Identification of three human type-II classic cadherins and frequent heterophilic interactions between different subclasses of type-II classic cadherins

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We identified three novel human type-II classic cadherins, cadherin-7, -9 and -10, by cDNA cloning and sequencing, and confirmed that they interact with catenins and function in cell–cell adhesion as do other classic cadherins. Cell–cell binding activities of the eight human type-II classic cadherins, including the three new molecules, were evaluated by long-term cell-aggregation experiments using mouse L fibroblast clones transfected with the individual cadherins. The experiments indicated that all the type-II cadherins appeared to possess similar binding strength, which was virtually equivalent to that of E-cadherin. We next examined the binding specificities of the type-II cadherins using the mixed cell-aggregation assay. Although all of the type-II cadherins exhibited binding specificities distinct from that of E-cadherin, heterophilic interactions ranging from incomplete to complete were frequently observed among them. The combinations of cadherin-6 and -9, cadherin-7 and -14, cadherin-8 and -11, and cadherin-9 and -10 interacted in a complete manner, and in particular cadherin-7 and -14, and cadherin-8 and -11 showed an indistinguishable binding specificity against other cadherin subclasses, at least in this assay system. Although these data were obtained from an *in vitro* study, they should be useful for understanding cadherin-mediated mechanisms of development, morphogenesis and cell–cell interactions *in vivo*.

Key words: binding specificity, catenin, cell–cell adhesion, L cell.

INTRODUCTION

Classic cadherins are Ca^{2+}-dependent cell–cell adhesion molecules. They are transmembrane glycoproteins that consist of a signal peptide and a prosequence, which are both removed by intracellular proteolytic processing, a long extracellular domain, one transmembrane domain, and a rather short and highly conserved cytoplasmic domain. The cytoplasmic domain is essential for association with catenins, the ensuing linkage to the cytoskeleton, and full functioning as a cell–cell adhesion molecule [1–5]. The extracellular domain of classic cadherins serves as an interface responsible for cell–cell binding and determination of binding specificity, and can be divided into five homologous subdomains [6], which are also called cadherin repeats. Recent structural analyses have revealed that these subdomains indeed form structural units and also that the dimerization of the extracellular domain, especially the first subdomain, is crucially important for adhesive activity [7–9]. In the last decade, numerous molecules sharing this subdomain structure have been discovered, and it is generally accepted that these molecules constitute a large gene family, the cadherin superfamily [10]. Apart from the classic cadherins, however, the exact biological functions of these newly identified cadherins remain elusive.

Many classic cadherin molecules have been discovered in various vertebrates. With respect to human classic cadherins, 11 independent molecules have been identified so far by full cDNA cloning and sequencing analyses; these are E-, N- and P-cadherin, and cadherin-4 (R-cadherin), -5 (V-cadherin), -6 (K-cadherin), -8, -11 (OB-cadherin), -12, -14 and -15 [11–19]. On the basis of their overall similarities, and the conservation of several motifs and aromatic amino acid residues in the extracellular domains, Suzuki et al. [13] first proposed grouping the classic cadherins into two subgroups, type I and type II. At present, however, we consider that the human classic cadherins can be divided into more than two groups: type I, which includes E-, N- and P-cadherins and cadherin-4, type II, which includes cadherin-6, -8, -11, -12 and -14, and the others, cadherin-5 and -15, which do not show high similarities to either type-I or type-II cadherins, or to each other [19]. No subclasses with high similarity to cadherin-5 or -15 have been identified so far in humans.

As compared with non-classic-type cadherins, classic cadherins have been well characterized both functionally and structurally. However, most of these characteristics have been based on type-I classic cadherins, especially E- and N-cadherin, and it remains uncertain whether the characteristics of the type-I classic cadherins are true of the non-type-I classic cadherins. As for cell–cell binding activity, it has been suggested that some of the non-type-I classic cadherins mediate weaker cell–cell adhesion than do the type-I cadherins, since they are expressed in loosely associated cells [20]. It has also been reported that cadherin-5 and -8 did not

Abbreviations used: CMFDA, 5-chloromethylfluorescein diacetate; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DMEM, Dulbecco's modified Eagle's medium; EC, extracellular subdomain.

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Nucleotide sequences of a human cadherin-7 cDNA, a human cadherin-9 cDNA and a human cadherin-10 cDNA have been submitted to the DDBJ/EMBL/GenBank® nucleotide sequence databases under the accession numbers AB035301, AB035302 and AB035303, respectively.

show significant cell-adhesion activity in cell-aggregation experiments [21,22]. These observations have led us to investigate the molecular nature of the non-type-I classic cadherins, and compare it with that of the type-I cadherins. We recently identified three human non-type-I classic cadherins, cadherin-6, -14 and -15, and characterized them using an L fibroblast cDNA transfection system [17–19,23]. In order to evaluate the binding strength of each cadherin as precisely as possible, we employed a long-term cell-aggregation assay focusing particularly on the expression level of β-catenin by the cadherin-transfected L cells, and reached the conclusion that all three non-type-I cadherins possess cell–cell binding activity similar to that of E-cadherin [19,23]. We found, however, that unlike type-I cadherins they were not protected by Ca^{2+} from trypsin degradation [19,23], although resistance to trypsin in the presence of Ca^{2+} had been considered as one of the characteristic features of classic cadherins [1].

As mentioned above, 11 classic cadherin molecules, including four type-I cadherins, five type-II cadherins and two non-type-I or -II cadherins, have been identified in humans. In addition, it is likely that there are at least three type-II cadherin molecules in humans, cadherin-7, -9 and -10, of which the full sequences are not yet available [13]. In order to analyse and clarify the molecular features of non-type-I, especially type-II, classic cadherins, in this study we first determined the full coding sequences of these three human cadherin molecules. We then assessed the cell–cell binding activities of all the eight human type-II cadherins using an L fibroblast cDNA transfection system and a long-term cell-aggregation assay. All of the cadherins exhibited cell–cell binding activity comparable with that of E-cadherin. Moreover, we frequently observed cross-reactivity of the type-II cadherins, which was incomplete or complete depending on the particular combination.

