HHS Public Access

Author manuscript

Int J Biochem Cell Biol. Author manuscript; available in PMC 2019 January 01.

Published in final edited form as:

Int J Biochem Cell Biol. 2018 January ; 94: 31–39. doi:10.1016/j.biocel.2017.11.010.

Functional Analysis of Corin Protein Domains Required for PCSK6-mediated Activation

Shenghan Chen[a,b], **Hao Wang**[b], **Heng Li**[a], **Yue Zhang**[c], and **Qingyu Wu**[b,c,*]

[a]Human Aging Research Institute and School of Life Science, Nanchang University, Nanchang, China

[b]Department of Molecular Cardiology, Cleveland Clinic, Cleveland, Ohio, USA

[c]Cyrus Tang Hematology Center, Collaborative Innovation Center of Hematology, Soochow University, Suzhou, China

Abstract

Atrial natriuretic peptide (ANP) is a cardiac hormone essential for normal blood pressure and cardiac function. Corin is a transmembrane serine protease that activates ANP. Recently, we identified proprotein convertase subtilisin/kexin-6 (PCSK6), also called PACE4, as the long-sought corin activator. Both corin and PCSK6 are expressed in cardiomyocytes, but corin activation occurs only on the cell surface. It remains unknown if cell membrane association is needed for PCSK6 to activate corin. Here we expressed corin deletion mutants in HEK293 cells to analyze the domain structures required for PCSK6-mediated activation. Our results show that soluble corin lacking the transmembrane domain was activated by PCSK6 in the conditioned medium but not intracellularly. Recombinant PCSK6 also activated the soluble corin under cell-free conditions. Moreover, PCSK6-mediated corin activation was not enhanced by cell membrane fractions. These results indicate that cell membrane association is unnecessary for PCSK6 to activate corin. Experiments with monensin that blocks PCSK6 secretion and immunostaining indicated that the soluble corin and PCSK6 were secreted via different intracellular pathways, which may explain the lack of corin activation inside the cell. We also found that the protein domains in the corin pro-peptide region were dispensable for PCSK6-mediated activation and that addition of heparan sulfate and chondroitin sulfate or treatment with heparinase or chondroitinase did not alter corin activation by PCSK6 in HEK293 cells. Together, our results provide important insights into the molecular and cellular mechanisms underlying PCSK6-mediated corin activation that is critical for cardiovascular homeostasis.

[*]Corresponding author at: Molecular Cardiology, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA, wuq@ccf.org (Q. Wu).

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Keywords

corin; PCSK6; transmembrane domain; type II transmembrane serine protease; zymogen activation

1. Introduction

Atrial natriuretic peptide (ANP) is a cardiac hormone essential for cardiovascular homeostasis (de Bold 2011; Kuhn 2016; Song et al. 2015; Theilig and Wu 2015). ANP regulates salt-water reabsorption and vascular tension to maintain normal blood volume and pressure. Moreover, ANP participates in vessel remodeling and lipid metabolism (Chen et al. 2016; Cui et al. 2012; Ramos et al. 2015; Schlueter et al. 2014; Tokudome et al. 2009). Variants in the *NPPA* gene, encoding ANP, have been associated with cardiovascular and metabolic diseases (Fox et al. 2009; Lynch et al. 2009; Newton-Cheh et al. 2009; Rubattu et al. 2014; Song et al. 2015). Corin is a transmembrane serine protease that converts the ANP precursor, *i.e.* pro-ANP, to mature ANP (Armaly et al. 2013; Li et al. 2017). In mouse models, disruption of the *Corin* gene prevents the conversion of pro-ANP to ANP (Chan et al. 2005). Corin-deficient mice on high-salt diets had impaired renal sodium excretion and developed salt-sensitive hypertension and cardiac hypertrophy, indicating the importance of corin in regulating sodium homeostasis and cardiovascular function (Buckley and Stokes 2011; Chan et al. 2005; Nigrovic et al. 2008; Wang et al. 2012b).

A trypsin-like serine protease, corin is synthesized as a single-chain zymogen with no detectable catalytic activity. Proteolytic cleavage at a conserved activation site, R801↓I802, converts corin into a two-chain active enzyme. Naturally occurring *CORIN* variants that impair corin zymogen activation have been identified in patients with hypertension and heart disease (Dong et al. 2013; Dong et al. 2014; Dries et al. 2005; Rame et al. 2009; Wang et al. 2008; Zhang et al. 2014). Most recently, we reported that proprotein convertase subtilisin/kexin-6 (PCSK6), also called PACE4 (Kiefer et al. 1991; Seidah et al. 2013), is the long-sought protease responsible for corin activation *in vivo* (Chen et al. 2015). In PCSK6 knockout mice, corin activation and pro-ANP processing were abolished (Chen et al. 2015). The mice exhibited a hypertensive phenotype similar to that in corin knockout mice. A PCSK6 variant with impaired corin activation activity was also found in hypertensive patients (Chen et al. 2015). These results indicate that PCSK6-mediated corin activation is critical for pro-ANP processing and normal blood pressure.

PCSK6 belongs to the proprotein convertase family that includes nine members, which are important for processing growth factors, hormones, adhesion molecules and cell surface receptors (Seidah and Prat 2012; Seidah et al. 2013; Turpeinen et al. 2013). Many of the PCSKs share similar substrate specificities, cleaving after single or paired basic residues (Rockwell et al. 2002; Seidah et al. 2013). The subcellular location of the PCSKs, however, varies widely; some are packaged in secretary granules; some are constitutively secreted; and some are membrane-bound (Seidah and Prat 2012; Seidah et al. 2013; Turpeinen et al. 2013; Zhou et al. 1999). PCSK6 is a secreted protein and expressed in many cell types including cardiomyocytes and human embryonic kidney (HEK) 293 cells (Beaubien et al.

1995; Chen et al. 2015; Mayer et al. 2008; Nakagawa et al. 1993; Seidah et al. 2013; Tsuji et al. 1999). Previously, we found that PCSK6 activated corin on the cell surface but not inside the cell (Chen et al. 2015), leading to the question if the cell membrane association is required for PCSK6 to activate corin.

In this study, we conducted site-directed mutagenesis, cellular and biochemical experiments to examine the importance of the transmembrane domain and other extracellular domains of corin in PCSK6-mediated activation. Previous reports indicate that the binding to proteoglycans on the cell surface enhances PCSK6 activity (Mayer et al. 2008; Nour et al. 2005; Tsuji et al. 2003). In this study, we also examined the effects of heparan and chondroitin on PCSK6-mediated corin activation. Findings for these studies should help to understand the biochemical and cellular mechanisms underlying corin activation.

2. Materials and methods

2.1. Cell culture

HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). HL-1 cardiomyocytes were a gift from William Claycomb of Louisiana State University Medical Center and cultured in Claycomb medium (Sigma) with 10% FBS and 4 mM L-glutamine (Claycomb et al. 1998). All cells were cultured at 37°C in humidified incubators with 5% CO_2.

2.2. Plasmid constructs

Plasmids expressing human corin wild-type (WT) and mutants including the activation cleavage site mutant R801A, pro-peptide domain deletion mutants and soluble forms of corin WT and the active site mutant S985A were described previously (Knappe et al. 2003; Liao et al. 2007). Plasmid expressing human PCSK6 was described recently (Chen et al. 2015). Recombinant corin and PCSK6 proteins encoded by these plasmids contained a C-terminal V5 or flag tag for protein detection.

2.3. Transfection, immunoprecipitation and Western blotting

Plasmids expressing corin WT and mutants or PCSK6 were transfected into HEK293 cells using FuGENE HD (Promega) reagents. The transfected cells were cultured at 37°C for 48–72 h and lysed in a solution containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, and 1% (v/v) protease inhibitor cocktail (Sigma). Recombinant corin and PCSK6 proteins in the cell lysates were analyzed by SDS-PAGE and Western blotting using antibodies against V5 (Invitrogen, R96025) and flag (Agilent Technologies, 200471) tags. To analyze corin and PCSK6 proteins in the conditioned medium from the transfected cells, immunoprecipitation was performed with protein A-Sepharose beads (Thermo Fisher) and an anti-V5 antibody (Invitrogen, R96025) or an anti-PCSK6 antibody (Abcam, ab151562) followed by SDS-PAGE and Western blotting under reducing (with 2.5% β-mercaptoethanol in sample buffer) or non-reducing (without β-mercaptoethanol) conditions. In Western blotting, glycerol-dehyde-3-phophate dehydrogenase (GAPDH) protein levels were analyzed using an antibody (Millipore, MAB374) as a control for protein sample loading. Western blots were exposed to X-ray films and protein bands were analyzed

by densitometry to quantify protein expression levels. The percentages of corin activation, *i.e.* the ratio of the cleaved corin protease domain fragment band *vs.* the corin zymogen bands, were calculated.

