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Motility Enhancement by Tumor-Derived Mutant E-Cadherin Is Sensitive to Treatment with Epidermal Growth Factor Receptor and Phosphatidylinositol 3-Kinase Inhibitors

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Diffuse-type gastric and lobular breast cancers are characterized by frequent mutations in the cell adhesion molecule E-cadherin. Here we report that tumor-associated mutations of E-cadherin enhanced random cell movement of transfected MDA-MB-435S mammary carcinoma cells as compared to wild-type (*wt*) E-cadherin-expressing cells. The mutations included *in frame* deletions of exons 8 or 9 and a point mutation in exon 8 which all affect putative calcium-binding sites within the linker region of the second and third extracellular domain. Motility enhancement by mutant E-cadherin was investigated by time-lapse laser scanning microscopy. Increased cell motility stimulated by mutant E-cadherin was influenced by cell–matrix interactions. The motility-increasing activity of mutant E-cadherin was blocked by application of pharmacological inhibitors of epidermal growth factor receptor and phosphatidylinositol (PI) 3-kinase. Investigation of the activation status of PI 3-kinase and the downstream signaling molecules Akt/protein kinase B and MAP kinase p44/42 showed that these kinases are not more strongly activated in mutant E-cadherin-expressing cells than in *wt* E-cadherin-expressing cells. Instead, the basal level of PI 3-kinase is necessary for mutant E-cadherin-enhanced cell motility. Our data suggest a critical role of E-cadherin mutations for the fine tuning of tumor cell motility.

Key Words: **E-cadherin; cell motility; EGF receptor; PI 3-kinase; MAP kinase; Akt/protein kinase B.**

INTRODUCTION

The cell adhesion molecule E-cadherin is a transmembrane receptor protein which mediates adhesive interactions between epithelial cells and regulates the organization of the actin cytoskeleton via its cytoplasmic binding partners, the catenins [1, 2]. E-cadherin acts as a suppressor of tumor invasion and is often downregulated or mutated in invasive and metastatic tumors [3, 4]. Somatic E-cadherin mutations were found in diffuse-type gastric carcinomas which are characterized by scattered tumor cell morphology and poor prognosis [5–8] and in breast and ovarian carcinomas [9]. Germline E-cadherin mutations have been identified in families with diffuse-type gastric carcinoma [10–13]. Recently, E-cadherin has been shown to be part of signal transduction pathways although the molecule lacks intrinsic enzymatic activity [14–16]. Moreover, this adhesive receptor inhibits cell proliferation [17] and upregulates the cyclin-dependent kinase inhibitor p27 [18].

Accumulating evidence suggests that E-cadherin plays an important role in outside-in signal transduction. For instance, E-cadherins activate MAP kinase through EGFR[2] [16]. Moreover, formation of E-cadherin-based adherens junctions triggers activation of the PI 3-kinase–Akt/PKB pathway [14]. So far, there is no evidence that cadherins signal by themselves; instead, associated signaling proteins are likely to mediate the effects.

We have recently identified E-cadherin mutations in diffuse-type gastric carcinomas [5, 6] and investigated

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[2] Abbreviations used: PKB, Akt/protein kinase B; *del 8* E-cadherin, E-cadherin with deletion of exon 8; *del 9* E-cadherin, E-cadherin with deletion of exon 9; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCS, fetal calf serum; *p8* E-cadherin, E-cadherin with point mutation in exon 8; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; TLC, thin-layer chromatography; *wt* E-cadherin, wild-type E-cadherin.

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the functional effects of the mutant molecules in cell culture [19, 20]. The mutated E-cadherin molecules resulted in decreased cell adhesion and aggregation and in enhanced migration of MDA-MB-435S mammary carcinoma cells and L929 fibroblasts in wound-healing assays as compared to cells expressing *wt* E-cadherin. By these wound-healing experiments, we found that the region within the E-cadherin molecule responsible for its migration suppressor function resides within the linker region between domains 2 and 3. Mutant E-cadherin molecules were partially perinuclearly localized and caused perinuclear localization of its cytoplasmic binding partner β-catenin. An epithelial to mesenchymal transition upon expression of mutant E-cadherin was indicated morphologically.

In the present study, an increase in cell motility and cell speed of individual MDA-MB-435S mammary carcinoma cells expressing E-cadherin mutated within the extracellular domain compared to cells expressing *wt* E-cadherin was shown by time-lapse laser scanning microscopy. Enhanced cell motility stimulated by mutant E-cadherin was dependent on cell–matrix interactions and sensitive to treatment with EGFR and PI 3-kinase inhibitors. Our findings indicate that tumor-associated mutations in E-cadherin play a critical role in tumor cell motility.

MATERIALS AND METHODS

Cell cultivation and transfection. The human E-cadherin-negative mammary carcinoma cell line MDA-MB-435S (ATCC, Rockeville, USA) and the E-cadherin–cDNA transfected derivatives that were described by Handschuh *et al.* [19] were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany) and penicillin–streptomycin (50 IU/ml and 50 μg/ml; Life Technologies) at 37°C and 5% CO_2.

Cell motility studies. For time-lapse laser scanning microscopy, cells were cultivated in a microscope-coupled incubation chamber (Zeiss, Jena, Germany) at 37°C under 5% CO_2. Cells were seeded at a density of 2×10^5 cells per 3.5-cm plate with a glass bottom; these plates were purchased either uncoated or coated with polylysine from MatTek Corp. (Ashland, MA). Uncoated plates were coated for 4 h at 37°C with collagen I (100 μg/ml; Sigma, Deisenhofen, Germany) or overnight at 4°C with fibronectin (10 μg/ml; Sigma) or vitronectin (10 μg/ml; Becton–Dickinson, Bedford, USA). Kinase inhibitors were used at final concentrations of 50 μM (PD 98059; Sigma), 40 μM (LY 294002; Calbiochem, Schwalbach, Germany), or 6.3 μM (Tyrphostin AG 1478; Sigma). EGF was used at a concentration of 100 ng/ml (Sigma).

Phase-contrast images were taken at 3-min intervals with an Axiovert laser scanning microscope LSM 510 (Zeiss) with lens PNF 20×/0.4 PH2 and a helium–neon laser at 543 nm in transmission scanning mode. The percentage of motile cells was measured by drawing the outlines of cells on the screen and counting the cells which moved completely out of the initial area within the recording time of 7 h. Semiautomatic tracing of cell nuclei using the laser scanning microscope software from Zeiss allowed determination of the individual cell speed. The calculation of the cell speed is based on the division of the displacement of an individual cell divided by the total time of recording.

Cell adhesion assay. Flat-bottomed 96-well microtiter plates (Nunc, Wiesbaden-Biebrich, Germany) were coated overnight at 4°C with poly-L-lysine (150 $\mu g/cm^2$), collagen I (10 $\mu g/cm^2$), fibronectin (0.9 $\mu g/cm^2$), or vitronectin (0.45 $\mu g/cm^2$). Poly-L-lysine, collagen I, fibronectin, and vitronectin were purchased from Sigma. Cells were treated with versene (0.53 mM EDTA in phosphate-buffered saline (PBS); Life Technologies) to preserve cell surface receptors and then seeded on the different matrices at a density of 10^4 cells per well in 100 μl DMEM without FCS. Cells were allowed to adhere to the substrata for 20 min at 37°C and 5% CO_2 in a cell culture incubator. Unattached cells were removed by washing two times with Dulbecco's PBS without calcium and magnesium (PAA Laboratories, Cölbe, Germany). Fresh medium was added to the residual attached cells and cell viability was determined by XTT-cell proliferation and viability assay (Roche Molecular Biochemicals, Mannheim, Germany); 50 μl XTT labeling mixture was added to each well. The cleavage of the tetrazolium salt XTT to form a formazan dye that occurs in metabolically active viable cells was quantified spectrophotometrically by measuring the absorbance of the formazan product at 450 nm by an enzyme-linked immunosorbent assay plate reader. The absorbance values obtained when culture medium without cells was measured were subtracted from the values obtained with cells. Quadruplicate determinations were performed for each value.

