

Merlin Phosphorylation by p21-activated Kinase 2 and Effects of Phosphorylation on Merlin Localization*

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The *Nf2* tumor suppressor gene product merlin is related to the membrane-cytoskeleton linker proteins of the band 4.1 superfamily, including ezrin, radixin, and moesin (ERMs). Merlin is regulated by phosphorylation in a *Rac/cdc42*-dependent fashion. We report that the phosphorylation of merlin at serine 518 is induced by the p21-activated kinase PAK2. This is demonstrated by biochemical fractionation, use of active and dominant-negative mutants of PAK2, and immunodepletion. By using wild-type and mutated forms of merlin and phospho-directed antibodies, we show that phosphorylation of merlin at serine 518 leads to dramatic protein relocalization.

Neurofibromatosis type 2 (NF2)¹ is an inherited disorder characterized by the development of Schwann cell tumors of the eighth cranial nerve. Mutations and loss of heterozygosity of the *NF2* gene have been detected in NF2 patients and in various sporadic tumors, including schwannomas, meningiomas, and ependymomas (1). In further support of a role for *NF2* in tumor suppression, mice heterozygous for an *Nf2* mutation are predisposed to a wide variety of tumors with high metastatic potential (2). In a separate model in which *Nf2* is inactivated specifically in Schwann cells, mice develop schwannomas and Schwann cell hyperplasia (3).

The longest and predominant splice form of the *Nf2* gene codes for a 595-amino acid protein highly similar to the band 4.1 family of proteins. It is most closely related to the ERM proteins, moesin, ezrin, and radixin. The ERM proteins are thought to function as cell membrane-cytoskeleton linkers and are localized to cortical actin structures near the plasma membrane such as microvilli, membrane ruffles, and lamellipodia (4, 5). Likewise, merlin is localized to cortical actin structures, in patterns that partially overlap with the ERMs (1). It has been proposed that intramolecular binding of the N-terminal and C-terminal domains conformationally regulates the ERM proteins by masking binding sites for interacting proteins. The ERMs can also form homodimers and heterodimers, among themselves and with merlin, adding an additional level of com-

plexity to the regulation of these proteins (6). The recently solved crystal structure of the moesin N/C-terminal complex strengthens this model of conformational regulation (7). Given the sequence and, most likely, structural similarities of merlin to the ERM proteins, it is possible that merlin itself could be regulated in a similar fashion.

Recent studies (8, 9) have implicated additional factors in the regulation of the ERMs, including phospholipids and phosphorylation. Previous work from our group and others (10, 11) has shown that merlin is differentially phosphorylated as well and that merlin protein levels are affected by growth conditions such as cell confluency, loss of adhesion, or serum deprivation. Merlin is found in an hypophosphorylated form when the combination of cellular and environmental conditions are growth-inhibitory (10). ERMs can be phosphorylated by Rho kinase, and this phosphorylation can affect intramolecular association and cellular localization. Phosphorylation and/or phospholipids may promote the transition of the proteins to an active form by “opening” intra- and intermolecular associations. These active monomers can then bind to other interacting proteins and the actin cytoskeleton and induce actin-rich membrane projections (5, 8, 12, 13). The induction of merlin phosphorylation by activated alleles of the Rho family GTPases has also been examined. Interestingly, although activated Rho did not induce noticeable phosphorylation of merlin, activated forms of *Rac* and *cdc42* did. The site of *Rac*-induced phosphorylation was determined to be a serine at position 518; mutation of serine 518 results in reduced basal phosphorylation and eliminated *Rac*-induced phosphorylation (11).

Although *Rac* and *cdc42* are implicated in the regulation of many pathways, they are most associated with regulation of cytoskeleton reorganization and gene expression (for recent reviews see Refs. 14–16). In light of the data demonstrating that activated *Rac/cdc42* leads to phosphorylation and possible inactivation of merlin, the elucidation of the responsible effector pathways and their effects on merlin function are of major importance. Understanding this regulation of merlin could lead to a more complete appreciation of the effects of merlin loss in tumors.

