Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane

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Introduction of activated p21-activated kinase (PAK) is sufficient to release primary endothelial cells from contact inhibition of growth. Confluent cells display deficient activation of PAK and translocation of Rac to the plasma membrane at matrix adhesions. Targeting Rac to the plasma membrane rescues these cells from contact inhibition. PAK's ability to release human umbilical vein endothelial cells from contact inhibition is blocked by an unphosphorylatable form of its target Merlin, suggesting that PAK promotes mitogenesis by phosphorylating, and thus inactivating, Merlin. Merlin mutants, which are presumed to exert a dominant-negative effect, enable recruitment of Rac to matrix adhesions and promote mitogenesis in confluent cells. Small interference RNA–mediated knockdown of Merlin exerts the same effects. Dominant-negative Rac blocks PAK-mediated release from contact inhibition, implying that PAK functions upstream of Rac in this signaling pathway. These results provide a framework for understanding the tumor suppressor function of Merlin and indicate that Merlin mediates contact inhibition of growth by suppressing recruitment of Rac to matrix adhesions.

Introduction

Normal cells cease to proliferate upon establishing cell–cell adhesions (Dubbecco and Stoker, 1970). This contact-mediated inhibition of growth is critical for tissue organization and contributes to limiting the size of tissues and organs according to body plan (Gottardi and Gumbiner, 2001). Loss of contact inhibition enhances the ability of cancer cells to invade host tissues and ultimately metastasize (Hanahan and Weinberg, 2000).

It is likely that the homophilic cell adhesion proteins' cadherins initiate contact inhibition of growth, but the mechanisms are not clear. The cadherins mediate assembly of adherens junctions by associating with the actin cytoskeleton through β-catenin. Because β-catenin also transduces proliferative Wnt signaling to the nucleus, the cadherins may inhibit proliferation by reducing the levels of β-catenin available for Wnt signaling (for review see Gottardi and Gumbiner, 2001). Other studies suggest that cell–cell adhesion inhibits growth factor receptor signaling (Rahimi and Kazlauskas, 1999; Qian et al., 2004) by segregating receptor tyrosine kinases (RTKs) from their cognate ligands (Vermeer et al., 2003) or by inducing dephosphorylation of their cytoplasmic tail (Lampugnani et al., 2003). In spite of these important insights, contact inhibition of growth remains a poorly understood phenomenon.

The ezrin–radixin–moesin (ERM) protein Merlin is the protein product of the NF-2 tumor suppressor gene that is inactivated in familiar type II neurofibromatosis, as well as in sporadic Schwannomas, meningiomas, and mesotheliomas (for review see Lim et al., 2000). Mouse genetics studies indicate that Merlin may have a broader role in tumor invasion and metastasis than manifested from its specific inactivation in Schwann cells (McClatchey et al., 1998; Giovannini et al., 2000). The biochemical function of Merlin and the mechanism by which its loss contributes to tumorigenesis are not completely understood. It has been proposed that Merlin associates with the cytoplasmic tail of CD44, a hyaluronic acid receptor, to mediate contact inhibition of growth (Morrison et al., 2001). However, genetic ablation of CD44 does not result in the loss of contact inhibition, whereas knockout of Merlin induces this effect, but it does cause destabilization of cadherin-dependent adhesions (Lallemand et al., 2003). In addition, Merlin binds to paxillin and appears to modify integrin-dependent organization of the actin cytoskeleton in a density-dependent manner (Fernandez-Valle et al., 2002). Finally, there is evidence that
Merlin suppresses signaling by the small GTPase Rac (Shaw et al., 2001). This effect has been attributed to the ability of Merlin to interact with and to inhibit the Rac target effector p21-activated kinase (PAK; Kissil et al., 2003). On the other hand, PAK phosphorylates the COOH terminus of Merlin and thereby inactivates the growth-suppressive function of the protein (Kissil et al., 2002; Xiao et al., 2002). Thus, there is evidence for both inhibition of PAK by Merlin and inhibition of Merlin by PAK. It is not clear which of these two opposing functions is prevalent in the cell or, if both are relevant, how they are regulated. In addition, the mechanism by which Merlin inhibits Rac signaling and its relevance to contact inhibition are not known.

Rac is required for normal cell proliferation and migration and plays a key role in cancer progression (Sahai and Marshall, 2002). To activate its target effectors and exert its biological functions, Rac needs to translocate to the plasma membrane and dissociate from Rho-GDI (Etienne-Manneville and Hall, 2002). Prior studies have provided evidence that integrin signaling promotes recruitment of Rac to the membrane by inducing the dissociation of Rac from Rho-GDI (del Pozo et al., 2002) and/or tyrosine phosphorylation of CrkII (Abassi and Vuori, 2002). In spite of these advances, our knowledge of the signals that control recruitment of Rac and other Rho-GTPases to the plasma membrane remains fragmentary.

We provide evidence that activation of PAK is sufficient to release endothelial cells from contact inhibition. PAK exerts this effect by phosphorylating, and thus inactivating, Merlin. Biochemical and imaging studies indicate that dephosphorylated Merlin suppresses recruitment of Rac to the plasma membrane in contact-inhibited cells. These results indicate that Merlin mediates contact inhibition of growth with a signaling mechanism.

