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E-CADHERIN PEPTIDE SEQUENCE RECOGNITION BY ANTI-E-CADHERIN ANTIBODY

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Cadherins are calcium dependent glycoproteins involved in homophilic cell-cell adhesion. To further elucidate the interaction between cadherins we have developed an immobilized peptide ELISA to quickly scan the extracellular domains of E-cadherin. Peptides displaying antigenic reactivity to anti-E-cadherin antibody are presumably on the surface and thus may be involved in cadherin-cadherin interaction. We have found three peptides from the EC-1 domain which are recognized by the anti-E-cadherin antibody. These peptides are in the two important regions of the EC-1 domain that was deduced from its secondary structure.

Cadherins are a molecular family of glycoproteins essential for the calcium dependent process of cell-cell adhesion (1-3). Cadherins are present on the cell surface and mediate cellular junctions. The cadherin family is divided into subclasses which exhibit different tissue distribution patterns and generally mediate homotypic cell-cell adhesion. The cell-cell adhesion becomes loose and may be disrupted if calcium is removed from the extracellular environment (3). Transfection of L-cells with E-cadherin cDNA results in their calcium dependent homophilic adhesion (1).

The highly conserved extracellular domain contains three or more major internal repeats. The first major internal repeat, EC-1, is important for the specificity of binding. This region contains a conserved His-Ala-Val (HAV) sequence. Mutation of specific amino acids adjacent to the HAV sequence alters specificity of cadherins by various degrees (4). Synthetic peptide LRAHAVDVNG-(NH_2) from the EC-1 region of N-cadherin inhibits the compaction of mouse embryos and neurite growth by the inhibition of cell-cell adhesion (5). It has been shown that resealing cellular junctions is inhibited by anti-E-cadherin antibodies, presumably the antibodies recognize the amino acid sequence responsible for homophilic interactions (6).

We have developed an immobilized peptide ELISA in order to scan the extracellular domains of cadherin in a short period of time (7). In this paper, we identify

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sequences of the EC-1 region of E-cadherin which presumably are responsible for the cadherin-cadherin interactions.

Understanding which sequences are involved in cadherin-cadherin recognition will enable us to design selective peptides for potential use as cell-cell adhesion inhibitors. Our peptides, which display antigenic reactivity to the anti-E-cadherin antibody, should be on the surface or belong to a mobile segment of EC-1. Our results are in agreement with the secondary structure of EC-1 (8), thus confirming the reliability of our immobilized assay. In this paper, we further demonstrate the validity of our immobilized peptide ELISA by comparing the E-cadherin peptide sequences to their N-cadherin peptide counterparts in our previous paper (7).

MATERIALS AND METHODS

Peptide Synthesis

The immobilized peptides **1-12** (Figure 3, 4 & 5) were synthesized using Fmoc (9-fluorenylmethyloxycarbonyl) amino acid chemistry on controlled pore glass , CPG, (118 Å pore diameter, CPG-10, Electro-Nucleonics Inc.) which was functionalized with an aminopropyl group (9). A linker of six ε-aminocaproic acids was synthesized between the CPG and the peptides using Fmoc-aminocaproic acid. Diisopropyl-carbodiimide (DIC) and hydroxybenzotriazole (HOBT) in dimethylformamide (DMF) were used in the amino acid coupling reaction. Each amino acid was coupled until completion and confirmed using the ninhydrin test (10); and then the Fmoc group was deprotected with 20% piperidine in DMF for 30 minutes. After completion of the synthesis, the last residue was deprotected with 20% piperidine in DMF. The side chain protections were then removed by TFA with phenol used as a scavenger. The glass beads were thoroughly washed with DMF, 50% dichloromethane (DCM)/MEOH, and DCM, respectively. The peptide's amino acid composition and the load per gram on the glass beads were determined by amino acid analysis at the Biochemical Research Service Laboratory (BRSL) at the University of Kansas. The peptides' concentrations were also determined by quantitation of the free amine by the ninhydrin test using a standard curve for amine concentration.

Horse Radish Peroxidase (HRP) Labeling ofAnti-E-cadherin Antibody

HRP (horse radish peroxidase) was coupled to the monoclonal anti-E-cadherin antibody,(uvomorulin rat IgG1 isotype; Sigma Inc.), by using the two step glutaraldehyde coupling procedure (11). The final solution contained three species, the uncoupled HRP, the uncoupled antibody, and the anti-E-cadherin-HRP antibody conjugate. The mixture was passed through a size exclusion column, sephadex G-150 (Sigma Inc.) with PBS buffer 7.4. Figure 1 illustrates the Western Blot obtained for identification of the antibody conjugate. Figure 1A represents addition of chemilumenescence HRP substrate to lane 1 (HRP) and to lane 2 (anti-E-cadherin-HRP antibody). Figure 1B represents the inactivation of HRP in these two lanes followed by probing with goat anti-rat IgG coupled to HRP. This was followed by reapplication of the HRP substrate which identified our antibody in lane 2.

ELISA

Enzyme linked immunosorbent assay (ELISA) was used to determine the binding between the peptides and the anti-E-cadherin antibody. All peptides were analyzed by our immobilized peptide ELISA method. In the immobilized method, 6 mg of peptide beads (loading 60 nM/mg) were weighed and placed in siliconized test

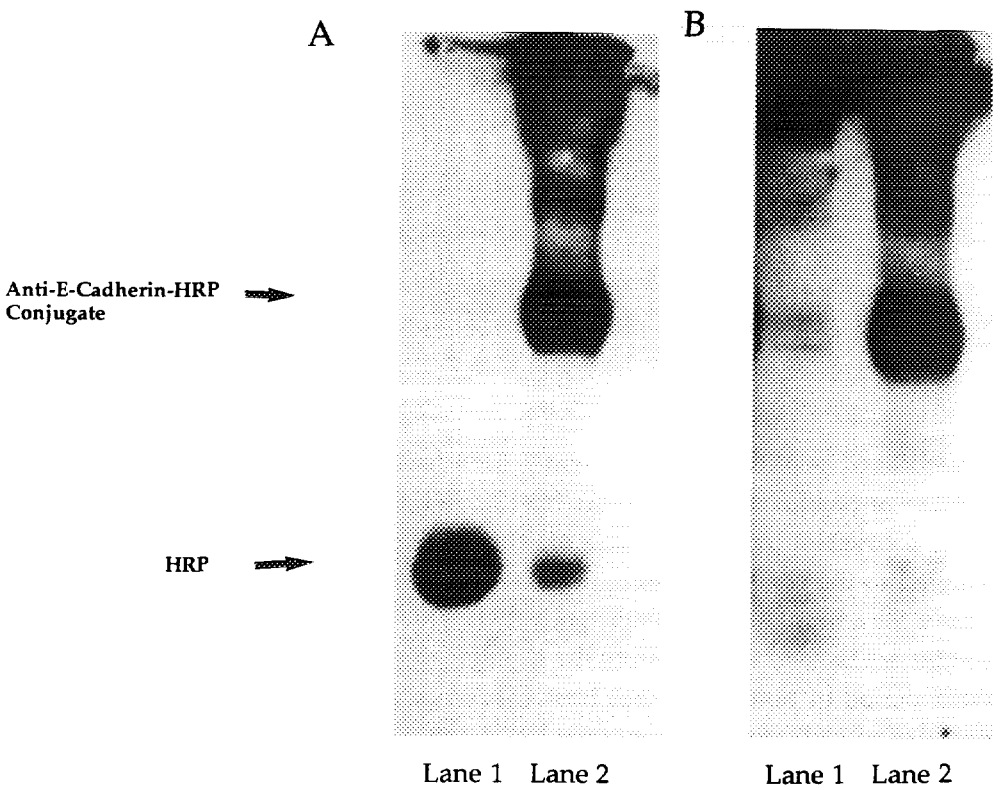

Figure 1. Western Blot obtained for the identification of anti-E-cadherin-HRP antibody.

tubes. 3 ml of BSA (Bovine Serum Albumin, 1%, Pierce) blocking buffer was added to all the peptides and incubated at 4 °C for 24 hours. The samples were washed with 2 ml of PBS pH 7.4/.05% Tween20 (Pierce) three times and 0.692 μg/ml of anti-E-cadherin-HRP antibody was added. The samples were then incubated for 1 hr at 37 °C. The samples were washed again three times , filtered and reweighed. Then, 200 μL of one step Turbo TMB (Pierce), was added for 10 minutes. 200μL of 1M Sulfuric acid was added to stop the reaction; the samples were then read by an ELISA plate reader at 450 nm. The experiments were repeated at least three times, and the standard deviation and coefficient of variation were calculated.

RESULTS AND DISCUSSION

We are interested in determining which sequences on the surface of the E-cadherin are important for cadherin-cadherin interactions. Not all of the exposed regions on the surface of a protein are important and display antigenic reactivity. Our aim is to scan the extracellular domains of E-cadherin for antigenic reactivity, presumably these antigenic reactive sequences are involved in cadherin-cadherin recognition. Anti-E-cadherin antibody was used since it has been shown to inhibit cadherin mediated cell-cell adhesion. We have developed an ELISA for the recognition of immobilized cadherin peptides on glass beads by anti-E-cadherin antibody.

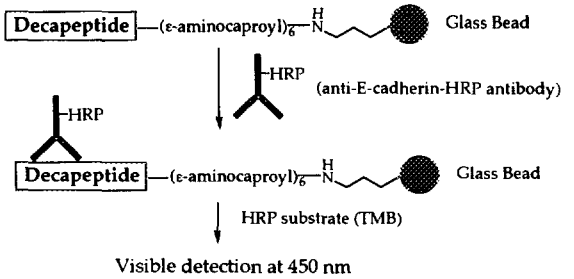

Figure 2. ELISA of anti-E-cadherin-HRP antibody binding to the various cadherin decapeptides immobilized on glass beads.

Immobilized peptides have frequently been used as a means to synthesize a large number of peptides in a short time (12-13). In our study, peptides were immobilized using a covalent bond to a linker of six ε-aminocaproic acid moieties which was coupled to the controlled-pore glass (14) (Figure 2). The ε-aminocaproic acid linker allows for a more natural interaction between the peptide and the antibody. Because we did not have to further purify the peptides, we could synthesize these peptides in a short period of time. Thus our immobilized assay allows us to quickly scan all the domains of cadherin. We therefore can determine important sequences involved in cadherin-cadherin regulation which will enable us to potentially design selective peptides for the inhibition of cell-cell adhesion.

The sequences for the synthetic peptides and their secondary structures in the intact EC-1 domain are shown in Figure 3 (8). Immobilized decapeptides **1-9** represent a 90 amino acid sequence in the N- to C-terminal direction of EC-1 (Gly^{15} to Asn^{104}) of E-cadherin. Peptide **10** is the $(\varepsilon\text{-aminocaproic acid})_6$ linker used as a negative control peptide. We used an indirect ELISA in which the anti-E-cadherin antibody was labeled with HRP through a glutaraldehyde conjugation. This eliminated the use of a secondary antibody and therefore minimized the background interference. The binding of peptides **1-10** to anti-E-cadherin antibody is represented in Figure 4. Peptides **7** and

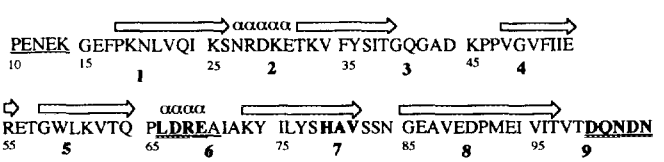

Figure 3. Decapeptides **1-9** represent the 90 amino acid sequence in the EC-1 domain of E-cadherin. Peptides were synthesized in the N- to C-terminal direction. Peptides **7** and **9** displayed high binding to anti-E-cadherin-HRP antibody and peptide **1** displayed moderate binding. **Bold** letters represent conserved sequences among the cadherins. Arrows represent β-sheet structure. α represents helical structure. Underline represents sequences proposed to be involved in the calcium binding pocket. The number at the beginning of each decapeptide corresponds to the amino acid position in the intact E-cadherin.

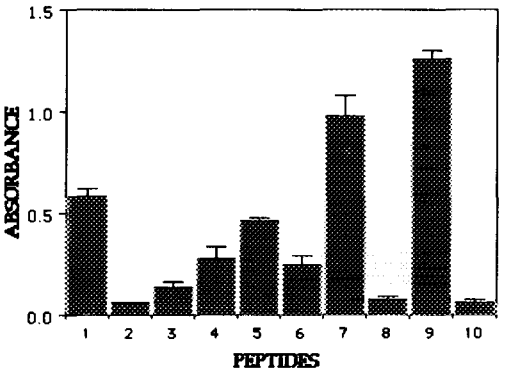

Figure 4. The binding of immobilized peptides to anti-E-cadherin-HRP antibody.

9 display high binding to the anti-E-cadherin antibody, and peptide 1 displays moderate binding. The remaining peptides (2, 3, 4, 5, 6, 8 and 10) do not display appreciable binding.

Peptide 7 contains a His^{79}-Ala^{80}-Val^{81} sequence which is the most conserved sequence among cadherins. A synthetic peptide containing the HAV sequence from N-cadherin inhibits the compaction of mouse embryos and neurite growth by cell-cell adhesion (5). Also, altering residues around HAV can alter the specificity of cadherins (15). We have previously shown three other synthetic HAV peptides from N- and E-cadherin to display significant antigenic reactivity to anti-E-cadherin antibody (7). The His^{79} and Val^{81} residues of E-cadherin are solvent exposed amino acids on the surface of EC-1 and it has been inferred that this region confers homophilic specificity on cadherins (8). We therefore mutated His^{79} and Val^{81} with alanine to confirm the importance of this region. Figure 5 illustrates the alanine mutagenesis. Both residues were susceptible to mutagenesis.

