Cadherin-Mediated Cell–Cell Adhesion Is Perturbed by v-*src* Tyrosine Phosphorylation in Metastatic Fibroblasts

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Abstract. Rat 3Y1 cells acquire metastatic potential when transformed with v-*src*, and this potential is enhanced by double transformation with v-*src* and v-*fos* (Taniguchi, S., T. Kawano, T. Mitsudomi, G. Kimura, and T. Baba. 1986. *Jpn. J. Cancer Res.* 77:1193–1197). We compared the activity of cadherin cell adhesion molecules of normal 3Y1 cells with that of v-*src* transformed (SR3Y1) and v-*src* and v-*fos* double transformed (fosSR3Y1) 3Y1 cells. These cells expressed similar amounts of P-cadherin, and showed similar rates of cadherin-mediated aggregation under suspended conditions. However, the aggregates or colonies of these cells were morphologically distinct. Normal 3Y1 cells formed compacted aggregates in which cells are firmly connected with each other, whereas the transformed cells were more loosely associated, and could freely migrate out of the colonies. Overexpression of exogenous E-cadherin in these transformed cells had no significant effect on their adhesive properties. We then found that herbimycin A, a tyrosine kinase inhibitor, induced tighter cell–cell associations in the aggregates of the transformed cells. In contrast, vanadate, a tyrosine phosphatase inhibitor, inhibited the cadherin-mediated aggregation of SR3Y1 and fosSR3Y1 cells but had little effect on that of normal 3Y1 cells. These results suggest that v-*src*–mediated tyrosine phosphorylation perturbs cadherin function directly or indirectly, and the inhibition of tyrosine phosphorylation restores cadherin action to the normal state. We next studied tyrosine phosphorylation on cadherins and the cadherin-associated proteins, catenins. While similar amounts of catenins were expressed in all of these cells, the 98-kD catenin was strongly tyrosine phosphorylated only in SR3Y1 and fosSR3Y1 cells. Cadherins were also weakly tyrosine phosphorylated only in the transformed cells. The tyrosine phosphorylation of these proteins was enhanced by vanadate, and inhibited by herbimycin A. Thus, the tyrosine phosphorylation of the cadherin–catenin system itself might affect its function, causing instable cell–cell adhesion.

An initial process in metastasis is the detachment of cells from primary tumors. For understanding the mechanisms of tumor invasion and metastasis, it is important to unravel how cell detachment occurs. In normal tissues, cells are tightly associated with each other, so that they are generally not allowed to migrate freely. The adhesion of cells is mediated by a variety of surface molecules. Among them, cadherins play a crucial role in tight association of cells (Takeichi, 1991; Edelman and Crossin, 1991). Cadherins are a family of transmembrane glycoproteins involved in Ca^{2+}-dependent cell–cell adhesion and are expressed in all the cells that constitute solid tissues, except blood cells. Evidence has been accumulated that inactivation of cadherins causes the disruption of cell–cell adhesion (Takeichi, 1988, 1990). Conversely, cells to which additional cadherins are introduced by means of cDNA transfection become more adhesive to each other (Takeichi, 1990). These observations led us to propose that invasiveness or metastatic activity of tumor cells might be enhanced if cadherin function is perturbed (Hashimoto et al., 1989; Takeichi, 1991). This idea has been supported by in vitro observations. For example, MDCK cells acquire invasiveness when their E-cadherin is inactivated with antibodies (Behrens et al., 1989), and the invasiveness of several human carcinoma lines is suppressed by E-cadherin cDNA transfection (Frixen et al., 1991). Similar analyses support these findings (Vleminckx et al., 1991; Chen and Obrink, 1991; Navarro et al., 1991).

The above hypothesis was tested by examining the expression of cadherins in human carcinomas, such as gastric adenocarcinoma (Shimoyama and Hirohashi, 1991*a*; Takeichi, 1991; Shiozaki et al., 1991), hepatoma (Shimoyama and

Hirohashi, 1991*b*), esophageal carcinoma (Shiozaki et al., 1991), and mammary carcinoma (Shiozaki et al., 1991). In these studies, a clear trend was observed that the tumors which preserve epithelial morphology, classified as undifferentiated type, express high amounts of cadherins, whereas the tumors which exhibit scattered morphology, classified as undifferentiated type, have reduced amounts of these molecules. Generally, the latter type of tumors are more invasive into neighboring tissues than the former. These observations are in accord with the idea that the loss of cadherins may promote tumor invasion.

However, contradictory phenomena have been observed. The differentiated-type gastric adenocarcinoma, which expresses high amounts of E-cadherin, often metastasizes to remote organs, although is not necessarily invasive into neighboring tissues (Shimoyama and Hirohashi, 1991*a*). The cells that metastasize from these tumors generally retain the expression of this molecule in host organs. These phenomena imply that, in certain carcinomas, cells can disrupt their intercellular adhesion even in the presence of cadherins. This would occur if cadherin function were impaired by some mechanism. Consistent with this notion, it is important to note that a certain group of scattered-type tumors contain cells that express considerable amounts of E-cadherin but never form multicellular clusters (Shimoyama and Hirohashi, 1991*a*; Takeichi, 1991), probably representing an extreme case of cadherin functional suppression.

Cadherins are concentrated in the adherens-type intercellular junctions (AJs,[1] zonula adherentes) (Boller et al., 1985; Geiger et al., 1987; Volberg et al., 1991). In these junctions, cadherins form a complex with cytoskeletal proteins (Hirano et al., 1987; Nelson et al., 1990; Matsuzaki et al., 1990). Molecules directly associated with cadherins include catenins α, β, and γ (Vesteweber and Kemler, 1984; Peyrieras et al., 1985; Ozawa et al., 1989; Ngafuchi and Takeichi, 1989). Recently, catenin α and β were cloned, and found to be similar to vinculin (Nagafuchi et al., 1991; Herrenknecht et al., 1991) and plakoglobin (McCrea et al., 1991), respectively. Other work demonstrated that the association of cadherins with catenins is essential for their cell-binding function; that is, E- or N-cadherin with partial deletions of cytoplasmic domains cannot function either in catenin-binding or in cell–cell adhesion (Nagafuchi and Takeichi, 1988, 1989; Ozawa et al., 1990; Fujimori et al., 1990). Thus, catenins are considered to be key modulators of cadherin action, and their loss or aberrant action may interfere with cadherin-mediated cell–cell adhesion. If such changes occur in tumors, cell detachment could be induced even in the presence of cadherins.

According to recent work, the transformation of cells with the v-*src* oncogene product $p60^{v\text{-}src}$ leads to the disruption of AJs. MDCK epithelial cells lose their firm cell–cell association when transformed with $p60^{v\text{-}src}$, and coincidentally lose AJs, although other junctions such as tight junctions and desmosomes are left intact (Warren and Nelson, 1987). Similarly, chicken lens cells alter their epithelial morphology to a fibroblastic shape and lose AJs when transformed with Rous sarcoma virus (Volberg et al., 1991). It is also known that cell–cell contacts are the major sites of tyrosine phosphorylation (Maher et al., 1985; Maher and Pasquale, 1988; Takata and Singer, 1988). These findings suggest that over tyrosine-phosphorylation directly or indirectly prevents AJ formation, and that AJs are required for stable cell–cell connections.

The aim of the present study is to examine whether the function of cadherins or catenins is affected by the expression of $p60^{v\text{-}src}$ in rat fibroblasts. We compared the adhesive and invasive properties of normal rat 3Y1 cells with those of 3Y1 cells transformed with v-*src* (SR3Y1) and also double transformed with v-*src* and v-*fos* oncogenes (fosSR3Y1). Previous studies demonstrated that the latter two cell lines exhibit spontaneous lung metastases when transplanted subcutaneously in host animals, and the fosSR3Y1 cells show a higher metastatic activity (Taniguchi et al., 1986; Kawano et al., 1987). The results of the present study demonstrate that these transformed cells, in particular fosSR3Y1, cannot retain stable cell–cell associations and scatter into the culture matrix, even though they express functional cadherins. We then show that intracellular tyrosine phosphorylation perturbs cadherin function, and that catenins and cadherins themselves are tyrosine-phosphorylated in the v-*src* transformed 3Y1 cells.

