Loss of Epithelial Differentiation and Gain of Invasiveness Correlates with Tyrosine Phosphorylation of the E-Cadherin/β-Catenin Complex in Cells Transformed with a Temperature-sensitive v-SRC Gene

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Abstract. Loss of histotypic organization of epithelial cells is a common feature in normal development as well as in the invasion of carcinomas. Here we show that the v-src oncogene is a potent effector of epithelial differentiation and invasiveness. MDCK epithelial cells transformed with a temperature-sensitive mutant of v-src exhibit a strictly epithelial phenotype at the nonpermissive temperature for pp60^{v-src} activity (40.5°C) but rapidly loose cell-to-cell contacts and acquire a fibroblast-like morphology after culture at the permissive temperature (35°C). Furthermore, the invasiveness of the cells into collagen gels or into chick heart fragments was increased at the permissive temperature. The profound effects of v-src on intercellular adhesion were not linked to changes in the levels of expression of the epithelial cell adhesion molecule E-cadherin. Rather, we observed an increase in tyrosine phosphorylation of E-cadherin and, in particular, of the associated protein β-catenin. These results suggest a mechanism by which v-src counteracts junctional assembly and thereby promotes invasiveness and dedifferentiation of epithelial cells through phosphorylation of the E-cadherin/catenin complex.

THE formation of tight tissue sheets depends on strong intercellular adhesion and represents a prerequisite for the generation of characteristic properties of epithelia. To achieve intercellular adhesion, epithelial cells possess various cell junctions, the composition and function of which must be tightly regulated and, conceivably, depend on multiple molecular interactions. In recent years it became apparent that the epithelial cell adhesion molecule E-cadherin is one potent regulator of epithelial junction formation. For instance, forced expression of E-cadherin in nonepithelial cells by cDNA transfection induces junction formation, and disturbance of E-cadherin function in epithelial cells by specific antibodies leads to loss of junctions and to a fibroblast-like morphology (Behrens et al., 1985; Gumbiner and Simons, 1986; Nagafuchi et al., 1987; Matsuzaki et al., 1990; McNeill et al., 1990; see also Takeichi, 1991, for a review). E-cadherin, a 120-kD transmembrane glycoprotein, is enriched in the adherens junctions of epithelial cells and interacts with the cytoskeleton via associated cytoplasmic molecules, the catenins (Boller et al., 1985; Behrens et al., 1985; Nagafuchi and Takeichi, 1988; Ozawa et al., 1989, 1990; Ozawa and Kemler, 1992). The cDNAs for α- and β-catenin have recently been characterized (α in the mouse,

β in the *Xenopus* system); they show sequence similarities to the cDNAs of other well known junction-associated proteins, i.e., of vinculin and plakoglobin, respectively (Nagafuchi et al., 1991; Herrenknecht et al., 1991; McCrea and Gumbiner, 1991; McCrea et al., 1991). β-Catenin of *Xenopus* is homologous to the *armadillo* gene product of *Drosophila* (McCrea et al., 1991). E-cadherin expression is frequently downregulated in highly invasive, poorly differentiated carcinomas (Behrens et al., 1989; Schipper et al., 1991; Frixen et al., 1991; Shiozaki et al., 1991; Shimoyama and Hirohashi, 1991a,b), and re-expression of E-cadherin by cDNA transfection in poorly differentiated carcinoma cell lines inhibits invasiveness (Frixen et al., 1991; Vleminckx et al., 1991; Chen and Öbrink, 1991; Navarro et al., 1991). Thus, E-cadherin seems to act as a kind of master molecule for maintaining the differentiation of normal epithelial cells, and loss of expression or function of E-cadherin in transformed epithelial cells appears to be a key step in the progression of the cells to a malignant phenotype (see Behrens et al., 1992 for a review).

Besides E-cadherin, other molecular components have been identified as modulators of the epithelial phenotype. For instance, motility and growth factors like scatter fac-

tor/hepatocyte growth factor (Weidner et al., 1990, 1991) and acidic FGF (Jouanneau et al., 1991), or extracellular matrix molecules such as syndecan (Leppä et al., 1992) and laminin (Klein et al., 1988) counteract or promote epithelial differentiation, respectively. In addition, antibodies against desmosomal glycoproteins inhibit the formation of desmosomal plaques (Cowin et al., 1984); the involved proteins, the desmocollins and desmogleins, have recently been identified as members of the cadherin family of cell adhesion molecules (Koch et al., 1990; Collins et al., 1991; see also Buxton and Magee, 1992, for a review). Another putative epithelial regulator is pp60^{src}, a tyrosine kinase which has been localized to the adherens junction undercoat of various types of cells (Rohrschneider, 1980; Tsukita et al., 1991). In RSV-transformed fibroblasts, the v-src product interferes with cell–substratum adhesion, possibly via phosphorylation of integrin receptors and associated proteins (Hirst et al., 1986; Pasquale et al., 1986). In epithelial cells, low-level expression of v-src has been associated with disturbance of the junctional complex, in particular of the zonula adherens (Warren and Nelson, 1987).

It is thus remarkable that some of the effectors of epithelial junction formation are tyrosine protein kinases, e.g., the scatter factor/hepatocyte growth factor receptor c-*Met* (Naldini et al., 1991), the FGF receptors (cf. Jouanneau et al., 1991) and the pp60^{src} kinase. Furthermore, junctional complexes have been identified as major sites of tyrosine phosphorylation (Maher and Pasquale, 1988; Takata and Singer, 1988; Tsukita et al., 1991; Volberg et al., 1992). To study in more detail the possible role of pp60^{src} in epithelial junction formation, we have transformed MDCK epithelial cells with a temperature-dependent mutant of v-src. We show here that activation of the pp60^{v-src} tyrosine kinase at the permissive temperature leads to profound and rapid changes in the morphology of the cells, primarily affecting the intercellular junction system. In addition, the transformed cells at the permissive temperature acquire invasive properties in vitro. Concomitantly with these changes, we noticed increased tyrosine phosphorylation of the E-cadherin/catenin complex, indicating that the E-cadherin–mediated adhesion system might be subject to negative regulation by the pp60^{v-src} tyrosine kinase.

Materials and Methods

Cell Lines, Morphological Analysis, Electrical Resistance Measurements, and Invasion Assays

The ts-src MDCK cell lines were generated in our lab (R. Friis). MDCK cells (clone MDCK-1; see Behrens et al., 1989) were infected with a murine leukemia retroviral construct in which the v-src gene of LA ts 31 (Wyke and Lineal, 1973) was inserted in place of the pol-env genes (ts 31-src MuLV, a generous gift of Dr. Steven Anderson, Merck, Sharpe and Dohm, Rahway, NJ). ts 31-src MuLV was propagated on the defective amphitrophic helper cell line PA 317 (Miller and Buttimore, 1986), and the MDCK cells were infected at a multiplicity of 0.1 with the defective virus in the presence of Polybrene (Aldrich Chemical Co., Milwaukee, WI) at a concentration of 8 μg/ml. 1 d after infection, the cells were suspended in 0.25% soft agar culture (10⁴ cells/60-mm dish). After 18 d culture at 35°C, colonies were aspirated with finely drawn-out Pasteur pipettes, and grown up in regular tissue culture. Stock cultures of the infected MDCK cells were routinely maintained at 35°C in DME containing 10% FCS. Selected clones, ts 31 clone 1 and clone 2, were again suspended in soft agar at densities of 10⁴ and 10⁵ cells/plate at both 35°C (the permissive) and 40.5°C (the nonpermissive temperature). After a 14-d incubation, colony numbers were counted macroscopically.

