Akt Phosphorylation of Merlin Enhances Its Binding to Phosphatidylinositol and Inhibits the Tumor-Suppressive Activities of Merlin

Masashi Okada, Yanru Wang, Sung-Wuk Jang, Xiaoling Tang, Luca M. Neri, and Keqiang Ye

Abstract

The NF2 tumor suppressor gene encodes an intracellular membrane-associated protein, called merlin, which belongs to the band 4.1 family of cytoskeleton-associated proteins that link cell surface glycoproteins to the actin cytoskeleton. Merlin suppresses phosphatidylinositol 3-kinase (PI3K)/Akt signaling by directly binding and inhibiting the stimulatory activity of PIKE-L on PI3K. Akt feeds back and phosphorylates merlin and provokes its polyubiquitination and degradation. Here, we show that Akt phosphorylation and P(3,4,5)P_3 binding mediate the tumor-suppressive activity of merlin. The extreme NH2 terminus of merlin directly interacts with phosphatidylinositols, for which the unfolded conformation is required. Moreover, Akt phosphorylation enhances merlin binding affinity to phosphatidylinositols and inhibits its proapoptotic actions. Furthermore, Akt phosphorylation and phosphatidylinositols increase merlin binding to CD44. Epidermal growth factor treatment and Akt phosphorylation provoke merlin to aggregate in the ruffled plasma membrane and promote cell migration. Thus, these results suggest that PI3K signaling regulates the tumor-suppressive activity of merlin via both Akt phosphorylation and phosphatidylinositol lipids binding to merlin. [Cancer Res 2009;69(9):4043–51]

Introduction

The NF2 tumor suppressor protein merlin is structurally related to the protein 4.1 family of molecules and, specifically, a subgroup including ezrin, radixin, and moesin (ERM proteins). Like the ERM proteins, merlin contains an NH2 terminal domain (NTD; residues 1–302), which is highly conserved among all members of the protein 4.1 family and is thought to mediate interactions with the cytoplasmic tail of cell surface glycoproteins, such as glycoporphin C and CD44 (1). The second half of the molecule contains a predicted α-helical domain (residues 303–478) and a unique COOH terminal domain (CTD; residues 479–595). ERM proteins link the actin cytoskeleton to cell surface glycoproteins. Merlin can associate with polymerized actin in vitro by virtue of an amino (NH2) terminal actin binding domain, including residues 178 to 367. Merlin actin binding is not affected by natural NF2 patient mutations or alternatively spliced isoforms (2). Phosphatidylinositol lipids have been implicated in ERM protein activation. For example, P(4,5)P_2 enhances the ability of full-length radixin, but not its NTD, to bind the cytoplasmic tail of CD44. However, the tail of CD44 does not bind P(4,5)P_3, suggestive of an activating effect on full-length radixin (3). Analysis of recombinant moesin T588D, which mimics phosphorylated moesin, suggests an auxiliary role for P(4,5)P_3 in unmasking the COOH terminal filamentous actin (F-actin) binding site. Recent work using either unphosphorylated or T558-phosphorylated moesin purified from platelets revealed a dual requirement for T558 phosphorylation and the presence of a detergent and a phospholipid, such as P(4,5)P_2, for F-actin binding (4). In support of a two-step activation process wherein P(4,5)P_2 renders moesin susceptible to COOH terminal phosphorylation, transfection studies have suggested that enhanced phosphatidylinositol 4-phosphate 5-kinase activity results in enhanced phosphorylation of the COOH terminal threonine (5).