Materials and Methods

Cells

The following cell lines were used: normal rat fibroblast cell line 3Y1-B clone 1-6 (3Y1), v-*src* transformant SR-3Y1-2 (SR3Y1) which was established by infection of 3Y1 with Schmidt-Ruppin D strain Rous sarcoma virus, and double-oncogene transformant fos-SR-3Y1-202 (fosSR3Y1) which was established by transfection of SR3Y1 with the cloned v-*fos* gene (Taniguchi et al., 1986). The cells were cultured in a 1:1 mixture of DME and Ham's F12 medium supplemented with 10% FCS (DH10).

Antibodies

The following monoclonal and polyclonal antibodies were used: a rabbit antiserum to mouse P-cadherin (Nose et al., 1987), rat mAb PCD-1 to mouse P-cadherin (Nose and Takeichi, 1986), rat mAb ECCD-2 to mouse E-cadherin (Shirayoshi et al., 1986), mouse mAb PY-20 to phosphotyrosine (ICN Immuno Biologicals, Lisle, IL), and a rabbit antiserum raised against α-catenin or CAP102 (Nagafuchi et al., 1991). The rabbit anti-P-cadherin serum was treated at 56°C for 30 min to inactivate complement when used in cell cultures. Our preliminary studies showed that this antiserum specifically inhibits P-cadherin-mediated cell aggregation, but does not inhibit the cell aggregation mediated by other cadherins. Anti-*armadillo* antibody was kindly provided by M. Peifer.

Cell Aggregation

Cadherin-mediated cell aggregation was assayed as described (Takeichi, 1977). Briefly, cells were treated with 0.01% crystallized trypsin in the presence of 2 mM $CaCl_2$ at 37°C for 20 min, and then washed with Ca^{2+}- and Mg^{2+}-free Hepes-buffered (pH 7.4) HBSS (HCMF) to obtain single cell suspensions. Cells suspended in HCMF with or without 1 mM $CaCl_2$ were placed in wells of a 24-well plate (model 143982; Nunclon, Roskilde, Denmark) and incubated to allow aggregation for 30 min at 37°C on a gyratory shaker rotating at 80 rpm. The degree of cell aggregation was represented by the index N_{30}/N_0, where N_0 is the total cell number per well and N_{30} is the total particle (single cells plus cell clusters) number per well at 30 min of incubation.

For culturing cells in suspension for longer periods, cells prepared as above were suspended in DH10, placed in 3.5- or 5-cm plastic dishes and incubated on the gyratory shaker in a 5% CO_2 incubator.

Collagen Embedded Culture and Dispersion Index

Type I collagen stock solution (Cell Matrix Type I-A; Nitta Gelatin, Osaka,

1. *Abbreviations used in this paper*: AJ, adherens-type intercellular junctions; TBS-Ca, TBS containing 1 mM $CaCl_2$.

Japan) was mixed with 5× concentrated DME and a reconstituting buffer to achieve a 0.2% (wt/vol) final concentration of collagen, according to the manufacturer's instructions, and 10% FCS was added to this mixture. 0.5 ml of this solution was placed on a 5-cm dish as the basal layer. After polymerization at 37°C for 30 min, 3 ml of the same collagen solution but containing 5×10^2 cells/ml was overlaid on the basal collagen layer, and again allowed to polymerize at 37°C for 30 min. 3 ml of DH10 was then added to the top of collagen gel, and cells were cultured.

In the collagen gel cultures, each cell grew to form a three-dimensional colony. To measure the degree of cell dispersion in the colonies, the number of particles (isolated cells plus cell clusters) constituting each colony was counted under an inverted microscope after 7–13 d in culture. This number was divided by total cell number per colony, which was estimated by cell counting after digestion of the collagen with collagenase, and the obtained values were defined as the dispersion index. In this assay, colonies with 100% dispersion have a value of one, and those with lower degrees of cell dispersion give smaller values. Colonies with ~30 cells were chosen for this assay.

Assay of Cell Migration

Cover slips were coated with 0.03% collagen dissolved in 0.02 N acetic acid for 12 h at room temperature and dried. These cover slips were further coated with colloidal gold particles according to Albrecht-Buehler and Goldman (1976) and thoroughly rinsed with HCMF. Onto these coverslips, cells were seeded in a density of 1×10^3 cells per cover slip and cultured with DH10 for 12 or 24 h. The areas where cells had removed colloidal gold particles were measured using an image analyzer (Digitizer I/O 9864A; Hewlett-Packard, Palo Alto, CA). Single and paired cells, the latter generated by cell division, were chosen in the samples incubated for 12 and 24 h, respectively.

E-Cadherin cDNA Transfection

Cells were cotransfected with the plasmid pSV2hph encoding the hygromycin resistance gene (Gritz and Davies, 1983) and pBATEM2 encoding E-cadherin (Nose et al., 1988) or pBATEM21 encoding a mutant E-cadherin whose carboxy-terminal 37 amino acids were deleted (Nagafuchi and Takeichi, 1989) at a molar ratio of 1:10 using a calcium phosphate or electroporation method. Stable transformants were then selected by hygromycin, and screened for E-cadherin expression.

Immunofluorescence Cytochemistry

Cells cultured on coverslips were briefly rinsed with HCMF containing 1 mM $CaCl_2$ (HMF) and fixed with 3.5% paraformaldehyde in HMF for 30 min at 4°C. After rinsing with 50 mM TBS, pH 7.4, containing 1 mM $CaCl_2$ (TBS-Ca), the fixed cells were extracted with methanol at −20°C for 10 min, and rinsed again with TBS-Ca. Cells were then treated with 5% skim milk in TBS-Ca for 30 min, and subsequently incubated with antibodies against cadherins in TBS-Ca containing 5% skim milk for 60 min at room temperature. After washing with TBS-Ca, the samples were finally incubated with fluorescence-labeled second antibodies diluted with 5% skim milk in TBS-Ca for 60 min at room temperature. After washing with TBS-Ca and then briefly with distilled water, the preparations were mounted with 90% glycerol-10% TBS-Ca containing paraphenylenediamine to prevent bleaching (Johnson and Noguera-Araujo, 1981), and examined using a Zeiss fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

Immunoprecipitation

To detect radiolabeled cadherins and cadherin-associated proteins by immunoprecipitation, cells in a 5-cm dish were incubated in 2 ml of methionine-free MEM supplemented with 2% FCS and 100 μCi/ml of [^{35}S]methionine for 12 h at 37°C. They were lysed with 1 ml of the extraction buffer, 1% Triton X-100, 1% NP-40, 1 mM $CaCl_2$, 2 mM PMSF, and 20 mg/ml leupeptin in 50 mM TBS, pH 7.4, and centrifuged at 100,000 rpm for 30 min. To immunoprecipitate E-cadherin, the supernatant was preabsorbed by incubation with 100 μl of anti-rat Ig conjugated Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) for 30 min, and after removing the beads, the solution was mixed with 10 μl of the rat mAb ECCD-2 solution and placed for 60 min, and then incubated with 100 μl of the anti-rat Ig conjugated Sepharose for 60 min. The beads were collected by centrifugation, washed 5 times with the extraction buffer, then suspended in 100 μl of a SDS-lysis buffer with 5% mercaptoethanol, and boiled for 5 min. The released materials were analyzed by SDS-PAGE. The gels were then subjected to fluorography using the DMSO-PPO (2,5-diphenyloxazole) enhancing system as described previously (Bonner and Lasky, 1974).

To detect phosphotyrosine in cadherin–catenin complexes, cells confluent in a 10-cm dish were pretreated with 0.2–1 mM sodium orthovanadate in the presence or absence of 3 mM hydrogen peroxide added to DH10 for varying times at 37°C and lysed with the above extraction buffer supplemented with 1 mM orthovanadate and 3 mM hydrogen peroxide. These cells were subjected to immunoprecipitation as described above, and further subjected to immunoblotting.

Immunoblotting

Samples were separated by SDS-PAGE using 7.5% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose sheets. The transferred sheets were incubated with mAbs or polyclonal antisera, followed by incubation with ^{125}I-labeled anti-mouse Ig, anti-rat Ig or Protein A (Amersham International, Amersham, UK) depending on the type of primary antibody. Radiolabeled electrophoretic bands were visualized by subjecting the transfers to autoradiography.