2.4. Stable cell lines expressing corin domain deletion mutants

Plasmids expressing corin deletion mutants were transfected into HEK293 cells cultured in 6-well plates. After 12–24 h at 37°C, the cells were treated with 0.5% trypsin-EDTA, split into 100 mm-dishes, and cultured in DMEM with G418 (600 μg/mL). The culture medium was replaced every other day. After one week in culture, individual cell colonies were picked and expanded. The stable cells were analyzed by Western blotting to verify mutant corin protein expression using an antibody against a V5-tag, as described above.

2.5. Cell surface protein labeling

Transfected HEK293 cells expressing recombinant corin proteins in 6-well plates were washed with phosphate-buffered saline (PBS) and incubated with 1.5 mL (200 μM) of membrane impermeable Sulfo-NHS-SS-biotin (Thermo Scientific) for 5 min. A quenching solution (100 mM glycine, 2 mL) was added to the cells on ice. After 10 min, the cells were gently scraped into the solution, transferred into 15-mL tubes and centrifuged at 500 × *g* for 3 min. The cells were washed with 1 mL of PBS and lysed with 100 μL of the lysis buffer containing 1% of protease inhibitor cocktail (Sigma). The lysed cells were kept on ice for 30 min and centrifuged at 10,000 × *g* for 2 min at 4°C. The cell lysates were incubated with NeutrAvidin Agarose beads (Thermo Scientific) on a rocking platform for 1 h at room temperature. The beads were washed 3 times with 200 μL of a washing solution containing 1% of protease inhibitors. Proteins bound to the beads were eluted with a Laemmli buffer (Bio-Rad) with 50 mM DTT. Protein samples were analyzed by SDS-PAGE and Western blotting to examine corin proteins.

2.6. Monensin treatment

Monensin, which disrupts protein trafficking in the secretory pathway, was used to inhibit endogenous PCSK6 secretion from HEK293 cells. HEK293 cells were incubated with monensin (0.2 and 2 μM, Sigma) at 37°C. After 8 h, the medium was replaced with fresh monensin-containing medium. After 40 h, the conditioned medium was collected and the cell lysate was prepared, as described above. PCSK6 protein in the conditioned medium and cell lysates was analyzed by Western blotting. To examine the effect of monensin on soluble corin activation by endogenous PCSK6 protein, HEK293 cells were transfected with the plasmid expressing soluble WT (sWT) corin and cultured at 37°C in the presence of monensin (0.2 and 2 μM) for 40 h. Soluble corin protein in the conditioned medium was analyzed by immunoprecipitation and Western blotting, as described above.

2.7. Immunostaining

To visualize recombinant PCSK6 and corin proteins in cardiomyocytes, cultured HL-1 cells were transfected with plasmids expressing human PCSK6 and WT or sWT corin. After 24 h, the cells were fixed with 3% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min, and incubated with an anti-flag tag antibody (Cell Signaling, 14793) for

recombinant PCSK6 or an anti-V5 tag antibody (ThermoFisher, R96025) for recombinant corin proteins at 4°C for 12 h. Secondary antibodies used were Alexa 488 or 594-labeld antibodies (ThermoFisher, A11008 and A21203). The slides were mounted in a medium with DAPI to stain nuclei (Vector Laboratories, H1500). The stained cells were examined under a confocal microscope (Leica DM2500).

2.8. Activation of soluble corin by recombinant PCSK6 in cultured medium

HEK293 cells expressing soluble corin were cultured in DMEM with 10% FBS and monensin (2 μM) for 40 h. The presence of monensin inhibited PCSK6 secretion from the cells, thereby blocking soluble corin activation by endogenous PCSK6. In parallel, HEK293 cells expressing recombinant PCSK6 were cultured in separate plates for 48 h. The conditioned medium was collected from both cell cultures and centrifuged to remove cell debris. The conditioned medium containing soluble corin was mixed with the conditioned medium containing recombinant PCSK6 and incubated at 37°C for 2 h. Corin protein fragments in the conditioned medium were immunoprecipitated and analyzed by SDS-PAGE and Western blotting.

2.9. Preparation of cell membrane fractions

Parental HEK293 cells were scraped off from culture dishes in the presence of a solution containing 25 mM Tris-HCl (pH 7.4) and 5 mM EDTA (pH 8.0). The cells were homogenized in a tube on ice. After agitation at 4°C for 30 min, the homogenate was centrifuged at 2,000 rpm for 5 min to remove cell debris. The supernatant was collected and centrifuged at 200,000 × *g* at 4°C for 1 h. The pellet containing cell membrane fractions was washed and re-suspended in a solution with 150 mM NaCl, 50 mM Tris-HCl (pH 8.0) and 1% NP-40. Protein concentrations were determined by a Bradford assay, as described previously (Chen et al. 2010).

2.10. Effect of cell membrane fractions on soluble corin activation by PCSK6

HEK293 cells expressing soluble corin were cultured with 2 μM of monensin for 40 h. The conditioned medium containing soluble corin was collected and incubated with cell membrane fractions (1 and 10 μg) and the conditioned medium containing recombinant PCSK6 at 37°C. After 2 h, soluble corin proteins were immunoprecipitated and analyzed by Western blotting.

2.11. Effects of heparan and chondroitin on PCSK6-mediated corin activation in HEK293 cells

HEK293 cells expressing WT corin were cultured in 6-well plates at 37°C in the presence of monensin (2 μM). After 48 h, the cells were washed with PBS. The conditioned medium containing recombinant PCSK6 was added to the cell culture together with heparan sulfate (Sigma H7640, 5 and 50 μg/mL) or chondroitin sulfate (Sigma C4384, 5 and 50 μg/mL). After incubation at 37°C for 2 h, the cells were washed and lysed. Corin protein fragments in the cell lysates were examined by SDS-PAGE and Western blotting. To verify the effects of heparan and chondroitin on protease activity, a tryptase assay was performed (Anower et al. 2013), in which 5 μg/mL of haparan sulfate or chondroitin sulfate was added to a 100 μL

of reaction mixtures with 5 ng of tryptase (Sigma) and 50 nM of chromogenic substrate S-2288 (Chromogenix). The reaction mixtures were incubated at 37°C for different time periods, during which tryptase activity was assayed by monitoring the absorbance at 405 nm in a plate reader. Each data point was assayed in quadruplicate in six experiments.

2.12. Effects of heparinase and chondroitinase on PCSK6-mediated corin activation in HEK293 cells

HEK293 cells expressing WT corin were cultured in 6-well plates at 37°C in the presence of monensin (2 μM). After 48 h, the cells were washed with PBS. The conditioned medium containing recombinant PCSK6 was added to the cell culture together with heparinase (I and III blend), which removes heparan sulfate chains (Sigma H3917, 0.015, 0.15 and 1.5 U/mL) or chondroitinase ABC, which removes chondroitin sulfate chains (Sigma C3667, 1.5 U/mL). After incubation at 37°C for 2 h, the cells were washed and lysed. Corin protein fragments in the cell lysates were examined by SDS-PAGE and Western blotting.

2.13. Statistical analysis

Statistical analysis was done using the GraphPad Prism software. Comparisons between two groups were done by Student's *t* test. One-way ANOVA followed by Tukey's post test was used for comparisons among three or more groups. A P value of <0.05 was considered to be statistically significant. All data are expressed as means ± S.D.

3. Results

3.1. Activation of soluble corin in HEK293 cells

PCSK6 cleaves corin at Arg-801, converting corin into an active enzyme that consists of two chains linked by a disulfide bond (Fig. 1A) (Chen et al. 2015). When human WT corin was expressed in HEK293 cells and analyzed by Western blotting, the cleaved protease domain fragment (corin-p) appeared as an ~40-kDa band under reducing conditions (Fig. 1B). In corin R801A mutant (Fig. 1A), abolishing the activation cleavage site prevented the cleavage by PCSK6 and no corin-p band was detected on Western blots (Fig. 1B). The corin-p band, therefore, was used as an indicator for corin activation cleavage in our experiments. As reported previously, the corin activation cleavage was inhibited in HEK293 cells that were transfected with small interference RNA (siRNA) targeting the PCSK6 gene (Fig. 1C), indicating the importance of PCSK6 in corin activation (Chen et al. 2015).

Corin is a type II transmembrane protein with its C-terminal protease domain on the cell surface (Yan et al. 1999). Previously, PCSK6 was shown to activate corin on the cell surface (Chen et al. 2015). To examine if the cell membrane association is required for PCSK6-mediated corin activation, we studied a soluble corin (sWT), in which the cytoplasmic tail and the transmembrane domain were replaced by a signal peptide from immunoglobulin κ chain (Fig. 1A and D). We included a soluble negative control, sS985A corin, in which the catalytic residue Ser was replaced by Ala (Fig. 1A). We expressed WT corin and mutants R801A, sWT and sS985A in HEK293 cells and analyzed corin proteins by Western blotting under reducing conditions. In cell lysates from the transfected HEK293 cells (Fig. 1E), both

sWT and sS985A were detected as a single band of ~130-kDa without the ~40-kDa corin-p band, indicating that sWT and sS985A were not activated inside the cell.

In parallel experiments, we biotin-labeled cell surface proteins in the transfected HEK293 cells and analyzed corin proteins by Western blotting. We detected the activated and zymogen WT corin and single-chain R801A mutant in the biotin-labeled protein samples (Fig. 1F). No corin bands were detected in samples from sWT and sS985A (Fig. 1F), indicating that sWT and sS985A proteins were secreted and not present on the cell surface. In the conditioned medium from the same sets of the transfected cells, corin zymogen and corin-p bands from WT and corin zymogen band from R801A were detected (Fig. 1G). These fragments were derived from ADAM-mediated shedding of cell surface corin in the juxtamembrane region (Fig. 1A, arrowhead in WT), as reported previously (Jiang et al. 2011). Similar corin zymogen and corin-p bands were also detected in the conditioned medium from the cells expressing sWT and the inactive sS985A (Fig. 1G), indicating that the secreted soluble corin proteins were activated in the conditioned medium.

3.2. Monensin blocked PCSK6, but not soluble corin, secretion in HEK293 cells

Monensin is an antibiotic that disturbs Golgi structures (Dinter and Berger 1998). Consistent with our previous findings (Chen et al. 2015), monensin inhibited PCSK6 secretion from HEK293 cells, as indicated by decreased PCSK6 levels in the conditioned medium, but not in cell lysates, in Western blotting analysis (Fig. 2A and B, lower panels). However, similar monensin treatment did not inhibit sWT secretion, as indicated by sWT protein in the conditioned medium, which appeared as a single zymogen band without the corin-p band (Fig. 2B, top panel). The results indicate that monensin blocked PCSK6 secretion, thereby preventing sWT corin activation in the conditioned medium.

3.3. Activation of soluble corin by exogenous PCSK6

To examine if PCSK6-mediated corin activation requires co-expression of PCSK6 and sWT corin in the same cells and to verify that sWT from the monensin-treated cells remains cleavable by PCSK6, we tested recombinant PCSK6 for sWT corin activation. HEK293 cells expressing sWT corin were treated with monensin to block endogenous PCKS6 secretion. The conditioned medium containing recombinant human PCSK6 was added to the cells and incubated at 37°C for 2 h. As indicated by Western blotting analysis, sWT corin activation increased in the presence of increasing volumes of recombinant PCSK6-containing medium (Fig. 2C and D). Similar results were also observed with the inactive sS985A corin (Figs. 1A, 2C and D), which was used as a negative control to exclude the possibility of corin autocleavage in the conditioned medium. The results indicate that the soluble corin from the monensin-treated cells remained cleavable by PCSK6 and that PCSK6-mediated corin activation did not require co-expression of these two proteins in the same cells.

To determine if sWT corin can be activated by PCSK6 in the absence of HEK293 cells, we incubated the conditioned medium containing sWT corin with the conditioned medium containing recombinant PCSK6. Western blotting analysis showed increased sWT corin activation with increasing volumes of PCSK6-containing medium (Fig. 3A), indicating that the presence of the cells is unnecessary for PCSK6-mediated corin activation.

We next examined if the cell membrane enhances PCSK6-mediated corin activation. The conditioned medium containing sWT corin and recombinant PCSK6 were incubated in the presence of crude cell membrane fractions isolated from parental HEK293 cells. In Western blotting analysis, sWT corin activation was detected when PCSK6-containing medium was added (Fig. 3B and C). The activation, however, was not enhanced in the presence of the crude cell membrane fractions (Fig. 3B and C).

3.4. Immunostaining of recombinant PCSK6 and corin proteins in HL-1 cardiomyocytes

The findings that sWT corin was not activated inside the cell and that monensin inhibited PCSK6, but not sWT, secretion suggested that PCSK6 and corin proteins may not encounter inside the cell. To test this hypothesis, we expressed recombinant human PCSK6 and WT or sWT corin in HL-1 cardiomyocytes. In immunostaining experiments, co-staining of PCSK6 and WT or sWT corin proteins was found mostly near cell nuclei, possibly in the ER (Fig. 4). Toward the cell periphery, there was little overlapping staining of PCSK6 and corin proteins in these cells (Fig. 4).

3.5. Activation of corin deletion mutants in HEK293 cells expressing recombinant PCSK6

Between the N-terminal transmembrane domain and the C-terminal serine protease domain of corin, there are two frizzled (Fz) domains, eight LDL receptor-like (LDLR) repeats and a scavenger receptor-like (SR) domain (Fig. 5A). To examine if these protein domains are required for PCSK6-mediated corin activation, we expressed corin deletion mutants lacking Fz, LDLR and SR domains, individually or in combination (Fig. 5A), in HEK293 cells stably expressing recombinant human PCSK6. Western blotting analysis of cell lysates from the transfected cells showed similar levels of the corin-p band in corin WT and mutants ΔFz1, ΔR1-4, ΔFz2 and ΔFz1-SR (Fig. 5B), indicating that Fz, LDLR and SR domains in the pro-peptide region of corin are not required for PCSK6 to activate corin.

3.6. Effects of heparan and chondroitin on PCSK6-mediated corin activation in HEK293 cells

It has been reported that the binding of PCSK6 to heparan sulfate proteoglycans on the cell surface or in the extracellular matrix enhances PCSK6 activity (Mayer et al. 2008; Nour et al. 2005; Tsuji et al. 2003). We examined the effect of heparan sulfate and chondroitin sulfate on PCSK6-mediated corin activation. HEK293 cells expressing WT corin were incubated with recombinant PCSK6 in the presence or absence of exogenous heparan sulfate and chondroitin sulfate. In Western blotting analysis of cell lysates, corin activation was detected when the corin-expressing cells were cultured with PCSK6, as indicated by the corin-p band (Fig. 6A). When the cells were incubated with PCSK6 and heparan sulfate or chondroitin sulfate, corin activation was not enhanced, as indicated by similar levels of the corin-p band (Fig. 6A and B). As a control, heparan sulfate and chondroitin sulfate enhanced the activity of tryptase toward a peptide substrate (Fig. 6C), which was consistent with a previous report (Anower et al. 2013).

3.7. Effects of heparinase and chondroitinase on PCSK6-mediated corin activation in HEK293 cells

It is possible that HEK293 cells express endogenous proteoglycans that are sufficient to enhance corin activation by PCSK6 under our experimental conditions. We next examined the effects of heparinase and chondroitinase on PCSK6-mediated corin activation in HEK293 cells. HEK293 cells expressing corin WT were incubated with recombinant PCSK6 in the presence or absence of heparinase and chondroitinase. In Western analysis of cell lysates, similar levels of the corin-p band were observed in the cells treated with or without heparinase and chondroitinase (Fig. 7A and B), indicating that heparinase or chondroitinase treatment did not inhibit corin activation by PCSK6.

4. Discussion

Zymogen activation is a key step in regulating the catalytic activity of trypsin-like proteases (Neurath 1984; Perona and Craik 1995). For most secreted serine proteases, zymogen activation occurs distantly from the protein production site. Trypsinogen, for example, is made in the pancreas but activated in the gut. Such a mechanism limits proteolytic reactions at intended locations. Corin is a member of the type II transmembrane serine protease (TTSP) family (Antalis et al. 2011; Bugge et al. 2009). All TTSPs have an N-terminal single-span transmembrane domain that anchors the C-terminal protease domain on the cell surface (Antalis et al. 2011; Bugge et al. 2009). Despite their structural similarities, the TTSPs appear to have distinct zymogen activation mechanisms. For example, matriptase, a TTSP essential for epithelial barrier function, is activated by prostasin, a glycosylphosphatidylinositol-anchored serine protease, in a reciprocal manner (Chen et al. 2008; Friis et al. 2016; Netzel-Arnett et al. 2006; Szabo et al. 2016). Matriptase-2, a hepatic TTSP critical for iron metabolism, is autoactivated (Jiang et al. 2014; Stirnberg et al. 2010; Velasco et al. 2002). Our recent discovery of PCSK6 as a primary corin activator provides the first example of a PCSK family member in activating a TTSP (Chen et al. 2015).

PCSK6 is present in the trans-Golgi network and secreted through the secretory pathway (Nour et al. 2005; Tsuji et al. 2003). Previously, we found that PCSK6-mediated corin activation occurred on the cell surface but not intracellularly (Chen et al. 2015). It was unclear what prevents PCSK6 from activating corin inside the cell. It is possible that the cell membrane is required to enhance PCSK6-dependent corin activation. In blood coagulation, cell membrane association is known to enhance the activation of clotting serine proteases (Davie 2003; Mann et al. 2006). Alternatively, PCSK6 and corin may travel via different intracellular pathways, thereby preventing their encounter before reaching the cell surface.

