Involvement of Rabphilin3 in Endocytosis through Interaction with Rabaptin5

(Received for publication, August 19, 1997, and in revised form, October 20, 1997)

Takeshi Ohya, Takuya Sasaki, Masaki Kato, and Yoshimi Takai

From the Department of Molecular Biology and Biochemistry, Osaka University Medical School, Suita 565, Japan

Rabphilin3 and rabaptin5 are downstream target molecules of the Rab3 and -5 subfamily small G proteins that are implicated in exocytosis and endocytosis, respectively. We examined here the physical and functional relationship between the Rab3-rabphilin3 and Rab5-rabaptin5 systems. Rabphilin3 interacted with rabaptin5 at the N-terminal region (amino acids 1–280), which GTP-Rab3A interacted with. The interaction of rabphilin3 with rabaptin5 was inhibited by guanosine 5′-(3-O-thio)triphosphate-Rab3A. Overexpression of the N-terminal fragment of rabphilin3 (amino acids 1–280) inhibited the receptor-mediated endocytosis of transferrin, and this inhibition was overcome by co-transfection with a dominant active mutant of Rab3A or rabaptin5 in PC12 and HeLa cells. These results suggest that rabphilin3, free of GTP-Rab3A, regulates endocytosis through interaction with rabaptin5 after rabphilin3 complexed with GTP-Rab3A regulates exocytosis.

Endocytosis is often coupled with exocytosis, particularly at nerve terminals (for reviews, see Refs. 1 and 2). Molecular mechanisms of exocytosis and endocytosis have extensively been investigated, but the coupling mechanism between these two events is unknown. The Rab family small G proteins are implicated in intracellular vesicle trafficking, such as exocytosis and endocytosis (for reviews, see Refs. 3–5). The Rab3 subfamily consists of at least two functionally different domains: the N-terminal C1-like domain interacting with rabphilin3. Rabphilin3 consists of at least two functionally different domains: the N-terminal C1-like domain responsible for interaction with GTP-Rab3, and the C-terminal two C2-like domains responsible for interaction with Ca2+ and phospholipid (20, 21). We have found that β-adducin, known to be involved in the assembly of spectrin-actin complexes (for a review, see Ref. 22), interacts with the C-terminal region of rabphilin3 in the presence of Ca2+ and phospholipid (23, 24) and that α-actinin, known to cross-link actin filaments into a bundle (for reviews, see Refs. 25 and 26), interacts with the N-terminal region of rabphilin3 in a manner competitive with GTP-Rab3 (27). Here, we have found that rabphilin3 interacts with rabaptin5 at the N-terminal region and that this interaction is inhibited by GTP-Rab3A. We have furthermore shown that rabphilin3 is involved in endocytosis.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Bovine Rab3A and rabphilin3 were purified from the membrane fraction of Spodoptera frugiperda Sf9 cells (Sf9 cells) overexpressing each protein (28, 29). GDP-Rab3A and GTP-S-Rab3A were prepared as described (30). Truncated rat rabaptin5, which corresponds to an amino acid position from 385 to 753 of human rabaptin5 (GenBank accession number X91141), was expressed as a GST-fusion protein and purified from overexpressing Escherichia coli. Anti-HA and anti-myc mouse monoclonal antibodies and an anti-rabphilin3 rabbit polyclonal antibody were prepared as described (31–33).

Construction of Expression Vectors—Mammalian expression plasmids, pEFOBS-HA and pEFOBS-myc, were generated to express fusion proteins with the N-terminal HA and myc epitopes, respectively (33, 34).
Another mammalian expression plasmid, pGEM-HA, was generated to express a fusion protein with the N-terminal HA epitope, using the T7 RNA polymerase recombinant vaccinia virus (LO-T7) (35). The cDNA fragments encoding full-length rabphilin3 and its N-terminal fragment (1-280 aa) were inserted into pGEM-HA and pEFBOS-HA, respectively. The DNA fragment encoding truncated rabaptin5 was inserted into pGEM-HA and pEFBOS-myc. The cDNA fragments encoding human transferrin receptor and a dominant active mutant of Rab3A (Rab3AQ81L) were inserted into pEFBOS-myc.

