

V-src Kinase Shifts the Cadherin-based Cell Adhesion from the Strong to the Weak State and β Catenin Is Not Required for the Shift

Hiroshi Takeda,*[‡] Akira Nagafuchi,*[¶] Shigenobu Yonemura,*[¶] Sachiko Tsukita,*[§] Jürgen Behrens,^{||} Walter Birchmeier,^{||} and Shoichiro Tsukita*[¶]

*Laboratory of Cell Biology, Department of Information Physiology, National Institute for Physiological Sciences, Myodaiji-cho, Okazaki 444, Japan; [‡]Department of Physiological Sciences, School of Life Sciences, The Graduate University of Advanced Studies, Myodaiji-cho, Okazaki, Aichi 444, Japan; [§]College of Medical Technology, Kyoto University, Sakyo-ku, Kyoto 606, Japan; [¶]Max-Delbrück-Center for Molecular Medicine, 13122 Berlin, Germany; and ^{||}Department of Cell Biology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

Abstract. The elevation of tyrosine phosphorylation level is thought to induce the dysfunction of cadherin through the tyrosine phosphorylation of β catenin. We evaluated this assumption using two cell lines. First, using temperature-sensitive v-src-transfected MDCK cells, we analyzed the modulation of cadherin-based cell adhesion by tyrosine phosphorylation. Cell aggregation and dissociation assays at nonpermissive and permissive temperatures indicated that elevation of the tyrosine phosphorylation does not totally affect the cell adhesion ability of cadherin but shifts it from a strong to a weak state. The tyrosine phosphorylation levels of β catenin, ZO-1, ERM (ezrin/radixin/moesin), but not α catenin,

vinculin, and α -actinin, were elevated in the weak state.

To evaluate the involvement of the tyrosine phosphorylation of β catenin in this shift of cadherin-based cell adhesion, we introduced v-src kinase into L fibroblasts expressing the cadherin- α catenin fusion protein, in which β catenin is not involved in cell adhesion. The introduction of v-src kinase in these cells shifted their adhesion from a strong to a weak state. These findings indicated that the tyrosine phosphorylation of β catenin is not required for the strong-to-weak state shift of cadherin-based cell adhesion, but that the tyrosine phosphorylation of other junctional proteins, ERM, ZO-1 or unidentified proteins is involved.

CADHERINS are a family of transmembrane glycoproteins responsible for calcium-dependent cell-cell adhesion (Takeichi, 1988, 1991). The functions of cadherins are regulated from the cytoplasmic side, and these regulations are thought to be of great importance in the development of organs and in carcinogenesis/metastasis.

Tyrosine phosphorylation appears to be an important regulatory signal for cadherins. A relationship between cadherins and tyrosine phosphorylation has been demonstrated by Warren and Nelson (1987) and Kellie (1988). They showed that in v-src tyrosine kinase transfectants, v-src kinase suppresses the cadherin-based cell adhesion with the concomitant destruction or structural modification of adherens junctions (AJ),¹ where cadherins act as adhesion molecules. This was confirmed with RSV-transformed cul-

tured chick lens cells (Volberg et al., 1992). Using an antiphosphotyrosine antibody, Takata and Singer (1988) demonstrated at the electron microscopic level, that the phosphotyrosine-modified proteins were highly concentrated at AJ in various tissues, and Tsukita et al. (1991) found that the specific proto-oncogenic tyrosine kinases, c-src, and c-yes kinases, were enriched in AJ. Furthermore, incubation of the cells with vanadate/H₂O₂, a potent inhibitor of tyrosine phosphatases, induces the destruction of AJ (Volberg et al., 1991).

Cadherin is closely associated with several cytoplasmic proteins, α , β , and γ catenin and p120 (Vestweber and Kemler, 1984; Peyrieras et al., 1985; Ozawa et al., 1989; Shibamoto et al., 1995). Furthermore, EGF receptor tyrosine kinase is associated with cadherin via β catenin (Hoschuetzky et al., 1994). Studies using v-src transfected cells have revealed that in the cadherin-catenin complex, β catenin was preferably tyrosine phosphorylated, and that increased tyrosine phosphorylation of β catenin appeared to be associated with the dysfunction of cadherin (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993).

Address all correspondence to Sh. Tsukita, Department of Cell Biology, Kyoto University Faculty of Medicine, Konoe-Yoshida, Sakyo-ku, Kyoto 606, Japan. Ph.: 81-75-753-4372. Fax: 81-75-753-4660.

1. *Abbreviation used in this paper:* AJ, adherens junction.

These findings suggest that the elevation of tyrosine phosphorylation level induces the dysfunction of cadherin through the tyrosine phosphorylation of β catenin. However, this idea is not valid until the following two points are addressed. (a) What is the "dysfunction" state of cadherin? Our knowledge on the tyrosine phosphorylation-induced dysfunction state of cadherin remains fragmentary. (b) Is tyrosine phosphorylation of β catenin required for the dysfunction of cadherin? So far a correlation was obtained only between the tyrosine phosphorylation of β catenin and the dysfunction of cadherins.

To address the first point, we used an MDCK stable cell line transfected with a temperature-sensitive mutant of v-src (ts-v-src MDCK) (Behrens et al., 1993). This ts-v-src MDCK cell line exhibits an epithelial phenotype at the nonpermissive temperature, but rapidly loses cell-cell contact and acquires a fibroblast-like morphology at the permissive temperature. Therefore, this cell line can be used to analyze the tyrosine phosphorylation-induced dysfunction state of cadherins. By cell aggregation and dissociation assay with this cell line, we found that elevation of the tyrosine phosphorylation level does not totally affect the cadherin-based cell adhesion, but shifts it from a strong to a weak state. In the latter state, the activation of ts-v-src increases the tyrosine phosphorylation level not only of β catenin but also of other undercoat-constitutive proteins such as ZO-1, ezrin, radixin, and moesin.

To address the second point, we used a mouse L fibroblast cell line expressing the E-cadherin- α catenin fusion protein (Nagafuchi et al., 1994). Without the interaction of β catenin, this fusion protein acts as an adhesion molecule in the strong state of cell adhesion. We introduced v-src kinase into this cell line, and found that the elevation of tyrosine phosphorylation shifts the cell adhesion from a strong to a weak state. This indicated that the tyrosine phosphorylation of β catenin is not required for the tyrosine phosphorylation-dependent shift of cadherin-based cell adhesion from the strong to the weak state.

Materials and Methods

Cells and Antibodies

Temperature sensitive-v-src-transfected MDCK cells (Behrens et al., 1993) were grown in a mixture of Ham's F12 and DME supplemented with 10% FCS at 40°C (nonpermissive temperature) or at 35°C (permissive temperature). Mouse L cell transfectants expressing E-cadherin (EL β 1; Nose et al., 1988), or a fusion protein (nE α C) between a carboxyl terminus-truncated cadherin and the carboxyl-terminal half of α catenin (nE α CL; Nagafuchi et al., 1994) were grown in DME supplemented with 10% FCS and 150 μ g/ml of G418.

Mouse anti-ZO-1 mAb (T8-754) (Itoh et al., 1991), rat anti- α catenin mAb (α 18) (Nagafuchi and Tsukita, 1994), rat anti- β catenin mAb (2D4) (Nagafuchi et al., 1994), and rabbit anti-ERM pAb (I1) (Tsukita et al., 1989) were obtained and characterized as described. Rat anti-E-cadherin mAb (ECCD-1 and ECCD-2) and rat anti-P-cadherin mAb (PCD-1) were gifts from Dr. M. Takeichi (Kyoto University, Kyoto, Japan). Mouse mAbs against phosphotyrosine (pT66), vinculin (VIN11-5), and α -actinin (BM75.2) were purchased from Sigma Chemical Co. (St. Louis, MO), and mouse mAb p60^{v-src} (mAb 327) was purchased from Oncogene Science (Manhasset, NY).