EXPERIMENTAL

cDNA cloning and DNA sequence analysis

To obtain cDNA fragments of human cadherin-9 and -10, degenerative reverse transcriptase PCR was performed using human brain poly(A)$^+$ RNA (Clontech, Palo Alto, CA, U.S.A.) as described previously [17]. The PCR products were subcloned into pCR®2.1 using a TA Cloning® kit (Invitrogen, San Diego, CA, U.S.A.). Clones were sequenced one by one, and those that corresponded with the partial nucleotide sequences of cadherin-9 and -10 reported previously [13] were selected. These clones were re-amplified by PCR with [α-^{32}P]dCTP, and used as probes for the following cDNA clonings. A human fetal brain Uni-ZAP® XR cDNA library (Stratagene, La Jolla, CA, U.S.A.), which was prepared from a pool of male and female whole brain samples ranging from 19 to 23 weeks of gestational age, was screened using the above probes by a plaque-hybridization technique as described previously [11], and positive clones were plaque-purified. The cDNA sequences of both ends of the inserts were determined by an ABI Prism® 377 DNA sequencer (Perkin-Elmer, Foster City, CA, U.S.A.) using a Dye Primer Cycle Sequencing kit (Perkin-Elmer), and compared with the cDNA sequences of known human classic cadherins. cDNA clones considered to contain start codons for complete forms of classic cadherins and to be of sufficient length were selected, and their entire nucleotide sequences were determined. The sequences were determined on both strands by an ABI Prism® 377 DNA sequencer using oligonucleotide primers that annealed the cDNAs and a Prism® Ready Reaction Terminator Cycle Sequencing kit (Perkin-Elmer).

For cDNA cloning of human cadherin-7, we utilized chicken cadherin-7 cDNA [24], which we expected to cross-hybridize with the human homologue. We first obtained a 223-bp fragment of chicken cadherin-7 cDNA corresponding to nucleotide positions 429–651 of the reported cDNA [24], which encodes the signal peptide and precursor region, by PCR using chicken brain QUICK-Clone® cDNA (Clontech) as a template. The fragment was re-amplified by PCR using the same primer set with [α-^{32}P]dCTP, and used as a probe to screen the above cDNA library. A positive clone was plaque-purified, and sequenced as described above.

Other cDNAs of known human type-II cadherins, except for cadherin-6 and -14, for which we have already reported the cDNA cloning and sequencing [17,18], were obtained as follows. For cadherin-8 and -12, we prepared radiolabelled PCR probes referring to the previously reported cDNA sequences [16] as we did for the cadherin-7 cloning, and cloned each cDNA containing the entire coding sequence from the same library as above. For cadherin-11, a 2493-bp cDNA containing the whole coding region corresponding to nucleotide positions 381–2873 of the reported cDNA [15] was amplified by reverse transcriptase PCR from poly(A)$^+$ RNA of MRC-5 cells, a normal human fibroblast strain derived from embryonic lung [25]. These cadherin cDNAs were also sequenced on both strands as mentioned above to confirm their integrity.

Nucleotide and deduced amino acid sequences were analysed using the GeneWorks software package (IntelliGenetics, Mountain View, CA, U.S.A.).

Expression-vector construction and transfection

For cadherin-6 and -14, we have already reported construction of the expression vectors and the establishment of L transfectant clones [23]. The transfectant clones designated L6-33 and L14-4, which express human cadherin-6 and -14, respectively, were also used in this study. To express other human type-II classic cadherins in mouse L fibroblasts that lack cadherin-mediated cell–cell adhesion activity [26] and to analyse the cell–cell adhesion properties of each cadherin, expression vectors were constructed by introducing each cadherin cDNA containing the entire open reading frame into the multicloning site of an expression vector pBAX [23] in the right direction. The resulting expression vectors were cleaved by digestion with *Bgl*II, *Pvu*I or *Sca*I, and used for the following transfection.

Transfection of the expression vectors into L cells was performed using Lipofectamine® reagent (Life Technologies, Gaithersburg, MD, U.S.A.) 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 % CO_2/95 % air at 37 °C for about 2 weeks. Then, the G418-resistant colonies were isolated, screened for cadherin expression, and maintained under the same conditions.

Immunoblotting and immunoprecipitation

Immunoblotting of β-catenin and immunoprecipitation of the cadherin–catenin complex from cadherin-transfected L cells were performed as described previously [19,23] using an anti-β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY, U.S.A.).

Long-term cell-aggregation assay

Completely dispersed cell suspensions were obtained by treating the cells with PBS containing 0.05 % trypsin and 0.02 % EDTA at 37 °C for 15 min. The cells were washed twice with DMEM

supplemented with 10% calf serum, and then resuspended in DMEM supplemented with 10% calf serum and 70 units/ml DNase I (Takara, Shiga, Japan) at a cell density of 2×10^5 cells/ml. The cell suspensions were added to a 24-well plastic plate (10^5 cells in 0.5 ml per well; Ultra Low Cluster, Corning Costar, Cambridge, MA, U.S.A.) and allowed to aggregate at 37 °C for 48 h at 100 rev./min in a humidified atmosphere comprising 5% CO_2/95% 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 labelled with 40 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, U.S.A.) in DMEM supplemented with 10% calf serum or 10 μM 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes) in serum-free DMEM for 1 h, and the other was unlabelled. 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.

RESULTS

cDNA cloning and sequence analysis of human cadherin-7, -9 and -10

cDNA clones for cadherin-7, -9 or -10, which appeared to have the complete coding regions as compared with the cDNA sequences of known human classic cadherins, were isolated. These clones were subjected to full DNA sequence analysis. The cadherin-7 cDNA consisted of 2828 nucleotides covering the complete coding region for the classic cadherin, and a poly(A)$^+$ tail. The deduced amino acid sequence comprised 785 amino acid residues. The N-terminal 47 amino acids were predicted to be removed by signal peptidases and endopeptidases [23], and the resulting mature form of 738 amino acids, which was predicted to be expressed on the cell surface and to function as a cell–cell adhesion connector, had the complete features of the type-II classic cadherin. The cadherin-9 and -10 cDNA clones consisted of 3060 and 3261 nucleotides, respectively, with poly(A)$^+$ tails, and also encoded complete forms of the typical type-II classic cadherin. The amino acid sequences of cadherin-7 and -10 exhibited high homologies, 94 and 96%, with those of the reported chicken cadherin-7 [24] and -10 [27], respectively, indicating that these clones indeed encode human cadherin-7 and -10. The deduced amino acid sequences of the putative mature forms after post-transcriptional proteolytic processing are shown in Figure 1 together with those of other human type-II classic cadherins. Besides the above clones, we also isolated several cDNA clones for cadherin-9 and -10, which appeared to encode various truncated forms (results not shown). These are under investigation.

