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Probing the Interaction between cHAVc3 Peptide and the EC1 Domain of E-cadherin using NMR and Molecular Dynamics Simulations

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Abstract

The goal of this work is to probe the interaction between cyclic cHAVc3 peptide and the EC1 domain of human E-cadherin protein. Cyclic cHAVc3 peptide (cyclo(1,6)Ac-CSHAVC-NH₂) binds to the EC1 domain as shown by chemical shift perturbations in the 2D ¹H,-¹⁵N-HSQC NMR spectrum. The molecular dynamics (MD) simulations of the EC1 domain showed folding of the C-terminal tail region into the main head region of the EC1 domain. For cHAVc3 peptide, replica exchange molecular dynamics (REMD) simulations generated five structural clusters of cHAVc3 peptide. Representative structures of cHAVc3 and the EC1 structure from MD simulations were used in molecular docking experiments with NMR-constraints to determine the binding site of the peptide on EC1. The results suggest that cHAVc3 binds to EC1 around residues Y36, S37, I38, I53, F77, S78, H79, and I94. The dissociation constants (K_d values) of cHAVc3 peptide to EC1 were estimated using the NMR chemical shifts data and the estimated K_ds are in the range of 0.5 × 10⁻⁵ to 7.0 × 10⁻⁵ M.

Keywords

blood-brain barrier; Cyclic HAV peptide; E-cadherin binding; NMR; molecular dynamics

Introduction

Brain diseases are difficult to treat because drug molecules cannot be easily transported from the bloodstream to the brain. The endothelial microvessels can deliver nutrients to the brain; however, these microvessels also comprise the blood-brain barrier (BBB), which is a selective barricade that prevents unwanted molecules from entering the brain from the

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bloodstream. Many drug and diagnostic molecules cannot cross the BBB; therefore, there is a need to develop new methods for delivery of drugs and diagnostic molecules across the BBB to treat diseases of the central nervous system (CNS). Only very few methods, such as using brain osmotic solutions, have been successful in improving brain delivery in a non-invasive manner (Dean *et al.*, 1999; Neuwelt *et al.*, 1979). In that approach, the hyperosmolarity of the osmotic solution, that is used to deliver the drug, modulates the intercellular junctions of the BBB by shrinking the vascular endothelial cells. Because the current choices are so limited, any new alternative and selective methods to deliver therapeutic and diagnostic molecules to the brain would help patients with brain diseases. Our group and others have developed peptides to enhance delivery of molecules to the brain by modulating protein-protein interactions (i.e., those of occludins, claudins, cadherins) in the intercellular junctions of the BBB (Kiptoo *et al.*, 2011; Laksitorini *et al.*, 2014; Laksitorini *et al.*, 2015; On *et al.*, 2014; Zwanziger *et al.*, 2012).

The HAV6 peptide (Ac-SHAVSS-NH₂) derived originally from the first domain (EC1) of E-cadherin enhances the brain delivery of a paracellular marker molecule (i.e., ¹⁴C-mannitol) across *in vitro* cell culture monolayers of Madin-Darby canine kidney cells (MDCK) (Makagiansar *et al.*, 2001). The HAV6 peptide can also increase the brain delivery of ¹⁴C-mannitol and ³H-daunomycin in the *in-situ* rat brain perfusion model as well as Gd-DTPA, R800 near IR (NIR) dye, and R800cw-polyethylene glycol (25 kDa) in Balb/c mice *in vivo* upon intravenous (i.v.) administration (Kiptoo *et al.*, 2011; On *et al.*, 2014). Recently, the cyclic cHAVc3 peptide (cyclo-(1,6)Ac-CSHAVC-NH₂) was shown to have better activity than linear HAV4 peptide (Ac-SHAVAS-NH₂) in modulating the intercellular junctions of MDCK cell monolayers and in enhancing *in vivo* brain delivery of Gd-DTPA in Balb/c mice (Alaofi *et al.*, 2015). In addition, cyclic cHAVc3 has better plasma stability than the linear HAV4 peptide (Alaofi *et al.*, 2015).

The proposed hypothesis for the general mechanism of action of synthetic HAV peptides is that they modulate the BBB via binding to the EC repeat domain(s) of E-cadherin; as a result, the peptides disrupt cadherin-cadherin interactions in the intercellular junctions of the BBB. The disruption of cadherin interactions increases pore sizes in the intercellular junctions of the BBB and enhances the penetration of molecules through the BBB. Because the EC1 domain is known to be one of the most important repeat domains in cadherins, we have proposed that HAV peptides bind to the EC1 domain to modulate E-cadherin homophilic interactions (Lutz *et al.*, 1997; Makagiansar *et al.*, 2002; Zheng *et al.*, 2006). The results from this study will provide guidance in designing better BBB modulators for improving delivery of molecules into the brain.

