III.   Results

III.1   Design and Biochemical Characterization of Soluble Corin

Corin is a cardiac enzyme that was discovered in a functional genomics study. Structurally, corin belongs to the family of TTSPs (Yan *et al.*, 1999). Like many genomic studies, the challenge was to identify the biological function of this new enzyme. When the study for my thesis was initiated in 2000, corin had already been shown to process the cardiac peptide hormone pro-ANP in cell-based assays (Yan *et al.*, 2000). There was, however, no direct experimental evidence to demonstrate that corin is the physiological pro-ANP convertase. In the past, other serine proteases such as trypsin, kallikrein and thrombin also were reported to process pro-ANP *in vitro* (Currie *et al.*, 1984a; Currie *et al.*, 1984b; Michener *et al.*, 1987). Because the initial functional studies of corin by Yan *et al.* were conducted in cell-based experiments, it was possible that the corin-mediated pro-ANP processing may not be direct. In principle, corin could activate another unknown protease present in the cell culture, which in turn activates pro-ANP. Furthermore, the enzymatic properties of corin had not been thoroughly characterized.

The goal of the first part of my thesis was to design, express, purify, and characterize a soluble corin protein that contains all extracellular domains. As a first step, we examined whether the transmembrane domain (see section III.1.1) and the conserved activation cleavage site at amino acid residue Arg801 (see section III.1.2) are required for the functional activity of corin in pro-ANP processing.

III.1.1.   Requirement of the transmembrane domain of corin for its function

The TTSPs are characterized by a single span transmembrane domain at the N-terminus and an extracellular region that contains two or more structural modules including the protease domain at the C-terminus. The transmembrane domain of corin is located between amino acids 46-66, which includes many hydrophobic residues. Clusters of hydrophobic amino acids, however, often reduce the water solubility of the protein, making protein purification more difficult. One way to circumvent this problem is to generate a soluble corin that lacks the transmembrane domain. To assess the feasibility of this approach, we needed to determine if the transmembrane domain is essential for the biological activity of corin.
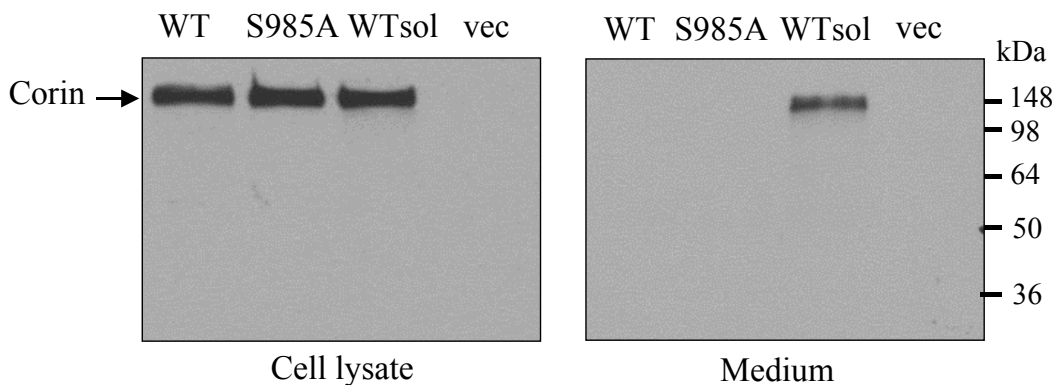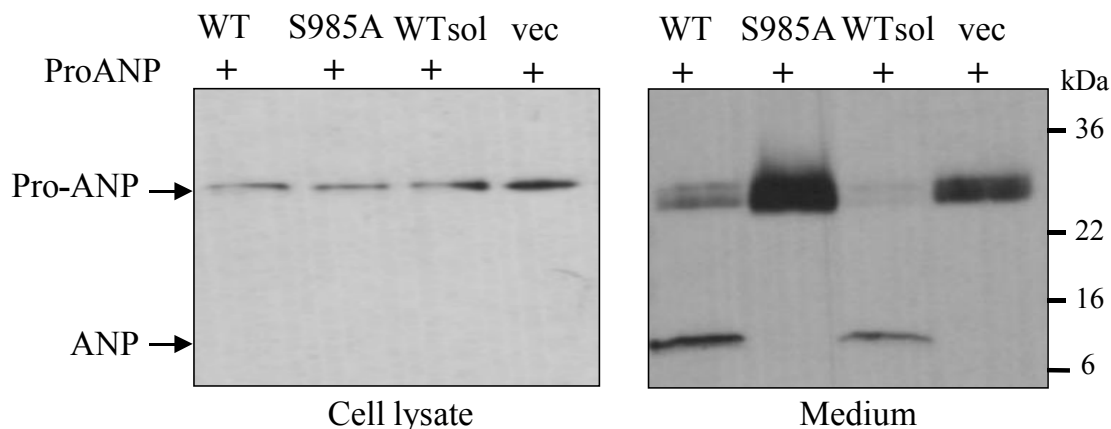
We constructed a plasmid that encodes a soluble form of human corin (WTsolCorin) containing all the extracellular domains (Fig. 3.1A). Recombinant WT corin, active site mutant corin S985A, and the soluble corin, WTsolCorin, were transiently expressed in HEK 293 cells. WT corin and the active site mutant corin S985A were detected by Western analysis in the cell lysate but not in the conditioned medium (Fig. 3.1B), consistent with corin being a transmembrane protein. In contrast, WTsolCorin was detected in both the cell lysate and conditioned medium (Fig. 3.1B), confirming that the soluble corin was secreted from the cells. We then examined the activity of these recombinant corins in pro-ANP processing in a co-transfection experiment. HEK 293 cells were co-transfected with a plasmid expressing human pro-ANP and plasmids expressing either WT corin, mutant corin S985A, or WTsolCorin. Pro-ANP and its derivatives in the conditioned medium were analyzed by Western blotting.

As shown in Fig. 3.1C, pro-ANP, but not ANP was detected in the cell lysate, indicating that pro-ANP was not processed by an intracellular enzyme. In the conditioned medium, conversion of pro-ANP to ANP was observed when cells were transfected with the pro-ANP expressing plasmid together with plasmids expressing WT corin or WTsolCorin (Fig. 3.1C). As negative controls, the cells were also co-transfected with the pro-ANP-expressing construct and a plasmid expressing either active site mutant corin S985A or a parental vector.

In the absence of a plasmid expressing an active corin, no pro-ANP processing was detected in the conditioned medium (Fig. 3.1C), 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. These findings encouraged a design of a soluble form of corin for future studies of this enzyme.

A
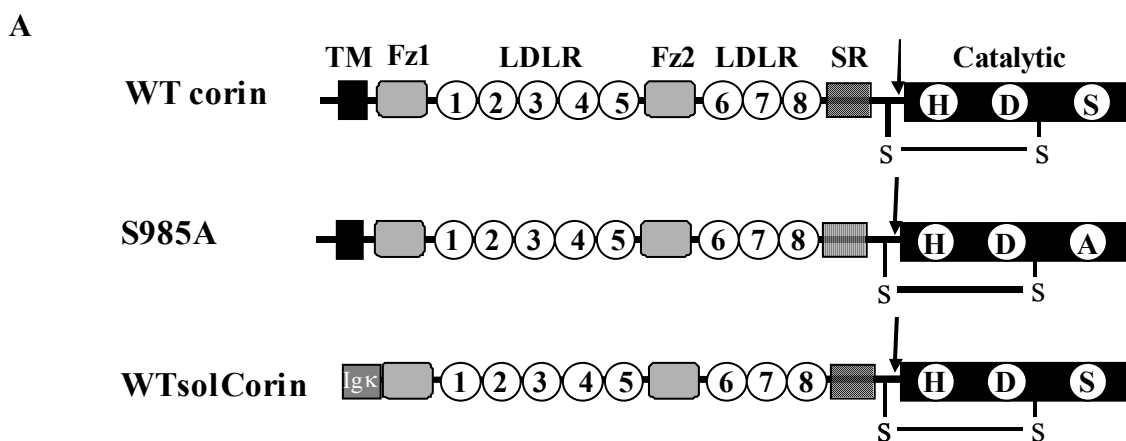
Figure 3.1. Processing of pro-ANP by soluble corin, WTsolCorin. **(A)** Schematic presentation of the domain structure of WT corin and mutant corins. The transmembrane domain (*TM*), Fz cysteine-rich domains (*Fz*), LDLR repeats (*LDLR*) are numbered from 1 to 8, SR cysteine-rich domain (*SR*), and protease catalytic domain (*Catalytic*) with active-site residues histidine (*H*), aspartate (*D*) and serine (*S*) are indicated. The conserved activation cleavage 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; WTsolCorin, a soluble corin that consists of a signal peptide sequence derived from human Igκ chain followed by the extracellular domains of corin.

B

WT   S985A WTsol   vec   WT   S985A WTsol   vec   kDa

Corin →

- 148
- 98
- 64
- 50
- 36

Cell lysate   Medium

C

WT   S985A WTsol   vec   WT   S985A WTsol   vec   kDa

ProANP   +   +   +   +   +   +   +   +

- 36

Pro-ANP →

- 22
- 16

ANP →

- 6

Cell lysate   Medium

Figure 3.1. Processing of pro-ANP by soluble corin, WTsolCorin. (B) Transfection experiments were performed in HEK 293 cells using plasmids expressing human WT corin (*WT*), active site mutant corin S985A (*S985A*), the soluble WTsolCorin (*WTsol*), or a control vector (*vec*) as described under "Methods".   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. **(C)** To examine pro-ANP processing by these forms of corin, HEK 293 cells were co-transfected with a plasmid expressing pro-ANP (*ProANP*) together with plasmids expressing WT corin (*WT*), mutant corin S985A (*S985A*), the soluble 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.

III.1.2   Pro-ANP processing activity of activation site mutant R801A

Like most serine proteases of the trypsin superfamily, corin is predicted to be expressed as an inactive zymogen that requires proteolytic cleavage at a specific activation site. In human corin protein, a conserved activation cleavage sequence Arg801-Ile802-Leu803-Gly804-Gly805 was identified by amino acid alignment with other trypsin-like serine proteases (Yan *et al.,* 1999). To examine the functional importance of the conserved activation cleavage sequence in human corin, we constructed a plasmid expressing an activation cleavage site mutant corin, R801A, that is predicted to prevent the cleavage at this site (Fig. 3.2A). Co-transfection experiments were performed using a plasmid expressing pro-ANP together with plasmids expressing either WT 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, as determined by Western analysis (Fig. 3.2B). Recombinant pro-ANP was detected in the cell lysate after transfection with the pro-ANP expressing plasmid (Fig. 3.2C, *left panel*). In the conditioned medium, processing of pro-ANP to ANP was observed when cells were co-transfected with a plasmid encoding WT corin but not those encoding mutant corins S985A and R801A or a control vector (Fig. 3.2B, *right panel*). The results demonstrate that proteolytic cleavage at Arg801 is required for the pro-ANP processing activity of corin.

The fact that WT 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. 3.1 and 3.2), however, we were unable to detect the cleaved protease fragment from WT 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 small fraction of the overall amount of corin protein. It is unknown, however, which enzyme(s) is responsible for this limited activation of corin in the cell-based experiments.
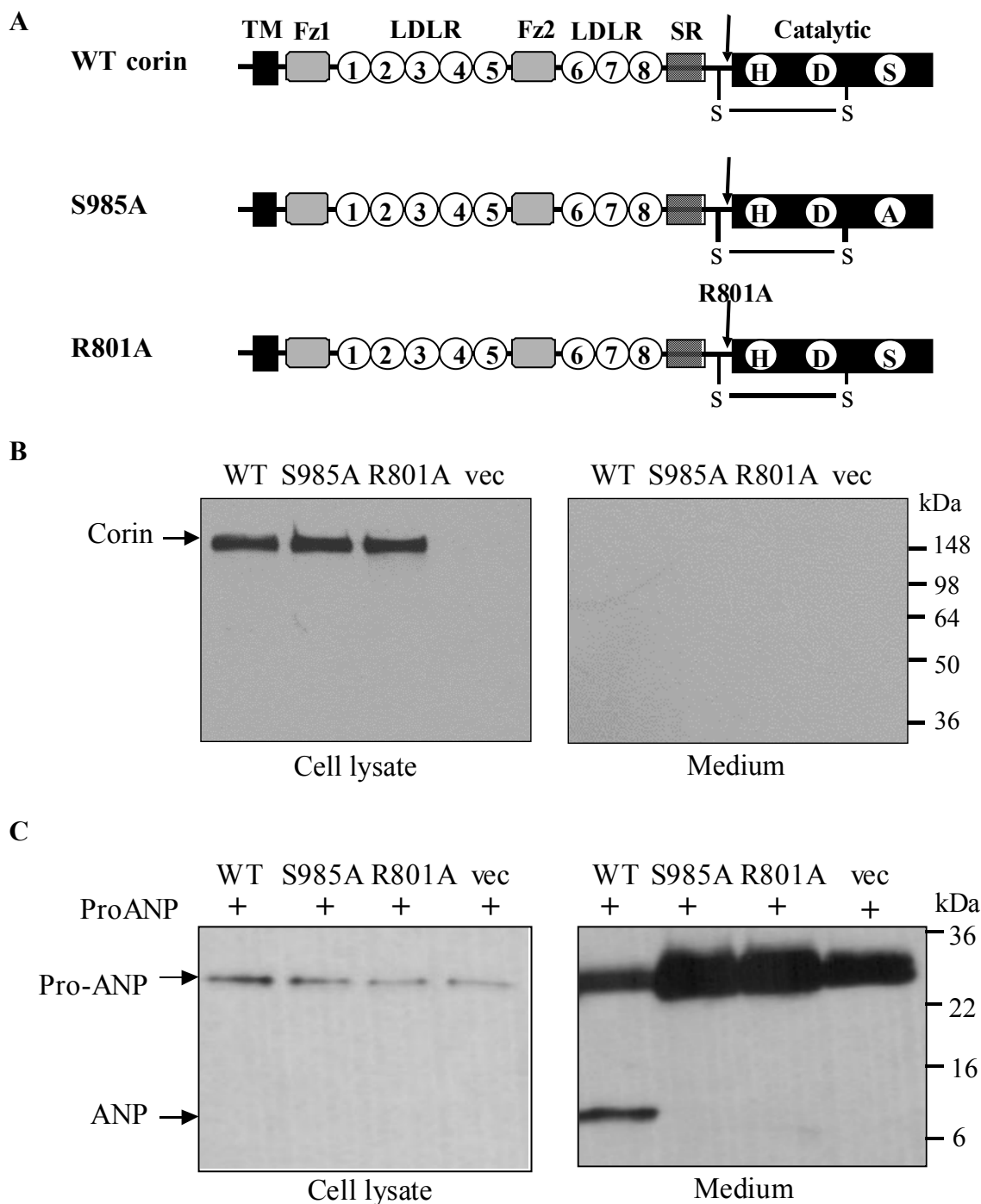
Figure 3.2. Processing of pro-ANP by activation cleavage mutant corin R801A. (**A**) Schematic presentation of the domain structure of WT corin and mutant corins. Co-transfection experiments were performed in HEK 293 cells using a plasmid expressing human pro-ANP (*ProANP*) together with plasmids expressing WT corin (*WT*), active site mutant corin S985A (*S985A*), activation cleavage site mutant corin R801A (*R801A*) or a control vector (*vec*) as described under "Methods". Recombinant corin proteins (**B**), pro-ANP and its derivatives (**C**) in cell lysate (*left panels*) and the conditioned media (*right panels*) were analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody.

