A Novel Family of Cyclic Peptide Antagonists Suggests That N-cadherin Specificity Is Determined by Amino Acids That Flank the HAV Motif*  

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The classical cadherins (e.g. N-, E-, and P- cadherin) are well established homophilic adhesion molecules, however, the mechanism that governs cadherin specificity remains contentious. The classical cadherins contain an evolutionarily conserved His-Ala-Val (HAV) sequence, and linear peptides harboring this motif are capable of inhibiting a variety of cadherin-dependent processes. We now demonstrate that short cyclic HAV peptides can inhibit N-cadherin function. Interestingly, the nature of the amino acids that flank the HAV motif determine both the activity and specificity of the peptides. For example, when the HAV motif is flanked by a single aspartic acid, which mimics the natural HAVD sequence of N-cadherin, the peptide becomes a much more effective inhibitor of N-cadherin function. In contrast, when the HAV motif is flanked by a single serine, which mimics the natural HAVS sequence of E-cadherin, it loses its ability to inhibit the N-cadherin response. Our results demonstrate that subtle changes in the amino acids that flank the HAV motif can account for cadherin specificity and that small cyclic peptides can inhibit cadherin function. An emerging role for cadherins in a number of pathological processes suggests that the cyclic peptides reported in this study might be developed as therapeutic agents.

The classical cadherins are composed of five extracellular domains, a single transmembrane domain, and two cytoplasmic domains (1, 16, 17). The first extracellular domain (ECD1) of these cadherins contains an evolutionarily conserved His-Ala-Val (HAV) motif (6, 18), and several lines of evidence suggest that this sequence is critical for function. In this context, synthetic peptides containing the HAV motif (for example, N-Ac-LRAHAVDING-NH₂) have been shown to be capable of inhibiting cadherin-dependent biological processes, such as myoblast fusion (19), neurite outgrowth (8), and embryo compaction (18). Furthermore, antibodies directed against the HAV sequence are also capable of disrupting cadherin-dependent cell adhesion (5, 20, 21).

Crystal studies have demonstrated that N-cadherin ECD1 monomers will form two types of dimeric structure, one that might reflect a trans adhesion interface, and one that might reflect the formation of a cis dimer (22). Interestingly, the HAV motif is part of a βFG strand that forms one face of the trans adhesion dimer; however, only the histidine and valine make lattice contacts, and these account for less than 5% of the adhesion interface. Furthermore, the alanine at position 80 lines a hydrophobic acceptor pocket for the side chain of tryptophan 2 on a second N-cadherin monomer, and this might underpin the formation of a cis or strand dimer (22). Mutational studies that demonstrate the importance of alanine 80 for stable cell adhesion have been interpreted in support of the hypothesis that formation of the cis dimer is a prerequisite for trans adhesion (23), and this challenges the view that the HAV motif is conserved within cadherins because it is an integral part of the trans adhesion interface (18). However, although there is an emerging view that lateral dimerization of cadherins is required for strong adhesion, structural studies on E-cadherin do not support the strand dimer model (24), and although mutation of alanine 80 inhibits trans binding of E-cadherin, it has no effect on a cis interaction (24). Thus, despite the large number of structural studies and additional mutagenesis data, the precise basis of cadherin homophilic interactions remains contentious (25).

The HAV motif is present in all of the classical cadherins, and it is self-evident that on its own it cannot account for cadherin specificity. One possibility is that the HAV sequence is a primary binding motif for all of the classical cadherins and that selectivity is determined by the flanking amino acids, which differ among the classical cadherins (26, 27). In
the present study we have addressed this issue by testing a large family of small cyclic peptides for their ability to inhibit N-cadherin function.

We have previously demonstrated that neurons extend longer neurites when cultured on a monolayer of transfected 3T3 cells that express physiological levels of N-cadherin, as compared with untransfected 3T3 cells (8, 28, 29). This response is driven by the homophilic binding of N-cadherin in the neurons to the transfected N-cadherin present in the 3T3 cell monolayers, and this model is one of the few quantitative assays that measures a biological response to physiological levels of N-cadherin. In the present study we demonstrate that a simple cyclic HAV peptide can act as an N-cadherin antagonist; however, cyclic HAV peptides containing flanking amino acids found in N-cadherin are more potent inhibitors of N-cadherin function. In contrast, cyclic HAV peptides that contain flanking amino acids found in E-cadherin do not inhibit N-cadherin function. Collectively, these data show that the HAV motif is a functional recognition motif within cadherins and demonstrate the pivotal role played by the flanking amino acids in determining cadherin specificity.

EXPERIMENTAL PROCEDURES

Cell Culture and Neurite Outgrowth Assays—Co-cultures of cerebellar neurons on monolayers of control 3T3 cells and monolayers of transfected 3T3 cells that express physiological levels of chick N-cadherin or human L1 were established as described previously (29). In brief, 80,000 3T3 cells (control and transfected) were plated into individual chambers of an 8-chamber tissue culture slide coated with poly-lysine and fibronectin and cultured in Dulbecco’s modified Eagle’s medium, 10% FCS. After 24 h, when confluent monolayers had formed, the medium was removed, and 3000 cerebellar neurons isolated from postnatal day 2–3 rats were plated into each well in SATO media (30). The medium was removed, and 3000 cerebellar neurons isolated from postnatal day 2–3 rats were plated into each well in SATO/2% FCS. All of the test peptides were added immediately before the neurons as a 2 x stock prepared in SATO/2% FCS. The co-cultures were maintained for 16–18 h, at which time they were fixed and immunostained for GAP-43 which is present only in the neurons and delineates the neuritic processes. The mean length of the longest neurite per cell was measured from ~150 neurons sampled as described previously (29). The percentage inhibition of neurite outgrowth at various peptide concentrations was calculated as the average of at least three independent experiments. Dose-response curves were plotted, and the IC50 values (± S.E.) were determined.

Peptide Synthesis—All peptides were synthesized using the solid-phase method (31, 32). The peptides were assembled on methylenzydylamine resin for the C-terminal amide peptides, and the traditional Merrifield resins were used for the C-terminal acid peptides. Acetylation of the N-terminal was performed by reacting the peptide resins with a solution of acetic anhydride in dichloromethane in the presence of triethylamine. Peptides were generally prepared as a stock solution at a concentration of 5–10 mg/ml and stored in small aliquots at 70 °C. The N-Ac-CHAVDIC-NH2 peptide (where the underlined residue indicates a cyclic peptide) was made up in tissue culture grade DMEM supplemented with 2% FCS. All of the test peptides were added immediately before the neurons as a 2 x stock prepared in SATO/2% FCS. The co-cultures were maintained for 16–18 h, at which time they were fixed and immunostained for GAP-43 which is present only in the neurons and delineates the neuritic processes. The mean length of the longest neurite per cell was measured from ~150 neurons sampled as described previously (29). The percentage inhibition of neurite outgrowth at various peptide concentrations was calculated as the average of at least three independent experiments. Dose-response curves were plotted, and the IC50 values (± S.E.) were determined.

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Molecular Modeling—Molecular modeling was based on crystals of the EC1D of N-cadherin, which have been solved as monomeric structures and also found to exist as dimers with protein-protein interfaces that might reflect a cis dimer interface and a trans adhesion interface (Protein Data Base codes 1DHI and 1DHI) and NMR (Protein Data Base code 1SUH). MSI and Swiss PDB software packages were used to isolate the HAV motifs from the intact structures and to make structural and docking orientation predictions for the cyclic peptides. The percentage accessible surface area for individual amino acids at the adhesion interface of the trans dimer of N-cadherin was calculated by submitting the 1NCH structure to the University College London protein-protein interaction server.
N-cadherin function when tested at 250 μg/ml (see Fig. 2); however, the N-Ac-CHAVIDC-NH₂ was the only peptide that retained activity when tested at 125 μg/ml.

