The NF2 Tumor Suppressor Merlin and the ERM Proteins Interact with N-WASP and Regulate Its Actin Polymerization Function

Nitasha Manchanda§§, Anna Lyubimova§§, Hsin-Yi Henry Ho¶, Marianne F. James‡, James F. Gusella‡‡, Narayanawamy Ramesh*, Scott B. Snapper†††, and Vijaya Ramesh§§§

From the §Molecular Neurogenetics Unit, Center for Human Genetic Research, Massachusetts General Hospital, Charlestown, Massachusetts 02129, the ‡Department of Genetics, ¶Department of Cell Biology, and †††Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, the ‡‡Gastrointestinal Unit (Medical Services) and the Center for Inflammatory Bowel Disease, Massachusetts General Hospital, Boston, Massachusetts 02114, and the *Division of Immunology, Children’s Hospital, Boston, Massachusetts 02115

Received for publication, December 15, 2004, and in revised form, February 3, 2005. Published, JBC Papers in Press, February 7, 2005, DOI 10.1074/jbc.C400583200

The function of the NF2 tumor suppressor merlin has remained elusive despite increasing evidence for its role in actin cytoskeleton reorganization. The closely related ERM proteins (ezrin, radixin, and moesin) act as linkers between the cell membrane and cytoskeleton, and have also been implicated as active actin reorganizers. We report here that merlin and the ERM proteins can interact with and regulate N-WASP, a critical regulator of actin dynamics. Merlin and moesin were found to inhibit N-WASP-mediated actin assembly in vitro, a function that appears independent of their ability to bind actin. Furthermore, exogenous expression of a constitutively active ERM inhibits N-WASP-dependent Shigella tail formation, suggesting that the ERM proteins may function as inhibitors of N-WASP function in vivo. This novel function of merlin and the ERM proteins illustrates a mechanism by which these proteins directly exert their effects on actin reorganization and also provides new insight into N-WASP regulation.

Ezrin, radixin, and moesin, known as ERM1 proteins, act as membrane-cytoskeletal linkers that play important roles in cell morphology, polarity, and signal transduction (1). ERM proteins bind membrane proteins through their N-terminal FERM domain and actin filaments through their C terminus, thus linking the cell membrane to the underlying cytoskeleton. Merlin, the protein product of the Neurofibromatosis type 2 (NF2) tumor suppressor gene, is closely related to the ERM proteins (2, 3). The FERM domain of merlin and ERM proteins is the region of highest homology and can interact with various membrane proteins. The C terminus of ERM proteins contains a high affinity actin-binding site that is not conserved in merlin. However, merlin also directly binds F-actin through its FERM domain and stabilizes actin filaments in vitro (4, 5). ERMs and merlin are regulated by an intramolecular head-to-tail association between the FERM domain and the C terminus that prevents binding of membrane partners as well as F-actin (6). The autoinhibited conformation of the ERM proteins can be disrupted by polyphosphatidylinositol 4,5-bisphosphate (PIP2) binding or phosphorylation at a conserved C-terminal threonine.

The role of ERM proteins and merlin in actin cytoskeleton reorganization has been well documented. In cultured cells, they localize to actin-rich regions such as microvilli, lamellipodia, filopodia, and neuronal growth cones. Antisense suppression of ERM proteins can disrupt microvilli and slow growth cone advancement (7, 8). The absence of merlin, either in schwannoma cells from NF2 patients, or mouse embryo fibroblasts from NF2-deficient mice, results in increased membrane ruffling and cell motility (9, 10). Thus merlin may negatively regulate actin remodeling in vivo. These cellular phenotypes caused by merlin or ERM loss can be partially explained by their structural role as membrane-cytoskeleton linkers. In addition, merlin and ERM proteins are regulated by Rho family GTPases via phosphorylation and also alter GTPase function through a negative feedback mechanism, thereby regulating the actin cytoskeleton indirectly (9, 11). However, it remains unknown whether these proteins can directly alter actin nucleation and assembly downstream of Rho, Rac, or Cdc42.

We examined whether merlin and ERM proteins directly regulate the actin polymerization pathway downstream of these GTPases. The WASP (Wiskott-Aldrich syndrome protein) family proteins, upon activation by Rac and Cdc42, are capable of activating actin assembly by the actin nucleating Arp2/3 complex (12). Cdc42 binds and activates N-WASP, a ubiquitously expressed member of the WASP family, which in turn stimulates Arp2/3 complex activity. WIP (WASP interacting protein) interacts with the WH1 domain of N-WASP and inhibits its function in vitro and in vivo (13, 14). We report here that merlin and ERM proteins are able to directly bind N-WASP. Furthermore, merlin and ERM proteins regulate N-WASP function in vitro and in vivo, demonstrating a novel, direct mechanism for their effect on actin reorganization and a new mode of N-WASP regulation.