III.1.3   Effects of thrombin, factor Xa and kallikrein on corin activation

To examine if plasma-derived serine proteases could possibly activate corin, a study was performed in our laboratory to examine the effects of thrombin, blood clotting factor Xa, and kallikrein on corin activation. Recombinant human WT 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. Cell lysates were prepared and analyzed by Western blotting under reducing conditions. The results showed that recombinant human corin was not cleaved at the predicted activation site in cells treated with either thrombin, factor Xa or kallikrein (data not shown). We also added thrombin, factor Xa or kallikrein directly to HEK 293 cell lysates containing recombinant corin and analyzed the corin protein by SDS/PAGE and Western blotting. Again, no activation cleavage of corin was detected in these experiments (data not shown). Thus, it is unlikely that thrombin, factor Xa, and kallikrein could act as the corin activator.

### III.1.4   Generation and characterization of EKsolCorin

In the two previous sections we showed that the transmembrane domain is not required for the pro-ANP processing activity of corin, and that activation cleavage at Arg801 is necessary to convert the corin zymogen to a functional protease. To produce an active recombinant soluble corin for further biochemical studies, we designed a mutant corin, EKsolCorin, that lacked 123 amino acids on its N-terminal end, which were substituted by an Igκ signal sequence. In EKsolCorin, an EK recognition sequence (DDDDK) was used to replace the activation cleavage sequence RMNKR of human corin at residues 797-801 (Yan *et al.*, 1999). Because EK cleaves this sequence in a highly specific manner (Anderson *et al.*, 1977), the EK recognition sequence is commonly used for zymogen activation or release of fusion tags from recombinant proteins. To facilitate purification and detection of the EKsolCorin protein, a viral V5 tag and 6xHis tag are included on the C-terminus.

III.1.4.1   Expression, purification and activation of EKsolCorin protein

We established stable HEK 293 cell lines that secreted recombinant EKsolCorin to final concentrations of ~2 mg/L in conditioned medium. The conditioned medium was collected and EKsolCorin was purified by nickel affinity and ion exchange chromatography. The purified protein was quantified by UV spectrophotometry at 280 nm using an extinction coefficient (1 mg/mL) of 1.45 calculated from the protein sequence. The two-step purification of 7 L of conditioned medium yielded ~7 mg EKsolCorin protein.

SDS/PAGE analysis 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 non-reducing conditions (Figs. 3.3B and C). The results were consistent with the calculated mass of ~108 kDa for EKsolCorin, which also contains 19 potential N-linked glycosylation sites (Yan *et al.*, 1999). HPLC-based gel filtration chromatography showed that the purified EKsolCorin appeared as a single peak and had a purity of >98 % (Fig. 3.3D). The N-terminal sequence of EKsolCorin was confirmed by protein sequencing.
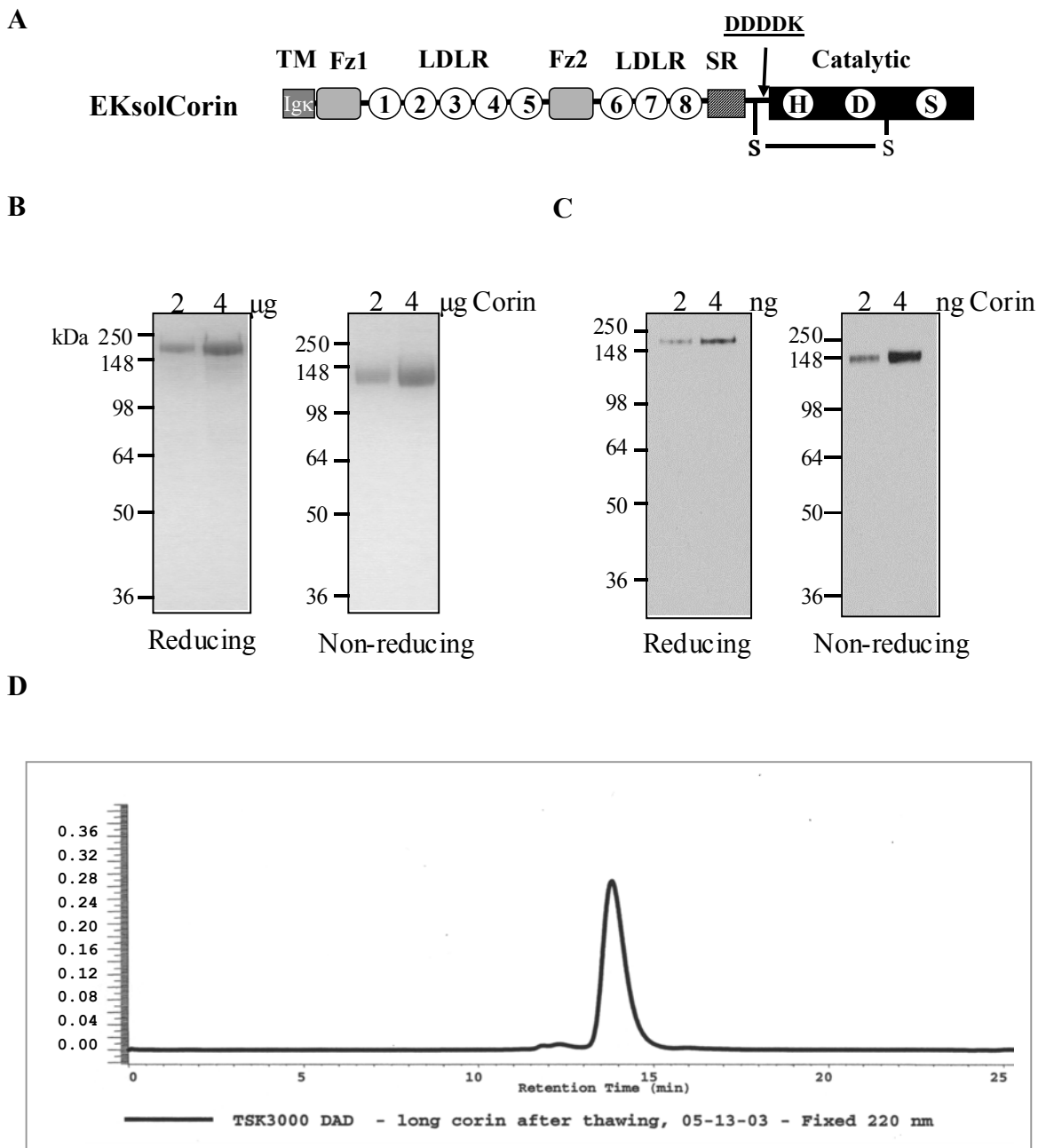
Figure 3.3. Analysis of purified EKsolCorin protein. The soluble corin, EKsolCorin, was stably expressed in HEK cells and purified by affinity and ion exchange chromatography as described under "Methods". **(A)** Schematic structure of EKsolCorin. **(B)** Protein samples (2 and 4 μg) were separated by SDS-PAGE under reducing and non-reducing conditions and stained with Coomassie blue. **(C)** In a separate experiment, 2 and 4 ng of purified EKsolCorin were analyzed by SDS-PAGE under reducing and non-reducing conditions followed by Western blotting using an anti-V5 antibody. **(D)** A sample of ~10 μg purified EKsolCorin was analyzed by analytical SEC.

III.1.4.2   Dose-dependent activation of EKsolCorin by EK

Purified EKsolCorin protein was activated with increasing concentrations of recombinant EK at 25 °C for 2 h as described under "Methods". Protein samples were analyzed by SDS-PAGE under reducing and non-reducing conditions followed by Western analysis using an anti-V5 antibody. As shown in Fig. 3.4, EKsolCorin was activated by recombinant EK in a dose-dependent manner. An amount of 10 units/mL EK was needed to completely activate the EKsolCorin protein. Under non-reducing conditions, EKsolCorin appeared as a single band of ~145 kDa (*right panel*). Under reducing conditions, activated EKsolCorin migrated as two fragments, an N-terminal propeptide (~115 kDa) and a C-terminal protease domain (~35 kDa). 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 (*left panel*). These observations are consistent with the presence of a disulfide bond between Cys790 and Cys912, which connects the protease domain with the propeptide after the proteolytic cleavage at DDDDK (residues 797-801) of EKsolCorin (Fig. 3.3A).
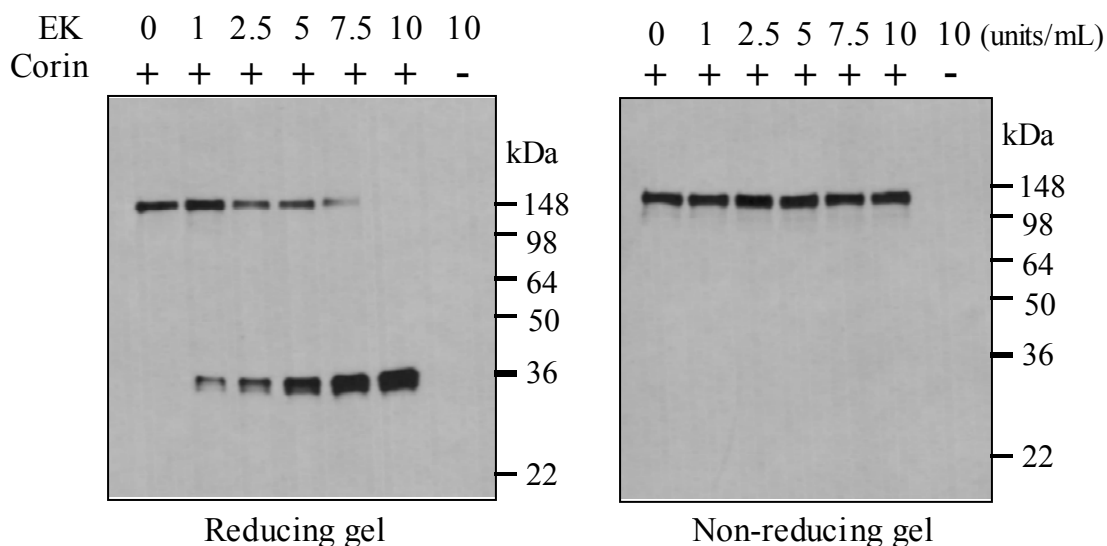

Figure 3.4. Dose-dependent activation of EKsolCorin by EK. Purified EKsolCorin was incubated with increasing concentrations of recombinant bovine EK at 25 °C for 2 h as described under "Methods". Protein samples were analyzed by SDS-PAGE under reducing (*left panel*) and non-reducing (*right panel*) conditions followed by Western blotting using an anti-V5 antibody.

III.1.4.3   Catalytic activity of EKsolCorin

III.1.4.3.1   Screening for small molecule substrates for EKsolCorin

To identify suitable small molecule substrates to study the kinetic properties of corin, a panel of commercially available chromogenic substrates was screened as described under "Methods". Table 2.1 lists names and peptide sequences of the substrates tested. The selection of the substrates was based on the amino acid sequence at the cleavage site of corin substrates pro-ANP (Thr95-Ala96-Pro97-Arg98↓Ser99) and pro-BNP (Arg99-Ala100-Pro101-Arg102↓Ser103), another natriuretic peptide processed by corin in cell-based assays (Yan *et al.*, 2000). We also included substrates that were unlikely to be cleaved by corin such as kallikrein or elastase substrates.

For this assay, fixed final concentrations for the activated enzyme and substrate were used. Two controls included were a non-activated corin sample and an assay buffer control that underwent the same activation and EK removal procedure as the activated corin batch. Fig. 3.5 shows the rates of substrate conversion by EKsolCorin in mOD/min, which was measured at 405 nm over 30 min. To choose substrates for the determination of K_m and k_{cat} values of corin, we looked for substrates that were converted with highest rates by corin but had only minimal readings for non-activated corin and the buffer control. According to these criteria, we selected the substrates S-2222, S-2302, S-2366, S-2403 and S-2444 for detailed enzyme kinetic studies.

