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Defining the Roles of β-catenin and Plakoglobin in Cell-cell Adhesion: Isolation of β-catenin/plakoglobin-deficient F9 Cells

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ABSTRACT. **F9 teratocarcinoma cells in which β-catenin and/or plakoglobin genes are knocked-out were generated and investigated in an effort to define the role of β-catenin and plakoglobin in cell adhesion. Loss of β-catenin expression only did not affect cadherin-mediated cell adhesion activity. Loss of both β-catenin and plakoglobin expression, however, severely affected the strong cell adhesion activity of cadherin. In β-catenin-deficient cells, the amount of plakoglobin associated with E-cadherin dramatically increased. In β-catenin/plakoglobin-deficient cells, the level of E-cadherin and α-catenin markedly decreased. In these cells, E-cadherin formed large aggregates in cytoplasm and membrane localization of α-catenin was barely detected. These data confirmed that β-catenin or plakoglobin is required for α-catenin to form complex with E-cadherin. It was also demonstrated that plakoglobin can compensate for the absence of β-catenin. Moreover it was suggested that β-catenin or plakoglobin is required not only for the cell adhesion activity but also for the stable expression and cell surface localization of E-cadherin.**

Key words: F9/β-catenin/plakoglobin/targeting/cadherin/cell adhesion

Introduction

Cadherin-mediated cell adhesion plays a pivotal role in animal morphogenesis (Takeichi, 1991). β-Catenin is a cytoplasmic protein that directly interacts with the C-terminal cytoplasmic domain of cadherin. β-Catenin also directly interacts with α-catenin, which associates with actin-based cytoskeletons directly or indirectly (Imamura *et al.*, 1999; Orsulic and Peifer, 1996; Rimm *et al.*, 1995). Thus, β-catenin links cadherin to actin-based cytoskeletons through α-catenin. The cadherin-catenin complex forms a typical cell-cell junction referred to the adherens junction. Plakoglobin is a close homologue of β-catenin and also interacts directly with cadherin and α-catenin (Sacco *et al.*, 1995). Unlike the case with β-catenin, plakoglobin plays a fundamental role as a link between desmosomal cadherins and the intermediate filament cytoskeletons. Desmosomal cadherins are major constituents of another cell-cell adhesive junction referred to as desmosomes. Thus, plakoglobin could be a component of both adherens junctions and desmosomes, although this molecule preferentially incorporates into desmosomes in cells that assemble both junctions (Cowin *et al.*, 1986). β-Catenin and plakoglobin also play an important role in animal development by acting as cytoplasmic signaling molecules in the Wnt signaling pathway. It remains to be determined whether β-catenin and plakoglobin play a role in the cross-talk between the cell adhesion system and the Wnt signaling pathway (Ben-Ze'ev and Geiger, 1998).

It was reported that armadillo, a β-catenin/plakoglobin homologue in *Drosophila*, is indispensable for cell-cell adhesion (Peifer *et al.*, 1993). Maternal/zygotic mutants for a strong allele of armadillo undergo widespread cell dissociation following gastrulation. In contrast, inactivation of HMP-2, a *C. elegans* homologue of β-catenin, failed to disrupt early embryonic cell adhesion (Costa *et al.*, 1998; Korswagen *et al.*, 2000). Knock-out (KO) experiments of β-catenin and plakoglobin in mice have been reported. β-Catenin-deficient mouse embryos showed early embryonic lethality, and displayed a defect in anterior-posterior axis formation at embryonic day 5.5, probably due to the lack of Wnt signaling (Huelsken *et al.*, 2000). Plakoglobin KO mice exhibited an embryonic lethal phenotype due to cell

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dissociation in the heart muscle probably due to the destruction of desmosomes (Ruiz *et al.*, 1996). Thus, the use of animal models has mainly demonstrated the importance of β-catenin and plakoglobin in Wnt signaling and desmosome formation, respectively. The importance of β-catenin and plakoglobin in cadherin-mediated cell adhesion especially in mammalian cells remains to be delineated.

We previously reported that the E-cadherin-catenin complex mediated strong cell adhesion activity in the absence of plakoglobin (Nagafuchi *et al.*, 1987). This indicated that plakoglobin is unnecessary for cadherin-mediated cell adhesion in the presence of β-catenin. On the other hand, it remains to be determined whether plakoglobin can compensate for the absence of β-catenin in the cadherin-mediated cell adhesion system. Analysis of β-catenin KO mice yielded confusing results. Haegel *et al.*, (1995) reported that plakoglobin could not compensate for the absence of β-catenin in cadherin-mediated cell adhesion. In contrast, Huelsken *et al.*, (2000) showed a normal distribution of E-cadherin and α-catenin and normal junctional complex formation in the absence of β-catenin. A homozygous deletion of the β-catenin gene was reported in the human malignant mesothelioma cell line NCI-H28 (Usami *et al.*, 2003). The cell adhesion properties of this cell line, however, were not described. HSC-39, a human gastric cancer cell line, expresses aberrant β-catenin which lacks the α-catenin-binding domain. Since these cells express E-cadherin, α-catenin and plakoglobin, but do not form cell-cell adhesion, it was supposed that plakoglobin could not compensate for the absence of β-catenin in cadherin-mediated cell adhesion. The reexpression of β-catenin in these cells, however, could not necessarily restore strong cell adhesion activity (Kawanishi *et al.*, 1995; Oyama *et al.*, 1994). It is possible that truncated β-catenin in HSC-39 cells functions as a dominant-negative molecule for β-catenin and plakoglobin. It is also possible that an unidentified mutation might prevent cadherin-mediated cell adhesion in these cells (Peng *et al.*, 2002). Notwithstanding experiments reported to date pertaining to cultured cell lines, the role of β-catenin and plakoglobin in the cell adhesion system remains unclear.

