The cardiac serine protease corin is the pro-atrial natriuretic peptide convertase. Corin is made as a zymogen, which is activated by proteolytic cleavage. Previous studies showed that recombinant human corin expressed in HEK 293 cells was biologically active, but activated corin fragments were not detectable, making it difficult to study corin activation. In this study, we showed that recombinant corin was activated in HEK 293 cells, murine HL-1 cardiomyocytes, and rat neonatal cardiomyocytes. In these cells, activated corin represented a small fraction of the total corin molecules. The activation of recombinant rat corin was inhibited by small molecule trypsin inhibitors but not inhibitors for matrix metalloproteinases or cysteine proteinases, suggesting that a trypsin-like protease activated corin in these cells. Glycosidase digestion showed that rat and human corin proteins contained substantial $N$-glycans but little $O$-glycans. Treatment of HEK 293 cells expressing rat corin with tunicamycin prevented corin activation and inhibited its pro-atrial natriuretic peptide processing activity. Similar effects of tunicamycin on endogenous corin activity were found in HL-1 cells. Mutations altering the two $N$-glycosylation sites in the protease domain of rat corin prevented its activation in HEK 293 and HL-1 cells. Our results indicate that $N$-linked oligosaccharides play an important role in corin activation.

Corin is a serine protease made primarily in atrial and ventricular cardiomyocytes (1–3). Corin mRNA expression also has been detected in scar myofibroblasts in a rat model of myocardial infarction (4). In cardiomyocytes, corin converts pro-atrial natriuretic peptide (pro-ANP) to active ANP (5), a hormone that regulates blood pressure by promoting natriuresis, diuresis, and vasodilation (6–8). ANP also suppresses renin and endothelin release (9–11), which represents an additional mechanism regulating vascular tone. In corin null mice, pro-ANP to ANP conversion was abolished, demonstrating that corin is the long sought physiological pro-ANP convertase (12). Corin null mice develop hypertension, indicating the importance of corin in maintaining normal blood pressure in vivo (12). Most recently, nonsynonymous single nucleotide polymorphisms in a corin gene allele were found to be associated with an increased risk for hypertension in African-Americans (13). Patients with this gene allele also exhibited an enhanced cardiac hypertrophic response to high blood pressure, as indicated by a greater left ventricular mass that is disproportional to their systolic blood pressure (14, 15). These data suggest that corin might be involved in hypertensive disease in humans.

Structurally, corin belongs to the type II transmembrane serine protease family (16–18). Corin has a short N-terminal cytoplasmic tail and an integral transmembrane domain. In its extracellular region, there are two frizzled-like cysteine-rich domains, eight low density lipoprotein receptor repeats, a scavenger receptor-like cysteine-rich domain, and a trypsin-like protease domain at the C terminus (1, 3). Like most trypsin-like proteases, corin is made as a single chain zymogen, as shown by Western blots under reducing conditions (1, 19, 20). Human corin contains a conserved activation cleavage sequence Arg↓Ile-Leu-Gly-Gly at residues 801–805 (3). Mutant corin R801A, in which Arg-801 was replaced by an Ala, had no detectable activity in functional assays, indicating that proteolytic cleavage at Arg-801 is required to convert corin zymogen to an active enzyme (19). For many proteolytic enzymes, zymogen activation is one of the most important steps in regulating their biological activities. To date, however, the physiological corin activator has not been identified. Little is known about how corin activity is controlled in vivo.

The calculated mass for human corin protein is $\sim$116 kDa (3). In Western blotting analysis, recombinant human corin from human embryonic kidney (HEK) 293 cells appeared as a band of $\sim$150 kDa (19, 20). Similar molecular mass also was observed in Western blotting of native corin from human hearts (1). The difference between the calculated and actually observed values in molecular mass suggests that corin protein may be extensively glycosylated. This is consistent with 19 predicted $N$-linked glycosylation sites in the extracellular region of human corin (3). Most of these $N$-glycosylation sites are conserved in rat and mouse corin proteins (21, 22). To date, the extent of $N$-linked oligosaccharides on corin protein and their functional importance have not been examined. Studies of other type II transmembrane serine proteases such as matriptase (23) and enteropeptidase (24, 25) have shown that $N$-glycosylation is critical for zymogen activation and subcellular targeting. It is possible that carbohydrate moieties may play a similar role in corin.

