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p60$^{v\text{-}src}$ causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system

Michinari Hamaguchi[3], Norihisa Matsuyoshi[1], Yukano Ohnishi, Bin Gotoh, Masatoshi Takeichi[2] and Yoshiyuki Nagai

Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Tsurumai-cho 65, Shouwa-ku, Nagoya 466, [1]Department of Dermatology, Faculty of Medicine and [2]Department of Biophysics, Faculty of Science, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-11, Japan

[3]Corresponding author

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Transformation of chick embryonic fibroblasts with Rous sarcoma virus strongly suppresses N-cadherin-mediated cell-cell adhesion, without inhibiting its expression. This suppression is correlated with tyrosine phosphorylation of N-cadherin and catenins, the cadherin-associated proteins, which are known to regulate cadherin function. Experiments with non-myristylation and temperature-sensitive mutants of RSV and with herbimycin A, a potent inhibitor of tyrosine kinases, suggest that both the suppression of cell adhesion and tyrosine phosphorylation of catenins are highly transformation-specific.

Key words: cadherin/catenin/p60$^{v\text{-}src}$/transformation/tyrosine phosphorylation

Introduction

Studies on cell-cell interaction in oncogenesis are important for clarifying the molecular mechanism of not only neoplastic transformation, but also tumour invasion and metastasis. Stable association between cells in vertebrate tissues is established by a family of Ca^{2+}-dependent homophilic adhesion molecules, cadherins (Takeichi, 1991). While the inactivation of cadherins with antibodies induces invasiveness in epithelial cells (Behrens *et al.*, 1989), the transfection of invasive carcinoma with cadherin cDNAs suppresses their invasive activity (Frixen *et al.*, 1991; Vleminckx *et al.*, 1991). Moreover, down-regulation of cadherin expression was observed in human invasive carcinoma (Shimoyama and Hirohashi, 1991a,b; Shiozaki *et al.*, 1991). All these observations suggest that the suppression of cadherin function may facilitate tumour invasion and metastasis.

To analyse directly the effect of oncogene expression on cadherin-mediated cell adhesion, we have examined cadherin activity in Rous sarcoma virus (RSV)-transformed cells. Cell transformation by RSV is mediated by the v-*src* gene product, p60$^{v\text{-}src}$, a membrane-bound tyrosine protein kinase (Collet and Erikson, 1978; Levinson *et al.*, 1978) that is associated with cytoskeletal structures (Burr *et al.*, 1978; Hamaguchi and Hanafusa, 1987). Non-myristylated mutants of p60$^{v\text{-}src}$ such as NY314, which are active in protein kinase but defective in membrane association, were found to lack transforming activity (Cross *et al.*, 1984; Kamps *et al.*, 1985), suggesting that the key substrates for cell transformation can only be accessible in the vicinity of plasma membrane. p60$^{v\text{-}src}$ is further known to perturb intercellular contacts and communication (Atkinson *et al.*, 1981; Azarnia and Loewenstein, 1987; Azarnia *et al.*, 1988) and to induce a highly metastatic phenotype (Egan *et al.*, 1987). Given these findings, we postulate that cadherins could be functionally modified by v-*src* transformation. Here, we show that RSV transformation strongly suppresses N-cadherin-mediated cell adhesion by inducing tyrosine-specific phosphorylation of N-cadherin and its associated proteins, catenins, in a transformation-specific manner.

Results

Cadherin-mediated aggregation of normal and RSV-transformed cells

We compared the cadherin-mediated aggregation of normal chicken embryonic fibroblasts (CEF) with that of CEF that had been transformed with RSV or infected with non-myristylation RSV mutant NY314 (Cross *et al.*, 1984). Monolayer cultures of these cells were dissociated by treatment with trypsin in the presence of $CaCl_2$ (TC-treatment), which is known to leave cadherins intact, but remove all other cell-cell adhesion molecules temporarily (Takeichi, 1977). The cells were then allowed to reaggregate. The results showed that the aggregation of CEF was strongly inhibited by RSV transformation but not by infection with NY314 (Figure 1A-C and Table I).

To compare directly the cadherin activity in RSV-transformed and uninfected CEF, these cells were dissociated by TC-treatment, then mixed and shaken to allow aggregation. The cells placed on adherent culture dishes were fixed and stained with anti-phosphotyrosine (PTYR) antibody (Hamiguchi *et al.*, 1988, 1990) to identify the RSV-transformed cells. While uninfected CEF were found to form clusters by homologous cell-cell interactions, RSV-transformed cells remained dispersed and were excluded from the uninfected CEF clusters (Figure 1D and E). As a control, the cells were treated with trypsin in the presence of EDTA (TE-treatment), which is known to temporarily remove all cadherins from the cell surface (Takeichi, 1977). Cells treated in this way did not show any segregated distribution when mixed (Figure 1F and G). These results confirm that cadherin activity is strongly inhibited in RSV-transformed cells.