Results

Cadherin Expression and Activity in 3Y1 Cells and Their Transformants

To determine which types of cadherin are expressed in 3Y1 cells, their reactivity to antibodies to various cadherins was tested by immunoblot analysis. Antibodies to mouse P-cadherin recognized a single 120-kD band in the blots of lysates of these cells (Fig. 1, lane *1*). An antiserum raised against the cytoplasmic domain of E-cadherin, which is known to recognize multiple types of cadherin, also showed a single 120-kD band. Other anti-cadherin antibodies did not react with 3Y1 cells. P-cadherin, therefore, seems to be a major cadherin expressed in this cell line. Cells of the 3Y1 line transformed with v-*src* (SR3Y1) or double transformed with v-*src* and v-*fos* (fosSR3Y1) expressed similar amounts of P-cadherin (Fig. 1, lanes *2* and *3*). We also found that these three cell lines expressed similar amounts of α-catenin, a cadherin-associated protein (Fig. 1, lanes *4–6*).

The cell-binding activity of cadherins expressed in these cell lines was assayed. Cell lysates were dispersed into single cells by treatment with trypsin in the presence of Ca^{2+} (TC-treatment), which is known to leave cadherins intact but to temporally remove all other cell–cell adhesion molecules (Takeichi, 1977, 1988), and they were allowed to reaggregate for 30 min on a gyratory shaker. Cells of all the lines aggregated in the presence of 1 mM Ca^{2+}, but did not aggregate in the absence of this ion (Fig. 2 *A*, ■). Anti-P-cadherin antibody partly inhibited the Ca^{2+}-dependent aggregation of all of these cells (Fig. 2 *A*, ■). The incomplete inhibitory effect of this antibody is probably due to either its insufficient titer or the presence of other cadherins which were not detected in the above immunoblot analysis. The transformed lines tended to show lower aggregation rates than the normal line, but this tendency fluctuated from experiment to experiment. Between the two transformed lines, there was no difference in the aggregation rate.

When these cells were incubated overnight in DH10 medium under suspension conditions on a gyratory shaker, normal 3Y1 cells formed compacted aggregates with smooth surfaces (Fig. 2 *B*, *a*). When the antiserum specific to P-cadherin was added to these cultures, cells tended to become rounded (Fig. 2 *B*, *b*), although not dispersed probably because of the incomplete inhibitory effect of this antiserum (see above). Decompaction was also induced by transferring

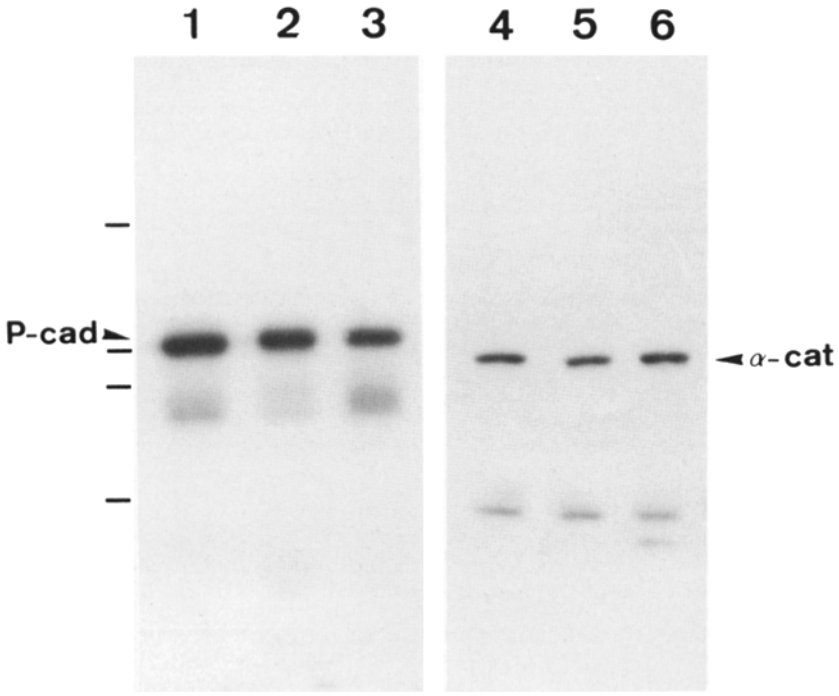

Figure 1. Immunoblot detection of P-cadherin and α catenin. Lanes *1* and *4*, 3Y1; lanes *2* and *5*, SR3Y1; lanes *3* and *6*, fosSR3Y1. Lower molecular weight bands are degradation products, or proteins with which the antibodies reacted nonspecifically. P-cad, P-cadherin; α-cat, α catenin. Molecular weight markers of 200, 116, 97.4, and 66.2 kD are indicated by lines at left.

these aggregates in a Ca^{2+}-free medium (data not shown). These results suggest that P-cadherin and other unidentified cadherins, if present, is involved in compacting 3Y1 cell aggregates. SR3Y1 and fosSR3Y1 cells aggregated much more loosely than normal cells. Cells in those aggregates were round and the outlines of the cells could easily be seen (Fig. 2 *B*, *c* and *d*). Anti–P-cadherin antiserum as well as Ca^{2+} removal had no obvious effect on their morphology. These results suggest that the transformed cells cannot use cadherins for their compaction, although they can use them for their initial aggregation, as shown above.

Colony Morphology in Collagen Gels

To assess the invasiveness of these cells, we cultured them in collagen gels. When they were embedded in collagen gels at a low density, each cell grew into a three-dimensional colony. Morphology of the colonies was, however, different among the cell lines. Normal 3Y1 cells gave rise to colonies with a dendritic morphology and with smooth surfaces, in which the cells appeared to be tightly associated with each other (Fig. 3 *a*). Cells rarely migrated out of the colonies. SR3Y1 also formed dendritic colonies. In these colonies, however, the outlines of individual cells could be seen, suggesting that their associations are loose. Moreover, some cells were released from the main body of colonies and migrated out into the matrix (Fig. 3 *c*). fosSR3Y1 cells showed more dispersed colonies (Fig. 3 *d*). They consisted of scattered single cells or small cell clusters. To quantify these phenomena, we counted the number of single cells and cell clusters comprising each colony. The results of this analysis confirmed the above morphological observations (Fig. 4, ■). These differences among the cell lines were always found during their clonal growth. In summary, normal 3Y1 cells achieved firm intercellular adhesion, SR3Y1 cells were loosely associated with each other, and fosSR3Y1 cells scattered in collagen gels.

To examine how P-cadherin is involved in the formation of colonies in collagen gels, we tested the effect of anti–P-cadherin antiserum. When this antibody was added to the collagen gel cultures, 3Y1 colonies were dissociated into single cells or small cell clusters (Fig. 3 *b*) although preimmune serum had no effect. SR3Y1 and fosSR3Y1 colonies did not clearly respond to the antiserum, probably because their colonies were already dissociated. These results again indicate that P-cadherin is functioning in normal 3Y1 cells, but not effectively used by SR3Y1 and fosSR3Y1 cells.

The scattering behavior of the transformed cells might have been due to high migratory activity. To estimate such activity of these cell lines, cells were plated at low density onto coverslips coated with collagen and then with colloidal gold particles. Cells remove colloidal gold particles in the areas where they move, as reported previously (Albrecht-Buehler and Goldman, 1976). We measured such "clean" areas formed by a single cell or a pair of cells after certain culture periods; under these conditions, the effect of cell–cell contact on cell mobility was minimized. The results of this assay showed that the transformed cells migrated to produce only slightly wider areas than the normal cells (Fig. 5). When the two transformed lines were compared, SR3Y1 migrated more actively than fosSR3Y1. Thus, the migratory activity of these cells did not correlate with their scattering activity.

All of the results described above were obtained using a clonal line of v-*src* transformed 3Y1 cells and its derivatives. To establish the generality of these observations, we performed the same series of experiments using two other independent clones of v-*src* transformed 3Y1 cells and obtained essentially the same results (data not shown).

