Biochemical and Biophysical Research Communications **288,** 87–90 (2001)
doi:10.1006/bbrc.2001.5740, available online at http://www.idealibrary.com on IDEAL®

Distinct Phosphoinositide Binding Specificity of the GAP1 Family Proteins: Characterization of the Pleckstrin Homology Domains of MRASAL and KIAA0538[1]

Tetsuya Minagawa,* Mitsunori Fukuda,*,†,[2] and Katsuhiko Mikoshiba*,†,‡

**International Joint Project "Calcium Oscillation," Japan Science and Technology Corporation, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; †Laboratory for Developmental Neurobiology, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; and ‡Division of Molecular Neurobiology, Department of Basic Medical Science, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*

Received September 12, 2001

GAP1, one of the Ras GTPase-activating protein families, includes four distinct genes (GAP1^m, GAP1^{IP4BP}, MRASAL (murine Ras GTPase-activating-like), and KIAA0538). It contains an amino-terminal tandem C2 domain, a GAP-related domain, and a carboxyl-terminal pleckstrin homology (PH) domain. Although the PH domains of GAP1^m and GAP1^{IP4BP} have been shown to be essential for membrane targeting via binding of specific phospholipids, little is known about the functions of the PH domains of MRASAL and KIAA0538. Herein, we show that the PH domain of MRASAL has binding activity toward PI(4,5)P_2 and PI(3,4,5)P_3, while the PH domain of KIAA0538 does not bind these phospholipids due to an amino acid substitution at position 592 (Leu-592). Mutation of the corresponding position of MRASAL (Arg-to-Leu substitution at position 591) resulted in loss of the phospholipid binding activity. MRASAL proteins were localized at the plasma membrane in NIH3T3 cells, and this plasma membrane association was unchanged even after cytochalasin B or wortmannin treatment. By contrast, KIAA0538 and MRASAL (R591L) proteins were present in the cytosol. Our data indicate that the distinct phosphoinositide binding specificity of the PH domain is attributable to the distinct subcellular localization of the GAP1 family.

Key Words: **pleckstrin homology domain; GAP1; RasGAP; phosphoinositide; plasma membrane localization.**

Abbreviations used: Btk, Bruton's tyrosine kinase; GAP, GTPase-activating protein; GST, glutathione *S*-transferase; GRD, GAP-related domain; IP_4, inositol 1,3,4,5-tetrakisphosphate; MRASAL, murine Ras GTPase-activating-like; PC, phosphatidylcholine; PH, pleckstrin homology; PI, phosphatidylinositol.

[1] This work was supported in part by grants from the Science and Technology Agency to Japan (to K.M.) and Grants 13780624 from the Ministry of Education, Science, and Culture of Japan (to M.F.).

[2] To whom correspondence should be addressed at Laboratory for Developmental Neurobiology, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Fax: +81-48-467-9744. E-mail: mnfukuda@brain.riken.go.jp.

The GAP1 family is one of the Ras GTPase-activating protein families (GAP) and now consists of four proteins (1, 2), GAP1^m (3, 4), GAP1^{IP4BP}/GAPIII/R-Ras GAP (5–7) MRASAL (murine Ras GTPase-activating-like) (8) and KIAA0538 (9) in mammals (Fig. 1A). All members contain five protein motifs, amino-terminal Ca^{2+}-binding C2A and C2B domains, a central GAP-related domain (GRD), a carboxyl-terminal pleckstrin homology (PH) domain and a Bruton's tyrosine kinase (Btk) motif (3, 10, 11). The PH domain is a less conserved small protein module (approximately 100 amino acids) often found in proteins involved in signal transduction and cytoskeletal structures (reviewed in Refs. 12 and 13). It is thought to be essential for membrane anchoring by binding of specific phosphoinositides and/or specific proteins (e.g., actin and G$\beta\gamma$) (12, 13).