Further Investigation of the Effects of the Flanking Amino Acids at the C-terminal Side of the HAV Motif—The results in Fig. 2 demonstrate that addition of an aspartic acid to the C terminus increases the activity of the simple cyclic HAV peptide, whereas addition of an alanine to the N terminus decreases activity. Based on this result, a number of peptides...
were made that were progressively extended by single amino acids only at the C-terminal side of the HAV motif. Full dose-response curves are shown for the three most active peptides in Fig. 3. The cyclic HAVD peptide was substantially more active than the cyclic HAV peptide (IC50 0.114 ± 0.006 mM as compared with 0.323 mM). Activity was further increased by the addition of an isoleucine to give N-Ac-CHAVDIC-NH2 (IC50 of 0.065 ± 0.005 mM), but there was no further benefit when an asparagine was added (IC50 of 0.110 ± 0.006 mM, data not shown). A peptide with an additional glycine (N-Ac-CHAVDINGC-NH2) was inactive (a 1.5 ± 2.2% inhibition of the N-cadherin response at 125 μg/ml, mean ± S.E., n = 3). There was an approximate 7-fold reduction in the activity of the most active analogue when it was tested as a linear peptide (IC50 of 0.005 mM), but there was no further benefit when an asparagine was added (IC50 of 0.110 ± 0.006 mM, data not shown). A peptide with an additional glycine (N-Ac-CHAVDINGC-NH2) was inactive (a 1.5 ± 2.2% inhibition of the N-cadherin response at 125 μg/ml, mean ± S.E., n = 3). There was an approximate 7-fold reduction in the activity of the most active analogue when it was tested as a linear peptide (IC50 of 0.005 mM), but there was no further benefit when an asparagine was added (IC50 of 0.110 ± 0.006 mM, data not shown). A peptide with an additional glycine (N-Ac-CHAVDINGC-NH2) was inactive (a 1.5 ± 2.2% inhibition of the N-cadherin response at 125 μg/ml, mean ± S.E., n = 3). There was an approximate 7-fold reduction in the activity of the most active analogue when it was tested as a linear peptide (IC50 of 0.005 mM), but there was no further benefit when an asparagine was added (IC50 of 0.110 ± 0.006 mM, data not shown). A peptide with an additional glycine (N-Ac-CHAVDINGC-NH2) was inactive (a 1.5 ± 2.2% inhibition of the N-cadherin response at 125 μg/ml, mean ± S.E., n = 3). There was an approximate 7-fold reduction in the activity of the most active analogue when it was tested as a linear peptide (IC50 of 0.005 mM), but there was no further benefit when an asparagine was added (IC50 of 0.110 ± 0.006 mM, data not shown).

The effect of cyclic E-cadherin peptides on the N-cadherin response. The effects of a family of cyclic E-cadherin peptides (as indicated) on the N-cadherin response was determined as in Fig. 1. The results show the percent inhibition of the N-cadherin response for the peptides tested at a concentration of 250 μg/ml. For comparison, the effect of 125 μg/ml of the most active N-cadherin antagonist is shown (A). Each value is the mean ± S.E. from three experiments. None of the peptides had a significant effect on neurite outgrowth over the control 3T3 cells (data not shown).

DISCUSSION

Despite an extensive series of structural studies on N- and E-cadherin, there remains no consensus on the mechanisms that govern the specificity of cadherin binding (25). Although peptide competition studies support the notion that the HAV motif plays a direct role in binding (8, 18), the peptides that were used in the studies contained a number of amino acids other than HAV, and it could be argued that these might have been responsible for the inhibition. Furthermore, although mutation of alanine 80 inhibits stable trans adhesion, this might be an indirect effect as this mutation can prevent the docking of tropoehan 2 from either the same or a second ECD1 monomer into a hydrophobic pocket lined by alanine 80 (23, 24).

If the HAV motif is directly involved in trans adhesion, peptide mimetics might be expected to act as cadherin antagonists. One way to increase the efficacy of peptide inhibitors is to make them cyclic and thereby constrain structure. In the present study we have shown that a relatively simple cyclic HAV peptide (N-Ac-CHAVC-NH2) can inhibit the N-cadherin component of neurite outgrowth over 3T3 cells in the absence of any effect on the integrin component of growth over control 3T3 cells. This demonstrates that the cyclic peptide is an effective N-cadherin antagonist and suggests that the cyclic peptide can adopt an active binding conformation. Interestingly, a cyclic HAV peptide with a free amino group at the N-terminal region (H-CHAVC-NH2) was inactive, as was a peptide with an alanine → glycine substitution (N-Ac-CHGVC-NH2). These observations highlight the fact that very subtle structural modifications of a cyclic peptide can affect efficacy, presumably by...
constraining the peptide in a manner that restricts adoption of the active configuration.