Antigenic reactivity was slightly increased when His^{79} was mutated to Ala (peptide 11); however, this HAV region showed a significant decrease in antigenic reactivity when Val^{81} is mutated to Ala (peptide 12). This data is consistent with the structure of E-cadherin in which His^{79} and Val^{81} are exposed because they are susceptible to mutagenesis. This furthermore supports the importance of the residues flanking HAV because 11 and 12 still show appreciable binding to the anti-E-cadherin-HRP antibody. The HAV region of E-cadherin (ILYSHAVSSNG) has a β-sheet (ILYSHAV) conformation followed by a β-turn at SSNG (8). We studied the secondary structure of this HAV region in N-cadherin (LRAHAVDVNG) by nuclear magnetic resonance (NMR), circular dichroism (CD) and molecular dynamics. These studies show a β-sheet conformation at LRAHAV followed by a βI-turn at DVNG (15). This β-turn conformation and the difference in the amino acid sequence surrounding the HAV region could be important for the selectivity between N- and E-cadherin.

Peptide 9 contains the conserved putative calcium binding sequence Asp^{100}-Gln^{101}-Asn^{102}-Asp^{103}. The EC-1 domain of E-cadherin has been shown to bind calcium

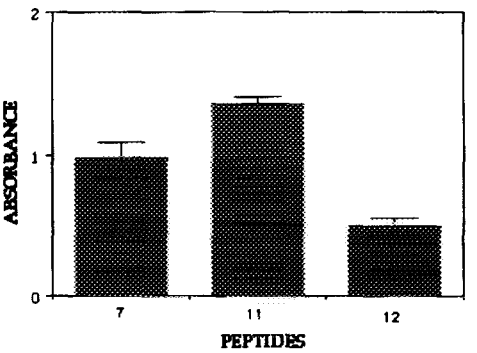

Figure 5. The effect of alanine mutagenesis on anti-E-cadherin-HRP antibody binding to His^{79} and Val^{81} mutated peptides. Peptide 11 is (ILYSAAVSSN) and peptide 12 is (ILYSHAASSN).

(16). It has also been shown that a single mutation in another putative calcium binding region in the amino terminal domain of E-cadherin can completely abolish cell adhesion activity (17). Thus, we were not surprised to observe the high antigenic reactivity of peptide 9. Furthermore, NMR studies indicated that this calcium binding sequence undergoes chemical shift changes between the calcium bound and unbound forms (8). The sequence Pro^{10}-Glu^{11}-Asn^{12}-Glu^{13}-Lys^{14} also undergoes chemical shift changes in the calcium bound forms of EC-1 of cadherin and is suggested to be part of this calcium binding pocket (8). Adjacent to this sequence is Gly^{15}-Glu^{16}-Phe^{17} (peptide 1) which is a loop extending from the proposed calcium binding pocket. The remaining seven residues (17-23) of peptide 1 are incorporated into a β-sheet. When residues 13, 16, and 19 were mutated with their P-cadherin sequence counterparts, the E-cadherin mutant would not react with the anti-E-cadherin antibody suggesting that this region is important for specificity (4). Therefore, 1 may be part of a continuous epitope extending from PENEK and/or may be part of a discontinuous epitope with the proposed calcium binding pocket. The moderate binding of 1 compared to 7 and 9 is probably due to incomplete residues needed for antigenic reactivity (Pro^{10}-Lys^{14}).

We are encouraged that our active peptides' sequences appear to be on the surface of the EC-1 domain and are located in the proposed homophilic specificity region and calcium binding pocket region deduced by Overduin and coworkers (8). This further strengthens the reliability of our immobilized assay. Our previous HAV peptides from E- and N-cadherin displayed similar binding as peptide 7 which also strengthens the reliability of our assay(7). We are currently using our immobilized assay to scan the EC-2 and EC-3 domains of E-cadherin. Determining antigenic reactive sequences will help us elucidate cadherin-cadherin interactions and develop potential cadherin peptide inhibitors of cell-cell adhesion.

In conclusion, we have found a rapid and reliable method to analyze peptide sequences in cadherin which are responsible for cadherin-cadherin interactions. This method can also be applied to other systems for rapid analysis of peptide sequences which are responsible for peptide-receptor interactions.

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