In this study, the binding properties of the cHAVc3 peptide to the EC1 domain of human E-cadherin were evaluated using ¹H-, ¹⁵N-heteronuclear single quantum correlation (HSQC) NMR spectroscopy, molecular dynamics simulation, and molecular docking experiments (Stark and Powers, 2012; Williamson, 2013). The amino acid assignments in the EC1 domain were completed previously using 3D NMR spectroscopy (Prasasty *et al.*, 2015). The EC1 domain was titrated with the peptide, and the NMR chemical shift perturbations (CSP) for the affected residues were monitored to determine the peptide-binding site on the EC1 domain (Williamson, 2013). CSP has also been used to estimate the dissociation constant

(K_d) of cHAVc3 to the EC1 domain (Fielding, 2003). The conformations of cHAVc3 peptide and the EC1 domain were evaluated with molecular dynamics simulations, and the potential peptide-binding site was determined in molecular docking experiments using residues that were affected by the peptide titration.

Materials and Methods

Peptide Synthesis and Cyclization

The linear precursor of cyclic cHAVc3 peptide was synthesized using a solid-phase method with Fmoc chemistry in a peptide synthesizer (Pioneer, PerSeptive Biosystems). The linear peptide was cleaved from the resin and purified by reversed-phase HPLC using a C18 column. The cyclization reaction was done by bubbling air into a dilute solution of peptide in sodium bicarbonate buffer at pH 8.5 as previously described (Alaofi *et al.*, 2015). The peptide was purified with semi-preparative HPLC using a C18 column. Mass spectrometry was used to confirm the molecular weight of the cHAVc3 peptide (Kiptoo *et al.*, 2011).

Protein Expression and Purification

Expression and purification of the ¹⁵N-labeled EC1 domain protein with 138 residues were accomplished using our previously published protocol (Prasasty *et al.*, 2015). The protein contains 110 residues from the EC1 domain and 28 residues at the C-terminus from the sequence of the EC2 domain. The N-terminus was connected to Streptag I sequence (WSHPQFEK) via a Factor Xa sequence (IEGR) for affinity purification using a Strep Tactin II column with size 5.0 × 0.6 cm (GE Healthcare Life Sciences, Pittsburgh, PA). The cDNA of the 138 residues protein (BlueHeron, Bothell, WA) was subcloned into pASK-IBA6 plasmid (Genosys, Woodland, TX). The cDNA vector of 2 μL was added in sterile technique to 100 μL of BL21 cells for 30 min in ice. Then the mixture was placed in a water bath at 42°C for 30 s to allow cDNA to enter the cells; this was followed by 3 min in ice. 200 μL of SOC medium (1.55 g yeast, 0.25 mL of 1M KCL, 0.5 mL of 1 M MgCl₂, 0.5 mL of 1 M MgSO₄, 1 mL of 1 M glucose in ddH₂O) was added to the mixture. The mixture was shaken at 250 RPM and 37°C for 0.5–1 h. Two aliquots of the mixture (50 and 100 μL) were added to different agar plates followed by incubation for 12–16 h at 37°C. One or two colonies were selected from either a 50- or 100-μL-plate and added to 10–20 mL of LB medium (10 g NaCl, 10 g peptone, 5 g yeast up to 1 L of dd H₂O). Ampicillin solution (10–20 μL of 100 mg/mL) was added into the small-scale expression followed by overnight incubation at 37°C. The 20 mL LB medium was added to 1 L of 5× M9 medium (200 mL M9 minimal medium with ¹⁵N-NH₄Cl as a nitrogen source, 2 mL of 1 M MgSO₄, 10 mL of 40% glucose, 0.5 mL of 1 M CaCl₂, 1 mL of 1% FeSO₄, 1 mL of 1% thiamine, 1 mL of antibiotic 100 mg/mL) until the cells density was 0.6–0.8 at OD₆₀₀ nm. Cell growth was induced using 50 μL anhydrotetracycline (2 mg/mL, Promega Inc., Madison, WI) to express the ¹⁵N-labeled EC1 followed by incubation for 6 h at 30°C. The resulting cells were harvested by centrifugation at 10000–12000 RPM, and cell pellets were immediately stored at –80°C.

Prior to the NMR experiment, the ¹⁵N-labeled EC1-containing cells were taken from the –80°C freezer and subjected to lysing by sonication (Sonic Dismemberator) for 10 s every

min for 30 min at 65 Hz in binding (or B) buffer (100 mM Tris, 150 mM NaCl, 1 M EDTA, 1 mM DTT, 0.02% NaN₃, pH 8). Then, the lysed cells were centrifuged at 14000 RPM for 1 h at 4°C. The supernatant was passed through a 0.2 µm sterile filter and concentrated by centrifugation using Amicon Ultra tubes (EMD Millipore, Billerica, MA) with 10,000 or 3,000 Da molecular weight cutoff. The concentrated ¹⁵N-labeled EC1 was purified using a StrepTactin II column. The column was equilibrated and washed with B buffer before and after protein solution exposure to the column at 5 mL/min flow rate. Pure protein was then eluted from the column by elution buffer (B buffer + 2.5 mM desthiobiotin) at a low flow rate of 2 mL/min. Tris-Bis SDS-PAGE (4–12%) was used to check the purity of the protein fractions. The protein was concentrated to 0.18–0.3 mM for NMR studies with Amicon Ultra tubes by centrifugation, and the protein concentration was monitored with a UV spectrophotometer at 280 nm using molar absorptivity of 19480 M⁻¹ cm⁻¹ (Makagiansar *et al.*, 2002). The EC1 domain in elution buffer was dialyzed overnight with 20 mM phosphate buffer containing 5 mM DTT. Two hours before the NMR experiment, the DTT was removed from the EC1 buffer by dialysis using 20 mM phosphate buffer or by centrifuging with Amicon Ultra tubes with 3,000 Da molecular weight cutoff for 15–20 min. DTT was removed to prevent reduction of the disulfide bond in the cyclic cHAVc3 peptide during the titration procedure.

