The classical cadherins, definitive proteins of the cadherin superfamily, are characterized functionally by their ability to mediate calcium-dependent cell aggregation in vitro. To test hypothetical mechanisms of adhesion, we have constructed two mutants of the chicken E-cadherin protein, one with the highly conserved His-Ala-Val (HAV) sequence motif reversed to Val-Ala-His (VAH), the other lacking the first extracellular domain (EC1). The inversion of HAV to VAH has no effect on the capacity of E-cadherin to mediate adhesion. Deletion of EC1 completely eliminates the ability of E-cadherin to mediate homophilic adhesion, but the deletion mutant is capable of adhering heterophilically to both unmutated E-cadherin and to the HAV/VAH mutant. These results demonstrate that the conserved HAV sequence motif is not involved in cadherin-mediated adhesion as has been suggested previously and supports the idea that in the context of the cell surface, cadherin-mediated cell–cell adhesion involves an interaction of EC1 with other domains of the cadherin extracellular moiety and not the "linear zipper" model, which posits trans interactions only between EC1 on apposing cell surfaces.

Classical cadherins were defined initially by their ability to mediate calcium-dependent cell–cell adhesion (1–5). Both the extracellular and the intracellular portions of the cadherin play critical roles in mediating adhesion (6). Perturbation and expression experiments have demonstrated that cadherin-mediated adhesion promotes a range of cellular processes subsequent to adhesion, including epithelial polarization (7, 8), blastula compaction (2), neurite outgrowth (9, 10), and formation of desmosomes (11, 12) and gap junctions (13–16).

Classical cadherin proteins at the cell surface consist of five extracellular domains (ECs), a transmembrane sequence, and a cytoplasmic domain (Fig. 1). Each cadherin extracellular domain shares a folding topology of seven β-strands arranged in two β-sheets forming a barrel with hydrophobic amino acid side chains largely sequestered within the interior (17–20). The amino and carboxyl termini emerge from opposite ends of each folded domain, maintaining an orientation with the amino terminus directed away from the cell. Conserved amino acid residues that are capable of coordinating calcium ions are present at the ends of each barrel (17–20). Binding of calcium at these sites maintains the structural integrity of the cadherin ECs, giving the protein a rigid conformation that allows it to mediate cell–cell adhesion. A single amino acid substitution in a calcium binding site between EC1 and EC2 or between EC2 and EC3 can abolish adhesion and increase motility, whereas mutations in other calcium-binding elements do not affect these behaviors (21, 22).

Cadherins expressed on adjacent cells homoassociate through an antiparallel, trans interaction of their extracellular regions. The bonds that they form across the intercellular space hold the plasma membranes of adjacent cells in close proximity. Adhesive strength is also dependent on a cis interaction between cadherins of the same cell (23) mediated at least in part by the transmembrane domain (24). These stable interactions can be rapidly disassembled or reorganized in response to extracellular signals such as growth factors (25) and can occur without extreme alterations in the makeup of the adhesive complex or the amount of complex at the junction (26).

The mechanism by which the extracellular domains mediate adhesion is poorly understood. The amino acid residues responsible for the trans interaction are currently unknown, although there is solid evidence that the first domain (EC1) is important in forming specific cadherin interactions (27, 28). Several different crystal structures have yielded different interaction interfaces (18–20, 29), and an NMR solution structure of EC1 indicates that it does not dimerize in solution (17). It has also been suggested that the highly conserved His-Ala-Val motif (HAV) of the first domain is important for binding (30, 31). E-cadherin peptides containing the HAV sequence have been shown to induce epithelial cell invasion, implying that the adhesive region flanks the HAV sequence (32). However, there is doubt that this motif is crucial because many nonclassical cadherins, and some classical ones, do not have HAV but are still capable of trans adhesion (33, 34).

To characterize the role of the HAV motif and of the whole EC1 in adhesion we evaluated the capability of two mutant forms of E-cadherin to mediate adhesion in a set of well defined cell–cell aggregation assays. One mutant has had the conserved HAV motif reversed to VAH, and the other mutant has the entire EC1 domain deleted. The results of these experiments, taken in conjunction with recent reports on human cadherin 4 (R-cadherin) (28) and Xenopus laevis C-cadherin (35), demonstrate that the HAV sequence is not essential for adhesion and that EC1 interacts in trans with EC domains other than EC1 to mediate cell–cell adhesion, consistent with data obtained by

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The abbreviations used are: ECs, extracellular domain(s); CMV, cytomegalovirus; HAV/VAH, mutant with inversion of His-Ala-Val to Val-Ala-His; Δ1, mutant with first extracellular domain deleted; TBS, Tris-buffered saline.
atomic force measurements (36–38) and contrary to the linear zipper model, which is based on the interfaces predicted from crystal structures (18, 20) (Fig. 1A). A, schematic representation of the trans interactions between cadherin molecules in two models of interaction, the linear zipper model (top) and the deep intercalation model (bottom). B, diagram of the unmutated and mutated forms of E-cadherin used in this study, with the ECs represented as ovals. The functional tertiary structure of classical cadherins is maintained by coordination of calcium ions between domains (small ovals between ECs). One adhesive interface is located within the EC1, which contains the highly conserved HAV peptide sequence. C–F, immunofluorescence microscopy was performed on S180L-11 (C), untransfected S180 (D), HAVVAH-1 (E), and ΔI-1 cells (F), using a polyclonal anti-E-cadherin. In all three transfectants the staining is concentrated at the cell surface in regions of cell-cell contact. In the unmutated E-cadherin and the HAVVAH mutants, the contact interfaces have a crenelated appearance that has previously been found to be the result of deep intercalation of interlocked membrane processes (arrows in C and E). The ΔI mutant cell lines do not appear to form these contact structures (arrow in F). The scale bar is 10 μm.