Results
Mitogenic activation of Ras proceeds normally, but signaling to ERK is attenuated in contact-inhibited endothelial cells
To avoid the potential effects of immortalization on growth control, we studied contact inhibition of growth in primary cultures of human umbilical vein endothelial cells (HUVEC). These cells undergo growth arrest and fail to respond to bFGF, EGF, and insulin as they become confluent and assemble vertebral endothelial (VE)-cadherin–dependent junctions (Fig. S1, A–C, available at http://www.jcb.org/cgi/content/full/jcb.200503165/DC1). Assembly of tight junctions may prevent diffusion of peptide growth factors from the medium to the basolateral surface of polarized epithelial cells in vivo (Vermeer et al., 2003). However, experiments on cells plated on Transwell filters showed that contact inhibition of HUVEC is not caused by a segregation of RTKs from their cognate ligands (Fig. S1, A and C). In addition, GST pull-down assays with the Ras-binding domain of Raf (GST-Raf-RBD) provided evidence that cell–cell contact does not interfere with joint integrin–RTK signaling to Ras (Fig. S1 D), suggesting that the reported effect of VE-cadherin on growth factor receptor activation (Lampugnani et al., 2003) is not necessary for contact inhibition. Interestingly, extracellular signal-related protein kinase (ERK) was activated in a less sustained manner in confluent cells than in sparse cells (Fig. S1 E). We note that cell–cell contact may induce attenuation of Ras to ERK signaling through inhibition of PAK (Fig. 1 C), as PAK functions downstream of Ras to promote activation of ERK (King et al., 1998). These observations indicate that cell contact does not inhibit signaling to Ras but attenuates activation of ERK in HUVEC.

PAK activation is sufficient to release endothelial cells from contact inhibition
To identify the mechanism by which cell–cell contact inhibits cell proliferation, we used a functional complementation
approach. HUVEC were transiently transfected with plasmids encoding GFP in combination with the activated versions of various signaling proteins. BrdU incorporation was used to evaluate the ability of each activated signaling protein, and certain combinations thereof, to promote proliferation of confluent HUVEC. Notably, activated PAK (PAK-CAAX) effectively rescued progression through G1 and entry into S phase in confluent HUVEC (Fig. 1 A), and it did so in a dose-dependent manner (Fig. 1 B). None of the other activated signaling proteins tested were able to exert this effect as efficiently as activated PAK (Fig. 1 A). In particular, membrane-targeted p110 (myr-p110) and Akt (myr-Akt), as well as unphosphorylatable, stabilized β-catenin (S35/37/41/45A-β-catenin–S4xA-β-catenin), did not promote entry into S phase in contact-inhibited HUVEC, suggesting that inhibition of phosphatidylinositol-3 kinase or β-catenin does not play a significant role in contact inhibition of growth. Activated β-catenin’s lack of effect is consistent with the observation that genetic ablation of β-catenin does not induce growth arrest but, instead, releases HUVEC from contact inhibition (Lampugnani et al., 2003). Similarly negative results were obtained with activated versions of Cdc42 (Cdc42-V12) and Rho A (Rho-L63; Fig. 1 A). By contrast, activated Rac (Rac-L61), Raf (Raf-CAAX), and MEK (MEK-ΔN3) exerted a partial effect (Fig. 1 A), suggesting that inhibition of Rac and ERK may contribute to contact inhibition of growth. Control experiments indicated that none of the constructs tested induced significant apoptosis (>5%) in confluent HUVEC. We note that VE-cadherin–mediated adhesion protects HUVEC from apoptosis (Carmeliet et al., 1999). These results indicate that activation of PAK is sufficient to release HUVEC from contact inhibition.

To examine the effect of cell–cell contact on PAK activation, HUVEC were plated on fibronectin (FN) under either sparse or confluent conditions in the presence or absence of growth factors and subjected to PAK assay. In agreement with prior results (del Pozo et al., 2000), adhesion to FN induced strong activation of PAK in sparse cells, whereas growth factor stimulation did not exert this effect in suspended cells (Fig. 1 B), suggesting that integrin engagement is necessary and sufficient for activation of PAK. Notably, PAK was not activated in cells plated on FN under confluent conditions, whether they were treated with growth factors or not (Fig. 1 C), indicating that cell contact suppresses activation of PAK.

To examine whether PAK activation is necessary for normal cell proliferation, we used a dominant-negative approach. As shown in Fig. 1 B, expression of a kinase-dead version of PAK (PAK-CAAX-KD) prevented the entry into S phase of HUVEC plated on FN under sparse conditions. These results suggest that integrin-mediated activation of PAK is necessary for cell cycle progression and imply that cell–cell adhesion inhibits cell proliferation by preventing the activation of PAK.
Contact inhibition proceeds through suppression of recruitment of Rac to the membrane

PAK is a well established target effector of Rac (Etienne-Manneville and Hall, 2002). However, constitutively active Rac-L61 did not fully rescue cell cycle progression in confluent HUVEC, whereas activated PAK did (Fig. 1 A). We considered the possibility that Rac-L61 did not exert a proproliferative effect in confluent cells because of incorrect targeting. To examine whether cell–cell contact interferes with the recruitment of Rac to the plasma membrane, we used biochemical fractionation and optical imaging methods. HUVEC were plated on FN under either sparse or confluent conditions for 4 h and treated with growth factors. At various times after mitogenic stimulation the cells were subjected to biochemical fractionation and immunoblotting. As shown in Fig. 2 A, the crude membrane fraction of cells plated on FN under sparse conditions contained a significant amount of Rac (~25% of the total). Mitogenic stimulation did not modify the proportion of Rac in the crude membrane fraction of these cells. These observations are in agreement with the model that RTKs induce GTP-loading on Rac, whereas integrins mediate its recruitment to the membrane and coupling to target effectors (del Pozo et al., 2000, 2002). Parenthetically, prolonged mitogenic stimulation led to increased levels of Rac in both the cytosolic and the crude membrane fraction of sparse cells, suggesting that Rac levels increase in mid-to-late G1 in HUVEC. Interestingly, most of the total Rac (>95%) remained in the cytosolic fraction in confluent cells, indicating that the translocation of Rac to the membrane fraction is impaired in confluent cells (Fig. 2 A). This result indicates that cell contact suppresses recruitment of Rac to the plasma membrane.

