IV. Discussion

The biological importance of membrane-bound serine proteases has been recognized for many years. EK, the first known TTSP, was discovered by Ivan Pavlov, who won the Nobel Prize in Physiology or Medicine in 1904. EK is an essential enzyme to activate trypsinogen, thereby initiating the protease reaction in food digestion. In more recent years, other membrane-associated serine proteases have been identified including hepsin, matriptases, and TMPRSS2-4 (Hooper *et al*., 2001; Wu, 2003; Szabo *et al*., 2003). Although studies have suggested that these enzymes may be involved in a broad range of biological processes such as development and cancer progression, the physiological substrates for these proteases remain poorly defined.

Corin cDNA was cloned from the human heart in 1999, encoding a new member of the TTSP family (Yan *et al*., 1999). The mosaic structure predicted for the corin protein consisted of two Fz domains, eight LDLR repeats, one SR domain, and a trypsin-like serine protease domain. The combination of domain structures was distinct from the few other TTSPs that had been identified at that time. In particular, the presence of two Fz domains represented a novelty among TTSPs and tryspin-like serine proteases in general.

Based on the structural domains found in corin, predictions about the physiological function of corin were made. Fz domains, for example, are usually found in Frizzled receptors or their soluble counterparts, which both interact with Wnt proteins, important signaling molecules in animal development. It was conceivable that the Fz domains in corin also are involved in protein-protein interactions. It remains unclear if corin interacts with Wnt proteins. On the other hand, the presence of two clusters of LDLR repeats suggested a role of corin in lipoprotein metabolism (Tomita *et al*., 1998). Experimentally, however, no lipoprotein binding to corin expressed on the surface of transfected COS cells was detected,

indicating that corin does not participate in interactions with lipoproteins. The potential function of the LDLR repeats in corin remained undefined.

Examination of the tissue distribution of corin indicated a role for corin in the cardiovascular system. High levels of corin mRNA were found in the human heart, whereas other human tissues that are rich in muscles including uterus, small intestine and bladder did not express corin. Corin mRNA was also detected in the developing hearts of mouse embryos, as early as E9.5 and in high levels by E15.5 (Yan *et al*., 1999). This expression pattern suggested a function of corin in the heart.

The tissue expression pattern and the substrate specificity of trypsin-like serine proteases for basic residues suggested that corin might be the long-sought convertase for pro-ANP, the precursor of the cardiac hormone ANP that plays a role in the regulation of blood pressure. To determine if corin is indeed the pro-ANP convertase, cell-based experiments were performed. HEK 293 cells co-transfected with corin and pro-ANP expressing plasmids processed pro-ANP to a peptide with a size expected for ANP, which was subsequently shown to stimulate cGMP production in BHK cells (Yan *et al*., 2000). Later, these findings were extended by a study using the murine cardiomyocyte cell line HL5 that expresses corin endogenously. In RNA interference-based gene silencing experiments, small interfering RNA directed against the *corin* gene abolished the pro-ANP processing activity in the cardiomyocytes, indicating that corin is the endogenous pro-ANP convertase in cardiomyocytes (Wu *et al*., 2002). In these cell-based assays, however, the possibility remained that corin was only indirectly involved in pro-ANP processing. For instance, corin could activate another protease that subsequently cleaves pro-ANP.

In transfection experiments, recombinant corin protein was expressed in HEK 293 cells and analyzed by SDS-PAGE under reducing conditions followed by Western blotting (Yan *et al*., 2000). Corin protein appeared as a single band with a molecular mass of ~150 kDa, the size expected for the one-chain form of corin. At that time, it was not known if corin requires the conversion into a two-chain form by activation cleavage at the conserved site Arg801 for its enzymatic activity.

The observed molecular mass of ~150 kDa of recombinant corin was consistent with the calculated mass of ~116 kDa for human corin that contains 19 potential N-linked glycosylation sites in its extracellular region. The prediction that corin is a transmembrane protein was also supported by cell-based experiments. In transfected HEK 293 cells, recombinant corin protein was detected in cell lysates and membrane fractions but not in the conditioned cell medium (Yan *et al*., 2000). It was not known, however, if corin requires the transmembrane domain for its function.

