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Raf Kinase Inhibitor Protein Positively Regulates Cell–Substratum Adhesion While Negatively Regulating Cell–Cell Adhesion

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Abstract Raf kinase inhibitor protein (RKIP) regulates a number of cellular processes, including cell migration. Exploring the role of RKIP in cell adhesion, we found that overexpression of RKIP in Madin-Darby canine kidney (MDCK) epithelial cells increases adhesion to the substratum, while decreasing adhesion of the cells to one another. The level of the adherens junction protein E-cadherin declines profoundly, and there is loss of normal localization of the tight junction protein ZO-1, while expression of the cell–substratum adhesion protein β1 integrin dramatically increases. The cells also display increased adhesion and spreading on multiple substrata, including collagen, gelatin, fibronectin and laminin. In three-dimensional culture, RKIP overexpression leads to marked cell elongation and extension of long membrane protrusions into the surrounding matrix, and the cells do not form hollow cysts. RKIP-overexpressing cells generate considerably more contractile traction force than do control cells. In contrast, RNA interference-based silencing of RKIP expression results in decreased cell–substratum adhesion in both MDCK and MCF7 human breast adenocarcinoma cells. Treatment of MDCK and MCF7 cells with locostatin, a direct inhibitor of RKIP and cell migration, also reduces cell–substratum adhesion. Silencing of RKIP expression in MCF7 cells leads to a reduction in the rate of wound closure in a scratch-wound assay, although not as pronounced as that previously reported for RKIP-knockdown MDCK cells. These results suggest that RKIP has important roles in the regulation of cell adhesion, positively controlling cell–substratum adhesion while negatively controlling cell–cell adhesion, and underscore the complex functions of RKIP in cell physiology. J. Cell. Biochem. 103: 972–985, 2008.

Key words: Raf kinase inhibitor protein; cell adhesion; extracellular matrix; cell migration

We have previously shown that the small molecule locostatin is an inhibitor of cell migration [Mc Henry et al., 2002] that covalently binds to and directly inhibits the function of Raf kinase inhibitor protein (RKIP) [Zhu et al., 2005]. RKIP is an endogenous inhibitor of the activity of multiple kinases, including Raf-1 kinase, G protein-coupled receptor kinase 2 and kinases involved in nuclear factor κB (NFκB) signaling [for reviews, see refs. Odabaei et al., 2004; Keller et al., 2005; Trakul and Rosner, 2005]. Locostatin prevents RKIP from binding Raf-1 kinase [Zhu et al., 2005]. We have found also that overexpression of RKIP in Madin-Darby canine kidney (MDCK) epithelial cells, which are widely used as a model for the behavior of differentiated epithelia, results in

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loss of cell–cell contacts and transformation of the cells to a highly migratory, fibroblast-like phenotype [Zhu et al., 2005]. Conversely, small interfering RNA (siRNA)-mediated silencing of RKIP expression reduces the rate of cell migration in MDCK cells [Zhu et al., 2005].

RKIP was originally identified as a protein that bound the lipid phosphatidylethanolamine [Bernier et al., 1986], and so it is also referred to as phosphatidylethanolamine-binding protein in the literature. Subsequent demonstration of its ability to bind and inhibit Raf-1 [Yeung et al., 1999, 2000] and other kinases [Yeung et al., 2001; Corbit et al., 2003; Lorenz et al., 2003; Park et al., 2005; Trakul et al., 2005], and questions about whether its interaction with phosphatidylethanolamine is physiologically relevant at all, has led to a consensus that its main intracellular role is as a modulator of kinase signaling.

RKIP appears to play a role in a number of biological processes, including nervous and cardiac functions, membrane biogenesis and spermatogenesis [for reviews, see refs. Odabaei et al., 2004; Keller et al., 2005; Trakul and Rosner, 2005]. Another important role for RKIP is as the precursor of hippocampal cholinergic neurostimulating peptide [for review, see ref. Ojika et al., 2000]. RKIP-knockout mice are viable and appear normal but do develop an olfaction defect that is consistent with the expression pattern of the RKIP gene in the brain [Theroux et al., 2007]. RKIP has been implicated as a suppressor of tumor cell metastasis in prostate cancer, breast cancer, melanoma, hepatocellular carcinoma, and colorectal cancer cells, based on a number of expression studies [Fu et al., 2003, 2006; Schuierer et al., 2004, 2006a; Hagan et al., 2005; Al-Mulla et al., 2006; Lee et al., 2006; Minoo et al., 2007]. In contrast, RKIP expression is higher in tumorigenic and metastatic fibrosarcoma cells than in their less aggressive parental cells [Hayashi et al., 2005]. Following initial solid tumor formation and neovascularization, metastasis is a complex process involving not only cell motility but also changes in cell adhesion, secretion of a range of proteases and the development of distant metastases [for reviews, see Engers and Gabbert, 2000; MacDonald et al., 2002; Bogenrieder and Herlyn, 2003; Kopfstein and Christofori, 2006; Eccles and Welch, 2007]. Furthermore, RKIP has been shown to regulate Aurora B kinase, and it has been suggested that cells that have lost RKIP expression could have chromosomal abnormalities that would provide a basis for their increased metastatic potential [Eves et al., 2006]. It is not yet known exactly which metastasis-related processes are affected by changes in RKIP expression in cancer cells.

Adhesion of cells to extracellular matrix (ECM) is a dynamic process that is critical to the mechanical coupling of intracellular and extracellular environments required for cell migration [for a review, see Li et al., 2005]. Overexpression of RKIP has been shown to promote macrophage differentiation by modulating NFκB signaling, resulting in increased expression of leukocyte integrin CD11c [Schuierer et al., 2006b]. These results thus imply a link between RKIP and the expression of cell–substratum adhesion proteins.

Here we report that, based on multiple lines of evidence, RKIP appears to have a positive function in regulating cell–substratum adhesion in MDCK cells and MCF7 human breast adenocarcinoma cells. MDCK cells overexpressing RKIP exhibit increased β1 integrin expression and cell adhesion on multiple substrata, including glass in the presence of serum and non-tissue culture-treated dishes coated with defined ECM components. RKIP also increases the ability of cells to generate productive traction force. Knockdown of RKIP in both MDCK and MCF7 cells results in reduced cell–substratum adhesion. In contrast, RKIP appears to play a negative role in the control of cell–cell adhesion, regulating the expression of the adherens junction protein E-cadherin and the localization of the tight junction protein ZO-1 in MDCK cells. Finally, we show that knockdown of RKIP in MCF7 cells leads to a slight decrease in the rate of wound closure in a scratch-wound assay, an effect consistent with, although not as strong as, that of RKIP knockdown in MDCK cells [Zhu et al., 2005].

