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Characterization of cadherin-4 and cadherin-5 reveals new aspects of cadherins

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SUMMARY

Several properties of cadherin-4 and cadherin-5 were characterized by using the cDNA transfection approach. The proteins of both cadherins had a relative molecular mass of about 130 kDa and were present at the cell periphery, especially at cell-cell contact sites. These cadherins were easily digested with trypsin, and Ca^{2+} protected cadherin-4, but not cadherin-5, from the digestion. In immunoprecipitation, cadherin-4 co-precipitated with two major proteins of 105 kDa and 95 kDa, respectively. The 105 kDa and the 95 kDa proteins are likely to correspond to α- and β-catenins. Cadherin-5 co-precipitated with only one major protein of 95 kDa, but seems to associate with the 105 kDa protein. On the other hand, plakoglobin or γ-catenin did not co-precipitate well with either cadherin-4 or cadherin-5 in immunoprecipitation, but plakoglobin also appears to associate weakly with these cadherins. Cadherin-4 transfectants aggregated within 30 minutes in a cell aggregation assay, but cadherin-5 transfectants did not aggregate under the same conditions. Furthermore, the transfectants of chimeric cadherin-4 with cadherin-5 cytoplasmic domain showed cell aggregation activity comparable to that of wild-type cadherin-4 transfectants, whereas the transfectants of chimeric cadherin-5 with cadherin-4 cytoplasmic domain did not show appreciable cell aggregation, suggesting that the extracellular domains of cadherins, in conjunction with their cytoplasmic domains, play an important role in cell aggregation activity. These results show that cadherin-4 is very similar to the classical cadherins, whereas cadherin-5 is functionally as well as structurally distinct from classical cadherins.

Key words: cadherin, catenin, cell adhesion, cytoplasmic domain, extracellular domain, motif

INTRODUCTION

A group of cell membrane proteins, termed cadherins, mediate Ca^{2+}-dependent and highly specific cell-cell adhesion, and are thought to play an important role during morphogenesis. So far, more than a dozen cadherins have been identified in different tissues from a variety of organisms. E-cadherin and N-cadherin, which are also known by other names, have been studied in detail and their structural and functional properties have been characterized extensively (for review, see Takeichi, 1991; Geiger and Ayalon, 1992). Most other cadherins have not yet been characterized well. Cloning studies, however, have already revealed that some of these cadherins have unique structural features that have not been reported for the classical cadherins, suggesting that these cadherins constitute a cadherin subfamily, type II cadherins: type II cadherins show low homology with classical cadherins and share common sequence features, such as characteristic amino acid deletions or additions and distinctive amino acid substitution at various sites, which are not found in classical cadherins (Suzuki et al., 1991; Tanihara et al., 1993).

Cadherin-4 is a classical type cadherin, or type I cadherin, and is likely to be a mammalian homologue of chicken R-cadherin, judging from their amino acid sequences (Inuzuka et al., 1991a; Suzuki et al., 1991). Cadherin-4 and R-cadherin have very high homology with N-cadherin, and their expression is somewhat complementary to that of N-cadherin in chicken retina (Inuzuka et al., 1991a; Redies et al., 1992). Although cadherin-4 and R-cadherin were originally isolated from nervous tissue, they are also expressed in other tissues (Inuzuka et al., 1991b; Suzuki et al., 1991; Davidson et al., unpublished observation). Cadherin-5, on the other hand, is one of the cadherins isolated recently by polymerase chain reaction (PCR) (Suzuki et al., 1991). This cadherin is a type II cadherin, and its expression appears to be limited to vascular endothelial cells. Recently, Lampugnani et al. (1992) reported a membrane protein from vascular endothelial cells, and it is likely that this is the same protein as cadherin-5. Heimark et

al. (1990) reported a cadherin named V-cadherin in vascular endothelial cells, but cadherin-5 does not appear to correspond to V-cadherin (Lampugnani et al., 1992; Heimark et al., unpublished observation). The biological roles of cadherin-4 and cadherin-5 are not clear at present.

Cadherin-4 and cadherin-5 apparently belong to two structurally different cadherin subgroups, but many of their properties have not yet been characterized. Accordingly, we examined and compared some of the basic properties of these cadherins using their cDNA transfectants. Our results showed that most of the properties of cadherin-4 were very similar to those of classical cadherins, but some features of cadherin-5 were unique among the various cadherins, suggesting that cadherin-5 is functionally as well as structurally distinct from the classical cadherins. Furthermore, the present results of experiments with chimeric cadherins highlight the importance of the extracellular domains of cadherins in cell adhesion activity. In this report, we describe our results and discuss the possible biological implications of these findings.