Merlin is phosphorylated on both serine and threonine residues, and the phosphorylation status of merlin varies in response to growth conditions (6). PAK2 phosphorylates S518 and abolishes merlin NH2/COOH terminal binding. PAK2 also impairs the ability of merlin to bind to two interacting proteins, CD44 and HRS, both critical for merlin growth suppression (7). In addition to the phosphorylation of S518, PKA has recently been shown to phosphorylate S10 and affect the actin cytoskeleton, mediating cell migration (8). Most recently, we show that Akt phosphorylates merlin on residues T230 and S315, which abolishes merlin intramolecular NTD/CTD interaction and merlin binding to PIKE-L and other binding partners, leading to merlin degradation by polyubiquitination (9). Phosphorylation and P(4,5)P_2 binding coordinately mediate the activation of ERM proteins (5, 10). However, whether the dual regulatory machinery also controls merlin activity remains elusive. In this report, we show that the NTD of merlin associates directly with phosphatidylinositols, for which the “open” conformation is required. Moreover, Akt phosphorylation enhances the interaction between merlin and phosphatidylinositols. Akt phosphorylation blocks its proapoptotic activity. Finally, we show that Akt phosphorylation of merlin substantially inhibits its proapoptotic activity and blocks its tumor-suppressive activity on cell migration.

Materials and Methods

Cells and reagents. Both 5, and 6, which are RT4-D6P2T schwannoma cells stably transfected with wild-type merlin (merlin T230DS315D and T230AS315A stably expressed cells), were maintained in DMEM including 10% fetal bovine serum, 100 units of penicillin-streptomycin, 500 μg/mL of G-418, and 1 μg/mL of puromycin. L64P, S518A, and S518D cells are patient-derived mutant (L64P), and S518 phosphorylation mutant stably transfected RT4-D6P2T cells. Merlin proteins were induced by 1 μg/mL of doxycycline treatment and then incubated for 24 h. HCT116 and HCT116 PTEN−/− cells...
were maintained in McCoy’s 5A, including 10% fetal bovine serum and 100 units of penicillin-streptomycin. All cells were maintained at 37°C with 5% CO2 atmosphere in a humidified incubator. Wortmannin, LY294002, and glutathione S-transferase (GST)–horseradish peroxidase were from Sigma. Akt1/2 inhibitor was from VWR, and PAK inhibitor IPA-3 was from Sigma. Phosphatidylinositol-conjugated beads and various phosphatidylinositols diC16 were from Echelon, Inc.

Cosedimentation of merlin recombinant proteins with lipid vesicles. The analysis of merlin-lipid interactions by cosedimentation of proteins with multilamellar liposomes was performed as described (11). Multilamellar liposomes were prepared from phosphatidylcholine and PI(3,4,5)P3, PI(4,5)P2, and phosphatidylinositol in a buffer containing 20 mmol/L of HEPES (pH 7.4) and 0.2 mmol/L of EGTA. Proteins were subsequently incubated for 15 min at 25°C in the absence of liposomes for 15 min. The proteins were subsequently incubated for 15 min at 25°C. Multilamellar liposomes were prepared from phosphatidylcholine and PI(3,4,5)P3, PI(4,5)P2, PI(3,4)P2, and phosphatidylinositol in a buffer containing 20 mmol/L of HEPES (pH 7.4) and 0.2 mmol/L of EGTA.

In vitro binding assay between GST-CD44 CTD and merlin in the presence of lipid micelles. The glutathione beads binding to GST-CD44 CTD or GST alone were pretreated with radioimmunoprecipitation assay buffer [0.1% SDS, 6.5% deoxycholic acid, 1% NP40, 50 mmol/L Tris–HCl (pH 8.0), and 150 mmol/L NaCl] for 20 min at room temperature. They were washed with 20 vol of buffer containing 10 mmol/L of HEPES (pH 7.5), 1 mmol/L of MgCl2, and 40 mmol/L of NaCl. After brief centrifugation, the beads were resuspended in 130 μL of buffer containing 50 μg/mL of phosphatidyserine and a variety of 5 μg/mL phosphatidylinositols. The mixture was sonicated, and the resulting vesicles were mixed with the glutathione beads and 2.6 μg of purified merlin. The reaction mixture was incubated at room temperature with constant rotation, followed by immunoblotting.

Wound-healing assays. RT4 schwannoma cells were stably transfected with inducible T230DS15D and T230AS15A mutant constructs. Three independent clones for each mutant were used for the migration assay. The cells were induced by doxycycline and split into 10-cm dishes and incubated until 90% to 100% confluent. After dishes were scratched by blue tip (0 h), cells were incubated for different time courses. The pictures were taken at 0, 6, 12, and 18 h, respectively. Phase contrast images were taken by Olympus IX71. Measurement of the wound-healing gap distance was performed using the computer program Image J. Results were expressed as mean ± SE. P ≤ 0.05 was considered significant (Student’s t test).