Effect of Overexpression of E-cadherin

The above results suggest that P-cadherin cannot maintain stable contacts between the transformed cells, in particular

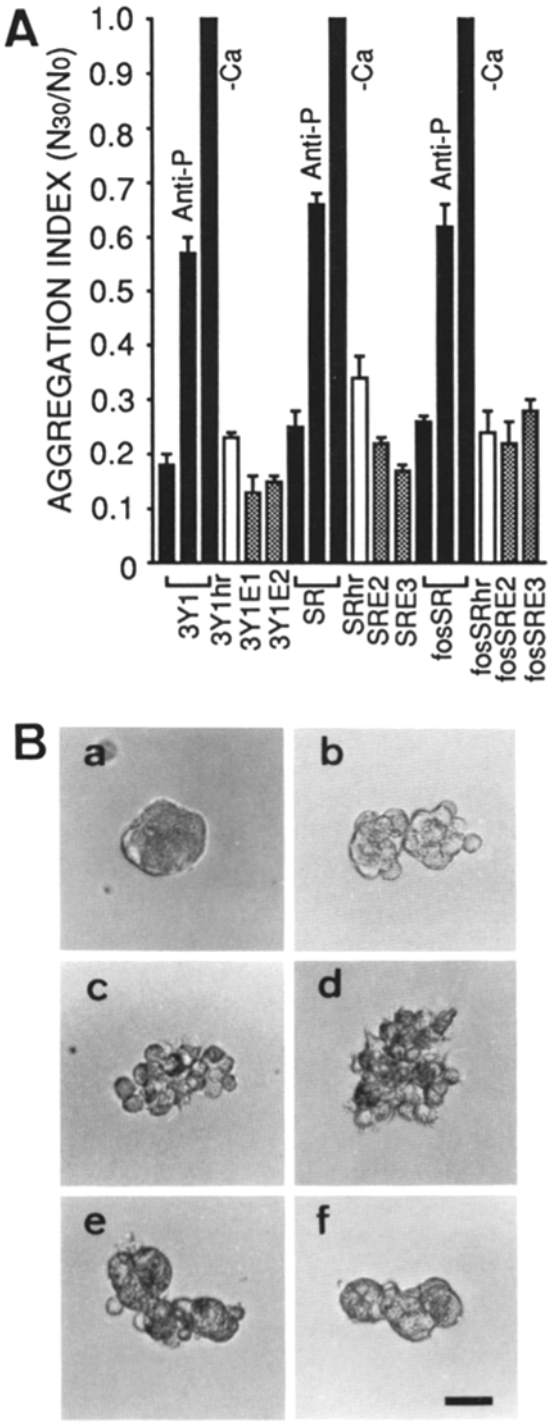

Figure 2. Aggregation of cells. (*A*) Aggregation of TC-treated 3Y1, SR3Y1 (*SR*) and fosSR3Y1 (*fosSR*) cells. Culture medium contained 1 mM $CaCl_2$, except in the cases shown as *−Ca*. *Anti-P* represents the medium that was supplemented with 500 μg/ml anti-P-cadherin antibodies. (■) Untransfected parent lines; (□) control hygromycin-resistant lines without E-cadherin cDNA; (▩) E-cadherin-transfected lines. Values represent the mean of 6 wells with SE. (*B*) Cell aggregates cultured overnight in suspension. (*a*) 3Y1; (*b*) 3Y1 in the presence of anti-P-cadherin antiserum diluted 1 to 100, containing ~100 μg/ml antibodies; (*c*) SR3Y1; (*d*) fosSR3Y1; (*e*) SR3Y1 incubated with herbimycin A; and (*f*) fosSR3Y1 incubated with herbimycin A. Bar, 50 μm.

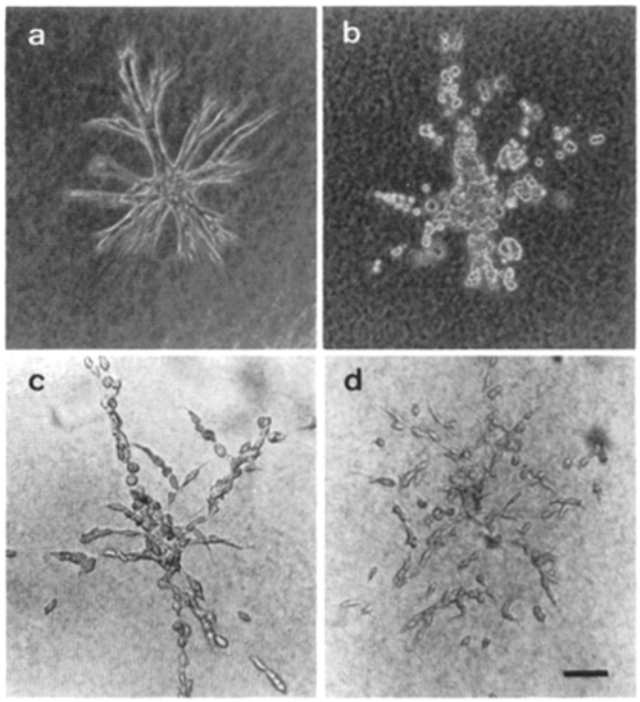

Figure 3. Cell colonies in collagen gels. (*a*) A colony of 3Y1 cells cultured for 13 d. (*b*) The same colony as in *a* was incubated for 24 h in the presence of anti-P-cadherin antiserum. (*c*) A colony of SR3Y1 cells. (*d*) A colony of fosSR3Y1 cells. The transformed lines were cultured for 7 d. Bar, 100 μm.

between fosSR3Y1 cells. We then examined whether stable cadherin-mediated contacts can be restored by overexpression of exogenous cadherins in the transformed cells. Cells of each line were transfected with mouse E-cadherin cDNA, and clones expressing exogenous E-cadherin were isolated. These clones included 3Y1E1 and 3Y1E2 obtained from 3Y1 cells, SRE1, SRE2, and SRE3 from SR3Y1 cells, and fosSRE1, fosSRE2, and fosSRE3 from fosSR3Y1 cells. Transfected lines with the hygromycin-resistant gene but without E-cadherin cDNA were also isolated as controls, designated as 3Y1hr, SRhr, and fosSRhr for each parent line. The use of E-cadherin is of advantage in distinguishing between the exogenous and endogenous cadherins, since these lines normally lack this particular cadherin.

Fig. 6 shows an immunoblot analysis of the expression of exogenous E-cadherin and endogenous P-cadherin in the parent and transfected lines. All the E-cadherin cDNA transfected lines shown in the figure expressed considerable amounts of this molecule together with endogenous P-cadherin. It was observed, however, that the expression of exogenous E-cadherin affected that of endogenous P-cadherin in some lines. P-cadherin expression tended to be suppressed and its band on immunoblot became a doublet if large amounts of E-cadherin were expressed (for example, lanes SRE1 and fosSRE1 in Fig. 6). These results suggest that some interference occurs between the expression of these two proteins.

Exogenous E-cadherin was expressed on the surface of the transfected lines, as judged from the observations that the Ca^{2+}-dependent aggregation of all these E-cadherin transfectants was partially inhibited by anti-E-cadherin antibod-

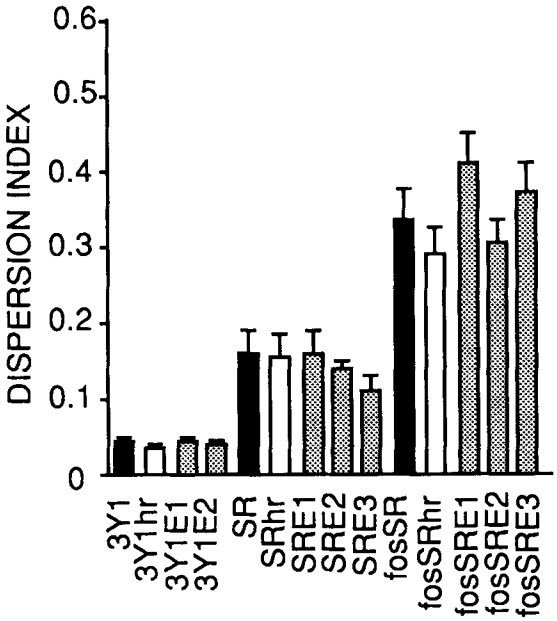

Figure 4. Dispersion of 3Y1, SR3Y1 (*SR*), and fosSR3Y1 (*fosSR*) cells in collagen gels. (■) Untransfected lines; (□) control hygromycin-resistant lines without E-cadherin cDNA; (▨) E-cadherin-transfected lines. Values represent the mean of 20 colonies with SE. See Materials and Methods for definition of the dispersion index.

