Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme

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Edited by Philip W. Majerus, Washington University School of Medicine, St. Louis, MO, and approved May 23, 2000 (received for review April 4, 2000)

Atrial natriuretic peptide (ANP) is a cardiac hormone essential for the regulation of blood pressure. In cardiac myocytes, ANP is synthesized as a precursor, pro-ANP, that is converted to biologically active ANP by an unknown membrane-associated protease. Recently, we cloned a transmembrane serine protease, corin, that is highly expressed in the heart. In this study, we examine effects of corin on pro-ANP processing. Our results show that recombinant human corin converts pro-ANP to ANP and that the cleavage in pro-ANP by corin is highly sequence specific. Our findings suggest that corin is the long-sought pro-ANP-converting enzyme and that the corin-mediated pro-ANP activation may play a role in regulating blood pressure.

Hypertension is the most prevalent cardiovascular disease in the United States, afflicting more than 15% of the entire adult population (1). Atrial natriuretic peptide (ANP), also called atrial natriuretic factor, is a cardiac hormone that reduces high blood pressure by promoting salt excretion, decreasing blood volume, and relaxing vessel tension in a receptor-dependent manner (2–9). It is implicated in major cardiovascular diseases such hypertension and congestive heart failure (10). In knockout mice, deficiency in either ANP or its receptor causes spontaneous hypertension (11–13). In cardiac myocytes, ANP is synthesized as a precursor, pro-ANP (14, 15), which is converted to biologically active ANP by a proteolytic cleavage (16–19). This activation step is critical in the regulation of ANP activity. Previous studies have suggested that the enzyme responsible is a trypsin-like protease associated with the membrane of cardiac myocytes (20–22). To date, however, the identity of this enzyme remains unknown.

Recently, we cloned a cardiac serine protease, corin, that has the predicted structure of a type II transmembrane protein (23). At its amino terminus, corin contains a short cytoplasmic domain and a transmembrane sequence. In the extracellular region of corin, there are two frizzled-like cysteine-rich motifs, seven low density lipoprotein receptor repeats, a macrophage scavenger receptor-like domain, and a trypsin-like protease domain at the carboxyl terminus. In Northern and *in situ* hybridization analyses, corin mRNA was detected in the human heart, where its expression was most abundant in cardiac myocytes of the atrium (23). Corin mRNA also was detected in developing kidney and bones (23). The tissue expression pattern of corin mRNA is very similar to that of natriuretic peptides (24). In addition, corin mRNA was present in endometrial carcinoma cell lines HEC-1-A, AN3 CA, and RL95–2, leiomyosarcoma cell line SK-LMS-1, as well as in osteosarcoma cell line U2-OS (23). Reverse transcriptase–PCR analysis showed that ANP mRNA also was present in each of these cell lines (unpublished data). The striking similarity in tissue and cellular distribution of corin and ANP mRNA led to our hypothesis that corin is the pro-ANP-converting enzyme.

In this study, we expressed human recombinant corin and examined effects of corin on pro-ANP processing. Our results show that human corin converts pro-ANP to ANP and that the cleavage in pro-ANP by corin is highly sequence specific. Our data suggest that corin is most likely the long-sought pro-ANP-converting enzyme.