MATERIALS AND METHODS

Cell Line—The S180 cell line was originally derived from the axial tip of a transplanted mouse sarcoma (39). S180 cells are spindle-shaped, do not undergo calcium-dependent cell-cell adhesion, and do not express any known cadherin proteins, making them ideal for observing the effects of cadherin transfection. S180 cells and all transfectants were grown in Dulbecco’s modified Eagle’s medium with 15% fetal calf serum in a 10% CO₂ incubator at 37 °C. For passaging, cells were released from the plate by incubation with PBS (150 mM NaCl, 2 mM Na₂HPO₄, 10 mM NaH₂PO₄, pH 7.4) and 5 mM EDTA at 37 °C, counted using a Coulter counter, and replated in culture medium at desired cell densities, which released them from the plates as single cells, counted using a Coulter counter, and replated in culture medium at desired cell densities, usually 10⁵ cells/10-cm culture dish in 10 ml of medium.

Construction of E-cadherin Expression Plasmid—The 5’-end of the E-cadherin reading frame was amplified from chicken liver cDNA using primers WJG1003 and WJG1004 (Table I) and digested with Nhel and Xhol. An Xhol/BamHI fragment containing the majority of the E-cadherin coding region was isolated from plasmid pEC320 (40). pBK-CMV expression vector (Stratagene) was modified by digestion with EcoRI and KpnI followed by polishing with mung bean nuclease and ligation to remove part of the polylinker. The resulting vector (pBK-CMV-E/K) was digested with Nhel and BamHI, and a three-fragment ligation was performed. The entire insert of the resulting plasmid was sequenced completely to confirm that the plasmid had the correct sequence. A schematic drawing of the mature protein encoded by this plasmid is shown in Fig. 1B.

Construction of HAVVAH Plasmid—The HAVVAH mutant was created by overlapping PCR mutagenesis (41). Two fragments were amplified from unmutated plasmid template using primers WJG1034 and WJG1036, and primers WJG1045 and WJG1042 (Table I). The resulting PCR products were gel purified, and a mixture was used as a template, amplified with primers WJG1034 and WJG1042. The resulting PCR product was digested with Xhol and KpnI, ligated into the wild-type plasmid that had also been digested with Xhol and KpnI, and a cloned plasmid was isolated. The sequence of the resulting insert was determined to confirm that only the designed mutation, inversion of the HAV sequence to VAH, had been introduced into the E-cadherin sequence. A schematic drawing of the mature protein encoded by this plasmid is shown in Fig. 1B.

Construction of ΔI Plasmid—The ΔI mutation was created by overlapping PCR mutagenesis (41). As described for the ΔI mutant, using primers WJG1034 and WJG1046 and primers WJG1047 and WJG1042. There were two undesigned mutations found, one converting an arginine to a proline in the propeptide sequence and the other converting valine to isoleucine in the second extracellular domain. A schematic drawing of the mature protein encoded by this plasmid is shown in Fig. 1B.

Production of Stable Transfectants—S180 cells were transfected with plasmids using an optimized calcium coprecipitation method (42) and selected in medium supplemented with 400 μg/ml Geneticin (G418, Invitrogen). Two weeks after transfection, cells were selected for their ability to express high levels of E-cadherin using magnetic activated cell sorting (Miltenyi Biotec). Cells were released from the culture plate and incubated with rabbit anti-E-cadherin antibody (40 μg/ml) in PBS, 2 mM EDTA, 0.5% (w/v) bovine serum albumin, then with magnetic beads coupled to goat anti-rabbit antibody (20 μl of bead suspension/10⁶ cells). Cells expressing E-cadherin were retained on a high gradient magnetic separation column and then rinsed from the column upon removal of the magnetic field. Clones were isolated from the separated cells by limiting dilution. Expression of E-cadherin was confirmed by immunofluorescent staining with a polyclonal rabbit anti-E-cadherin antibody. Only a single clone from each primary transfection plate was saved, ensuring that the clones are independently derived. Cultures of stable transfectants were maintained in 200 μg/ml G418 in Dulbecco’s modified Eagle’s medium and 15% (w/v) fetal calf serum.

Verification of the Transfectants—Genomic DNA was isolated from confluent 10-cm dishes of S180, S180L-11, HAVVAH-1, HAVVAH-2, HAVVAH-4, ΔI-1, and ΔI-2 cells. DNA was isolated from the cells (43), and E-cadherin sequences were amplified from the DNA of each cell line by PCR, using primers WJG1024 and WJG1042. PCR products were purified and sequenced.