MATERIALS AND METHODS

Transfection of cadherin-4, cadherin-5 and chimeric cadherins

The cDNAs that contained the entire coding sequences of cadherin-4 and cadherin-5 were cut out by *Hin*dIII digestion, followed by blunting and *Spe*I digestion or *Eco*RV and *Xba*I digestion, and subcloned into the *Spe*I-blunted *Xba*I site or the blunted *Hin*dIII-*Xba*I site of pRc/RSV. Chimeric constructs of cadherin-4 and cadherin-5 with different cytoplasmic domains were prepared as follows. A *Mro*I sequence was introduced near the N terminus of the cadherin-4 cytoplasmic domain that corresponds to the *Mro*I site of the cadherin-5 cytoplasmic domain by PCR using an upstream primer that corresponds to the 5′ flanking region, and a downstream primer that corresponds to the region near the N terminus of the cytoplasmic domain and contains a *Mro*I sequence at the 5′ end. The PCR product was subcloned into the *Sma*I site of Bluescript SK. Then, the cDNA that corresponds to the cytoplasmic domain of cadherin-5 was cut out by *Spe*I digestion followed by blunting and *Mro*I digestion, and subcloned into the *Mro*I-*Eco*RV site of the above construct. The authentic cadherin-5 construct was cut with *Mro*I and *Xba*I and purified by agarose gel electrophoresis. Then, the cadherin-4 cytoplasmic sequence that corresponds to the *Mro*I-*Xba*I fragment of cadherin-5 cytoplasmic domain was synthesized by PCR using primers that contained *Mro*I or *Xba*I at the 5′ end, and subcloned into the *Mro*I-*Xba*I site of the above cadherin-5 construct. The resultant constructs were subcloned into the multicloning site of pRc/RSV vector and introduced into *Escherichia coli* NM522 cells, and the plasmids were isolated from *E. coli* cells by using the Quiagen plasmid purification kit. L cells were then transfected with the resultant DNAs by the calcium phosphate method using a Pharmacia CellPhect transfection kit. The transfectant cells were isolated by a combination of G418 selection and fluorescence activated cell sorting (FACS), as described by Ozawa et al. (1989).

Northern blot analysis

Total RNA preparations were made by a guanidium isothiocyanate method (Sambrook et al., 1989). The resultant samples were separated by electrophoresis on a 0.8% agarose gel under denaturing conditions and transferred to a nitrocellulose filter. Northern blot analysis was performed by the method of Thomas (1980).

Antibodies

The cDNAs that correspond to EC-1 through EC-3 of cadherin-4 and EC-2 through EC-4 of cadherin-5 were prepared by PCR and subcloned into the *Eco*RI-*Xba*I site of an expression vector, pMal-CRI (New England Biolab.). NM522 cells were then transformed with the resultant DNAs by a single-step method (Chung et al., 1989). The fusion proteins were induced by the addition of isopropyl-β-D-thiogalactopyranoside, and were purified from the extract by amylose resin affinity column chromatography, as described by the manufacturer. Polyclonal antibodies were prepared by injecting rabbits with 0.5 mg of the respective fusion proteins in complete Freund's adjuvant at four subcutaneous sites. After subsequent booster injection, the sera were collected and the antibodies were purified from the sera using a Sepharose column coupled with the above fusion proteins.

Monoclonal anti-α-catenin antibody (α18) and anti-β-catenin antibody (12F7) were gifts from Dr S. Tsukita and Dr K. A. Knudsen, respectively, and monoclonal anti-plakoglobin antibody (PG5.1) was the product of IBL Research Product Corporation. Alkaline phosphatase-conjugated anti-rabbit, anti-rat and anti-mouse antibodies were obtained from Promega. FITC-conjugated anti-rabbit, anti-rat and anti-mouse antibodies were purchased from Cappel.

Immunoblotting and immunoprecipitation

After washing the cultured cells with phosphate-buffered saline (PBS), the SDS-PAGE sample buffer was applied directly to the cultured cells and samples were made. SDS-PAGE was carried out by using Laemmli's (1970) buffer system and the separated proteins were blotted electrophoretically onto a PVDF membrane (Matsudaira, 1987). The resultant membranes were incubated for 2 hours at room temperature in Tris-buffered saline (TBS) containing 5% skim milk, and then for 1 hour at room temperature with primary antibody in TBS containing 0.05% Tween-20. After washing the membranes four times (5 minutes each) with TBS containing 0.05% Tween-20, the membranes were incubated for 1 hour at room temperature with alkaline phosphatase-conjugated anti-IgG antibody (Promega) in TBS that contained 0.05% Tween-20. The membranes were then washed again four times (5 minutes each) at room temperature with TBS that contained 0.05% Tween-20 and then developed using Promega Western Blue.

The cultured cells were metabolically labeled overnight by incubation in Dulbecco's modified Eagle's medium (DMEM) containing [^{35}S]methionine (50 μCi/ml). The cells were then washed with PBS and solubilized by incubation in Triton X-100-containing PBS (Ozawa et al., 1989). After centrifugation, the extract was precleared with nonspecific antibody and incubated with specific antibody for 1 hour at room temperature. The immunocomplex was then collected with Protein A-Sepharose and washed with PBS four times by centrifugation. The resultant samples were run on SDS-PAGE and subjected to fluorography. Pharmacia molecular mass calibration kit proteins were used as size markers.

Immunofluorescence microscopy

Transfectant cells were grown on coverslips precoated with fibronectin or laminin, or without coating. For α-catenin staining, the cells were fixed with 4% paraformaldehyde for 10-20 minutes and then permeabilized with 0.2% Triton X-100 for 15 minutes at room temperature (Fey et al., 1984). For β-catenin and plakoglobin staining, the cells were fixed in cold methanol for 20 minutes. After washing with TBS, cells were incubated for 1 hour with TBS containing 4% bovine serum albumin (BSA) and then reacted with first antibody in TBS containing 1% BSA and 2% goat serum. The cells were washed three times, 5 minutes each, with TBS containing 0.1% BSA and reacted for 60 minutes at room temperature with FITC-conjugated anti-IgG antibody in TBS containing 0.1% BSA. The cells were washed again with TBS containing 0.1% BSA, mounted and subjected to immunofluorescence microscopy.

