Biochemical Characterization and Functional Analysis of Two Type II Classic Cadherins, Cadherin-6 and -14, and Comparison with E-cadherin*

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Classic cadherins can be grouped based on their deduced primary structures. Among them the type I cadherins have been well characterized; however, little is known about non-type I cadherins. In this study we characterized two human type II cadherins, cadherin-6 and cadherin-14, using a cDNA transfection system. They were each detected as two bands electrophoretically, were expressed on the external cell surface at cell-cell contact sites, and were associated with catenins. Direct sequencing of the N-terminal amino acids showed that the two bands of cadherin-14 corresponded to precursor and mature forms, whereas the two bands of cadherin-6 both had the N-terminal sequence of the mature form. Unlike type I cadherins, both cadherin-6 and -14 were not protected from trypsin degradation by Ca2⁺. We evaluated their adhesive functions by a long term cell aggregation method. The results suggest that both cadherin-6 and -14 have cell-cell binding strengths virtually equivalent to that of E-cadherin and that their binding specificities are distinct from that of E-cadherin. Cadherin-6 and -14 interacted with each other in an incomplete manner. They have a QAI tripeptide in the first extracellular subdomain instead of the HAV motif that is characteristic of type I cadherins and is intimately involved in the adhesive function. The QAI tripeptide, however, appeared not to be involved in the adhesive functions of cadherin-6 and -14.

Cadherin was originally identified as a cell-cell adhesion molecule that functions in a Ca2⁺-dependent and homophilic manner and that is involved in various morphogenetic events during development (1). The first cadherins to be identified are known as the classic cadherins. In the last decade, numerous molecules that share the extracellular subdomain (EC)1 structure (cadherin repeat) of the classic cadherins have been discovered in both vertebrates and invertebrates, and they are now considered to constitute a large gene family, the cadherin superfamily. Besides the classic cadherins this family includes truncated type cadherins, desmosomal cadherins, protocadherins and protocadherin-related proteins, and HPT/II-cadherins (2). The biological functions of most of these non-classic cadherins remain elusive.

Classic cadherins share a common primary structure that consists of a signal peptide and a prosequence, which are both removed by intracellular proteolytic processing, five cadherin repeats, a transmembrane domain, and a highly conserved cytoplasmic domain, which is essential for association with catenins, the ensuing linkage to the cytoskeleton, and full functioning as a Ca2⁺-dependent cell-cell adhesion molecule (3–5). Full cDNA cloning of 11 human classic cadherin molecules has been accomplished so far as follows: E-, N-, and P-cadherin, cadherin-4 (R-cadherin), -5, -6, -8, -11 (OB-cadherin), -12, -14, and -15 (M-cadherin) (6–14). Suzuki (2) proposed that the classic cadherins were divided into two subgroups, type I and type II, on the basis of their overall sequence similarities and the conservation of several motifs and aromatic amino acid residues in their extracellular domains. The human classic cadherins E-, N-, and P-cadherin and cadherin-4 have been classified as type I, and cadherin-6, -8, -11, -12, and -14 are classified as type II. Although cadherin-5 and -15 (M-cadherin) have been proposed to be type II and I, respectively (2), we think that they do not clearly belong to either subgroup (14).

The type I classic cadherins, especially E- and N-cadherin, have been well characterized both functionally and structurally. By contrast, characterization of the non-type I classic cadherins has just begun, and it is still not clear whether they behave as do E- and N-cadherin. It has been suggested that some of them have weaker cell-cell binding strengths than the type I cadherins because they are expressed in loosely associated cells (15). In fact, Suzuki and colleagues (2, 16) reported that cadherin-5 and -8 appear to lack strong cell-cell adhesion activity. Some of the type II cadherins, however, were reported to mediate definite cell-cell adhesion (11, 17). Thus, the relation of the molecular characteristics and functions of these non-type I classic cadherins to their roles in various tissues is intriguing.

We recently cloned two novel human type II cadherins, cadherin-6 and -14 (12, 13). In the present study we analyzed the two molecules in detail using an L fibroblast cDNA transfection system, and we compared them with E-cadherin. Friedlander et al. (18) and Steinberg and Takeichi (19) showed that the expression level of a cadherin can influence cadherin-mediated cell sorting, suggesting that it is important to check the relative expression level of each cadherin. However, a method for quan-

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‡ The abbreviations used are: EC, extracellular subdomain; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate.
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Characterizing the expression of individual cadherins on the cell surface has not been established, so it is difficult to compare their binding strengths precisely. In this context, we have paid particular attention to the expression level of β-catenin protein by our cadherin transfectants in order to semi-quantify the expression of cadherins and to evaluate their binding strengths and specificities. In this report we describe and discuss the functional characteristics of cadherin-6 and -14 together with their biochemical properties.

