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# *N*-Glycosylation modulates the cell-surface expression and catalytic activity of corin

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ABSTRACT

*N*-Glycosylation may influence the subcellular localization and biological activity of the pro-ANP convertase, corin. In HEK293–corin cells, the inhibition of *N*-glycosylation, with tunicamycin, reduced the cell-surface expression of murine corin, but did not alter the total expression. Therefore, tunicamycin treatment likely caused the intracellular accumulation of non-glycosylated corin. Tunicamycin treatment also significantly reduced corin activity (pro-ANP cleavage) in these cells. We developed an assay to measure the effect of *N*-glycosylation on corin activity, independent of its effect on corin localization. We determined that the reduction in corin activity was due to a direct effect of *N*-glycosylation, and was not secondary to the effect of *N*-glycosylation on corin cell-surface expression. Our data provide evidence that *N*-glycosylation is essential for the cell-surface expression of murine corin and modulates its functional activity. *N*-Glycosylation represents a possible mechanism for the regulation of native corin on the surface of cardiomyocytes.



The pro-atrial natriuretic peptide (pro-ANP) converting enzyme, corin [1], and its murine and rat homologs [2,3], are expressed primarily in the heart, and are localized on the external membrane surface of cardiomyocytes [4,5]. Recent studies implicate corin in experimental heart failure and cardiac hypertrophy in humans [1].

Corin is a type II transmembrane serine protease, which consists of intracellular, transmembrane, and extracellular fragments (Fig. 1A) [2,4]. The extracellular fragment contains the trypsin-like serine protease domain and a multi-domain extracellular stem region [2,4].

Recombinant and native corin proteins undergo post-translational processing, including zymogen activation and glycosylation [5,6]. The predicted *N*-glycosylation sites, 19 in human corin and 16 in murine and rat corin, are conserved between species [2–4]. The recombinant rat and human corin proteins are *N*-glycosylated, but contain few *O*-glycans [6]. The native murine corin protein is also extensively *N*-glycosylated [5]. Murine corin is composed of 1113 amino acid residues with a predicted molecular mass of 123 kDa [2]. However, native corin was detected as a 205–210 kDa protein in mouse heart tissue and murine HL-1 cell extracts [5]. The discrepancy between the predicted and the actual molecular mass is attributed to glycosylation of the protein [5].

Studies of recombinant rat corin indicate that the two *N*-glycosylation sites in the protease domain are necessary for corin zymogen activation, but are not required for the expression of corin on the cell surface [6]. In addition to these two glycosylation sites in the protease domain, the murine/rat corin extracellular fragment also contains 14 *N*-glycosylation sites in the stem domain [2,3]. Deglycosylation of the whole native rat corin molecule, both the protease and stem domains, reduced corin expression on the neonatal cardiomyocyte surface, suggesting that *N*-glycosylation of the stem domain may be important for corin cell-surface expression [5].

The cell-surface expression of corin is a prerequisite for its enzymatic activity; therefore, this study was performed to determine the role of *N*-glycosylation in murine corin cell-surface expression and functional activity. Wu and coworkers showed that tunicamycin, a well known inhibitor of *N*-glycosylation, did not alter the total expression of recombinant rat corin in HEK293 cells, but did suppress corin's pro-ANP cleavage activity [6]. Therefore, we used HEK293 cells stably transfected with recombinant murine corin in our study. Using HEK293–corin cells, we show that, *in vitro*, *N*-glycosylation is essential for the cell-surface expression of murine corin and directly modulates its functional activity.

## Materials and methods

*Cloning of the corin and pro-ANP expression vectors.* Recombinant murine corin fragments [2] were cloned by PCR from a total mouse

*Abbreviations:* ANP, atrial natriuretic peptide; FITC-FPR-CMK, Fluorescein-Phe-Pro-Arg-chloromethyl ketone; MAbs, Mouse monoclonal antibodies.