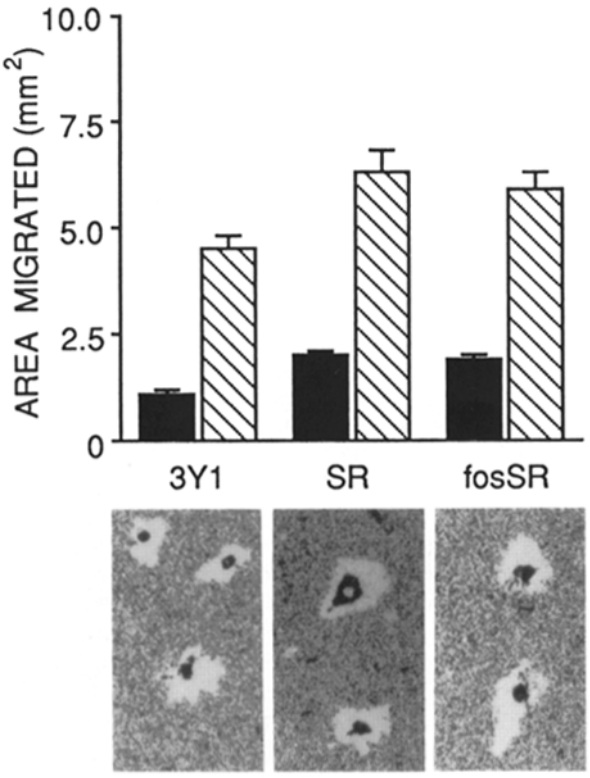

Figure 5. Migration of cells on collagen- and colloidal gold-coated dishes. (■) Cells cultured for 12 h, and the areas where single cells had migrated were measured. (▨) cells cultured for 24 h, and the areas where pairs of divided cells had migrated were measured. Photographs show examples of these cultures. Values represent the mean of 50 areas with SE. (*SR*) SR3Y1; (*fosSR*) fosSR3Y1.

ies (data not shown). Cadherin-mediated aggregation was, however, not significantly enhanced by E-cadherin expression (Fig. 2, ▨). These E-cadherin-transfected normal and v-*src* transformed cells were cultured under suspended conditions overnight. Their aggregates showed essentially the same morphology as the parental cells' aggregates. These cells were also cultured in collagen gels. The expression of exogenous E-cadherin, however, did not alter the dispersing nature of the v-*src* transformed lines (Fig. 4, ▨). Thus, the addition of exogenous E-cadherin had little effect on the adhesive properties of these cells, suggesting that the activity of cadherins is already as high as possible in the parental cell lines.

Immunofluorescent Localization of Cadherins

On plastic culture dishes, 3Y1 cells formed contact-inhibited monolayers, in which the cells were closely associated with each other. In contrast, SR3Y1 and fosSR3Y1 formed more dispersed colonies. E-cadherin-transfected cells showed the same morphology as the corresponding parental lines (Fig. 7, *a–c*).

Monolayer cultures of these cell lines were immunostained for E- or P-cadherin. These molecules were concentrated at cell-cell contact sites in all of the cell lines examined, but their staining patterns differed with the lines. Normal 3Y1 or its E-cadherin transfectants showed complex immunoreactive cell-cell boundaries. Their edges in the cell-cell contact sites overlapped with fine processes or filopodia, and the overlapping zones of cell surface exhibited strong P- or E-cadherin staining (Fig. 7 *d*). SR3Y1 or its E-cadherin transfectants also showed irregular immunoreactive cell-cell boundaries with overlapping fine processes, but the overall pattern of staining was less complex than in normal 3Y1 cells (Fig. 7 *e*). The morphology of cell-cell contacts in fosSR3Y1 or its E-cadherin transfectants was the simplest; immunoreactive lines that demarcate the apposed cells were sharp and smooth, and there was no overlapping of cell edges (Fig. 7 *f*). These observations suggest that normal 3Y1 cells can establish the largest cell-cell contact areas via multiple cell-cell contacts, and such areas are reduced in SR3Y1 and especially in fosSR3Y1 cells. We also found a tendency that more cadherin molecules distribute on free cell edges in the transformed cells.

Effect of Herbimycin A and Vanadate on Cell Aggregation

To examine whether the peculiar adhesive properties of v-*src* transformed 3Y1 cells are linked with the action of the $p60^{v\text{-}src}$ tyrosine kinase, we tested the effect of herbimycin A, a specific inhibitor of tyrosine kinases (Uehara et al., 1988, 1989; Murakami et al., 1988), on cadherin function. SR3Y1 and fosSR3Y1 cells which had been dispersed were cultured overnight in suspension in the presence of 0.2 mg/ml herbimycin A, under the same conditions as described for Fig. 2 *B*, *c* and *d*. These cells aggregated normally during the incubation, but the morphology of the resultant aggregates was quite distinct from that of the aggregates formed in the absence of herbimycin A. This antibiotic induced compaction in the v-*src* transformed 3Y1 cell aggregates (Fig. 2 *B*, *e* and *f*), suggesting that cadherins now can normally function in these cells.

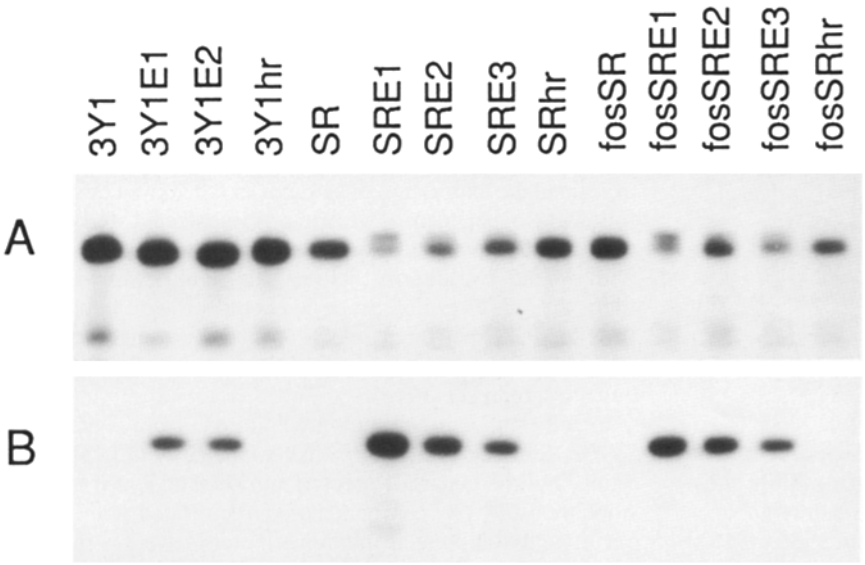

Figure 6. Immunoblot detection of cadherins in cells transfected with E-cadherin cDNA. (*A*) Endogenous P-cadherin. (*B*) Exogenous E-cadherin. Total proteins in each sample were adjusted to be equal for all lanes. (*SR*) SR3Y1; (*fosSR*) fos-SR3Y1. *E1*, *E2*, and *E3* represent clones with exogenous E-cadherin, and *hr* represents hygromycin-resistant clones without E-cadherin.