MATERIALS AND METHODS

Reagents

Locostatin was synthesized according to previously published procedures [Mc Henry et al., 2002]. Cell culture media were from Invitrogen/Gibco. Mouse anti-E-cadherin, mouse anti-ZO-1 and mouse anti-RKIP (N-terminal) antibodies were purchased from Invitrogen/Zymed. Mouse anti-β-actin antibody was from Sigma–Aldrich. Rat anti-β1 integrin antibody was from the

Developmental Studies Hybridoma Bank (University of Iowa). Fluorophore-conjugated goat anti-mouse IgG secondary antibodies were from Invitrogen/Molecular Probes. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rat IgG secondary antibodies were from Bio-Rad Laboratories. Fibronectin and "ultrapure" laminin were from BD Biosciences. Gelatin was from Merck (Darmstadt, Germany).

Cells and Cell Culture

MDCK cells were cultured in Minimum Essential Medium (MEM, with Earle's balanced salts, non-essential amino acids, L-glutamine and sodium pyruvate) supplemented with 10% (v/v) newborn calf serum (NCS) or fetal bovine serum (FBS). The stable transfectants constitutively expressing enhanced GFP-RKIP under the control of the CMV promoter have been described previously, as have the RKIP-knockdown MDCK cells stably expressing an RKIP-specific siRNA from a small hairpin RNA (shRNA) expression vector [Zhu et al., 2005]. MCF7 cells were cultured in MEM with 10% FBS. All cells were maintained at 37°C and 5% CO_2. For experiments, cells were plated onto BD Falcon tissue culture-treated multiwell plates, glass coverslips or Bioptechs Delta T chambers. No culture exceeding 19 passages from thawing was used.

Immunofluorescent Staining

Cells were fixed with ethanol at 4°C for 30 min, followed by ice-cold acetone for 3 min. Samples were blocked for 30 min in 1× phosphate-buffered saline (PBS) with 0.2% bovine serum albumin (BSA) and 2% goat serum. Primary antibody was added for a 1-h incubation, followed by secondary antibody. Slides were washed three times in PBS and mounted with Mowiol before examination by fluorescence microscopy on a Zeiss Axiovert 200 inverted microscope.

Western Blot Analysis

Cells were grown in 75-cm^2 tissue culture flasks until ~60–80% confluent and then treated with trypsin. The resulting cell suspension was then counted on a hemocytometer. Cells were suspended to a density of 1.0×10^6 cells/ml in 2× PBS, and then an equal volume of 2× SDS sample buffer was added to samples for Western blot analysis. The samples were boiled for 5 min and equivalent loadings were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels. Following blotting to polyvinylidene difluoride membranes (Millipore), proteins were detected using the appropriate primary and secondary antibodies and the enhanced chemiluminescence detection system, according to the manufacturer's instructions (Amersham Biosciences/GE Healthcare).

Cell Adhesion on Glass in the Presence of Serum

MDCK or MCF7 were plated at a density of 3×10^5 cells/ml on glass coverslips with locostatin or dimethyl sulfoxide (DMSO) carrier solvent alone at corresponding concentrations in MEM with 10% NCS (MDCK cells) or 10% FBS (MCF7 cells). After allowing 1 h for attachment at 37°C and 5% CO_2, cells were rinsed twice with 1× PBS, fixed with freshly prepared 4% paraformaldehyde and mounted on microscope slides. We captured five random fields of view per slide as digital images. Cell number for each field of view was determined.

Cell Adhesion and Spreading on Different ECM Proteins

Bacteriological (non-tissue culture-treated) 35-mm-diameter BD Falcon dishes were incubated at 37°C with either type I collagen from rat tail tendons (prepared as described in Montesano and Orci [1985]), gelatin, fibronectin or laminin, all diluted in double distilled water to 30 μg/ml. After 30 min, the dishes were washed with Ca^{2+}- and Mg^{2+}-free PBS ($PBS^{-/-}$) and incubated with 0.5% BSA in $PBS^{-/-}$ for 30 min at 37°C to saturate non-specific cell-binding sites. In control dishes, the coating step was replaced by incubation in $PBS^{-/-}$ alone. MDCK cells were harvested with trypsin/EDTA, resuspended in serum-free Dulbecco's Modified Eagle's Medium/F12 medium supplemented with 0.5% BSA and plated at a density of 1×10^5 cells/dish. After 1–3-h incubation at 37°C, the medium and unattached cells were removed. The cultures were then fixed in 2.5% glutaraldehyde in PBS and imaged on a Nikon Diaphot TMD inverted microscope and a 20× phase-contrast objective.

Cell Culture in Three-Dimensional Collagen Gels

MDCK cells were harvested with trypsin/EDTA and mixed with a type I collagen solution, as previously described [Montesano et al., 1991], for a density of 3×10^5 cells/ml. Aliquots

of the cell suspension (2.5 ml) were then dispensed into the 35-mm-diameter wells of 6-well Costar plates. After a 10-min incubation at 37°C to allow collagen gelation, 2.5 ml of medium was added above the gels. Collagen gel cultures were examined and imaged on a Nikon Diaphot TMD inverted microscope and a 20× phase-contrast objective.

To evaluate the ability of MDCK cells to contract the collagen matrix, MDCK cells were suspended in collagen gels and cast into the 35-mm-diameter wells of 6-well plates. The gels were then gently loosened from the walls and the bottom of the wells by passing a curved-tip metallic spatula around their circumference and were allowed to float in the medium [Montesano and Orci, 1988]. The plates were imaged 24 h later.

Silencing of RKIP Expression in MCF7 Cells

Preparation of the RKIP-knockdown MDCK cells have been previously described [Zhu et al., 2005]. RKIP-knockdown MCF7 cells were prepared as follows. An RKIP-specific shRNA expression construct was generated by cloning the annealed oligonucleotides 5′-GATCCCC GATTCAGGGAAGCTCTACATTCAAGAGATGTAGAGCTTCCCTGAATCTTTTTA-3′ and 5′-AGCTTAAAAAGATTCAGGGAAGCTCTACATCTCTTGAATGTAGAGCTTCCCTGAATCGGG-3′ into pSUPER.retro-puro (OligoEngine) according to the manufacturer's instructions (human RKIP target sequences are underlined above). A control shRNA expression construct specific for luciferase was made by cloning the annealed oligonucleotides 5′-GATCCCCCGTACGCGGAATACTTCGATTCAAGAGATCGAAGTATTCCGCGTACGTTTTTA-3′ and 5′-AGCTTAAAAACGTACGCGGAATACTTCGATCTCTTGAATCGAAGTATTCCGCGTACGGGG-3′ into the same retroviral vector (luciferase sequences are underlined). Infectious retroviruses were generated using the 293GPG packaging cell line as previously described [Ory et al., 1996]. Following infection, MCF7 cells were cultured in puromycin selection medium for 2 days, and drug-resistant cells were pooled.