To address these questions, we studied a soluble corin, in which the transmembrane domain was replaced by a signal peptide. In transfected HEK293 cells, the soluble corin remained as a one-chain zymogen in cell lysates (Fig. 1E) and activated soluble corin was detected in the conditioned medium (Fig. 1G). These results show that the transmembrane domain is unnecessary for corin activation. The results also suggest that lack of corin activation inside the cell is likely due to different intracellular pathways that separate PCSK6 and corin before they reach the cell surface. We verified this hypothesis with monensin, a Golgi-disturbing compound (Dinter and Berger 1998), which blocks PCSK6 secretion (Chen et al. 2015). In

transfected HEK293 cells, monensin inhibited PCSK6, but not soluble corin, secretion (Fig. 2B). Moreover, the secreted soluble corin remained as one-chain zymogen in the conditioned medium (Fig. 2B), indicating that blocking endogenous PCSK6 secretion inhibited corin activation. When we added exogenous recombinant PCSK6 to the monensin-treated HEK293 cells, soluble corin activation was observed (Fig. 2C). The results are consistent with our previous findings that monensin inhibited PCSK6-meidated activation of WT corin with the transmembrane domain (Chen et al. 2015). These data indicate that corin, even in a soluble form, travels through an intracellular pathway that differs from that of PCSK6. Consistent with this idea, overlapping immunostaining of PCSK6 and WT or sWT corin proteins was found mostly near the nuclei, but not in the peripheral zones, in transfected HL-1 cardiomyocytes (Fig. 4).

If the transmembrane domain is not required for corin activation by PCSK6, will cell membranes enhance PCSK6-mediated corin activation? We examined soluble corin activation by PCSK6 in solution with or without crude membrane fractions from HEK293 cells. We did not observe enhanced corin activation in the presence of the crude cell membrane fractions (Fig. 3B and C), suggesting that the primary function of the corin transmembrane domain is to anchor the protease on the cell surface but not to enhance zymogen activation or protease catalytic activity. Consistent with this idea, previous studies have shown that the transmembrane domain is not required for the catalytic activity of other TTSPs, such as enteropeptidase (Zheng et al. 1999), matriptase (Lee et al. 2000; Takeuchi et al. 2000), hepsin (Xuan et al. 2006), TMPRSS2 (Meyer et al. 2013), and human airway trypsin-like protease (Liu et al. 2013).

Human corin consists of 1,042 amino acids that are organized in distinct domains, including the Fz, LDLR and SR domains in the pro-peptide region (Hooper et al. 2000; Yan et al. 1999). The functional significance of these protein domains remains unclear. In other trypsin-like proteases, protein domains in the pro-peptide region may interact with their activators. For example, the kringle 2 domain in prothrombin interacts with clotting factor Xa, which activates prothrombin (Taneda et al. 1994). In TMPRSS3, another TTSP, several naturally occurring mutations identified in deaf patients were located in the pro-peptide region, which prevented TMPRSS3 zymogen activation (Guipponi et al. 2002). We and others also reported naturally occurring mutations in hypertensive patients that are located in the corin pro-peptide region (Dong et al. 2013; Dong et al. 2014; Dries et al. 2005; Zhang et al. 2014). In functional studies, some of those corin mutants had reduced activities due to ER retention, poor intracellular trafficking and/or impaired zymogen activation (Dong et al. 2013; Dong et al. 2014; Wang et al. 2008; Zhang et al. 2014). For example, the corin variant T555I/Q568P identified in African Americans with hypertension had reduced activity *in vitro* and *in vivo* (Dries et al. 2005; Wang et al. 2012a; Wang et al. 2008). The T555I/Q568P substitutions are located in the corin Fz2 domain. In this study, however, domain deletions in the corin pro-peptide region, including the Fz2 domain, did not prevent corin activation by PCSK6 in transfected HEK293 cells (Fig. 5). The biochemical mechanism by which the T555I/Q568P variant impairs corin activity remains unclear. It is possible that the protein domains in the corin pro-peptide region are not necessary for PCSK6-mediated activation. However, mutations in the corin pro-peptide region may alter the overall corin structure,

thereby impairing corin zymogen activation. Further studies are required to test such a hypothesis.

In many serine proteases, the binding to heparan sulfate proteoglycans alters the enzyme activity and substrate specificity (Liu et al. 2002; Marcum and Rosenberg 1987). It has been shown that PCSK6, via its C-terminal cysteine-rich domain, binds to heparan sulfate proteoglycans on the cell surface or in the extracellular matrix, which enhances PCSK6 activity (Mayer et al. 2008; Nour et al. 2005; Tsuji et al. 2003). We examined the effect of heparan sulfate and chondroitin sulfate on PCSK6-mediated corin activation. In the transfected HEK293 cells expressing PCSK6, corin activation was not enhanced when heparan sulfate or chondoitin sulfate was added (Fig. 6). As controls, heparan sulfate or chondoitin sulfate did enhance the tryptase activity in a peptide substrate assay (Fig. 6). To test if HEK293 cells contain endogenous heparan or chondroitin sulfate proteoglycans that may be sufficient to promote PCSK6 activity, we treated HEK293 with heparinase, which removes heparan sulfate chains, or chondroitinase, which removes chondroitin sulfate chains. As indicated by Western blotting analysis, heparinase or chondroitinase treatment did not significantly alter PCSK6-mediated corin activation (Fig. 7). Additional studies are important to understand if and how PCSK6-mediated corin activation is regulated under physiological and pathological conditions.

In summary, we examined corin protein domains that are required for PCSK6-mediated activation. Our data show that in transfected HEK293 cells, the soluble corin lacking the transmembrane domain was activated by PCSK6 in the conditioned medium but not inside the cell, indicating that cell membrane association is unnecessary for PCSK6-mediated corin activation. The data support a cellular mechanism, in which corin and PCSK6 travel via separate intracellular pathways before their encounter on the cell surface. We also found that the protein domains in the corin pro-peptide region are not required for PCSK6-medicated activation. Moreover, addition of heparan sulfate and chondroitin sulfate or treatment with heparinase or chondroitiniase did not alter corin activation by PCSK6 in the transfected HEK293 cells. Together, our results provide new insights into the biochemical and cellular mechanisms underlying PCSK6-mediated corin activation that is important in regulating blood volume and pressure. Further studies are required to verify our findings *in vivo* such as transgenic or knock-in mouse models.

Acknowledgments

This work was supported, in whole or in part, by grants from the National Institutes of Health (HL126697) and grants from the National Natural Science Foundation (81671485, 91639116), Postgraduate Innovative Program of Jiangsu (KYZZ15_0337), the Priority Academic Program Development of Jiangsu Higher Education Institutions, and the Seed Grant of Nanchang University for Biomedical Science (9202-0210210807), China.

References

Anower EKMF, Habuchi H, Nagai N, Habuchi O, Yokochi T, Kimata K. Heparan sulfate 6-O-sulfotransferase isoform-dependent regulatory effects of heparin on the activities of various proteases in mast cells and the biosynthesis of 6-O-sulfated heparin. J Biol Chem. 2013; 288(6): 3705–17. [PubMed: 23223449]

Antalis TM, Bugge TH, Wu Q. Membrane-anchored serine proteases in health and disease. Prog Mol Biol Transl Sci. 2011; 99:1–50. [PubMed: 21238933]

Armaly Z, Assady S, Abassi Z. Corin: a new player in the regulation of salt-water balance and blood pressure. Curr Opin Nephrol Hypertens. 2013; 22(6):713–22. [PubMed: 24100222]

Beaubien G, et al. The distinct gene expression of the pro-hormone convertases in the rat heart suggests potential substrates. Cell Tissue Res. 1995; 279(3):539–49. [PubMed: 7736551]

Buckley CL, Stokes AJ. Corin-deficient W-sh mice poorly tolerate increased cardiac afterload. Regul Pept. 2011; 172(1–3):44–50. [PubMed: 21903139]

Bugge TH, Antalis TM, Wu Q. Type II transmembrane serine proteases. J Biol Chem. 2009; 284(35): 23177–81. [PubMed: 19487698]

Chan JC, Knudson O, Wu F, Morser J, Dole WP, Wu Q. Hypertension in mice lacking the proatrial natriuretic peptide convertase corin. Proc Natl Acad Sci U S A. 2005; 102(3):785–90. [PubMed: 15637153]

Chen M, Chen LM, Lin CY, Chai KX. The epidermal growth factor receptor (EGFR) is proteolytically modified by the Matriptase-Prostasin serine protease cascade in cultured epithelial cells. Biochim Biophys Acta. 2008; 1783(5):896–903. [PubMed: 18054338]

Chen S, et al. PCSK6-mediated corin activation is essential for normal blood pressure. Nat Med. 2015; 21(9):1048–53. [PubMed: 26259032]

Chen S, Sen S, Young D, Wang W, Moravec CS, Wu Q. Protease corin expression and activity in failing hearts. Am J Physiol Heart Circ Physiol. 2010; 299(5):H1687–92. [PubMed: 20802129]

Chen W, et al. Endothelial Actions of ANP Enhance Myocardial Inflammatory Infiltration in the Early Phase After Acute Infarction. Circ Res. 2016; 119(2):237–48. [PubMed: 27142162]

Claycomb WC, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci U S A. 1998; 95(6):2979–84. [PubMed: 9501201]

