Association of Ash/Grb-2 with Dynamin through the Src Homology 3 Domain* 

(Received for publication, October 27, 1993, and in revised form, December 10, 1993)

Takao Nakatani, Koji Murota, Koozi Matuoka, Takao Nakata, Nobutaka Hirokawa, Satoshi Orita, Kozo Kalibuchi, Yoshimi Takai, and Tadaomi Takenawa

From the Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, the Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Sasebo, Itohashi-ku, Tokyo 173, the Department of Anatomy and Cell Biology, School of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, and the Department of Biochemistry, School of Medicine, University of Kobe, Kobe 650, Japan

Ash/Grb-2 is an adaptor protein composed only of Src homology (SH) 2 and SH3 domains that is considered to be essential for Ras activation. To clarify the downstream of Ash signaling, we investigated Ash-bound proteins. Ash-glutathione S-transferase (GST) fusion proteins were used to affinity-purify proteins bound to Ash. We found 180-, 150-, 100-, and 70-kDa proteins bound to GST-Ash, among which the 100-kDa protein was found to be dynamin by amino acid sequencing and Western blot with anti-dynamin antibody. Next, the in vitro and in vivo associations between Ash and dynamin were examined using PC12 cells. Dynamin in PC12 cell lysates bound to GST-Ash independent of NGF treatment. Also, Ash and dynamin co-precipitated when cell lysates of PC12 were immunoprecipitated with anti-Ash antibody or anti-dynamin antibody. Using various GST-Ash constructs, we studied the importance of the individual domains in binding and found that the SH3 domain is necessary for binding. This binding was inhibited by a synthetic peptide (GPPPVQPSRNRC, amino acids 827-838 in dynamin). These data show that Ash SH3 domains bind to the proline-rich region of dynamin. Considering the function of dynamin in membrane trafficking, Ash may regulate endocytosis in addition to Ras activation.

The Ash/Grb-2 gene, encoding a 25-kDa protein composed entirely of Src homology (SH) 1 and 2 and SH3 domains in the order SH3-SH2-SH3, has been cloned from human and rat cDNA libraries (1, 2). The gene product has been found to be the mammalian homolog of Caenorhabditis elegans Sem-5 (3), and shown to bind the autophosphorylated EGF receptor and other phosphotyrosine-containing proteins such as Shc and insulin receptor substrate 1 through its SH2 domain (1, 2, 3-6). More recently, Ash/Grb-2 has been demonstrated to bind mSos1 (7-12), a mammalian homolog of the Drosophila guanine nucleotide-releasing factor for Ras (7), which is essential for Ras signaling by EGF receptor and sevenless protein (13, 14). Moreover, the overexpression of Ash/Grb-2 in mammalian cells has been found to potentiate EGF-induced activation of Ras and mitogen-activated protein kinase by enhancing the rate of guanine nucleotide exchange on Ras (9). The interaction of mSos1 and Ash is thought to be mediated through the SH3 domains of Ash and proline-rich carboxyl-terminal region of mSos1 (10, 11). In addition, Ash seems to play important roles not only in mitogenesis through Ras activation, but also in the reorganization of actin assembly to ruffle formation (15), showing the possibility that signals received at the SH2 domain of Ash are transmitted to several proteins through its SH3 domains.

Dynamin was first identified as a protein of relative molecular mass 100,000 that induces nucleotide-dependent microtubule-binding proteins (16), and whose GTPase activity is stimulated by microtubules (17, 18). It is also closely related to the product of the shibire gene in Drosophila that exhibits a general endocytosis defect (19, 20). From these results, two disparate properties have been suggested: involvement in microtubule-based motility and microtubule-independent membrane trafficking. However, recent detailed analyses suggest it is unlikely that dynamin acts as a microtubule-based motor (21). It has been also shown that dynamin is phosphorylated by protein kinase C and that the GTPase activity of phosphorylated dynamin increases markedly (22), indicating its important role in the rapid endocytosis of synaptic vesicles after neurotransmitter release.

Interestingly, dynamin has proline-rich sequences near its carboxyl terminus that are recognized by the SH3 domains of the PI 3-kinase 85-kDa subunit and phosphatidylinositol 3-kinase (23). These results suggest that in addition to its regulation by protein kinase C, dynamin is regulated by tyrosine kinases through SH2- and SH3-containing proteins.