Comparison of deduced amino acid sequences of human type-II classic cadherins

Percentage homologies among the type-II cadherins and with E-cadherin, as a representative of the type-I cadherins, are shown in Table 1. Previously we reported the cDNA cloning and sequence determination of cadherin-6 and -14 [17,18], and these sequences are shown in Figure 1. We also obtained cDNA clones encoding the complete proteins of cadherin-8, -11 and -12 in this study, and sequenced them. Amino acid sequences deduced from those cDNA clones are also shown in Figure 1 and Table 1. Although the deduced amino acid sequence of cadherin-11 completely agreed with the reported sequence [15], the sequences of cadherin-8 and -12 differed from the previously reported ones [16] at several points, as follows. As compared with the previously reported human cadherin-8 protein, our cadherin-8 cDNA clone encoded a longer molecule at the N-terminus: seven amino acids, Met-Pro-Glu-Arg-Leu-Ala-Glu, were added. Moreover, Asp at amino acid position 348 in the third extracellular subdomain (EC3) was changed into Val, and Gln at 641 in the cytoplasmic domain was deleted in our cadherin-8 protein. As for cadherin-12, the amino acid sequence deduced from our cDNA differed from the previously reported sequence at three points. Gly at amino acid position 416 in the EC4, His at 644 and Ile at 733 in the cytoplasmic domain were replaced by Ser, Asp and Tyr, respectively, in our cadherin-12 protein.

As shown in Figure 1, the type-II cadherins share many amino acid residues. In the extracellular domains, 180 amino acid residues are conserved among the type-II cadherins, and 65 of these residues are conserved among all of the human classic cadherins (Figure 1A). These include DRE and DXND in the EC1, AXDXDD and DXNXN in the EC2, PXF at the end of EC1, 2 and 3, and four Cys residues in the EC5. On the other hand, 21 out of the 180 conserved residues are specific for the type-II cadherins, but they do not appear to form specific clusters (Figure 1A). The HAV motif, which is considered to be intimately involved in the adhesive functions of type-I classic cadherins [28–30], is replaced by a QAI tripeptide in cadherin-6, -10 and -14, a QAL tripeptide in cadherin-7, a QAV tripeptide in cadherin-8, -11 and -12, and a KAI tripeptide in cadherin-9 (Figure 1A). In the cytoplasmic domains, 52 amino acid residues are conserved among the type-II cadherins (Figure 1C). Although 26 of these residues are conserved among all of the human classic cadherins, only three residues are specific for the type-II cadherins. The alignment of amino acid sequences of the human classic cadherins revealed that the transmembrane regions of the type-II cadherins are conspicuous in comparison with those of other classic cadherins. Figure 1(B) shows the alignment of the transmembrane sequences of the type-II cadherins with other known human classic cadherins. The transmembrane domains of all of the type-II cadherins consist of exactly 33 amino acids and are well conserved, whereas those of other cadherins vary in size and do not show high similarities to each other or to those of the type-II cadherins (Figure 1B).

Expression of type-II classic cadherins

To examine the cell–cell binding activities of the type-II classic cadherins, the cadherin-7, -8, -9, -10, -11 and -12 cDNA clones, ligated into the eukaryotic pBAX expression vector, were introduced into L cells, a mouse fibroblast strain deficient in cadherin activity [26]. A lipofection method was used, and transfected clones expressing the individual cadherins were obtained. Since antibodies for these cadherins were not available, transfected clones were selected on the basis of the expression level of β-catenin, which is considered to reflect the expression level of a transfected cadherin in this system [19,23]. Establishment of the L cell clones L6-33 and L14-4, which express cadherin-6 and -14, respectively, has already been reported [23]. These clones and LE-1 cells, an E-cadherin transfectant [19], were also used in this study.

Thus transfected clones L7-3, L8-10, L11-3 and L12-6, for cadherin-7, -8, -11 and -12, respectively, were selected on the basis that they express similar amounts of β-catenin protein as the LE-1, L6-33 and L14-4 transfectants. We have reported in a previous study that LE-1, L6-33 and L14-4 cells express almost equivalent amounts of β-catenin protein. For cadherin-9 and -10, we selected over 30 transfected clones for each cadherin, but

