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Rac1 and Rho contribute to the migratory and invasive phenotype associated with somatic E-cadherin mutation

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Recent evidence suggests a close association between extracellular E-cadherin mutation in diffuse-type gastric carcinoma and the acquisition of a migratory phenotype of tumour cells. To characterize the cellular machinery that mediates the gain of motility of tumour cells with mutant E-cadherin, we turned to the small Rho GTPases Rac1 and Rho because they have been implicated in pathological processes including tumour cell migration and invasion. In the present study, we analyse the activity of Rac1 and Rho in relation to E-cadherin harbouring an in-frame deletion of exon 8 and prove for the first time that the mutation reduces the ability of E-cadherin to activate Rac1 and to inhibit Rho. We provide evidence that the lack of Rac1 activation observed in response to mutant E-cadherin influences the downstream signalling of Rac1, as is shown by the decrease in the binding of the Rac1 effector protein IQGAP1 to Rac1-GTP. Moreover, reduced membranous localization of p120-catenin in mutant E-cadherin expressing cells provides an explanation for the lack of negative regulation of Rho by mutant E-cadherin. Further, we show by time-lapse laser scanning microscopy and invasion assay that the enhanced motility and invasion associated with mutant E-cadherin is sensitive to the inhibition of Rac1 and Rho. Together, these findings present evidence that the mutation of E-cadherin influences Rac1 and Rho activation in opposite directions and that Rac1 and Rho are involved in the establishment of the migratory and invasive phenotype of tumour cells that have an E-cadherin mutation.

INTRODUCTION

E-cadherin belongs to the major class of cell–cell adhesion molecules that mediate intercellular adhesion by calcium-dependent homophilic interactions (1). In epithelial cells, E-cadherin is concentrated within adherens junctions, where the components of the adherens complex interact with the actin cytoskeleton (2). Furthermore, E-cadherin plays an important role in the maintenance of tissue integrity and is frequently downregulated or mutated in human invasive and metastatic tumours (3,4).

Somatic E-cadherin mutations have been identified in ~50% of diffuse-type gastric carcinomas and lobular breast cancers (5–9). Gastric carcinoma-derived somatic E-cadherin mutations were preferentially detected in a mutational hotspot region comprising exons 8 or 9 (6). These mutations are predominantly splice-site mutations that lead to exon skipping or missense mutations. They frequently affect calcium binding

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sites within the second and third extracellular domains of E-cadherin that are essential for binding to external calcium ions (10). As a result, the mutants are partially defective in calcium-mediated cell–cell adhesion but retain an intact cytosolic domain. Expression of E-cadherin harbouring mutations that are derived from diffuse-type gastric carcinomas is associated with decreased cell adhesion and increased cell motility, indicating an epithelial-to-mesenchymal transition (10,11). In contrast, E-cadherin is inactivated in a majority of sporadic invasive human lobular breast cancers by truncation mutations throughout its extracellular domain (9,12,13). In gynecological and thyroid tumours, somatic E-cadherin mutations have been found at a low frequency (14,15). Germline E-cadherin mutations have been identified in hereditary diffuse-type gastric carcinoma as the molecular genetic cause for a hereditary gastric cancer syndrome (16–19). Furthermore, an association of sporadic lobular breast carinoma with germline E-cadherin mutations has been described and a significant over-representation of lobular breast carcinomas in diffuse-type gastric cancer families with E-cadherin germline mutations has been demonstrated (20,21).

Rho GTPases contribute to physiological processes, including cytoskeletal dynamics, cell cycle progression, transcriptional regulation and apoptosis, and to cancer progression by regulating cell migration, invasion and metastasis (22,23). The Rho GTPase family includes, among several others, the prototype subfamilies Rac and Rho (24). The Rac subfamily comprises Rac1, Rac2, Rac3 and RhoG, whereas the Rho subfamily consists of RhoA, RhoB and RhoC. Rho GTPases are guanine nucleotide-binding proteins that regulate signal transduction pathways by functioning as intracellular binary switches, cycling between a GTP-bound active state and a GDP-bound inactive state. Their activity is increased by guanine nucleotide exchange factors (GEFs), which promote the release of GDP and binding of GTP, and decreased by GTPase-activating proteins (GAPs), which stimulate the hydrolysis of GTP (25).

Adherens junction formation requires the re-organization of the actin cytoskeleton, which is mediated by Rho GTPases (26–28). RhoA and Rac1 localize to E-cadherin-mediated cell–cell contacts and regulate the cytoskeletal dynamics during the assembly of adherens junctions (27). The formation of junctional complexes triggers activation of the phosphatidyl-inositol 3-kinase (PI 3-kinase) Akt/PKB pathway (29). Recruitment of PI 3-kinase leads to formation of phosphatidylinositol-(3,4,5)-triphosphate (PIP3). Subsequently, GEFs containing PIP3-binding pleckstrin-homology domains are recruited to the membrane and activate Rac1 (30,31). Rac1 activation results in stimulation of several downstream pathways through a large number of effector molecules, for instance the actin-binding protein IQGAP1 that serves as a link between Rac1 and the actin cytoskeleton (32). In contrast, activity levels of Rho were reduced by homophilic engagement of E-cadherin in several cell lines (33,34).

Through its role in the signal transduction of the small Rho GTPases Rac1 and Rho, E-cadherin has a direct effect on signalling pathways that are involved in tumour development and progression. In the present study, we investigate the hypothesis that the Rac1 and Rho activity levels are modulated by somatic E-cadherin mutations in a way that promotes tumour cell migration and invasion. We analysed the Rac1 and Rho activity levels in motile and invasive MDA-MB-435S cells expressing E-cadherin with an in-frame deletion of exon 8 that leads to a structural alteration within the extracellular domain of the protein. The role of Rac1 and Rho in mutant E-cadherin-associated tumour cell migration and invasion was investigated applying the Rac-inhibiting Toxin B from the variant *Clostridium difficile* serotype F strain 1470 (TcdBF) (35) and the Rho-inhibiting exoenzyme C3 from *Clostridium botulinum* (C3). Our data provide evidence that Rac1 and Rho play a role in the establishment of the migratory and invasive phenotype associated with the deletion of exon 8 of E-cadherin.

RESULTS

The effect of E-cadherin mutations on Rac1 expression, localization and activation

Several groups have shown that Rac1 is activated by homophilic ligation of E-cadherin (36–38), which raises the question as to whether somatic in-frame deletions of exon 8 (*del 8*) influence the ability of E-cadherin to activate Rac1. The effect of *del 8* E-cadherin on the expression, localization and activation of Rac1 was assessed in the E-cadherin-negative human MDA-MB-435S carcinoma cell line, after stable transfection with wild-type or *del 8* E-cadherin (10). Empty-vector transfection was used as control. As reported previously, the migration pattern of *del 8* E-cadherin in western blots is different from that of the wild-type protein, because the deletion leads to a protein that is smaller than 120 kDa. In addition, the deletion renders E-cadherin more accessible to proteolytic degradation, and therefore, a smaller fragment of ~80 kDa was detectable [Fig. 1A and (10)]. The likely explanation for the appearance of the smaller fragment is that the deletion affects a putative calcium-binding motif, presumably leading to reduced protease resistance of the protein (39). In line with this explanation, we had demonstrated by flow cytometric analysis, reduced surface localization of *del 8* when compared with wild-type E-cadherin, which was due to enhanced internalization, again suggesting that the mutation influences the endocytosis of E-cadherin and destabilizes the protein at the cellular membrane (40).