NMR Experiments for Titration of EC1 with cHAVc3 Peptide

Two-dimensional (2D) ¹H,-¹⁵N-HSQC NMR experiments were carried out using a Bruker Avance 800 MHz NMR spectrometer (Billerica, MA) equipped with TCI cryoprobes. The effects of titration of cHAVc3 peptide on the ¹⁵N-labeled EC1-domain were evaluated in buffer containing 10% D₂O. The HSQC NMR spectra were acquired with 10 or 16 scans, depending on the EC1 domain concentration. The peptide-to-protein ratios (cHAVc3: EC1) were from 0.3:1.0 to 2.5:1.0 for the low range and from 1:1 to 10:1 for the high range. The lower range titration ratios were used to estimate the K_d values of cHAVc3 peptide. For each titration point, 10 µL of cHAVc3 peptide was added to a sample of 500 µL of EC1. Then the data for each titration point were processed using NMRPipe program (nmrDraw and view2D.tcl). The chemical shift perturbation (CSP) and broadening of the ¹H or ¹⁵N peaks from the EC1 residues were used to determine the potential binding site and the dissociation constant (K_d) of the cHAVc3 peptide. The CSP was calculated using the following equations (Skinner and Laurence, 2010) :

$$\Delta F = [(\Delta\delta^1\text{H} * (800.234 \text{ Hz/ppm}))^2 + (\Delta\delta^{15}\text{N} * (81.096 \text{ Hz/ppm}))^2]^{1/2}, \quad (1)$$

where F represents fraction bound of protein and $\delta^1\text{H}$ or $\delta^{15}\text{N}$ represents the difference between free and bound protein for the EC1 domain for each NMR nucleus. Then, F was plotted against the peptide concentration, and the K_d was calculated using non-linear regression for best fitting (Fielding, 2003):

$$\Delta_{\text{obs}} = \Delta_{\text{max}} ((P_o + K_d + L_o) - [(P_o + K_d + L_o)^2 - 4 P_o L_o]^{1/2}) / 2 P_o, \quad (2)$$

where Δ_{obs} is the difference between bound and free protein, Δ_{max} is the penultimate titration, and P_0 and L_0 are total EC1 concentration and total cHAVc3 concentration, respectively.

Molecular Dynamics Simulations

MD Simulation of the EC1 Domain—Molecular dynamics (MD) simulations were carried out to evaluate the dynamic movements of the EC1 domain and its C-terminal tail region. The X-ray structure of the EC1 domain (PDB file 2O72 from the Protein Data Bank) was used as the starting structure (Parisini *et al.*, 2007). The EC1 structure was solvated in a cubic box of 35307 TIP3P water of size 10.27578 nm. To simulate the ionic strength to 0.15 M, 86 Cl^- and 98 Na^+ ions were added by replacing water molecules. Ionization states of titratable residues were assigned corresponding to pH 7.0 conditions. After energy minimization, a brief constrained MD simulation and 100 ps unconstrained equilibration of EC1 were done. The EC1 structure was then subjected to an unconstrained MD simulation for 100 ns at a constant temperature of 300 K. The final structure from the MD simulations was used as a model for the solution structure of the EC1 domain and for molecular docking simulations with cHAVc3 peptide. The simulations were done using GROMACS 4.6 program (Berendsen *et al.*, 1995; Hess *et al.*, 2008) with the CHARMM27 force field and nonbonded interactions. The long-range electrostatic interaction was computed by PME (not cutoff) with a distance of 1.3 nm for short-range non-bonded cutoff. The temperature of 300 K was maintained by the v-rescale method.

Replica Exchange Molecular Dynamics (REMD) Simulations for Linear HAVc3 and Cyclic cHAVc3

Simulation of the Linear HAVc3 Peptide as the Precursor of Cyclic cHAVc3—

The structures of cyclic peptide cHAVc3 used in this study were built the molecular dynamics simulations and energy minimizations. In this case, the linear HAVc3 peptide (Ac-CSHAVC- NH_2) was built using the CHARMM program (Brooks *et al.*, 2009) with acetylated N-terminus and amidated C-terminus. Both linear HAVc3 peptide without a disulfide bond and cyclic cHAVc3 peptide (cyclo(1,6)Ac-CSHAVC- NH_2) with a disulfide bond were simulated in a box of water (TIP3P) with eight ions (4 ions each for Cl^- and Na^+). REMD simulations were performed for HAVc3 with 15 replicas in a temperature range of 320–480 K over 50 ns. The rate for exchange attempts between neighboring replicas was every 1 ps; the observed exchange probabilities were in a range of 0.07–0.23 with cubic box size of 3.55578 nm and 1447 TIP3P waters. A GROMACS 4.5.6 program was used to simulate the peptide with CHARMM27 force field and non-bonded interactions as in the modeling of EC1. The structures sampled in the 320 K REMD were used to generate the cyclic peptide structure as described below.