It is well accepted that β-catenin and plakoglobin play a crucial role in the cadherin-based cell adhesion system. The molecular details of the mechanism employed by β-catenin and plakoglobin remain unknown. To resolve this problem, we isolated β-catenin-deficient (βT) and β-catenin/plakoglobin-deficient (BPD) mouse teratocarcinoma F9 cells by gene targeting. Using these cell lines, we showed that β-catenin or plakoglobin, acting as a linker between E-cadherin and α-catenin, are indispensable for the strong cell-cell adhesion activity mediated by cadherin. We also demonstrated the functional redundancy between β-catenin and plakoglobin in the cell adhesion system. Moreover, we suggested that β-catenin and plakoglobin are involved in the stabilization and cell surface localization of E-cadherin.

Materials and Methods

Cell culture and phase contrast images

Mouse F9 EC cells and their derivatives were cultured in Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal calf serum (GIBCO BRL). Culture dishes or cover slips were pre-coated with 0.2% gelatin for 15 min or 10 μg/ml fibronectin for 60 min. Phase contrast microscopic images were acquired using a microscope (ECLIPSE TS100, Nikon) equipped with a digital camera (Coolpix 990, Nikon).

Targeting vector, gene targeting and Southern blot analysis

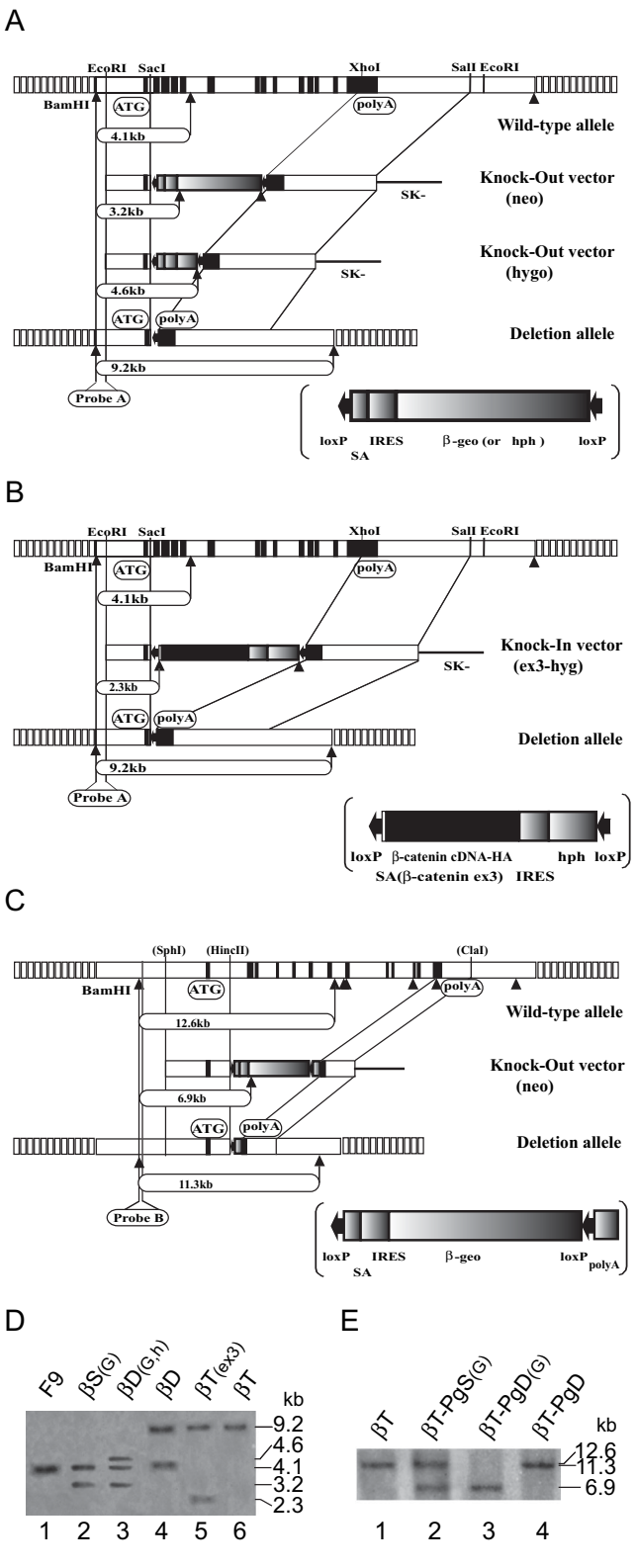
β-Catenin and plakoglobin genomic clones were isolated from a 129/Sv mouse genomic library (kindly provided by Dr. O. Chisaka of Kyoto University, Japan).

The targeting vector construct for β-catenin, pβT2-RE-PSIBP, is composed of the following 5 distinct fragments: 1) a 28 bp repressor element, 2) a 1.8 kb EcoRI-SacI fragment including an ATG exon, 3) the PSIBP trap-selection cassette (Maeno *et al.*, 1999), 4) a 4.8 kb XhoI-SalI fragment including a polyA exon and 5) a pBluescript SK- plasmid. The repressor element, derived from the type II sodium channel gene (Kraner *et al.*, 1992), was expected to repress expression of the drug-resistance gene when the targeting vector was randomly integrated in the genome DNA. Other targeting vectors, pβT2-RE-PSIhP and pβT2-RE-Pex3βH-IhP, are similar to pβT2-RE-PSIBP but utilize the PSIhP trap-selection and Pex3βH-IhP knock-in trap cassettes, respectively. In PSIhP, the β-geo sequence in PSIBP was replaced with the hygromycin resistance gene. In Pex3βH-IhP, the splicing acceptor of PSIhP was replaced with the splicing acceptor of β-catenin exon 3, followed by the β-catenin cDNA with a HA-tag sequence.

The targeting vector construct for plakoglobin, pPgT1-RE-PSIBPA, is composed of the following 5 distinct fragments: 1) a 28 bp repressor element, 2) a 4.3 kb SphI-HincII fragment including an ATG exon, 3) the PSIBPAdelSal trap-selection cassette, 4) a 2.2 kb BamHI-ClaI fragment including a poly-A exon and 5) a pBluescript SK- plasmid. PSIBPAdelSal is a derivative of the PSIBP trap selection cassette, in which an additional poly-A additional sequence was added after the second loxP sequence.