Thus, in this study we wanted to address several questions. First, we wanted to know if the transmembrane domain of corin is critical for enzymatic and biological activities and if corin requires activation cleavage at the conserved site Arg801 for its function. The answers to these questions allowed us to assess the feasibility of generating a soluble corin that can be purified and tested for its pro-ANP processing activity and its basic biochemical properties such as substrate specificity, kinetic constants or sensitivity to inhibitors. In addition, we wanted to examine the importance of the propeptide of corin and identify regions within the propeptide that might be involved in pro-ANP processing.

IV.1 Requirement of the Transmembrane Domain of Corin

As a member of the TTSP family, corin contains an integral transmembrane domain near its N-terminus. In this study, we showed that a soluble corin that consists of only the extracellular region was capable of processing pro-ANP in cell-based assays. In transfection experiments, both membrane bound WT corin and the soluble corin, WTsolCorin, had similar activities in processing human pro-ANP (Fig. 3.1), indicating that the transmembrane domain is not necessary for corin activity.

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 (Davie *et al*., 1991; Furie and Furie, 1988; Mann *et al*., 1990). For corin, however, the transmembrane domain appears to be dispensable. The results are

consistent with reports of other TTSPs including EK (Lu *et al*., 1997), matriptases (Velasco *et al*., 2002; Lin *et al*., 1999; Takeuchi *et al*., 2000), hepsin (Kurachi *et al*., 1994; Wu, 2001), spinesin (Yamaguchi *et al*., 2002), HAT (Yamaoka *et al*., 1998), polyserase-I (Cal *et al*., 2003), showing that soluble enzymes that lack the transmembrane domain can be catalytically active. It appears, therefore, that the main function of the transmembrane domain in the proteases of this family is either to retain the activity of these enzymes in specific tissues or to localize the activity to specific subcellular sites.

IV.2 Activation Cleavage of Corin

Most trypsin-like proteases are synthesized as a one-chain zymogen. Proteolytic cleavage at a conserved activation site converts the zymogen to an active enzyme (Stroud, 1974; Neurath, 1984; Kraut, 1977). Usually, the activation cleavage occurs at a canonical sequence of Arg↓Ile-Val-Gly-Gly. In human corin, the predicted cleavage is located at Arg801-Ile802 (Yan *et al*., 1999). In previous cell-based experiments, human WT corin was shown to be capable of converting pro-ANP to ANP (Yan *et al*., 2000; Wu *et al*., 2002). By Western analysis, however, we did not detect the activated form of corin, which should migrate as a two-chain molecule under reducing conditions. This implies that either the single chain form of corin has sufficient catalytic activity or only a very small fraction of corin was activated in the transfected cells but was undetectable by Western blotting.

To test the importance of the activation cleavage in corin, we made a mutant corin R801A, which is expected to abolish the cleavage at Arg801-Ile802. In Western blots, recombinant corin R801A remained as a one-chain molecule under reducing conditions (Fig. 3.2). In cell based assays, we found that mutant corin R801A was inactive in processing pro-ANP (Fig. 3.2), demonstrating that the cleavage at Arg801 is critical for the activity of corin and that the one-chain zymogen form of corin does not possess detectable activity by the techniques we used in this study. In this regard, corin appears to be different from tissue-type plasminogen activator, which has significant catalytic activity in its one-chain form (Madison *et al*., 1993; Stubbs *et al*., 1998).

At this time, the physiological activator of corin has not yet been identified, making the production of catalytically active corin a challenge. Based on the activation cleavage sequence of corin (Arg801-Ile802), the corin activator is most likely a trypsin-like serine protease. We attempted to activate corin with some soluble plasma serine proteases (thrombin, factor Xa, and kallikrein) but were unsuccessful. To circumvent this problem, we made a soluble form of corin, EKsolCorin in which the activation cleavage site was replaced by the EK recognition sequence DDDDK. Because this EK recognition sequence is highly specific (Anderson *et al*., 1977), this strategy should allow us to activate recombinant corin in a specific manner.