A

B
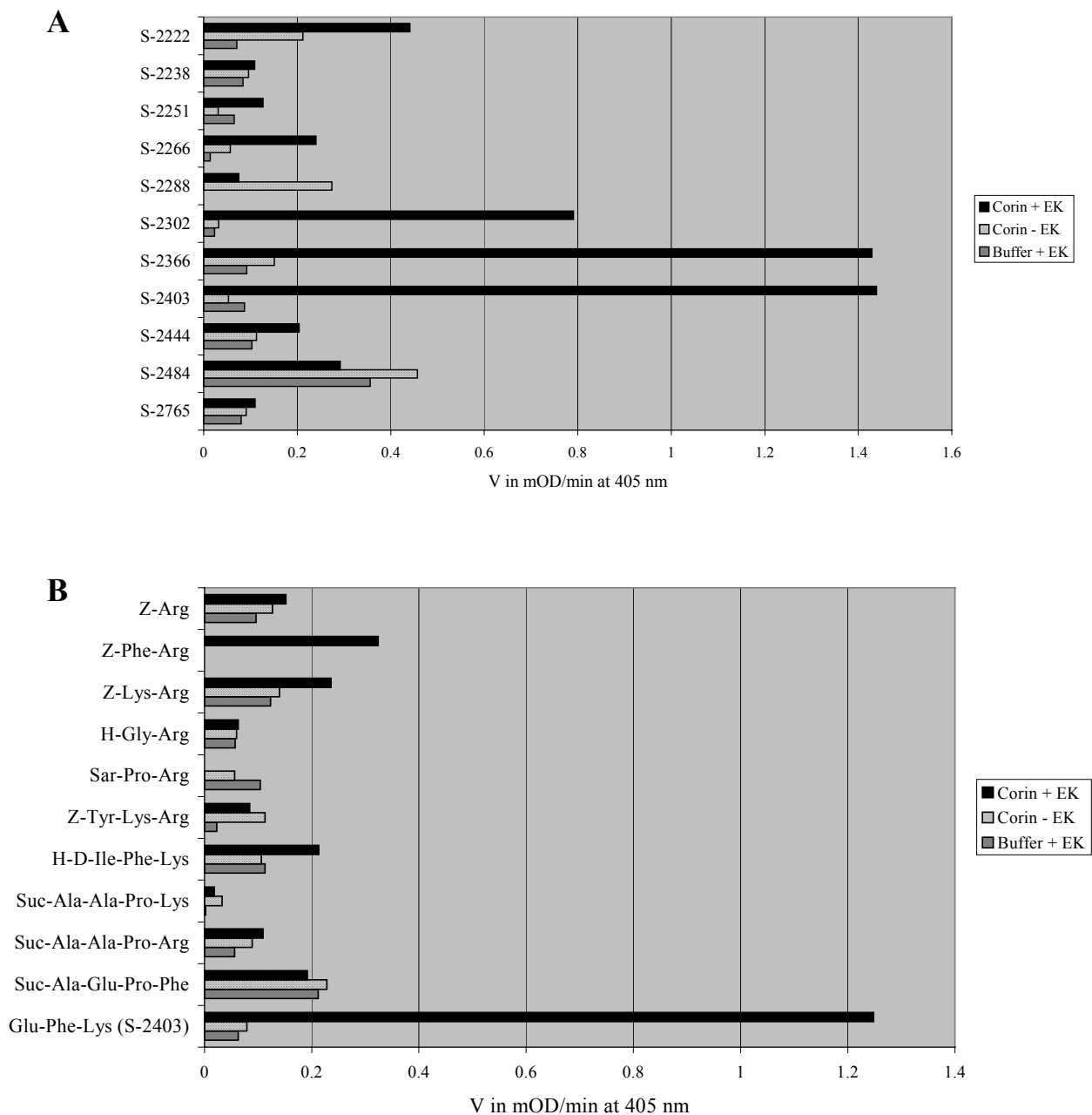
Figure 3.5. Selection of small molecule substrates for enzyme kinetic studies. Activated EKsolCorin protein (*Corin+EK*) at a final concentration of 40 nM was used to select a panel of chromogenic substrates from Chromogenix **(A)** and Bachem Biosciences **(B)**. For all substrates, the final concentration was 500 μM. The kinetic rate was measured at 405 nm wavelength in 2-min intervals over 30 min. Purified non-activated EKsolCorin (*Corin–EK*) and a buffer control that had been treated with EK (*Buffer+EK*) were used as negative controls.

III.1.4.3.2   Determination of kinetic constants and substrate specificity of EKsolCorin

Enzymes can perform up to several million catalytic reactions per second. To determine the maximum speed of an enzymatic reaction, the substrate concentration is increased until a constant rate of product formation is achieved. This is the maximum velocity (V_{max}) of the enzyme. Since the substrate concentration at V_{max} cannot be measured exactly, enzymes are characterized by the substrate concentration at which the rate of reaction is half of its maximum. This substrate concentration [S] is called the Michaelis-Menten constant (K_m). Another valuable constant that describes the catalytic reaction of an enzyme is k_{cat}, or turnover number. The turnover number is the number of converted substrate molecules per enzyme molecule per second. The value for the ratio of k_{cat}/K_m provides the best estimate, how an enzyme performs when [S] is below K_m, which is the case for most physiological processes.

Kinetic constants of EKsolCorin-mediated hydrolysis for the selected peptide substrates S-2222, S-2302, S-2366, S-2403 and S-2444 (see section III.1.4.3.1) were determined using purified, activated EKsolCorin. For the assay, different concentrations of substrates ranging from 0.125 to 10 mM were prepared and mixed with purified EKsolCorin. As negative controls, EKsolCorin without EK activation and a buffer that was treated with EK were included. The initial rate of substrate conversion was measured at 405 nm wavelength. The results were plotted as a graph of initial rate or velocity (V) against substrate concentration [S]. As an example, Fig. 3.6A shows the relation between the rate of the corin-catalyzed hydrolysis of substrate S-2366 and the concentration of S-2366. For each data point, V for activated EksolCorin was corrected by subtracting the averaged background reading of the two negative controls. Then the reciprocal of V (1/V) was plotted versus the reciprocal of the [S] (1/[S]), known as the Lineweaver-Burk plot. As shown in Fig. 6B, the Lineweaver-Burk plot yields a linear graph that has a slope of K_m/V_{max} and an intersection with the ordinate at $1/V_{max}$. V_{max} values (converted from mOD/min to μM/s) were used to calculate the k_{cat} value using the equation $k_{cat}= V_{max}/[E]$, where [E] is the enzyme concentration.

A

B
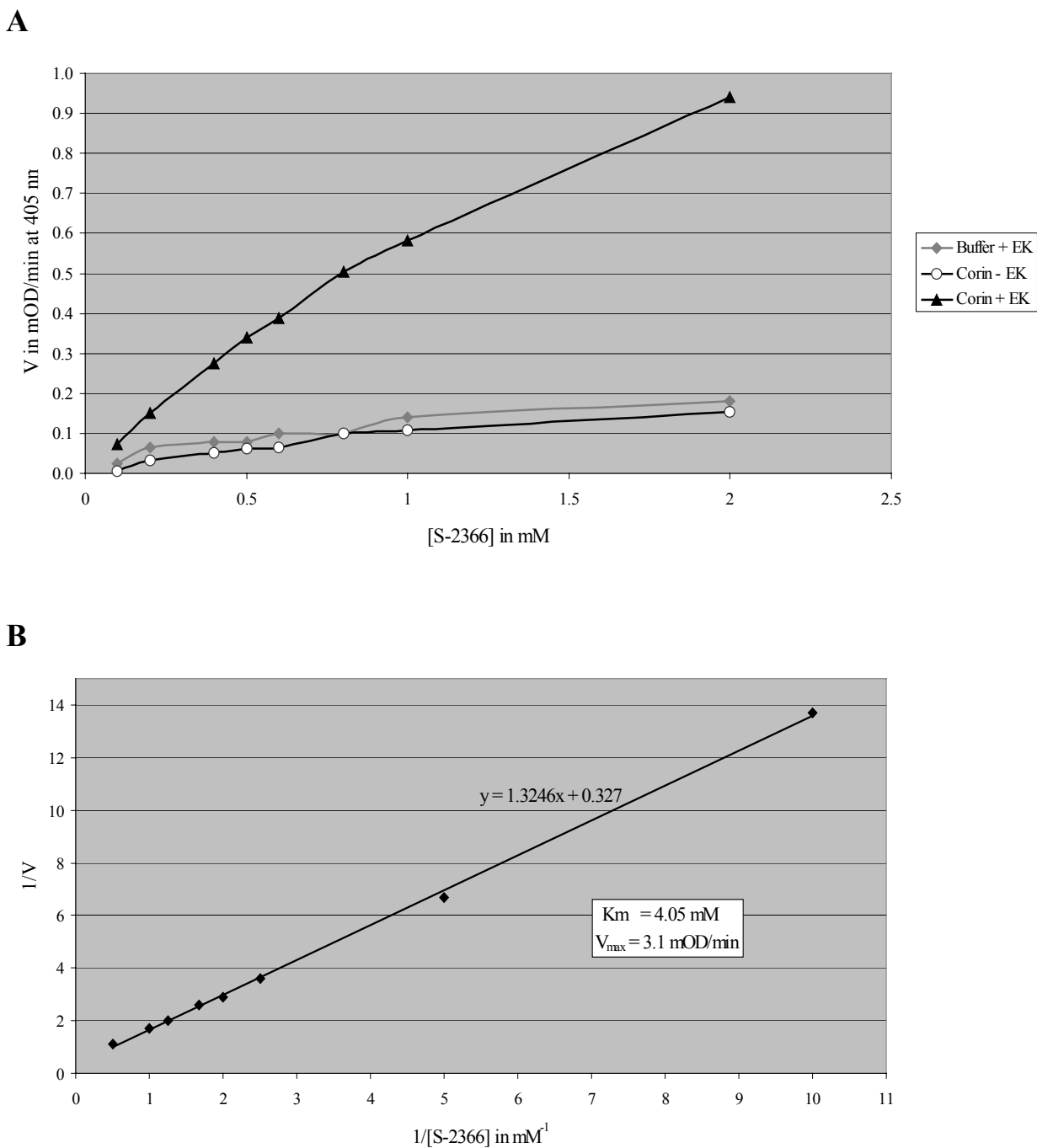
Figure 3.6. Determination of K_m values for the corin-catalyzed hyrdrolysis of substrate S-2366 (pyroGlu-Pro-Arg-pNA·HCl). The initial rate of hydrolysis of S-2366 (0.125 to 2 mM) by corin was tested in a chromogenic assay using purified and activated EKsolCorin (*Corin+EK*). Controls included were non-activated EKsolCorin (*Corin-EK*) and EK-treated buffer (*Buffer+EK*). Data are presented in a Michealis-Menten diagram (**A**) and a double-reciprocal or Lineweaver-Burk plot (**B**).

To confirm the results described in section III.1.4.3.1, the kinetic assays were also performed for substrates S-2238 (H-D-Phe-Pip-Arg-pNA·2HCl), S-2251 (H-D-Val-Leu-Lys-pNA·2HCl), S-2266 (H-D-Val-Leu-Arg-pNA·2HCl), S-2288 (H-D-Ile-Pro-Arg-pNA·2HCl), S-2484 (pyroGlu-Pro-Val-pNA), and S-2765 (N-α-Z-D-Arg-Gly-Arg-pNA·2HCl). Again, no significant hydrolysis of these substrates by corin was detected (K_m values >50 mM).

The results showed that EKsolCorin cleaved peptide substrates with either Arg or Lys at the P1 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 P2 position, and a pyro-Glu residue, an analog of small neutral amino acids, was preferred at the P3 position. The overall results are consistent with the corin cleavage sequence in human pro-ANP (Thr-Ala-Pro-Arg↓Ser) (Koller and Goeddel, 1992).

Table 3.1. Kinetic parameters of activated EKsolCorin for selected chromogenic substrates

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1}·s^{-1})
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

Kinetic constants were determined as described under "Methods". The data are presented as the mean ± standard deviation (S.D.) from at least three independent experiments.

III.1.4.4   Effects of protease inhibitors on EKsolCorin

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 3.2, the activity of EKsolCorin was inhibited dose-dependently by non-specific trypsin-like serine protease inhibitors including benzamidine, PMSF, antipain, leupeptin, aprotinin, TLCK, 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 TPCK (100 µM), or metallo- and cysteine-protease inhibitors such as phosphoramidon (1 mM), EDTA (20 mM), pepstatin (100 µM), and bestatin (100 µM).   This data is consistent with corin being a trypsin-like serine protease based on its protein sequence.

Table 3.2. Effects of protease inhibitors on the hydrolytic activity of EKsolCorin.

Inhibitor	Concentration	% Inhibition
Phosphoramidon	1 mM	0
EDTA	20 mM	0
Pepstatin	100 μM	0
Bestatin	100 μM	0
Chymostatin	100 μM	0
TPCK	1 mM	16.2 ± 1.0
	100 μM	0
TLCK	1 mM	59.1 ± 1.9
	100 μM	6.7 ± 3.1
Benzamidine	10 mM	97.4 ± 0.3
	100 μM	47.4 ± 6.0
PMSF	10 mM	96.1 ± 3.1
	100 μM	5.7 ± 0.4
Antipain	100 μM	68.6 ± 1.4
	10 μM	19.5 ± 0.5
Leupeptin	100 μM	76.5 ± 4.7
	10 μM	24.1 ± 0.1
Aprotinin	1 μM	97.0 ± 1.6
	0.1 μM	70.5 ± 1.4
Soybean trypsin inhibitor	1 μM	96.5 ± 1.3
	0.1 μM	88.4 ± 1.6

Effects of protease inhibitors on the hydrolytic activity of EKsolCorin were determined as described under "Methods." The data are presented as the mean ± S.D. from at least three independent experiments.

III.1.4.5   Processing of pro-ANP by purified EKsolCorin

Previously, corin had been shown to process pro-ANP in cell-based experiments (Yan *et al.*, 2000). In such experiments, the possibility that other proteases might also contribute to the observed pro-ANP processing can not be excluded completely. Here we wanted to show directly that purified and activated EKsolCorin indeed has the pro-ANP processing activity. If EKsolCorin cleaves pro-ANP, we also wanted to show that the corin-mediated pro-ANP processing is sequence-specific. In the atrium, pro-ANP is primarily cleaved at Arg98 to yield the 28-amino acid mature ANP (Fig. 3.8A), which is the principal circulation form of ANP. In other tissues such as kidney, brain, and testis, alternatively processed forms of ANP have also been reported. For example, pro-ANP was cleaved at Ala95 in the testis and at Arg101 or Arg102 in the brain (Pandey *et al.*, 1987; Pandey and Orgebin-Christ, 1991; Shiono *et al.*, 1986). At this time, the physiological significance of the reported alternative processing remains unknown.

To demonstrate the pro-ANP processing activity of purified and activated EKsolCorin, recombinant human WT pro-ANP and mutant pro-ANPs 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. 3.7B, activated EKsolCorin converted WT 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 the zymogen 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 (Yan *et al.*, 2000). The data also show that the introduction of an EK recognition sequence did not alter the sequence specificity of corin for its substrate pro-ANP.