To confirm and extend this result, we used optical imaging. HUVEC were transiently transduced with GFP fused to wild-type Rac under conditions inducing low levels of expression (~10-fold lower than endogenous Rac by immunoblotting), plated on FN under sparse or confluent conditions, and imaged by confocal microscopy. As expected, GFP-Rac localized to lamellipodia and membrane ruffles in sparse cells. Analysis of Z-sections indicated that most of the protein associated with the ventral membrane in these cells. By contrast, GFP-Rac appeared predominantly distributed throughout the cytoplasm in confluent cells (unpublished data). To obtain more quantitative data and to determine if, and to what extent, cell contact specifically suppresses recruitment of Rac to matrix adhesions, we applied FN- or control poly-l-lysine (PL)–coated beads to sparse or confluent HUVEC. As shown in Fig. 2 B, the FN-coated beads caused efficient recruitment of GFP-Rac in sparse cells, but they did not exert this effect in confluent cells. The PL beads promoted negligible recruitment of Rac in sparse cells, as anticipated. These results indicate that cell–cell contact suppresses integrin-dependent recruitment of Rac to the plasma membrane.

To examine the effect of cell contact on activation of Rac, we conducted pull-down experiments with GST-PAK. We observed that cell contact inhibits growth factor–induced activation of Rac only partially, whereas loss of matrix adhesion suppresses this event to a significant extent (Fig. 2 C). Although we cannot exclude other mechanisms, we posit that the partial decrease of GTP loading on Rac observed in confluent cells is a consequence of reduced recruitment to the plasma membrane, and hence activation, of Rac by RTKs. Prior studies have shown that a fraction of Rac is activated transiently during the formation of cell–cell junctions (Ehrlich et al., 2002). We believe it is unlikely that this local and transient activation of Rac influenced the outcome of our experiment because the cells were plated under confluent conditions for 4 h before growth factor treatment and GST pull-down assay, and control experiments had indicated that this time is sufficient for assembly of cell–cell junctions. These results are consistent with the hypothesis that cell contact inhibits Rac signaling predominantly by inhibiting localization of Rac to the membrane.

Rac signaling promotes progression through G1 in HUVEC (Mettouchi et al., 2001) and several other cell types (Etienne-Manneville and Hall, 2002). If Rac needs to associate with the plasma membrane to be properly coupled to its target effectors, inhibition of this process may underlie contact inhibition. To examine this hypothesis, we tested whether targeting wild-type Rac to the membrane was sufficient to release HUVEC from contact inhibition. Cells were transfected with Myr-Rac, which carries a Src myristoylation site at its NH₂ terminus, but no additional mutations. BrdU incorporation indicated that this membrane-targeted form of Rac rescues a large fraction of HUVEC from contact inhibition whereas Rac-L61 was much less effective (Fig. 2 D). Because Myr-Rac does not carry mutations reducing its GTPase activity, its ability to promote proliferation of confluent HUVEC indicates that targeting Rac to the plasma membrane is sufficient to release cells from contact inhibition of growth. Together, these observations imply that contact inhibition of growth involves the inhibition of membrane recruitment of Rac.

Activated PAK releases HUVEC from contact inhibition through the phosphorylation of Merlin

The aforementioned results suggest that contact inhibition proceeds through the suppression of membrane recruitment of Rac and the inactivation of PAK, but they do not explain why inhibition of PAK causes growth arrest. To address this question, we sought to identify the target effector through which PAK releases HUVEC from contact inhibition. We focused on the PAK substrate Merlin because it is a tumor suppressor protein and its growth-suppressing activity is regulated by phosphorylation (Bretscher et al., 2002). In contrast, the other known target effectors of PAK (with the exception of Raf; see poor rescue by activated Raf in Fig. 1 A) regulate the actin cytoskeleton (Bokoch, 2003).