Transfection

L cell transfectants, EL β 1 and nE α CL, were plated at a density of 1×10^5 cells/35 mm dish, and then cotransfected with an avian v-src expression

vector (pMv-src) (Paul et al., 1985) and pSV2bsr (for blasticidin-S resistance) (Funakoshi Pharmaceutical Co., Tokyo) by lipofection (Life Technologies, Inc., Grand Island, NY). The cells were then replated on three 9 cm dishes and cultured in the presence of 20 μ g/ml blasticidin-S. Colonies of blasticidin-S-resistant cells were isolated and recloned. Using anti-p60^{v-src} mAb (mAb327), we isolated several stable clones expressing p60^{v-src} for EL β 1 and nE α CL cells. The plasmid pMv-src was a generous gift from Dr. Motoharu Seiki (Kanazawa University, Kanazawa, Japan).

Cell Aggregation and Dissociation

For cell aggregation studies, ts-v-src MDCK cells (see Fig. 1) were cultured in 35 mm plastic dishes at 35°C for 12 h, and then detached using a rubber policeman. They were passed through Pasteur pipettes several times to obtain single cells, then 0.3 ml of the cell suspension was placed in 15 mm plastic dishes covered with the culture medium containing 1% agar. The dishes were placed on a gyratory shaker and rotated at 80 rpm for several hours at 35° or 40°C to allow aggregation. In some experiments, during rotation culture, 3 mM EGTA or a mixture of anti-E-cadherin mAb and anti-P-cadherin mAb was added to the medium. For cell aggregation using L transfectants, the cells were digested with 0.1% trypsin in the presence of 1 mM CaCl₂ at 37°C for 20 min, and then washed three times with Ca²⁺/Mg²⁺-free Hepes-buffered saline (HCMF; pH 7.4) to obtain single cell suspensions. Cells suspended in DME medium were placed in 1% agar-coated four-well plates and rotated for 3 h at 37°C on a gyratory shaker at 80 rpm.

For the cell dissociation assay, confluent ts-v-src MDCK cells (see Fig. 3) in monolayers were cultured in 35 mm plastic dishes at 40°C for 12 h, incubated at 35°C for 0–12 h, and then detached using a rubber policeman. The cell suspension was passed through Pasteur pipettes 30 times, and then fixed with 1% glutaraldehyde in PBS. The extent of cell dissociation was represented by the index N_p/N_c , where N_p and N_c are the total numbers of particles and cells per dish, respectively. For cell dissociation studies using L cell transfectants, aggregates formed on a gyratory shaker were passed through Pasteur pipettes 30 times.

Immunoprecipitation

Confluent monolayers of ts-v-src MDCK cells were maintained on 100 mm dishes at 40°C for 3 d, and then incubated at 35°C for 0, 5, 10, 30, or 60 min. The cells were washed three times with ice-cold PBS, lysed and incubated in 1 ml of RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 10 mM Na₂P₂O₇, 4 mM EDTA, 1 mM PMSF, 1% Nonidet P-40, 0.1% SDS). The cells were scraped from the dish using a rubber policeman, incubated for additional 30 min on ice, and then clarified by centrifugation at 10,000 g for 30 min. The supernatant was collected as the soluble fraction. The pellet was suspended in 100 μ l of SDS immunoprecipitation buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% SDS) (Hinck et al., 1994), incubated at 100°C for 10 min, diluted up to 1 ml with RIPA buffer, and then used as the insoluble fraction. Anti-phosphotyrosine mAb (or control mouse IgG) was added to the soluble or insoluble fraction and allowed to form immune complexes for 3 h on ice. Protein G-Sepharose 4B (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) (50 μ l) was added to the sample, and incubated for an additional 3 h on ice. Protein G-Sepharose 4B-bound immune complexes were washed three times with RIPA buffer. Immune complexes were then eluted by boiling in 150 μ l of SDS sample buffer.

L cell transfectants cultured in 60 mm dishes were grown to confluence, washed three times in ice-cold PBS, lysed in lysis buffer (1% Triton X-100, 1% NP-40, 1 mM CaCl₂, 2 mM PMSF, 1 mM Na₃VO₄, 50 mM NaF, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4) (Matsuyoshi et al., 1992), and then centrifuged at 15,000 rpm for 30 min at 4°C. The clarified lysates were immunoprecipitated with 10 μ l of rat mAb ECCD-2 and 50 μ l of protein G-Sepharose (Zymed Laboratories, Inc., San Francisco, CA). Immunoprecipitates were washed three times with lysis buffer and solubilized with SDS sample buffer containing 1 mM Na₃VO₄.

Immunoblotting

The eluted immune complexes were resolved by one-dimensional SDS-PAGE (7.5%) (Laemmli, 1970), and then electrophoretically transferred from gels to nitrocellulose membranes, which were incubated with first antibodies. These were detected using a blotting detection kit (Amersham Corp., Arlington Heights, IL).

Results

Two States of Cadherin-based Cell Adhesion in *ts-v-src* MDCK Cells and Their Modulation by Tyrosine Phosphorylation

As reported (Behrens et al., 1993), when *ts-v-src* MDCK cells were cultured on a plastic dish at 35°C (the permissive temperature) at a low density, they exhibited fibroblast-like morphology (Fig. 1 *a*). These cells were detached from the substratum using a rubber policeman, then passed several times through Pasteur pipettes, which resulted in single dissociated cells (Fig. 1 *b*). When these cells were allowed to aggregate at 40°C (the nonpermissive temperature) for 3 h under gentle gyration, compact aggregates were formed