In this study, we tested this hypothesis by examining corin zymogen activation in HEK 293 cells, mouse atrial cardiomyocyte HL-1 cells (26), and primary rat neonatal cardiomyocytes.
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Our results showed that a fraction of rat corin zymogen was converted to biologically active enzyme in these cells and that N-glycosylation was required for this activation process. Treatment of HEK 293 and HL-1 cells with tunicamycin or mutations altering the N-glycosylation sites in the protease domain prevented rat corin zymogen activation. Our data indicate that N-glycans are important in regulating corin biosynthesis and activity.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK 293 and its derived cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). The murine atrial cardiomyocyte cell line HL-1, a generous gift from Dr. William C. Claycomb (Louisiana State University Medical Center, New Orleans), was cultured in Claycomb medium with 10% FBS, 100 μM norepinephrine, and 4 mM L-glutamine in gelatin/fibronectin-coated flasks or plates. Primary neonatal cardiomyocytes were isolated from hearts of 1-day-old rats and cultured in DMEM/F-12 medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin, as described previously (27). The use of new born rats was approved by the local Institutional Animal Care and Use Committee and in compliance with the Guide for the Care and Use of Laboratory Animals, National Institutes of Health. All cells were cultured at 37 °C in humidified incubators with 5% CO2 and 95% air.

Corin Expression Vectors—The full-length rat corin cDNA was cloned from a heart library. First, total RNA was isolated from adult Sprague-Dawley rat hearts using TRIzol reagents (Invitrogen). First strand cDNAs were synthesized using Super-Script III reverse transcriptase (Invitrogen). The full-length cDNA was amplified by PCR using Pfu polymerase (Stratagene, La Jolla, CA) and oligonucleotide primers based on the published rat corin cDNA sequence (21). The cDNA was subjected to a second round PCR using Phusion High Fidelity polymerase (New England Biolabs, Inc., Ipswich, MA) with sense primer 5′-CAG TCA TGG GCA GGG TTT CTT TCA-3′ and antisense primer 5′-TCC TTG GGA TTT CTT TTG GAG AAA GGT C-3′ to delete the original stop codon. The PCR product was gel-purified and made an overhanging 3′-adenine by a 20-min incubation at 72 °C with Taq polymerase and a dNTP mixture (1 mmol/liter), and cloned into pcDNA3.1-V5–6xHis TOPO vector (Invitrogen). The resultant plasmid, pcDNAratCorin, encoded rat corin protein containing a viral 6xHis TOPOvector, and the resultant plasmid, pcDNAhumanCorin, encoded human corin with a V5 tag at the C terminus. All expression plasmids used in this study were verified by restriction enzyme digestion and DNA sequencing.

To express recombinant rat corin in primary cardiomyocytes, a lentiviral vector and packaging system, a generous gift from the Stark laboratory (Cleveland Clinic Foundation), was used. The plasmid pcDNAratCorin was digested with HindIII and PmeI to release the cDNA insert, which was blunted with Klenow enzyme and inserted into pLV-uro vector to yield vector pLVratCorin. Lentivirus was packaged in HEK 293T cells by co-transfection of pLVratCorin with packaging plasmid pCMVΔR8.74 and envelope plasmid pDM2G (28). Primary rat neonatal cardiomyocytes were infected twice with the lentivirus in the conditioned medium from transfected 293T packaging cells.

Transfection and Western Blotting—HEK 293 and HL-1 cells were transfected with expression plasmids using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instruction. Expressed proteins were detected by Western blotting. Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100 (v/v), 10% glycerol (v/v), and a protease inhibitor mixture (1:100 dilution; Sigma). Protein samples were mixed with a loading buffer with (reducing) or without (nonreducing) 2% β-mercaptoethanol and boiled at 100 °C for 5 min before being loaded onto an SDS-polyacrylamide gel. Western blotting of recombinant corin and pro-ANP was done using a horseradish peroxidase conjugated anti-V5 (anti-V5-HRP) antibody, as described previously (5, 20). For stable transfection, transfected cells were selected in DMEM containing G418 (500 μg/ml, Sigma). Cell clones expressing corin were identified by Western blotting. To quantify activated corin fragments, x-ray films from Western blotting were scanned by a densitometer (The PharsosFx System, Bio-Rad). The optical density of the bands representing corin zymogen and the protease domain was measured, and the percentage of the activated molecules was calculated using the Quantity One one-dimensional analysis software (Bio-Rad).

