respectively (26). The RSRLLR↓D cleavage site matches the consensus furin recognition sequence, RXXR↓ (31–35), indicating that CNP-53 is probably the product produced by the furin-mediated processing. In our SDS-PAGE and Western analyses, the processed recombinant CNP form appeared as a ~9-kDa band, consistent with the calculated molecular mass of 5.8 kDa for human CNP-53 plus a carboxyl-terminal V5 and His tag (~4 kDa). On Western blots, however, we did not detect the CNP-22 form that, without the tag, has a calculated molecular mass of 2.2 kDa, indicating that the enzyme responsible for generating the CNP-22 form is not present in the cells we tested. The data indicate that CNP-53 is the major secreted form of CNP generated by furin processing and that CNP-22 may be generated subsequently from CNP-53 by another extracellular enzyme whose identity and tissue distribution are unknown at this time (Fig. 8). This conclusion is also supported by a recent study in which the amino-terminal pro-CNP peptide 1–50, a product from the proteolytic cleavage at the RSRLLR↓D site, was detected as the only major cleaved propeptide from pro-CNP in circulating human plasma (42). Apparently, both pro-CNP peptide 1–50 and mature CNP-53 are secreted into the circulation after pro-CNP is processed by furin (Fig. 8). Functionally, CNP-53 and CNP-22 seem to be similar. In anesthetized rats, intravenous injection of CNP-53 and CNP-22 elicits similar natriuretic responses, although their activities are ~100 times less potent than that of ANP (26, 38). Thus, the furin-mediated processing of pro-CNP is a critical step in converting the precursor to a biologically active hormone. The functional importance of further conversion of CNP-53 to CNP-22 remains to be determined.

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