Simulation of Cyclic cHAVc3—To allow HAVc3 peptide cyclization, a brief constrained MD was done for linear HAVc3 peptide structures from REMD simulations to bring two sulfur atoms to a distance of $<2 \text{ \AA}$. After forming a disulfide bond to make cyclic cHAVc3, a 50 ns REMD simulation was performed with conditions analogous to those of linear HAVc3 (see above), and exchange probabilities in the range of 0.1–0.18 were observed. Clustering was performed from the 5000 conformations sampled in the 320 K replica trajectory of

cHAVc3 using GROMOS algorithm (Daura *et al.*, 1999). The central structures from the five most populated clusters were selected to represent 95% of all sampled structures, and these structures were used for docking experiments with the EC1 domain of E-cadherin.

Docking of cHAVc3 to the EC1 Domain using HADDOCK

The coordinates for the human EC1 domain model were extracted from the final structure in the 100 ns EC1 MD simulation. Coordinates for five representative structures of cHAVc3 from REMD simulations were used for the docking experiments. They were docked to the EC1 domain structure from the 100 ns EC1 structure using HADDOCK server with the Easy Interface option. Both blind and NMR-constraints docking experiments were performed. The blind docking was carried out using all 138 residues of the EC1 as the potential binding site, while NMR-constraints docking experiments were done using the C9, Y36, I38, T63, F77, S78, I94, D103, and V112 residues as constraints for the potential binding site(s). The NMR constraint residues were selected from the chemical shift perturbation data of peptide titration experiments, and the HADDOCK clusters of docking were selected based on the highest HADDOCK scores (de Vries *et al.*, 2010; Wassenaar *et al.*, 2012).

Results

NMR Studies

Determination of the Binding Properties of cHAVc3 on the EC1 Domain—To identify the EC1 residues that showed CSP changes (i.e., interactions) when titrated with cHAVc3, the NMRPipe program was used to overlay the 2D ^1H - ^{15}N -HSQC NMR spectrum of the free and peptide-titrated EC1 domain (Figures 1A-B). The peptide titration generated different magnitudes of CSP (Figure 1C) and ^{15}N -shift changes (Figure 1D) on the EC1 domain, including C9, N12, L21, Y36, S37, I38, I52, I53, T63, R68, F77, S78, H79, S82, S83, I94, D103, T109, V112, and F113 residues. These residues were selected based on noticeable changes upon overlaying the NH crosspeaks of the free and titrated EC1 domain. The EC1 residues that have F values higher than the total average F value are considered to be residues that are involved in direct interactions with the peptide. The residues that had F values higher than the total average F were C9, Y36, I38, T63, F77, S78, I94, D103, and V112. The residues that had F values lower than the total average were N12, L21, S37, I52, I53, R68, H79, S82, S83, T109, and F113, and these were considered to be insignificant changes. These residues were assumed not to be involved in direct binding, and the chemical shift changes (or CSP) were due to conformational changes in the EC1 domain away from the primary peptide-binding site.

Determination of Dissociation Constant (K_d) of cHAVc3 to the EC1 domain—

The dissociation constant (K_d) of cHAVc3 peptide to the EC1 domain was determined using NMR data from the peptide titration experiments at the peptide-to-protein ratios of 0.3:1 to 2.5:1. The estimated K_d values were determined using obs and max using equation 2 on different residues (i.e., C9, R68, F77, S78, I94, D103, and V112) with F values (Equation 1) as represented by the F77, S78, and V112 residues in Figure 2. The K_d values of the R68 and I94 residues were measured using only the chemical shift changes in ^{15}N nucleus:

$\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{obs}} - \delta^{15}\text{N}_{\text{free}}$. The calculated K_d values from various residues ranged from 0.5×10^{-5} to 7.0×10^{-5} M (Table 1).

Molecular Modeling Studies of Binding between cHAVc3 and the EC1 domain

Molecular Dynamics for the EC1 domain—To evaluate the dynamic properties of the EC1 domain used in the NMR studies, the starting structure had 138 amino acid residues that are derived from the sequence of the EC1 domain (110 residues as the “head region”) plus 28 residues from the EC2 domain sequence of the “tail region.” Because the starting structure was derived from the X-ray structure of the EC1-EC2 domains of human E-cadherin, the tail region extended away from the head EC1 domain (Figure 3A). At 100 ns MD simulations, the tail region moved closer to and interacted with the head region (EC1, Figure 3B). The movement of the tail group was due to the absence of crystal packing in the starting X-ray structure. The starting structure and the final MD structure were overlaid using the C-alpha of the backbone residues 1 to 90. This comparison showed a root-mean-square-deviation (RMSD) of 0.746 Å (Figure 3C), indicating a stable head region. This study suggests that the EC1 structure in solution has the head region retained in its folded structure while the flexible tail region moves closer to the head region. The MD structure of EC1 domain showed large RMSD changes (> 10 Å) in the tail residues, which were observed from the residue 102 to the residue 138 (Figure 3D). The largest RMSD change of 76 Å was found at residue 120 (Figure 3D).