Corin-mediated Pro-ANP Processing—A cell-based assay was used to examine corin-mediated pro-ANP processing, as described previously (20, 29). HEK 293 cells in 6-well plates were co-transfected with plasmids expressing human pro-ANP (pcDNAproANP) (20) and rat or human corin.
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(pcDNAratCorin or pcDNAhumanCorin) and incubated for 16 h. Conditioned medium was collected and centrifuged at 13,000 rpm for 10 min to remove cell debris. Pro-ANP and its derivatives in the conditioned medium were analyzed by immunoprecipitation using an anti-V5 antibody against the tag attached to the C terminus of recombinant pro-ANP/ANP followed by Western blotting using the anti-V5-HRP antibody. On Western blots, recombinant human pro-ANP and ANP with the V5 tag appeared as bands of ~23 and ~11 kDa, respectively, as described previously (20, 29).

In some experiments, conditioned medium containing pro-ANP was first prepared in transfected HEK 293 cells, then added (2 ml/well) to HL-1 cells or HEK 293 cells expressing wild-type or mutant corin in 6-well plates, and incubated at 37 °C for 1 h. Pro-ANP and its derivatives in the conditioned medium were analyzed by immunoprecipitation and Western blotting, as described above.

Effects of Small Molecule Inhibitors—Transfected HEK 293 and HL-1 cells expressing recombinant rat corin were grown in DMEM. Small molecule inhibitors, including trypsin-like protease inhibitors (10 mM benzamidine and 100 μM leupeptin), a metalloproteinase inhibitor (20 μM GM 6001) (30), and a cysteine protease inhibitor (100 μM N-acetyl-leucyl-leucyl-methionine (ALLM)) (31) were added to the cells in separate wells and incubated for 16 h. Recombinant corin and its derived fragments were analyzed by Western blotting, as described above.

Glycosidase Digestion—To analyze the carbohydrate contents in rat and human corin proteins, glycosidase digestion was done using a deglycosylation kit from Prozyme (San Leandro, CA). Briefly, HEK 293 cells expressing rat or human corin in 10-cm dishes were washed twice with phosphate-buffered saline (PBS), collected, and lysed in 100 μl of a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% Nonidet P-40. Cell lysate containing ~100 μg of protein was incubated at 100 °C for 5 min in 42.5 μl of a buffer containing 50 mM sodium phosphate, pH 7.0, 0.1% SDS, and 50 mM β-mercaptoethanol. Denatured and reduced proteins were digested without (control) or with 5 × 10^{-3} units of N-glycanase, O-glycanase, and sialidase A, either individually or in combination, at 37 °C for 16 h. Protein samples were analyzed by Western blotting using an anti-V5 antibody.

Effects of Tunicamycin—Effects of tunicamycin on corin zymogen activation and pro-ANP processing activity were examined in HEK 293 cells expressing rat corin. Confluent (~95%) cells in 6-well plates were cultured in DMEM with 10% FBS with or without tunicamycin (1 μg/ml; Sigma). After 16 h, the medium was removed, and the cells were washed with serum-free medium. Conditioned medium containing recombinant pro-ANP was added to the cells and incubated at 37 °C for 1 h. As a control for potential direct effect of tunicamycin on corin or pro-ANP, tunicamycin (1 μg/ml) was added to pro-ANP containing medium and incubated for 1 h with HEK 293 that were not pretreated with the antibiotic. Pro-ANP and its derivatives in the conditioned medium were analyzed by immunoprecipitation and Western blotting. Recombinant corin expression in HEK 293 cells with or without tunicamycin treatment was verified by Western blotting of cell lysate.

Experiments were done with HL-1 cells, which appeared to be more resistant to tunicamycin inhibition. As a result, a higher concentration (10 μg/ml) of tunicamycin was used during the 16-h incubation period. Pro-ANP processing was examined as described above.