EXPERIMENTAL PROCEDURES

Expression Vector Construction and Transfection—To express human cadherin-6 and -14 in mouse L fibroblasts, the expression vectors pBAT6H and pBAT14H were constructed by replacing the mouse E-cadherin cDNA of pBATEM2 (20) with cDNA fragments covering the entire open reading frames of cadherin-6 and -14, respectively. Transfection of the expression vectors into L cells was performed using LipofectAMINE reagent (Life Technologies, Inc.) together with pSTneoB, which carries the neomycin resistance gene (21), according to the manufacturer’s instructions. The transfected cells were selected in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum in the presence of 400 μg/ml G418 in a humidified atmosphere comprising 5% CO2:95% air at 37 °C for about 2 weeks. Then, the G418-resistant colonies were isolated, screened for cadherin-6 and -14 expression and maintained under the same conditions. Mouse E-cadherin transfectants were also obtained using pBATEM2 together with pSTneoB or pMAM2-BS2 (Kaken Pharma Co.) and used as controls. When pMAM2-BS2 was used, clones of the transfected cells were maintained in the presence of 5 μg/ml blasticidin S hydrochloride (Kaken Pharma Co.) instead of G418. Mouse L fibroblasts (LTK−) were supplied by the Riken Gene Bank.

We also used a newly reconstructed expression vector, designated pBAX, to prepare cadherin expression vectors and establish cadherin transfectants. pBAX was constructed by replacing the promoter region of pcdNAS (Invitrogen Corp.) with that of pBATEM2 and reconstructing L and HindIII sites. pBAX expresses cadherin cDNAs under the control of the promoter of the 11.9 kb PvuII-HindIII fragment of cDNA of cadherin-6 (data not shown).

To analyze the proteolytic processing of cadherin-6 and the functional significance of the Q1A1 tripeptide in the first EC (ECH1) of cadherin-6 and -14, amino acid substitutions were introduced into cadherin-6 and -14 by site-directed mutagenesis using the QuikChange Site-directed Mutagenesis Kit (Stratagene). The mutant molecules were expressed in L cells at similar levels to pBATEM2 and its derivatives (data not shown).

Antibodies—To produce a cadherin-6-specific antibody we generated a cadherin-6 fusion protein. A cadherin-6 cDNA fragment corresponding to EC5, a region with low homology to the other classic cadherins (12–14), and containing 0.95 trypsin and 0.02% EDTA at 37 °C for 15 min. Then the cells were washed twice with DMEM supplemented with 10% calf serum and were resuspended in DMEM supplemented with 10% calf serum and 70 units/ml DNase I (Takara) at a cell density of 2 × 10^6 cells/ml. The cell suspensions were added to a 24-well plastic plate (10^5 cells/well; Ultra Low Cluster; Corning Costar Corp.) and allowed to aggregate at 37 °C for 48 h at 100 rpm in a humidified atmosphere comprising 5% CO2, 95% air. To examine the heterotypic interactions between the cadherin subclasses, mixed cell-type aggregation experiments were performed using two transfectant cell lines expressing different cadherins; one line was labeled with 10 μg/ml 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) in DMEM supplemented with 10% calf serum for 1 h and the other was unlabeled. The cells were suspended as described above, and equal numbers of cells of the two cell lines (5 × 10^4 in 0.25 ml each) were mixed and allowed to aggregate for 12 h as described above.

Immunoblotting and Immunocytochemistry—Immunoblot analysis was carried out as described previously (4) except that the antigens were detected using the ECL system (Amersham Pharmacia Biotech). To quantify the amount of β-catenin protein in the cells, the density of each band of β-catenin was determined by densitometry and normalized for those of the parent L cells and the LE-5 transfectant cell line, which expresses mouse E-cadherin at a high level.