Flow cytometry. Cells were harvested with versene, and 5×10^5 cells were incubated with 4 μg/ml monoclonal antibodies directed to α1 or β1 integrin (Chemicon, Temecula, CA) for 1 h on ice in PBS, washed with 0.1% sodium azide and 0.1% bovine serum albumin (Sigma), and stained with DTAF-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, USA) for 1 h on ice. Purified mouse IgG1 (Pharmingen, Heidelberg, Germany) were used as κ immunoglobulin isotype controls. Cells were analyzed on a Beckman Coulter Epics XL (Beckman Coulter, Krefeld, Germany).

Western blot. For immunoblot analysis, cells were seeded at a density of 6×10^5 cells per 10-cm tissue culture dish and lysed 5 h later with 500 μl L-CAM buffer (140 mM NaCl, 4.7 mM KCI, 0.7 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM Hepes, pH 7.4, containing 1% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF)) [21]. Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis followed by transfer to nitrocellulose (Schleicher & Schuell, Dassel, Germany) or PVDF (Bio-Rad Laboratories, Munich, Germany) membranes. Polyclonal antibodies against total or activated kinases were purchased from New England Biolabs (Frankfurt, Germany): Akt antibody (No. 9272), detecting total Akt kinase levels; phospho-Akt antibody (Ser473, No. 9270), detecting phosphorylated Ser 473 in Akt1, Akt2, and Akt3; MAP kinase antibody (No. 9102), detecting total MAP kinase; and phospho-p44/42 MAP kinase (No. 9101), detecting activated MAP kinase phosphorylated at Thr 202/Thr 204. For signal detection the enhanced chemoluminescence system (Amersham Pharmacia Biotech, Braunschweig, Germany) was used. Densitometric analysis was performed with Scion Image Software from Scion Corp. (Frederick, USA).

PI 3-kinase assay. For the detection of PI 3-kinase activity, a protocol from Upstate Biotechnology (Lake Placid, USA) was used. Cells were seeded at a density of 1.6×10^6 cells per 15-cm tissue culture plate and grown for 5 h in DMEM supplemented with 10% FCS. As a positive control, cells were treated with 100 ng/ml EGF for 2 min prior to lysis. Cells were lysed with 137 mM NaCl, 20 mM Tris–HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1% Nonidet-P40, and 1 mM PMSF. After preclearing with protein A agarose beads (Amersham Pharmacia Biotech), anti-PI 3-kinase p85 antibody (No. 06–195; Upstate Biotechnology) was added to the lysates for 2 h at 4°C; then protein A agarose beads were added for 1 h at 4°C and the immunoprecipitates were collected by centrifugation. Each pellet was washed three times with lysis buffer, three times with 0.1 M Tris–HCl, pH 7.4, 5 mM LiCl, 0.1 mM sodium orthovanadate, and two times with TNE buffer (0.1 M Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium orthovanadate) and resuspended in 50 μl TNE buffer. After addition of 20 μg phosphatidylinositol (Sigma) and 10 μl 100 mM $MgCl_2$, the reactions were

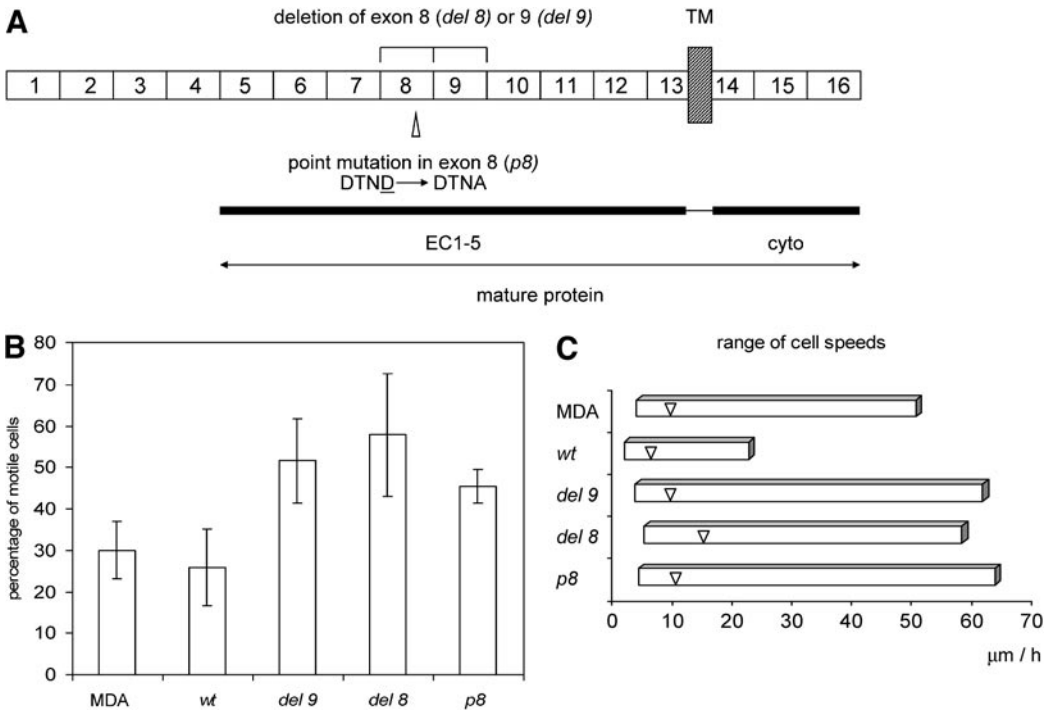

FIG. 1. Enhancement of cell motility by mutant E-cadherin. (A) Schematic drawing showing the exon structure of E-cadherin–cDNA used for transfection of MDA-MB-435S cells. The point mutation in exon 8 changes the codon GAT (position 370; clone HSECAD, Genbank/EMBL Z13009) to GCT (aspartic acid to alanine), thereby mutating the putative calcium-binding site DTND to DTNA. TM, transmembrane domain; cyto, cytoplasmic domain; EC1-5, extracellular domain 1 to 5. (B) Nontransfected MDA-MB-435S cells (MDA) and transfected MDA-MB-435S cells expressing *wt* or mutant (*del 9, del 8, p8*) E-cadherin–cDNAs were plated on collagen I-coated glass plates. Phase-contrast images were taken every 3 min for 7 h with a laser scanning microscope equipped with a temperature- and CO_2-controlled incubation chamber, starting 2 h after plating. The percentage of motile cells was determined by counting cells of a microscopic field which moved completely out of the initial area within the time of the record. Only attached nondividing cells that did not leave the observation field during the period of investigation were analyzed. Each bar represents the mean ± SD of at least three independent experiments. A total of at least 60 cells was investigated for each cell line in at least three independent experiments. (C) Semiautomatic tracing of cell nuclei allowed determination of the individual speed of 60 cells for each cell line derived from at least three different microscopic fields. Calculation of the cell speed is based on the division of the displacement of an individual cell divided by the total time of recording. The bars represent the range between the minimal and the maximal cell speed in a population of 60 cells per cell line derived from three independent microscopic fields. Arrowheads indicate the median of cell speeds.