The targeting vector was linearized with SalI and electroporated into F9 cells in 0.4 ml of HEPES-buffered saline at 250 V and 960 μF using a gene pulser. Cells were then subjected to G418 selection at 400 μg/ml for two weeks or hygromycin selection at 400 μg/ml.

Genomic DNA preparation and Southern blot analysis were performed as previously described (Maeno *et al.*, 1999; Saitou *et al.*, 1997). Correct targeting insertion was confirmed by hybridization with probe A for β-catenin and probe B for plakoglobin (Fig. 1). Single integration of the targeting vector in cells was checked by hybridization with an IRES probe (data not shown). Excision of the trap-cassettes was performed by transient expression of the Cre-pac plasmid as previously reported (Taniguchi *et al.*, 1998).



Antibodies

The following primary antibodies were used for immunocytochemical and immunoblot analyses: rat anti-mouse E-cadherin mAb (ECCD-2; Shirayoshi *et al.*, 1986), rat anti-mouse α-catenin mAb (α18; Nagafuchi and Tsukita, 1994), mouse anti-β-catenin mAb, mouse anti-γ-catenin mAb (Transduction Laboratories). As secondary antibodies, FITC-conjugated donkey anti-mouse IgG, FITC-conjugated donkey anti-rat IgG, Cy3-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rat IgG (Jackson Immnoresearch, West Grove, PA) were used.

Western blot and immunoprecipitation analyses

SDS-PAGE and immunoblotting were performed as previously described (Imamura *et al.*, 1999). Samples solubilized in SDS sample buffer were separated by SDS-PAGE. For the immunoblotting, proteins were electrophoretically transferred onto nitrocellulose sheets. Nitrocellulose membranes were then incubated with primary antibody. Antibody detection was performed using an Amersham biotin-streptavidin kit according to the manufacturer's instructions.

For the immunoprecipitation analysis, cells harvested from a confluent culture in 9-cm dishes were lysed in 1 ml of extraction buffer (0.5% NP-40, 1 mM $CaCl_2$, 2 mM Pefabloc SC Plus (Roche), 10 μg/ml leupeptin and 10 μg/ml aprotinin in HEPES-buffered Mg^{2+}-free saline) and centrifuged at 15,000 rpm for 20 min. The cell extract was preadsorbed three times with 50 μl anti-rat Ig beads, then adsorbed with 10 μl beads preincubated with ECCD-2 antibody. Beads were extensively washed with the extraction buffer and then suspended in SDS lysis buffer to prepare the sample for SDS-PAGE.

Fig. 1. Isolation of β-catenin-deficient and β-catenin/plakoglobin-deficient F9 cells. A: Structure of the wild-type β-catenin allele, the β-catenin KO targeting vectors, targeted alleles, deletion allele following Cre treatment and the PSIBP (or PSIhP) trap-selection cassette are shown together with the pertinent restriction enzyme sites. B: Structure of the wild-type β-catenin allele, the β-catenin knock-in targeting vector, targeted allele, deletion allele following Cre treatment and the Pex3βH-IhP trap-selection cassette are shown together with the pertinent restriction enzyme sites. C: Structure of the wild-type plakoglobin allele, the plakoglobin KO targeting vector, targeted allele, deletion allele following Cre treatment and PSIBPA trap-selection cassette are shown together with the pertinent restriction enzyme sites. In panel A–C, exons including the ATG initiation codon and termination codon are also indicated. The probes for Southern blot are shown as probe A for β-catenin and probe B for plakoglobin. The length of the BamHI fragments used for the Southern blot analysis is also shown. D and E: Southern blot analysis of DNA derived from each cell clone. Genomic DNA was digested with BamHI and hybridized with probe A and probe B, respectively. F9: parental F9 cells. βS(G); β-catenin single KO cells. βD(G,h); β-catenin double KO cells. βD; β-catenin double KO cells lacking trap-selection cassettes following Cre treatment. βT(ex3); β-catenin knock-in cells. βT; β-catenin triple KO cells following Cre treatment. βT-PgS(G); β-catenin-deficient and plakoglobin single KO cells. βT-PgD(G); β-catenin-deficient and plakoglobin double KO cells. βT-PgD; β-catenin- and plakoglobin-deficient cells following Cre treatment (BPD cells).

Cell fractionation

Cells harvested from a confluent culture in a 9-cm dish were suspended in 80 μl of HEPES-buffered Mg^{2+}-free saline. The cell suspension was frozen in liquid N_2, thawed and then centrifuged at 1,500 g for 10 min. To the supernatants and pellets was added SDS lysis buffer to generate equal sample volumes for SDS-PAGE and used as the cytosolic and membrane fractions, respectively.

Trypsin treatment and dissociation of Cells

Cells were trypsinized using two different methods for the differential removal of E-cadherin, as previously described (Takeichi, 1977). In brief, cells were treated with 0.01% trypsin in the presence of 1 mM $CaCl_2$ (TC treatment) or 1 mM EGTA (TE treatment) at 37°C for 30 min. Generally, cadherins remain intact following TC treatment, but are digested following TE treatment. For the cell dissociation assay, confluent cultures were treated with TC and TE and dissociated by pipetting 10 times (Nagafuchi *et al.*, 1994). The extent of cell dissociation was represented by N_{TC}/N_{TE}, where N_{TC} and N_{TE} represent the total particle number following TC and TE treatment, respectively.

Expression vectors and transfection

To construct the expression vector for the Histone H1.1-GFP fusion pCAG-Histone-GFP, the EcoRI-XhoI fragment of Histone H1.1 cDNA was inserted into the EcoRI-XhoI site of pCAG-CGFP (Matsuda *et al.*, 2004). This full-length Histone H1.1 cDNA was isolated by PCR using genome DNA derived from F9 cells using the primer pairs (Histone H1.1: **GAATTC**TCATC**ATG**TCTGAGGCTGCT and **CTCGAG**CTTTTTCTTGGCTGCAACCT; Bold letters show the EcoRI site, initiation methionine codon and the XhoI site). In this cDNA, the EcoRI site was inserted 5 bases before the initiation methione codon. The stop codon was replaced with a XhoI site.