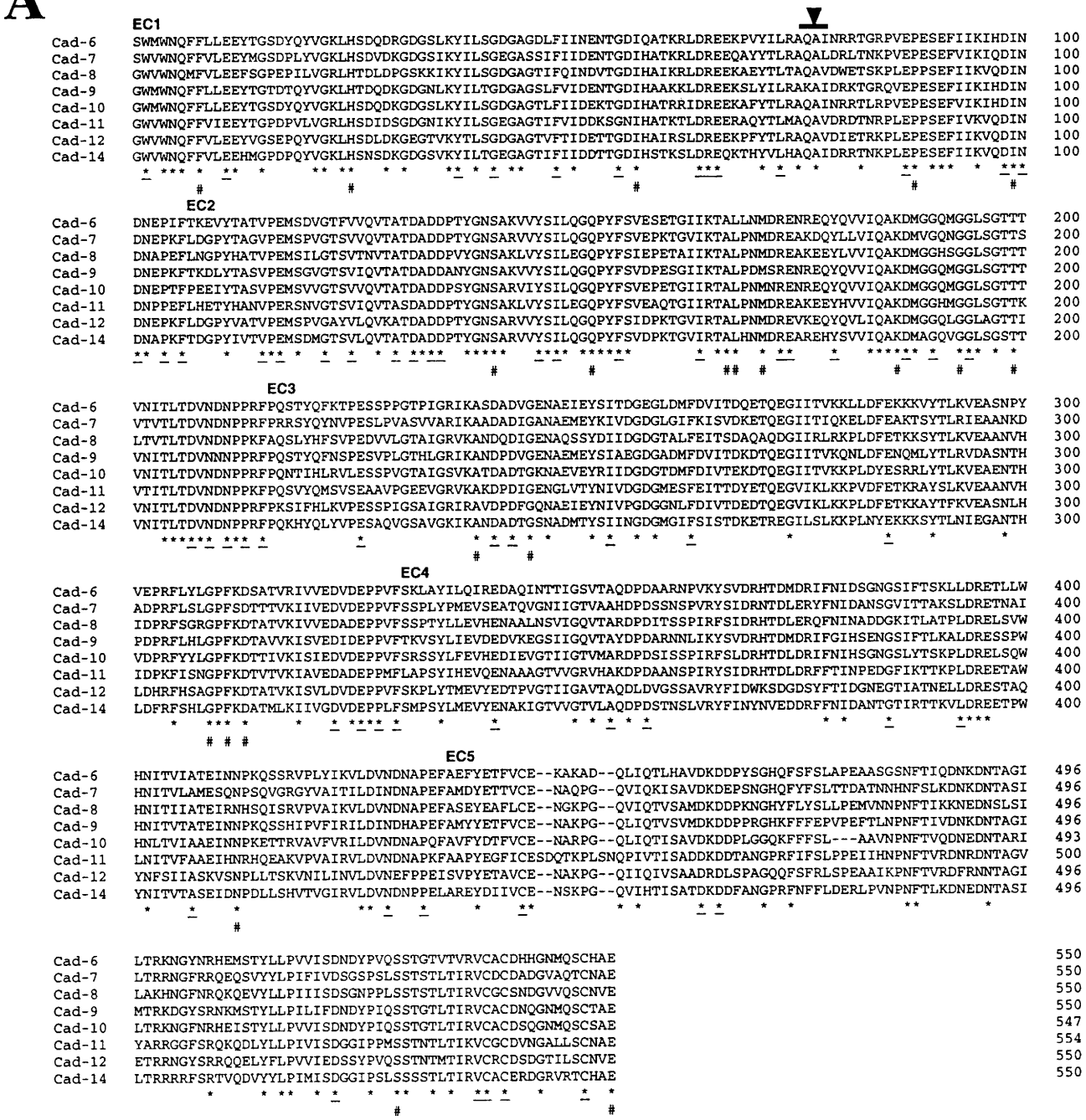

Figure 1 For legend see opposite.

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Cad-6    RRQRKKEPLIISKE-DIRDNIVSYNDEGGGEEDTQAFDIGTLRNPEAIEDNKLRRDIVPEALFLPRR-TPTARDN-TDVRDFINQRLKENDTDPTAPPYDS  98
Cad-7    RRRKK-EPLIFDEERDIRENIVRYDDEGGGEEDTEAFDMAALRNLNVIRDTKTRRDVTPEIQFLSRPAFKSIPDN-VIFREFIWERLKEADVDPGAPPYDS  99
Cad-8    RRHKN-EPLIIKDDEDVRENIIRYDDEGGGEEDTEAFDIATLQNPDGINGFLPRKDIKPDLQFMPRQ-GLAPVPNGVDVDEFINVRLHEADNDPTAPPYDS  99
Cad-9    KRQRKKEPLIISKD-DVRDNIVTYNDEGGGEEDTQAFDIGTLRNPEAREDSKLRRDVMPETIFQIRR-TVPLWEN-IDVQDFIHRRLKENDADPSAPPYDS  98
Cad-10   KRQRKKEPLILSKE-DIRDNIVSYNDEGGGEEDTQAFDIGTLRNPAAIEEKKLRRDIIPETLFIPRR-TPTAPDN-TDVRDFINERLKEHDLDPTAPPYDS  98
Cad-11   RRQKK-EPLIVFEEEDVRENIITYDDEGGGEEDTEAFDIATLQNPDGINGFIPRKDIKPEYQYMPRP-GLRPAPNSVDVDDFINTRIQEADNDPTAPPYDS  99
Cad-12   RRQKKKDTLMTSKE-DIRDNVIHYDDEGGGEEDTQAFDIGALRNPKVIEENKIRRDIKPDSLCLPRQ-RPPMEDN-TDIRDFIHQRLQENDVDPTAPPYDS  98
Cad-14   RRSKK-EPLIISEE-DVRENVVTYDDEGGGEEDTEAFDITALRNPSAAEELKYRRDIRPEVKLTPRHQTSSTLES-IDVQEFIKQRLAEADLDPSVPPYDS  98
          *      *      * * *   * *********** ***   * *         * *  *      *               **  *  * * **  *****

Cad-6    LATYAYEGTGSVADSLSSLESVTTDADQDYDYLSDWGPRFKKLADMYGGVDSDKDS                                              154
Cad-7    LQTYAFEGNGSVAESLSSLDSISSNSDQNYDYLSDWGPRFKRLADMYGTGQESLYS                                              155
Cad-8    IQIYGYEGRGSVAGSLSSLESTTSDSDQNFDYLSDWGPRFKRLGELYSVGESDKET                                              155
Cad-9    LATYAYEGNDSIADSLSSLESLTADCNQDYDYLSDWGPRFKKLADMYGGDDSDRD                                               153
Cad-10   LATYAYEGNDSIAESLSSLESGTTEGDQNYDYLREWGPRFNKLAEMYGGGESDKDS                                              154
Cad-11   IQIYGYEGRGSVAGSLSSLESATTDSDLDYDYLQNWGPRFKKLADLYGSKDTFDDDS                                             156
Cad-12   LATYAYEGSGSVAESLSSIDSLTTEADQDYDYLTDWGPRFKVLADMFGEEESYNPDKVT                                           157
Cad-14   LQTYAYEGQRSEAGSISSLDSATTQSDQDYHYLGDWGPEFKKLAELYGEIESERTT                                              154
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Figure 1 Alignment of deduced amino acid sequences of eight human type-II classic cadherins

(**A**) Alignment of extracellular domains. The signal peptide and precursor region of each cadherin are omitted from this Figure. The position of tripeptides corresponding to the HAV motif of type-I cadherins is marked by an arrowhead (▼) and a line. The beginning of each extracellular subdomain (EC) is indicated by EC1–EC5. (**B**) Alignment of transmembrane domains. Transmembrane domains of other human classic cadherins are also shown in alignment. (**C**) Alignment of cytoplasmic domains. The amino acid residues conserved among all the type-II cadherins are marked by asterisks. In (**A**) and (**C**), out of these conserved amino acid residues, those that are also conserved in the four human type-I classic cadherins and in cadherin-5 and -15, and those that are not conserved in any of them, are indicated by underlining and #, respectively.

Table 1 Homologies among eight human type-II cadherins

Homologies among the putative mature forms of eight human type-II cadherins (6–14) and E-cadherin (E) were calculated and are shown as percentages.

