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Merlin, the Product of the Nf2 Tumor Suppressor Gene, Is an Inhibitor of the p21-Activated Kinase, Pak1

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Summary

The Nf2 tumor suppressor gene codes for merlin, a protein whose function has been elusive. We describe a novel interaction between merlin and p21-activated kinase 1 (Pak1), which is dynamic and facilitated upon increased cellular confluence. Merlin inhibits the activation of Pak1, as the loss of merlin expression results in the inappropriate activation of Pak1 under conditions associated with low basal activity. Conversely, the overexpression of merlin in cells that display a high basal activity of Pak1 resulted in the inhibition of Pak1 activation. This inhibitory function of merlin is mediated through its binding to the Pak1 PBD and by inhibiting Pak1 recruitment to focal adhesions. This link provides a possible mechanism for the effect of loss of merlin expression in tumorigenesis.

Introduction

Neurofibromatosis type 2 is an inherited disorder, characterized by development of Schwann cell tumors of the eighth cranial nerve. Mutations and loss of heterozygosity of the *NF2* locus have been detected at high frequency in various tumors of the nervous system, including schwannomas, meningiomas, and ependymomas (Gusella et al., 1999). Mice heterozygous for an *Nf2* mutation are predisposed to a wide variety of malignant tumors (McClatchey et al., 1998). Inactivation of *Nf2* specifically in Schwann cells leads to development of schwannomas and Schwann cell hyperplasia in mice (Giovannini et al., 2000). The *NF2* gene codes for a 595 amino acid protein, termed merlin, which is highly related to the ERM proteins ezrin, radixin, and moesin.

Recent work has shown that merlin protein levels and phosphorylation are affected by growth conditions such as cell confluence, loss of adhesion, or serum deprivation. One site of phosphorylation of merlin is serine 518, and phosphorylation at this site can be induced by active forms of Rac and cdc42 but not Rho (Shaw et al., 2001). Rac/cdc42-induced phosphorylation at merlin serine 518 is mediated by p21-activated kinase (Pak) (Kissil et al., 2002; Xiao et al., 2002). Such phosphorylation can disrupt merlin intramolecular interactions and its association with the actin cytoskeleton and induces a shift in the subcellular localization of merlin in LLC-PK1 cells (Kissil et al., 2002; Shaw et al., 2001).

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The p21-activated kinases (Pak1 through 3) are immediate downstream effectors of Rac/cdc42. They comprise a subgroup of serine/theronine kinases, termed the "group I" Paks, belonging to a larger protein family, which also contains the "group II" kinases (Pak4, 5, and 6). The group I Paks, which have been studied in more detail, have been shown to mediate signals to cytoskeletal reorganization and transcriptional activation (reviewed by Bagrodia and Cerione, 1999; Jaffer and Chernoff, 2002). The Paks are regulated by diverse mechanisms. Based on three-dimensional structure analysis, it has been suggested that inactive Pak is in a conformation in which the autoinhibitory domain interacts with the kinase domain. The binding of active Rac/cdc42 to Pak alleviates this inhibition and enables Pak activation. Once the inhibition is relieved, Pak undergoes autophosphorylation, and this prevents a conformational switch back into an inactive state (Li et al., 2001). Several additional mechanisms and molecules have been shown to regulate Pak activation, including phospholipids and proteolysis. In addition, membrane localization via NCK, localization to focal adhesions via p95/PKL-Cool/Pix, and signals converging from both growth factor receptors and integrins can all effect Pak activation (Brown et al., 2002; del Pozo et al., 2000; Turner et al., 1999).

Recently, merlin has been implicated as a negative regulator of Rac signaling. The overexpression of merlin inhibited Rac-induced activation of c-Jun N-terminal kinase (JNK) and activation of the AP-1 transcriptional activator. Conversely, in Nf2-deficient fibroblasts, basal JNK activity was found to be elevated, as was the activity of AP-1 (Shaw et al., 2001). Thus, it would seem that merlin both is regulated by the Rac/cdc42 signaling pathway and can serve as an inhibitor of this pathway. Here we describe the interaction of merlin with Pak1, a critical mediator of Rac/cdc42 signaling, and the effect of this interaction on the activity of the kinase.

Results and Discussion

Direct Interaction between Merlin and Pak1

Based on the observations that merlin inhibits Rac signaling at some level, we assessed the possibility of a stable interaction between merlin and Pak. NIH3T3 cells were cotransfected with expression vectors for merlin and Pak1, and association of the proteins was assessed by coimmunoprecipitations. As shown in Figure 1A, immunoprecipitation of merlin led to coimmunoprecipitation of Pak1. Likewise, immunoprecipitates of Pak1 contained merlin (Figure 1A). To assess the interaction in an additional cell type, a rat schwannoma cell line (RT4-DP6) was examined. These cells express relatively low levels of endogenous merlin and detectable endogenous levels of Pak1. Neither Pak2 nor Pak3 was detected in these cells by Western blot analysis (data not shown). As in the case of NIH3T3 cells, association of the proteins was assessed by coimmunoprecipitations. As shown in Figure 1A, the immunoprecipitation of merlin also precipitated endogenous Pak1. In the reciprocal experi-