Two unplanned mutations are present in the sequence of the ΔI construct. In the precursor sequence a Pro was substituted for an Arg residue. This may have an effect on processing of the protein and transport to the membrane. However, the mutant E-cadherin is expressed at the cell surface (Fig. 1F) and is largely correctly processed by cleavage of the precursor peptide (Fig. 2A). The second mutation is the 44th amino acid in the second domain in which Ile is substituted for Val. This mutation is unlikely to be significant because the residue that was replaced has properties similar to the one that was replaced (a large,
The unmutated E-cadherin and the HAVVAH mutant comigrated, indicating that the simple inversion of amino acid sequence has no effect on post-translational processing of the protein. The Δ1 mutant protein has two bands, one migrating more slowly and one more rapidly than the unmutated E-cadherin. C. Western blot of cell surface-exposed proteins isolated by surface biotinylation and avidin precipitation. Neither the HAVVAH mutation nor the Δ1 mutation prevents transport of E-cadherin to the plasma membrane. The processed (lower molecular weight) Δ1 E-cadherin is preferentially transported to the cell surface.

branched, nonpolar side chain). The side chain faces outward from the domain, not into the hydrophobic interior of the globular domain, so there is no effect on side chain packing in the interior of the domain, and the mutated residue is at a site that is highly variable in other domains and other cadherins (alignment not shown) and which does not correlate with adhesive specificity or subfamily identity.

Antibodies—Polyclonal goat antibody was raised against the trypsin-released extracellular fragment of chicken E-cadherin. Polyclonal rabbit antibody was raised against a fusion protein encompassing most of the E-cadherin extracellular region. Fab' fragments were prepared by overnight digestion of the whole IgG with papain followed by treatment with β-mercaptoethanol and iodoacetamide (44).

Immunofluorescent Staining—Cells were grown on glass coverslips. Confluent and subconfluent cultures were fixed with 4% paraformaldehyde in PBS, 0.5 mM CaCl₂, 0.5 mM MgCl₂, at room temperature for 15–30 min and quenched with 0.1 M glycine in PBS. Cells were permeabilized and further blocked in Tris/PO₄/carrageenan/Triton X-100 (41 mM Tris, 4.4 mM Na₂HPO₄, 1.8 mM NaH₂PO₄, 120 mM NaCl, 0.5% (v/v) Triton X-100, 0.7% (v/v) Lambda Carrageenan, 30 mM NaCl) (45), followed by incubation overnight with 4 μg/ml rabbit anti-E-cadherin IgG or 30 μg/ml goat anti-E-cadherin IgG in Tris/PO₄/carrageenan/Triton X-100 solution, five 10-min washes with Tris/PO₄, a 1-h incubation with fluorescein isothiocyanate-conjugated or Texas Red-conjugated secondary antibodies, and five 10-min washes with Tris/PO₄.

Coverslips were mounted on slides with MOWIOL/DABCO (46) and examined by epifluorescence microscopy and confocal microscopy.

Western Blotting—Confluent cultures were rinsed with 10 ml of TBS, 1 mM phenylmethylsulfonyl fluoride and extracted with 1 ml of 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 62.5 mM Tris-HCl, pH 6.8, and the resulting extract was stored frozen at -20 °C. 200 μl of the SDS extract was mixed with 1 ml of HS buffer (0.1% SDS, 1% sodium deoxycholate, 0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 10 mM EDTA) and clarified by centrifugation for 30 min at maximum speed in a microcentrifuge. The clarified supernatant was transferred to a fresh microcentrifuge tube with 50 μl of 1:1 slurry of avidin-coupled agarose beads (Pierce). The samples were mixed by gentle rocking at 4 °C for 2 h, and then the beads were pelleted and washed once with HS buffer, once with high salt buffer (HS buffer with 1 mM NaCl), and once with low salt buffer (2 mM EDTA) (45). The beads were then incubated in 25 μl of 2× Laemmli SDS sample buffer, 100 mM dithiothreitol, heated in a boiling water bath for 5 min, and 20 μl of the resulting extract was resolved on a 6% Laemmli SDS gel and visualized by Western blotting.

Aggregation Assays—Aggregation assays were performed according to Hoffman (48). Cells were released from dishes with PBS, 5 mM EDTA, 2% (v/v) fetal calf serum and incubated on ice in Eagle’s Spinner medium (Invitrogen) for 1 h to allow full dissociation. Aliquots of 2 × 10⁶ cells were incubated with anti-E-cadherin or nonimmune Fab' fragments (300 μg of Fab' fragments/2 × 10⁶ cells) in HDBF buffer (137 mM NaCl, 5 mM KCl, 5.5 mM glucose, 4 mM NaHCO₃, 2 mM EDTA, pH 7.5) in a minimum of 30 h on ice. The assay was initiated by suspending the cells to a final volume of 2 ml in prewarmed Eagle’s medium, transferring them to glass scintillation vials, and shaking at ~80 rpm at 37 °C. At 0, 20, 40, and 60 min after the start of incubation, aliquots of cell suspension were fixed with 1% glutaraldehyde in PBS, pH 7.5. Aliquots of the cell suspension were counted by diluting 1:20 into Isoton II (Beckman/Coulter Electronics) and counting particles within the range of single suspended S180 cell sizes (10–20 μm) with a Z2 Coulter counter. Percentage aggregation for each vial was calculated by the formula 100 × (Nₐ/N₀ − Nₛ/N₀), where Nₛ equals number of particles at time 0, and N₀ is the number of particles at the time the cells were sampled. Each point on each graph represents the average of three replicate wells, three cultures, treated with the same Fab'. Each assay was performed at least three times.