Analysis of Biotin-labeled Cell Surface Protein Expression—HEK 293 cells expressing recombinant rat corin in 60-mm dishes were washed three times with PBS and incubated with 3 ml of PBS, pH 8.0, containing 1 mM sulfo-NHS-biotin (Pierce) at room temperature for 30 min. Reactions were quenched by a 10-min incubation in 3 ml of PBS with 100 mM glycine. The cells were washed with PBS and lysed on ice for 30 min in 300 μl of a lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (v/v), and a protease inhibitor mixture (1:100 dilution). Cell lysate was centrifuged at 4 °C at 13,000 rpm for 10 min, and protein concentration was adjusted to 1 μg/μl using the lysis buffer. Streptavidin-Sepharose beads (50 μl in a 50:50 (beads/buffer volume) slurry) were added to 300 μl of cell lysate, and the mixture was rocked at 4 °C for 2 h. The beads were washed three times with the lysis buffer and boiled in 50 μl of sample loading buffer. Protein eluates were analyzed by Western blotting.

RESULTS

Expression of Rat Corin in HEK 293 Cells—The full-length rat corin cDNA was cloned from a Sprague-Dawley rat heart library by PCR based on the published sequence (21). A plasmid vector was made to express recombinant rat corin with a V5 tag at the C terminus (Fig. 1A). To test the activity of recombinant rat corin, HEK 293 cells were co-transfected with a pro-ANP expression vector and the rat corin expression vector. Western blotting of the conditioned medium indicated that pro-ANP was converted to ANP in cells expressing rat corin (data not shown), confirming that recombinant rat corin was biologically active.

N-Linked Glycosylation in Rat and Human Corin—HEK 293 cell lysates containing recombinant rat or human corin were digested with N-glycanase, O-glycanase, and sialidase A and analyzed by Western blotting using an antibody against the V5 tag at the C termini of recombinant rat and human corin. Under reducing conditions, rat corin appeared as a major band of ~170 kDa (Fig. 1B, lane 1), whereas human corin appeared as two bands of ~150 and ~130 kDa, respectively (Fig. 1B, lane 7). The result was consistent with rat corin being 69 amino acids longer than human corin (1111 versus 1042 amino acids) (3, 21). Peptide-N-glycosidase (PNGase) F digestion reduced the apparent mass of rat corin to ~130 kDa (Fig. 1B, lane 2) and that of human corin to ~110 kDa (Fig. 1B, lane 8). It appeared that the ~150- and ~130-kDa species of human corin were differentially N-glycosylated products. Digestion with O-glycanase and sialidase A, either individually or in combination, did not yield noticeable reduction in the apparent mass of rat and human corin (Fig. 1B, lanes 3–6, and 9). The results indicate that the majority, if not all, of carbohydrate moieties on corin are N-linked oligosaccharides.

Interestingly, a minor band of ~40 kDa was detected in cell lysate with rat, but not human, corin (Fig. 1B, lane 1). Upon
Detection of Cleaved Rat Corin Protease Fragments in HEK 293 and HL-1 Cells, and Neonatal Cardiomyocytes—If the observed ~40-kDa band in lysate from HEK cells expressing rat corin was the activated corin protease fragment, it is expected to be attached to the propeptide region by a disulfide bond connecting Cys residues 855 and 977 (Fig. 1A) (3, 21). On Western blots, the propeptide and the activated protease domain should appear as a single band under nonreducing conditions. This was confirmed upon further studies. The ~40-kDa band was detected by Western blotting under reducing but not nonreducing conditions in HEK 293 cells expressing rat corin (Fig. 2A). To examine if the activation cleavage occurs in physiologically relevant cardiomyocytes, recombinant rat corin was expressed in murine atrial cell line HL-1 and rat primary neonatal cardiomyocytes. Western blotting analysis of cell lysate showed a similar ~40-kDa band in HL-1 cells and rat neonatal cardiomyocytes under reducing but not nonreducing conditions (Fig. 2A). This band represented a small fraction of overall corin protein in these cells, as estimated by densitometry. In lysate from HEK 293 cells expressing human corin, this ~40-kDa band was barely visible (Fig. 2B). The results were consistent with our previous observation that only a very low percentage of human corin molecules was activated in HEK 293 cells.