For the morphological analysis, ts-src MDCK cells were cultured for 14 h either at 35 or at 40.5°C in 6-well plates (10⁶ cells per well, for light microscopy; Falcon Plastics, Cockeysville, MD) or on 13-mm Thermanox coverslips (10⁴ cells per coverslip, for EM; Nunc Roskilde, Denmark). For electron microscopical analysis, cells were fixed with 2.5% glutaraldehyde in 0.1 M NaPO₄, pH 7.5, postfixed in 2% OsO₄, and embedded in an Araldite/Epon mixture. After polymerization, the Thermanox dishes were removed by dipping into liquid nitrogen. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with a Philips 400 electron microscope (Philips Electronic Instruments, Mahwah, NJ). For time lapse video recording, cells in tissue culture flasks were observed with an inverted microscope (Carl Zeiss, Oberkochen, Germany), and recordings were performed with a high resolution camera (WV-1850C; National Panasonic, Tokyo, Japan) and a video recorder (U-matic VO-5850P; Sony, Tokyo, Japan). The temperature inside the culture vessel was changed with a heating stage (TRZ 3700; Carl Zeiss) mounted on the inverse microscope, and was monitored with a thermistor probe (Telethermometer; YSI, Yellow Springs, OH). Electrical-resistance measurements were performed on cells growing on 24-mm polycarbonate membranes in Costar Transwells (Cambridge, MA) using a Millicell-ERS instrument (Millipore Continental Water Systems, Bedford, MA) as described (Strange et al., 1991).

Invasion in organ culture at the different temperatures was assessed as described previously (Mareel et al., 1979). Briefly, precultured embryonic chick heart fragments were confronted on top of semisolid agar with monolayer fragments of the MDCK cell lines, and the cocultures were incubated in six-well plates at the indicated temperatures. After 4 and 7 d, cultures were processed for histology and examined by immunohistochemical staining with antibodies against chick heart (Mareel et al., 1981) or with antibodies against MDCK cells (Behrens et al., 1989). Invasion was evaluated according to Bracke et al. (1984). Collagen invasion assays using 10⁵ cells per well in six-well plates were performed as described (Behrens et al., 1989). Cultures were incubated at the permissive or nonpermissive temperature, and after 24 h invasive cells were counted in the light microscope.

Immunofluorescence Staining, Immunoprecipitation, and Western Blotting

Immunofluorescence staining of ts-src MDCK cells for E-cadherin at the different temperatures was performed using a rabbit-anti-canine E-cadherin antiserum (Behrens et al., 1989). For immunoprecipitation experiments, 10⁶ cells were precultured in 10-cm tissue culture dishes at 40.5°C for 24 h and further cultured for 15 h at either 40.5 or at 35°C. Cells were then labeled at both temperatures for 3 h with either 0.2 mCi/ml inorganic ³²P or 50 μCi/ml of [³⁵S]methionine, lysed in nonionic detergent buffer (L-CAM assay buffer –1% Triton X-100 – 1mM PMSF – 0.2 mM NaVO₃; see Cunningham et al., 1984 for L-CAM assay buffer), and subjected to immunoprecipitation using anti-canine E-cadherin antibodies as described (Behrens et al., 1989). For comparison of different samples, lysates containing equal amounts of TCA-precipitable radioactivity were used. Enrichment for phosphotyrosine over serine/threonine labeling was achieved by treatment of polyacrylamide gels with hot KOH solution (Cooper et al., 1983). Alternatively, tyrosine phosphorylation in the immunoprecipitated E-cadherin/catenin complex (from 4 × 10⁶ non-labeled cells) was monitored by Western blotting using an anti-phosphotyrosine mAb according to the protocol suggested by the manufacturer (Upstate Biotechnology, Inc., NY).

Tyrosine Kinase Assay

ts-src MDCK cells were grown for 24 h at 40.5°C, at which time half of the plates were shifted to 35°C. After incubation for a further 4 h, samples were lysed in 50 mM KH₂PO₄–150 mM NaCl-1% Triton X-100, pH 7.4, debris was pelleted in an Eppendorf centrifuge (Brinkman Instruments Inc., Westbury, NY), and pp60^{src} was immunoprecipitated with tumor-bearing rabbit serum number 8, as described in Fig. 2 of Ziemiecki and Friis (1980). This antiserum exhibits high reactivity with Prague strain pp60^{src}. A kinase reaction was performed with γ-³²P-labeled ATP, and phosphorylated proteins were resolved by SDS-PAGE and autoradiography.

Results

Reversible Loss of Epithelial Phenotype after Transformation of MDCK Cells by a Temperature-sensitive v-src Gene
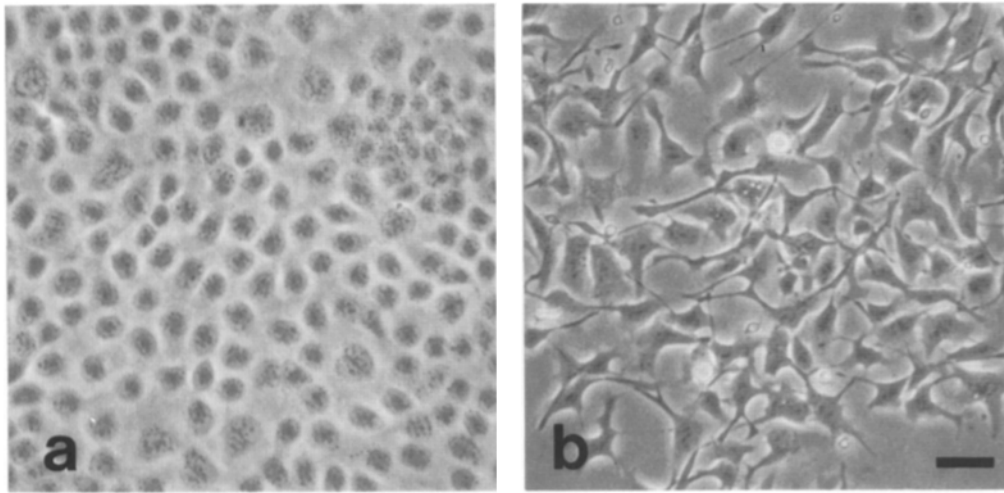
MDCK cells transformed with a temperature-sensitive v-src

