I. Introduction

I.1 Natriuretic Peptides and Their Roles in the Cardiovascular System

I.1.1 Discovery of natriuretic peptides

In the early 1600s, William Harvey provided the first accurate description of the human blood circulation and the central role of the heart as a dynamic pump that moves the entire blood volume through the vessels. This revolutionary view of the heart replaced the Galenic concept of two separate types of blood, the venous and the arterial, with distinct pathways and tasks. Harvey's conception of cardiac function remained unchanged for more than 300 years.

Additional functions for the heart were recognized in the 1950s, when a role of the cardiac atrium in regulating diuretic and natriuretic actions of the kidney was proposed. Henry and colleagues showed that the stimulation of the left atrium by inflating a balloon induces an increase in urinary flow rate in dogs (Henry *et al.*, 1956). Thus, they suggested a mechanism in which atrial mechanoreceptors mediate the response of the kidney.

In the same year, studies of guinea pig hearts by electronic microscope revealed a morphological difference between atrial and ventricular cardiomyocytes (Kisch, 1956). The atrial cells contained dense granules that were structurally similar to storage granules found in peptide hormone-producing cells in endocrine organs. At that time, the physiological function of these granules in the atrial cells and the nature of the substance contained in the granules were unknown.

In the 1970s, Adolfo J. de Bold devoted himself to isolate and analyze the contents of the atrial granules (de Bold and Bencomse, 1973; de Bold *et al.,* 1978; de Bold, 1979). The breakthrough came in 1981 when he and his co-workers demonstrated that injection of granule-enriched atrial extracts into rats caused a rapid and substantial increase in sodium excretion and urine volume (de Bold *et al.*, 1981). This experiment provided the first evidence that heart and kidney are linked through a humoral factor, which is produced in the atrium. The idea of the heart as an endocrine organ started to take shape (de Bold, 1985).

De Bold's pioneering observation initiated a period of active research in laboratories around the world, racing to identify the cardiac hormone. Shortly after, several groups isolated a peptide hormone from human (Kangawa and Matsuo, 1984) and rat (Flynn *et al.*, 1983) hearts. The hormone was designated atrial natriuretic peptide (ANP) or factor (reviewed in Rosenzweig and Seidman, 1991). The isolation and sequencing of the cDNA encoding human (Oikawa *et al.*, 1984; Maki *et al.*, 1984), rat (Kangawa *et al.*, 1984; Yamanaka *et al.*, 1984), and mouse ANP precursor, pro-ANP (Seidman *et al.*, 1984) soon followed.

After ANP, structurally related molecules were also discovered: brain- or B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) were first isolated from porcine brain (Sudoh *et al.*, 1988; 1990). Later, however, it was shown that BNP is predominantly expressed in the ventricle of the heart (Minamino *et al*., 1988; Hosoda *et al.*, 1991; Mukoyama *et al.*, 1991). Based on their expression patterns, ANP and BNP are also referred to as the cardiac natriuretic peptides. Most recently, Dendroaspis natriuretic peptide was isolated from the venom of the green mamba, but its existence in mammalian species has not yet been confirmed (Schweitz *et al.*, 1992). The common structural feature of the natriuretic peptides is a disulfide bond, which shapes the peptide into a ring form. The ring consists of 17 amino acid residues, most of them being highly conserved. The schematic structures of human ANP, BNP, and CNP are shown in Figure 1.1 (Suzuki *et al.*, 2001).

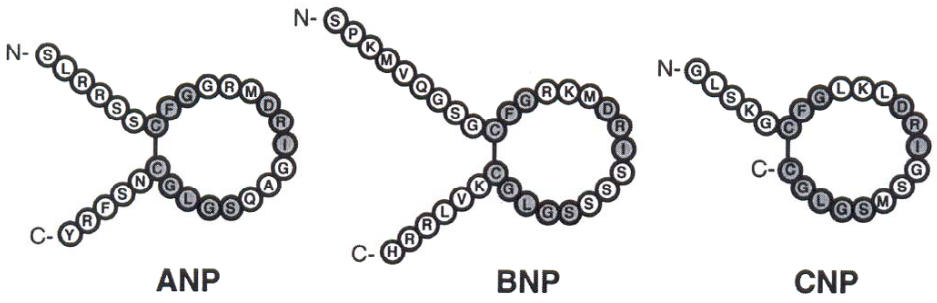

Figure 1.1. Primary structure of ANP, BNP, and CNP. Human ANP, BNP, and CNP consist of 28, 32, and 22 amino acids, respectively. Conserved residues are shaded. The disulfide bonds, which form the ring-structure are indicated by a black line.

I.1.2 The natriuretic peptide receptors

When released into circulation, all three natriuretic peptides exert their effects by binding to specific cell surface guanylyl cyclase (GC) receptors. ANP and BNP bind to the same receptor, GC-A (Chinkers *et al*., 1989), also referred to as natriuretic peptide receptor type A (NPR-A). CNP, instead, binds to GC-B or NPR-B (Koller *et al.*, 1991). The binding event activates the carboxy-terminal (C-terminal) GC of the receptors to generate the second messenger cyclic guanosine monophosphate (cGMP), which in turn modulates the activity of cGMP-dependent protein kinases, cGMP-gated ion channels, and cGMP-regulated phosphodiesterase (reviewed in Lucas *et al.*, 2000; Potter and Hunter, 2001). These effectors play a role in the regulation of a variety of physiological processes in the cardiovascular, gastrointestinal, and nervous systems as well as in the kidney, bone, and many other tissues (Kishimoto *et al*., 1996; Kone, 2001; Levin *et al*., 1998; Silberbach and Roberts, 2001).

There is a third receptor subtype, NPR-C, which binds ANP, BNP and CNP with high affinity (Suga *et al.*, 1992a.) but lacks the GC activity. NPR-C serves as a clearance receptor for all three natriuretic peptides. The kidney, vascular endothelium, and lung are the tissues rich in NPR-C and contribute most to the elimination of natriuretic peptides (Fuller *et al.*, 1988). Other mechanisms for the inactivation and elimination of natriuretic peptides include

the degradation by neutral endopeptidase present in renal tubular and vascular cells, and renal filtration of the peptides (Yandle, 1994).

I.1.3 The function of natriuretic peptides

The importance of the ANP/NPR-A system for cardiovascular homeostasis has been demonstrated by studies in different genetic mouse models. Targeted deletion of the peptide (ANP-/-) or its receptor (NPR-A-/-) leads to severe, chronic arterial hypertension, cardiac hypertrophy and sudden death (John *et al.*, 1995; Lopez *et al.*, 1995; 1997; Oliver *et al.*, 1997). The hypotensive/hypovolemic effect of ANP is mediated by an interplay of different responses that include inhibition of the renin-angiotensin-aldosterone and sympathetic systems, stimulation of natriuresis and diuresis, modulation of endothelial permeability, and vasodilatation (Brenner *et al.*, 1990).