Cell aggregation assay

Cell aggregation activity was assayed essentially according to the

method of Urushihara et al. (1979), with some modification (Ozawa et al., 1990). Briefly, single cell suspensions were prepared by treatment with 0.01% trypsin in the presence of 2 mM EGTA for 25 minutes, followed by pipetting. The resultant cells were collected by centrifugation and washed with Ca^{2+}-free Hepes-buffered saline (HBS). The collected cells were suspended in HBS containing 0.5% BSA. This simple method produces good single cell suspensions. The cells were then incubated in HBS containing 0.5% BSA, 2 mM $CaCl_2$ and 20 μg/ml of DNase for 20-60 minutes, or in a 1:1 mixture of the above HBS and DMEM for incubation for longer periods of time on a rotary shaker at 37°C. Parental L cells did not show appreciable cell aggregation for at least 3 hours under these conditions.

RESULTS

Expression of cadherin-4 and cadherin-5 in L cells

To examine the properties of cadherin-4 and cadherin-5, these cadherins were expressed in L cells from their cDNAs. Among the many transfectants, several clones were isolated that showed a high level of cadherin expression. Subsequent experiments were carried out using multiple independent clones for each cadherin, and similar results were obtained. Both transfectants expressed the expected sizes of mRNAs (about 3 and 3.2 kb) on northern blot analysis (Fig. 1A) and antibodies against cadherin-4 and cadherin-5 stained a band of about 130 kDa in respective transfectants on immunoblotting (Fig. 1B, lanes a and c). The anti-cadherin-5 antibody stained a band of similar size, but the anti-cadherin-4 antibody stained a band slightly smaller than the transfectant band in the rat brain extract in immunoblotting (Fig. 1B, lanes b and d). The size difference may be attributable to a difference in glycosylation.

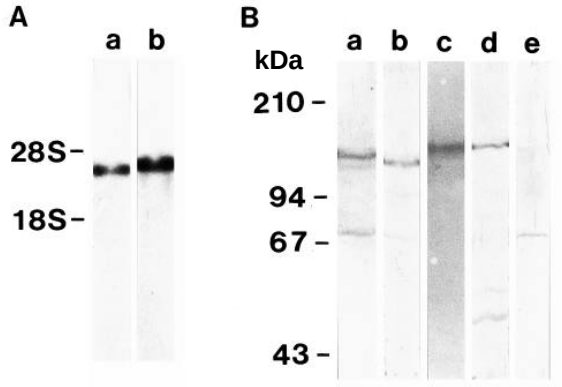

Fig. 1. Northern and western blot analysis of cadherin-4 and cadherin-5. (A) Total RNA preparations were separated electrophoretically on an agarose gel under denaturing conditions and transferred onto a nitrocellulose filter. Then, the filter was hybridized with ^{32}P-labeled cadherin-4 (a) or cadherin-5 (b) probe. The sizes of cadherin-4 and cadherin-5 messages were about 3.0 kb and 3.2 kb, respectively. Calf liver ribosomal RNAs were used for size markers. (B) The cell extracts were separated by SDS-PAGE, blotted onto a PVDF membrane and stained with antibodies. Anti-cadherin-4 antibody (a,b,e) and anti-cadherin-5 antibody (c,d) stained a band of about 130 kDa in the cadherin-4 (a) and cadherin-5 (c) transfectants and rat brain (b,d), but not in parental L cells (e). Myosin, phosphorylase *b*, albumin and ovalbumin were used as size markers.

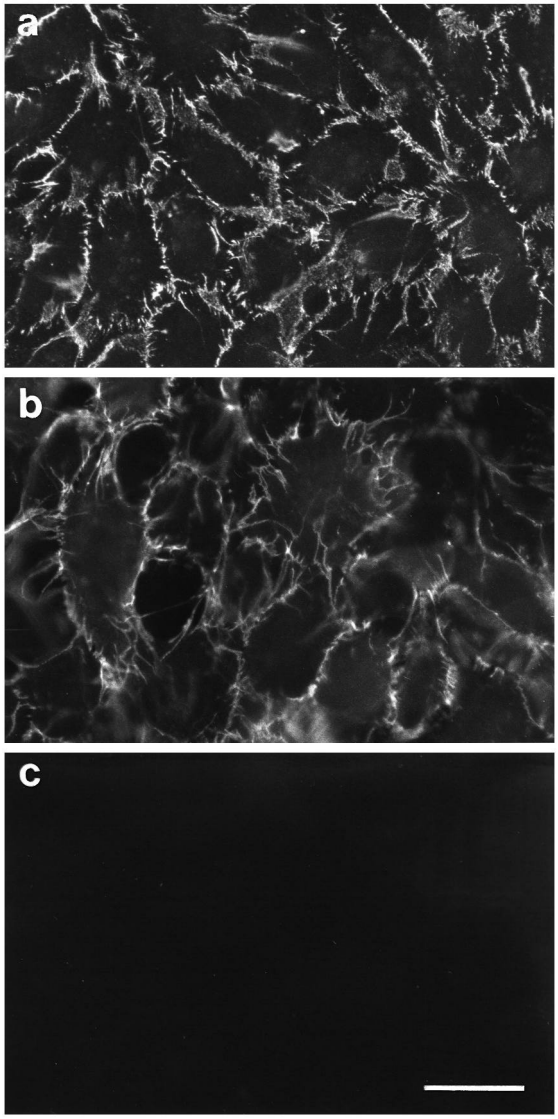

Fig. 2. Immunofluorescence staining of cadherin-4 and cadherin-5. Transfectants grown on coverslips were fixed with paraformaldehyde and stained with anti-cadherin-4 (a) or anti-cadherin-5 antibody (b). The antibodies stained primarily cell periphery or cell-cell contact sites of the transfectants of cadherin-4 (a) or cadherin-5 (b), but non-immune serum did not stain these sites (c). Bar, 20 μm.