A

$$Thr^{95}-Ala^{96}-Pro^{97}-Arg^{98}-Ser^{99}-Leu^{100}-Arg^{101}-Arg^{102}-Ser^{103}$$
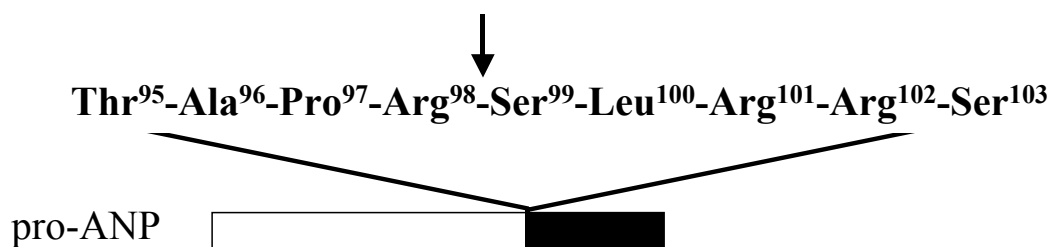
pro-ANP

B
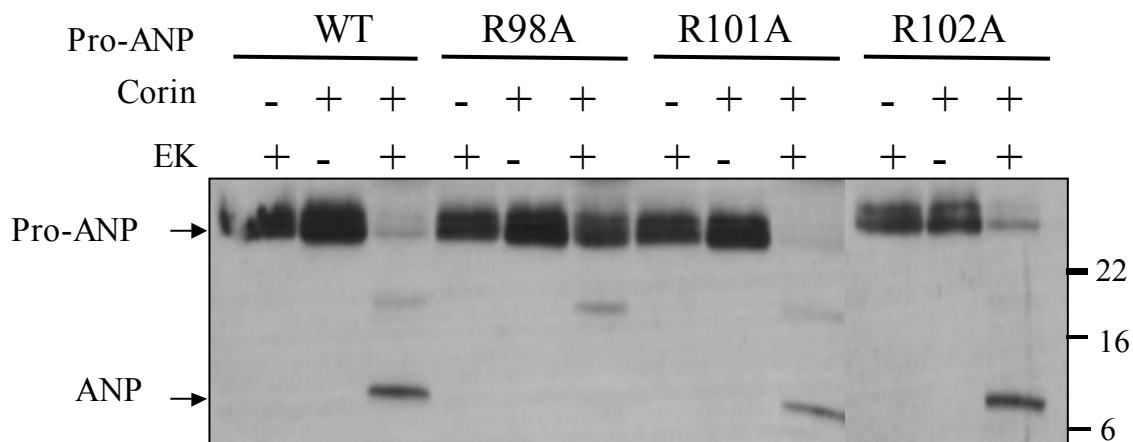
Figure 3.7 Processing of WT and mutant pro-ANPs by EKsolCorin. **(A)** A schematic structure of human pro-ANP is shown. The activation cleavage site at Arg98 is indicated by an arrow. **(B)** Processing of human WT pro-ANP (*WT*) and mutant pro-ANPs R98A, R101A, and 102A by activated EKsolCorin was analyzed by SDS-PAGE under reducing conditions followed by Western analysis using an anti-V5 antibody. As controls, recombinant EK and the zymogen form of EKsolCorin were included in the experiment.

III.1.4.6   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 if EKsolCorin-processed recombinant ANP is biologically active, a BHK cell-based cGMP assay was performed.

As shown in Fig. 3.8, little cGMP-stimulating activity was detected in the conditioned medium from HEK 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 WT 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. 3.7B) and demonstrate that EKsolCorin-processed recombinant ANP is biologically active.
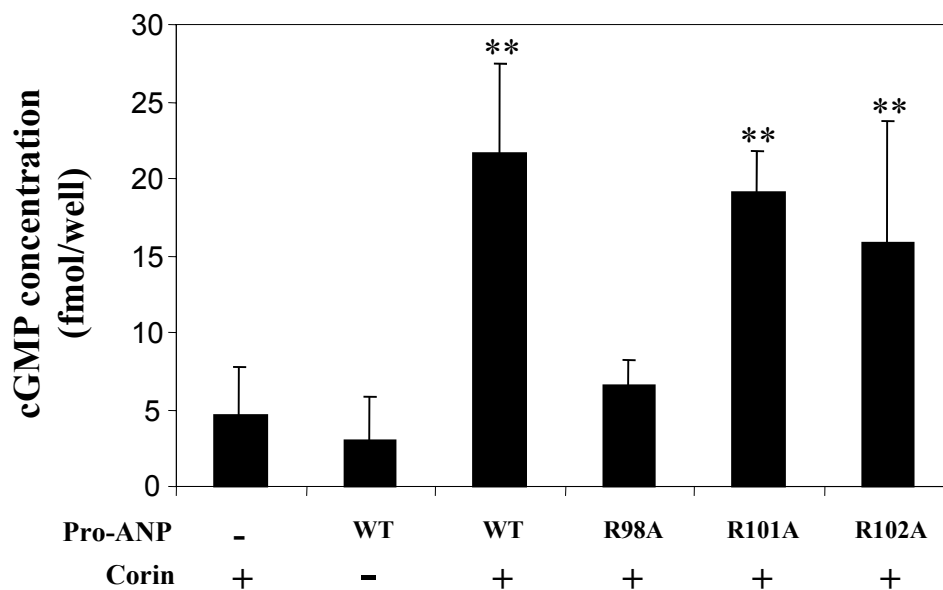
Figure 3.8. Stimulation of intracellular cGMP production. The conditioned media containing WT pro-ANP (*WT*) and mutant pro-ANP R98A (*R98A*), R101A (*R101*A),or R102A (*R102A*) were incubated with activated EKsolCorin (*corin*) at 37 °C for 2 h, then added to BHK cells cultured in 96-well plates and incubated at 37 °C for 10 min. As controls, the cells were also treated with the conditioned medium containing only WT pro-ANP or activated EKsolCorin. The cells were lysed by addition of a buffer containing 2 % dodecyl trimethylammonium and 50 mM sodium acetate, pH 5.8. The intracellular concentrations of cGMP in BHK cells were measured with the Biotrak EIA kit as described under "Methods". The intracellular concentrations of cGMP in untreated BHK cells, which were minimal, were subtracted as the background. Each experimental condition was assayed in triplicate.

III.1.4.7   Effects of human plasma on EKsolCorin activity

Plasma from human blood contains a variety of serpins. Some serpins such as AT-III are present in human plasma in concentrations of up to 2.5 μM. Members of the serpin family act to control major proteolytic cascades in blood coagulation, (AT-III and heparin cofactor II), fibrinolysis (α-2-antiplasmin and plasminogen activator inhibitor-1), inflammation (α-1-antitrypsin), and complement activation (C1-inhibitor). In addition, other serine proteases that are not present in human plasma can be inhibited by plasma serpins *in vitro*. The inhibitory effect of some serpins, AT-III in particular, can be enhanced more than one thousand times in the presence of heparin. At this time, it is not known if there is an inhibitor present in plasma that inhibits the activity of corin.

To examine the effect of human plasma on the pro-ANP processing activity of corin, we incubated purified, activated EKsolCorin (40 nM) with 0, 25, 50, and 75 % human plasma in the absence or presence of heparin (5 or 50 units/mL). Negative controls used were non-activated EKsolCorin and an EK-treated buffer, which were incubated with 0 and 75 % plasma.   Conditioned medium containing recombinant pro-ANP was incubated with EKsolCorin in a reaction mixture containing human plasma at 37 °C for 4 h. Corin protein, pro-ANP and its derivatives were analyzed by immunoprecipitation with an anti-V5 antibody, followed by SDS-PAGE and Western blotting with an HRP-conjugated anti-V5 antibody (Fig. 3.9). The results demonstrated that plasma in a concentration of up to 75 % does not inhibit the pro-ANP processing activity of corin (data not shown). Only activated EKsolCorin converts pro-ANP to ANP, whereas non-activated EKsolCorin or EK-treated buffer did not process pro-ANP. No difference was observed between plasma samples containing 5 units (not shown) or 50 units/mL of heparin. However, it was observed that with an increasing concentration of plasma in the sample, the total amount of detected protein was reduced. We suspect that the high protein content of plasma interferes with the antibody binding during the immunoprecipitation. The data indicate that the activity of corin is not regulated by serpins derived from human plasma.
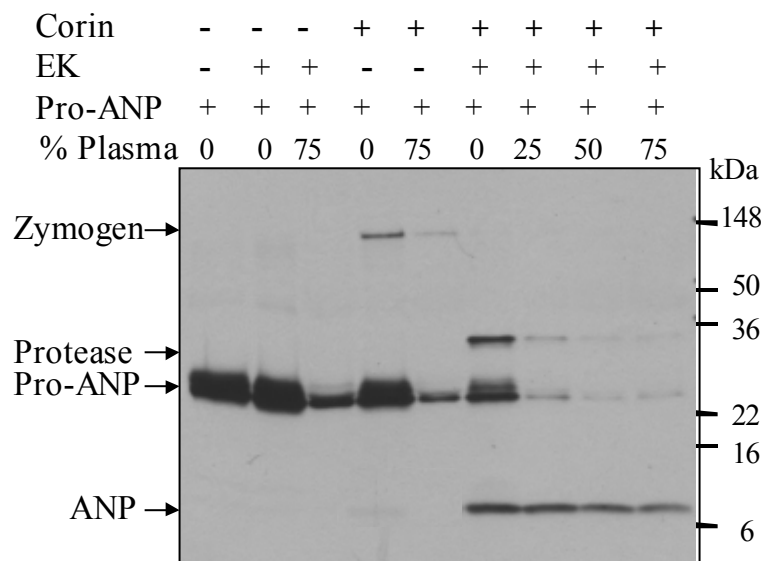
Figure 3.9. Effect of human plasma on the pro-ANP processing activity of EKsolCorin. Purified, activated EKsolCorin protein (*Corin*) was incubated with 0 to 75 % plasma containing heparin (50 units/mL) as described under "Methods". Functional activity of EKsolCorin was tested by incubating corin-plasma samples with conditioned medium containing pro-ANP for 4 h at 37 °C. Corin, pro-ANP and ANP proteins were immunoprecipitated, and analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody. Pro-ANP processing activity of activated EKsolCorin was not reduced in the presence of up to 75 % plasma containing heparin. In control samples, EKsolCorin zymogen (*-EK*) and EK-treated buffer (*+EK*), no ANP formation was observed.

III.2   Requirement of the Propeptide of Corin for Pro-ANP Processing

Previous studies have shown that non-catalytic domains in the extracellular region of several other TTSPs are important for their biological functions. For example, Lu *et al.* have reported that a soluble bovine EK consisting of only the protease domain is catalytically active toward small peptide substrates but fails to activate trypsinogen, indicating that the propeptide of EK plays a role in recognizing trypsinogen (Lu *et al.*, 1997). The propeptide of corin comprises five modular domains but their functional significance was not known. To determine the importance of the propeptide of corin for pro-ANP processing activity, we generated another soluble form of corin, EKshortCorin, that contains only the catalytic domain and examined its activity.

III.2.1   Expression and purification of EKshortCorin

We designed a soluble corin protein, EKshortCorin, that contains the amino acids 787-1042 of the human corin sequence. This region comprises the catalytic domain as well as the residues Cys790 and Cys912, which form a disulfide bond between the catalytic domain and the propeptide (Fig 3.10A). The expression vector, pSECEKshortCorin, encodes a protein that contains an Igκ signal peptide at the N-terminus followed by the corin protease domain and a V5 viral tag and 6xHis tag at the C-terminus. Like EKsolCorin described in preceding sections, EKshortCorin also contains an EK cleavage site (DDDDK) at the conserved activation site, which allows the activation of the zymogen by EK.

Recombinant EKshortCorin protein was expressed in stably transfected HEK 293 cells. The expression level of EKshortCorin in the conditioned medium was significantly lower than that for EKsolCorin (~0.5 vs. ~2 mg/L). The conditioned medium was collected and purified by nickel affinity chromatography. Protein yield was estimated by SDS-PAGE followed by Western blotting using an anti-V5 antibody and a V5-tagged protein of known concentration