In this study, recombinant GST-Ash fusion protein was used to purify several Ash-binding proteins from bovine brain extracts. By partial amino acid sequencing, one of the purified proteins was found to be dynamin. Accordingly, it was demonstrated that GST-Ash binds dynamin independent of NGF treatment when PC12 cell lysates are used. Moreover, dynamin was co-immunoprecipitated with Ash when PC12 cell lysates were treated with anti-dynamin antibody or anti-Ash antibody. This binding appeared to occur through the SH3 regions of Ash and the proline-rich regions of dynamin. Ash, therefore, may link tyrosine kinases and dynamin in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Glutathione-agarose beads were from Pharmacia LKB Biotechnology, Inc. Dynamin was purified from rat brain as described previously (17), and rabbit anti-Ash antisera was produced by the method described previously (24). Anti-Ash antibody was prepared as described (15). Anti-phosphotyrosine antibody (PY20) was from ICN. NGF-2 SS was from Boehringer Mannheim. Nitrocellulose filters were from Schleicher & Schuell. Nylon membranes were from ATTO Corp.
Alkaline phosphatase-conjugated secondary antibodies were from Promega. Protein A-agarose beads were from Pierce Chemical Co. A peptide (GPPQQYSPSRPNIC, amino acids 827–838 of dynamin with C added at the carboxyl end) was synthesized with an Applied Biosystems peptide synthesizer and purified by high performance liquid chromatography on a C18 reverse phase column. Polypropylene peptide was from Sigma.

Affinity Chromatography and Peptide Sequence Analysis—Recombinant GST-Ash fusion protein, coupled to glutathione-agarose, was used to affinity-purify several Ash-binding proteins from the cytosol fractions of bovine brain. Bovine brains were homogenized in buffer A containing 20 mM Tris/HCl (pH 7.5), 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and diisopropyl fluorophosphate. The resulting homogenate was centrifuged at 10,000 × g for 30 min, and the supernatant was frozen overnight. The thawed extracts were reconstituted at 100,000 × g for 60 min. The resulting supernatants were used as bovine brain cytosol fractions. To remove endogenous glutathione S-transferase (GST) from the brain cytosol fractions, the supernatants were first applied to a glutathione-agarose column. The flow through fractions were next applied on GST-Ash-glutathione beads column. The column was washed with buffer A containing 1% Triton X-100, and then eluted with 50 mM glutathione-containing buffer A. Ash-binding proteins eluted from the GST-Ash-glutathione column were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nylon membranes, and the 100-kDa region was cut out and digested with lysylendopeptidase. The resulting peptides were separated on a reverse-phase C18 column with a 0–60% gradient of acetonitrile in 0.1% trifluoroacetic acid and sequenced with an Applied Biosystems Sequencer.

GST Fusion Proteins—The bacterial expression plasmids coding GST-Ash AB (1–161) were prepared as described previously (1). The full-length GST-Ash expressing plasmid was made by polymerase chain reaction using the synthetic oligonucleotide primers 5’-CAGGCTGCT-GAGCACTGATCCGGCAGAAATGAAACCACATCCGTGG-3’ and 5’-TCAGCTGTCGACTTTATATATATTATATATCTGTCCTGTC-3’, which correspond, respectively, to the NH2-terminal and COOH-terminal regions of Ash, and then the product was digested with BamHI and inserted into the BamHI site of pGEX 3-X. The plasmid expressing GST-Ash AB (1–161) was digested by the GST-Ash expressing plasmid with SpI and EcoRI to excise the COOH-terminal SH3-coding region and ligating it. The plasmid expressing GST-Ash B (55–150) was made by polymerase chain reaction using the synthetic oligonucleotide primers 5’-TGAGACTAAAGAAACACATCTGCGTGCAGC-3’ and 5’-GACGAATTCGTCGAGGAGGAGATCGTGC-3’, which correspond, respectively, to the region between the NH2-terminal SH3 and the SH2 and the region between the SH2 and the COOH-terminal SH3 of Ash, and then the product was digested with BamHI and EcoRI and inserted into the BamHI and EcoRI sites of pGEX 3-X. These plasmids were transformed into the XL I-Blue strain of Escherichia coli and induced with isopropyl-l-thio-β-D-galactopyranoside to produce GST fusion proteins. The bacteria were collected by centrifugation and resuspended in the E. coli lysis buffer (40 mM Tris/HCl (pH 7.5), 5 mM EDTA, 0.1 mM PMSF, diisopropyl fluorophosphate, and 1% Triton X-100). Vigorous sonication was performed before centrifugation at 100,000 × g for 30 min. The resulting supernatants were saved as crude extracts containing GST fusion proteins.

