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Interactions of Drebrin and Gephyrin with Profilin[1]

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Profilin is an actin monomer-binding protein which stimulates actin polymerization. Recent studies have revealed that profilin interacts with VASP, Mena, Bni1p, Bnr1p, and mDia, all of which have the proline-rich domain. Here, we isolated three profilin-binding proteins from rat brain cytosol by glutathione *S*-transferase-profilin affinity column chromatography and identified them as Mena, drebrin, and gephyrin. These proteins had a proline-rich domain and directly interacted with profilin.

Profilin was originally isolated as an actin monomer-binding protein (for a review, see Ref. 1). It was previously thought that profilin sequestered actin monomer and thereby inhibited actin polymerization. However, since the discovery of thymosinβ4, profilin has been shown to stimulate the ADP/ATP exchange reaction of actin monomer and thereby to make it ready to be incorporated into filamentous actin (1). Profilin is currently considered to stimulate actin polymerization.

It has been shown that profilin directly interacts with many cytoskeletal proteins, such as VASP (2,3), Mena (4), Bni1p (5-7), Bnr1p (7), and mDia (8), all of which are known to have the proline-rich domain. VASP (3), Mena (4), Bni1p (7), and Bnr1p (7) have been shown to interact with profilin through this domain (for a review, see Ref. 9), although this domain has also been shown to interact with the SH3 domain of other proteins (10,11).

Another series of experiments have clarified that the Rho family small G proteins regulate various actin cytoskeleton-dependent cell functions, such as cell shape change, cell motility, and cytokinesis (for reviews, see Refs. 12-16), and that at least three profilin-binding proteins, Bni1p (5-7), Bnr1p (7), and mDia (8), are downstream target molecules of the Rho family members.

Thus, to understand the molecular mechanism of dynamic reorganization of the actin cytoskeleton, it is important to identify profilin-binding proteins. In this study, we attempted to identify new profilin-binding proteins from rat brain.

MATERIALS AND METHODS

Expression and production of recombinant proteins. The cDNA fragment encoding human profilin-1 with *Bam*HI sites upstream of the methionine codon and downstream of the termination codon were obtained by polymerase chain reaction amplification from QUICK-Clone cDNA of human brain (Clontech) (17). This fragment was digested by *Bam*HI and inserted into the *Bam*HI site of pGEX-2T plasmid (Pharmacia Biotech Inc.). GST-profilin was expressed in *Esche---ri-chia coli* and purified by affinity column chromatography as described (18). The cDNA fragment of rat drebrin A was inserted into an *Autographa california* baculovirus transfer vector, pAcYM1-*myc,* to express its fusion protein with the N-terminal Myc epitope under the control of the polyhedrin promoter in *Spodoptera frugiperda* cells (Sf9 cells) (19,20). Myc-tagged drebrin was prepared from the cytosol fraction of Sf9 cells by immunoprecipitation with an anti-Myc monoclonal antibody.

Purification of profilin-binding proteins. All the purification procedures were performed at 4°C. Ten rat brains were homogenized in a Potter-Elvehjem Teflon-glass homogenizer with twelve strokes in 40 ml of Buffer A [5 mM Tris/HCl at pH 7.5, 10% sucrose, and 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride] and diluted to 10%(w/v) in Buffer A. The homogenate was centrifuged at 1,000 × g for 20 min, and the supernatant was further centrifuged at 100,000 × g for 1 hr to separate the membrane and cytosol fractions. The membrane

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Abbreviations used: GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; aa, amino acid(s).

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fraction was suspended with 15 ml of Buffer B (20 mM HEPES/NaOH at pH 7.4 and 1 mM dithiothreitol) containing 2 M NaCl, sonicated for 30 sec three times at 1-min intervals, and incubated for 1 hr. The NaCl extract was prepared by centrifugation at 100,000 × g for 1 hr. The extracted membrane fraction was resuspended with 15 ml of Buffer B containing 2%(w/v) Triton X-100, sonicated for 30 sec three times at 1-min intervals, and incubated for 1 hr. The Triton X-100 extract was prepared by centrifugation at 100,000 × g for 1 hr. Each fraction was applied to GST-profilin-bound glutathione-Sepharose beads. After extensive washing, the proteins bound to GST-profilin were eluted with 5 mM reduced glutathione.