Materials and Methods

Construction of Expression Vectors. To express recombinant human corin, the full-length corin cDNA (23) was cloned into the mammalian expression vector pcDNA3.1 (Invitrogen) to yield plasmid pcDNACorin. To facilitate detection of recombinant corin, a similar plasmid, pcDNACorinV5, was constructed by cloning the entire corin-coding sequence into the vector pcDNA3.1/V5-His/TOPO (Invitrogen) to express recombinant corin that contained viral V5 and His tags at the carboxyl terminus. The recombinant protein can be detected by anti-V5 or anti-His antibodies. To express human pro-ANP, the coding region of pro-ANP cDNA (25) was amplified from a human heart cDNA library (CLONTECH) by PCR using sense primer (5′-GAGAGACAGAGCAGCAAGCAGTG-3′) and antisense primer (5′-GTCCCGGAAGCTGTTACAGCCC-3′). The cDNA fragment was then cloned into the pcDNA3.1/V5-His/TOPO vector to yield plasmid pcDNAproANP. Similarly, the coding region of human pro-brain natriuretic peptide (BNP) cDNA (26) was amplified by PCR from a human brain cDNA library (CLONTECH) using sense primer (5′-GGGAAGCAAACCCGGACGCATCGC-3′) and antisense primer (5′-ATGCCGCCTCAGCACTTTGCAGCC-3′). The PCR fragment was cloned into the pcDNA3.1/V5-His/TOPO vector to yield plasmid pcDNAproBNP. All of the expression constructs were verified by restriction digestion and DNA sequencing.

Site-Directed Mutagenesis. Plasmid constructs for corin active site mutant S985A and mutant pro-ANPs R98G, R101A, and R102A were generated by site-directed mutagenesis (QuikChange kit, Stratagene) using pcDNACorinV5 or pcDNAproANP as templates. The oligonucleotide primers used for mutant corin S985A, mutant pro-ANPs R98G, R101A, and R102A were 5′-GATTCATGCATGGGTGACGCCGGTGGGCCTCTTGTTTGT-3′, 5′-CTGCTCACTGCCCCTGGGAGCCTGCGGAGATCC-3′, 5′-TGCCCCTCGGAGCCTGGCGAGATCCAGCTGCTT-3′, and 5′-TGCCCCTCGGAGCCTGCGGGCATCCAGCTGCTT-3′, respectively. The constructs were confirmed by DNA sequencing.

Transfection and Western Analysis. Human embryonic kidney 293 cells were cultured in MEM α (Life Technologies) containing 10% fetal bovine serum. Transient transfection was performed with Lipofectin (Life Technologies) according to the manufacturer's instructions. The conditioned medium was collected 48 or 72 h after transfection. Preparation of cell lysate and membrane fractions was based on a method described previously (27). In brief, transfected cells were washed and detached from culture

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.150149097. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.150149097