Cadherin	E	7	8	9	10	11	12	14
6	36	63	62	75	76	60	64	60
7	34	–	62	61	64	60	60	64
8	35	–	–	58	61	69	58	59
9	32	–	–	–	73	58	60	60
10	33	–	–	–	–	60	64	62
11	33	–	–	–	–	–	58	61
12	36	–	–	–	–	–	–	61
14	35	–	–	–	–	–	–	–

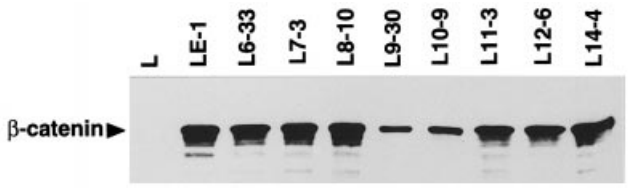

Figure 2 Immunoblot analysis of β-catenin expressed by cadherin transfectants

Whole-cell lysates of cadherin transfectants and L cells (20 μg of protein/lane) were separated by SDS/PAGE (7.5% gel) and transferred to a PVDF membrane. β-Catenin was then detected with an anti-β-catenin monoclonal antibody using an ECL® system (Amersham Life Science, Bucks., U.K.). Faint bands below the main bands probably indicate degradation of β-catenin.

failed to obtain any transfectant expressing β-catenin protein at a similar level to that of the other cadherin transfectants used in this study. We then used transfectant clones L9-30 and L10-9, for cadherin-9 and -10, respectively, which express the highest levels of β-catenin among each cadherin transfectant group. The levels of β-catenin expression in these cadherin transfectants, and in parental L cells, were analysed by immunoblotting, and are shown in Figure 2. As mentioned above, the cadherin-transfected clones, except for L9-30 and L10-9, expressed β-catenin at almost similar levels (Figure 2). Compared with these transfectants, L9-30 cells expressed considerably lower and L10-9 cells expressed slightly lower amounts of β-catenin, and β-catenin protein could hardly be detected in parental L cells under these experimental conditions (Figure 2).

Cadherin-7, -9 and -10 molecules, the human type-II classic cadherins newly identified in this study, were visualized as bands of approx. 116, 107 and 112 kDa, respectively, in an immunoprecipitation experiment (Figure 3). This experiment also confirmed that the three newly identified cadherins interact not only with β-catenin but also with α-catenin, similar to known classic cadherin molecules. In fact, the cadherin transfectants presented here exhibited elevated expression levels of α-catenin protein as compared with L cells, a similar result to that obtained with β-catenin (results not shown).

Cell–cell binding activities of type-II cadherins

Cell–cell binding activities of the eight human type-II classic cadherins were evaluated by long-term cell-aggregation assays using the aforementioned cadherin transfectants and the parental L cells. Although cell-aggregation experiments for 30 min–3 h have been used widely to evaluate cadherin activity, we did not employ this type of short-term assay. The reason is that in the short-term assay cells are usually dispersed into single-cell suspensions by trypsin in the presence of Ca^{2+}, which is known to leave type-I cadherins intact; however, we found the functional forms of cadherin-6 and -14 to be destroyed by this treatment

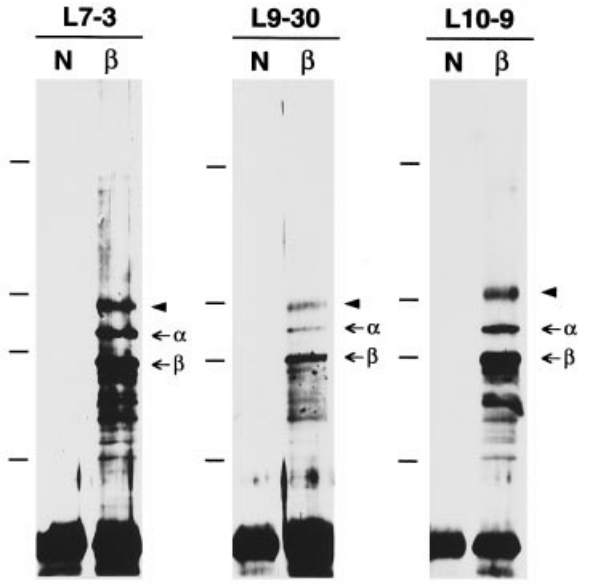

Figure 3 Immunoprecipitation of cadherin-7, -9 and -10 with an anti-β-catenin monoclonal antibody

Cadherin-7, -9 and -10 molecules were co-immunoprecipitated with an anti-β-catenin monoclonal antibody (β) from L7-3, L9-30 and L10-9 cell lysates, respectively, based on the formation of cadherin–catenin complexes in cadherin-positive cells. As a control, immunoprecipitations with normal mouse IgG (N) were performed simultaneously. The precipitates were denatured, separated by SDS/PAGE (7.5% gel), transferred on to a PVDF membrane and stained with AuroDye® forte (Amersham Life Science). Cadherins and α- and β-catenins are indicated by arrowheads and arrows, respectively. Bands below the β-catenin bands probably indicate degradation of β-catenin. Bars on the left indicate the mobilities of molecular-mass markers (200, 116, 97.4 and 66.2 kDa).