started by adding 5 μl per sample of [γ-^{33}P]ATP solution (Amersham Pharmacia Biotech; 0.88 mM ATP containing 20 μCi [γ-^{33}P]ATP, 2000 Ci per mmole, and 20 mM $MgCl_2$). As a control, 20 μM LY 294002 was included in the reaction sample. After incubation for 10 min at 37°C, the reaction was stopped with 6 N HCl. Chloroform/methanol (1:1, v/v) was added to extract the radiolabeled lipid. The organic phase was spotted on a thin-layer chromatography (TLC) plate (Merck, Darmstadt, Germany) and the TLC plate was developed in chloroform/methanol/H_2O/NH_4OH (43:38:7:5). Radiolabeled lipids were visualized by autoradiography.

RESULTS

Expression of Mutant E-Cadherin Enhances Random Cell Movement as Compared to wt E-Cadherin

In the present study, MDA-MB-435S mammary carcinoma cell transfectants expressing either *wt* or mutant E-cadherin cloned from diffuse-type gastric carcinomas were compared with respect to their individual motile behavior. As outlined in Fig. 1A, the mutations were deletions of exons 8 (*del 8*) or 9 (*del 9*) and a point mutation in exon 8 (*p8*) as described elsewhere in detail [19]. Expression of *wt* or mutant E-cadherin–cDNA after transfection of E-cadherin-negative MDA-MB-435S cells was recently shown by Western blot analysis and immunofluorescence staining [19]. Enhanced migration of MDA-MB-435S cells expressing mutant E-cadherin as compared to *wt* E-cadherin was demonstrated by wound-healing assay [19]. In the wound-healing assay, the observed cell migration results from a number of combined effects, such as cell–cell and cell–matrix interactions, cell proliferation and secretion of growth factors, and extracellular matrix (ECM) components. Therefore, in the present study, we analyzed the motility of individual E-cadherin-expressing cells by time-lapse laser scanning microscopy. At least two independent cell clones were investigated for each E-cadherin expression construct used in this study.

Cells were brought in suspension by disruption of cell–cell and cell–matrix interactions and seeded on

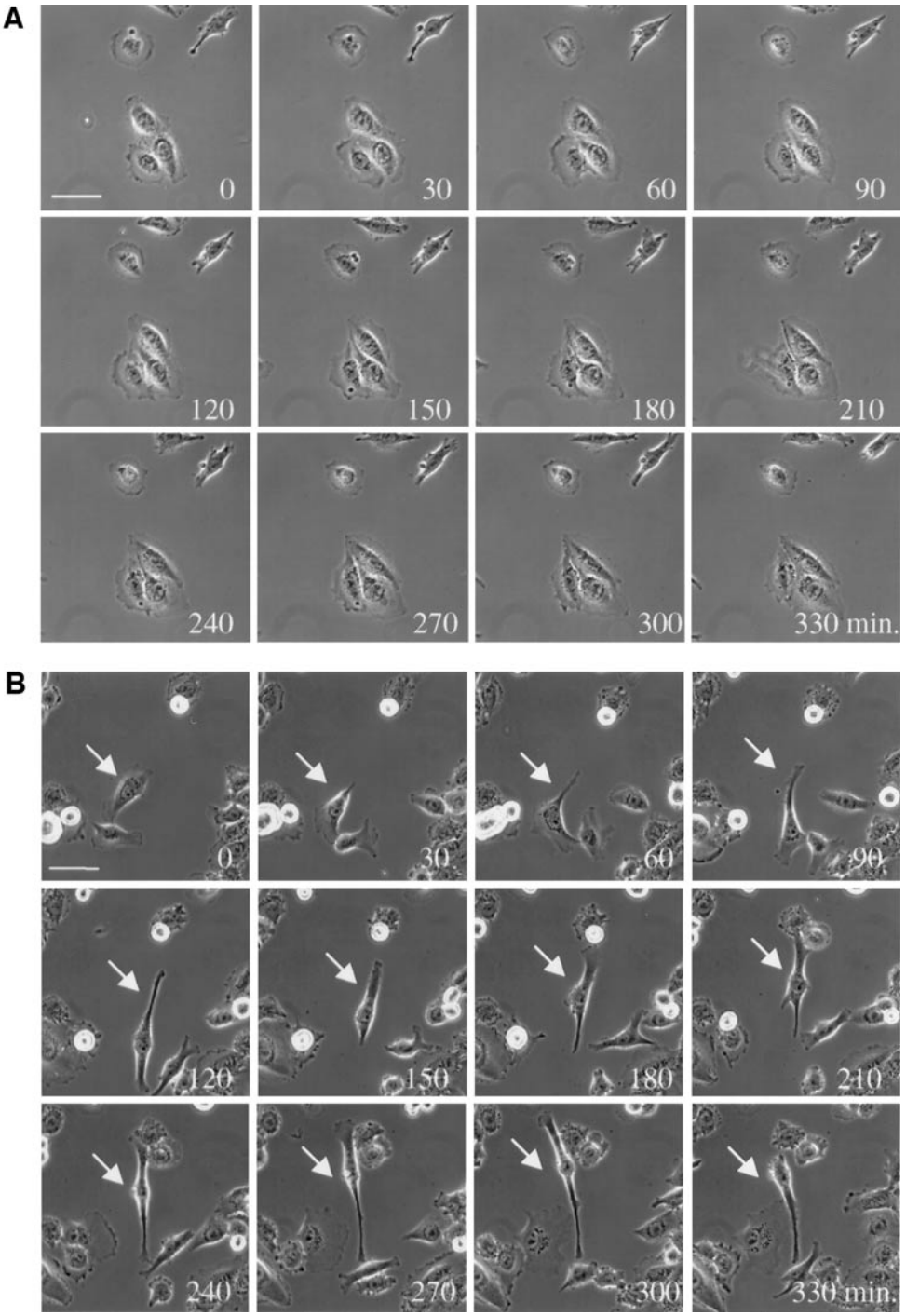

FIG. 2. Mutant E-cadherin affects the style of cell movement. Cells were plated on type I collagen-coated dishes and examined by laser scanning microscopy. Individual cells attached on collagen I-coated dishes were tracked for the indicated time points, starting 2 h after plating. Shown are MDA-MB-435S cells expressing *wt* E-cadherin (A) or *del 8* E-cadherin (B); one motile cell is marked by an arrow. The bars represent 50 μm.

glass plates precoated with collagen type I. Cell motility was analyzed by long-term recordings of a duration of 7 h starting 2 h after plating using a laser scanning microscope coupled with a temperature- and CO_2-controlled incubation chamber. Motile cells were defined as cells which were able to move out of their initial space within 7 h according to a method described by Marks *et al.* [22]. The percentage of motile cells was similar in nontransfected (30%) or *wt* E-cadherin-expressing (26%) MDA-MB-435S cells but elevated upon