Wound Closure Assay

Wound closure experiments were conducted and analyzed as previously described [Mc Henry et al., 2002; Zhu et al., 2005].

RESULTS AND DISCUSSION

RKIP Negatively Regulates Cell–Cell Adhesion

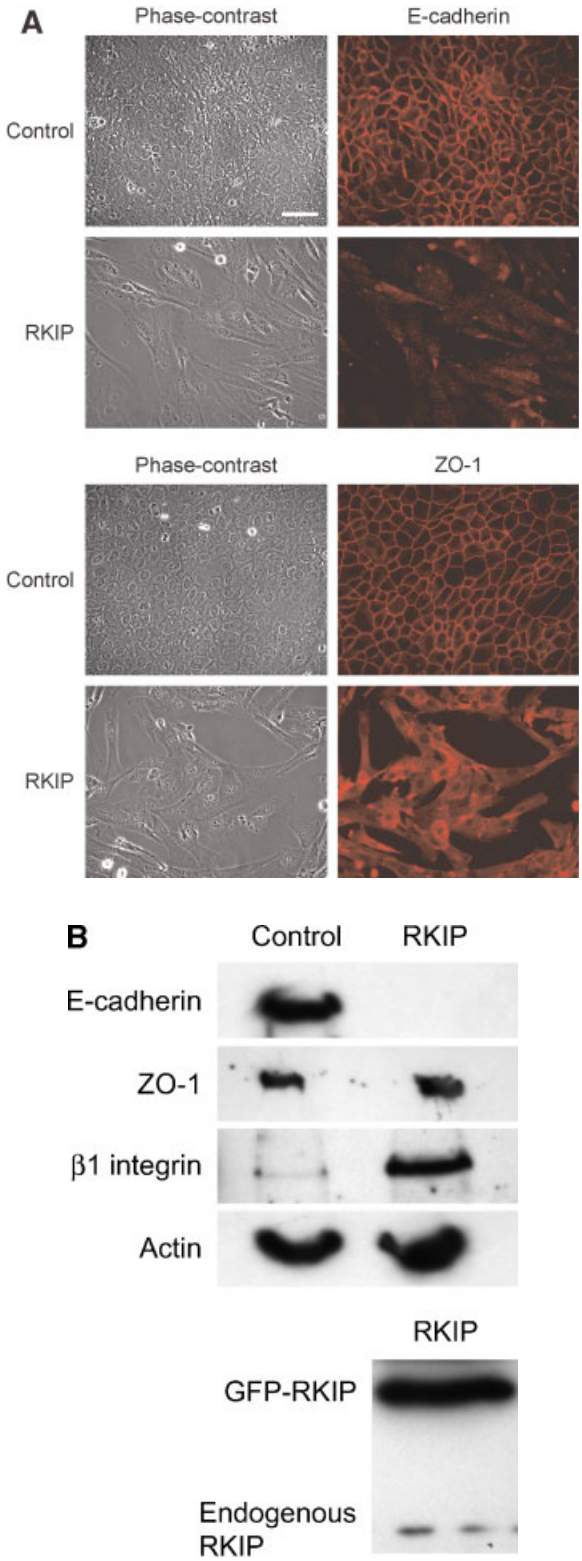
Expression of GFP-RKIP in Madin-Darby canine kidney (MDCK) cells results in a dramatic change in morphology from that of a normal cuboidal epithelium to a much more spread fibroblast-like state with loss of cell–cell adhesions; RKIP-overexpressing cells also exhibit a faster rate of cell migration than control cells and migrate as individuals rather than a continuous cell sheet [Zhu et al., 2005]. The cells also grow as individuals without cell–cell contacts rather than in islands of cells adhering to one another. The change in phenotype resembles the epithelial-mesenchymal-like transition associated with treatment of MDCK cells in monolayer culture with hepatocyte growth factor/scatter factor (HGF/SF), characterized by loss of cell–cell contacts, acquisition of a highly migratory behavior and dispersal of the cells [Stoker and Perryman, 1985; Stoker et al., 1987; Stoker, 1989; Weidner et al., 1990]. Treatment of RKIP-overexpressing cells with the direct RKIP inhibitor locostatin results in a partial reversion back to a more epithelial state [Zhu et al., 2005]. Locostatin also inhibits HGF/SF-induced scatter of MDCK cells [Zhu et al., 2005].

To further characterize changes in cell–cell interactions with RKIP overexpression, we immunofluorescently stained both GFP-RKIP-expressing MDCK cells and control MDCK cells expressing GFP alone with antibodies against the cell–cell adhesion protein E-cadherin, found in epithelial adherens junctions, and the tight junction-associated protein ZO-1. (These cell lines have been described previously [Zhu et al., 2005].) We observed loss of specific localization of both E-cadherin and ZO-1 in RKIP-overexpressing cells compared to control cells (Fig. 1A). In particular, E-cadherin levels appeared much lower in the RKIP-overexpressing cells. These results demonstrate a failure to properly assemble cell–cell junctional complexes with overexpression of RKIP.

RKIP Overexpression Downregulates E-Cadherin but Upregulates β1-Integrin

We evaluated the levels of E-cadherin, ZO-1, β1 integrin and the cytoskeletal protein actin in whole-cell lysates from control and RKIP-overexpressing cells. We found that E-cadherin

levels decreased dramatically with RKIP overexpression, while β1 integrin strongly increased (Fig. 1B). Therefore, overexpression of RKIP affects either the gene expression or protein stability of these different molecules in opposing ways. In contrast, there was little or no difference in the levels of ZO-1 or actin. However, ZO-1 localization becomes more diffuse with loss of normal epithelial tight junctions (Fig. 1A).