The PH domain of GAP1^{IP4BP} showed binding activity toward inositol 1,3,4,5-tetrakisphosphate (IP_4), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) and PI(3,4,5)P_3 (5, 14), while the PH domain of GAP1^m showed binding activity toward IP_4 and PI(3,4,5)P_3 (4, 15). Because of the differences in the specificities for phosphoinositides, GAP1^{IP4BP} is constitutively localized at the plasma membrane via binding to PI(4,5)P_2, whereas GAP1^m is mainly localized in the cytosol and translocated to the plasma membrane in a PI(3,4,5)P_3-dependent manner (15, 16). By analogy with the structure of other PH domains, combined with mutational analysis, the amino-terminal positively charged amino acid cluster (KKR) in the PH domain (Fig. 1B) is essential for high-affinity phosphoinositide binding (4,

0006-291X/01 $35.00

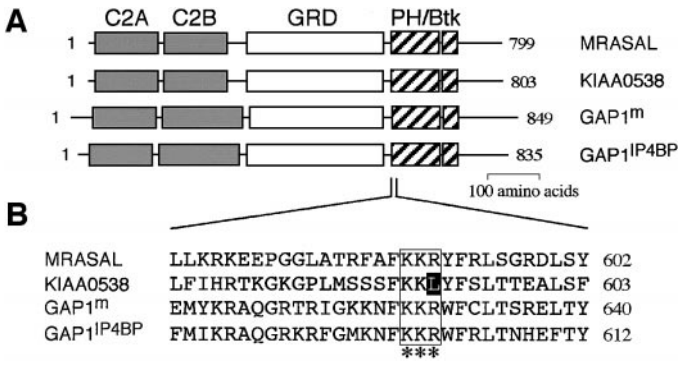

FIG. 1. Structure of the GAP1 family. (A) A schematic representation of GAP1 family proteins, MRASAL, KIAA0538, GAP1^{m}, and GAP1^{IP4BP}. They consist of the amino-terminal C2A and C2B domains (shaded boxes), a central GAP-related domain (GRD) (open boxes), and a carboxyl-terminal PH domain and a Btk motif (slashed boxes). (B) Alignment of the amino-terminal region of the PH domain in GAP1 family proteins. Asterisks indicate the essential residues for high affinity phosphoinositide binding in GAP1^{m}, GAP1^{IP4BP}, and Btk (4, 10, 17, 25). Note the Leu occupying at amino acid position 592 instead of Arg in KIAA0538 (black background). Amino acid numbers are given on right side.

17, 18). Mutation of these amino acids (e.g., KKR to QQQ) resulted in the cytosolic localization of GAP1^{IP4BP} mutant proteins (15) as well as loss of phosphoinositide binding (17). Thus, the PH domains of GAP1^{IP4BP} and GAP1^{m} are crucial for membrane anchoring via binding of phosphoinositides.

Nucleotide sequences of two other GAP1 members (MRASAL and KIAA0538) were recently reported (8, 9), but their functions and subcellular localizations remain to be completely unknown. No research has focused on the function of the PH domains of these GAP1 members to date. In this paper we characterized the PH domain of MRASAL, its point mutant (R591L) and KIAA0538 in terms of phosphoinositide binding, and examined their subcellular localizations in NIH3T3 cells. The MRASAL PH domains showed phosphoinositide binding activity similar to that of the GAP1^{IP4BP} PH domain, whereas the KIAA0538 PH domain completely lacked this activity, resulting in cytosolic localization of the KIAA0538 protein. Based on our results, we discussed the functional diversity of the GAP1 family.

MATERIALS AND METHODS

Materials. A full-length human cDNA clone of KIAA0538 (GenBank Accession No. AB011110) was obtained from Kazusa DNA Research Institute (Chiba, Japan). KIAA0538 is a 6169-bp cDNA with an 18-bp poly(A)$^{+}$ stretch, which was inserted at the *Sal*I–*Not*I site of the pBluescript II SK^{+} vector (Stratagene, La Jolla, CA). Phosphatidylcholine (PC) was purchased from Sigma (St. Louis, MO). PI(3,4)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 were from Alexis Biochemicals (San Diego, CA). All other chemicals were commercial products of reagent grade.