Strains and Media—Yeast strain L40 (MATa trp1 leu2 his3 lys2::lexA-HIS3 ura3::lexA-lacZ) was grown on YPDAU medium that contained 2% glucose (Difco), 1% Bacto-peptone (Difco), 0.04% adenine sulfate, and 0.02% uracil. Yeast transformations were performed by the lithium acetate method (36). Transformants were selected on SD medium that contained 2% glucose and 0.7% yeast nitrogen base without amino acids, and amino acids (Difco) were supplemented to SD medium when required. Standard yeast genetic manipulations were performed as described (37). An E. coli strain DH5α was used for construction and propagation of plasmids.

Screening for Rabphilin3-interacting Molecules by the 2-hybrid Method—A strain L40 was transformed with a derivative of pBTM116 bearing the N-terminal fragment (1-280 aa) of rabphilin3 fused to the LexA DNA-binding domain: pBTM116-rabphilin3-N. A strain L40 carrying pBTM116-rabphilin3-N was transformed with the library DNA (Mouse brain library: Clontech, Inc.) or a plasmid library in pGAD10, CLONTECH). Approximately 1.1 × 10^8 transformants were screened for the growth on SD plate media lacking tryptophan, leucine, and histidine, but containing 0.5 μm 3-amino-1,2,4-triazole, which is a specific indicator of the HIS3 gene product. His+ colonies were then plated on the nitrocellulose filter and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for β-galactosidase activity as described (38). From the 50 His+ and lacZ+ positive clones obtained at this screening, library plasmids were recovered through E. coli transformation. The recovered plasmids were transformed again into L40 containing pBTM116-rabphilin3-N, and it was found that 14 clones conferred the His+ and lacZ+ phenotypes on L40 containing pBTM116-rabphilin3-N. The nucleotide sequences of the insert DNA of these 14 clones were determined.

Assay for Rabphilin3-Rabaptin5 Interactions in an Intact Cell System—PC12 cells were plated at a density of 1.5 × 10^5 cells/35-mm dish and then incubated for 18 h at 37°C. The cells were infected with T7 RNA polymerase recombinant vaccinia virus (LO-T7) for 30 min and then transfected with 2 μg of pGEM-HA-rabaptin5, 2 μg of pGEM-HA-rabphilin3, or both the plasmids, by use of LipofectAMINE reagent (Life Technologies, Inc.) (35). Immunoprecipitation was performed at 4 h after the transfection. The cells were washed with phosphate-buffered saline and lysed in 0.5 ml per dish of a lysis buffer containing 10 mM Tris/HCl at pH 7.5, 150 mM NaCl, 1.5% Nonidet P-40, 0.1% sodium deoxycholate, and 1% EGTA. The cell lysate was subjected to immunoprecipitation with 10 μmol of the anti-rabphilin3 antibody bound to 25 μl of protein A-Sepharose (Pharmacia Biotech Inc.). Comparable amounts of the cell pellets were subjected to SDS-PAGE, followed by immunoblot analysis with the anti-HA antibody.

Assay for Rabphilin3-rabaptin5 Interactions in a Cell-free System—Full-length rabphilin3 (50 pmol) was incubated with GST or GST-rabaptin5 (100 pmol) bound to glutathione-Sepharose in a buffer containing 20 mM Tris/HCl at pH 7.5, 1 mM dithiothreitol, 5 mM MgCl2, 0.5% Nonidet P-40, and 20 mM NaCl for 90 min at 4°C in the presence or absence of 0.4 μM GDP-Rab3A or GTPγS-Rab3A. After the beads were washed four times with the same buffer, the protein beads were eluted by addition of 100 μl of a buffer containing 20 mM Tris/HCl at pH 8.0, 1 mM dithiothreitol, 5 mM MgCl2, 0.5% Nonidet P-40, 20 mM NaCl, and 10 mM reduced glutathione. The eluates were subjected to SDS-PAGE, followed by protein staining with Coomassie Brilliant Blue.