The downregulation of E-cadherin may explain the loss of cell–cell interactions with elevated RKIP levels, as E-cadherin is the primary cell–cell adhesion molecule in MDCK [Imhof et al., 1983; Behrens et al., 1985] and other epithelial cells [for reviews, see refs. Braga, 2002; Perez-Moreno et al., 2003]. The increase in β1 integrin levels, in contrast, implies that adhesion of the cells to the underlying substratum could be enhanced with RKIP overexpression, a possibility explored below. β1 integrin, as a dimer with α integrins such as α2 and α3, is an important ECM receptor and is involved in cell–substratum adhesion and morphogenesis in MDCK [Saelman et al., 1995; Schwimmer and Ojakian, 1995; Jiang et al., 2001; Yu et al., 2005] and other epithelial cell types [Tournier et al., 1992; Zutter et al., 1999; Lindberg et al., 2002; Massoumi et al., 2003].

In neuronal cells, the cadherin and integrin systems are believed to engage in crosstalk, with the cadherin system upstream of the integrin [Lilien et al., 1999]. Furthermore, overexpression of E-cadherin has been shown to reduce cell–substratum adhesion to fibronectin through α5β1 integrin in breast carcinoma cells [Wu et al., 2006]. Decrease in cadherin activity with function-blocking antibodies prevents loss of both α6 and β1 integrins expression in terminally differentiated keratinocytes [Hodivala and Watt, 1994]. A dominant-negative E-cadherin fusion protein results in increased

Fig. 1. Overexpression of RKIP results in dissolution of cell–cell junctional complexes in MDCK epithelial cell cultures, downregulation of E-cadherin and upregulation of β1-integrin. **A**: Immunofluorescent staining for E-cadherin and ZO-1 is shown for control MDCK cells stably expressing GFP alone (control) and MDCK cells stably expressing GFP-RKIP (RKIP). Transfection, selection and preliminary characterization of these cells have been described previously [Zhu et al., 2005]. Scale bar = 50 μm. **B**: Western blot analyses showing levels of E-cadherin, ZO-1, β1 integrin and β-actin from equivalent loadings of whole-cell lysates prepared from control and RKIP-overexpressing MDCK cells, as well as the levels of endogenous RKIP and GFP-RKIP in the RKIP-overexpressing MDCK cells (equivalent loadings of two different whole-cell lysates prepared from the same stably transfected cell line).

expression of α2, α3, and β1 integrins and conversion of keratinocytes to an invasive phenotype [Zhang et al., 2006]. During macrophage differentiation, overexpression of RKIP results in increased mRNA levels and cell-surface expression of the integrin CD11c [Schuierer et al., 2006b]. The increase in β1 integrin levels we observed upon RKIP overexpression is consistent with these results.

RKIP Positively Regulates Adhesion on Glass in the Presence of Serum

We have previously identified the small molecule locostatin as an inhibitor of cell migration in MDCK cells whose relevant cellular target is RKIP [Zhu et al., 2005]. Locostatin specifically and covalently binds RKIP, blocking its ability to associate with Raf-1 [Zhu et al., 2005]. To investigate whether RKIP regulates cell–substratum adhesion, we compared adhesion of control cells and RKIP-overexpressing MDCK cells. All adhesion experiments were preformed 1 h after plating out to eliminate any change in cell number due to cell proliferation. The apparent strength of cell adhesion on glass coverslips after 1 h increased with overexpression of RKIP and decreased when RKIP function was inhibited in a dose-dependent manner by treatment with the RKIP inhibitor locostatin (Fig. 2). Furthermore, RKIP overexpression reduced the sensitivity of the cells to locostatin (Fig. 2), in agreement with our previous results showing that locostatin has far less of an anti-migratory effect on RKIP-overexpressing cells than it does on normal MDCK cells [Zhu et al., 2005]. This is presumably due to the fact that the overexpressed RKIP effectively "titrates" out locostatin. In addition, locostatin leads to a partial reversion of RKIP-overexpressing MDCK cells to a more normal epithelial phenotype with reestablishment of cell–cell contacts [Zhu et al., 2005].

The adhesion experiments above were performed on glass coverslips in the presence of serum, which have long been known to contain plasma fibronectin and vitronectin that adsorb onto glass or tissue culture-treated plastic to promote adhesion and spreading [Grinnell, 1976; Hook et al., 1977; Thom et al., 1978; Knox and Griffiths, 1979; Thom et al., 1979; Barnes et al., 1980; Hayman et al., 1983]. Furthermore, MDCK cells produce various ECM components, including collagen, fibronectin and laminin [Caplan et al., 1987; Salas et al., 1987; Smith et al., 1988; Zuk et al., 1989; Ecay and Valentich, 1992; Cook and Van Buskirk, 1994; Low et al., 1994; Fiorino and Zvibel, 1996; Altieri et al., 1998; Inoue et al., 1999, 2001; Jiang et al., 1999, 2000, 2001; Erickson and Couchman, 2001; O'Brien et al., 2001; Shimazu et al., 2001; Wohlfarth et al., 2003; Liu et al., 2005; Yu et al., 2005]. Secreted ECM proteins accumulate and are deposited onto the underlying support

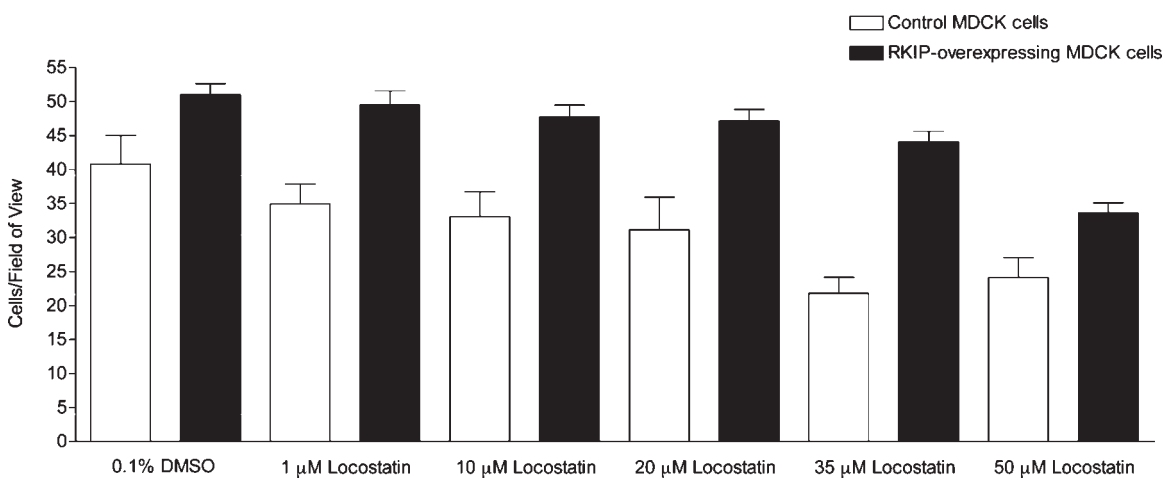


Fig. 2. Overexpression of RKIP increases adhesion of MDCK cells and results in reduced sensitivity to the RKIP inhibitor locostatin. Control cells expressing GFP alone and GFP-RKIP-expressing cells were plated in the presence of the indicated concentrations of locostatin, and the number of adherent cells 1 h after plating in the presence of serum was determined as described in Materials and Methods. Values represent mean ± SEM for adherent cells per field of view (n = 20–30 fields of view for each treatment).