IV.3 Biochemical Properties of a Soluble Corin

As described in chapter III section 1.4, we designed, expressed, and purified a soluble corin, EKsolCorin, that contains an EK recognition sequence at the conserved activation site. We showed that purified EKsolCorin was inactive when tested in chromogenic assays but was readily activated by EK in a dose-dependent manner. Western analysis under non-reducing and reducing conditions indicated that EKsolCorin was cleaved by EK at the predicted site (Fig. 3.4).

In chromogenic substrate assays, activated EKsolCorin exhibited hydrolytic activities with a preference for Arg/Lys residues at the P1, Pro/Phe/Gly at the P2, and pyro-Glu, which is an analog of small neutral amino acids, at the P3 position. For example, the lowest K_m values and highest k_{cat} values were found for the hydrolysis of chromogenic substrates S-2403 (pyroGlu-Phe-Lys-pNA·HCl) and S-2366 (pyroGlu-Pro-Arg-pNA·HCl) (Table 3.1). This substrate profile of corin is consistent with the cleavage sequence of Ala-Pro-Arg↓Ser in human pro-ANP (Koller and Goeddel, 1992). The observed k_{cat}/K_m values of EKsolCorin for peptide substrates were similar to those reported for other TTSPs such as human polyserase-I (Cal *et al*., 2003) and mouse matriptase (Cho *et al*., 2001) but lower than those for human matriptase (Takeuchi *et al*., 1999) and EK (Lu *et al*., 1999).

Importantly, we showed that purified and activated EKsolCorin cleaves human WT pro-ANP and mutant pro-ANPs R101A and R102A but not mutant pro-ANP R98A (Fig. 3.7), indicating that EKsolCorin cleaves pro-ANP specifically at Arg98 but not at the adjacent residues Arg101 or Arg102. We also showed that EKsolCorin-processed recombinant ANP was biologically active as measured in the cell-based cGMP-stimulating activity assay (Fig. 3.8). The stringent sequence specificity observed for EKsolCorin was similar to the results from WT corin in previous cell-based studies (Yan *et al*., 2000; Wu *et al*., 2002). The results from this study demonstrate that introduction of the EK cleavage site did not alter the substrate specificity of the soluble corin, and that purified active EKsolCorin was indeed capable of processing pro-ANP.

Under physiological conditions, the activity of proteases is strictly regulated, in part by their cognate inhibitors. In this study, we examined the effect of protease inhibitors on the catalytic activity of corin in chromogenic substrate-based assays. We found that the activity of EKsolCorin was inhibited by non-specific serine protease inhibitors such as benzamidine, PMSF, and aprotinin but not by inhibitors of chymotrypsin-like, aspartic- or metallo-proteases such as chymostatin, pepstatin, and EDTA. The overall results were consistent with corin being a trypsin-like serine protease, as predicted by its amino acid residues (Asp979, Gly1007, and Gly1018) in the protease domain, which forms the substrate binding pocket.

One surprising finding was the inhibition of EKsolCorin by soybean trypsin inhibitor. In previous cell-based experiments, the presence of soybean trypsin inhibitor at up to 600 μM in culture medium had little effects on the processing of pro-ANP mediated by either recombinant corin in transfected HEK 293 cells (Yan *et al*., 2000) or endogenous corin in cultured cardiomyocytes (Wu *et al*., 2002). In this assay, however, the catalytic activity of EKsolCorin was inhibited by soybean trypsin inhibitor at 0.1 μM (Table 3.2). Such different effects of soybean trypsin inhibitor on soluble and membrane forms of proteases have also been reported for other TTSPs. For example, soybean trypsin inhibitor has been shown to inhibit the activity of soluble hepsin in chromogenic substrate assays but has little effect on that of cell surface hepsin in factor VII activation assays (Zhukov *et al*., 1997; Kazama *et al*.,

1995). 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 TTSPs may serve as a regulatory mechanism in their interactions with cognate inhibitors.

At the present time, it is not known if physiological corin inhibitors exist. In pro-ANP processing assays, we found that human plasma had little effect on the activity of soluble corin (Fig. 3.9), 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. In this aspect, corin seems to differ from some TTSPs, which have been suggested to be regulated by specific inhibitors. Matriptase, for example, was shown to be inhibited by hepatocyte growth factor activator inhibitor-1 (HAI-1), a Kunitz-type serine protease inhibitor (Lin *et al*., 1999; Friedrich *et al*., 2002). Most recently, the murine DESC-1 protein, another TTSP, was reported to form stable protease-serpin complexes with plasminogen activator inhibitior-1 and protein C inhibitor, both abundant in DESC-1 expressing tissues such as skin, salivary glands, and testis (Hobson *et al*., 2004).

IV.4 The Requirement of the Propeptide of Corin for Pro-ANP Processing Activity

Unlike trypsin, which consists of a simple protease domain, TTSPs contain a variety of protein domain structures in their propeptide region. For corin, there are two Fz domains, eight LDLR repeats and a SR domain in its propeptide. In this study, we examined the functional importance of the domain structures in the propeptide of corin for pro-ANP processing. We expressed a soluble corin, EKshortCorin, that consists of only the serine protease domain and contains an EK recognition sequence at the conserved activation cleavage site, a design similar to that for the full-length soluble corin, EKsolCorin.

We showed that EKshortCorin was activated by EK and retained the catalytic activity when examined in chromogenic substrate-based assays. EKshortCorin and EKsolCorin had similar K_m and k_{cat} values for chromogenic substrates S-2403 and S-2366. In addition, EKshortCorin and EKsolCorin were inhibited similarly by, for example, benzamidine, leupeptin, and soybean trypsin inhibitor. These results indicate that the protease domain of EKshortCorin is folded correctly in the absence of the domain structures in the propeptide. However, unlike the full-length soluble corin, EKsolCorin, which is biologically active, EKshortCorin failed to cleave pro-ANP under similar conditions (Fig. 3.11), indicating that certain domain structures in the propeptide are required for this activity of corin.

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 and co-workers 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). To date, EK and corin are the only two members from the TTSP family for which physiological substrates have been identified. Our data suggest that the propeptide in other TTSPs may also have an important function in interacting with their physiological substrates.

IV.5 Domain Structures in the Propeptide of Corin Essential for Pro-ANP Processing

As discussed above, corin contains several distinct structural domains in its propeptide, but their function is not known. In other TTSPs, functional importance for specific domains within the propeptide have been indicated. For example, the SEA domain of EK has been shown to be critical for targeting EK to the apical cell surface (Zheng and Sadler, 2002), whereas activation of matriptase requires intact LDLR repeats and a glycosylated CUB domain (Oberst *et al*., 2003). In this study, we examined if the Fz domains and LDLR repeats in the propeptide of corin are required for pro-ANP processing.

We first constructed a series of corin mutants by deleting increasing numbers of domain structures starting at the N-terminus of the extracellular region (Fig. 3.13). We showed that deletion of Fz1 domain reduced the pro-ANP processing activity of corin by ~60 %, whereas deletion of Fz1 domain and the LDLR repeats 1-5 together abolished the activity (Fig. 3.14). We also showed loss of activity for a corin mutant lacking LDLR repeats 1-5 alone (Fig. 3.14). These findings indicated important roles for Fz1 domain and LDLR repeats 1-5 of corin for pro-ANP processing.

We further verified these results by generating a series of corin mutants lacking Fz1 domain in addition to an increasing number of LDLR repeats starting with LDLR repeat 1 (Fig. 3.15). We showed that all mutants of this series, ΔFz1R1, ΔFz1R12, ΔFz1R1-3 and ΔFz1R1-4, were inactive in pro-ANP processing, further supporting the requirement of Fz1 domain and suggesting a crucial role for LDLR repeat 1 for pro-ANP processing.

We then analyzed an additional series of corin mutants that lacked an increasing number of LDLR repeats (R1-R5) but contained Fz1 domain. We showed that mutant corins ΔR1, ΔR12, ΔR1-3, and ΔR1-4 had ~49, ~26, 0 and 0 % pro-ANP processing activity, respectively (Fig. 3.16), confirming that LDLR repeats 1-5 are critical for pro-ANP processing. In addition, these results suggest that three consecutive LDLR repeats in LDLR-CI is the minimum requirement for the pro-ANP processing activity of corin.