Figure 1. Dissociation of the epithelial monolayer of ts-src MDCK cells after the switch from the nonpermissive to the permissive temperature. MDCK cells transformed with a temperature sensitive v-src gene (ts-src MDCK, ts 31 clone 1) were cultured either at 40.5°C (*a*) or at 35°C (*b*) for 14 h. Note the loss of cell contacts and the fibroblast-like morphology of cells cultured at 35°C, the temperature permissive for src activity. Bar, 40 μm.

gene (ts-src MDCK; see Materials and Methods) were cultured either at 40.5°C (the nonpermissive temperature for ts-src activity) or at 35°C (the permissive temperature). At 40.5°C, the cells formed tight epithelial colonies and exhibited the characteristic cobblestone morphology of nontransformed MDCK cells (Fig. 1 *a*). In contrast, when cultured at 35°C, pronounced changes in cell morphology were apparent: the cells dissociated from each other and they acquired a spindle shape morphology (Fig. 1 *b*, as shown for

clone ts-31-1, similar morphology changes were seen with clone ts-31-2). These effects were rapid being detectable within one hour after shifting the cells from the nonpermissive to the permissive temperature (Fig. 2, *top*). The epithelial phenotype was completely restored after incubation of cells at the nonpermissive temperature (Fig. 2, *bottom*). Under similar conditions, the parental MDCK cells did not exhibit any temperature-dependent alterations in morphology (data not shown). The morphology changes of ts-src MDCK
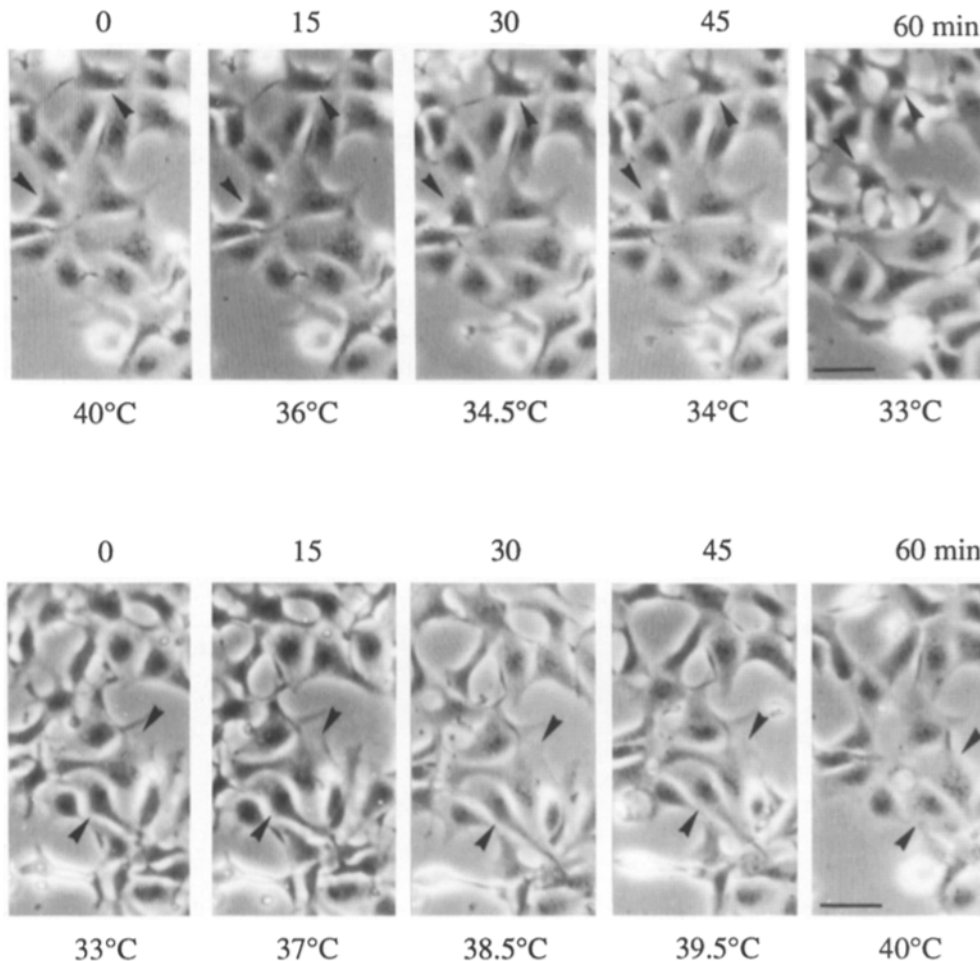

Figure 2. Time course of morphological changes of ts-src MDCK cells when switched from the nonpermissive to the permissive temperature, and vice versa. A subconfluent culture of ts-src MDCK cells at 40°C was cooled to 33°C (*top*) and warmed again to 40°C (*bottom*; see Materials and Methods). Time-lapse video recordings of a single microscopic field at intervals of 15-min are shown. The temperature inside the culture flask was continuously measured and is indicated below each panel. Arrowheads indicate typical changes from an epithelial to a fibroblast-like morphology and vice versa. Bar, 20 μm.
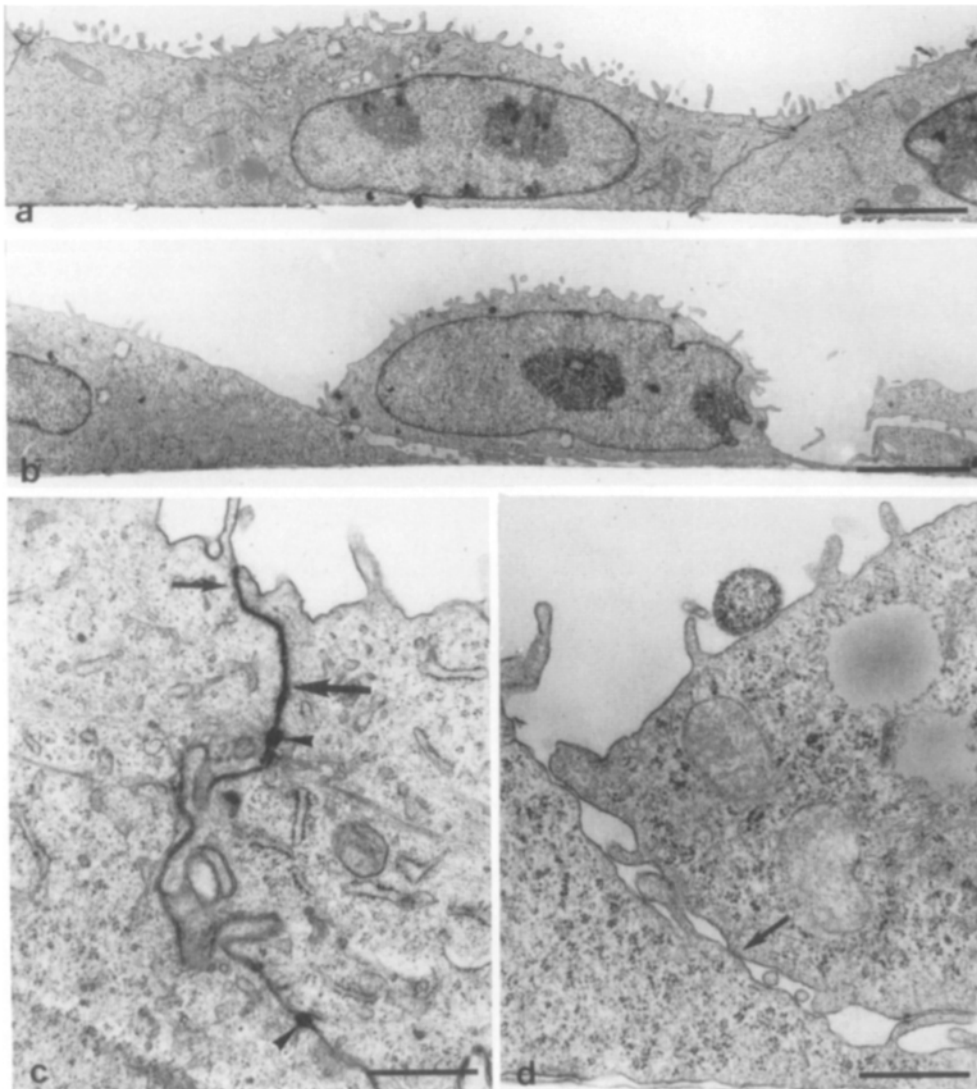
Figure 3. Transmission EM of ts-src MDCK cells grown at the nonpermissive and permissive temperatures. ts-src MDCK cells cultured at 40.5°C (*a* and *c*) or 35°C (*b* and *d*) were processed for EM as described in Materials and Methods. Note that the cells at 40.5°C show closely apposed lateral membranes with a well developed junctional complex composed of tight (*small arrow*) and adherens junctions (*large arrow*) as well as desmosomes (*arrowheads*). At 35°C lateral cell membranes are not closely apposed and the cells lack fully developed junctions and form irregular intercellular space with only undifferentiated contacts (*arrow*). Bars: (*a* and *b*) 3 μm; (*c* and *d*) 0.7 μm.

cells were also examined by transmission EM: At the nonpermissive temperature, the cells showed closely apposed plasma membranes at their lateral faces with proper formation of tight and adherens junctions as well as of desmosomes (Fig. 3, *a* and *c*). At the permissive temperature, the lateral membranes of touching cells were not closely apposed and the cells rarely showed any sign of junction formation (Fig. 3, *b* and *d*).

The ts-src MDCK cells showed clear temperature-dependence of growth in semisolid agar (Table I). Thus, while non-transformed MDCK cells did not grow in the semisolid medium, the two inspected ts-src MDCK cell lines exhibited a significant cloning efficiency in agar at 35°C, which was 20- to 40-fold lower at 40.5°C (also remember that the ts-src MDCK cell lines were initially selected on the basis of this property, see Materials and Methods). It has been shown before that monolayers of normal MDCK can be cultured under conditions that they build up transepithelial electrical resistance (cf. also Gumbiner and Simons, 1986). Similarly, ts-src MDCK cells build up such transepithelial resistance when plated at 40.5°C; however, this property is rapidly lost following the shift of the cells to 35°C (Fig. 4).

Non-transformed MDCK cells remain resistant while shifted to 35°C. Furthermore, we measured the tyrosine kinase activity of immunoprecipitated v-src protein of ts-src MDCK cells cultured at both temperatures (Fig. 5). Clearly, autophosphorylation of ts-src is seen at 35° but not at 40.5°C (*arrowheads*), and phosphorylation of coprecipitated Ig

Table I. Colony Forming Efficiency of Normal and ts-src MDCK Cells in Soft Agar Culture

	Colonies,* percent of total cells suspended	
Cells	35°C	40.5°C
Normal MDCK cells	<0.002	<0.002
ts-src MDCK cells clone ts-31-1	2.4	0.06
ts-src MDCK cells clone ts-31-2	7.1	0.33

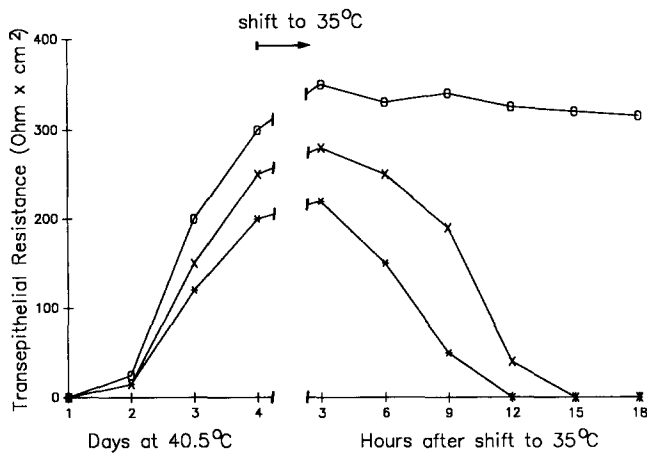* A colony constituted a cell aggregate of more than 100 cells as monitored by microscopic inspection.

Figure 4. Transepithelial electrical resistance of monolayers of ts-src MDCK cells plated at 40.5°C and then shifted to 35°C. Non-transformed MDCK cells (o) and ts-src MDCK cells clone 1 (x) and clone 2 (*) were cultured for 4 d at 40.5°C until electrical resistance was established. After the shift to 35°C electrical resistance was monitored for the next 18 h.

Figure 5. In vitro tyrosine autophosphorylation and Ig heavy chain phosphorylation of immunoprecipitated pp60^{src}. ts-src MDCK and control cells were grown at 40.5° and 35°C, pp60^{src} was immunoprecipitated, and a kinase reaction with γ^{32}P-ATP was performed followed by SDS-gel electrophoresis and autoradiography as described in Materials and Methods. (Lane *1*) Serum control without cell lysate; (lane *2*) *ts* 31 MDCK clone 1 at 40.5°C; (lane *3*) *ts* 31 MDCK clone 1 at 35°C; (lane *4*) *ts* 31 MDCK clone 2 at 40.5°C; (lane *5*) *ts* 31 MDCK clone 2 at 35°C; (lane *6*) uninfected MDCK cells at 40.5°C; (lane *7*) uninfected MDCK cells at 35°C; (lane *8*) ts 68 NIH 3T3 cells at 40.5°C; and (lane *9*) ts 68 NIH 3T3 cells at 35°C. The 53-kD heavy chain of coprecipitated IgG is indicated with an arrow. Arrowheads identify pp60^{src} autophosphorylation visible for the MDCK cells infected with the ts RSV Prague strain. The pp60^{src} band of NIH 3T3 cells infected with the RSV Schmidt-Ruppin strain is not resolved because it is located just above the IgG heavy chain. Autophosphorylation of the cellular pp60^{src} is not apparent.

heavy chain (53 kD, *arrow*) was also much higher at the permissive temperature.

Invasiveness of ts-src MDCK Cells In Vitro

Previous work from our group has shown that the generation of fibroblast-like, dedifferentiated phenotypes of epithelial cells correlates with increased invasiveness of these cells in vitro (Behrens et al., 1989; Frixen et al., 1991). We therefore tested the ts-src MDCK cells for invasion into chick heart fragments and into collagen gels. When confronted with precultured chick heart fragments, the ts-src MDCK cells exhibited a temperature dependent invasive behavior: at the permissive temperature, 8 out of 15 cocultures were scored as invasive (see an example in Fig. 6, *a–c*), i.e., single cells and cell clusters were seen inside the heart fragment (*arrowheads*) while the remnant cells formed an unorganized structure at the periphery of the heart fragment. At the nonpermissive temperature, no invasion was found (six cocultures were tested) and the cells were organized as a monolayered epithelium around the heart tissue (Fig. 6, *d–f*). At both temperatures, non-infected MDCK cells were noninvasive. In the collagen invasion assay, the ts-src MDCK cells also showed a significant increase in invasiveness at the permissive as compared to the nonpermissive temperature (Fig. 7). Again, non-transformed MDCK cells were virtually noninvasive at both temperatures.

Tyrosine Phosphorylation of the E-Cadherin/Catenin Complex in ts-src MDCK Cells

The effects of v-src on MDCK cells resemble the phenotypic changes induced by disturbance of E-cadherin function via anti-E–cadherin antibodies (Behrens et al., 1989). We therefore tested whether v-src activity affects the E-cadherin adhesion system. Immunofluorescence staining showed a more diffuse distribution of E-cadherin on ts-src MDCK cells grown at the permissive temperature as compared to cells grown at the nonpermissive temperature (Fig. 8). In certain cells, some intercellular E-cadherin staining was ap-
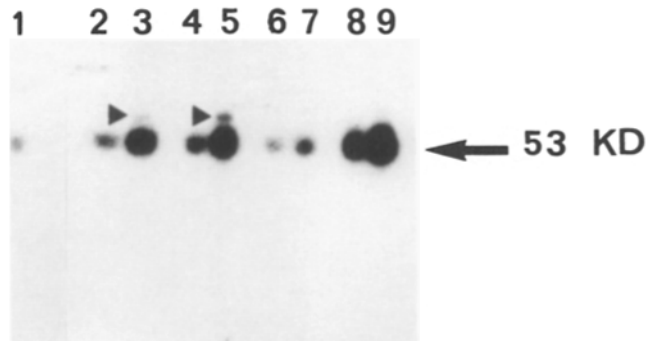
parent at the permissive temperature (Fig. 8 *b*). This staining was not seen when cells were not permeabilized after fixation (data not shown), and probably represents internalized E-cadherin. However, the overall intensity of surface labeling appeared similar at both temperatures. Furthermore, the total amount of E-cadherin and of the associated α-, β-, and γ-catenins was similar at both temperatures, as revealed by both immunoprecipitation and by Western blotting using anti-E–cadherin antibodies (shown for immunoprecipitation in Fig. 9 B, lanes *c* and *c'*). In addition, the transcriptional activity of the recently characterized −178/+92-bp E-cadherin promoter fragment cloned in front of a CAT reporter gene (cf. Behrens et al., 1991) was similar in ts-src MDCK cells at both temperatures (data not shown). These combined results indicate that pp60^{v-src} activation following the shift of ts-src MDCK cells to the permissive temperature does not significantly change the level of E-cadherin expression.

To assess whether phosphorylation of E-cadherin is altered by the pp60^{v-src} protein kinase, we prepared extracts of ts-src MDCK cells labeled with ^{32}P$_i$ at both the permissive and nonpermissive temperature and performed immunoprecipitation experiments using anti-E cadherin antibodies. We found that both E-cadherin and the coprecipitated α- and β-catenins were ^{32}P-labeled, and that the level of overall phosphorylation was similar at the nonpermissive and the permissive temperature (Fig. 9 *A*, lanes *a* and *a'*). However, clear differences were observed when specific phosphorylation on tyrosine residues was examined. Hot alkali treatment of the gels revealed increased tyrosine phosphorylation of β-catenin and, to a lesser extent, of E-cadherin at the permis-
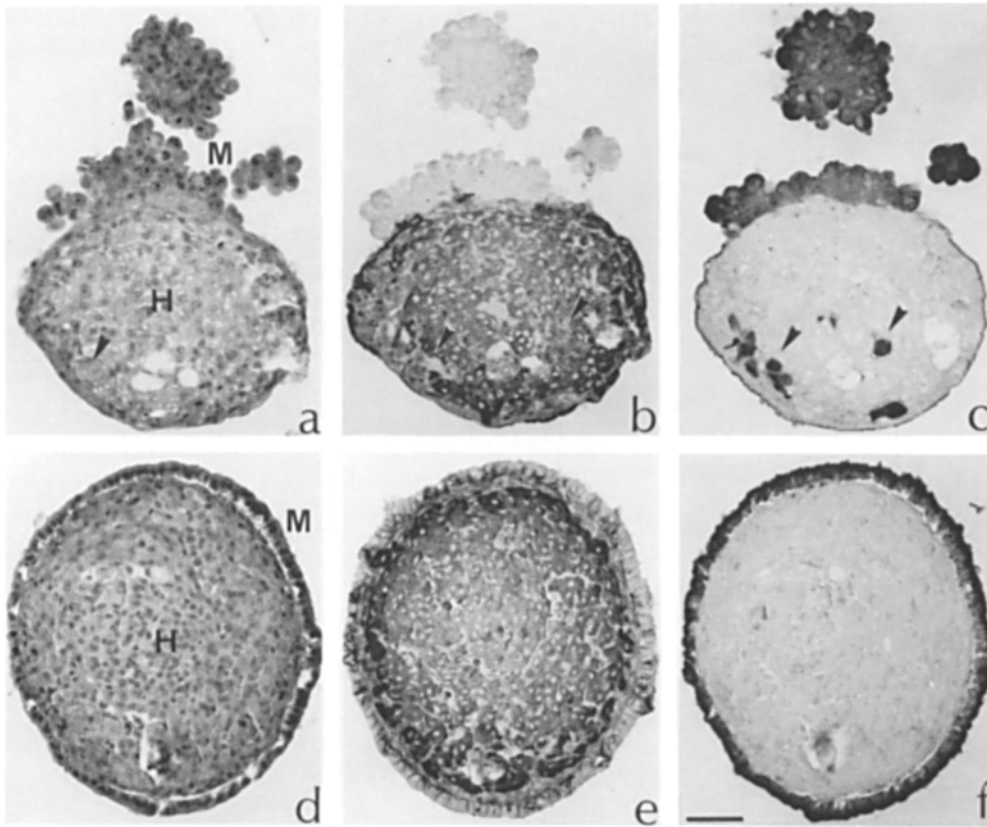
Figure 6. Sections of 4-d-old confrontation cultures between ts-src MDCK cells and embryonic chick heart kept at the permissive and the nonpermissive temperature. Cultures were either incubated at 35°C (*a–c*) or at 39.5°C (*d* and *f*). Consecutive sections were stained with hematoxyline-eosine (*left*), anti-chick heart antiserum (*middle*), and anti-MDCK cell antiserum (*right*). M, MDCK cells; H, heart tissue. Arrowheads in *a* to *c* mark invasive MDCK cells. Bar, 100 µm.

