Cobra CRISP Functions as an Inflammatory Modulator via a Novel Zn\(^{2+}\) and Heparan Sulfate-dependent Transcriptional Regulation of Endothelial Cell Adhesion Molecules*

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Cysteine-rich secretory proteins (CRISPs) have been identified as a toxin family in most animal venoms with biological functions mainly associated with the ion channel activity of cysteine-rich domain (CRD). CRISPs also bind to Zn\(^{2+}\) at their N-terminal pathogenesis-related (PR-1) domain, but their function remains unknown. Interestingly, similar the Zn\(^{2+}\)-binding site exists in all CRISP family, including those identified in a wide range of organisms. Here, we report that the CRISP from *Naja atra* (natrin) could induce expression of vascular endothelial cell adhesion molecules, *i.e.*, intercellular adhesion molecule-1, vascular adhesion molecule-1, and E-selectin, to promote mononuclear cell adhesion in a heparan sulfate (HS)- and Zn\(^{2+}\)-dependent manner. Using specific inhibitors and small interfering RNAs, the activation mechanisms are shown to involve both mitogen-activated protein kinases and nuclear factor-κB. Biophysical characterization of natrin by using fluorescence, circular dichroism, and x-ray crystallographic methods further reveals the presence of two Zn\(^{2+}\)-binding sites for natrin. The strong binding site is located near the putative Ser-His-Glu catalytic triad of the N-terminal domain. The weak binding site remains to be characterized, but it may modulate HS binding by enhancing its interaction with long chain HS. Our results strongly suggest that natrin may serve as an inflammatory modulator that could perturb the wound-healing process of the bitten victim by regulating adhesion molecule expression in endothelial cells. Our finding uncovers a new aspect of the biological role of CRISP family in immune response and is expected to facilitate future development of new therapeutic strategy for the envenomed victims.

The cysteine-rich secretory proteins (CRISPs)\(^{3}\) belong to the Crisp, antigen 5, and pathogenesis-related proteins superfamily

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3 The abbreviations used are: CRISP, cysteine-rich secretory protein; CRD, cysteine-rich domain; DS, dermatan sulfate; EC, endothelial cell; GAGs, glycosaminoglycans; HS, heparan sulfate; HSPGs, HS proteoglycans; ICAM-1, intercellular adhesion molecule-1; PR-1, pathogenesis-related domain-1; SPR, surface plasmon resonance; VCAM-1, vascular adhesion molecule-1; FOM, figure of merit; dp, depolymerization.
Recent advances in studies on the structure and function of cobra venom components have attempted to explain the perturbed wound-healing processes by showing that many non-Arg-Gly-Glu cationic cobra toxins, including cardiotoxins, snake venom matrix metalloprotease, and phospholipase A2, bind strongly to glycosaminoglycans (GAGs) and/or integrins in the extracellular matrix of cell membranes (23–25). We proposed that natrin, a Zn2+-binding CRISP, can bind to heparan sulfate (HS) to modulate pro-inflammatory processes in wound healing by regulating adhesion molecule expression in vascular endothelial cells (ECs). In this study we characterized the structure of natrin in the presence and absence of Zn2+ binding by x-ray diffraction and demonstrated that natrin can induce intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), and E-selectin expressions in ECs through the Zn2+ and HS modifications in mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB pathways. Our results provide insights into the molecular basis of mechanisms by which the snake CRISP serves as a proinflammatory modulator and suggest that CRISPs may act synergistically with other members of the snake toxin family, such as snake venom matrix metalloprotease and phospholipase A2, to interfere with wound healing and inflammatory processes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal antibodies (mAbs) against ICAM-1, VCAM-1, and E-selectin were purchased from R&D Systems (Minneapolis, MN). Mouse mAbs against extracellular-signal regulated kinase 2 (ERK2; sc-1647), phospho-ERK1/2 (sc-7383), c-Jun-N-terminal kinase 2 (JNK2; sc-7345), phospho-JNK (sc-6254), and the NF-κB subunit p65 (sc-8008) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against p38 MAPK and mouse mAb against phospho-p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA). PD98059, SP600125, and SB203580 were obtained from Calbiochem. The ERK-, JNK-, and p38-specific small interfering RNA (siRNA) and control siRNA were purchased from Invitrogen. All other chemicals of reagent grade were obtained from Sigma unless otherwise noted.