cDNA cloning of MRASAL. The full-length cDNA of MRASAL was amplified by polymerase chain reaction from adult mouse brain Marathon-Ready cDNA (Clontech Laboratories, Inc., Palo Alto, CA) (19) using the following primers: primer 1 (sense, amino acid residues 1–6), 5′-GGATCCATGGCCAAGAGCGGCTC-3′; primer 2 (sense, amino acid residues 98-104), 5′-GGATCCCCTCGAGGGATCGACAGCTGG-3′; primer 3 (sense, amino acid residues 220–224), 5′-GGATCCCAGACCCTGCAGCA-3′; primer 4 (sense, amino acid residues 322–329), 5′-GGATCCTCTAGATTATCTCACAAGGCGTGAG-3′; primer 5 (sense, amino acid residues 572–577), 5′-GGATCCCTGCTGAGCGCAAGGAG-3′; primer 6 (antisense, amino acid residues 215–219), 5′-TGGGGTGAACTCCACC-3′; primer 7 (antisense, amino acid residues 421–428), 5′-GAGCTCACGATGGCGTCCACAAC-3′; primer 8 (antisense, amino acid residues 565–571), 5′-GAAGCCTTTCTCGAACAATGGTTG-3′; and primer 9 (antisense, amino acid residues 795–799), 5′-CTATCCCCGTTTCTGGAAC-3′.

Site-directed mutagenesis. The phospholipid binding negative mutant of the MRASAL PH domain carrying an Arg-to-Leu substitution at amino acid position 591 (R591L) was generated by polymerase chain reaction with the following two primers containing the desired mutation as described previously (20): 5′-CTTCAAGAAGCTCTACTTCCGGC-3′ and 5′-GCCGGAAGTAGAGCTTCTTGAAG-3′.

Protein synthesis and purification. The cDNAs of the MRASAL, MRASAL(R591L) and KIAA0538 PH domains were subcloned into the pGEX 4T-3 vector (Amersham Pharmacia Biotech, Buckinghamshire, UK). They were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* JM109 and purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's notes.

Phospholipid binding assay. Liposomes (phosphatidylcholine (PC), 50 μg) containing 2 μg of various phosphoinositides were mixed with 5 μg of the GST-PH domain and cosedimented essentially as described previously (21, 22).

Cell culture, transfection, and immunocytochemistry. NIH3T3 cells were cultured in Dulbecco's modified Eagle medium (Sigma) containing 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C. Full-length MRASAL, MRASAL(R591L), and KIAA0538 were subcloned into the T7-tagged pEF-BOS mammalian expression vector as described previously (19, 23, 24). These constructs were transfected into NIH3T3 cells by the lipofection method according to the manufacturer's instructions (Life Technologies, Rockville, MD), and cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Twenty four hours after transfection, cells cultured on coverslips were fixed with 3.7% formaldehyde in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and permeabilized with 0.2% Triton X-100 in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline for 5 min. The cells were incubated with anti-T7 monoclonal antibody (1/1000 dilution; Novagen, Madison, WI) visualized by a second antibody (1/10,000 dilution; anti-mouse Alexa 488 antibodies; Molecular Probes, Eugene, OR) or Texas Red-conjugated phalloidin (1/200 dilution; Molecular Probes) for 1 h at room temperature. The cells were then observed with a confocal fluorescence microscope (Fluoview, Olympus, Tokyo, Japan).

RESULTS AND DISCUSSION

Distinct Phosphoinositide Binding Activity of the PH Domains of MRASAL and KIAA0538

To examine the phosphoinositide binding properties of the PH domains of MRASAL and KIAA0538, we produced their PH domains as GST fusion proteins and performed phosphoinositide binding assays by a conventional sedimentation method (21, 22). In brief,

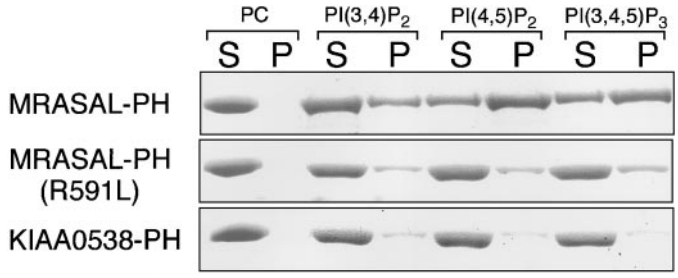

FIG. 2. Specific binding of PH domains of MRASAL, MRASAL(R591L), and KIAA0538 to phospholipids. Cosedimentation assays were performed essentially as described previously (21, 22). Liposomes (PC, 50 μg) containing 2 μg of each of the indicated phosphoinositides were mixed with 5 μg of GST-PH domains and cosedimented by centrifugation. Proteins in the supernatant (S; nonbinding fraction) and precipitate (P; phosphoinositide binding fraction) were analyzed by SDS–polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue R-250.