Bearing in mind that cadherin proteins were expressed in L cells as described above, we also used a newly reconstructed expression vector, designated pBAX, to prepare cadherin expression vectors and establish cadherin transfectants. pBAX was constructed by replacing the promoter region of pcdNAS (Invitrogen Corp.) with that of pBATEM2 and reconstructing L and HindIII sites. pBAX expresses cadherin cDNAs under the control of the promoter of the 11.9 kb PvuII-HindIII fragment of cDNA of cadherin-6 (data not shown). The resultant plasmid was introduced into an Escherichia coli strain, BL21(DE3)pLysS (Novagen), and expression of the fusion protein was induced by 1 mM isopropyl-β-D-thiogalactoside.

When pMAM2-BS2 was used, clones of the transfected cells were maintained in the presence of 5 μg/ml blasticidin S hydrochloride (Kaken Pharma Co.) instead of G418. Mouse L fibroblasts (LTK−) were supplied by the Riken Gene Bank.

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Antibodies—To produce a cadherin-6-specific antibody we generated a cadherin-6 fusion protein. A cadherin-6 cDNA fragment corresponding to EC5, a region with low homology to the other classic cadherins (12–14), and containing KpnI and HindIII sites at the 5′ and 3′ ends, respectively, was amplified by polymerase chain reaction, digested with KpnI and HindIII, and ligated to a prokaryotic expression vector, plasmid pET B (Invitrogen Corp.) that had been cleaved with the same enzymes. The resultant plasmid was introduced into an Escherichia coli strain, BL21(DE3)pLysS (Novagen), and expression of the fusion protein was induced by 1 mM isoprropyl-β-D-thiogalactoside. The protein was purified from bacterial lysates using a metal affinity resin (Talon, CLONTECH). A polyclonal anti-cadherin-6 antibody was then raised by immunizing rabbits with the fusion protein and was affinity purified using the same protein. A polyclonal anti-cadherin-14 antibody was raised by immunizing rabbits with a synthetic peptide, corresponding to the 15 C-terminal amino acids, conjugated to keyhole limpet hemocyanin, and affinity purified using the same peptide.

An anti-β-catenin monoclonal antibody (Transduction Laboratories) was used to detect β-catenin by immunoblotting and to immunoprecipitate cadherin-catenin complexes.

Short Term Cell Aggregation—Dispersed cell suspensions were obtained by treating the cells with phosphate-buffered saline containing 0.05% trypsin and 0.02% EDTA at 37 °C for 15 min. Then the cells were washed twice with DMEM supplemented with 10% calf serum and were resuspended in DMEM supplemented with 10% calf serum and 70 units/ml DNase I (Takara) at a cell density of 2 × 10^6 cells/ml. The cell suspensions were added to a 24-well plastic plate (10^5 cells/well; Ultra Low Cluster; Corning Costar Corp.) and allowed to aggregate at 37 °C for 48 h at 100 rpm in a humidified atmosphere comprising 5% CO2, 95% air. To examine the heterotypic interactions between the cadherin subclasses, mixed cell-type aggregation experiments were performed using two transfectant cell lines expressing different cadherins; one line was labeled with 10 μg/ml 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) in DMEM supplemented with 10% calf serum for 1 h and the other was unlabeled. The cells were suspended as described above, and equal numbers of cells of the two cell lines (5 × 10^4 in 0.25 ml each) were mixed and allowed to aggregate for 12 h as described above.

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Results

Expression of Cadherin-6 and -14 by L Cells—L cells, which are mouse lung fibroblasts deficient in cadherin activity (22), were transfected with pSTneoB and either pBAT6H or pBAT14H. Over 30 transfectant clones of each cadherin were isolated after G418 selection and screened for cadherin expression. Concomitantly, transfectant clones of cells transfected with pSTneoB, pBAX, or pMAM2-BS2, the control clones, were isolated; they were indistinguishable from the parent L cells based on the tests used in this study (data not shown).

