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,II and Shoichiro Tsukita*.I

*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; klMax-Delbrück-Center for Molecular Medicine, 13122 Berlin, Germany; andlDepartment 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 cultured 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).

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 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 elevation of the tyrosine phosphorylation level does not affect cadherin-based cell adhesion, but shifts it 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 35°C (nonpermissive temperature) or at 35°C (permissive temperature). Mouse L cell transfectants expressing E-cadherin (ELB1; Nose et al., 1988), or a fusion protein (nEoC) between a carboxy terminus-truncated cadherin and the carboxy-terminal half of α catenin (nEoCl; Nagafuchi et al., 1994) were grown in DME supplemented with 10% FSC and 150 μg/ml of G418.

Mouse anti-ZO-1 mAb (T-8-754) (Itoh et al., 1991), rat anti-α catenin mAb (a18) (Nagafuchi and Tatsuka, 1994), rat anti-β catenin mAb (Z01) (Nagafuchi et al., 1994), and rabbit anti-ERM pAb (11) (Tatsuka 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 phosphoryosine p(166) vinculin (VIN11-5), and α-actinin (BM75.2) were purchased from Sigma Chemical Co. (St. Louis, MO), and mouse mAb p60^src (mAb 327) was purchased from Oncogene Science (Manhasset, NY).

**Transfection**

L cell transfectants, ELB1 and nEoCl, were plated at a density of 1 x 10⁶ 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 μg/ml blasticidin-S. Colonies of blasticidin-S-resistant cells were isolated and redenoned. Using anti-p60^src mAb (mAb327), we isolated several stable clones expressing p60^src for ELB1 and nEoCl 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 gyra-tory shaker and rotated at 80 rpm for several hours at 35°C 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 Heps-buffered saline (HCMF; pH 7.4) to obtain single cell suspensions. Cells suspended in DME medium were placed in 1% agaro-coated four-well plates and rotated for 3 h at 37°C on a gyration 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 Np/Nc, where Np and Nc are the total numbers of particles and cells per dish, respectively. For cell dissociation studies of L cell transfectants, aggregates formed on a gyration 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, 1 mM NaVO₄, 10 mM NaF, 10 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 μl of SDS-immunoprecipitation buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% SDS) for 3 h at 4°C, and then centrifuged at 10,000 g for 30 min. The supernatant was collected as the insoluble fraction. Anti-phosphoosine 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 μl) was added to the sample, and incubated for an additional 3 h on ice. Protein G-Sepharose 4B-bound immune complexes were then eluted by boiling in 150 μl of SDS sample buffer.

L cell transfectants cultured in 100 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 NaVO₄, 30 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 μl of rat mAb ECCD-2 and 50 μl 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 NaVO₄.

**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 (Fig. 1 c). This aggregation was completely suppressed in the absence of Ca²⁺ 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 Ca²⁺: 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 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 Ca²⁺ 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.

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.
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 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, ERM (from the top, arrows correspond to ezrin, radixin, and moesin), vinculin, and α-actinin.
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, nEaC, 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 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 6. Expression of pp60src in stable transfectants. Total lysates (20 μg/lane) from ts-v-src MDCK cells (lane 1), parent nEaCL cells (lane 2), three independent clones of pp60src-introduced nEaCL cells (lanes 3–5), parent ELB1 cells (lane 6), or three independent clones of pp60src-introduced ELB1 cells (lanes 7–9) were separated by SDS-PAGE and immunoblotted with anti-pp60src mAb. In addition to the pp60src band (arrows), one or two nonspecifically stained bands were detected. The positions of molecular mass markers are indicated on the left (kD).

Figure 7. Phase-contrast microscopic images of nEaCL cells (a), ELB1 cells (b), nEaCL cells expressing pp60src (c), and ELB1 cells expressing pp60src (d). Bar, 100 μm.
ylation of each junctional component was the same as cells (expressing this fusion molecule (nEoC) was just as minal half of ct catenin was directly linked to the nonfunc-

The carboxyl terminus-truncated cadherin has no cell ad-

rescued as an adhesion molecule, when the carboxyl-ter-

role of tyrosine phosphorylation of 13 catenin in cadherin

also other junctional proteins such as ZO-1 and ERM are

However, the E-cadherin-ct catenin fusion protein is involved in the ty-

then diluted with RIPA buffer to decrease the SDS con-

Then, these data were consistent with the notion that the ty-

These data were consistent with the notion that the ty-

Introduction of v-src Kinase into nEoCL Cells

The carboxyl terminus–truncated cadherin has no cell ad-

The introduction of v-src kinase into nEoCL cells did not

Tyrosine Phosphorylation in the E-Cadherin

As reported, when we immunoprecipitated ELB1 cells with anti-E-cadherin mAb, both endogenous α and β catenins were coimmunoprecipitated with intact E-cadherin mole-

As described, when ELB1 cells with anti-E-cadherin mAb, both endogenous α and β catenins were coimmunoprecipitated with intact E-cadherin mole-

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

Comparison, three clones of ELB1 cells expressing v-src ki-

nase were also established. Expression of v-src kinase in-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

Tyrosine Phosphorylation in the E-Cadherin

Immuno precipitates from v-src Kinase-expressing nEoCL or ELB1 Cells

As reported, when we immunoprecipitated ELB1 cells with anti-E-cadherin mAb, both endogenous α and β catenins were coimmunoprecipitated with intact E-cadherin mole-

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-

In sharp contrast, when the same aggregation experiment

As described, when nEoC cells were incubated for 3 h under gentle gyration, they formed compact aggregates, in which individual cells were hardly recognizable by phase-
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 Nelson, 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 catenins, 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. 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
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 nEaCCL cells, neither endogenous α nor β catenin was detected, probably because the nEaC fusion molecule had no ability to associate with either α or β catenin (Nagafuchi et al., 1994). The introduction of v-src kinase into nEaCCL cells resulted in a shift of the cadherin-based cell adhesion from the strong to the weak state. Considering that the nEaC fusion molecule itself did not include a portion of β catenin, and that the nEaC 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 (Takeuchi, 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 phosphorylation 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; Pankonen 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., 1993; 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. Moosuker. 1988. Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. J. Cell Biol.