rapidly after plating; for instance, even in serum-free media, fibronectin is detectable on the cell surface within 10 min of initial cell attachment of human fibroblasts to tissue culture dishes, and at the cell–substratum interface within an hour, by which time the cells are completely spread [Grinnell and Feld, 1979]. This suggests that when plated on glass, by the time of the experiments shown in Figures 2 and 6, the cells have produced and secreted ECM components upon to which they specifically adhere.

RKIP Positively Regulates Adhesion and Spreading on Defined ECM Components in the Absence of Serum

We next sought to more specifically assess cell adhesion to defined ECM components in the absence of serum-derived factors. To this end, we seeded the cells in non-tissue culture-treated dishes coated with either type I collagen, gelatin, fibronectin or laminin in serum-free medium. After seeding onto dishes coated with type I collagen, gelatin or fibronectin and incubating for 1 h, control MDCK cells displayed either a fried-egg or fan-shaped morphology, suggestive of an initial stage of spreading. In contrast, at the same time point, RKIP-overexpressing cells had undergone full spreading and exhibited an elongated asymmetrical shape with long cell processes (Fig. 3). Surprisingly, on a laminin coating, both control and RKIP-overexpressing cells showed very little spreading after 60 min (data not shown). However, after a longer incubation period (3 h), RKIP-overexpressing cells began to spread on laminin, whereas control cells remained rounded (Fig. 3). The increased spreading ability of RKIP-overexpressing cells was even more evident in dishes coated with suboptimal concentrations of type I collagen (100 ng/ml) or gelatin (1 μg/ml). In this experimental setting, control cells were able to attach to the substrate, but maintained a rounded shape after 1 h of incubation (Fig. 3). In contrast, most RKIP-overexpressing cells had already undergone considerable spreading (Fig. 3). Finally, when the adhesion assay was carried out under restrictive conditions (dishes coated with 100 ng/ml gelatin), a 5.6-fold greater number of RKIP-overexpressing cells (133 ± 28 cells/field, mean $\pm$ standard error of the mean (SEM); $n = 9$ fields of view) than control cells (24 ± 3 cells/field, mean $\pm$ SEM; $n = 9$; $P < 0.0025$ by Student's t-test) attached to the dishes.

Cell Behavior in Three-Dimensional Collagen Gels

To assess potential differences in cell-ECM interactions in a three-dimensional environment, MDCK were grown in collagen gels. After being suspended in a collagen gel for 4 h, control cells still maintained their original spherical shape (Fig. 4A). In striking contrast, at the same time point, RKIP-overexpressing cells had undergone a marked elongation and sent out long membrane extensions into the surrounding collagen matrix (Fig. 4B). During the next few days of culture in collagen gels, control MDCK cells progressively formed hollow cysts, as previously reported [Montesano et al., 1991]. In contrast RKIP-overexpressing cells maintained a very elongated shape and did not organize into multicellular structures (data not shown), again consistent with a negative role for RKIP in cell–cell adhesion.

To determine whether the marked elongation of RKIP-overexpressing MDCK cells in collagen gels was associated with the ability to generate traction force against the matrix, the gels were released from the 35-mm-diameter wells and allowed to float in the medium [Montesano and Orci, 1988]. Under these conditions, gels populated by RKIP-overexpressing cells contracted within 24 h into dense collagen discs with a diameter of ~18 mm, whereas gels containing the same number of control cells did not contract appreciably (Fig. 5). This increased ability to generate traction force may in large part explain the increased rate of cell migration we previously observed in the RKIP-overexpressing cells [Zhu et al., 2005].

Taken together, these findings indicate that overexpression of RKIP profoundly modifies cell-ECM interactions and increases the ability of cells to generate traction force against the substratum. Since RKIP overexpression increases β1 integrin expression, RKIP may positively regulate the cell-surface expression of integrin receptors. Integrin-based adhesion complexes are involved in the biochemical and mechanical coupling of the contractile actomyosin cytoskeleton to the ECM. These results are consistent with the increased spreading of cells that occurs with RKIP overexpression, as previously published [Zhu et al., 2005].