Assay for the Receptor-mediated Endocytosis in PC12 and HeLa Cells—PC12 or HeLa cells were transfected with the plasmids encoding the indicated proteins by use of Lipofectin reagent (Life Technologies, Inc.). After 48 h, the cells were incubated for 60 min at 37°C with serum-free Dulbecco’s modified Eagle’s medium containing 0.1 mg/ml FITC-transferin (Molecular Probes, Inc.). The cells were then fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. The receptors were detected by use of anti-HA and anti-myc antibodies as the first antibodies, respectively, and rhodamine-conjugated anti-mouse donkey antibody (Chemicon International) as the second antibody. To examine the co-expression of the HA- and myc-tagged proteins, rhodamine-conjugated anti-HA antibody and FITC-conjugated anti-myc antibodies were used. The cells were analyzed with a LSM 410 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). To examine whether the expression level of the indicated protein was affected by co-expression of the other proteins, transfected HeLa cells were lysed, and the expression levels of the HA-tagged and myc-tagged proteins were examined by immunoblot analysis of whole cell lysates using the anti-HA and anti-myc antibodies, respectively.

RESULTS

Interaction of Rabphilin3 with Rabaptin5 Estimated by the Yeast Two-Hybrid System.—We first attempted to isolate rabphilin3-interacting proteins by use of the yeast two-hybrid system with the N-terminal fragment or the C-terminal fragment of rabphilin3 as a bait from a rat brain cDNA library. We did not isolate any clones with the C-terminal fragment as a bait. But with the N-terminal fragment as a bait, we isolated several clones and selected one clone (N22) encoding a protein, which corresponded to a human cDNA clone from 385 to 753 of human rabaptin5, because rabaptin5 is known to be the downstream target molecule of the Rab5 subfamily implicated in endocytosis (3, 15–18). The isolated clone indeed interacted with the N-terminal fragment of rabphilin3 by the yeast two-hybrid method, and this interaction was comparable to the interaction of Rab3A with rabphilin3 (Fig. 1A). This clone also

---

FIG. 1. Rabphilin3-rabaptin5 interactions estimated by the yeast two-hybrid system. A, interaction of rabphilin3 with isolated clone N22. Yeast strains expressing LexA-N-terminal fragment (1-280 aa) of rabphilin3 or LexA alone were transformed with a plasmid encoding GAD, GAD-clone N22, or GAD-Rab3A. The resultant transformants were stained with β-galactosidase activity. B, interaction of rabaptin5 with full-length rabphilin3 and its N-terminal fragment but not with its C-terminal fragment. Yeast strains expressing LexA-N-terminal fragment (1-280 aa), LexA-rabphilin3 (1-704 aa), or LexA-C-terminal fragment (396-704 aa) were transformed with a plasmid encoding GAD-clone N22. The resultant transformants were stained with β-galactosidase activity. The results shown are representative of three independent experiments.
interacted with full-length rabphilin3 but not with its C-terminal fragment (Fig. 1B).

Rabphilin3-Rabaptin5 Interactions in an Intact Cell System—The rabphilin3-rabaptin5 interactions were confirmed by co-immunoprecipitation of HA-tagged full-length rabphilin3 and HA-tagged truncated rabaptin5 from cultured PC12 cells. PC12 cells were transiently transfected with the plasmid encoding HA-tagged rabphilin3 alone or HA-tagged rabaptin5 alone, or with both the plasmids. The cells were lysed, and the lysates were incubated with the anti-rabphilin3 antibody, which was immobilized on protein A-Sepharose. The immunoprecipitates were subjected to SDS-PAGE, followed by immunoblotting with the anti-HA antibody. Lane 1, with rabaptin5 alone; lane 2, with rabphilin3 alone; lane 3, with rabaptin5 and rabphilin3; arrowhead, HA-tagged rabaptin5; arrow, HA-tagged rabphilin3. The results shown are representative of three independent experiments.

Inhibition by GTPγS-Rab3A of the Rabphilin3-Rabaptin5 Interactions—The rabphilin3-rabaptin5 interactions were further confirmed by use of the recombinant proteins. GST-rabaptin5 was prebound to glutathione-Sepharose, and the binding of rabphilin3 to this prebound protein was analyzed. Rabaptin5 indeed interacted with rabphilin3 (Fig. 2, lanes 1 and 2).