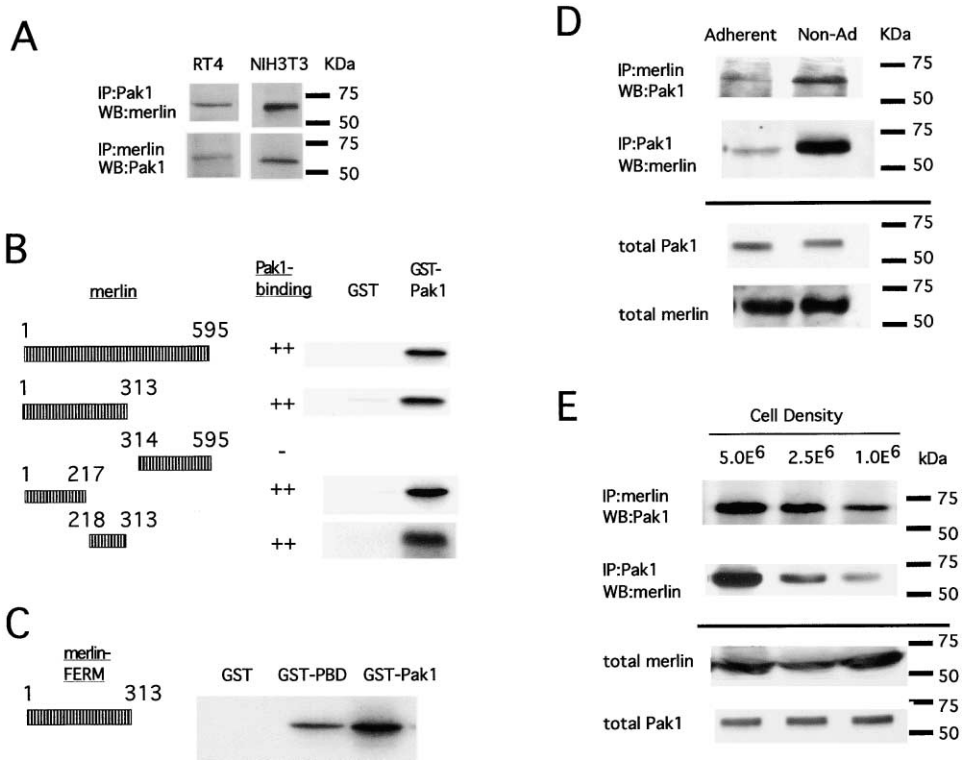

Figure 1. Merlin and Pak1 Interact In Vitro and In Vivo

Western blot analysis of merlin and Pak1 immunoprecipitates from NIH3T3 cells transfected with expression vectors for merlin and Pak1, and from RT4 Schwann cells expressing endogenous levels of merlin and Pak1 (A). In vitro interactions of GST only or GST-Pak1 proteins with S^{35}-labeled full-length merlin (1–595), FERM domain (1–313), C-terminal half (314–595), FERM lobes F1-F2 (1–217), and lobe F3 (218–313) (B). In vitro interactions of the merlin FERM domain with GST, GST-Pak1 (1–545), and GST-PBD (70–143) (C). Western blot analysis of merlin and Pak1 immunoprecipitates and total expression levels of protein from RT4-67 grown under adherent or nonadherent growth conditions for 4 hr prior to harvesting (D) or grown at increasing cellular densities as indicated (E).

ment, the immunoprecipitation of endogenous Pak1 also resulted in the coimmunoprecipitation of endogenous or exogenous merlin (Figure 1A).

To determine if merlin and Pak can interact directly, the association of the two proteins was assessed in vitro. Full-length Pak1 was produced in bacteria as a GST-fusion protein and purified on glutathione- agarose beads. Merlin was produced by in vitro transcription/translation. The S^{35}-labeled merlin protein was incubated with either GST-Pak1 or GST bound to the agarose beads. The interaction of the proteins was assessed by separation of the proteins by SDS-PAGE and autoradiography. As shown in Figure 1B, GST-Pak1 bound to merlin, whereas GST alone did not. Thus, the interaction between Pak and merlin is likely to be direct.

To further delineate the regions of Pak1 that mediate interaction with merlin, we assessed the binding of different merlin domains to full length Pak1 in vitro. The N-terminal FERM domain (1–313) and the C-terminal tail of merlin (314–595) were transcribed and translated in vitro and tested for their ability to interact with GST-Pak1. While the N-terminal FERM domain interacted efficiently with GST-Pak1, the C-terminal fragment failed to interact (Figure 1B). This was not due to different levels of expression, as all merlin fragments were produced at similar levels (data not shown). Trying to further narrow down the interacting domains in the merlin FERM, we tested the ability of either the F1-F2 domain (1–217) or the F3 domain (218–313) (Pearson et al., 2000) to bind to Pak1. Both domains interacted well with the kinase, indicating there are multiple binding sites in the FERM domain involved in the interaction of merlin with Pak1 (Figure 1B). The interaction of both the F1-F2 and F3 fragments with GST-Pak1 appeared to be stronger than that of the entire FERM domain (F1-F3). This could be due to intramolecular associations within the intact FERM domain (Gutmann et al., 1999) that partially mask the Pak1 interaction sites. We next tested the possibility that the FERM domain could bind to the N-terminal regulatory domain of Pak1 (70–143), which contains the cdc42/Rac binding domain (PBD). The FERM domain interacted with the PBD, although the interaction was weaker than the interaction with full-length Pak1.