The dihedral angles (ϕ ; ψ) of D100, D103, and N104 from 0 ns (X-ray structure) to 100 ns (MD structure) were shown in Figure 4 and the ψ of D103 was dramatically changed during simulations (Figure 4A). On the other hand, the ϕ of D103 (Figure 4A) as well as the ϕ and ψ D100 and N104 (Figures 4A and B) did not change dramatically during the MD run. This suggests that the D103 residue behaves as a hinge residue to move the tail region closer to the head region of EC1 (Figure 3C).

Molecular Dynamics of cHAVc3

For docking studies, the structure of cyclic cHAVc3 was generated using CHARMM from the structure of linear HAVc3, which was subjected to 100 ps REMD simulations in a box of water molecules. The resulting structures of linear HAVc3 have an RMSD of 0.1 Å, which was close to that of the initial structure. Then, a 50 ns REMD was carried out on linear HAVc3 with increasing constraints to pull the two sulfur atoms of the Cys residues to a distance of less than 0.2 nm. A disulfide bond was formed between two sulfur atoms of the cysteine residues of linear HAVc3 to make the cyclic cHAVc3 structure, followed by energy minimization. The cyclic cHAVc3 structure was then subjected to 100 ps REMD simulations to give acceptable probability exchange. A 50 ns REMD simulation at 320 K was carried out to provide the top five structural clusters, and the central conformer from each cluster was used to represent each cluster (Figure 5). The phi and psi angles of each residue are shown in Table 2. The structures in both cluster 1 (Figure 5A) and cluster 2 (Figure 5B) contain type-I and type-VII β -turns, respectively, at His3-Ala4-Val5-Cys6, with a potential hydrogen bond from the NH of Cys6 to the C=O of His3. The structures in cluster 3 (Figure 5C) and cluster 5 (Figure 5E) have type-I and type-I' β -turns, respectively, at Cys1-Ser2-His3-Ala4, with a potential hydrogen bond from the NH of Ala4 to the C=O of Cys1. Finally, cluster 4 (Figure

4D) has structures with a γ -turn at Ala4-Val5-Cys6 with a potential hydrogen bond from the NH of Cys6 to the C=O of Ala4.

Molecular Docking of cHAVc3 to the EC1 Domain

The purpose of the docking studies was to propose a working model of binding between the cHAVc3 peptide and the EC1 domain. The potential binding site(s) was searched with the help of experimental data from peptide titration observed by 2D-NMR. The NMR-constraints docking experiment showed that the cHAVc3 structures with the highest HADDOCK scores were clustered around the F77 and S78 residues on the EC1 domain. This suggests that the region around F77 and S78 residues is the potential binding site for cyclic cHAVc3 peptide (Figures 6A–B). The second highest docking score was found in a cluster of molecules that were docked on the tail region around the V112 residue. Finally, the third highest docking score was in a cluster of molecules that docked on the region around the K105 and D103 residues. The NMR data from titration experiments showed that the highest F value was from the residue S78, supporting the suggestion that the potential binding site of cHAVc3 is around the S78 and F77 residues (Figure 6C). The detailed interaction between cHAVc3 on the EC1 around both F77 and S78 is shown in Figure 6D, where hydrophobic pockets bind to the Ala4 and the disulfide bond of the cHAVc3 peptide, respectively. For the blind docking experiments, the highest scoring clusters were found around the K105/D103 residues. Finally, the third cluster was found around the V112 residue region (data not shown).

Discussion

The NMR assignments of amino acids in the EC1 domain of human E-cadherin (h-E-cadherin) were done using the ^{15}N -labeled and $^{13}\text{C}/^{15}\text{N}$ -labeled EC1 domain (Prasasty *et al.*, 2015). These assignments were accomplished with the help of the NMR assignments of the EC1 domain of mouse E-cadherin (Overduin *et al.*, 1995; Prasasty *et al.*, 2015). The difference between the human EC1 (h-EC1) domain studied here and the mouse EC1 (m-EC1) domain is that the h-EC1 domain has an additional 28 amino acid residues from the EC2 domain at the C-terminal, which is called the tail region. The function of the tail region is to stabilize the conformation of the h-EC1 domain. Although the CD spectrum of the h-EC1 domain without the tail region showed a folded structure with a high beta-sheet secondary structure (Trivedi *et al.*, 2009; Trivedi *et al.*, 2012), the NMR spectrum of the EC1 domain did not show well-spread crosspeaks in the HSQC spectrum. This suggests that without this tail region the structure of the EC1 domain is folded but rather dynamic in nature. Many attempts to change the solution conditions (i.e., pH, ionic strengths, various buffers) to stabilize the EC1 domain without the tail region for NMR study were not successful (Trivedi *et al.*, 2009; Trivedi *et al.*, 2012). In contrast, the m-EC1 domain has a stable conformation in solution and produces well-spread crosspeaks in the NMR spectrum for structural determination (Overduin *et al.*, 1995).