**Preparation and Characterization of Natrin**—The crude venom (15 ml) was collected directly from about 100 Naja atra cobras at a snake farm in Tainan City, Taiwan, and lyophilized before further use. The detailed procedure of purification of natrin was previously described (26). Briefly, natrin protein was purified using a three-step chromatography procedure consisting of Sephadex C25, DEAE-Sepharose fast flow, and CM-Sepharose fast flow (GE Healthcare), with ion-exchange chromatography on a fast protein liquid chromatography system (ÄKTA purifier, GE Healthcare) at 4 °C. The natrin was desalted and concentrated (4000 rpm) using Centricron Plus (Millipore, Billerica, MA) centrifugal filter devices. The yield of the protein was ~5 mg, and the purity was better than 95% as analyzed by 10% SDS-polyacrylamide gel electrophoresis and UV-visible spectra. Protein concentrations were determined by BCA assay (Pierce).

**EC Culture**—ECs were isolated from fresh human umbilical cords by means of the collagenase perfusion technique (27) and grown in Petri dishes in medium 199 (M199, Invitrogen) supplemented with 20% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen) for 3 days. Secondary cultures (1–2 × 10⁵ cells/cm²) were used in the experiments.

**Monocytic Cell Adhesion Assay**—The human monocytic cell line U937 was obtained from American Type Culture Collection (Rockville, MD) and maintained in culture medium RPMI 1640 (Invitrogen) supplemented with 10% FBS. Before adhesion experiments, U937 cells were suspended in RPMI 1640 containing 0.1% FBS and labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI; Molecular Probes, Eugene, OR) for 20 min. The labeled U937 cells (1 × 10⁵ cells/ml) were added onto ECs, with subsequent incubation for 30 min. In parallel experiments ECs were pretreated with natrin at various concentrations (50, 100, and 1000 nm) for 6 h. Non-adherent cells were quickly removed by washing with RPMI. The adherent U937 cells on the EC surface were identified and counted in 10 randomly selected microscopic fields (1.37 × 1.07 mm) under an inverted epifluorescence microscope (Axiovert 200 M, Zeiss, Germany) with 10× objective (NA = 0.4, LD Achroplan, Zeiss), and adhesion was expressed as cells/mm².

**RNA Isolation and Quantitative Real-time PCR**—Total RNA was isolated by the guanidine isothiocyanate/phenol-chloroform method, and cDNA was synthesized using the Superscript II reverse transcriptase system and oligo-dT primers (Invitrogen). The cDNA was amplified through PCR on a LightCycler (Roche Diagnostics) using LightCycler FastStart DNA MasterPlus SYBR Green I (Roche Diagnostics) with 0.5 μM primers of ICAM-1 (sense, 5'-CTTTCTCACCGTGACTGG-3'; antisense, 5'-AGGCTAGGTGTAAGGTTCRGC-3'; product length, 90 bp), VCAM-1 (sense, 5'-TGGACATAAGAACACTGGAAAGG-3'; antisense, 5'-CCACTCATCTGATTTCTTGAG-3'; product length, 78 bp), and E-selectin (sense, 5'-TGTCCTGCTTCTCTACAGGGAGG-3'; antisense, 5'-GTGCTAATGTTCAGGAGGGAGA-3'; product length, 100 bp) genes. PCR was performed in triplicate at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 5 s, and extension at 72 °C for 8 s, and single signal acquisition for 10 s. The α-tubulin gene expression was used as an internal control (sense, 5'-TTACCTGACTGCTTCTCACAGGGAGG-3'; antisense, 5'-GGATGGAGATGCCTCAGCG-3'; product length, 107 bp). The PCR conditions were optimized to obtain a PCR product with a single peak on melting curve analysis on the LightCycler. PCR product was also run on a 1% agarose gel and stained with ethidium bromide to obtain a single band of the expected size. Raw data collected from the LightCycler were analyzed using LightCycler Software Version 3.5 (Roche Diagnostics). The adhesion molecule gene expression levels were normalized with α-tubulin gene expression levels in the same sample.

**Flow Cytometry**—ECs were washed with M199, detached with Versene buffer containing EDTA, and centrifuged. Each sample (1 × 10⁶ cells) was washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and resuspended in 0.2 ml of PBS containing a mouse mAb against ICAM-1, VCAM-1, or E-selectin (10 μg/ml). After incubation at 4 °C for 30 min, the cells were centrifuged at 1500 rpm for 5
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min and washed with PBS to remove the unbound antibody. The ECs were then incubated with anti-mouse IgG (Cappel, West Chester, PA), conjugated with FITC for 30 min at 4 °C, and resuspended in 0.5 ml of PBS containing 10% FBS. Fluorescein-labeled cells (10 $\times$ 10$^4$ cells/sample) were analyzed with the flow cytofluorometer (FACScan, BD Biosciences). Cells incubated with FITC-conjugated antibody alone were used as negative controls.

**Western Blot Analysis**—ECs were lysed with a buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). The total cell lysate (100 $\mu$g of protein) was separated by SDS-PAGE (12% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-$\mu$m pore size). The membrane was then incubated with the designated antibodies. Immunodetection was performed by using the Western Light chemiluminescent detection system (Applied Biosystems, Foster City, CA).