Under physiological conditions, plasma concentrations of circulating BNP are similar to that of ANP (10-100 pg/mL) (Ruskoaho, 2003). Although BNP also shares its receptor and most of its physiological properties with ANP, BNP-deficient mice (BNP-/-) displayed a different phenotype. Instead of hypertension or cardiac hypertrophy, the BNP knockout mice showed ventricular fibrotic lesions and up-regulation of the factors known to promote ventricular fibrosis such as angiotension-converting enzyme and pro-α1 (I) collagen (Tamura *et al.*, 2000). These results indicate that the physiological functions of ANP and BNP are not identical and suggest that these peptides may play complementary roles in maintaining cardiovascular homeostasis.

CNP is highly expressed in the central nervous system and in the vascular endothelium (Ogawa *et al.*, 1992; Suga *et al.*, 1992b; Chen and Burnett, 1998), but is also present in lower amounts in the kidney, reproductive organs, and other tissues (Mattingly *et al.*, 1994; Suzuki *et al.*, 1993). Plasma levels of circulating CNP are below those of ANP and BNP (Yandle, 1994; Igaki *et al.*, 1996). Therefore, CNP is more likely to mediate its responses in an autocrine/paracrine fashion in tissues. By stimulating the NPR-B, which is abundant in the

vasculature, CNP induces vasodilatation, albeit to a far lesser extent than that induced by ANP (Lopez *et al.*, 1997; Wei *et al.*, 1994). Physiological functions suggested for CNP are the regulation of vascular tone and modulation of vascular regeneration (Komatsu *et al.*, 1996; Yamahara *et al.*, 2003) among others. CNP has also been shown to inhibit the proliferation of vascular smooth muscle cells in culture (Furuya *et al.*, 1991; Hutchinson *et al.*, 1997) and to prevent balloon injury-induced coronary artery restenosis in animal models (Brown *et al.*, 1997; Shinomiya *et al.*, 1994; Ueno *et al.*, 1997). A role for CNP beyond the cardiovascular system became apparent when targeted disruption in mice of either the CNP gene or the NPR-B gene produced a phenotype of severe dwarfism (Chusho *et al.*, 2001). This observation indicates that CNP has a regulatory function in the bone, where it stimulates chondrocyte proliferation and differentiation in growth plate.

I.1.4 Biosynthesis and processing of natriuretic peptides

In endocrine cells, most peptide hormones are synthesized as larger, inactive precursors. Limited endoproteolysis at specific amino acid residues is required to convert the precursor to a biologically active, mature hormone. In general, most peptide hormones are processed by a family of intracellular subtilisin-like enzymes called prohormone convertases (PC). The PC family includes furin, PC1/3, PC2, PC4, PACE4, PC5/6, and PC7/LPC (reviewed in Steiner, 1998; Zhou *et al.*, 1999; Seidah and Chretien, 1999). The endoprotease furin, for example, is located on the trans-Golgi network of virtually all cell types and is known to process numerous substrates such as pro-factor X (Wallin *et al.*, 1994), pro-endothelin (Denault *et al.*, 1995), insulin pro-receptor (Bravo *et al.*, 1994), and membrane type 1-matrix metalloproteinase (Sato *et al.*, 1996). The proteolytic cleavage is not only an important part of the biosynthetic process, but also a regulatory step in various physiological processes such as blood coagulation, fibrinolysis, and complement activation.

I.1.4.1 Biosynthesis and processing of ANP

The human pro-ANP gene (*Nppa*) resides on chromosome 1p36.2 and comprises three exons and two introns that span ~2 kb (Yang-Feng *et al.*, 1985). Transcription of the ANP gene yields an ~0.8 kb-long mRNA that encodes a 151-amino-acid peptide, prepro-ANP (Maki *et al.*, 1984; Oikawa *et al.*, 1984; Seidman *et al.*, 1984, Nakayama *et al.*, 1984; Yamanaka *et al.*, 1984). After cleavage of a 25-amino-acid hydrophobic signal sequence by the signal peptidase, the prohormone pro-ANP (1-126) is stored in the dense granules of atrial cardiomyocytes (Thibault *et al.*, 1987). The primary stimuli for pro-ANP secretion from the granules are atrial stretch and/or volume overload as shown by several *in vivo* and *in vitro* studies (Katsube *et al.*, 1985; Lang *et al*., 1985; Ledsome *et al.*, 1986; Ruskoaho *et al*., 1986; Schiebinger and Linden, 1986). Some hormones and neurotransmitters may also play a role in stimulating ANP release. The peptide hormone endothelin, for example, was shown to stimulate ANP secretion in superfused rat atria (Schiebinger and Gomez-Sanchez, 1990). Administration of the neurotransmitter norepinephrine to isolated rat hearts also resulted in an increase of ANP release (Currie and Newman, 1986).

The proteolytic cleavage of pro-ANP at Arg98 leads to the generation of the C-terminal mature ANP (99-126) and the amino-terminal (N-terminal) inactive cleavage fragment (1-98), both circulating in the blood (Flynn *et al.*, 1983; Michener *et al.*, 1986; Schwartz *et al.*, 1985; Thibault *et al.*, 1985). For many years, the enzyme responsible for the proteolytic cleavage of pro-ANP in the heart remained poorly defined. In the mid 1980s, several reports were published describing enzymes that cleaved pro-ANP *in vitro* including trypsin (Currie *et al.*, 1984a), kallikrein (Currie *et al.*, 1984b), and thrombin (Michener *et al.*, 1986). Cromlish and colleagues isolated a porcine pituitary protease called IRCM-serine protease 1 that displayed trypsin-like substrate specificity (Cromlish *et al.*, 1986a; 1986b). Later, a rat homologue of this protease was isolated from heart atria and ventricles. Rat IRCM-serine protease 1 cleaved pro-ANP at three different sites, Arg98, Arg101, and Arg102 (Seidah *et al.*, 1986). Another protease, designated atrioactivase, was partially purified from fractionated bovine atria. This enzyme also displayed general properties of a serine protease

and was able to convert bovine pro-ANP to ANP by specific cleavage at Arg98. Based on size exclusion chromatography, the enzyme appeared to have a high molecular weight (MW) of 580 kDa, whereas by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis a cluster of several bands with MWs of around 30 kDa was observed (Imada *et al.*, 1987; 1988). A more detailed characterization of this potential pro-ANP-converting enzyme has never been reported. For the next few years, the identity of the physiological pro-ANP convertase remained unknown.