Coaggregation Assays—Coaggregation was performed, with slight modifications, according to Friedlander et al. (49). Cells were loaded with a 3 mg/ml solution of either Texas Red-labeled fixable dextran or fluorescein isothiocyanate-labeled fixable dextran using the Influx method (Molecular Probes). Cells were then maintained in normal medium until harvesting for the experiment (usually ~8 h). Labeled cells were released from the culture dish with PBS and 5 mM EDTA. After counting, cells were diluted to 10⁶ cells/50 μl in HDBF with appropriate Fab' fragments (20 μg/10⁶ cells). The assay was initiated by combining both cell types in a final volume of 700 μl with minimum EDTA medium and incubating for 1 h at 37 °C on aagua platform shaker. The cell suspension was pipetted off and washed on a rotary shaker at ~80 rpm. Cells were fixed after the 60-min incubation period by addition of the aliquots to 4% (v/v) paraformaldehyde PBS, pH 7.5. To calculate overall levels of aggregation, aliquots were taken from each well at times 0 and 60 min and evaluated using a Coulter counter, as above. Preliminary experiments indicated that the maximum of aggregation was achieved in 1–2 h of incubation, leaving essentially no visible aggregates to score. Also, if S180 aggregation was high, it was attributable to excess cellular debris aggregating cells in a cadherin-independent manner. When the negative control was contaminated in this way, the experiment was abandoned because any of the other wells could be contaminated in the same way.

The number of Texas Red-labeled cell aggregates in a cell cluster was counted and expressed as a percentage of the total number of cells in that cluster. Comparisons of percentages of the three replicate wells were performed using single factor analysis of variance. Data from 20 coaggregates from three replicate wells were pooled and graphed on a
histogram with bin sizes of 10%. Selected clusters were also photographed using confocal microscopy.

**RESULTS**

_Characterization of Transfectants—_S180 cells were transfected with the plasmids encoding wild-type E-cadherin (S180L), the mutant with the His-Ala-Val sequence inverted to Val-Ala-His (HAV/VAH), and the mutant with deletion of EC1 (Δ1). Three clonal cultures of unmutated E-cadherin transfec-
tants, three clonal cultures of HAV/VAH transfec-
tants, and two clonal cultures of Δ1 transfec-
tants were isolated for study. PCR of genomic DNA from each line yielded the expected PCR product. The identity of the transfected construct was confirmed by direct sequencing of the PCR products (data not shown).

S180 cells at low density appear spindle-shaped, usually with filopodia spreading on substrate. When the culture reaches confluence, the cells become packed together, forming more than one layer of cells. S180 cells transfected with E-
cadherin (S180L) retain the tendency to form multiple layers, but the layers tend to be sheets of cells that are connected through cadherin-mediated adhesion. Immunofluorescence re-
veals that wild-type E-cadherin localizes to the plasma mem-
brane at sites of cell-cell interaction (Fig. 1C). Contact regions are somewhat flattened with distinctive spikes of concentrated staining that indicate deep intercalation of the apposing adhe-
sive membranes, as described previously for another S180 E-
cadherin-transfected cell line (13). HAV/VAH-1, -2, and -4 have the same morphology as S180L cells, in that the fluorescence is present in intense spiky structures along flattened areas of contact between adjacent cells (Fig. 1E).

Δ1 cadherin mutant protein is localized to the plasma mem-
brane at regions of contact, but not as intensely as the unmu-
tated E-cadherin and HAV/VAH clones (Fig. 1F). In particular, the spiky areas of intense staining that represent convoluted intercalations of adhering cell surfaces are absent in these cell lines, although uneven staining on free edges of the cells is observed.