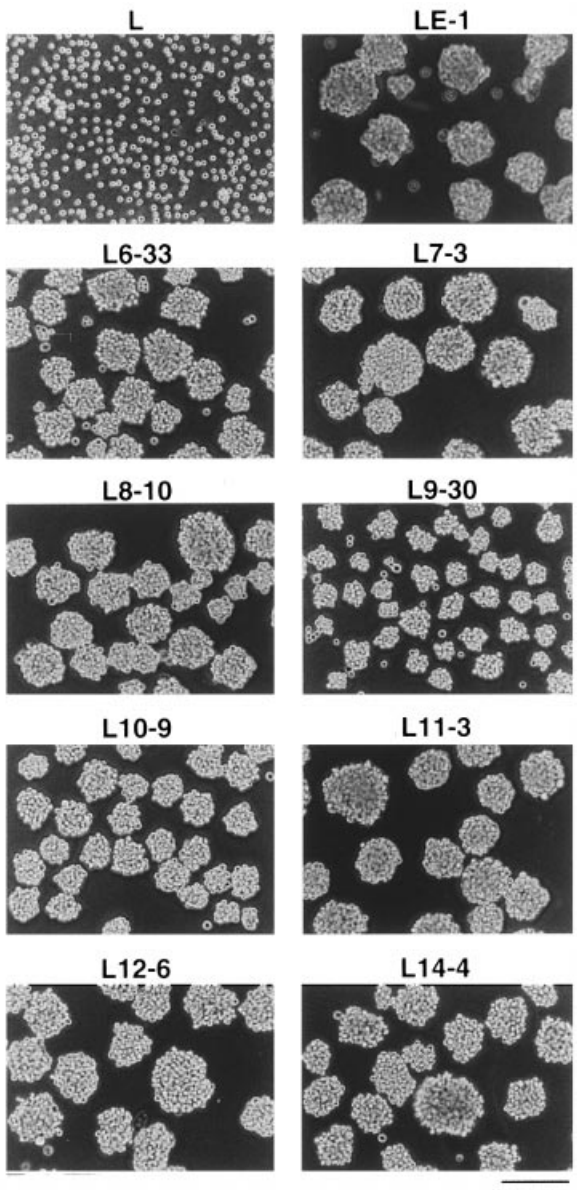

Figure 4 Long-term cell aggregation of cadherin transfectants

L, LE-1, L6-33, L7-3, L8-10, L9-30, L10-9, L11-3, L12-6 and L14-4 cells were trypsinized completely in the presence of EDTA to obtain single cells, and allowed to aggregate at 37 °C for 48 h at 100 rev./min in a CO_2 incubator. Then phase-contrast micrographs were taken. Note the sizes of the individual aggregates. The scale bar at the bottom represents 200 μm.

[23]. We therefore treated the cadherin transfectants with trypsin in the absence of Ca^{2+}, which clears the functional cadherin molecules from the external cell surface, and allowed the resulting single-cell suspensions to aggregate for 12–48 h. Since we know that expression of cadherin molecules returns to initial levels within 3 h of treatment with trypsin and EDTA [23], the influence of the trypsin pretreatment was considered to be negligible in the long-term assay. Thus the long-term assay appeared to be suitable for evaluating the cell–cell binding activities of the type-II cadherins, and also for comparison of cell–cell binding activities irrespective of cadherin type.

The results of the long-term (48 h) cell-aggregation experiments are shown in Figure 4. As can be seen, the parental L cells hardly aggregated under the assay conditions, and most of the cells remained dispersed even after incubation for 48 h. In contrast, all of the cadherin transfectants formed definite aggregates. Although these aggregates could not be discriminated from one another in terms of cell–cell adhesiveness, they varied in size. LE-1, L6-33, L7-3, L8-10, L11-3, L12-6 and L14-4 cells formed almost indistinguishable aggregates, whereas L9-30 aggregates were much smaller and L10-9 aggregates were slightly smaller, than those of other transfectants. The sizes of the aggregates corresponded well to the expression level of β-catenin protein in the individual transfectants (Figure 2).

Binding specificities of type-II cadherins

In order to elucidate the binding specificities of the type-II cadherins, one line was labelled with DiI or CMFDA fluorescent reagent, mixed with another unlabelled line, and allowed to aggregate in a similar way to the long-term assays. In these experiments, the nine cadherin transfectants mentioned above, including the E-cadherin transfectant LE-1, and the parental L cells were used, and cells were incubated for 12 h in order to provide intelligible micrographs. The results after incubation for up to 48 h were the same as those indicated here.

First, each cadherin transfectant was mixed with the parental L cells and allowed to aggregate. L cells were excluded completely from every aggregate, and chimaeric aggregates were never found (results not shown), indicating that all of the type-II cadherins mediate cell–cell adhesion in a homophilic manner but not in a ligand–receptor interaction. The results are summarized

Table 2 Interactions between eight human type-II cadherins and E-cadherin

Two out of the nine cadherin transfectants, LE-1, L6-33, L7-3, L8-10, L9-30, L10-9, L11-3, L12-6 and L14-4, which express E-cadherin and cadherin-6, -7, -8, -9, -10, -11, -12, and -14, respectively, were mixed, and allowed to aggregate for 12 h. The resultant aggregates were then observed using an Olympus IX70 phase-contrast microscope equipped with a fluorescence system. Interactions between cadherins were classified into — (no interaction), + (incomplete interaction) and ++ (complete interaction) categories.

Cadherin	E	6	7	8	9	10	11	12	14
E	++*	−*	−	−	−	−	−	−	−*
6	−*	++*	+	−	++	+	−	−	+*
7	−	+	++	−	+	−	−	+	++
8	−	−	−	++	−	−	++	−	−
9	−	++	+	−	++	++	−	−	+
10	−	+	−	−	++	++	−	−	−
11	−	−	−	++	−	−	++	−	−
12	−	−	+	−	−	−	−	++	+
14	−*	+*	++	−	+	−	−	+	++*

* These data were reported previously [23].

in Table 2. When LE-1 cells were mixed with one of the type-II cadherin transfectants, they always aggregated separately, and chimaeric aggregates were never formed. This observation indicated that all of the type-II cadherins have binding specificities distinct from that of E-cadherin, a representative of the type-I cadherins. In contrast, and surprisingly, chimaeric aggregates, which varied in their extent of heterogeneous mixing, were observed frequently when two lines of the eight type-II cadherin transfectants were mixed. In particular, with combinations of L6-33 and L9-30, L7-3 and L14-4, L8-10 and L11-3, or L9-30 and L10-9, each pair of transfectants formed the same randomly intermixed aggregates as those observed when labelled and unlabelled cells of one line were mixed (Table 2). These complete heterophilic interactions suggested that cadherin-6 and -9, cadherin-7 and -14, cadherin-8 and -11, or cadherin-9 and -10 cannot discriminate between each other, at least in this assay system. Moreover, in six other combinations, including a combination of L6-33 and L14-4 [23], incomplete heterogeneous aggregates, which were composed of clusters of each cell type, were formed (Table 2), suggesting that in these combinations the two cadherin types share part of their binding specificities, enabling incomplete heterophilic interactions. Representative results are shown in Figure 5.