Cui Y, et al. Role of corin in trophoblast invasion and uterine spiral artery remodelling in pregnancy. Nature. 2012; 484(7393):246–250. [PubMed: 22437503]

Davie EW. A brief historical review of the waterfall/cascade of blood coagulation. J Biol Chem. 2003; 278(51):50819–32. [PubMed: 14570883]

de Bold AJ. Thirty years of research on atrial natriuretic factor: historical background and emerging concepts. Can J Physiol Pharmacol. 2011; 89(8):527–31. [PubMed: 21671768]

Dinter A, Berger EG. Golgi-disturbing agents. Histochem Cell Biol. 1998; 109(5–6):571–90. [PubMed: 9681636]

Dong N, et al. Corin mutation R539C from hypertensive patients impairs zymogen activation and generates an inactive alternative ectodomain fragment. J Biol Chem. 2013; 288(11):7867–74. [PubMed: 23372161]

Dong N, et al. Corin mutations K317E and S472G from preeclamptic patients alter zymogen activation and cell surface targeting. J Biol Chem. 2014; 289(25):17909–16. [PubMed: 24828501]

Dries DL, et al. Corin gene minor allele defined by 2 missense mutations is common in blacks and associated with high blood pressure and hypertension. Circulation. 2005; 112(16):2403–10. [PubMed: 16216958]

Fox AA, et al. Natriuretic peptide system gene variants are associated with ventricular dysfunction after coronary artery bypass grafting. Anesthesiology. 2009; 110(4):738–47. [PubMed: 19326473]

Friis S, Madsen DH, Bugge TH. Distinct Developmental Functions of Prostasin (CAP1/PRSS8) Zymogen and Activated Prostasin. J Biol Chem. 2016; 291(6):2577–82. [PubMed: 26719335]

Guipponi M, et al. The transmembrane serine protease (TMPRSS3) mutated in deafness DFNB8/10 activates the epithelial sodium channel (ENaC) in vitro. Hum Mol Genet. 2002; 11(23):2829–36. [PubMed: 12393794]

Hooper JD, Scarman AL, Clarke BE, Normyle JF, Antalis TM. Localization of the mosaic transmembrane serine protease corin to heart myocytes. Eur J Biochem. 2000; 267(23):6931–7. [PubMed: 11082206]

Jiang J, et al. Ectodomain shedding and autocleavage of the cardiac membrane protease corin. J Biol Chem. 2011; 286(12):10066–72. [PubMed: 21288900]

Jiang J, et al. N-glycosylation is required for matriptase-2 autoactivation and ectodomain shedding. J Biol Chem. 2014; 289(28):19500–7. [PubMed: 24867957]

Kiefer MC, Tucker JE, Joh R, Landsberg KE, Saltman D, Barr PJ. Identification of a second human subtilisin-like protease gene in the fes/fps region of chromosome 15. DNA Cell Biol. 1991; 10(10): 757–69. [PubMed: 1741956]

Knappe S, Wu F, Masikat MR, Morser J, Wu Q. Functional analysis of the transmembrane domain and activation cleavage of human corin: design and characterization of a soluble corin. J Biol Chem. 2003; 278(52):52363–70. [PubMed: 14559895]

Kuhn M. Molecular Physiology of Membrane Guanylyl Cyclase Receptors. Physiol Rev. 2016; 96(2): 751–804. [PubMed: 27030537]

Lee SL, Dickson RB, Lin CY. Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. J Biol Chem. 2000; 275(47): 36720–5. [PubMed: 10962009]

Li H, Zhang Y, Wu Q. Role of corin in the regulation of blood pressure. Curr Opin Nephrol Hypertens. 2017; 26(2):67–73. [PubMed: 27898523]

Liao X, Wang W, Chen S, Wu Q. Role of glycosylation in corin zymogen activation. J Biol Chem. 2007; 282(38):27728–35. [PubMed: 17660514]

Liu C, Li Q, Zhou X, Kolosov VP, Perelman JM. Human airway trypsin-like protease induces mucin5AC hypersecretion via a protease-activated receptor 2-mediated pathway in human airway epithelial cells. Arch Biochem Biophys. 2013; 535(2):234–40. [PubMed: 23602830]

Liu D, Shriver Z, Qi Y, Venkataraman G, Sasisekharan R. Dynamic regulation of tumor growth and metastasis by heparan sulfate glycosaminoglycans. Semin Thromb Hemost. 2002; 28(1):67–78. [PubMed: 11885027]

Lynch AI, Claas SA, Arnett DK. A review of the role of atrial natriuretic peptide gene polymorphisms in hypertension and its sequelae. Curr Hypertens Rep. 2009; 11(1):35–42. [PubMed: 19146799]

Mann KG, Brummel-Ziedins K, Orfeo T, Butenas S. Models of blood coagulation. Blood Cells Mol Dis. 2006; 36(2):108–17. [PubMed: 16500122]

Marcum JA, Rosenberg RD. Anticoagulantly active heparan sulfate proteoglycan and the vascular endothelium. Semin Thromb Hemost. 1987; 13(4):464–74. [PubMed: 2962288]

Mayer G, et al. The regulated cell surface zymogen activation of the proprotein convertase PC5A directs the processing of its secretory substrates. J Biol Chem. 2008; 283(4):2373–84. [PubMed: 18039650]

Meyer D, Sielaff F, Hammami M, Bottcher-Friebertshauser E, Garten W, Steinmetzer T. Identification of the first synthetic inhibitors of the type II transmembrane serine protease TMPRSS2 suitable for inhibition of influenza virus activation. Biochem J. 2013; 452(2):331–43. [PubMed: 23527573]

Nakagawa T, Hosaka M, Torii S, Watanabe T, Murakami K, Nakayama K. Identification and functional expression of a new member of the mammalian Kex2-like processing endoprotease family: its striking structural similarity to PACE4. J Biochem. 1993; 113(2):132–5. [PubMed: 8468318]

Netzel-Arnett S, et al. Evidence for a matriptase-prostasin proteolytic cascade regulating terminal epidermal differentiation. J Biol Chem. 2006; 281(44):32941–5. [PubMed: 16980306]

Neurath H. Evolution of proteolytic enzymes. Science. 1984; 224(4647):350–7. [PubMed: 6369538]

Newton-Cheh C, et al. Association of common variants in NPPA and NPPB with circulating natriuretic peptides and blood pressure. Nat Genet. 2009; 41(3):348–53. [PubMed: 19219041]

Nigrovic PA, et al. Genetic inversion in mast cell-deficient (Wsh) mice interrupts corin and manifests as hematopoietic and cardiac aberrancy. Am J Pathol. 2008; 173(6):1693–701. [PubMed: 18988802]

Nour N, et al. The cysteine-rich domain of the secreted proprotein convertases PC5A and PACE4 functions as a cell surface anchor and interacts with tissue inhibitors of metalloproteinases. Mol Biol Cell. 2005; 16(11):5215–26. [PubMed: 16135528]

Perona JJ, Craik CS. Structural basis of substrate specificity in the serine proteases. Protein Sci. 1995; 4(3):337–60. [PubMed: 7795518]

Rame JE, et al. Dysfunctional corin I555(P568) allele is associated with impaired brain natriuretic peptide processing and adverse outcomes in blacks with systolic heart failure: results from the Genetic Risk Assessment in Heart Failure substudy. Circ Heart Fail. 2009; 2(6):541–8. [PubMed: 19919978]

Ramos HR, Birkenfeld AL, de Bold AJ. INTERACTING DISCIPLINES: Cardiac natriuretic peptides and obesity: perspectives from an endocrinologist and a cardiologist. Endocr Connect. 2015; 4(3):R25–36. [PubMed: 26115665]

Rockwell NC, Krysan DJ, Komiyama T, Fuller RS. Precursor processing by kex2/furin proteases. Chem Rev. 2002; 102(12):4525–48. [PubMed: 12475200]

Rubattu S, Sciarretta S, Volpe M. Atrial natriuretic peptide gene variants and circulating levels: implications in cardiovascular diseases. Clin Sci (Lond). 2014; 127(1):1–13. [PubMed: 24611929]

Schlueter N, de Sterke A, Willmes DM, Spranger J, Jordan J, Birkenfeld AL. Metabolic actions of natriuretic peptides and therapeutic potential in the metabolic syndrome. Pharmacol Ther. 2014; 144(1):12–27. [PubMed: 24780848]

Seidah NG, Prat A. The biology and therapeutic targeting of the proprotein convertases. Nat Rev Drug Discov. 2012; 11(5):367–83. [PubMed: 22679642]

Seidah NG, Sadr MS, Chretien M, Mbikay M. The multifaceted proprotein convertases: their unique, redundant, complementary, and opposite functions. J Biol Chem. 2013; 288(30):21473–81. [PubMed: 23775089]

Song W, Wang H, Wu Q. Atrial natriuretic peptide in cardiovascular biology and disease (NPPA). Gene. 2015; 569(1):1–6. [PubMed: 26074089]