Lack of Activation Cleavage in Mutant Rat Corin R866A—To confirm that the observed ~40-kDa band indeed was generated by cleavage at the conserved activation site, a plasmid construct was made to express mutant rat corin R866A, in which Arg-866 was replaced by an Ala to abolish the activation site (21). HEK 293 cells were transfected with plasmids expressing rat wild-type corin or mutant corin R866A, and cell lysate was analyzed by Western blotting. As shown in Fig. 3, the ~40-kDa band was detected in lysate with wild-type corin but not mutant corin R866A under reducing conditions, supporting the idea that the ~40-kDa band was derived from the activation cleavage at Arg-866.

In functional assays, pro-ANP was converted to ANP in HEK 293 cells co-transfected with plasmids expressing pro-ANP and wild-type corin but not mutant corin R866A (Fig. 4A). Similar results were obtained in a differently designed experiment, in which conditioned medium containing human pro-ANP was added to the transfected HEK 293 cells expressing either wild-type or R866A mutant corin. Pro-ANP processing was detected in cells expressing wild-type corin but not mutant corin R866A or control parental HEK 293 cells (Fig. 4B). These results indicate that mutation at Arg-866 prevented corin zymogen activation and that rat corin zymogen had no detectable pro-ANP processing activity in these assays.

Effects of Small Molecule Inhibitors on Corin Activation Cleavage—Because the activation cleavage of rat corin occurred at Arg-866, it is likely...
that the corin-activating enzyme is a trypsin-like protease, which favors single basic residues such as Arg and Lys. To test this hypothesis, we examined the effect of a set of small molecule inhibitors in HEK 293 and HL-1 cells expressing rat corin. As shown in Fig. 5, the intensity of the ~40-kDa band was markedly reduced when HEK 293 and HL-1 cells were treated with nonspecific trypsin inhibitors (10 mM benzamidine and 100 μM leupeptin) but not a matrix metalloproteinase inhibitor (20 μM GM6001) or a cysteine protease inhibitor (100 μM ALLM). The results indicate that an unknown trypsin-like protease was responsible for activating a small fraction of corin in HEK 293 and HL-1 cells.

Inhibition of Corin Activation in Tunicamycin-treated Cells—
To assess the role of N-glycosylation in corin activation, stable HEK 293 cells expressing rat corin were cultured with or without tunicamycin, which blocks the protein N-glycosidic linkages by inhibiting the transfer of N-acetylglycosamine 1-phosphate to dolichol monophosphate (32). After a 16-h incubation at 37 °C, the cells were lysed, and protein samples were analyzed by Western blotting under reducing and nonreducing conditions. As shown in Fig. 6, the ~160-kDa corin band was reduced to ~130 kDa in tunicamycin-treated cells, indicating the inhibition of N-glycosylation by tunicamycin. The results were consistent with the data from glycosidase digestion experiments (Fig. 1B). More importantly, the ~40-kDa activated corin protease fragment was not detected under...
The results indicate that inhibited pro-ANP processing by endogenous corin (Fig. 8). Similarly, treatment of HL-1 cells with tunicamycin (Fig. 7, lane 4), tunicamycin was added to pro-ANP-containing medium (Tun added to medium) during a 1-h incubation with HEK 293 cells that were not treated with tunicamycin. Pro-ANP and its derivatives were analyzed by immunoprecipitation and Western blotting (top panel). As a control for rat corin expression and cleavage in HEK 293 cells, cell lysates were prepared. Corin protein (Corin) and its cleaved protease fragment (Corin-p) were analyzed by Western blotting (lower panel).

The presence of tunicamycin in the conditioned medium during the 1-h pro-ANP processing assay did not block corin zymogen activation, as indicated by the presence of the ~40-kDa band in the cell lysate (Fig. 7, lower panel, lane 4). In other controls, no pro-ANP processing was detected in parental HEK 293 cells with or without tunicamycin treatment (Fig. 7, top panel, lanes 5 and 6). Similarly, treatment of HL-1 cells with tunicamycin inhibited pro-ANP processing by endogenous corin (Fig. 8). The results indicate that N-glycosylation is essential for corin zymogen activation and pro-ANP processing activity in HEK 293 and HL-1 cells.