Here, MD simulations were done to observe the dynamic behavior of the EC1 molecule, especially the role of the tail region in stabilizing the structure of EC1. The starting structure of the EC1 domain was from the X-ray structure of h-E-cadherin with the extended tail

region derived from the sequence of the EC2 domain and without interaction with the head region (Figure 3A). Because the NMR data showed that the tail region was important in stabilizing the solution structure of the head region of EC1, molecular dynamics simulations were carried out in an explicit water environment. The results showed that the tail region swings into and interacts with the head region of EC1 as indicated by the dramatic changes of the RMSD of the tail residues (Figures 3 B–C). This indicates that these interactions stabilize the structure of the EC1 domain in solution. After MD simulations, the RMSD was determined using alignments of C-alpha backbone from residues 1–90 for EC1 from MD simulation and X-ray as well as m-EC1 from the NMR study (Figure 3D). The results show a small magnitude of the RMSD, indicating that there is limited change in the head region of the h-EC1 domain during MD simulations. The majority of the change occurred in the tail region that swung closer to and interacted with the head region of EC1 (Figure 3D).

The calcium-binding region in E-cadherin was found at the interface between the EC1 and EC2 domains, and the D103 and the tail region of EC1 studied here contained part of the calcium-binding site. It has been shown previously that cadherin molecules form a rod-like structure (or extended structure) due to the coordination of calcium at the interface region. In the absence of calcium, the rod-like structure collapses into a globular cadherin structure. In this study, the tail region swinging to interact with the head region of EC1 could be a natural behavior of this protein in the absence of calcium, and it is reasonable to speculate that the D103 residue is also involved in the dynamic conversion of a rod-like structure in the presence of calcium to a globular structure of cadherin in the absence of calcium.

The structure of the EC1 domain from MD simulations was used to determine the binding site of cHAVc3 by implementing the NMR constraints from the CSP data upon peptide titration. The observed CSP could also be the result of local conformational changes in the EC1 as a consequence of peptide binding (Cui *et al.*, 2003; Stark and Powers, 2012; Williamson *et al.*, 1997). Although the magnitude of CSP varied from residue to residue, the residues that have CSP values higher than average are C9, Y36, I38, T63, F77, S78, I94, D103, and V112 (Figure 6C). The high CSP changes were attributed to binding to the EC1 domain, and these were used as NMR-constraints in the docking experiments to search for binding site(s) of the peptide (Cui *et al.*, 2003). The docking model shows that the cHAVc3 peptide interacts with residues Y36, I38, F77, S78, and I94 with CSP values higher than average, as well as with S37, I53, and H79 with CSP values lower than average (Figure 6B).

From the docking experiments, the residues on the EC1 domain that are strongly influenced by peptide titration in the NMR data are shown in Figure 6C and they are consistent with the docking data. Residues that are far away from the binding site can also be affected by peptide titration in the NMR study. These chemical shift changes in these residues are normally less dramatic and can be attributed to conformational change upon peptide binding. The docking results show that the peptide interacts with residues that are strongly affected by peptide titration. The methyl groups of the Ala4 and Val5 residues in the peptide interact with Ser78 and Pro91, respectively in the EC1 domain (Figure 6D). The side chain His3 residue in the peptide interacts with the Ser37 and H79 on EC1. The side chain of the Ser2 residue is located around Ile38 and Asp44 on the EC1 domain and the backbone NHs of

Cys1 and Ser2 on the peptide form hydrogen bonds to the side chain carbonyl group of Asp44 of EC1.

There are also residues with low and high CSP values that are far away from the binding site; these residues include D103, T109, V112, and F113 (Figures 1C & 6C). The observed CSPs in these residues were attributed to conformational changes on the tail region of EC1 during peptide binding. It is interesting to find that the large change in chemical shift of D103 was also observed upon peptide titration; however, it is not clear if it can be attributed to the conformational swing of the tail region to stabilize its interaction with the head region (i.e., EC1) upon peptide titration (Figure 3C). The D103 residue can be categorized as the hinge residue for the dynamic movement of the tail region to interact with the head region. In addition, the CSP comparison among the S78, D103, and V122 residues at the initial titration points shows that the CSP values of the D103 and V112 residues are less sensitive than that of S78 when titrated with the peptide (Figure 1C), suggesting that cHAVc3 more likely binds to the region of the S78 residue. T109, V112, and F113 from the tail region were additional residues that were affected by peptide titration (Figure 6C); these residues from the tail region are clearly interacting with the head region of the protein. The observed CSP values in the tail region are due to the conformational changes in the tail region upon peptide binding. This result also supports the idea that the tail region folded into the head region of EC1 as indicated by the MD simulation results.

The K_d values were estimated by curve fitting simulations using NMR data of chemical shift changes from several residues (i.e., C9, F77, S78, I94, D103, and V112) at different peptide:protein ratios (<2.5:1.0). The low range K_d s of cHAVc3 binding to EC1 were estimated to be around $0.5 \times 10^{-5} - 1.0 \times 10^{-5}$ M using the F77, C9, R68, and D103 residues (Table 1). The medium range K_d was estimated using the S78 residue, and the high range K_d s were estimated from the V112 and I94 residues. For the R68 and I94 residues, the chemical shift changes in ^{15}N as [PL] complex ($\delta^{15}\text{N}$; 1D) was fitted in the titration curve.