This paper is available on line at http://www.jbc.org

12517
**Experimental Procedures**

Materials and Antibodies—HA-radixin full-length (FL) and N-term and C-term plasmids were a gift from F. Solomon (MIT, Cambridge, MA). Mutants FL HA-radixin (T564E and T564A) were provided by S. Tsukita (Kyoto University, Kyoto, Japan). Myc-tagged N-WASP constructs were described previously (15). Mini-N-WASP was provided by W. Lim (University of California, San Francisco, CA). The anti-WAVE, anti-N-WASP, and anti-WIP antibodies have been described (13, 16, 17). Anti-NHE-RF IC270 and anti-merlin antibodies 1C4 and C26H were described previously (18, 19). Anti-Nck and anti-Myc 9E10 antibodies were purchased from BD Transduction Laboratories and Developmental Studies Hybridoma Bank, respectively. Phospho-ERM antibody anti-ERM was provided by M. Mayne (University of Colorado, Denver, CO) and supplemental Fig. S1). WAVE, another FERM protein that regulates N-WASP function through its proline-rich domain (20, 21), did not precipitate along with N-WASP (Fig. 1B). WH1 and WGP (WH1, G protein-binding region, proline-rich domain) polypeptides were capable of binding, whereas the VCA (verprolin homology, cofilin homology, acidic) was not, indicating that the WH1 domain is sufficient. This is a region conserved between N-WASP and WASP, but not WAVE/Scar proteins, which supports our finding that WAVE does not interact with merlin or moesin.

To determine whether the FERM-N-WASP interaction is direct, we performed a solution binding assay using purified GST-moesin and purified, untagged N-WASP, followed by precipitation of GST fusion proteins. Both moesin FERM domain and FL moesin showed direct binding as compared with C-term alone (Fig. 1C) and FERM moesin bound FL N-WASP in a dose-dependent manner (Fig. 1D).

To examine the interaction between merlin and N-WASP in vivo, we performed co-immunoprecipitation using 293T cell lysates. Anti-merlin antibody specifically precipitated endogenous N-WASP along with endogenous merlin and conversely, the N-WASP antibody precipitated both merlin and N-WASP (Fig. 1E). Although moesin and merlin were both capable of interacting with N-WASP in pull-down assays, we were unable to detect any ERMs in N-WASP immunoprecipitates, suggesting that their tightly closed conformation in vivo renders them inaccessible. Phosphorylation of a conserved C-terminal threonine has been shown to stabilize the open conformation of ERMs (22, 23). To enrich phosphorylated ERMs, we treated HeLa cells with a Ser/Thr phosphatase inhibitor (calyculinA) and used an ERM antibody that recognizes the conserved C-terminal phospho-Thr. Using the N-WASP antibody for immunoprecipitation, we detected a significant association between the phosphorylated, active ERMs and N-WASP in vivo (Fig. 1F). We also performed co-immunoprecipitation experiments using FLAG-merlin FERM domain and Myc-N-WASP WH1 domain, which confirmed that these regions were sufficient to mediate the interaction between merlin and N-WASP.
The ERMs are more similar to each other within the FERM domain (87% identity) than to merlin (63%). Given this high degree of similarity, and the fact that two ERMs and merlin can co-immunoprecipitate with N-WASP, it is likely that all three ERMs interact similarly with N-WASP. Therefore, we assume that any functional consequence of FERM domain-N-WASP binding will apply to ezrin, radixin, and moesin.

To assess the relevance of the interaction between merlin/ERMs and N-WASP, we examined the effect of these proteins on N-WASP-induced Arp2/3 complex-mediated actin polymerization in vitro. The kinetics of actin polymerization can be monitored using pyrene-labeled actin, which displays an increase in fluorescence intensity when incorporated into filaments. Full-length N-WASP, being auto-inhibited in vitro, requires binding of activators such as Cdc42 or PI(4,5)P2 (16). We examined the effect of merlin/ERMs on full-length N-WASP activated by Cdc42 and found that merlin and moesin proteins that contained the FERM domain inhibited the rate of actin assembly. The merlin FERM domain caused a dose-dependent decrease in the rate of actin polymerization when added to actin, Arp2/3 complex, N-WASP, and Cdc42 (Fig. 2A). FERM domain and FL moesin also functioned similarly in this assay (Fig. 2B) as did FL merlin (not depicted). Despite FERM domain binding N-WASP more robustly than FL proteins in the pull-down assays, their effect appears similar in kinetics, probably due to the higher sensitivity of the actin polymerization assay. Thus, all proteins that demonstrated binding to N-WASP in the pull-down assay were able to inhibit N-WASP-mediated actin polymerization. The C term...
mini of moesin (Fig. 2C) and merlin (not depicted), which cannot bind N-WASP, had no effect, confirming the specificity of this result. The C terminus of the ERMs contains the F-actin-binding site. The fact that we observed no effect of this region in our assay indicates that F-actin binding does not contribute to the inhibition we observed, and indeed it is the FERM domain binding to N-WASP that mediates this function.