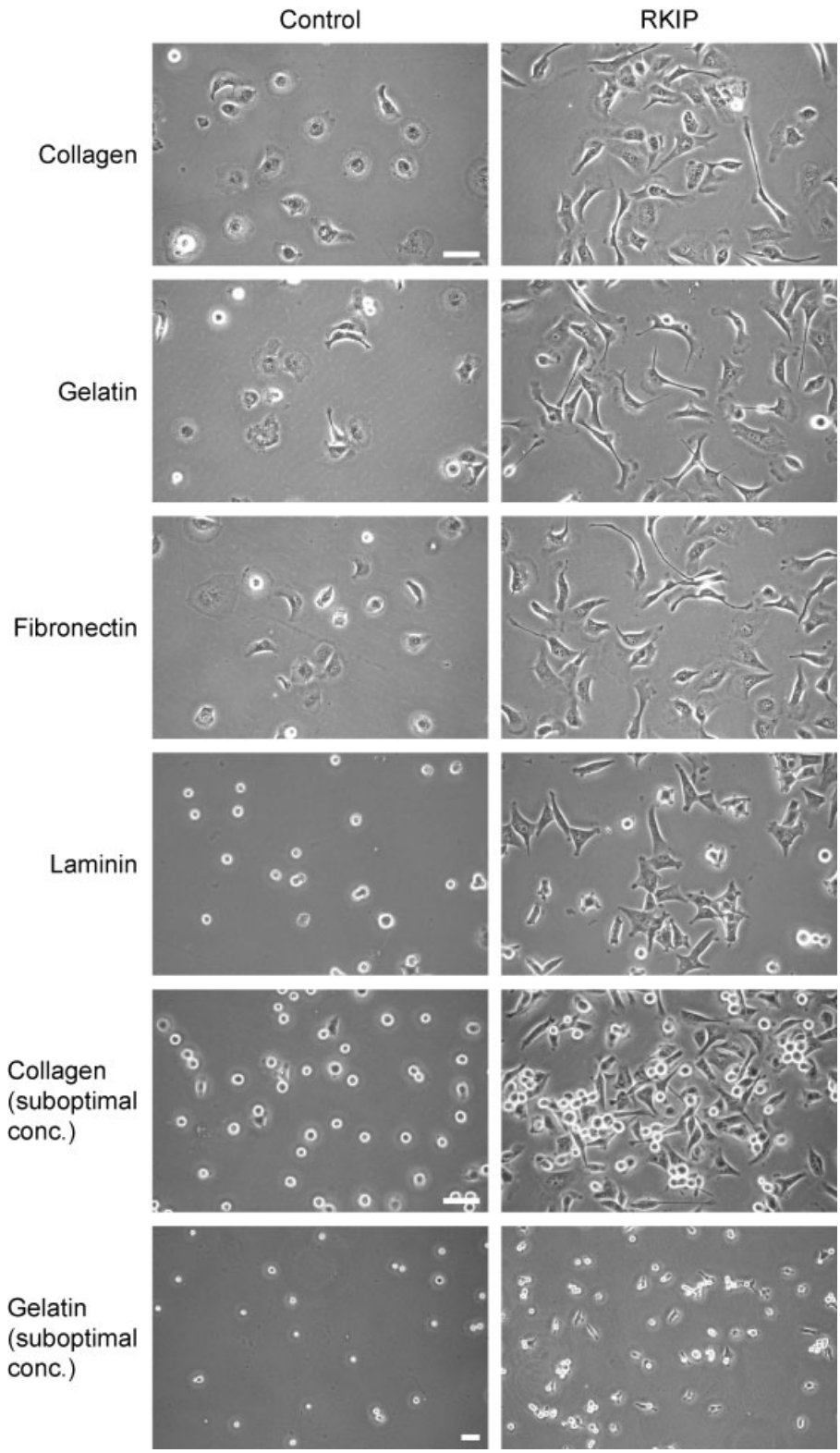


Fig. 3. Overexpression of RKIP increases cell adhesion and cell spreading on defined ECM substrata. Control cells expressing GFP alone (control) and GFP-RKIP-expressing cells (RKIP) were seeded on non-tissue culture-treated dishes coated with type I collagen (30 μg/ml), gelatin (30 μg/ml), fibronectin (30 μg/ml) or laminin (30 μg/ml). Images were taken 1 h after plating on type I collagen, gelatin or fibronectin, and 3 h after plating on laminin. On all these substrata, RKIP-overexpressing MDCK cells were considerably more spread than control cells. When seeded on dishes coated with suboptimal concentrations of either type I collagen (100 ng/ml) or gelatin (1 μg/ml), control cells maintained a rounded shape after 1 h of incubation, whereas most RKIP-overexpressing cells were already well spread. Both control and RKIP-overexpressing cells showed virtually no attachment to dishes incubated with the non-adhesive protein BSA alone. Scale bar = 50 μm.

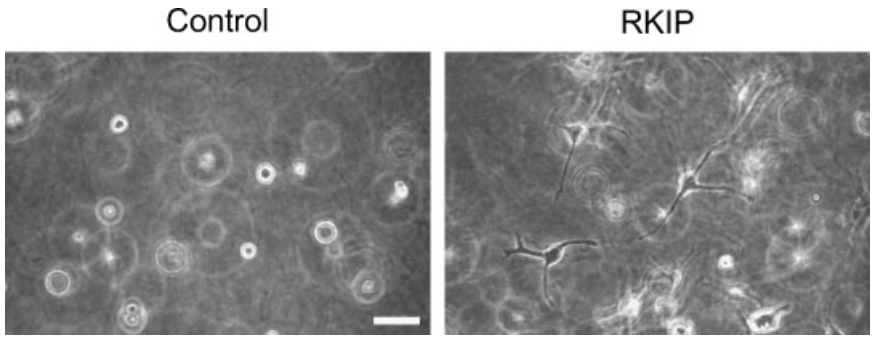


Fig. 4. RKIP-overexpressing MDCK cells extend long processes in three-dimensional collagen gels. After being suspended in a collagen gel for 4 h, control cells expressing GFP alone (control) maintain a spherical shape, whereas GFP-RKIP-expressing cells (RKIP) have and elongated shape and send out long membrane extensions into the surrounding collagen matrix. Scale bar = 50 μm.

Silencing of RKIP Expression Reduces Cell Adhesion

We performed the opposite manipulation—downregulation of RKIP expression—and obtained results that were again consistent with a positive function for RKIP in cell–substratum adhesion. siRNA-mediated knockdown of RKIP expression resulted in slightly diminished adhesion of RKIP-knockdown MDCK cells 1 h after plating on glass coverslips (Fig. 6A). The efficiency of silencing was ~73% in cells expressing an RKIP-specific siRNA from an shRNA expression vector, based on Western blot analysis. This degree of silencing was not as great as previously published for the same stably transfected cells [Zhu et al., 2005], possibly because of genetic selection with continued culture due to the very slow growth rate of the knockdown cells. This may account for the relatively modest effect in Figure 6A. In a more transformed cell line of epithelial origin, MCF7 human breast adenocarcinoma cells, we prepared cells with more complete silencing of RKIP expression (~95% silencing efficiency) and observed stronger reduction in adhesion on glass with knockdown of RKIP (Fig. 6B). In both cases, locostatin treatment resulted in further inhibition of adhesion (compare black bars), presumably due to inhibition of the remaining expressed RKIP (see Western blots in Fig. 6).

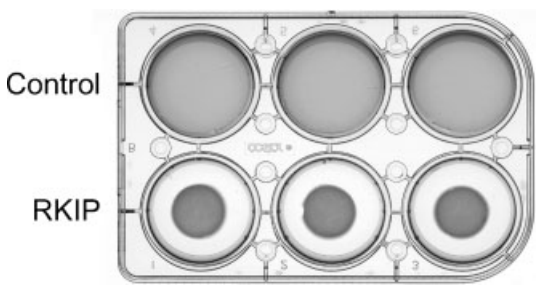


Fig. 5. Overexpression of RKIP causes MDCK cells to strongly contract collagen gels. After cell suspension, the gels were detached from the walls of 35-mm-diameter wells and allowed to float. After 24 h, gels containing control cells expressing GFP alone have not contracted (upper row). In striking contrast, gels populated with GFP-RKIP-expressing cells have undergone a marked contraction, resulting in the formation of compact collagen discs with a greatly reduced diameter (~18 mm, lower row). This experiment was repeated several times with similar results.

RKIP and Cell Motility

In a scratch-wound assay, RKIP-knockdown MCF7 cells exhibit slightly slower rates of wound closure at later times after wounding than control MCF7 cells expressing an inert siRNA (Fig. 7). This is consistent with our previously published results for RKIP-knockdown MDCK cells, although the negative effect on cell migration with knockdown is much more pronounced and manifests itself at the earliest stages of wound closure in MDCK cells [Zhu et al., 2005]. In addition, silencing of RKIP expression in MCF7 cells makes the cells insensitive to the anti-migratory activity of locostatin (Table I). Interestingly, even the control MCF7 cells are not highly sensitive to locostatin, with only 23% inhibition of wound closure at 50 μM locostatin (Table I), compared to 96% inhibition at 50 μM locostatin for MDCK cells [Zhu et al., 2005]. The highly invasive and metastatic mouse melanoma cell line B16-BL6 also exhibits considerably greater sensitivity to locostatin's anti-migratory activity than do MCF7 cells, with 79% inhibition of wound

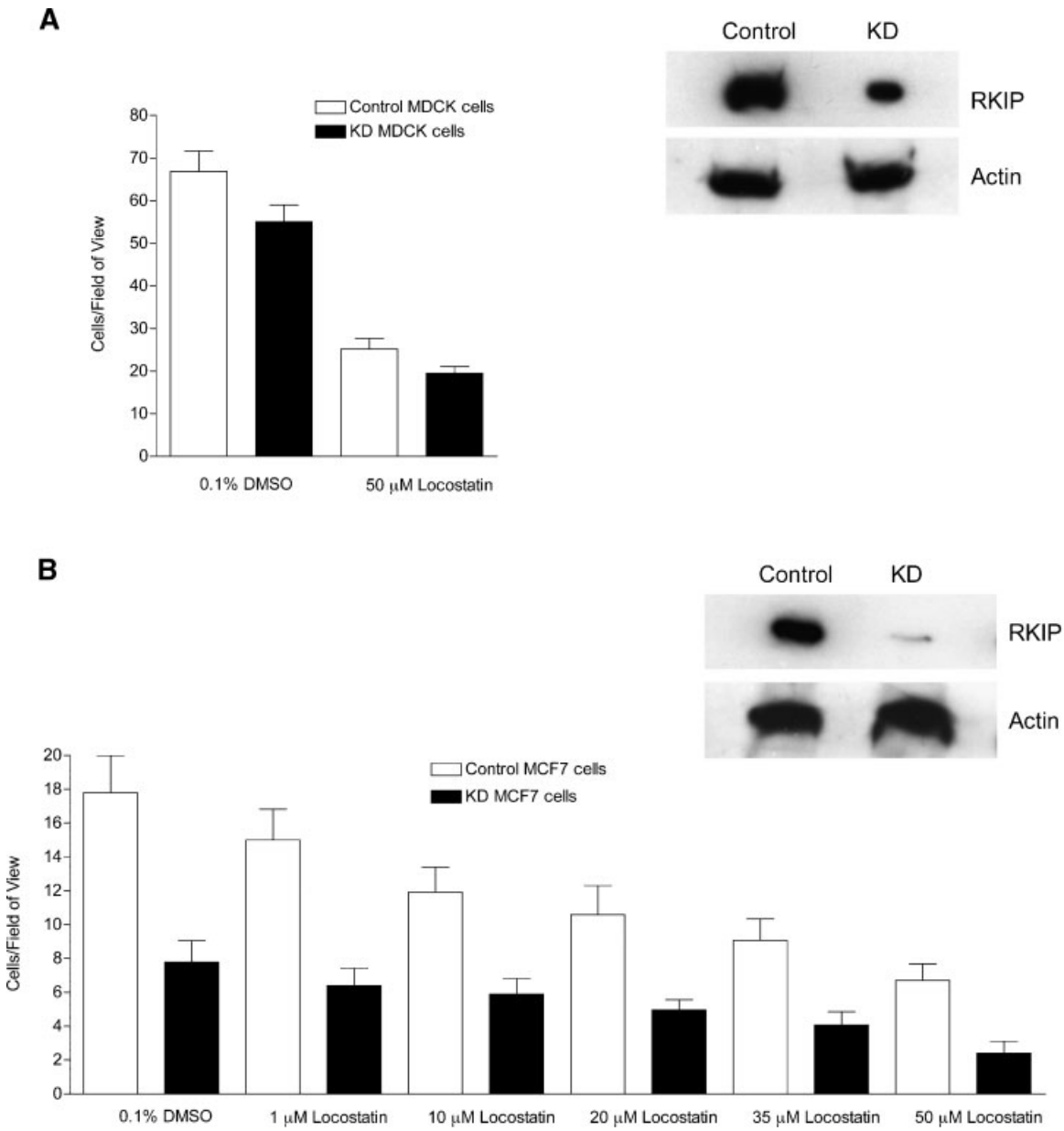


Fig. 6. siRNA-mediated silencing of RKIP expression leads to reduced adhesion in MDCK and MCF7 cells. **A**: Control MDCK cells expressing an inert siRNA and RKIP-knockdown MDCK cells (KD MDCK cells). **B**: Control MCF7 cells expressing an inert siRNA and RKIP-knockdown MCF7 cells (KD MCF7 cells). For both cell types, cells were plated in the presence of the indicated concentrations of the direct RKIP inhibitor locostatin, which specifically and covalently binds RKIP and prevents its association with Raf-1 kinase [Zhu et al., 2005]. The number of attached cells 1 h after plating in the presence of serum was determined. Values represent mean ± SEM for adherent cells per field of view (n = 20–30 fields of view for each treatment). Western blots of equivalent loadings of samples from control and RKIP-knockdown cells for each cell type were probed with an anti-RKIP antibody and are shown beside each corresponding graph; the efficiency of silencing was ~73% for the knockdown MDCK cells and ~95% for the knockdown MCF7 cells compared to the control cells. The loading control was β-actin in both cases.

closure at 50 μM locostatin [Zhu et al., 2005]. Knockdown of RKIP in MCF7 cells leads to only a mild reduction in the rate of wound closure (Table I). Therefore, either locostatin treatment or knockdown of RKIP in MCF7 cells leads to only a small decrease in wound closure rate, implying that RKIP is not as important a mediator of cell migration in MCF7 cells as it is in MDCK cells [Zhu et al., 2005].