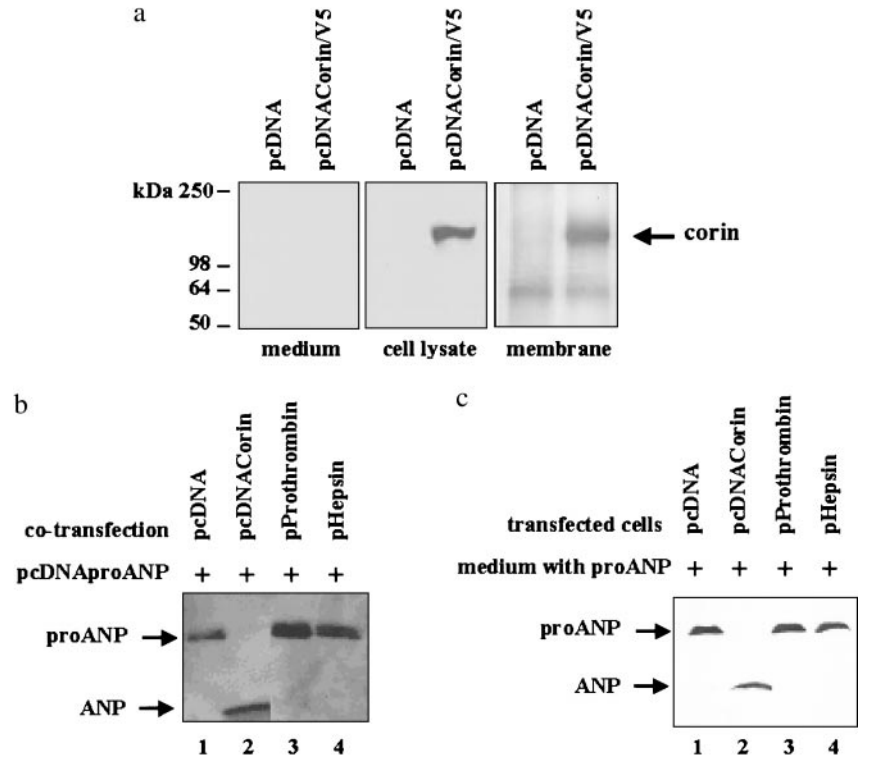

Fig. 1. Cleavage of pro-ANP by corin. (*a*) Western analysis of recombinant corin. Human embryonic kidney 293 cells were stably transfected with corin expression vector pcDNACorin/V5 or a control vector pcDNA. Conditioned medium, total cell lysate, and membrane fractions were prepared based on methods described previously (27). Expression of corin was detected by SDS/PAGE and Western blotting using an anti-V5 antibody. (*b*) Cotransfection of pro-ANP and corin expression vectors. Pro-ANP expression vector pcDNAproANP was cotransfected into 293 cells with vectors expressing corin (pcDNACorin, lane 2), prothrombin (pProthrombin, lane 3), hepsin (pHepsin, lane 4), or a control vector (pcDNA, lane 1). Conditioned medium was collected and processing of pro-ANP was analyzed by Western blotting using an anti-V5 antibody. (*c*) Incubation of conditioned medium containing pro-ANP with transfected cells. Conditioned medium from pcDNAproANP transfected cells was collected and incubated with 293 cells transfected with vectors expressing corin (pcDNACorin, lane 2), prothrombin (pProthrombin, lane 3), hepsin (pHepsin, lane 4), or a control vector (pcDNA, lane 1). Processing of pro-ANP was analyzed by Western blotting.

flasks in a buffer (10 mM Hepes, pH 7.4/137 mM NaCl/4 mM KCl/11 mM glucose) supplemented with 2 mM EDTA. Approximately 1×10^6 cells were suspended in 0.5 ml of the same buffer, and total cell lysate was prepared by sequential passage of cells through a 25-gauge needle. Membrane fractions were prepared by ultracentrifugation, as described (27). Expression of recombinant proteins was analyzed by SDS/PAGE and Western blotting using an anti-V5 mAb (Invitrogen). To analyze the processing of pro-ANP, recombinant pro-ANP and its derivatives in the conditioned medium were immunoprecipitated by the anti-V5 mAb. Proteins were separated by SDS/PAGE and analyzed by Western blotting using a horseradish peroxidase-conjugated anti-V5 antibody. For expression of corin in stable cell lines, 293 cells were transfected with pcDNACorin or pcDNACorinV5 and selected in the presence of G418 (Life Technologies). At least three independent cell clones were selected and used in this study.

Effects of Protease Inhibitors on Corin-Mediated Pro-ANP Processing. Stable 293 cells expressing corin were incubated with conditioned medium containing pro-ANP at 37°C for 4 h in the presence of one of the following: 25 mg/ml apoptinin, 20 mg/ml benzamidine, 25 mg/ml leupeptin, or 12.5 mg/ml soybean trypsin inhibitor. The conditioned medium was collected, and processing of pro-ANP was analyzed by SDS/PAGE and Western blotting. Conversion of pro-ANP to ANP in the presence or absence of protease inhibitors was quantified by scanning of Western blots with a densitometer.

Results

Expression of Recombinant Human Corin. To study the function of corin, recombinant corin was expressed in human embryonic kidney 293 cells. Western blotting showed recombinant human corin as a single band with a molecular mass of ≈150 kDa (Fig. 1*a*). The results was consistent with the calculated mass of 116 kDa for human corin, which also contained 19 potential N-linked glycosylation sites in its extracellular domains (23). The recombinant protein was detected only in total cell lysate and membrane fractions but not in conditioned medium (Fig. 1*a*), consistent with the prediction from sequence analysis that corin is a transmembrane protein.