To determine whether interaction with the WH1 domain mediated this inhibition, we utilized a mini-N-WASP protein. This protein lacks the WH1 and proline-rich domains but is still capable of auto-inhibitory binding of the GBD to the VCA, which is relieved by Cdc42, enabling Arp2/3 complex activation (24). Unlike full-length N-WASP which was inhibited strongly (Fig. 2, A and B), mini-N-WASP was unaffected by the same concentration of FERM merlin and FL moesin (Fig. 2D). We also tested the ability of merlin and moesin to inhibit VCA domain function and found none (not depicted), confirming that N-WASP polypeptides that are able to activate Arp2/3 complex but lack the WH1 domain are not inhibited. This also demonstrates that merlin and moesin have no effect on other components including Cdc42, the Arp2/3 complex, and actin itself in this assay. Interestingly, WIP and its related proteins (CR16 and WICH) also bind the WH1 domain and inhibit N-WASP mediated actin polymerization in a similar assay (13, 25, 26). In summary, merlin and moesin inhibit N-WASP function in actin assembly in vitro through binding of their FERM domains to the WH1 domain of N-WASP. These results provide the first example of proteins unrelated to WIP binding the WH1 domain and suppressing N-WASP activity.

The enteroinvasive pathogen S. flexneri uses host cell actin polymerization machinery to propel itself within and between cells. The Shigella surface protein IcsA recruits host cell N-WASP to activate the Arp2/3 complex and form an actin comet tail (27). This process is completely dependent on the presence of N-WASP as N-WASP-deficient cells permit Shigella entry but not subsequent tail formation and motility (28, 29) providing a reliable assay to study the effect of ERMs on N-WASP function in vivo. Ezrin, a member of the ERM family, is required for Shigella entry into HeLa cells. Overexpressing its FERM domain, which exerts a dominant negative effect on ezrin function, causes a decrease in Shigella entry (30).

We evaluated the in vivo relevance of the ERM-N-WASP interaction by examining the effect of exogenous ERMs on actin tail formation. ERMs were chosen because merlin and moesin FERM domains behaved similarly in binding and inhibiting N-WASP in vitro. Moreover, the physical separation of N-WASP and actin-binding domains in ERMs, unlike merlin, ensured that any effect of direct actin binding on tail formation would be evident. Radixin was chosen as we had access to several expression constructs and because we observed no difference between the various FERM domains with respect to N-WASP binding and regulation (Figs. 1 and 2). We used HA-tagged FL, N-term, C-term, and active and inactive mutants of radixin. Constitutively active radixin T564E is a phosphomimetic mutant that renders the protein open, whereas the inactive T564A mutant cannot be phosphorylated and is closed.
After transfection of radixin constructs into fibroblasts and infection with *Shigella*, cells were fixed and processed for immunofluorescence. Due to the marked inhibition of *Shigella* entry caused by FERM domain overexpression (30), which we also confirmed in our experiments, we were unable to evaluate whether this construct inhibited tail formation. All other radixin constructs did not significantly alter *Shigella* entry and were evaluated for their effect on tail formation. We found that constitutively active radixin T564E inhibited actin tail formation by 60% (Fig. 3A). Constitutively inactive T564A had no such inhibitory effect (Fig. 3B). FL, wild-type radixin, which is probably in the closed conformation *in vivo*, did not significantly inhibit tail formation (Fig. 3C). Our co-immunoprecipitation results indicated that only phospho-ERMs bind N-WASP (Fig. 1F), suggesting that the phosphomimetic ERM is the only transfected construct able to bind N-WASP and thereby inhibit tail formation. The free radixin C-term, which binds actin had no effect despite co-localizing with F-actin in the tails (supplemental Fig. S2), supporting our conclusion that the inhibition is probably mediated through N-WASP binding.

To confirm that the active mutant inhibited tail formation by inhibiting N-WASP, we co-transfected it with N-WASP and observed a large increase in tail formation compared with mutant T564E alone (Fig. 3C). Exogenous N-WASP alone has been shown to have no significant effect on *Shigella* tail formation (31). Thus the inhibition we observe is likely a consequence of ERM inhibition of N-WASP and not an indirect effect of overexpressing active radixin. Rho, Rac, and Cdc42 activity is dispensable for tail formation (32, 33), supporting the conclusion that active ERM is not functioning by altering the activity of these GTPases. Together, these results suggest that constitutively active radixin inhibits N-WASP dependent *Shigella* tail formation through their interaction *in vivo*.

These results confirm the observations made using the actin assembly assays. The *in vitro* assays facilitated our ability to assess free FERM domain function because the dominant negative effect seen *in vivo* was eliminated. Interestingly, FL ERM was able to inhibit N-WASP *in vitro* (Fig. 2B), whereas FL wild-type ERM did not have a significant effect *in vivo*. This apparent contradiction can be explained by the fact that GST fusion ERMs can be less tightly closed than ERMs *in vivo*. Therefore, we observe GST-FL ERM binding and inhibiting N-WASP *in vitro*, whereas *in vivo*, there is no detectable effect on tail formation due to the inability of N-WASP to bind FL ERMs unless they are phosphorylated. We were able to address
this problem by using the phosphomimetic, active FL ERM, which was found to inhibit tail formation. Use of this T564E mutant also enabled us to assess the effect of the FERM domain on tail formation because, unlike the free FERM domain, this construct does not inhibit bacterial entry.

N-WASP recruitment to the surface of Shigella is mediated through the interaction of its WH1 and GBD domains with IcsA (29). Since the FERM domains of merlin/ERMs bind the WH1 domain of N-WASP, we examined whether active radixin disrupted the recruitment of N-WASP, thereby inhibiting tail formation. However, N-WASP was still able to localize correctly to the pole of bacteria in cells transfected with active mutant. This does not, therefore, appear to be the mechanism of ERM inhibition. It is possible that FERM domain binding causes a conformational change within N-WASP and/or interferes with binding of specific activators.

N-WASP activity was previously thought to be regulated by the auto-inhibitory interactions between the GBD and VCA domains. It has recently emerged, however, that WIP-WH1 binding of specific activators. This does not, therefore, appear to be the mechanism of ERM inhibition. Most mutations in Wiskott-Aldrich syndrome patients map to the WH1 domain of WASP, suggesting that loss of the inhibition imposed by this domain may be the mechanism of disease initiation.

Previously, the effect of merlin and ERMs on the actin cytoskeleton was attributed to either binding to actin or to an indirect effect on Rho GTPase signaling. Here, we report that direct binding between the FERM domain of merlin/ERMs and N-WASP can regulate actin assembly independent of their actin binding and GTPase regulation functions. Thus our finding that merlin and ERM proteins can directly regulate N-WASP provides a novel mechanism by which these proteins affect actin reorganization and also represents an alternate means of N-WASP regulation.

Acknowledgment—We thank Roberta Beauchamp for critical reading of the manuscript.

REFERENCES

1. Bretscher, A., Edwards, K., and Feehon, R. G. (2002) Nat. Rev. Mol. Cell. Biol. 3, 586–599
2. Trofatter, J. A., MacCollin, M. M., Rutter, J. L., Murrell, J. R., Dayno, M. P., Parry, D. M., Eldridge, R., Kley, N., Menon, A. G., Pulaski, K., Haase, V. H., Ambrose, C. M., Munroe, D., Rove, C., Haines, J. L., Martuza, R. L., MacDonald, M. E., Seizinger, B. R., Short, M. P., Buckler, A. J., and Guggenheim, A. J. (1999) Cell 72, 791–800
3. Ramesh, V. (2004) Nat. Rev. Neurosci. 5, 462–470
4. Brault, E., Gautreau, A., Lamarine, M., Callebaut, I., Thomas, G., and Gout, P. (2003) J. Cell Sci. 116, 1901–1912
5. James, M. F., Manchaiah, N., González-Agosti, C., Hartwig, J. H., and Ramesh, V. (2001) Biochem. J. 356, 377–386
6. Hirao, M., Sato, N., Kondo, T., Yonemura, S., Mondon, M., Sasaki, T., Takai, Y., Tsukita, S., and Tsukita, S. (1996) J. Cell Biol. 135, 37–51
7. Takeuchi, K., Sato, N., Kasahara, H., Funayama, N., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S. (1994) J. Cell Biol. 125, 1371–1384
8. Paglino, G., Kunda, P., Quiroga, S., Kosik, K., and Caceres, A. (1998) J. Cell Biol. 143, 445–455
9. Shaw, R. J., Paez, J. G., Curto, M., Morris, P., Sato, T., Yoneyama, M., Sato, Y., and Fehon, G. R. (1998) Cell 90, 26448–26452
10. Bashour, A. M., Menez, J. M., Ip, W., MacCollin, M., and Ratner, N. (2002) Mol. Cell. Biol. 22, 1150–1157
11. Speck, O., Hughes, S. C., Noren, N. K., Kulikauskas, R. M., and Fehon, R. G. (2002) Nature 418, 799–808
12. Millard, T. H., Sharp, S. J., and Machovsky, L. (2004) Biochem. J. 380, 1–17
13. Martinez-Quiles, N., Rohatgi, R., Anton, I. M., Medina, M., Saville, S. P., Miki, H., Yamaguchi, H., Takenawa, T., Hartwig, J. H., Geha, R. S., and Ramesh, N. (2001) Nat. Cell Biol. 3, 484–491
14. Ho, H. Y., Rohatgi, R., Lebedezkin, A. M., Le, M., Li, J., Gygi, S. P., and Kirschner, M. W. (2004) Cell 118, 203–216
15. Rohatgi, R., Ho, H. Y., and Kirschner, M. W. (2000) J. Cell Biol. 150, 1299–1310
16. Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M. W. (1999) Cell 97, 221–231
17. Eden, S., Rohatgi, R., Poutillechkov, A. V., Mann, M., and Kirschner, M. W. (2002) Nature 418, 799–808
18. Gonzalez-Agosti, C., Wiederhold, T., Herndon, M. E., Gussela, J., and Ramesh, V. (1999) J. Biol. Chem. 274, 34438–34442
19. Wiedherhold, T., Lee, M. F., James, M., Neubauer, R., Smith, N., Murthy, A., Hartwig, J., Gussela, J. P., and Ramesh, V. (2004) Oncogene 23, 8815–8825
20. Rohatgi, R., Nollau, P., Ho, H. Y., Kirschner, M. W., and Mayer, B. J. (2001) J. Biol. Chem. 276, 26448–26452
21. Fischknecht, F., Moreau, V., Rottger, S., von Gröll, S., Reckmann, I., Soper, F., and Way, M. (1999) Nature 401, 926–929
22. Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaikuchi, K., Tsukita, S., and Tsukita, S. (1998) J. Cell Biol. 140, 647–657
23. Fievet, B. T., Gautreau, A., Roy, C., Del Maestro, L., Mangeat, P., Louvard, D., and Arpin, M. (2004) J. Cell Biol. 164, 653–659
24. Prehoda, K. E., Scott, J. A., Mullins, R. D., and Lim, W. A. (2000) Science 290, 801–806
25. Kato, M., Miki, H., Kurita, S., Endo, T., Nakagawa, H., Miyamoto, S., and Takenawa, T. (2002) Biochem. Biophys. Res. Commun. 291, 41–47
26. Ho, H. Y., Rohatgi, R., Ma, L., and Kirschner, M. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11306–11311
27. Egile, C., Loisel, T. P., Laurent, V., Li, R., Pantaloni, D., Carlier, M. F., and Sansonetti, P. J. (1999) Dev. Cell. 1, 1319–1332
28. Snapper, S. B., Takeshima, F., Anton, I., Liu, C. H., Thomas, S. M., Nguyen, D., Dudley, D., Fraser, H., Purich, D., Lopez-Ilasaca, M., Klein, C., Davidson, L., Bronson, R., Mulligan, R. C., Southwick, F., Geha, R., Goldberg, M. B., Rosen, F. S., Hartwig, J. H., and Alt, F. W. (2001) Nat. Cell Biol. 3, 897–904
29. Lommel, S., Benesch, S., Rettner, K., Franz, T., Wehland, J., and Kuhn, R. (2001) EMBO Rep. 2, 850–857
30. Skoudy, A., Nhieu, G. T., Manzi, N., Arpin, M., Mounier, J., Geonon, P., and Sansonetti, P. J. (1999) J. Cell Biol. 122, 2059–2068
31. Moreau, V., Frischknecht, F., Beckman, I., Vincentelli, R., Rabut, G., Stewart, D., and Way, M. (2000) Nat. Cell Biol. 2, 441–448
32. Mounier, J., Laurent, V., Hall, A., Fort, P., Carlier, M. F., Sansonetti, P. J., and Egile, C. (1999) J. Cell Biol. 142, 2069–2080
33. Shihata, T., Takeda, F., Chen, F., Alt, F. W., and Snapper, S. B. (2002) Curr. Biol. 12, 341–345