We attempted to identify additional merlin binding sites on Pak1 by generating additional truncation mutants; however, we were unable to obtain these mutants due to the high toxicity of the Pak1 kinase domain in bacteria (J. Chernoff and E. Manser, personal communication). Thus, it remains possible that additional interaction domains exist between merlin and Pak1. However, the identification of an interaction between the FERM domain with the PBD is of functional significance, as it implicates merlin in a regulatory role for Pak1 (see below).

The Interaction of Merlin and Pak Is Dynamic

To assess whether the interaction between Pak and merlin is dynamic, we examined the effect of cell adhesion and confluence on the interaction. Exogenous expression of merlin was employed in these experiments to circumvent the fact that merlin expression is regulated by different cellular growth conditions (Shaw et al., 1998). Toward this aim, the RT4-67 cell line was employed. The RT4-67 cells were constructed from the RT4-DP6 rat schwannoma cells and harbor a tetracycline-inducible allele of NF2 (Morrison et al., 2001). RT4-67 cells were grown in the presence of doxycycline and placed into suspension by plating them on poly-HEME coated dishes, which prevents the cells from adhering to the plastic (Folkman and Moscona, 1978). Four hours after being placed into suspension, the cells were harvested, and either merlin or Pak1 was immunoprecipitated. The precipitates were resolved by SDS-PAGE and Western blotting and compared to precipitates from adherent RT4-67 cells. While merlin and Pak1 could be coimmunoprecipitated under adherent growth conditions, the interaction between the two proteins was greatly enhanced when adhesion was lost (Figure 1D).

To test the effect of cell confluence on the merlin-Pak1 interaction, protein extracts were prepared from RT4-67 cells grown at high or low confluence. The cells were plated at increasing densities in the presence of doxycycline and were harvested 24 hr after plating. Merlin or Pak1 was immunoprecipitated from the cellular extracts and resolved by SDS-PAGE. Both merlin and Pak1 can reciprocally coimmunoprecipitate under conditions of either low or high confluence. However, the interaction between merlin and Pak1 was enhanced when cells were grown to a higher density (Figure 1E). Importantly, the observed differences in the precipitated levels of merlin or Pak1 are not due to differences in the expression levels of these proteins. The levels merlin and Pak1 were not altered in the RT4-67 Schwann cells, whether the cells were adherent or nonadherent or grown at high or low cellular densities.

These experiments indicate that the interaction of merlin and Pak1 is dynamic and influenced by cellular adhesion and cell density. The interaction of merlin and Pak was enhanced under conditions demonstrated to be inhibitory to Pak activation in NIH3T3 cells (del Pozo et al., 2000) and in the RT4-67 Rat schwannoma cells (J.L.K. and T.J., unpublished data). The regulation of the Paks is complex and involves many different factors (discussed below). However, the localization of Pak to specific regions at the plasma membrane might be an important determinant of its activation. Earlier work has shown that the SH2/SH3 domain protein NCK is required for the recruitment of Pak to the cell membrane (Lu et al., 1997; Sells et al., 1997). Interestingly, NCK interaction with Pak is enhanced upon adhesion and lost when cells are suspended (Howe, 2001), which is opposite to the pattern of interaction of merlin and Pak shown here.

Merlin Inhibits the Pak1-Rac and Pak1-Paxillin Interactions

As merlin bound the Pak1 PBD domain, we tested the possibility that merlin can inhibit the interaction between Rac and Pak1. RT4-67 cells were grown in the presence or absence of doxycycline, and Pak1-Rac interaction was examined by immnuoprecipitations. Overexpression of merlin inhibited the interaction between Pak1 and Rac, as demonstrated by the reduced levels of Rac coimmunoprecipitated with Pak1 and vice versa. The overall reduction in this interaction was approximately 4-fold in the presence of merlin (Figure 2A). While the levels of Rac1 were not altered in the presence or absence of merlin (Figure 2A), the levels of the GTP-bound form of Rac1 were decreased by approximately 2-fold in cells expressing merlin (Figure 2A). These reduced levels of Rac-GTP could explain in part the lower levels of Rac-Pak1 complexes in the presence of merlin.

We also examined the effect of merlin expression on the interactions of Pak1 with other adaptor proteins. This was done by immunoprecipitation and Western blot analysis of the relevant interactions. Merlin expression did not affect the interaction between Pak1 and NCK, Pak1 and β-pix, or Pak1 and p95PKL, as similar amounts of Pak1 and the various binding proteins were coimmunoprecipitated in the presence or absence of increased merlin expression (Figures 3B–3D). Merlin expression also did not alter the overall expression levels of these proteins (data not shown). In contrast, the interaction of Pak1 and paxillin was greatly reduced upon increased merlin expression (Figure 3A). When merlin was overexpressed in the RT4-67 cell lines, an average of 10-fold reduction in the Pak1-paxillin interaction was evident from the reduced levels of paxillin coimmunoprecipitated with Pak1 and vice versa. Again, expression of merlin did not affect the overall levels of paxillin or Pak1 expression in the cells (Figure 3A).