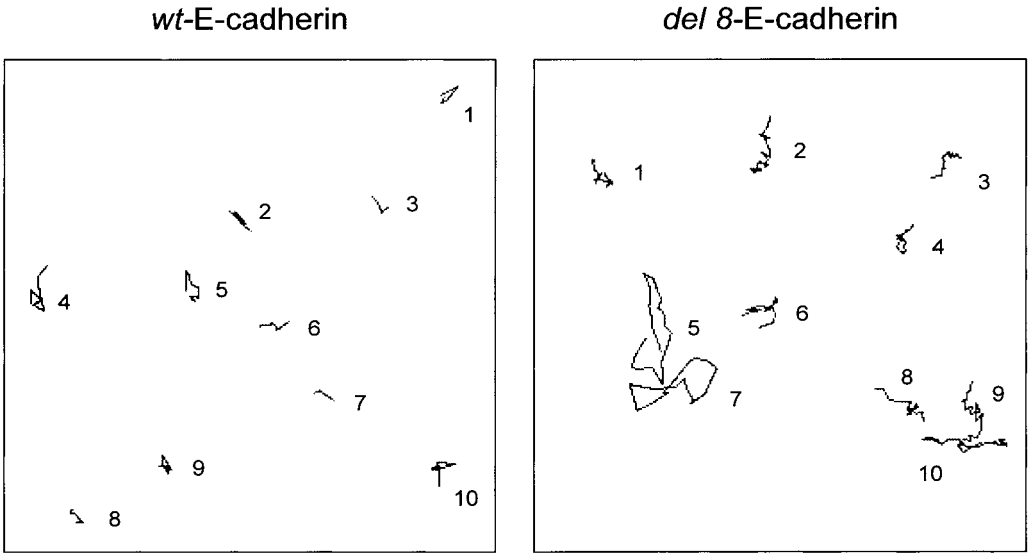

FIG. 3. Mutant E-cadherin induces random cell movement. MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin were recorded for 7 h starting 2 h after plating on collagen I-coated dishes. Cell nuclei were traced semiautomatically using the Zeiss LSM software. Shown are the paths of 10 randomly chosen cells within a microscopic field (460 × 460 μm).

expression of mutant E-cadherin: *del 9* (52%), *del 8* (58%), and *p8* (45%) (Fig. 1B).

It has been shown for a number of cell lines that the motile behavior of individual cells is variable and therefore it is advisable to investigate a large number of cells to obtain a reliable determination of cellular velocity [23]. To determine the motility of individual cells, the center of the nucleus was semiautomatically traced and the speed of 60 cells per cell line derived from at least three independent experiments was calculated. The range between the minimal and the maximal cell speed and the median of cell speeds were used as motility parameters. Expression of mutant E-cadherin induced broad speed ranges and higher maximal speeds in MDA-MB-435S cells expressing *del 9* (3.0–58.1 μm/h), *del 8* (4.6–53.0 μm/h), or *p8* (3.6–59.5 μm/h) E-cadherin compared to *wt* E-cadherin-expressing (1.2–20.8 μm/h) or parental (3.4–46.6 μm/h) cells (Fig. 1C). The medians of cell speeds were higher in cells expressing *del 9* (10.4 μm/h), *del 8* (16.0 μm/h), or *p8* (10.7 μm/h) E-cadherin or in nontransfected parental cells (10.4 μm/h) than in *wt* E-cadherin-expressing cells (7.0 μm/h) (Fig. 1C). These data suggest that expression of *wt* E-cadherin, in contrast to mutant E-cadherin, considerably decreases the median of cell speeds of a cell population, presumably by increasing cell-to-cell contacts which counteract cell migration.

In the following experiments, *del 8* E-cadherin, as a prototype of mutant E-cadherin, was compared to *wt* E-cadherin. The motile behavior of cells in the absence of a chemotactic agent was described as random movement [24]. As shown in Fig. 2A, MDA-MB-435S cells expressing *wt* E-cadherin formed small colonies even at low density and revealed low locomotion activity. In contrast, cells expressing *del 8* E-cadherin had a strong tendency to separate from each other and to form lamellipodial protrusions (Fig. 2B). A significant number of cells underwent random cell migration which was characterized by frequent and abrupt changes of the direction of movement. Often, *del 8* E-cadherin-expressing cells separated from the surrounding cells immediately after cell division rather than attaching to other cells and forming colonies. Plots derived from the paths of 10 randomly chosen cells show that mutant E-cadherin affects the style of cell movement and enhances random cell migration (Fig. 3).

Enhancement of Cell Motility by Mutant E-Cadherin Is Dependent on ECM Conditions

Cell adhesion to the substrate plays a critical role in cell migration. Therefore, we determined the influence of extracellular matrices on cell motility and adhesion. Cells plated on polylysine, fibronectin, or vitronectin were well spread, but only cells expressing *del 8* E-cadherin plated on collagen I showed the typical morphology of migrating cells with broad lamellipodiae at the leading edge and a trailing end.

To determine the adhesion of cells on the various ECM proteins, MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin were allowed to adhere for 20 min on cell culture plates precoated with polylysine, collagen I, vitronectin, or fibronectin, and the attached viable cells were quantified (Fig. 4A). Cell adhesion on polylysine and vitronectin was stronger than that on collagen I or fibronectin and within the same range for *wt* or *del 8*