Expression vectors were transfected using the Lipofectamin 2000 system (GIBCO BRL). Cells were subjected to G418 selection at 400 μg/ml for two weeks to facilitate the isolation of stable transfectants.

Aggregate formation and tissue spreading analysis

For the aggregate formation of F9 cells or their derivatives, cells were allowed to form an aggregate for three days using the hanging drop method (Oyamada *et al.*, 1996). For morphological observation, we used an aggregate composed of about 1000 cells. An aggregate for tissue spreading analysis (Steinberg and Takeichi, 1994) was composed of about 50 cells. For this analysis, two aggregates were allowed to be present in one hanging drop for one day, then fused aggregates were cultured in EOG-sterilized polystyrene petri dishes for another two days. Images of fused aggregates were acquired using the MetaMorph system equipped with a microscope (model Axiovert 200 M; Carl Zeiss, Inc.) and a CoolSNAP-cooled CCD camera with a 10x/0.30NA Plan-Neofluar objective.

Immunohistochemistry

For the immunofluorescent staining, cells cultured on 15-mm fibronectin-coated cover slips were washed, fixed, and then incubated with primary and secondary antibodies as previously described (Imamura *et al.*, 1999). Cells were fixed with 1% paraformaldehyde for 15 min. Samples were embedded using a SlowFade Light Antifade Kit (Molecular Probes). Images were acquired using the AxioVision 3.0.6 equipped with a microscope (model Axioskop 2; Carl Zeiss, Inc.) and AxioCAM-cooled CCD camera with a 40x/0.75NA Plan-Neofluar objective and a 63x/1.40NA oil Plan-Apochromat objective. Adjustment of brightness, contrast, color balance and final image size was achieved using Adobe Photoshop CS.

Results

Targeted disruption of the β-catenin and plakoglobin genes in F9 cells

The F9 cell line was electroporated with the targeting construct pβT2-RE-PSIBP to generate cells possessing a disrupted β-catenin gene (Fig. 1A). Following electroporation, Southern blot analysis indicated that 5 out of 58 G418-resistant F9 clones had one targeted β-catenin allele. A comparison of the signals of the wild-type allele band with those of the KO allele band suggested that there might be three β-catenin alleles in F9 cells (see Fig. 1D lane 2). Reports have described karyotype disorders in F9 cells and showed that some genes possess an additional allele (Stephens *et al.*, 1993). One β-catenin single KO cell line (clone 6–5) was electroporated with the targeting construct pβT2-RE-PSIhP in an effort to disrupt the second β-catenin allele. After a second electroporation, 1 out of 16 hygromycin and G418 double resistant clones possessed the second targeting allele with the wild-type allele as expected (Fig. 1D lane 3). One β-catenin double KO cell line (clone 22) was transiently transfected with a Cre recombinase expression construct (Cre-pac, Taniguchi *et al.*, 1998). Southern blot analysis revealed that correct excision of the resistance gene cassette occurred in most of the clones isolated (see Fig. 1D lane 4). The third allele of the β-catenin gene was targeted in the β-catenin double KO cell line using the knock-in-type targeting construct pβT2-RE-Pex3βH-IhP (see Materials and Methods, Fig. 1B). Following electroporation, Western blot analysis indicated that 2 out of 71 hygromycin-resistant clones expressed only HA-tagged β-catenin and no endogenous β-catenin proteins (data not shown). Southern blot analysis showed that these clones were positive for the desired recombination event (see Fig. 1D lane 5). One β-catenin triple KO cell line (clone 1–5) was treated with the Cre-pac method and cell lines were eventually isolated

in which all β-catenin alleles were disrupted and no drug resistance genes remained (see Fig. 1D lane 6). Southern blot analysis using an IRES probe confirmed that unexpected recombinations had not occurred (data not shown). This β-catenin triple KO cell line was referred to as βT.

To establish F9 cells in which both β-catenin and plakoglobin genes had been inactivated, βT cells were electroporated with the pPgT1-RE-PSIBPA targeting construct (Fig. 1C). Southern blot analysis revealed that 6 out of 182 G418-resistant clones had one targeted plakoglobin allele (see Fig. 1E lane 2). One plakoglobin single KO βT cell line (clone C-1) was cultured in the presence of a relatively high concentration of G418 (5 mg/ml) to isolate cells in which both alleles were disrupted. Following selection, double KO cell clones were isolated (see Fig. 1E lane 3). One plakoglobin KO cell line (clone 10) was treated with the Crepac method and cell lines in which all β-catenin and plakoglobin alleles were disrupted with no drug resistance genes being present were eventually isolated (clone 10–13; see Fig. 1E lane 4). Southern blot analysis using an IRES probe confirmed that unexpected recombinations had not occurred (data not shown). This β-catenin/plakoglobin-deficient cell line was referred to as BPD.

Role of β-catenin and plakoglobin in cadherin-mediated cell adhesion activity

The effect of loss of β-catenin and plakoglobin expression on cell adhesion activity was examined. For this purpose, the process of aggregate formation was observed using the hanging drop method (Fig. 2A). Following 6 hours culture, aggregates of BPD cells displayed different morphology from that of parental F9 cells. BPD cells formed loose aggregates in which individual cells were clearly distinguished under phase contrast microscopy. In contrast, F9 cells formed compact aggregates where cell boundaries became unclear. Following 3 days of culture in the hanging drop, BPD formed spherical aggregates, similar to those of F9 cells. The surface of the BPD cell aggregates, however, was less smooth than that of the F9 cell aggregates. Mechanical resistance to vigorous pipetting was significantly lower for BPD cell aggregates compared with F9 cell aggregates. Following pipetting for thirty times, BPD cell aggregates were easily broken and associated with the production of single isolated cells or fragments of aggregates. In contrast, F9 cell aggregates following 3 days of culture remained almost unaffected. βT cells displayed a similar phenotype to F9 cells (data not shown). These results confirmed that β-catenin or plakoglobin is necessary for strong cell-cell adhesion, although F9 cells could form cell aggregates in the absence of a normal cadherin-catenin system.