Interestingly, the effect of deleting one or two LDLR repeats (ΔR1 and ΔR12) was less drastic than the deletion of Fz1 domain and LDLR repeats together (ΔFz1R1 and ΔFz1R12). This finding indicates that Fz1 domain might serve as a spacer between the cell membrane and the region of corin that is critical for pro-ANP processing.

In this study, we also examined the individual contribution of LDLR repeats 1-5 and Fz2 domain of corin in pro-ANP processing. By analyzing additional corin mutants that either lacked individual LDLR repeats, contained point mutations at a conserved Ca^{2+}-binding site in the LDLR repeats, or lacked the Fz2 domain, we showed that a region spanning from the Fz1 to the Fz2 domain is critical for the activity of corin in processing pro-ANP. Fig. 3.20

summarizes the corin propeptide mutants described in section II.3 and their activities in pro-ANP processing. In the following paragraphs, we will discuss the importance of the Fz domains and LDLR repeats.

IV.5.1 Importance of Fz domains in corin for pro-ANP processing

Corin contains two Fz domains in its propeptide, which is unusual among trypsin-like serine proteases. Fz domains are ~120 amino acids in length and contain 10 conserved cysteine residues. They were first discovered in members of the Frizzled family of seven-transmembrane receptors for Wnt signaling proteins (Vinson *et al*., 1989; Bhanot *et al*., 1996). Subsequently, the Fz cysteine-rich domain also has been found in soluble Frizzled-related proteins that act as antagonists of Wnt signaling (Rattner *et al*., 1997; Finch *et al*., 1997; Leyns *et al*., 1997). Several studies have indicated direct interaction between Wnt proteins and the Fz domain of Frizzled or soluble Frizzled-related proteins (Bhanot *et al*., 1996; Rattner *et al*., 1997; Leyns *et al*., 1997; Hsieh *et al*., 1999a and b). These protein-protein interactions are likely to be mediated by patches of amino acids exposed on the surface of the Fz domain, as shown by *in vitro* binding assays and mutagenesis studies (Dann *et al*., 2001).

In addition to Wnt receptors and inhibitors, other proteins such as human carboxypeptidase Z (Song and Fricker, 1997), mouse collagen (XVIII) α1 chain (Rehn and Pihlajaniemi, 1995), and several receptor tyrosine kinases including muscle-specific kinase and Smoothened (Masiakowski and Yancopoulos, 1998; Xu and Nusse, 1998) also contain Fz domains. A recent study has indicated that the Fz domain in chick carboxypeptidase Z binds to Wnt proteins and plays a role in the formation of the skeleton (Moeller *et al*., 2003). At this time, the functional significance of the Fz domain in collagen (XVIII) α1 chain, muscle-specific kinase and Smoothened is not known.

In this study, we examined the effect of Fz domain deletions on the pro-ANP processing activity of corin. We showed that corin mutants lacking either the Fz1 or Fz2 domain had

~40 % and ~32 % activity in pro-ANP processing, respectively, indicating that Fz domains are involved in the interaction of corin with pro-ANP. These findings are consistent with a recent genetic study in which single nucleotide polymorphisms were identified within Fz2 domain of corin that were associated with a hypertensive phenotype in African-American population (Dries *et al*., unpublished data).

To date, the only other proteolytic enzyme known to contain a Fz domain is CPZ, a secreted enzyme that is associated with the ECM (Novikova *et al.*, 2000). In an *in vitro* assay, CPZ was found to enhance Wnt-dependent induction of the homeobox gene *CDX1*. In immunoprecipitation experiments, the Fz domain of CPZ was shown to serve as a binding region for Wnt-4, suggesting a role of CPZ in Wnt-signaling (Moeller *et al*., 2003). At this time, it is not known if the Fz domains in corin interact with Wnt proteins. Our data from this study show that the Fz domains in corin are important for substrate recognition. Together, the data suggest that Fz domains have a much broader role in protein-protein interactions.