Western blots were performed to confirm that protein of the correct size was synthesized and to compare protein expression levels between the various transfectants (Fig. 2). The HAV/
VAH mutant protein comigrates with the unmutated E-
cadherin, indicating that the mutation did not interfere with the post-translational modification of the protein. Two distinct bands are visible in both Δ1 cadherin transfec-
tants. One migrates faster than wild-type E-cadherin and is consistent with the expected difference in mobility of the fully processed mu-
tant protein. Note that the difference in molecular mass does not appear to be the expected 12 kDa; however, the mobility of the cadherins in Laemmli gels is consistently anomalous (40, 50–52), so the relative mobility cannot be taken to be an accu-
rate reflection of molecular mass. The slower migrating species of Δ1 likely represents Δ1 precursors, with the ~10-kDa propep-
tide uncleaved. It is possible that the unintentional substitu-
tion of Pro for Arg in the precursor sequence has affected Δ1 post-translational processing. However, a similar defect in processing has recently been reported (35) in an EC1 and EC2 deletion of _Xenopus laevis_ C-cadherin without any changes in the propeptide sequence, so this incomplete cleavage may be solely the result of an artifact fusing the propeptide to an internal site in the cadherin protein sequence. We have also previously seen increased levels of the precursor form in other constructs that have had part of the propeptide altered by replacement of the propeptide and part of the propeptide with the amino terminus of the neural cell adhesion molecule (N-
CAM) (52). Note that the presence of the uncleaved form of the full-length E-cadherin had no effect on the ability of the cleaved form to mediate adhesion (52), i.e. this uncleaved protein does not manifest a dominant negative phenotype. Also (see below), the presence of the uncleaved precursor form does not pre-
vent coaggregation with cells expressing full-length E-cadherin.

Surface biotinylation of the cells, followed by avidin selection and Western blotting (Fig. 2C), indicates that the HAV/VAH mutation does not prevent transport of the mutant protein to the cell surface. The Δ1 mutation also does not prevent expres-
sion of E-cadherin at the cell surface, even in the presence of the uncleaved precursor form; quantitative scanning of band intensity indicates that the surface-exposed E-cadherin is in fact enriched in the cleaved form, suggesting that the uncleaved form may be retained preferentially inside the cell until it is either cleaved or degraded.

Quantitation of band intensities from several independent Western blots demonstrated that the amount of E-cadherin did not vary by more than a factor of 2 between any of the trans-
fected cell lines used in this study, usually less (Table II). Significantly, the levels of Δ1 mutant protein are generally equal to or higher than that of the unmutated protein, so the lack of aggregation observed between the Δ1 mutant-express-
ing cells (see below) cannot be caused simply by low cadherin expression levels.

**Effect of the HAV/VAH Mutation on Homophilic Cell Adhe-
sion—_**Short term aggregation assays are functional tests of cell adhesion activity. Although immunofluorescent staining in a monolayer can mean that the cadherin is more concentrated at the lateral plasma membrane, indicating a possible adhesive interaction, it is uninformative about the ability of the cells to initiate and maintain stable adhesive contact under the stress of shearing forces. Thus, the more stringent aggregation assay is necessary to evaluate the functional capacity of the two mutants.

HAV/VAH cells are highly adhesive, usually aggregating to the level of wild-type E-cadherin-expressing clones, or higher, within 60 min. Fig. 3A depicts the result of a typical aggrega-
tion assay of HAV/VAH cells, compared with S180L cells and untransfected S180 cells. Three independently derived clones aggregated to consistently high levels as exemplified by the graph in Fig. 3A. The observed adhesion in all three HAV/VAH clones was E-cadherin-dependent because preincubation of the cells with anti-E-cadherin Fab’ fragments consistently reduced aggregation to a level comparable with the untransfected S180 cells (~10%). Table III shows the levels of aggregation at 60 min in representative assays of each clone. Pairwise comparis-
ons of the mutant transfectants with S180 and unmutated E-cadherin were made incorporating data from the three inde-
pendent replications of the experiments for each clone.

Although the HAV/VAH mutant E-cadherin is capable of mediating homotypic interactions resulting in aggregation, it is still possible that the mutation may have impaired their ability to cross-adhere with the wild-type E-cadherin. Mutation of

| Cell line | Relative expression |
|-----------|---------------------|
| L-CAM-11  | 1.00                |
| L-CAM-13  | 1.45                |
| L-CAM-14  | 1.60                |
| HAV-1     | 1.04                |
| HAV-2     | 1.08                |
| Δ1-1 upper | 0.79                |
| Δ1-1 lower | 1.32                |
| Δ1-2 upper | 0.28                |
| Δ1-2 lower | 0.76                |
with that in the presence of nonimmune antibodies. and the aggregation in the presence of specific antibodies is comparable in inhibited by specific antibodies.

tion is comparable with that of unmutated E-cadherin and is completely panel/ function-blocking epitopes of anti-E-cadherin Fab’ fragments (right panel). A, aggregation of mutant HAV/VAH-1 (▲). HAV/VAH aggregation is comparable with that of unmutated E-cadherin and is completely inhibited by specific antibodies. B, aggregation of mutant clone ΔI-1 (▲). ΔI fails to aggregate significantly more than untransfected S180 cells, and the aggregation in the presence of specific antibodies is comparable with that in the presence of nonimmune antibodies.