(data not shown). Approximately 1 mg of EKshortCorin protein was purified from 5.7 L of the conditioned medium. In SDS-PAGE followed by silver staining (Fig. 3.10B) and Western blotting (Fig. 3.10C), EKshortCorin appeared as a double band at ~38 kDa under reducing conditions. The observed size is consistent with the calculated mass of ~33 kDa for EKshortCorin, which also contains one potential glycosylation site. Protein sequence analysis indicated that there is an alternative proteolytic cleavage site in the Igκ signal peptide, which may explain the double band on SDS-PAGE.
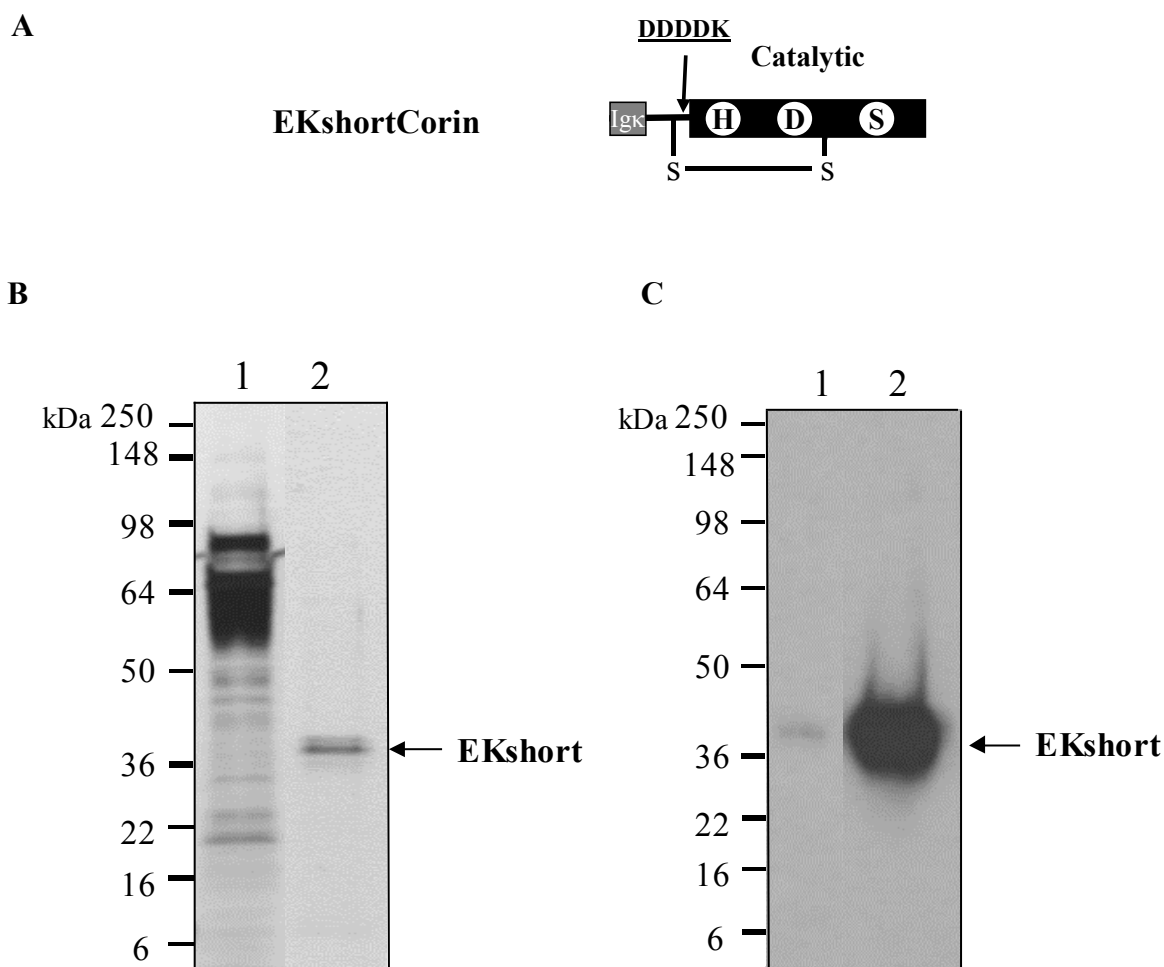
Figure 3.10. Analysis of purified EKshortCorin protein. The soluble corin, EKshortCorin, was stably expressed in HEK cells and purified from the conditioned medium by affinity chromatography, as described under "Methods." (**A**) Schematic structure of EKshortCorin. Samples of 15 μL of conditioned medium containing EKsolCorin protein (*lane 1*) and 2 μL of purified EKshortCorin protein (*lane 2*) were separated by SDS-PAGE under reducing conditions and visualized by Silver staining (**B**) and Western blotting using an anti-V5 antibody (**C**).

III.2.2   Pro-ANP processing activity of EKshortCorin

To assess the importance of the propeptide of corin in pro-ANP processing, we tested the two soluble corin proteins, EKsolCorin and EKshortCorin, side by side in a pro-ANP processing assay as described under "Methods". Purified EKsolCorin and EKshortCorin proteins were activated with increasing amounts of EK and then incubated with the conditioned medium containing recombinant pro-ANP. The protein samples were separated by SDS-PAGE in the presence of the reducing agent β-ME. Corin, pro-ANP and its derivatives were detected by Western blot analysis using an anti-V5 antibody.

As shown in Fig. 3.11B and C, zymogen forms of EKsolCorin and EKshortCorin migrate as single bands of ~150 kDa and ~38 kDa, respectively. When treated with EK, corin zymogens were converted to a ~35 kDa band in a dose-dependent manner. This band is consistent with the predicted size for the corin protease domain under reducing conditions. As indicated in Fig. 3.11A, corin contains a disulfide bond between Cys790 and Cys912. After proteolytic activation by EK at residue 801, the disulfide bond connects the protease domain to its propeptide. Under reducing conditions in the presence of β-ME, the V5-tagged C-terminal fragments can be visualized using an anti-V5 antibody, whereas the N-terminal fragments were not detected. Conversion of pro-ANP (~24 kDa) to ANP (~7 kDa) was detected in the presence of the activated EKsolCorin but not activated EKshortCorin. These data indicate that the propeptide of corin is required for its interaction with the substrate pro-ANP.
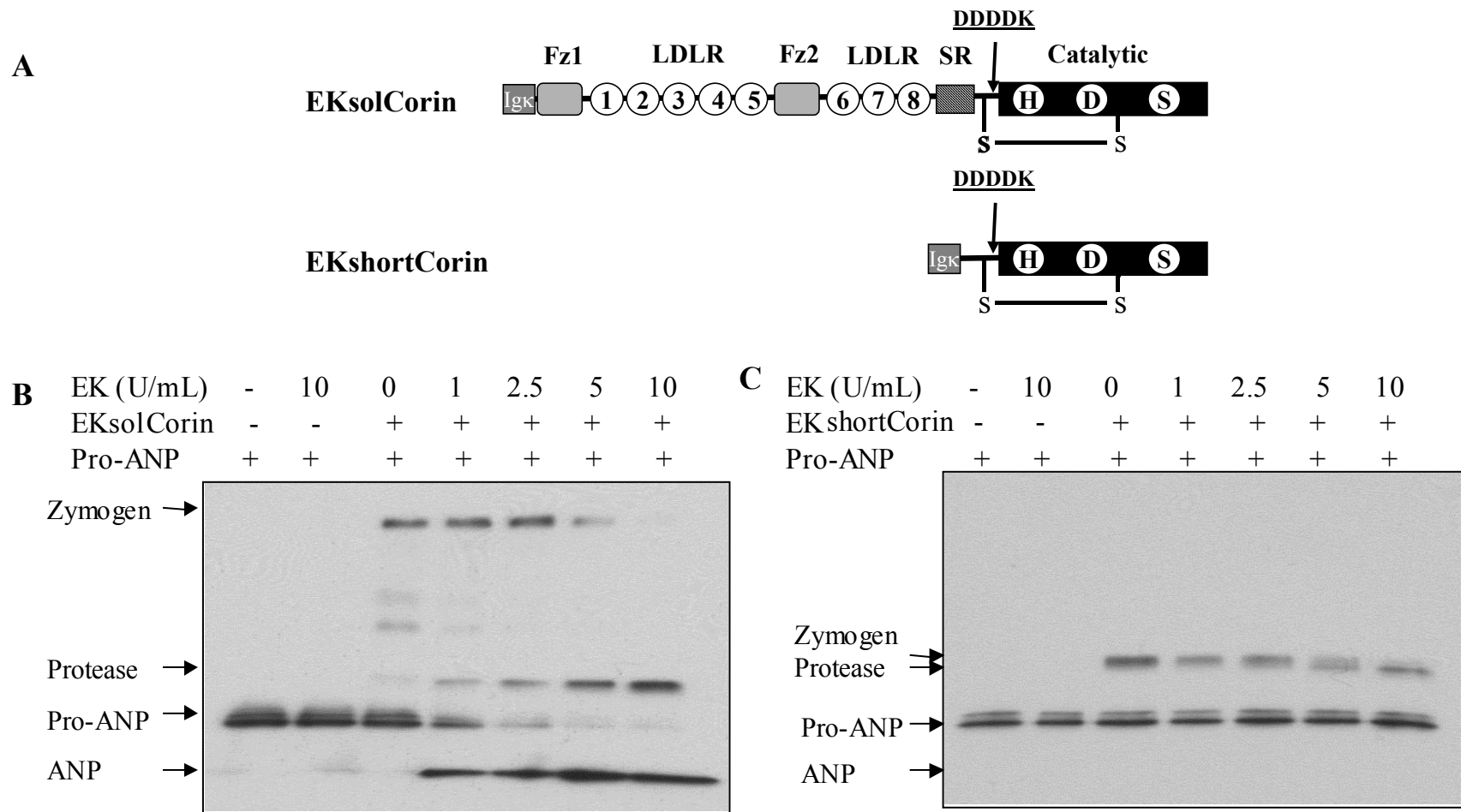
Figure 3.11. Processing of pro-ANP by EKsolCorin and EKshortCorin. **(A)** Schematic diagrams of the two soluble corin proteins. Purified EKsolCorin **(B)** and EKshortCorin **(C)** were activated by increasing concentrations of EK and added to conditioned medium containing pro-ANP. Recombinant corin, pro-ANP, and ANP were analyzed by immunoprecipitation and SDS-PAGE under reducing conditions followed by Western blotting using an anti-V5 antibody. The bands representing corin zymogen forms (*Zymogen*), the protease domain after the activation cleavage (*Protease*), pro-ANP, and ANP, respectively, are indicated. At high resolution, two bands of pro-ANP were detected on the Western blot possibly caused by differences in glycosylation in transfected cells.

III.2.3   **Comparison of kinetic constants of EKsolCorin and EKshortCorin**

One possibility for the failure of EKshortCorin to cleave pro-ANP could be that the protein was not properly folded in the absence of the propeptide, making it catalytically inactive. We then compared enzyme kinetics of EKshortCorin and EKsolCorin, which is biologically active. In assays using the chromogenic substrates S-2366 and S-2403, the K_m values for EKshortCorin were similar to those of EKsolCorin (3.63 ± 0.48 vs. 3.29 ± 0.85 mM for S-2366 and 1.52 ± 0.20 vs. 1.40 ± 0.38 mM for S-2403). Similar k_{cat} values also were found for EKshortCorin and EKsolCorin (0.50 ± 0.06 vs. 0.43 ± 0.14 s^{-1} for S-2403 and 0.46 ± 0.06 vs. 0.51 ± 0.12 s^{-1} for S-2366). The results are consistent with the data presented in section III.1.4.3.2 for EKsolCorin and show that EKshortCorin is catalytically active toward small peptide substrates.

III.2.4   **Effects of inhibitors on EKsolCorin and EKshortCorin**

We next examined the effects of protease inhibitors on the catalytic activity of EKshortCorin and EKsolCorin in a chromogenic substrate-based assay. As summarized in Table 3.3, the catalytic activity of EKshortCorin and EKsolCorin was inhibited similarly by trypsin-like serine protease inhibitors including benzamidine, PMSF, leupeptin, and soybean trypsin inhibitor. Neither EKshortCorin nor EKsolCorin was inhibited by metallo- and aspartic-protease inhibitors such as EDTA and pepstatin. The data show that the active center of EKshortCorin is properly formed, consistent with the results from the kinetic studies described above. Together, these data indicate that the propeptide of corin is not necessary for its interactions with small peptide substrates and inhibitors, but is required for its interaction with the physiologic substrate pro-ANP.

Table 3.3. Effects of protease inhibitors on the hydrolytic activity of EKshortCorin and EKsolCorin. Inhibitor assays were performed as described under "Methods". The data are presented as the means ± S.D. from at least three independent experiments.

Inhibitor	Concentration	% Inhibition EKshortCorin	% Inhibition EKsolCorin
EDTA	20 mM	0	0
Pepstatin	50 uM	0	0
Benzamidine	5 mM	92.9 ± 2.3	90.8 ± 0.6
	50 μM	42.9 ± 1.1	41.0 ± 1.6
PMSF	5 mM	92.2 ± 2.5	91.9 ± 2.1
	50 μM	5.3 ± 0.7	6.3 ± 0.6
Leupeptin	50 μM	76.1 ± 2.8	75.0 ± 1.3
	5 μM	22.9 ± 3.1	22.0 ± 1.8
Soybean trypsin inhibitor	0.5 μM	93.0 ± 2.1	93.5 ± 0.6
	0.05 μM	83.4 ± 0.8	81.8 ± 1.5

III.3 Identification of Functional Domains in the Propeptide of Corin

The results presented in the section III.2 show that the extracellular propeptide of corin is not required for its interactions with small peptide substrates and inhibitors, but is necessary for its interaction with its physiological substrate pro-ANP. The propeptide of corin consists of a combination of modular domains including two clusters of LDLR repeats, two Fz domains, and a SR domain (Yan *et al.*, 1999) (Fig. 3.12). The presence of the Fz domains is unusual for a serine protease. Usually, Fz motifs are found in Wnt-interaction proteins that are involved in developmental processes.
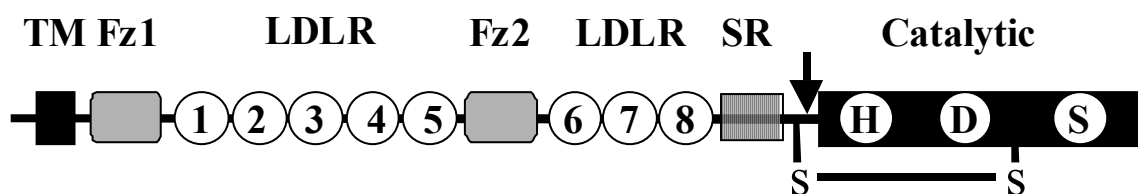
Figure 3.12. Domain structure of human corin protein. Schematic presentation of the structural domains of corin. Corin contains a transmembrane domain (*TM*), Fz cysteine-rich domains (*Fz*), clusters of LDLR class A motifs (*LDLR*), in which individual repeats are numbered with 1 to 8, a SR cysteine-rich domain (*SR*), and a protease catalytic domain (*Catalytic*) with active-site residues histidine (*H*), aspartate (*D*), and serine (*S*). The activation site is indicated by an arrow. The disulfide bond (*S-S*) connecting two polypeptide chains after the activation is shown.

To assess the functional importance of the domain structures in the propeptide of corin for pro-ANP processing, we generated several series of corin mutants with deletions or single amino acid changes in the different structural motifs and tested them in cell-based pro-ANP processing assays.

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III.3.1   Sequential truncations of modular domains in corin

To identify functional domains in the propeptide of corin, we constructed a first series of five deletion mutants that lacked increasing numbers of individual domains, starting with the deletion of Fz1 (ΔFz1) followed by deletion of Fz1 and the LDLR cluster containing repeats 1-5 (ΔFz1R1-5), and so forth. The shortest mutant consisted of the protease domain only (ΔFz12R1-8SR) (Fig. 3.13A). All corin mutants contained the transmembrane domain.

