Rho family proteins are essential for the formation of adherens junctions, which are required for the maintenance of epithelial integrity. Activated Rac and the Rac exchange factor Tiam1 have been shown to promote the formation of adherens junctions and the accompanying induction of an epithelioid phenotype in a number of cell lines. Here we show that Madin-Darby canine kidney II cells in which Tiam1 was down-regulated using short interfering RNA disassembled their cadherin-based adhesions and acquired a flattened, migratory, and mesenchymal morphology. In addition, the expression of E1A in mesenchymal V12Ras-transformed Madin-Darby canine kidney II cells led simultaneously to the up-regulation of the Tiam1 protein, the activation of Rac, the formation of cadherin-based adhesions, and reversion to an epithelial phenotype. This finding suggests that E1A induces an epithelial morphology through the up-regulation of Tiam1 and, thereby, the activation of Rac and the formation of cadherin-based adhesions. Indeed, we found that E1A is able to induce an epithelial-like morphology accompanied by the formation of cadherin-based adhesions only in wild-type but not in Tiam1-deficient primary mouse embryonic fibroblasts. These studies indicate that the Rac activator Tiam1 is essential for the formation as well as the maintenance of cadherin-based adhesions.

The acquisition of a migratory and invasive ability, a hallmark of malignant epithelial cells, is predominantly caused by the loss of cell-cell adhesion. In healthy tissues, pronounced junctional complexes such as tight junctions, adherens junctions (AJs), and desmosomes are localized to the lateral membranes of adjacent epithelial cells. These complexes mediate stable cell-cell adhesion, limit solute diffusion, and permit the development of cellular polarity. The primary adhesion molecules within AJs are cadherins whose extracellular domains interact homotypically, depending upon the presence of Ca\(^{2+}\) ions. The cytoplasmic tails of cadherin molecules recruit adaptor proteins termed catenins to the plasma membrane that, in turn, physically link cadherins with cortical actin filaments.

The Rac exchange factor Tiam1 is required for the establishment and maintenance of cadherin-based adhesions.*

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The abbreviations used are: AJ, adherens junction; GST, glutathione S-transferase; MDCK, Madin-Darby canine kidney; MEF, mouse embryonic fibroblast; MET, mesenchymal to epithelial; siRNA, short interfering RNA; WT, wild-type.

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1 The abbreviations used are: AJ, adherens junction; GST, glutathione S-transferase; MDCK, Madin-Darby canine kidney; MEF, mouse embryonic fibroblast; MET, mesenchymal to epithelial; siRNA, short interfering RNA; WT, wild-type.

(Reviewed in Ref. 1). The maintenance of AJs is indispensable for epithelial integrity. Disassembly of AJs occurs during epithelial-mesenchymal transition and is required for embryogenesis (e.g., during mesoderm or neural crest formation) and wound healing. Deregulated epithelial-mesenchymal transition underlies malignant conversion. Disassembly of AJs can be elicited by growth factor signaling, activation of certain oncproteins, or direct inhibition of AJ components and is accompanied by increased cell migration and invasiveness (reviewed in Ref. 2).

The Rho-like molecules Cdc42, Rac, and Rho regulate the establishment and maintenance of AJs in epithelial cells (3–5). Rac activity in particular appears to be essential for the translocation of cadherins and catenins to sites of cell-cell contact and also for the recruitment of filamentous (F)-actin to AJs (5–7). Rho-like molecules are members of the small GTP-binding protein Ras superfamily. Like Ras, Rho-like molecules cycle between an active GTP-bound form, which is capable of binding effector molecules, and an inactive GDP-bound form. Guanine nucleotide exchange factors activate Rho proteins by promoting GTP loading. We identified the guanine nucleotide exchange factor Tiam1 as a specific activator of Rac (8–10). In Madin-Darby canine kidney (MDCK) II epithelial cells the endogenous Tiam1 protein is localized to intercellular adhesions. Tiam1-mediated Rac activation stimulates cadherin-mediated cell-cell adhesion in both mesenchymal like Ras-transformed MDCKII (MDCK-R3) cells and NIH3T3 fibroblasts, limiting their migration and invasiveness (7, 10). Moreover, Rac transformation of MDCKII cells leads to down-regulation of Tiam1 and, consequently, to reduced Rac activity and a mesenchymal phenotype (11). These findings imply a role for Tiam1 in promoting cadherin-based adhesion and reveal a correlation between the acquisition of a mesenchymal phenotype and the down-regulation of Tiam1. However it is unclear whether the down-regulation of Tiam1 is responsible for the mesenchymal phenotype or merely its consequence. Nor is it known whether endogenous Tiam1 plays an essential role in the maintenance of adherens junctions.