MATERIALS AND METHODS

Plasmids and Transfections—Wild-type merlin and S518A were subcloned from previously described vectors (10) into the *Bam*HI-*Eco*RI sites of pCDNA3 (Invitrogen). The GST-merlin recombinant substrates were prepared by PCR amplification of the 60 amino acid coding sequences from the wild-type and S518A versions of merlin (primer sequences are available upon request) and subcloned into *Eco*RI and *Xho*I sites of pGEX5-3x. Wild-type PAK2 was amplified and cloned into the *Bam*HI and *Xho*I sites of pCDNA3 (Invitrogen). Activated PAK2 was made by inserting the T402E mutation by site-directed mutagenesis using the Quick-change kit, as instructed by the manufacturer (Stratagene). The PAK2 auto-inhibitory domain (amino acids 82–146) was amplified and cloned into pCDNA3. All plasmids were verified by DNA sequencing. Transfections were done with LipofectAMINE (Invitrogen).

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¹ The abbreviations used are: NF2, neurofibromatosis type 2; ERM, ezrin, radixin, and moesin; PAK, p21-activated kinase; GST, glutathione *S*-transferase; AID, auto-inhibitory domain.

Antibodies—The antibodies used are as follows: commercial merlin antibody SC331 (Santa Cruz Biotechnology); PAK1-SC882, PAK2-SC1872, and PAK3-SC1871 (Santa Cruz Biotechnology). SC1871 was demonstrated to be specific by Western blot comparison of extracts from cells overexpressing PAK1–3 (not shown). SC882 has been shown to be specific for PAK1 using PAK1^{-/-} mouse embryo fibroblasts.² SC1872 may also react with PAK1; however, this does not affect the conclusion drawn from the immunodepletion experiments shown in Fig. 3.

In Vitro Kinase Assay and Immunodepletion—Total cellular extracts were prepared by lysing the cells in cell lysis buffer: 50 mM HEPES, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 25 mM NaF, 20 mM β -glycerophosphate, 1 mM EDTA, and protease inhibitors. The extracts were added into reactions containing 200 ng of GST substrate, 2 mM MgCl₂, 2 mM DTT, 100 μ M cold ATP, and 10 μ Ci of [γ -³²P]ATP. For the immunodepletion experiments extracts were incubated with the relevant antibody for 3 h at 4 °C. After four sequential exchanges of antibody, the presence of the protein in question was determined by Western blot analysis of both supernatant and the precipitate. Kinase reactions were carried out at 30 °C for 20 min. The substrate was washed 3 times in lysis buffer at 4 °C, and the reaction was terminated with protein sample buffer and boiling for 5 min. The samples were then resolved by SDS-PAGE and exposed to film.

Merlin Phosphorylation—In the *in vivo* studies, cells were transfected with various expression vectors and harvested after 48 h into SDS boiling buffer (10 mM Tris, pH 7.5, 50 mM NaF, 1% SDS). Cells were scraped off the plates and boiled for 5 min; protein concentration was determined by the BCA method (Pierce); and proteins were resolved by 9% SDS-PAGE and detected by Western blot analysis. Phosphatase treatment was described previously (10).

Production of Merlin Phospho-specific Antibodies—A chemically phosphorylated peptide (H-CKDTDMKRLS*MEIE-NH₂; * = serine is phosphorylated) was coupled to SulfoLink coupling gel (Pierce) and used to immunize rabbits. A standard protocol of immunization was employed. Sera were purified by first passing it over an affinity column of the phospho-antigen (binding antibodies recognizing the peptide, phosphorylated and unphosphorylated). In a second step, the bound fraction was applied to a second column of the unphosphorylated peptide. The flow-through was collected and found to contain only phospho-specific antibodies.