In addition, the localization of the pro-ANP convertase was also a subject of investigation. Mechanisms of pro-ANP processing within the atrial granules (Wypij and Harris, 1988), in or on cardiomyocytes concurrently with secretion, and postsecretional cleavage on the myocyte surface had been suggested (Shields and Glembotski, 1988; Sei *et al.*, 1992). The coexistence of pro-ANP and its converting-enzyme inside the atrial granules was soon disproved by a report in which lysates of isolated atrial granules failed to process pro-ANP (Corthorn *et al.*, 1991). Despite the continuous progress in ANP research, the pro-ANP-converting enzyme remained unknown for several more years. Figure 1.2 summarizes the essential steps that lead to the generation of biologically active ANP (99-126).

I.1.4.2 Biosynthesis and processing of BNP

The human BNP gene *Nppb* is located 8 kb downstream of the *Nppa* gene on chromosome 1 and, like the *Nppa* gene, contains three exons (Steinhelper, 1993; Arden *et al.*, 1995). The *Nppb* mRNA encodes the 132-amino-acid precursor prepro-BNP, which, after removal of the signal peptide by the signal peptidase, yields the propeptide pro-BNP (1-108). Specific cleavage of pro-BNP at Arg76 results in the biologically active BNP (77-108). Unlike ANP, the mature peptide BNP but not the propeptide pro-BNP is the major form of the hormone present in the cardiomyocytes (Kambayashi *et al.*, 1990). The ubiquitous endoprotease furin was indicated to be the major converting enzyme responsible for the pro-BNP processing step. In stretch-induced hypertrophic neonatal ventricular cardiocytes, the endogenous pro-BNP was converted to BNP as assessed by high pressure liquid chromotography (HPLC)

analysis. The pro-BNP processing activity was inhibited by furin-specific inhibitors but not by inhibitors against other proteases such as trypsin or thrombin (Sawada *et al.*, 1997). However, pro-BNP has also been detected in the conditioned medium from transfected cultured cells (Yan *et al.*, 2000) and in plasma from patients with congestive heart failure, indicating that pro-BNP is not completely processed intracellularly. To date, it is not clear if other proteases also contribute to the processing of pro-BNP.

I.1.4.3 Biosynthesis and processing of CNP

The gene encoding human CNP (*Nppc*) is localized to chromosome 2 and comprises two exons and one intron. The protein is a prepropeptide of 126 amino acids that first undergoes the removal of the signal peptide by the signal peptidase. The resulting 103-amino-acid propeptide, pro-CNP, is further processed by specific cleavage of the peptide bond Arg50-Asp51 generating an inactive N-terminal propeptide (1-50) and a C-terminal fragment CNP-53 (51-103) (Tawaragi *et al.*, 1991). Both cleavage products are secreted. In cell-based experiments, furin was demonstrated to be a critical enzyme for pro-CNP processing (Wu *et al.*, 2003). Human embryonic kidney (HEK) 293 cells and chondrosarcoma SW 1353 cells processed pro-CNP intracellularly as detected by Western analysis. The pro-CNP processing activity was inhibited in a dose-dependent manner when the cells were incubated with a furin-specific inhibitor, whereas inhibitors of matrix metalloproteases or tumor-necrosis factor-α convertase had no effect. The same study showed that in the furin-deficient cell line LoVo the ability to process pro-CNP was abolished. This effect could be restored by transfection of LoVo cells with recombinant furin (Wu *et al.*, 2003). CNP-53, was shown to be the major molecular form in porcine brain (Minamino *et al.*, 1990). However, CNP-53 can undergo an additional processing step at Lys80-Gly81 (referring to the pro-CNP amino acid sequence) producing the C-terminal fragment CNP-22, the form that is usually referred to as CNP. The extracellular enzyme responsible for CNP-53 conversion is still unknown.

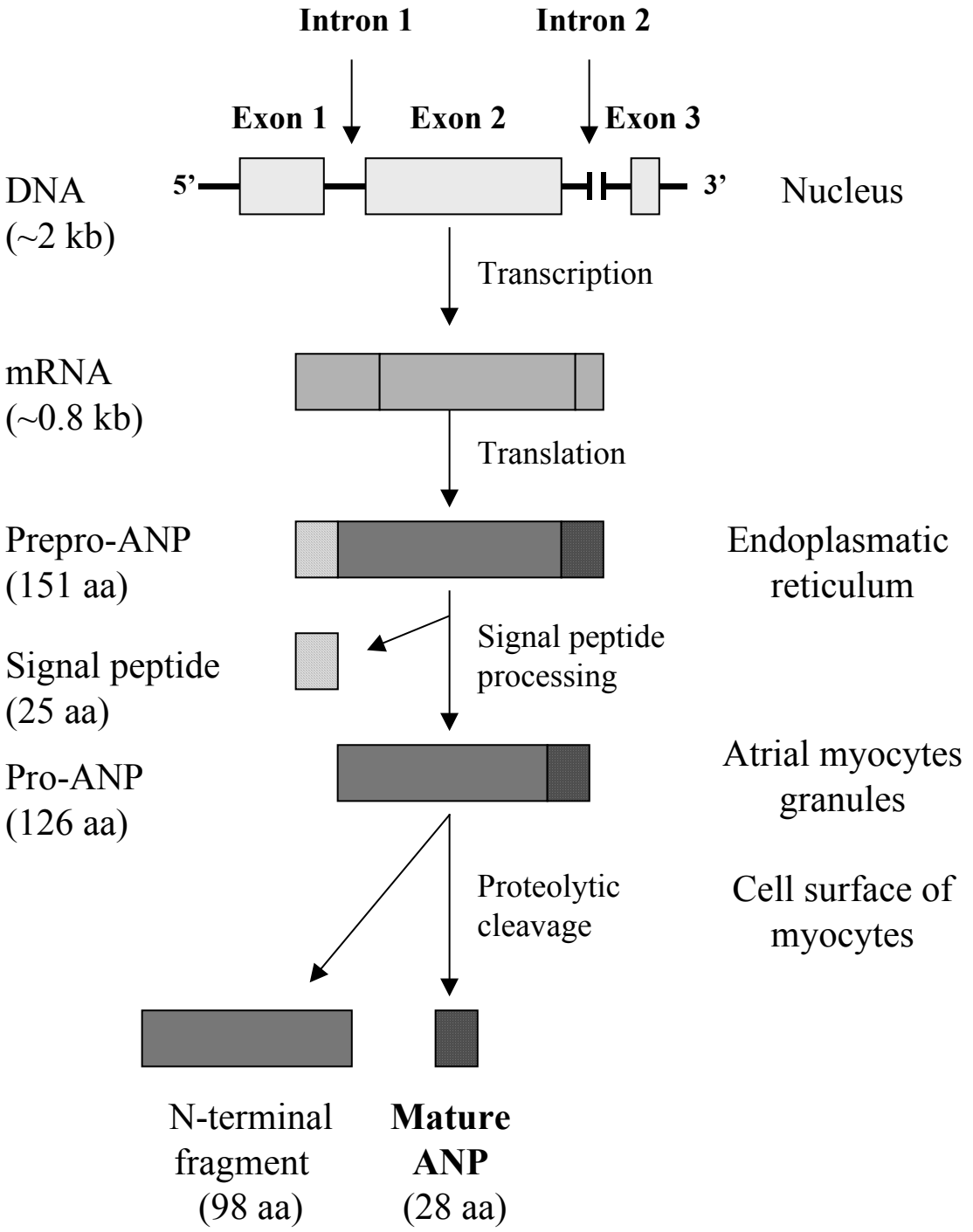

Figure 1.2. Diagram of the essential steps leading to the generation of biologically active ANP.