IV.5.2 Importance of LDLR repeats in corin for pro-ANP processing

In the extracellular region of corin, there are eight LDLR repeats in two separate clusters. This type of repeat, which is ~40 amino acids in length and contains 6 conserved cysteine residues, was first identified as lipoprotein-binding motif in the LDLR (Goldstein *et al*., 1985; Brown and Goldstein, 1986). To date, at least 150 different mutations in the human LDLR gene have been reported that are responsible for altering the function of the LDLR and causing familial hypercholesterolemia, a disease that is characterized by elevated plasma LDL-cholesterol levels (Hobbs *et al*., 1992). Many of those mutations affect the lipoprotein-binding domain of the LDLR, demonstrating the importance of this region for the function of the receptor. In addition to cell surface receptors of the LDLR protein family such as VLDLR, LRP, or megalin, LDLR repeats are found in numerous other proteins such as complement component C9, HAI-1, and the membrane bound adhesion protein SEZ-12 (Stanley *et al*., 1985; Shimomura *et al*., 1997; Kajiwara *et al*., 1996). LDLR repeats are also

present in many human TTSPs such as polyserase, EK, matriptases, MSPL, and TMPRSS2-4. Matriptase, for example, contains a cluster of four LDLR repeats. A mutational study of matriptase showed that point mutations of a conserved Asp residue in each individual LDLR repeat resulted in an impairment of matriptase activation (Oberst *et al*., 2003).

In this study, we identified the LDLR-CI containing repeats 1-5 as a critical region for pro-ANP processing. We generated corin mutants lacking single LDLR repeats 1-5 and tested their pro-ANP processing activity. Mutant corins ΔR1, ΔR2, ΔR3, ΔR4, and ΔR5 exhibited ~47, ~13, ~50, ~71, and ~80 % of pro-ANP processing activity, respectively, compared to that of the full-length corin. These results indicate that each LDLR repeat within LDLR-CI contributes to the substrate recognition with repeat 2 being the most critical and repeats 4 and 5 being less important.

To further verify the importance of each individual LDLR repeat, we made single amino acid mutations at a conserved Ca^{2+}-binding Asp residue in LDLR repeats 1, 2, 3, and 4 (D300Y, D336Y, D373Y, and D410Y). The mutation is expected to affect only the function of the individual LDLR repeat in which it resides but not the overall protein structure (North and Blacklow, 1999; 2000). Unlike the deletion mutants, the mutants with a single amino acid substitution shall maintain the distance between the cell membrane and individual LDLR repeats, which may be important for the binding of macromolecular substrates. In pro-ANP processing assays, mutants D300Y, D336Y, D373Y, and D410Y had ~25, ~11, ~16, and ~75 % activities, respectively. Overall, the data are consistent with the results from corin deletion mutants, showing that LDLR repeat 2 is most crucial for pro-ANP processing, whereas repeat 4 is less important.

Interestingly, there appears to be a difference in pro-ANP processing activity between the deletion and point mutation of LDLR repeats 1 and 3: ΔR1 and ΔR3 have ~47 and ~ 50% activity, respectively; but D300Y and D373Y have only ~25 and ~16 % pro-ANP processing activity, respectively. In both cases, point mutations caused a greater reduction in activity than deletion of the entire repeat. The reason for this apparent difference is not clear. One

possible explanation might be that in the deletion mutants the function of LDLR repeats 1 and 3 was partially compensated by adjacent repeats that replaced the original position of repeat 1 and 3.

The observation that individual LDLR repeats within a cluster do not function equivalently was first reported by Esser in collaboration with Michael S. Brown and Joseph L. Goldstein, who shared the Nobel Prize in Physiology or Medicine in 1985 for their discoveries concerning the regulation of the cholesterol metabolism (Esser *et al*., 1988). In that study, the mutational dissection of the eight consecutive LDLR repeats in the ligand binding domain of LDLR pointed to a specific role for repeat 5 in the binding of β-VLDL and important roles for repeats 3-7 in the binding of LDL. These findings indicate that different combinations of LDLR repeats within a LDLR cluster are required for binding of specific ligands. The modular character of LDLR repeats and their contributions to ligand binding have been confirmed in several studies of other LDLR family members (Neels *et al*., 1999; Mikhailenko *et al*., 1999). For example, Rettenberger and colleagues examined the binding properties of a naturally occurring variant of VLDLR that is encoded by alternatively spliced mRNA. This variant VLDLR lacks the third LDLR repeat in a cluster of eight repeats. In binding assays, this VLDLR variant, VLDLR-III, displayed a ~4-fold lower binding affinity to receptor-associated protein (RAP) (Rettenberger *et al*., 1999).

At this time, the specific amino acid residues in the Fz domains and LDLR repeats 1-5 of corin that make contact with pro-ANP are not known. Andersen *et al.* reported specific amino acid residues of the LRP that are directly involved in ligand-receptor interactions (Andersen *et al*., 2000b). By ligand blotting and surface plasmon resonance analysis, two Glu acid residues (Glu903 and Glu924) in LDLR repeat 4 of LRP were identified that interact with two Lys residues of α2-macrogobulin. In corin, LDLR repeats 1-5, which are required for pro-ANP processing, do not have negatively charged amino acids at the corresponding positions. Thus, it is unlikely that pro-ANP processing by corin depends on these particular amino acid residues.

In a separate study, Andersen *et al*. also showed that a conserved Asp residue present in LDLR repeats 3 through 9 directly interacts with RAP (Andersen et al., 2000a). In corin, LDLR repeats 1, 2, and 5 have a Tyr or Ser residue in the corresponding position (Tyr292, Tyr328, and Ser440, respectively). However, in LDLR repeat 3 and 4 of corin, Asp residues are located at amino acids 365 and 402, respectively, suggesting that these residues might be directly involved in pro-ANP binding.

Several other studies have suggested that ligand binding to LDLR repeats depends on interactions between acidic residues of the receptor and basic residues on the surface of the ligand (Lohse *et al*., 1991; Wardell *et al*., 1991; Horn et al., 1997). Interestingly, pro-ANP contains highly conserved clusters of basic residues (R82, K87, K89, and R91) located near the cleavage site at Arg98, indicating that these residues possibly are involved in enzyme-substrate interactions. Further studies with site-directed mutagenesis should help us to understand how corin interacts with its physiological substrate pro-ANP.

IV.6 Proposed Model of Substrate Recognition by Corin

Large receptors of the LDLR family such as LRP and megalin contain four clusters of LDLR repeats. The multiplicity of structural domains in these receptors allow binding of ~30 different ligands by employing different combinations of LDLR repeats (Croy *et al*., 2003; Herz and Strickland, 2001; Hussain *et al*., 1999;). For a proteolytic enzyme like corin, interactions with such a variety of proteins is unlikely. We have shown that corin-mediated pro-ANP processing is highly sequence-specific. In a previous study, corin was shown to cleave pro-ANP but not pro-CNP (Wu *et al*., 2003), which contains the two cleavage sites, Ser46-Arg47-Leu48-Leu49-Arg50↓Asp51 and Lys76-Gly77-Ala78-Asn78-Lys80↓Gly81 (Tawaragi *et al*., 1991). In a protease, however, a propeptide with a complex modular architecture may have multiple contact sites for the specific binding of a single substrate.

Based on our results we suggest a model in which corin binds to its physiological substrate pro-ANP via multiple contact sites located in Fz1 domain, LDLR repeats 1-5, and Fz2

domain prior to proteolytic cleavage. Based on our data, LDLR repeats 1-3 are more critical than repeats 4 and 5 in interacting with pro-ANP (Fig. 4.1). The binding of pro-ANP to this region of corin may allow the protease domain to cleave the substrate more efficiently. It is equally possible that the binding induces conformational changes in pro-ANP, making its activation cleavage site accessible to the protease domain of corin. Once pro-ANP is cleaved by corin, converting it to an N-terminal cleavage fragment and a C-terminal mature ANP, the binding affinity to these two separate peptides is expected to reduce, allowing the cleaved products to be released from the enzyme. This model may explain why EKshortCorin, which lacks the propeptide domain, failed to cleave pro-ANP.