In this study we have made use of a recently developed siRNA technique (12) to down-regulate endogenous Tiam1 and demonstrate its requirement in adherens junction maintenance. Furthermore, to study the requirement of Tiam1 in the establishment of cadherin-based adhesions we have used the ability of the 12 S product of adenoviral E1A to induce mesenchymal to epithelial transition (MET). E1A has been shown to induce epithelial morphology in neoplastically transformed cells and cells of mesenchymal origin (reviewed in Ref. 13). E1A-induced MET corresponds to the (re)emergence of intercellular junctional complexes, reorganization of the cytoskeleton, and the restoration/institution of contact inhibition (14, 15). We found that, following the introduction of E1A into fibroblasts or MDCK-R3 cells, de novo Tiam1 expression was induced concomitantly with Rac1 activation and the formation of...
cadherin-based adhesions. Furthermore, by using Tiam1-deficient cells we demonstrate that Tiam1/Rac signaling is essential for the E1A-mediated formation of cadherin-based adhesions and MET, reinforcing a governing role for Tiam1/Rac in determining epithelial cell behavior.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Constructs, and Transfection—**MDCKII cells, MDCCK3 cells, and mouse embryoblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (both from Invitrogen). NIH3T3 cells were cultured in Dulbecco's modified Eagle's with 10% newborn calf serum (Invitrogen). The construction of pCMV1A12S to direct the expression of the wild-type (WT) E1A 12 S protein and confer neomycin-resistance was described previously (16) and was a kind gift of Dr. R. Bernards (The Netherlands Cancer Institute). Cells were transfected with pCMV1A12S using Effectene (Invitrogen) according to the manufacturer's instructions. Following transfection, cells were cultured for 24 h in fresh medium prior to selection in a medium containing 0.8 mg/ml neomycin (Sigma).

**In Vitro Wounding Assay—**Cells were grown in the presence of 10% fetal bovine serum to ~90% confluency in six wells. Subsequently, a scratch of ~1 mm was made, and a fresh, serum-containing medium was added. Closure of the scratch was determined by phase-contrast microscopy, and images were taken 0 and 24 h after the scratch was made. Migration was quantified by measuring the relative areas that were closed by migrated cells. Means and S.E. of three independent experiments are shown.

**RESULTS**

**Tiam1 Is Essential for the Maintenance of Adherens Junctions—**Tiam1 is localized to adherens junctions in a number of epithelial cell lines, and its overexpression leads to increased E-cadherin-based adhesions (7). This fact indicates that Tiam1 may play an essential role in the maintenance of adherens junctions. We used siRNA to study the role of Tiam1 in the maintenance of adherens junctions in epithelial MDCKII cells. We prepared and incubated purified GST-PARK-C (for determination of Rac1 activation status) or GST-C21 (for RhoA) bound to glutathione-coupled Sepharose beads. Following pull-down and SDS-PAGE, GTP-Rac1 or GTP-RhoA were detected by immunoblottting with anti-Rac1 (Pharmingen/BD Transduction Laboratories) or anti-RhoA (Santa Cruz Biotechnology) mouse monoclonal antibodies, respectively, and subsequently by horseradish peroxidase-conjugated rabbit anti-mouse IgG (Amersham Biosciences). Reactive proteins were visualized by enhanced chemiluminescence.

**Rac and Rho Activity Assays—**Rac1 and RhoA activity assays were performed as described previously (10). Briefly, lysates from an equivalent number of cells were prepared and incubated with purified GST-PARK-C (for determination of Rac1 activation status) or GST-C21 (for RhoA) bound to glutathione-coupled Sepharose beads. Following pull-down and SDS-PAGE, GTP-Rac1 or GTP-RhoA were detected by immunoblotting with anti-Rac1 (Pharmingen/BD Transduction Laboratories) or anti-RhoA (Santa Cruz Biotechnology) mouse monoclonal antibodies, respectively, and subsequently by horseradish peroxidase-conjugated rabbit anti-mouse IgG (Amersham Biosciences). Reactive proteins were visualized by enhanced chemiluminescence.
not shown). These findings demonstrate that Tiam1-Rac signaling is essential for the maintenance of E-cadherin mediated cell-cell adhesions and are consistent with earlier observations that the ectopic expression of Tiam1 promotes the formation of E-cadherin-based adhesions in MDCKII cells (7).

E1A Expression Restores Adherens Junctions in Mesenchymal MDCK-f3 Cells and Induces Tiam1/Rac Signaling—E1A induces epithelial characteristics in a number of human mesenchymal tumor cell lines through its ability to genetically reprogram cells to (re)express epithelial cell determinants, including tight, adherens, and desmosomal junctional components, and to simultaneously lose expression of non-epithelial genes (e.g. desmin, tyrosinase, and cardiac β-actin) (15). In this study we examined whether E1A is able to induce epithelial characteristics in V12Ras-transformed MDCK-f3 cells with the intention to use it as a means to study the requirement of Tiam1/Rac signaling in the establishment of cadherin-based adhesion. MDCK-f3 cells exhibit a mesenchymal transformed phenotype and display increased motility and invasiveness compared with parental cells (21, 22). We have demonstrated previously that epithelial-mesenchymal transition in these cells is caused by Ras-mediated down-regulation of Tiam1-Rac signaling (11). Analysis of cell populations and a number of MDCK-f3 clones selected for sustained expression of E1A showed that they appeared to be morphologically reverted toward a more epithelial phenotype resembling that of the original MDCKII cells (Fig. 2, a and b). Importantly, these cells still express oncogenic Ras, demonstrating that their epithelioid phenotype is not the result of selection for clones that had lost exogenous V12Ras expression (Fig. 2b). The epithelioid phenotype induced by E1A is associated with the re-establishment of cadherin-based adhesions as shown by E-cadherin staining (Fig. 2c). These results demonstrate that E1A is capable of restoring E-cadherin-containing cell-cell adhesions in MDCK-f3 cells and, as a consequence, reinstate an epithelial phenotype in these cells.
We have shown previously that overexpression of Tiam1 or constitutively active Rac1 (V12Rac1) in MDCK-f3 cells leads to the emergence of an epithelioid morphology by stimulating the formation of cadherin-mediated cell-cell contacts. Moreover, following overexpression in MDCK-f3 cells Tiam1 co-localizes with E-cadherin at sites of cell-cell contact (7). To examine whether E1A induces MET through the modulation of Tiam1/Rac signaling, we determined Tiam1 protein levels in several MDCK-f3 E1A-expressing clones and cell populations, compared those levels with the corresponding levels in MDCK-f3 control clones, and analyzed the amount of active Rac in these clones. As shown in Fig. 2, MDCK-f3 cells expressing E1A showed increased expression of Tiam1 accompanied by increased Rac activity (Fig. 2e). Increased Rac1 activity was associated with decreased RhoA activity (Fig. 2e) as observed previously (10, 11). These data suggest that the epithelioid phenotype of MDCK-f3 E1A-expressing cells is the result of the up-regulation of Tiam1 and, consequently, of increased Rac activity.