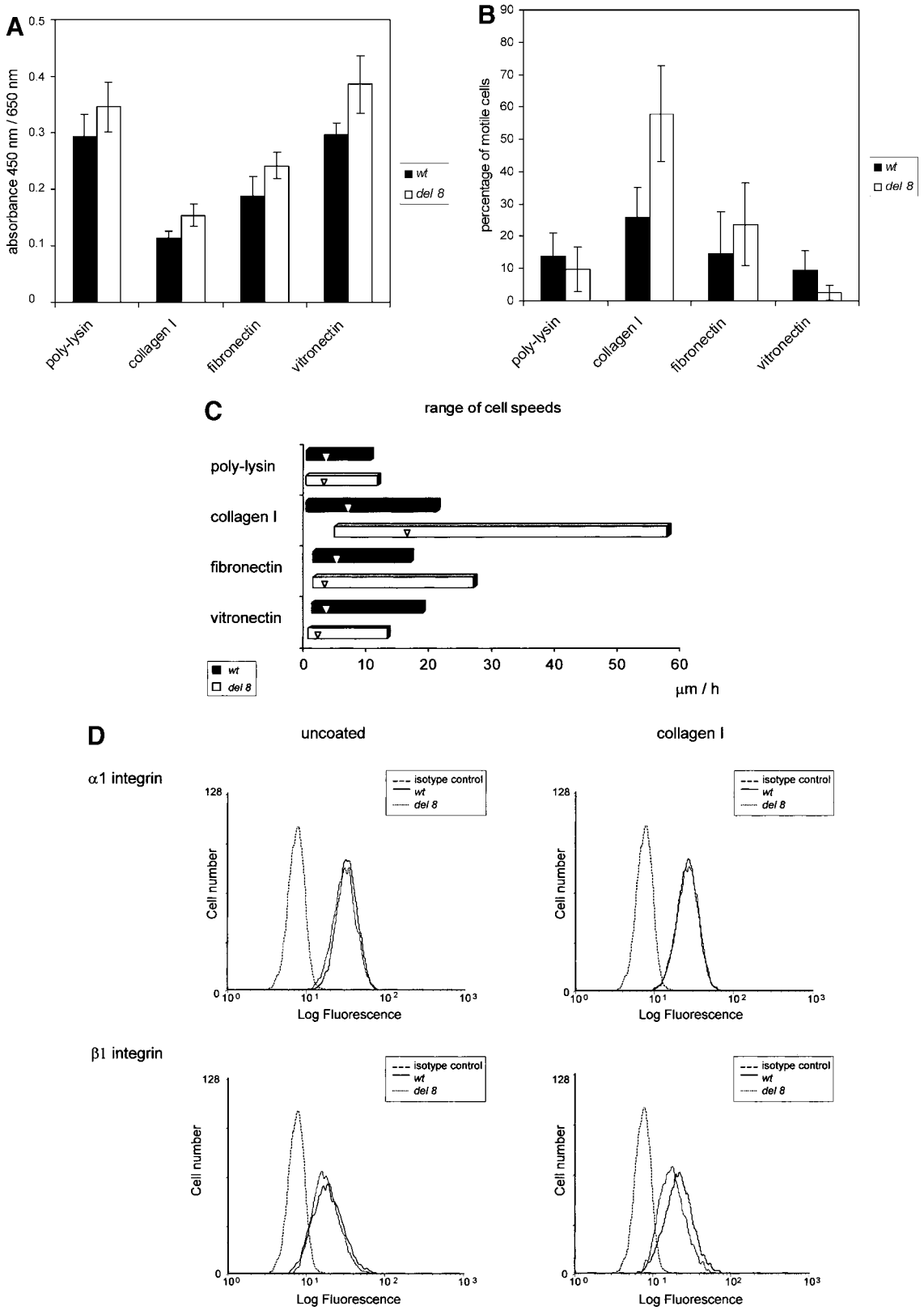

FIG. 4. Cell adhesion and motility of MDA-MB-435S cells expressing *wt* or *del 8*-E-cadherin–cDNA on different ECM proteins. (A) Cells were seeded on polylysine-, collagen I-, fibronectin-, or vitronectin-coated plates and allowed to adhere to the different purified ECM proteins for 20 min at 37°C and 5% CO_2 in a cell culture incubator. Nonadherent cells were removed after 20 min and cell viability of the residual

E-cadherin-expressing cells. Next, the relationship between adhesion to the ECM and cell motility was examined (Fig. 4B). Cell motility was inversely correlated with cell–matrix adhesion. Only *del 8* E-cadherin-expressing cells plated on collagen I were able to migrate significantly (Figs. 4B and 4C). Flow cytometric analysis of collagen-specific integrin $\alpha 1$ which pairs with the integrin $\beta 1$ subunit carried out with nontransfected, *wt*, and mutant E-cadherin-expressing MDA-MB-435S cells revealed that both integrins are expressed at similar levels in all tested cell lines (Fig. 4D). Taken together, these data suggest that ECM conditions influence the migratory behavior of cells and that strong cell–substrate adhesive interactions counteract cell migration. *wt* E-cadherin-expressing MDA-MB-435S cells show low locomotion activity on all ECM proteins tested, indicating that strong cell–cell adhesive forces also block cell motility.

Motility Enhancement by Mutant E-Cadherin Is Sensitive to Inhibitors of EGFR and PI 3-Kinase

Cell migration is influenced by a number of factors including cell–cell and cell–matrix interactions and transduction of extracellular signals into cells. Next, the contribution of the cell motility-associated EGFR pathway to mutant E-cadherin-enhanced cell motility was investigated. The percentage of motile *del 8* E-cadherin-expressing MDA-MB-435S cells and the median and range of cell speeds decreased to those levels of *wt* E-cadherin-expressing cells upon treatment with the specific EGFR kinase inhibitor Tyrphostin AG 1478 (Figs. 5A and 5B) [25].

EGFR activates diverse downstream signaling molecules including PI 3-kinase, Akt/PKB, and MAP kinase [26]. To determine the importance of these signaling molecules for mutant E-cadherin-enhanced cell motility, cell tracking experiments were performed in the presence of MAP kinase kinase inhibitor PD 98059, which prevents threonine and tyrosine phosphorylation of MAP kinase [27, 28], or the synthetic PI-3 kinase inhibitor LY 294002 [29]. Cell motility could be blocked by LY 294002, whereas PD 98059 was less effective (Figs. 5A and 5B). To determine whether PI 3-kinase was activated by mutant E-cadherin, PI 3-kinase activity was measured. Similar activation levels were observed for all tested cell lines, suggesting that a basic activation level rather than stimulation of PI 3-kinase by mutant E-cadherin is necessary for enhanced cell motility stimulated by mutant E-cadherin (Fig. 6). PI 3-kinase activation was sensitive to LY 294002 (Fig. 6).

Next, the effect of EGF on the motility of MDA-MB-435S transfectants was tested. The percentage of motile *wt* E-cadherin-expressing MDA-MB-435S cells was increased to the level of *del 8* E-cadherin-expressing cells in response to EGF treatment (Fig. 5A). EGF-treated cells developed filopodia and lost cell-to-cell contacts (Fig. 5C). The influence of PD 98059, LY 294002, Tyrphostin AG 1478, and EGF on cell morphology is shown in Fig. 5C.

The specificity of the kinase inhibitors used in this study was tested by Western blot analysis using phosphorylation-specific antibodies. Tyrphostin AG 1478 completely blocked EGF-induced activation of MAP and Akt/PKB kinases (data not shown). LY 294002 inhibited EGF-induced Akt/PKB activation to a similar extent and PD 98059 effectively blocked MAP kinase phosphorylation (data not shown).

Akt/PKB Activity Is Increased by Expression of wt as Compared to Mutant E-Cadherin, Whereas Members of the MAP Kinase Family Are Not Affected

The importance of MAP kinase activation for cell adhesion, spreading, and motility has recently been demonstrated [30–32]. To further investigate the role of p44/42 MAP kinase, a downstream signaling molecule of PI 3-kinase, in mutant E-cadherin-enhanced cell motility, p44/42 MAP kinase expression levels and activities were measured in nontransfected and *wt* or mutant E-cadherin–cDNA-expressing MDA-MB-435S cells.

Western blot analysis performed with antibodies specific for the phosphorylated, active forms of MAP kinases p44 and p42 or detecting total MAP kinase revealed a slight increase of p44 and p42 MAP kinase activity in MDA-MB-435S cells expressing *wt* or mutant E-cadherin as compared to nontransfected parental cells on uncoated or collagen I-coated tissue cell culture plates. This increase was accompanied by elevated MAP kinase steady-state expression levels (Fig. 7A).

Next, activity and expression of Akt/PKB, another downstream molecule of PI 3-kinase, were investigated

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attached cells was determined as described for XTT-cell proliferation and viability assay. Quadruplicate determinations were performed for each value and the mean + SD is shown. The figure shows one representative of three independent experiments. (B) Cells were plated on glass plates coated with polylysine, collagen I, fibronectin, or vitronectin and cell motility was analyzed as described in the legend to Fig. 1B. Each bar represents the mean ± SD of at least three independent experiments. (C) Shown are the medians and ranges of cell speeds, determined as described in the legend to Fig. 1C. (D) Flow cytometric analysis was carried out on nontransfected, *wt*, and mutant (*del 9*, *del 8*, *p8*) E-cadherin-expressing cells stained with monoclonal antibodies to $\alpha 1$ and $\beta 1$ integrins and DTAF-conjugated anti-mouse IgG or isotype control. Similar results were obtained for all tested cell lines; only the results for *wt* and *del 8* E-cadherin-expressing cells are shown.