Results

The NH2 terminus of merlin binds phosphatidylinositols. To examine whether phosphatidylinositol lipids are involved in binding to merlin, we performed in vitro binding assays. PI(3,4,5)P3 selectively interacted with both NTD and full-length merlin, but not CTD or negative control GST. By contrast, PI(4,5)P2 weakly associated with both NTD and CTD, but not full-length merlin (Fig. 1A). Truncation assay showed the extreme NH2 terminal 1–82 and 1–132 fragments associated with PI(3,4,5)P3; in contrast, all the other truncates lacking the NH2 terminal 1–132 region failed to bind PI(3,4,5)P3 (Fig. 1B, top).

As a measure for bivalent association, cosedimentation of proteins with liposomes containing 20% of a variety of phosphatidylinositols lipids and 80% phosphatidylincholine was analyzed. Control values of protein sedimenting in the absence of lipids were subtracted. Lipid interactions were measured under physiologic ionic strength conditions, as described in Materials and Methods. GST-merlin NTD strongly cosedimented with PI(3,4,5)P3 liposomes (25 ± 5%). It revealed comparable affinity to both PI(4,5)P2 and PI(3,4)P2 with 18 ± 4% and 20 ± 3%, respectively. However, its affinity to PI(3)P was substantially decreased to 6 ± 2%. By contrast, GST-merlin CTD almost did not bind to 3’-phosphorylated phosphatidylinositols lipids, but it potently cosedimented with PI(4,5)P2 (26 ± 6%). GST alone revealed negligible cosedimentation activity to all liposomes (Fig. 1C). Therefore, these findings support that merlin NTD possesses much stronger PI(3,4,5)P3 binding affinity than its CTD, which, instead, reveals tighter affinity to PI(4,5)P2.

![Figure 1](https://example.com/f1.png)

**Figure 1.** NH2 terminus of merlin binds to phosphatidylinositol lipids. **A**, NTD of merlin directly associates phosphatidylinositol lipids. Purified GST and GST-merlin recombinant fusion proteins (5 μg each) were mixed with 30 μL of PI(3,4,5)P3-conjugated or PI(4,5)P2-conjugated beads for 3 h at 4°C, respectively. Lipid-protein complexes were separated by SDS-PAGE and analyzed by immunoblotting (IB) against GST. **B**, PI(3,4,5)P3 binds to the extreme NH2 terminus of merlin. **C**, quantitative analysis of cosedimentation of purified GST-merlin recombinant proteins with liposomes. The amount of protein present in the pellets and the supernatant was quantified by scanning the bands of the Coomassie blue–stained gels. The presented data stand for [protein in the pellets / total protein with liposomes [%] – [proteins in the pellets / total proteins without liposomes [%]]. The amount of proteins in the pellets in the absence of liposomes was subtracted from the amount of proteins in the presence of liposomes. Data are presented as mean ± SD of five independent experiments.
The unfolded conformation is required for the association between merlin and phosphatidylinositols. The NH2 terminus of ezrin reveals more potent binding activity to PI(4,5)P2 than full-length ezrin in the presence of physiologic ionic conditions, suggesting that folding conformation mediates its binding affinity to phosphatidylinositol lipids. To explore whether merlin folding conformation mediates its binding effect to phosphatidylinositol lipids, we used three forms of well-characterized merlin mutants: L64P, S518D, and S518A. The patient-derived L64P mutant displays an open inactive conformation, and so does S518D. However, S518A possesses a folded active conformation. We examined the binding activity in RT4-D6P2T schwannaoma cell lines stably transfected with inducible wild-type and mutated merlins. The parental RT4-D6P2T schwannaoma cell line expresses a negligible amount of merlin (7). Interestingly, no binding activity was observed with S518A. Surprisingly, S518D robustly associated with phosphatidylinositol lipids (Fig. 2, left, second row). Both wild-type and L64P merlin interacted with PI(4,5)P2, PI(3,4)P2, and PI(3,4,5)P3. However, wild-type merlin in 5× cells displayed the strongest binding activity to PI(4,5)P2. By contrast, L64P revealed the most prominent interaction with PI(3,4,5)P3. These observations strongly support the notion that PI(3,4,5)P3 or PI(3,4)P2 robustly bind to open merlin whereas PI(4,5)P2 preferentially interacts with native merlin.