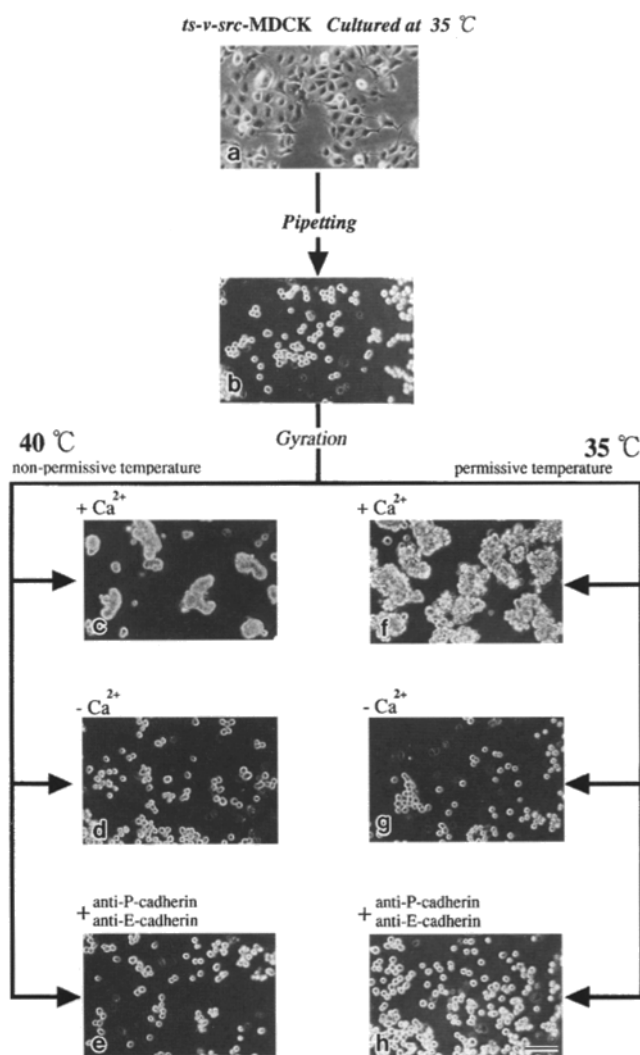


Figure 1. Two states of cadherin-based cell adhesion in *ts-v-src* MDCK cells. At the permissive temperature, *ts-v-src* MDCK cells exhibited a fibroblast-like morphology at a low density (*a*). These cells were easily dissociated into single cells by pipetting (*b*). These cells were allowed to aggregate at the nonpermissive (*c–e*) or permissive (*f–h*) temperature in the presence (*c, e, f,* and *h*) or absence (*d* and *g*) of Ca^{2+} ions in the culture medium. In *e* and *h*, a mixture of anti-E-cadherin and anti-P-cadherin antibodies was added to the medium. Bar, 10 μm .

(Fig. 1 *c*). This aggregation was completely suppressed in the absence of Ca^{2+} ions as well as in the presence of anti-E- and P-cadherin antibodies, indicating that it was mediated by cadherins (Fig. 1, *d* and *e*). When the same aggregation experiment was performed at the permissive temperature, loose cell aggregates were formed, in which individual cells were easily recognized (Fig. 1 *f*). This type of cell aggregation was again completely inhibited by Ca-removal or anti-E- and P-cadherin antibodies, indicating that these looser aggregates were also maintained by cadherin-based adhesion (Fig. 1, *g* and *h*). Incubating the compact aggregates at the permissive temperature resulted in the looser type, which again became compact at the nonpermissive temperature (Fig. 2, *a* and *b*).

To evaluate the state of the cell adhesion in the compact and looser aggregates, they were passed several times through Pasteur pipettes. As shown in Fig. 2 (*c* and *d*), the compact aggregates were hardly affected, whereas the looser type was easily dissociated into single cells. We concluded that cadherin-based cell adhesion can assume two states in *ts-v-src* MDCK cells in the presence of extracellular Ca^{2+} ; a strong adhesion state at the nonpermissive temperature and a weak adhesion state at the permissive temperature.

Using confluent *ts-v-src* MDCK cells, we quantified the level of the tyrosine phosphorylation-dependent shift of cadherin-based cell adhesion from the strong to the weak by the cell dissociation assay (Fig. 3). Confluent cell sheets were detached from the substratum with a rubber policeman and passed through Pasteur pipettes several times. As shown in Fig. 3 (*a–d*), the cell sheet at the nonpermissive temperature was hardly affected, whereas that at the permissive temperature easily dissociated into single cells. The time course of the shift from the strong to the weak

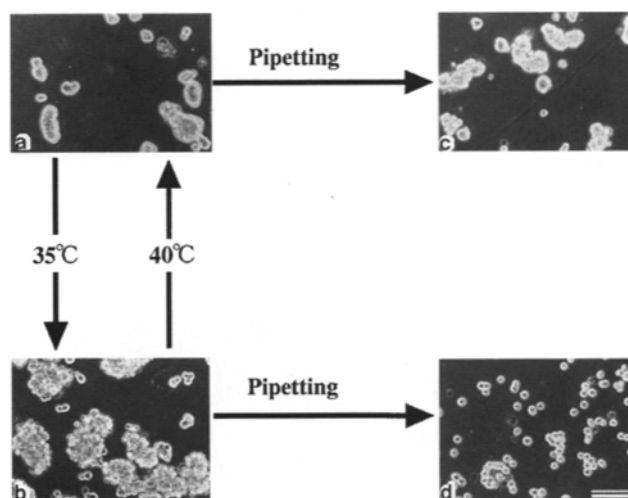


Figure 2. Transition between compact and loose aggregates of *ts-v-src* MDCK cells. Incubating the compact aggregates (*a*) at the permissive temperature resulted in the looser type (*b*), which again became compact at the nonpermissive temperature. By pipetting, the compact aggregates were hardly dissociated (*c*), whereas the looser type was easily dissociated into single cells (*d*). Bar, 10 μm .

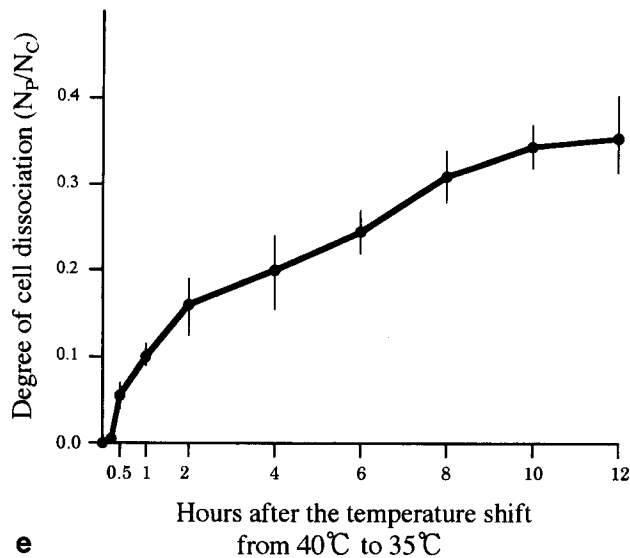
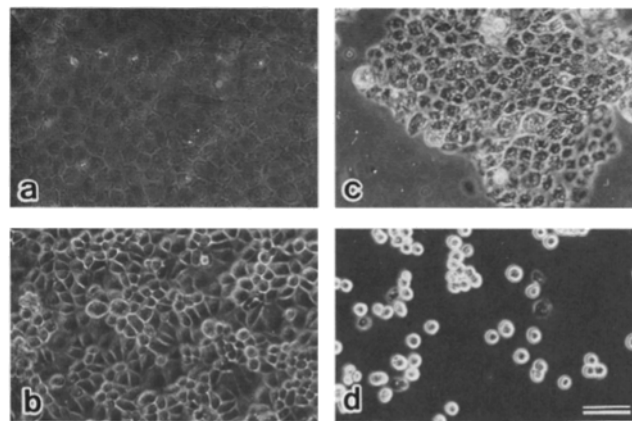


Figure 3. Dissociation assay of confluent ts-v-src MDCK cells. Confluent monolayers were detached from dishes using a rubber policeman, and the cell suspension was passed through Pasteur pipettes 30 times (see Materials and Methods). The cell sheet at the nonpermissive temperature (*a*) was basically unaffected (*c*), whereas that at permissive temperature (*b*) easily dissociated into single cells (*d*). In *e*, the degree of cell dissociation (N_p/N_c) at 0–12 h after the shift from the nonpermissive to the permissive temperature was quantified, where N_p and N_c are the total number of particles and cells per dish, respectively. Bar, 10 μ m.

adhesion state after the temperature change was quantified and is presented in Fig. 3 *e*.

Tyrosine Phosphorylation of Junctional Proteins in ts-v-src MDCK Cells at the Permissive Temperature

Confluent ts-v-src MDCK cells cultured at the nonpermissive temperature were transferred to the permissive temperature, then 5, 10, 30, and 60 min after the temperature shift, the cells were processed for immunoblotting with antiphosphotyrosine mAb (Fig. 4 *a*). The tyrosine phosphorylation level in whole cells appeared to increase in a linear fashion until 30 min, reaching a plateau around 60 min after the temperature shift.

To investigate the tyrosine phosphorylation level of each junctional protein after the temperature shift, at various

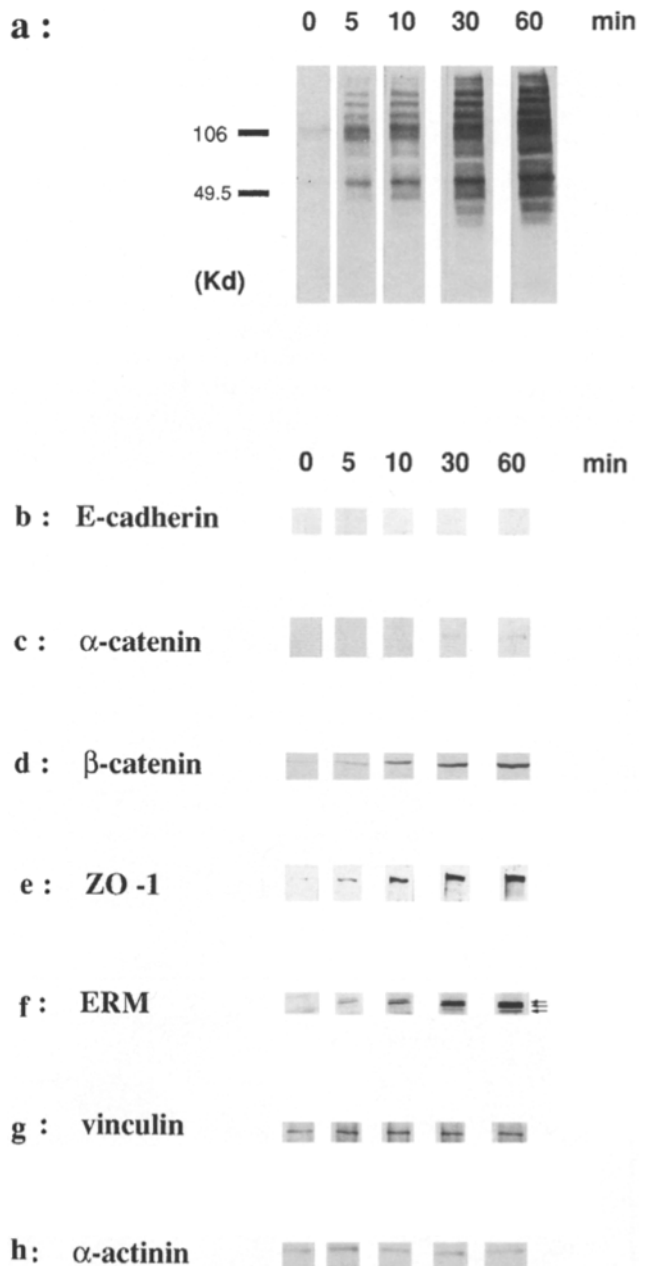


Figure 4. Tyrosine phosphorylation of junctional proteins in ts-v-src MDCK cells at the permissive temperature. (*a*) Confluent cells cultured at the nonpermissive temperature for 12 h were transferred to the permissive temperature, then 5, 10, 30, and 60 min after the temperature shift, they were processed for immunoblotting with anti-phosphotyrosine mAb. (*b–h*) At various times after the temperature shift, the cells were lysed with RIPA buffer containing 0.1% SDS, immunoprecipitated with anti-phosphotyrosine mAb, and then immunoblotted with antibodies specific for E-cadherin, α catenin, β catenin, ZO-1, ERM (from the top, arrows correspond to ezrin, radixin, and moesin), vinculin, and α -actinin.

times the cells were lysed with RIPA buffer containing 1% Nonidet P-40 and 0.1% SDS, and immunoprecipitated with antiphosphotyrosine antibody. Thereafter, the immunoprecipitate was immunoblotted with an antibody specific for E-cadherin, α catenin, β catenin, ZO-1, ezrin/ra-

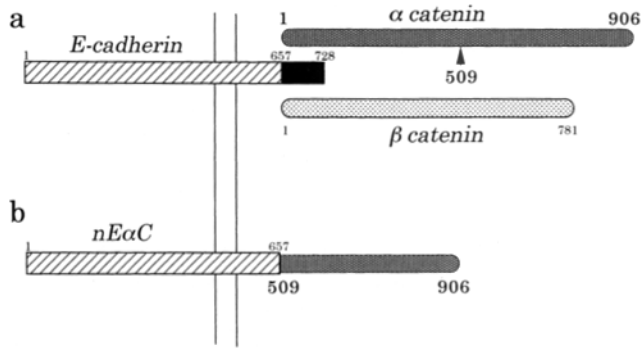


Figure 5. E-cadherin- α catenin fusion protein constructs. (a) E-cadherin/ α catenin/ β catenin complex. The carboxy-terminal 70 amino acids of E-cadherin (closed box) constitute a catenin-binding site. (b) The fusion protein, nE α C, of E-cadherin lacking the catenin-binding site with the carboxyl-terminal half of α catenin.

dixin/moesin (ERM), vinculin, or α -actinin (Fig. 4, b-h). As reported (Behrens et al., 1993), among the E-cadherin-catenin complex components, β catenin was gradually and heavily tyrosine phosphorylated, while the tyrosine phosphorylation of α catenin was not so remarkable. Tyrosine-phosphorylated E-cadherin was hardly detectable even 60 min after the temperature shift. ZO-1 and ERM were significantly tyrosine phosphorylated with the same time course as that of β catenin after the temperature shift. By contrast, vinculin and α -actinin were constitutively tyrosine

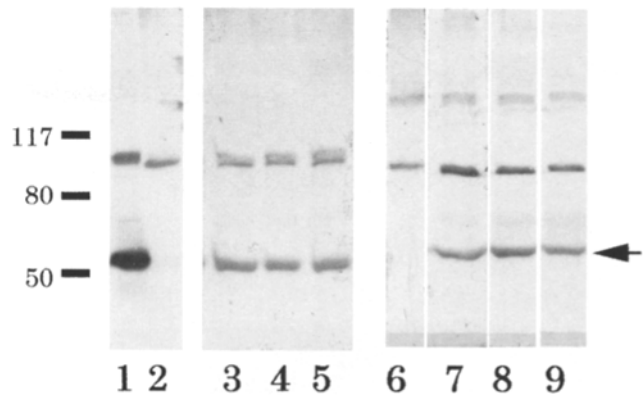


Figure 6. Expression of pp60^{v-src} in stable transfectants. Total lysates (20 μ g/lane) from ts-v-src MDCK cells (lane 1), parent nE α CL cells (lane 2), three independent clones of pp60^{v-src}-introduced nE α CL cells (lanes 3-5), parent EL β 1 cells (lane 6), or three independent clones of pp60^{v-src}-introduced EL β 1 cells (lanes 7-9) were separated by SDS-PAGE and immunoblotted with anti-pp60^{v-src} mAb. In addition to the pp60^{v-src} band (arrows), one or two nonspecifically stained bands were detected. The positions of molecular mass markers are indicated on the left (kD).

phosphorylated at the nonpermissive temperature, and the temperature shift did not significantly increase the tyrosine phosphorylation level of these proteins.