The cell morphology of F9 cells and their derivatives was then examined (Fig. 2B). Phase contrast microscopic analysis showed that the morphology of βT cells was indistinguishable from that of parental F9 cells (Fig. 2B compare βT to F9). F9 and βT cells adhered to each other and formed tightly-packed colonies following 1 day of culture on a dish. In contrast, BPD cells displayed non-adhesive morphology. Each cell grew sparsely on a dish without making colonies. The cell dissociation assay demonstrated that the strong cell adhesion activity was correlated with colony formation activity (Fig. 2C). F9 and βT cells hardly dissociated following vigorous pipetting. BPD cells were easily dissociated following pipetting even in the presence

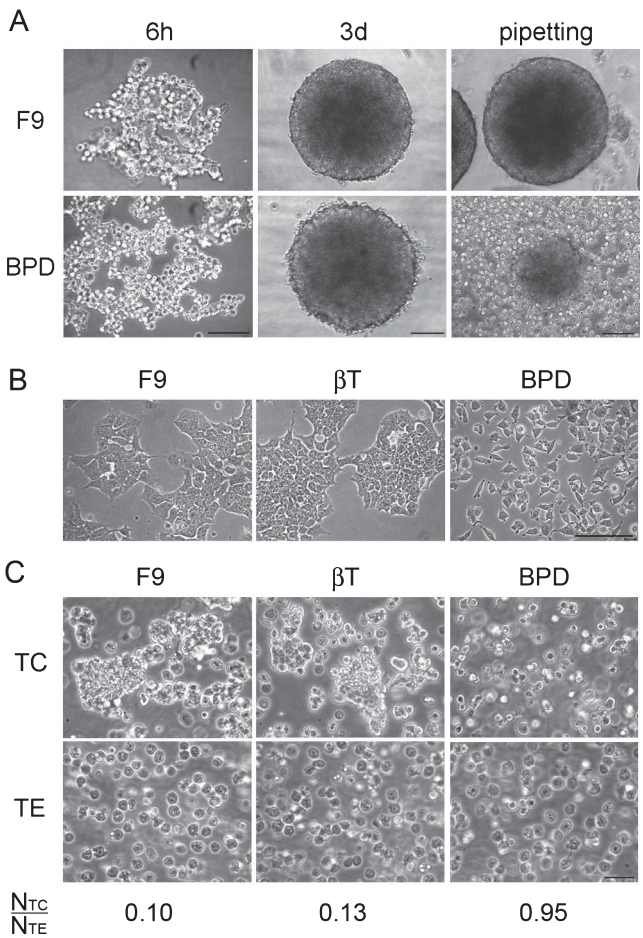


Fig. 2. Morphology and cell adhesion activity of F9, βT and BPD cells under suspension and monolayer culture conditions. A: Morphology under suspension culture condition. Aggregate formation in hanging drops following 6 hours (6h) and 3 days (3d) and 3 day aggregates following pipetting for 30 times. Note that BPD cell aggregates were easily dissociated into single cells following pipetting. Bar, 100 μm. B: Phase contrast images. Note that BPD cells could not form compact colonies. Bar, 100 μm. C: Cell dissociation assay. The confluent cell sheet of each cell line was treated with 0.01% trypsin in the presence (TC) or absence (TE) of Ca^{2+}, and then dissociated by pipetting several times. The cell dissociation index (N_{TC}/N_{TE}; see Materials and Methods) is shown under phase contrast images. The lower the value of the index, the higher the activity of cell adhesion. Note that BPD cells were easily dissociated even in the presence of Ca^{2+}. Bar, 100 μm

of Ca^{2+}. The dissociation index of BPD cells was significantly higher than that of F9 and βT cells. The re-expression of β-catenin or plakoglobin in BPD cells could restore the aggregate formation, colony formation and cadherin-mediated strong cell adhesion activity (YF and AN, unpublished observations). These results demonstrated that β-catenin or plakoglobin was required for the strong cell adhesion activity of cadherin. It was also demonstrated that plakoglobin was able to support cadherin-based cell-cell adhesion activity in the absence of β-catenin.

Role of β-catenin and plakoglobin in the configuration change of cell aggregates

Since cells of every line could form compact cell aggregates, tissue-spreading analysis was employed in an effort to compare the adhesion properties of each cell line (Steinberg and Takeichi, 1994). In this experiment two cell aggregates, one of which was labeled with GFP, were allowed to fuse. It was expected that, under specific conditions, one aggregate composed of more adhesive cells would be surrounded by another composed of less adhesive cells to form a "sphere within a sphere" aggregate. When a BPD cell aggregate was fused to a F9 cell aggregate, the former surrounded the latter and formed a partial "sphere within a sphere" aggregate (Fig. 3b). When two F9 cell aggregates or BPD cell aggregates were fused, they formed large aggregates that maintained their boundaries (a "half and half " aggregate) (Fig. 3a and c). Thus, two aggregates composed of the same cell type formed a "half and half" aggregate. Then, βT cell aggregate was fused to a F9 cell aggregate in an effort to compare the adhesion properties of β-catenin-deficient cells. βT cells formed "half and half" aggregates with F9 cells (Fig. 3d). Thus, the cadherin-based cell adhesion activity supported by plakoglobin could not be distinguished from that of β-catenin.