The transfectant clones designated L6-33 and L14-4 showed the highest levels of cadherin-6 and -14 expression, respectively, in immunoblots (Fig. 1). Two bands of protein of approximately 125 and 120 kDa were labeled by the anti-cadherin-6 polyclonal antibody (Fig. 1A). The two bands of protein of approximately 112 and 107 kDa were labeled by the anti-cadherin-14 polyclonal antibody (Fig. 1B). As reported previously (23), these cadherin transfectants exhibited elevated levels of β-catenin protein (Fig. 1C) as well as α-catenin protein (data not shown), suggesting that both cadherin-6 and -14 interact with catenins, form cadherin-catenin complexes, and stabilize catenins, as do other classic cadherins (24). The asso-
We wondered whether one of the two cadherin-6 peptides was an N-terminally blocked non-mature form, and this result was due to contamination of this band by the mature form, because the two bands were contiguous (Figs. 1A and 2). We repeated the analysis and obtained the same result. Next we prepared a mutant cadherin-6 molecule with a single amino acid substitution of Arg-53 to Gly, which would not be cleaved to the mature form of cadherin-6 and which would correspond to a precursor form of cadherin-6. To do this we replaced an A with a G at nucleotide position 276, expressed the mutant in L cells, and compared the gene product with the two molecular forms of cadherin-6 by immunoblotting (Fig. 3). The immunoblot analysis showed that the mutant cadherin-6 expressed in an L cell transfectant clone, designated L6PRE-2, also resulted in two protein bands on SDS-PAGE, with higher molecular masses than the wild-type cadherin-6 molecule expressed by L6-33 cells. These findings show that the larger wild-type cadherin-6 is not a precursor form, unlike the larger cadherin-14 protein, and suggest that both bands of cadherin-6 were mature forms.

We previously reported that the deduced cadherin-6 protein sequence contains five possible N-linked glycosylation sites (12). To examine the influence of glycosylation on the mobility of cadherin-6, N-linked oligosaccharides were removed enzymatically. Fig. 4 shows the N-deglycosylation effect of various concentrations of glycopeptidase F on cadherin-6, the molecular masses of the pair of cadherin-6 bands were reduced stepwise as the enzyme concentration increased; however, the two-band pattern remained unchanged. We also used tunicamycin, an inhibitor of N-linked oligosaccharide synthesis, but the two-band pattern remained unchanged (data not shown).

The subcellular distributions of cadherin-6 and -14 in the transfected clones L6-33 and L14-4, respectively, were examined immunocytochemically (Fig. 5). As expected, both cadherin-6 and -14 were concentrated at cell-cell contact sites. Furthermore, exposure of cadherin-6 and -14 molecules on the external cell surface was confirmed by extracellular biotin labeling of the cadherins (Fig. 6). Unexpectedly, this experiment showed that not only the mature forms of cadherin-6 and -14 but also the precursor form of cadherin-14 were exposed on the cell surface.

Cell-Cell Binding Activities and Biochemical Properties of
Fig. 3. Immunoblot analysis of wild-type and mutant cadherin-6. Cell lysates of L6-33, L6PRE-2, and L6HAV-6 cells were separated by 7.5% SDS-PAGE, transferred to PVDF membranes, and probed with an anti-cadherin-6 polyclonal antibody. The amount of protein in each lane was not adjusted in this blot. The lower bands in all the lanes probably indicate degradation of cadherin-6. Bars on the left indicate the mobilities of molecular mass markers (200, 116, 97.4, and 66.2 kDa).

Fig. 4. N-Deglycosylation effect of glycopeptidase F on cadherin-6. L6-33 cell lysate was treated with glycopeptidase F at 0 (lane 1), 0.005 (lane 2), 0.02 (lane 3), 0.05 (lane 4), 0.2 (lane 5), 0.5 (lane 6), 2.0 (lane 7), 5.0 (lane 8), and 50 milliunits/ml (lane 9) at 37 °C for 18 h and then analyzed by immunoblotting with an anti-cadherin-6 polyclonal antibody. Bars on the left indicate the mobilities of molecular mass markers (200, 116, and 97.4 kDa).

Cadherin-6 and -14—The exposure of cadherin-6 and -14 at cell-cell contact sites, as shown in Figs. 5 and 6, suggests that these molecules function as intercellular connectors, as do type I cadherins. We first examined their cell-cell binding activities using a conventional method, the short term cell aggregation assay. We also examined the up-regulation of β-catenin protein expression in L cells with introduced cadherins (Fig. 1C). Assuming that the different cadherins interact with β-catenin and contribute to its preservation in a similar manner, the binding strengths of the cadherins can be compared using transfectants that express equal amounts of β-catenin protein. Both the L6-33 and L14-4 cells, however, exhibited only weak Ca\(^{2+}\)-dependent cell-cell aggregation in this assay system as compared with LE-1 cells and other E-cadherin transfectants (data not shown) that express almost the same amount of β-catenin (Table 1).