The cell debris resistant against the RIPA extraction was further solubilized with a solution containing 1% SDS, and

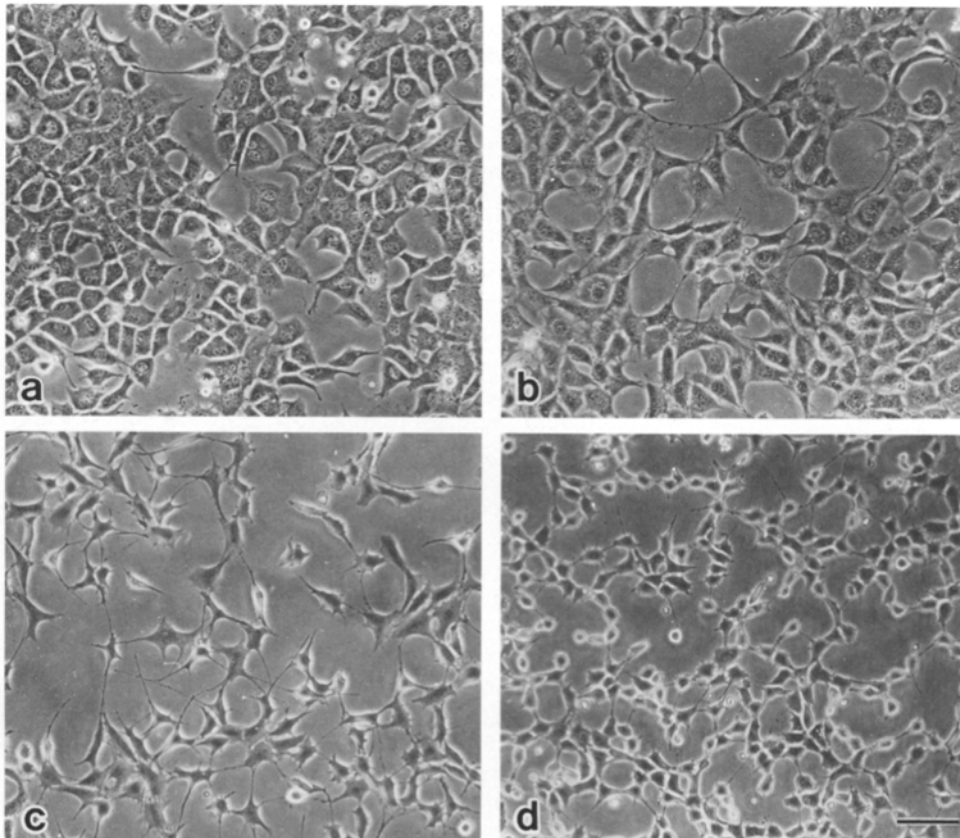


Figure 7. Phase-contrast microscopic images of nE α CL cells (a), EL β 1 cells (b), nE α CL cells expressing pp60^{v-src} (c), and EL β 1 cells expressing pp60^{v-src} (d). Bar, 100 μ m.

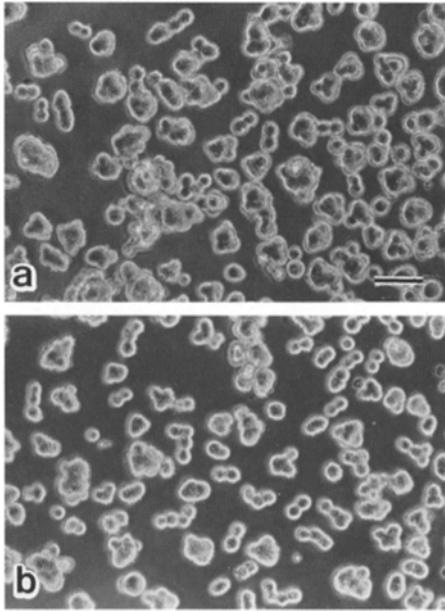


Figure 8. Cell aggregation (a) and dissociation (b) assays of nE α CL cells. Single nE α CL cells were rotated for 3 h on a gyratory shaker at 80 rpm to form tightly compact aggregates, in which individual cells were hardly recognizable (a). When these aggregates were passed several times through Pasteur pipettes, there were few single cells (b). Bar, 50 μ m.

then diluted with RIPA buffer to decrease the SDS concentration to $\sim 0.1\%$, which allowed immunoprecipitation with antiphosphotyrosine mAb. The antiphosphotyrosine immunoprecipitate was immunoblotted with antibodies as described above. The time course of the tyrosine phosphorylation of each junctional component was the same as shown in Fig. 4 (data not shown).

These data were consistent with the notion that the tyrosine phosphorylation of β catenin is involved in the "dysfunction" of cadherin molecules (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993). On the other hand, our data showed that not only β catenin, but also other junctional proteins such as ZO-1 and ERM are heavily tyrosine phosphorylated in a time-dependent manner after the activation of v-src kinase. To evaluate the role of tyrosine phosphorylation of β catenin in cadherin regulation, we analyzed the effects of tyrosine phosphorylation on the cell adhesion ability of nE α CL cells.

Introduction of v-src Kinase into nE α CL Cells

The carboxyl terminus-truncated cadherin has no cell adhesion activity because of its failure to associate with catenins. As described, this nonfunctional cadherin was rescued as an adhesion molecule, when the carboxyl-terminal half of α catenin was directly linked to the nonfunctional E-cadherin (Fig. 5; Nagafuchi et al., 1994). The adhesion activity of the mouse L cell transfectants (nE α CL cells) expressing this fusion molecule (nE α C) was just as cadherin dependent as the L cell transfectants expressing intact E-cadherin (EL β 1 cells). In nE α CL cells, nE α C molecules were not associated with β catenin, indicating that they can function as adhesion molecules in the absence of

β catenin. Therefore, if nE α CL cells could express v-src kinase, they would be useful to check whether or not the tyrosine phosphorylation of β catenin is involved in the tyrosine phosphorylation-dependent shift of cadherin-based cell adhesion from the strong to the weak state.

An expression vector for v-src kinase was introduced into nE α CL cells, and those stably expressing v-src kinase were selected and examined by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-v-src kinase mAb (Fig. 6). Three stable clones expressing a relatively large amount of v-src kinase were obtained. For comparison, three clones of EL β 1 cells expressing v-src kinase were also established. Expression of v-src kinase induced significant morphological changes in both nE α CL and EL β 1 cells (Fig. 7). Cells rounded up and adhered with neighboring cells by means of thin protrusions.

Cell Aggregation and Dissociation Assay of v-src Kinase-introduced nE α CL Cells

As described, when nE α CL cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-contrast microscopy (Fig. 8). Removing Ca^{2+} ion from the medium or anti-E-cadherin mAb inhibited the aggregation of these cells.

In sharp contrast, when the same aggregation experiment was performed using v-src kinase-expressing nE α CL cells, loose aggregates were formed, in which individual cells were easily recognizable (Fig. 9 a). EL β 1 cells expressing v-src kinase also formed similar loose cell aggregates (Fig. 9 b). This type of cell aggregation was completely inhibited by Ca removal or anti-E-cadherin mAb (data not shown). When these aggregates were passed through Pasteur pipettes several times, they were easily dissociated into single cells, indicating that in the presence of v-src kinase, nE α C as well as E-cadherin assume the weak adhesion state (Fig. 9, c and d).

Tyrosine Phosphorylation in the E-Cadherin Immunoprecipitates from v-src Kinase-expressing nE α CL or EL β 1 Cells

As reported, when we immunoprecipitated EL β 1 cells with anti-E-cadherin mAb, both endogenous α and β catenins were coimmunoprecipitated with intact E-cadherin molecules (Nagafuchi et al., 1991). In contrast, under the same conditions, the E-cadherin- α catenin fusion protein that immunoprecipitated from nE α CL cells was not associated either with α or β catenins (Nagafuchi et al., 1994). The introduction of v-src kinase into EL β 1 or nE α CL cells did not change the composition of the E-cadherin immunoprecipitates (E-cadherin/catenin complex or E-cadherin- α catenin fusion protein) (data not shown). Using an antiphosphotyrosine mAb, we tried to detect tyrosine phosphorylated polypeptides in these immunoprecipitated E-cadherin- α / β catenin complexes or E-cadherin- α catenin fusion proteins in the presence of v-src kinase (Fig. 10). In the E-cadherin- α / β catenins complex immunoprecipitated from v-src kinase-expressing EL β 1 cells, β catenin was predominantly tyrosine phosphorylated. However, the E-cadherin- α catenin fusion protein from nE α CL cells was hardly tyrosine phosphorylated. In the absence of v-src kinase, no tyrosine

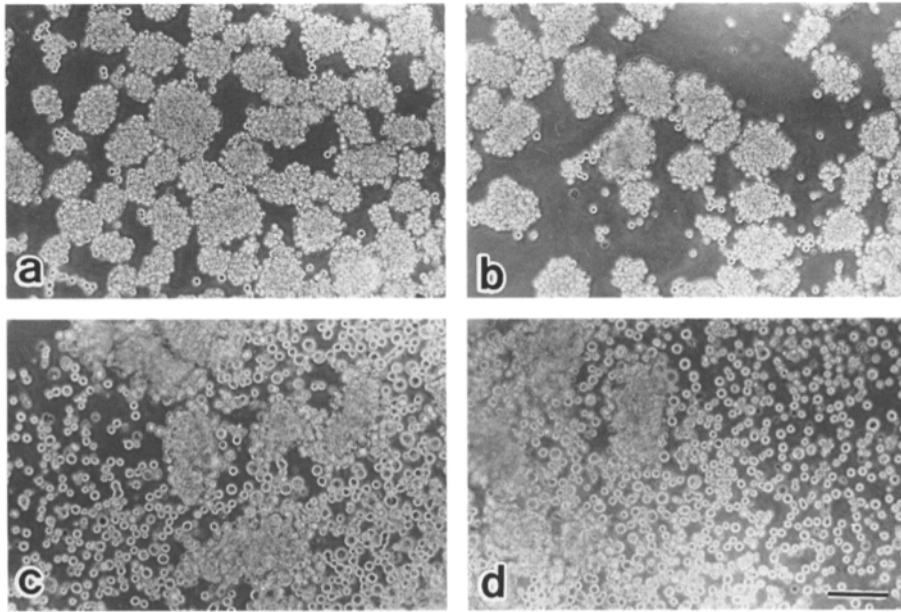


Figure 9. Cell aggregation (*a* and *b*) and dissociation (*c* and *d*) assays of pp60^{v-src}-expressing nEαCL (*a* and *c*) and pp60^{v-src}-expressing ELβ1 (*b* and *d*) cells. Single cells were rotated for 3 h on a gyratory shaker at 80 rpm. Both types of cells formed loose cell aggregates, in which individual cells were easily recognized. When these aggregates were passed several times through Pasteur pipettes, they readily dissociated into single cells. Bar, 50 μm.

phosphorylated polypeptides were detected in E-cadherin immunoprecipitates either from ELβ1 or nEαCL cells.

Discussion

Two States of Cadherin-based Cell–Cell Adhesion

So far, the function of cadherin molecules has been evaluated by means of the so called cell aggregation assay (Takeichi, 1977). Some investigators have described that in this type of assay, the cell aggregates assume two distinct morphologies, compact and loose, but knowledge of how these are formed has been limited (Matsuyoshi et al., 1992). In this study, we found that ts-v-src MDCK cells showed these distinct cell aggregates under nonpermissive and permissive conditions, and that judging from the effects of Ca²⁺-chelating reagents and anti-cadherin antibodies, both types of aggregation were mediated by cadherins. Furthermore, we found that under conditions of high shearing, such as vigorous pipetting, the loose aggregates were completely dissociated into single cells, whereas the compact form was fairly stable. Thus, we concluded that cells assumed two states of cadherin-based cell adhesion, strong and weak, and that these were experimentally produced in ts-v-src MDCK cells at nonpermissive (normal levels of tyrosine phosphorylation) and permissive temperatures (elevated levels of tyrosine phosphorylation), respectively. These two states are difficult to be distinguished by cell aggregation rates in the classical aggregation assay, since both states of ts-v-src MDCK cells showed the same rate of aggregation.

The transition from the weak to the strong state of cadherin-based cell–cell adhesion occurs *in vivo* as the phenomenon referred to as “compaction” at the eight-cell stage of mammalian embryogenesis. During this process, cadherins aggregate on the cell surface to form cell-to-cell AJ (Ducibella and Anderson, 1975; Vestweber et al., 1987). On the other hand, as shown by others (Warren and Nel-

son, 1987; Kellie, 1988; Volberg et al., 1992), the elevation of tyrosine phosphorylation induced destruction of the cell-to-cell AJ. Therefore, the weak and strong states of cadherin-based cell adhesion appear to correspond to the degree of aggregation of cadherin molecules on the cell surface. If so, we speculate that the elevated level of tyrosine phosphorylation in the cytoplasm disaggregates cadherins on the cell surface through the tyrosine phosphorylation of plasmalemmal undercoat-constitutive proteins, which changes cadherin-based cell adhesion from the strong to the weak state.

Tyrosine Phosphorylation of β Catenin

The molecular mechanism involved in the “dysfunction” (which has not been distinguished from the “weak state” so far) of cadherin-based cell adhesion induced by tyrosine phosphorylation has been intensively investigated. Using v-src transfected fibroblasts, Matsuyoshi et al. (1992) have revealed that in a P-cadherin immunoprecipitate consisting of P-cadherin, α and β catenin, only the latter was tyrosine phosphorylated in accordance with a P-cadherin dysfunction. Furthermore, Hamaguchi et al. (1993) have also suggested from studies using a similar system in the chick, that the tyrosine phosphorylation of β catenin is important for E-cadherin dysfunction. When ts-v-src MDCK cells are shifted from the nonpermissive to the permissive temperature, among E-cadherin, α and β catenins, only the latter was gradually and heavily tyrosine phosphorylated (Behrens et al., 1993). This was confirmed here, indicating that the tyrosine phosphorylation of β catenin correlated well with the shift of cadherin-based cell adhesion from the strong to the weak state, not only from the phosphorylation level, but also from the time course.

In mouse L cells, α and β catenins are not detectable. When E-cadherin is introduced into L cells (ELβ1), both α and β catenins become detectable as cadherin-associated proteins (Nagafuchi et al., 1991, 1994). We found here that

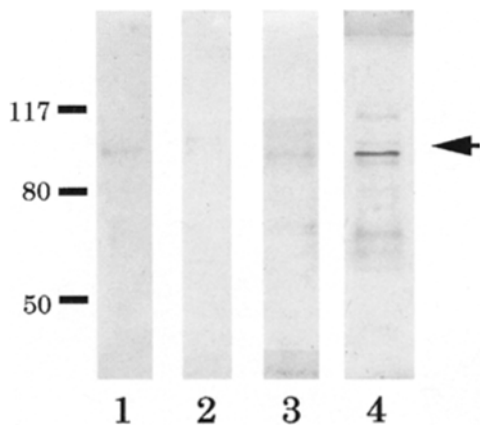


Figure 10. Tyrosine phosphorylation of E-cadherin immunoprecipitates. Total cell lysates from nEαCL cells (lane 1), nEαCL cells expressing pp60^{v-src} (lane 2), ELβ1 cells (lane 3), ELβ1 cells expressing pp60^{v-src} (lane 4) were immunoprecipitated with anti-E-cadherin mAb, resolved by SDS-PAGE, and then immunoblotted with an antiphosphotyrosine mAb. Only β catenin in the E-cadherin immunoprecipitate from ELβ1 cells expressing pp60^{v-src} was tyrosine phosphorylated (arrow). The positions of molecular mass markers are indicated on the left (kD).

the transfection of v-src kinase in ELβ1 cells shifted their cadherin-based cell adhesion from the strong to the weak state with the concomitant tyrosine phosphorylation of β catenin. In nEαCL cells, neither endogenous α nor β catenin was detected, probably because the nEαC fusion molecule has no ability to associate with either α or β catenin (Nagafuchi et al., 1994). The introduction of v-src kinase into nEαCL cells resulted in a shift of the cadherin-based cell adhesion from the strong to the weak state. Considering that the nEαC fusion molecule itself did not include a portion of β catenin, and that the nEαC fusion molecule itself was not tyrosine phosphorylated by v-src kinase, we concluded that without the tyrosine phosphorylation of β catenin, the elevation of the tyrosine phosphorylation level at the cytoplasm shifts the cadherin-based cell adhesion from the strong to the weak state.

Tyrosine Phosphorylation of ERM and ZO-1

Not only catenins but also other junctional proteins such as vinculin, α-actinin, ERM, and ZO-1 are thought to be involved in the regulation of cadherin-based cell adhesion (Takeichi, 1991; Tsukita et al., 1992). Therefore, these proteins are also likely tyrosine phosphorylation-dependent regulators of cadherin-based cell adhesion.

Vinculin and α-actinin are concentrated at cadherin-based cell adhesion sites, and are thought to directly or indirectly interact with cadherins (Geiger, 1979; Lazarides and Burridge, 1975). Vinculin is reportedly tyrosine phosphorylated (Sefton et al., 1981; Ito et al., 1983). In ts-v-src MDCK cells, both vinculin and α-actinin were constitutively tyrosine phosphorylated, showing no correlation with the cadherin-based adhesion shift. This suggests that the tyrosine phosphorylation of either vinculin or α-actinin is not involved in the dysfunction of cadherin-based cell adhesion. Of course, it is possible that, although the overall tyrosine phosphoryla-

tion level of these proteins does not change, the site of phosphorylation is different, resulting in different activity.

Members of the ERM family consisting of three closely related proteins, ezrin, radixin, and moesin, are thought to play an important role in the interaction of actin filaments with plasma membranes in general (Tsukita et al., 1992). Recent studies with antisense oligonucleotides have revealed that ERM proteins play a pivotal role in maintaining both integrin- and cadherin-based cell adhesion (Tsukita et al., 1993; Takeuchi et al., 1994). Among ERM proteins, ezrin is reportedly a good *in vivo* substrate for tyrosine kinases such as EGF receptors (Bretscher, 1983, 1989; Gould et al., 1986; Hunter and Cooper, 1981, 1983; Pakkanen et al., 1987). Two tyrosine residues in ezrin molecules have been identified as phosphorylation sites, and one of them is conserved in radixin and in moesin (Krieg and Hunter, 1992). This study revealed that after the temperature shift of ts-v-src MDCK cells, radixin, moesin and ezrin were heavily tyrosine phosphorylated in a time course similar to that of β catenin. Taking the results obtained with antisense oligonucleotides into consideration, we speculate that the tyrosine phosphorylation of ERM proteins is involved in the regulation of cadherin-based cell adhesion.

ZO-1 was originally reported to be exclusively localized at tight junctions, but it was later shown to be colocalized with various types of cadherins in cells lacking tight junctions (Stevenson et al., 1986; Anderson et al., 1988; Itoh et al., 1991, 1993). ZO-1 is reportedly serine/threonine phosphorylated (Stevenson et al., 1989; Singer et al., 1994), and tyrosine phosphorylated (Van Itallie et al., 1995). The time course of ZO-1 tyrosine phosphorylation in ts-v-src MDCK cells after the temperature shift was very similar to that of β catenin and ERM proteins. Although the function of ZO-1 in cadherin-based cell adhesion is unknown, ZO-1 is also a potential tyrosine phosphorylation-dependent regulator of cadherin-based cell adhesion.

Of course, it is possible that other cytoplasmic or undercoat-constitutive proteins play central roles in the tyrosine phosphorylation-dependent regulation of cadherin-based cell adhesion. As the role of the tyrosine phosphorylation of β catenin was evaluated in this study by genetic engineering means, we should next evaluate ERM proteins and ZO-1. Studies are currently being conducted along these lines in our laboratory.

We would like to thank all the members of our laboratory (Department of Cell Biology, Faculty of Medicine, Kyoto University, Kyoto, Japan) for their helpful discussions. Our thanks are also due to Dr. M. Takeichi (Kyoto University) and Dr. M. Seiki (Kanazawa University, Kanazawa, Japan) for supplying the anti-cadherin mAbs, ECCD-1 and PCD-1 and the pMv-src, respectively.

This work was supported in part by a Grant-in-Aid for Cancer Research and a Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Science and Culture of Japan, and by research grants from the Yamada Science Foundation, the Mitsubishi Foundation, and the Toray Science Foundation (to Sh. Tsukita).

Received for publication 4 July 1995 and in revised form 15 September 1995.

References

- Anderson, J. M., B. R. Stevenson, L. A. Jesaitis, D. A. Goodenough, and M. S. Mooseker. 1988. Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. *J. Cell Biol.*

- 106:1141-1149.
- Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. van Roy, M. M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlate with tyrosine phosphorylation of the E-cadherin/ β -catenin complex in cells transformed with a temperature-sensitive v-src gene. *J. Cell Biol.* 120:757-766.
- Bretscher, A. 1983. Purification of an 80,000-dalton protein that is a component of the isolated microvilli cytoskeleton, and its localization in nonmuscle cells. *J. Cell Biol.* 108:921-930.
- Bretscher, A. 1989. Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor. *J. Cell Biol.* 108:921-930.
- Ducibella, T., and E. Anderson. 1975. Cell shape and membrane changes in eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. *Dev. Biol.* 47:45-58.
- Geiger, B. 1979. A 130k protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193-205.
- Gould, K. L., J. A. Cooper, A. Bretscher, and T. Hunter. 1986. The protein-tyrosine kinase substrate, p81, is homologous to a chicken microvillar core protein. *J. Cell Biol.* 102:660-669.
- Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, and Y. Nagai. 1993. p60^{src} causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO J.* 12:307-314.
- Hinck, L., I. S. Nathke, J. Papkoff, and W. J. Nelson. 1994. Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J. Cell Biol.* 125:1327-1340.
- Hoschuetzky, H., H. Aberle, and R. Kemler. 1994. β -catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J. Cell Biol.* 127:1375-1380.
- Hunter, T., and J. A. Cooper. 1981. Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. *Cell.* 24:741-752.
- Hunter, T., and J. A. Cooper. 1983. Role of tyrosine phosphorylation in malignant transformation by viruses and in cellular growth control. *Prog. Nucleic Acid Res. Mol. Biol.* 29:221-233.
- Ito, S., D. K. Werth, N. D. Richert, and I. Pastan. 1983. Vinculin phosphorylation by the src kinase. *J. Biol. Chem.* 258:14626-14631.
- Itoh, M., S. Yonemura, A. Nagafuchi, Sa. Tsukita, and Sh. Tsukita. 1991. A 220-kD undercoat-constitutive protein: its specific localization at cadherin-based cell-cell adhesion sites. *J. Cell Biol.* 115:1449-1462.
- Itoh, M., A. Nagafuchi, S. Yonemura, T. Kitani-Yasuda, Sa. Tsukita, and Sh. Tsukita. 1993. The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J. Cell Biol.* 121:491-502.
- Kellie, S. 1988. Cellular transformation, tyrosine kinase oncogenes, and the cellular adhesion plaque. *BioEssays.* 8:25-29.
- Krieg, J., and T. Hunter. 1992. Identification of the two major, EGF-induced tyrosine phosphorylation sites in the microvillar core protein ezrin. *J. Biol. Chem.* 267:19258-19265.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lazarides, E., and K. Burridge. 1975. α -actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell.* 6:289-298.
- Matsuyoshi, N., M. Hamaguchi, S. Taniguchi, A. Nagafuchi, Sh. Tsukita, and M. Takeichi. 1992. Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.* 118:703-714.
- Nagafuchi, A., and Sh. Tsukita. 1994. The loss of the expression of α catenin, the 102kD cadherin associated protein, in central nervous tissues during development. *Dev. Growth & Differ.* 36:759-761.
- Nagafuchi, A., M. Takeichi, and Sh. Tsukita. 1991. The 102kDa cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. *Cell.* 65:849-857.
- Nagafuchi, A., S. Ishihara, and Sh. Tsukita. 1994. The roles of catenins in the cadherin-mediated cell adhesion: functional analysis of E-cadherin- α catenin fusion molecules. *J. Cell Biol.* 127:235-245.
- Nose, A., A. Nagafuchi, and M. Takeichi. 1988. Expressed recombinant cadherins mediate cell sorting in model system. *Cell.* 54:993-1001.
- Ozawa, M., H. Baribault, and R. Kemler. 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J.* 8:1711-1717.
- Pakkanen, R., K. Hedman, O. Turunen, T. Wahlstrom, and A. Vaheri. 1987. Microvillus-specific Mr 75,000 plasma membrane protein of human chorio carcinoma cells. *J. Histochem. Cytochem.* 135:809-816.
- Paul, J. J., M. C. Paul, V. D. Annette, and S. Davis. 1985. Overexpressed pp60^{src} can induce focus formation without complete transformation of NIH3T3 cells. *Mol. Cell. Biol.* 5:1073-1083.
- Peyrieras, N., D. Louvard, and F. Jacob. 1985. Characterization of antigens recognizing by monoclonal and polyclonal antibodies directed against uvomorulin. *Proc. Natl. Acad. Sci. USA.* 82:8067-8071.
- Sefton, B. M., T. Hunter, E. H. Ball, and S. J. Singer. 1981. Vinculin: a cytoskeletal target of the transforming protein of Rous Sarcoma Virus. *Cell.* 24:165-174.
- Shibamoto, S., M. Hayakawa, K. Takeuchi, T. Hori, K. Miyazawa, N. Kitamura, K. R. Johnson, M. J. Wheelock, N. Matsuyoshi, M. Takeichi, and F. Ito. 1995. Association of p120, a tyrosine kinase substrate, with E-cadherin/catenin complexes. *J. Cell Biol.* 128:949-957.
- Singer, K. L., B. R. Stevenson, P. L. Woo, and G. L. Firestone. 1994. Relationship of serine/threonine phosphorylation/dephosphorylation signaling to glucocorticoid regulation of tight junction permeability and ZO-1 distribution in nontransformed mammary epithelial cells. *J. Biol. Chem.* 269:16108-16115.
- Stevenson, B. R., J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough. 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* 103:755-766.
- Stevenson, B. R., J. M. Anderson, I. D. Braun, and M. S. Mooseker. 1989. Phosphorylation of the tight-junction protein ZO-1 in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance. *Biochem. J.* 263:597-599.
- Takata, K., and S. J. Singer. 1988. Phosphotyrosine-modified proteins are concentrated at the membranes of epithelial and endothelial cells during tissue development in chick embryos. *J. Cell Biol.* 106:1757-1764.
- Takeichi, M. 1977. Functional correlation between cell adhesive properties and some cell surface proteins. *J. Cell Biol.* 75:464-474.
- Takeichi, M. 1988. The cadherin: cell-cell adhesion molecules controlling animal morphogenesis. *Development.* 102:639-655.
- Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science (Wash. DC)* 251:1451-1455.
- Takeuchi, K., N. Sato, H. Kasahara, N. Funayama, A. Nagafuchi, S. Yonemura, Sa. Tsukita, and Sh. Tsukita. 1994. Perturbation of cell adhesion and microvilli formation by antisense oligonucleotides to ERM family members. *J. Cell Biol.* 125:1371-1384.
- Tsukita, Sa., Y. Hieda, and Sh. Tsukita. 1989. A new 82 kd-barbed end capping protein localized in the cell-to-cell adherens junction: Purification and characterization. *J. Cell Biol.* 108:2369-2382.
- Tsukita, Sa., K. Oishi, T. Akiyama, Y. Yamanashi, T. Yamamoto, and Sh. Tsukita. 1991. Specific proto-oncogenic tyrosine kinases of src family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. *J. Cell Biol.* 113:867-879.
- Tsukita, Sh., M. Itoh, A. Nagafuchi, S. Yonemura, and Sa. Tsukita. 1993. Submembranous junctional plaque proteins include potential tumor suppressor molecules. *J. Cell Biol.* 123:1049-1053.
- Tsukita, Sh., Sa. Tsukita, A. Nagafuchi, and S. Yonemura. 1992. Molecular linkage between cadherins and actin filaments in cell-to-cell adherens junctions. *Curr. Opin. Cell Biol.* 4:834-839.
- Van Itallie, C. M., M. S. Balda, and J. M. Anderson. 1995. Epidermal growth factor induces tyrosine phosphorylation and reorganization of the tight junction protein ZO-1 in A431 cells. *J. Cell Sci.* 108:1735-1742.
- Vestweber, D., and R. Kemler. 1984. Some structural and functional aspects of the cell adhesion molecule uvomorulin. *Cell Diff.* 15:269-273.
- Vestweber, D., A. Gossler, K. Boller, and R. Kemler. 1987. Expression and distribution of cell adhesion molecule uvomorulin in mouse preimplantation embryos. *Dev. Biol.* 124:451-456.
- Volberg, T., B. Geiger, R. Dror, and Y. Zick. 1991. Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells. *Cell Regulation.* 2:105-120.
- Volberg, T., Y. Zick, R. Dror, I. Sabanay, C. Gilon, A. Levitzki, and B. Geiger. 1992. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO J.* 11:1733-1742.
- Warren, S. L., and W. J. Nelson. 1987. Nonmitogenic morphoregulatory action of pp60^{src} on multicellular epithelial structure. *Mol. Cell. Biol.* 7:1326-1337.