Processing of Pro-ANP by Corin. To examine processing of pro-ANP by corin, a pro-ANP expression vector was constructed and cotransfected with the corin expression vector into 293 cells. Cleavage of human pro-ANP was analyzed by Western blotting using antibodies against peptide tags attached to recombinant pro-ANP or corin. Cotransfection of corin and pro-ANP expression vectors resulted in cleavage of pro-ANP, producing an ANP fragment of the predicted molecular mass (≈3 kDa) in the conditioned culture medium (Fig. 1*b*). Similar cleavage of pro-ANP also was detected when the transfection experiments were performed using BHK and COS-7 cells (data not shown). In an independent experiment, pro-ANP and corin expression vectors were transfected into separate 293 cells. When conditioned medium from pro-ANP vector transfected cells was incubated with corin expressing cells, a similar

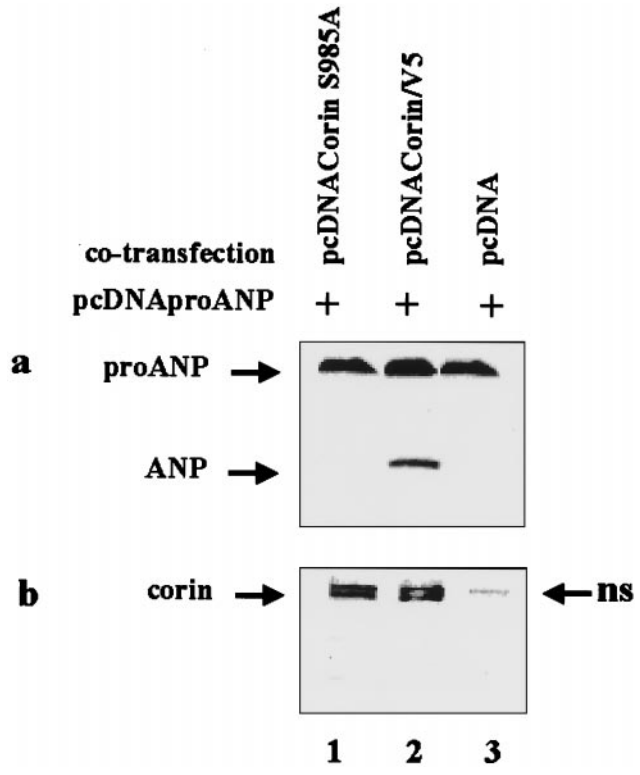

Fig. 2. Cleavage of pro-ANP depended on the catalytic activity of corin. (*a*) Pro-ANP expression vector pcDNAproANP was cotransfected into 293 cells with vectors expressing wild-type corin (pcDNACorin, lane 2) and mutant corin S985A (pcDNACorin S985A, lane 1) or a control vector (pcDNA, lane 3). Pro-ANP and its derivatives were detected by Western blotting using an anti-V5 antibody. (*b*) Expression of recombinant corin in transfected 293 cells. To verify expression of recombinant corin in transfected cells, cell lysate was prepared and analyzed by SDS/PAGE and Western blotting. Expression of recombinant corin was detected in pcDNACorinV5 and pcDNACorin S985A but not pcDNA, transfected cells. A nonspecific (ns) band was present in samples from pcDNACorinV5, pcDNACorinS985A, and pcDNA transfected 293 cells.

cleavage of pro-ANP was detected in the medium (Fig. 1*c*), confirming the processing of pro-ANP by corin. The results also indicated that the protease catalytic domain of corin was exposed on the cell surface as predicted by protein sequence analysis. In Western analysis using cell lysates from cells transfected with both corin and pro-ANP expression constructs, most pro-ANP remained unprocessed, suggesting that the processing of pro-ANP by corin is extracellular (data not shown). This is consistent with previous reports that ANP remained as a propeptide in the dense granules of cardiomyocytes and the proteolytic activation occurred upon secretion (22). In controls using plasmids expressing the hepatic transmembrane serine protease hepsin (28–30) or prothrombin (31), no cleavage in pro-ANP was detected in either the cotransfection or the incubation experiments (Fig. 1 *b* and *c*). These results show that corin, but not hepsin or prothrombin, processes pro-ANP under the experimental conditions.