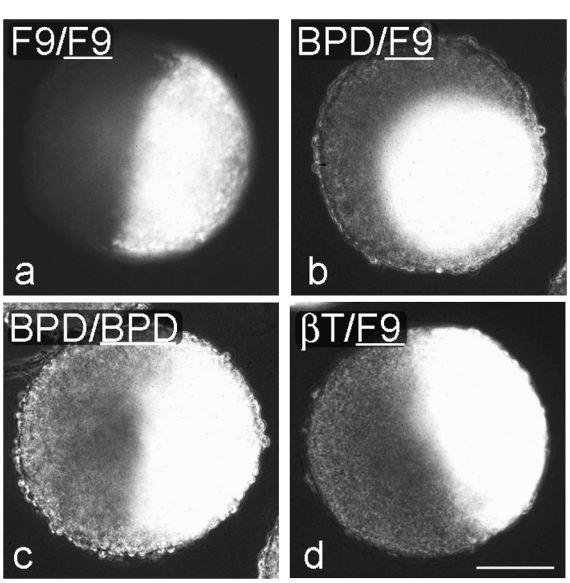


Fig. 3. Tissue spreading analysis. Configuration changes of parental F9, βT and BPD cells. Two aggregates composed of cells indicated in each photo were allowed to fuse. Underlines indicate the cells labeled with Histon-GFP. Note that BPD cells surrounded F9 cells but βT cells formed "half and half" aggregates with F9 cells. Bar, 100 μm

Expression of E-cadherin, α-catenin, β-catenin and plakoglobin in F9, βT and BPD cells

Immunoblot analysis was employed to examine the expression of E-cadherin, α-catenin, β-catenin and plakoglobin in F9, βT and BPD cells (Fig. 4A). In βT cells, expression of β-catenin was completely lost while that of plakoglobin increased (Fig. 4A lane 2). The expression level of E-cadherin and α-catenin was not affected (Fig. 4A compare lane 2 with 1). In BPD cells, the expression of both β-catenin and plakoglobin was completely lost (Fig. 4A lane 3), while the level of E-cadherin markedly decreased to under 30% compared to that in parental F9 cells (Fig. 4A compare lane 3 with lane 1 or 2). The expression level of α-catenin protein also decreased to ca. 40% compared to that in parental F9 cells (Fig. 4A compare lane 3 with lane 1 or 2). Complex formation between E-cadherin, α-catenin and β-catenin was then examined by immunoprecipitation analysis using anti-E-cadherin antibodies (Fig. 4B). In F9 cells, β-catenin mainly co-precipitated with E-cadherin. Only trace amounts of plakoglobin were detected in immunoprecipitates (Fig. 4B lane 1). In βT cells, the amount of plakoglobin associated with E-cadherin dramatically increased. The amount of α-catenin in immunoprecipitates of βT cells was similar to that of parental F9 cells (Fig. 4B compare lane 2 with 1). In immunoprecipitates of BPD cells, β-catenin, plakoglobin and α-catenin were not detected (Fig. 4B lane 3). The amount of cadherin-catenin complex components in the cytosolic and membrane fractions was then compared (Fig. 4C). E-cadherin was detected mainly in the membrane fraction of all the cell lines. Half or more of β-catenin and plakoglobin, when expressed, were detected in the membrane fraction. The amount of plakoglobin in the membrane fraction of βT cells was significantly higher than that in parental F9 cells (Fig. 4C compare lane 4 to 2). Half of the amount of α-catenin detected was found in the membrane fraction of F9 and βT cells (Fig. 4C lanes 1–4). In contrast, most of the α-catenin detected was found in the cytosolic fraction of BPD cells (Fig. 4C lane 5 and 6). These data demonstrated that 1) plakoglobin could efficiently be included in the E-cadherin-catenin complex in the absence of β-catenin, 2) both β-catenin and plakoglobin are functional for cadherin-catenin complex formation, 3) β-catenin or plakoglobin is required for the stable expression of E-cadherin and 4) β-catenin or plakoglobin is required for α-catenin to be included in the membrane fraction.

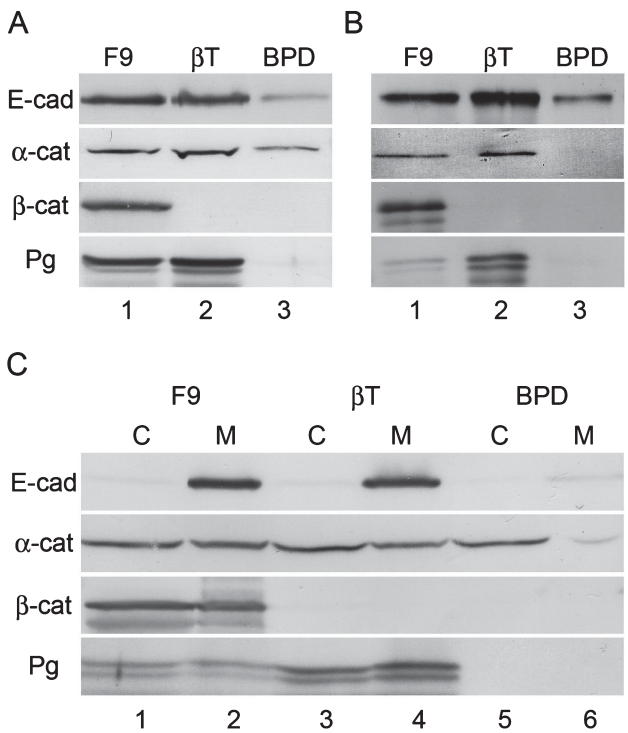


Fig. 4. Expression and subcellular localization of cadherin-catenin complex components. Western blot analysis of parental F9, β-catenin-deficient F9 (βT) cells and β-catenin/plakoglobin-deficient F9 (BPD) cells with anti-E-cadherin (E-cad), anti-α-catenin (α-cat), anti-β-catenin (β-cat) and anti-plakoglobin (Pg) mAbs. A: Total cell lysates. Note that the expression level of E-cadherin was reduced in BPD cells. B: Immunoprecipitation using anti-E-cadherin mAb. Note that the amount of plakoglobin in samples derived from βT cells is larger than that of parental F9 cells and no α-catenin was detected in BPD cells. C: Cadherin-catenin complex components in cytosolic (C) and membrane (M) fractions. Note that α-catenin in BPD cells was negligibly detected in the membrane fraction.