sive temperature as compared to the nonpermissive temperature (Fig. 9 A, lanes b and b'). Tyrosine phosphorylation of α-catenin was similar at both conditions. In non-infected MDCK cells, no differences in the overall and the tyrosine-specific phosphorylation pattern of the E-cadherin catenin complex was observed (Fig. 9 B, lanes a and a', and b and b'). To further demonstrate src-induced tyrosine phosphorylation of the E-cadherin catenin complex, non-labeled E-cadherin immunoprecipitates were Western blotted using anti-phosphotyrosine antibodies. Again, increased tyrosine phosphorylation at the permissive temperature was observed, particularly on β-catenin and to a lesser degree also on E-cadherin (Fig. 10 A, lanes a and a'). Time course studies showed that increased tyrosine phosphorylation on β-catenin could be detected as early as 10 min after the shift of cells from the nonpermissive to the permissive temperature (Fig. 10 B).

Discussion

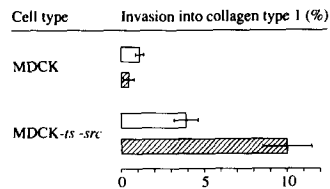By introducing a temperature sensitive mutant of the v-src

Figure 7. Invasion of ts-src MDCK cells into collagen gels. Invasion into collagen gels after 24 h at 35°C (⬛) and at 39.5°C (□) is expressed as the percentage of the cells that have migrated into the gel over the total number of cells counted. For the ts-src MDCK cells invasion is significantly higher ($P < 0.01$) at the permissive temperature. Statistical analysis was based on chisquare calculations.

gene, we have generated transformed MDCK epithelial cells that could be rapidly and reversibly shifted between the noninvasive, epithelial and the invasive, fibroblast-like state. The identification of the E-cadherin/catenin complex as a target for src-induced tyrosine phosphorylation suggests a regulatory pathway by which the function of a cell–cell adhesion molecule and its associated cytoplasmic components is controlled via intracellular posttranslational modification. Since the cellular pp60src kinase is physically associated with the junctional complex, these results suggest an important role of the src gene in normal junctional development as well as in the establishment and maintenance of epithelial differentiation.

A prominent feature of our ts-src MDCK cells is the junctional disassembly at the permissive, i.e., the transforming temperature of ts-v-src. In the past years, various components of junctional organelles of epithelial cells have been characterized at the molecular level. However, the sequence of events that lead to junctional assembly and disassembly are still not well defined. This is in part due to the lack of appropriate in vitro systems to study the involved processes. For instance, withdrawal of Ca^{2+} from the culture medium has been mainly used to achieve controlled dissociation of epithelial cells (e.g., Green et al., 1987; Duden and Franke, 1988). Low extracellular Ca^{2+} concentrations, as applied in these studies, are unlikely to occur in vivo, indicating that the observed splitting of junctions is a rather nonphysiological event. In the ts-src MDCK cell line described here, junctional disassembly is triggered by activation of a physiologically relevant component, the src oncogene product. Furthermore, both cellular and viral pp60src tyrosine kinases are located at the cell junctions (Rohrschneider, 1980;
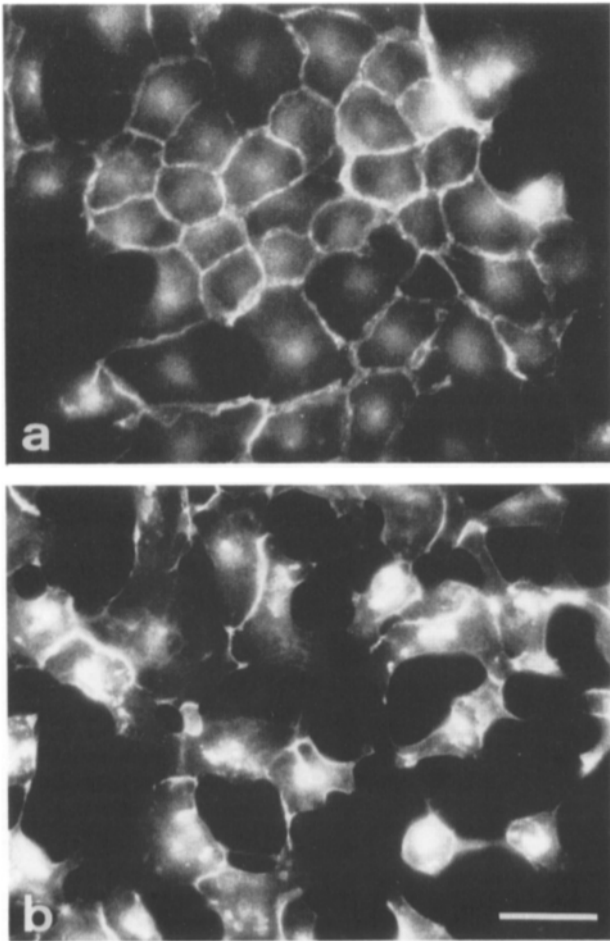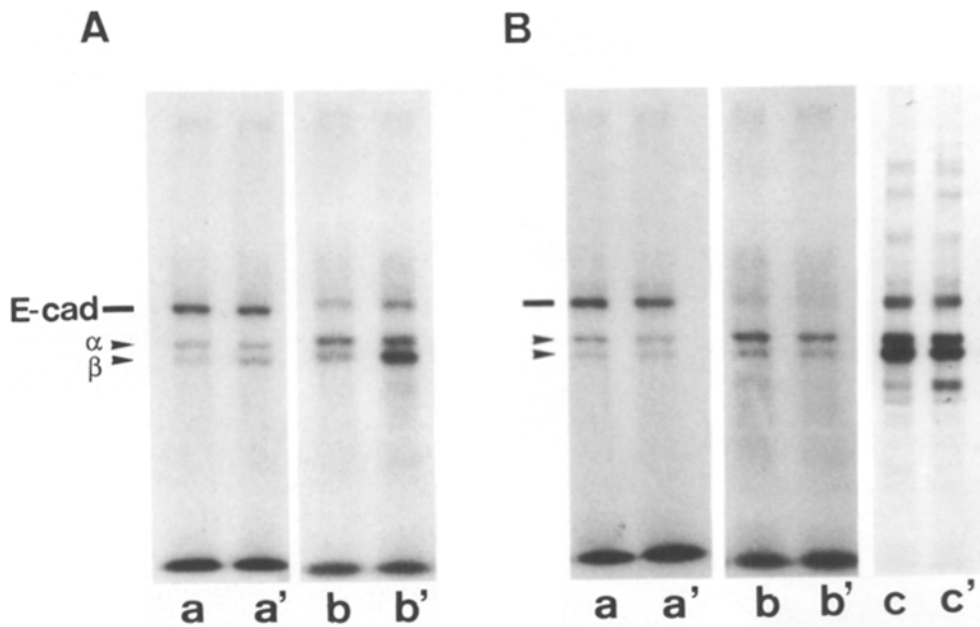
Figure 8. Immunofluorescence staining of E-cadherin in ts-src MDCK cells. Immunofluorescence staining was performed on ts-src MDCK cells cultured at 40.5°C (*a*) or at 35°C (*b*) using anti-E-cadherin antibodies. E-cadherin is concentrated at the contact areas of cells at the nonpermissive temperature, whereas it is more diffusely distributed over the surface of dissociated cells at the permissive temperature. Bar, 30 μm.