Dependence of Pro-ANP Processing on the Catalytic Activity of Corin. Previous studies showed that the processing of pro-ANP in cardiac myocytes depended on a proteolytic activity that was inhibited by certain types of protease inhibitors (21). To show that pro-ANP processing depends on the proteolytic activity of corin, a mutant corin S985A was constructed in which the protease active site residue Ser-985 was replaced with an Ala.

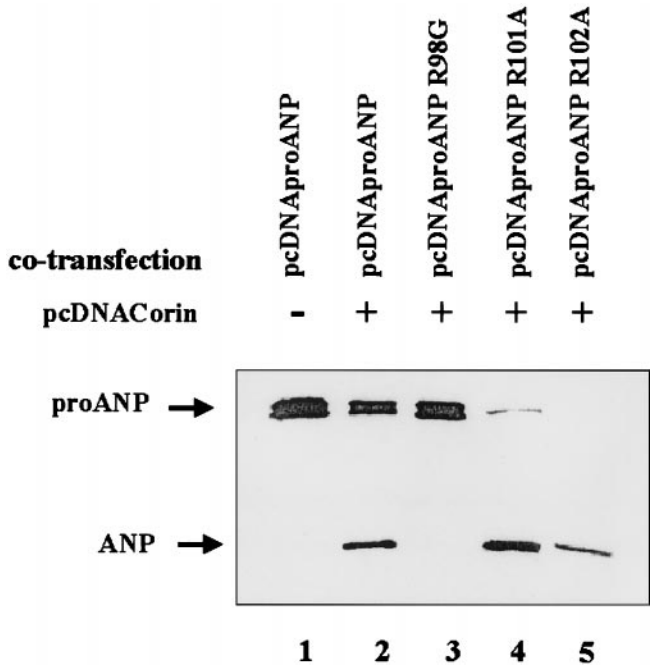

Fig. 3. Determination of the corin cleavage site in pro-ANP. Expression vectors for wild-type pro-ANP or mutant pro-ANPs R98G, R101A, and R102A were cotransfected into 293 cells with corin expression vector, pcDNACorin. Pro-ANP and its derivatives in conditioned medium were analyzed by Western blotting using an anti-V5 antibody. At high resolution, two specific bands of pro-ANP were detected on the Western blot possibly caused by differences in glycosylation in transiently transfected 293 cells.

The mutation is expected to abolish the enzymatic activity of corin. The mutant construct was used in a cotransfection experiment with the pro-ANP expression vector. In contrast to wild-type corin, mutant corin S985A failed to cleave pro-ANP (Fig. 2*a*). A similar expression level for mutant corin S985A and wild-type corin proteins in the transfected cells was shown by Western analysis (Fig. 2*b*). The results demonstrated that processing of pro-ANP depended on the catalytic activity of corin.

Effect of Serine Protease Inhibitors on Corin-Mediated Pro-ANP Processing. To examine the effect of serine protease inhibitors on corin-mediated pro-ANP processing, a cotransfection experiment was performed in the presence of protease inhibitors. In controls, cotransfection of corin and pro-ANP expression vector resulted in >70% of conversion of pro-ANP to ANP. In the presence of aprotinin, benzamidine, leupeptin, or soybean trypsin inhibitor, the corin-mediated cleavage of pro-ANP was inhibited by 99%, 87%, 96%, and 17%, respectively. The results were consistent with the previous study, in which high concentrations (100 mg/ml) of benzamidine, leupeptin, and aprotinin but not soybean trypsin inhibitor inhibited the processing of pro-ANP by a protease activity partially purified from membrane fractions (21).