Based on these observations, we propose that merlin can inhibit Pak1 activation by binding directly to the Pak1 PBD and interfere with the binding of active Rac to the Pak1 PBD. Merlin might also function by directly reducing the level of active Rac1 that is available to bind and activate Pak1. Similarly, merlin could interfere directly with the interaction of Pak1 to paxillin, or this effect could be a consequence in the reduction of active Rac, which is required for the recruitment of Pak1 to focal adhesion complexes (Brown et al., 2002).

Loss of Merlin Results in Increased Pak Activity

We next tested the possibility that merlin expression might affect Pak1 activation. The phosphorylation status of Pak1 serves as a direct indication of the activation status of the kinase (Buchwald et al., 2001; Chong et al., 2001). To examine differences in the phosphorylation state of Pak1, we used 2-dimensional gel analysis to separate the different forms of activated Pak1 (Garcia Arguinzonis et al., 2002). MEFs were serum starved for 24 hr and then treated for 5 min with PDGF. Extracts were prepared from cells directly into sample buffer and quantified. Equal amounts of protein were separated by isoelectric focusing on an immobilized 4–7 pH gradient. Subsequently, the extracts were separated by SDS-PAGE in the second dimension, transferred to membranes, and analyzed by Western blotting using an anti-Pak1 antibody. As shown in Figure 4B, under conditions of serum starvation only two forms of Pak1 were detected in the extracts, most likely corresponding to non- or hypophosphorylated forms of Pak1. When extracts

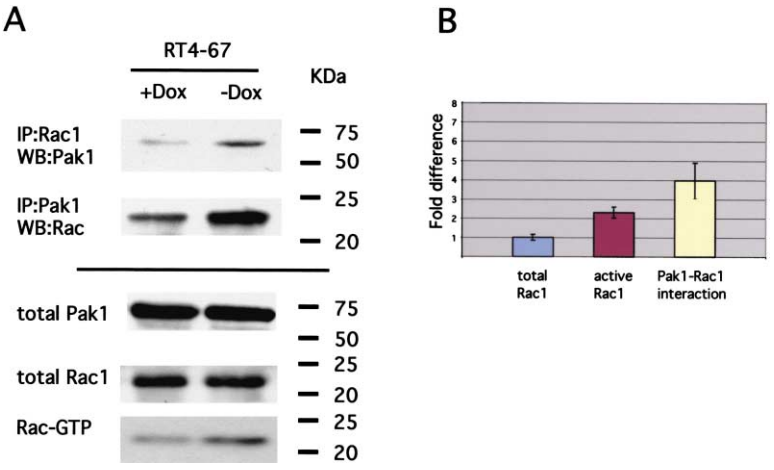

Figure 2. Merlin Reduces Levels of Rac-GTP and Interferes with Pak1-Rac Interactions

Western blot analysis of total protein levels and immunopreciptates from RT4-67 Schwann cells grown in the presence or absence of doxycycline. Pak1 and Rac1 (A). Quantification of the fold-differences in the levels of Rac1 and Rac1-GTP and in the interaction between Rac1 and Pak1 (B). The data represent the average of five independent experiments.

prepared from PDGF-treated MEFs were examined, however, several additional spots were detected. These spots correspond to hyperphosphorylated forms of Pak1, as they appear to be more acidic than the forms of Pak1 found in the serum-starved cells and separate in a signature pattern of a phospho-protein with multiple phosphorylation sites (Garcia Arguinzonis et al., 2002). This also correlated with the increased kinase activity of Pak1, as assessed directly by an in vitro kinase assay employing MBP (myelin basic protein) as a substrate (Figure 4A). To confirm that the additional Pak1 forms are due to phosphorylation, extracts of PDGF-treated cells were incubated with protein phosphatase 1 (PP1) in the presence or absence of protein phosphatase inhibitors. The protein phosphatase treatment resulted in the disappearance of the additional acidic forms of the protein that appear after the PDGF treatment (see Figure 4B), indicating that the additional spots appearing after PDGF stimulation are phosphorylated forms of Pak1. As expected, the inclusion of phosphatase inhibitors in the reaction prevented the loss of the phosphorylated species (data not shown).

To address the effect of merlin on Pak1 activation in vivo, the consequence of loss of merlin expression in MEFs was examined. Mouse embryo fibroblasts (MEFs) were prepared from animals carrying a conditional knockout (floxed) allele of *Nf2* (*Nf2*^{flox2}) (Giovannini et al., 2000). In addition to the *Nf2*^{flox2} allele, the cells carried either a wild-type *Nf2* allele (*Nf2*$^{flox2/+}$) or a *Nf2* deletion allele (*Nf2*$^{flox2/-}$) (McClatchey et al., 1998). The MEFs were then infected with adenovirus expressing Cre-recombinase (ad-Cre), which led to the inactivation of the floxed *Nf2* allele. To test for loss of merlin expression in the ad-Cre treated Nf2$^{flox2/-}$ MEFs, extracts were prepared 96 hr after infection. One milligram of extract was used to immunoprecipitate merlin, and merlin levels were examined by Western blotting. The ad-Cre-treated Nf2$^{flox2/-}$ MEFs lacked detectable merlin protein 96 hr after infection, while merlin levels were not altered in ad-Cre Nf2$^{flox2/+}$ MEFs (Figure 5B). As a control, infection of the

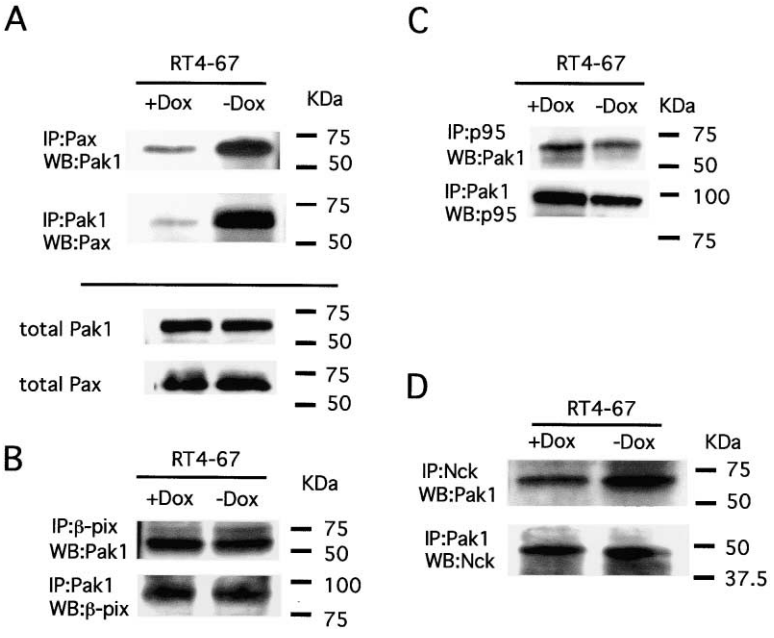

Figure 3. Merlin Interferes with Pak1-Paxillin Interactions

Western blot analysis of total protein levels and immunopreciptates from RT4-67 Schwann cells grown in the presence or absence of doxycycline: Pak1 and paxillin (A), Pak1 and β-pix (B), Pak1 and p95PKL (C), and Pak1 and Nck (D).

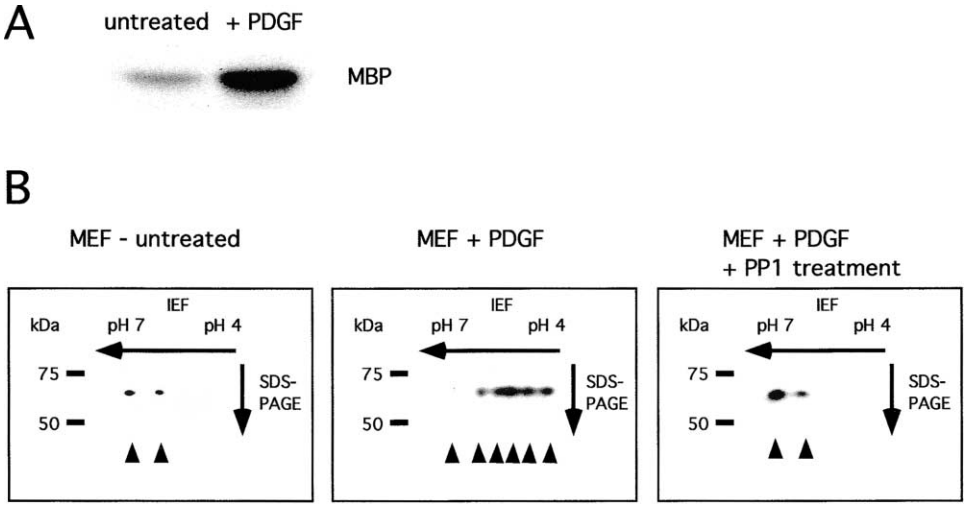

Figure 4. Analysis of Pak1 Phosphorylation by 2-Dimensional Gel Analysis

In vitro kinase assay of Pak1 immunoprecipitated from serum-starved or PDGF-treated (5 ng/ml) NIH3T3 cells, employing MBP as a substrate (A). Western blot analysis of Pak1 in MEFs that were serum starved (left), treated with PDGF (5 ng/ml) (center), and treated with protein phosphatase 1 (2.5 U/ml) (right).

cells with an adenovirus expressing the LacZ gene did not alter merlin levels (data not shown). The status of Pak1 was then analyzed in the MEFs. The ad-Cre-treated and adenovirus control-treated $Nf2^{flox2/-}$ and ad-Cre-treated $Nf2^{flox2/+}$ MEFs were plated at the same cellular densities and allowed to adhere to the tissue culture dish. The MEFs were then serum starved for 24 hr and extracted into sample buffer, and the status of Pak1 phosphorylation was analyzed by 2D gel analysis. Under conditions of serum starvation, Pak1 was not activated in control-treated MEFs, as demonstrated by detection of only hypophosphorylated forms of Pak1 (Figure 5A). However, in the ad-Cre treated $Nf2^{flox2/-}$ MEFs, which had lost the expression of merlin, a marked activation of Pak1 was observed, as indicated by the appearance of several phosphorylated forms of the kinase (Figure 5A). Thus, loss of merlin expression in MEFs promoted Pak1 activation under conditions normally associated with inactivity. These data are consistent with merlin functioning as an inhibitor of Pak1.

