The Redistribution of Cortactin into Cell-Matrix Contact Sites in Human Carcinoma Cells with 11q13 Amplification Is Associated with Both Overexpression and Post-translational Modification*

(Received for publication, July 17, 1996, and in revised form, November 12, 1996)

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The EMS1 gene, located at the chromosome 11q13 region, is the human homologue of p80/p85 cortactin, a chicken pp60src tyrosine kinase substrate. In cells derived from breast carcinomas and squamous carcinomas of the head and neck, DNA amplification of this region results in overexpression of cortactin. Overexpression is accompanied by a partial redistribution of cortactin from the cytoplasm into cell-matrix contact sites. To investigate whether overexpression only is sufficient for this redistribution, we performed biochemical analysis of human cortactin derived from carcinoma cell lines with either normal levels (UMSCC8) or with excessive levels of cortactin due to chromosome 11q13 amplification (UMSCC2). Pulse-chase experiments performed with UMSCC2 cells revealed that p85 originated from p80 by post-translational modifications. However, the conversion of p80 into p85 was hardly observed in UMSCC8 cells, indicating a different processing of the two isoforms in cells with a normal expression level of cortactin. Western blot analysis showed that treatment of UMSCC2 cells with cycloheximide, serum, epidermal growth factor, or vanadate resulted in the disappearance of the p80 form and conversion into p85. Conversion of p80 into p85 was accompanied by a redistribution of cortactin from cytoplasm to cell-matrix contact sites. In UMSCC8 cells, these treatments had no effect on the p80/p85 ratio, and cortactin remained in the cytoplasm. Conversion into p85 therefore is correlated with a relocalization of cortactin to the cell periphery. In addition, p85 from epidermal growth factor- or vanadate-treated UMSCC2 cells showed a significant enhancement in phosphorylation compared with p85 in UMSCC8 cells. Our findings demonstrate that in carcinoma cells with 11q13 amplification not only overexpression but also post-translational modifications of cortactin coincides with the redistribution from the cytoplasm into cell-matrix contact sites.

related with a poor prognosis of patients, which is most likely caused by an elevated invasive potential of these cells (3). On the molecular level, however, little is known about how cells originating from these carcinomas have become increasingly invasive. Overexpression of cyclin D1 in transfection experiments has been reported to shorten the G1 phase and to extend the S phase of the cell cycle (4). Overexpression also gave rise to an altered complex formation of cyclin D1 with preferentially two protein kinases, CDK4 and CDK6 (5). Several reports showed that cyclin D1 overexpression has a stimulating effect on tumor development in cooperation with other oncogenes (4, 6). Finally, in human mantle cell lymphomas, cyclin D1 is overexpressed as a result of a specific chromosomal translocation (7).

However, a role in tumor development has not yet been explored in detail for the overexpressed EMS1 gene. Indications that the EMS1 gene might be an important factor in the process of adhesion or metastasis come from various observations. (i) The EMS1 gene product is designated as the human homologue of cortactin, which appears in two forms (80 and 85 kDa) (8) and which was initially identified as a substrate for the tyrosine kinase pp60src in chicken embryo fibroblasts (9, 10). (ii) Cortactin contains a 7-fold repeat motif that is responsible for the in vitro binding to F-actin (11). In addition, the protein contains a src homology 3 domain that is found in several membrane- and cytoskeleton-associated proteins and that is able to bind to specific proline-rich sequence motifs (12–14). Therefore, cortactin has been suggested to be an important linker protein between membrane-bound receptors and the microfilament system. (iii) Chicken embryo fibroblasts, transformed as a result of Rous sarcoma virus infection (10), showed relocation and accumulation of cortactin from cytoplasm to modified focal adhesion sites called podosomes, which are believed to deregulate the cellular adhesive properties (15). The same localization in podosome-like cell-matrix contact sites was reported in squamous carcinoma cell lines in which human cortactin was overexpressed due to 11q13 amplification. Squamous carcinoma cell lines without 11q13 amplification showed a cytoplasmic distribution of the protein (8).