**Electrophoretic Mobility Shift Assay (EMSA)**—ECs were collected by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in cold buffer A (containing 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF) for 15 min. The cells were lysed by adding Nonidet P-40 to a final concentration of 0.4% and then centrifuged at 6000 rpm to obtain pellets of nuclei. The nuclear pellets were resuspended in cold buffer B (containing 20 mM HEPES, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 400 mM NaCl), vigorously agitated, and then centrifuged. The supernatant containing the nuclear proteins was used for the EMSA or stored at −70 °C until used. Double-stranded consensus oligonucleotides (5'-AGTGGAGGGGACTTTCCCAGGC-3'; Promega Corp., Madison, WI) containing the DNA-binding site for NF-κB were end-labeled with [γ-$^{32}$P]ATP. The extracted nuclear proteins (10 $\mu$g) were incubated with 0.1 ng of $^{32}$P-labeled DNA for 15 min at room temperature in 25 $\mu$l of binding buffer containing 1 $\mu$g of poly(dI-dC). In the antibody supershift assay, an antibody against NF-κB subunit p65 (1 $\mu$g) was incubated with the mixture for 10 min at room temperature followed by the addition of the labeled probe. The mixtures were electrophoresed on 6% nondenaturing polyacrylamide gels. The gels were dried and imaged by autoradiography.

**Surface Plasmon Resonance (SPR) Assay**—The experiments were carried out on a Biacore 3000 instrument (GE Healthcare). All experiments were performed at 25 °C. For binding analysis, biotinylated heparin was immobilized on streptavidin-coated SPR biosensor chip (SA chip; GE Healthcare) at a flow rate of 5 $\mu$l/min. The streptavidin-coated surface without immobilized heparin was used as a reference. For competitive studies, natrin (5 $\mu$m) preincubated with different GAGs (i.e. heparin, HS, dermatan sulfate (DS), chondroitin sulfate A and C, keratin sulfate, and hyaluronic acid) and different lengths of heparin (i.e. disaccharide (dp2), tetrasaccharide (dp4), hexasaccharide (dp6), octasaccharide (dp8), decasaccharide (dp10), dodecasaccharide (dp12), and tetradecasaccharide (dp14)) were injected over the heparin surfaces at a flow rate of 40 $\mu$l/min. Surfaces were regenerated by injection of 0.02% (w/v) SDS and 1.0 mM NaCl. The resulting sensorgrams were fitted using Biaevaluation 3.2 software (GE Healthcare).

**Fluorescence and Circular Dichroism (CD) Measurements of Zinc Binding**—CD spectra for natrin were recorded on a spectropolarimeter (AVIV 62A DS, Lakewood, NJ) at 25 °C as previously described (28). Briefly, CD spectra were acquired using quartz cells (path length 0.1 cm) at a protein concentration of 10 $\mu$m in 10 mM Tris buffer, pH 7.4, and 1 mM ZnCl$_2$. Spectra, averaged from three trials, were obtained on scanning from 260 to 190 nm. For measurements of intrinsic fluorescence intensity of tryptophan from natrin, the excitation and emission wavelengths were set at 280 and 340 nm, respectively, on a fluorescence spectrometer (HITACHI F4500, Japan). To determine the dissociation constant ($K_d$), we performed computer curve-fitting based on the equation $K_d = [P][Zn]/[PZn]$, where [P], [Zn], and [PZn] represent the concentrations of natrin, cation, and natrin/cation complex, respectively. Complete (100%) binding were assumed to occur when the fluorescence intensity reached a plateau.

**Crystal Structure Determination and Refinement**—The crystallization of native natrin and data collection were performed as previously reported (26). X-ray diffraction data to resolution 1.58 Å from a frozen native crystal were collected at 110 K using a detector (Quantum 4R CCD, ADSC) at wavelength 1.0 Å on the Taiwan contract beamline BL12B2 at SPring-8 in Japan. The natrin structure (PDB ID 1XTA) was determined with the sulfur single-wavelength anomalous dispersion (S-SAD) method. The S-SAD data were collected to resolution 2.6 Å with a long wavelength 1.743 Å at 110 K at the beamline BL17B2 equipped with a detector (Q210 CCD, ADSC) at the National Synchrotron Radiation Research Center (NSRRC) in Hsinchu, Taiwan. The anomalous-difference Patterson map at 3.5 Å showed four disulfide sites that were directed to the program SOLVE 2.06 (29), yielding an initial figure of merit (FOM) of 0.37–3.5 Å resolution; the phases were then improved with a FOM 0.51 using RESOLVE 30. Additional two disulfide positions were found in an anomalous difference Fourier map, and eventually six disulfide features were input to SOLVE 2.06 to generate the initial phases with a FOM 0.41 at 3.5 Å, and phases were improved with non-crystallographic 2-fold symmetry averaging in RESOLVE with a final FOM 0.71 at 2.6 Å. An initial structure with ~71% complete model in an asymmetric unit was automatically traced into electron density maps, and the remaining model was manually built with O (31). After rigid-body refinement and simulated annealing with CNS (32), several rounds of model building with O and refinement with CNS were performed to improve the quality and completeness of structure. At this stage all data to resolution 1.58 Å were included for further refinement. The refinement then proceeded through several cycles of simulated annealing and individual B-factor refinement and, finally, the PICKWATER subroutine from CNS to define peaks in difference maps (3σ cut-off
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FIGURE 1. Natrin induces human monocytic U937 cell adhesion to ECs. ECs were kept as controls (CL) or incubated with natrin at different concentrations of 50, 100, and 1000 nm for 6 h. ECs treated with TNF-\(\alpha\) (100 units/ml) were used as positive controls. The numbers of adherent U937 cells were measured as described under “Experimental Procedures.” Data are the mean ± S.E. relative to adhesion to untreated control cells from triplicate independent experiments. * p < 0.05 versus untreated control cells.