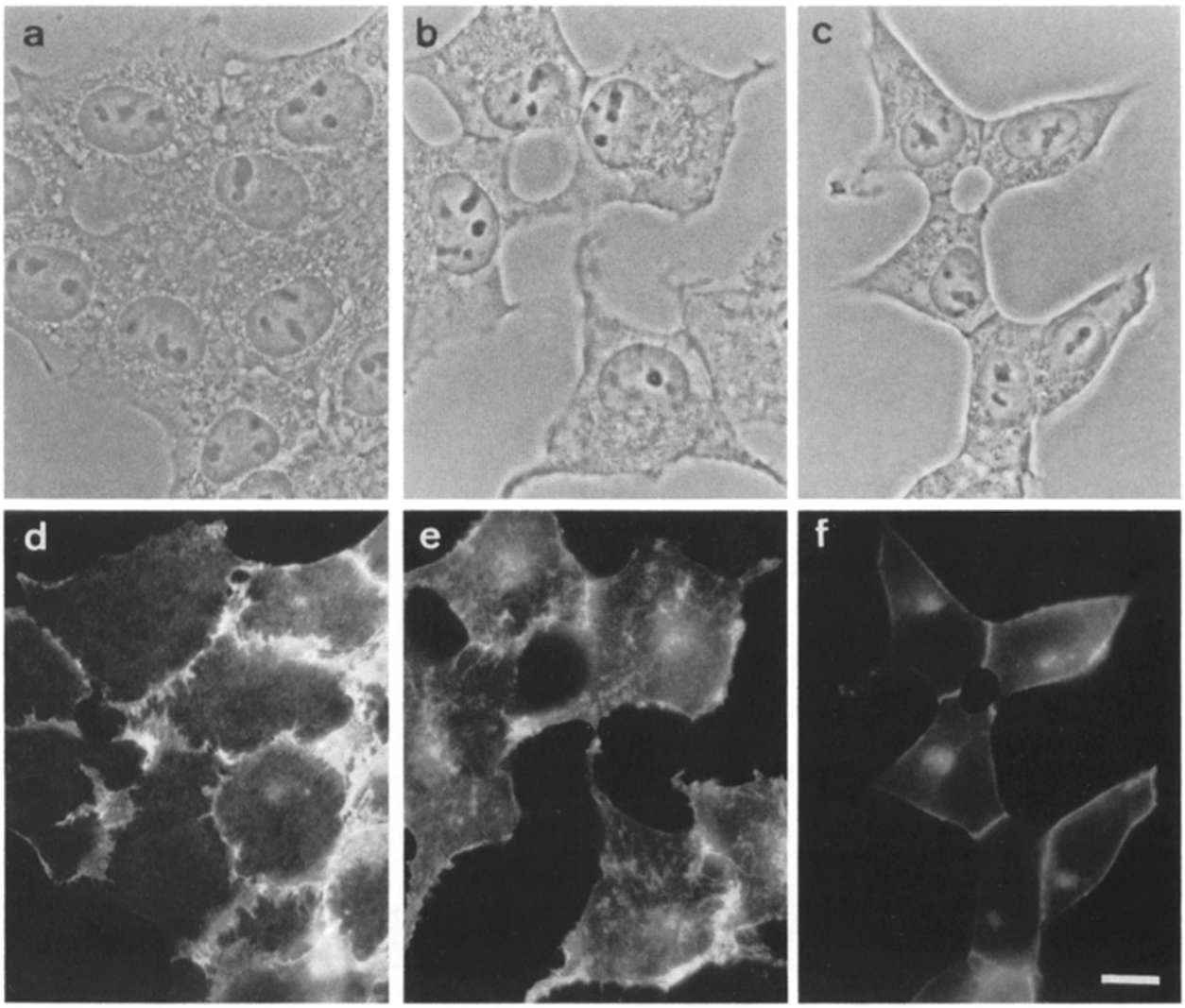

Figure 7. Immunofluorescent staining for E-cadherin. (*a* and *d*) Normal 3Y1E2 cells. (*b* and *e*) SRE2 cells. (*c* and *f*) fosSRE2 cells. (*a*, *b*, and *c*) Phase-contrast microphotographs. (*d*, *e*, and *f*) Immunofluorescence microphotographs of the same fields as in *a*, *b*, and *c*. Similar results were obtained by staining for endogenous P-cadherin. Bars, 10 μm.

Table I. Effect of Vanadate on Cadherin-mediated Cell Aggregation

Concentration of vanadate	Percent inhibition of aggregation*		
	3Y1	SR3Y1	fosSR3Y1
mM			
0.2	6.8 ± 1.8	56.4 ± 2.7	74.7 ± 4.1
0.5	10.3 ± 3.7	74.8 ± 5.2	84.1 ± 1.3

* Cells were preincubated in DH10 medium containing vanadate for 2 h, dissociated by TC-treatment and allowed to aggregate for 30 min in the absence of vanadate. After obtaining the aggregation index N_{30}/N_0, percent inhibition of aggregation was calculated as described by Urushihara et al. (1979). Values are the means of triplicate cultures with SE.

Next, we designed an opposite type of experiment. The treatment of cells with vanadate is known to inhibit phosphotyrosyl protein phosphatases, and consequently enhance tyrosine phosphorylation (Brown and Gordon, 1986). To test the effect of this reagent on cadherin activity, normal and v-*src*-transformed 3Y1 cells in monolayer cultures were treated with 0.2–0.5 mM vanadate in DME for 2 h, dissociated by TC-treatment, and used for cell aggregation assay. As summarized in Table I while this pretreatment of cells showed only small effects on cadherin-mediated aggregation of normal 3Y1 cells, it strongly inhibited that of SR3Y1 and fosSR3Y1 cells; stronger effects were observed for fosSR3Y1. Thus, the enhancement of tyrosine phosphorylation led to a more severe perturbation of cadherin function.

The effect of herbimycin A and vanadate on the invasiveness of these cells could not be tested, since these reagents were slightly toxic to 3Y1 cells and their transformants in long-term cultures.

Tyrosine Phosphorylation of Catenins and Cadherins

Cadherin-mediated cell adhesion is thought to be regulated by a group of cytoplasmic proteins called catenins. We thus examined the expression of catenins in the above cells. In the following experiments, we used untransfected and E-cadherin–transfected lines for analyzing catenins associated with P- and E-cadherin, respectively, and obtained essentially the same results. Since we could provide better controls for the experiments of E-cadherin by using its mutant molecule, the results obtained with this cadherin are shown below as representatives.

E-cadherin was immunoprecipitated from lysates of 3Y1E2, SR3E2, and fosSRE2 cells labeled with [^{35}S]methionine, and the materials precipitated were analyzed by electrophoresis. A few components coprecipitated with E-cadherin, and their electrophoretic patterns were similar in normal and transformed cells (Fig. 8 *A*). Of the coprecipitated materials, the major 102- and 98-kD bands probably correspond to catenin α and β, respectively, as inferred from their molecular weight (Ozawa et al., 1989). To confirm this identification, we used an antiserum against catenin α, and found that it reacted with a 102-kD band in the immunoprecipitates, not only in normal 3Y1E2 cells but also in the transformed lines (Fig. 8 *B* and also see Fig. 1, lanes *4–6*). We obtained essentially the same results by immunoprecipitating P-cadherin from untransfected lines. Thus, we could not detect significant differences in catenin expression among the cell lines used.

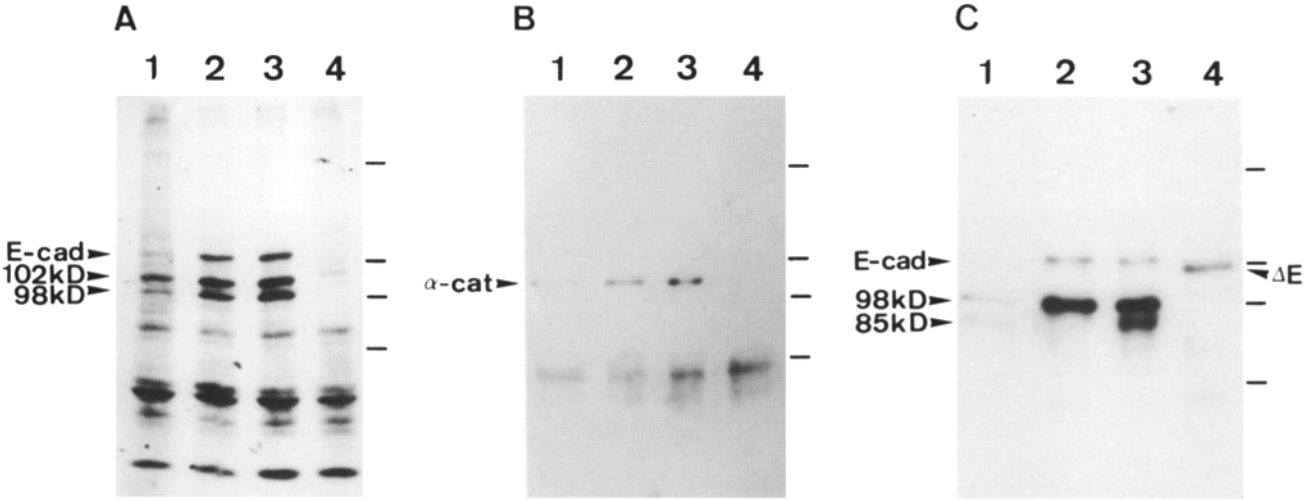

Figure 8. Electrophoretic detection of cadherin-associated proteins. (*A*) Fluorography of ^{35}S-labeled materials coprecipitated with E-cadherin from 3Y1E2 (lane *1*), SRE2 (lane *2*), and fosSRE2 (lane *3*). Lane *4* shows materials which nonspecifically bound to the immunoadsorbents in the absence of anti–E-cadherin antibody. Immunoprecipitates for each lane were collected from the same number of cells. (*B*) Immunoblot detection of α catenin in the E-cadherin–catenin complexes. Nonradiolabeled E-cadherin–catenin complexes were immunoprecipitated, transferred onto a nitrocellulose filter, and subjected to immunoblotting. Lanes are the same as in *A*, except lane *4* which contains materials coprecipitated with a truncated form of E-cadherin whose catenin-binding sites were deleted. The cells line with this mutant E-cadherin, SRΔE, was obtained by transfecting SR3Y1 cells with pBATEM21. Note no α catenin on this lane. Lower molecular weight bands represent nonspecific reactions of the antibody. (*C*) Immunoblot detection of phosphotyrosine in the E-cadherin–catenin complexes using PY-20 antibody. Lane *1*, 3Y1E2; lane *2*, SRE2; lane *3*, fosSRE2; lane *4*, SRΔE expressing the truncated E-cadherin without the catenin-binding sites. In *C*, the amount of proteins loaded was so adjusted that each lane contains the same amount of E-cadherin. (*E-cad*) E-cadherin; (ΔE) the truncated E-cadherin; (α-cat) α catenin. Molecular weight markers of 200, 116, 97.4 and 66.2 kD are indicated by bars at right of each panel.

We next examined if cadherins and catenins are phosphorylated at tyrosine residues in these cells, using the same E-cadherin transfected lines as above. To detect tyrosine phosphorylation with a high sensitivity, cells were preincubated with 1 mM vanadate and 3 mM hydrogen peroxide for 20 min for inhibiting phosphatases, as recommended by Volberg et al. (1991), and then E-cadherin–catenin complexes were immunoprecipitated, blotted, and examined for reactivity to mAb PY-20 that specifically recognized phosphotyrosine (Fig. 8 *c*). In normal cells, the PY-20 antibody recognized two faint bands of 98 and 85 kD. In SRE2 and fosSRE2 cells, however, the 98-kD band very strongly reacted with the antibody. This 98-kD band is probably β-catenin, because it comigrated with a band recognized by anti-*Drosophila armadillo* antibody which was recently shown to cross react with this catenin (Peifer et al., 1992). The 85-kD band also intensely reacted with the PY-20 antibody in fosSRE2. Furthermore, E-cadherin weakly reacted with this antibody in both SRE2 and fosSRE2. As a control, materials coprecipitating with a mutated E-cadherin whose catenin-binding sites are deleted were also examined (Fig. 8 *C*, lane *4*). With this molecule, no positive bands were detected at the putative positions for catenins, confirming that the above bands are cadherin-associated proteins. In addition, we found that this deleted E-cadherin more strongly reacted with the antibody to phosphotyrosine than normal E-cadherin. To confirm that the PY-20 antibody indeed recognized phosphotyrosine in the above immunoblotting, we tested the effect of free phosphotyrosine added to the antibody solution. 5 mM phosphotyrosine completely inhibited the reaction of PY-20 antibody to the above bands, whereas the same concentration of phosphoserine and phosphothreonine had no effect.

We then tested whether the preincubation of cells with vanadate, that was shown to suppress cadherin action (Table I), affects tyrosine phosphorylation on cadherin and catenins. SRE2 and fosSRE2 cells were treated for 2 h with or without 0.5 mM vanadate under the same conditions that its effect on cadherin activity was assayed; hydrogen peroxide was not included in the present test solution. As shown in Fig. 9 *A*, the PY-20 antibody did not react with the E-cadherin–catenin complex isolated from the cells not treated with vanadate, whereas it recognized the 98-kD catenin and also weakly detected the 85-kD catenin in the cells incubated with vanadate.

We also tested the effect of herbimycin A. SRE2 and fosSRE2 cells were incubated overnight with or without 0.2 μg herbimycin A. The results showed that this antibiotic abolished tyrosine phosphorylation of catenins (Fig. 9 *B*). Less tyrosine phosphorylation in these samples as compared with those in Fig. 8 *C* is probably due to the absence of hydrogen peroxide in the solutions used for cell treatments.

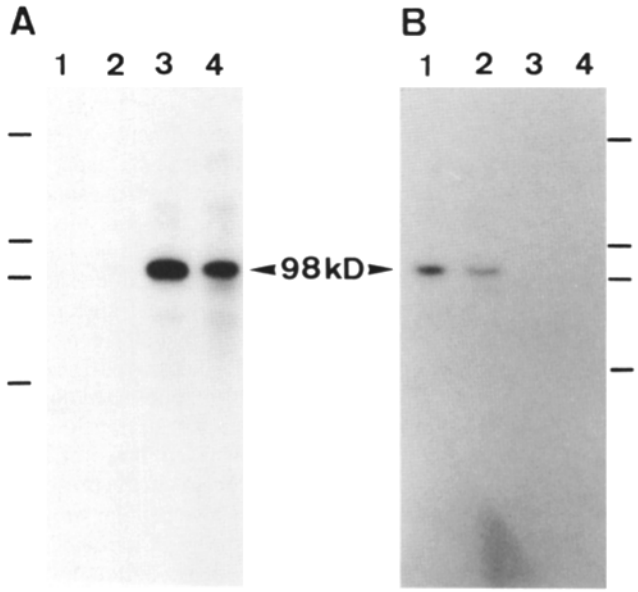

Figure 9. Effect of vanadate and herbimycin A on tyrosine phosphorylation of the E-cadherin–catenin complexes. E-cadherin immunoprecipitates were collected from cells after the following treatments and analyzed by immunoblotting using PY-20 antibody. (*A*) Cells were incubated without (lanes *1* and *2*) or with (lanes *3* and *4*) 0.5 mM vanadate for 2 h. (*B*) Cells were incubated overnight without (lanes *1* and *2*) or with (lanes *3* and *4*) 0.2 μg herbimycin A. Before harvesting the cells, 0.5 mM vanadate was added and incubated for 2 h. Lanes *1* and *3*, SRE2; lanes *2* and 4, fosSRE2. Molecular weight markers of 200, 116, 97.4, and 66.2 kD are indicated by bars to the side of each panel.

Discussion

Generally, cells are tightly associated with each other when cadherins are active, but their associations are loosened if these adhesion molecules are inactivated or removed (Takeichi, 1991). Such phenomena were in fact observed with normal 3Y1 cells in the present study. They formed compact aggregates, but this compacted state was lost by the treatment with antibodies to P-cadherin. The adhesive properties of v-*src* transformed 3Y1 cell lines were, however, unusual. Even though these cells expressed P-cadherin, they could not form compact aggregates, and moreover, their colonies were spontaneously dissociated in collagen gel cultures. This tendency was enhanced by introduction of the v-*fos* gene into these cells. The expression of exogenous E-cadherin in these v-*src* transformed cells via cDNA transfection produced no effects on their invasive behavior. This finding is in contrast with the results using other cell lines such as L and carcinoma cells. L cells that have no endogenous cadherin grow as dispersed colonies, but they form nondispersed colonies when transfected with cadherin cDNAs (Nagafuchi et al., 1987; Hatta et al., 1988; McNeill et al., 1990; Chen and Obrink, 1991), and certain lines of invasive tumor cells lose their invasiveness when transfected with E-cadherin cDNA (Frixen et al., 1991; Vleminckx et al., 1991). These observations imply that v-*src* and v-*src*/v-*fos* transformed 3Y1 cells are provided with some mechanism that interferes with cadherin function.