I.2 Discovery of Corin

In 1999, the search for the high-MW serine protease in the heart responsible for pro-ANP conversion took an unexpected turn. Yan *et al.* cloned and characterized a 1042-amino-acid-long, membrane-spanning serine protease from the heart. The enzyme possessed a unique structural architecture consisting of a variety of modular domains, which are described in section I.3. For its abundant expression in the heart, this novel protease was designated corin (Cor means "heart" in Latin.).

I.2.1 Cloning of corin

Human corin was first identified as an expressed sequence tag (EST) clone from a heart cDNA library during a search for novel serine proteases in the cardiovascular system (Yan *et al.*, 1999). In Northern analysis, the EST-derived probes were used to detect an ~5 kb transcript in mRNA sample from the human heart, but not other tissues such as the liver, lung, brain, skeletal muscle, spleen, and placenta. The size of detected mRNA was consistent with the size of the isolated full-length corin cDNA of ~4.9 kb. In mice, corin mRNA expression was detected in embryonic heart as early as day E9.5, which became highly abundant by day E15.5. Corin mRNA expression was also detected in the pregnant uterus, developing bones and kidneys of mice. In an independent study, human corin cDNA was also cloned by Hooper and co-workers in a search for novel serine proteases related to cancer (Hooper *et al.*, 2000). Tomita and colleagues cloned murine corin cDNA. The cDNA-deduced amino acid sequence revealed the presence of eight low-density lipoprotein receptor (LDLR) repeats in mouse corin protein. Therefore, it was initially considered as a member of the LDLR protein family and termed LDLR-related protein (LRP) 4 (Tomita *et al.*, 1998). Most recently, the cloning of the full-length cDNA for rat corin has also been reported (Langenickel *et al.*, 2004).

I.2.2 Structural elements of the *corin* gene

By fluorescence in situ hybridization analysis, the human *corin* gene has been mapped to chromosome 4 (4p12-13) (Yan *et al.*, 1999). Both the mouse and human *corin* gene comprise 22 exons and span >200 kb. In a study of the human and mouse *corin* gene promoters, two conserved GATA elements were found to be major transcriptional elements for *corin* gene expression (Pan *et al.*, 2002). A mutation within the GATA sequence resulted in a significant decrease of promoter activity in transfected cardiomyocytes. Furthermore, an electrophoretic mobility shift assay was performed to show that transcription factor GATA-4, but not other GATA proteins such as GATA-1, -3, and –6, bind to the GATA elements of the *corin* gene. Interestingly, GATA-4 also plays a critical role in the cardiac expression of the ANP and BNP genes, suggesting that these three genes have a similar regulation mechanism (Durocher and Nemer, 1998; Charron and Nemer, 1999; Grepin *et al.*, 1994). In addition, the *corin* gene contains other putative regulatory regions including two TBX5-binding sites (Bruneau *et al.*, 2001), two GT-boxes for the Krüppel-like factors (Bieker, 2001), one NKX2.5-binding site (Chen and Schwartz, 1995), and a TATA box (Bucher, 1990).

I.2.3 Corin as a new candidate for the pro-ANP convertase

Corin displays an expression pattern and a mechanism for gene regulation similar to that of pro-ANP. In addition, the catalytic domain of corin showed the characteristics of trypsin-like serine proteases, which will be discussed in more detail in section I.3.2.4. The homology to other members of the trypsin superfamily was determined by amino acid sequence alignment. For instance, the amino acid sequence of the catalytic domain of corin was 40 % identical to the trypsin-like serine proteases prekallikrein or factor XI (Yan *et al.*, 1999). Trypsin-like enzymes have a preference for cleaving their substrates after basic amino acid residues such as arginine or lysine. The processing of pro-ANP to biologically active ANP also requires proteolytic cleavage at a basic residue, Arg98. These observations led to the hypothesis that corin could be a candidate for the pro-ANP-converting enzyme.

Initial co-expression experiments of pro-ANP and corin in HEK 293 cells provided first evidence to support this hypothesis. Transfection of recombinant human corin led to the processing of the prohormone to a smaller peptide of the size predicted for ANP. Cells expressing wild-type (WT) corin cleaved pro-ANP specifically at the Arg residue within the activation cleavage site Leu94-Tyr95-Ala96-Pro97-Arg98↓Ser99, whereas no cleavage occurred when the corin active site mutant S985A was tested (Yan *et al.*, 2000).

The activation cleavage site of pro-BNP (Leu72-Arg73-Ala74-Pro75-Arg76↓Ser77) is similar to that of pro-ANP. To determine the processing ability of corin for pro-BNP, cells were also co-transfected with corin and pro-BNP. Cells transfected with corin processed the prohormone to a peptide with the size expected for BNP. However, the corin-mediated processing of pro-BNP was less efficient than the processing of pro-ANP (Yan *et al.*, 2000). The results suggest that corin-mediated pro-BNP conversion might be an alternative mechanism for pro-BNP processing, which is also carried out by furin as described in section I.1.4.2.

To further demonstrate that corin is the endogenous protease in cardiac myocytes that is responsible for the processing of pro-ANP, its functional properties were studied in a murine cardiac myocytic cell line called HL-5. The HL-5 cell line is derived from the lineage of mouse atrial cardiomyocyte tumor (AT-1) cells (White *et al.*, 2004). AT-1 cells, on the other hand, were originally obtained from transgenic mice in which the transcriptional regulatory sequences of ANP were fused with those encoding the SV40 T antigen (Field, 1988). AT-1 cells displayed the phenotype of cardiomyocytes but could not be cultured continuously. A few years ago, AT-1 cells were used to develop the first differentiated cardiac cell line, HL-1, that could be maintained in cell culture (Claycomb *et al.*, 1998). HL-1 and HL-5 cells were demonstrated to share similar properties (White *et al.*, 2004). Both cell lines maintain the differentiated phenotype of cardiomyocytes including the presence of dense granules containing pro-ANP, making them an ideal cell system to study pro-ANP processing.