Stirnberg M, et al. Proteolytic processing of the serine protease matriptase-2: identification of the cleavage sites required for its autocatalytic release from the cell surface. Biochem J. 2010; 430(1): 87–95. [PubMed: 20518742]

Szabo R, Lantsman T, Peters DE, Bugge TH. Delineation of proteolytic and non-proteolytic functions of the membrane-anchored serine protease prostasin. Development. 2016; 143(15):2818–28. [PubMed: 27385010]

Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, Craik CS. Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. J Biol Chem. 2000; 275(34):26333–42. [PubMed: 10831593]

Taneda H, Andoh K, Nishioka J, Takeya H, Suzuki K. Blood coagulation factor Xa interacts with a linear sequence of the kringle 2 domain of prothrombin. J Biochem. 1994; 116(3):589–97. [PubMed: 7852276]

Theilig F, Wu Q. ANP-induced signaling cascade and its implications in renal pathophysiology. Am J Physiol Renal Physiol. 2015; 308(10):F1047–55. [PubMed: 25651559]

Tokudome T, et al. Impaired recovery of blood flow after hind-limb ischemia in mice lacking guanylyl cyclase-A, a receptor for atrial and brain natriuretic peptides. Arterioscler Thromb Vasc Biol. 2009; 29(10):1516–21. [PubMed: 19628785]

Tsuji A, et al. Secretory proprotein convertases PACE4 and PC6A are heparin-binding proteins which are localized in the extracellular matrix. Potential role of PACE4 in the activation of proproteins in the extracellular matrix. Biochim Biophys Acta. 2003; 1645(1):95–104. [PubMed: 12535616]

Tsuji A, et al. Human subtilisin-like proprotein convertase, PACE4 (SPC4) gene expression is highly regulated through E-box elements in HepG2 and GH4C1 cells. J Biochem. 1999; 126(3):494–502. [PubMed: 10467164]

Turpeinen H, Ortutay Z, Pesu M. Genetics of the first seven proprotein convertase enzymes in health and disease. Curr Genomics. 2013; 14(7):453–67. [PubMed: 24396277]

Velasco G, Cal S, Quesada V, Sanchez LM, Lopez-Otin C. Matriptase-2, a membrane-bound mosaic serine proteinase predominantly expressed in human liver and showing degrading activity against extracellular matrix proteins. J Biol Chem. 2002; 277(40):37637–46. [PubMed: 12149247]

Wang W, et al. Salt-sensitive hypertension and cardiac hypertrophy in transgenic mice expressing a corin variant identified in blacks. Hypertension. 2012a; 60(5):1352–8. [PubMed: 22987923]

Wang W, et al. Corin variant associated with hypertension and cardiac hypertrophy exhibits impaired zymogen activation and natriuretic peptide processing activity. Circ Res. 2008; 103(5):502–8. [PubMed: 18669922]

Wang W, et al. Impaired sodium excretion and salt-sensitive hypertension in corin-deficient mice. Kidney Int. 2012b; 82(1):26–33. [PubMed: 22418978]

Xuan JA, et al. Antibodies neutralizing hepsin protease activity do not impact cell growth but inhibit invasion of prostate and ovarian tumor cells in culture. Cancer Res. 2006; 66(7):3611–9. [PubMed: 16585186]

Yan W, Sheng N, Seto M, Morser J, Wu Q. Corin, a mosaic transmembrane serine protease encoded by a novel cDNA from human heart. J Biol Chem. 1999; 274(21):14926–35. [PubMed: 10329693]

Zhang Y, et al. A corin variant identified in hypertensive patients that alters cytoplasmic tail and reduces cell surface expression and activity. Sci Rep. 2014; 4:7378. [PubMed: 25488193]

Zheng X, Lu D, Sadler JE. Apical sorting of bovine enteropeptidase does not involve detergent-resistant association with sphingolipid-cholesterol rafts. J Biol Chem. 1999; 274(3):1596–605. [PubMed: 9880538]

Zhou A, Webb G, Zhu X, Steiner DF. Proteolytic processing in the secretory pathway. J Biol Chem. 1999; 274(30):20745–8. [PubMed: 10409610]

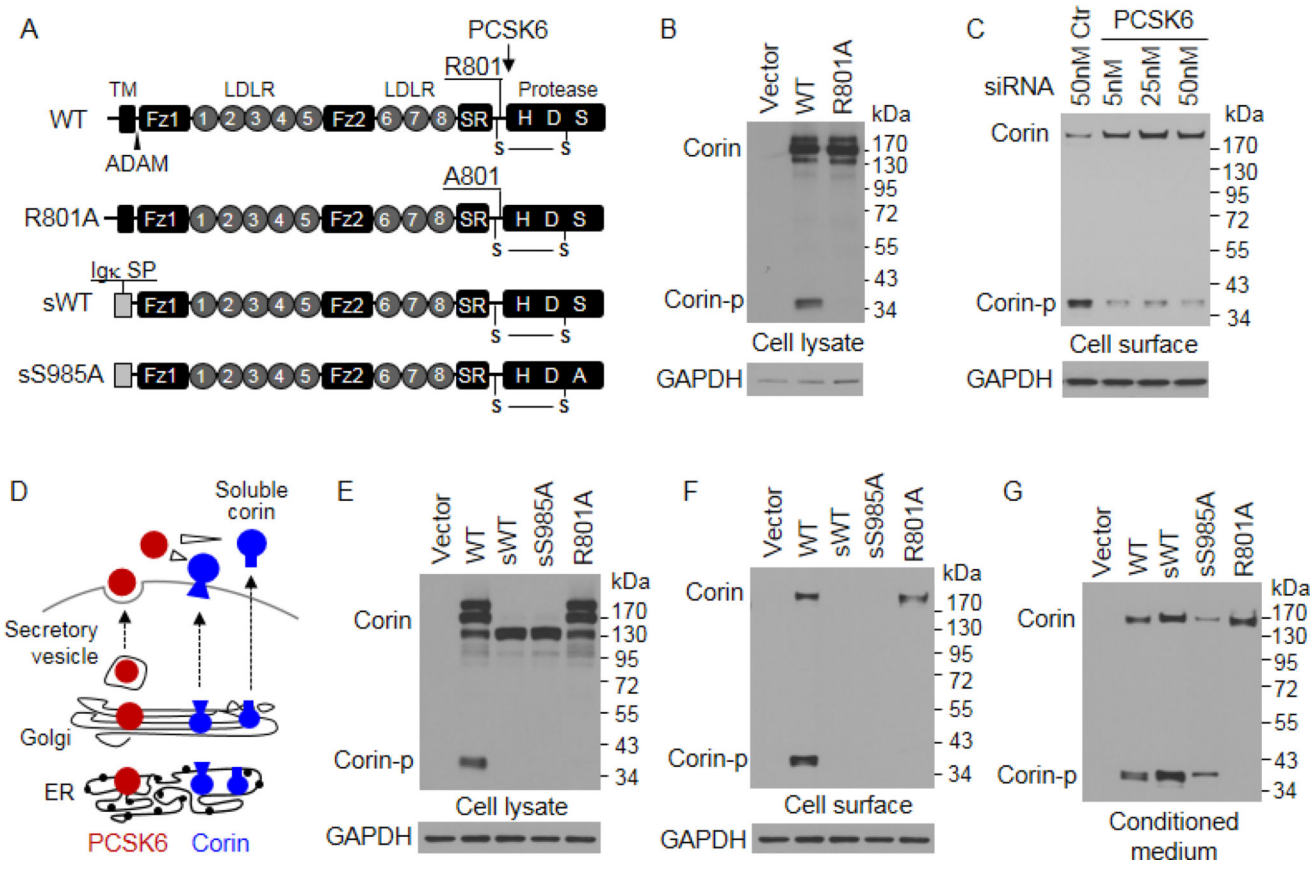

Fig. 1.

Analysis of soluble corin proteins expressed in HEK293 cells. (A) Schematic illustration of corin WT, R801A mutant, and soluble corin proteins sWT and sS985A that lacked the transmembrane domain. The transmembrane (TM), frizzled (Fz), LDL receptor (LDLR), scavenger receptor (SR) and protease domains are indicated. The catalytic residues His (H), Asp (D) and Ser (S) are shown. PCSK6 cleaves and activates corin at R801 (arrow). The cleaved protease domain fragment is connected to the pro-peptide region by a disulfide bond (s-s). ADAM-mediated shedding site in the juxtamembrane region is indicated by an arrowhead. In sWT and sS985A, the cytoplasmic tail and the transmembrane domain are replaced by the signal peptide (SP) from Igκ. (B) Western blotting analysis of WT and R801A corin proteins in HEK293 cell lysates under reducing conditions. (C) Western blotting analysis of corin activation in HEK293 cells transfected with non-targeting control siRNA (Ctr) or siRNA targeting the PCSK6 gene under reducing conditions. (D) Illustration of intracellular trafficking of PCSK6 and membrane-bound or soluble corin. (E) Western blotting analysis of WT, sWT, sS985A and R801A corin proteins in HEK293 cell lysates under reducing conditions. (F) Western blotting analysis of biotin-labeled cell surface corin in HEK293 cells under reducing conditions. (G) Western blotting analysis of corin proteins in the conditioned medium from HEK293 cells under reducing conditions. Data are representative of at least three independent experiments.