Cadherin-4 and cadherin-5 transfectants showed a fibroblast morphology similar to that of the parental L cells, although the transfectants tended to show a slightly flattened shape. At low cell density, the cadherin-4 and cadherin-5 transfectants were loosely connected to one another, mainly via processes, but at high cell density they showed close contact. The cellular localization of the expressed cadherins was examined by immunofluorescence microscopy (Fig. 2). The polyclonal antibody against each cadherin stained primarily at the cell periphery, and when the cells at higher cell density formed close contacts, the antibody stained the cell-cell contact sites. Occasionally, dot-like staining was observed on the cell surface. It is unclear what was stained, but similar staining has been reported in other cadherin transfectants (Nagafuchi et al., 1987). Since

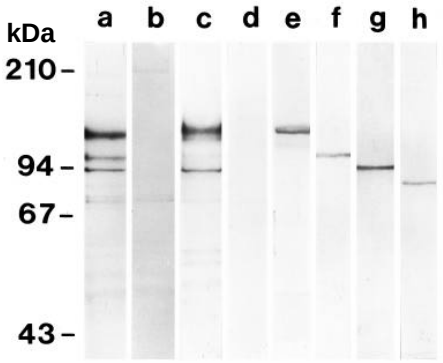

Fig. 3. Immunoprecipitation of cadherin-4 and cadherin-5. Metabolically labeled cells were solubilized with Triton X-100 and NP40 and immunoprecipitated with anti-cadherin-4 (a,b) and anti-cadherin-5 (c,d) antibodies. Cadherin-4 (a) co-precipitated with a 105 kDa and a 95 kDa band, whereas cadherin-5 (c) co-precipitated with only a 95 kDa band. b,d, control L cells. In immunoblot analysis of the immunoprecipitated samples of cadherin-5 transfectants (e-h), anti-cadherin-5 antibody (e), anti-α-catenin antibody (f), anti-β-catenin antibody (g) and anti-plakoglobin antibody (h) stained the 130 kDa, 105 kDa, 95 kDa and 85 kDa bands, respectively.

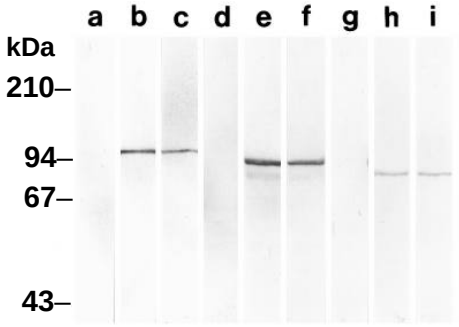

Fig. 4. Increase in the protein levels of α-catenin, β-catenin and plakoglobin in cadherin-4 and cadherin-5 transfectants. The protein levels of α-catenin (a,b,c), β-catenin (d,e,f) and plakoglobin (g,h,i) increased in cadherin-4 (b,e,h) and cadherin-5 (c,f,i) transfectants compared with their parental L cells (a,d,g). The same amount of extracts (10 μg) was used for each lane. The experimental procedures were essentially the same as described in Fig. 1B.

both antibodies are against the extracellular portion of the cadherins, and no permeabilization treatment was necessary for the staining, the expressed cadherins are likely to be located on the cell surface.

Association of cadherin-4 and cadherin-5 with catenins

Cadherin-4 co-precipitated with two major proteins of 105 kDa and 95 kDa molecular mass, respectively, in immunoprecipitation (Fig. 3a), as do the classical cadherins. In contrast, under the same conditions cadherin-5 co-precipitated with only one major protein of about 95 kDa molecular mass (Fig. 3c). This is not a transfectant-specific result, since similar results were obtained using human umbilical cord vein endothelial cells (Heimark et al., unpublished observation). When parental cells or non-immune sera were used instead of the transfectants or specific antibodies in the immunoprecipitation experiments, these bands were not precipitated.

The 105 kDa and the 95 kDa bands appear to correspond to α-catenin and β-catenin, respectively, since highly homologous N-cadherin co-precipitates with α-catenin and β-catenin in immunoprecipitation and their sizes are comparable to the 105 kDa or the 95 kDa protein (Ozawa et al., 1990; Hirano et al., 1992). Furthermore, anti-α-catenin and anti-β-catenin antibodies stained bands of 105 kDa and 95 kDa in immunoblot analysis of immunoprecipitated samples. Although the 105 kDa band did not co-precipitate well with cadherin-5, a comparable band was detected weakly in the immunoprecipitated sample with anti-α-catenin antibody (Fig. 3f), suggesting that cadherin-5 weakly interacts with α-catenin. The band that corresponds to plakoglobin or γ-catenin was not obtained in most of the immunoprecipitation experiments. However, anti-plakoglobin antibody did stain a faint band on immunoblotting of the immunoprecipitated samples, suggesting a weak association between plakoglobin or a closely related protein and cadherin-4 and cadherin-5 cytoplasmic domains. Furthermore, the protein levels of α-catenin, β-catenin and plakoglobin increased in the transfectants of cadherin-4 and cadherin-5 as compared with parental L cells (Fig. 4), as reported previously for α-catenin in E-cadherin transfectants (Nagafuchi et al., 1991). Cadherin-4 and cadherin-5 co-localized with α-catenin, β-catenin and plakoglobin in immunofluorescence microscopy of the transfectants (Fig. 5). All of these results support the view that cadherin-4 and cadherin-5 interact with these catenins, although the strength of the interaction between the two cadherins and catenins varies.

Cell aggregation activity of cadherin-4 and cadherin-5

Ca^{2+} protects classical cadherins from trypsin digestion (Takeichi, 1977), and this property has been used to prepare single cells for the conventional cell aggregation assay of cadherins (Urushihara et al., 1979). Accordingly, the effect of Ca^{2+} on trypsin treatment of cadherin-4 and cadherin-5 was examined before the cell aggregation assay. As shown in Fig. 6, Ca^{2+} protected cadherin-4 from trypsin digestion, as in the case of classical cadherins, but cadherin-5 was digested easily with trypsin, even in the presence of 5 mM of Ca^{2+}. A similar result was reported for cadherin-5 of human umbilical cord vein endothelial cells (Lampugnani et al., 1992).

Because of this result, trypsin treatment in the presence of EGTA was used mainly to prepare single cells for the cell aggregation assay in this study. In the cell aggregation assay, cadherin-4 transfectants began to aggregate after about 30 minutes of incubation, in a Ca^{2+}-dependent manner, and formed relatively large aggregates within 1 hour (Fig. 7a). The cell aggregation was partially inhibited by the addition of anti-cadherin-4 antibody to the incubation mixture. On the other hand, cadherin-5 transfectants, as well as parental L cells, did not aggregate under the same conditions (Fig. 7b and c). Even after prolonged incubation (up to 3 hours), the cadherin-5 transfectants did not show significant cell aggregation.

Chimeric cadherin-4 and cadherin-5 with different cytoplasmic domains

In contrast to classical cadherins, cadherin-5 did not co-pre-

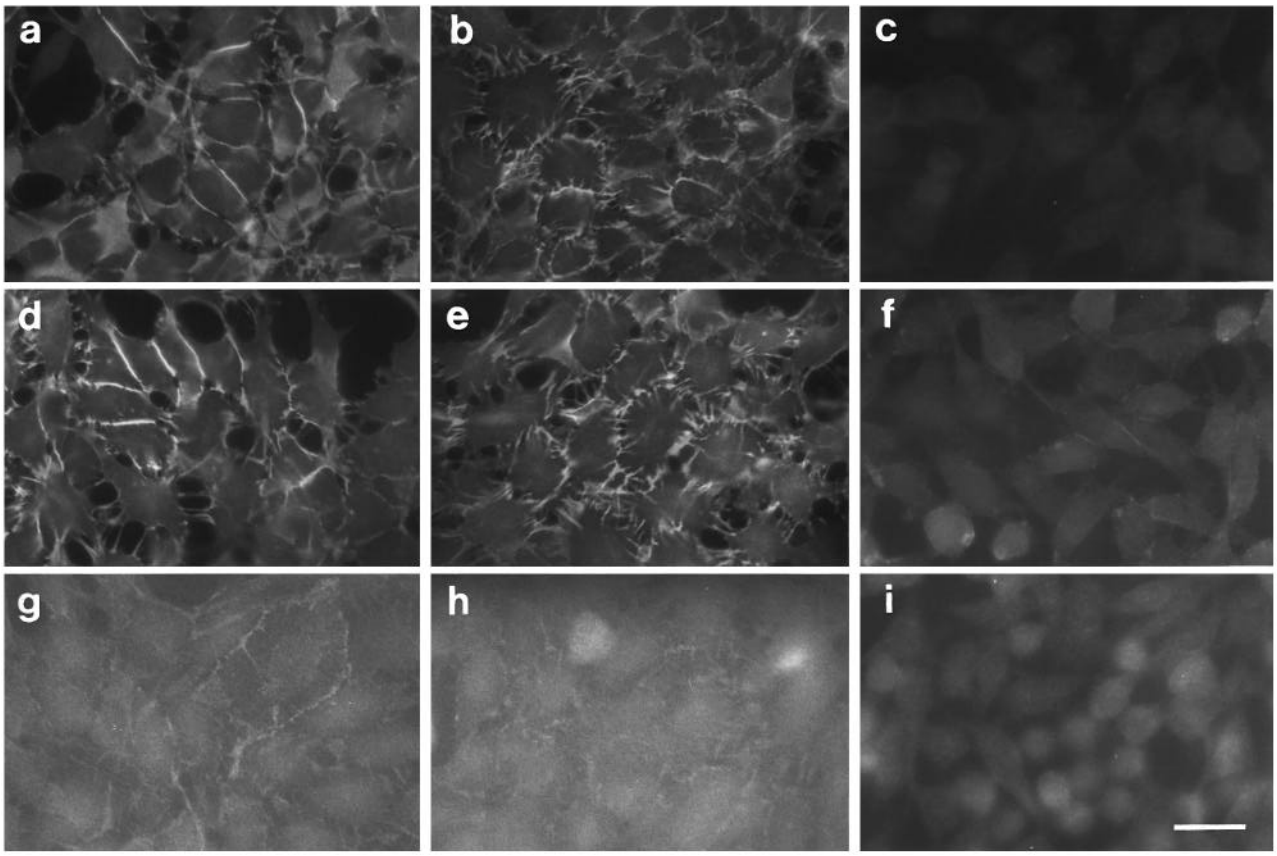

Fig. 5. Immunofluorescence staining of α-catenin, β-catenin and plakoglobin. Plakoglobin (g-i), α-catenin (a-c) and β-catenin (d-f) co-localized with cadherin-4 (a,d,g) and cadherin-5 (b,e,h) in the respective transfectants. The parental L cells did not stain well with these antibodies (c,f,i). The experimental procedures were essentially the same as described for Fig. 2. Bar, 20 μm.

cipitate well with a 105 kDa protein in immunoprecipitation and the transfectants did not show significant cell adhesion activity, as noted above. Therefore, the cytoplasmic domains of cadherin-5 and cadherin-4 were replaced with those of cadherin-4 and cadherin-5, respectively, expressed in L cells and the properties of the transfectants were examined. The joint site of the two sequences corresponds to the fifth amino acid from the N terminus of the cytoplasmic domain. We used transfectants that expressed chimeric cadherins at levels similar to those of wild-type cadherin-4 and cadherin-5 transfectants used in the previous section.

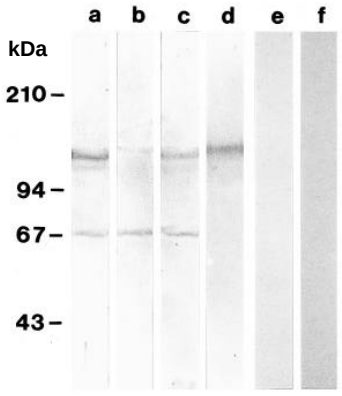

Fig. 6. Effect of Ca^{2+} on the trypsin digestion of cadherin-4 and cadherin-5. Cadherin-4 (a-c) and cadherin-5 (d-f) transfectants were treated with 0.01% trypsin for 30 minutes at 37°C in the presence (c,f) and absence (b,e) of 2 mM Ca^{2+}. The resultant cells were washed once with PBS containing soybean trypsin inhibitor, dissolved in SDS-PAGE sample buffer solution and analyzed by immunoblotting. Ca^{2+} protected cadherin-4 from trypsin digestion, but did not protect cadherin-5. The a and d lanes are untreated samples.

The resultant transfectants of chimeric cadherin-4 with the cytoplasmic domain of cadherin-5 (C4/C5) and chimeric cadherin-5 with the cytoplasmic domain of cadherin-4 (C5/C4) resembled parental L cells in morphology. The expressed chimeric cadherins were about 130 kDa in relative molecular mass (Fig. 8a and b) and were localized at the cell periphery, similar to those of wild-type cadherin-4 and cadherin-5 transfectants (Fig. 9a and c); the chimeric cadherin C4/C5 co-precipitated with only one major protein of about 95 kDa, whereas C5/C4 co-precipitated with two major proteins of 105 kDa and 95 kDa in immunoprecipitation (Fig. 8c and d). These results are consistent with the above conclusion that the cytoplasmic domain of cadherin-4 can co-precipitate with the 95 kDa and the 105 kDa proteins, whereas that of cadherin-5 can co-precipitate with only the 95 kDa protein. Interestingly, C4/C5 transfectants showed cell aggregation activity comparable to that of wild-type cadherin-4 transfectants, but C5/C4 transfectants did not show significant cell adhesion activity, even after several hours of incubation (Fig. 9b and d). Thus, cadherin-4 cytoplasmic domain is replaceable with that of cadherin-5, at least in terms of strong cell aggregation activity, despite the difference in the catenin association property.

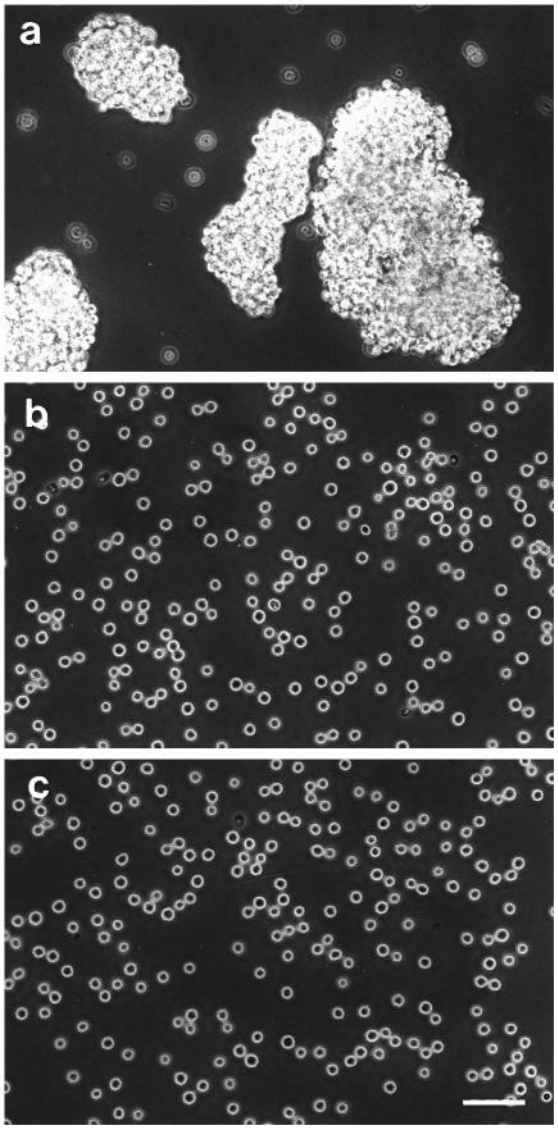

Fig. 7. Cell aggregation of cadherin-4 and cadherin-5. Transfectants were treated with 0.01% trypsin and 1 mM EGTA, and single cells were collected by centrifugation. After washing, the cells were incubated in HBS containing 2 mM $CaCl_2$, 1% BSA and 20 μg/ml of deoxynucleotidase for 1 hour on a rotary shaker. Cadherin-4 transfectants (a) started to aggregate after about 30 minutes, but cadherin-5 transfectants (b) and parental L cells (c) did not form aggregates even after 1 hour. Bar, 200 μm.

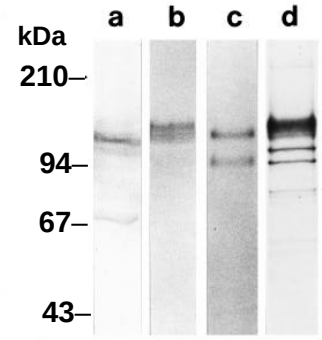

Fig. 8. Immunoblotting and immunoprecipitation of chimeric cadherins C4/C5 and C5/C4. Chimeric cadherins C4/C5 (a) and C5/C4 (b) showed an approx. 130 kDa band in immunoblot analysis. C4/C5 co-precipitated with only one major band of 95 kDa (c), whereas C5/C4 co-precipitated with a 105 kDa and a 95 kDa band (d) in immunoprecipitation. The experimental procedures were described for Fig. 1 and Fig. 3.

DISCUSSION

Previous studies indicated that cadherins can be classified into at least two structurally distinct types, classical type I cadherins and newly identified type II cadherins, based on their amino acid sequences (Suzuki et al., 1991; Tanihara et al., 1993). The present comparison of some properties of cadherin-4 and cadherin-5 transfectants indicates that cadherin-4, a type I cadherin, shows properties very similar to those of classical cadherins, whereas cadherin-5, a type II cadherin, has several unique features, in addition to the properties common to the classical type I cadherins.

One striking result is that the cadherin-5 transfectants did not show clear cell aggregation activity, as do the classical type I cadherins, including cadherin-4, despite the fact that the overall sequence is very similar to that of classical cadherins. One may argue that the negative result of cadherin-5 cell aggregation assay may be attributable to the failure of cadherin-5 recovery on the cell surface within a couple of hours of incubation, since trypsin-EGTA treatment that digests cadherins on the cell surface was used to prepare single cell suspensions in this study. However, this may not be the case, because we could detect trypsin-sensitive cadherin-5 as well as cadherin-4 after about 1 hour of incubation. One may also argue that the expression of cadherin-5 in the transfectants was much less than that of cadherin-4 transfectants, and the cadherin-5 transfectants could not aggregate. At present, there is no good method for measuring the relative amounts of cadherin-4 and cadherin-5. On immunoblotting, however, anti-cadherin-5 antibody and anti-pancadherin antibody stained similarly a band of about 130 kDa in cadherin-5 and C5/C4 transfectants, and cadherin-4 and C5/C4, respectively. The results suggest that the expression level of cadherin-5 was not significantly lower than that of cadherin-4 in the transfectants used. Furthermore, the immunoprecipitation results, showing that similar amounts of β-catenin were co-precipitated with cadherin-4, cadherin-5 and chimeric cadherins C4/C5 and C5/C4 under the same conditions, are consistent with this notion. Taken together, it is likely that cadherin-5 lacks strong cell aggregation activity, at least in L cells.

The cytoplasmic domain of cadherin-4 was replaceable with that of cadherin-5 in terms of cell aggregation activity, as shown by the chimeric cadherin experiments. On the other hand, C5/C4, in which the cytoplasmic domain of cadherin-5 was replaced with that of cadherin-4, did not show significant cell adhesion activity either, despite the fact that C5/C4 co-precipitated with α- and β-catenins. Thus, the extracellular domain of cadherin-5 appears to be unable to support strong cell aggregation activity. Blaschuk et al. (1990) suggested an involvement of the HAV sequence in EC-1 of classical cadherins as the site of cell-cell interaction. EC-1 sequences of type II cadherins are not as well conserved as in the classical cadherins and do not have the HAV sequence (Tanihara et al., 1993). The difference may also be responsible, at least in part, for the poor cell aggregation activity by cadherin-5 transfectant cells. We cannot, however, exclude completely some other possibility at present: cell adhesion activity of cadherin-5 may