**E1A Induces Tiam1/Rac Signaling and Cadherin-based Cell-Cell Adhesions in Fibroblasts**—The previous data show that E1A induces up-regulation of Tiam1/Rac signaling and cell-cell adhesions in cells expressing active V12Ras. To study the effects of E1A in cells lacking active Ras, we expressed E1A in NIH3T3 cells. These are immortalized mouse fibroblasts with a characteristic mesenchymal morphology and migratory behavior. Following transfection with E1A, cell populations and several independent clones of NIH3T3 cells with sustained expression of E1A protein were isolated. Compared with cells transfected with empty vector alone, the morphology of E1A-expressing cells appeared to be epithelioid (Fig. 3a), as has been described previously (23, 24). Even at low plating density, E1A-expressing cells grew preferentially in tightly packed colonies rather than dispersing as parental cells.

**NIH3T3 cells express N- and P-cadherins but not E-cadherin** (25). Immunocytochemical staining for N- and P-cadherins (with an anti-pan-cadherin antibody) revealed increased localization of these molecules to the induced intercellular contacts in E1A-expressing clones (Fig. 3b). This finding was not due to increased steady-state protein expression levels of cadherins, β-catenin, and γ-catenin as detected by immunoblotting (Fig. 3c). Rather, following E1A expression, cadherins showed reduced solubility in Nonidet P-40 lysis buffer (see “Experimental Procedures”).
Procedures" (Fig. 3d), implying that cadherins were stabilized at the plasma membrane at sites of cell-cell contact through intimate association with the cytoskeleton (Nonidet P-40 insoluble fraction). Because the anti-pan-cadherin antibody recognizes different cadherins, we checked whether individual cadherins were up-regulated by E1A using specific antibodies. Fig. 3e shows that E-cadherin is not expressed in NIH3T3 cells and that its expression is not up-regulated by E1A. In addition, the expression of N-cadherin, the major cadherin in NIH3T3 cells, remains unchanged. Interestingly, as in E1A-expressing MDCK-f3 cells, NIH3T3 cells up-regulate Tiam1 expression and Rac1 activity upon the expression of E1A (Fig. 3, f and g). The up-regulation of Tiam1 in these cells is at the level of transcription (Fig. 3h). NIH3T3 cells also express significant levels of the closest homologue of Tiam1, the Rac exchange factor STEF. STEF mRNA is not induced by E1A expression, which suggests that Tiam1 is an important exchange factor for Rac during the E1A-induced formation of cell-cell adhesions in fibroblasts (Fig. 3h).

Tiam1 Is Essential for E1A Mediated Mesenchymal-to-Epithelial Transition—The previous results further support the hypothesis that Tiam1/Rac signaling is important for the E1A-induced formation of cell-cell adhesions. To determine whether Tiam1 is essential for the establishment of these adhesions, we expressed E1A in MEFs derived from WT mice or mice in which the Tiam1 gene had been ablated by homologous recombination (Tiam1−/− mice) (17). The majority of E1A-expressing WT MEF cell populations and clones resembled their E1A-expressing NIH3T3 counterparts, growing in tightly packed colonies with few scattered cells at the periphery. In contrast, E1A-expressing Tiam1−/− MEFs grew in irregular colonies; detached and dispersed cells were frequently observed at the edges of colonies (Fig. 4a). As in MDCK-f3 and NIH3T3 cells, the introduction of E1A induced the expression of Tiam1 in WT MEFs.
Obviously, no expression of Tiam1 was found in Tiam1-deficient MEFs in the presence or absence of E1A (Fig. 4b). Immunofluorescence staining revealed a pronounced localization of cadherins and β-catenin at sites of cell-cell contact in E1A-expressing WT MEFs that was not seen in E1A-expressing Tiam1−/− MEFs (Fig. 4c) or control-transfected MEFs of either genotype. The fact that E1A is unable to induce an epithelial-like phenotype in Tiam1-deficient cells indicates that Tiam1 is essential for the induction of cadherin-based adhesions and an epithelial phenotype by E1A. Taken together, from the presented studies we conclude that Tiam1 plays a key role in the formation as well as in the maintenance of the cadherin-based adhesions required for an epithelial phenotype.