Sequence Specificity of Corin-Dependent Cleavage in Pro-ANP. In the atrium, pro-ANP is primarily cleaved at Arg-98 to yield the 28-aa mature ANP that is the principal circulating form of ANP. In other tissues, such as kidney, brain, and testis, alternatively processed forms of ANP also exist whose physiological significance is unknown. For example, pro-ANP was cleaved at Ala-95 in the testis and at Arg-101 or Arg-102 in the brain (32–34). By Western analysis, we showed a corin-dependent proteolytic cleavage in pro-ANP. The results, however, did not distinguish the cleavage at Arg-98 from alternative cleavages. To determine

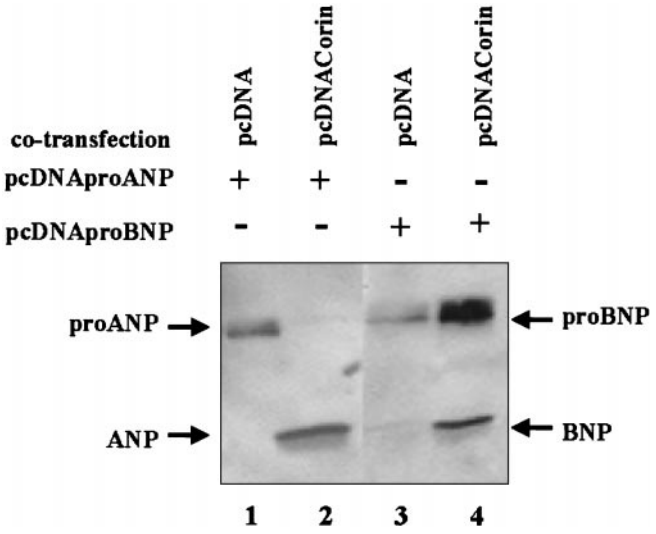

Fig. 4. Cleavage of pro-BNP by corin. Expression vectors for human pro-ANP or pro-BNP were cotransfected into 293 cells with corin expression vector, pcDNACorin, or a control vector pcDNA. Pro-ANP, pro-BNP, and their derivatives in conditioned medium were analyzed by Western blotting using an anti-V5 antibody.

whether corin cleaves pro-ANP at Arg-98, a vector was constructed to express mutant pro-ANP R98G in which residue Arg-98 was replaced with a glycine. The mutation is expected to prevent cleavage at residue Arg-98 by a trypsin-like protease that cleaves after arginine and lysine residues. Mutant pro-ANP R98G and wild-type pro-ANP expression vectors were cotransfected with the corin expression vector. Western analysis of the conditioned medium showed that the cotransfection resulted in cleavage of wild-type pro-ANP but not mutant pro-ANP R98G (Fig. 3). In contrast, in the cotransfection experiments using two other mutant constructs, pro-ANP R101A and pro-ANP R102A, replacement of arginine residues at 101 or 102 in pro-ANP with an alanine did not prevent the corin-mediated processing of pro-ANP (Fig. 3). These results indicated that corin cleaved pro-ANP specifically at Arg-98 but not at other adjacent arginine residues such as Arg-101 and Arg-102.

Processing of Pro-BNP by Corin. In addition to ANP, there is a closely related member of the natriuretic peptide family, namely BNP (or B-type natriuretic peptide) (3–9). BNP plays a role similar to that of ANP in regulating blood pressure. Like ANP, BNP is synthesized as a precursor and cleavage is required to produce the mature, active peptide. The activation cleavage sequence in pro-BNP (Leu-Arg-Ala-Pro-Arg-Ser, residues 72–77) is similar to that of pro-ANP (Leu-Tyr-Ala-Pro-Arg-Ser, residues 94–99). To determine whether corin also is involved in the processing of pro-BNP, a pro-BNP expression vector was constructed and used for cotransfection experiments. As shown in Fig. 4, cotransfection of pro-BNP and corin expression vectors resulted in cleavage of pro-BNP, generating a small fragment with the predicted molecular weight of BNP. In controls, no proteolytic cleavage of pro-BNP was detected after cotransfection of the pro-BNP expression vector with a control vector. These results suggested that corin also can activate pro-BNP.