level) to locate 268 water molecules automatically. The refinement converged to a final R-factor value 22.4% (\(R_{free} = 25.1\%\)) for all data at a resolution 1.58 Å.

The natrin-zinc complex was crystallized using the hanging-drop vapor-diffusion method at 291 K. 2-\(\mu\)l drops of purified protein (8 mg/ml) in 0.1 M HEPES buffer, pH 7.4, were mixed with equal volumes of the reservoir solution containing 20% (w/v) PEG 8000 and equilibrated against 0.5 ml of reservoir solution in a 24-well ADX plate (Hampton Research, Aliso Viejo, CA). X-ray diffraction data were collected at 110 K using the synchrotron radiation on the beamline BL13B at National Synchrotron Radiation Research Center. The structure of natrin-zinc complex was determined by molecular replacement as implemented in CCP4 (33) using the \(\text{N. atra} \) natrin monomer structure (PDB code 1XTA) as a search model. Two natrin molecules were located in the asymmetric unit after rotation and translation searches. Several cyclic model refinements were performed in accordance with density maps of \(2F_o - F_c \) and \(F_o - F_c \) coefficients with CNS Version 1.2 (32) and the Maximum-Likelihood method using REFMAC5 (34). After rigid-body refinement and simulated annealing with CNS, several rounds of model building with \(O\) and refinement with CNS were carried out to improve structure quality. The B-factor refinement and PICKWATER subroutine from CNS were used to locate 193 water molecules. The refinement converged to a final R-factor value of 18.2% (\(R_{free} = 24.1\%\)) for all data at a resolution of 2.7 Å. The crystallographic data and refinement statistics are shown in Table 1.

Coordinates—Atomic coordinates for the crystal structures of natrin in the absence or presence of Zn\(^{2+}\) binding have been deposited in the Protein Data Bank (accession code 1XTA and 3MZ8, respectively).

Statistical Analysis—Results are expressed as the mean ± S.E. from three-four independent experiments. Statistical analysis was performed by Student’s \(t\) test for two groups of data and one-way analysis of variance followed by Scheffe’s test for multiple comparisons. * p < 0.05 was considered statistically significant.

RESULTS

Natrin Induces Monocytic Cell Adhesion to ECs by Inducing Their Expression of Adhesion Molecules—To investigate the possible inflammatory effects of natrin on ECs, we first investi-
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**FIGURE 2.** Natrin induces ICAM-1, VCAM-1, and E-selectin expressions in ECs. A, total mRNAs of ECs were isolated, and real-time PCR was performed using specific primers to human ICAM-1, VCAM-1, and E-selectin, as described under “Experimental Procedures.” ECs were kept as controls (CL) or incubated with natrin (100 nm) for 1, 3, and 6 h. Data are the mean ± S.E. and are presented as fold relative to controls from triplicate independent experiments. *p < 0.05 versus untreated control cells. B, ECs were incubated with natrin at concentrations of 50, 100, and 1000 nm for 3 h. C, ECs were treated with natrin at a concentration of 1000 nm for 3 h. The mean fluorescence intensities of ICAM-1, VCAM-1, and E-selectin were 291, 894, and 419 compared with 197, 361, and 115 for untreated control cells (CL), respectively. ECs incubated only with FITC-conjugated antibody were used as blank controls (negative controls). 30,000 cells per experimental set were analyzed.