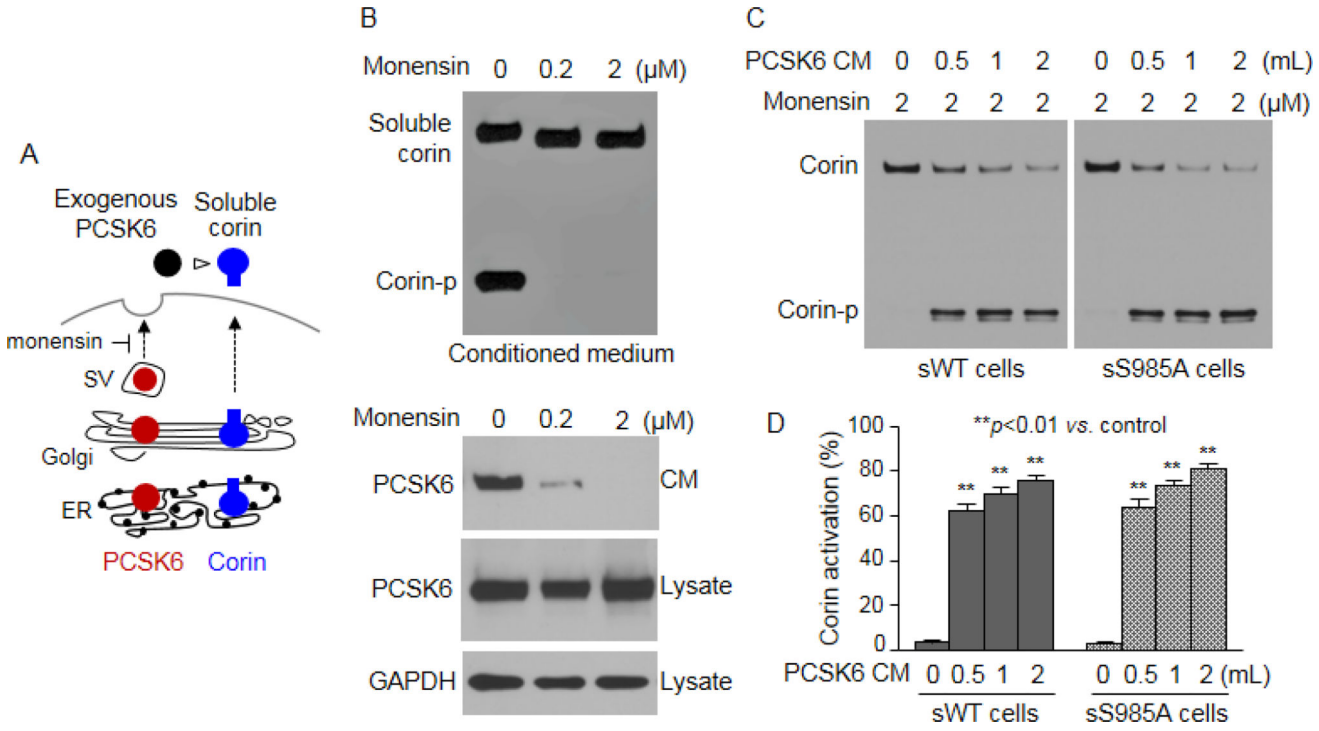

Fig. 2.

Secretion and activation of soluble corin in monensin-treated HEK293 cells. (A) Illustration of monensin inhibition of endogenous PCSK6 and soluble corin activation by exogenous PCSK6. SV, secretory vesicle. (B) Monensin blocked PCSK6 (lower panels), but not soluble corin (top panel), secretion in HEK293 cells, as indicated by Western blotting analysis. (C) Activation cleavage of sWT and sS985A corin in HEK293 cells treated with monensin and incubated with exogenous PCSK6. (D) Percentages of corin activation were calculated based on the densitometric analysis of corin zymogen and corin-p bands on Western blots. Values are mean ± S.D. (n=3 per group). ** $p<0.01$ *vs.* control (0 mL PCSK6 CM).

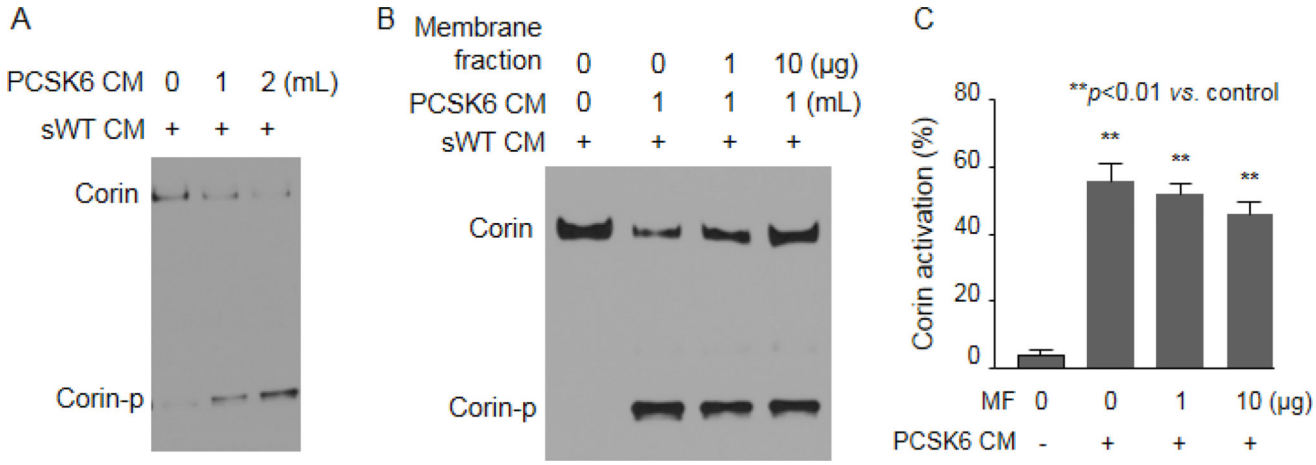

Fig. 3.

PCSK6-mediated activation of sWT corin in solution with or without crude cell membrane fractions. (A) Western blotting analysis of sWT corin activation by PCSK6 in the conditioned medium. (B) Western blotting analysis of sWT corin activation by PCSK6 in solution in the presence of crude membrane fractions from parental HEK293 cells. (C) Percentages of sWT corin activation were calculated based on the densitometric analysis of corin zymogen and corin-p bands on Western blots. Values are mean ± S.D. (n=3 per group). ** $p<0.01$ *vs.* control (0 μg membrane fractions, MF).

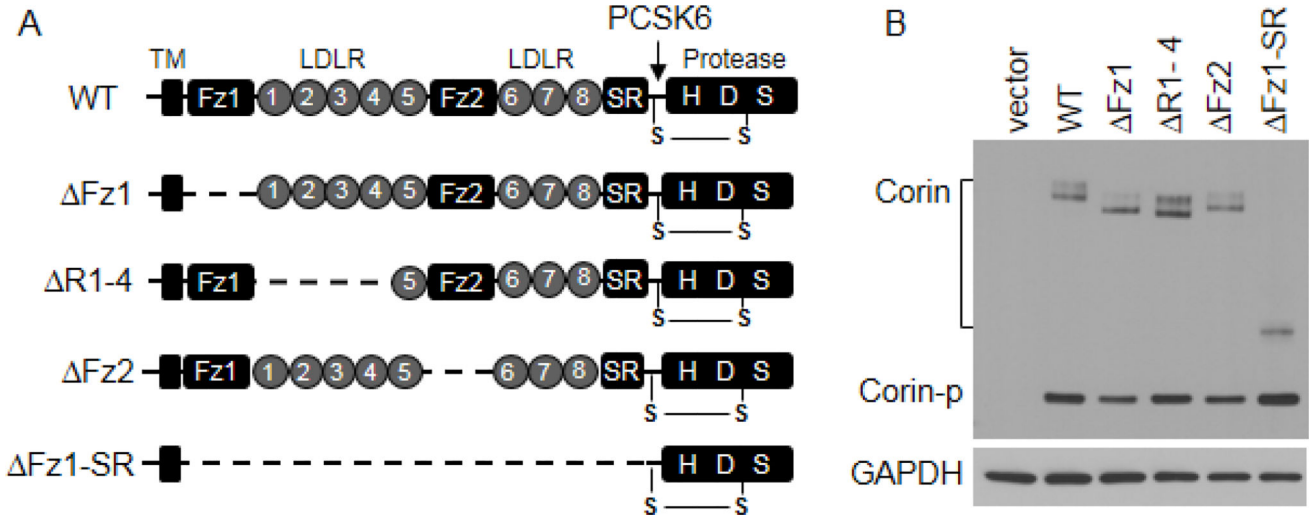

Fig. 4.