electrophoretically pure GST-MRASAL-PH (or GST-KIAA0538-PH) and liposomes containing the respective phosphoinositides ($PI(3,4)P_2$, $PI(4,5)P_2$ or $PI(3,4,5)P_3$) were incubated at room temperature for 15 min. Phosphoinositide binding (pellets (P) in Fig. 2) and nonbinding (supernatant (S) in Fig. 2) fractions were separated by centrifugation, and analyzed by SDS–polyacrylamide gel electrophoresis. The PH domain of MRASAL bound liposomes containing either $PI(4,5)P_2$ or $PI(3,4,5)P_3$ (Fig. 2, top panel), as did $GAP1^{IP4BP}$ (14). To our surprise, however, the PH domain of KIAA0538 completely lacked such activity (Fig. 2, bottom panel), even though the PH domains of MRASAL and KIAA0538 showed high sequence similarity (more than 49% identity). To identify the amino acid responsible for the loss of phosphoinositide binding in KIAA0538, we compared the amino acid sequences of the PH domains of GAP1 family members (Fig. 1B). In our previous study, we determined that the amino-terminal basic cluster (KKR) is essential for high affinity phosphoinositide binding in $GAP1^m$ (4) and Btk (10, 25). We noticed that in KIAA0538 Leu occupies amino acid position 592 instead of Arg (Fig. 1B, black background). To further examine whether Arg-to-Leu substitution is sufficient for the loss of phosphoinositide binding activity, we introduced a point mutation in MRASAL (R591L). As expected, the PH domain of MRASAL (R591L) showed no phosphoinositide binding activity (Fig. 2, second panel). Thus, the phosphoinositide binding activity loss of KIAA0538 can be explained by the point mutation of a critical basic residue in the amino-terminus of the PH domain. Since the same variation was observed in the murine orthologue of KIAA0538 in the EST sequence databases (accession number AK014220), lack of phosphoinositide binding activity is not a unique event in humans, and is probably present, at least, in other mammals.

Distinct Subcellular Localization of MRASAL and KIAA0538 in NIH3T3 Cells

Next, we examined the relationship between the phosphoinositide binding activity and subcellular localization of MRASAL and KIAA0538 proteins in the cell. When pEF-T7-MRASAL, -KIAA0538, or -MRASAL(R591L) was transiently transfected into NIH3T3, MRASAL proteins were localized at the plasma membrane, whereas KIAA0538 and MRASAL(R591L) proteins were localized in the cytosol (Fig. 3A, left panels), suggesting an impor-