PAK phosphorylates Merlin at Serine 518, disrupting the interaction between the NH₂-terminal protein 4.1 ERM domain and the COOH-terminal tail of the protein (Shaw et al., 2001; Kissil et al., 2002; Xiao et al., 2002). To examine the potential role of PAK's phosphorylation of Merlin in release from contact inhibition, HUVEC were transduced with HA-tagged Merlin in combination with empty vector, activated PAK (PAK-CAAX), or dominant-negative PAK (PAK-CAAX-KD) and were plated
previous studies have suggested that the "closed," dephosphorylated form of Merlin and the bottom band to the unphosphorylated form. (B) Total lysates prepared as in A were subjected to standard SDS-PAGE and immunoblotting with anti–phospho-Merlin, anti-Merlin or vector alone. As shown in Fig. 3 B, most complete dephosphorylation of the protein in sparse cells under either sparse or confluent conditions in the presence of growth factors. High resolution SDS-PAGE followed by immunoblotting with anti-HA was used to monitor the mobility shift caused by the phosphorylation of Merlin (Shaw et al., 2001). Control transfectants plated under confluent conditions contained more dephosphorylated Merlin as compared with those plated under sparse conditions (Fig. 3 A). In addition, activated PAK-induced significant phosphorylation of Merlin in confluent cells, whereas dominant-negative PAK caused an almost complete dephosphorylation of the protein in sparse cells (Fig. 3 A). Immunoblotting with an antibody that specifically recognizes Merlin phosphorylated at Serine 518 showed that Merlin is significantly phosphorylated at this site in cells plated under sparse conditions, but not in those plated under confluent conditions (Fig. 3 B). In addition, activated PAK caused significant phosphorylation of Merlin at Serine 518 in confluent and in suspended cells, whereas kinase-dead PAK did not exert this effect (Fig. 3 B). These results identify a potential role for Merlin phosphorylation at Serine 518 in exit from contact inhibition.

Overexpression of mutant forms of Merlin that are presumed to disrupt head-to-tail association of the protein causes hyperproliferation in the fly wing (LaJeunesse et al., 1998) and transforms rodent fibroblasts (Johnson et al., 2002). It has been argued that these mutants exert a dominant-negative effect on the growth-suppressive function of endogenous wild-type protein (Bretscher et al., 2002). To examine if PAK controls membrane recruitment of Rac through Merlin, we used two such mutants: Merlin-ΔBB, which carries a deletion of the Blue Box, which is a short amino acid segment in the protein 4.1 ERM domain thought to be involved in head-to-tail association (LaJeunesse et al., 1998); and Merlin-S518D, which carries a serine to aspartic acid permutation at the PAK phosphorylation site and thus mimics the phosphorylated form of Merlin (Johnson et al., 2002).

HUVEC were transfected with vector alone or HA-tagged forms of wild-type Merlin, Merlin-ΔBB, Merlin-S518D, and Merlin-S518A (which cannot be phosphorylated by PAK) and plated under confluent conditions in the presence of growth factors. BrdU incorporation experiments indicated that Merlin-ΔBB and Merlin-S518D induce a significant fraction of confluent HUVEC to progress through G1 and enter into S phase (Fig. 3 C). By contrast, wild-type Merlin and Merlin-S518A did not exert this effect. It is likely that Merlin-ΔBB and Merlin-S518D rescue HUVEC from contact inhibition by exerting a dominant-negative effect on the growth-suppressive function of endogenous wild-type protein. In addition, or instead, these mutants may directly stimulate cell proliferation. These findings are consistent with the hypothesis that phosphorylation of Merlin mediates exit from contact inhibition.

We next used BrdU incorporation to examine the ability of Merlin-S518A to interfere with PAK’s ability to rescue HUVEC from contact inhibition. HUVEC were transfected with activated PAK in combination with HA-tagged Merlin-S518A or, as controls, HA-tagged Merlin-WT, HA-tagged Merlin-S518D, or vector alone. As shown in Fig. 3 D, Merlin-S518A inhibited PAK’s ability to rescue cell proliferation in confluent HUVEC, whereas wild-type Merlin did not exert this effect, and Merlin-S518D enhanced the proliferative effect of activated PAK. These findings imply that PAK releases HUVEC from contact inhibition of growth through phosphorylation of Merlin.

Merlin mediates contact inhibition by suppressing membrane recruitment of Rac

Previous studies have suggested that the “closed,” dephosphorylated form of Merlin suppresses Rac signaling (Shaw et al., 2001; Kissil et al., 2002), raising the possibility that PAK func-
tions upstream of Rac during release from contact inhibition. We thus examined if Merlin suppresses recruitment of Rac to matrix adhesions in confluent cells. HUVEC were transfected with GFP-Rac, alone or in combination with HA-tagged Merlin-S518D, Merlin-ΔBB (BB), Merlin-WT, and Merlin-S518A. FN-coated beads were applied for 25 min to G0-synchronized cells plated on FN under confluent conditions. The cells were then fixed and stained with anti-HA to detect HA-Merlin (red) and DAPI (blue). The graph shows the percentage of GFP-Rac–positive beads under the indicated conditions. Arrows point to FN-coated beads that had induced recruitment of GFP-Rac. (B) Cells were transfected with vectors encoding HA-tagged Merlin-S518D or Merlin-S518A. GO-synchronized cells were plated on FN under confluent conditions and subjected to double staining with antibodies to β-catenin (red) and HA (green). The inset shows that Merlin-S518D localizes in part to adherens junctions, but does not disrupt them. Error bar represents the mean ± SD.