One factor that guides the redistribution of human cortactin might be tyrosine phosphorylation because chicken embryo fibroblasts transformed with Rous sarcoma virus (10) and mouse embryo fibroblasts that lack the pp60src inhibitor gene csk (16) showed cytoskeletal reorganization, disruption of focal contacts, and formation of podosomes and concomitant redistribution to these podosomes of several focal adhesion-associated proteins, including cortactin. This redistribution was accompanied by increased pp60src tyrosine kinase activity and enhanced tyrosine phosphorylation of cortactin, paxillin, and tensin (10, 16). However, it is not known whether tyrosine phosphorylation directly triggers the redistribution of cortactin

* This work was supported by Grant RUL93-550 from The Dutch Cancer Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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or whether a multistep process is responsible for this phenomenon.

To investigate whether the redistribution observed in human carcinoma cells with 11q13 amplification is due solely to overexpression or whether additional post-translational modifications of the protein are needed for the redistribution, we performed the biochemical characterization of human p80/p85 cortactin. For this purpose we compared the various biochemical properties of the protein between carcinoma cells with a normal expression and carcinoma cells with overexpression of the protein. We report here that in p80/p85 cortactin-overexpressing cells, the addition of serum, cycloheximide, EGF, or vanadate resulted in the conversion of the p80 isoform of cortactin into p85 and also resulted in the subcellular relocalization of cortactin into cell-matrix contact sites. In addition, we observed that during this conversion p85 was phosphorylated. We suppose that in cells with 11q13 amplification not only overexpression but post-translational modifications coincide with the redistribution of human cortactin from the cytoplasm into cell-matrix contact sites.

MATERIALS AND METHODS

**Cell Culture and Antibodies**—The squamous carcinoma cell lines UMSCC2 and UMSCC8 were provided by T. Carey (Department of Otolaryngology, University of Michigan, Ann Arbor). Unless indicated otherwise, cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin (50 units/ml), streptomycin (50 µg/ml), and 10% FCS (all from Life Technologies, Inc.). A rabbit anti-human cortactin antisera RA23 was generated by injecting bovine cortactin, the separate p80 and p85 forms of human cortactin were excised from PVDF membrane and directly subjected to hydrolysis as described previously (18).

**Immunofluorescence**—All procedures were performed at room temperature. Coverslips on glass coverslips were first coated with PBS containing 1 mM CaCl₂, 1 mM MgCl₂, and fixed in PBS including 3.7% formaldehyde (Merck), 1 mM CaCl₂, 1 mM MgCl₂, for 10 min. The fixed cells were rinsed three times for 5 min in PBS and two times in PBS containing 20 mM glucose. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min followed by rinsing three times with PBS. The fixed and permeabilized cells were then pretreated with 1% bovine serum albumin/PBS for 0.5 h and incubated with antibodies diluted in 1% bovine serum albumin/PBS. After washing three times with PBS, the secondary antibodies (anti-rabbit or anti-mouse), conjugated with fluorescein isothiocyanate, were added. Actin was stained with tetramethylrhodamine B isothiocyanate-phalloidin. After three final washing steps in PBS, the glass coverslips were mounted in 20 mM Tris-HCl, pH 8.0, containing 80% glycerol and 0.2 M NaCl in 1.4-diazabicyclo-(Janssen Chimica, Geel, Belgium). Coverslips were sealed with nail polish and analyzed with confocal laser scan microscopy (Bio-Rad).

**RESULTS**

The p80 Form of Human Cortactin in UMSCC2 Cells with 11q13 Amplification Is Rapidly Converted into p85—To study the processing of the p80 and p85 forms of human cortactin, we performed pulse-chase experiments with two squamous carcinoma cell lines, UMSCC8 and UMSCC2. In UMSCC8 cells, which have a normal expression of cortactin, both [32P]methionine/cysteine-labeled p80 and p85 immunoprecipitated just after the 15-min pulse labeling interval. In the presence of unlabeled methionine/cysteine during the chase interval, the ratio between the two labeled forms changed only slightly in favor of p85, whereas the labeled p80 form was still visible after 72 h (Fig. 1). It seemed that the ratio between the intensity of both forms present in these cells was very stable. Remarkably, in UMSCC2 cells containing 11q13 amplification and a 10-fold overexpression of cortactin, only labeled p80 was present after the pulse interval, but following a certain lag time during the chase period, labeled p80 rapidly disappeared synchronously with the appearance of labeled p85 (Fig. 1). The half-life of p80 in UMSCC2 cells was estimated to be 1 h, whereas in UMSCC8 cells the half-life of p80 was approximately 30 h. We concluded that in UMSCC2 cells, p80 is rapidly turned over into p85.