We recently reported that the susceptibility of cadherin-15 to trypsin digestion in the presence of Ca\(^{2+}\) causes a low aggregation rate in the short term assay (14). We therefore examined the trypsin sensitivities of cadherin-6 and -14 (Fig. 7). Both molecular forms of cadherin-6 were digested by trypsin treatment, even in the presence of Ca\(^{2+}\), whereas most of the mature form of cadherin-14 was digested by trypsin but a small fragment remained regardless of the presence of Ca\(^{2+}\). The mature form of cadherin-14 appeared to be slightly more stable in the presence of Ca\(^{2+}\) than in the absence of Ca\(^{2+}\), and partially digested molecules were detected only in the presence of Ca\(^{2+}\). Interestingly, a fragment of the precursor form of cadherin-14 was detected after the trypsin treatment. These results suggest that the low Ca\(^{2+}\)-dependent cell-cell aggregation rates described above were due to the digestion of cadherin-6 and -14 by trypsin and thus that the conventional aggregation assay cannot be used to evaluate the cell-cell adhesion activities of these cadherins correctly.

After treatment with trypsin and EDTA to destroy most of the E-cadherin, cadherin-6, and cadherin-14 molecules expressed by the transfected L cells, the expression levels of these cadherins returned to the initial levels within 3 h (data not shown). Therefore, we repeated the cell aggregation assay using a 48-h incubation period to compare the cell-cell binding activity of each cadherin. The influence of trypsin pretreatment to disperse the cells was considered to be negligible in these assays. The results are shown in Fig. 8. After 48-h incubations L6-33 and L14-4 cells formed aggregates that were almost indistinguishable in both size and cell-cell adhesiveness from LE-1 aggregates, whereas virtually no parent L cell aggregation was observed under the same conditions (Fig. 8). Therefore, both cadherin-6 and -14 do function as cell-cell connectors. Taking account of the almost equivalent level of β-catenin...
protein expressed in these three transfectant cell lines (Fig. 1 and Table I) and assuming that these lines express almost equivalent numbers of cadherin molecules per cell, these results suggest that the cell-cell binding strengths of these three cadherins are virtually the same. Our observations that LE-5 cells with a higher β-catenin expression level (Fig. 1 and Table I) formed larger and tighter aggregates (Fig. 8) and that many transfectant lines that expressed β-catenin at lower levels than the HAV motif in the EC1 of type I classic cadherins is intimately involved in their adhesive functions (27–29), whereas the HAV motif is replaced by a QAI tripeptide in cadherin-6 and cadherin-14. To determine the significance of the QAI tripeptide to the adhesive functions of cadherin-6 and -14, mutant cadherin-6 and -14 cDNAs that encoded HAV instead of the QAI tripeptide were constructed by replacing the HAV motif with (Ca(++) + 5 mM CaCl₂) in triplicate and allowed to aggregate at 37 °C for 60 min at 80 rpm. The extent of cell aggregation is represented by the aggregation index $(n_p/n_0)^{-1}$, where $n_p$ and $n_0$ are the mean total numbers of particles after incubation for 60 min and at the start of incubation, respectively. Values are the means of at least three separate experiments.

TABLE I

| Cell line | Aggregation index | β-Catenin expression level |
|-----------|-------------------|---------------------------|
|           | Ca (−)            | Ca (+)                    |
| L         | 0                  | 0                          |
| L6-33     | 0                  | 20                         |
| L14-4     | 0                  | 4                           |
| LE-1      | 0                  | 82                          |
| LE-5      | 0                  | 89                          |
| L6PRE-2   | ND⁺               | ND                          |
| L6HAV-6   | ND⁺               | ND                          |
| L14HAV-6  | ND⁺               | ND                          |

For quantitative detection of β-catenin, 20 μg of protein of each cell lysate was applied to SDS-PAGE and immunoblotted with an anti-β-catenin monoclonal antibody. L and LE-5 cell lysates were loaded on each gel as controls without exception. The band density of the β-catenin protein of each cell line was determined by densitometry. The densities of L and LE-5 cells were normalized to 0 and 100, respectively, and the expression level of β-catenin protein of each cell line was calculated for ease of comparison. Values are the means of at least three separate experiments.

ND, not determined.