Figure 4. Open forms of Merlin promote recruitment of Rac to matrix adhesions. (A) HUVEC were transfected with GFP-Rac in combination with empty vector or vectors encoding HA-tagged Merlin-S518D, Merlin-ΔBB (BB), Merlin-WT, and Merlin-S518A. FN-coated beads were applied for 25 min to GO-synchronized cells plated on FN under confluent conditions. The cells were then fixed and stained with anti-HA to detect HA-Merlin (red) and DAPI (blue). The graph shows the percentage of GFP-Rac–positive beads under the indicated conditions. Arrows point to FN-coated beads that had induced recruitment of GFP-Rac. (B) Cells were transfected with vectors encoding HA-tagged Merlin-S518D or Merlin-S518A. GO-synchronized cells were plated on FN under confluent conditions and subjected to double staining with antibodies to β-catenin (red) and HA (green). The inset shows that Merlin-S518D localizes in part to adherens junctions, but does not disrupt them. Error bar represents the mean ± SD.

siRNA-mediated knockdown of Merlin promotes recruitment of Rac to matrix adhesions and releases cells from contact inhibition

The aforementioned experiments are consistent with the model that the dephosphorylated, closed form of Merlin suppresses recruitment of Rac to the membrane, but they do not exclude the possibility that the phosphorylated, “open” form of Merlin exerts an independent and positive effect on cell proliferation. To directly test if Merlin mediates contact inhibition of growth by suppressing recruitment of Rac to matrix adhesions, we used RNA interference. Transfection of a small interference RNA (siRNA) targeting human Merlin was sufficient to rescue integrin-dependent recruitment of GFP-Rac to the plasma membrane in confluent HUVEC (Fig. 5 A). Notably, knockdown of Merlin induced a large fraction of confluent HUVEC to progress through G1 and enter into S phase after mitogenic stimulation (Fig. 5 B). Immunoblotting revealed that the siRNA inhibited Merlin expression by >80%. As expected, the Merlin knockdown cells escaping contact inhibition had elevated levels of cyclin D1 and decreased levels of p27 (Fig. 5 B). However, cyclin D1 was not up-regulated and p27 was not down-regulated as effectively as they normally are in sparse cells. We attribute this partial result to the incomplete suppression of Merlin expression after siRNA transfection.

To control for potential off-target effects of the siRNA or activation of the interferon response, we used a rescue approach. The HUVEC were transfected with a cDNA encoding mouse Merlin that is insensitive to the siRNA we used to inhibit human Merlin and were subjected to RNA interference. As shown in Fig. 5 C, BrdU incorporation and immunoblotting with anti–cyclin D1 and anti-p27 indicated that reexpression of Merlin restores contact inhibition of growth in Merlin knockdown HUVEC. These results provide evidence that Merlin is necessary and sufficient to block membrane recruitment of Rac.
transfection of Merlin or control siRNA, HUVEC were plated under confluent conditions and incubated with mitogens and BrdU for 20 h. The percentage of transfected cells entering S phase was determined as described in Fig. 2. PAK-CAAX was tagged with HA and hence detected by immunoblotting with anti-HA, whereas Rac-N17 was tagged with Myc and thus detected with anti-Myc. (B) HUVEC were transfected with GFP-Rac in combination with empty vector or vector encoding activated PAK (PAK-CAAX). They were then synchronized in G0 and plated on FN under sparse or confluent conditions. FN-coated beads were applied for 25 min. The cells were fixed and stained with anti-HA to detect PAK-CAAX (red) and DAPI to stain nuclei (blue). Arrows point to FN-coated beads that induced recruitment of GFP-Rac. The graph shows the percentage of GFP-Rac-positive beads under the indicated conditions. Error bars represent the mean ± SD.

Finally, we wanted to obtain additional evidence that suppression of Merlin enhances Rac signaling in HUVEC. After transfection of Merlin or control siRNA, HUVEC were plated under sparse conditions to facilitate detection of Rac-dependent structures, such as lamellipodia and ruffles, and subjected to double immunofluorescent staining with anti-VE-cadherin antibodies and phalloidin. As shown in Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200503165/DC1), suppression of Merlin expression caused a significant loss of focal adhesions and stress fibers in HUVEC. Concomitantly, the cells formed extensive lamellipodia and ruffles. These observations suggest that loss of Merlin enhances Rac signaling in HUVEC.

Figure 6. PAK promotes recruitment of Rac to matrix adhesions. (A) Cells were cotransfected with GFP and empty vector, or vector encoding activated PAK (PAK-CAAX) or Pak-CAAX-H83/86L, in combination or not with dominant-negative Rac (Rac-N17). G0-synchronized cells were plated on FN under confluent conditions and incubated with mitogens and BrdU for 20 h. The percentage of transfected cells entering S phase was determined as described in Fig. 2. PAK-CAAX was tagged with HA and hence detected by immunoblotting with anti-HA, whereas Rac-N17 was tagged with Myc and thus detected with anti-Myc. (B) HUVEC were transfected with GFP-Rac in combination with empty vector or vector encoding activated PAK (PAK-CAAX). They were then synchronized in G0 and plated on FN under sparse or confluent conditions. FN-coated beads were applied for 25 min. The cells were fixed and stained with anti-HA to detect PAK-CAAX (red) and DAPI to stain nuclei (blue). Arrows point to FN-coated beads that induced recruitment of GFP-Rac. The graph shows the percentage of GFP-Rac-positive beads under the indicated conditions. Error bars represent the mean ± SD.

PAK controls recruitment of Rac to matrix adhesions
PAK is a key target effector of Rac, but it can also function upstream of Rac (Bokoch, 2003), raising the possibility that PAK functions in proliferative signaling through Rac-mediated activation of target effectors other than PAK. This data supports the hypothesis that PAK regulates Rac translocation to the membrane through phosphorylation and inactivation of Merlin. To confirm that PAK functions upstream of Rac during release from contact inhibition, we first tested if PAK rescues cells from contact inhibition through Rac. HUVEC were transfected with activated PAK, alone or in combination with dominant-negative Rac. As shown in Fig. 6 A, dominant-negative Rac
(Hac-N17) suppressed PAK’s ability to release confluent HUVEC from contact inhibition, suggesting that PAK functions upstream of Rac in this process. A mutation crippling the CRIB domain (H83/86L) did not impair the ability of activated PAK to rescue HUVEC from contact inhibition, indicating that this effect does not require Rac binding to PAK. As expected, dominant-negative Rac also interfered with the ability of the CRIB domain mutant form of activated PAK to release cells from contact inhibition (Fig. 6A). These findings indicate that PAK functions upstream of Rac in release from contact inhibition.