In agreement with the pulse-chase experiments, p80 in UMSCC2 cells disappeared within 2 h upon addition of cycloheximide, which inhibited de novo biosynthesis of p80 (Fig. 2). The appearance of p85 remained the same for 6 h, which was not caused by new protein synthesis. The conversion of p80 into p85 was not observed in cycloheximide-treated UMSCC8 cells, which is in good agreement with the pulse-chase experiments. These results demonstrate that p85 is rapidly formed out of p80 by a post-translational modification and that this conversion was observed only in cortactin-overexpressing UMSCC2 cells.
The Amount of p85 Cortactin in UMSCC2 Cells Is Dependent on Cell Density and EGF—Since a significant part of cortactin in cells with 11q13 amplification is localized in cell-matrix contact sites (8), we investigated the effect of the amount of available contact sites on the cellular ratio between p80 and p85 by Western blot analysis. When UMSCC2 cells were grown to full confluence, a situation in which cells reduce their amount of cell-matrix contact sites, the p80/p85 ratio changed in favor of p80 (Fig. 3). UMSCC8 cells also showed some fluctuations, but they were much less pronounced than in UMSCC2 cells. We also tested whether the ratio between p80 and p85 was dependent on growth stimulation. When UMSCC2 cells were serum-starved for 16 h the amount of p80 was much higher than the amount of p85 (Fig. 4). Treatment of these cells with FCS for 2 h induced a rapid shift to p85, and the p80/p85 ratio remained disturbed for 6 h (not shown). In UMSCC8 cells, however, a lot of p85 was still present after serum starvation, whereas addition of FCS led to a quick restoration of the normal p80/p85 ratio (Fig. 4, lane 3). The requirement of serum factors for the conversion into p85 might indicate that growth factors contributed to the accumulation of p85 in UMSCC2 cells by a yet unknown mechanism. Indeed, when serum-starved UMSCC2 cells were incubated with EGF for different time periods and cortactin was immunoprecipitated, p80 was almost completely converted into p85 within 15 min, whereas in UMSCC8 cells this conversion was not seen and p80 was still present (Fig. 5, upper panels). The changed p80/p85 ratio was not the result of preferential immunoprecipitation of p85, because Western blotting of the total lysates gave the same p80/p85 ratios (data not shown). Therefore, we concluded that formation of p85 in UMSCC2 cells is dependent on the number of available cell-matrix contact sites and on growth stimulation induced by EGF.

Conversion from p80 to p85 Is Accompanied by Phosphorylation—To study whether differences in p80/p85 ratios are due to differences in phosphorylation levels, we first investigated the tyrosine phosphorylation status of cortactin in UMSCC8 and UMSCC2 cells with normal and high expression of cortactin, respectively, by analyzing cortactin immunoprecipitates with the anti-phosphotyrosine antibody PY20. Under normal tissue culture conditions (in the presence of FCS), tyrosine phosphorylation of human cortactin in both cell lines was not or was only poorly observed (Fig. 6, lower panels, lane 1). Furthermore, cortactin could not be detected in anti-phosphotyrosine (PY20) immunoprecipitates, as judged by immunostaining with anti-cortactin antibodies (data not shown). The lack or low levels of tyrosine phosphorylation under normal tissue culture conditions could be due to severe tyrosine phosphatase activity. Indeed, the addition to cells of vanadate, which prevents tyrosine dephosphorylation, strongly elevated the amount of tyrosine-phosphorylated cortactin in both cell lines and at similar levels (Fig. 6, lower panels). These results indicate that human cortactin can serve as a substrate for tyrosine phosphorylation in both cell lines, a situation similar to that reported in other studies performed with murine and chicken cell lines (9, 20).