Using information about the binding site of cHAVc3 peptide on the EC1 domain, we propose a potential mechanism of action of cHAVc3 peptide in modulating cadherin-cadherin interactions in the intercellular junctions of the BBB. Using the X-ray structure of C-cadherin ((Boggon *et al.*, 2002), the potential interaction of E-cadherin proteins in the intercellular junctions of the BBB was modeled in Figure 7. As in C-cadherin, E-cadherin proteins are protruding from one cell membrane surface (parallel arrangement) to form organized *cis*-interactions, where the EC1 domain of one E-cadherin molecule interacts with the EC2 domain of another neighboring E-cadherin molecule (Figure 7A). The many *cis*-interactions can be formed continuously using parallel E-cadherin proteins on the same cell surface membranes to generate an organized cluster of E-cadherin proteins. Next, *trans*-interactions of E-cadherin proteins from the opposing cell membranes (antiparallel arrangement) are formed by the EC1-EC1 domain interaction. The EC1-EC1 antiparallel interaction is generated via domain swapping of the N-terminus of one EC1 into the hydrophobic pocket of the opposing EC1 (Figure 7B). The cHAVc3 peptide binds to the EC1 domain at the interface region between EC1 and EC2 domains from the neighboring or parallel E-cadherins; thus, cHAVc3 peptide blocks the *cis*-interactions of E-cadherin proteins (Figure 7A). The peptide disrupts *cis*-interactions of E-cadherins while maintaining

the *trans*-interactions of cadherin proteins via domain swapping (Figure 7). As a result, the dissociations of *cis*-interactions increase the porosity or permeability of the intercellular junctions of the BBB to allow paracellular penetration of molecule into the brain.

Conclusions

The NMR results showed that the cHAVc3 peptide binds to the EC1 domain of E-cadherin, providing support for the BBB intercellular junction modulation hypothesis (mentioned above) by cHAVc3 peptide. The titration data between cHAVc3 and EC1 from NMR experiments showed that cHAVc3 peptide falls into the weak binding category with one favorable binding site on the EC1 domain. The MD simulations data of EC1 showed that the tail part of the protein interacted with the head part of EC1 domain in a box of water, which might be a mimic for EC1 dynamics in a solution system. The D103 residue was suggested to work as hinge residue in which the tail part of the protein is sandwiched to the head of EC1 domain. Free docking calculations yielded several possible models for cHAVc3:EC1 complexes. However, the availability of NMR CSP data allowed generation of more reliable microscopic models by docking with NMR-constraints. Finally, the potential mechanism of action of cHAVc3 to increase the paracellular permeability of the BBB is via its binding to the EC1 domain at the interface of EC1-EC2 interaction of two parallel E-cadherins, which increases porosity of the intercellular junctions.

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Figure 1A

Figure 1B

Figure 1C

Figure 1D

Figure 1.

The chemical shift changes of residues in the 2D ^1H - ^{15}N -HSQC NMR spectra of the free and peptide-titrated EC1 domain collected using Bruker Avance 800 MHz spectrometer. **(A)** The overlay of partial 2D NMR spectrum of the EC1 domain (red) and titrated EC-1 domain at the highest peptide concentration (black). **(B)** The overlay of crosspeak shifts from the NH of Ser78 (S78, left) and Asp103 (D103, right) upon titration with cHAVc3 peptide from 0.3:1 to 2.5:1 peptide-to-protein molar ratios. **(C)** The observed chemical shift perturbation (CSP) of the EC1 residues weighted for both ^1H , and ^{15}N using equation 1 when titrated with cHAVc3 peptide at a peptide/protein ratio of 2.5:1. **(D)** The ^{15}N chemical shift changes in the EC1 residues upon titration with 2.5:1 peptide/protein ratio.

Figure 2A

Figure 2B

Figure 2C

Figure 2.

The ^1H , ^{15}N -weighted CSP changes (F) from the NMR spectrum as a function of peptide/protein ratios for representative residues in the EC1 domain: (A) S78, (B) V112, and (C) F77. The K_d was estimated using nonlinear regression for the simulated curve (-.-.). The saturation point was estimated because of small changes in chemical shift between the last and penultimate titration points.

Figure 3D

Figure 3.

The comparison between **(A)** the starting structure, taken from the X-ray structure of the EC1 domain with a small fragment from the EC2 domain, and **(B)** the final structure after 100 ns molecular dynamics simulations. **(C)** The overlay of the starting structure (magenta) and the final structure from MD simulations (pink). The structure from the MD simulations shows the C-terminal tail that swings into the main region of the EC1 domain as indicated by the curved arrow. After MD simulations, the alignment of the residues 1 to 90 between the X-ray structure and the MD simulation structure has RMSD < 1.0 and the tail is close to the head part of the protein. **(D)** The RMSD per residues between the X-ray and MD structures of the EC1 domain as RMSD > 10 Å are accounted for mostly by tail residues. The higher RMSD values start from residue 102 to residue 138 with the maximum RMSD (76 Å) at the residue 120.

Figure 4A

Figure 4B

Figure 4.