In transfection experiments with HL-5 cells, an endogenous enzyme processed recombinant pro-ANP to ANP by specific cleavage at Arg98 (Wu *et al.*, 2002). When the active site corin mutant S985A was overexpressed in HL-5 cells, the processing of pro-ANP was inhibited, indicating a dominant inhibitory effect of mutant corin S985A on the endogenous pro-ANP convertase. Furthermore, transfection of small interfering RNA (siRNA) duplexes directed against the mouse *corin* gene blocked the processing of pro-ANP in HL-5 cells completely. Taken together, these findings indicated that corin is indeed the pro-ANP convertase in cardiomyocytes (Wu *et al.*, 2002).

I.3 Structure of Corin

I.3.1 Cell membrane expression of corin protein

Most trypsin-like serine proteases are synthesized as secreted proteins. Corin, however, is anchored in the cell membrane by a short sequence of 21 amino acids near the N-terminus, which were identified by hydropathy plots (Kyte and Doolittle, 1982; Yan *et al.*, 1999). Based on the presence of positively charged amino acids preceding this hydrophobic region, the N-terminus of corin is predicted to be cytoplasmic and the C-terminus to be extracellular (Hartmann *et al.*, 1989; Yan et al, 1999). The extracellular domain of corin also contains 19 potential N-linked glycosylation sites, consistent with the predicted orientation of the protein on the cell membrane. A recombinant form of human corin, which contains a C-terminal epitope tag, was expressed in HEK 293 cells. It appeared as a single band with a molecular mass of ~150 kDa when analyzed by SDS-PAGE and Western blotting. The protein was detected in total cell lysates and membrane fractions but not in the conditioned cell culture medium. These results were in agreement with the prediction that corin is a transmembrane protein (Yan *et al.*, 2000).

Based on its topology, corin belongs to the recently defined class of type II transmembrane serine proteases (TTSPs). To date, the following members of this protease class have been identified: enterokinase (EK) also called enteropeptidase (Kitamoto *et al.*, 1995), matriptases (Lin *et al.*, 1999; Takeuchi *et al.*, 1999, Velasco *et al.*, 2002), hepsin (Leytus *et al.*, 1988), TMPRSS2-4 (Paoloni-Giacobino *et al.*, 1997; Scott *et al.*, 2001; Wallrapp *et al.*, 2000), human airway trypsin-like protease (HAT) (Yamaoka *et al.*, 1998), MSPL (Kim *et al.*, 2001), differentially expressed in squamous cell carcinoma (DESC) 1 protein (Lang and Schuller, 2001), spinesin (Yamaguchi *et al.,* 2002), and polyserase-1 (Cal *et al.*, 2003). All members share a basic architecture as shown in Figure 1.3: a short N-terminal tail in the cytoplasm, a single span transmembrane domain, and an extracellular region, which includes the protease domain(s) at the C-terminus and at least one additional structural module between the transmembrane domain and the protease domain. The location of TTSPs on the cell surface promotes interaction with other membrane proteins or soluble proteins from the extracellular environment.

The specific physiological function of most TTSPs is not well understood. For some members, however, functions have been indicated or demonstrated. For example, EK plays a key role in the protease cascade of the digestive system. It converts trypsinogen to active trypsin, which in turn activates other digestive enzymes including chymotrypsinogen and proelastase (Kitamoto *et al.*, 1995; Lu *et al.*, 1997). Other TTSPs have been implicated in a variety of biological processes such as participation in signaling cascades via protease-activated receptors (Takeuchi *et al.*, 2000), activation of growth factors (Lee *et al.*, 2000), blastocyst hatching during embryonic development (Vu *et al.*, 1997), and host defense in the mucous membrane (Yamaoka *et al.*, 1998).

I.3.2 The modular architecture of corin

Many proteins are composed of a certain number of structural units called domains or modules that are assembled in a specific sequence. The unique composition in each protein allows for specificity in its interactions with proteins including cell surface receptors, intracellular signaling molecules, and extracellular matrix (ECM) proteins. Several thousand different kinds of modules exist, and their size varies from 25 to 500 amino acids (Campbell, 2003). One example for an extracellular formation of modular proteins is the growth factor/receptor signaling complex of erythropoietin (Wilson *et al.*, 1999). In the TTSP family, all members are of modular nature. Corin and EK have the most complex mosaic structure among the TTSPs and exceed a length of 1000 amino acids. Besides the serine protease domain(s), TTSPs contain additional structural motifs in the extracellular region such as LDLR class A -, frizzled-like (Fz) cysteine-rich -, CUB- (complement, urchin embryonic growth factor and bone morphogenic protein), or macrophage scavenger receptor (SR) cysteine-rich domains. These different domains are commonly found in modular proteins of other families, where they contribute to enzyme regulation or ligand binding. However, little is known about the role of these non-catalytic domains in proteins of the TTSP family. Figure 1.3 shows the schematic modular structure of all human members of the TTSP family reported to date.