To examine the effect of natrin on the mRNA stability of ICAM-1, VCAM-1, and E-selectin, ECs were initially treated with natrin for 3 h and followed by actinomycin D (10 μg/ml) treatment for another 4 h, then the ICAM-1, VCAM-1, and E-selectin mRNA levels were quantified at 1, 2, and 4 h later. The mRNA transcription levels were initially down-regulated within 1 h after actinomycin D treatment; however, the expressions became stabilized from 1–4 h (Fig. 3B), indicating that natrin-induced ICAM-1, VCAM-1, and E-selectin expressions are stable and regulated at the transcriptional level in ECs. The results that natrin regulations of adhesion molecule expression in ECs are transcriptional events were confirmed by the transfections of ECs with the promoter constructs containing the promoter regions of ICAM-1 (~850 bp) or E-selectin (~540) and the reporter gene luciferase (40), which showed that ECs treated with natrin significantly increase the promoter activities of these adhesion molecules in ECs compared with static control cells (data not shown).

**Natrin Activates ERK, JNK, and p38 MAPK in ECs**—Despite the demonstration of functionality of natrin in up-regulating the EC expressions of ICAM-1, VCAM-1, and E-selectin, the major signaling pathways that regulate natrin inductions of these adhesion molecules remain to be explored. Because MAPKs are involved in virtually all aspects of immune responses (41), we first investigated the effect of natrin on the activations of ERK, JNK, and p38 MAPK in ECs. ECs treated with TNF-α (100 units/ml) for 30 min that induced MAPK phosphorylations were used as positive controls. Although natrin (100 nm) induced a rapid increase in the phosphorylations for ERK and p38 MAPK within 10 min, it took about 30 min for JNK (Fig. 4A). The increased levels of phosphorylations were maintained at the same levels for ERK up to 24 h; however, only a transient phosphorylation was observed for JNK and p38 MAPK upon natrin treatment up to 24 h. Our results indicate that, upon natrin treatment to the EC membrane surface, cell signaling of MAPK pathways can be activated, which may be involved in regulating further transcriptional activities.

**Increased NF-κB Binding Activity in the Nucleus of ECs by Natrin**—Because the promoter regions of ICAM-1, VCAM-1, and E-selectin genes are known to contain the NF-κB-binding sites for gene expression (42, 43), we investigated whether natrin can regulate the NF-κB binding activity in the nucleus of ECs. This was done by using the nuclear protein extracts from ECs treated with natrin (100 nm) for 30 min and 1, 3, 6, and 24 h. By incubating protein extracts from EC nuclei with the end-labeled oligonucleotides corresponding to the NF-κB-binding sites, the EMSA results showed that treatment of ECs with natrin causes an increase in NF-κB binding activity in the EC nuclei around 3 h after stimulation (Fig. 4B). The increased levels of NF-κB binding activity were then reduced to the basal levels around 6 h of stimulation. The specificity of this binding for NF-κB was confirmed by the super-shifting in gel mobility of the NF-κB-oligonucleotide complex after preincubation of nuclear proteins with an antibody against p65.
MAPKs and NF-κB Are Involved in Natrin-induced Adhesion Molecule Expression in ECs—Given our findings that natrin induces the expressions of ICAM-1, VCAM-1, and E-selectin and activations of MAPKs and NF-κB in ECs, we investigated whether MAPKs and NF-κB are involved in natrin-induced EC ICAM-1, VCAM-1, and E-selectin expressions. ECs were transfected with ERK-, JNK-, and p38-specific siRNA (compared with control siRNA; 40 nM for each) (Fig. 5A), which could inhibit their respective protein expression by 70% (Fig. 5B), and then treated with natrin (100 nM) for 3 h. The results showed that the natrin-induced EC expression of these adhesion molecules is inhibited by transfecting ECs with ERK-, JNK-, and p38-specific siRNA.

The involvement of MAPKs in natrin inductions of ICAM-1, VCAM-1, and E-selectin in ECs was confirmed by the inhibitions in natrin induction of these adhesion molecules by pretreating ECs with specific inhibitors of ERK (PD98059; 30 μM), JNK (SP600125; 20 μM), and p38 MAPK (SB203580; 10 μM) (Fig. 5C). The EC expressions of ICAM-1, VCAM-1, and E-selectin induced by natrin were also inhibited by pretreating the cells with NF-κB inhibitor lactacystin (20 μM), indicating that NF-κB is also involved in natrin-induced ICAM-1, VCAM-1, and E-selectin expressions in ECs (Fig. 5C).

Role of Cell Surface HS in Modulating EC Responses to Natrin—HS is known to play important roles in modulating many
inflammatory processes, including cytokine-induced cell signaling events (44). It is, therefore, interesting to see whether HS on the EC surfaces could play a role in mediating natrin induction of adhesion molecule expression in ECs.