**Importance of N-Glycosylation Sites in the Corin Protease Domain for Zymogen Activation**—Studies of other serine proteases have shown that N-glycosylation sites in the protease domain are important for intracellular processing and zymogen activation (23, 33). The rat corin protease domain has two potential N-glycosylation sites, Asn-968 and Asn-1087, in the protease domain are indicated. B, cleavage of recombinant mutant corin proteins in HEK 293 and HL-1 cells. Wild-type (WT) rat corin and mutants N968S, N1087S, and N968S/N1087S were expressed in HEK 293 (left panel) and HL-1 (right panel) cells. Cell lysates were prepared. Corin protein (Corin) and its cleaved protease fragment (Corin-p) were analyzed by Western blotting under reducing conditions.

In other controls, no pro-ANP processing was detected in parental HEK 293 cells with or without tunicamycin treatment (Fig. 7, top panel, lanes 5 and 6). Similarly, treatment of HL-1 cells with tunicamycin inhibited pro-ANP processing by endogenous corin (Fig. 8). The results indicate that N-glycosylation is essential for corin zymogen activation and pro-ANP processing activity in HEK 293 and HL-1 cells.

**Importance of N-Glycosylation Sites in the Corin Protease Domain for Zymogen Activation**—Studies of other serine proteases have shown that N-glycosylation sites in the protease domain are important for intracellular processing and zymogen activation (23, 33). The rat corin protease domain has two potential N-glycosylation sites at residues Asn-968 and Asn-1087 (Fig. 9A) (21). The corresponding residues in human corin are Ser-903 and Asn-1022, with the latter being a potential N-glycosylation site (3). To examine the functional importance of the two Asn residues in the rat corin protease domain, we made plasmids in which one or both Asn residues were replaced by Ser residues (Fig. 9A). The plasmids were transfected into HEK 293 and HL-1 cells, and recombinant rat corin protein was analyzed by Western blotting under reducing conditions. As shown in Fig. 9B, the activated corin protease fragment was detected from wild-type corin and single mutants.
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N968S and N1087S but not double mutant N968S/N1087S in both HEK 293 and HL-1 cells, suggesting that at least one N-glycosylation site in the protease domain is required for corin zymogen activation.

Cell Surface Expression of Corin Mutants—As a type II transmembrane protease, corin is expressed on the cell surface. We further investigated if the alterations of the N-glycosylation sites in the corin protease domain prevented its cell surface expression. Total cell surface proteins in HEK 293 cells expressing rat wild-type and corin mutants were labeled with biotin. The labeled cell surface proteins were analyzed by precipitation with streptavidin-Sepharose beads followed by Western blotting. Biotin-labeled corin, as represented by three bands of ~170, ~140, and ~40 kDa, respectively, was detected in HEK 293 cells expressing wild-type and mutants N968S and N1087S. In HEK 293 cells expressing double mutant corin N968S/N1087S, both ~150- and ~130-kDa bands were labeled by biotin (Fig. 10, top left panel). As a control for total corin protein expression, the presence of corin protein was confirmed in each sample of cell lysate (Fig. 10, lower panels). As another control for biotinylation of cell surface proteins, Western blotting of GAPDH, a cytoplasmic protein, in streptavidin-pulldown samples (lower, left panel) and cell lysates (lower, right panel) was performed.

DISCUSSION

Corin is a cardiac serine protease and functions as the pro-ANP convertase, essential for maintaining normal blood pressure (2, 12, 34). Like many newly discovered type II transmembrane serine proteases, activation cleavage is required to convert corin zymogen to an active enzyme (19), but the mechanism responsible for the zymogen activation is unknown. In our previous studies of human corin in HEK 293 cells, we consistently observed the corin activity in processing pro-ANP, but we were unable to detect the activated protease fragment by Western blotting, indicating that the number of activated corin molecules must be a low fraction of the overall amount of recombinant corin under the experimental conditions (19).

In this study, we cloned rat corin cDNA and expressed recombinant rat corin in HEK 293 cells. In Western blotting under reducing conditions, a distinct band of ~40 kDa was detected, which likely represented the activated corin protease fragment (Figs. 1 and 2). This prediction was confirmed using an activation cleavage site mutant rat corin R866A. In HEK 293 cells expressing mutant corin R866A, the ~40-kDa band was not detected and neither was pro-ANP processing activity (Figs. 3 and 4), indicating that mutation at Arg-866 prevented corin zymogen activation. Consistent with our previous results, a similar ~40-kDa band was barely detectable in Western blotting of human corin in HEK 293 cells (Fig. 2B). It appeared that corin was activated more readily than human corin in HEK 293 cells, but the reason for this apparent difference is not clear. Rat and human corin activation cleavage sites share identical sequences of ~20 amino acids on the either side of the scissile bond. Thus, other determinants on corin, which may be distant to the activation cleavage site, may influence its activation.

The observed activation cleavage of rat corin was not unique to HEK 293 cells. A similar activated protease fragment from recombinant rat corin was also detected when the protein was expressed in primary rat neonatal cardiomyocytes and murine cardiomyocyte HL-1 cells, which were derived from a murine atrial tumor (26) (Fig. 2). These cells are physiologically relevant, as corin is expressed primarily in cardiomyocytes. At this time, the identity of the enzyme(s) responsible for corin activation in HEK 293 and cardiomyocytes is not known. Most likely, the enzyme is a trypsin-like protease because the activation cleavage was inhibited by small molecule inhibitors for trypsin-like proteases but not by inhibitors for matrix metalloproteinases and cysteine proteases in both HEK 293 and HL-1 cells (Fig. 5).

It was noted that in all three cell types tested in this study, the activated corin represented a small fraction of the total corin molecules, as estimated by densitometry (Fig. 2). Currently, antibodies that recognize native mouse or rat corin are not available. We tested several anti-human corin antibodies that are commercially available by Western blotting and immunoprecipitation but were unable to detect endogenous corin in murine and rat cardiomyocytes and heart tissues. It is possible that under normal circumstance only a small percentage of corin is active in cardiomyocytes. When corin activity is needed, for example, under high blood pressure, corin zymogen activation may be increased to promote the ANP-mediated pathway to lower blood pressure. Consistent with this idea, an early study showed that human corin was expressed predominantly as a zymogen form in the heart (1). Studies also showed that corin mRNA expression was up-regulated in left ventricular myocardium in animal models of heart failure (35, 36). Further identification and isolation of the corin activator will be
important to understand how corin activity is regulated under physiological and pathological conditions.

Previous biochemical studies of corin have suggested that the protein may be glycosylated (20). In this study, we showed that corin is extensively glycosylated, mostly with N-linked oligosaccharides. PNGase F digestion reduced the apparent mass of both rat and human corin, whereas O-glycosidase or sialidase A digestion did not cause any noticeable reduction in the apparent mass of these proteins (Fig. 1). In this regard, corin differs from enteropeptidase that contains a mucin-like domain where O-linked oligosaccharides are abundant (24, 25). Our results indicate that the N-glycosylation is required for corin zymogen activation. Treatment of corin-expressing HEK 293 cells with tunicamycin prevented corin activation and inhibited its pro-ANP processing activity (Figs. 6 and 7). Similar inhibition of pro-ANP processing also was observed in HL-1 cardiomyocytes treated with tunicamycin (Fig. 8). By site-directed mutagenesis, we further showed that the N-glycosylation sites at Asn-968 and Asn-1087 in the protease domain are critical for the activation of matriptase, a type II transmembrane serine protease (26). Asn-968 in the protease domain is important for rat corin zymogen activation but not cell surface expression (Figs. 9 and 10). Our results are consistent with findings in other transmembrane serine proteases. Oberst et al. (23) have shown that an N-glycosylation site in the protease domain is critical for the activation of matriptase, a type II transmembrane serine protease essential for the development of multiple epithelial tissues (37–39). Similarly, Zheng et al. (24, 25) have shown that N-glycans in the enteropeptidase protease domain are important for the apical targeting of this digestive enzyme. Therefore, it appears that N-glycans may have a general role in regulating the biosynthesis and activation of type II transmembrane serine proteases.

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