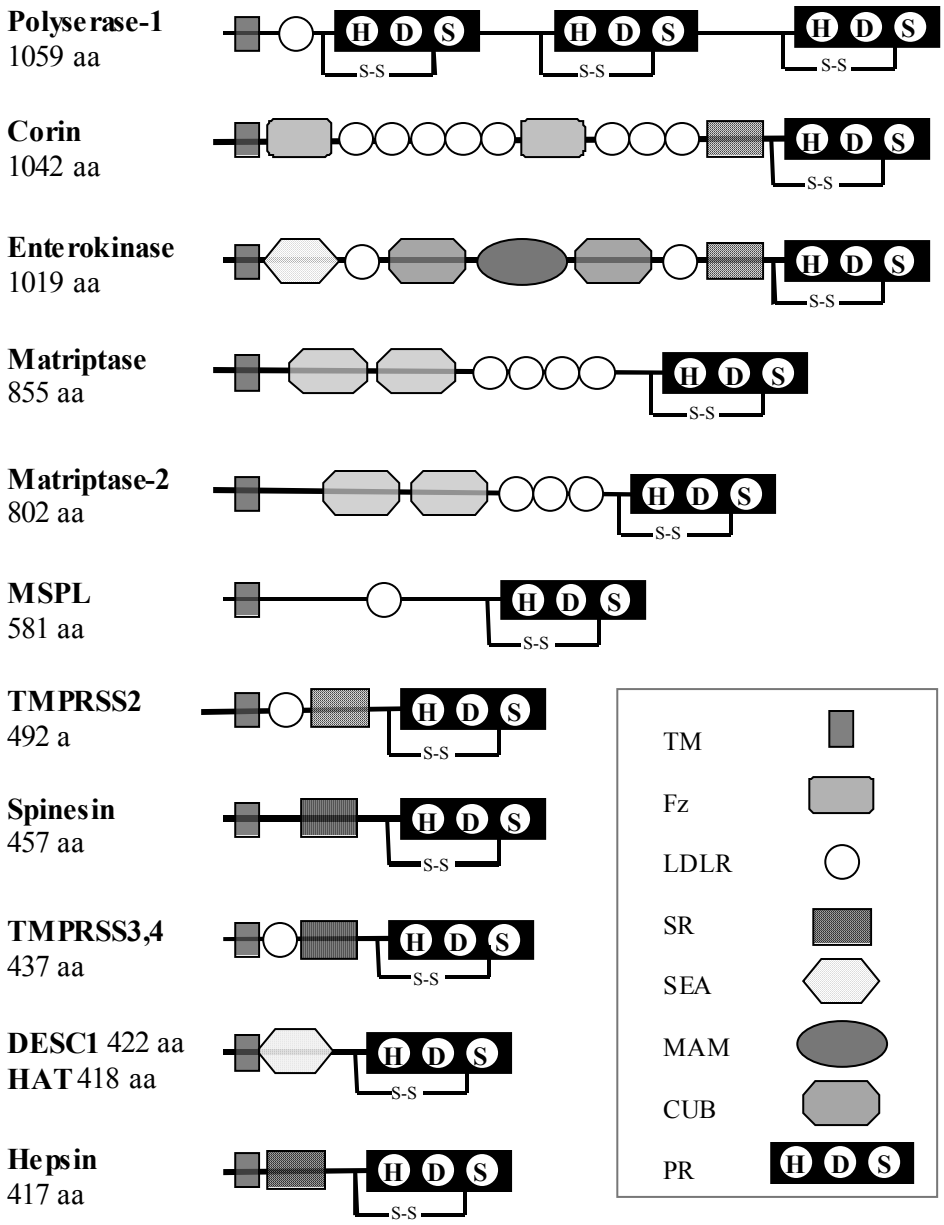

Figure 1.3. Schematic presentation of the modular structure of the TTSPs in human. All members of this class contain a transmembrane domain (*TM*) at the N-terminus and a protease catalytic domain (*PR*) at the C-terminus. The active site residues His (*H*), Asp (*D*), and Ser (*S*) are indicated. Additional domain structures are present in the extracellular region including Fz modules (*Fz*), LDLR repeats (*LDLR*), SR domains (*SR*), sea urchin sperm protein domains (*SEA*), meprin-like domains (*MAM*), and CUB repeats (*CUB*). *S-S* indicates the disulfide bond that connects the propeptide with the protease domain.

I.3.2.1 Frizzled domains

One unique structural feature of corin is the presence of two Fz cysteine-rich domains that span the amino acid sequences 134-259 and 454-573. Amino acid sequence alignment of these regions with the corresponding domain of Frizzled family members showed a highly conserved pattern of 10 cysteine residues (Yan *et al.*, 1999). Crystal structure studies of Fz domains in Frizzled 8 and secreted Frizzled-related protein 3 have shown that these cysteine residues form disulfide bonds in the following pattern: Cys1-5, Cys2-4, Cys3-8, Cys6-10, and Cys7-9. Furthermore, the structural studies revealed predominantly α-helical structures and two short β-strands at the N-terminus. Mutagenesis-based experiments have identified regions on the surface of the Fz domain for interactions with Wnt signaling proteins (Dann *et al.*, 2001).

Generally, the Fz module is found in a group of proteins that is encoded by the *Frizzled* gene family, and is widely expressed in many animal species. The seven-pass transmembrane proteins of the Frizzled family serve as receptors for Wnt signaling proteins (Bhanot *et al.*, 1996). Wnt proteins, on the other hand, are known to play important roles as intercellular signaling molecules in differentiation and development (Nusse and Varmus, 1992; Wodarz and Nusse, 1998). In addition, the Fz domain is present in several secreted Frizzled-related proteins (Rattner *et al.*, 1997), a number of receptor tyrosine kinases (Xu and Nusse, 1998), mouse collagen α1 (XVIII) chain (Rehn and Pihlajaniemi, 1995) and human metallocarboxypeptidase Z (CPZ), a secreted zinc-dependent enzyme (Song and Fricker, 1997). To date, corin is the only serine protease reported that contains the Fz cysteine-rich motifs.

I.3.2.2 Low-density lipoprotein receptor repeats

Corin contains two clusters of LDLR repeats, LDLR-CI and LDLR-CII, which span the amino acid sequences 268-452 and 579-689, respectively. LDLR-I consists of five LDLR repeats, each comprising about 38 amino acids. The first four repeats include 6 highly conserved cysteine residues and a cluster of acidic residues at the C-terminal end. As determined by crystal structure of the ligand binding repeat 5 of the LDLR, four of these acidic amino acids as well as two additional residues coordinate a Ca^{2+} atom in an octahedral arrangement, which is essential for correct protein folding (Fass *et al.*, 1997). In the fifth LDLR repeat of corin, however, the cysteine pattern is less conserved, and it lacks four of the five conserved negatively charged amino acids. LDLR repeats 6 to 8 of corin are located in LDLR-CII, and their sequences are well conserved (Yan *et al.*, 1999).

The group of LDLR-like receptors contains LDLR repeats as common building blocks in the extracellular region. In 1984, the prototype of this receptor family, the LDLR itself, was first cloned and characterized by Yamamoto and colleagues (Yamamoto *et al*., 1984). In humans, the LDLR family also includes the LRP, the very low-density lipoprotein receptor (VLDLR), the apoE receptor 2 (apoER2), megalin/gp330, LRP5, LRP6 (Brown *et al*., 1998; Hey *et al*., 1998, Hussain, 1999; Nykjaer and Willnow, 2002), and LRP7/LR3 (Dong *et al*., 1998) as well as the two most recently discovered members, LRP8 and LRP9 (Riddell *et al.*, 1999; Sugiyama *et al.*, 2000). The LDLR mediates the cellular uptake of lipoproteins containing cholesterol by a process called receptor-mediated endocytosis. Other members of this receptor class also display lipoprotein-binding ability. More recently, new molecular interactions for members of the LDLR family have emerged. Among other roles, LDLR-like receptors bind proteases, protease inhibitors, or lipases and act as signal transducers in neuronal migration processes and mediate vitamin uptake (Gliemann, 1998; Herz and Strickland, 2001). The LDLR motif is also found in proteins that are not functionally related to the LDLR family, e.g. the complement component C9 (Stanley *et al.*, 1985). Besides corin, one or more LDLR repeats have been identified in other TTSPs including matriptases, EK, TMPRSS2-4 and MSPL (see Figure 1.3). The function of LDLR repeats in these TTSPs is still unclear.