We examined if activated PAK rescues recruitment of Rac to integrin-dependent adhesions in confluent cells. HUVEC were transfected with GFP-Rac, alone or in combination with activated PAK, plated under confluent conditions, and treated with FN-coated beads. Fig. 6B shows that activated PAK promotes recruitment of Rac to FN-coated beads in confluent cells. Interestingly, activated PAK accumulated underneath FN-coated beads in “rescued” cells, which is in agreement with the hypothesis that it promotes cell proliferation by inducing Rac to matrix adhesions (Fig. 7B). These observations indicate that PAK controls recruitment of Rac to matrix adhesions by a signaling mechanism and imply that intercellular contact interferes with this process.

**Discussion**

Rac promotes normal cell proliferation and cancer invasion (Sahai and Marshall, 2002). To exert its functions, Rac needs to dissociate from Rho-GDI and attach to the inner aspect of the plasma membrane. Our study provides evidence that the tumor suppressor Merlin mediates contact inhibition of growth by blocking recruitment of Rac to matrix adhesions. Upon activation, PAK reverses this inhibition by phosphorylating, and thus inactivating, Merlin. These results place Merlin at the center of a novel tumor suppressor–oncogene circuit and provide a framework to interpret its function in tumorigenesis.

We have observed that PAK signaling is sufficient to release endothelial cells from contact inhibition. By contrast, activation of other mitogenic signaling pathways, including those involving β-catenin, phosphatidylinositol-3 kinase, and ERK, does not exert this effect. Upon introduction in sparse cells, dominant-negative PAK promotes growth arrest, suggesting that PAK kinase activity is required for normal cell cycle progression. Activated PAK promotes recruitment of Rac to matrix adhesions in confluent cells, whereas dominant-negative Rac blocks PAK’s ability to release cells from contact inhibition, indicating that PAK promotes mitogenesis by recruiting Rac to matrix adhesions. Two distinct mutant forms of Merlin, Merlin-ΔBB and Merlin-S518D, which are presumed to exert a dominant-negative effect, promote recruitment of Rac to matrix adhesions and release cells from contact inhibition. By contrast, an unphosphorylatable form of Merlin, Merlin-S518A, suppresses PAK’s ability to release HUVEC from contact inhibition. These observations indicate that PAK promotes recruitment of Rac to matrix adhesions and mitogenesis by phosphorylating and thereby inactivating Merlin.

Several lines of evidence indicate that Merlin’s ability to suppress Rac function is necessary and sufficient for contact inhibition of primary endothelial cells. Constitutively active PAK, dominant-negative Merlin, and membrane-targeted Rac are each sufficient to release HUVEC from contact inhibition. Conversely, dominant-negative PAK promotes growth arrest when introduced in cells that are not contact inhibited. In addition, siRNA-mediated knockdown of Merlin is sufficient to promote recruitment of Rac to matrix adhesions and mitogenesis of confluent HUVEC, excluding the possibility that the open form of Merlin directly promotes recruitment of Rac to the membrane and mitogenesis. Thus, our results support the hypothesis that the closed, dephosphorylated form of Merlin inhibits proliferation and mediates contact inhibition by suppressing activation of Rac, whereas the open, phosphorylated form does not exert this effect. In addition, our results suggest that the open, phosphorylated form may inhibit the growth-suppressive function of the closed, dephosphorylated form. Future studies will be required to elucidate the molecular basis of this effect. Finally, we cannot exclude the possibility that the open form of Merlin has additional functions, as it has been reported that Merlin-S518D enhances filopodia formation in rat Schwannoma cells (Surace et al., 2004).

Under growth-permissive conditions, joint integrin-RTK signaling causes recruitment and activation of Rac and, thus, of PAK. We propose that, upon phosphorylation by PAK, Merlin loses its ability to inhibit recruitment of Rac to the membrane, thus facilitating Rac–PAK signaling. This positive feed-forward mechanism of signal amplification would cause robust activation of Rac. Finally, activated Rac would promote cell proliferation through target effectors that were distinct from PAK. We speculate that, upon cell–cell contact cadherin signaling suppresses activation of PAK, leading to the accumulation of unphosphorylated Merlin. This form of Merlin would finally block proliferation by suppressing recruitment of Rac to the membrane (Fig. 7). Several aspects of this model require further experimental examination. Specifically, it will be important to address the mechanism by which cadherin-dependent adhesion suppresses PAK and the mechanism by which Merlin inhibits recruitment of Rac to the membrane. In
addition, future studies will have to examine whether the growth-suppressive pathway identified here also operates in other normal cell types.