Treatment of cells with EGF after serum starvation led to a slightly enhanced tyrosine phosphorylation of both p80 and p85 in UMSCC8 cells that decreased again after 1 h (Fig. 5, lower panels). In UMSCC2 cells, tyrosine phosphorylation upon EGF treatment was observed after 15 min only in p85, most proba-
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Fig. 5. p85 cortactin accumulates in UMSCC2 cells as a result of EGF treatment. UMSCC8 and UMSCC2 cells were serum-starved for 16 h prior to treatment with 100 ng/ml EGF at different time periods. Immunoprecipitates were separated by 7.5% SDS-PAGE and transferred to blotting membrane. Each lane contained the same amount of immunoprecipitated cortactin. Experiments were performed four times. Screening was performed with anti-cortactin antiserum ra23 and anti-phosphotyrosine antibody PY20. The p80 and p85 forms of cortactin and the immunoglobulin heavy chain (IgH) are indicated. 

Fig. 6. Tyrosine phosphorylation of p80 and p85 cortactin upon addition of vanadate. UMSCC8 and UMSCC2 cells were treated with increasing concentrations of vanadate for 2 h in DMEM including 10% FCS. Vanadate concentrations are 0 μM (lane 1), 50 μM (lane 2), 100 μM (lane 3), 200 μM (lane 4), 1000 μM (lane 5), and 2000 μM (lane 6). Experiments were performed four times. Proteins were immunoprecipitated with the monoclonal anti-cortactin antibody 4F11. Immunoprecipitates were separated by 7.5% SDS-PAGE and transferred to blotting membrane. Screening was performed with anti-cortactin antisera ra23 and anti-phosphotyrosine antibody PY20. The p80 and p85 forms of cortactin and the immunoglobulin heavy chain (IgH) are indicated.

bly because at that time no p80 was present and all p80 was converted into p85 (Fig. 5, upper panels). The EGF-induced tyrosine phosphorylation of cortactin was considerably lower compared with that in vanadate-treated cells. As in EGF-treated samples, immunoprecipitation of cortactin from UMSCC2 cell lysates pretreated with vanadate showed a shift from p80 to p85. This shift was observed most clearly at higher vanadate concentrations (Fig. 6, upper panels). This indicated that under tyrosine phosphatase-inhibiting conditions, p85 is again the predominant form in UMSCC2 cells. In contrast to UMSCC2 cells, UMSCC8 cells did not show any changes in p80/p85 ratios upon treatment with vanadate, whereas tyrosine phosphorylation was explicitly observed in both forms. Since the level of tyrosine phosphorylation followed the amount of p80 and/or p85 present, it is clear that there is no preferential tyrosine phosphorylation of one of the isoforms. These results indicate that tyrosine phosphorylation cannot fully account for the mobility shift between p80 and p85.

In order to compare the total phosphorylated state of cortactin between UMSCC8 and UMSCC2 cells, we performed metabolic phosphate-labeling experiments and loaded equal amounts of immunoprecipitated cortactin on protein gel. In UMSCC8 as well as in UMSCC2 cells both p80 and p85 were radiolabeled with phosphate (Fig. 7A). Compared with UMSCC8 cells, EGF or vanadate treatment of UMSCC2 cells resulted in a much stronger elevation (5–11-fold) of phosphorylation (Fig. 7A). Under all conditions, even in serum-starved UMSCC2 cells that contained significantly more p80 than p85 (see Figs. 4 and 5), the most strongly phosphorylated form was p85. In UMSCC2 cells, EGF or vanadate treatment resulted in a nearly complete conversion of p80 into p85 (see Figs. 4 and 5). Therefore, the strong enhancement of phosphorylation under these two conditions in UMSCC2 cells can be nearly ascribed to phosphorylation of only p85. In order to identify the amino acid residues that became phosphorylated, p80 and p85 from serum-starved, p85 from EGF-treated, and p85 from vanadate-treated UMSCC2 cells were excised from blotting membrane and subjected to phosphoamino acid analysis. The p80 form derived from serum-starved cells as well as p85 derived from EGF-treated cells contained predominantly phosphoserine and phosphothreonine residues (Fig. 7C). Phosphotyrosine residues were found only in p85 derived from vanadate-treated cells. Phosphotyrosine residues were not detected in EGF-treated UMSCC2 cells even after long exposure times, suggesting that the enhancement of phosphorylation of immunoprecipitated cortactin in EGF-treated UMSCC2 cells is predominantly due to an increase in serine/threonine phosphorylation.