**DISCUSSION**

Several studies to date have implicated Rac in the establishment and maintenance of adherens junctions. The inhibition of Rac by overexpression of a dominant negative Rac mutant (N17Rac1) hinders the establishment of cadherin-mediated cell-cell contacts and can also result in the selective dislocation of AJ components and F-actin from preformed adhesions in both primary keratinocytes and MDCKII cells (5, 6). Similarly, Rac activity is required in vivo for actin accumulation at the AJs of adjacent epithelial cells in Drosophila wing imaginal discs (26). However, little is known about the signaling pathways that stimulate Rac, resulting in the establishment or maintenance of adherens junctions. Previously, we have shown that overexpression of a stabilized form of Tiam1 (C1199-Tiam1) can, similarly to dominant active Rac, promote the accumulation of AJ components and F-actin at sites of cell-cell contact in MDCKII, MDCK-F3, or NIH3T3 cells (7, 10). Indeed, C1199-Tiam1 and endogenous Tiam1 co-localize with AJ components (10). In this way, Tiam1/Rac signaling enhances cell-cell adhesion while limiting hepatocyte growth factor-induced or Ras-induced cell migration and invasion (7, 27).

The data presented here demonstrate that endogenous levels of Tiam1 are required for the maintenance of AJs in MDCKII cells. Furthermore, by exploiting the MET-inducing properties of E1A we have shown that induction of the expression of endogenous Tiam1 is required for the establishment of cell-cell adhesions. Recently we have found that Ras-induced skin tumors progressed to malignancy more frequently in Tiam1−/− mice than in wild-type mice, which might be explained by the requirement of Tiam1 in the formation and maintenance of cadherin junctions (17). Malignant conversion is normally associated with loss of cell-cell adhesions and increased migration, as was also observed in MDCKII cells in which Tiam1 was down-regulated. In addition, Tiam1−/− mice develop smaller tumors than wild-type control mice, which is consistent with the decreased growth rate observed in MDCKII cells that express Tiam1 siRNA (1).

The MET-inducing property of E1A protein, although well documented (13, 28), is not well understood. E1A expression in neoplastic cells and mesenchymal cells is envisaged as inhibiting non-epithelial gene expression by squelching the transcriptional co-activators (e.g. CBP/p300) required by cell type-specific transcription factors (e.g. MyoD) while simultaneously de-repressing epithelial gene expression. Epithelial proteins induced by E1A include desmoglein and desmoplakin, two desmosomal components, plakoglobin, which is both an AJ and desmosomal component, and ZO-1, a major structural component of tight junctions. Surprisingly, in most cell types tested the expression of E-cadherin, a major component of AJs, was not up-regulated by E1A (15) (Fig. 3c).

We have demonstrated that the expression of E1A in Ras-transformed MDCK-F3 cells, NIH3T3 cells, or immortalized MEFs induces MET by promoting the formation of cadherin-based cell-cell contacts. However, this did not require the de novo synthesis of AJ components (as revealed by examining the steady-state levels of cadherin and β- and γ-catenins in NIH3T3 cells). MET in the above cell types corresponded with increased Tiam1 expression and, consequently, increased Rac1 activity and reduced RhoA activity. Significantly, E1A failed to...
induce MET in MEFs derived from Tiam1−/− mice, showing that induction of Tiam1 expression and the ensuing activation of Rac1 are prerequisites for the E1A-stimulated formation of cadherin-based cell-cell contacts and the emergence of an epithelioid morphology.

The capacity of E1A to induce MET maps to its C terminus, encoded by exon 2 of the 12 S isoform (29). To date, the C-terminal binding protein is the only cellular factor identified that binds the C terminus of E1A (30, 31). The C-terminal binding protein is a zinc finger-containing nuclear protein that is capable of interacting with other nuclear transcription factors. In reporter assays and during fly and mouse development, the C terminus-binding protein functions as a transcriptional co-repressor (32–34). Indeed, the C-terminal binding protein is a strong candidate for an activity capable of co-repressing epithelial genes that is sequestered and inactivated by E1A (29, 35). Thus far, the region of E1A responsible for inducing Tiam1 expression is unknown. Mapping this region and identifying the cellular factors that bind to it may help to elucidate the signaling cascade(s) that regulate Tiam1 expression during E1A-induced mesenchymal to epithelial transition.

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