DISCUSSION

In the present study, we identified three novel human type-II classic cadherins, cadherin-7, -9 and -10, by cDNA cloning and sequencing. The alignment of deduced amino acid sequences of

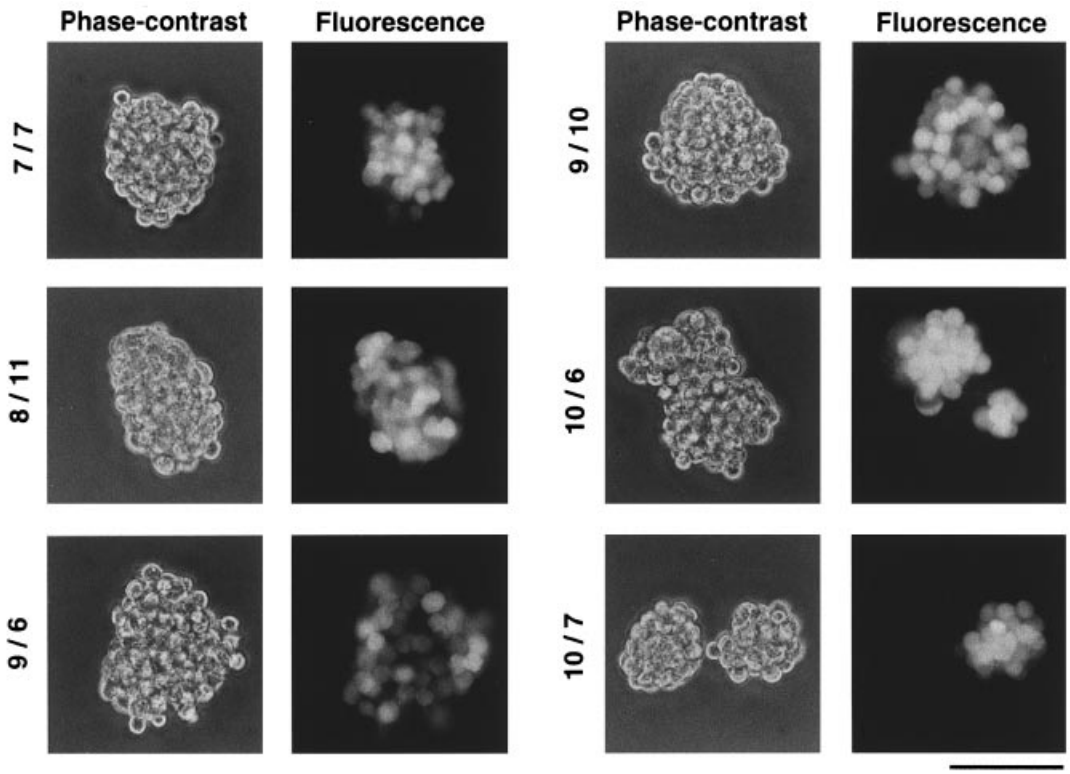

Figure 5 Mixed cell aggregation of cadherin transfectants

Equal numbers of labelled L7-3 and unlabelled L7-3 cells (7/7), labelled L8-10 and unlabelled L11-3 cells (8/11), labelled L9-30 and unlabelled L6-33 cells (9/6), labelled L9-30 and unlabelled L10-9 cells (9/10), labelled L10-9 and unlabelled L6-33 cells (10/6), and labelled L10-7 and unlabelled L7-3 cells (10/7) were mixed and allowed to aggregate for 12 h. Phase-contrast and fluorescence micrographs of the aggregates in the same fields were taken. Cells shown here were labelled with CMFDA reagent. Note that in 8/11, 9/6 and 9/10 the labelled and unlabelled cells form completely intermixed aggregates similar to that in 7/7. In contrast, the labelled and unlabelled cells were completely segregated in 10/7. In 10/6 the aggregate is composed of two clusters of labelled L10-9 cells surrounded by unlabelled L6-33 cells. This pattern of heterogeneous intermixing was interpreted as incomplete heterophilic interaction. The scale bar at the bottom represents 100 μm.

the type-II cadherins, including the three novel molecules, and comparison of the type-II cadherins with other classic cadherins confirmed the identity of this grouping. As shown in Table 1, the sequence similarities of the putative mature forms among the type-II cadherins are high, ranging from 58 to 76%, whereas all of the type-II cadherins are less similar to every other known human classic cadherin. For example, sequence similarities to E-cadherin are also shown in Table 1, and all of these are below 40%. In the extracellular domains, which are composed of about 550 amino acids, 21 out of 180 conserved residues among the type-II cadherins are specific for type-II cadherins and are not found in any other human classic cadherin. In contrast, in the cytoplasmic domains, only three out of 52 conserved residues among the type-II cadherins are specific for type II, indicating that the type-II cadherins are more characteristic in the extracellular domains, which form interfaces for cell–cell adhesion, than in the cytoplasmic domains, which link to the cytoskeleton via catenins. However, even in the extracellular domains we could not identify any motifs specific for type-II cadherins. Unexpectedly, the most impressive sequence characteristic for the type-II cadherins was found in the transmembrane domains, which have never been considered to be of significance in the field of cadherin research. As compared with the transmembrane domains of the non-type-II cadherins, those of the type-II cadherins were markedly conserved, both in length and in amino acid sequence (Figure 1B).

To our knowledge, besides the eight molecules presented here, no other human classic cadherins of type II have been identified. Kremmidiotis et al. [31] recently reported sequencing of a partial cDNA clone homologous to chicken cadherin-7, and registered the sequence as human cadherin-7 in the GenBank® nucleotide sequence database with the accession number AF047826. However, the deduced amino acid sequence of 317 residues, corresponding to a region from the end of the EC4 to the C-terminus, shares only 54 and 53% identities with the corresponding region of human cadherin-7 presented here and with chicken cadherin-7, respectively. In the same region, our human cadherin-7 is much more similar to chicken cadherin-7 (92% similarity). These findings strongly suggest that the cDNA clone obtained in the present study by cross-hybridization with a chicken cadherin-7 cDNA probe actually encodes an authentic human counterpart of chicken cadherin-7, and that the cDNA fragment obtained by Kremmidiotis et al. indicates the existence of an unknown human classic cadherin. The partial amino acid sequence of clone number AF047826 shows higher similarity to the type-II cadherins (43–46%, except for cadherin-7) than to the non-type-II cadherins (below 40%), and possesses the 33-amino acid transmembrane domain. However, this clone, of which only a restricted sequence is available, conserves only one residue out of the five residues defined as specific for type-II cadherins in the restricted region (results not shown), and shows relatively low similarities to the type-II cadherins described above. These findings suggest that the clone is likely to encode a novel human type-II classic cadherin that is somewhat diversified from the eight type-II cadherins identified so far.

Cadherin-7, -9 and -10 were expressed in mouse L fibroblasts by a cDNA-transfection method, and the individual transfectant clones were selected. As has been demonstrated for other classic cadherins, these cadherins interacted with α- and β-catenins, and retarded their degradation (Figures 2 and 3). We obtained new L transfectant clones for cadherin-8, -11 and -12 in this study, and also used L transfectant clones for cadherin-6 and -14 that we reported previously [23]. We then evaluated cell–cell binding activities of the eight human type-II classic cadherins by cell-aggregation experiments over 48 h with particular attention to the expression level of β-catenin protein by the transfectants. As we proposed recently [19,23], the expression level of β-catenin is considered to reflect the expression level of a transfected cadherin in this system. This is based on the observation that β-catenin is hardly detectable in L cells at the protein level because of its rapid turnover rate. However, once L cells begin to express classic cadherin molecules, upregulation of β-catenin protein occurs at the same time, possibly because of stabilization of the β-catenin protein by its association with the cytoplasmic domain of classic cadherin. Assuming that all classic cadherin subclasses influence the preservation of β-catenin protein in L cells to a similar extent, the relative expression level of a cadherin in an L transfectant clone could be estimated from the expression level of β-catenin protein in the clone.

As shown in Figure 4, transfectant clones for E-cadherin and cadherin-6, -7, -8, -11, -12 and -14, all of which express similar amounts of β-catenin protein (Figure 2) and therefore are expected to express a similar number of cadherin molecules per cell, formed aggregates hardly distinguishable from one another both in size and in cell–cell adhesiveness. The cadherin-9 and -10 transfectant clones, which show lower expression of β-catenin protein than the other transfectants (Figure 2), formed smaller aggregates (Figure 4). Transfectants of other cadherin types expressing similar amounts of β-catenin protein to the cadherin-9 or -10 transfectant clones formed aggregates indistinguishable from those of the cadherin-9 or -10 transfectants (results not shown). These findings strongly suggest that all of the type-II cadherins possess a similar cell–cell binding ability that is almost equivalent to that of E-cadherin.

Although Suzuki and colleagues have reported that cadherin-5 and -8 did not show significant cell-adhesion activity on cell-aggregation experiments [21,22], we demonstrated in this study that cadherin-8 mediates cell–cell binding comparable with that of other classic cadherins, and we confirmed recently that cadherin-5 also exhibits definite cell–cell binding activity in the same assay system as used in this study (Y. Shimoyama, G. Tsujimoto, M. Kitajima and M. Natori, unpublished work). This discrepancy may be explained by the difference in the assay system. Alternatively, as mentioned in the Results section, their cadherin-8 cDNA clone encoded a molecule that was seven amino acids shorter at the N-terminus as compared with the clone used in this study; this might cause derangement of the cleavage of the signal peptide and the precursor region, or of the transport of the cadherin to the cell surface, leading to malfunction of the cadherin in their study. Interestingly, a cadherin-5 cDNA clone that was isolated recently from a human adult skeletal-muscle cDNA library in our laboratory contained an open reading frame that was 12 nucleotides longer (corresponding to Met-Gln-Arg-Leu) than that previously reported by Suzuki and colleagues [16] (Y. Shimoyama, G. Tsujimoto, M. Kitajima and M. Natori, unpublished work). This might suggest that the truncated N-terminal sequence was the cause of the reduced cell–cell binding activity. The nucleotide sequences of the human cadherin-5 and -8 cDNAs isolated in our laboratory have also been submitted to the DDBJ/EMBL/GenBank® nucleotide sequence databases with the accession numbers AB035304 and AB035305, respectively.

It has been postulated that some non-type-I classic cadherins, including the type-II cadherins, mediate weaker cell–cell adhesion than do type-I cadherins, since they are expressed in loosely associated cells [20]. In fact, we have confirmed recently that various human fibroblast strains express multiple cadherin molecules including type-II cadherins (Y. Shimoyama, G. Tsujimoto, M. Kitajima and M. Natori, unpublished work). It could be speculated, however, that mesenchymal cells, such as

fibroblasts lack other components of the cell–cell binding machinery besides the cadherin cell-adhesion system. In any case, the data presented here indicate clearly that all of the type-II cadherins function as cell–cell binding connectors, as do type-I cadherins.

In a recent study, we found that cadherin-6 and -14 interacted with each other, although in an incomplete manner [23]. This finding prompted us to verify the heterophilic interactions among the eight type-II cadherins, and we performed aggregation experiments with mixed cell types. Surprisingly, the formation of chimaeric aggregates was observed in various combinations (Figure 5 and Table 2). Completely intermixed aggregates were formed with combinations of L transfectant clones expressing cadherin-6 and -9, cadherin-7 and -14, cadherin-8 and -11, and cadherin-9 and -10, indicating that in these combinations the two cadherin subclasses exhibit equal binding specificities. Moreover, partially intermixed aggregates similar to the heterogeneous aggregates between cadherin-6 and -14 [23] were observed with combinations of L transfectant clones expressing cadherin-6 and -7, cadherin-6 and -10, cadherin-7 and -9, cadherin-7 and -12, cadherin-9 and -14, and cadherin-12 and -14, indicating that in these combinations the two cadherin subclasses share a part of their binding specificities. In particular, either cadherin-7 and -14 or cadherin-8 and -11 showed an indistinguishable binding specificity against other cadherin subclasses, at least in this assay system (Table 2).

Similar complete and incomplete heterophilic interactions between different cadherin subclasses from the same species have been described for B-cadherin and L-CAM (liver cell-adhesion molecule) in chicken [32], for N- and R-cadherin in chicken and mouse [33,34], and for cadherin-6B and -7 in chicken [24]. However, the frequency of heterophilic interactions among the human type-II classic cadherins was unexpected. Although these results might offer significant clues for identification of the sites responsible for the binding specificities, we could not determine the sites or motifs from comparison and analysis of the primary structures. We demonstrated previously that the QAI tripeptide, corresponding to the HAV motif in type-I cadherins that is known to be intimately involved in the adhesive function and binding specificity [28–30], is itself unlikely to be responsible for the adhesive functions and binding specificities of cadherin-6 and -14 [23]. As mentioned above, cadherin-6 and -9, cadherin-7 and -14, cadherin-8 and -11, and cadherin-9 and -10 cross-reacted completely in the mixed cell-aggregation experiments; however, in every combination the tripeptide corresponding to the HAV motif of the two cadherin subclasses does not coincide (Figure 1A). Future studies on the three-dimensional structures would clarify this issue.

Although this study demonstrating frequent heterophilic interactions among the type-II classic cadherins used an assay system *in vitro*, we believe that the results presented here will be useful in the future for understanding cadherin-mediated mechanisms of development, morphogenesis and cell–cell interactions *in vivo*.

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