While a number of expression studies suggest that RKIP can function as a suppressor of tumor cell metastasis in prostate cancer, breast

TABLE I. Anti-Migratory Activity of Locostatin in Control and RKIP-Knockdown MCF7 Cells

Cells	IC_{50}[a]	Activity[b]
Control MCF7 (expressing inert siRNA)	19.2 μM	23% (50 μM)
RKIP knockdown MCF7	No anti-migratory activity	NA

By way of comparison, corresponding data for the following cell types are: "wild-type" MCF7 cells ($IC_{50} = 24.1$ μM; activity = 29% at 75 μM; MDCK cells, $IC_{50} = 17.9$ μM; activity = 96% at 50 μM); B16-BL6 mouse melanoma cells ($IC_{50} = 16.7$ μM; activity = 79% at 50 μM); A549 human lung adenocarcinoma cells ($IC_{50} > 50$ μM; activity = 29% at 75 μM) [Zhu et al., 2005].
[a]IC_{50} was calculated for inhibition of wound closure at 12 h post-wounding with normalization to parallel controls using five different subtoxic concentrations in concentration–response experiments. Each concentration involved at least three separate experiments, each with multiple replicates, and was considered a bioactive concentration if there was statistically significant inhibition with $P < 0.05$ by unpaired two-tailed Student's *t*-test. Experiments were performed in serum-containing medium. At the end of the experiment, cytotoxicity was evaluated with the Trypan blue dye exclusion assay, as well as observation of any rounding up of cells. While we observed no subtoxic activity of locostatin in the RKIP-knockdown MCF7 cells, locostatin exhibited signs of cytotoxicity at concentrations >50 μM.
[b]Value corresponds to the percent inhibition of wound closure normalized to parallel controls for the highest subtoxic concentrations, as indicated in parentheses. The higher the percentage, the more inhibitory the compound is relative to parallel controls.

cancer, melanoma, hepatocellular carcinoma, and colorectal cancer cells [Fu et al., 2003, 2006; Schuierer et al., 2004, 2006a; Hagan et al., 2005; Al-Mulla et al., 2006; Lee et al., 2006; Minoo et al., 2007], another study found that RKIP expression is higher in tumorigenic and metastatic fibrosarcoma cells than in their less aggressive parental cells [Hayashi et al., 2005]. These investigations, however, have largely focused on cancer metastasis in vivo or on the degree of cell invasiveness in vitro and not more narrowly on cell migration per se, with the exception of a report showing that ectopic expression of RKIP in a hepatocellular carcinoma cell line with low RKIP expression decreases both cell migration and proliferation [Lee et al., 2006].

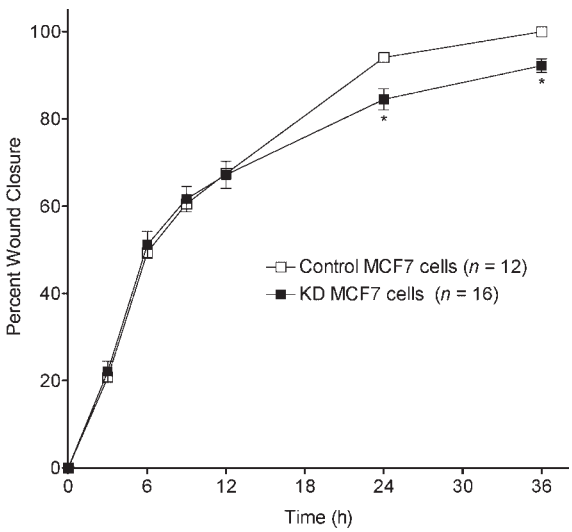


Fig. 7. siRNA-mediated silencing of RKIP expression slightly reduces wound closure at later stages in MCF7 cells. Wound closure curves are shown for control MCF7 cells expressing an inert siRNA and RKIP-knockdown (KD) MCF7 cells. Values represent mean ± SEM, with asterisks indicating a significant difference ($P < 0.05$ by unpaired two-tailed Student's *t*-test). A similar experiment with control and RKIP-knockdown MDCK cells has been previously published and shows a much more pronounced and early effect of delayed cell migration after wounding with knockdown of RKIP [Zhu et al., 2005].

Cell migration is necessary but not sufficient for cancer cell invasion and metastasis to occur. Once a solid tumor is formed through neoplastic transformation and angiogenesis, subsequent invasion of cancer cells into surrounding tissues and metastasis to distant sites involves not only cell migration but also other factors such as changes in cell adhesion and secretion of matrix metalloproteinases and other proteases; in addition, survival and growth of the secondary tumor cells are necessary for formation of detectable metastases [for reviews, see Engers and Gabbert, 2000; MacDonald et al., 2002; Bogenrieder and Herlyn, 2003; Kopfstein and Christofori, 2006; Eccles and Welch, 2007]. Therefore, the metastasis-suppressing activity of RKIP may not be directly related to cell migration. RKIP could be a positive regulator of cell migration, at least in some cell types, while at the same time being a negative regulator of another process that is also limiting in metastasis. For example, RKIP has been found to regulate Aurora B kinase and spindle checkpoint control, leading to the suggestion that cells lacking RKIP may display increased chromosomal abnormalities and genetic changes that could account for their enhanced metastatic potential [Eves et al., 2006]. It is not clear at this time which specific metastasis-implicated processes are affected by changes in RKIP expression in cancer cells. In aggregate, the data appear to suggest that RKIP has complex, cell-type- and context-specific functions that still remain to be elucidated.

SUMMARY

We have demonstrated that overexpression of RKIP causes dissociation of cell–cell contacts between MDCK epithelial cells, downregulation of E-cadherin and loss of normal localization of ZO-1. In contrast, the level of β1 integrin increases, and there is greater cell adhesion and spreading in both two- and three-dimensional cultures on a number of different substrata, including glass in the presence of serum and non-tissue culture-treated dishes coated with collagen, gelatin, fibronectin or laminin. RKIP-overexpressing cells appear to generate greater traction force than do control cells, which could result from the increased integrin expression in these cells. This may explain the increased rate of cell migration with RKIP overexpression that we previously reported [Zhu et al., 2005]. We also found that pharmacological inhibition of RKIP or siRNA-mediated downregulation of its expression results in reduced cell–substratum adhesion in MDCK and MCF7 cells and rates of cell motility during wound closure. Our results thus suggest that RKIP has important roles in cell adhesion, negatively regulating cell–cell adhesion while positively regulating cell–substratum adhesion.

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