Under all conditions, immunoprecipitates also contained small amounts of several other phosphorylated proteins around 50–60 kDa in both cell lines, whereas EGF-treated UMSCC2 cells contained additional co-immunoprecipitated proteins around 65–68 kDa (Fig. 7A). One of the 50–60 kDa proteins could be identified as pp60src (Fig. 7B). Binding of pp60src seemed not significantly altered by the various treatments. The origin of the other co-immunoprecipitated proteins is presently under investigation.

Shift from p80 to p85 Is Accompanied by an Altered Immunolocalization of Cortactin—To link the effect of the disappearance of p80 and accumulation of p85 to the redistribution of cortactin, we performed confocal laser scan immunofluorescence experiments in the presence of EGF or vanadate. As shown in Fig. 8D, UMSCC2 cells normally showed an intense staining of cortactin around the nucleus in the cytoplasm, and near the border of adhesion sites. Treatment of cells with EGF or vanadate induced redistribution of cortactin from the perinuclear space (Fig. 8, E and F, TOP) to the cell-matrix contact sites in UMSCC2 cells (Fig. 8, E and F, BOTTOM). UMSCC8 cells retained their cytoplasmic cortactin staining under all conditions (Fig. 8, A–C). Besides its tyrosine dephosphorylation-blocking effect, vanadate has been reported to induce focal adhesions and formation of stress fibers (21). UMSCC2 cells showed dramatic morphological changes upon vanadate treatment, including stretching, the formation of stress fibers, and the presence of microspikes (Fig. 8F, BOTTOM). Enhancement of tyrosine phosphorylation, especially at the cell boundaries, was observed in both UMSCC8 and UMSCC2 cells upon vanadate treatment and subsequent staining with the antibody PY20 (data not shown). However, only in UMSCC2 cells was a significant part of cortactin localized in cell-matrix contact sites. There were no indications of any redistribution in UMSCC8 cells, although vanadate treatment also enhanced tyrosine phosphorylation of cortactin in these cells (see Fig. 6). Cortactin in vanadate-treated UMSCC2 cells was mainly found to be co-localized with F-actin at the cell cortex but not with the stress fibers itself (Fig. 9). We conclude that p85 accumulation in cells with cortactin overexpression accompanied the redistribution of the protein to actin microfilaments present in cell-matrix contact sites.

**DISCUSSION**

The control of adhesion of cells to their extracellular matrix is generally dependent on cell-matrix contact sites consisting of
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FIG. 7. Phosphorylation pattern, co-immunoprecipitation of pp60<sup>src</sup>, and phosphoamino acid analysis of p80 and p85 cortactin in UMSCC2 and UMSCC8 cells. A, cells were serum-starved for 16 h and then labeled with 
<sup>32</sup>Porthophosphate for 2 h in the absence (O) or in the presence of 100 ng/ml EGF. Labeling in the presence of 1 mM vanadate was performed for 2 h in the presence of 10% dialyzed FCS (VAN). Subsequently, cells were lysed and p80/p85 was immunoprecipitated with the monoclonal anti-cortactin antibody 4F11. Immunoprecipitates were separated by 7.5% SDS-PAGE and blotted on PVDF membrane, and incorporation of labeled phosphate was analyzed by PhosphoImager analysis. All lanes were exposed for the same period of time. The amount of immunoprecipitated cortactin was the same in each lane, as checked by Western blotting with anti-cortactin antibodies (data not shown). Molecular mass markers (in kDa) are indicated on the left. The increase in the amount of label incorporated into cortactin was calculated as the ratio between the amount of label found in cortactin (p80 + p85) from EGF- or vanadate-treated cells relative to the amount of label found in cortactin (p80 + p85) derived from serum-starved samples. The relative increase was measured to be 2.2- (EGF) and 2.5-fold (vanadate) in UMSCC8 cells and 11- (EGF) and 4.5-fold (vanadate) in UMSCC2 cells. The 60-kDa protein is indicated by the arrow and was further analyzed in panel B by screening with the monoclonal anti-src antibody EMS1. pp60<sup>src</sup> and the immunoglobulin heavy (IgH) and light chains (IgL) are indicated. In panel C the p80 and p85 form derived from serum-starved UMSCC2 cells as well as the p85 forms derived from EGF- and vanadate-treated UMSCC2 cells were excised from the membrane and determined separately on radiolabeled phosphoamino acid incorporation by phosphoamino acid analysis (18). T, phosphothreonine; S, phosphoserine; Y, phosphotyrosine; P, free radiolabeled phosphate. Dashed circles indicate the position of unleaved phosphotyrosine. Spots on the right of the panels are partially hydrolyzed phosphopeptides.