Results of representative aggregation assays

| Exp. no. | Cell line | Nonimmune Fab’ fragments | Anti-E-cadherin Fab’ fragments |
|----------|-----------|---------------------------|-------------------------------|
| 1        | HAV/VAH-1 | 55 +/- 2                  | 8 +/- 14                      |
| 1        | S180L-11  | 57 +/- 4                  | 7 +/- 7                       |
| 1        | S180     | 12 +/- 2                  | 8 +/- 3                       |
| 2        | HAV/VAH-2 | 69 +/- 7                  | 11 +/- 5                      |
| 2        | S180L-11  | 38 +/- 4                  | 15 +/- 4                      |
| 2        | S180     | 11 +/- 3                  | 7 +/- 9                       |
| 3        | HAV/VAH-4 | 54 +/- 4                  | 5 +/- 4                       |
| 3        | S180L-11  | 45 +/- 3                  | 20 +/- 3                      |
| 3        | S180     | 14 +/- 4                  | 7 +/- 7                       |
| 4        | ΔI-1      | 7 +/- 1                   | 15 +/- 7                      |
| 4        | S180L-11  | 45 +/- 3                  | 5 +/- 6                       |
| 4        | S180     | 1 +/- 3                   | 2 +/- 4                       |
| 5        | ΔI-2      | 12 +/- 5                  | 18 +/- 5                      |
| 5        | S180L-11  | 35 +/- 2                  | 5 +/- 8                       |
| 5        | S180     | 7 +/- 7                   | 14 +/- 4                      |

* After 60 min.

HAV to VAH might not disrupt the ability of the cadherin to mediate adhesion if the HAV motif interacts only with the HAV motif on the complementary binding surface. However, if this were the case, then the HAV/VAH mutant protein would not be able to interact with the unmutated E-cadherin, which would result in an inability to coaggregate with cells transfected with the unmutated E-cadherin.

The HAV/VAH clones were able to coaggregate with S180L lines (Fig. 4 and Table IV). They did not coaggregate with untransfected S180 cells. Coaggregation with S180L lines was inhibited by anti-E-cadherin Fab’ fragments, showing that this aggregation was cadherin-dependent. HAV/VAH coaggregated with S180L in a 1:1 ratio, indicating that the heterotypic adhesion between HAV/VAH-expressing cells and unmutated E-cadherin-expressing cells was as probable as the homotypic

Results of representative co-aggregation assays

| Exp. no. | Texas Red-labeled cells/ fluorescein-labeled cells | % Texas Red-labeled cells/ total cells in a cluster |
|----------|---------------------------------------------------|-----------------------------------------------|
| 1        | HAV-1/HAV-1                                       | 51                                            |
| 1        | HAV-1/S180L-11                                    | 56                                            |
| 1        | HAV-1/S180                                        | 85                                            |
| 1        | HAV-1/ΔI-1                                        | 87                                            |
| 2        | HAV-2/HAV-2                                       | 70                                            |
| 2        | HAV-2/S180L-11                                    | 70                                            |
| 2        | HAV-2/S180                                        | 57                                            |
| 3        | HAV-3/HAV-4                                       | 45                                            |
| 3        | HAV-3/S180L-11                                    | 45                                            |
| 3        | HAV-3/S180                                        | 90                                            |
| 3        | HAV-3/ΔI-2                                        | 61                                            |
| 3        | HAV-3/ΔI-2                                        | 85                                            |
| 3        | HAV-3/ΔI-2                                        | 67                                            |
| 3        | HAV-3/ΔI-2                                        | 67                                            |

with S180L in a 1:1 ratio, indicating that the heterotypic adhesion between HAV/VAH-expressing cells and unmutated E-cadherin-expressing cells was as probable as the homotypic
adhesion. Also, when the three HAVVAH mutant clones were coaggregated with S180 cells, HAVVAH cells constituted 90–100% of each aggregate, indicating that the presence of a functional cadherin molecule was required for the coaggregation with HAVVAH to occur.

Effect of the ΔI Mutation on Coaggregation—Unlike HAV/VAH, the ΔI mutant protein does not detectably mediate aggregation in a short term suspension assay (Fig. 3B). The level of aggregation of cells expressing the ΔI mutant protein was indistinguishable from untransfected S180 cells, regardless of whether the cells were preincubated with anti-E-cadherin or nonimmune Fab’ fragments. Statistical analysis of the triplicate replications of the aggregations assays of cells expressing ΔI confirms these conclusions (Table III). The inability of ΔI to aggregate is consistent with previous data indicating a crucial role for EC1 in cell-cell adhesion (27).

The commonly accepted model for cadherin-mediated cell-cell adhesion, the linear zipper model (18), involves a trans interaction between the first domains of cadherins of adjacent cells. However, recent evidence suggests that the trans interaction is more deeply intercalated, involving interactions between EC1 and EC5, implying the possibility of two or more adhesive contact surfaces on each cadherin molecule (36–38). The fact that the ΔI deletion E-cadherin does not mediate cell-cell adhesion is not sufficient evidence to distinguish these two models; that result only indicates that EC1 participates in the strong interaction of adhesion, not that it necessarily binds to itself.

When cells expressing the ΔI mutant E-cadherin are incubated with either cells expressing unmutated E-cadherin or cells expressing the HAVVAH mutant E-cadherin they coaggregate, at levels that are significantly higher than the background levels of untransfected S180 cells (Fig. 4 and Table IV). Unlike cells expressing the HAVVAH mutant E-cadherin, however, the clusters that formed during these experiments usually contained 10–40% ΔI cells. Thus, the ΔI-mediated adhesion to fully functional E-cadherin is less favorable than the wild-type, implying that the interaction between ΔI and wild-type E-cadherin is not as strong or stable as the interaction between two wild-type cells. ΔI also interacts with HAVVAH to the same extent it does with wild-type E-cadherin (10–40% ΔI in a cluster). Coaggregation of HAVVAH with ΔI has a peak range that is distinct from the negative control, S180 and the positive control, HAVVAH.