Tsukita et al., 1991), and the former might thus also play a role in the control of normal contact formation. Importantly, v-src activity in the ts-src MDCK cells can be rapidly modulated by temperature adjustment, thereby allowing early biochemical effects of pp60^{v-src} on the junctions to be detected. Because of the complete reversibility of cell dissociation at the nonpermissive temperature, the rebuilding of junctions from junction-deficient cells can be studied as well. Thus, a detailed investigation of the localization and phosphorylation pattern of the various junctional components in our ts-src MDCK cells will give new insights in the organization and dynamics of the junctional machinery.

In a previous report, v-src was constitutively expressed in MDCK cell lines, resulting in some deficiencies of cell–cell contacts, mainly at the zonula adherens (Warren and Nelson, 1987). Our results with the temperature-sensitive v-src mutant confirm and extend these studies. In particular, they reveal the rapidity of src effects on cell–cell contacts, and they establish induction of invasiveness as a major consequence of v-src activity in epithelial cells. In the study by Warren and
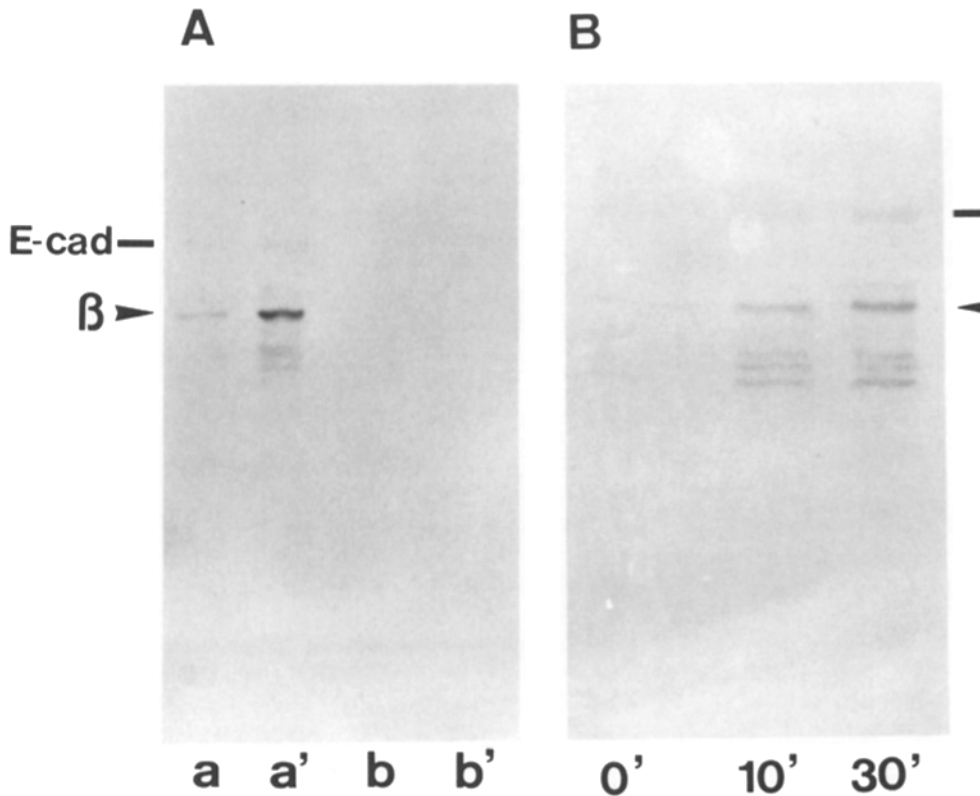
Nelson (1987), v-src–transformed MDCK cells formed distorted cyst-like structures inside collagen gels, but did not show invasion. Furthermore, the disturbance of cell–cell contacts was much less dramatic than in the case of our ts-src MDCK cells. These differences might be explained by the fact that MDCK cells expressing low levels of pp60^{v-src} were analyzed by Warren and Nelson, indicating that v-src effects on epithelial phenotype are dose dependent. Interestingly, overexpression of c-src in MDCK cells did not grossly alter cell–cell adhesion (Warren et al., 1988). After completion of this manuscript, a paper by Matsuyoshi et al. (1992) described alterations of cell–cell adhesion of P-cadherin-expressing rat 3Y1 fibroblasts after introduction of the v-src gene. After aggregation of the v-src–transfected cells in suspension, the degree of compaction was decreased compared to the parental cells. Furthermore, the cadherin/catenin complex was tyrosine phosphorylated in the v-src–expressing fibroblasts. This suggests that although the cell adhesion machinery differs considerably between fibroblasts and epithelial cells, tyrosine phosphorylation of cadherins and catenins might negatively affect cell adhesion in both systems (see also below).

Cell dissociation of our ts-src MDCK cells at the permissive temperature is a fast process, the alterations in cell contacts being visible as early as 15 min after the temperature shift. This indicates that short-term enzymatic reactions rather than changes in transcriptional or translational control are involved in the initial steps of the process. In fact, when studying E-cadherin as a likely target for v-src action, we found that its synthesis or steady state level was unchanged in the dissociated cells. However, we show here that pp60^{v-src} activation leads to increased tyrosine phosphorylation of E-cadherin and in particular, of the associated β-catenin. It is known that E-cadherin function crucially depends on its association with at least three cytoplasmic protein components, the α-, β-, and γ-catenins. Deletion of parts of the cytoplasmic portion of E-cadherin abolishes both the binding of catenins and the cell adhesive function of E-cadherin in transfected fibroblasts (Nagafuchi and Takeichi, 1988, 1989; Ozawa et al., 1989, 1990). Furthermore, overexpression of a mutant form of N-cadherin that lacks the extracellular domain in *Xenopus* embryos disturbed cell–cell adhesion and inhibited catenin binding to E-cadherin (Kintner, 1992). We might therefore hypothesize that tyrosine phosphorylation of β-catenin alters its interaction with E-cadherin and thereby interferes with E-cadherin function. Interestingly, it has also been shown that β-catenin interacts more directly with E-cadherin than the other catenins (McCrea and Gumbiner, 1991; Ozawa and Kemler, 1992). Alternatively, the proposed function of the catenins as mediators of E-cadherin/cytoskeleton interactions could be disturbed by the phosphorylation reaction. The finding that both the E-cadherin/catenin complex and the c-src product are located at the zonula adherens (Boller et al., 1985; Tsukita et al., 1991) supports the idea of a functional relationship between the tyrosine kinase and the cell adhesion system. Since β-catenin is the homolog of the segment polarity gene product *armadillo* of *Drosophila* (McCrea et al., 1991), one could also envisage developmental mechanisms by which tyrosine kinases regulate intercellular adhesion via modification of catenins. A role of tyrosine phosphorylation in the control of junction formation was also suggested by a

Figure 9. Immunoprecipitation of the E-cadherin/catenin complex from ts-src MDCK cells. (*A*) E-cadherin was immunoprecipitated with anti-E-cadherin antibody from ^{32}P-labeled ts-src MDCK cells cultured at 40.5°C (lanes *a* and *b*) or at 35°C (lanes *a'* and *b'*). After SDS-PAGE the gel was exposed for autoradiography before (lanes *a* and *a'*) and after treatment with hot KOH (lanes *b* and *b'*). Bands representing E-cadherin and the coimmunoprecipitated α- and β-catenins are indicated. (*B*) E-cadherin immunoprecipitation from ^{32}P-labeled noninfected MDCK cells (lanes *a*, *a'*, *b*, and *b'*) and [^{35}S]methionine–labeled ts-src MDCK cells (lanes *c* and *c'*) cultured at 40.5°C (lanes *a*, *b*, and *c*) or at 35°C (lanes *a'*, *b'*, and *c'*). In lanes (*a* and *a'*) and (*b* and *b'*) the gel was exposed for autoradiography before and after KOH treatment, respectively. E-cadherin and catenins are indicated as in (*A*).

Figure 10. Tyrosine phosphorylation of the E-cadherin/catenin complex in ts-src MDCK cells as revealed by anti-phosphotyrosine antibody labeling. (*A*) Immunoprecipitates from extracts of ts-src MDCK cells cultured either at 40.5°C (lanes *a* and *b*) or at 35°C (lanes *a'* and *b'*) were subjected to SDS-PAGE, blotted onto nitrocellulose, and probed with anti-phosphotyrosine antibodies. Immunoprecipitation reactions were performed using anti-E–cadherin antiserum (lanes *a* and *a'*) or preimmune serum (lanes *b* and *b'*). Bands representing E-cadherin and β-catenin were identified by comparison with radioactively labeled immunoprecipitates run in adjacent lanes and by Western blot analysis using anti-β–catenin antibodies (not shown). Addition of 5 mM phosphotyrosine but not of phosphoserine or phosphothreonine completely blocked reactivity of the anti-phosphotyrosine antibodies (not shown). (*B*) Anti-E-cadherin was immunoprecipitated form ts-src MDCK cells that have been either cultured at 40.5°C (lane *0'*) or shifted from 40.5° to 35°C for 10 and 30 min, respectively (lanes *10'* and *30'*). Reaction with anti-phosphotyrosine antibodies was performed as in *A*. Bands representing E-cadherin and β-catenin are indicated. The identity of labeled proteins running below the β-catenin band (which were mainly observed after short time temperature shifts) is not known.

recent study in which MDCK cells were treated with tyrosine phosphatase inhibitors (Volberg et al., 1992). This treatment led to an increase of phosphotyrosine-containing proteins at the cell contact areas and to the subsequent disruption of the adherens junctions. Interestingly, certain growth and motility factors that activate receptor tyrosine kinases also disrupt epithelial cell–cell adhesion (see Behrens et al., 1992 for a review). In particular, scatter factor/hepatocyte growth factor acts as a potent dissociating agent of epithelial monolayers in vitro, apparently by activating the

c-*Met* receptor tyrosine kinase (Weidner et al., 1991; Bottaro et al., 1991; Naldini et al., 1991). Similarly, aFGF can disrupt cell–cell adhesion of bladder carcinoma cells by activating a tyrosine kinase receptor (Jouanneau et al., 1991). It will be interesting to analyze whether these receptor-type tyrosine kinases, of which a junctional localization has not yet been shown, also exert their anti-adhesive effects through phosphorylation of the E-cadherin/catenin complex. Since the cloned cDNA of β-catenin has recently become available (McCrea et al., 1991), the potential role of tyrosine phosphorylation can now be studied more directly, e.g. through functional analysis of β-catenin molecules that are mutated at the phosphorylation sites.

What other components of the junctional machinery might be targets of the v-src tyrosine kinase and thus be involved in junctional breakdown in the ts-src MDCK cells? For example, desmosomal proteins like desmogleins and desmocollins (which are also cadherins, cf. Koch et al., 1990; Collins et al., 1991; see Buxton and Magee, 1992, for a review) and desmoplakin might be candidates, although no localization of tyrosine kinases at desmosomes has so far been reported. Vinculin, a component of both cell–substrate as well as cell–cell junctions, appears to be phosphorylated by pp60^{v-src} in fibroblasts (Sefton et al., 1981). Interestingly, tyrosine phosphorylation of the E-cadherin associate α-catenin, which is homologous to vinculin (Nagafuchi et al., 1991; Herrenknecht et al., 1991), is not detectably altered by v-src in the ts-src MDCK cells. Breakdown of gap junctional communication has been observed in src-transformed fibroblasts and is correlated with increased tyrosine phosphorylation of connexins (Azarnia et al., 1988; Filson et al., 1990). However, there is no indication that gap junctions mediate direct adhesion in epithelial cells.

Loss of E-cadherin expression in carcinomas has been correlated with reduced differentiation and increased invasiveness of tumors (Behrens et al., 1989; Frixen et al., 1991; Schipper et al., 1991; Mareel et al., 1991; Shiozaki et al., 1991). However, in certain cases of dedifferentiated tumors E-cadherin expression was apparently normal, yet the tumor cells exhibited reduced intercellular adhesiveness (Shimoyama and Hirohashi, 1991a). In the present study, we suggest that disturbance of intercellular adhesion and induction of in vitro invasion of MDCK cells by v-src is achieved through tyrosine phosphorylation of the E-cadherin/catenin complex. Thus, in dedifferentiated tumors that show normal E-cadherin expression, its function might be abolished by similar biochemical modifications. Because of the reversibility of phosphorylation reactions, differentiated epithelial structures could reform in the metastases after appropriate dephosphorylation of the adhesion system. In fact, metastases of carcinomas can exhibit a differentiated phenotype and produce functional junctions (Weinstein et al., 1976; Gabbert et al., 1985; Schipper et al., 1991). As a consequence of our work, carcinomas should now also be screened for aberrant catenin expression. If misregulation of E-cadherin function through disturbances of catenins is indeed important for invasiveness of carcinoma cells, posttranslational modification, mutation, or loss of expression of catenins could be a frequent event in these tumors.

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