Expression and subcellular localization of cadherin in RSV-transformed CEF

CEF express N-cadherin, as they react with the monoclonal antibody NCD-2 to this molecule (Hatta and Takeichi, 1986), giving a 127 kDa band in immunoblots (Figure 2A, lane 1), and N-cadherin seems to be the major cadherin type expressed in CEF, since their cadherin-mediated aggregation is almost completely inhibited by anti-N-cadherin

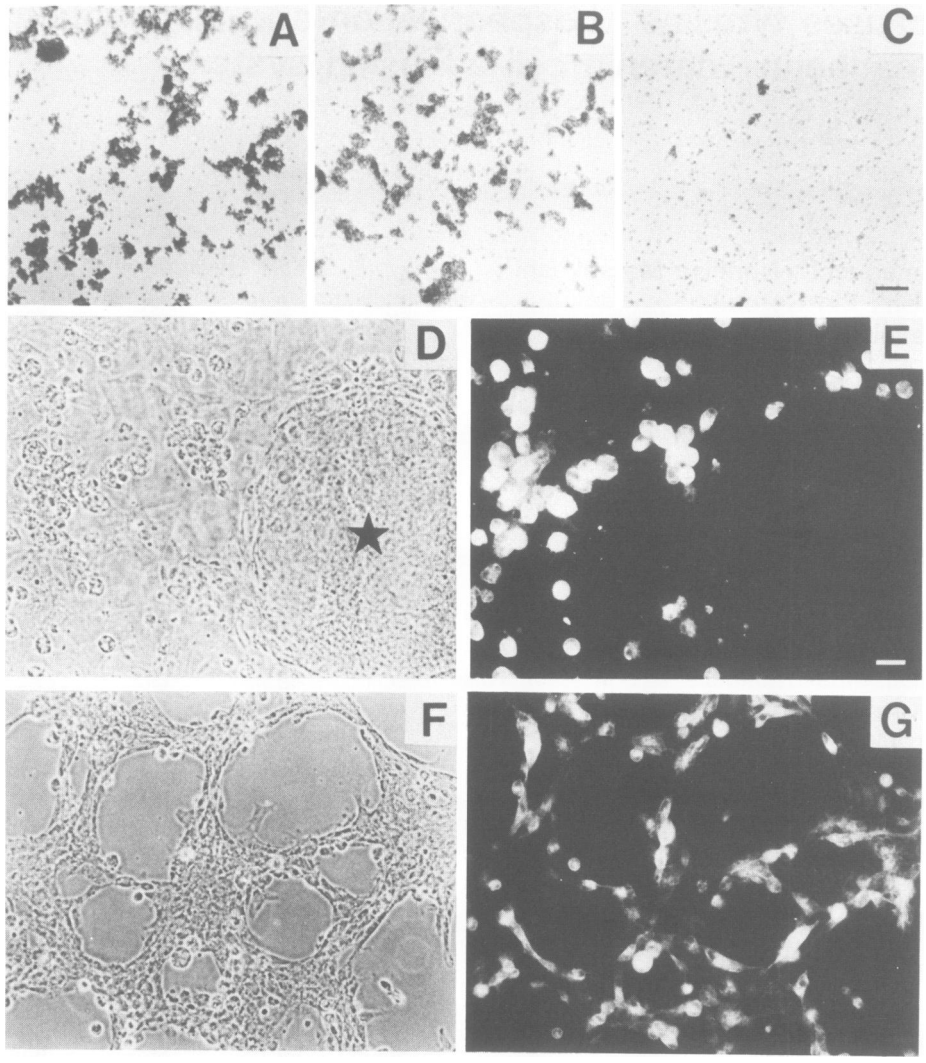

Fig. 1. Aggregation of normal and transformed CEF. (**A**, **B** and **C**) Monolayer cultures of CEF were dissociated into single cells by incubation with 0.01% trypsin plus 1 mM $CaCl_2$ for 20 min (TC-treatment) and allowed to reaggregate for 30 min in the presence of 1 mM $CaCl_2$, as described in Materials and methods. (A) Uninfected CEF; (B) NY314-infected CEF; (C) RSV-transformed CEF. (**D** and **E**) Uninfected and RSV-transformed CEF were dissociated by TC-treatment, mixed in a 1:1 ratio, allowed to aggregate for 60 min and then placed on dishes for an additional 60 min. Cells were then fixed and immunofluorescently stained with anti-PTYR antibodies. (**F** and **G**) Cells dissociated by TE-treatment to remove cadherins (Takeichi, 1977) were mixed and allowed to aggregate as above. D and F, phase-contrast micrographs; E and G, immunofluorescent micrographs of the corresponding fields: Note: anti-PTYR-positive RSV-transformed cells are excluded from a cluster of uninfected CEF (*) in D, but inter-mixed with them in F. Bars: 100 μm for A–C, 10 μm for D–G.

antibodies (data not shown). NY314-infected CEF also express this molecule (Figure 2A, lane 2). Interestingly, RSV-transformed cells expressed a slightly higher amount of N-cadherin than the others (Figure 2A, lane 3). TC-treated cells used in the above aggregation experiments showed the same pattern of N-cadherin expression as that of non-trypsinized cells (Figure 2B).

To study the subcellular localization of N-cadherin, plasma membrane fractions of normal and RSV-transformed CEF were purified. Both in uninfected and RSV-transformed CEF, N-cadherin was found predominantly in the light membrane fraction of sucrose gradients, which is known to be enriched with the plasma membrane (Krueger *et al.*, 1983; Hamaguchi and Hanafusa, 1989), suggesting that the association of N-cadherin with the plasma membrane is not affected by transformation (Figure 2C). Immunostaining using NCD-2 also showed that N-cadherin is expressed on the surface of all these cells (data not shown). These results suggest that N-cadherin is expressed on the surface of RSV-transformed cells, but cannot function normally.