I.3.2.3 Scavenger receptor domain

The third type of module in the structure of corin is a class A SR cysteine-rich domain that is located between amino acids 713-789. The amino acid sequence of this domain shares homology with the respective domain of human EK, sea urchin speract receptor, and human SR-AI (Yan *et al.*, 1999). However, the SR domain of corin is a truncated version of the one found in the SR-AI, which comprises ~110 amino acids (Kodama *et al.*, 1990). The structure of a full-length SR domain contains three disulfide bridges between three pairs of cysteine residues (Resnick *et al.*, 1996). Crystal structure studies of SR domains of the Mac-2 binding protein revealed six β-strands cradling an α-helix (Hohenester, 1999). In contrast, the truncated SR domain of corin contains only four cysteine residues, which are predicted to form two disulfide bridges. This difference might result in changes of the overall structure of the SR module in corin.

SR-AI serves as the eponym for the family of macrophage scavenger receptors, a group of trimeric membrane glycoproteins that bind polyanionic macromolecules such as modified lipoproteins, surface lipids of bacterial origin, or polynucleotides (reviewed in de Winther *et al.*, 2000). Class A SRs play a role in atherosclerosis, cell adhesion, and host defense (Matsumoto *et al.*, 1990; Suzuki *et al.*, 1997; Thomas *et al.*, 2000). In addition, the SR motif has been found in cell surface and secreted proteins such as neurotrypsin (Proba *et al.*, 1998), lysyl oxidase-like 3 protein (Huang *et al.*, 2001), and the lymphocyte cell surface receptor CD6 (Resnick *et al.*, 1994) among many others. In the family of TTSPs, the SR motif is also widely distributed as shown in Figure 1.3. The function of the SR domains in these diverse proteins is for the most part still unclear. To date, only one study has linked a ligand-binding event directly to a SR motif. In cell and protein binding assays, it was demonstrated that CD6 binds the activated leukocyte cell adhesion molecule via a membrane-proximal SR domain (Whitney *et al.* 1995).

I.3.2.4 Serine protease domain

The protease domain at the C-terminus of corin (amino acids 790-1042) shares high sequence homology with the catalytic domain of trypsin-like proteases (see section I.2.3). Serine proteases represent the most extensively studied group of proteolytic enzymes. They are involved in a wide range of biological processes such as food digestion, blood coagulation, complement activation, and hormone processing (Neurath, 1984; Davie *et al.*, 1991). In corin, all the characteristic residues that contribute to enzymatic catalysis and substrate recognition are conserved (Yan *et al.*, 1999). This includes the catalytic triad (His843, Asp892, and Ser985) and residues that shape the substrate specificity pocket (Asp979, Gly1007, and Gly1018).

Mammalian trypsin-like serine proteases are usually synthesized as inactive proenzymes, i.e. zymogens, consisting of a single polypeptide chain. Activation occurs when the zymogen is cleaved at one or several specific sites. Most commonly such cleavage is accomplished by the action of another protease. In corin, the conserved activation cleavage sequence Arg801↓Ile802-Leu803-Gly804-Gly805 is located between the SR domain and the protease domain. A disulfide bond at Cys790-Cys912 bridges these two domains and allows an attachment of the protease domain to the stem region after activation cleavage. The activation cleavage at the peptide bond between Arg801-Ile802 predicts that the corin activator is another trypsin-like enzyme. To date, however, the corin activator has not been identified.

The Cys790-Cys912 disulfide bond is one of five pairs of conserved cysteine residues that are predicted to form the secondary protein structure of the protease domain in corin. Surprisingly, there are two additional cysteine residues (Cys817 and Cys830) in corin, a unique feature among vertebrate serine proteases. Based on database search, the only other serine protease containing a cysteine pair at the corresponding positions identified at that time was a chymotrypsin-like enzyme from the lugworm, *Arenicola marina* (J. Eberhardt, GenBank™ accession number G1160388). Computational modeling of the protease domain

of corin predicted that these two cysteines connect two β-sheets in the core of the protease domain (Yan *et al.*, 1999). In the course of my diploma project, the importance of these two cysteine residues was assessed. A double mutant corin C817/830S was generated by site-directed mutagenesis and co-transfected with pro-ANP in HEK 293 cells. Western blot analysis showed that the pro-ANP processing activity of the mutant corin C817/830S was completely abolished, whereas WT corin converted pro-ANP to ANP. The mutational study of residues Cys817 and Cys830 demonstrated that the unique disulfide bond in corin is required for corin function. It is possible that the mutation C817/830S alters the overall folding of the corin protein, thereby interfering with the protein trafficking to the cell membrane (Knappe, 1999).

I.4 Corin in Human Diseases

I.4.1 Corin and hypertension

Hypertension is a leading cause for morbidity and mortality worldwide. According to the American Heart Association, in the United States alone more than 50 million people have the disease. The physiological significance of the ANP-mediated pathway for regulating blood pressure has been demonstrated by many studies. In knockout mice, for example, ANP or NPR-A deficiency resulted in spontaneous hypertension (John *et al.*, 1995; Lopez *et al.*, 1995), whereas in transgenic mice, overexpression of the respective genes had hypotensive effects (Steinhelper *et al.* 1990; Oliver *et al.*, 1998). Corin is proposed to function as an up-stream regulator of the ANP signaling pathway. Therefore, reduction or absence of corin expression is expected to lead to hypertension through the decrease in ANP generation. To test this hypothesis, a corin-deficient mouse was generated by homologous recombination. Blood pressure was examined by indwelling telemetry in the animal. Compared to WT mice, corin-deficient mice showed an increased systolic and diastolic blood pressure that was further enhanced when the animals were fed a high-salt diet. Importantly, increased levels of pro-ANP but not mature ANP were detected in atrial tissues from corin-deficient mice by

HPLC and Western analysis, indicating that the lack of corin prevents the processing of pro-ANP. These findings confirmed that corin is in fact the physiological pro-ANP-converting enzyme and plays a critical role in regulating blood pressure (Chan *et al.*, 2004).

I.4.2 Corin in congestive heart failure

Hypertension is a common cause for congestive heart failure (CHF) (Levy, 1996), a serious condition defined as the failure of the heart to function normally, leading to excessive retention of water and salt, which eventually results in fluid build-up in the lungs. As a result, patients with CHF experience shortness of breath and fatigue. Worldwide millions of people are affected by CHF, which has a mortality rate of approximately 20 percent each year. In the United States alone, five million people suffer from CHF and each year about 550,000 new cases are diagnosed (American Heart Association, 2004). Heart failure can result from multiple factors such as coronary artery disease (Fox *et al.*, 2001), hypertension, and metabolic diseases such as thyroid disorders (Ladenson *et al.*, 1992). Regardless of the cause, the end stage of CHF has similar pathophysiological features. In early stages of CHF, cardiac enlargement (hypertrophy) is an adaptive mechanism that comes into effect as the heart attempts to improve output. As the disease progresses, however, the heart over-compensates for declining systolic performance and hypertrophy reaches a pathological state (Francis *et al.*, 1995).

