p21-activated Kinase Links Rac/Cdc42 Signaling to Merlin*

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The neurofibromatosis type 2 tumor suppressor gene, NF2, is mutated in the germ line of NF2 patients and predisposes affected individuals to tumors of neuroectodermal origin (1, 2). However, the cellular significant homology to the highly conserved ezrin-radixin-moesin family of proteins. However, the mechanism by which merlin exerts its tumor suppressor activity is not well understood. In this investigation, we show that merlin is phosphorylated in response to expression of activated Rac and activated Cdc42 in mammalian cells. Furthermore, we demonstrate that merlin phosphorylation is mediated by p21-activated kinase (Pak), a common downstream target of both Rac and Cdc42. Both in vivo and in vitro kinase assays demonstrated that Pak can directly phosphorylate merlin at serine 518, a site that affects merlin activity and localization. These biochemical investigations provide insights into the regulation of merlin function and establish a framework for elucidating tumorigenic mechanisms involved in neoplasms associated with merlin inactivation.

The neurofibromatosis type 2 tumor suppressor gene, NF2, is mutated in the germ line of NF2 patients and predisposes affected individuals to tumors of neuroectodermal origin (1, 2). NF2 encodes a 595-amino acid protein (merlin), which exhibits significant homology to the highly conserved ezrin-radixin-moesin (ERM) family of proteins. However, the cellular function and regulation of merlin is not well understood.

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† The abbreviations used are: ERM, ezrin-radixin-moesin; Pak, p21-activated kinase; MLK, mixed lineage kinase; CIP, calcium intestinal phosphatase; AID, autoinhibitory domain; HA, hemagglutinin; GST, glutathione S-transferase; JNK, c-Jun NH2-terminal kinase.

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Because of the similarity between merlin and ERM proteins, and the fact that ERM proteins are phosphorylated by Rho GTPase-mediated signaling (4, 5), we tested whether merlin is also regulated by members of the Rho family of GTPases. Our investigations have determined that merlin is phosphorylated in response to constitutively active Rac1 and, to a lesser extent, Cdc42, and this phosphorylation is mediated by the Rac/Cdc42 effector, p21-activated kinase (Pak).

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—A human NF2 expression plasmid was created by inserting a hemagglutinin (HA) epitope tag after the first methionine of full-length human NF2 cDNA (from D. H. Gutmann, Washington University, St. Louis, MO) and cloned into pcDNA3 vector (Invitrogen). NF2 S518A and NF2 S518D mutant constructs were made by polymerase chain reaction using a QuickChange site-directed mutagenesis kit (Stratagene). The oligonucleotide primers designed to introduce the mutations were as follows (odon changes are underlined): forward primer S518A, 5′-GAGCATTAGGCGCTTGGCCAATGATAGAGAAAG-3′; reverse primer S518A, 5′-CTTCTCTCATCTCCATGATAGAGAAAG-3′; forward primer S518D, 5′-GAGCATTAGGCGCTTGGCAGATAGAGAAAG-3′; reverse primer S518D, 5′-CTTCTCTCATCTCCATGATAGAGAAAG-3′. A HA-tagged NF2-C construct, encoding the carboxyl terminus (amino acids 299–595) of merlin was generated by PCR and cloned into pcDNA3 vector. Then the HA-NF2-C was used as a template to create truncated forms of NF2, containing S518A (HA-NF2-C Ala518) or S518D (HA-NF2-C Asp518), by site-directed mutagenesis as described above. Authenticity of various wild-type and mutant forms of NF2 was verified by nucleotide sequencing. Other plasmids included constitutively active Rho GTPases: pCMV-Myc-RhoA Q63L, pCGN-HA-Rac1 V12H40 (both from D. Bar-Sagi, State University of New York, Stony Brook, NY); FLAG-tagged wild-type and kinase dead MLK3 (both from J. R. Woodgett, Ontario Cancer Institute, Toronto, Canada); HA-tagged wild-type and constitutively active Pak1 (from E. Manser, Glaxo-IMCB Group, Singapore); and active Rac1 effector mutants: pCGT-Rac1 V12L37 and pCGT-Rac1 V12H40 (both from D. Bar-Sagi, State University of New York, Stony Brook, NY); PLK (p21-activated kinase)-negative dominant negative Pak1 (pEBG-Pak1, auto-inhibitory domain (AID) (pEBG Pak1, 83–149); inactive dominant negative Pak1: pEBG-Pak1-L107F (6); dominant negative Pak1: pEBG-Pak1, auto-inhibitory domain (AID) (pEBG Pak1, 83–149); inactive dominant negative Pak1: pEBG-Pak1-L107F (pEBG-Pak1, 83–149 L107F); and pCMV-Pak1 was constructed by inserting Pak6-DNA into the BglII/EcoRI cloning sites of a pCMV6-Myc mammalian expression vector.

Cell Culture, Transfections, and Immunoblotting—NIH3T3 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum or 10% fetal bovine serum, respectively. Plasmid DNA was transfected into cells using GenePorter reagent (Gene Therapy System). After 24 h, cells were solubilized with lysis buffer (20 mm Tris-HCl, pH 7.5, 137 mm NaCl, 1% Nonidet P-40, 10 mm NaF, 1 mm Na2VO4, 1 mm sodium pyrophosphate, 25 mm β-glycerophosphate, 2 mm EDTA, 1 mm dithiothreitol, 0.5 mm phenylmethylsulfonyl fluoride, 5 mm/l mprtin, 2 mm/l peuprotein). Samples were then centrifuged at 14,000 × g for 10 min at 4 °C, and the supernatants were collected. For phosphatase treatment, lysates from NIH3T3 cells transfected with HA-NF2 and Rac1 Leu15 (7) were precipitated with HA.11 monoclonal antibody (Babeo). Aliquots of the precipitated protein were treated with buffer alone, with 10 units of calf intestinal phosphatase (CIP, New England Biolabs) alone or with CIP plus 1 mm sodium vanadate and 10 mm NaF at 37 °C for 10 min. Reactions were terminated by adding SDS-PAGE sample buffer. Cell lysates or immunoprecipitates were electrophoresed on a 6% SDS-PAGE gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Inc.) for Western blotting. Antibodies included anti-Myc 9E10 and anti-FLAG M5 (both from Sigma), anti-GST (Santa Cruz), anti-Rac1 (Upstate Biotechnology), and anti-phospho-JNK (Cell Signaling). Antibody detection was by means of an ECL Western analysis system (Amersham Biosciences, Inc.).
In Vitro Phosphorylation of Merlin by Pak—Human Pak2 cDNA was subcloned into the pET28 bacterial expression vector (Invitrogen). BL21 (DE3) cells were transformed with this plasmid, and protein expression was induced by incubating a log phase culture in 0.5 mM isopropyl-β-D-thiogalactopyranoside for 6 h at 30 °C. The recombinant protein was recovered by chromatographic purification on a Talon column (CLONTECH) using standard methods. The recombinant His6-tagged Pak2 was stored in 10-μl aliquots at −80 °C.

Ser518 (wild-type), Ala518, and Asp518 forms of NF2-C (amino acid residues 299–595) were transiently expressed in HeLa cells and immunoprecipitated with anti-HA antibody. Each immunoprecipitate was washed extensively and then incubated in 25 μl of protein kinase buffer (40 mM Hepes, pH 7.4, 10 mM NaCl, 1 mM MgCl2, 1 mM MnCl2) containing 20 μM ATP, 2.5 μCi of [γ-32P]ATP, and 60 ng of activated recombinant Pak2. The reaction was incubated at 30 °C for 30 min and terminated by adding SDS-PAGE sample buffer. The products were separated by SDS-PAGE, and the autoradiogram was made from the dried gel. Western blot analysis was performed using anti-HA antibody to verify equivalent amounts of merlin substrate among reactions.

RESULTS AND DISCUSSION

Previous studies have demonstrated that merlin is phosphorylated on serine and threonine residues and that merlin phosphorylation decreases with serum starvation, high cell density, or loss of adhesion (7). The ERM proteins play a role in cell surface dynamics and structure by linking the cytoskeleton to the plasma membrane (8, 9) and are regulated by Rho signaling (4, 5, 10). The Rho GTPases play crucial roles in regulating the organization of the actin cytoskeleton in mammalian cells, and Rho GTPases have been shown to regulate both cell-cell and cell-matrix adhesions and can influence the motile and invasive properties of tumor cells in vitro (11). Thus, we postulated...
Phosphorylation of Merlin by Pak

**Fig. 3. Merlin Ser518 is the phosphoacceptor site for Pak.** A, HA-tagged full-length NF2 Ser518, NF2 Ala518, or NF2 Asp518 was transiently transfected into HeLa cells or cotransfected with either Rac1 Leu61 or Pak1 165 followed by immunoblotting with HA antibody. Although merlin Ser518 shows faster and slower mobility bands in response to active forms of Rac1 or Pak1, NF2 Ala518 migrates consistently as a single mobility form. Expression of Pak1 165 and Rac1 was verified by immunoblotting using anti-Myc or anti-HA antibodies. B, HA-tagged truncated forms of NF2 were transiently transfected into HeLa cells or cotransfected with Pak1 165 followed by immunoblotting with HA antibody. Truncated merlin Ser518, but not the mutant forms Ala518 or Asp518, was phosphorylated by Pak1 165. Expression of Pak1 165 was verified by immunoblotting using anti-Myc antibody. C, in vitro phosphorylation of merlin by active Pak. HA-tagged truncated forms of NF2 were transiently expressed in HeLa cells and immunoprecipitated with anti-HA antibody, and the in vitro kinase assay was performed as described under “Materials and Methods.” Note that wild-type merlin, but not mutant forms of merlin, are phosphorylated in the presence of Pak.

that Rho GTPase signaling could regulate the phosphorylation status of merlin. NIH3T3 cells and HeLa cells were transiently cotransfected with HA-tagged NF2 and individual constitutively active Rho GTPase constructs. Cells were lysed 24 h after transfection, and expression of merlin was examined by immunoblotting with anti-HA antibody. As shown in Fig. 1A, exogenous merlin was detected as two mobility forms. In cells transfected with active RhoA (RhoA Q63L), merlin migrated as a single mobility form. However, in cells transfected with active Rac1 (Rac1 Q61L), and to a lesser extent, in cells transfected with active Cdc42 (Cdc42 R40L), merlin migrated as a doublet consisting of faster and slower mobility bands. To investigate the nature of the slower migrating form of merlin, lysates from NIH3T3 cells cotransfected with HA-NF2 and Rac1 Leu61 were precipitated with anti-HA antibody. CIP treatment eliminated the slower migrating form of merlin, whereas phosphatase inhibitors reversed the dephosphorylation effect of CIP (Fig. 1B). These data indicate that the slower migrating band represents the phosphorylated form of merlin, which is induced by either active Rac1 or Cdc42.

Earlier work identified several downstream target molecules of Rac. MLK3, a member of the mixed lineage kinase family, is able to associate with Rac and Cdc42 and, in turn, to activate JNK (12). Pak1, another effector of Rac, plays an important role in cell morphology and motility (6, 13-15). To examine which effector mediates merlin phosphorylation by Rac, NIH3T3 cells and HeLa cells were transiently cotransfected with HA-NF2, Rac1 Leu61, and a dominant negative form of MLK3 or Pak1 (Pak1-AID). Immunoblot analysis was performed as shown in Fig. 2A. The slower mobility phosphorylated form of merlin induced by active Rac1 was inhibited by a dominant negative form of Pak1 containing the autoinhibitory domain (Pak1-AID) (16, 17), but not by Pak1-AID L107F, which is known to block the autoinhibitory effect of Pak1-AID (17).

To confirm the involvement of Pak in merlin phosphorylation, we cotransfected HA-NF2 with the Rac effector mutant Rac1 V12L37, which is able to activate Pak1, or Rac1 V12H40, which cannot bind or activate Pak1 (18). Rac1 V12L37, but not Rac1 V12H40, stimulated merlin phosphorylation (Fig. 2C). Next, we cotransfected HeLa cells with HA-NF2 and individual active forms of Pak1 (Pak1 L107F, Pak1 T423E, Pak1 165) or Pak2 (Pak2 T403E), or wild-type Pak6, each of which was able to stimulate phosphorylation of merlin (Fig. 2D). Similar results were obtained with NIH3T3 cells (data not shown). These results show that both group I Paks and group II Paks can induce phosphorylation of merlin.