Fig. 7. Trypsin sensitivity of cadherin-6 and -14. L6-33 and L14-4 cells were treated with HCMF containing 5 mM CaCl₂ (N), or 0.01% trypsin and 1 mM EDTA (TE), or 0.01% trypsin and 5 mM CaCl₂ (TC) at 37 °C for 15 min and were analyzed by immunoblotting with an anti-cadherin-6 polyclonal antibody and an anti-cadherin-14 polyclonal antibody, respectively. Whole cell lysates derived from the same number of cells were loaded onto each lane. A possible precursor form of cadherin-14, which was reduced in molecular mass by trypsin treatment, was detected (arrow). A partially digested fragment of cadherin-14 was also detected (arrowhead). Bars on the left indicate the mobilities of molecular mass markers (200, 116, 97.4, and 66.2 kDa).

Fig. 8. Long term cell aggregation of cadherin transfectants. L (A), L6-33 (B), L14-4 (C), LE-1 (D), LE-5 (E), L6PRE-2 (F), L6HAV-6 (G), and L14HAV-6 (H) cells were trypsinized completely in the presence of EDTA to obtain single cells, which were suspended in DMEM supplemented with 10% calf serum and 70 units/ml DNase I. The cells (10⁵ cells in 0.5 ml per well) were plated into 24-well plastic plate and allowed to aggregate at 37 °C for 48 h at 100 rpm in a CO₂ incubator. Then phase-contrast micrographs were taken. Scale bar, 200 μm.

-14. First, L6-33 cells or L14-4 cells were mixed with L cells and allowed to aggregate; both the L6-33 cells and L14-4 cells formed aggregates that excluded the parent L cells (data not shown), indicating that cadherin-6 and -14 mediate cell-cell adhesion in a homophilic manner, as do other classic cadherins (1, 14). Next, one cell line was labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) reagent, mixed with another unlabeled line, and allowed to aggregate for 12 h. Representative results are shown in Fig. 9. When DiI-labeled and unlabeled cells of the same cell line were mixed, randomly intermixed aggregates were formed (Fig. 9, A and B). When LE-1 cells were mixed with L6-33 or L14-4 cells, the LE-1 cells aggregated separately from the L6-33 or L14-4 cells, and chimeric aggregates were not found (Fig. 9, C and D). Interestingly, when L6-33 and L14-4 cells were mixed, chimeric aggregates composed of clusters of each cell type were formed (Fig. 9, E and F). We performed this set of experiments repeatedly and always observed similarly intermixed aggregates. These findings indicate that cadherin-6 and -14 possess binding specificities distinct from that of E-cadherin and that they partially interact with each other.

The HAV motif in the EC1 of type I classic cadherins is intimately involved in their adhesive functions (27–29), whereas the HAV motif is replaced by a QAI tripeptide in cadherin-6 and cadherin-14. To determine the significance of the QAI tripeptide to the adhesive functions of cadherin-6 and -14, mutant cadherin-6 and -14 cDNAs that encoded HAV instead of the QAI tripeptide were constructed by replacing the A at nucleotide positions 507 and 511 (12) with C and G, respectively, in cadherin-6 and by replacing the A at nucleotide positions 700 and 704 (13) with C and G, respectively, in
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FIG. 9. Mixed cell aggregation of cadherin transfectants. Dil-labeled L6-33 cells (5 \times 10^5) were mixed with an equal number of unlabeled L6-33 (A and B), LE-1 (C and D), or L14-4 cells (E and F) in DMEM supplemented with 10% calf serum and 70 units/ml DNase I and were allowed to aggregate, as described in the legend to Fig. 8, for 12 h. Phase-contrast (A, C, and E) and fluorescence (B, D, and F) micrographs of the same fields are shown. Dil-labeled and unlabeled L6-33 cells formed randomly mixed aggregates (A and B). When Dil-labeled L6-33 cells were mixed with unlabeled LE-1 cells, mixed cell aggregates were not seen (C and D), whereas L6-33 and L14-4 cells formed chimeric aggregates composed of clusters of each cell type (E and F). Scale bar, 100 \mu m.

FIG. 10. Effect of a mutation of QAI to HAV in cadherin-6 and -14 on mixed cell aggregation. Equal numbers (5 \times 10^5) of Dil-labeled L6HAV-6 and unlabeled L6-33 cells (A and B), Dil-labeled L6HAV-6 and unlabeled L14-4 cells (C and D), Dil-labeled L14HAV-6 and unlabeled L14-4 cells (E and F), or Dil-labeled L14HAV-6 and unlabeled L6-33 cells (G and H) were mixed and allowed to aggregate as in Fig. 9. Phase-contrast (A, C, E, and G) and fluorescence (B, D, F, and H) micrographs of the same fields are shown. Scale bar, 100 \mu m.