Akt phosphorylation of merlin increases its affinity to phosphatidylinositols. To explore whether Akt phosphorylation of merlin mediates its binding activity to phosphatidylinositol lipids, we conducted an in vitro binding assay. Under control conditions, the NTD strongly bound to PI(3,4,5)P3, whereas full-length merlin barely associated with PI(3,4,5)P3. In the presence of active Akt, both the NTD and full-length merlin displayed substantially elevated binding affinities to PI(3,4,5)P3 (Fig. 3A). To examine the effect of Akt phosphorylation on merlin’s binding behavior to phosphatidylinositols in intact cells, we infected 5× schwannaoma cells with adenovirus expressing plasma membrane–localized active myristoylated Akt and short hairpin RNA of Akt1. Under control conditions (Fig. 3B, bottom left), wild-type merlin exhibited a binding effect to various phosphatidylinositols, with the strongest activity to PI(4,5)P2, a phenomena similar to what was observed in Fig. 2A. However, when Akt1 was depleted, merlin lost its binding activity to both PI(3,4)P2 and PI(3,4,5)P3, but it still retained affinity to PI(4,5)P2, indicating that Akt phosphorylation is not required for merlin to bind to PI(4,5)P2 (Fig. 3B, middle left). In the presence of active Akt, merlin displayed obviously increased binding effect to both PI(3,4,5)P3 and PI(3,4)P2, whereas merlin remained comparable affinity to PI(4,5)P2 as the control condition (Fig. 3B, top left), supporting that Akt phosphorylation provokes merlin binding to 3′-phosphorylated phosphatidylinositols. Both GSK3β and merlin S315 phosphorylation tightly correlated with Akt1 expression and activation profiles (Fig. 3B, right). Compared with wild-type NTD, purified recombinant GST-merlin 1–332 (T230DS315D) revealed much stronger binding activity to PI(3,4,5)P3 (Fig. 3C). We have made similar observations with mammalian cells transfected with green fluorescent protein (GFP)–merlin. The phosphorylation mimetic merlin (T230DS315D) displayed markedly higher affinity to both PI(4,5)P2 and PI(3,4,5)P3 than wild-type merlin. By contrast, unphosphorylated merlin (T230AS315A) failed to bind either lipid (Fig. 3D), similar with S518A. These data further support that Akt phosphorylation is indispensable for merlin to interact with PI(3,4,5)P3. Thus, Akt plays a critical role in dictating the binding of merlin to phosphatidylinositols.

PI(3,4,5)P3 lipid enhances merlin binding affinity to CD44. To explore whether phosphatidylinositols regulate the interaction between merlin and CD44, we conducted an in vitro binding assay in the presence of phosphatidylerine or PIPx/phosphatidylerine micelles. Under the control condition (50 μg/mL phosphatidylerine), GST-CD44 CTD, but not GST, selectively interacted with merlin (Fig. 4A, lanes 9 and 10). Phosphatidylinositol did not significantly elevate the interaction between merlin and CD44 CTD, whereas PI(3,4)P2 and PI(4,5)P2 notably augmented the association (Fig. 4A). Remarkably, PI(3,4,5)P3 elicited the strongest binding affinity between CD44 CTD and merlin (lane 8), indicating that
phosphatidylinositol 3-kinase (PI3K) might up-regulate the merlin-CD44 association.