To test the pro-ANP processing activity of these corin deletion mutants, expression vectors for corin and pro-ANP were co-transfected in HEK 293 cells, and pro-ANP processing was analyzed by immunoprecipitation and Western blot analysis of the conditioned media as described in "Methods". We found that cells transfected with full-length corin or corin mutant ΔFz1 converted pro-ANP completely to ANP when the conditioned medium was examined 16 h after the transfection (Fig. 3.13B, *lower panel)*. The cells transfected with plasmids expressing corin mutants ΔFz1R1-5, Fz12R1-5, ΔFz1R1-8, and ΔFz1R1-8SR were unable to process pro-ANP under similar conditions. The results indicate that Fz1 domain is not absolutely required for the pro-ANP processing of corin. These findings are consistent with a preliminary functional study of this corin mutant series (Knappe, 1999). We also confirmed recombinant corin protein expression in cell lysates by Western blot analysis using an anti-Xpress antibody (Fig. 3.13B, *upper panel*).

A

B
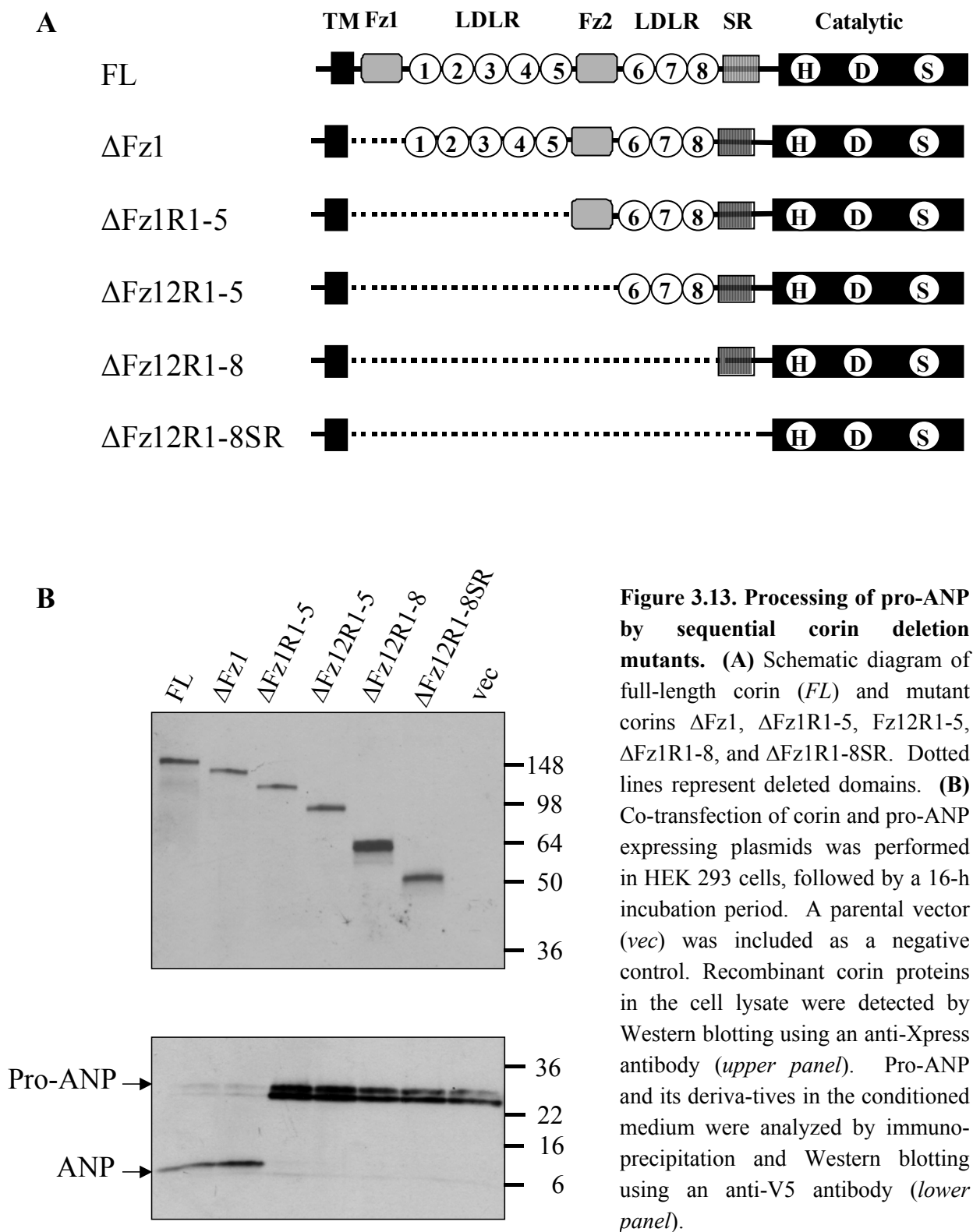
Figure 3.13. Processing of pro-ANP by sequential corin deletion mutants. (A) Schematic diagram of full-length corin (*FL*) and mutant corins ΔFz1, ΔFz1R1-5, Fz12R1-5, ΔFz1R1-8, and ΔFz1R1-8SR. Dotted lines represent deleted domains. **(B)** Co-transfection of corin and pro-ANP expressing plasmids was performed in HEK 293 cells, followed by a 16-h incubation period. A parental vector (*vec*) was included as a negative control. Recombinant corin proteins in the cell lysate were detected by Western blotting using an anti-Xpress antibody (*upper panel*). Pro-ANP and its deriva-tives in the conditioned medium were analyzed by immuno-precipitation and Western blotting using an anti-V5 antibody (*lower panel*).

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III.3.2   Pro-ANP processing activity of corin mutants lacking Fz1 domain or LDLR repeats 1-5

To further quantify the results from section III.3.1, we repeated the pro-ANP processing experiment for full-length corin and corin deletion mutants ΔFz1 and ΔFz1R1-5. We also wanted to know whether the loss of function of corin deletion mutant ΔFz1R1-5 was caused by the reduction of the overall length of the corin protein or by the absence of LDLR repeats 1-5.

To answer this question, we constructed a plasmid that encoded a membrane-bound protein lacking LDLR repeats 1-5, ΔR1-5 corin (Fig. 3.14A). Plasmids encoding full-length corin and the corin mutants ΔFz1, ΔFz1R1-5, or ΔR1-5, or the parental vector were transfected in HEK 293 cells. The transfected cells were incubated with conditioned medium containing recombinant pro-ANP for 4 h. Pro-ANP processing was examined by Western blotting using an anti-V5 antibody (Fig. 3.14B, *lower panel*). Expression of recombinant corin proteins in the cell lysate was confirmed by Western blotting with an anti-X-press antibody (Fig. 14B, *upper panel*). Unlike the pro-ANP processing assay performed overnight (Fig. 3.13), the results from the pro-ANP processing assay performed in 4 h revealed that ΔFz1 corin had reduced (~40 %) activity in processing pro-ANP in comparison to full-length corin. Corin mutants lacking either Fz1 together with LDLR repeats 1-5 (ΔFz1R1-5) or only LDLR repeats 1-5 (ΔR1-5) had no detectable activity of pro-ANP processing. These results indicate that the region containing Fz1 domain and LDLR repeats 1-5 is critical for the function of corin in pro-ANP processing.

III.3.3　　　　Expression of corin mutant proteins on the cell surface

To exclude the possibility that the corin deletion mutants lost their activity due to the lack of cell surface expression, we examined the cell surface expression using a biotin-labeling method. HEK 293 cells were transiently transfected with plasmids encoding the full-length corin or corin mutants ΔFz1, ΔFz1R1-5, and ΔR1-5. Cell surface proteins were then labeled with biotin. Cells transfected with plasmids encoding soluble EKsolCorin and EKshortCorin, or a parental vector were included as controls. Cells transfected with a plasmid expressing the full-length corin but not biotinylated were used as an additional control. By SDS-PAGE and Western blotting, we showed that the full-length corin and corin mutants ΔFz1, ΔFz1R1-5, ΔR1-5, EKsolCorin, and EKshortCorin proteins were present in cell lysate (Fig. 3.14C, *upper panels*). When the same blots were re-probed with HRP-conjugated streptavidin, which binds to biotin-labeled cell surface proteins, the full-length corin and mutant corin ΔFz1, ΔFz1R1-5 and ΔR1-5 proteins, but not control soluble EKsolCorin and EKshortCorin, were detected (Fig. 2C, *lower panels*). No specific bands were detected in cells transfected with the plasmid encoding the full-length corin but had not been biotinylated, or cells transfected with a control vector and had been biotinylated (Fig. 3.14C, *lower panels*). The results show that mutant corin ΔFz1, ΔFz1R1-5 and Δ R1-5 proteins were indeed present on the cell surface.
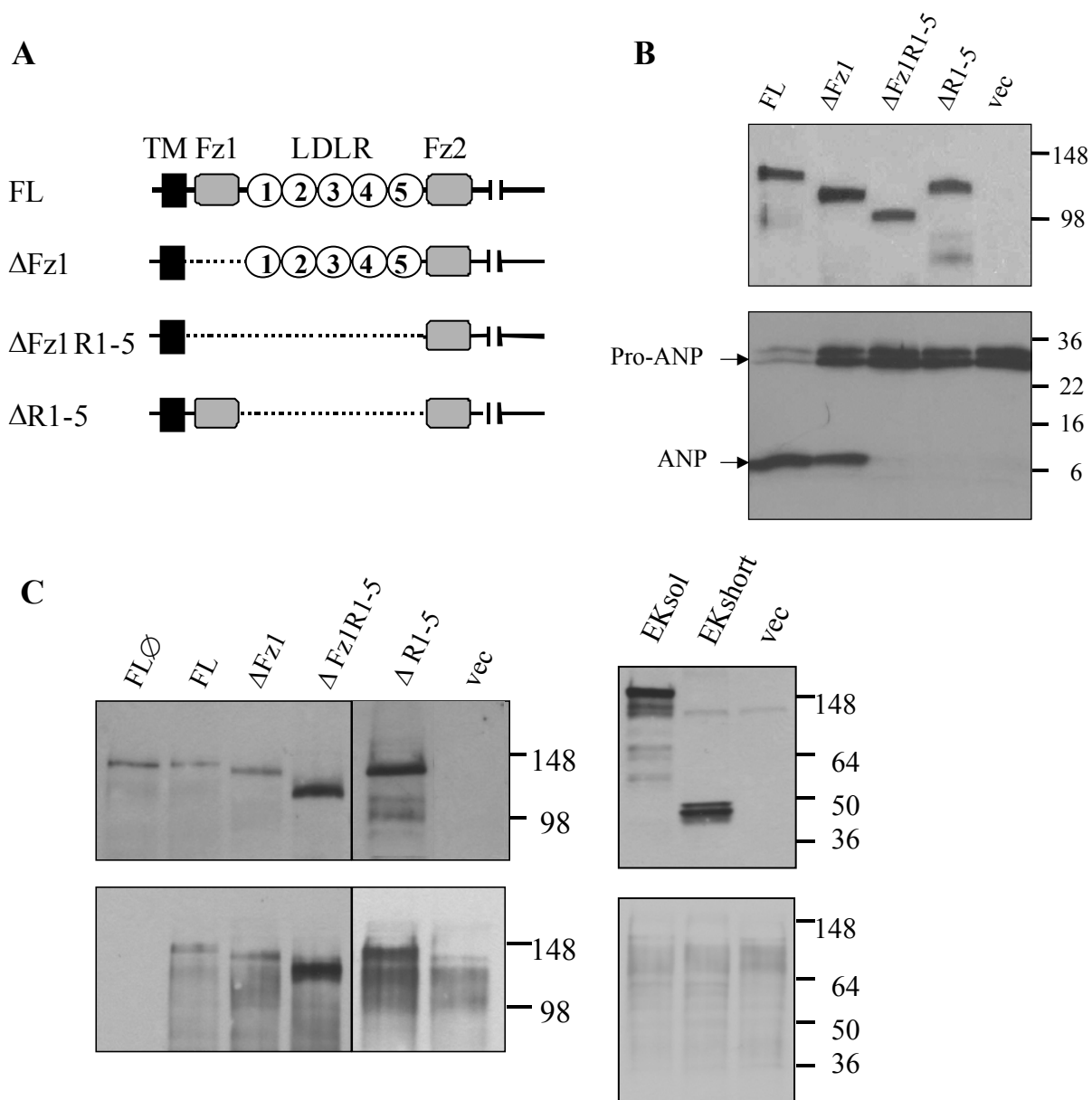
Figure 3.14. Expression and activity of corin mutants lacking Fz1 domain and LDLR repeats 1-5. **(A)** Schematic diagrams of the full-length corin (*FL*) and mutant corins ΔFz1, ΔFz1R1-5, and ΔR1-5. Dotted lines represent deleted domains. The domain structures C-terminal to the Fz2 domain are omitted. **(B)** Recombinant corin proteins in HEK 293 cells transfected with plasmids encoding full-length (*FL*) corin and corin mutants ΔFz1, ΔFz1R1-5, and ΔR1-5, or a control vector (*vec*) were detected by Western blotting using an anti-Xpress antibody (*upper panel*). Pro-ANP and its derivatives in the conditioned medium were analyzed by immunoprecipitation and Western blotting using an anti-V5 antibody (*lower panel*). **(C)** Cell surface proteins in transfected HEK 293 cells were biotinylated. Total recombinant corin proteins in cell lysate were detected by immunoprecipitation and Western blotting (*upper panels*). The same blots were re-probed with HRP-conjugated streptavidin to detect the corin proteins that were present on the cell surface. Cells transfected with the plasmid expressing the full-length corin but were not biotinylated (*FL∅*) or transfected with plasmids expressing soluble EKsolCorin and EKshortCorin or a control vector and biotinylated were included as controls (*lower panels*).

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III.3.4   Functional analysis of LDLR repeats 1-5

The LDLR-CI of corin consists of five individual LDLR repeats, each about 38 amino acids in length. LDLR repeats 1-4 contain 6 highly conserved cysteine residues and a cluster of acidic residues at the C-terminal end. The cysteine residue pattern in LDLR repeat 5, however, is less conserved and the repeat lacks four conserved negatively charged amino acids (Yan *et al.* 1999). For cell surface receptors of the LDLR family, the repeats have been shown to contribute to ligand binding.