Merlin Expression Inhibits Pak Activation

As loss of merlin expression resulted in the appearance of activated forms of Pak1, we examined the possibility that the overexpression of merlin would inhibit Pak1 activation. In the RT4-67 Schwann cells basal levels of merlin are extremely low (Morrison et al., 2001). To assess Pak1 activity in these cells, RT4-67 cells were serum starved for 24 hr, protein was extracted, and the activation status of Pak1 was examined by 2D gel analysis. As shown in Figure 5C, the basal activity of Pak1 was relatively high in the RT4 cells. To assess whether the expression of merlin would affect the activity of Pak1 in these cells, the expression of merlin was induced by the addition of doxycycline into the growth media 48 hr prior to the harvesting of the cells, in the same manner as described above. Indeed, increased expression of merlin significantly reduced the levels of activated forms of Pak1 (Figure 5C). Thus, the reintroduction of merlin into the RT4 schwannoma cells, which display a high level of basal Pak1 activity, results in inhibition of Pak1 activation.

The data reported here are in agreement with previous work from us and others implicating merlin as a negative regulator of Rac-signaling. Specifically, overexpression of merlin has been shown to inhibit Rac-induced activation of c-Jun N-terminal kinase (JNK) and AP-1 transcription, while loss of merlin further resulted in elevated JNK basal activity and activation of AP-1. Loss of merlin also induced cytoskeleton changes that are phenotypically consistent with Rac activation, including membrane ruffling and increased cellular motility (Shaw et al., 2001). This is similar to the higher motility of cells expressing activated Rac alleles. While these data support a functional connection between merlin and Rac, they do not establish where in the Rac pathway merlin might act. Our data indicate that merlin can act both at the level of Rac activation and downstream of Rac, at the level of Pak activation.

The overexpression of Rac can induce transformation and anchorage-independent growth of cells (Khosravi-Far et al., 1995; Qiu et al., 1995). As Rac effectors, PAKs have been shown to mediate some of these signals. Using activated or dominant-negative forms of the proteins, the Paks have been shown to be involved in focal complex formation and membrane ruffling in various cell types (Daniels et al., 1998; Manser et al., 1997; Sells et al., 1997). Paks also have a role in signal transduction from Rac to JNK. Some reports have concluded that activated Pak1 or Pak3 can lead to upregulation of JNK activity; however, further studies are required to fully establish this connection (Brown et al., 1996; Zhang et al., 1995). In addition, recent data point to involvement of Pak in the regulation of the MAPK pathway. Pak can phosphorylate Raf-1 on serine 338 and induce phosphorylation of Mek1 on serine 298 (Diaz et al., 1997; Frost et al., 1997; King et al., 1998). Moreover, Rac or