Because rabaptin5 interacted with the N-terminal region of rabphilin3, with which GTP-Rab3A interacts (20, 21, 27), the effect of GTPγS-Rab3A on the rabphilin3-rabaptin5 interactions was examined next. Rabphilin3 was incubated with rabaptin5 in the presence of GTPγS-Rab3A or GDP-Rab3A, and the interaction of rabphilin3 with rabaptin5 was analyzed. Only a small amount of rabphilin3 interacted with rabaptin5 in the presence of GTPγS-Rab3A, indicating that GTP-Rab3A inhibited the interaction of rabphilin3 with rabaptin5 (Fig. 3, lanes 2 and 4). Because GDP-Rab3A weakly bound to rabphilin3 (11), the rabphilin3-rabaptin5 interactions were slightly inhibited by GDP-Rab3A (Fig. 3, lanes 2 and 3).

Inhibition of the Receptor-mediated Endocytosis of Transferrin by Rabphilin3—Finally, effect of rabphilin3 on the receptor-mediated endocytosis of transferrin was examined. PC12 cells were transiently transfected with the plasmid encoding myc-tagged human transferrin receptor alone or co-transfected with the plasmid encoding the HA-tagged N-terminal fragment of rabphilin3 and then incubated with FITC-transferrin. The expressed myc-tagged human transferrin receptor and HA-tagged N-terminal fragment of rabphilin3 were detected by confocal immunofluorescence microscopy using the anti-myc and anti-HA antibodies, respectively. The FITC-transferrin incorporated into the cells by endocytosis was examined by confocal immunofluorescence microscopy. In the cells expressing myc-tagged human transferrin receptor alone, FITC-transferrin was markedly incorporated into the cells (Fig. 4A, 1 and 2). However, in the cells co-expressing both myc-tagged human transferrin receptor and the HA-tagged N-terminal fragment of rabphilin3, the incorporation of FITC-transferrin into the cells was not detected (Fig. 4A, 3 and 4). This inhibitory action of the N-terminal fragment of rabphilin3 was overcome by co-expression with a dominant active mutant of Rab3A (Rab3AQ81L) (Fig. 4A, 5 and 6) or rabaptin5 (Fig. 4A, 7 and 8). These data were obtained in about 90% of the cells expressing the HA-tagged N-terminal fragment of rabphilin3.

The co-expression of the HA-tagged N-terminal fragment of rabphilin3 and myc-tagged human transferrin receptor was confirmed by the anti-HA and anti-myc antibodies, respectively (Fig. 4A, 9 and 10). The expression of the Rab3A mutant and rabaptin5 was confirmed by confocal immunofluorescence microscopy using the anti-myc antibody (data not shown).

Similar results were obtained in HeLa cells. Because this cell line expresses endogenous human transferrin receptor, FITC-transferrin was incorporated into the cells by this endogenous receptor. In the cells expressing the HA-tagged N-terminal fragment of rabphilin3, the incorporation of FITC-transferrin into the cells was not detected, whereas FITC-transferrin was incorporated into the non-expressing cells (Fig. 4B, 1 and 2). This inhibitory action was overcome by co-expression with a dominant active mutant of Rab3A (Rab3AQ81L) (Fig. 4B, 3 and 4) or rabaptin5 (Fig. 4B, 5 and 6). The expression level of the HA-tagged N-terminal fragment of rabphilin3 was not impaired by co-expression of the Rab3A mutant or rabaptin5 (Fig. 4C). Overexpression of the HA-tagged C-terminal fragment of rabphilin3 alone did not inhibit the incorporation of FITC-transferrin into the cells (Fig. 4B, 7 and 8).

**DISCUSSION**

We have shown here by use of three different methods, the yeast two-hybrid, affinity column chromatography, and immunoprecipitation, that rabphilin3 directly interacts with rabaptin5. Rabaptin5 interacts with the N-terminal region of rabphilin3, and this interaction is inhibited by GTP·γ·S-Rab3A. The precise mode of action of the Rab3-rabphilin3 system in Ca²⁺-
Rabphilin3 and Endocytosis