Table II
Summary of interactions between cadherins

|       | 6A | 6HAV | 14 | 14HAV | E |
|-------|----|------|----|-------|---|
| 6     | ++ | +   | +  | +     | − |
| 6HAV  | ++ | +   | +  | +     | − |
| 14    | ++ | ++  | +  | +     | − |
| 14HAV | ++ | +   | +  | +     | − |
| E     | +  | +   | +  | +     | + |

* a 6, 6HAV, 14, 14HAV, and E represent cadherin-6, mutant cadherin-6 with the HAV motif, cadherin-14, mutant cadherin-14 with the HAV motif, and E-cadherin, respectively.

DISCUSSION

Many members of the cadherin superfamily have been discovered in the last decade; over 20 classic cadherins have been identified in various vertebrates. To our knowledge, in humans 11 classic cadherins have been characterized so far by full cDNA cloning and sequencing. The classic cadherins can be divided into subtypes (type I, type II, and others) on the basis of their deduced amino acid sequences (2, 14). Among the classic cadherins, E- and N-cadherin, both of which are type I cadherins, have been investigated most thoroughly and have been well characterized; however, little is known about the other, especially the non-type I, cadherins. We therefore planned to analyze the molecular nature of cadherin-6 and -14, both of which are classified as type II classic cadherins.

Cadherin-6 and -14 cDNAs were introduced into mouse L fibroblasts, and transfectant clones stably expressing each cad-...
herin were isolated. Immunoprecipitation and immunoblot experiments showed that these cadherins interacted with and stabilized catenins, as does E-cadherin. On SDS-PAGE cadherin-6 and -14 were seen as two bands, 125 and 120 kDa, and 112 and 105 kDa proteins, respectively. It is widely thought that transcribed cadherin molecules are cleaved by signal peptidases on the endoplasmic reticulum membranes and that the resultant precursor forms undergo further proteolytic processing by endoproteases to generate the functional mature forms (26). Indeed, a faint band is often observed just above the main band of a cadherin on immunoblotting, which may correspond to the precursor form (30). Here, we demonstrated that the larger of the two cadherin-14 proteins seen on SDS-PAGE was indeed a precursor that had been cleaved just after the signal peptide and that at least part of the precursor was exposed on the cell surface similar to the mature form. The significance of the cadherin-14 precursor on the cell surface remains unclear at present. We also detected two cadherin-6-positive bands; however, direct sequencing of each protein showed that they had the same N terminus as the putative mature form of cadherin-6; both were expressed on the external cell surface, and they had similar trypsin sensitivities and detergent solubilities. Interestingly, cadherin-6 expressed in various human cell lines also shows the same two-band pattern.

The phosphorylation of a protein often alters its mobility on SDS-PAGE. We therefore examined, by potato acid phosphatase treatment (31) and immunoblot analysis using antibodies against phosphoamino acid residues (32), whether the two forms of cadherin-6 are phosphorylated and non-phosphorylated forms of the mature cadherin-6 protein, but phosphorylation of cadherin-6 does not seem to alter its electrophoretic mobility. It is also unlikely that there is post-translational modification of the C-terminal region, because anti-pan cadherin rabbit serum (Sigma) raised against the C-terminal sequence of chicken N-cadherin detects both bands on immunoblots.

It is well known that cadherins undergo sugar modifications (33, 34) and that cadherin-6 has five consensus sites for N-linked glycosylation (12). Therefore, we suspected that the two molecular forms of cadherin-6 might represent different stages of glycosylation. However, the two bands were not replaced by a single band after treatment with glycopeptidase F or tunica-mycin, although these experiments could not rule out the above possibility completely. Note that in the N-deglycosylation experiment using glycopeptidase F, the relative molecular mass of cadherin-6 became smaller in a stepwise manner as the enzyme concentration increased (Fig. 4). For example, the upper band of cadherin-6 is clearly seen in the 0.5 milliunit/ml glycopeptidase F sample, but this band becomes faint at 2 milliunits/ml glycopeptidase F and is absent at 5 milliunits/ml glycopeptidase F; the cadherin-6 molecule corresponding to the upper band at 0.5 milliunit/ml glycopeptidase F appears to be migrating at the same rate as the lower band with the advance of deglycosylation (Fig. 4). If this is the case, the difference between the two molecular forms of mature cadherin-6 may result from one N-linked oligosaccharide chain that is resistant to experimental deglycosylation. At present, this issue and the difference in adhesive function between the two forms remains to be resolved.