Merlin's function as a tumor suppressor has been investigated intensively. Merlin \(^{-/-}\) fibroblasts and keratinocytes display defective cadherin-dependent junctions and do not undergo growth arrest upon becoming confluent (Lallemand et al., 2003), suggesting that Merlin exerts its tumor suppressor function by stabilizing cell–cell junctions. In this model, Merlin functions upstream of cadherins, either by facilitating their assembly or by preventing their disassembly, and it thus inhibits proliferative signaling indirectly. In contrast, we have observed that dominant-negative or siRNA-mediated inhibition of Merlin rescues HUVEC from contact inhibition of growth without disrupting cell–cell junctions. We attribute this apparent discrepancy to the use of different methods of inhibiting Merlin function and/or different cell types. The effect of genetic ablation is permanent and complete, whereas the effect of dominant-negative inhibition or siRNA-mediated knockdown is transient and often incomplete. In addition, it is known that Rac signaling participates in both the assembly and the disassembly of cell–cell junctions, and the extent to which it promotes these contrasting functions may vary with cell type (Braga, 2002). It is thus possible, and indeed likely, that genetic ablation of Merlin causes a more robust up-regulation of Rac signaling than dominant-negative or siRNA-mediated inhibition. In addition, fibroblasts and keratinocytes may be more sensitive to the effect of loss of Merlin function on cell–cell junctions than endothelial cells. Regardless of what specific mechanism explains this apparent discrepancy, our results indicate that inhibition of Merlin can rescue cells from contact inhibition without disrupting their junctions, supporting the hypothesis that Merlin regulates proliferation by a direct signaling mechanism. Similarly, studies in *Drosophila Melanogaster* support the notion that the ERM protein Moesin promotes epithelial organization by inhibiting Rho signaling rather than by functioning as a cytoskeletal linker (Speck et al., 2003).

The biochemical function of Merlin is poorly understood. Merlin interacts with the integrin signaling component paxillin (Fernandez-Valle et al., 2002) that functions, together with PKL, as a scaffold to recruit PAK and PIX, a GEF for Rac, to focal complexes (Turner, 2000). Hence, Merlin may inhibit recruitment of Rac by the paxillin–PKL–PIX complex until it becomes phosphorylated and thus inactivated by PAK. In addition, PAK phosphorylates Rho-GDI at Ser 10/17, promoting its dissociation from Rac (DerMardirossian et al., 2004). Although this mechanism can contribute to the recruitment of Rac to matrix adhesions, our results clearly show that PAK functions in this process also by phosphorylating Merlin. Merlin has been shown to bind to Rho-GDI in vitro (Maeda et al., 1999), raising the possibility that Merlin stabilizes the association of Rac with Rho-GDI. However, we have failed to detect association of endogenous Merlin and Rho-GDI in endothelial cells by communoprecipitation (unpublished data). Finally, recent studies suggest that integrins control recruitment of Rac to the membrane by organizing cholesterol-rich membrane microdomains (del Pozo et al., 2004), and dephosphorylated Merlin tends to accumulate in these microdomains (Stickney et al., 2004), suggesting the possibility that Merlin inhibits recruitment of Rac to these membrane domains.

Rho family GTPases promote cell proliferation and migration, and they mediate Ras transformation in vitro. However, unlike Ras proteins, Rho GTPases do not acquire mutations that impair their GTPase activity and cause constitutive activation in human cancer (Sahai and Marshall, 2002). It is possible that these mutations are not sufficient to cause transformation because they do not overcome the barrier imposed by cytoplasmic segregation. PAK1 is amplified in some ovarian and breast cancers (Bekri et al., 1997; Schram et al., 2003). In addition, elevated levels of PAK1 have been detected in breast and colorectal cancer (Balasenthil et al., 2004; Carter et al., 2004). Finally, PAK4 is overexpressed in various carcinoma lines (Callow et al., 2002). Based on our results, we speculate that PAK may promote tumorigenesis by functionally inactivating Merlin and elevating Rac signaling.

**Materials and methods**

**Cell culture and materials**

HUVEC were obtained from Clonetics. Cells were synchronized in GO by growth factor deprivation, detached, and replated on dishes or coverslips coated with 15 μg/ml of human plasma FN (BD Biosciences) at 2.5 × 10^4 per cm^2 (sparse) or 1.5 × 10^5 per cm^2 (confluent). After incubation in serum-free medium [SFM; GIBCO BRL] for 4 h, the cells were stimulated with growth factors [20 ng/ml bFGF, 1 μg/ml heparin, 10 ng/ml EGF, 10 μg/ml insulin, 10 μg/ml transferrin, and 1 μg/ml selenious acid] for the times indicated in the figure legends. For some experiments, the cells were cultured on FN-coated polycarbonate filters (0.4-μm pore size; Transwell Costar). The following antibodies were purchased: anti-Rac1 (Upstate Biotechnology); anti-RhoGDI, anti-cyclin D1 [A-12, anti-p27 (C-19), anti-Erk2 (C-14), anti-PAK (N-20), and anti-Merlin (A-19, C-18; Santa Cruz Biotechnology, Inc.); anti-β-actin (Sigma-Aldrich); anti-phospho-ERK (Cell Signaling); anti-Ras and anti-VE-cadherin (BD Biosciences); and anti-β-catenin (Babco). Rabbit antibodies to β-catenin and to phosphorylated Ser18 in Merlin were described previously (McCrean et al., 1993; Kissil et al., 2002).

**Expression vectors**

We used expression vectors encoding wild-type and mutant forms of Rac1, pDNA3 Rac-L61, Rac-N17, Myr-Rac [a gift from M. del Pozo, Centro Nacional Investigaciones Cardiovasculares, Madrid, Spain], pEGFP-Rac [Rac1 was subcloned into pEGFP vector (CLONTECH laboratories, Inc.)], and pX40-HA-Rac, Cdc42 (pZip Cdc42-V12), RhoA (pDNA3 RhoA-L63), p110-PI3K (pCG-myr-p110), MEK1 (pMCL-MEK1-ΔN3, S222D), Raf (pZipRafCAAX), Sos (pDNA3 Myr-Sos1), PAK (pX40-PakCAAX), Akt (pCMV-Myr-Akt), β-catenin [a gift from C. Gotardi, Northwestern University, Chicago, IL; pCS1-HA-S35/37/41/45A-β-catenin or HA-S4xA-β-catenin], and Merlin (pX40-HA-Merlin; full-length Merlin was subcloned from pBluscript II [Merlin obtained from S. Tsukita, Kyoto University, Kyoto, Japan]). The rest of the constructs were previously described (Mettochi et al., 2001). The Merlin I mutants S518D, S518A, and S68 (deletion of amino acid residues 177–183) and PAK mutants PAK-CAAX-K298R and PAK-CAAX-H3/86L were generated by site-directed mutagenesis using the Quickchange kit (Stratagene) according to the manufacturer's instructions.

**Cell proliferation**

For transfection, 5 × 10^5 cells were resuspended in 300 μl SFM with the indicated amounts of expression vectors in combination with pEGFP-F [encoding farnesylated EGFP] and electroporated at 300 V and 450 μF. Cells were allowed to recover in complete medium for 12 h. They were then synchronized in GO, detached, and replated on FN-coated coverslips under confluent conditions. After 4 h in SFM, cells were incubated with growth factors and 10 μM BrdU (Boehringer) for 20 h. After fixation, they
were stained with anti-BrDU mAb (Boehringer) and TRITC-conjugated anti-mouse IgGs (The Jackson Laboratory) to measure the percentage of GFP-positive cells that had incorporated BrdU.

**Biochemistry**

Pak was immunoprecipitated from cell lysates with anti-Pak1 antibodies (N-20; Santa Cruz Biotechnology, Inc.) and subjected to kinase assay with myelin basic protein as a substrate, as previously described (Yablonski et al., 1998). The samples were also probing by immunoblotting with the same antibody. GTP-Rac and GTP-Pak were pulled down from 800 μg of total proteins using GST-Raf-RBD or GST-Pak67-150, respectively, before immunoblotting with specific antibodies. For subcellular fractionation, cells were homogenized with a Dounce homogenizer in cold hypotonic buffer (10 mM Tris, pH 7.4, 1.5 mM MgCl2, 5 mM KCl, 1 mM EDTA, and protease inhibitors). After pelleting nuclei and intact cells by centrifugation at 750 g for 10 min, the supernatants were spun at 10,000 g for 1 h. The resulting supernatants containing the cytosolic fraction were then kept aside, whereas the pellets were extracted with radiimmunoprecipitation buffer and briefly clarified at 10,000 g for 5 min to recover the crude membrane fraction. An equal amount of proteins from the cytosolic or membrane fractions were analyzed by immunoblotting.

**Optical imaging**

Cells were transfected with a vector encoding GFP-Rac (pEGFP-Rac1) in combination with constructs encoding various forms of Pak or Merlin. 12 h after transfection, cells were starved for 24 h, detached and replated on Fn-coated coverslips in SFM without growth factors. After an additional 4 h, 6.5-μm polystyrene latex beads (Interfacial Dynamics Corp.) were applied to the cells for 25 min at a beads to cell ratio of 40:1 (del Pozo et al., 2002). Beads were washed with bicarbonate buffer, incubated with 50 μg/ml FN or 100 μg/ml F1 at RT for 1 h, and washed with PBS. Cells were fixed in 4% PFA, permeabilized in 0.2% Triton X-100/PBS, blocked in 2.5% BSA/goat serum, and incubated with primary anti-bodies or rhodamine-conjugated phalloidin (0.1 μg/ml Sigma-Aldrich). Samples were examined on an inverted microscope using 40 x and 63 x 1.5 oil immersion lenses (Axiovert 200M; Carl Zeiss MicroImaging, Inc.). Confocal analysis was performed with an LSM510 instrument (Carl Zeiss MicroImaging, Inc.).

**RNA interference**

The siRNA oligonucleotide TGGCCAACGAAGCACTGAT was designed in combination with constructs encoding various forms of Pak or Merlin. After transfection, cells were starved for 24 h, detached and replated on Fn-coated coverslips in SFM without growth factors. After an additional 4 h, 6.5-μm polystyrene latex beads (Interfacial Dynamics Corp.) were applied to the cells for 25 min at a beads to cell ratio of 40:1 (del Pozo et al., 2002). Beads were washed with bicarbonate buffer, incubated with 50 μg/ml FN or 100 μg/ml F1 at RT for 1 h, and washed with PBS. Cells were fixed in 4% PFA, permeabilized in 0.2% Triton X-100/PBS, blocked in 2.5% BSA/goat serum, and incubated with primary anti-bodies or rhodamine-conjugated phalloidin (0.1 μg/ml Sigma-Aldrich). Samples were examined on an inverted microscope using 40 x and 63 x 1.5 oil immersion lenses (Axiovert 200M; Carl Zeiss MicroImaging, Inc.). Confocal analysis was performed with an LSM510 instrument (Carl Zeiss MicroImaging, Inc.).

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