Biochemical Fractionation of Merlin Kinase Activity—For ion-exchange chromatography, a Q-Sepharose (Amersham Biosciences) 10 \times 10 column was run at pH 8.5 (diethanolamine buffer) and a linear NaCl gradient, with maximal activity eluting around 120–130 mM. A 60-fold enrichment was achieved with overall recovery of about 70%. For dye-ligand chromatography a Matrex Red A dye (Millipore) 10 \times 10 column was used (pH 8, 0–500 mM KCl linear gradient), which gave 10-fold enrichment with 25% recovery.

Immunofluorescence—NIH3T3 or LLC-PK1 cells were plated on glass coverslips and transfected with various expression vectors. 24-h post-transfection cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with Triton X-100 for 10 min. Sc331 was used at 1:1000 dilution, and HM2175 was used at 1.0 μ g/ml.

RESULTS

In Vivo Phosphorylation of Merlin by PAKs—Merlin phosphorylation is regulated in response to various stimuli (10), and phosphorylation of serine 518 can be induced in a Rac/cdc42-dependent manner (11). To identify the kinase that phosphorylates merlin on serine 518, NIH3T3 cells were co-transfected with expression vectors for merlin and the constitutively active forms of Rac/cdc42 kinase effectors. These included PAK1, PAK2, PAK3, MLK3, LIM kinase, MEKK1, and JNK. Extracts were made 48 h post-transfection and analyzed by Western blot analysis. As shown in Fig. 1A, PAK1–3 induced an increase in the slower migrating and hyperphosphorylated form of merlin (see also Ref. 11). All other effectors tested did not induce merlin phosphorylation and were confirmed as active with an independent substrate (not shown). This phosphorylation is serine 518-specific as the merlin S518A mutant, in which serine 518 was changed to alanine, runs as a single band corresponding to the hypophosphorylated form of wild-type merlin in the presence of the activated PAK kinases (Fig. 1A).

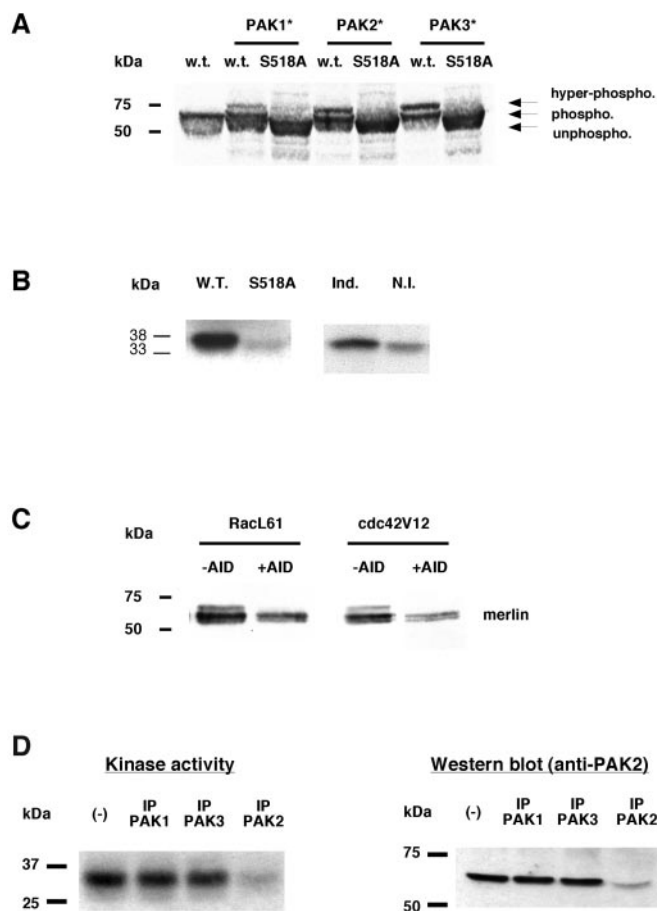


FIG. 1. *In vivo* analysis of merlin phosphorylation by PAKs. A, merlin Western blot analysis of extracts from NIH3T3 cells transfected with expression vectors for wild-type (*w.t.*) or S518A merlin along with activated (*) PAK1–3. Arrows indicate the different phosphorylated (*phospho.*) forms of merlin. B, *in vitro* kinase assay with extracts from NIH3T3 cells that were serum-stimulated (*Ind.*) or not stimulated (*N.I.*). The merlin kinase activity was determined using the wild-type (*W.T.*) GST-merlin recombinant substrate or the S518A mutant version of this substrate. C, merlin Western blot analysis of extracts prepared from NIH3T3 cells transfected with active Rac mutant (*RacL61*) or active cdc42 (*cdc42V12*) and full-length merlin, alone or in combination with the PAK2 autoinhibitory domain (*AID*). D, NIH3T3 extracts were immunodepleted with PAK1, PAK2, or PAK3 antibodies and analyzed by Western blot with a PAK2 antibody. The merlin kinase activity in the treated extracts was determined by an *in vitro* kinase assay with the merlin GST-pseudo-substrate and resolved by SDS-PAGE (See “Materials and Methods” for details of immunodepletion). *IP*, immunoprecipitation.

PAK2 Levels Correlate with Merlin Kinase Activity—To discriminate the different PAKs as candidate merlin kinases in NIH3T3 cells, we set out to purify the kinase activity over a series of chromatographic steps employing extracts from untransfected NIH3T3 cells that were serum-stimulated. Toward this goal, an *in vitro* kinase assay was established. The assay employs a recombinant substrate composed of residues 478–535 of merlin fused to glutathione *S*-transferase (GST). The recombinant substrate was purified from bacteria and then used in an *in vitro* kinase assay bound to glutathione-agarose beads. Activity is determined by SDS-PAGE and autoradiography. This assay is highly specific for the merlin kinase, as the S518A version of the substrate was not phosphorylated to a significant extent under conditions that have been shown to induce merlin phosphorylation (Fig. 1B). After an initial step of ammonium sulfate precipitation, the precipitate containing the activity was separated by ion-exchange chromatography on

² J. Chernoff (Fox Chase Cancer Center), personal communication.