First, we examined whether E-cadherin affects the expression level and subcellular localization of Rac1. By western blotting, we found that the total cellular levels of Rac1 were unchanged (Fig. 1A). Then, we visualized the subcellular staining pattern of Rac1 in wild-type or *del 8* E-cadherin positive cells, as well as in control cells, by immunofluorescence (Fig. 1B). Rac1 was localized predominantly at the plasma membrane at cell–cell contacts in wild-type E-cadherin positive cells, reflecting the distribution pattern of wild-type E-cadherin (10). In contrast, Rac1 was found mainly in the cytoplasm and at residual cell–cell contacts in *del 8* cells, resembling the previously characterized staining pattern of mutant E-cadherin (10,41). In control cells, Rac1 also accumulated in the cytoplasm and at residual cell–cell contacts established by adhesion molecules that are different from E-cadherin [for instance N-cadherin, which is expressed in MDA-MB-435S cells (10)]. Together, these findings suggest that the subcellular localization of E-cadherin (wild-type or mutant) had an apparent effect on the subcellular

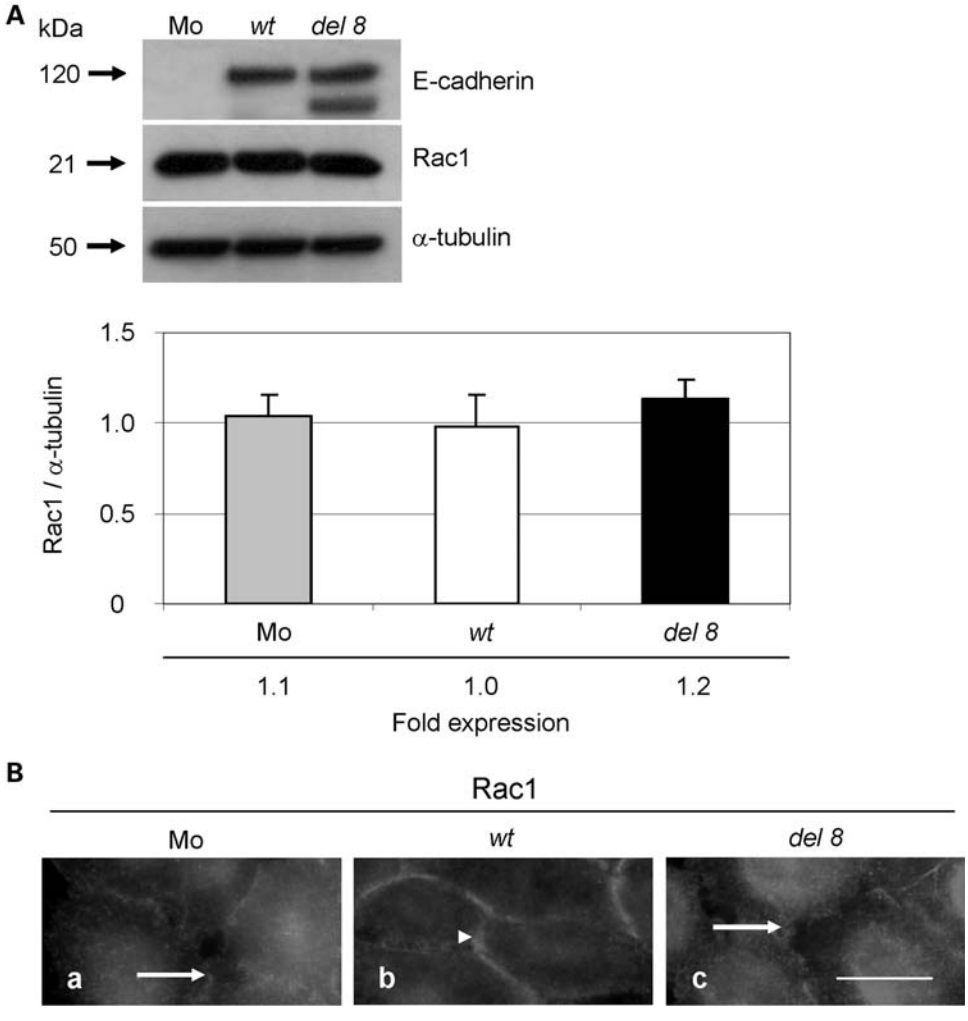

Figure 1. Expression and localization of Rac1 was studied in MDA-MB-435S cells that were expressing ectopic wild-type (*wt*) or mutant E-cadherin stably. (**A**) E-cadherin and Rac1 were detected in total lysates of MDA-MB-435S cells that were previously transfected stably with the empty vector pBATEM as a mock (Mo) control or with *wt* or *del 8* E-cadherin (10) by western blot, using mouse-anti-E-cadherin or mouse-anti-Rac1 Ab, respectively. Mouse anti-α-tubulin Ab was used as a loading control. Of note, flow cytometric analysis, used as quality control, had revealed the absence of E-cadherin in the parental MDA-MB-435S cell line and expression of either the *wt* or the mutant form of E-cadherin in transfected cell lines in more than 95% of the cells (74). The lower E-cadherin band of ~80 kDa in the cellular lysates that were derived from the *del 8* cells, most likely represents a proteolytic fragment. Rac1 expression levels were not influenced by the expression of E-cadherin (*wt* or *del 8*). The results presented are representative of three independent experiments. Mean expression levels of Rac1 were quantified using densitometric analysis and were calculated in relation to α-tubulin (+SD). The statistical analysis was performed with the *post hoc* test. (**B**) Rac1 was visualized by immunofluorescence in MDA-MB-435S cells expressing *wt*, *del 8* E-cadherin expressing or vector-transfected control cells after fixation and permeabilization using mouse anti-Rac1 Ab, and the signals were detected using a secondary FITC-conjugated goat anti-mouse IgG. The arrowhead points to Rac1 staining at cell–cell contacts in *wt* E-cadherin transfectants, whereas the arrows mark Rac1 localization at residual contact points in *del 8* and control cells. Images were acquired using a Zeiss Axiovert 135. Scale bar: 25 μm.

distribution of Rac1 in these particular cell lines, whereas the expression level of Rac1 was not affected by E-cadherin.

Using pull-down assay for the detection of Rac1 activity, we next demonstrated that the expression of wild-type E-cadherin results in a statistically significant, 40–50% increase in endogenous Rac1-GTP levels, compared with vector-transfected ($P = 0.015$) or *del 8* E-cadherin expressing cells ($P = 0.006$), whereas the total expression levels of Rac1 were unchanged, as shown above (Fig. 2). These data strongly indicate that the increase in Rac1 activity which is normally observed as cells form wild-type E-cadherin-mediated cell–cell contacts (34) was lost upon somatic mutation of E-cadherin.

Binding of Rac1 to its effector protein IQGAP1

Rac1 activation results in the stimulation of multiple pathways through a large number of effector molecules, including IQGAP1, an actin-binding protein that serves as a link between Rac1 and the actin cytoskeleton (32). To investigate whether the lack of Rac1 activation by *del 8* E-cadherin influences the IQGAP1-driven Rac1 downstream signalling, the interaction of Rac1-GTP with IQGAP1 was analysed using immunoprecipitation. We demonstrate that the cellular level of the Rac1–IQGAP1 complex is increased by 40–60%, which is statistically significant, in wild-type E-cadherin expressing cells compared with vector-transfected ($P = 0.004$)

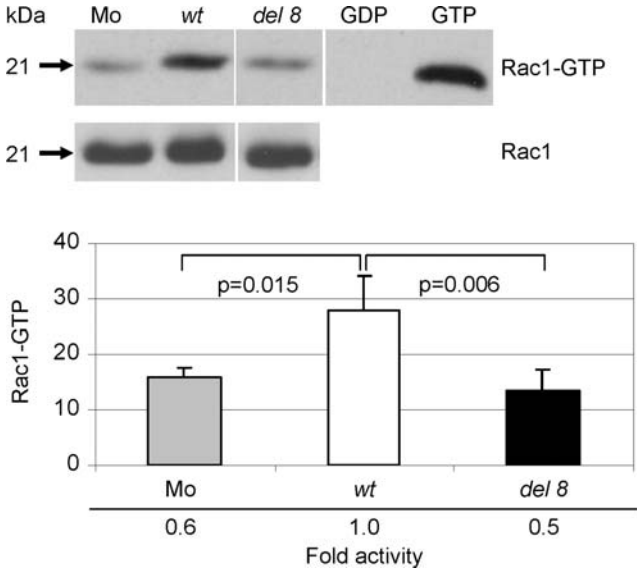

Figure 2. The activity of Rac1 was studied in MDA-MB-435S cells that were transfected with *wt* or mutant E-cadherin stably. A non-radioactive assay (75) was used to measure the activation of Rac1 by affinity precipitation of Rac1-GTP with the p21-binding domain of p21-activated kinase (PAK) and subsequent detection of Rac1 by western blot in MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin and in vector-transfected control cells. The GTP control represents the activated, GTP-bound form of Rac1, whereas GDP represents the inactive, GDP-bound form of Rac1. Rac1 activity increased in response to *wt* E-cadherin compared with control cells, whereas the expression of *del 8* E-cadherin caused no increase in Rac1 activation. Immunoblots were quantified by densitometric analysis using Scion Image Software. Mean Rac1 activity levels (+SD) were calculated using three independent experiments. The statistical analysis was performed with the *post hoc* test.