There might be several factors that could theoretically perturb cadherin-mediated cell–cell contacts, four examples of which follow. First, if cells are highly mobile, they might disrupt their intercellular contacts by the force of movement. It was shown that fosSR3Y1 cells change their shape more frequently than SR3Y1 cells (Taniguchi et al., 1989). Our assay of the migration of these cell lines, however, showed no correlation between the mobility and scattering abilities of cells. Second, higher adhesiveness of cells to substrates might facilitate cell dispersion. A previous study, however, showed that SR3Y1 and fosSR3Y1 are similar in their ability

to attach to Matrigel, collagen, and laminin (Taniguchi et al., 1989). Third, the colonies of fosSR3Y1 cells are morphologically reminiscent of those of cells treated with "scatter factor" (Stoker et al., 1987), whose receptor is now known to be the *met* tyrosine kinase. A recent report describes the data that scatter factor induces cell dispersion without affecting cadherin expression or overall cadherin phosphorylation (Weidner et al., 1990). These results are similar to the present findings. However, we could not obtain any evidence that the transformed 3Y1 cells produce such a factor; that is, culture supernatants of v-*src*-transformed cells had no effect on morphology of normal 3Y1 cells and also MDCK cells which are known to be sensitive to scatter factor (N. Matsuyoshi and M. Takeichi, unpublished observations). Fourth, if proteases are more active in transformed cells, they might digest cadherins. We showed, however, that scattering of the transformed 3Y1 cells occurs in the presence of intact cadherins. Thus, none of the above cellular properties or factors seem to play a primary role in inducing the dispersion of v-*src*-transformed 3Y1 cells, although they might be indirectly involved in such a process.

We, then, have to consider the possibility that the cadherin-mediated adhesion mechanism itself is impaired by v-*src* and v-*src*/v-*fos* transformation. v-*src* transformed 3Y1 cells can aggregate via cadherins as normal cells do in suspension cultures, indicating that these molecules can normally function for initial cell aggregations. Morphological observations, however, suggest that the cadherin adhesion system is not entirely normal in the transformed cells. In aggregation cultures, normal 3Y1 cells spread onto other cells after their initial attachment, forming compact aggregates. This spreading is mediated by cadherins, because antibodies to them induced decompaction and rounding up of the cells. In contrast, v-*src*-transformed cells never formed such compact aggregates. Immunostaining also demonstrated that normal 3Y1 cells form complex cell–cell junctions with their cadherin-positive processes, but fosSR3Y1 cells did not show such structures; their intercellular adhesions were mediated by the simple apposition of smooth cell surfaces. SR3Y1 cells exhibited an intermediate morphology.

The rounded shape of v-*src* transformed cells in their aggregates is reminiscent of the cell morphology observed when cells assemble with Ca^{2+}-independent cell–cell adhesion molecules, such as Ig superfamily adhesion molecules (Edelman and Crossin, 1991), under conditions where cadherins are inactivated, as shown in this and other studies (Takeichi, 1977; Atsumi and Takeichi, 1980; Ogou et al., 1982; Shirayoshi et al., 1983; Yoshida-Noro et al., 1984). These observations suggest that the cadherin adhesion system generally induces the spreading of cells onto other cells, which allows cells to maximize their cell–cell contact area, but this mechanism does not seem to operate in v-*src*-transformed 3Y1 cells. The results of the effect of two reagents, herbimycin A and vanadate, on cell–cell adhesion strongly suggest that the abnormality in cadherin action in v-*src* transformed 3Y1 cells is somehow linked with intracellular tyrosine phosphorylation. The v-*src* transformed 3Y1 cells could form compacted aggregates, when treated with herbimycin A which specifically inhibits tyrosine kinases (Uehara et al., 1988, 1989; Murakami et al., 1988), suggesting that normal cadherin-dependent adhesion was restored by a reduction of tyrosine phosphorylation. Conversely, vanadate, which is known to enhance tyrosine phosphorylation (Brown and Gordon, 1986), inhibited cadherin-mediated aggregation of the v-*src* transformed cells. It is thus obvious that high levels of intracellular tyrosine phosphorylation interfere with cadherin action. Previous studies demonstrated that the AJ which are the sites for cadherin localization disappear in cells transformed with the v-*src* gene (Warren and Nelson, 1987; Volberg et al., 1991), suggesting that the cadherin adhesion system is structurally disrupted by over tyrosine phosphorylation. The cadherin system under such conditions may not be able to exert its full function, resulting in uncompacted and reversible cell–cell associations as observed in this study.

Cadherins are associated with cytoskeletal proteins, and this association seems to be mediated by catenins (Nagafuchi and Takeichi, 1989; Ozawa et al., 1990). Mutated cadherin molecules whose catenin-binding sites are deleted cannot bind to the cytoskeleton and concomitantly lose their cell binding function. This indicates that catenins play a central role in cadherin function. Interestingly, we found that some catenins and cadherins themselves can be tyrosine phosphorylated in v-*src*-transformed 3Y1 cells. Tyrosine phosphorylation of the 98-kD catenin was especially interesting, because it was induced when cells were treated with vanadate, and, on the contrary, it was abolished by herbimycin A treatment. Recently, we found that v-*src* transformation of chicken fibroblasts strongly inhibits N-cadherin action and also induces tyrosine phosphorylation of catenins and N-cadherin (M. Hamaguchi, N. Matsuyoshi, and M. Takeichi, unpublished results). Thus, there is a correlation between the suppression of cadherin function and the tyrosine-specific phosphorylation of catenins in v-*src* transformed cells. It is therefore possible that the tyrosine-specific phosphorylation of catenin–cadherin complexes themselves might be involved in the inhibition of this cell adhesion system. We cannot, however, exclude the possibility that tyrosine phosphorylation of other cellular components, such as vinculin (Sefton et al., 1981), is also involved in perturbing the cadherin system. It also should be noted that tyrosine phosphorylation of catenins and cadherins was detected only in the presence of vanadate in the present study. This finding suggests that phosphatases rapidly dephosphorylate these proteins under physiological conditions. A dynamic balance of phosphorylation/dephosphorylation, thus, may play a role in cadherin-mediated cell–cell interactions.

Cadherins and catenins incorporate phosphate into nontyrosine residues even in nontransformed cells (Lagunowich and Grunwald, 1991; M. Hamaguchi, N. Matsuyoshi and M. Takeichi, unpublished results), and such phosphorylation has not been correlated with the dissociation of epithelial colonies induced by scatter factor (Weidner et al., 1990). Therefore, the phosphorylation of nontyrosine residues does not seem to be associated with cadherin inhibition. The role of v-*fos* in inducing increased tyrosine-specific phosphorylation, found in the present study, is unknown. It would be interesting to elucidate in the future how the expression of v-*fos* results in such enhancement of tyrosine phosphorylation.

We found other interesting phenomena in the present study, such as a competition between exogenous and endogenous cadherin expressions, and an increased tyrosine-specific phosphorylation of E-cadherin whose catenin-binding sites were deleted. The former implies that there is

a regulatory mechanism to maintain a certain level of cadherin expression in a cell. In which step does such regulation take place during the expression of cadherin molecules? We detected a larger size of endogenous cadherin when exogenous cadherin is overexpressed. This would be a precursor cadherin molecule which has remained unprocessed, suggesting that some posttranslational regulation exists to prevent cells from overexpressing the mature cadherin polypeptides. The increased tyrosine phosphorylation on the E-cadherin with a deletion of the catenin-binding site suggests that phosphorylation is regulated by interactions among different regions of the cytoplasmic domain of this molecule.

Many of cadherin-positive human carcinoma cells can metastasize (Shimoyama and Hirohashi, 1991*a*,*b*). It is possible that the same mechanism as found in this study may operate in such human carcinoma and enhance their detachment from primary tumor sites. Thus, there would be at least two ways to inhibit cadherin-mediated cell adhesion in tumors, downregulation of cadherin expression and biochemical suppression of the action of cadherins or their associated proteins. Both need to be taken into account when we consider the possible involvement of cadherins in tumor invasion and metastasis.

We thank Professor Sadao Imamura for encouragement during this work. We also thank Drs. Y. Uehara for herbimycin A, M. Peifer for anti-*armadillo* antibody, H. Yamagishi for the image analyzer, K. M. Yamada for critical comments on the manuscript, and Shinji Hirano and Masayuki Hatta for technical advice.

This work was supported by research grants from the Ministry of Education, Science, and Culture of Japan.

Received for publication 3 March 1992 and in revised form 28 April 1992.

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