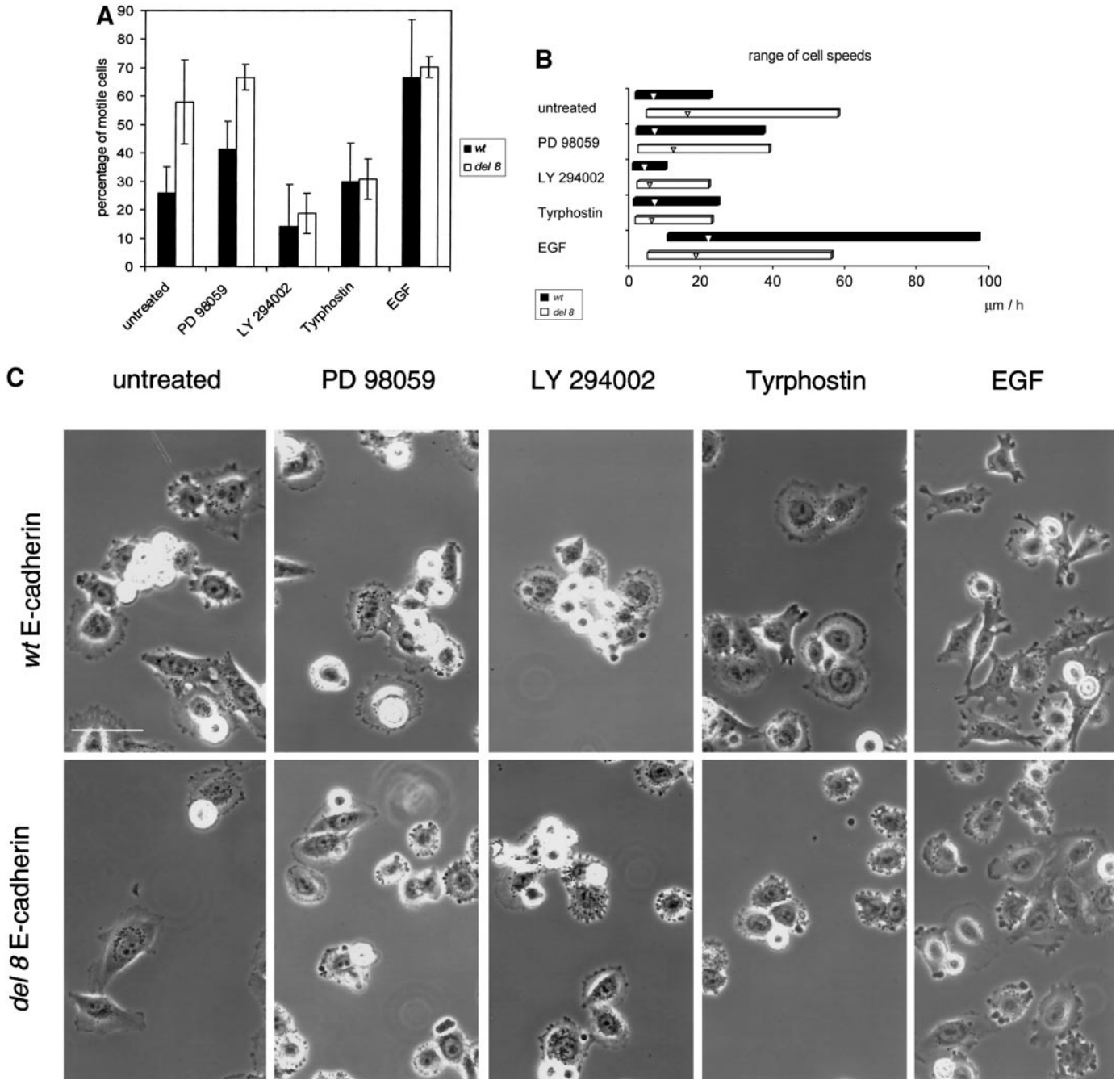

FIG. 5. Influence of PD 98059, LY 294002, Tyrphostin AG 1478, and EGF on cell motility. Comparison of the percentage of motile cells (A) and medians and ranges of cell speeds (B) of human MDA-MB-435S transfected with *wt* or *del 8* E-cadherin–cDNA in the presence of PD 98059, LY 294002, Tyrphostin AG 1478, and EGF. Cells were plated onto a collagen I matrix and traced for 7 h under a phase-contrast microscope as described in the legend to Fig. 1. Only attached nondividing cells were analyzed. Each bar in A represents the mean ± SD of three independent experiments. (C) Shown are MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin plated on glass plates coated with collagen I in the presence of PD 98059, LY 294002, Tyrphostin AG 1478, and EGF 2 h after plating. Concentrations: PD 98059, 50 μM; LY 294002, 40 μM; Tyrphostin AG 1478, 6.3 μM; EGF, 100 ng /ml. The bar represents 50 μm.

in nontransfected and *wt* or mutant E-cadherin–cDNA-expressing MDA-MB-435S cells (Fig. 7B). By densitometric analysis of Western blots, we found that Akt/PKB was around twofold more strongly activated in cells expressing *wt* E-cadherin than cells expressing mutant E-cadherin or nontransfected parental cells on uncoated and collagen I-coated plates, and the effect was not due to variations of the expression level.

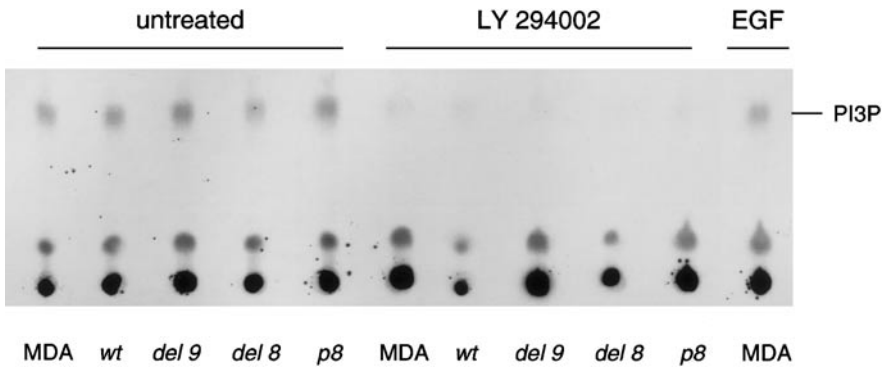

FIG. 6. Detection of PI 3-kinase activity. PI 3-kinase activity was assayed in anti-PI 3-kinase p85 immunoprecipitates from cell lysates of nontransfected (MDA), *wt*, *del 9*, *del 8*, or *p8* E-cadherin–cDNA-expressing MDA-MB-435S cells. The reaction product ^{33}P-labeled phosphatidylinositol 3-phosphate (PI3P) is indicated. As a negative control, LY 294002 was included into the reaction. As a positive control, serum-starved cells were treated with EGF for 2 min. Concentrations: LY 294002, 20 μM; EGF, 100 ng/ml.