Table I. Cadherin-mediated aggregation of CEF and the transformants

Experiment no.	Aggregation (N_{30}/N_0)		
	Uninfected CEF	NY314-infected CEF	RSV-infected CEF
1	0.310	0.412	0.810
2	0.205	0.283	0.677
3	0.339	0.295	0.641

Cells were dissociated by TC-treatment and allowed to reaggregate under the same conditions as in Figure 1A–C.

Cadherin-associated proteins, catenins, in RSV-transformed CEF

Previous reports suggest that cadherins require their association with the cytoskeleton for their function

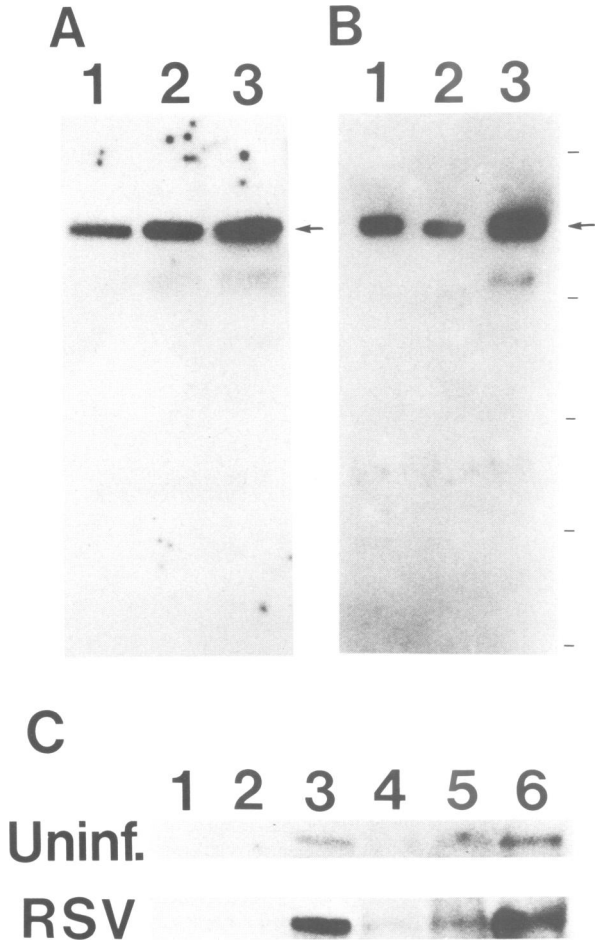

Fig. 2. Immunoblot detection of N-cadherin. (**A** and **B**) Uninfected (lane 1), NY314-infected (lane 2) and RSV-transformed (lane 3) CEF of untrypsinized (A) or TC-treated (B) cells were lysed with a 2% SDS sample buffer containing 5% mercaptoethanol. 100 μg protein of each cell lysate was analysed by immunoblotting using the N-cadherin-specific monoclonal antibody, NCD-2, as described in Materials and methods. (**C**) Uninfected CEF (Uninf.) and RSV-transformed CEF (RSV) were fractionated into crude nuclear (lane 1), S100 (lane 2) and P100 (lane 3) fractions by hypotonic disruption followed by differential centrifugation, as described in Materials and methods. The P100 fraction was further fractionated by centrifugation on a discontinuous sucrose gradient. Membrane fractions at sucrose interfaces: 50%/40% (heavy fraction, lane 4), 40%/35% (intermediate fraction, lane 5) and 35%/20% (light fraction, lane 6) were collected and N-cadherin was detected using NCD-2 in each fraction. Molecular mass markers of 200, 97.4, 68 and 43 kDa are shown.

(Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1989; Fujimori *et al.*, 1990), and this association appears to be mediated by catenins (Nagafuchi and Takeichi, 1989; Ozawa *et al.*, 1990). Catenins were originally identified as proteins that coimmunoprecipitate with E-cadherin (uvomorulin) (Vestweber and Kemler, 1984; Peyrieras *et al.*, 1985) and similar proteins were also found for N-cadherin (Wheelock and Knudsen, 1991). Cadherins lose cell binding activity if the portions of their cytoplasmic domain to which catenins bind are deleted (Nagafuchi and Takeichi, 1989), suggesting that catenins play a crucial role in cadherin function. We thus postulated that the expression or function of catenins might be perturbed in RSV-transformed cells. To test this possibility, N-cadherin and its associated proteins were immunoprecipitated from ^{35}S-labelled cell lysates using NCD-2 and analysed by 7.5% SDS−PAGE. Two major polypeptides of 102 and 94 kDa coprecipitated with N-cadherin in RSV-transformed CEF as well as untransformed CEF (Figure 3A). These two molecules are similar in molecular mass to catenin α and β (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1989; Fujimori *et al.*, 1990), respectively, and the 102 kDa band reacted with an antiserum against catenin α (CAP102) associated with E-cadherin (Nagafuchi *et al.*, 1991) (Figure 3B), suggesting that they are the homologues of the E-cadherin-associated catenins. As the ratio of N-cadherin to the coprecipitated proteins in RSV-transformed CEF is similar to those in uninfected and NY314-infected CEF, the association of N-cadherin with these proteins is obviously unaffected by transformation. A few other minor components coprecipitated with N-cadherin and one of them with a molecular mass of 90 kDa was found only in RSV-transformed CEF (Figure 3A, lane 3).