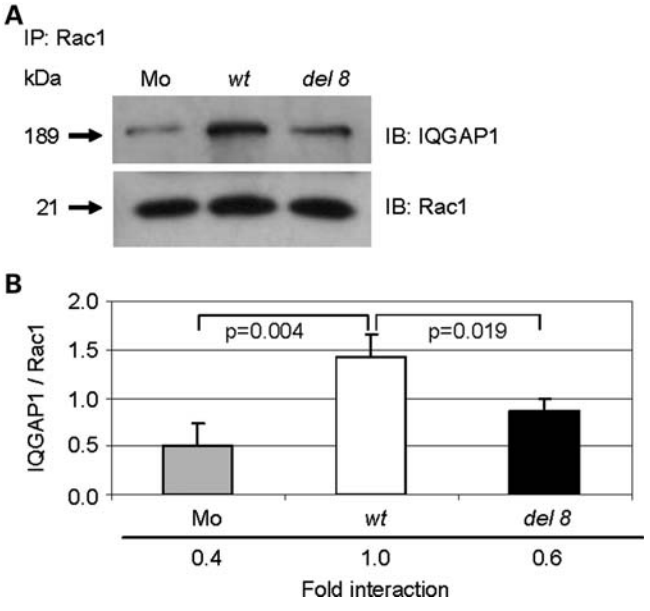

Figure 3. Association of Rac1 with the effector protein IQGAP1 was examined in MDA-MB-435S cells stably expressing *wt* or mutant E-cadherin. Extracts of *wt* or *del 8* E-cadherin expressing or empty vector-transfected MDA-MB-435S cells were immunoprecipitated with anti-Rac1 antibody, followed by western blotting for the detection of the Rac1-GTP–IQGAP1 complex using the anti-IQGAP1 antibody. The results presented are representative of three independent experiments. Complex formation between Rac1-GTP and IQGAP1 significantly increased in response to *wt* E-cadherin compared with control cells, whereas the expression of *del 8* E-cadherin caused a weaker increase in Rac1-GTP–IQGAP1. Western blots were quantified by densitometric analysis using Scion Image Software. Mean IQGAP1 levels obtained from three independent experiments were calculated in relation to Rac1 expression. Bars represent the mean (+SD). The statistical analysis was performed with the *post hoc* test.

or *del 8* E-cadherin expressing cells ($P = 0.019$), whereas the total Rac1 expression levels remained constant as shown before (Fig. 3). This result clearly indicates that the lack of Rac1 activation, which is observed in response to mutant E-cadherin, affects IQGAP1 signalling. Interestingly, western blot analysis revealed a significant 20% increase in the cellular expression levels of IQGAP1 in wild-type cells, compared either with *del 8* E-cadherin expressing cells ($P = 0.029$) or with E-cadherin-negative control cells (Fig. 4A, $P = 0.024$).

To elucidate whether the distribution pattern of IQGAP1 was influenced by E-cadherin, we visualized IQGAP1 using immunofluorescence in wild-type or *del 8* E-cadherin positive MDA-MB-435S cells, as well as in negative control cells. IQGAP1 was localized predominantly at the cellular membrane at cell–cell contacts in wild-type E-cadherin expressing cells, while it was detected in residual cell–cell contact sites and in lamellipodial structures in *del 8* cells, which are indicative of the motile phenotype of the cells (Fig. 4B). In vector-transfected control cells, IQGAP1 was detectable at cell–cell contacts as well as in the lamellipodia (Fig. 4B). From this staining pattern, we conclude that the subcellular localization of IQGAP1 is influenced by the expression and mutation of E-cadherin. Together, our results indicate that the lack of Rac1 activation that is observed in response to mutant E-cadherin influences the downstream signalling of Rac1, as shown by the decrease of binding of the Rac1 effector protein IQGAP1 to Rac1-GTP in *del 8* compared with wild-type E-cadherin positive cells.

The effect of E-cadherin mutations on Rho expression and activation

Recent evidence suggests that Rho activity decreases after the homophilic engagement of E-cadherin in several different cell lines (34,42). Next, we determined whether E-cadherin mutation affects the expression or activity level of Rho. Using pull-down assay, we determined that expression of wild-type E-cadherin resulted in a statistically significant 63% decrease in endogenous Rho-GTP levels, when compared with negative control cells (Fig. 5, $P < 0.001$, which is in line with the above-mentioned reports. Remarkably, expression of the E-cadherin deletion mutant resulted only in a 19% decrease in the Rho activity levels, when compared with the control cells, a reduction which also reached, however, statistical significance (Fig. 5, $P = 0.017$). Furthermore, statistical analysis revealed that Rho activity levels are significantly lower in wild-type cells when compared with *del 8* E-cadherin expressing cells (Fig. 5, $P < 0.001$). The total Rho expression levels were unchanged (Fig. 5). Together, this result clearly indicates that the mutation reduces the ability of E-cadherin to inhibit Rho activity. On the basis of these results, deletion