**DISCUSSION**

Cadherins are responsible for calcium-dependent interactions between membranes of adjacent cells. A combination of cis interactions between cadherins on the same cell and trans interactions between cadherins on apposing cells is necessary for productive cell-cell binding. However, it is unclear how the extracellular domains are involved in mediating these interactions. There is considerable evidence that isolated extracellular domains do not interact in solution in a way that mimics the trans adhesive interaction (17, 35). Cells expressing mutant cadherins that have had the intracellular region that is responsible for β-catenin binding deleted do not aggregate (6). However, if a cadherin construct that can be inducibly dimerized through cytoplasmic domain interactions is expressed in Chinese hamster ovary cells, it will mediate adhesion in a dimer-dependent manner, indicating that sufficient affinity for cell aggregation depends on cadherins having a multimeric form at the cell surface (53). Thus, the function of cadherins critically depends on the cellular context in which they are expressed.

To date, structure-function analysis of the interaction interfaces of cadherins has largely focused on EC1. This domain was shown to be important in defining the specificity of the trans interaction (27) in aggregation assays. A highly conserved tripeptide HAV, located in the first domain, has been proposed to be a cadherin-type adhesion recognition peptide, similar to the RGD sequence in fibronectin (30, 31). Also, the first crystal structure of an EC1 domain, solved for N-cadherin EC1, had two extensive interfaces of interaction in the crystal form and a strand interchange of the amino terminus between adjacent domains (18). The combination of these results led to a generally accepted idea that the trans interaction between cadherins was based on an EC1-EC1 interaction between cadherins on apposed cells, the linear zipper model (18). This model was strengthened substantially by the recent determination of the crystal structure of the C-cadherin ectodomain, comprised of all five EC domains, which has a strand interchange between EC1 domains in an apparently trans orientation. However, atomic force measurement data (36–38) have provided evidence for an alternative hypothesis, that cadherins are fully intercalated, with trans interactions between EC1 and EC5, and possibly between EC2 and EC4, and EC3 and EC3. The atomic force data also indicate that there can be significant interactions between domains EC1 and EC4, and possibly EC2 and EC3.

This deep intercalation model also predicts an intermembrane distance that is much closer to that observed in vivo than that predicted by the linear zipper model.

Our experiments were designed to test these hypotheses of cadherin interaction mechanism in the context of intact cells. The cellular context is particularly important because there is ample evidence that the individual cadherin-cadherin interactions are energetically very weak and that the strong cadherin-dependent cell-cell interactions depend on a high level of cooperativity that is controlled by the organization of a large number of cadherin monomers at the adhesive surface. The fact that multiple interaction interfaces are found in different crystal structures suggests that there are several interaction surfaces that can mediate interactions between the cadherin monomers during the process of crystallization but that are not relevant to in vivo cadherin interactions. Therefore, the various models of interaction that are derived from crystallographic studies must be validated in a cellular context.

Two mutations of E-cadherin were constructed: one was a mutant with the putative trans binding site HAV (residues 78, 79, and 80 of the mature protein) inverted to VAH and the other, a clean deletion of the first extracellular cadherin domain at a highly conserved interdomain proline residue (54). These mutants and an unmutated construct were transfected into S180 cells. Both HAVVAH and ΔI mutant proteins were expressed at the plasma membrane and were concentrated at areas of cell-cell contact. Thus, we have created cell lines that differ only in the extracellular structure of the cadherin expressed on their surfaces.

The short term suspension aggregation assay is the definitive test for cell adhesion activity (3, 55, 56). Results of the aggregation and coaggregation assays revealed that the strength and specificity of adhesion of HAVVAH-transfected cells are indistinguishable from those of the unmutated E-cadherin transfectants (Figs. 3 and 4). If HAV is an essential part of the adhesive interface, then there are two possible scenarios: it must interact directly either with the HAV of the cadherin of an adjacent cell or with some other part of the ectodomain.

In the first scenario, direct HAV-HAV interaction, HAVVAH mutant cells could be expected to aggregate with other HAVVAH cells because the adhesive interface could still be complementary (i.e. His still interacts with Val, Ala with Ala, and Val with His). In this case, however, HAVVAH would not be able to associate with unmutated E-cadherin, without the complemen-
In addition, it has been shown recently that a peptide based on the HAV motif is conserved in most of the characterized classical cadherins of vertebrates. Clearly, any selection pressure to maintain this motif is based on conservation of some function other than primary adhesion. One likely possibility is that this motif is involved in postadhesion signaling. Treatment of cell cultures with peptides including the HAV motif and adjacent sequence can inhibit a number of processes that occur consequent to adhesion, including embryo compaction and neurite outgrowth (30, 57, 58), and it has been suggested that the HAV motif is essential for postadhesion signaling, via a trimeric G protein and calcium channels, which cause neurite outgrowth (57, 58). In addition, it has been shown recently that a peptide based on the homologous motif in desmosomal cadherins will interfere with desmosome-dependent cell-cell signaling (59).