Tyrosine-specific phosphorylation of cadherin and catenins in RSV-transformed CEF

We next examined the tyrosine-specific phosphorylation of N-cadherin or its associated catenins by RSV transformation, which may cause inactivation of these molecules. N-cadherin−catenin complexes were immunoprecipitated with NCD-2 from cell lysates and these materials were analysed for reactivity to the anti-PTYR by immunoblotting. Two strong bands corresponding to the 102 and 94 kDa catenins were found to react with the antibody only in RSV-transformed cells (Figure 3C). In addition, the antibody very weakly detected N-cadherin and the 90 kDa protein in the transformed CEF. No bands reacted with anti-PTYR in the samples of cadherin−catenin complexes obtained from uninfected CEF or NY314-infected CEF, although many cellular proteins were tyrosine-phosphorylated in NY314-infected cells (Figure 3D).

Metabolic labelling of the cells with ^{32}P followed by immunoprecipitation with NCD-2, showed that N-cadherin was phosphorylated in both normal and transformed cells (Figure 4A), as found in other studies (Lagunowich and Grunwald, 1991) and this protein was most intensely labelled in RSV-transformed cells. The 102 and 94 kDa bands were also phosphorylated in NY314 and RSV-infected cells. The pattern of the phosphorylation of catenins at non-tyrosine residues did not correlate with the inactivation of this adhesion system (not shown). Phosphoamino acid analysis of the ^{32}P-labelled bands from RSV-transformed cells confirmed that both N-cadherin and the 102 and 94 kDa catenins contained phosphotyrosines (Figure 4B).

Immunoprecipitation of tyrosine-phosphorylated cadherin and catenins

We determined the extent of tyrosine phosphorylation of cadherin and its associated catenins by immunoprecipitation with anti-PTYR antibodies. N-cadherin−catenin complexes were immunoprecipitated with NCD-2 from ^{35}S-labelled cell lysates of RSV-transformed cells, and these complexes were denatured and dissociated completely by treatment with SDS, mercaptoethanol and urea, as described in Materials and methods. Tyrosine-phosphorylated forms of these denatured cadherin and catenins were immunoprecipitated with anti-PTYR antibody and analysed by SDS−PAGE. As shown in Figure 5, catenins precipitated with anti-PTYR