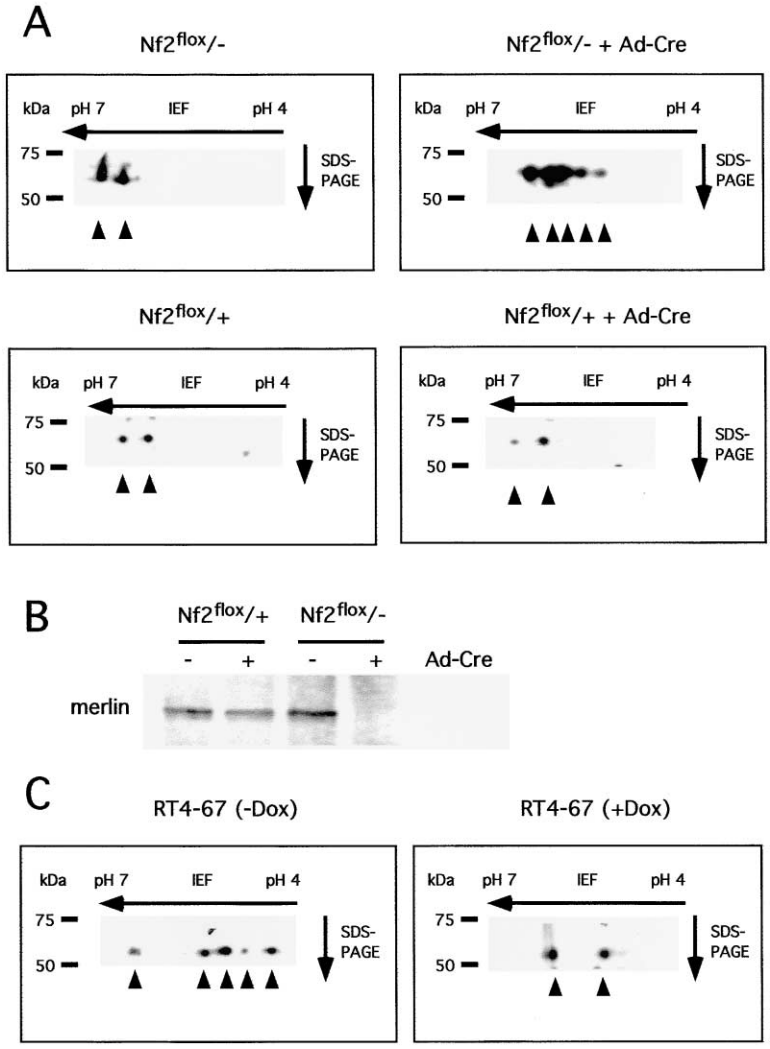

Figure 5. Analysis of Pak1 Phosphorylation by 2-Dimensional Gel Analysis

Western blot analysis of Pak1 in serum-starved untreated MEF clones that express merlin ($Nf2^{flox2/-}$, $Nf2^{flox2/+}$), serum-starved clones infected with adenovirus-Cre that express merlin ($Nf2^{flox2/+}$ + ad-Cre), and serum-starved clones infected with adenovirus-Cre that does not express merlin ($Nf2^{flox2/-}$ + ad-Cre). The various phosphorylated forms of Pak1 are indicated by arrows (A). IP-Western blot analysis of merlin expression in non-treated and ad-Cre-treated MEF lines (B). Western blot analysis of Pak1 in RT4 Schwann cells that are serum starved and either untreated (−Dox) or overexpressing merlin (+Dox). The various phosphorylated forms of Pak1 are indicated by arrows (C).

cdc42 has been reported to be required for the full activation of Ras by Raf. Pak may mediate the effect of Rac/cdc42 through phosphorylation of Raf (Li et al., 2001; Macara et al., 1996).

Merlin might exert its inhibitory function by several different mechanisms, which could be overlapping. As we have shown, expression of merlin in the RT-4 inducible cell line resulted in reduced levels of Rac-GTP, without affecting the levels of total Rac1. Merlin, interestingly, binds RhoGDI (Maeda et al., 1999) and therefore may link between RhoGDI and Rac1, thus stabilizing the inactive state of Rac1. Alternatively, merlin might act by affecting the activity of β-pix, which is a Rac GEF (Manser et al., 1998). We have also demonstrated here that merlin binds to the PBD domain of Pak1, and this interaction could well disrupt association with Rac and the subsequent activation of Pak1.

The localization of Pak is important in the regulation of its activity. Pak1 activation requires signals converging from both growth factor receptors and integrins. One possible site of physical integration of these pathways is the scaffold protein paxillin (Turner, 2000). Pak has been shown to bind to paxillin via the p95PKL-Cool/Pix protein complex (Turner et al., 1999). The recruitment of Pak1-PIX-p95PKL to paxillin is triggered by binding of Rac-GTP to Pak1 and activation of the adaptor function of Pak1 (Brown et al., 2002). We have demonstrated that merlin interferes with the Pak1-paxillin interaction, but does not affect the interaction between Pak1 and β-pix, p95PKL, or NCK. It is probable that NCK and p95/PKL-Cool/Pix target Pak to different subcellualr locations, where Pak is activated by different stimuli. Thus, it is possible that merlin can specifically inhibit the cellular fraction of Pak1 that is to be recruited to focal adhesion complexes. Interestingly, merlin has been demonstrated to bind to paxillin and to β-integrin (Fernandez-Valle et al., 2002; Obremski et al., 1998). Therefore, merlin may bind Pak while it is bound to paxillin and act to both release it from paxillin and inhibit its full activation by Rac. Alternatively, there could exist separate pools of merlin with distinct functions. One pool of merlin may function by binding to Pak1 prior to its association with paxillin and prevent the association. Another fraction of merlin, which is associated with paxillin, might have other, additional functions. The existence of pools of merlin with different functions can be demonstrated with the identification of merlin mutants impaired in their ability to bind Pak1 and the functional comparison of these

mutants with the mutants of merlin unable to bind paxillin (Fernandez-Valle et al., 2002). It is important to note that the localization and function of merlin is also likely to be affected by phosphorylation at serine 518 (Kissil et al., 2002; Shaw et al., 2001). The effects of different merlin mutants, including those affecting serine 518, on the interaction with and inhibition of Pak are currently being investigated.

The data described here, combined with the fact that Pak phosphorylates and, perhaps, inactivates merlin supports a "feedforward" signaling model (Kissil et al., 2002; Xiao et al., 2002). In such a model, merlin would function normally to downregulate Rac/cdc42-induced signaling. Once activated, Rac/cdc42 can stimulate Pak activity, which in turn would lead to merlin phosphorylation and relief from its inhibitory effect. Given the fact that Rac signaling is necessary, and in some cases sufficient, for transformation, it is possible that merlin's inhibition of Rac/cdc42-signaling represents its tumor suppressor function. Studies employing dominant-negative mutants have shown that Rac is required for cellular transformation by Ras (Khosravi-Far et al., 1995; Qiu et al., 1995; Ridley et al., 1992; Roux et al., 1997). Rac has also been shown to regulate cell motility and invasiveness (del Peso et al., 1997; Evers et al., 2000; Habets et al., 1994; Sahai et al., 2001). The fact that $Nf2^{+/-}$ mice develop highly metastatic tumors, which display loss of the wild-type Nf2 allele, is also consistent with a merlin-Rac functional connection (McClatchey et al., 1998).