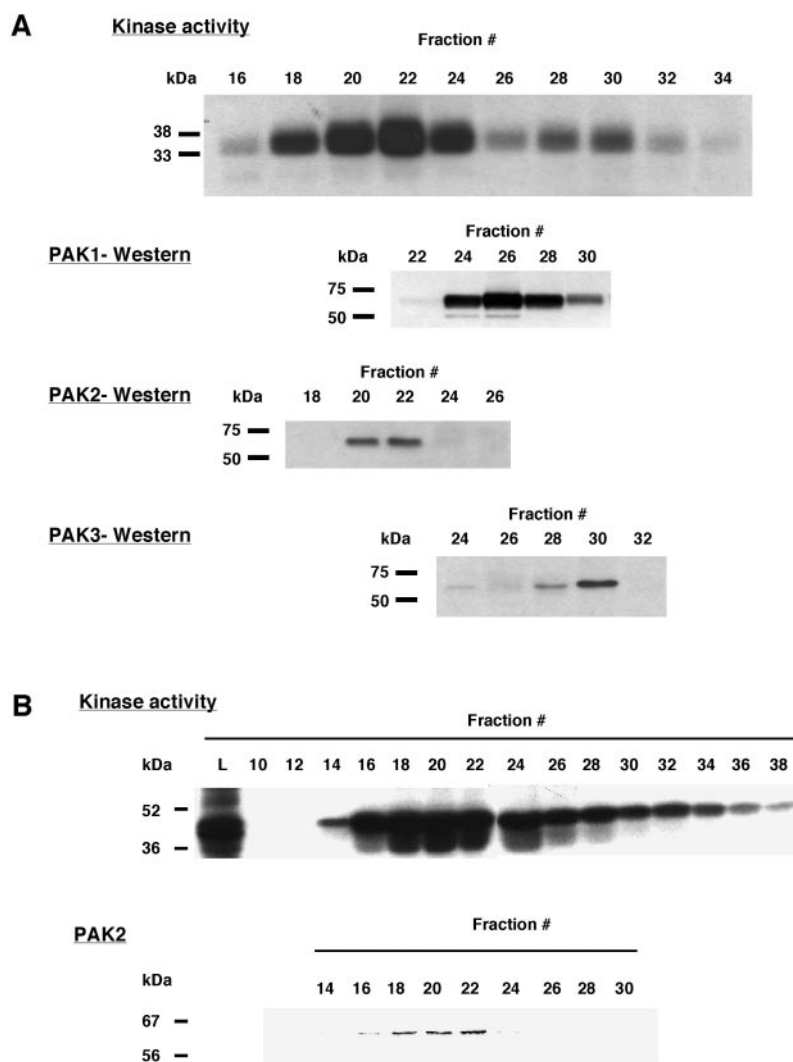


FIG. 2. Chromatographic separation of merlin kinase activity. Merlin kinase activity was separated on a Q-Sepharose 10×10 column. *A*, fractions were monitored for activity by *in vitro* kinase assay using the GST-merlin recombinant substrate and by Western blot analysis for PAK1, PAK2, and PAK3. *B*, in a subsequent step, fractions 20–24 were further resolved on a Matrex Red dye-ligand column. Kinase activity and PAK2 levels were followed. *L*, load fraction.

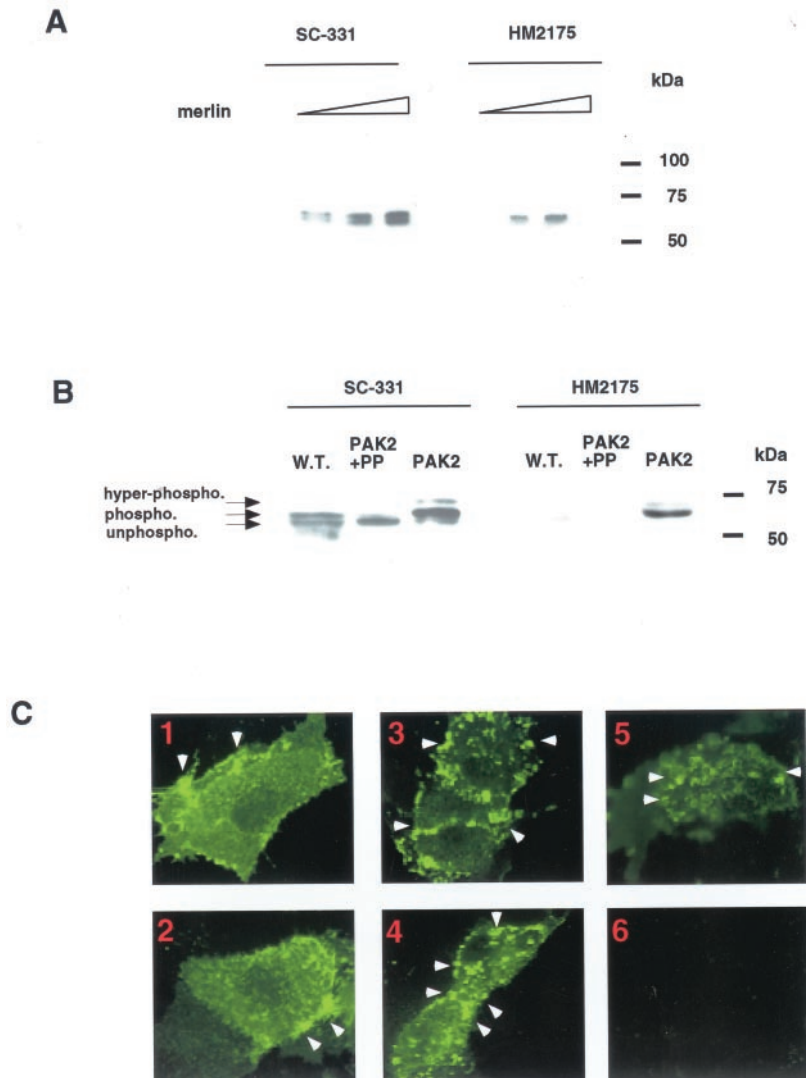
a Q-Sepharose column. Fractions were then examined for merlin kinase activity using the *in vitro* kinase assay and for PAK1, PAK2, and PAK3 levels by Western blotting. As shown in Fig. 2*A*, the maximal activity of merlin kinase was found in fractions 20–22 in this separation. PAK2 levels also peaked in fractions 20–22, whereas PAK1 levels peaked in fractions 23–27, and PAK3 levels peaked around fraction 30. Fractions 20–22 were taken from the ion exchange and used in a subsequent step of dye-ligand chromatography. Maximal activity of merlin kinase was observed in fractions 18–22. Again, PAK2 was present in a pattern that fully overlaps with the peak of merlin kinase activity (Fig. 2*B*), whereas PAK1 and PAK3 were no longer evident in these fractions (not shown). After four total steps of enrichment, which included ammonium sulfate precipitation, ion-exchange, dye-ligand chromatography, and gel filtration, more than 1200-fold enrichment of the kinase activity was achieved. When assessing the fractions of the various chromatographic steps by Western blot analysis, PAK2 levels consistently paralleled merlin kinase activity (Fig. 2 and not shown). Thus, by chromatographic separation, we were able to separate PAK1–3 and demonstrate that the merlin kinase activity from NIH3T3 cells co-fractionates with PAK2.