Because dominant negative MLK3 partially blocked merlin phosphorylation induced by Rac (Fig. 2A), we tested whether merlin can also be phosphorylated via MLK3 signaling. As shown in Fig. 2E, overexpression of MLK3 failed to stimulate merlin phosphorylation. Since MLK3 contains a partial CRIB (Cdc42-Rac interaction and binding) motif, the inhibitory effect of dominant negative MLK3 could be due to sequestering Rac from Pak. In addition, we determined that ROK, a downstream target of Rho, is also unable to stimulate merlin phosphorylation (Fig. 2E). Taken together, these findings indicate that merlin is phosphorylated by Rac specifically via Pak signaling.

Shaw et al. (19) has also recently observed that merlin functions in Rac/Cdc42-dependent signaling, although that work did not link Rac and merlin signaling with Pak. Their investigation also demonstrated that Rac-induced phosphorylation of merlin, at Ser518, regulates its activity by weakening both its head-to-tail interaction and its association with the cytoskeleton. Since our data indicated that merlin is phosphorylated by Rac through Pak, we sought to determine whether Pak also phosphorylates merlin at Ser518. To test this possibility, merlin Ser518 was mutated to an alanine (NF2 S518A), which could no longer be phosphorylated at this site, or aspartic acid (NF2 S518D), which could mimic the effect of phosphorylation. Active Rac1 or Pak1 was transiently cotransfected with wild-type NF2, NF2 S518A, or NF2 S518D, followed by immunoblotting. As shown in Fig. 3A, mutation of this site had no effect on the level of expression of merlin. However, in cells transfected with either active Rac1 or Pak1, the NF2 S518A mutant was refractory to phosphorylation as indicated by a single mobility form, whereas NF2 S518D consistently migrated as a doublet whether cotransfected with active Rac1 or Pak1 or when transfected alone. Next, we transfected constructs encoding truncated forms of merlin into HeLa cells (Fig. 3B). Western blot analysis confirmed that truncated merlin Ser518, but not the mutant forms Ala518 or Asp518, is phosphorylated by Pak1 165. Collectively, these data indicate that merlin Ser518 is the phosphoacceptor site mediated not only by Rac, but also by Pak.

To determine whether merlin can be phosphorylated by Pak in vitro, we performed an immunocomplex kinase assay. Because full-length merlin comigrates with Pak, we used truncated forms of merlin (NF2-C Ser518 NF2-C Ala518 NF2-C Asp518) as substrates. As demonstrated in Fig. 3C, truncated merlin Ser518 was phosphorylated in the presence, but not in the absence, of active, recombinant Pak. Furthermore, truncated mutant forms of merlin (Ala518 and Asp518) were not phosphorylated by Pak. Collectively, these data indicate that Pak can directly phosphorylate merlin at serine 518.

Previous work indicates that overexpression of merlin in rat schwannoma cells inhibits their growth (20) and impairs cell motility, adhesion, and spreading (21). Merlin is hypophospho-
Phosphorylation of Merlin by Pak

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rylated in connection with serum deprivation, high cell density, or loss of adhesion (7). At low cell density, merlin is phosphorylated, growth-permissive, and exists in a complex with ezrin, moesin, and the hyaluronic acid receptor CD44 (22). These data indicate that the phosphorylation status of merlin specifies cell growth arrest or proliferation: i.e. hypophosphorylated merlin is growth-inhibitory and represents the functionally active tumor suppressor form of the protein, whereas hyperphosphorylation inactivates merlin and is growth-permissive.

Merlin loss has been associated with a high metastatic potential in an animal model reported by McClatchey et al. (23). The signal transduction studies presented here establish a framework for elucidating tumorigenic mechanisms involved in neoplasms associated with merlin inactivation. Interestingly, somatic mutations of merlin are common in human malignant mesothelioma (24, 25), a highly invasive and metastatic tumor type. Merlin loss of function by either phosphorylation or biallelic inactivation may contribute to tumor growth and invasive/metastatic. Thus, our characterization of merlin phosphorylation by Rac through Pak provides new insights into the regulation of its tumor suppressor function. Importantly, Pak has been shown to regulate motility in mammalian cells (6), and activated Paks can be transforming (26, 27). These data raise the intriguing possibility that merlin inactivation by Pak may play a role in tumor cell spreading and metastasis.

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