At an early stage of cadherin research the resistance of cadherin to trypsin degradation in the presence of Ca\(^{2+}\) was considered a hallmark of the cadherin family (1). However, the present study has demonstrated clearly that both cadherin-6 and -14 were not fully protected from trypsin by Ca\(^{2+}\). We reported recently that cadherin-15, a human homologue of mouse M-cadherin, is also sensitive to trypsin in the presence of Ca\(^{2+}\) (14) and Tanihara et al. (16) described similar trypsin sensitivity for cadherin-5. Thus, protection from trypsin degradation by Ca\(^{2+}\) may not be a common feature of cadherin family members, particularly for non-type I classic cadherins.

Cell-cell adhesion activities of cadherins have been evaluated mainly by short term cell aggregation assays, in which cells dispersed by trypsin treatment in the presence of Ca\(^{2+}\) were studied (35). However, we have found that this assay system does not always accurately evaluate the cell-cell adhesive functions of cadherins. Therefore, we used a long term cell aggregation assay in this study. Another important point to be considered when evaluating cadherin function is how individual cadherin molecules are quantified. Therefore, we measured the expression level of \(\beta\)-catenin protein in the cadherin transfectants used in this study. We assumed that in the L fibroblast cDNA transfection system each classic cadherin subclass interacts with and stabilizes \(\beta\)-catenin in a similar manner and that, therefore, the binding strength and specificity of each cadherin subclass can be compared using transfectants that express equal amounts of \(\beta\)-catenin protein. Thus, we used cadherin-6, cadherin-14, and E-cadherin transfectants that expressed almost the same amount of \(\beta\)-catenin protein to evaluate the adhesive functions of these cadherins. In the long term cell aggregation experiments, all of the transfectants formed aggregates for the same size in both cell-cell adhesiveness, suggesting that the cell-cell binding strengths of cadherin-6, cadherin-14, and E-cadherin are virtually equivalent. Moreover, the mixed cell aggregation experiments showed that both cadherin-6 and -14 had binding specificities distinct from that of E-cadherin. Interestingly, these experiments also showed that cadherin-6 and -14 can interact with each other in an incomplete manner. Similar heterophilic interactions between cadherins from the same species have been described for N-cadherin and R-cadherin (cadherin-4) (36, 37), chick B-cadherin and liver cell adhesion molecule (L-CAM) (38), and chick cadherin-6B and -7 (17). It is possible that heterophilic interactions between cadherins of different subclasses are responsible for the interaction of adjacent tissues in vivo, because in the mixed cell aggregation experiments referred to above chimeric aggregates composed of clusters of each cell type were observed, except for the combination of chick B-cadherin and L-CAM. Much more work is necessary, however, to elucidate the significance of these interactions.

It is widely accepted that the HAV tripeptide motif, which resides in EC1 of type I classic cadherins, and its flanking amino acids is intimately involved in the adhesive function and binding specificity (27–29). In non-type I classic cadherins, however, the HAV motif is replaced by other tripeptides (14), but it is not known whether these tripeptides are involved in the binding functions of the cadherin. Both cadherin-6 and -14 have a QAI tripeptide instead of the HAV motif (12, 13), prompting us to investigate whether the QAI motif is involved in the binding specificities of cadherin-6 and -14, especially the partial interaction between cadherin-6 and -14. We constructed mutant cadherin-6 and -14 molecules that had the HAV motif instead of QAI, but this mutation had no effect on the binding specificities of cadherin-6 and -14. We also examined the effects of synthetic peptides, including the QAI tripeptide, on cell aggregation of cadherin-6 and cadherin-14 transfectants, but they did not show any inhibitory effects. Thus, we did not observe any evidence that the QAI tripeptide and its flanking amino acids are involved in the adhesive functions of cadherin-6 and -14 and the region that is responsible for the

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2 Y. Shimoyama, H. Takeda, S. Yoshihara, M. Kitajima, and S. Hirohashi, unpublished observations.
adhesive functions of the non-type I classic cadherins remains to be identified. More detailed studies including structural analyses will provide answers to this question.

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