Functional Analysis of the Transmembrane Domain and Activation Cleavage of Human Corin

DESIGN AND CHARACTERIZATION OF A SOLUBLE CORIN*

Corin is a cardiac transmembrane serine protease. In cell-based studies, corin converted pro-atrial natriuretic peptide (pro-ANP) to mature ANP, suggesting that corin is potentially the pro-ANP convertase. In this study, we evaluated the importance of the transmembrane domain and activation cleavage in human corin. We showed that a soluble corin that consists of only the extracellular domain was capable of processing recombinant human pro-ANP in cell-based assays. In contrast, a mutation at the conserved activation cleavage site, R801A, abolished the function of corin, demonstrating that the activation cleavage is essential for corin activity. These results allowed us to design, express, and purify a mutant soluble corin, EKsolCorin, that contains an enterokinase recognition sequence at the activation cleavage site. Purified EKsolCorin was activated by enterokinase in a dose-dependent manner. Activated EKsolCorin had hydrolytic activity toward peptide substrates with a preference for Arg and Lys residues in the P-1 position. This activity of EKsolCorin was inhibited by trypsin-like serine protease inhibitors but not inhibitors of chymotrypsin-like, cysteine-, or metallo-proteases. In pro-ANP processing assays, purified active EKsolCorin converted recombinant human pro-ANP to biologically active ANP in a highly sequence-specific manner. The pro-ANP processing activity of EKsolCorin was not inhibited by human plasma. Together, our data indicate that the transmembrane domain is not necessary for the biological activity of corin but may be a mechanism to localize corin at specific sites, whereas the proteolytic cleavage at the activation site is an essential step in controlling the activity of corin.

Corin is a mosaic serine protease that was recently identified from the human heart (1, 2). It consists of 1,042 amino acids and contains an integral transmembrane domain near the N terminus. In the extracellular region of corin, there are two frizzled-like cysteine-rich domains, eight low density lipoprotein receptor type A repeats, a scavenger receptor-like cysteine-rich domain, and a C-terminal trypsin-like protease domain. Topologically, corin belongs to the newly defined type II transmembrane serine protease family (3–6), which includes enterokinase (EK) (7), hepsin (8), matriptases (9–12), trypsin-like serine proteases, because corin is the only serine protease identified so far that contains frizzled-like cysteine-rich domains.

Corin mRNA and protein are abundantly expressed in the heart (1, 2), suggesting that corin might have a role in the cardiovascular system. In cell-based experiments, we showed that recombinant human corin mediated the conversion of pro-atrial natriuretic peptide (pro-ANP) and pro-brain natriuretic peptide to mature ANP and brain natriuretic peptide (21), both of which are cardiac hormones important in maintaining normal blood pressure and electrolyte homeostasis (22–24). Corin, however, does not convert pro-C-type natriuretic peptide to mature C-type natriuretic peptide (25), the third member of the natriuretic peptide family (26), which may play a role in angiogenesis and arterial restenosis. The results from these experiments suggest that corin is the pro-ANP/pro-brain natriuretic peptide convertase in the heart. This hypothesis is further supported by additional experiments in which overexpression of an active site mutant corin or transfection of small interfering RNA duplexes directed against the corin gene completely blocked pro-ANP processing in cultured cardiomyocytes (27). To date, however, the reported studies of corin were performed in cell-based experiments, which do not allow elimination of the possibility that other proteins or enzymes might contribute to the observed pro-ANP processing activities. It is important, therefore, to demonstrate directly that corin itself possesses the pro-ANP processing activity by using purified active corin.

To test the hypothesis that purified active corin is able to process pro-ANP, we first examined the importance of the transmembrane domain and activation cleavage in corin for its biological activity. Our results showed that the transmembrane domain is not required for corin to process pro-ANP, but proteolytic cleavage of corin at its conserved activation site is essential. Based on these results, we designed, expressed, and purified a soluble form of human corin and studied its biochemical properties. Our results showed that activated soluble human corin hydrolyzed synthetic peptidic substrates and activated human pro-ANP in a highly sequence-specific manner.

EXPERIMENTAL PROCEDURES

Materials—Cell culture medium, G418, anti-V5 antibody, transfection reagent LipofectAMINE 2000 (Invitrogen), and expression vector pSecTag/FRT/V5-His-ToPO were purchased from Invitrogen. Fetal bovine serum was from SeraCare Life Sciences, Inc. (Oceanside, CA). Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection and maintained at the Core Facility at Berlex Biosciences.
Oligonucleotide primers were synthesized by BIOSOURCE International Inc. (Camarillo, CA). Restriction enzymes and DNA polymerases were obtained from New England Biolabs Inc. (Beverly, MA). Recombinant bovine light chain EK and EK capture beads (EKapture) were from Novagen Inc. (Madison, WI). Chromogenic substrates were purchased from DiaPharma (West Chester, OH). Phenylmethylsulfonyl fluoride and tosyl-lys-chloromethylketone were from Bachem Biosciences Inc. (King of Prussia, PA). Protease inhibitors antipain, pepstatin, bestatin, chymostatin, phosphoramidon, leupeptin, and aprotinin were purchased from Roche Applied Science. Heparin was from U. S. Biochemical Corp. All other chemical reagents were obtained from Sigma.

Expression Vectors—Expression plasmids encoding human wild-type corin (pcDNACorin), active site mutant corin cDNA (pcDNAcorin), human wild-type pro-ANP (pcDNAproANP), and mutant pro-ANPs R98A (pcDNAproANPR98A), R101A (pcDNAproANPR101A), and R102A (pcDNAproANPR102A) were described previously (21, 27). A plasmid vector encoding corin activation cleavage site mutant R801A (pcDNAcorinR801A) was constructed by a PCR-based mutagenesis method (QuiikChange site-directed mutagenesis kit; Stratagene, La Jolla, CA) using pcDNAcorin as a template and oligonucleotide primer 5'–CCG AAT GAA CAA AGC AAT CCT TGG AGG TGC-3'. To construct a plasmid expressing a soluble corin, a cDNA fragment containing nucleotides 463–3219 of human corin cDNA (1) was amplified by PCR and inserted into the expression vector pSEC to yield the plasmid pSECcorin. This plasmid encodes a protein, WTcorin, consisting of the IgG signal peptide at the N terminus followed by a 919-amino acid sequence from the extracellular region of corin (residues 124–1042) and a viral V5 tag at the C terminus (see Fig. 1). To construct a plasmid encoding a soluble corin that can be activated by EK, PCR-based site-directed mutagenesis was performed using plasmid pSECcorin as a template. Nucleotides 2402–2496 (CGA ATG AAC AAA AGG) of human corin cDNA were replaced by nucleotides GAC GAT GAC GAT AAG. The resulting plasmid, pSEC EcKcorin, encodes a soluble corin, EKcorin, with the amino acid sequence DDDDK replacing the original sequence RMNKR at the conserved activation cleavage site (see Fig. 1). All plasmid constructs were verified by restriction enzyme digest and direct DNA sequencing.

Characterization of a Soluble Corin

Expression

HEK 293 cells were cultured in α-minimum essential medium supplemented with 10% fetal bovine serum. Transient transfection was performed using LipofectAMINE 2000 according to the manufacturer’s instructions. The conditioned medium was collected 12–16 h after transfection and subjected to centrifugation at 15,000 rpm to remove cell debris. The cells were lysed in a buffer containing 100 mM Tris-HCl, pH 7.5, and 1% Triton X-100. To analyze pro-ANP processing, the conditioned medium containing recombinant wild-type or mutant pro-ANPs was incubated with purified soluble corin (1.8 μg/ml) at 37 °C for 4 h. Recombinant human pro-ANP and its derivatives in the conditioned medium were immunoprecipitated by an anti-V5 antibody. The protein sample was separated by SDS-PAGE and analyzed by Western blotting using a horseradish peroxidase-conjugated anti-V5 antibody.

Effects of Protease Inhibitors—Effects of protease inhibitors on EKsolCorin were tested in an assay using the chromogenic substrate S-2403. In each experiment, 45 μl of activated EKsolCorin (final concentration of 104 μM) was mixed with 5 μl of an inhibitor (final concentrations ranging from 0.1 μM to 20 μM) and incubated at 37 °C for 30 min. To measure the remaining hydrolytic activity of EKsolCorin, 50 μl of S-2403 (final concentration of 500 μM) was added to the mixture, and the absorbance was measured at 405 nm after 2 h. Each experiment was performed in triplicate and repeated at least twice.

cGMP Assay—To examine the biological activity of corin-processed recombinant ANP, a cGMP assay was performed using an enzyme immunoassay kit (Biotrak; Amersham Biosciences), as described previously (27). In these experiments, synthetic human ANP (Peninsula Laboratories Inc., San Carlos, CA) was used as positive controls under the conditions recommended by the manufacturer. The Km and Vmax values were determined by Lineeweaver-Burk double-reciprocal plot. Each enzymatic assay was carried out in triplicate and repeated at least twice.

RESULTS

Processing of Pro-ANP by Wild Type and a Soluble Corin, WTsolCorin—To examine the importance of the transmembrane domain of corin for its pro-ANP processing activity, we constructed a plasmid that encodes a soluble form of human corin containing all of the extracellular domains (Fig. 1). Recombinant wild-type corin, active site mutant corin S985A, and the soluble corin, WTsolCorin, were expressed transiently in HEK 293 cells. Wild-type corin and active site mutant corin S985A were detected by Western analysis in the cell lysate but not in the conditioned medium (Fig. 2A), consistent with corin being a transmembrane protein. In contrast, WTsolCorin was detected in both the cell lysate and conditioned medium (Fig. 2A), confirming that the soluble corin was secreted from the cells. We determined the activity of these recombinant corins in pro-ANP processing in co-transfection experiments using a plasmid expressing human pro-ANP together with plasmids expressing either wild-type corin, mutant corin S985A, or WTsolCorin. Pro-ANP and its derivatives in the conditioned medium were analyzed by Western blotting. As shown in Fig. 2B, pro-ANP, but not ANP, was detected in the cell lysate. In the conditioned medium, conversion of pro-ANP to ANP was ob-
The conserved activation cleavage site is indicated by an arrow. The disulfide bond (S — S) that connects two polypeptide chains after the activation cleavage is also shown. WT corin, wild-type corin; corin S985A, a mutant corin in which the active-site Ser is replaced by Ala; corin R801A, a mutant corin in which the cleavage site Arg is replaced by Ala; WTsolCorin, a soluble corin that consists of a signal peptide sequence derived from human Ig heavy chain followed by the extracellular domains of corin; EKsolCorin, a soluble corin that contains an EK recognition sequence (DDDDK) at the activation cleavage site.

**Fig. 1.** A schematic presentation of the domain structure of wild-type and mutant corins. The transmembrane domain (TM), frizzled-like cysteine-rich domains (Fz), low density lipoprotein receptor type A repeats (LDLR), scavenger receptor cysteine-rich domain (SR), and protease catalytic domain (Catalytic) with active-site residues histidine (H), aspartate (D), and serine (S) are indicated. The conserved activation cleavage sequence Arg-Ile-Leu-Val of wild-type corin contains an EK recognition site. The human corin protein contains a conserved activation cleavage sequence Arg-Ile-Leu-Gly-Gly at residues 801–805 (1). To examine the functional importance of the activation cleavage of human corin, we constructed a plasmid expressing a mutant that would have impaired activation (activation cleavage site mutant corin R801A; Fig. 1). Co-transfection experiments were performed using a plasmid expressing pro-ANP together with plasmids expressing either wild-type corin, active site mutant corin S985A, or activation cleavage site mutant corin R801A. Recombinant corin proteins were present in the cell lysate but not in the conditioned medium (Fig. 3A). Recombinant pro-ANP was detected in the cell lysate after transfection with the pro-ANP expressing plasmid (Fig. 3B, *left panel*). In the conditioned medium, processing of pro-ANP to ANP was observed when cells were co-transfected with a plasmid encoding wild-type corin but not those encoding mutant corins S985A and R801A or a control vector (Fig. 3B, *right panel*). The results demonstrate that proteolytic cleavage at Arg801 is required for the pro-ANP processing activity of corin. The fact that wild-type corin was capable of processing pro-ANP in cell-based transfection experiments indicates that some corin molecules must be activated. In the Western analysis (Figs. 2 and 3), however, we were unable to detect the cleaved protease fragment from wild-type corin or mutant corin S985A, which is expected to migrate as a band at ~35 kDa under reducing conditions. This would suggest that the number of activated corin molecules is a low fraction of the overall amount of corin.

**Effects of Thrombin, Factor Xa, and Kallikrein on Corin Activation**—To examine whether plasma-derived serine proteases could activate corin, we examined the effects of thrombin, blood clotting factor Xa, and kallikrein on corin activation. Recombinant human wild-type corin was stably expressed in HEK 293 cells. Purified human plasma thrombin, factor Xa, or kallikrein was added to the cell culture and incubated at 37 °C for 1 h. The cell lysates were prepared and analyzed by Western blotting under reducing conditions. The results showed that recombinant human corin was not cleaved in cells treated with either thrombin, factor Xa, or kallikrein (data not shown). We also added thrombin, factor Xa, or kallikrein directly to 293 cell lysates containing recombinant corin and analyzed the

**Fig. 2.** Processing of pro-ANP by soluble corin, WTsolCorin. *A*, transfection experiments were performed in HEK 293 cells using plasmids expressing human wild-type corin (WT), active site mutant corin S985A (S985A), the soluble corin WTsolCorin (WTsol), or a control vector (vec) as described under “Experimental Procedures.” Recombinant corin proteins in cell lysate (left panel) and the conditioned medium (right panel) were analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody. *B*, to examine pro-ANP processing by these forms of corin, HEK 293 cells were co-transfected with a plasmid expressing pro-ANP (pProANP) together with plasmids expressing wild-type corin (WT), mutant corin S985A (S985A), the soluble corin WTsolCorin (WTsol), or a control vector (vec). Recombinant pro-ANP and its derivatives in cell lysates (left panel) and conditioned medium (right panel) were analyzed by Western blotting using an anti-V5 antibody.

Observed when cells were transfected with the pro-ANP expressing plasmid together with plasmids expressing wild-type corin or WTsolCorin. As controls, the cells were co-transfected with the pro-ANP expressing construct and a plasmid expressing either active site mutant corin S985A or a control vector. Without the presence of a plasmid expressing an active corin, no pro-ANP processing was detected, showing that the cells do not contain any detectable endogenous pro-ANP processing activity. The results indicate that the transmembrane domain of corin is not necessary for the pro-ANP processing activity in this cell-based assay.
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Expression and Purification of Soluble EK-activatable Corin, EKsolCorin—To produce an active soluble corin for further biochemical studies, we designed a mutant corin, EKsolCorin, in which an EK recognition sequence (DDDDK) (7) was used to replace the activation cleavage sequence of human corin (RMNKR) (1). Stable cell lines expressing EKsolCorin were established. The conditioned medium from these cells was collected, and EKsolCorin was purified by nickel affinity and ion exchange chromatography. SDS-PAGE followed by Coomassie Blue staining and Western blotting using an anti-V5 antibody showed that EKsolCorin migrated as a single band at ~150 kDa under reducing conditions and at ~145 kDa under nonreducing conditions (Fig. 4). The results were consistent with the calculated mass of ~108 kDa for EKsolCorin, which also contained 19 potential N-linked glycosylation sites (1). HPLC-based gel filtration chromatography showed that the purified EKsolCorin had a purity of ~98% (data not shown). The N-terminal sequence of EKsolCorin was confirmed by protein sequencing. The purified protein was quantified by UV spectrometry at 280 nm using an extinction coefficient (1 mg/ml) of 1.45 calculated from the protein sequence.

Activation of EKsolCorin by EK—Purified EKsolCorin protein was activated with increasing concentrations of recombinant EK at 25 °C for 2 h. The protein samples were analyzed by SDS-PAGE under reducing and nonreducing conditions followed by Western analysis using an anti-V5 antibody. As shown in Fig. 5, EKsolCorin was activated by recombinant EK in a dose-dependent manner. Under nonreducing conditions, EKsolCorin appeared as a single band of ~145 kDa. Under reducing conditions, activated EKsolCorin migrated as two fragments: an N-terminal propeptide (~115 kDa) and a C-terminal protease domain (~35 kDa) (Fig. 1). Because the V5 tag is located at the C terminus, the anti-V5 antibody detected only the C-terminal protease domain once EKsolCorin was activated (Fig. 5).

Enzymatic Properties of Soluble Corin—Substrate specificity and kinetic constants of EKsolCorin for a panel of selected peptide substrates were determined using purified and activated EKsolCorin. As controls, EKsolCorin without EK activation and a buffer that was treated with EK were included. Initial experiments showed that activated EKsolCorin, but not thezymogen form of EKsolCorin or the buffer control, hydrolyzed some chromogenic substrates (S-2222, S-2302, S-2366, S-2403, and S-2444). No significant hydrolysis of substrates S-2238 (H-D-Ile-Pro-Arg-pNA2HCl), S-2251 (H-D-Val-Leu-Lys-pNA2HCl), S-2266 (H-D-Val-Leu-Arg-pNA2HCl), S-2288 (H-D-Ile-Pro-Arg-pNA2HCl), S-2248 (pyroGlu-Pro-Val-pNA), and S-2765 (N-o-Z-D-Arg-Gly-Arg-pNA2HCl) was detected (K_m values were >50 mM). This profile was very different from that of recombinant bovine light chain EK used in this study (data not shown), indicating that the observed activity was not derived from any potential contamination of EK. We further determined the kinetics of EKsolCorin-mediated hydrolysis of the substrates S-2403, S-2366, S-2302, S-2222, and S-2444.
The kinetic constants were determined as described under “Experimental Procedures.” The data are presented as the means ± S.D. from at least three independent experiments.

| Substrate | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|------------|-------|-----------|---------------|
| S-2403, pyroGlu-Phe-Lys-pNA-HCl | 1.28 ± 0.46 | 0.47 ± 0.10 | 389.1 ± 74.2 |
| S-2366, pyroGlu-Pro-Arg-pNA-HCl | 3.52 ± 1.07 | 0.48 ± 0.21 | 138.6 ± 56.7 |
| S-2302, H-D-Pro-Phe-Arg-pNA-2HCl | 2.95 ± 0.90 | 0.23 ± 0.04 | 79.9 ± 14.0 |
| S-2222, Bz-Ile-Glu-(γ-OR)-Gly-Arg-pNA-HCl | 1.92 ± 0.53 | 0.09 ± 0.01 | 50.8 ± 8.5 |
| S-2444, pyroGlu-Gly-Arg-pNA-HCl | 16.0 ± 3.77 | 0.40 ± 0.08 | 25.0 ± 3.4 |

The effects of protease inhibitors on the hydrolytic activity of EKsolCorin were determined as described under “Experimental Procedures.” The data are presented as the means ± S.D. from at least three independent experiments.

| Inhibitor | Concentration | Inhibition |
|-----------|---------------|------------|
| Phosphoramidon | 1 mM | 0 |
| EDTA | 20 mM | 0 |
| Pepstatin | 100 μM | 0 |
| Bestatin | 100 μM | 0 |
| Chymostatin | 100 μM | 0 |
| TPKC* | 1 mM | 16.2 ± 1.0 |
| Tosyl-Lys-chloromethylketone | 1 mM | 59.1 ± 1.9 |
| Benzanidine | 10 mM | 67.1 ± 3.1 |
| Phenylmethylsulfonyl fluoride | 100 μM | 97.4 ± 0.3 |
| Antipain | 100 μM | 68.6 ± 1.4 |
| Leupeptin | 100 μM | 76.5 ± 4.7 |
| Aprotinin | 1 mM | 97.0 ± 1.6 |
| Soybean trypsin inhibitor | 1 mM | 98.5 ± 2.3 |

* TPKC, Tosyl-Phe-chloromethylketone.

The results showed that EKsolCorin cleaved peptide substrates with either Arg or Lys at the P-1 position. For example, the $K_m$ values were 1.28 ± 0.46 and 3.52 ± 1.07 mM for S-2403 and S-2366, respectively. Pro, Phe, and Gly residues appeared to be preferred at the P-2 position, and a pyro-Glu residue, an analog of small neutral amino acids, seemed to be preferred at the P-3 position. The overall results are consistent with the corin cleavage sequence (Thr-Ala-Pro-Arg ↓ Ser) in human pro-ANP (28).

Effects of Protease Inhibitors—To examine the effects of protease inhibitors on corin, the hydrolysis of the chromogenic substrate S-2403 by activated EKsolCorin was monitored in the presence of various protease inhibitors. As shown in Table II, the activity of EKsolCorin was inhibited dose-dependently by nonselective trypsin-like serine protease inhibitors including benzamidine, phenylmethylsulfonyl fluoride, antipain, leupeptin, aprotinin, tosyl-Lys-chloromethylketone, and soybean trypsin inhibitor. In contrast, the activity of EKsolCorin was not inhibited by inhibitors of chymotrypsin-like serine proteases such as chymostatin (100 μM) and Tosyl-Phe-chloromethylketone (100 μM) or metallo- and cysteine-protease inhibitors such as phosphoramidon (1 mM), EDTA (20 mM), pepstatin (100 μM), and bestatin (100 μM). These data are consistent with corin being a trypsin-like serine protease based on its protein sequence.

Processing of Pro-ANP by EKsolCorin—To demonstrate the pro-ANP processing activity of purified and activated EKsolCorin, recombinant human wild-type pro-ANP and mutant pro-ANP R98A, R101A, and R102A were expressed in HEK 293 cells. Conditioned media were collected and incubated with purified EKsolCorin. Processing of pro-ANP was analyzed by Western blotting. As shown in Fig. 6, activated EKsolCorin converted wild-type pro-ANP and mutant pro-ANPs R101A and R102A, but not mutant pro-ANP R98A, to mature peptides. An additional weak band of ~20 kDa was also observed in these experiments, which represented a degradation fragment derived from EKsolCorin (data not shown). This fragment is biologically inactive as measured by cell-based cGMP assay (see below). As controls, recombinant EK or thezymogen form of EKsolCorin did not cleave recombinant pro-ANPs. The results are consistent with our previous finding that corin cleaves human pro-ANP specifically at residue Arg98 but not at the adjacent arginine residues 101 or 102. The results also show that the introduction of an EK recognition sequence did not alter the sequence specificity of corin for its physiological substrate.

The Activity of EKsolCorin-processed Recombinant ANP—The biological function of ANP is mediated through its receptor that has guanylyl cyclase activity. Binding of ANP to its receptor stimulates the guanylyl cyclase activity, leading to production of intracellular cGMP. To determine whether EKsolCorin-processed recombinant ANP is biologically active, a BHK cell-based cGMP assay was performed. As shown in Fig. 7, little cGMP-stimulating activity was detected in the conditioned medium from 293 cells containing either pro-ANP or activated EKsolCorin. The cGMP-stimulating activity was significantly increased when activated EKsolCorin was added to the conditioned medium containing wild-type pro-ANP or mutant pro-ANPs R101A and R102A. In contrast, there was no significant increase in the cGMP-stimulating activity when activated EKsolCorin was added to the conditioned medium containing mutant pro-ANP R98A. These data are consistent with the results showing that mutation at Arg98 in pro-ANP prevented the conversion of pro-ANP to mature ANP (Fig. 6) and demonstrate that EKsolCorin-processed recombinant ANP is biologically active.