The PAK Auto-inhibitory Domain Inhibits the Rac-dependent Phosphorylation of Merlin—The N-terminal regulatory domain of the PAKs has been shown to contain an auto-inhibitory domain (AID), which can act *in trans* (17). The inhibitory frag-

ment in PAK2 resides between residues 82 and 146 (equivalent to the 83–149 AID of PAK1). To investigate further the role of PAK2 in induction of merlin phosphorylation in response to Rac/cdc42, we assessed the ability of the AID to inhibit this activity. NIH3T3 cells were co-transfected with expression vectors for full-length merlin, activated Rac or cdc42, and the PAK2 AID. The inclusion of the PAK2 AID significantly reduced the phosphorylation of merlin, as evident by the 3–4-fold decrease in the ratio of hyperphosphorylated to hypophosphorylated merlin in cells expressing the AID (Fig. 1*C*). These data indicate that merlin kinase activity induced by Rac/cdc42 is sensitive to PAK inhibition and strengthen the claim that PAK2 is the responsible kinase downstream of Rac in NIH3T3 cells.

Reduced Merlin Kinase Activity following Immunodepletion of PAK2—As another approach to address whether PAK2 is directly responsible for merlin phosphorylation in this cell system, the enzyme was immunodepleted from extracts of serum-treated NIH3T3 cells. After four sequential rounds of immunoprecipitation, the amount of PAK2 in the extract was reduced, on average, by 2–3-fold (Fig. 1*D*). Treated extracts were then used in kinase assays with the GST-merlin recombinant substrate; activity was determined by SDS-PAGE and autoradiography. As shown in Fig. 1*D*, extracts immunodepleted for PAK2 (IP-PAK2) show a 2–3-fold reduction in kinase activity compared with those treated in a similar fashion without antibody

FIG. 3. Phosphoserine 518 antibodies and subcellular localization of merlin. Western blot analysis of exogenous wild-type merlin expressed in LLC-PK1 cells. **A**, Western blot analysis of increasing amounts of protein extract from transfected LLC-PK1 cell showing the specificity of the HM2175 antibody toward the slower migrating, phosphorylated form of merlin. **B**, Western blot analysis of merlin immunoprecipitated from NIH3T3 cells transfected with a vector for wild-type merlin alone (*W.T.*) or with a vector for active PAK2 (*PAK2*). Half of the protein from the merlin plus active-PAK2 immunoprecipitate was treated with phosphatase (*PAK2 + PP*). **C**, immunolocalization of merlin with SC331 and wild-type merlin (*panel 1*), merlin S518A (*panel 2*), merlin S518D (*panel 3*), wild-type merlin plus active PAK2 (*panel 4*) and staining with HM2175 of wild-type merlin (*panel 5*) and merlin S518A (*panel 6*). *SC331*, commercial antibodies (Santa Cruz Biotechnology); *HM2175*, phospho-directed antibodies. *Arrows* indicate the membrane protrusions to which merlin relocalized.



added. Also, the immunodepletion of PAK3 (IP-PAK3) or PAK1 (IP-PAK1) from these extracts had little or no effect on merlin kinase levels of activity (Fig. 1D). This demonstrates the requirement for PAK2 for the phosphorylation of merlin serine 518 under these conditions.