ECs were treated with heparinase III (1 units/ml) to selectively cleave HS from extracellular matrix and then stimulated with natrin. Pretreatment of ECs with heparinase III resulted in inhibitions in natrin inductions of ICAM-1, VCAM-1, and E-selectin mRNAs (Fig. 6A) and proteins (Fig. 6B) and activations of different MAPKs (Fig. 6C) and binding activity of NF-κB (Fig. 6D). These results suggest that HS-containing molecules on membrane surfaces, e.g. heparan sulfate proteoglycans (HSPGs) of syndecan or glypican, may play important roles in modulating natrin-induced MAPK and NF-κB signaling and ICAM-1, VCAM-1, and E-selectin expressions in ECs.

The sensitivity of natrin-induced proinflammatory response to cell surface HS implies that natrin should bind to HS with a certain degree of specificity. We tested the binding specificity of natrin toward different GAGs by using SPR binding assay. We first allowed natrin bound to different types of GAGs and then applied it to the heparin chip surface to carry out competition experiment. As shown in Fig. 7A, the binding specificity of natrin toward heparin, HS, and DS was found to be more effective than that toward the other GAGs, including chondroitin sulfate A and C, keratan sulfate, and hyaluronic acid. Thus, both results on biochemical and cell signaling studies support the notion that binding of natrin to HS on EC surfaces plays important roles in modulating natrin-induced signaling events and adhesion molecule expression in ECs.

**Effects of Zn²⁺**—Zn²⁺ ion is critical for the functional and structural integrity of cells and contributes to a number of important processes including gene expression. Recent studies demonstrated that Zn²⁺ ion might play important roles in modulating natrin-induced signaling events and adhesion molecule expression in ECs. Whether Zn²⁺ binding affects the heparin binding activity by performing SPR experiment in the presence and absence of Zn²⁺. As shown in Fig. 7B, by plotting the response of natrin binding to heparin surface as a function of natrin concentration.

![FIGURE 6. Roles of heparinase III in natrin-induced MAPK and NF-κB signaling activations and adhesion molecule expression in ECs.](image-url)
ranging from 0.5 to 10 μM, the results clearly indicated that the presence of Zn²⁺ indeed enhances the binding of natrin to heparin surface.

The binding isotherms revealed by the SPR experiments exhibited a sigmoid binding behavior within micromolar-concentration range of natrin in the absence of Zn²⁺, suggesting that a cooperative binding behavior might occur. More interestingly, although natrin binding to heparin surface can be reversed after extensive wash, the presence of Zn²⁺ retains natrin binding to the heparin surface in a significant amount (binding sensograms inset in Fig. 7B).

We tested the heparin-binding site by applying heparin-derived mimetics with different carbohydrate chain length prepared by depolymerization (dp) method for competition experiment. Indeed, as shown in Fig. 7C, Zn²⁺ was seen to enhance the retention ability of long chain heparin binding to natrin. Specifically, more heparin with chain lengths between 6 and 14 carbohydrate moieties was required to compete out natrin binding to the heparin surface in the presence of Zn²⁺. Our results indicated that there may be more than one heparin-binding site in natrin when its binds to heparin surface in the presence of Zn²⁺.

Moreover, we investigated whether Zn²⁺ binding to natrin could affect the natrin-induced expressions of ICAM-1, VCAM-1, and E-selectin in ECs. ECs were stimulated by natrin for 3 h in the presence of Zn²⁺ ion (0.3, 0.5, and 1 μM), and their ICAM-1, VCAM-1, and E-selectin mRNA expression was examined by real-time PCR. As shown in Fig. 8A, the addition of Zn²⁺ ion at concentration of 1 μM can significantly enhance E-selectin expression, whereas a more prominent effect was detectable for ICAM-1 and VCAM-1 at 0.5 μM. These Zn²⁺-
mediated augmentations of natrin inductions of ICAM-1, VCAM-1, and E-selectin were confirmed by flow cytometric analysis, which showed that the addition of Zn$^{2+}$ ion also enhances EC surface protein expression of these adhesion molecules (Fig. 8B).

Two Zn$^{2+}$-binding sites with distinct heparin binding sensitivity — Although snake CRISPs are known to bind Zn$^{2+}$, there has been no report to show Zn$^{2+}$ binding strength of natrin. The total concentration of Zn$^{2+}$ ion in eukaryotic cells is $\sim 200 \, \mu M$ (46). In normal physiological conditions, most of the Zn$^{2+}$ ion in the cells are conjugated with proteins, with free Zn$^{2+}$ ion at a concentration of 100 pm or even lower in the cytoplasm (47, 48). However, Zn$^{2+}$ ion could be released from necrosis cells, resulting in much higher local concentration of Zn$^{2+}$ up to a submillimolar range outside the cells. We then determined the Zn$^{2+}$ binding constant by monitoring the intrinsic tryptophan fluorescence of natrin as a function of Zn$^{2+}$ concentration. As shown in Fig. 9, A and B, two apparent binding constants with $K_d$ values of $\sim 2$ and $\sim 490 \, \mu M$ were determined. Interestingly, whereas no effect on both strong and weak binding curves could be observed in the presence of short chain heparin-derived mimetics (dp6), the long chain heparin-derived mimetics (dp12) affect the weak binding but not the strong binding. This is consistent with the SPR experiments that high concentration of Zn$^{2+}$ could enhance the binding of natrin to the long chain, but not the short chain, heparin.