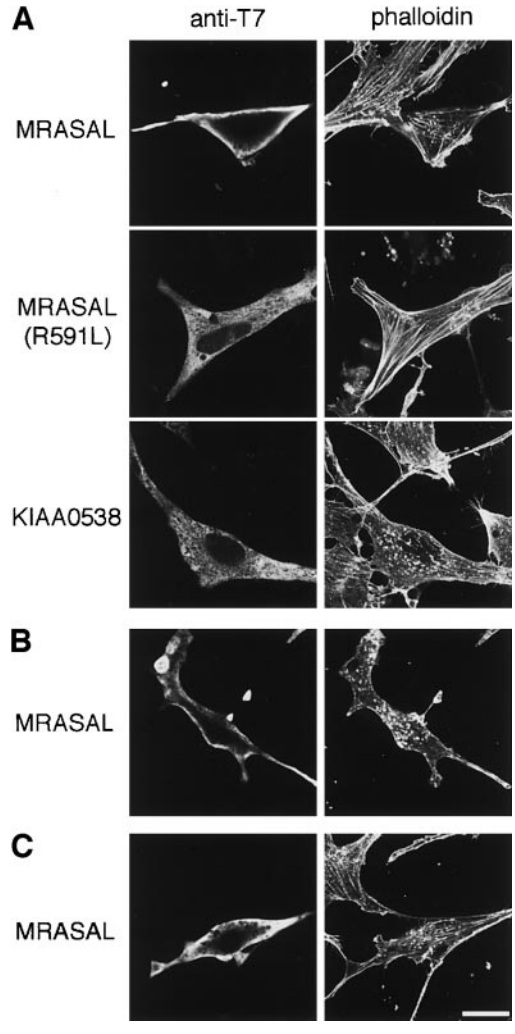

FIG. 3. Expressions of full length MRASAL, MRASAL(R591L), and KIAA0538 in NIH3T3 cells. (A) The T7-tagged full length MRASAL, KIAA0538, and MRASAL(R591L) were expressed in NIH3T3 cells. The cells were stained with anti-T7 monoclonal antibody (left) and Texas Red-conjugated phalloidin (right). (B) The T7-tagged full length of MRASAL was expressed in NIH3T3 cells. After treatment with 500 nM cyotochalasin B for 1 h, the cells were fixed with 3.8% formaldehyde in PBS and stained with anti-T7 monoclonal antibody (left) and Texas Red-conjugated phalloidin (right). Note that the localizations of MRASAL proteins were unchanged in the NIH3T3 cells even after cytochalasin B treatment. (C) The localizations of MRASAL proteins (left) and actin (right) in the NIH3T3 cells after treatment with 10 μM wortmannin for 1.5 h. Scale bar, 20 μm.

tant role of phosphoinositide binding activity in membrane anchoring. Recently, however, it was reported that the PH domain of Btk binds F-actin via its N-terminal basic region, which is also important for phosphoinositide binding (26), suggesting another possibility that MRASAL proteins localize to the membrane by binding to F-actin at the PH domain. Indeed, a portion of MRASAL immunostainings did overlap with Texas Red phalloidin (Fig. 3A, right panels). To rule out this possibility, we treated the cells with cytochalasin B which is known to disassemble F-actin. As shown in Fig. 3B, the localizations of MRASAL proteins were unchanged in NIH3T3 cells even after cytochalasin B treatment. Furthermore, the localizations of MRASAL proteins were insensitive to wortmannin, a PI-3 kinase inhibitor, treatment (Fig. 3C). Thus, we concluded that MRASAL proteins localize to the plasma membrane in a phospholipid ($PI(4,5)P_2$)-dependent manner via the PH domain.

CONCLUSIONS

In this study we characterized the phosphoinositide binding activity of two newly-identified members of the GAP1 family. Our results indicated that GAP1 family proteins can be divided into three groups based on the phosphoinositide binding capacities of their PH domains. The first group contains $GAP1^{IP4BP}$ and MRASAL, both of which show binding activity toward $PI(4,5)P_2$ and $PI(3,4,5)P_3$ with similar affinity and are constitutively localized at the plasma membrane by binding to $PI(4,5)P_2$ (ref. 14 and this study). The second group consists of $GAP1^m$, which shows binding activity toward $PI(3,4,5)P_3$, but not $PI(4,5)P_2$ and is translocated to the plasma membrane in a $PI(3,4,5)P_3$-dependent manner (Ref. 14). KIAA0538, which is included in the third group, lacks phosphoinositide binding capacity, due to the amino acid substitution at 592 (Leu-592) (this study). Based on these findings, we propose that although the GAP1 family proteins share the same domain structure, they appear to regulate Ras differently because of their distinct phosphoinositide binding capacities and distinct plasma membrane targeting mechanisms. In the course of preparing the manuscript, Lockyer *et al.* reported that KIAA0538/CAPRI (Ca^{2+}-promoted Ras inactivator) is present in the cytosol which is consistent with our results, but is translocated to the plasma membrane via its C2 domain following a stimulus that elevates intracellular Ca^{2+} (27). Thus, GAP1 family members may act differently as Ras effectors in response to the specific stimuli (e.g., elevation of $PI(3,4,5)P_3$ and Ca^{2+}).

ACKNOWLEDGMENTS

We thank Drs. T. Nagase and O. Ohara (Kazusa DNA Research Institute, Kisarazu, Chiba, Japan) for kindly donating a cDNA clone of KIAA0538.