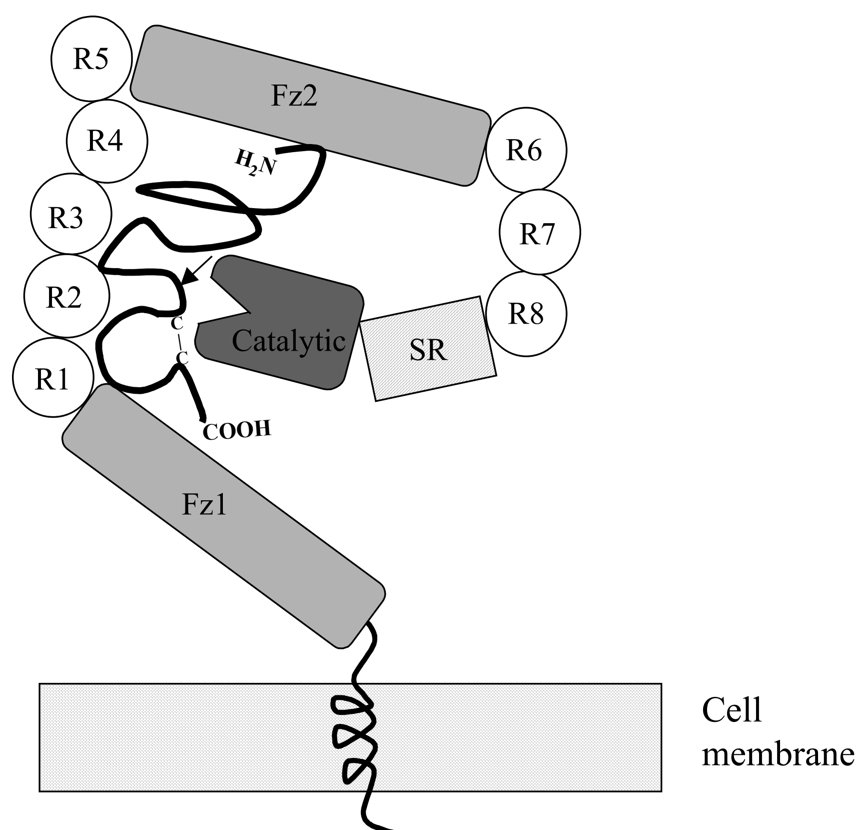

Figure 4.1. A proposed model for the interaction of corin and pro-ANP. As a type II transmembrane protein, corin is present on the cell surface. Upon release from the dense granules of cardiomyocytes, pro-ANP (*solid line*) binds to a region that includes Fz1 domain, LDLR repeats 1-5, and Fz2 domain of corin, which in turn cleaves pro-ANP (indicated by an arrow), converting it to mature ANP. The Fz domains (*Fz*), LDLR repeats 1-8 (*R1-R8*), SR domain (*SR*), and protease catalytic domain (*catalytic*) of corin are indicated.

In eukaryotes, many proteins are composed of multiple modular domains, some of which are catalytic (e.g. proteases and kinases), but the majority of which mediate interactions between protein binding partners. Protein-protein interactions often involve contacts at multiple sites. Over the years, much has been learned about the interactions between proteins with simple structures, but little is known about how proteins with multiple domains interact with other macromolecules (reviewed in Campbell, 2003; Doolittle, 1995). Here we present the first model of a TTSP to explain potential interactions between the enzyme and its substrate. We understand that this model is simplified and requires further testing and refinement. The possibility certainly exists that the recognition of pro-ANP depends on more complex three-dimensional arrangements of the domain structures within the corin propeptide. For example, the two Fz domains in corin may be dimerized, resulting in a correct conformation of the protein, which allows pro-ANP to bind. This idea is supported by crystal structure studies of the Fz domains of Frizzled 8 and secreted Frizzled-related protein 3, which form homologous dimers by intertwining C-terminal ends (Dann *et al.*, 2001). Further mutagenesis experiments and crystal structure analysis should help us to better understand how corin recognizes and processes pro-ANP. Our results may also provide new insights into the mechanism by which other members of the TTSP family interact with their physiological substrates.

As the pro-ANP convertase, corin plays a critical role in maintaining normal blood pressure and is involved in human diseases including hypertension, congestive heart failure, and cancer (see section I.4). In this study, we have shown that domain structures within the propeptide of corin are necessary for pro-ANP processing. Thus, mutations in the propeptide region may reduce or abolish the function of corin, causing cardiovascular diseases. Further studies will be important to screen potential mutations in the *corin* gene of patients with cardiovascular complications such as hypertension and CHF.