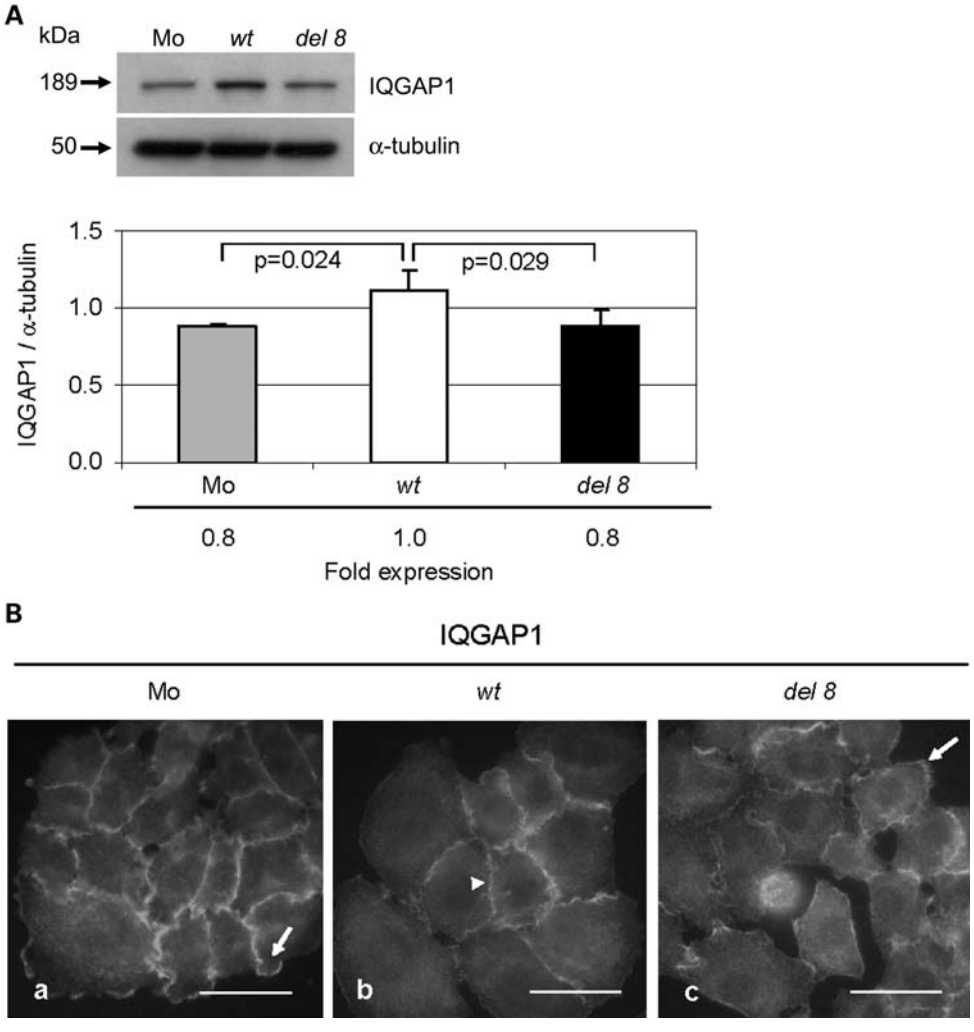

Figure 4. Expression and localization of IQGAP1 were investigated in MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin stably. (**A**) IQGAP1 expression was increased in total lysates of MDA-MB-435S cells expressing wild-type E-cadherin when compared with control cells. In contrast, no change in IQGAP1 expression was detectable in *del 8* E-cadherin expressing cells when compared with control cells. Mouse anti-α-tubulin Ab was used as a loading control. The results presented are representative for three independent experiments. Mean expression levels of IQGAP1 were quantified by densitometric analysis and calculated in relation to α-tubulin (+SD). The statistical analysis was performed with the *post hoc* test. (**B**) IQGAP1 was visualized in MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin or vector-transfected control cells. The cells were cultivated for 2 days and analysed by immunofluorescence, after fixation and permeabilization using mouse anti-IQGAP1 Ab, and the signals were detected using a secondary FITC-conjugated goat anti-mouse IgG. The arrowhead points to IQGAP1 staining at cell–cell contacts in *wt* E-cadherin transfectants, while the arrows mark IQGAP1 localized at lamellipodia in *del 8* and control cells. Images were acquired using a Zeiss Axiovert 135. Scale bar: 50 μm.

of exon 8 of E-cadherin is associated with deregulation of the activity of Rac1 and Rho.

The effect of E-cadherin mutations on the localization of p120-catenin

Rac is able to negatively regulate Rho via a molecularly characterized signalling pathway (hereafter, the 'Bar-Sagi' pathway) (43). The mechanism that coordinates the antagonism between Rac and Rho is mediated by p120-catenin and nucleated by cadherin complexes. Specifically, Rac activity induces translocation of p190RhoGAP to cadherin complexes, where it transiently interacts with p120-catenin and mediates the local inhibition of Rho (44). To investigate whether this mechanism was influenced by the E-cadherin mutation, the cellular distribution of p120-catenin was analysed using immunofluorescence. Our results prove that wild-type E-cadherin signalling leads to an increase of Rac1 activity and a decrease of Rho activity, suggesting an antagonism between Rac1 and Rho in our cell system. Indeed, p120-catenin was localized mainly at cell–cell contacts at the plasma membrane in wild-type E-cadherin expressing cells (Fig. 6). In exon 8-deleted E-cadherin expressing cells, p120-catenin was detectable only at residual cell–cell contacts, indicating that the mutation strongly influences the cortical localization of p120-catenin (Fig. 6). In vector-transfected control cells, p120-catenin was also localized to residual cell–cell contacts (Fig. 6). The p120-catenin signal was weaker in the control cells when compared with wild-type or mutant E-cadherin expressing cells. This is in accordance with our previous observation that the steady-state level of p120-catenin increased in

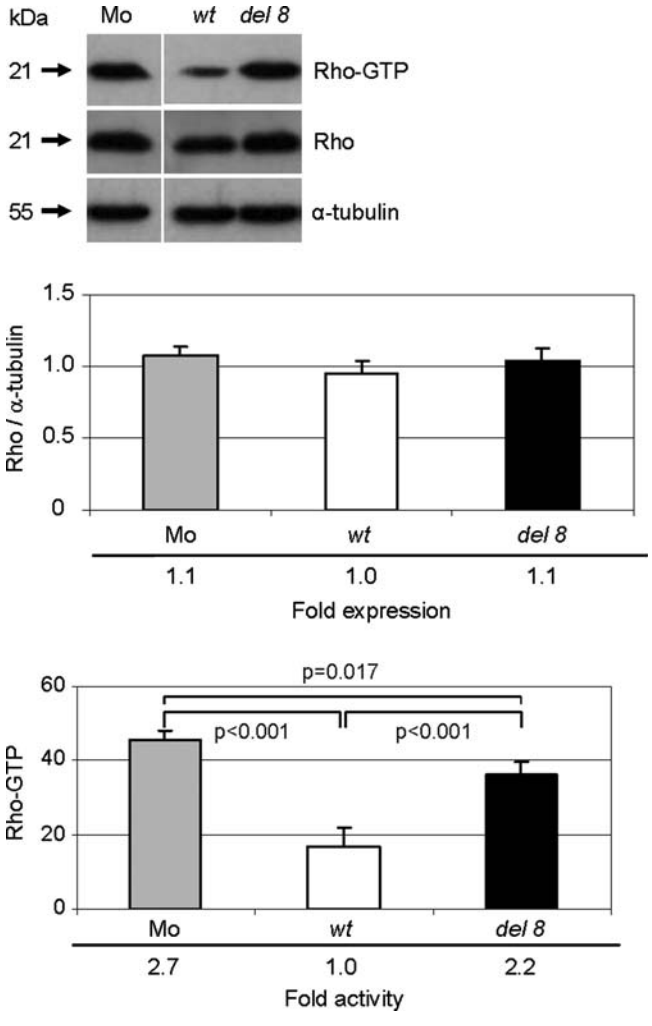

Figure 5. Expression and activity of Rho were examined in MDA-MB-435S cells transfected stably with *wt* or mutant E-cadherin. Rho expression levels were not influenced by expression of the wild-type or *del 8* E-cadherin in MDA-MB-435S cells. A non-radioactive assay was used to measure the activation of Rho by affinity precipitation of Rho-GTP with the Rho-binding domain (RBD) of Rhotekin, and by subsequent detection of Rho using western blotting in MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin and in vector-transfected control cells. Rho activity strongly decreased in response to *wt* E-cadherin when compared with control cells, whereas *del 8* E-cadherin expression caused only a moderate decrease in Rho activation. The results presented represent three independent experiments. The immunoblots were quantified by densitometric analysis using Scion Image Software. Mean Rho expression and activity levels obtained from three independent experiments were calculated (+SD). The statistical analysis was performed using the *post hoc* test.

response to E-cadherin (wild-type or mutant) in MDA-MB-435S cells (41). Taken together, the changed localization of p120-catenin likely abolished the Rac-dependent suppression of Rho activity through the 'Bar-Sagi' pathway. This offers an explanation for the higher relative Rho activity in *del 8* cells.