**Fig. 4. Inhibition of the receptor-mediated endocytosis of transferrin by rabphilin3.** PC12 or HeLa cells, which were transiently transfected with the plasmids encoding the indicated proteins, were incubated with FITC-transferrin. The cells were examined by the confocal immunofluorescence microscopy. A, PC12 cells. Shown are panels 1 and 2, with myc-tagged human transferrin receptor (myc-hTR) alone; 3 and 4, with myc-hTR and the HA-tagged N-terminal fragment of rabphilin3 (HA-Rp-N); 5 and 6, with myc-hTR, HA-Rp-N, and the myc-tagged dominant active mutant of Rab3A (myc-Rab3AQ81L); 7 and 8, with myc-hTR, HA-Rp-N, and myc-tagged rabaptin5; 9 and 10, with myc-hTR and HA-Rp-N; panels 1 and 10, for the myc epitope; 2, 4, 6, and 8, for FITC-transferrin; 3, 5, 7, and 9, for the HA epitope. B, HeLa cells. Shown are panels 1 and 2, with HA-Rp-N; 3 and 4, with HA-Rp-N and myc-Rab3AQ81L; 5 and 6, with HA-Rp-N and myc-tagged rabaptin5; 7 and 8, with the HA-tagged C-terminal fragment of rabphilin3; 9, 5, 5, and 7, for the HA epitope. C, the expression levels of the HA-tagged N-terminal fragment of rabphilin3, the myc-tagged dominant active mutant of Rab3A, and myc-tagged rabaptin5. HeLa cells were transiently transfected with the plasmid encoding the HA-tagged N-terminal fragment of rabphilin3 alone or co-transfected with the plasmid encoding the myc-tagged dominant active mutant of Rab3A or myc-tagged rabaptin5. The cells were lysed, and the expression levels of the HA-tagged and myc-tagged proteins were examined by the immunoblot analysis of whole cell lysates using the anti-HA and anti-myc antibodies, respectively. Shown are lane 1, with HA-Rp-N; lane 2, with HA-Rp-N and myc-Rab3AQ81L; lane 3, with HA-Rp-N and myc-tagged rabaptin5; arrow, HA-Rp-N; closed arrowhead, myc-tagged rabaptin5; and open arrowhead, myc-Rab3AQ81L. The results shown are representative of three independent experiments.

Endocytosis is often coupled with exocytosis, especially at nerve terminals (1, 2). The mechanism of this type of endocytosis remains a matter of debate, but several lines of evidence implicate that endocytosis of synaptic vesicles appears to resemble receptor-mediated endocytosis mediated by latyrin-coated vesicles in non-neuronal cells (for a review, see Ref. 41). However, since endocytosis of synaptic vesicles is faster than receptor-mediated endocytosis in non-neuronal cells and is not inhibited by decrease in the pH or the K+ concentration which disrupt receptor-mediated endocytosis (42), endocytosis of synaptic vesicles is considered a specialization of receptor-mediated endocytosis. Therefore, our present results used here indicate that the Rab3-rabphilin3 system is involved in receptor-mediated endocytosis, but it is not known whether rabphilin3 is implicated in endocytosis coupled with Ca2+-dependent exocytosis from nerve terminals. Further study is necessary for the precise role of rabphilin3 in endocytosis.

**Acknowledgments**—We thank Dr. Michinori Kohara (The Tokyo Metropolitan Institute of Medical Science, Tokyo), Dr. Shigekazu Nagata (Osaka University Medical School, Suita), and Dr. Satoshi Orita (Shionogi Institute for Medical Science, Settsu) for providing the T7 RNA polymerase recombinant vaccinia virus (LO-T7), plasmid pEF-BOS, and the cDNA of human transferrin receptor, respectively.

**REFERENCES**

1. Schweizer, F. E., Betz, H., and Augustine, G. J. (1995) Neuron 14, 689–696
2. De Camilli, P., and Takei, K. (1996) Neuron 16, 481–486
3. Simon, K., and Zerial, M. (1993) Neuron 11, 789–799
4. Nueffer, C., and Balch, W. E. (1994) Annu. Rev. Biochem. 63, 949–990
5. Pfeffer, S. R. (1994) Curr. Opin. Cell Biol. 6, 522–526
6. Takai, Y., Sauka, T., Shirataki, H., and Nakanishi, H. (1996) Genes Cells 1, 615–632
7. Kikuchi, A., Yamashita, T., Kawata, M., Yamamoto, K., Ikeda, K., Tanimoto, T., and Takai, Y. (1986) J. Biol. Chem. 261, 2977–2984
8. Matsui, Y., Kikuchi, A., Araki, S., