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^{1}H, ^{13}C and ^{15}N Backbone Assignment of the EC-1 Domain of Human E-Cadherin

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Abstract

The EC1 domain of E-cadherin has been shown to be important for cadherin-cadherin homophilic interactions. Cadherins are responsible for calcium-mediated cell-cell adhesion located at the adherens junction of the biological barriers (i.e., intestinal mucosa and the blood-brain barrier (BBB). Cadherin peptides can modulate cadherin interactions to improve drug delivery through the blood-brain barriers (BBB). However, the mechanism of modulating the E-cadherin interactions by cadherin peptides has not been fully elucidated. To provide a basis for subsequent examination of the structure and peptide-binding properties of the EC1 domain of human E-cadherin using solution NMR spectroscopy, the ^{1}H, ^{13}C and ^{15}N backbone resonance of the uniformly labeled-EC1 were assigned and the secondary structure was determined based on the chemical shift values. These resonance assignments are essential for assessing protein-ligand interactions and are reported here.

Keywords

human E-cadherin; EC1; backbone resonance assignment; multidimensional NMR

Biological Context

E-cadherin has an important role in regulating calcium-dependent cell-cell adhesion at the adherens junctions of critical biological barriers such as the intestinal mucosa and blood-brain barrier (BBB). Cell adhesion is mediated by homotypic interaction of E-cadherins. E-cadherin has cytoplasmic, trans-membrane, and extracellular (EC) regions. The extracellular

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The authors declare that they have no conflict of interest.

Ethical Standard

The authors declare that the experiments comply with the current laws of the United States.

region is composed of five repeat domains called EC1 to EC5 (Makagiansar et al. 2002; Zhang et al. 2009). The EC1 domain has a role in selective homotypic cadherin-cadherin interactions and it is also responsible for heterotypic interactions between E-cadherin and $\alpha_E\beta_7$ integrin to achieve cell-cell adhesion (Cepek et al. 1994; Trivedi et al. 2012; Shiraishi et al. 2005). E-cadherin peptides derived from the conserved sequence of EC1 have been applied to enhance the BBB permeation of ^{14}C-mannitol and ^{3}H-daunomycin through the paracellular pathways in *in-situ* rat brain perfusion model (Kiptoo et al. 2011). This result suggests that E-cadherin peptides modulate the cadherin-cadherin interactions in the adherens junctions of the BBB to increase the porosities of the paracellular pathways of the BBB. It is proposed that the activity of the cadherin peptide is due to competitive binding to the EC1 domain of E-cadherin.

The high-resolution structure of human E-cadherin has not been solved, but the solution structure of the EC1 domain of mouse E-cadherin has been previously determined using NMR and it has a similar fold with the immunoglobulin protein (Overduin et al. 1996). Human EC-1 is 84% identical to the mouse homologue. Here, the backbone resonance assignments of the EC1 domain of human E-cadherin were determined and the chemical shifts for H^N, N, CA, CB and CO are reported. The chemical shift index (CSI) was also applied to predict the EC1 secondary structure.

Method and Experiments

Overexpression and purification of human EC1 domain

The cDNA corresponding to the 110 residues from human E-cadherin 1 domain (EC1) plus 28 residues extending into EC2 to encode the calcium binding region was synthesized and subcloned into the pASK-IBA6 plasmid (BlueHeron) to generate a protein containing the first 138 residues of E-cadherin. pASK-IBA6 has 3681 bp DNA and carries AmpR, f1 origin and Multi Cloning Site 1 (MCS 1) gene regions (Genosys, Woodland, TX). 5′ Xba 1 and 3′ Xho 1 restriction sites were used to insert the gene. In total, the protein product consists of 150 residues, including Streptag I (WSHPQFEK), Factor Xa and EC1 sequences. After the EC1 cDNA was successfully subcloned into the plasmid, it was transformed into the DH5α competent cells, spread on the LB agar plates containing 100 mg/L ampicillin, then incubated at 37°C overnight. The plasmid DNA was isolated and purified using Qiagen Spin Miniprep Kit (Stratagene). The DNA concentration was determined using UV absorbance on a Spectrometer Carry 300 at 260 nm (A_{260}).

To obtain ^{15}N-^{13}C-labeled protein for 3D NMR analysis, EC1 was expressed in *E.coli* BL21 (DE3) cells grown in M9 minimal media containing ^{15}N-labeled ammonium chloride and ^{13}C-labeled glucose. A 10 mL starter culture was grown for 16 hours. A 10 mL aliquot of the starter cell culture was added to each of 4×1 L culture flasks containing 250 mL of M9 minimal of cell culture. The cells were grown at 37°C to an OD_{600} of 0.5– 0.6; then anhydrotetracycline was added at a final concentration of 250 mL (Promega Inc., Madison, WI) to induce the expression of the EC1 protein. After 6 h of additional growth at 30° C, cells were harvested by centrifugation at 4500xg and cell pellets were stored at −80°C prior to use.

Cell pellets containing the overexpressed EC1 were resuspended in 10 mL of 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3 at pH 8.0 (Buffer B) and lysed by three passes through a French Pressure cell at 20,000 psi. To remove cellular debris, the lysate was centrifuged at 21,000xg for 1 hour at 4 °C. The supernatant containing soluble EC1 was diluted with an equal volume of buffer B then concentrated via centrifugation at 4,500 rpm for 20 minutes, using a 10,000 kDa cut-off Amicon Ultra concentrator (Millipore, Billerica, MA). The resulting solution was loaded onto a Strep Tactin II affinity column with size 5.0×0.6 cm (GE Healthcare Life Sciences, Pittsburgh, PA) attached to a peristaltic pump after equilibration with buffer B at room temperature. The column was then washed with buffer B at 2 mL/min before eluting EC1 with buffer E (buffer E contains 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3, 2.5 mM desthiobiotin and 1 mM diothiothreitol (DTT) at pH 8.0 with a flow of 2 mL/min. The protein fractions were pooled and concentrated to 1.5 mL. The purity of each EC1 fraction was determined by 4–12% Tris-Bis SDS-PAGE. The protein concentration was determined from UV absorbance at 280 nm and calculated using the predicted molar absorption coefficient of 19,480 M^{-1} cm^{-1} (Makagiansar et al. 2002). Because cell binding experiments confirm the N-terminal tag does not interfere with EC1 function (unpublished data), the tagged protein was studied. Final pure EC1 was stored in the buffer E at 4 °C.

In preparation for 3D NMR experiments, 600 μL of purified protein was dialyzed overnight against 1 L NMR buffer (20 mM Potassium Phosphate, 5 mM DTT, 50 mM KCl, 5 mM $CaCl_2$, 50 μM Sodium Azide) at pH 6.8 and 4 °C in Slide-a-Lyzer dialysis cassette (5,000 kDa cut-off, Thermo Scientific, PA) (Overduin et al. 1996).

NMR Spectroscopy

All triple resonance 3D ^{1}H-^{15}N-^{13}C were performed on a 1 mM protein sample using a Bruker Avance 800 MHz NMR spectrometer equipped with a cryoprobe, and spectra were recorded on the uniformly $^{13}C/^{15}N$-labeled sample in 95% H_2O/5% D_2O using the following pulse programs HNCACB (Wittekind and Mueller 1993), CBCA(CO)NH (Grzesiek and Bax 1992), HNCO (Ikura et al. 1990), HNCA (Ikura et al. 1990) and HNCOCA (Bax et al. 1991).

Data were processed using NMRpipe (Delaglio et al. 1995), and the NMR assignment program Sparky was used to determine position of peaks in the spectra acquired (Goddard TD, Kneller DG).

Assignments and data deposition

Approximately 90% of human EC1's resonances were assigned using the standard sequential backbone assignment method. The data was submitted to the PINE server for semiautomated protein backbone assignment and rechecked for accuracy. The assignment data are deposited in BioMagResBank (http://www.bmrb.wisc.edu under accession number 19443). Figure 1 depicts the ^{15}N-HSQC of the EC1 domain. Most of the peaks in the 2D HSQC spectrum were assigned. Of the 138 residues in EC1, E64, L66, V81, I96, Q101, E111, M128, L122, A135 and D136 could not be assigned. The vast majority of the residues in the conserved region of EC1 domain were assigned; these sites are important for binding

to cadherin peptides. Residues 111–138 comprise the calcium-binding region adjacent to the core domain. Without this extension, EC1 was not sufficiently well behaved in solution to achieve a well-resolved HSQC. Five residues from this region could not be assigned. Six of 12 residues within the tag sequence of EC1 were also assigned, including W(-11), S(-10), Q(-7), F(-6), G(-1), R(0). The tag sequence is needed to help EC1 purification using single step streptactin column chromatograpy and it does not interfere with EC1 function. Moreover, the N-terminal extension in EC1 prevented the native dimerization of EC1 domain when it was produced in the host cell by fusing it from cytoplasm into periplasm space as a monomeric EC1 domain. Side chain amide NH_2 groups were not assigned but are identified in Figure 1 with lines connecting the paired peaks. Three tryptophan residues are present in EC1, and the NH indole moieties from these residues are circled in Figure 1. A few peaks appear for which no assignment could be made, and these peaks are enclosed in black boxes.

Despite having optimized the construct and conditions for successfully accomplishing backbone assignment, experiments to identify side chain resonances beyond CB resulted in poor data and a limited number of additional side chain assignments. Based on these results, a high-resolution three-dimensional structure cannot be determined under these conditions. As such, chemical shift index (CSI) analysis was performed using the chemical shifts of H^N, N, CA, CO, and CB atoms (Figure 2) to predict the protein's secondary structure (Wishart and Sykes 1994; Wishart et al. 1992). The result shows that the majority of the secondary structure in EC1 is beta sheet with one alpha helix; this structural prediction is consistent the X-ray crystal structure and the NMR structure of the EC1 domain of mouse E-cadherin (Figure 3).

The backbone NMR assignments of EC1 were determined and used to elucidate the secondary structure of the protein. The study performed here is important because it serves as a baseline for assessing the function of human EC1. The data set provides the foundation for carrying out perturbation experiments, which enable examination of the binding properties of the EC1 domain of human E-cadherin with itself, E-cadherin-derived peptides and other binding partners that are responsible for regulating adherens junctions.