The specificity of cadherin binding can be dramatically altered by mutating residues that immediately flank the HAV motif in cadherins. For example, if the amino acids at position 2 and 1 (relative to HAV) in E-cadherin are replaced by P-cadherin residues, the mutant protein acquires the ability to bind P-cadherin (26). In the present study we made the observation that specificity can also be built into short cyclic peptides. In this context, the addition of a single amino acid to HAV that results in an N-cadherin-specific sequence (N-Ac-CHAVDC-NH₂) generated a much more potent N-cadherin antagonist (a decrease in the IC₅₀ from 0.32 to 0.114 mM), with a clear benefit being seen on further extending the peptide to N-Ac-CHAVDIC-NH₂ (IC₅₀, 0.064 mM). In stark contrast, modifications to the amino side of the HAV motif had little effect on peptide activity or were detrimental. For example, removal of the acetyl group from the N-Ac-CHAVC-NH₂ or the N-Ac-CAHAVDIC-NH₂ peptides resulted in a complete loss of antagonist activity. Likewise, addition of an alanine to the N-Ac-CHAVDC-NH₂ peptide (to give N-Ac-CAHAVDC-NH₂) also resulted in a loss of activity. Interestingly, addition of an alanine had little effect when added to the N-Ac-CHAVDIC-NH₂ peptide (to give N-Ac-CAHAVDIC-NH₂). Overall, the picture that emerges is one where there appears to be a tussle between the forces on the N and C terminus of the HAV motif in relation to peptide activity, and this initially suggested to us that much of the structure-function relationship between this family of peptides might be accounted for by the precise ordering of the side chains of the histidine and valine within the cyclic peptide (however, see below). The specificity of this class of peptide is reinforced by the observation that the most active N-cadherin antagonists do not inhibit neurite outgrowth stimulated by L1, despite the fact that following the homophilic binding step, both molecules promote neurite outgrowth by activating the same FGF receptor-dependent signal transduction cascade (28, 29).

Our results have shown that when we turn the cyclic HAV peptide into an N-cadherin peptide by incorporating flanking amino acids that are specific to N-cadherin, we can substantially increase the antagonistic properties of the peptide. Remarkably, we find the opposite result when we turn the peptide into an E-cadherin peptide. Whereas addition of N-cadherin amino acids at position −1 or +1 (relative to HAV) has little effect or increases peptide activity, addition of the corresponding amino acids from E-cadherin (to give N-Ac-CSHAVC-NH₂ and N-Ac-CSHAVSC-NH₂) generated peptides that no longer inhibit N-cadherin function. Likewise, three other cyclic HAV
peptides that contained flanking amino acids from E-cadherin had little or no effect on the N-cadherin response. The same panel of cyclic peptides have been tested in assays that measure E-cadherin function, in contrast to the above results the E-cadherin set of peptides inhibit E-cadherin function, and the N-cadherin set of peptides have little or no effect. 

The possibility that the flanking amino acids confer specificity by differentially orienting the side chains of the histidine and valine within E- and N-cadherin is attractive; however, there appears to be no obvious difference in the structure of the HAV motif in E- and N-cadherin (Fig. 6E) nor any evidence for a change in conformation of the HAV motif within N-cadherin following homophilic binding (Fig. 6F). An alternative possibility is that the flanking amino acids contribute directly to the binding energy. In this context, the aspartic acid and isoleucine of the HAVDI motif both contribute to the adhesion face, with the isoleucine in particular accounting for a significant percentage of the interface surface (7.4%). Although this might explain why the cyclic HAVDI peptide is a very good N-cadherin antagonist (IC₅₀ ~0.064 mM) relative to the cyclic HAV peptide (IC₅₀ ~0.32 mM), it does not explain why the cyclic HAVS peptide has no activity when tested at up to 250 μM. One possibility is that flanking amino acids can additionally hinder the docking of the histidine and valine side chains to an inappropriate cadherin.

In summary, a novel family of cyclic peptides containing the HAV motif has been developed as N-cadherin antagonists. Our studies indicate that specific N-cadherin antagonists can be developed based on the incorporation of 1 or 2 flanking amino acids from native N-cadherin onto the HAV motif. The results obtained from this study demonstrate the importance of the flanking amino acids in determining the specificity of cadherin interactions and provide novel insights into the mechanisms that govern the specificity of cadherin binding.

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