The effect of specific inhibitors of Rac1 and Rho activity on migration and invasion

E-cadherin mutation in the extracellular region is intimately connected with the acquisition of a migratory phenotype by MDA-MB-435S cells (10,11,45). To migrate, cells become polarized, elongate and extend the leading edge which attaches to the extracellular matrix (46). Subsequently, contraction of the cell body leads to movement of the cell. Cell migration results from a continuous cycle of these steps (46). During this process, Rac1 regulates actin polymerization during the lamellipodial extension at the leading edge of the migrating cells, whereas Rho regulates the contractile forces that are required for the movement of the cell body (47). Migration was analysed using time-lapse laser scanning microscopy. Mutation of E-cadherin results in enhanced migration of MDA-MB-435S cells (11) (Fig. 7A, $P = 0.005$). To determine whether Rac1 and Rho are involved in mediating the cytoskeletal changes that are required for the migration of *del 8* E-cadherin expressing MDA-MB-435S cells, we used the Rac1 inhibitor TcdBF and the Rho inhibitor C3. Both inhibitors effectively blocked Rac1 or Rho activity in our cells, respectively (Supplementary Material, Fig. S1). Treatment of *del 8* cells with Rac1 inhibitor TcdBF effectively inhibited cell migration (from 58 to 25% motile cells) down to the level of wild-type E-cadherin expressing cells (26%, Fig. 7A, $P = 0.007$). In contrast, wild-type E-cadherin expressing cells reacted to the inhibitor with an increase in cellular motility, from 26 to 40% motile cells, which was not significant statistically (Fig. 7A). Morphologically, the inhibition of Rac1 did not result in the rounding of the cells or in the detachment in any of the cell lines during the observation time. However, TcdBF caused a reduction in the number of cell–cell contacts in cells expressing wild-type E-cadherin. In contrast, TcdBF caused no significant morphological changes in *del 8* E-cadherin expressing cells (Fig. 7B). The Rho inhibitor C3 strongly reduced the motility of *del 8* cells (from 58 to 12% motile cells, Fig. 7A, $P = 0.001$) and slightly reduced the motility of wild-type E-cadherin cells (from 26 to 15% motile cells). Treatment with the Rho inhibitor provoked no detectable changes in cellular morphology (Fig. 7B). Taken together, our data show that enhanced cell motility associated with mutant E-cadherin is sensitive to the inhibition of Rac1 and Rho.

E-cadherin suppresses epithelial tumour invasion. Regulation of the actin cytoskeleton by Rac1 and Rho is also involved in various aspects of cell invasion. The effect of the Rac1 and Rho inhibitors on the invasion of wild-type or *del 8* E-cadherin expressing MDA-MB-435S cells was examined. Using the Matrigel invasion assay, we demonstrated for the first time that the exon 8 deletion of E-cadherin increases the ability of tumour cells to invade. Treatment with either of both inhibitors blocked the ability of *del 8* E-cadherin expressing cells to invade (TcdBF, $P = 0.008$, significant; C3: $P = 0.066$, trend, Fig. 8). No significant differences were observed between untreated and treated wild-type E-cadherin expressing or vector-transfected cells (Fig. 8). Taken together, we demonstrate that enhanced cell motility and invasion of *del 8* E-cadherin expressing cells depend on the activity of both Rac1 and Rho.

DISCUSSION

The mutation of E-cadherin influences Rac1 and Rho activity levels in opposite directions

Rac1 and Rho, members of the family of small Rho GTPases, are major regulators of E-cadherin-mediated cell–cell adhesion and

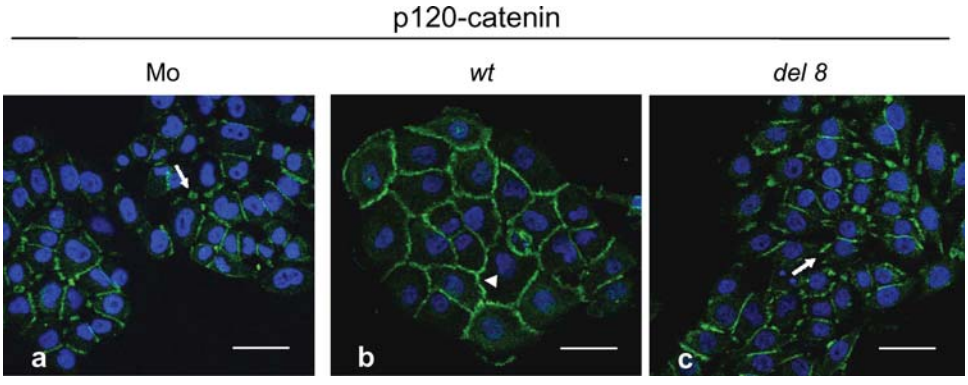

Figure 6. Localization of p120-catenin was investigated in MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin stably. p120-Catenin was detected in MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin or vector-transfected control cells by immunofluorescence after fixation and permeabilization using mouse anti-p120-catenin Ab, and the signals were detected using a secondary FITC-conjugated goat anti-mouse IgG. The arrowhead points to membraneous p120-catenin staining in *wt* E-cadherin transfectants, while the arrows indicate p120-catenin localized at residual cell–cell contact areas in the *del 8* and control cells. Images were acquired using an apotome that was implemented into the setup of a Zeiss Axiovert 200M epifluorescence microscope equipped with a charge-coupled device camera. Scale bar: 50 μm.

are involved in the induction, propagation and expansion of cell–cell contacts (48). Otherwise, they contribute to cellular processes that affect several stages of tumour development, including proliferation, motility, invasion and metastasis (22). Homophilic ligation of E-cadherin has been shown to activate Rac1 (26,28,49) and to inhibit Rho activity (34,42). Remarkably, E-cadherin is frequently absent or mutated in tumours. This tumour-specific alteration is associated with invasive tumour growth (3,9,10). E-cadherin mutations are mainly detected in diffuse-type gastric carcinoma, in a hotspot region comprising exons 8 and 9 (6,9). These mutations cause either a loss-of-function (decrease of cell adhesion and interference with the tumour-suppressive function of E-cadherin) or a gain-of-function (increase of motility, proliferation and proteolytic capacity) (10,11,50,51). In the present study, we demonstrate for the first time that the in-frame deletion of exon 8 of E-cadherin, a genomic alteration that is associated with diffuse-type gastric carcinoma, reduces the capacity of the protein to activate Rac1 and to inhibit Rho, which contributes to the acquisition of the migratory and invasive phenotype of tumour cells (Fig. 9). Our results consistently show that the enhanced motility and invasion associated with mutant E-cadherin is sensitive to inhibition of Rac1 and Rho.

The formation of adherens junctions requires reorganization of the actin cytoskeleton controlled by the Rho family of GTPases (52). We have confirmed earlier reports that wild-type E-cadherin activates Rac1 (36–38), whereas we observed no increase in active Rac1 in response to mutant E-cadherin. The predominant cytoplasmic localization of Rac1 in mutant E-cadherin cells supports this notion, because only active Rac1-GTP is localized at the cellular membrane. There are several reports that artificial E-cadherin mutations abrogate the ability of E-cadherin to activate Rac1, including a mutation of the cadherin cytoplasmic tail that uncouples binding of p120-catenin (36), a mutant that lacks the entire cytoplasmic domain of E-cadherin, and mutant ED134A that abolishes E-cadherin-mediated cell–cell adhesion levels (38). Our observation that Rac1 activity was not increased in response to E-cadherin that has a deletion of exon 8 is in line with the finding that Rac1 activation was sensitive to various E-cadherin mutations that decrease or abolish cell–cell adhesion. The significance of our finding, and the difference from other studies, is that the E-cadherin mutation that we investigate, is a naturally occurring, tumour-associated genetic alteration that we have proven, for the first time, to regulate Rac1 activity.

Rac1 activation was shown to inhibit endocytosis of E-cadherin and to stabilize the protein at the cellular membrane (53), thereby providing an explanation for the reduced surface localization of exon 8-deleted E-cadherin compared with the intact protein (40). The lack of increase in the level of active Rac1 in response to mutant E-cadherin, resulted in downregulated complex formation with IQGAP1, when compared with wild-type E-cadherin. Regulation also occurs on the expression level, because we observed a slight decrease in the total IQGAP1 expression levels in mutant E-cadherin cells. IQGAP1 is known to negatively regulate E-cadherin-mediated cell–cell adhesion by interacting with β-catenin, and thereby displacing it from the adherens complex (54). Active Rac1 can prevent this binding, thereby stabilizing cadherin-mediated cell–cell contacts. Accordingly, we believe that the lack of an increase in Rac1-GTP levels in response to mutant E-cadherin destabilizes the adherens complex. Interestingly, we detected IQGAP1 in lamellipodial structures in migrating exon 8-deleted cells, which is consistent with reports that IQGAP1 shifts from the cytosol to the membrane of migratory colorectal cancer cells (55). IQGAP1 has been suggested to influence cell motility at the leading edge of migrating cells, by increasing the levels of active Rac1 and Cdc42 (56). Although we observed only basal Rac1 activity in cells that were expressing mutant E-cadherin, we believe that this residual Rac1 activity plays a crucial role in mediating the necessary actin cytoskeletal changes during cell migration.

RhoA activity has been shown to decrease after homophilic engagement of E-cadherin (34,42) and to contribute to practically all of the stages of tumour progression, including migration, proliferation and disruption of epithelial polarity (22). By affinity precipitation, we determined that the deletion of exon 8 reduces the ability of E-cadherin to inhibit Rho

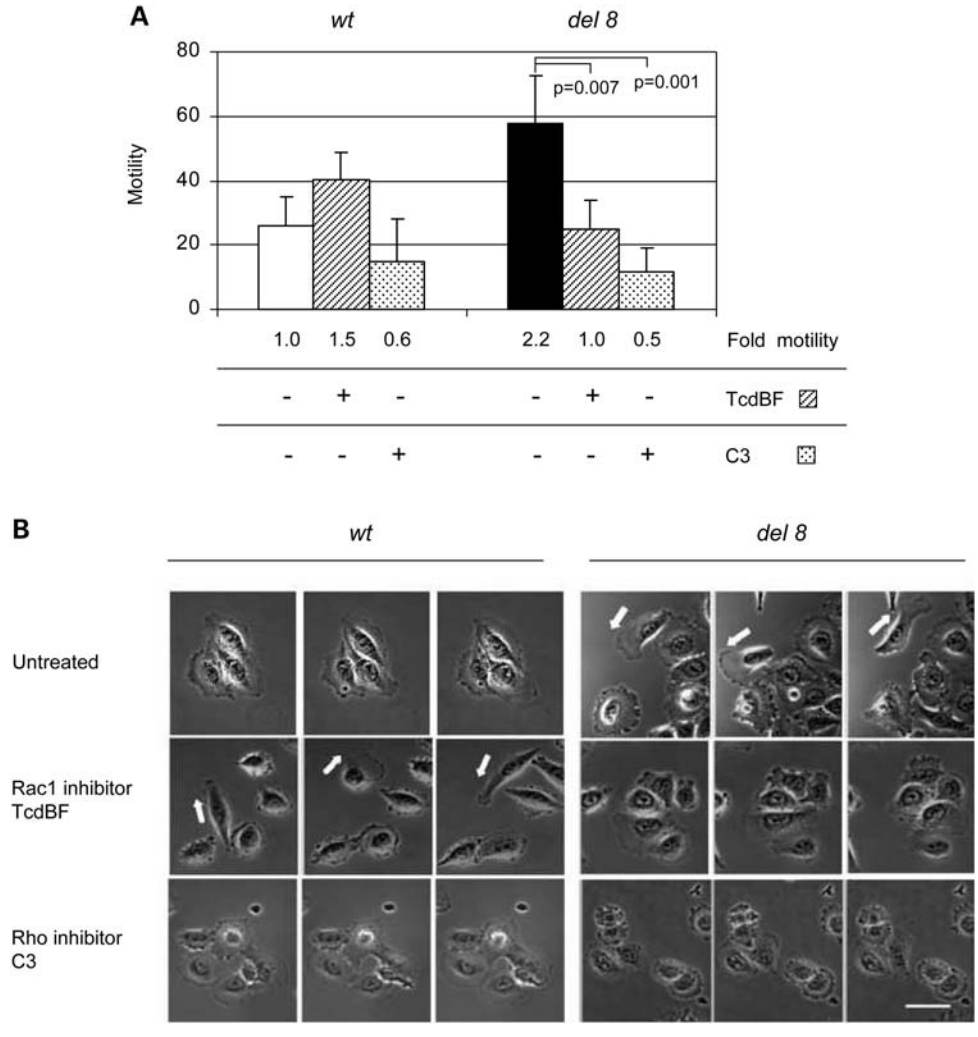

Figure 7. The effect of Rac1 and Rho inhibitors on cellular motility was determined in *wt* or *del 8* E-cadherin expressing MDA-MB-435S-cells. (**A**) MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin were seeded on plates with a glass bottom coated with collagen I. After 2 h of incubation, cells were treated with the Rac1 inhibitor TcdBF (0.4 ng/ml) or the Rho inhibitor C3 (6 μg/ml). Phase contrast images were taken every 3 min for 7 h with an Axiovert laser scanning microscope LSM 510. The percentage of motile cells (untreated and treated with the inhibitors) is shown. The cell motility of untreated *wt* and *del 8* cells was determined and published previously (11). Motility studies revealed that inhibition of Rac1 by TcdBF leads to increased motility of *wt* E-cadherin expressing cells (from 26 to 40% motile cells), whereas it leads decreased motility of *del 8* cells (from 58 to 25% motile cells). Application of the Rho inhibitor C3 decreases cell motility in both *wt* cells (from 58 to 12% motile cells) and *del 8* cells (from 26 to 15% motile cells). The percentage of motile cells was determined by counting cells in a microscopic field that moved completely out of the initial area within the time of the record. Only attached non-dividing cells that did not leave the observation field during the period of investigation were analysed. Each bar represents the mean (+SD) of at least three independent experiments. At least 60 cells were investigated for each cell line in at least three independent experiments. (**B**) Representative MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin untreated or in the presence of the Rac1 or Rho inhibitor are shown. The cells are shown at 30 min intervals, starting 150 min after the addition of the inhibitors. Inhibition of Rac1 did not lead to morphological changes (cell rounding, detachment or clustering) during the 7 h observation time, but a reduction of cell–cell contacts was observed in *wt* cells. Treatment with the Rho inhibitor did not affect cellular morphology in both cell lines. The motile cells are marked by an arrow. Scale bar: 50 μm.

activity. Crosstalk between Rac and Rho has been proposed, wherein Rac activity induces translocation of p190RhoGAP to cadherin complexes, where it transiently interacts with p120-catenin and mediates local inhibition of Rho (44). p120-Catenin binds to the juxtamembrane region of the cytoplasmic domain of E-cadherin (57) and this association has been proposed to stabilize E-cadherin at the plasma membrane and to prevent it from internalization and degradation (58). Exon 8 deletion leads to the reduced formation of functional E-cadherin–catenin complexes at the cellular membrane. Here we demonstrate that p120-catenin localizes only at residual contact points at the plasma membrane in cells that are expressing mutant E-cadherin, thus providing an explanation for the lack of negative regulation of Rho by mutant E-cadherin. Since the exon 8 deletion does not affect the intracellular domain of E-cadherin, p120-catenin was shown to be able to bind to mutant E-cadherin [data not shown, also (41)]. Taken together, our results indicate that wild-type E-cadherin signalling downregulates Rho while it increases Rac1 activity levels in our cell system, suggesting an antagonistic regulation of both GTPases by E-cadherin, as has been described previously for other cell systems (43). We provide evidence

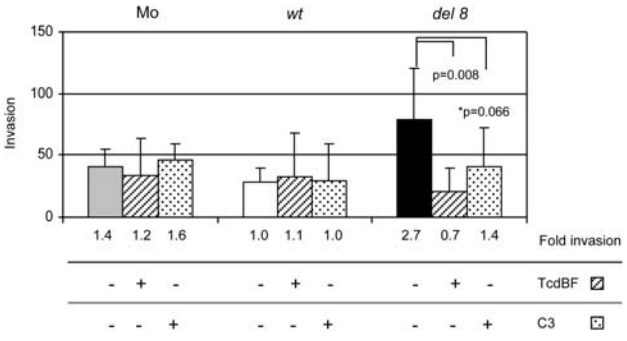

Figure 8. The influence of Rho and Rac1 inhibitors on the invasive behaviour of tumour cells was examined in MDA-MB-435S cells that were expressing *wt* or mutant E-cadherin stably. E-cadherin expressing or negative control cells were seeded in BioCoat™ Matrigel™ Invasion Chambers. After 22 h of incubation, cell invasion was determined by counting the cells which invaded through the Matrigel™ matrix. The number of invading cells was elevated in *del 8* compared with *wt* or vector-transfected control cells. Treatment with both inhibitors (TcdBF at 0.2 ng/ml and C3 at 6 μg/ml) decreased invasion of the *del 8* cells. In contrast, no change in the invasive behaviour was exhibited in the *wt* E and vector-transfected cells after treatment with the inhibitors. The average number of invasive cells (+SD) from two independent experiments, performed as triplicates, is shown.

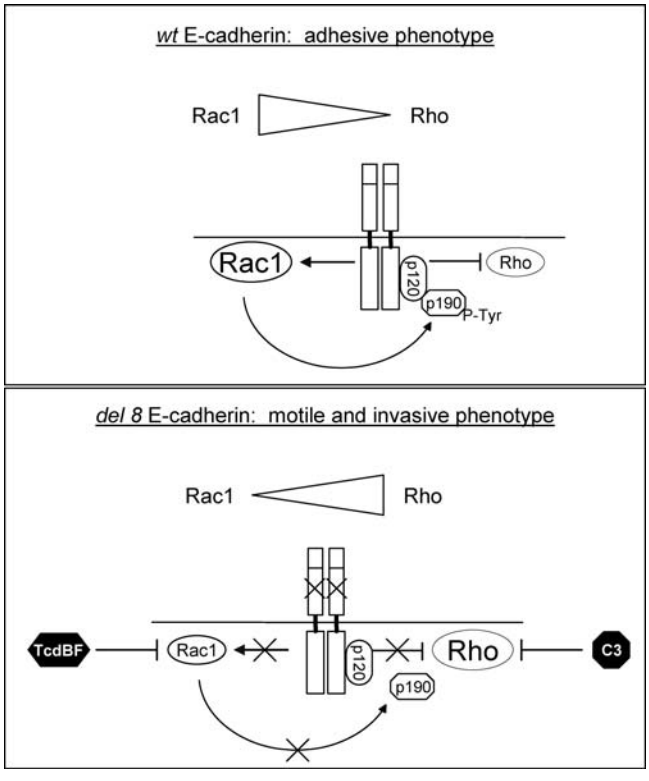

Figure 9. Schematic model. Activation of Rac1 in response to homophilic ligation of wild-type E-cadherin leads to negative regulation of Rho via the 'Bar-Sagi' pathway. p120-Catenin supports the cortical localization of p190RhoGAP which leads to local inhibition of Rho. The adhesive phenotype of cells expressing wild-type E-cadherin is associated with relatively high-Rac1 and low-Rho activity levels. Cells expressing E-cadherin with exon 8 deletion have lost the ability to activate Rac1 through homophilic ligation. In addition, the cortical localization of p120-catenin is reduced in *del 8* cells. Consequently, Rac1-dependent suppression of Rho activity is abolished. As a result, the motile and invasive phenotype of *del 8* cells is associated with relatively low-Rac1 and high-Rho activity levels. Taken together, the deletion of exon 8 of E-cadherin affects the balance between Rac1 and Rho which plays a crucial role for the coordination of cell–cell adhesion and cell motility and invasion. Inhibition of the basal Rac1 activity (by TcdBF) and Rho activity (by C3) strongly reduced cell motility and invasion of *del 8* cells, further underlining that both Rho GTPases contribute to the migratory and invasive phenotype that is associated with the somatic E-cadherin mutation.

that the deletion of exon 8 of E-cadherin affects this regulation and disturbs the balance between Rac1 and Rho that is critical for the coordination of cell–cell adhesion and cell motility (59).

A finely tuned balance of Rho and Rac1 activity controls migration and invasion

The acquisition of migratory and invasive properties are key events in the oncogenic progression of cells (60). Individual Rho GTPases are required for critical aspects of the migration and invasion of tumour cells (22). In cells expressing mutant E-cadherin, we observed that either migration or invasion are sensitive to inhibition by either TcdBF or C3, suggesting that the activity of either Rac1 or RhoA is critically required for either effect. Although Rac1 is likely to inhibit tumour cell motility and invasion through its capacity to regulate the cytoskeletal changes that are necessary for the establishment of epithelial cell–cell contacts, active Rac1 can promote a migratory phenotype in other situations and could therefore stimulate or inhibit tumour cell migration and invasion, depending on the cellular background (22,61).

We have shown here that the enhanced cell motility and the invasive capacity that is associated with mutant E-cadherin is sensitive to the inhibition of Rac1. The finding that the Rac1 inhibitor has such a strong effect was unexpected, because the Rac1 pathway is already strongly suppressed in these cells. However, we could show that the Rac1 inhibitor completely abolished even the residual Rac1 activity that is observed in these cells and likely accounts for the formation of movement-related actin structures. One may argue that it is unusual that Rac inhibition by TcdBF has such a strong effect on the invasion of these cells, as they exhibit already reduced Rac activity. This argument is based on the assumption that Rac1 activity correlates with invasive capacity in cells with mutant E-cadherin. This assumption may not be true, as Rac1 exerts opposing functions: on the one hand, Rac1 inhibits tumour cell motility and invasion through its capacity to regulate the cytoskeletal changes that are necessary for the establishment of epithelial cell–cell contacts. On the other hand, active Rac1 promotes a migratory phenotype and therefore stimulates tumour cell migration and invasion. We found that in cells with mutant E-cadherin, reduced Rac1 activity appears to be sufficient for a high-invasive capacity. In contrast, high Rac1 activity (as observed in cells expressing wild-type E-cadherin) does not result in a high-invasive capacity. The effect of Rac1 activation/inhibition on migration or invasion thus depends on the cellular background and is difficult (if possible) to predict.

Furthermore, we demonstrate that Rho is crucial for the cell motility and invasion associated with the exon 8 E-cadherin mutation, since inhibition of Rho activity leads to immobilization of the tumour cells. In our cell system, cell motility and

cell invasion were influenced in a similar way, whereas in CHO cells, expression of the T340A and A634V germline E-cadherin mutation was associated with an increased level of active Rho, and Rho inhibition resulted in the reduction of cell motility, although cell invasion was not influenced (62).

The significance of our findings in relation to cancer

Rac1 and Rho are overexpressed in various tumours, indicating that Rho GTPase signalling contributes to malignant transformation (reviewed in 63). Further, a splice variant of Rac1, Rac1b and few Rho GTPase mutations were described in some tumours (reviewed in 22). The genomic deletion of Rac1 in mice was lethal early in embryogenesis (47,64). Analysis of mouse embryonic fibroblasts that are defective in Rac1, has revealed that Rac1 is important for regulating the cell matrix interaction and cell spreading, and that Rac1-deficient cells were susceptible to apoptosis, suggesting that Rac1 plays a critical role in the regulation of survival (65).

Rac1 plays a role in the carcinogenesis of gastric cancer, because increased expression and activity of Rac1 has been related to tumour progression by several groups, including our own (66,67). In this study, we demonstrate that the mutation of E-cadherin influences Rac1 and Rho activation in opposite directions and that both Rho GTPases are critically involved in the establishment of the migratory and invasive tumour cell phenotype. Further investigations of tumour samples are necessary to clarify the importance of our findings for metastasis and for therapeutic intervention in gastric carcinoma patients.

MATERIALS AND METHODS

Cell cultivation and transfection

Wild-type and mutant E-cadherin cDNAs were isolated previously from non-tumourous gastric mucosa or somatic gastric carcinoma of the diffuse type and were expressed stably in MDA-MB-435S carcinoma cells (ATCC, Rockville, MD, USA) (10). Vector-transfected control MDA-MB-435S cells were obtained after transfection with the β-actin promoter-based pBATEM expression vector as described before (10). The cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Eggenstein, Germany) supplemented with 10% fetal calf serum (PAN-Biotech, Aidenbach, Germany) and penicillin–streptomycin (50 IU/ml and 50 μg/ml; Life Technologies) at 37°C and 5% CO_2. The MDA-MB-435S strain evolved from the parent line MDA-MB-435, which was isolated from the pleural effusion of a female with metastatic, ductal adenocarcinoma of the breast (68).