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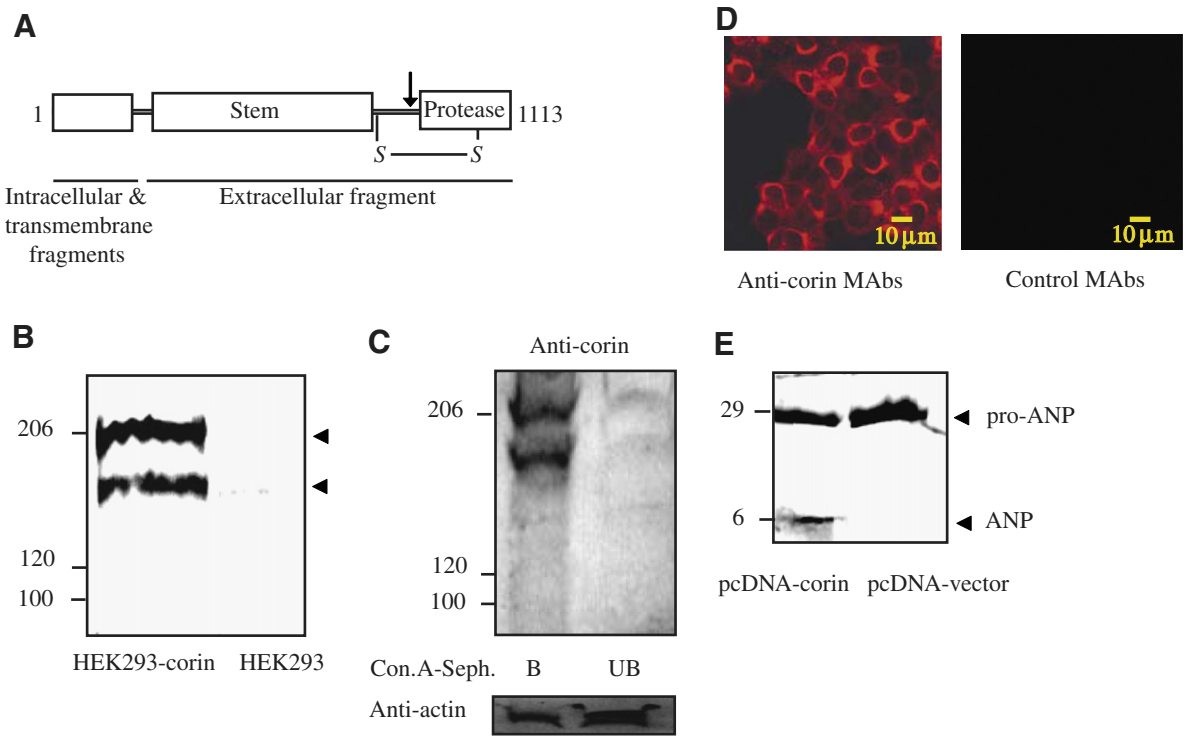


**Fig. 1.** Structure, expression, cell-surface localization, and enzymatic activity of recombinant mouse corin in transfected HEK293 cells. (A) Schematic diagram of the full-length corin protein [2]. The arrow indicates the activation (cleavage) site. The disulfide bond (s–s), connecting stem and protease domains, is shown. (B) HEK293 cells were transiently transfected with pcDNA–corin or empty vector. Western blotting was performed under reducing conditions with anti-corin stem domain antibody (R34). Arrows indicate corin protein. (C) HEK293–corin cells were lysed and subjected to affinity chromatography on Concanavalin A–Sepharose followed by Western blotting as described under (B). To confirm the equal sample loading the same blot was probed with anti-actin antibody. (D) Non-permeabilized stable HEK293–corin cells were stained with anti-corin pooled MAbs (12G8/16E6/14H1) or control MAbs, detected with Alexa Fluor 594-labeled secondary antibodies and analyzed by confocal microscopy. The anti-corin MAbs showed no cross-reactivity with HEK293 cells. (E) Stable HEK293–corin and control HEK293 cells were incubated with conditioned medium containing pro-ANP for 5 h at 37 °C. Pro-ANP/ANP cleavage was analyzed by immunoprecipitation of conditioned medium followed by Western blotting as described under Materials and methods. The protein standards in kilodalton are as indicated.

heart cDNA library (Clontech Lab. Inc., Palo Alto, CA) into pCR™ II vector (Invitrogen, Carlsbad, CA) using the following primers: for the intracellular + transmembrane fragment (residues 1–133), 5′-GGGCCCGCGGCCGCATGGGCAGGGTTTCCTTCAG-3′ (sense) and 5′-AGCATCTGAGGGAATCCGGCC-3′ (antisense); for the extracellular fragment (residues 134–1113), 5′-GAATTCATGCCTTTGTGGGAACA TTAA-3′ and 5′-GCGGCCGCTCCTTGGGATTTCTTTTG-3′ (Sigma-Genosys, The Woodlands, TX). The confirmed cDNAs were ligated into the pGEX-4T-2 (Amersham Biosciences, Piscataway, NJ) vector.