Immunostaining of recombinant PCSK6 and WT or sWT corin proteins in HL-1 cardiomyocytes. Transfected HL-1 cells expressing recombinant PCSK6 and WT (top panels) or sWT (bottom panels) were immunostained for PCSK6 (green) and corin (red). In the enlarged panels (left), red arrowheads indicate corin staining, green arrowheads indicate PCSK6 staining, and yellow arrowheads indicate PCSK6 and corin co-staining. Scale bars: 10 μm.

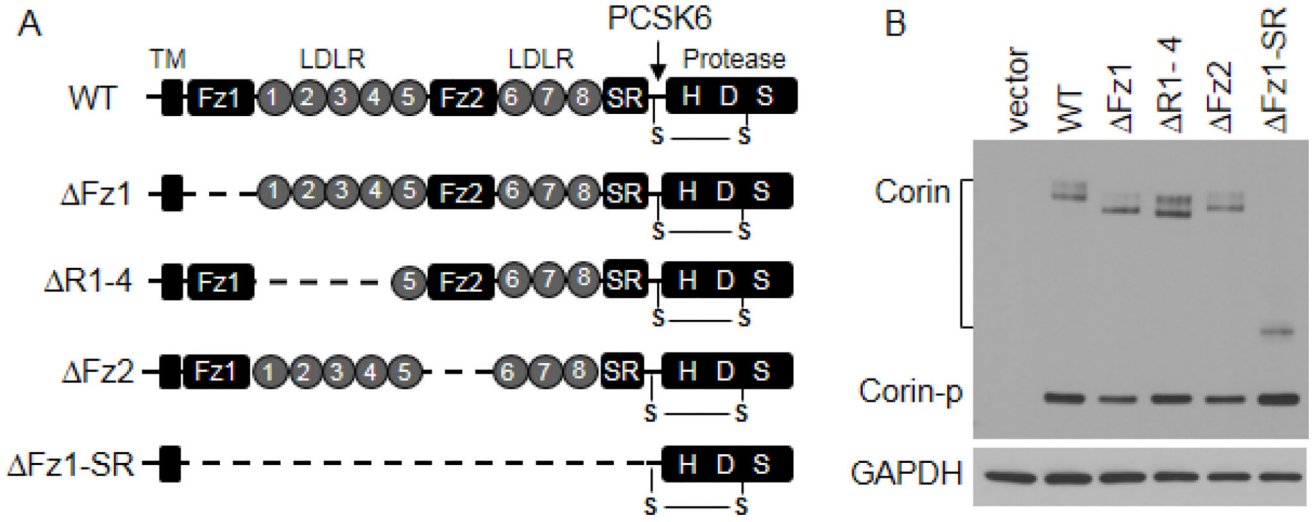

Fig. 5.

Activation cleavage of corin deletion mutants in HEK293 cells expressing recombinant PCSK6. (A) Illustration of corin mutants lacking protein domains in the pro-peptide region. (B) Western blotting analysis of corin proteins in cell lysates from HEK293 cells expressing recombinant PCSK6. Samples from vector-transfected HEK293 cells were used as a control. GAPDH levels were used as protein loading controls. Data are representative of at least five independent experiments.

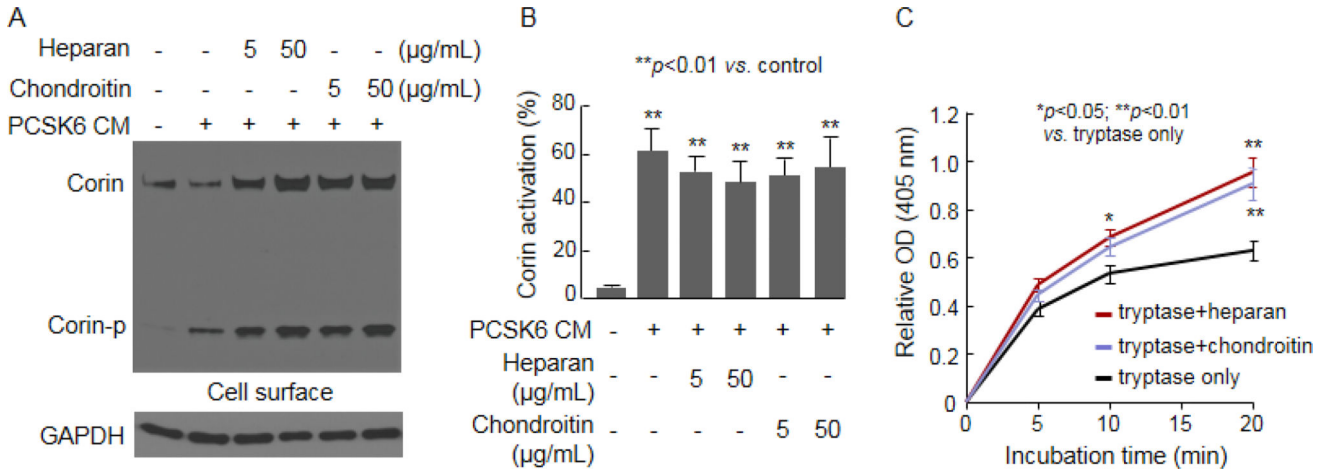

Fig. 6.

Effects of heparan sulfate and chondroitin sulfate on WT corin activation by PCSK6. (A) Western blotting analysis of corin activation on the cell surface in transfected HEK293 cells incubated with PCSK6 in the absence or presence of heparan sulfate or chondroitin sulfate. (B) Percentages of corin activation were calculated based on the densitometric analysis of corin zymogen and corin-p bands on Western blots. Values are mean ± S.D. (n=3 per group). ***p*<0.01 *vs.* control (without PCSK CM, heparan and chondroitin). (C) Tryptase activities, as indicated by optical density (OD) absorbance in a peptide substrate assay performed with or without heparan sulfide or chondroitin sulfide. **p*<0.05; ***p*<0.01 *vs.* tryptase only. Data are from six independent experiments.

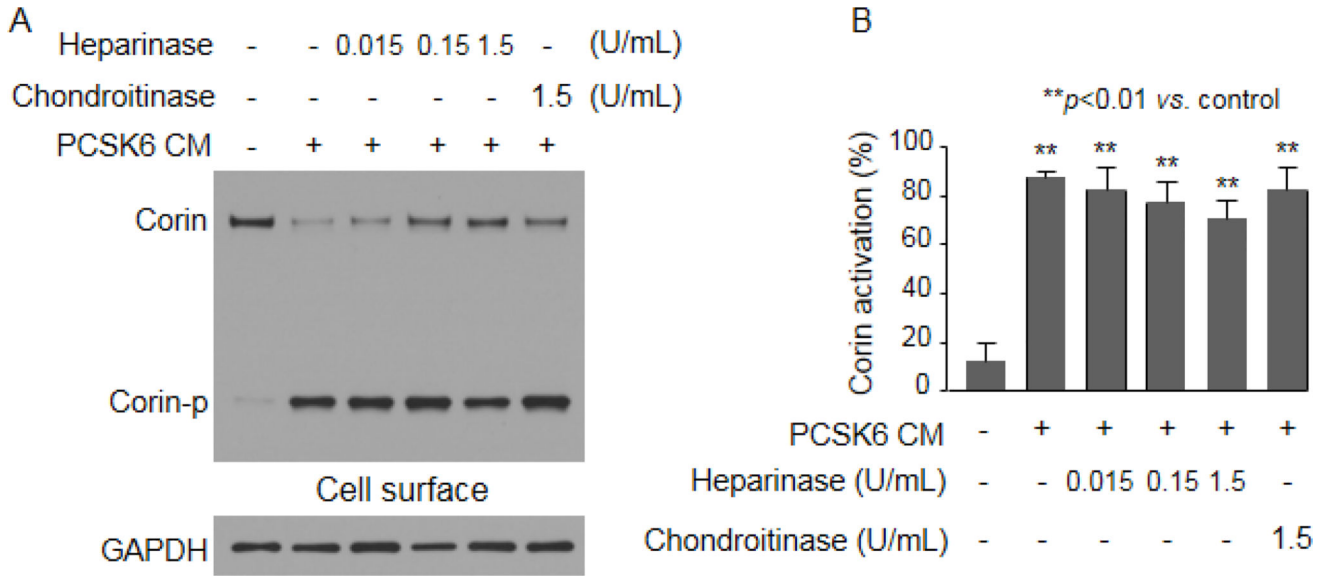

Fig. 7.

Effects of heparinase and chondroitinase treatment on WT corin activation by PCSK6 in HEK293 cells. (A) Western blotting analysis of corin activation on the cell surface in transfected HEK293 cells that were treated with heparinase or chondroitinase and incubated with PCSK6. (B) Percentages of corin activation were calculated based on the densitometric analysis of corin zymogen and corin-p bands on Western blots. Values are mean ± S.D. (n=3 per group). ** $p<0.01$ *vs.* control (without PCSK CM, heparinase and chondroitinase).