A hallmark of CHF is the activation of the cardiac endocrine system, the up-regulation of ANP and BNP in particular (Burnett *et al*., 1986; Wei *et al.*, 1993; Langenickel *et al.*, 2000). In CHF, the cardiac ventricles become the major source for the circulating ANP, which has been demonstrated in experimental animals (Thibault *et al.*, 1989) as well as in patients with heart failure and left ventricular hypertrophy (Yasue *et al.*, 1994). The natriuretic peptide response to heart failure is a beneficial compensatory response, counteracting the progression of the pathophysiological symptoms of CHF by mediating vasodilation, natriuresis, growth suppression, and inhibition of both the sympathetic nervous system and the renin-angiotensin-aldosterone axis. In animal models of heart failure, blocking of the NPR-A

caused an increase in plasma renin and aldosterone levels, and a reduction of urine flow rate and urinary sodium excretion (Wada *et al.*, 1994), whereas infusion of exogenous ANP prevented the reduction in urine output and sodium excretion (Lee *et al.*, 1989).

For diagnostic applications, plasma ANP and BNP levels have been used to diagnose the severity of CHF. BNP was found to better reflect the state of the disease because it has a longer plasma half-life than ANP (Mukoyama *et al.*, 1991). Therapeutically, ANP and BNP have been shown to be potential candidates for treatment of CHF. Recently, human recombinant BNP, marketed as "Natrecor", has been approved for treatment of patients with acutely decompensated heart failure and acute myocardial infarction to improve cardiac function (Colucci *et al.*, 2000).

Most recently, two groups studied the *corin* gene expression in experimental heart failure. Tran and colleagues exploited a rat model in which heart failure is induced by ligation of the left coronary artery (Tran *et al*., 2004). At different time points after surgery, the corin mRNA level in the non-infarcted left ventricle myocardium was determined by real-time quantitative reverse transcriptase-PCR (RT-PCR). No significant change in corin mRNA levels was found until 8 weeks after surgery when a three-fold increase of corin mRNA expression was observed. These findings were further supported by cell-based assays with rat neonatal cardiomyocytes, which were stimulated with phenylephrine (PE) to induce hypertrophy. Both, *corin* and *ANP* genes were up-regulated in the PE-stimulated cells as determined by real-time quantitative RT-PCR. In addition, PE-stimulated cardiomyocytes showed increased processing activity toward human recombinant pro-ANP that was added to the cells. This study indicated that hypertrophic stimuli induced corin expression in cardiomyocytes and that corin might contribute to the pathophysiological phenotype of cardiac hypertrophy and heart failure (Tran *et al.*, 2004). Independently, Langenickel and co-workers studied the *corin* gene expression in a rat model in which heart failure is induced by volume overload caused by an aortacaval shunt (Langenickel *et al*., 2004). Corin mRNA levels were determined in the atrium by Northern blot analysis at a single time point, 4 weeks after surgery. A slight decrease of 18 and 11 % in corin mRNA expression was observed for the left and right atrium, respectively. In this study, however, the corin mRNA expression in

ventricular cardiomyocytes was not examined (Langenickel *et al.*, 2004). Taken together, these two studies suggest that the regulation mechanism for the *corin* gene in the atrium might be different from that in the ventricle under the pathophysiological conditions of heart failure.

I.4.3 Implication of corin in cancer

Corin mRNA has also been found in cancer cells derived from osteosarcoma, leiomyosarcoma, endometrical carcinoma, and small cell lung cancer (SCLC) (Yan *et al.*, 1999; Wu and Wu, 2003). In patients with SCLC, hyponatremia is a common clinical syndrome, but its molecular mechanism is not well understood. This syndrome is an electrolyte disorder manifesting itself in inappropriately concentrated urine, increased urine Na^+ concentration, and increased intravascular volume. Because the high levels of antidiuretic hormone (ADH) are found in some SCLC patients with hyponatremia, ADH is considered a main cause of the condition. Therefore, the disease is also called the syndrome of inappropriate secretion of ADH (SIADH). SIADH can affect up to half of all SCLC patients (Marchioli and Graziano, 1997; Patel *et al.*, 1993).

Studies have found that plasma levels of ANP are also elevated in SIADH (Cogan *et al*., 1986; Donckier *et al*., 1986), and that most SCLC cells produce ANP (Bliss *et al.*, 1990; Campling *et al.*, 1995; Gross *et al*., 1993), suggesting that ANP may also contribute to SIADH. It was not known, however, how pro-ANP was processed in SCLC cells. A recent report indicates a role of corin as the pro-ANP convertase in SCLC (Wu and Wu, 2003). In this study, corin-expressing SCLC cells were shown to have the ability to process recombinant pro-ANP to biologically active ANP. Furthermore, using siRNA duplexes against the *corin* gene in cultured SCLC cells, it was demonstrated that the processing of pro-ANP was completely blocked. These findings demonstrated that corin is the major pro-ANP-converting enyzme in SCLC cells, and thus, might contribute to the development of cancer-associated SIADH (Wu and Wu, 2003).

I.4.4 Corin and total anomalous pulmonary venous return

A connection between corin and a rare condition called total anomalous pulmonary venous return (TAPVR) has also been considered. TAPVR is a congenital heart defect in which the pulmonary vein is abnormally connected to the right atrium. The disease locus and the *corin* gene are both mapped to the same region of chromosome 4 (Bleyl *et al.*, 1995; Yan *et al.*, 1999), suggesting a possible contribution of deficient or abnormal corin to the TAPVR phenotype. However, an immunohistochemical study analyzing heart tissue sections derived from a TAPVR patient for corin protein expression showed no apparent decrease of corin protein in these samples (Hooper *et al.*, 2000). Currently, no genetic studies have been reported that scan TAPVR patients for point mutations in the *corin* gene and other genes within the disease locus.

I.5 Objective of the Dissertation

In this study, we focused on the structure and function relationship of the cardiac TTSP corin. We wanted to know if the transmembrane domain of corin is necessary for enzymatic and biological activities and if activation cleavage at the conserved site Arg801 is required for corin function. The answers to these questions would allow us to judge the feasibility of generating a soluble corin that can be purified and analyzed for its pro-ANP processing activity and basic biochemical properties such as substrate specificity, kinetic constants, and sensitivity to inhibitors. In addition, we wanted to assess the requirement of the structural domains in the propeptide of corin for pro-ANP processing and possibly identify a specific region within the propeptide that is important for the recognition of pro-ANP.