Acknowledgments

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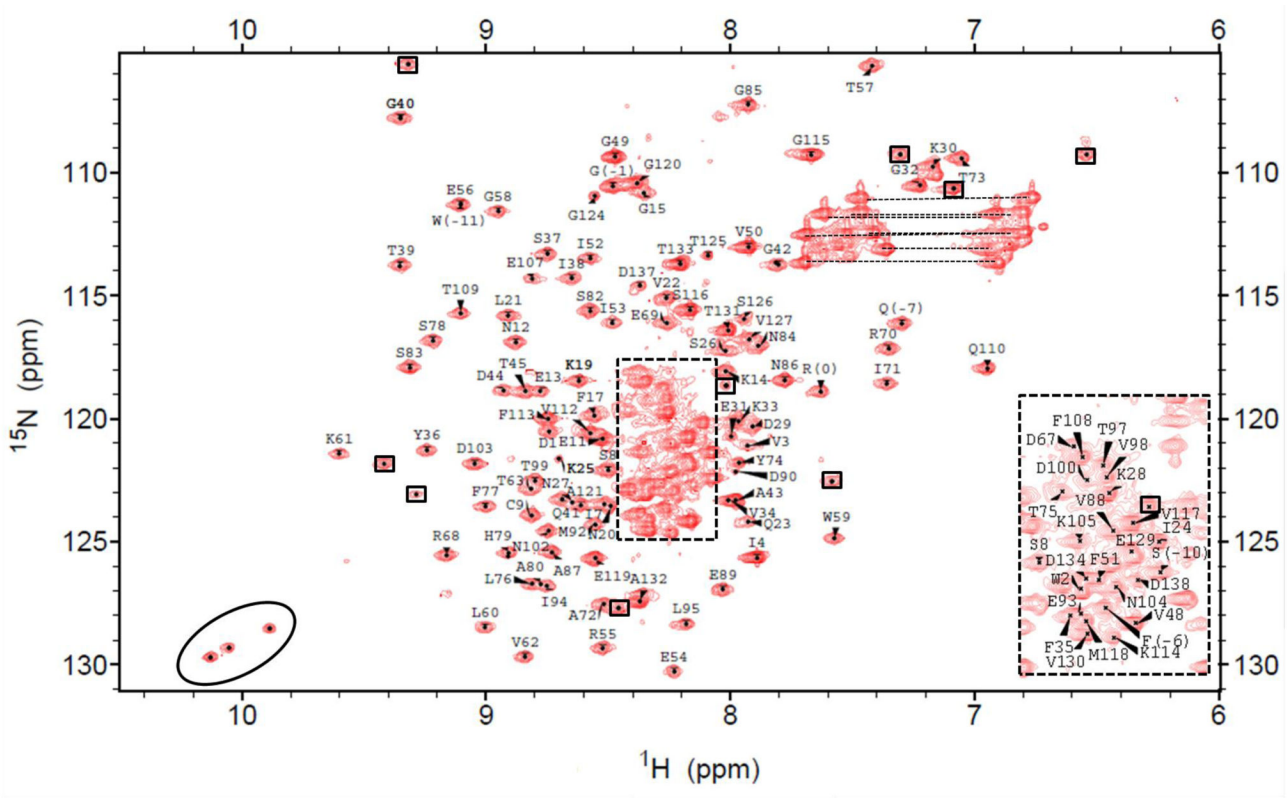

Figure 1.

Assigned 2D ^{15}N-HSQC spectrum of 1.0 mM EC1 in 20 mM potassium phosphate, pH 6.8 at 37°C, acquired on a Bruker Avance 800 MHz NMR spectrometer with a cryoprobe.

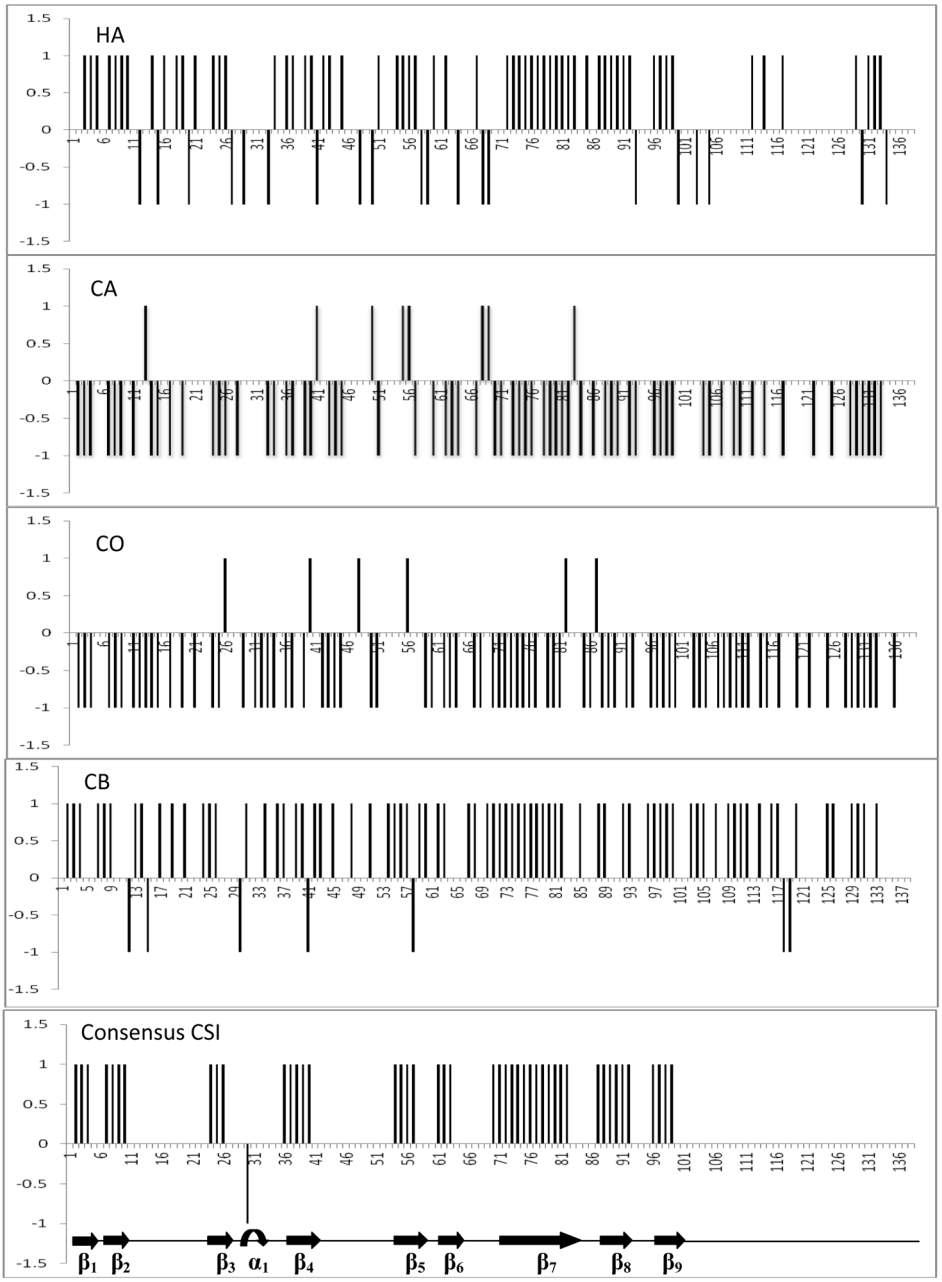

Figure 2.

The chemical shift index of individual nuclei and consensus plot were calculated from HA, CA, CO, and CB chemical shifts of the EC1 domain of human E-cadherin

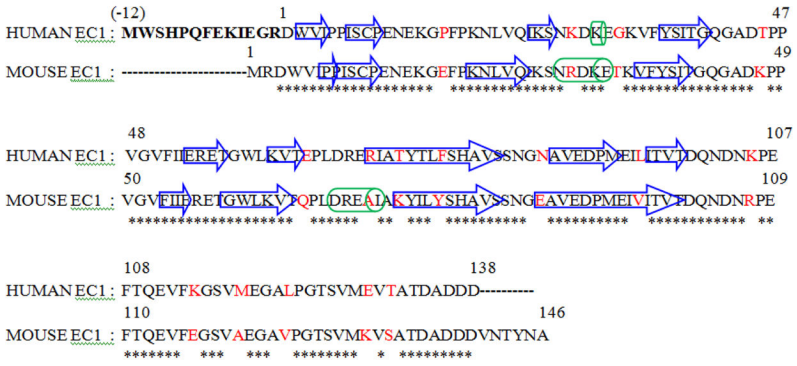

Figure 3.

Amino acid sequence alignment between human EC1 and mouse EC1 used for NMR assignment, with beta sheet regions in the blue arrow boxes and alpha helix regions in the green cylinder boxes. The bold residues are the tag sequence in human EC1. The red color residues are indicating the polymorpishm between human EC1 and mouse EC1. The asterisk residues are the similar residues between human EC1 and mouse EC1. These secondary structure prediction data are derived from the chemical shift index of the solution 3D NMR experiments (Wishart and Sykes, 1994).