DISCUSSION

In this study, we obtained evidence that mutant E-cadherins, as they were cloned from diffuse-type gastric carcinomas, enhance random cell movement of MDA-MB-435S breast cancer cells as compared to *wt* E-cadherin-expressing cells. The mutations affect the extracellular portion of the molecule and include deletions of exons 8 or 9 and a point mutation in exon 8. The motility-increasing activity of mutant E-cadherin was shown to be influenced by ECM proteins. Further-

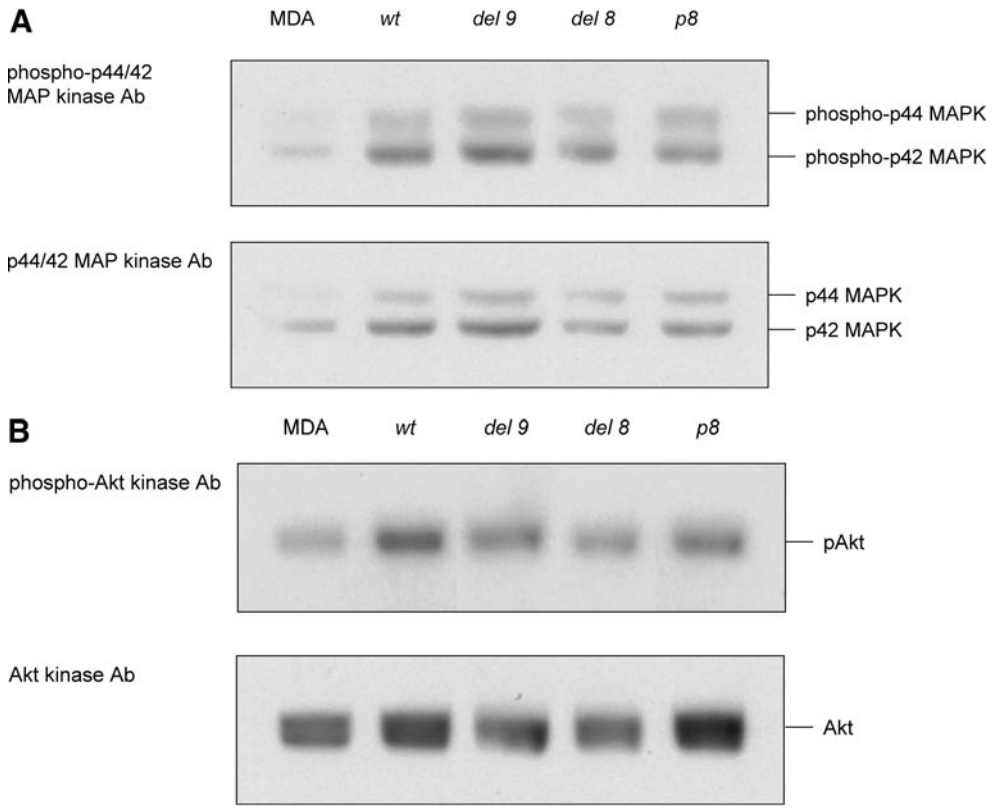

FIG. 7. Detection of activated and total MAP kinase and Akt/PKB levels. Expression levels and activities of MAP kinases p44 and p42 (A) and Akt/PKB (B) were investigated by immunoblot analysis of extracts from nontransfected (MDA), *wt*, *del 9*, *del 8*, or *p8* E-cadherin–cDNA-expressing MDA-MB-435S cells using the respective antibodies. (A) Phospho-p44/42 MAP kinase polyclonal antibody (Ab) detects activated p44/42 MAP kinase phosphorylated at threonine 202 and tyrosine 204. p44/42 MAP kinase polyclonal antibody detects total MAP kinase levels. Equal amounts of whole-cell lysates prepared from uncoated plates were used in each lane. (B) Phospho-Akt polyclonal antibody detects Akt1, Akt2, and Akt3 when phosphorylated at Ser 473. Akt kinase polyclonal antibody detects total Akt kinase levels. Equal amounts of whole-cell lysates prepared from uncoated plates were used in each lane.

more, we found that motility enhancement by mutant E-cadherin was sensitive to pharmacological inhibitors of EGFR- and PI 3-kinase-mediated signaling pathways. These data suggest that tumor-associated E-cadherin mutations are critical for tumor cell motility.

Despite the fact that the mutant E-cadherins investigated in this study were cloned from diffuse-type gastric carcinomas, we used MDA-MB-435S mammary carcinoma cells instead of gastric carcinoma cells as recipient cells. MDA-MB-435S cells lack endogeneous E-cadherin. Methylation-associated silencing of E-cadherin gene expression [33] and downregulation of E-cadherin gene expression by snail [34] have been suggested. MDA-MB-435S cells have therefore been used for studying E-cadherin function after transfection with E-cadherin expression constructs by us and other groups [19, 20, 35, 36]. Mutations in the E-cadherin gene have been identified in diffuse-type gastric carcinomas, lobular breast cancers, and gynecolocial tumors [5, 6, 9]. A mutational analysis of E-cadherin in human breast cancer cell lines revealed a deletion of exon 9 in cell line MPE600 [37, 38] which indicates that investigation of E-cadherin mutations in breast cancer cells is of physiological relevance.

Diffuse-type gastric carcinomas and lobular breast cancers invade surrounding tissues as single cells [3, 4, 39]. We have previously addressed the question whether E-cadherin mutations cloned from diffuse-type gastric carcinomas actively increase cell migration in a wound-healing assay or whether they only abolish E-cadherin function. We found that both are true: in highly motile L929 fibroblasts, *wt* E-cadherin caused a dramatic reduction of cell migration into the wound, whereas mutant E-cadherin caused only a partial reduction of cell migration [19]. These results indicate a partial loss of the migration-inhibiting function of E-cadherin by mutations in the extracellular domain. On the other hand, parental or *wt* E-cadherin-expressing MDA-MB-435S cells did hardly enter the wound, whereas 10 times more cells expressing *del 9* E-cadherin and 100 times more cells expressing *del 8* or *p8* E-cadherin moved into the wound. These results provided evidence that mutant E-cadherins result in a gain of function and actively enhance cell migration [19].

In the present study, parental and *wt* E-cadherin-expressing MDA-MB-435S cells showed a low percentage of motile cells (Fig. 1B; 30 and 26%, respectively), whereas cell motility was increased by expression of mutant E-cadherin (*del 9*, 52%; *del 8*, 58%; *p8*, 45%). These data are in accordance with the wound-healing assays. Despite the expression of *N*-cadherin on the RNA and protein levels [40], parental MDA-MB-435S cells were not highly motile in our experiments. The fact that the median of cell speeds of untransfected MDA-MB-435S cells was within the same range as that for mutant E-cadherin-expressing cells (Fig. 1C) is, on the first glance, contradictory. However, time-lapse laser scanning microscopy showed that parental MDA-MB-435S cells moved within a clone or rotated around their own axis, despite actively leaving clone-like mutant E-cadherin-expressing cells.