Role of β-catenin and plakoglobin in subcellular localization of E-cadherin-catenin complex

The localization of E-cadherin, α-catenin, β-catenin and plakoglobin was examined by immunocytochemical analysis (Fig. 5). In F9 cells, the localization of these proteins was detected at cell-cell contact sites, although that of plakoglobin was slightly unclear. Special localization of cytoplasmic β-catenin, plakoglobin and α-catenin was not observed. In βT cells, the β-catenin signal was completely lost and the localization of plakoglobin at cell boundaries became clear. On the other hand, the localization of E-cadherin and α-catenin remained unaffected. In BPD cells, the β-catenin and plakoglobin signals were lost and the localization of α-catenin at cell-cell contact sites was negligibly detected. The distribution of E-cadherin was severely affected. Although E-cadherin was detected at cell-cell boundaries, the signals were quite faint. In contrast, cytoplasmic aggregates of E-cadherin were frequently detected. These data indicated that β-catenin or plakoglobin was required for the stable expression and cell surface localization of E-cadherin.

Discussion

In this paper, F9 cells completely lacking β-catenin and plakoglobin expression were successfully isolated. Neither β-catenin nor plakoglobin were required for cell survival and growth of F9 cells under normal culture conditions. Using the β-catenin/plakoglobin-deficient F9 cells (BPD cells), functional analysis of β-catenin and plakoglobin in the cadherin-based cell adhesion system was performed. Here the role of β-catenin and plakoglobin in cadherin-mediated cell adhesion and the availability of BPD cells will be discussed.

Compensation of β-catenin function by plakoglobin in the cell adhesion system

Plakoglobin possesses a similar primary amino acid sequence to β-catenin. In fact, several experimental data indicated that plakoglobin binds to cadherin and α-catenin, not unlike the case with β-catenin. These data suggested that plakoglobin could compensate for the absence of β-catenin in the cell adhesion system. Some cancer cell lines which express aberrant β-catenin (Kawanishi *et al.*, 1995; Oyama *et al.*, 1994) or no β-catenin (Usami *et al.*, 2003), however, failed to show normal adhesion activity. Results stemming from investigations of β-catenin-deficient cell lines and β-catenin KO mice were also ambiguous (Haegel *et al.*, 1995; Huelsken *et al.*, 2000). In this paper, employment of the cell dissociation and tissue-spreading assays demonstrated that β-catenin-deficient F9 cells showed strong cadherin-mediated cell adhesion activity. The expression level and subcellular localization of E-cadherin and α-catenin remained unaffected with loss of β-catenin expression. In the absence of both β-catenin and plakoglobin, however, F9 cells lost strong cell adhesion activity. These data directly confirmed that β-catenin and plakoglobin are required for the cadherin-mediated cell adhesion system and demonstrated that plakoglobin could compensate for the absence of β-catenin in this system.

In F9 cells, immunoprecipitation analysis showed that only trace amounts of plakoglobin were associated with E-cadherin. In the absence of β-catenin, the amount of cadherin-associated plakoglobin dramatically increased. These data suggested that the affinity of β-catenin to E-cadherin was greater than that of plakoglobin. Such differences in affinity for cadherins might account for the fact that plakoglobin preferentially incorporates into desmosomes in cells that assemble both adherens junctions and desmosomes.