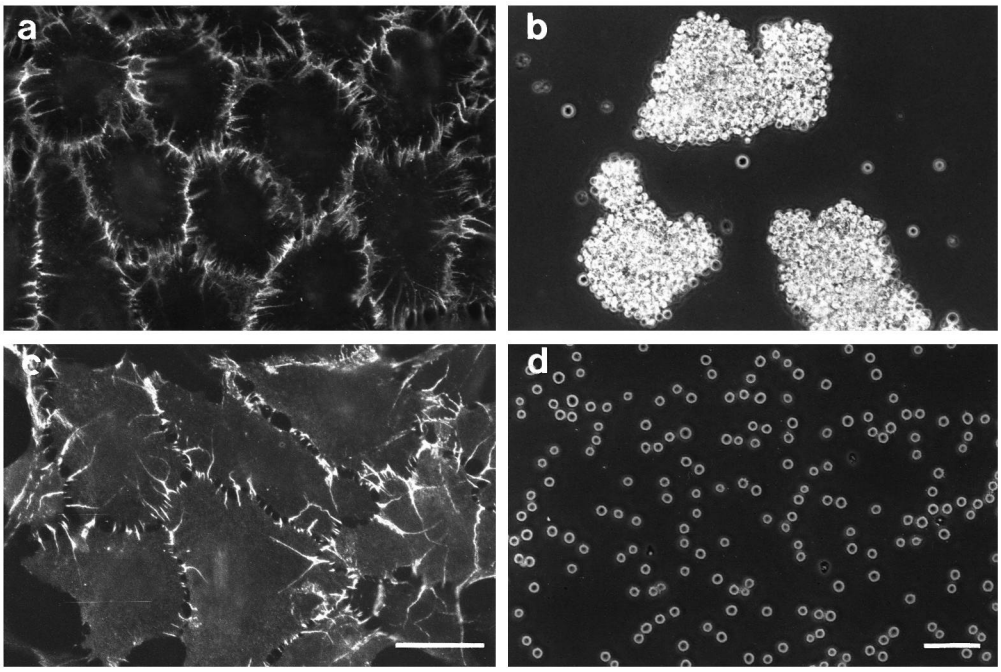

Fig. 9. Immunofluorescence staining and cell adhesion activity of chimeric cadherins C4/C5 and C5/C4. Anti-cadherin-4 (a) and anti-cadherin-5 (c) antibodies stained the cell periphery or cell-cell contact sites of C4/C5 transfectants (a) and C5/C4 transfectants (c). In the cell aggregation assay, C4/C5 transfectants (b) showed cell aggregation activity comparable to wild-type cadherin-4 transfectants, whereas C5/C4 transfectants (d) did not show appreciable activity. The experimental procedures were the same as described for Fig. 2 and Fig. 6. Bar, 20 μm (a,c) and 200 μm (b,d).

require an additional component necessary for the cell adhesion activity, but L cells may lack it; cell adhesion activity of cadherin-5 may be heterophilic, but L cells may not express the proper ligand for cadherin-5. Further experiments are necessary to clarify this issue. In any event, it is very conceivable that the extracellular domain, in conjunction with the cytoplasmic domain, has an important role in strong cell-cell interaction, since the extracellular domain has the interaction site.

Cadherins form a tight complex with cytoskeletal proteins that cannot be easily solubilized by detergents. Although the underlying mechanism is not known, it is thought that the tight complex is essential for the cell adhesion activity of cadherins and that catenins play a pivotal role in the interaction (for review, see Kemler, 1993). The finding that cadherin-5 did not co-precipitate well with α-catenin may explain the lack of strong cell aggregation activity of cadherin-5, since it has been reported that an association of the cytoplasmic domain of classical cadherins with α-catenin is essential for cell adhesion activity (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989; Hirano et al., 1992). However, our present study shows that the transfectants of chimeric cadherin C4/C5 did show cell adhesion activity, although C4/C5 did not co-precipitate well with α-catenin. If α-catenin is essential for the cell adhesion activity of cadherins, as other investigators have concluded (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989; Hirano et al., 1992), this result suggests that the cytoplasmic domain of cadherin-5 can interact with α-catenin, but that the interaction is so weak that cadherin-5 cannot co-precipitate with α-catenin in immunoprecipitation. Indeed, the protein level of α-catenin increased in the transfectants as compared with the parental L cells, and α-catenin was detected weakly in the immunoprecipitated cadherin-5 sample. Furthermore, cadherin-5 and α-catenin co-localized at cell-cell contact sites of cadherin-5 transfectants in immunofluorescence microscopy. Therefore, our findings suggest that strong interaction between α-catenin and cadherin cytoplasmic domain is not necessarily required for strong cell adhesion activity, and that α-catenin may mediate indirectly the interaction between cytoskeletal proteins and cadherin cytoplasmic domain. This is consistent with the conclusion reached by Ozawa and Kemler (1992), that the interaction of the cadherin cytoplasmic domain with α-catenin is rather indirect.

The cytoplasmic domain of cadherin-5 as well as cadherin-4 strongly interacts with β-catenin. Plakoglobin or a closely related protein, on the other hand, appears to associate weakly with the cytoplasmic domains of cadherin-4 and cadherin-5, although the evidence for this is circumstantial. The roles of β-catenin and plakoglobin in cadherin function are poorly understood at present. However, results of this study suggest that cadherins in general associate with β-catenin and plakoglobin. Furthermore, β-catenin and plakoglobin are highly homologous to the product of *armadillo*, a segment polarity gene of *Drosophila* (Franke et al., 1989; Peifer and Wieschaus, 1990; McCrea and Gumbiner, 1991; Knudsen and Wheelock, 1992), and it has recently been reported that β-catenin can associate with the product of the APC gene, a tumor suppressor gene (Rubinfeld et al., 1993; Su et al., 1993). It is tempting to speculate that β-catenin and plakoglobin play a regulatory or other important role in cadherin function.

In this study, we characterized some properties of cadherin-4 and cadherin-5. The results have revealed new aspects of cadherins that, to our knowledge, have not been reported before. In particular, cadherin-5, a type II cadherin, showed novel features. Interestingly, the transfectants of cadherin-8, which is another type II cadherin, showed properties similar to those of cadherin-5 in Ca^{2+} protection against trypsin digestion, cell adhesion activity, and catenin association (Kido et al., unpublished). Type II cadherins are structurally distinct from classical type I cadherins, and the type II cadherins may have characteristic sequence features in common. Although further experiments are necessary, the unique sequences

specific to type II cadherins may be responsible for the novel properties of cadherin-5.

As described in this study, cadherins appear to be functionally as well as structurally divergent proteins. The biological roles of cadherin-4, cadherin-5 and other recently found cadherins are not clear at present. However, these cadherins are likely to be involved in and have important roles in some cell-cell interactions, considering their properties. The information obtained by this study may provide the basis for further insight into their biological roles.

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