(A) The changes in psi (ψ) angle of D103 (◆), D100 (■) and N104 (▲) residues during the molecular dynamics run of EC1 domain in water. Comparing to the ψ^{D103} in the X-ray structure (0 ns) shows dramatic changes during the MD run. The ψ angles of D100 and N104 (flanking residues of D103) showed no dramatic changes between the X-ray and MD structures in comparison to ψ^{D103} . (B) The changes in phi (Φ) angles of D103 (◆), D100 (■) and N104 (▲) residues in which Φ^{D103} , Φ^{D100} and Φ^{N104} experienced similar changes during the MD run.

Figure 5.

The results from MD simulations to generate stable cyclic structures of cHAVc3 peptide. There are five different clusters of structures of cyclic cHAVc3 (A to E). Conformation A has the highest number of structures in a cluster, suggesting that it is the closest to the solution conformation of cHAVc3. Conformation A was used for docking experiments with the EC1 structure for the MD simulation. Other conformers (B–E) were also used for molecular docking with the EC1 domain, and they gave docking results on the EC1 domain similar to those of conformation A.

Figure 6A

Figure 6B

Figure 6C

Figure 6D

Figure 6.

(A) The docking model of conformation A of cHAVc3 to the EC1 domain (black arrow) to indicate the potential binding site of cHAVc3 peptide in EC1. (B) The interaction region of cHAVc3 (blue) on the residues of EC1 (cyan). The S78 residue has a hydrophobic pocket that interacts with the Ala4 residue of cHAVc3 peptide. The F77 residue has the same type

of interaction with the disulfide bond of the peptide. **(C)** The interaction network between cHAVc3 peptide and the EC1 domain from the docking structure. **(D)** The MD structure of the EC1 domain showing residues that had higher R^2 values (red) and residues with lower R^2 values (yellow) when the EC1 domain was titrated with cHAVc3 peptide. The pocket for cHAVc3 peptide in EC1 domain is represented by S78, F77, H79, Y36, S37, and I38, along with the surrounding I94 and I53 residues. Most chemical shift changes (red and yellow colors) are closed to the binding pocket of cHAVc3, indicating the binding region of cHAVc3 peptide. The chemical shift changes of the tail residues, including the D103 residue, can be attributed to the dramatic conformational changes of the tail region.

Figure 7.

Proposed mechanism of action of cHAVc3 peptide in modulating the intercellular junctions of the BBB to enhance the porosity or permeability of the paracellular pathway of the BBB. (A) The proposed E-cadherin interactions in the adherens junction of the BBB which was modeled using *cis*- and *trans*-interactions of C-cadherin in the X-ray structure (see Boggon *et al.*). In this case, the EC1 domain of an E-cadherin forms *cis*-interaction with the EC2 domain of a neighboring E-cadherin from the same cell membranes. The EC1 domain of an E-cadherin forms *trans*-interaction with the EC1 domain of E-cadherin from the opposing cell membranes and the EC1-EC1 interaction is due to domain swapping of the N-termini of two EC1 domains. The cHAVc3 peptide binds to the EC1 domain to block the *cis*-interaction of EC1-EC2 domain of neighboring E-cadherins to increase the porosity of the intercellular junctions. (B) Expansion of *cis*- and *trans*-interactions between EC1/EC2 and EC1/EC1 domains, respectively. The EC1-EC1 *trans*-interaction uses N-termini for domain swapping. The cHAVc3 peptide-binding site is at the interface between EC1 and EC2 that

forms *cis*-interaction of E-cadherin. Peptide binding to the EC1 domain dissociates the *cis*-interactions of cadherins to modulate the intercellular junction integrity.

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Table 1

The estimated K_d values of binding between cHAVc3 and the EC1 domain of E-cadherin determined using ^1H , ^{15}N CSP of different residues upon peptide titration with cHAVc3:EC1 ratios of 0.3:1 to 2.5:1.

Residue of Protein	K_d (1×10^{-5} M)
S78	2.5
F77	0.5
C9	1.0
*I94	7.0
*R68	1.0
V112	4.5
D103	0.5

The phi and psi angles for amino acid residues of cHAVc3 peptide in different stable clusters of conformers from MD simulations. Secondary structure(s) for each cluster were determined by phi and psi angles and either H-bond network

Table 2

Cluster Number	Dihedral Angles	Residues						Secondary Structure
		Cys1	Ser2	His3	Ala 4	Val5	Cys6	
Cluster 1	phi	-106.1	-108.5	-121.9	-63.5	-95.1	-124.9	β I-turn at H3-C6
	psi	144.2	139.0	-6.5	-39.9	-58.1	84.2	
Cluster 2	phi	-70.8	-70.5	-84.4	-59.5	-92.8	-73.6	β VII-turn at H3-C6 and Inv. γ -turn at C6
	psi	137.2	3.9	160.5	-41.3	126.7	77.4	
Cluster 3	phi	-116.3	-70.8	-107.5	-77.3	-108.3	-104.5	β I-turn at C1-A4
	psi	21.4	-41.5	-27.8	162.3	110.2	74.1	
Cluster 4	phi	-68.1	68.0	-145.9	-59.3	72.1	-67.6	γ -turn at V5
	psi	-36.2	43.3	169.3	-34.4	-47.3	-19.0	
Cluster 5	phi	-112.9	61.1	60.8	-141.5	-52.1	-84.9	β I'-turn at C1-A4
	psi	14.4	48.1	28.5	164.3	146.7	-7.3	