Generation of Merlin Phospho-specific Antibodies—To follow the function and localization of merlin phosphorylated at serine 518, we produced antibodies (HM2175) that preferentially recognize the phosphorylated form (see “Materials and Methods”). To assess the activity of this antiserum, protein extracts from LLC-PK1 cells ectopically expressing wild-type merlin were used in Western blot analysis with either SC331 or HM2175 antibodies. The SC331 antibody detected both the hypo- and hyperphosphorylated form of merlin, whereas the HM2175 antibody preferentially detected the hyperphosphorylated form of the protein (Fig. 3A). At high concentrations of merlin, HM2175 did recognize the hypophosphorylated form to some extent (Fig. 3A). To validate further the specificity of HM2175, merlin was ectopically expressed along with active PAK2 in NIH3T3 cells, immunoprecipitated, and analyzed by Western blot, before and after treatment with phosphatase. As shown in Fig. 3B, reactivity of the HM2175 antibody was significantly reduced following phosphatase treatment, whereas the non-phospho-directed SC331 antibody recognized merlin forms in all lanes.

The phospho-directed antibodies were tested for specificity in immunocytochemistry. LLC-PK1 cells were transfected

with vectors expressing either wild-type merlin or the merlin S518A mutant. Fixed cells were then incubated with either the SC331 or HM2175 antibodies. Although SC331 recognized both merlin forms (Fig. 3C, *panels 1* and *2*), the HM2175 antibody recognized only ectopic wild-type merlin (Fig. 3C, *panels 5* and *6*). This pattern was observed in many cell lines of both epithelial and fibroblastic origin (not shown). Thus, the HM2175 antibodies preferentially recognize the phosphorylated form of merlin both in Western blot analysis and immunocytochemistry.

Phosphorylation Leads to Changes in the Subcellular Localization of Merlin in LLC-PK1 Cells—To visualize subcellular localization of the different forms of merlin, LLC-PK1 epithelial cells were transfected with wild-type merlin or the merlin S518A or S518D (phospho-mimicking) mutants and incubated with the SC331 antibody. As shown in Fig. 3C (*panel 1*), wild-type merlin mainly localized to microvilli. Lower amounts of the protein were present in larger membrane protrusions and in cortical actin structures. Similar results were obtained with the S518A mutant (*panel 2*). However, the S518D mutant was localized predominantly in the larger membrane protrusions (*panel 3*). Likewise, a shift to the larger membrane protrusions was observed when wild-type merlin was co-transfected with active PAK2 to increase merlin phosphorylation (*panel 4*). Importantly, the localization of the S518A mutant was not affected by co-transfected PAK2 (not shown), indicating that phosphorylation of serine 518 is required for relocalization. To

confirm this observation, HM2175 was used to stain cells transfected only with wild-type merlin. As expected, the HM2175 antibody stained wild-type merlin mainly in the larger cellular protrusions (panel 5). This reinforces the observation that the phosphorylation of serine 518 is required for the redistribution of merlin and is not a consequence of simply mutating serine 518 to aspartic acid.

DISCUSSION

We present here several independent lines of evidence supporting PAK2 as the kinase responsible for phosphorylation of serine 518 on the *Nf2* tumor suppressor gene product, merlin. Of all *Rac/cdc42* kinase effectors tested only PAK1–3 induced the phosphorylation of merlin serine 518 *in vivo*. We were able to separate PAK2 from PAK1 and PAK3 biochemically and demonstrate that merlin kinase activity co-elutes in parallel with PAK2 in NIH3T3 cells. The use of the dominant-negative fragment (PAK2-AID) and immunodepletion of PAK2 directly reinforce the identification of PAK2 as the merlin kinase. Additional circumstantial evidence can be found in the amino acid context of the serine 518 in merlin (MKRLSM) and is similar to many PAK recognition sites such as the pHOX p47 site (RKRLSQ), which has been shown to be an *in vivo* substrate of the PAKs (18).