Recently, two lines of research have indicated a role for N-cadherin EC4 domain in cell signaling. Williams et al. (60) have identified a motif in EC4 of N-cadherin which participates in N-cadherin-dependent activation of fibroblast growth factor receptors. Also, Kim et al. (61) found that EC4 of N-cadherin is essential to the ability of N-cadherin to cause an epithelial to mesenchyme transition when N-cadherin is expressed in epithelial cells; replacement of EC4 in N-cadherin with EC4 from E-cadherin abolishes the transition-inducing activity. In a deep intercalation model of adhesion, the HAV sequence of EC1 is present near EC4 in the adhering surface. Thus, it is plausible that the highly conserved motif may participate not in adhesion, but in a cadherin-dependent signaling pathway that functions in parallel to the well studied cytoplasmic domain/β-catenin pathway. That EC4 swapping from E-cadherin to N-cadherin abolishes the N-cadherin transition activity and that forced expression of E-cadherin can counteract the N-cadherin activity (11) suggest that there may be a cadherin-type specificity in cell signaling which is based on structural elements that are different from those that are responsible for adhesive specificity.

The cadherins were initially identified by their primary role in mediating calcium-dependent cell-cell adhesion in vitro. Subsequent analyses of their role in cell-cell interactions in tissue in vitro and in vivo have shown that a number of intercellular signaling systems include or are modulated by cadherin interactions. Elements of the extracellular portion of the cadherin protein which are highly conserved are not necessarily involved in the adhesive interaction, however. Identification of amino acid ensembles that are highly conserved but that are not essential for adhesion is a first step in defining how the extracellular portion of the cadherins may mediate cell-cell signaling.

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REFERENCES
1. Hyafil, F., Babinet, C., and Jacob, F. (1983) Cell 26, 447–454
2. Hyafil, F., Morello, D., Babinet, C., and Jacob, F. (1989) Cell 21, 927–934
3. Gallin, W. J., Edelman, G. M., and Cunningham, B. A. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 5038–5042
4. Ogou, S.-I., Yoshida-Noro, C., and Takeichi, M. (1987) J. Cell Biol. 97, 944–948
5. Ogou, S.-I., Okada, T. S., and Takeichi, M. (1987) Dev. Biol. 129, 521–528
6. Nagafuchi, A., and Takeichi, M. (1988) EMBO J. 7, 3679–3684
7. Gumbiner, B., and Simons, K. (1986) J. Cell Biol. 102, 457–468
8. Nelson, W. J., Shore, E. M., Wang, A. Z., and Hammerton, R. W. (1990) J. Cell Biol. 110, 340–357
9. Drabza, J., and Lemmon, V. (1990) Dev. Biol. 138, 82–93
10. Tomaselli, K. J., Neugebauer, K. M., Bixby, J. L., Lilien, J., and Reichardt, L. F. (1988) Neuron 1, 53–63
11. Lai, Z., Gallin, W., and Pasdar, G. (1998) J. Cell Sci. 111, 1005–1019
12. Marrs, J. A., Andersson-Fisone, C., Jeong, M. C., Cohen-Gould, L., Zurzolo, C., Nabi, I. R., Rodriguez-Boulan, E., and Nelson, W. J. (1995) J. Cell Biol. 129, 507–519
13. Mege, R. M., Matsuzaki, F., Gallin, W. J., Goldberg, J. I., Cunningham, B. A., and Edelman, G. M. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 7274–7278
14. Nakamura, S., Cunningham, B. A., Edelman, G. M., and Goodenough, D. A. (1990) J. Cell Biol. 111, 2077–2088
15. Musil, L. S., and Goodenough, D. A. (1991) J. Cell Biol. 115, 1357–1374
16. Matsuzaki, F., Mege, R. M., Jaffe, S. H., Friedlander, D. R., Gallin, W. J., Goldberg, J. I., Cunningham, B. A., and Edelman, G. M. (1990) J. Cell Biol. 110, 1239–1252
17. Overduin, M., Harvey, T. S., Bagby, S., Tang, K. I., Yau, P., Takeichi, M., and Ikura, M. (1995) Science 267, 386–389
18. Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lehmann, M. S., Grubel, G., Legrand, J. F., Als-Nielsen, J., Colman, D. R., and Hendrickson, W. A. (1995) Nature 374, 327–337
19. Nagar, B., Overduin, M., Ikura, M., and Rini, J. M. (1996) Nature 380, 360–364
20. Boggon, T. J., Murray, J., Chappuis-Flament, S., Wang, E., Gumbiner, B. M., and Shapiro, L. (2002) Science 296, 1308–1313
21. Ozawa, M., Engel, J., and Kemler, R. (1990) Cell 63, 1033–1038
22. Handschuh, G., Luber, B., Hutxter, P., Hufer, H., and Becker, K. F. (2001) J. Mol. Biol. 314, 445–454
23. Brieher, W. M., Yap, A. S., and Gumbiner, B. M. (1996) J. Cell Biol. 135, 487–496