To express recombinant corin in mammalian cells, the full-length mouse corin cDNA was cloned into the pcDNA 3.1(−) vector (Invitrogen). The extracellular sequence was cut from the pGEX-4T-2 vector with EcoRI (blunted with Klenow enzyme) and NotI and cloned into the pcDNA3.1(−) vector. This pcDNA 3.1(−) extracellular sequence plasmid was then digested with ApaI and XhoI. The intracellular + transmembrane domain was cut from the pCR™ II vector with ApaI and XhoI and cloned into the pcDNA3.1(−) vector containing the extracellular sequence flanked by a XhoI at the 5′-end and a NotI site at the 3′-end. The constructs were verified by restriction digestion and DNA sequencing. Restriction enzymes were from New England Biolabs Inc. (Beverly, MA). Rapid DNA Ligation kit was from Roche Diagnostics (Mannheim, Germany).

Pro-ANP cDNA was amplified from a Human Adult 8 Tissue GenePools cDNA library (NT Omics, Inc San Mateo, CA) by PCR using sense primer (5′-GAGAGACAGAGCAGCAAGCAGTG-3′) and antisense primer (5′-GTCCCGGAAGCTGTTACAGCCC-3′) (Fisher Scientific Inc.) [4]. The pro-ANP cDNA was cloned into the pcDNA 3.1/V5-His-TOPO vector (Invitrogen) using the pcDNA3.1/V5-His-TOPO TA Expression Kit (Invitrogen) to yield the plasmid, pcDNA-pro-ANP.

*Polyclonal and monoclonal antibody*. Rabbit polyclonal anti-corin stem domain (R34) and mouse monoclonal anti-protease domain antibodies 12G8, 14H1, and 16E6 were generated and characterized recently [5].

*Stable transfected HEK 293–corin cell line.* Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were cultured in α-minimum essential medium (α -MEM, Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were adapted for growth in serum-free Opti-MEM (Invitrogen) and transfected with pcDNA–corin or empty vector plasmid (mock transfection), using the GenePORTER™ transfection reagent (Gene Therapy Systems Inc., San Diego, CA) according to the manufacturer's protocol. Stable pcDNA–corin clones were selected in α-MEM containing 500 μg/ml G418 (Invitrogen). Cells were screened for corin expression by Western blotting using anti-corin stem domain antibody (R34). The confluent cells were lysed in 50 mM Tris–HCl, pH.7.4, buffer containing 125 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Roche Diagnostics). The cell lysate was incubated for 1 h at 4 °C and centrifuged at 10,000*g* at 4 °C. The protein concentration was determined by the Protein Assay kit (Pierce, Rockford, IL). Cell extract was incubated or not with of Concanavalin A–Sepharose (Amersham Bioscience) and analyzed by Western blotting as previously described [5].

*Immunofluorescence staining.* HEK293–corin cells were cultured to confluence on eight-well glass chamber slides (Nalge Nunc International, Naperville, IL), under the conditions described above,

and immuno-stained according to the published protocol [5]. Slides were visualized on a LSM 510 Meta Confocal microscope with Argon-488, He–Neon 543, 633 lasers using 40× or 63× oil immersion lenses. Localization of labeled proteins was analyzed using Axio Vision software (Zeiss).

*Pro-ANP processing by stable HEK293–corin cells.* Recombinant pro-ANP was expressed in HEK293 cells using Lipofectin (Invitrogen) according to the manufacturer's protocol. Conditioned medium was collected 40 h after transfection and used for a cell-based assay to examine pro-ANP cleavage mediated by corin. Stable HEK293–corin cells were incubated with conditioned medium for 5 h at 37 °C. Cleaved and uncleaved pro-ANP forms were immunoprecipitated from the conditioned medium by a mouse monoclonal anti-V5 tag antibody (Invitrogen) coupled to protein A–Sepharose (Pierce). Immuno-precipitated proteins were solubilized in SDS–PAGE sample buffer and analyzed by Western blotting under reducing conditions with an anti-V5 tag rabbit polyclonal antibody (Immunology Consultants Laboratory Inc., Newberg, OR) followed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit/or goat anti-mouse antibody, and detection by ECF substrate (Amersham Biosciences).

*Flow cytometry analysis.* Non-permeabilized HEK293–corin cells were washed with ice-cold PBS containing 3% fetal bovine serum, collected, centrifuged at 2000*g* for 2 min, resuspended at a density of $4 \times 10^6$ cells/ml. The cells were stained with anti-corin pooled (12G8/16E6/14H1, 10 μg/ml) or non-specific control pooled (IgM/IgG/IgM, 10 μg/ml) according to the published protocol [5]. Fluorescence was measured in a flow cytometer (Becton–Dickinson FACSCalibur) with CellQuest software for acquisition and analysis.

Non-permeabilized HEK293–corin cells were stained with 0.5 μM FITC-FPR-CMK for 10 min followed by amplification with rabbit anti-FITC antibody (10 μg/ml, Invitrogen) for 35 min and goat anti-rabbit-FITC antibody (Sigma) for 35 min. HEK293–corin cells were double-labeled with anti-corin MAbs and FITC-FPR-CMK, using the same conditions described above.