GST-profilin blot overlay method. The eluate from the GST-profilin affinity column chromatography was subjected to SDS-PAGE and transferred to a nitrocellulose membrane sheet (0.45 μm pore size, Schleicher & Schuell). The sheet was blocked in TBS (20 mM Tris/HCl at pH 7.5 and 140 mM NaCl) containing 5%(w/v) defatted powder milk. The sheet was then incubated for 1 hr at 4°C with 1 μM GST-profilin or GST in the same buffer, followed by extensive washing. GST-profilin and GST bound to the sheet were detected by Western blotting using the anti-GST monoclonal antibody (sc-138) (Santa Cruz, CA).

Peptide mapping. The purified profilin-binding proteins were subjected to SDS-PAGE, the protein bands with *M*rs of 90, 130, and 140 kDa were excised from the gel and digested with lysyl en---dopeptidase, and the digested peptides were subjected to TSKgel ODS-80Ts column (4.6 × 150 mm, Tosoh) reversed-phase high performance liquid chromatography as described (21). The aa sequences of the peptides were determined with a peptide sequencer (HP G1005A protein sequencing system).

Other procedures. SDS-PAGE was performed as described (22). Protein concentrations were determined with bovine serum albumin as a reference protein as described (23).

RESULTS

We first prepared the cytosol and membrane fractions from rat brain. From the membrane fraction, proteins were furthermore extracted with 2 M NaCl, followed by extraction with 2% Triton X-100. Each fraction was applied to GST-profilin-bound glutathione-Sepharose beads. After extensive washing, the proteins bound to GST-profilin were eluted with reduced glutathione. An aliquot of each eluate was then subjected to SDS-PAGE, followed by Coomassie brilliant blue staining (Fig. 1). The proteins with *M*rs of 80, 90, 130, and 140 kDa (the 80, 90, 130, and 140 kDa proteins, respectively) were detected in the cytosol fraction, whereas the protein with a *M*r of 80 kDa was detected in both the NaCl extract and the Triton X-100 extract. Another aliquot of each eluate was subjected to SDS-PAGE. After the separated proteins were transferred to nitrocellulose membrane sheets, each sheet was overlayed with GST-profilin or GST as a control. After extensive washing, GST-profilin or GST bound to each protein was detected by the anti-GST-antibody. Of the four proteins of the cytosol fraction, the 90, 130, and 140 kDa proteins, but not the 80 kDa protein, bound to GST-profilin, but not to GST (Fig. 2). No protein in the NaCl extract or the Triton X-100 extract bound to GST-profilin or GST (data not shown).

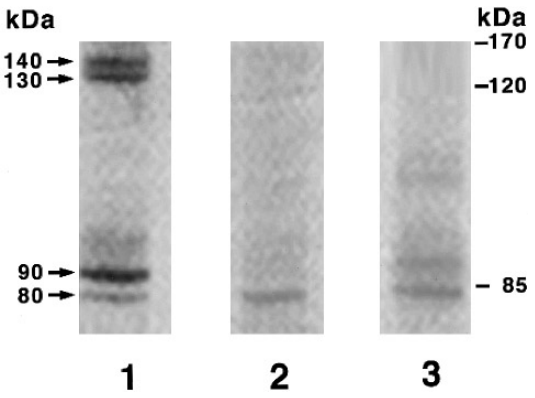

FIG. 1. Affinity-purified profilin-binding proteins. The cytosol fraction, the NaCl extract, and the Triton X-100 extract were applied to GST-profilin-bound glutathione-Sepharose beads. After washing, the bound proteins were eluted with reduced glutathione and subjected to SDS-PAGE, followed by Coomassie brilliant blue staining. *Lane 1,* the cytosol fraction; *lane 2,* the NaCl extract; *lane 3,* the Triton X-100 extract. *Arrows* indicate the 80, 90, 130, and 140 kDa proteins. The protein markers used were α2-macrogloblin (*M*r=170,000), β-galactosidase (*M*r=120,000), and bovine serum albumin (*M*r=85,000). The results shown are representative of three independent experiments.

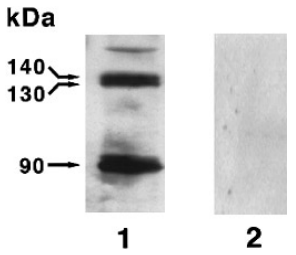

FIG. 2. GST-profilin blot overlay. The eluate from the GST-profilin affinity column chromatography of the cytosol fraction was subjected to SDS-PAGE, followed by GST-profilin blot overlay. *Lane 1,* GST-profilin blot overlay; *lane 2,* GST blot overlay. *Arrows* indicate the 90, 130, and 140 kDa proteins. The results shown are representative of three independent experiments.

The 90, 130, and 140 kDa proteins were accumulated by the same method as that used in Fig. 1. Each accumulated protein was then subjected to SDS-PAGE. Each band was excised and digested with lysyl endopeptidase. The peptides were eluted by the gel and subjected to C18 reversed-phase high performance liquid chromatography (Fig. 3). Of the many separated peptide peaks, the aa sequences of the several peaks were determined. The aa sequence of the 90 kDa protein was ENILRASHSAVDITK; the aa sequence of the 130 kDa protein was WVPAGGSTGFSRVHIYHHTGNNTFRVVGR; and the aa sequences of the 140 kDa protein were WVPAGGSTGFSRVHIY-HH-TGNNTF-RVVGR, LAASGEGGLQELSGHFENQK, DQSIFGDQRDEEEESQMK, and ALDARLRFEQERMEQERQEQEE. Computer homology search revealed that the 90 kDa protein was gephyrin (24); the 130 kDa protein was Mena (4); and the 140 kDa protein was drebrin and Mena (4,19).

Because the band of 140 kDa contained two proteins, drebrin and Mena, we confirmed the direct interaction of drebrin with profilin by use of recombinant drebrin. Myc-tagged drebrin was subjected to GST-profilin overlay assay as described above. Myc-tagged drebrin directly bound to GST-profilin, but not to GST (Fig. 4).

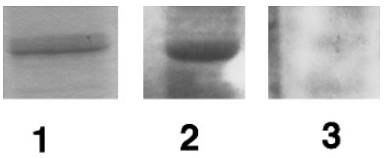

FIG. 4. Interaction of drebrin with profilin. Myc-tagged drebrin was precipitated from the cytosol fraction of baculovirally infected Sf9 cells with the anti-Myc monoclonal antibody and subjected to SDS-PAGE, followed by Coomassie brilliant blue staining and by GST-profilin blot overlay. *Lane 1,* Coomassie brilliant blue staining; *lane 2,* GST-profilin blot overlay; *lane 3,* GST blot overlay.

DISCUSSION

VASP (3), Mena (4), Bni1p (7), and Bnr1p (7) have been shown to bind to profilin through the proline-rich domain. We have confirmed that Mena binds to profilin as described (4), and furthermore found that drebrin and gephyrin bind to profilin. These two proteins indeed have a proline-rich domain at 410-419 aa in drebrin (19) and 187-201 aa in gephyrin (24). It is likely that profilin binds to this region of these proteins. Although the function of drebrin has not fully been understood, drebrin has been shown to directly interact with filamentous actin and has been considered to be one of the regulators of the neuronal morphogenesis (for a review, see Ref. 25). Gephyrin has been shown to interact with the glycine receptor and has been considered to anchor the glycine receptor to the submembranous cytoskeleton and thereby to induce its clustering (for reviews, see Refs. 26 and 27). Our present results suggest that both the proteins regulate these actin cytoskeleton-dependent cell functions through interaction with profilin.

Of the many profilin-binding proteins, Bni1p (5-7), Bnr1p (7), and mDia (8) are the Rho family member-binding proteins. These small G proteins have been shown to regulate reorganization of the actin cytoskeleton at least through interaction with profilin (7,8). Although it is unknown whether other profilin-binding proteins, such as VASP, drebrin, and gephyrin, interact with small G proteins, these proteins also regulate reorganization of actin filaments through interaction with profilin. We found here that neither drebrin nor gephyrin interacted with RhoA, Rac1, or Cdc42 small G protein (data not shown). Therefore, it is likely that profilin-binding proteins are classified into at least two groups: one group binds to small G proteins; and the other does not. Further studies are necessary for understanding of the function of many profilin-binding proteins.

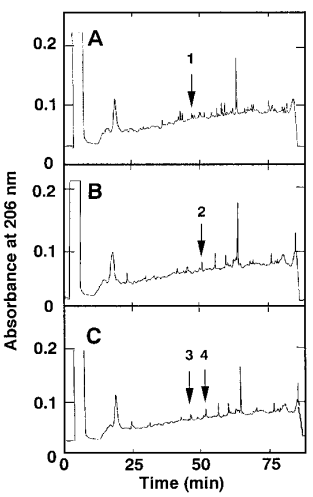

FIG. 3. The peptide map analyses of the 90, 130, and 140 kDa proteins. The profilin-binding proteins affinity-purified from the cytosol fraction were subjected to SDS-PAGE, the protein bands with *M*rs of 90, 130, and 140 kDa were excised from the gel and digested with lysyl endopeptidase, and the digested peptides were subjected to TSKgel ODS-80Ts column chromatography. (--), absorbance at 206 nm. The aa sequences of Peptide 1-4 were determined with a peptide sequencer. *A*, the 90 kDa protein; *B*, the 130 kDa protein; *C*, the 140 kDa protein.

REFERENCES

1. Sohn, R. H., and Goldschmidt-Clermont, P. J. (1994) *BioEssays* **16,** 465–472.
2. Reinhard, M., Halbrügge, M., Scheer, U., Wiegand, C., Jockusch, B. M., and Walter, U. (1992) *EMBO J.* **11,** 2063–2070.
3. Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., Jockusch, B. M., and Walter, U. (1995) *EMBO J.* **14,** 1583–1589.
4. Gertler, F. B., Niebuhr, K., Reinhard, M., Wehland, J., and Soriano, P. (1996) *Cell* **87,** 227–239.
5. Kohno, H., Tanaka, K., Mino, A., Umikawa, M., Imamura, H., Fujiwara, T., Fujita, Y., Hotta, K., Qadota, H., Watanabe, T., Ohya, Y., and Takai, Y. (1996) *EMBO J.* **15,** 6060–6068.
6. Evangelista, M., Blundell, K., Longtine, M. S., Chow, C. J., Adames, N., Pringle, J. R., Peter, M., and Boone, C. (1997) *Science* **276,** 118–122.
7. Imamura, H., Tanaka, K., Hihara, T., Umikawa, M., Kamei, T., Takahashi, K., Sasaki, T., and Takai, Y. (1997) *EMBO J.* **16,** 2745–2755.
8. Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M., and Narumiya, S. (1997) *EMBO J.* **16,** 3044–3056.
9. Frazier, J. F., and Field, C. M. (1997) *Curr. Biol.* **7,** R414-R417.
10. Lim, W. A., Richards, F. M., and Fox, R. O. (1994) *Nature* **372,** 375–379.
11. Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993) *Science* **259,** 1157–1161.
12. Hall, A. (1994) *Annu. Rev. Cell Biol.* **10,** 31–54.

13. Takai, Y., Sasaki, T., Tanaka, K., and Nakanishi, H. (1995) *Trends Biochem. Sci.* **20,** 227–231.
14. Machesky, L. M., and Hall, A. (1996) *Trends Cell Biol.* **6,** 304–310.
15. Symons, M. (1996) *Trends Biochem. Sci.* **21,** 178–181.
16. Zigmond, S. H. (1996) *Curr. Opin. Cell Biol.* **8,** 66–73.
17. Kwiatkowski, D. J., and Bruns, G. A. P. (1988) *J. Biol. Chem.* **263,** 5910–5915.
18. Kikuchi, A., Kuroda, S., Sasaki, T., Kotani, K., Hirata, K., Katayama, M., and Takai, Y. (1992) *J. Biol. Chem.* **267,** 14611–14615.
19. Shirao, T., Kojima, N., and Obata, K. (1992) *Neuroreport* **3,** 109–112.
20. Matsuura, Y., Possee, R. D., Overton, H. A., and Bishop, D. H. LD. H. (1987) *J. Gen. Virol.* **68,** 1233–1250.
21. Imazumi, K., Sasaki, T., Takahashi, K., and Takai, Y. (1994) *Biochem. Biophys. Res. Commun.* **205,** 1409–1416.
22. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
23. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
24. Prior, P., Schmitt, B., Grenningloh, G., Pribilla, I., Multhaup, G., Beyreuther, K., Maulet, Y., Werner, P., Langosch, D., Kirsch, J., and Betz, H. (1992) *Neuron* **8,** 1161–1170.
25. Shirao, T. (1995) *J. Biochem.* **117,** 231–236.
26. Kuhse, J., Betz, H., and Kirsch, J. (1995) *Curr. Opin. Neurobiol.* **5,** 318–323.
27. Garner, C. C., and Kindler, S. (1996) *Trends Cell. Biol.* **6,** 429–433.