III.3.4.1   Effects of deletions in LDLR repeats 1-5

To determine the functional importance of each individual LDLR repeat within the LDLR-CI, we first generated a series of plasmids expressing corin mutants lacking Fz1 domain and increasing numbers of LDLR repeats (ΔFz1R1, ΔFz1R12, ΔFz1R1-3, ΔFz1R1-4) (Fig. 3.15A). The plasmids were transfected into HEK 293 cells and the expression of mutant corin proteins was confirmed by Western analysis of the cell lysates (Fig. 3.15B, *upper panel*). Conditioned medium containing recombinant pro-ANP was incubated with these cells, and pro-ANP processing was analyzed by Western blotting. Consistent with data shown in Fig. 3.14B, mutant corin ΔFz1 had a reduced activity in pro-ANP processing (Fig. 3.15B, *lower panel*). No pro-ANP processing activity was detected in cells expressing corin mutants ΔFz1R1, ΔFz1R12, ΔFz1R1-3 and ΔFz1R1-4 (Fig. 3B, *lower panel*). These data show that in the absence of Fz1 domain, deletion of LDLR repeat 1 alone or together with LDLR repeats 2-4 abolished the pro-ANP processing activity of corin.

A

B
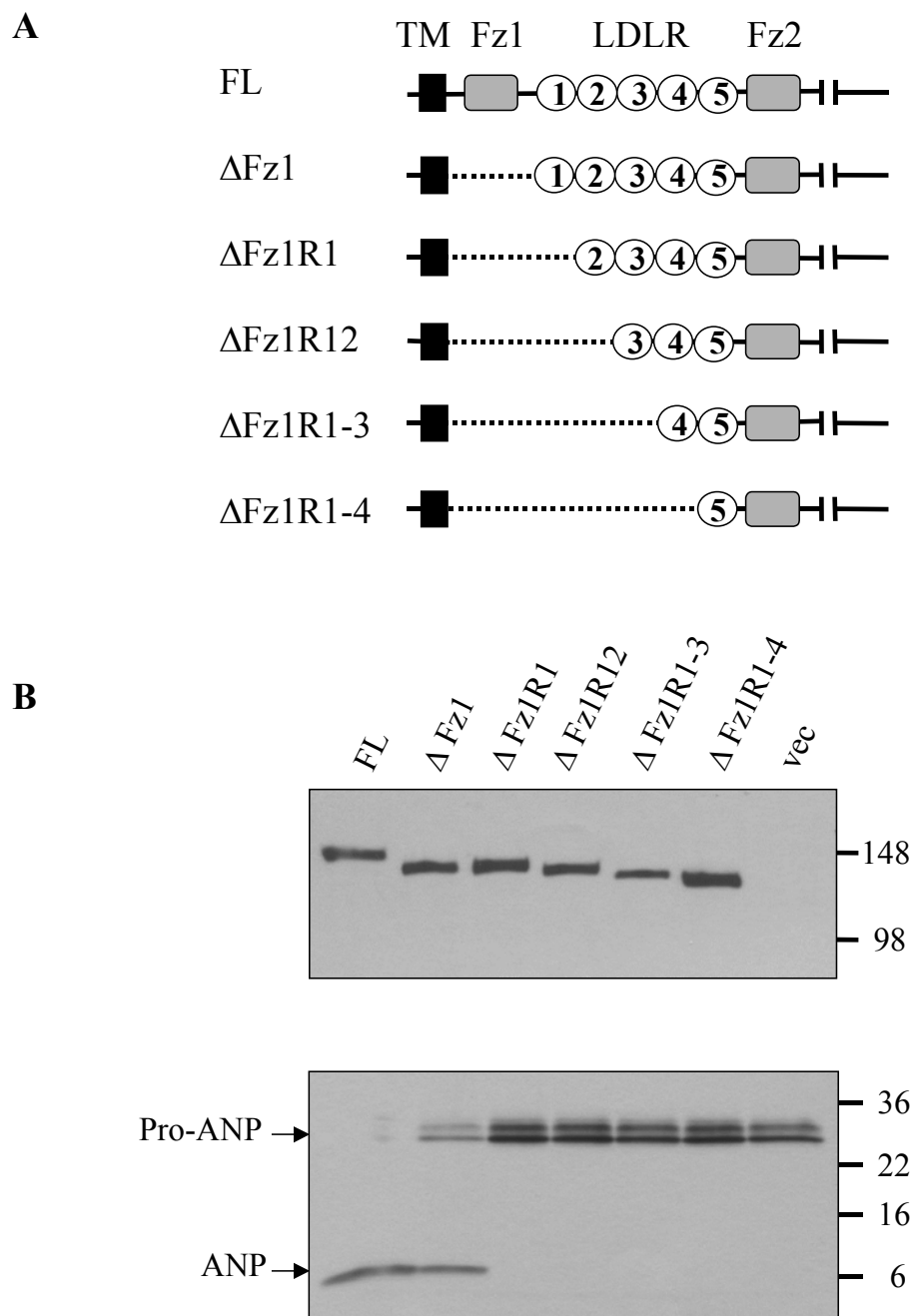
Figure 3.15. Pro-ANP processing by corin mutants lacking Fz1 domain and LDLR repeats 1-4.
(A) Schematic diagrams of the full-length corin (*FL*) and corin mutants ΔFz1, ΔFz1R1, ΔFz1R12, ΔFz1R1-3, and ΔFz1R1-4. Dotted lines represent deleted domains. **(B)** Recombinant corin proteins in HEK 293 cells transfected with plasmids encoding the full-length corin (*FL*) and corin mutants ΔFz1, ΔFz1R1, ΔFz1R12, ΔFz1R1-3, and ΔFz1R1-4, or a control vector (*vec*) were detected by Western blotting using an anti-Xpress antibody (*upper panel*). Pro-ANP and its derivatives in the conditioned medium were analyzed by immunoprecipitation and Western blotting using an anti-V5 antibody (*lower panel*).

We then constructed a second series of corin mutants that retained Fz1 domain but lacked increasing numbers of LDLR repeats 1-4 (ΔR1, ΔR12, ΔR1-3, and ΔR1-4) (Fig. 3.16A) and expressed them in HEK 293 cells (Fig. 3.16B, *upper panel*). As shown in pro-ANP processing assays, mutant corin ΔR1 had ~49 % activity, whereas mutant corin ΔR12 had ~26 % activity when compared with that of the full-length corin (Fig. 4B, *lower panel*). Mutants ΔR1-3 and ΔR1-4 had no detectable activity in pro-ANP processing (Fig. 3.16, *lower panel*). These data show that, in the presence of Fz1 domain, the amino acid sequence spanning LDLR repeats 1-3 is important for corin-mediated pro-ANP processing.

A

B
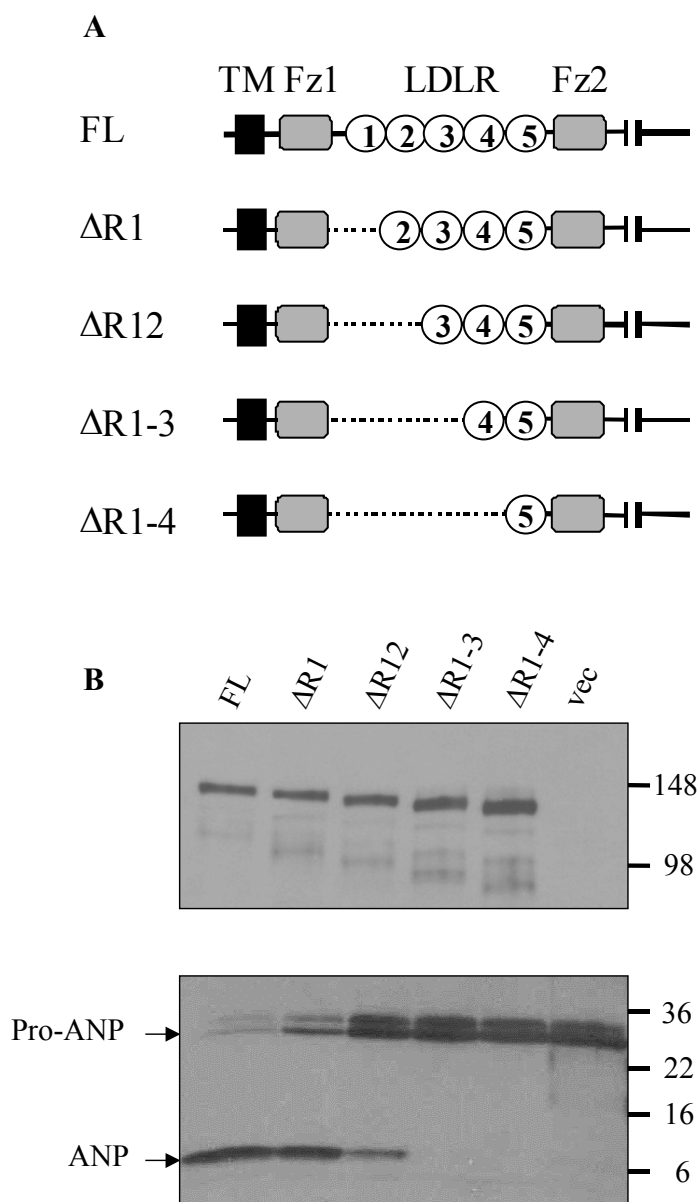
Figure 3.16. Pro-ANP processing by corin mutants lacking LDLR repeats 1-4. **(A)** Schematic diagrams of the full-length corin (*FL*) and corin mutants ΔR1, ΔR12, ΔR1-3, and ΔR1-4. Dotted lines represent deleted LDLR repeats. **(B)** Recombinant corin proteins in HEK 293 cells transfected with plasmids encoding the full-length (*FL*) corin and corin mutants ΔR1, ΔR12, ΔR1-3, and ΔR1-4, or a control vector (*vec*) were detected by Western blotting using an anti-Xpress antibody (*upper panel*). Pro-ANP and its derivatives in the conditioned medium were analyzed by immunoprecipitation and Western blotting using an anti-V5 antibody (*lower panel*).

To examine the importance of individual repeats within LDLR-CI for pro-ANP processing, we generated corin deletion mutants missing either LDLR repeat 1 (ΔR1), repeat 2 (ΔR2), repeat 3 (ΔR3), repeat 4 (ΔR4), or repeat 5 (ΔR5). The schematic structures of these mutants are shown in Fig. 3.17A. The 4-h pro-ANP processing assay (see previous paragraphs) was carried out to test the activity of the deletion mutants. Mutant corin proteins were detected in cell lysates with an anti-Xpress antibody (Fig. 3.17B, *upper panel*). The activity of these single LDLR repeat mutants in pro-ANP processing (Fig 3.17B, *lower panel*) was significantly different. As measured by densitometry, the activity of corin mutants relative to full-length corin was 47, 13, 50, 71 and 80 % for ΔR1, ΔR2, ΔR3, ΔR4, and ΔR5 corin, respectively. These data indicate that LDLR repeat 2 is the most critical unit in the first LDLR cluster, whereas repeats 4 and 5 are the least critical for pro-ANP processing.
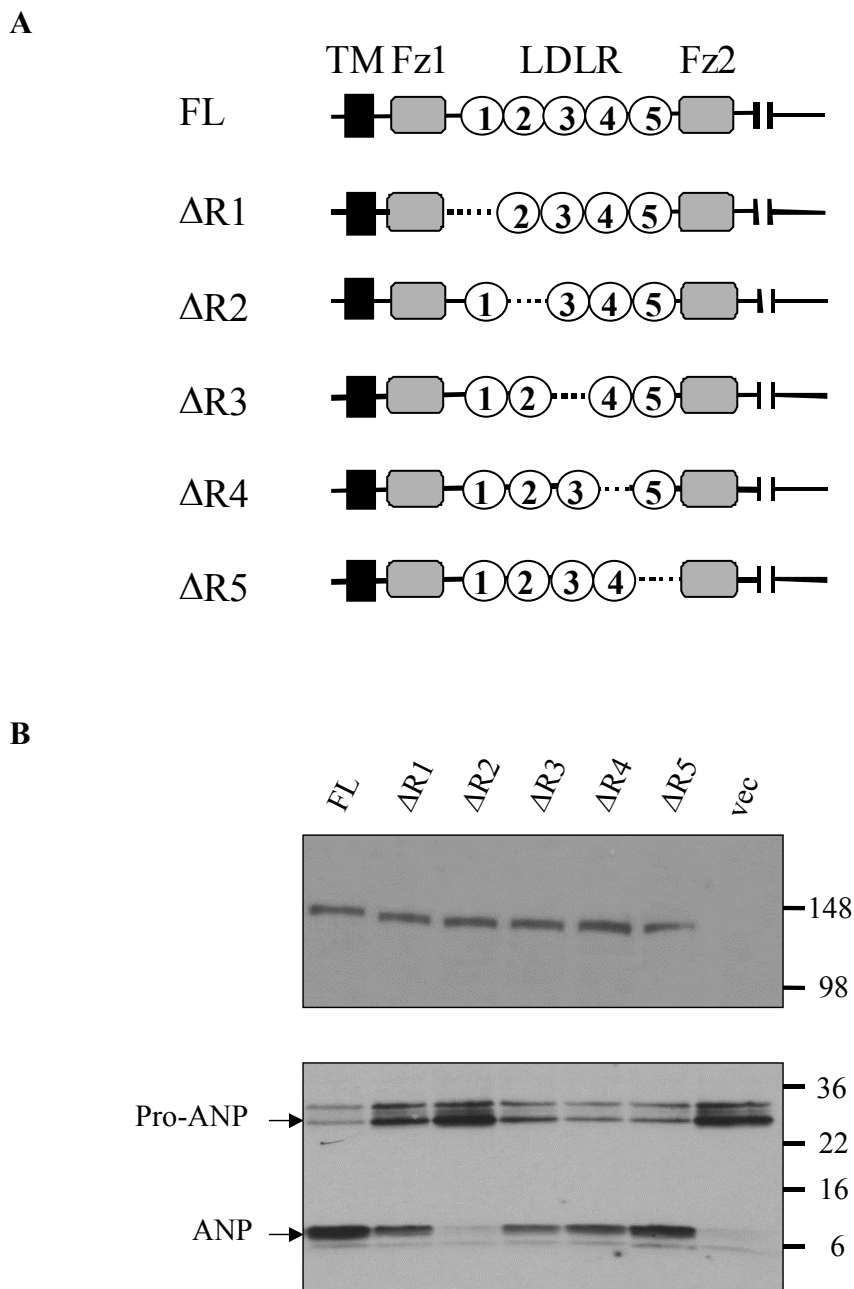
Figure 3.17. Processing of pro-ANP by corin mutants lacking individual LDLR repeats. **(A)** Schematic diagrams of the full-length corin (*FL*) and corin mutants ΔR1, ΔR2, ΔR3, ΔR4, and ΔR5. Dotted lines represent deleted LDLR repeats. **(B)** Recombinant corin proteins in HEK 293 cells transfected with plasmids encoding the full-length corin (*FL*) and corin mutants ΔR1, ΔR2, ΔR3, ΔR4, and ΔR5, or a control vector (*vec*) were detected by Western blotting using an anti-Xpress antibody (*upper panel*). Pro-ANP and its derivatives in the conditioned medium were analyzed by immunoprecipitation and Western blotting using an anti-V5 antibody (*lower panel*).

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III.3.4.2   Effects of point mutations in LDLR repeats 1-4

To further confirm the importance of LDLR repeats1-4, we constructed a new series of corin mutants by replacing a highly conserved Asp residue with a Tyr residue in LDLR repeats 1, 2, 3, or 4 (D300Y, D336Y, D373Y, and D410Y) (Figs. 3.18A and B). Repeat 5 lacks the corresponding Asp residue. In the LDLR, the corresponding Asp residue has been shown to coordinate Ca^{2+} binding (Fass *et al.*, 1997; Brown *et al.*, 1997) (Fig. 3.18A), which is required to maintain the structural integrity of the protein. A point mutation at the Asp residue is expected to alter the conformation of an individual LDLR repeat without causing structural perturbation to its neighboring repeats (North and Blacklow, 1999 and 2000). The mutant corin proteins were expressed in HEK 293 cells (Fig. 3.18C, *upper panel*) and their pro-ANP processing activities were analyzed by Western blotting (Fig. 3.18C, *lower panel*) and quantified by densitometry. Corin mutants D300Y, D336Y, D373Y, and D410Y had ~25, ~11, ~16, and ~75 % of pro-ANP processing activities, respectively, compared to that of full-length corin. These results are consistent with data from corin mutants with single LDLR repeat deletions (Fig. 3.17), demonstrating the individual contribution of LDLR repeats 1-4 in pro-ANP processing.

A

```
Repeat1  268   LCGRGEN   FLCASGICIP   GKLQCNGYND   CDDWSDEAHC
Repeat2  305   NC_SENL   FHCHTGKCLN   YSLVCDGYDD   CGDLSDEQNC
Repeat3  341   DCNPTTE   HRCGDR_CIA   MEWVCDGDHD   CVDKSDEVNC
Repeat4  378   SCHSQGL   VECRNGQCIP   STFQCDGDED   CKDGSDEENC
Repeat5  416   VIQTSCQ   EGDQRCLYNP   CLDSCGGSSL   CDPNNSLNNC
LDLR           C         F C   G CI      CD    D   CD   SDE   C
```

B

C
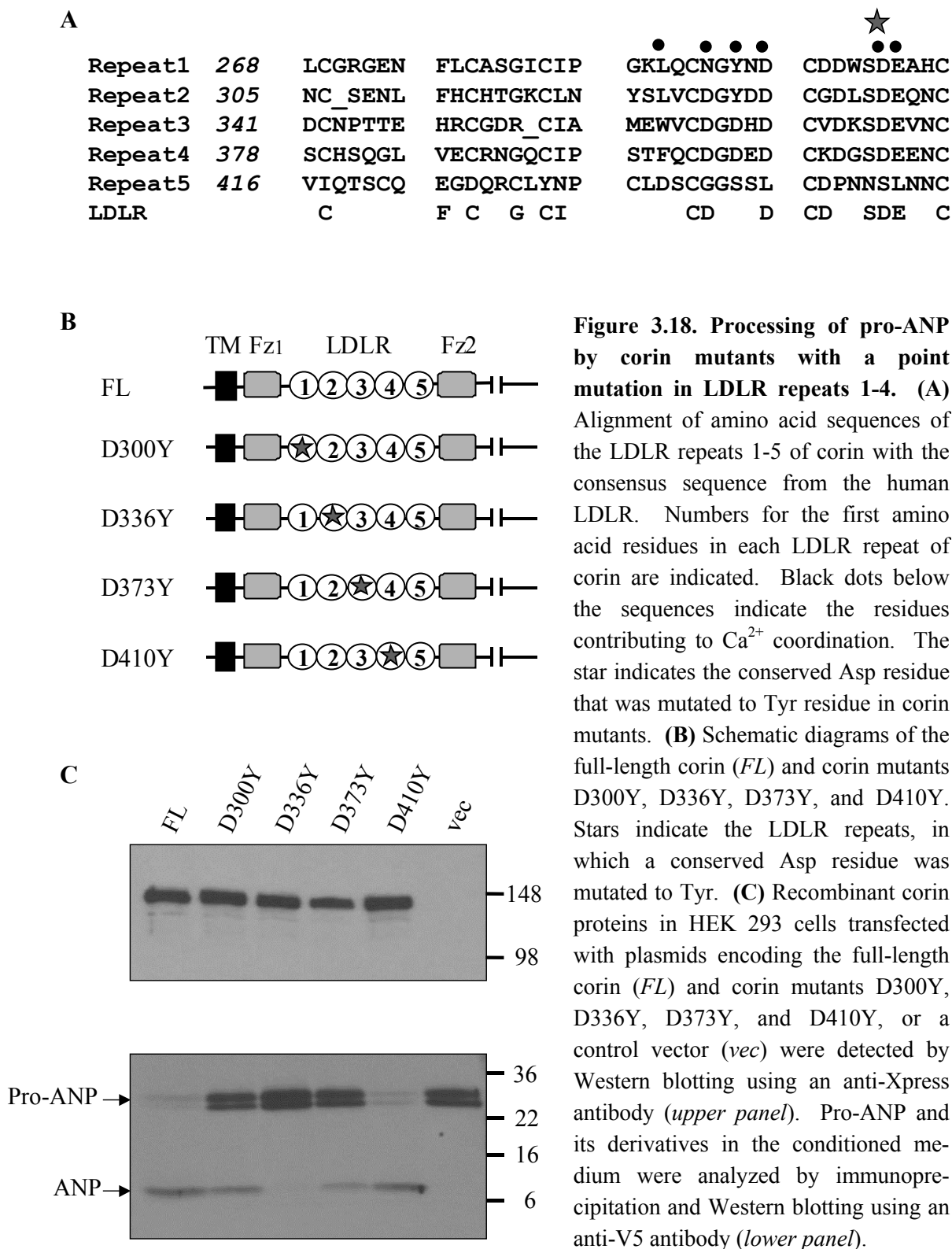
Figure 3.18. Processing of pro-ANP by corin mutants with a point mutation in LDLR repeats 1-4. (A) Alignment of amino acid sequences of the LDLR repeats 1-5 of corin with the consensus sequence from the human LDLR. Numbers for the first amino acid residues in each LDLR repeat of corin are indicated. Black dots below the sequences indicate the residues contributing to Ca^{2+} coordination. The star indicates the conserved Asp residue that was mutated to Tyr residue in corin mutants. **(B)** Schematic diagrams of the full-length corin (*FL*) and corin mutants D300Y, D336Y, D373Y, and D410Y. Stars indicate the LDLR repeats, in which a conserved Asp residue was mutated to Tyr. **(C)** Recombinant corin proteins in HEK 293 cells transfected with plasmids encoding the full-length corin (*FL*) and corin mutants D300Y, D336Y, D373Y, and D410Y, or a control vector (*vec*) were detected by Western blotting using an anti-Xpress antibody (*upper panel*). Pro-ANP and its derivatives in the conditioned medium were analyzed by immunoprecipitation and Western blotting using an anti-V5 antibody (*lower panel*).

III.3.5 Contribution of additional domains to the pro-ANP processing activity of corin

In the preceding experiments, a functional region within the propeptide of corin was identified that is required for pro-ANP processing. This region includes Fz1 domain and LDLR-CI in which LDLR repeats 1-3 are particularly important. Our data suggest that corin interacts with its substrate pro-ANP via multiple binding sites located in Fz1 and LDLR-CI. A proposed model will be discussed in section IV.
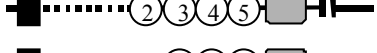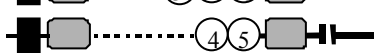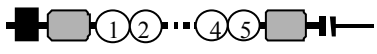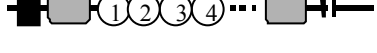
Most recently, a genetic study was carried out to determine if naturally occurring mutations/polymorphisms in the *corin* gene may contribute to cardiovascular diseases in human patients. The results showed that two single nucleotide polymorphisms T555I and Q568P on the same allele are present in 12.5 % of African-Americans and are associated with an increased risk for hypertension and cardiac hypertrophy (Dries *et al.*, unpublished data). Both amino acid residues, T555 and Q568, are located in Fz2 domain of corin, suggesting that this domain may also contribute to the interaction between corin and pro-ANP.

To test whether Fz2 domain is required for pro-ANP processing, we generated a corin deletion mutant, ΔFz2, that lacked amino acids 453-573. In a functional assay, we incubated the conditioned medium containing recombinant pro-ANP with cells transiently transfected with expression plasmids for full-length corin or corin mutants ΔFz1 and ΔFz2 (Fig. 3.19A). Cells transfected with a parental vector were used as a negative control. Corin proteins (Fig. 3.19B, *upper panel*) and pro-ANP and its derivatives (Fig. 3.19B, *lower panel*) were analyzed by SDS-PAGE and Western blotting using an anti-Xpress or an anti-V5 antibody. Consistent with data shown in Figs. 3.14 and 3.15, pro-ANP processing activity of mutant corin ΔFz1 was decreased (40 % activity) compared to that of full-length corin. A similar reduction in pro-ANP processing activity was observed for mutant corin ΔFz2, which had 32 % activity. These data suggest that both Fz domains are important in pro-ANP processing. Further studies will express mutant corin T555I and Q568P and test them in pro-ANP processing assays.

A

B
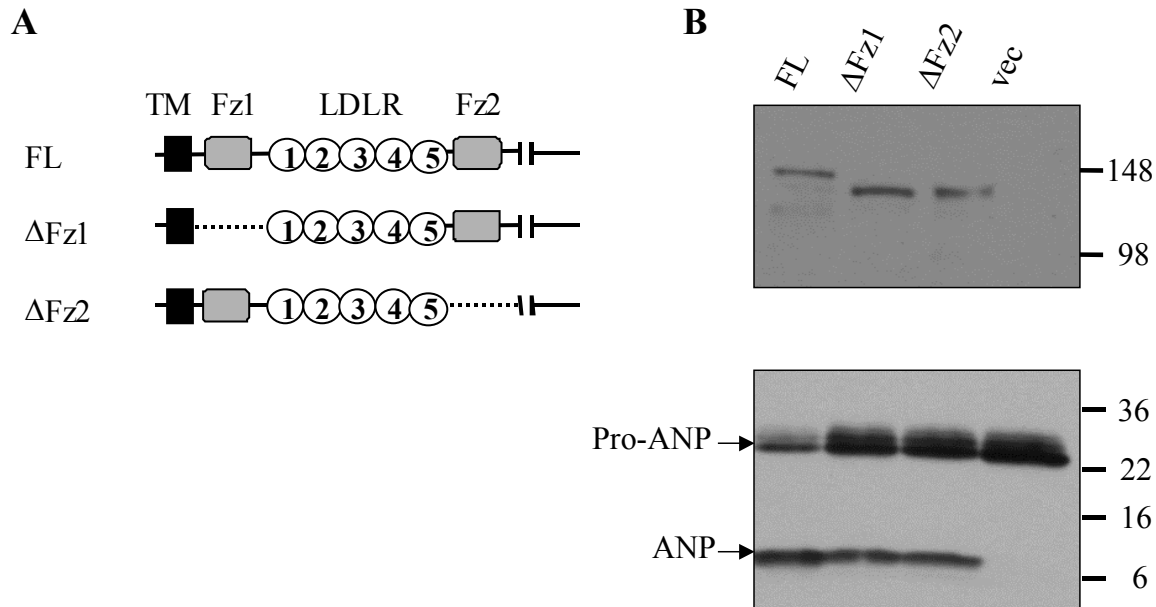
Figure 3.19. Comparison of pro-ANP processing activity of two corin mutants with Fz domain deletions. **(A)** Schematic diagrams of the full-length corin (*FL*) and corin mutants ΔFz1, and ΔFz2. Dotted lines represent deleted Fz domains. **(B)** Recombinant corin proteins in HEK 293 cells transfected with plasmids encoding the full-length corin (*FL*) and corin mutants ΔFz1, and ΔFz2, or a control vector (*vec*) were detected by Western blotting using an anti-Xpress antibody (*upper panel*). Pro-ANP and its derivatives in the conditioned medium were analyzed by immunoprecipitation and Western blotting using an anti-V5 antibody (*lower panel*).

In the third part of this study we examined the functional importance of the domain structures in the propeptide of corin for pro-ANP processing. Fig. 3.20 summarizes all transmembrane corin mutants described in the previous sections and their activities in pro-ANP processing.

Corin mutant	Relative activity (%)
FL	100
ΔFz1	40 ± 6
ΔFz1R1-5	0
ΔR1-5	0
ΔFz2	32 ± 3
ΔFzR1	0
ΔFzR12	0
ΔFzR1-3	0
ΔFzR1-4	0
ΔR1	47 ± 4
ΔR12	26 ± 6
ΔR1-3	0
ΔR1-4	0
ΔR2	13 ± 4
ΔR3	50 ± 6
ΔR4	71 ± 1
ΔR5	80 ± 8
D300Y	25 ± 4
D336Y	11 ± 1
D373Y	16 ± 3
D410Y	75 ± 2

Figure 3.20. A summary of corin mutants and their activities in pro-ANP processing. Schematic diagrams of the full-length corin (*FL*) and the mutant corins described in section III.3 are given on the *left*. The domain structures C-terminal to Fz2 domain are omitted. The pro-ANP processing activity was determined as described under "Methods." The activity for each mutant is expressed as a percentage of the value of the full-length corin. The data are presented as the means ± S.D. from at least four independent experiments.

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