Communication

NHE-RF, a Regulatory Cofactor for Na\(^+\)-H\(^+\) Exchange, Is a Common Interactor for Merlin and ERM (MERM) Proteins*  

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We have identified the human homologue of a regulatory cofactor of Na\(^+\)-H\(^+\) exchanger (NHE-RF) as a novel interactor for merlin, the neurofibromatosis 2 tumor suppressor protein. NHE-RF mediates protein kinase A regulation of Na\(^+\)-H\(^+\) exchanger NHE3 to which it is thought to bind via one of its two PDZ domains. The carboxyl-terminal region of NHE-RF, downstream of the PDZ domains, interacts with the amino-terminal protein 4.1 domain-containing segment of merlin in yeast two-hybrid assays. This interaction also occurs in affinity binding assays with full-length NHE-RF expressed in COS-7 cells. NHE-RF binds to the related ERM proteins, moesin and radixin. We have localized human NHE-RF to actin-rich structures such as membrane ruffles, microvilli, and filopodia in HeLa and COS-7 cells, where it co-localizes with merlin and moesin. These findings suggest that hNHE-RF and its binding partners may participate in a larger complex (one component of which might be a Na\(^+\)-H\(^+\) exchanger) that could be crucial for the actin filament assembly activated by the ERM proteins and for the tumor suppressor function of merlin.

Neurofibromatosis 2 (NF2),¹ is a dominantly inherited disorder characterized by bilateral occurrence of vestibular schwannomas and other brain tumors, especially meningiomas, and schwannomas of other cranial nerves and spinal nerve roots (1). The NF2 gene isolated by positional cloning encodes merlin, named for its striking similarity with moesin, ezrin, and radixin, three closely related proteins commonly referred to as the ERM family, a subclass of the protein 4.1 superfamily thought to link cytoskeletal components with proteins in the cell membrane (2, 3). The ERM proteins share ~78% amino acid identity with each other, and all three are ~45–47% identical to merlin (4).

In cultured cells, ERM proteins are highly concentrated in regions of contact between actin filaments and the plasma membrane, acting as possible linkers between integral membrane and cytoskeletal proteins (5–9). The carboxyl termini of both ezrin and moesin bind directly to actin in vitro (10, 11) via a conserved actin binding site present in ezrin, moesin, and radixin but not in merlin. These findings suggest that the carboxyl terminus of the ERM proteins is responsible for their association with the actin-based cytoskeleton. Recently, however, another actin binding site in the amino-terminal domain of ezrin has been characterized in vitro and shown to be conserved in moesin, radixin, and merlin (12). The highly conserved amino-terminal half of the ERM proteins also contains the domain responsible for interaction with membrane proteins, particularly the glycoprotein CD44 (13). ERM-CD44 complexes are also associated with RhoGDI (RhoGDP dissociation inhibitor) (14), and the ERM proteins have been directly implicated in Rho- and Rac-dependent cytoskeletal reorganization in permeabilized cells (15).

We have reported that endogenous merlin localizes to the actin-rich motile regions (i.e. leading and ruffling edges) in human fibroblast and meningioma cells where it co-localizes with actin but is not associated with stress fibers (16). Merlin when overexpressed in cells, however, localizes to membrane ruffles as well as to other actin-rich structures such as microvilli and filopodia thus resembling the ERM proteins (17). Given the similarity between merlin and the ERM proteins, it is likely that merlin’s normal function also involves interactions with membrane and cytoskeletal components. We have used the yeast two-hybrid interaction trap strategy to search for proteins that may associate with merlin. Here, we describe a human cDNA (merintc) that interacts not only with merlin, but also with the ERM proteins via their conserved amino-terminal domain. This cDNA encoded the carboxyl terminus of the human homologue of NHE-RF, which was originally identified as a cofactor that mediates protein kinase A inhibition of a renal brush-border membrane Na\(^+\)-H\(^+\) exchanger in rabbit (18). Exogenously expressed human NHE-RF also co-localizes with merlin and moesin. Our findings suggest that NHE-RF is a biologically significant interactor for the MERM (merlin and ERM) family of proteins and may be a participant in the activation of the Na\(^+\)-H\(^+\) exchange required for actin cytoskeleton reorganization. During the preparation of this manuscript a report appeared showing that NHE-RF binds to ezrin (19).

EXPERIMENTAL PROCEDURES

Molecular Cloning of Merintc Using the Yeast Two-hybrid System—DNA encoding the merlin sequence (as S–595, isoform 1) was polymerase chain reaction-amplified and cloned into the yeast LexA DNA binding vector pEG202 (20), and the plasmid was designated as pmerbait. To identify proteins interacting with merlin isoform 1, yeast strain EGY88 was sequentially transformed with the pmerbait and a human fetal frontal cortex interaction library fused to the activation domain of GAL4 in the plasmid pG4-5 and selected on ura–, his–, trp–, leu– plates. A partial merintc clone was obtained (see “Results”) and subsequently used as a probe to screen a human fetal brain library in Zap-Stratagene) by standard techniques to obtain a full-length clone. These clones were sequenced, and sequences were analyzed using software.

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been described previously (24). The hybridoma supernatant for the purified. The anti-merlin antibodies and the antibodies for moesin have been affinity-purified (NP1) by Research Genetics, Inc., Huntsville, AL. The antiserum obtained (NP1) was further affinity-purified against the carboxyl-terminal amino acids SLAMAKERAHQKR (aa 290–358) specific to human NHE-RF by Research Genetics, Inc., and using the standard method of Smith and Johnson (23) for moesin. Similarly full-length (aa 1–577), amino (aa 1–332), and carboxyl (aa 307–577) segments of human moesin were expressed as GST fusion proteins using the vector pGEX4T1. Expression and purification of the GST fusion proteins were performed as described previously for merlin (16) and using the standard method of Smith and Johnson (23) for moesin. For radixin, His6-tagged constructs were employed, and these have previously (24).

Protein Fusion Constructs—Full-length (aa 1–595) as well as the amino (aa 1–332) and carboxyl (aa 307–559) portions of merlin were expressed as GST fusion proteins using the vector pGEX4T1 (16). Another full-length merlin construct encoding a naturally occurring NF2 missense mutation, Asn220 → Tyr (22) was generated by site-directed mutagenesis (Stratagene) and expressed as a GST fusion protein. Similarly full-length (aa 1–577), amino (aa 1–332), and carboxyl (aa 307–577) segments of human moesin were expressed as GST fusion proteins using the vector pGEX4T1. Expression and purification of the GST fusion proteins were performed as described previously for merlin (16) and using the standard method of Smith and Johnson (23) for moesin. For radixin, His6-tagged constructs were employed, and these have been described previously (24).

Mammalian Expression—The entire coding sequence of hNHE-RF (1–358) engineered to have the influenza hemagglutinin (HA) epitope tag at the 5’ end was cloned into the mammalian expression vector pcDNA3. The entire coding sequence of merlin was expressed as a GFP (green fluorescent protein) fusion protein employing the vector pEGFP-N1 (CLONTECH). These expression constructs were transiently transfected in COS-7 cells and HeLa cells by the calcium phosphate method using the Cell Phect kit (Pharmacia Biotech Inc.) employing 10–20 μg of the plasmid DNA. Transient transfections were performed, and cells were harvested after 72 h.

Antibodies—An antipeptide rabbit polyclonal antibody was raised against the carboxyl-terminal amino acid codons SLAMKERAHQKR (aa 328–340) specific to human NHE-RF by Research Genetics, Inc., Huntsville, AL. The antiserum obtained (NP1) was further affinity-purified. The anti-merlin antibodies and the antibodies for moesin have been described previously (16, 25). The hybridoma supernatant between these sequences are boxed. The hNHE-RF sequence has been deposited in GenBank™, and the accession number is A5036241.

Affinity Precipitation of hNHE-RF from Cell Lysates—COS-7 cell lysates overexpressing hNHE-RF were incubated with 300 pmol of GST-merlin or GST-moesin immobilized on glutathione-Sepharose 4B (GSH) beads. The beads were washed with phosphate-buffered saline containing Pefabloc, resuspended in Laemmli loading buffer, subjected to 10% SDS-PAGE, and immunoblotted with anti-hNHE-RF (affinity-purified NP1). For radixin, COS-7 cell lysates overexpressing the interactome were incubated with 600 pmol of the His6-tagged full-length, amino-terminal, or carboxyl-terminal radixin polypeptides. The complexes were separated from the reaction mixture using Ni-NTA beads. The beads were washed (20 mCi imidazole, 50 mCi sodium phosphate, 300 mCi NaCl, pH 8.0), and the specifically bound complexes were eluted with the same buffer containing 400 mM imidazole separated on SDS-PAGE gel and detected with NP1 antibody as described above.

RESULTS AND DISCUSSION

To identify proteins that associate with merlin, we screened a human fetal frontal cortex interaction library with pmbrat. About 106 primary transformants were pooled and replated (at a multiplicity of 20) onto galactose leu− selection plates. Thirty-three colonies, which showed galactose-dependent growth and blue color on leu− plates and on 5-bromo-4-chloro-3-indolyl β-galactopyranoside medium, respectively, were identified, and plasmids containing the cDNA clones were isolated. Restriction mapping and hybridization experiments revealed that these cDNAs were clustered into three different groups of overlapping clones, representing three novel cDNAs. In this study we report the characterization of one of the cDNAs, an 880-base pair fragment which we refer to as merintc. We tested merintc for associations in yeast strains containing either the amino- (aa 1–341) or the carboxyl- (aa 342–595) terminal portion of hNHE-RF. The affinity-purified moesin antibody was used as described earlier (9). Cells were examined on a Nikon microscope using a 40 × 1.3 N.A. and 60 × 1.4 N.A. objectives. For confocal microscopy, cells were examined with Leica TCS-NT 4D scanning laser confocal microscope.

Provided by the Genetics Computer Group (GCG) and the BLAST network service at the National Center for Biotechnology Information (NCBI) (21).

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Affinity Precipitation of hNHE-RF from Cell Lysates—COS-7 cell lysates overexpressing hNHE-RF were incubated with 300 pmol of GST-merlin or GST-moesin immobilized on glutathione-Sepharose 4B (GSH) beads. The beads were washed with phosphate-buffered saline containing Pefabloc, resuspended in Laemmli loading buffer, subjected to 10% SDS-PAGE, and immunoblotted with anti-hNHE-RF (affinity-purified NP1). For radixin, COS-7 cell lysates overexpressing the interactome were incubated with 600 pmol of the His6-tagged full-length, amino-terminal, or carboxyl-terminal radixin polypeptides. The complexes were separated from the reaction mixture using Ni-NTA beads. The beads were washed (20 mCi imidazole, 50 mCi sodium phosphate, 300 mCi NaCl, pH 8.0), and the specifically bound complexes were eluted with the same buffer containing 400 mM imidazole separated on SDS-PAGE gel and detected with NP1 antibody as described above.

Immunofluorescence—The immunofluorescence staining was performed as described previously (16). Anti-HA monoclonal antibody (1:100) was used as a primary antibody to detect the localization of hNHE-RF. The affinity-purified moesin antibody was used as described earlier (9). Cells were examined on a Nikon microscope using a 40 × 1.3 N.A. and 60 × 1.4 N.A. objectives. For confocal microscopy, cells were examined with Leica TCS-NT 4D scanning laser confocal microscope.
hNHE-RF Interacts with MERM Proteins

Figure 2: Affinity precipitation of hNHE-RF from cell lysates. RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS containing a 1× protease inhibitor mixture) lysates from COS-7 cells overexpressing hNHE-RF were incubated with merlin or moesin expressed as GST fusion proteins and immobilized on GSH beads (A), and radixin expressed with a His-tag and eluted from Ni-NTA beads (B). The beads were extensively washed, separated on 10% SDS-PAGE, and immunoblotted with affinity-purified anti-hNHE-RF antibody (NP1). N and C represent the amino and carboxyl regions of merlin, moesin, and radixin. FL represents full-length merlin and radixin, and Mut-Mer is an NF2-associated missense mutation of merlin. GST protein expressed alone is shown as a control (GST). Total cell extracts from either COS-7 cells overexpressing full-length hNHE-RF or wild type COS-7 cells are also shown as controls. The supernatants that were not bound to the beads are shown to point out that the amino portion of moesin is completely bound by hNHE-RF (A). The arrow shows the hNHE-RF at 50 kDa.

Figure 3: Co-localization of hNHE-RF with merlin and moesin by double immunocytochemistry. A, merlin is visualized as GFP-tagged protein (a, d). Rhodamine-conjugated secondary anti-mouse antibody was used to detect hNHE-RF (b, e). The partial co-localization of these two proteins can be visualized in COS-7 cells at the ruffling membrane (small arrows), microvilli, and filopodia (large arrow, c), and similar co-localization in microvilli is observed in HeLa cells (f). Bar, 10 μm. B, fluorescein isothiocyanate-conjugated secondary anti-mouse antibody was used to detect hNHE-RFb (a, d), and rhodamine-conjugated secondary anti-mouse antibody was used to detect endogenous moesin (b, e) in HeLa cells. At the top of the cells (a–c), there is strong overlap of these two proteins in the microvilli (c). In the lower part of the cells close to substrate (d–f), the two proteins co-localize very clearly at ruffling membrane and filopodia. Bar, 20 μm.

crocelli, and filopodia reminiscent of the ERM proteins and of overexpressed merlin (Fig. 3). When we co-expressed both merlin and hNHE-RF in COS-7 and HeLa cells, we also observed the co-localization of these two proteins in membrane ruffles, microvilli, and filopodia (Fig. 3A). Double immunostaining of exogenous hNHE-RF and endogenous moesin in HeLa cells revealed co-localization in microvilli, ruffling membrane, and filopodia (Fig. 3B). However, hNHE-RF does not overlap with merlin and moesin in all microvilli and filopodia, and similarly merlin and moesin are not seen throughout the membrane ruffles where we observe hNHE-RF. The frequent co-localization of hNHE-RF with merlin and moesin is, however, consistent with the interaction of these proteins.

We demonstrate here that hNHE-RF, a widely expressed protein whose rabbit homologue was first identified by its involvement in mediating protein kinase A regulation of the renal brush-border Na⁺/H⁺ exchanger (18) interacts both with merlin and with its close relatives, moesin and radixin from the ERM family. Interestingly, while this manuscript was in preparation, Reczek et al. (19) identified hNHE-RF as a physiologically relevant interactor (which they named EBP50) for ezrin, the third ERM protein.

hNHE-RF possesses two PDZ domains, motifs thought to mediate protein-protein interactions, particularly at the plasma membrane during assembly of components involved in...
whether NHE-RF is also capable of regulating other Na\(^{+}\)H\(^{-}\) exchangers, particularly NHE1. Interestingly, increased Na\(^{+}\)H\(^{-}\) exchange in cytoskeletal rearrangement (26) suggests that Na\(^{+}\)H\(^{-}\) exchangers are sequestered to these regions by interacting with the cytoskeletal network (31). NHE1 is necessary for Rho-induced stress fiber formation (32).

Our demonstration of NHE-RF as a binding partner for MERM family members suggests a potential functional connection with the involvement of Na\(^{+}\)H\(^{-}\) exchange in cytoskeletal rearrangement (Fig. 4). Indeed, a recent study demonstrates that merlin-NHE-RF interaction is the basis of merlin’s tumor suppressor function since failure of the interaction might abrogate regulation of a Na\(^{+}\)-H\(^{-}\) exchanger in NF2 target cells and lead to formation of meningiomas and schwannomas.

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REFERENCES

1. Evans, D. G. R., Huson, S. M., Donnai, D., Neary, W., Blair, V., Teare, D., Newton, V., Strachan, T., Ramsden, R., and Harris, R. (1992) J. Med. Genet. 29, 841–846
2. Gusella, J. F., Ramesh, V., McColin, M., and Jacoby, L. B. (1996) Curr. Opin. Genet. Dev. 6, 87–92
3. Tsukita, S., Yonemura, S., and Tsukita, S. (1997) Curr. Opin. Cell Biol. 9, 76–80
4. Trofatter, J. A., McColin, M. M., Rutter, J. L., Murrell, J. R., Duyao, M. P., Parry, D. M., Eldridge, R., Kley, N., Monen, A. G., Pulasaki, K., Haase, V. H., Ambrose, C. M., Matroc, D., Beve, C., Haines, J. L., Martuza, R. L., MacDonald, M. E., Seizinger, B. R., Short, M. P., Buckley, A. J., and Gusella, J. F. (1993) Cell 72, 791–800
5. Bretcher, A. (1985) J. Cell Biol. 97, 425–432
6. Birbaut, E. (1981) Cytoskeletal Interactions of Ezrin in Different Cell Types. Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, MA
7. Sato, N., Yonemura, S., Ohnita, T., Tsukita, S., and Tsukita, S. (1991) J. Cell Biol. 113, 321–330
8. Sato, N., Panayama, N., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S. (1992) J. Cell Sci. 103, 131–143
9. Henry, M. D., Gonzalez-Agosti, C., and Solomon, F. (1995) J. Cell Biol. 129, 1907–1922
10. Turunen, O., Wahlström, T., and Vaheri, A. (1994) J. Cell Biol. 126, 1445–1453
11. Pensonjaap, K., Amieva, M. R., Strassel, C. P., Hansel, W. M., Furtmayer, H., and Luna, E. J. (1995) Mol. Biol. Cell 6, 247–259
12. Martin, M., Ray, C., Montcourrier, P., Sahaout, A., and Mangeat, P. (1997) Mol. Biol. Cell 8, 1543–1557
13. Tsukita, S., Oishi, K., Sato, N., Sagara, J., Kawai, A., and Tsukita, S. (1994) J. Cell Biol. 126, 391–401
14. Hirao, M., Sato, N., Kondo, T., Yonemura, S., Monden, M., Sasaki, T., Takai, Y., Tsukita, S., and Tsukita, S. (1996) J. Cell Biol. 135, 37–51
15. Mackay, D., Koch, F., Furtmayer, H., and Hall, A. (1997) J. Cell Biol. 138, 927–938
16. Gonzalez-Agosti, C., Xu, L., Pinney, D., Beauchamp, R., Hubs, W., Gusella, J., and Ramesh, V. (1996) Oncogene 13, 1239–1247
17. Xu, L., Gonzalez-Agosti, C., Beauchamp, R., Pinney, D., Sterner, C., and Ramesh, V. (1998) Exp. Cell Res., in press
18. Weinman, E. J., Dubinsky, W., and Shenolikar, S. (1995) J. Clin. Invest. 95, 2143–2149
19. Reczek, D., Broyerman, M., and Bretcher, A. (1997) J. Cell Biol. 139, 169–179
20. Gyruris, J., Golemis, E. A., Chernkov, H., and Brent, R. (1993) Cell 75, 791–803
21. Altchuk, S. F., Thomas, M. L., Alejando, S. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3342
22. McColin, M., Mohnuey, T., Trofatter, J., Werteleck, W., Ramesh, V., and Gusella, J. F. (1993) J. Am. Med. Assoc. 270, 2316–2320
23. Smith, D. B., and Johnson, K. S. (1988) Gene 67, 31–40
24. Magendanzita, M., Henry, M. D., Lander, A., and Solomon, F. (1995) J. Cell Biol. 127, 2529–2527
25. Winckler, B., Gonzalez-Agosti, C., Magendanzita, M., and Solomon, F. (1994) J. Clin. Cancer 107, 2523–2534
26. Tsukita, S., Yonemura, S., and Teate, D. (1997) Nature 388, 243–249
27. Fanning, A. S., and Anderson, J. M. (1996) Curr. Biol. 6, 1585–1588
28. Yun, C. H., Oh, S., Zizak, M., Steplock, D., Tsao, S., Tse, C. M., Weinman, E. J., and Denowitz, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3010–3015
29. Orlowski, J., and Grinstein, S. (1997) J. Biol. Chem. 272, 23737–23740
30. Yun, C. H., Tse, C. M., Naito, S. K., Levine, S. A., Brant, S. R., and Donowitz, M. (1995) Am. J. Physiol. 26, 31–40
31. Grinstein, S., Woodside, M., Waddell, T. K., Downey, G. P., Orlowski, J., Pouyssegur, J., Wong, D. C. P., and Foskett, J. K. (1993) EMBO J. 12, 5209–5218
32. Vexler, Z. S., Simons, M., and Barber, D. L. (1996) J. Biol. Chem. 271, 22281–22284
33. Harguny, S., Pedraz, J. L., Canero, R. G., Diego, J. P., and Cracog, E. J. (1995) Crit. Rev. Oncogene. 1, 1–33