Effect of Human Plasma on the Pro-ANP Processing Activity of EKsolCorin—To examine whether there are potential corin inhibitors present in human plasma, we tested the effects of human plasma on EKsolCorin-mediated processing of pro-ANP. The conditioned medium containing recombinant human pro-ANP was incubated with activated EKsolCorin in the presence of increasing concentrations of pooled human plasma. Processing of pro-ANP was analyzed by Western blotting. As shown in Fig. 8, no endogenous pro-ANP convertase activity was detected in the pooled human plasma. Activated EKsolCorin converted pro-ANP to ANP. When up to 75% human plasma was included in the reactions, we observed a reduction in corin-derived bands, suggesting that human plasma may interfere with the Western blots. Importantly, however, there was no inhibition of the pro-ANP convertase activity, indicating that EKsolCorin remained active in the presence of plasma. Furthermore, no inhibition of corin activity was observed when the experiment was performed in the presence of 5 or 50
units/ml of heparin (data not shown). The results indicate the absence of corin inhibitors in human plasma.

**DISCUSSION**

In this study, we examined the importance of the transmembrane domain and the activation cleavage of human corin for its activity in the processing of pro-ANP. We showed that a soluble corin that consists of only the extracellular domain was capable of processing pro-ANP in cell-based assays, indicating that the transmembrane domain is not necessary for corin activity. In contrast, a mutation at the conserved activation cleavage site, R801A, abolished the function of corin, demonstrating that the one-chain form of corin is not active but needs to be cleaved into the two-chain form for the protease domain to be active. These results led us to design, express, and purify a soluble corin, EKsolCorin, that contains an EK recognition sequence at the conserved activation site. We showed that purified EKsolCorin was activated by EK and that activated EKsolCorin was capable of hydrolyzing peptide substrates with a preference for Arg and Lys residues at the P-1 position.

As a member of the type II transmembrane serine protease family, corin contains an integral transmembrane domain near its N terminus. The importance of this transmembrane domain in corin had not previously been examined. For many soluble serine proteases such as blood clotting enzymes, binding to the cell surface through either phospholipids or integral membrane co-factors greatly enhances the rate of their catalytic reactions (29–31). For corin, however, the transmembrane domain appears to be dispensable. In transfection experiments, both membrane-bound wild-type corin and the soluble corin, WTsolCorin, had similar activities in processing human pro-ANP (Fig. 2). The results are consistent with reports of other type II transmembrane serine proteases including EK (32), matriptases (11, 33, 34), hepsin (35, 36), spinesin (16), human airway trpsin-like protease (17), and polyserase-I (20), showing that soluble enzymes lacking the transmembrane domain can be catalytically active. It appears, therefore, that the main func-
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600 μM in culture medium had little effect on the processing of pro-ANP mediated by either recombinant corin in transfected HEK 293 cells (21) or endogenous corin in cultured cardiomyocytes (27). Such different effects of soybean trypsin inhibitor, which has a molecular mass of ~20 kDa, on soluble and membrane forms of proteases have also been reported for other type II transmembrane serine proteases. For example, soybean trypsin inhibitor has been shown to inhibit the activity of soluble hepsin in chromogenic substrate assays but have little effect on that of cell-surface hepsin in factor VII activation assays (44, 45). It is possible that the presence of the transmembrane domain hinders the access of large molecule protease inhibitors to the protease active site, making these transmembrane proteases more resistant to protease inhibition. Thus, the transmembrane domain of the type II transmembrane serine proteases may serve as a regulatory mechanism in their interactions with cognate inhibitors. At the present time, it is not known whether physiological corin inhibitors exist. In pro-ANP processing assays, we found that human plasma had little effect on the activity of soluble corin (Fig. 8), indicating the absence of any corin inhibitors in human plasma. Together with the results from mutant corin R801A, it is most likely that corin activity is regulated physiologically by the activation cleavage rather than by inhibition of its activity.

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