Zn$^{2+}$-induced local conformational change of natrin — Because Zn$^{2+}$ binding has been observed to trigger the motion of CRD domain against PR-1 domain in the crystal structure (20), we examined this effect based on the CD spectroscopic study of natrin in solution. As shown in Fig. 9C, the CD spectra obtained from natrin were virtually the same in the presence and absence of Zn$^{2+}$ at a concentration of 1 mM, indicating that there is no significant conformational change at least from the secondary structural point of view. Despite the negative effect, it should be pointed out that CD is not a particularly good method to look for conformational changes when the major movements are in loops or movement of two domains relative to each other.

To determine the Zn$^{2+}$-binding sites, we diffused ZnCl$_2$ (with final concentration of Zn$^{2+}$ at 0.15 mM) into the solution containing natrin crystals and solved its three-dimensional structure at resolution 2.54 Å (Table 1). As shown in Fig. 10A, the asymmetric unit contains two natrin molecules with a non-crystallographic 2-fold symmetry. These two natrin molecules contain a surface area buried at an interface $\sim 486 \, \AA^2$.

Only one Zn$^{2+}$ per monomer was found to bind to the N$^2$-nitrogen of His-60 (Fig. 10B). By binding to His-60, it actually triggered a local conformational change to break its pre-existing hydrogen bond with the main chain of Ala-138 of the neighboring monomer, as observed in our natrin structure solved at 1.58 Å resolution. Because the same Zn$^{2+}$-binding site has also been reported in pseudocin as chelated by His-115 and an additional water molecule, we assign it as the strong binding site detected by our fluorescence measurement.

**DISCUSSION**

In the present study we demonstrated for the first time that CRISPs may function as a proinflammatory modulator via a novel Zn$^{2+}$- and HS-dependent mechanism. Apparently, a snake CRISP, *i.e.* natrin, can induce adhesion molecule ICAM-1, VCAM-1, and E-selectin expressions in ECs via the MAPKs- and NF-$\kappa$B-dependent transcriptional regulation. These natrin inductions of adhesion molecule expression in ECs were mediated by HS on the EC surface and can be augmented by the addition of Zn$^{2+}$. Our SPR experiments showed that Zn$^{2+}$ can enhance the binding of natrin to long chain, but
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Not short chain, heparin. Without further structural work, it is difficult to predict how Zn\(^{2+}\) binding could enhance natrin binding to the long chain heparin and then the enhanced expression of adhesion molecules in ECs. However, our three-dimensional structure of natrin in the presence of Zn\(^{2+}\) provides us a framework to discuss about the possible models.

We have previously reported a preliminary x-ray crystallographic analysis of natrin crystals with space group C222\(_1\) (26). Given the published three-dimensional structures of natrin, which showed that pseudochetoxin and pseudotetran are all determined in the crystals with different space group of P2\(_1\)2\(_1\)2\(_1\), it would be interesting to investigate whether 1) there is any conformational difference between the two crystal forms and 2) there are local conformational changes caused by Zn\(^{2+}\) binding.

Analysis of the three-dimensional structure of natrin in our present study (Fig. 10A) showed the occurrence of two molecules in the asymmetric unit related by proper 2-fold non-crystallographic symmetry. However, this symmetric structure does not imply dimer formation in solution, as these two molecules appear to have very little contact area, which is not likely to support dimer formation on its own. By using the Protein Interfaces, Surfaces, and Assemblies (PISA) server to analyze the structure of natrin, our recent study showed that the protein interfaces do not reveal specific interactions that could result in the formation of stable quaternary structures. Our recent study using size-exclusion chromatography to analyze the structure in solution in the presence of the dp12 further showed that no dimer formation in solution is observed. Thus, there has been no evidence to show dimer formation of natrin in solution in the presence of zinc or the dp12.

**Potential Heparin-binding Site**—We have shown that heparin binding does not affect the strong Zn\(^{2+}\)-binding site located near the concave side of natrin molecule. It provides us a clue to pin down the potential heparin-binding site based on the available positive charge domain in the three-dimensional structure of natrin. For instance, the positively charged residues proposed in pseudochetoxin for its interaction with ion channel turret are mainly located at the concave side and, therefore, should not play a role in heparin binding.

The surface electrostatic potential was calculated based on the determined three-dimensional structure and exhibited one major positively charged cluster (Arg-10, Arg-11, Lys-12, Lys-
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13, Lys-14, and Lys-16) located at the water-accessible helix I/III surface of the N-terminal PR-1 domain. Although not strictly conserved, some positively charged residues, e.g. Arg-10, Arg-11, Lys-12, Lys-16, and Lys-207, were also found in other snake venom CRISP. As shown in the model (Fig. 10C), the site can easily host heparin with chain length of six carbohydrate moieties to account for the relatively strong binding of dp6 against other shorter heparin derived mimetics observed by the SPR competition experiment in the absence of Zn$^{2+}$. It will be interesting to test the predicted HS-binding site experimentally in the future by either mutational analysis using a heterologous expression system or by trying to grow crystals in the presence and absence of HS and/or zinc. The later experiment is particularly interesting to see whether global conformational changes of natrin, such as movements of the CRD domain toward the PR-1 domain, also occur as a result of HS or Zn$^{2+}$ binding.

The SPR results (Fig. 7B) suggest that Zn$^{2+}$ creates an additional heparin-binding site in monomeric natrin or that Zn$^{2+}$ enhances the dimerization and/or oligomerization of natrin in the presence of heparin. These results are in agreement with the results of previous studies, which showed that heparin binding to many cytokines or chemokines induces their oligomerization for binding to cell surface receptor (49, 50).

Natrin is the only protein among CRISP family to possess a Ser–His–Glu catalytic triad because its amino acid residue at position 76 is Ser instead of Asn, as in all other CRISP homologues. As also shown in Fig. 10B, the side chain of Ser-76 is located near His-115, which is structurally cis-oriented by the side chain of Glu-96 by hydrogen bonds to both an imidazole ring and the main chain, as in the case of other typical Ser–His–Asp catalytic triad. The same side chain orientation was also observed in previously reported structure of natrin (13). The side chain orientation of Ser-76 does not allow its Oy to coordinate to His-115, but a simple rotation by 90 degree appears to be suitable for such an interaction to play a catalytic role. Unfortunately, we tested several substrates such as BSA, neuropetin, Tex31 substrate, and kenetensin but failed to find any detectable activity. One possible explanation is that the identified strong Zn$^{2+}$-binding site is also a good cation binding site to prevent the access of other possible peptide substrates for its enzymatic activity.

Potential Protein Receptor—Our present results showed that natrin-induced ICAM-1, VCAM-1, and E-selectin expressions are regulated at the transcriptional level through the MAPKs and NF-κB signaling pathways. However, the potential receptors responsible for these cell signaling and gene expressions remain to be determined. It is interesting to note that many members of TNF receptor superfamily and the interleukin-1 receptor, most of them also belonging to immunoglobulin superfamily, could mediate signal transduction induced by natrin as we reported here (51, 52). HSPGs, which are negatively charged proteins ubiquitously expressed on the cell surfaces and in the extracellular matrix, have been shown to interact with many different proteins such as cytokines, chemokines, and cell adhesion molecules to play important roles in leukocyte-endothelium trafficking (53). HSPGs on ECs have been shown to be crucial for the tethering, rolling, and adhesion of leukocytes to ECs and the subsequent transmigration of leukocytes (54, 55). The HSPGs on the surface of cells modulates the actions of a wide variety of HS-binding proteins. They act as co-receptors with signaling receptors and directly as endocytosis receptors. As co-receptors, they potentiate the action of low concentrations of ligands by enhancing the formation of ligand-receptor complexes. There is increasing evidence that HSPGs on the EC surface can modulate the inflammatory responses of ECs to various stimuli (56). Moreover, HSPGs on the cell surfaces have been shown to be involved in the modulation of signal transduction and gene expression in cells (57, 58).

The expression of CRISP-3 cDNA in B cell, neutrophils, and eosinophils is suggestive of a role in the innate immune system. CRISP-3 is up-regulated in a number of pathologies including prostate cancer (59, 60), chronic pancreatitis (61), Sjögren’s syndrome (62), and ectopic pregnancy (63). The present study demonstrated for the first time that natrin from the venoms of N. atra induces proinflammatory responses of ECs by inducing their adhesion molecule expression and, hence, mononuclear cell adhesion. This natrin induction of adhesion molecules was mediated by HS through activations of MAPKs and NF-κB and was augmented by the addition of zinc ion. Our findings not only provide new insights into the mechanisms of snake venom-induced inflammation but also shed light on the functional role of other CRISPs existed in different tissues and organisms, such as salivary glands, pancreas, prostate, and granules of neutrophils. A better understanding of the mechanisms underlying the proinflammatory role of CRISPs may identify new molecular or cellular targets that are responsible for snakebite envenomations and CRISP-related inflammatory diseases, thereby facilitating the development of new therapeutic strategies for these conditions and diseases.

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