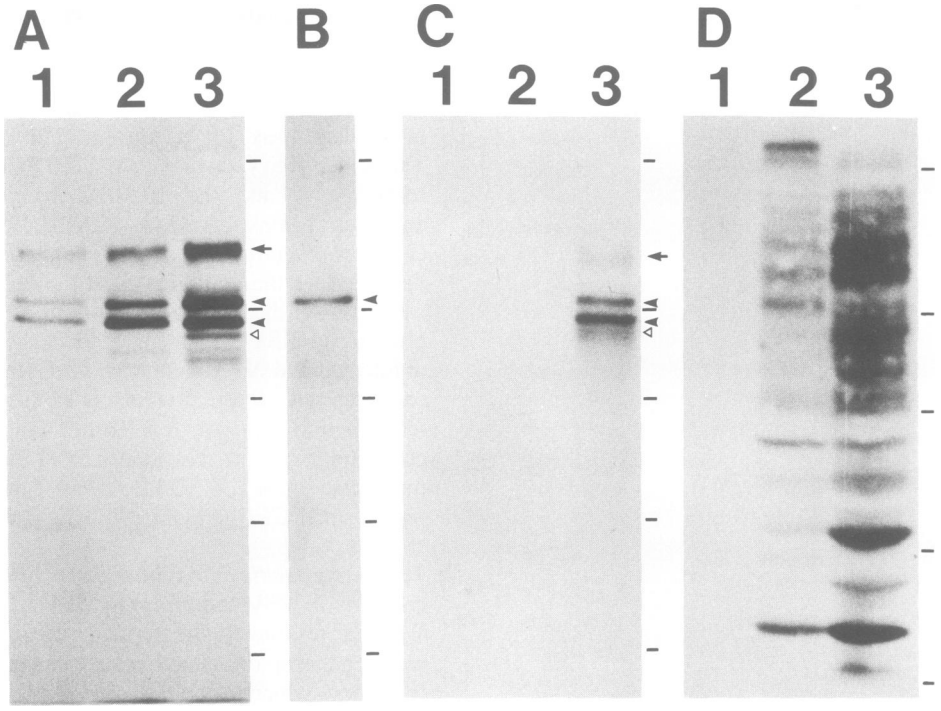

Fig. 3. Analyses of N-cadherin-associated proteins and tyrosine phosphorylation. (**A**) Uninfected (lane 1), NY314-infected (lane 2) and RSV-infected (lane 3) CEF were labelled with [^{35}S]methionine and lysed with an extraction buffer containing 1% NP-40, as described in Materials and methods. N-cadherin and its associated proteins were immunoprecipitated using NCD-2 from the lysates and subjected to SDS–PAGE and fluorography. (**B**) N-cadherin–catenin complexes were immunoprecipitated with NCD-2 from a lysate of RSV-transformed cells and catenin α in the complex was detected by immunoblotting with an antiserum specific for catenin α. (**C**) N-cadherin–catenin complexes were immunoprecipitated with NCD-2 from lysates of uninfected (lane 1), NY314-infected (lane 2) and RSV-infected CEF (lane 3). PTYR-containing proteins in the complex were analysed by immunoblotting with anti-PTYR antibodies. (**D**) PTYR-containing proteins in the whole lysates of uninfected (lane 1), NY314-infected (lane 2) and RSV-transformed (lane 3) CEF were analysed by immunoblotting with anti-PTYR antibody. Arrow, N-cadherin; closed arrowheads, 102 and 94 kDa catenins; open arrowhead, a 90 kDa N-cadherin-associated protein detected only in RSV-transformed cells. Molecular mass markers are as shown in Figure 2.

antibody (lanes 2 and 3) migrated slightly slower than the fractions of the complexes (lane 1) and the supernatant (lane 4), suggesting that these proteins were modified forms. Relative ratio of phosphorylation was estimated by Fujix Bioimage analyser, and we found 21% of the 102 kDa catenin and 25% of the 98 kDa catenin was tyrosine-phosphorylated. On the other hand, only 5% of cadherin was found to be phosphorylated.

Cell aggregation activity and phosphorylation of cadherin–catenin complexes in tsNY68-infected cells and RSV-transformed cells treated with herbimycin

To study further whether the suppression of cadherin function directly correlates with RSV transformation, we examined cells transformed with a temperature-sensitive RSV mutant, tsNY68 (Kawai and Hanafusa, 1971). When tsNY68-infected cells were maintained at the permissive temperature (36°C), their cadherin-mediated cell aggregation activity was strongly suppressed to a level similar to that of wild type RSV-infected cells. On the contrary, their cell aggregation activity was restored to the level of uninfected CEF under the non-permissive temperature (41°C) (Table II). Tyrosine phosphorylation of cadherin and catenins in tsNY68-infected cells was observed only at the permissive temperature and showed good correlation with the suppression of cell aggregation (Figure 6). Phosphorylation of cadherin and catenins occurred within 30 min after a down-shift of temperature (data not shown). We further examined the effects of herbimycin A, a potent inhibitor of tyrosine kinase (Murakami *et al.*, 1988; Uehara *et al.*, 1988, 1989) on cadherin function. When RSV-transformed cells were incubated with 0.5 μg/ml of herbimycin for 16 h, their cell aggregation activity was recovered to the level equivalent to that of the normal cells (Table III) with the concomitant dephosphorylation of cadherin and catenins (Figure 6).

These two lines of evidence obtained by the use of the ts mutant and the antibiotic complement each other well and strengthen our finding that a clear correlation exists between the suppression of cell aggregation activity and tyrosine phosphorylation of cadherin and catenins in RSV-transformed cells.

Discussion

The results presented here demonstrate that N-cadherin-dependent intercellular adhesion is substantially suppressed by transformation with RSV in a transformation-specific manner. Neither the expression nor intracellular transport of N-cadherin molecules were inhibited in RSV-transformed cells; rather, the expression of cadherin was increased by transformation. These results strongly suggest that cadherin is post-translationally modified.

In cells, cadherins form a complex with two or three other proteins, termed catenins (Vestweber and Kemler, 1984; Peyrieras *et al.*, 1985; Wheelock and Knudsen, 1991) and these cadherin–catenin complexes accumulate in a particular form at the cell–cell junctions, adherens junctions (AJs) (Boller *et al.*, 1985; Geiger *et al.*, 1987; Volberg *et al.*,

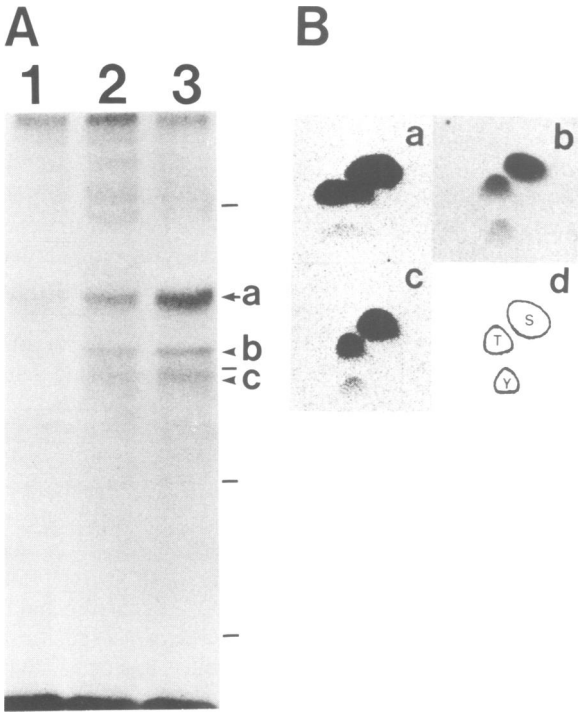

Fig. 4. Phosphoamino acid analysis of N-cadherin and its associated proteins. (**A**) Uninfected (lane 1), NY314-infected (lane 2) and RSV-transformed (lane 3) CEF were labelled with ^{32}P, and N-cadherin–catenin complexes were immunoprecipitated with NCD-2, and analysed by SDS–PAGE and autoradiography as described in Materials and methods. a, N-cadherin; b and c, 102 and 94 kDa catenins, respectively. Positions of molecular mass standards are the same as in Figure 2. (**B**) ^{32}P-Labelled N-cadherin (a) and 102 (b) and 94 kDa (c) catenins of RSV-transformed CEF were eluted from gels by tryptic digestion and subjected to acid hydrolysis. Phosphoamino acids of the hydrolysates were separated by two-dimensional electrophoresis on thin layer plates as described in Materials and methods. (d) Positions of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y).