Discussion

ANP was discovered 20 years ago as a cardiac hormone that regulates blood pressure (2). It is processed from its precursor, pro-ANP, by an enzyme that has not yet been identified. Recently, we cloned the transmembrane serine protease corin

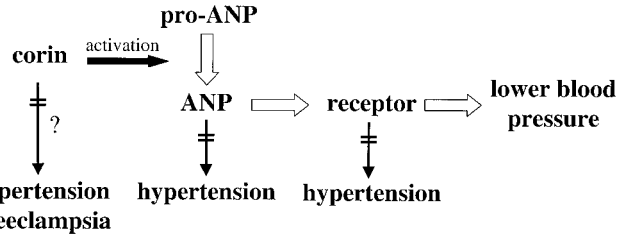

Fig. 5. Corin regulates the ANP-mediated pathway by activating pro-ANP. Deficiency in either ANP or its receptor causes hypertension (11–13), suggesting that defects in corin might also lead to hypertension and preeclampsia.

from the human heart (23). Based on the coexpression of pro-ANP and corin, we postulate that corin could be the enzyme that processes pro-ANP. To test this hypothesis, we expressed recombinant human corin and examined the function of corin in processing of pro-ANP. Our results showed that human corin, but not prothrombin and hepsin, converted pro-ANP to ANP in cell-based experiments (Fig. 1). The cleavage of pro-ANP by corin was prevented by a point mutation at Arg-98, but not Arg-101 or Arg-102, in pro-ANP, indicating that corin-mediated processing of pro-ANP was highly sequence specific (Fig. 3).

Previous studies have shown that the pro-ANP-converting enzyme is a membrane-associated high molecular weight serine protease present in cardiac myocytes (21) and that the cleavage of pro-ANP occurs on the surface of cardiac myocytes (22). To our knowledge, corin is the only transmembrane serine protease of the trypsin superfamily that is abundantly expressed in cardiac myocytes. Sequence analysis indicates that corin is a type II transmembrane protein with an extracellular protease catalytic domain (23). Consistent with this idea, Western blotting showed the recombinant corin was present in cell lysate and isolated membrane fractions but not in the conditioned medium (Fig. 1*a*). In addition, pro-ANP in the conditioned medium was cleaved after incubation with cells transfected with a corin-expressing vector (Fig. 1*c*), indicating that the catalytic domain of corin was present on the cell surface. Previous studies also showed that the activity of pro-ANP-converting enzyme was inhibited by aprotinin, benzamidine, and leupeptin but not soybean trypsin inhibitor (21). Similar effects of these protease inhibitors on corin-mediated processing of pro-ANP were observed in our studies. Thus, corin matches all known characteristics of the long-sought pro-ANP-converting enzyme. Further studies will determine the importance of corin in the processing of pro-ANP *in vivo*.

The physiological importance of ANP in controlling blood pressure has been well established. Identification of corin as the pro-ANP-converting enzyme suggests an addition to the regulatory mechanism for the ANP-mediated pathway (Fig. 5). By converting pro-ANP to biologically active ANP, corin acts at the top of the ANP-mediated molecular pathway. Because deficiency in both ANP and its receptor in mice causes spontaneous hypertension (11–13), deficiency in corin also might lead to hypertension. In addition to the heart, corin mRNA expression was detected in the pregnant, but not normal, uterus in mice (23). The biological significance of the up-regulation of corin in the uterus during pregnancy is not clear. ANP expression in the uterus has been reported (35). Plasma levels of ANP are increased significantly in pregnant women, especially in women with preeclampsia (36, 37). It is possible that corin is induced in the uterus during pregnancy to increase ANP production and prevent preeclampsia.

We thank Drs. B. Dole and D. Light for critical reading of the manuscript.

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