REFERENCES

1. Cullen, P. J. (1998) *Biochim. Biophys. Acta* **1436,** 35–47.
2. Fukuda, M., and Mikoshiba, K. (1997) *BioEssays* **19,** 593–603.
3. Maekawa, M., Li, S., Iwamatsu, A., Morishita, T., Yokota, K., Imai, Y., Kohsaka, S., Nakamura, S., and Hattori, S. (1994) *Mol. Cell. Biol.* **14,** 6879–6885.
4. Fukuda, M., and Mikoshiba, K, (1996) *J. Biol. Chem.* **271,** 18838–18842.
5. Cullen, P. J., Hsuan, J. J., Truong, O., Letcher, A. J., Jackson, T. R., Dawson, A. P., and Irvine, R. F. (1995) *Nature* **376,** 527–530.
6. Baba, H., Fuss, B., Urano, J., Poullet, P., Watson, J. B., Tamanoi, F., and Macklin, W. B. (1995) *J. Neurosci. Res.* **41,** 846–858.
7. Yamamoto, T., Matsui, T., Nakafuku, M., Iwamatsu, A., and Kaibuchi, K. (1995) *J. Biol. Chem.* **270,** 30557–30561.
8. Allen, M., Chu, S., Brill, S., Stotler, C., and Buckler, A. (1998) *Gene* **218,** 17–25.
9. Nagase, T., Ishikawa, K., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N., and Ohara, O. (1998) *DNA Res.* **5,** 31–39.
10. Fukuda, M., Kojima, T., Kabayama, H., and Mikoshiba, K (1996) *J. Biol. Chem.* **271,** 30303–30306.
11. Smith, C. I. E., Islam, T. C., Mattsson, P. T., Mohamed, A. J., Nore, B. F., and Vihinen, M. (2001) *BioEssays* **23,** 436–446.
12. Rebecchi, M. J., and Scarlata, S. (1998) *Annu. Rev. Biophys. Biomol. Struct.* **27,** 503–528.
13. Shaw, G. (1996) *BioEssays* **18,** 35–46.
14. Cozier, G. E., Lockyer, P. J., Reynolds, J. S., Kupzig, S., Bottomley, J. R., Millard, T. H., Banting, G., and Cullen, P. J. (2000) *J. Biol. Chem.* **275,** 28261–28268.
15. Lockyer, P. J., Bottomley, J. R., Reynolds, J. S., McNulty, T. J., Venkateswarlu, K., Potter, B. V., Dempsey, C. E., and Cullen, P. J. (1997) *Curr. Biol.* **7,** 1007–1010.
16. Lockyer, P. J., Wennstrom, S., Kupzig, S., Venkateswarlu, K., Downward, J., and Cullen, P. J. (1999) *Curr. Biol.* **9,** 265–268.
17. Bottomley, J. R., Reynolds, J. S., Lockyer, P. J., and Cullen, P. J. (1998) *Biochem. Biophys. Res. Commun.* **250,** 143–149.
18. Cozier, G., Sessions, R., Bottomley, J. R., Reynolds, J. S., and Cullen, P. J. (2000) *Biochem. J.* **349,** 333–342.
19. Fukuda, M., Kanno, E., and Mikoshiba, K. (1999) *J. Biol. Chem.* **274,** 31421–31427.
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) Current Protocols in Molecular Biology, Wiley, New York.
21. Fukuda, M., Kojima, T., and Mikoshiba K. (1996) *J. Biol. Chem.* **271,** 8430–8434.
22. Fukuda, M., Kojima, T., and Mikoshiba, K. (1997) *Biochem. J.* **323,** 421–425.
23. Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S., and Mikoshiba, K. (1994) *J. Biol. Chem.* **269,** 29206–29211.
24. Mizushima, S., and Nagata, S. (1990) *Nucleic Acids Res.* **18,** 5322.
25. Kojima, T., Fukuda, M., Watanabe, Y., Hamazato, F., and Mikoshiba, K. (1997) *Biochem. Biophys. Res. Commun.* **236,** 333–339.
26. Yao, L., Janmey, P., Frigeri, L. G., Han, W., Fujita, J., Kawakami, Y., Apgar, J. R., and Kawakami, T. (1999) *J. Biol. Chem.* **274,** 19752–19761.
27. Lockyer, P. J., Kupzig, S., and Cullen, P. J. (2001) *Curr. Biol.* **11,** 981–986.