2 E. Schuuring, H. van Damme, H. Brok, and E. Schuuring-Scholtes, unpublished observations.
that in the absence of EGF or vanadate, cortactin was not tyrosine-phosphorylated in UMSCC2 cells (Figs. 5 and 6) despite its partial localization in cell-matrix contact sites (Fig. 8). Furthermore, we did not observe a redistribution of cortactin after EGF or vanadate treatment in UMSCC8 cells, although cortactin was tyrosine-phosphorylated in this cell line.

One hypothesis for the rapid p80-p85 shift in UMSCC2 cells containing 11q13 amplification could be that due to the excessive amounts of cortactin in these cells, a large amount of the protein is no longer able to associate with its normal cellular counterparts, which are present in limiting amounts. This non-associated part of cortactin might then be rapidly available for modification into p85, initiated by a mechanism that is activated in cells with 11q13 amplification. Cooperative action of the other overexpressed gene from the amplified 11q13 region, cyclin D1, could be one of the factors in these cells that at least partially determine this effect. It is speculated that altered complex formation with the protein serine/threonine protein kinase CDK4 or CDK6, as has been reported in UMSCC2 cells (5), leads in some way to impaired control of the balance between p80 and p85 and to an excessive localization of cortactin in cell-matrix contact sites.

We showed that in UMSCC8 as well as in UMSCC2 cell lysates small amounts of pp60-src co-immunoprecipitated with antibodies against cortactin (see Fig. 7B). Although in UMSCC2 cells the p80/p85 ratios changed upon serum starvation or EGF treatment, we did not see any drastic changes in the association of pp60-src with cortactin (see Fig. 7, A and B). This suggests that there is no difference between p80 and p85 concerning the binding affinity for pp60-src. Another report showed that the src homology 2 domain of pp60-src facilitated the binding to tyrosine-phosphorylated cortactin (32). Furthermore, pp60-src can be recovered from a cytoskeletal cell fraction upon activation by EGF (33) or platelet-derived growth factor (34, 35). Therefore a possible role of tyrosine-phosphorylated cortactin could be the aggregation of pp60-src to the cytoskeleton, which was also a suggested mechanism in blood platelets (30). We therefore determined the pp60-src activity in total cell lysates or in cytoskeleton or membrane subcellular cell fractions, but there were no significant differences found between the two cell lines. Interestingly, cortactin showed a

3 B. Verbeek and G. Rijksen, unpublished observations.
very high homology with HS1/LckBP, a protein found in human and murine B- and T-cells (36–39). This protein has been reported to mediate signaling events upon B-/T-cell activation and has been shown to bind to the src-like tyrosine kinases p56<sup>lysrc</sup> and p56<sup>cri</sup> (36, 39).

Changes in cell-matrix contact sites sometimes resulted in the formation of podosomes, which were initially described in monocytes and macrophages (40) but are believed to be more widespread among different cell types, including virus-transformed fibroblasts (15), bone-resorbing osteoclasts (41), malignant B-cell lymphomas (42), and carcinomas (8). It is speculated that cortactin overexpression, modification, and transport to the cell-matrix contact sites occurs simultaneously with the appearance of podosomes representing structures with decreased adhesive capacity. One might expect that the large amounts of cortactin together with the accumulation of its p85 form deregulate important protein-protein interactions that are required for the proper formation of these cell-matrix contact sites. In good agreement with this hypothesis, we recently observed that cortactin overexpression, as a result of gene transfer, in particular cell lines resulted in anchorage-independent growth and distortion of cellular actin microfilaments. Since cortactin is overexpressed due to 11q13 amplification in many carcinomas, the EMS1 gene might be one of the genes that modulates the cellular adhesive properties.

Acknowledgments—We thank J. T. Parsons and J. Bartek for the generous gift of the monoclonal antibodies 4F11 and DCS-6, respectively, and B. Verbeek for the determination of pp60<sup>c-src</sup>-like tyrosine kinases that modulates the cellular adhesive properties.

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