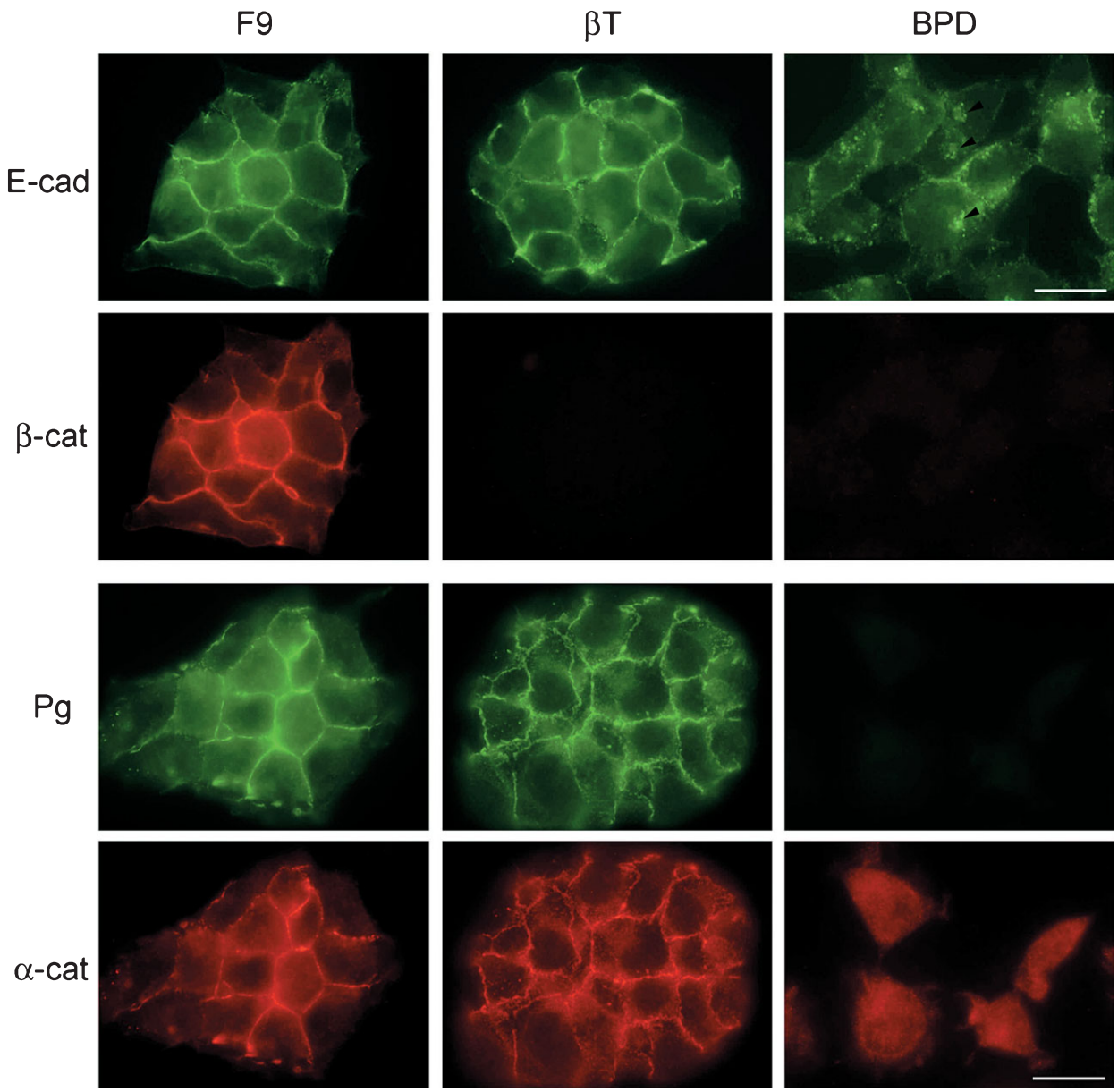


Fig. 5. Subcellular localization of cadherin-catenin complex components. The expression and distribution of E-cadherin (E-cad), β-catenin (β-cat), plakoglobin (Pg) and α-catenin (α-cat) in F9, βT and BPD cells were examined by immunofluorescent staining. Each of the cells was doubly-stained with anti-E-cadherin and anti-β-catenin mAbs or anti-plakoglobin and anti-α-catenin antibodies. Note that cytoplasmic aggregates (arrowheads) of E-cadherin signals were frequently observed in BPD cells. Bar, 20 μm

Cell adhesion activity in the absence of β-catenin and plakoglobin

E-cadherin was originally identified as a molecule involved in the "compaction" of the mouse early embryo (Hyafil *et al.*, 1980; Ogou *et al.*, 1982). Compact colony formation and the compaction phenotype of cell aggregates are considered as characteristic features of cadherin-mediated strong cell adhesion. Aggregate formation in a hanging drop revealed that cadherin-mediated strong cell adhesion activity is required for the compaction of cell aggregates at an early stage around 6 hrs. Following prolonged culture, however, cells could form compact aggregates even in the absence of β-catenin and plakoglobin. This is consistent with the observation that function-blocking antibodies against E-cadherin disrupted compaction of mouse 8 cell stage embryos but not that of 16 cell stage embryos (Shirayoshi *et al.*, 1983). Thus, strong cell adhesion activity

mediated by E-cadherin was not necessarily required for compact aggregate formation.

Role of β-catenin and plakoglobin in the stable expression of α-catenin

It was previously reported that expression of E-cadherin posttranscriptionally regulates the expression of α-catenin: the expression level of α-catenin protein was dramatically induced by the exogenous expression of E-cadherin in cadherin-deficient mouse L cells without the change of α-catenin mRNA expression (Nagafuchi *et al.*, 1991). In BPD cells, the expression level of α-catenin protein was reduced and α-catenin in membrane fraction was barely detected. These data indicated that the E-cadherin-β-catenin (or plakoglobin) complex is required for α-catenin to be included in the membrane fraction. α-Catenin in cytoplasmic fraction, however, was not severely affected by the loss of expression of β-catenin and plakoglobin. It was previously demonstrated that α-catenin was actively degraded in the absence of cadherins. It was also suggested that the expression of α-catenin was translationally regulated (Takahashi *et al.*, 2000). In F9 cells, the α-catenin degradation system might be inefficient compared to that in L cells. It was also possible that E-cadherin expressed in BPD cells supported active translation of α-catenin proteins. The regulatory mechanisms for α-catenin protein expression remain to be elucidated.

Role of β-catenin and plakoglobin in the stable expression of E-cadherin

In BPD cells, the level of E-cadherin decreased at cell-cell contact sites and cytoplasmic aggregates of E-cadherin were frequently observed. It was previously reported that mutant E-cadherin that lacked various cytoplasmic regions showed low-level transport to the cell surface and was not appropriately modified with carbohydrates in MDCK cells (Chen *et al.*, 1999). Since these mutant molecules could not bind to β-catenin, it was suspected that coupling assembly of E-cadherin and β-catenin is required for the release of E-cadherin from the endoplasmic reticulum (ER). It is accepted that carbohydrate modification and removal of the pro-region in Golgi apparatus are required for E-cadherin to become a mature protein with the appropriate molecular weight (Ozawa and Kemler, 1990; Shirayoshi *et al.*, 1986). The size of the E-cadherin protein expressed in BPD cells, however, was indistinguishable from the size of endogenous E-cadherin in parental F9 cells. Thus E-cadherin in BPD cells could be released from the ER and enter the Golgi apparatus in the absence of β-catenin and plakoglobin. The low level expression of E-cadherin protein in BPD cells, however, suggested that β-catenin or plakoglobin were involved in stable expression and cell surface localization of E-cadherin. Recently it was reported that cadherin turnover was regulated through p120 cadherin-binding protein (Chen *et al.*, 2003; Davis *et al.*, 2003; Xiao *et al.*, 2003). How were β-catenin and plakoglobin involved in the expression and localization of cadherin proteins? The molecular mechanisms remain to be elucidated.

We demonstrated the various functions of β-catenin and plakoglobin in the cadherin-mediated cell adhesion system using F9 cells possessing a complete loss of β-catenin and plakoglobin expression. β-Catenin is also known to play a crucial role as a cytoplasmic component in Wnt signaling. The role of plakoglobin in this signaling pathway remains unclear. Future use of the β-catenin/plakoglobin-deficient cells will help to elucidate the role of β-catenin and plakoglobin in Wnt signaling pathway. F9 cells are considered to be a good model of epithelial differentiation *in vitro*. The continued investigation of β-catenin/plakoglobin-deficient cells should contribute to our understanding of the role of cadherin-mediated cell adhesion and the Wnt signaling pathway in the differentiation and morphogenesis of epithelial tissue.

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