The data presented here demonstrate a direct connection between merlin and the Rac-signaling pathways, via the inhibition of Pak. The work ascribes a biochemical function to a tumor suppressor with previously unknown function. In addition, it identifies an established tumor suppressor in the process of Pak1 regulation, possibly linking Pak deregulation to tumorigenesis. Understanding the regulation of merlin by Rac/cdc42 and merlin's impact on these signaling pathways could lead to a more complete understanding for the role of merlin in tumor formation. Once these interactions are fully elucidated, the use of specific inhibitors can be assessed as therapeutic modalities for tumors bearing mutations in NF2.

Experimental Procedures

Cell Culture Conditions and Transfections

The RT4 Schwann cells and MEFs were grown in DME, 10% fetal calf serum, and antibiotics. In cases where merlin expression was involved, expression was induced by addition of 1 μg/ml Doxycycline for 48 hr prior to the experiment. All transfections were done with Lipofectamine (Invitrogen). For the experiments where Pak1 activation was examined, cells were serum starved by growing them for 24 hr in serum-free DME and antibiotics. Pak1 activation was stimulated by adding PDGF-BB (Sigma) at 5 ng/ml for 5 min. In experiments where cells were in suspension, tissue culture plates were coated with poly-HEME (Sigma) as previously described (Folkman and Moscona, 1978). Cells were trypsinized, treated with soybean Trypsin inhibitor (Sigma), and placed back onto regular tissue culture dishes or the poly-HEME coated plates for 4 hr before harvesting.

Plasmids and Antibodies

Expression plasmids used for transfection are pCDNA3-Nf2 (Kissil et al., 2002), pCMV-Pak1 (Sells et al., 1997), and pCDNA3-β-pix (Hashimoto et al., 2001). Plasmids for the in vitro translation were constructed by PCR of the FERM domain (1–313), C-terminal half (342–595), F1-F2 (1–217), and F3 (218–313) (sequences available upon request) and subcloning of the fragments into pCDNA3. Antibodies used were: for merlin, SC-331; Pak1, sc-881 and sc-882; β-pix, sc-10932; Nck, sc-290; Rac1, sc-217 (Santa Cruz Biotechnology); anti-paxillin mAb, anti-Rac1 mAb and anti-p95PKL mAb (BD Transduction Laboratories), and anti-paxillin pAb (Chemicon International).

Immunoprecipitations, Kinase Assays, and Rac Activation Assays

Cells were plated at 7.5×10^5/10 cm dish and transfected the next day. 48 hr after transfection the cells were serum starved for 24 hr and extracted into extraction buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.5% NP40, 0.1% deoxycholate, 1 mM $NaVO_4$, and protease inhibitors). Lysates were precleared for 1 hr, then incubated with the primary antibody for 3 hr at 4°C, and protein-A or protein-G beads were added for an additional 2 hr. Complexes were washed extensively with extraction buffer and separated by SDS-PAGE. Pak1 kinase assays were preformed as described (Kissil et al., 2002), except that Pak1 was immunoprecipitated from the extracts and MBP was added as substrate at 0.5 μg/ml. Quantification of active Rac was done employing the Rac activation assay kit, according to manufacturer's instructions (Upstate). Quantification of all Western blot experiments was done by densitometry analysis using NIH image v.1.63 of scanned data from at least three independent experiments.

In Vitro Binding Assays

Full-length and truncation mutants of merlin were produced using the TnT kit (Promega) with Methionine S^{35}. GST-Pak1 and GST-Pak1(70-143) were produced in bacteria as described (Thiel et al., 2002). The in vitro binding assays were preformed by incubation of 30 μl of GST, GST-Pak1, or GST-Pak1(70-143) bound to gluthathione beads (0.5 mg/ml) with equal amounts of in vitro translated merlin-S^{35} (determined beforehand by running 5% of each reaction on SDS-PAGE and autoradiography) in reaction buffer (50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 10 mM $MgCl_2$, 5% Glycerol, 1% Triton X-100) at 4°C for 3 hr and washed several times with the reaction buffer. The beads were then boiled, separated by SDS-PAGE, treated with Amplify (Amersham Pharmacia), dried, and exposed to film.

2-Dimensional Analysis of Pak1 Activity

Cells were harvested directly into sample buffer (9.8 M Urea, 2% CHAPS, 5 ml IPG buffer 4-7, DTT 15 mg/ml). Extracts were incubated on ice for 10 min and centrifuged 10 min, 14,000 × g, 4°C. Extracts (100 μg) were cup loaded onto 7 cm, pH 4–7 IPG strips (Amersham Pharmacia) and resolved at 50 mA/strip for 100V for 30 min, 200V for 30 min, 400V for 30 min, 1000V for 60 min, 3500V for 5 hr, and 500V to a total of 20,000V·hours using a IPGphor unit (Pharmacia biotech). Strips were then washed in wash solution (50 mM Tris-HCl [pH 8.8], 6 M Urea, 30% Glycerol, 2% SDS) supplemented with 20 mg/ml DTT for 10 min at RT and followed by a wash in wash buffer supplemented with 25 mg/ml iodoacetamide for 10 min. The strips were then loaded onto a standard SDS-PAGE, separated, and transferred to Immobilon (Millipore). The blots were then used in Western blot analysis. Equal loading of protein was determined by blotting with an actin antibody (Santa Cruz Biotechnology).

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