Western blot analysis

For western blot analysis, the cells were seeded at a density of 1.5×10^6 cells per 10 cm tissue culture dish and lysed 1 day later with 500 μl L-CAM buffer (140 mM NaCl, 4.7 mM KCl, 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 phenylmethylsulfonylfluoride (69)]. The proteins were separated by SDS–polyacrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Cell lysates were probed with monoclonal anti-E-cadherin antibody (Ab, dilution 1:5000), anti-Rac1 Ab (dilution 1:2500), anti-IQGAP1 Ab (dilution 1:1000) and anti-Rho Ab (dilution 1:250), all of which were purchased from BD Biosciences (Heidelberg, Germany). Monoclonal anti-α-tubulin Ab (Sigma, Deisenhofen, Germany, dilution 1:30 000) was used to stain α-tubulin as a loading control. The enhanced chemoluminescence system was used for signal detection (Amersham, Braunschweig, Germany). For signal quantification, the blots were scanned and densitometric analysis was performed with Scion Image Software from Scion Corporation (Version Beta 4.0.2, Frederick, USA).

Immunofluorescence staining

For immunofluorescence staining of Rac1, IQGAP1 or p120-catenin, the cells were grown on coverslips in six-well plates and, after 2 days of culture, were fixed with formaldehyde as described previously (10). The cells were stained with monoclonal anti-Rac1 Ab clone 23A8 (Upstate, part of Millipore, Schwalbach, Germany, dilution 1:250), anti-IQGAP1 Ab (BD Biosciences, dilution 1:300) or monoclonal anti-p120-catenin Ab (Sigma, dilution 1:100, 2 μg/ml). The cells were stained with FITC-coupled goat-anti-mouse Ab (Zymed, part of Invitrogen, Karlsruhe, Germany, dilution 1:500) after incubation at room temperature for 3 h. The cells were analysed using a Zeiss Axiovert 135 or a Zeiss Axiovert 200M epifluorescence microscope equipped with a charge-coupled device camera (Zeiss, Jena, Germany).

Immunoprecipitation

To examine the interaction of Rac1 with its effector protein IQGAP1, the cells were seeded at a density of 2×10^6 on 10-cm tissue culture plates and cultured for 24 h in DMEM with 10% FCS. For immunoprecipitation, 500 μg protein lysates and monoclonal anti-Rac1 antibody was used to precipitate Rac1 with *Catch and Release v2.0* Reversible Immunoprecipitation System (Upstate, part of Millipore) according to the manufacturer's instructions. For subsequent immunoblotting analysis of the eluated immunocomplexes, the antibodies anti-Rac1 and anti-IQGAP1 from BD Biosciences were used.

Rac1 activity assay

The Rac1 pull-down assay was performed using the EZ-Detect™ Rac1 Activation Kit (Pierce Biotechnology, Bonn, Germany) according to the manufacturer's instructions. Briefly, 2×10^6 cells were seeded per 10-cm dish and incubated for 24 h in DMEM containing 10% FCS. Cells were washed twice with PBS and lysed with 150 μl of the lysis buffer which was contained in the kit. Before use, phospatase- and protease inhibitors were added to the lysis buffer at the following concentrations: 2 mM phenylmethylsulfonylfluoride, 2 mM sodiumorthovanadate, 19 μg/ml aprotinin, 20 μg/ml leupeptin, 100 mM sodium fluoride and 10 mM sodium phosphate. The GST-Pak1-PDB pull-down assay uses a GST-fusion

protein containing the p21-binding domain of human Pak1 to specifically pull-down active Rac1. GST-Pak1-PDB was incubated with cell lysate in the presence of an immobilised glutathione disc. The pulled-down active Rac1 was detected using western blot analysis.

Rho activity assay

Rho activity levels were measured using the GST-Rhotekin-RDB pull-down assay (Upstate, now a part of Millipore) according to the manufacturer's instructions. Briefly, 2×10^6 cells were seeded per 10-cm dish and incubated for 24 h in DMEM containing 10% FCS. The cells were washed twice with PBS and lysed with 200 μl of FISCH-lysis buffer, which consists of 10% (w/v) glycerine, 2 mM $MgCl_2$, 100 mM NaCl, 1% (w/v) NP-40 and 50 mM Tris, pH 7.5. The assay uses a GST-fusion protein containing the Rho-binding domain of mouse Rhotekin to specifically pull-down active Rho. The pulled-down active Rho was detected by western blot analysis.

Specific inhibitors of Rac1 and Rho activity

Toxin B from the variant *C. difficile* serotype strain 1470 serotype (TcdBF), a glucosyltransferase that covalently modifies Rac1, but not Rho (A, B, C), was purified from clostridia as described (35). TcdBF mono-glucosylates Rac1 (not RhoA) at Thr-35, a pivotal amino acid residue in the effector region, resulting in impaired effector coupling (70–72). Impaired Rac1 signalling results in re-organization of the actin cytoskeleton, which is indicated by cell rounding, detachment from the substrate and by clustering. The stock concentration of TcdBF was 10 μg/ml. A kinetic assay of Toxin B concentration (10–0.1 ng/ml) revealed that very low concentrations of TcdBF (0.1 ng/ml) were sufficient to induce morphological changes (including cell rounding, clustering and detachment from the substrate) of the treated cells within 24 h (data not shown). Exoenzyme C3 (C3) from *C. botulinum* specifically inhibits Rho (A, B, C) by ADP-ribosylation at Asn-41 (73). C3 was obtained from Calbiochem (now a part of Merck, Darmstadt) and dissolved in H_2O to obtain a concentration of 50 μg/μl.

Time-lapse laser scanning microscopy

Time-lapse laser scanning microscopy was performed in a microscope-coupled incubation chamber (Zeiss) at 37°C under 5% CO_2 as described before (11). The cells were seeded at a density of 2×10^5 cells per plate with a glass bottom (MatTek Corporation, Ashland, MA, USA), which was coated for 1 h at 37°C with collagen I (100 μg/ml, Sigma) in DMEM containing 10% FCS. After 2 h of incubation, the cells were treated with the Rac1 inhibitor TcdBF (0.4 ng/ml) or the Rho inhibitor C3 (6 μg/ml). Phase contrast images were taken every 3 min for 7 h, using an Axiovert laser scanning microscope LSM 510. The percentage of motile cells was determined by counting the cells in a microscopic field that moved completely out of the initial area within the time of the record. Only attached non-dividing cells that did not leave the observation field during the period of investigation were analysed.

Invasion assay

BioCoat™ Matrigel™ Invasion Chambers (BD Biosciences) were used to study cell invasion, as recommended by the manufacturer. The BD Matrigel™ Matrix serves as a reconstituted basement membrane with a barrier function for non-invasive cells. In contrast, invading cells penetrate the Matrigel Matrix and migrate through the PET membrane. Cell suspensions containing 2.5×10^4 cells in 500 μl DMEM containing 10% FCS were added to the invasion chambers and incubated for 1 h, using DMEM containing 20% FCS as a chemoattractant. Subsequently, cells were treated with Rac1 inhibitor TcdBF (0.2 ng/ml) or Rho inhibitor C3 (6 μg/ml), and incubated for 22 h at 37°C under 5% CO_2. Then, the non-invading cells were removed from the upper surface of the membrane and all of the invading cells were fixed with ice-cold methanol for 10 min and stained with 4,6-diamidino-2-phenylindole (Sigma) at a concentration of 1 μg/ml (stock solution 1 mg/ml, dissolved in H_2O) for 10 min. All of the invading cells were counted in high power microscopic fields (magnification ×10, Axiovert 135, Zeiss). The average number of invading cells (+SD), from two independent experiments performed as triplicates, was determined.

Conflict of Interest statement. None declared.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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