Inactivating mutations of PTEN tumor suppressor gene are found in a wide range of human cancers. PTEN is a lipid phosphatase that converts the second messenger PI(3,4,5)P3 to PI(3,4)P2 (12). To further test whether PI(3,4,5)P3 mediates the interaction between CD44 and merlin in intact cells, we used the PTEN knockout HCT116 colon cell line, a previously described isogenic set of HCT116 cells in which PTEN genes had been deleted (13). We cultured the wild-type and PTEN-deleted cells at high density (100% confluence) and low density (50% confluence) and then immunoprecipitated merlin. The CD44-merlin association was substantially enhanced at the low-density culture compared with the high-density culture in wild-type HCT116 cells. Interestingly, significantly more CD44 was coprecipitated with merlin in highly confluent PTEN-null HCT116 cells than in the low-density cells (Fig. 4B, left). Akt phosphorylation was augmented in low-density HCT116 cells compared with high-density cells. As expected, Akt was highly phosphorylated in PTEN-null cells whether the cells were confluent or not. Accordingly, merlin S315 phosphorylation tightly correlated with Akt activation pattern (Fig. 4B, right). These data suggest that Akt phosphorylation and PI(3,4,5)P3 binding might contribute to the redistribution of merlin in the cytoplasm. However, in HCT116 PTEN-null cells, endogenous merlin was distributed in the whole cells, resembling the patterns observed in EGF-treated wild-type cells. It is worth noting that merlin was highly enriched in the microvilli and small protrusions (Fig. 4D, top, white arrows). By contrast, the actin filament was enriched in the cell boundaries. Strikingly, EGF provoked a tremendous number of small protrusions on the plasma membrane, where merlin and F-actin were aggregated and colocalized (Fig. 4D, bottom, white arrows). These results support that PI(3,4,5)P3 binding and Akt phosphorylation synergistically facilitates merlin relocation and regulates its cellular effect on F-actin organization. Immunostaining with anti-phospho-S315 antibody revealed similar results (data not shown).

Akt phosphorylation of merlin inhibits its proapoptotic activity. Transduction of merlin into human schwannoma cells decreases cell proliferation by inducing apoptosis (14). In Drosophila, merlin and expanded, similar to other components of the hippo pathway, are required for proliferation arrest and apoptosis in developing imaginal discs (15). Thus, merlin is somehow implicated in triggering apoptosis. To explore whether...
Figure 4. Akt phosphorylation and phosphatidylinositols enhance merlin binding to CD44. A, in vitro binding assay with GST-CD44 CTD. Glutathione beads–conjugated GST or GST-CD44 CTD were incubated with 2 μg of purified merlin in the presence of various vesicles containing phosphatidyserine, phosphatidyserine + PI, phosphatidyserine + PI(3,4)P₂, phosphatidyserine + PI(4,5)P₂, and phosphatidyserine + PI(3,4,5)P₃, respectively. The beads binding merlin were monitored by immunoblotting. B, Akt phosphorylation in PTEN-null cells up-regulates merlin-CD44 complex formation. C and D, EGF provokes merlin aggregation and colocalization with F-actin in PTEN-deficient HCT116 cells. In PTEN knockout cells, EGF triggered merlin to aggregate in the small protrusion, colocalizing with F-actin. Arrows, merlin accumulating in the dot-like structures (D).
the Akt phosphorylation of merlin mediates its proapoptotic effect, we cotransfected a variety of GFP-tagged merlin constructs into HEK293 cells with constitutively active Akt (Akt-CA) or kinase-dead Akt (Akt-KD) and monitored cell apoptosis. Compared with controls, overexpression of wild-type merlin provoked significant apoptosis, which was further elevated by unphosphorylated mutants (T230AS315A). By contrast, apoptosis was substantially decreased with the mutant (T230DS315D). Cotransfection of active Akt with wild-type merlin markedly diminished its proapoptotic actions. The apoptotic activity was recovered when merlin was cotransfected with Akt-KD (Fig. 5A). We also monitored cell death with trypan blue assay (Fig. 5B). Poly(ADP-ribose) polymerase (PARP) cleavage and caspase-3 activation were in alignment with the quantitative apoptotic results (Fig. 5C), underscoring that merlin phosphorylation by Akt is required to block its proapoptotic action.