Mutant E-Cadherin-Enhanced Cell Motility Is Dependent on Cell–Matrix Interactions

Eucaryotic cell motility plays a pivotal role in physiological and pathological processes, such as embryonic development, wound healing, tumor invasion, and metastasis. Cell migration requires interactions among the cellular adhesion molecules, the extracellular matrix at the leading edge of the cell, and the release of adhesive interactions at the trailing end [41]. MDA-MB-435S cells expressing mutant E-cadherin showed increased cellular motility compared to *wt* E-cadherin-expressing cells in a motility assay based on time-lapse laser scanning microscopy. As motility parameters, determination of the percentage of cells which leave the initial space within the observation time of 7 h and calculation of cellular velocity were used. Cell speeds within a transfected cell line differed up to 10-fold among individual cells. This might be due to the investigation of asynchronous cell populations because cells have been shown to exhibit differences in their motile behavior according to their cell cycle phases in a different study [23]. We observed maximal cell speeds in mutant E-cadherin–cDNA-expressing MDA-MB-435S cells, suggesting that reduced cell–cell adhesive interactions are critical for cellular motility *in vitro* and presumably also *in vivo*.

A mathematical relationship between cell–substratum adhesion and cell migration was defined by Palecek *et al.* [42]. Accordingly, the extent of cell adhesion to the ECM depends on the concentration of cell surface integrins and ECM proteins. Low integrin or ECM protein concentration resulted in weakly attached cells with low tractive forces. Increasing attachment led to an optimal rate of cell migration. With further attachment, however, cells displayed impaired motility, presumably due to the inability to cycle between the adherent and the nonadherent state. In our study, the extent of cell adhesion to different ECM proteins was inversely correlated with cell motility, suggesting that ECM proteins influence mutant E-cadherin-enhanced cell motility. Collagen I resulted in highest cell motilities of mutant E-cadherin-expressing MDA-MB-435S cells, presumably because this ECM protein resulted in sufficient attachment and detachment of cells to enable cell migration. MDA-MB-435S cells were found to express $\alpha 1$ and $\beta 1$ integrin which mediate as a heterodimer binding of collagen I. Mutations in E-cadherin did not influence the $\alpha 1$ and $\beta 1$ integrin expression patterns of MDA-MB-435S transfectants, ruling out the possibility that mutant E-cadherin tran-

scriptionally regulates the collagen I receptor expression pattern. Moreover, E-cadherin mutations did not alter the $\alpha 2$, $\alpha 3$, and αv integrin expression patterns (to be published elsewhere). Also, in our study E-cadherin expression was not transcriptionally downregulated by collagen I as observed by other authors [43], presumably because E-cadherin expression was driven by the β-actin promoter in our constructs and not by the native E-cadherin promoter.

Motility Enhancement by E-Cadherin Mutations Is Sensitive to the EGFR Kinase Inhibitor Tyrphostin AG 1478

Different members of the cadherin family have multiple functions. E-cadherin is implicated in the maintenance of an epithelial and noninvasive phenotype [35] and actively induces mesenchymal to epithelial transition [44]. In contrast to N-cadherin and cadherin-11, which are known to upregulate cell motility, E-cadherin is known to counteract cell motility and invasion [35]. However, our previous work revealed that E-cadherin, mutated within the linker region between extracellular domain 2 and 3, results in an increase in cell migration [19]. In the present study, we demonstrate that the motility-promoting activity of mutant E-cadherin is sensitive to treatment with the EGFR-specific inhibitor Tyrphostin AG 1478. EGFR has been shown to directly interact with the E-cadherin/catenin complex by other authors, the interaction being mediated by β-catenin, which is tyrosine phosphorylated in response to EGF treatment [45, 46]. A direct interaction between the mutant E-cadherin/catenin complex and the EGFR might activate cell motility by receptor cross talk.

The motility-promoting function of other members of the cadherin familiy and the involvement of cellular signaling pathways have been investigated in different studies. In contrast to E-cadherin, N-cadherin was suggested to induce an epithelial to mesenchymal transition and to promote motility, invasion, and metastasis of cancer cells [47, 48]. The extracellular domain 4 of N-cadherin was shown to mediate the epithelial to mesenchymal transition and to increase motility, which indicates that the motility-promoting activity of N-cadherin is distinct from the adhesive function which resides within extracellular domain 1 [49]. The motility-promoting function of N-cadherin has been shown to be dramatically enhanced by fibroblast growth factor (FGF)-2 [48]. The authors speculate that an interaction exists between the N-cadherin and the FGF receptor which leads to increased cell motility. This idea is supported by the results of other studies which suggest that N-cadherin can interact with FGF receptors [50, 51] and that N-cadherin-mediated cell motility of breast cancer cells can be blocked by an inhibitor of the FGF-mediated signaling pathway [47]. The relationship of another member of the cadherin family with a signaling pathway was also shown for VE-cadherin which forms a complex with β-catenin, PI 3-kinase, and VEGFR-2, thereby activating Akt kinase and endothelial cell survival [52].

Motility-Promoting Activity of Mutant E-Cadherin Is Sensitive to the PI-3 Kinase Inhibitor LY 294002

Stimulation of MDA-MB-435 cells with EGF has been demonstrated to activate PI 3-kinase, as shown by rapid recruitment of the p85 subunit of PI-3 kinase to the phosphotyrosine-containing cellular fraction [53]. In our study, EGF treatment of *wt* E-cadherin-expressing MDA-MB-435S cells enhanced cellular motility, which identifies the EGFR pathway as an important regulator of cell motility in our system. Enhancement of cell motility caused by mutant E-cadherin was inhibited by addition of the PI 3-kinase inhibitor LY 294002. PI 3-kinase was not more strongly activated in mutant E-cadherin-expressing cells than in *wt* E-cadherin-expressing cells, indicating that basal PI 3-kinase activity is necessary for the effect.

Akt/PKB is among the main effectors of PI 3-kinase [53]. Akt/PKB has recently been shown to be activated by the formation of E-cadherin-mediated cell–cell junctions [14]. By Western blots using phosphorylation-specific antibodies, Akt/PKB was found to be more strongly activated in MDA-MB-435S cells by *wt* than by mutant E-cadherin in our study. Treatment with the PI 3-kinase inhibitor LY 294002 did not detectably interfere with the phosphorylation status of Akt/PKB of *wt* or mutant E-cadherin–cDNA-expressing MDA-MB-435S cells (our unpublished observations), suggesting that in addition to PI 3-kinase other signaling molecules might be involved in the regulation of Akt/PKB activity. While Akt/PKB has been demonstrated to be involved in mediating the antiapoptotic effect of PI 3-kinase [54], its role in the regulation of cellular motility has yet to be defined.

MAP kinase p44/42 is among the main downstream effectors of PI-3 kinase [54] and was reported to regulate cell motility [30]. Several recent publications indicate that MAP kinase is also activated by cell–cell and cell–matrix interactions. For example, integrin engagement activates MAP kinase [32, 55] and E-cadherin signals to the MAP kinase pathway via EGFR engagement [16]. We failed to detect considerable differences in p44/42 MAP kinase activities and expression levels upon expression of *wt* or mutant E-cadherin–cDNAs. Also we observed no inhibitory effect of the MAP kinase inhibitor PD 98059 on cell motility of *del 8* E-cadherin-expressing MDA-MB-435S cells, suggesting that MAP kinase plays only a minor role in mutant E-cadherin-enhanced cell motility in these cells.

In conclusion, our findings suggest that E-cadherin mutations not only affect the adhesive functions but also influence the migratory behavior of MDA-MB-435S cells. We have found that increased cell motility stimulated by mutant E-cadherin is blocked by inhibitors of EGFR and PI 3-kinase. Inhibition of these signaling molecules with small-molecule drugs is a promising approach in treatment of malignant tumors with E-cadherin mutations.

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