*Inhibition of corin N-glycosylation in cell-based assay.* HEK293–corin cells were cultured until 80–90% confluence, pre-treated with 0.05% Trypsin–EDTA solution for 1 min to remove post-translationally modified proteins from the membrane surface, and washed with cold PBS. Cells were then treated with 0–40 μg/ml tunicamycin (Sigma) in supplemented culture medium for 30 h.

## Results

### *Expression and characterization of recombinant corin*

We recently reported that endogenous murine corin is an extensively glycosylated protease localized on the cell surface of pro-ANP-expressing cardiomyocytes [5]. To evaluate the impact of glycosylation on the cell-surface expression and enzymatic activity of corin, we expressed recombinant murine corin in mammalian cells, HEK293, which are capable of carrying out most post-translational protein processing [7]. Using Western blotting, we demonstrated that HEK293–corin cells express a protein that migrates as double bands with relative masses of 200–210 kDa and 180 kDa (Fig. 1B). The high molecular weight band is consistent with the size of the highly glycosylated, uncleaved corin protein in murine heart tissue [5]. The lower molecular weight band is comparable to the size of cleaved corin in murine heart tissue [5]. The glycosylation status of the corin expressed in these cells was assessed by the separation of the cell extract on Concanavalin A–Sepharose, which binds only glycosylated proteins. Both bands exhibit strong affinity toward Concanavalin A–Sepharose (Fig. 1C), confirming that they are glycosylated.

To examine the subcellular localization of recombinant corin in HEK293 cells, immunofluorescence and flow cytometric analyses of non-permeabilized HEK293–corin cells were performed using anti-corin MAbs. Confocal microscopic analysis (z-stack) showed that corin localized to the external membrane surface (Fig. 1D). Flow cytometric analysis confirmed the cell-surface localization of corin (Fig. 3A, upper left quadrant on the middle panel). Since mock-transfected HEK293 cells do not express corin (Fig. 1B), we did not include these cells in these analyses.

To verify that the recombinant mouse corin was catalytically active toward its natural substrate, pro-ANP, HEK293–corin cells were incubated with pro-ANP-containing medium and cleavage was analyzed. The corin expressed in HEK293–corin cells was able to cleave pro-ANP to a smaller fragment containing a V5 tag, while non-transfected HEK293 cells were not able to cleave pro-ANP (Fig. 1E).

### *Effect of tunicamycin on the cell-surface expression of murine corin*

The stable HEK293–corin cells were used to investigate the impact of the *N*-glycosylation of corin on physiologically important processes such as corin localization and catalytic activity. Cells were cultured with or without tunicamycin, which inhibits the first step in the lipid-linked pathway and prevents *N*-glycosylation [8]. To exclude the possibility that pre-treatment with tunicamycin would influence the corin expression level or cause degradation of the corin molecule, the total cell lysate, which contains both cytosol and membrane-bound proteins, was analyzed by Western blotting with an anti-corin antibody (Fig. 2A). Tunicamycin treatment did not reduce the corin expression level, but significantly reduced the size of the expressed proteins; a band consistent with the predicted molecular weight (123 kDa [2]) can be seen after tunicamycin treatment (Fig. 2A). This suggests that tunicamycin treatment did not cause degradation of the recombinant murine corin. However, tunicamycin treatment did have an effect on corin localization: tunicamycin-treated cells showed significantly less corin staining on the cell surface (Fig. 2B) compared with untreated cells, as assessed by immunofluorescence staining (Fig. 1D). Since the total corin expression level was normal, the tunicamycin treat-

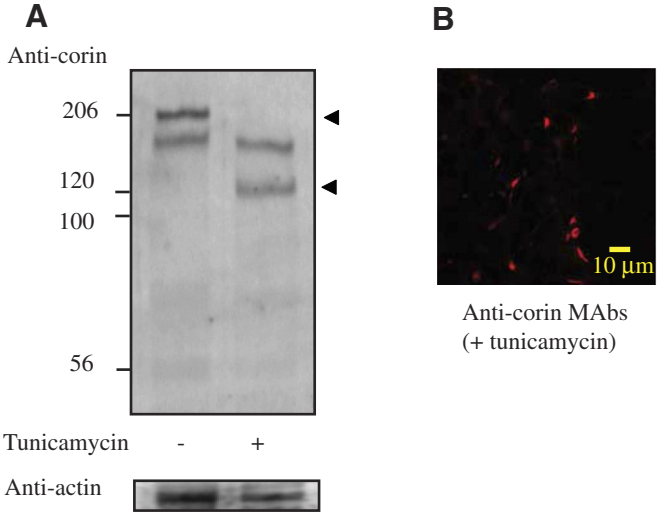


**Fig. 2.** Effect of tunicamycin on protein expression and cell-surface localization of mouse corin in stable HEK293–corin cells. (A,B) Stable HEK293–corin cells were treated (+) or not (−) with tunicamycin (20 μg/ml) and subjected to (A) Western blotting under reducing conditions with anti-corin stem domain antibody (R34) (the equal sample loading was confirmed by probing the same blot with anti-actin antibody) or (B) immunofluorescence staining as on Fig. 1D.

ment likely impaired the trafficking of corin to the cell surface, resulting in the intracellular accumulation of corin.

The expression of *N*-glycosylated and non-glycosylated corin on the membrane surface of stable HEK293–corin cells was also analyzed by flow cytometry (Fig. 3A). Corin-positive cells (upper left quadrant) were readily differentiated from unlabeled cells (low left quadrant, Fig. 3A). Treatment with tunicamycin decreased the percent of corin-positive cells (Fig. 3A, upper left quadrant on the right panel) in a dose-dependent manner (Fig. 3B). Taken together with the data from immunofluorescence staining (Fig. 2B) and Western blotting (Fig. 2A), these findings indicate that *N*-glycosylation is important for the cell-surface expression of recombinant corin.

*Effect of tunicamycin on catalytic activity of murine corin*

To examine whether glycosylation is essential for the catalytic activity of murine corin, a pro-ANP cleavage assay was performed. The effect of tunicamycin treatment on the ability of surface-localized corin to cleave pro-ANP was analyzed. Treatment of stable HEK293–corin cells with tunicamycin significantly reduced pro-ANP processing (Fig. 4A). Tunicamycin treatment does not directly inhibit corin activity or interaction with pro-ANP [6]. Tunicamycin treatment also reduces the cell-surface localization of corin (Figs. 2B and 3). These results suggest that *N*-glycosylation, either directly or indirectly, by regulating the cell-surface localization of corin, is necessary for murine corin to process pro-ANP in HEK293 cells.

To address this question, we designed an assay to measure the catalytic activity of surface-localized corin. Corin specific antibodies [5] were used to gate the population of cells expressing corin on the cell surface. The catalytic activity of surface-localized corin in HEK293–corin cells was analyzed by flow cytometry using FITC-FPR-CMK, an irreversible FITC-tagged chloromethyl ketone protease inhibitor [9]. FITC-FPR-CMK mimics the corin cleavage sequence in its natural substrate, pro-ANP, and is a specific and efficient inhibitor of native mouse corin on the surface of myocardial cells [5]. To amplify the FITC signal, rabbit anti-FITC antibodies were applied, followed by detection with FITC-labeled goat anti-rabbit antibodies (Fig. 4B). The FITC-FPR-CMK-bound cells (Fig. 4B, middle panel, lower right quadrant) could be differentiated from unstained cells that were exposed to the secondary anti-FITC antibodies without exposure to FITC-FPR-CMK (Fig. 4B, left panel, lower left quadrant). Thus, FITC staining was not due to the non-specific binding of rabbit anti-FITC or FITC-labeled goat anti-rabbit antibodies. When cells were stained with both FITC-FPR-CMK and anti-corin MAbs, the double stained cells represent cells that express *active corin* on their plasma membrane surface (Fig. 4B, right panel, upper right quadrant). The binding of FITC-FPR-CMK to cells caused the population of corin positive cells to shift to the right on the FITC axis (compare Fig. 4B, right panel, upper right quadrant to Fig. 3A, middle panel, upper left quadrant). Therefore, staining the FITC-FPR-CMK treated cells with anti-corin MAbs allowed us to distinguish the effect of *N*-glycosylation on pro-ANP cleavage from its influence on the cell-surface expression of corin.

Under these assay conditions, the majority of cell-surface expressed corin is catalytically active (Fig. 4B and C). Tunicamycin treatment caused a small, but statistically significant, reduction in the percent of corin-positive cells that bound FITC-FPR-CMK (Fig. 4C). These results indicate that *N*-glycosyl-

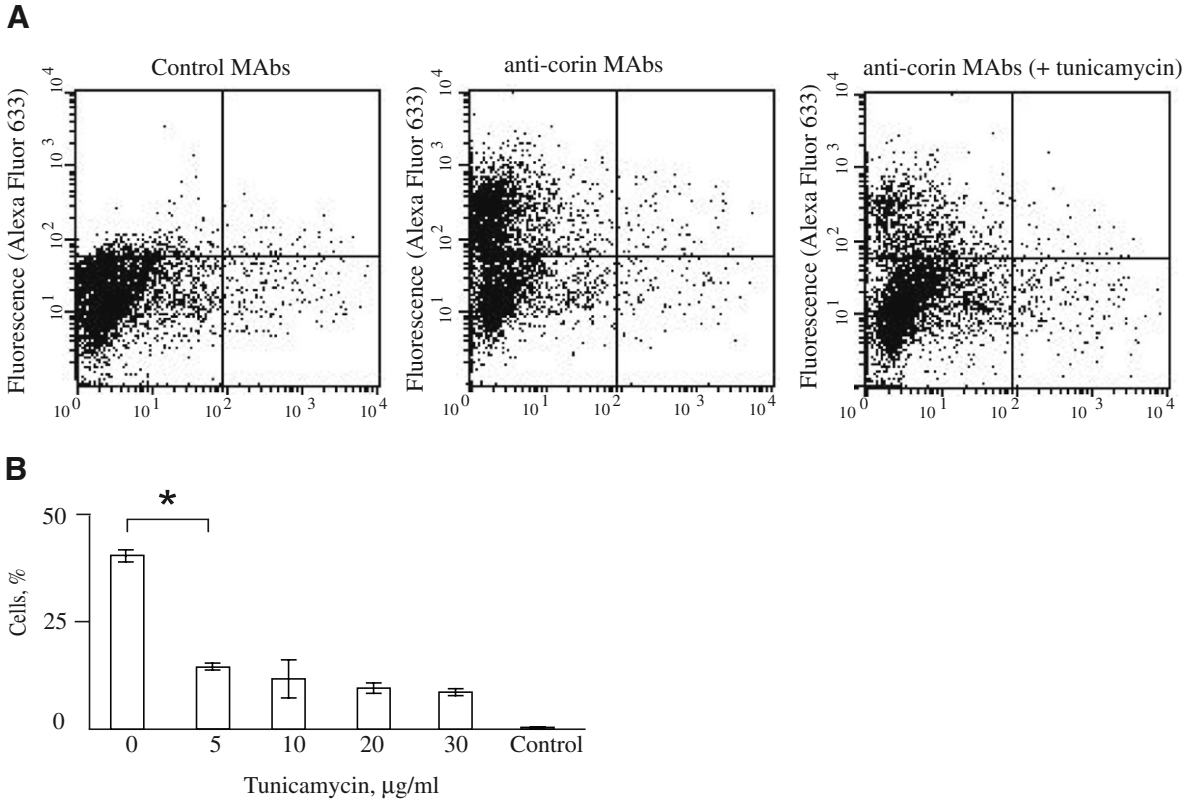


**Fig. 3.** Cell-surface localization of mouse corin analyzed by flow cytometry in stable HEK293–corin cells. (A) Cells were labeled with anti-corin MAbs and detected with Alexa Fluor 633 labeled secondary antibody (upper left quadrant on the middle panel). Cells were treated with tunicamycin and analyzed as above (upper left quadrant on the right panel). Subpopulations of unstained and control non-specific MAb-stained cells are overlapped (lower left quadrant). Panels shown are representative results of at least three independent experiments. (B) The percent of anti-corin MAb-positive cells was quantified after treatment with different doses of tunicamycin. Error bars indicate standard deviations. The differences in expression of anti-corin MAb binding after treatment with tunicamycin were statistically significant (paired *t* test, $^{*}P < 0.001$).

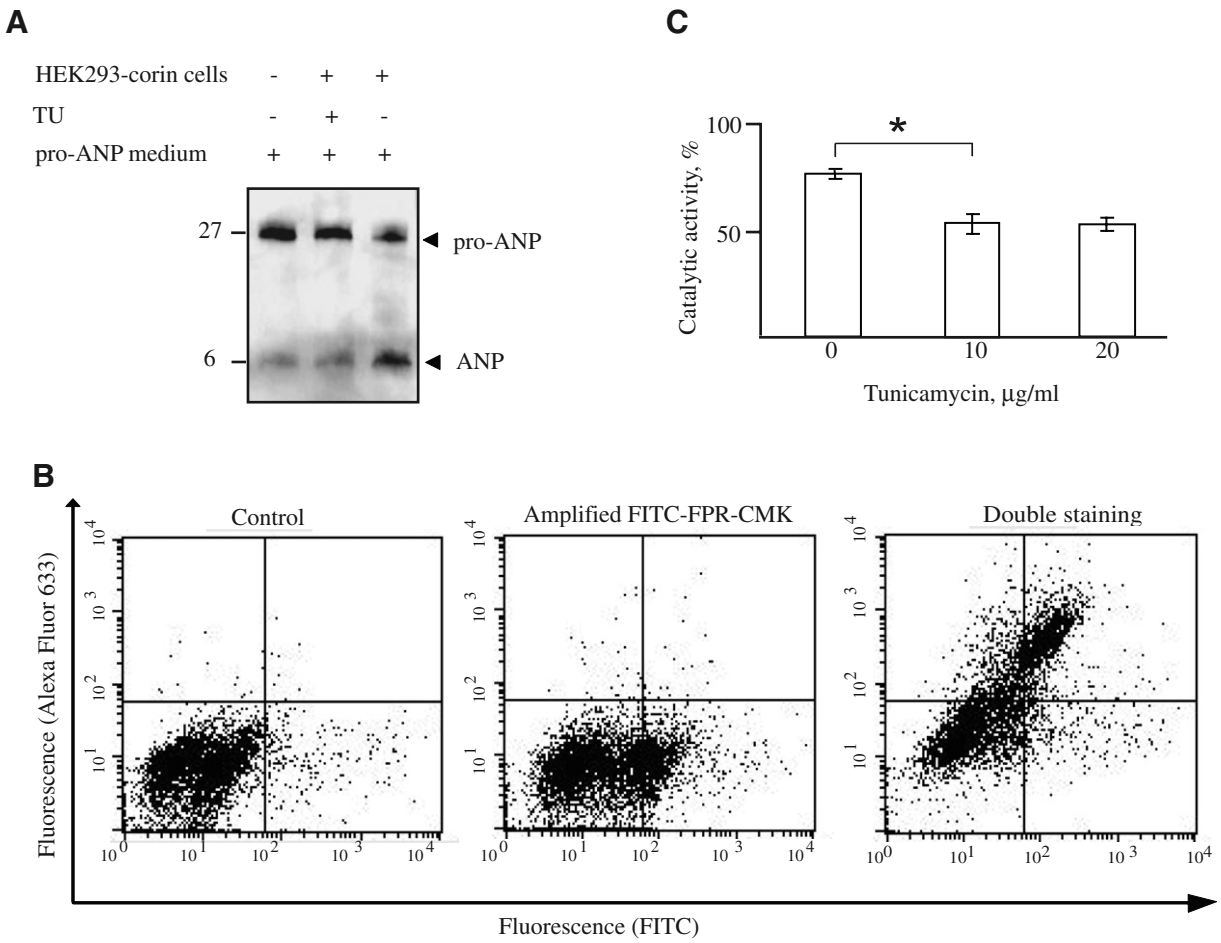


**Fig. 4.** Effect of tunicamycin on enzymatic activity of murine corin in stable HEK293–corin cells. (A) Stable HEK293–corin cells were cultured with (+) or without (−) tunicamycin (20 μg/ml) for 30 h and incubated with conditioned medium containing pro-ANP for 5 h at 37 °C. Pro-ANP/ANP cleavage was analyzed by immunoprecipitation of conditioned medium, followed by Western blotting under reducing conditions as described for Fig. 1E. (B,C) Enzymatic activity of mouse corin was analyzed by flow cytometry. (B) Cells were labeled with FITC-FPR-CMK. FITC signal was amplified by rabbit-anti-FITC antibody followed by detection with goat anti-rabbit antibody (middle panel, lower right quadrant). Control (left panel, lower left quadrant) represents cells stained with rabbit anti-FITC antibody and detected with goat anti-rabbit antibody. In separate experiments cells were double labeled with anti-corin MAbs and FITC-FPR-CMK and detected as above and as on Fig. 3A (right panel, upper right quadrant). Anti-corin MAb-stained cells appear in the upper left quadrant. Multiparameter dot plots representing FITC fluorescence versus Alexa Fluor 633 fluorescence. (C) The percent of anti-corin MAb-positive cells that bound FITC-FPR-CMK was quantified. Catalytic activity, %, indicates the percent of corin-positive (anti-corin MAb-positive, (B) right panel, upper left and upper right quadrants) cells that expressed the catalytically active corin ((B) right panel, upper right quadrant). Error bars indicate standard deviations. The observed reductions in expression of anti-corin MAb and FITC-FPR-CMK double-labeled corin after treatment with tunicamycin were statistically significant (paired *t* test, $^{*}P < 0.001$).

ation has an independent effect on corin enzymatic activity, separate from the influence on the cell-surface expression of corin molecule.

## Discussion

Corin (native murine and rat corin [5] and recombinant rat and human corin [6]) is localized on the cell-surface and undergoes post-translational modifications, including zymogen activation and *N*-glycosylation. In the murine/or rat corin protein, all 16 of the putative *N*-glycosylation sites are located in the extracellular region of the molecule which contains the stem and protease domains (Fig. 1A), [2,3]); therefore it is likely that glycosylation is required for cell-surface expression and biological activity. Direct mutation of the two *N*-glycosylation sites in the protease domain of rat corin reduced corin activity but did not alter corin expression on the cell surface [6]. However inhibiting *N*-glycosylation of the *whole* corin molecule reduced the expression of native rat corin on the neonatal cardiomyocyte membrane surface [5], suggesting that the *N*-glycosylation sites in the stem domain may play an important role in corin localization.

In order to be biologically active toward its natural substrate, pro-ANP, corin should be properly expressed on the cardiomyocyte surface. This study was designed to clarify the role of *N*-glycosylation in the cell-surface expression of corin, and to distinguish this effect from the direct effect of *N*-glycosylation on the enzymatic activity of corin.

Murine corin is expressed in stable HEK293–corin cells as a highly *N*-glycosylated 200–210 kDa protein (Figs. 1B,C and 2). Tunicamycin treatment suppressed the expression of the highly *N*-glycosylated form while allowing the expression of a molecular form which corresponds to the predicted molecular size of corin [2] (Fig. 2A). Since the HEK293–corin cells produced a highly *N*-glycosylated protein, with a molecular weight that is consistent with the native murine corin protein [5], we considered them a proper model for investigating the effect of *N*-glycosylation on the biological properties of mouse corin.

The strong corin staining that was observed on the cell-surface of non-permeabilized HEK293–corin cells (Figs. 1D and 3)

was reduced in tunicamycin-treated cells (Figs. 2B and 3A,B). The suppression of corin cell-surface expression by tunicamycin was not due to a decrease in total corin protein expression, degradation of corin, or to reduced affinity of the anti-corin MAbs to non-glycosylated forms of corin (Fig. 2A); most likely, tunicamycin caused an intracellular accumulation of non-glycosylated corin. Taken together, these findings suggest *N*-glycosylation of the whole corin molecule is important for corin expression on the surface of stable HEK293–corin cells. This confirms our previous finding that tunicamycin treatment suppressed the expression of the native rat corin protein on the surface of neonatal cardiomyocytes [5]. On the other hand, mutation of the two *N*-glycosylation sites in the protease domain of rat corin did not alter surface expression of recombinant rat corin [6]. Since 14 of the 16 putative *N*-glycosylation sites are located in the stem domain of the mouse/rat corin molecule [2,3], it is likely that *N*-glycosylation of the stem domain, but not the protease domain, plays critical role in cell-surface expression of corin protein. Future studies, which would use site-directed mutagenesis toward all or some of the *N*-glycosylation sites in the stem domain, are required.

Under our experimental conditions, only cell-surface expressed corin was able to interact with and cleave pro-ANP (Fig. 4A). Therefore, suppression of the cell-surface expression of corin by tunicamycin might indirectly suppress corin biological activity (pro-ANP cleavage). To explore this hypothesis, we designed a flow cytometric assay that allowed us to distinguish the direct effect of tunicamycin on the catalytic activity of corin from the indirect effect (through its influence on cell-surface expression). Stable HEK293–corin cells were analyzed for enzymatically active corin on their cell surface by double staining with anti-corin MAbs and the chloromethyl ketone inhibitor of corin, FITC-FPR-CMK [5]. Our anti-corin protease domain MAbs specifically stained total corin expressed on the cell-surface (Fig. 3A), while FITC-FPR-CMK [5] recognized only catalytically active corin on the external membrane surface of stable HEK293–corin cells (Fig. 4B). Treatment of HEK293–corin cells with increasing doses of tunicamycin caused a statistically significant reduction in the percent of corin-positive cells (corin on the cell-surface) that also bound FITC-FPR-CMK (Fig. 4C). Therefore, our results indicate that *N*-glycosylation directly modulates enzymatic activity of murine corin separately from the effect on cell-surface expression. Still, it should be noted that the catalytic activity of corin was examined against a synthetic peptide inhibitor, FITC-FPR-CMK. Therefore, our study does not address the impact of *N*-glycosylation on corin zymogen activation, or on the enzymatic activity of corin toward different physiological substrates or inhibitors.

In summary, our study provides strong evidence that, in cell-based assays, *N*-glycosylation is essential for the cell-surface expression of corin and might directly modulate its functional activity. If *N*-glycosylation plays a similar regulatory role *in vivo*, it may represent a mechanism responsible for regulating native corin activity on the surface of cardiomyocytes.

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