The extreme NH2 terminus of merlin is implicated in binding phosphatidylinositol lipids. It remains unknown whether this interaction is implicated in the proapoptotic action of merlin.

Wild-type merlin and phosphorylated/mutated merlin, such as S518A and S518D, display different binding affinities to PI(3,4,5)P3 and two different PIP2. To address whether lipid binding by merlin plays any role in mediating its proapoptotic action, we monitored cell survival activity. When merlin is in open conformation, like S518D, the proapoptotic action of merlin was substantially diminished as in T230DS315D transfected cells; in contrast, when merlin is in close conformation, like S518A, it displayed more potent apoptotic action than wild-type merlin. Interestingly, truncation of the 1–133 region in merlin had no effect on merlin's proapoptotic action (Fig. 5D). PARP cleavage and caspase-3 activation correlated with apoptosis results (data not shown). Hence, PIP2 or PI(3,4,5)P3 binding is not essential for the proapoptotic activity of merlin.

Merlin phosphorylation by Akt affects its subcellular distribution and cell migration. To explore whether Akt phosphorylation alone regulates merlin's subcellular residency, we transfected HEK293 cells with Myc-merlin constructs. T230DS315D mainly accumulated in the small protrusion structures on the

![Figure 5](image_url). Akt phosphorylation of merlin blocks its proapoptotic activity. A and B, quantitative analysis of merlin-provoked apoptosis. The transfected cells were analyzed under fluorescent microscope for apoptosis. Red, apoptotic cells. A, in total, ~500 GFP-positive cells were calculated. B, trypan blue assay. Columns, means calculated from five determinations; bars, SE (#, P < 0.05). C, immunoblotting analysis. PARP was cleaved, and caspase-3 was activated in wild-type merlin and merlin (T230AS315A) cells. Merlin p-S315 was confirmed (bottom). Verification of transfected GFP-merlin constructs (top). D, phosphatidylinositol binding is not required for the proapoptotic activity of merlin. Columns, means; bars, SE (*, P < 0.01, Student’s t test).
ruffled membrane, long cell extension, and cytoplasm in most of the transfected cells. Actin filaments strongly colocalized with merlin T230DS315D in the long cell extension (white arrow). In contrast, merlin mutants (T230AS315A) predominantly resided on the smooth plasma membrane without the aggregation in the protrusion (Fig. 6A). EGFR also provoked Myc-merlin accumulation in the ruffled membrane, which was blocked by wortmannin (Fig. 6B, top), underscoring that Akt phosphorylation of merlin dictates its subcellular distribution. Immunostaining with anti–phosphorylated merlin S315 confirmed the observation (Fig. 6B, middle). To further test the notion that Akt phosphorylation regulates merlin aggregation in the ruffled plasma membrane, we cotransfected GFP-merlin into HEK293 cells with constitutive active Akt-CA and Akt-KD. GFP-merlin was strongly phosphorylated by Akt-CA, but not by Akt-KD, and accumulated on the disheveled plasma membrane (Fig. 6C). We made a similar observation in GFP-merlin and RFP-Akt cotransfected cells (Supplementary Fig. S1). Therefore, Akt phosphorylation plays an essential role in regulating merlin's subcellular distribution.

To explore whether Akt phosphorylation regulates the effect of merlin in cell motility, we conducted a wound-healing assay with RT4 schwannoma cells. The stable clones were transfected with inducible T230DS315D and T230AS315A mutants. Both cell lines transfected with wild-type merlin (5A and 6A) loosely filled the wound. However, T230DS315D cells migrated faster and completely sealed the gap. Nevertheless, T230AS315A cells moved much slower than wild-type controls, and only about half of the gap was filled (Fig. 6D, left). These data support that Akt phosphorylation of merlin plays a critical role in mediating its effect on cell migration, fitting with our previous observation in Matrigel Boyden chamber (9).

Both Akt and PAK are implicated in phosphorylating merlin and regulate its binding to phosphatidylinositol lipids, but it remains unknown which signaling is essential for dictating the effect of merlin in cell migration. To quantitatively analyze this effect, we conducted a time course cell migration assay. In Supplementary Fig. S2, we show that merlin S518D cells migrated much faster than 5A cells. Blocking Akt by Akt1/2 or wortmannin inhibitor largely diminished both wild-type 5A and S518D cell migration; by contrast, inhibiting PAK3 markedly decreased wild-type, but not S518D, cell migration. This finding suggests that PI3K/Akt signaling is critical for schwannoma cell migration, for which merlin phosphorylation by Akt might be critical. On the other hand, S518 phosphorylation by PAK is also implicated in this event. Compared with merlin lacking V1 schwannoma control cells, induction of wild-type merlin markedly decreased cell migration in 5A cells in a time-dependent manner. Notably, induction of S518A further inhibited cell migration. As expected, phosphorylation of S518 significantly abolished the inhibitory effect of merlin in cell migration, as the migratory speed was substantially increased in S518D cells (Fig. 6D, right).

Discussion

In this report, we show that merlin directly binds phosphatidylinositol lipids, and this action is mediated by merlin folding conformation and Akt phosphorylation. Wild-type merlin reveals a stronger affinity to PI(4,5)P2 than PI(3,4,5)P3, whereas the patient-derived L64P, which possesses an unfolded conformation, exhibits a more potent affinity to PI(3,4,5)P3 than PI(4,5)P2. This observation was further supported by the finding with S518D, which also displays an open structure. By contrast, the folded S518A mutant fails to bind any of the phosphatidylinositol lipids, underscoring that the open inactive conformation is required for merlin to bind PI(3,4,5)P3. Moreover, we found that the interaction between merlin and PI(3,4,5)P3 was evidently enhanced by Akt phosphorylation. Nevertheless, Akt phosphorylation is dispensable for merlin to associate with PI(4,5)P2. Although binding to phosphatidylinositol lipids require merlin to maintain an inactive status, the functional relevance of this interaction remains obscure. Truncation of the 1–133 region in the NH2 terminus, which is implicated in binding to PI(3,4,5)P3, does not alter the proapoptotic action of merlin (Fig. 5D), suggesting that phosphatidylinositol binding to merlin is dispensable for this effect.

Previously, it has been reported that hypophosphorylated merlin, but not ezrin or moesin, binds the cytoplasmic tail of CD44 at high cell densities. At low cell densities, ezrin, moesin, and the phosphorylated form of merlin are associated with CD44, and this CD44-merlin association is likely to be indirect through ERM proteins (16). Consistently, we observed the increased interaction between CD44 and merlin in low-density HCT116 cells compared with high-density cells. Strikingly, the strongest CD44-merlin association occurred in high-density PTEN-null cells, which was slightly decreased when cell density was reduced (Fig. 5B). It remains unclear why, in PTEN-null cells, the association between CD44 and merlin was attenuated in low density compared with high density. Although the PI3K/Akt signaling cascade is markedly increased, when PTEN is depleted, other cell signalings that are dictated by cell/cell contact remain intact in PTEN-null cells. For instance, contact inhibition can be triggered by the addition of cell membrane preparations to dividing cells in vitro (17, 18). Adhesion molecules, including particular cadherins and integrins, induce cell cycle arrest upon contact with specific components of the extracellular matrix or with neighboring cells (19, 20). Presumably, these mechanisms operate in concert or in hierarchy to mediate cellular responses to contact with extracellular matrix and with other cells in addition to PI3K/Akt. This notion is indirectly supported by the cell migration observation that PI3K inhibitor wortmannin and Akt inhibitor Akt1/2 slightly but significantly decreased HCT116-PTEN cell migration; by contrast, PAK inhibitor exhibited a negligible effect (Supplementary Table S1). The weak inhibitory effect by Akt inhibitors might be due to the hyperactivity of Akt in PTEN-null cells.

To address the functional consequences of the enhanced binding affinity by Akt-phosphorylated merlin to CD44, we knocked down CD44 with its small interfering RNA in T230DS315D stably transfected cells, but we failed to observe any significant effect on cell migration compared with control small interfering RNA (data not shown). This finding indicates that the increased interaction between CD44 and Akt-phosphorylated merlin is not implicated in cell migration. This result is consistent with the previous finding that CD44 is not required for hyaluronan-induced vascular smooth muscle cell migration, which is dependent on PI3K-mediated Rac activation (21).