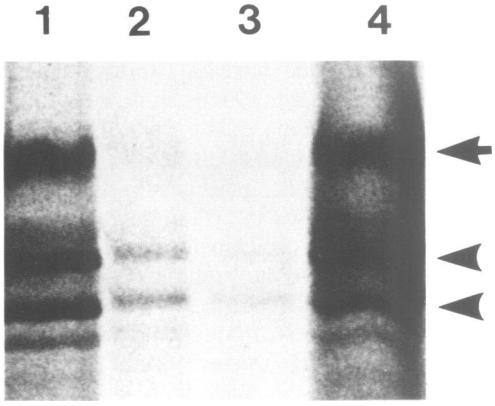

Fig. 5. Immunoprecipitation of tyrosine-phosphorylated cadherin and catenins. RSV-transformed CEF were labelled with [^{35}S]methionine and N-cadherin–catenin complexes were immunoprecipitated with NCD-2. Immunoprecipitated complexes were denatured and eluted by treatment with SDS, mercaptoethanol and urea as described in Materials and methods. These proteins were then immunoprecipitated twice with anti-PTYR antibody and analysed by SDS–PAGE. Immunoprecipitated cadherin–catenin complexes (lane 1); the first (lane 2) and second (lane 3) immunoprecipitated proteins with anti-PTYR antibody; supernatant fraction, which is not immunoprecipitated with anti-PTYR antibody (lane 4). Arrow, N-cadherin; closed arrowheads, 102 and 94 kDa catenins.

Table II. Cadherin-mediated aggregation of uninfected and tsNY68-infected CEF at the permissive (36°C) and non-permissive (41°C) temperatures

Experiment no.	Aggregation (N_{30}/N_0)			
	Uninfected CEF		tsNY68-infected CEF	
	36°C	41°C	36°C	41°C
1	0.325	0.413	0.709	0.500
2	0.351	0.429	0.727	0.507
3	0.359	0.500	0.791	0.540

The experimental conditions are the same as in Table I.

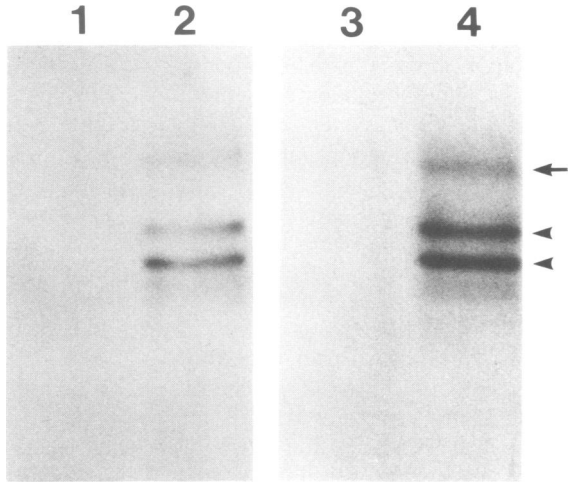

Fig. 6. Tyrosine phosphorylation of cadherin–catenin complexes in tsNY68-infected cells and wild type RSV-infected cells treated with herbimycin A. N-cadherin–catenin complexes of tsNY68-infected CEF incubated at the non-permissive (lane 1) or the permissive (lane 2) temperature and RSV-infected CEF treated with 0.5 μg/ml of herbimycin (lane 3) or untreated (lane 4) were immunoprecipitated and analysed with anti-PTYR antibody. Arrow, N-cadherin; closed arrowheads, 102 and 94 kDa catenins.

Table III. Cadherin-mediated aggregation of RSV-infected CEF untreated and treated with herbimycin A

Experiment no.	Aggregation (N_{30}/N_0)		
	Uninfected CEF	RSV-infected CEF	
		Treated[a]	Untreated
1	0.379	0.380	0.853
2	0.369	0.367	0.900

The experimental conditions are the same as in Table I.
[a]RSV-transformed CEF were treated with 0.5 μg/ml of herbimycin A for 16 h.

1991). However, when the cytoplasmic domain of E- or N-cadherin is deleted, cadherins lose association with the 94 kDa catenin and become diffusely distributed with a concomitant loss of AJ structure and stable cell–cell association. These findings suggest that catenins have a regulatory role in the assembly of cadherin into AJs and in cadherin-dependent cell–cell association. Similar diffuse distribution of E-cadherin and loss of AJ structure were observed in RSV-transformed chicken lens cells (Volberg *et al.*, 1991). In the present study, however, we found that association of N-cadherin with catenins was not disturbed

by RSV transformation, but cadherin and catenins were both tyrosine-phosphorylated. A significant amount of cadherin-associated catenins were phosphorylated, suggesting that tyrosine phosphorylation does not destroy the complex formation between cadherin and catenins. Experiments with *src* mutants suggest that phosphorylation of these proteins closely correlates with the suppression of cadherin-dependent cell aggregation. Tyrosine phosphorylation of cadherin and catenins was not detected in cells infected with a non-myristylation mutant of RSV, NY314, whose *src* protein is active in protein kinase but defective in cell transformation. In cells infected with tsNY68, both tyrosine phosphorylation of the cadherin–catenin complexes and suppression of cadherin-dependent cell aggregation are temperature-dependent. Moreover, treatment of transformed cells with herbimycin A, a potent inhibitor of tyrosine kinases, strongly inhibits the phosphorylation of cadherin and catenins and concomitantly recovered the cell aggregation activity to the level equivalent to that of uninfected cells. We also found that cadherin and catenins were phosphorylated in cells infected with Fujinami sarcoma virus and avian sarcoma virus Y73 (M.Hamaguchi, unpublished results). Thus, tyrosine phosphorylation of the cadherin–catenin complexes showed good correlation with the suppression of cell aggregation and thereby with the expression of the oncogenic, transformed phenotype of cells.

Previous studies suggested that the association of $p60^{v\text{-}src}$ with cytoskeletal structures was essential in its activity of morphological transformation (Hamaguchi and Hanafusa, 1987). Many potential substrates of $p60^{v\text{-}src}$ were localized in the membrane–matrix fraction, a cytoskeletal structure associated with the plasma membrane (Hamaguchi and Hanafusa, 1989). Although several cytoskeletal proteins, such as vinculin, ezrin, talin, calmodulin and p36, were known to be tyrosine-phosphorylated, the studies with non-myristylated mutants of $p60^{v\text{-}src}$ demonstrated that the tyrosine phosphorylation of these substrates alone is insufficient for the transformation (Kamps *et al.*, 1986). The results presented here suggest the importance of catenins and cadherins as the potential targets of $p60^{v\text{-}src}$ in its cell transformation.

However, from the results presented here, it is not clear whether phosphorylation of cadherin and catenins is required, or if some of them are enough for the oncogenic suppression of cell aggregation. The stoichiometry of tyrosine phosphorylation of cadherin and catenin was estimated by immunoprecipitation of anti-PTYR antibodies (Figure 5). We found that 5% of cadherin and 20–25% of catenins that were associated with cadherin were tyrosine-phosphorylated. It should be noted that catenins have the highest rate of stoichiometry of tyrosine phosphorylation among known substrates of $p60^{v\text{-}src}$. For instance, ~1% of vinculin (Sefton *et al.*, 1981), 1% of lactate dehydrogenase (Cooper *et al.*, 1983) and 3–6% of calpacitin I heavy chain (p36), enolase and phosphoglycerate mutase (Cooper and Hunter, 1983) are phosphorylated on tyrosine residues in RSV-transformed CEF. Phosphorylation of the fibronectin receptor was detectable only in cells that were pretreated with vanadate, a potent inhibitor of tyrosine phosphatase (Hirst *et al.*, 1986; M.Hamaguchi, unpublished results). Thus, our results suggest that phosphorylation of catenins is indeed of particular importance in the suppression of cadherin-dependent cell aggregation.

We also examined the effects of transformation on the function of other types of cadherin, P- and E-cadherins, using rat 3Y1 cells and v-*src*-transformed 3Y1, SR3Y1 (Matsuyoshi *et al.*, 1991). Both cells expressed P-cadherin associating with catenins, but 3Y1 cells showed compact aggregates, whereas SR3Y1 showed more loosely associated colonies, suggesting cadherin function is perturbed by transformation. However, we did not find such gross differences in the rates of cadherin-mediated cell aggregation activity, as described here under the suspension culture conditions employed. Moreover, over-expression of exogenous E-cadherin in these transformed cells had no significant effect on their adhesive properties. Interestingly, we found that in the SR3Y1 cells, tyrosine phosphorylation of the 102 kDa catenin was undetectable, while the cadherins and the 98 kDa catenin were both tyrosine-phosphorylated to a similar extent as we observed in RSV-transformed CEF. We could not clarify whether cell clones that had good cell adhesive properties and lacked the 102 kDa catenin phosphorylation were selected during the establishment of SR3Y1 cell lines, or both the phosphorylation of the 102 kDa catenin and the massive suppression of cell adhesiveness were specific for N-cadherin alone. However, these findings together with our present study strongly suggest that phosphorylation of the 102 kDa catenin probably plays a critical role in suppression of cadherin-dependent cell–cell aggregation in RSV-transformed CEF, a natural host of RSV.

Recently, Nagafuchi *et al.* (1991) reported the primary structure of the 102 kDa catenin and mentioned that this protein is similar to vinculin in its overall structure and has a sequence homologous to the self-association site of vinculin. More recently, Hirano *et al.* (1992) directly demonstrated by cDNA transfection experiments that the neural homologue of the 102 kDa catenin plays a critical role not only in E-cadherin-dependent cell–cell adhesion, but also in the organization of multicellular structures. Given these findings, identification of tyrosine phosphorylation sites in the 102 kDa catenin molecules and functional analysis of phosphorylated forms are important subjects for clarification.

It is reported that some types of human carcinoma cells, which are highly metastatic, have lost expression of cadherin (Shimoyama and Hirohashi, 1991b; Shiozaki *et al.*, 1991), but many human carcinoma cells expressing cadherin can metastasize (Shimoyama and Hirohashi, 1991a). These results suggest that a similar mechanism, as shown in this study, may operate in these cadherin-positive carcinoma cells and be involved in their detachment from primary tumour sites. Indeed, we found in our preliminary experiment that tyrosine phosphorylation of cadherin–catenin complexes does exist in certain types of human carcinoma cells with a concomitant loss of AJ structure. Thus, suppression of cadherin function by biochemical modification as shown in this study should be taken into account for the study of tumour invasion and metastasis.

Materials and methods

Cells and viruses

CEF were prepared, maintained and infected as described previously by Hanafusa (1969). The viruses analysed included a wild type RSV, Schmidt-Ruppin strain; non-myristylation mutant NY314 (Cross *et al.*, 1984); and temperature-sensitive mutant tsNY68 (Kawai and Hanafusa, 1971).

Cell aggregation study

Cadherin-mediated cell aggregation was assayed as described by Takeichi (1977). Briefly, cells were treated with 0.01% crystallized trypsin in the presence of 1 mM $CaCl_2$ at 37°C for 20 min and then washed with Ca^{2+}- and Mg^{2+}-free HEPES buffered saline (HCMF, pH 7.4) to obtain single cell suspensions. Cells suspended in HCMF with or without 1 mM $CaCl_2$ were replaced in wells of a 24-well plate and incubated to allow aggregation for 30 min at 37°C on a gyratory shaker rotating at 80 r.p.m. 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 immunofluorescent staining, cell suspensions were transferred to adherent culture dishes, incubated for a further 60 min, then fixed and stained with anti-PTYR antibody.

Cell fractionation

Cell fractionation by hypotonic disruption, followed by differential centrifugation was described previously by Krueger *et al.* (1983) and Hamaguchi and Hanafusa (1989). Briefly, cells were suspended in hypotonic buffer [20 mM Tris−HCl, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 2% Trasylol and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and homogenized. Nuclei were removed by low speed centrifugation. The postnuclear fraction was separated by centrifugation for 30 min at 100 000 *g* into S100 supernatant (cytosolic fraction) and P100 pellet (crude membrane fraction). The P100 fraction was resuspended in 40% sucrose, underlaid with 50% sucrose, overlaid with 35% and 20% sucrose and centrifuged at 200 000 *g* for 2 h at −5°C. Membrane fractions were collected from gradient interfaces and subjected to gel electrophoresis and immunoblotting.

Preparation of anti-PTYR antibody

Anti-PTYR antibody was prepared with v-*abl*-encoded protein expressed in bacteria, as described previously by Wang (1985) and Hamaguchi *et al.* (1988).

Immunoblotting

N-cadherin, 102 kDa catenin and PTYR-containing proteins were analysed by immunoblotting with NCD-2, anti-CAP102 and anti-PTYR antibody, respectively, as described previously by Hamaguchi *et al.* (1988).

Isotope labelling and immunoprecipitation of cadherin−catenin complex

Cells were labelled with [^{35}S]methionine (ICN; 100 μCi/ml) or [^{32}P]orthophosphate (ICN; 1 mCi/ml) for 15 h at 37°C and lysed with an extraction buffer (10 mM Tris−HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM Na_3VO_4, 0.1 mM Na_2MoO_4 and 1 mM PMSF) as described by Nagabuchi and Takeichi (1989). Cell lysates were clarified by centrifugation and the supernatants were immunoprecipitated with an anti-N-cadherin monoclonal antibody, NCD-2, as described by Hamaguchi *et al.* (1988). Immunoprecipitated cadherin−catenin complexes were subjected to SDS−PAGE and fluorography.

To assess the extent of tyrosine phosphorylation, cadherin−catenin complexes immunoprecipitated with NCD-2 were denatured and eluted from the antibody by boiling for 5 min in a buffer containing 10 mM Tris−HCl, pH 7.4, 2% SDS, 5% 2-mercaptoethanol and 0.5 mM Na_3VO_4 followed by incubation at 37°C for 30 min in the presence of 6 M urea. Denatured proteins were precipitated with ethanol, resolubilized by boiling in the presence of 1% SDS and 0.02% 2-mercaptoethanol, and diluted 10-fold with modified RIPA buffer (10 mM Tris−HCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5 mM Na_3VO_4, 0.1 mM Na_2MoO_4 and 1 mM PMSF). Tyrosine-phosphorylated cadherin and catenins were immunoprecipitated with anti-PTYR antibody and analysed by SDS−PAGE. Relative ratio of each protein band was measured by a Fujix Bioimage Analyzer (Tokyo, Japan).

Phosphoamino acid analysis

^{32}P-labelled protein bands were subjected to tryptic digestion followed by acid hydrolysis as described previously by Feldman *et al.* (1980). Hydrolysates were analysed by two-dimensional thin layer electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension as described previously by Hunter and Sefton (1980). ^{32}P-Labelled phosphoamino acids were detected by autoradiography.

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