By using the generic anti-merlin antibody SC331, wild-type protein and the S518A mutant were observed mostly localized to microvilli and to a small number of larger membrane protrusions and cortical structures in LLC-PK1 cells. However, when the merlin S518D mutant or wild-type merlin plus active PAK2 were introduced into these cells, a marked redistribution of merlin to membrane protrusions was observed. The HM2175 antibodies stained wild-type merlin in a similar pattern as the SC331-based staining of the merlin S518D mutant or wild-type merlin in the presence of active PAK2. The localization of phosphorylated merlin to such protrusions is not exclusive as phospho-merlin can still be observed in microvilli and cortical actin at a somewhat reduced level. The redistribution was dependent on phosphorylation of serine 518, because the merlin S518A mutant transfected with active PAK2 did not shift localization. This redistribution of merlin is highly reminiscent of the redistribution of ezrin in response to phosphorylation (12).

The data presented here are consistent and complementary to recent data demonstrating induction of serine 518 phosphorylation by *Rac/cdc42* (11). The identification of PAK2, which can be directly activated by *Rac/cdc42*, as the merlin kinase functionally connects merlin with the *Rac/cdc42* pathway. That merlin can inhibit *Rac*-induced signaling and that the immediate downstream effector, PAK2, phosphorylates and, perhaps, inactivates merlin reinforces the possibility of a “feed-forward” mechanism. In such a model, merlin would function at steady-state to down-regulate *Rac/cdc42*-induced signaling. Once activated, *Rac/cdc42* would activate PAK2, which in turn would phosphorylate merlin and relieve its inhibitory effect on *Rac/cdc42*. Functionally, this could be achieved by different mechanisms and impinge on these pathways at different levels (11). Merlin could inhibit signaling by acting upstream or upon effectors downstream to *Rac/cdc42*, or both. This would not be unprecedented as it has been suggested that activation of ERMs is required for the activation of Rho by lysophosphatidic acid and that Rho, in turn, induces phosphorylation of the ERMs (19). The observed shift in merlin localization after phosphorylation of serine 518 raises the possibility that merlin controls the subcellular localization of a molecule such as RhoGDI is worthy of consideration.

It is reasonable to suggest that the inhibitory function of merlin on *Rac/cdc42*-signaling is critical to its tumor suppressor

function, as *Rac* signaling is closely linked to cellular transformation (20). For example, *Rac* activation is required for the full transformed phenotype induced by Tiam and Ras (21–24). *Rac* and its regulators have also been shown to regulate cell motility and invasiveness (25). Examples include increased metastatic potential of cells expressing activated *Rac* (26) and identification of the *Rac*-GEF, Tiam, as a promoter of invasiveness (21, 27, 28). The fact that *Nf2*^{+/-} mice have highly metastatic tumors (2) with loss of the wild-type *Nf2* allele is also consistent with loss of merlin leading to activation of the *Rac* signaling pathways.

Although implicated in various cellular processes, the roles of the different members of the PAK family are not well defined. The identification of substrates specific to the different PAKs is one method of defining their specific functions. Presently, Raf-1 is a known PAK2 substrate and Bad and LIM kinase are specific substrates of PAK1 (29–31). This differential activity may reflect different cell types and/or environmental conditions used in specific experiments or may reflect true functional differences between the effector kinases. The identification of PAK2 as the kinase involved in merlin regulation and localization is a strong link between the well established *Rac/cdc42* signaling pathways with a tumor suppressor gene of unknown function. The further elucidation of these mechanisms will, undoubtedly, lead to a better understanding of merlin function and may suggest therapeutic strategies for *Nf2*-deficient tumors.

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