PI(4,5)P2 strongly binds full-length wild-type merlin from schwannoma 5A cells (Fig. 3), although it fails to interact with the purified recombinant full-length merlin (Fig. 1A). This discrepancy might result from posttranslational modification of merlin in 5A cells. For example, phosphorylation of merlin might alter its folding conformation, unveiling the binding motif for PI(4,5)P2 lipid. Unlike other ERM proteins, both the NTD and CTD of merlin bind to PI(4,5)P2, however, PI(3,4,5)P3 selectively associates with the NTD.
of merlin. Under subconfluent conditions, a portion of merlin might be phosphorylated by PAK on S518 in the CTD, leading to an unfolded conformation of merlin. PI(4,5)P2 binds merlin and docks it to the plasma membrane. When growth factors are introduced, Akt is immediately activated and translocated to the plasma membrane, wherein its PH domain binds to newly generated PI(3,4,5)P3 by PI3K, and the active Akt then attacks the NTD of merlin and phosphorylates it, resulting in a fully opened conformation. Figure 6.

Merlin phosphorylation by Akt regulates its subcellular distribution and cell migration. A, Akt phosphorylation of mimetic mutant T230DS315D aggregated in the ruffled plasma membrane and relocated in the cytoplasm. HEK293 cells were transfected with T230AS315A and T230DS315D constructs, respectively. The transfected cells were stained with rhodamine-phalloidin. T230DS315D colocalized with F-actin in long cell extension (arrow). It was also distributed in the cytoplasm. B, EGF treatment provoked S315-phosphorylated merlin to accumulate on the disheveled plasma membrane. C, Akt-CA, but not Akt-KD, promoted merlin S315 phosphorylation and aggregation on the plasma membrane. D, wound-healing assay. RT-4 schwannoma cells stably transfected with inducible merlin wild-type (S4 or S6), T230AS315A, or T230DS315D mutants were split in a 10-cm dish. Three independent clones for each mutant were used for the migration assay. One representative photo from three clones was presented (left). Quantitative analysis of the effect of merlin S518 phosphorylation in cell migration. Induction of wild-type merlin in S4 cells elicited a time-dependent inhibitory effect in cell migration (right). Columns, mean; bars, SE (*, P ≤ 0.01; #, P < 0.05, Student’s t test).
conformation. PI(3,4,5)P$_3$ subsequently tightly binds to the NTD, which in turn, further enhances its phosphorylation by Akt and many other kinases, including PKA, PAK, etc., leading to the full inactivation of merlin.

Merlin directly associates with PIKE-L GTPase that binds PI3K and enhances its kinase activity (22, 23). Merlin exerts its growth-inhibitory effect, at least in part, by inhibiting Akt/Akt signaling. This suppressive activity was achieved by blocking the stimulatory effect of PIKE-L on PI3K (23). Recently, we showed that Akt feeds back and robustly phosphorylates merlin on both T230 and S315 residues and unfolds merlin, leading to its polyubiquitination and degradation. Although either Akt or PAK phosphorylating merlin results in its binding to phosphatidylinositol lipids, merlin S518 phosphorylation by PAK cannot provoke merlin polyubiquitination and degradation (9). This finding suggests the unique aspect of the regulatory role of Akt in mediating merlin tumor suppressor. Nonetheless, it remains unclear whether phosphatidylinositol binding plays any role in triggering merlin degradation or plasma membrane residency. Here, we provide biochemical evidence revealing that PI(3,4,5)P$_3$, an essential second messenger generated by active PI3K, strongly binds Akt-phosphorylated merlin. Conceivably, this phosphatidylinositol lipid binding action might coordinately inactivate merlin with Akt. The dual regulation of merlin by phosphatidylinositol binding and Akt phosphorylation provides a novel mechanism explaining how the oncogenic PI3K signaling crosstalks with the tumor suppressor merlin.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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