Cell Culture and Preparation of Cell Lysates—PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum and 5% fetal bovine serum in 150-mm dishes in a humidified 5% CO2 atmosphere. Semiclone cells were treated with or without 10 ng/ml NGF for 3 min. After treatment, the cells were washed with ice-cold phosphate-buffered saline and then solubilized with 1 ml/dish harvest buffer (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1% Triton X-100). The extracts were sonicated briefly and rotated for 30 min before centrifugation at 10,000 × g for 10 min. The resulting supernatants were saved and used as PC12 cell lysates.

Immunoprecipitation—Cell lysates were incubated with the specified anti-dynamin antibodies. Following incubation, protein A-agarose beads were added and mixed for 2 h and the immune complexes bound to the beads were washed three times with harvest buffer and solubilized in SDS sample buffer. In some experiments, binding to bacterially expressed recombinant GST fusion proteins was assayed. Cell lysates were incubated with glutathione-agarose beads to which GST fusion proteins had been bound, and then washed with harvest buffer. In an experiment in which two peptides were tested for their capacity to inhibit Ash-dynamin association, GST-Ash-bound glutathione-agarose beads were precultivated with the indicated concentrations of peptides and then incubated with cell lysates. The beads were treated in the same way as described above.

RESULTS

GST Ash-binding Proteins—Recombinant GST or GST-Ash protein immobilized on glutathione-agarose beads was used to affinity-purify several proteins from bovine brain cytosol fractions. We found that 180-, 150-, 100-, and 70-kDa proteins bound specifically to GST-Ash (Fig. 1A). Among them, one protein (100 kDa) was cut from the SDS gel and digested with lysylendopeptidase. The resulting peptides were separated on a reverse-phase C18 column, and three were partially sequenced. Two of the peptides showed complete homology to rat dynamin (EVDPQQQRTIGV and EVDPDQQQ), and the other differed only in one amino acid (shown by underline, GIFFALFNTQQRNV). Furthermore, Western blot analysis revealed that the 100-kDa protein was recognized by anti-dynamin antisera (Fig. 1B). We therefore concluded that the 100-kDa protein was bovine dynamin.

In Vivo Association of Recombinant GST-Ash and Dynamin from PC12 Cells—In order to elucidate the in vivo association between Ash and dynamin, we chose PC12 cells because it is known that dynamin is strongly expressed in the nervous system. In fact, it was revealed by Western blot analysis with anti-dynamin antisera that PC12 cells contained much more dynamin than 3Y1 fibroblastic cells (data not shown). PC12 cell lysates treated with or without 10 ng/ml NGF were incubated with GST or GST-Ash immobilized on glutathione-agarose beads, and the bound proteins were analyzed by Western blot with anti-dynamin antisera and anti-phosphotyrosine antibody (PY20). As illustrated in Fig. 2, PC12 cell dynamin also binds to recombinant GST-Ash independent of NGF treatment. Western blot with PY20 showed that several proteins were phosphorylated by NGF on tyrosine residues, including a protein of about 100 kDa, although it was not clear whether this 100-kDa protein was dynamin or not.

In Vivo Association of Ash and Dynamin in PC12 Cells—Since we found that dynamin is expressed in PC12 cells and binds to recombinant GST-Ash, we next examined whether or not there is an association between Ash and dynamin in vivo.
We, therefore, attempted co-immunoprecipitation experiments in which PC12 cell lysates were immunoprecipitated with anti-Ash or anti-dynamin antiserum and the resulting immunoprecipitates were analyzed by Western blot with either antiserum. We detected significant amounts of Ash in the anti-dynamin immunoprecipitates and dynamin in the anti-Ash immunoprecipitates. These co-immunoprecipitations were not observed when preimmune sera were used (Fig. 3A). The amounts of Ash in anti-Ash immunoprecipitates and the amounts of dynamin in anti-dynamin immunoprecipitates were about 10% of those in the original cell lysates. On the other hand, the amounts of Ash in anti-dynamin immunoprecipitates and the amounts of dynamin in anti-Ash immunoprecipitates were smaller than 1% of those in the original cell lysates, indicating that less than 10% of Ash and dynamin exist as Ash-dynamin complex form. In order to clarify whether this Ash-dynamin complex plays a role in tyrosine kinase signaling process, we examined the association with tyrosine-phosphorylated proteins. PC12 cell lysates treated with or without 10 ng/ml NGF were immunoprecipitated with anti-Ash or anti-dynamin antiserum and resulting immunoprecipitates were analyzed by Western blot with anti-phosphotyrosine antibody (PY20). As shown in Fig. 3B, several tyrosine-phosphorylated proteins were detected in NGF-treated anti-dynamin immunoprecipitates. This result supports the possibility that Ash-dynamin complex moves toward the tyrosine-phosphorylated proteins and transmit the signals downstream.

**The SH3 Domain of Ash Binds to the Proline-rich Region of Dynamin**—In order to determine which regions of Ash and dynamin are responsible for binding, we performed the following two experiments. First, we constructed several GST fusion proteins and tested their ability to bind dynamin in vitro. GST or GST fusion proteins were immobilized on glutathione-agarose beads and incubated with cell lysates. The result protein complexes were analyzed by Western blot with anti-dynamin antiserum. GST-Ash AB (amino acids 1–161 of Ash, containing N-SH3 and SH2) and GST-Ash BC (amino acids 58–217 of Ash, containing SH2 and C-SH3) both could bind as much dynamin as GST-Ash; on the other hand, we could not find any association between dynamin and GST-Ash B (amino acids 55–150 of Ash, containing SH2). These data show that the SH3 domains are the dynamin binding sites of Ash. Second, a peptide corresponding to the proline-rich region of dynamin (GPPPPQVPSRPNRC: amino acids 827–838 with an additional C at the carboxyl end) was chemically synthesized and tested for its ability to inhibit the Ash-dynamin association. This synthetic peptide and a polyproline peptide (as a negative control) were preincubated with GST-Ash immobilized on glutathione-agarose beads at the indicated concentrations and their dynamin-binding capacities were assayed. As illustrated in Fig. 4B, the negative control polyproline peptide did not inhibit the association between recombinant GST-Ash and dynamin even at a concentration of 1 mM, while the synthetic peptide of the proline-rich region of dynamin inhibited the association completely at a concentration of 0.2 mM. This suggests that the proline-rich region of dynamin is responsible for the binding of
Ash bind to the proline-rich region of dynamin.tempted to characterize proteins that bind specifically to the complexes were analyzed in the same way as in the experiment shown in Fig. 2. The protein constructs used are indicated below the figure. The indicated concentrations of proline-rich peptide derived from dynamin or polyproline before incubation with cell lysates. The dynamin contents requires the SH3 domain. PC12 cell lysates were incubated with equal amounts of either GST or GST fusion proteins, including full-length Ash, GST-Ash B, which contains only the SH2 domain, did not bind dynamin. This strongly suggests that SH3 domains are the dynamin (less than 10%) form the Ash-dynamin complex, suggesting possibly involved in recognition. But only a part of Ash and dynamin (less than 10%) bind to the proline-rich region of Ash. These results show that the SH3 domains of Ash bind to the proline-rich region of dynamin.

**DISCUSSION**

To clarify the events downstream of Ash signaling, we attempted to characterize proteins that bind specifically to the SH3 domain of Ash. We detected at least four proteins bound to GST-Ash beads with molecular sizes of 180, 150, 100, and 70 kDa. One protein (180 kDa) was identified as mSos1 by the anti-mSos1 antibody. The other proteins were subjected to partial amino acid sequencing after lysylendopeptidase digestion. As a result, the 100-kDa protein was identified as dynamin.

GST-Ash beads bound dynamin in PC12 cell lysates regardless of NGF treatment, suggesting that the SH3 domain rather than the SH2 domain is important for binding. In experiments using various truncated constructs of Ash, both of the SH3 domains in Ash were found to bind dynamin. It is worth noting that GST-Ash B, which contains only the SH2 domain, did not bind dynamin. This strongly suggests that SH3 domains are the dynamin-binding sites of Ash. Since the association between Ash and dynamin was inhibited by the addition of synthetic proline-rich peptide (amino acids 827-838 in dynamin), this region is possibly involved in recognition. But only a part of Ash and dynamin (less than 10%) form the Ash-dynamin complex, suggesting that some other molecules bind to Ash or dynamin (e.g. mSos1 for Ash, 85-kDa subunit of PI 3-kinase for dynamin).

In nerve termini, dynamin is phosphorylated by protein kinase C and dephosphorylated upon excitation (22). Dynamin is therefore part of a group of nerve terminal phosphoproteins regulated by phosphorylation, suggesting its role in the endocytosis of synaptic vesicles. On the other hand, dynamin localizes not only in synapses but also in dendrites, cell bodies, and axons (24), indicating its involvement in various endocytotic processes. Our data concerning the association between Ash and dynamin in vivo and in vitro strongly support this idea. Based on these phenomena, the translocation of dynamin to membranes and the regulation of activity may be controlled by Ash, the Ash-dynamin complexes may then move toward tyrosine phosphorylation sites such as Y(P)INQ or Y(P)VNV sequences. These interactions may be important for receptor activation-induced membrane trafficking. These events and phosphorylation by protein kinase C may influence endocytosis. These problems remain to be solved in the future.

Addendum—After the submission of this work, similar findings were reported by Waterfield and colleagues (25).

**REFERENCES**

1. Matsuoka, K., Shibata, M., Yamakawa, A., and Takenawa, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9015-9019
2. Lowenstein, E. J., Batzer, A. G., Li, W., Margolis, B., Lammert, R., Ulrich, A., Skolnik, E. Y., Bar-Sagi, D., and Schlessinger, J. (1992) Cell 70, 431-442
3. Clark, S. G., Sien, M. J., and Horvitz, H. R. (1992) Nature 356, 340-344
4. Rozakis-Adrock, M., McClaide, J., Mhamdal, C., Pellici, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pellici, P. G., Schlessinger, J., and Pawson, T. (1992) Nature 360, 498-502
5. Seolnik, Y. E., Lee, C. H., Batzer, A., Vicentiiri, L. M., Zhou, M., Daly, R., Myers, M. J., Jr., Backer, J. M., Ulrich, A., White, M. F., and Schlessinger, J. (1993) EMBO J. 12, 1329-1336
6. Tobe, K., Matsuoka, K., Tanimoto, H., Ueki, K., Kaburagi, Y., Asai, S., Noguchi, T., Matsuuda, M., Tanaka, S., Hattori, S., Fukui, Y., Kanuma, Y., Yazaki, Y., Takekawa, T., and Kadowaki, T. (1993) J. Biol. Chem. 268, 11167-11171
7. Buday, L., and Downward, J. (1993) Cell 73, 611-620
8. Egan, S. E., Giddings, B. W., Brown, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) Nature 363, 45-50
9. Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J., and Bar-Sagi, D. (1993) Nature 363, 88-92
10. Rozakis-Adrock, M., Fennely, R., Wade, J., Pawson, T., and Bowtell, D. (1993) Nature 363, 83-85
11. Li, N., Batza, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993) Nature 363, 85-88
12. Chardin, P., Camonis, J. R., Gale, N. W., Alesi, L. V., Schlessinger, J., Wigler, M. H., and Bar-Sagi, D. (1993) Science 260, 1338-1343
13. Olivier, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mhamdal, C., Margolis, B., Schlessinger, J., and Pawson, T. (1993) Cell 73, 169-177
14. Simon, M. A., Dodaos, G. S., and Rubin, G. M. (1993) Cell 73, 169-177
15. Matsuoka, K., Shibata, M., Mhamdal, C., and Takenawa, T. (1993) EMBO J. 12, 3467-3473
16. Shpeter, H. S., and Valle, R. B. (1989) Cell 59, 421-432
17. Maeda, K., Nakata, T., Noda, Y., Sato-Yoshitake, R., and Hirokawa, N. (1992) Mol. Biol. Cell 3, 1181-1194
18. Shpeter, H. S., and Valle, R. B. (1992) Nature 353, 733-735
19. van der Bliek, A. M., and Megerowitz, E. M. (1991) Nature 351, 411-414
20. Chen, M. S., Ober, R. A., Schroeder, C. C., Austin, T. W., Fooey, C. A., Wadsworth, S. C., and Valle, R. B. (1991) Nature 351, 583-586
21. Nakata, Y., Sato-Yoshitake, R., Okada, Y., Noda, Y., and Hirokawa, N. (1994) Biophys. J., in press
22. Robinson, D. J., Sentac, J. M., Liu, J.-P., Fykse, E. M., Slaughter, C., McMahan, H., and Sudhof, T. C. (1993) Nature 365, 165-166
23. Koyama, S., Yu, H., Daigorn, D. C., Shin, T. B., Zydowsky, L. D., and Schreiber, S. C. (1993) Cell 72, 945-952
24. Noda, Y., Nakata, T., and Hirokawa, N. (1993) Neuroscience 55, 113-127
25. Gout, I., Dhand, W. H., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booth, G. W., Campbell, L. D., and Waterfield, M. D. (1993) Cell 75, 25-36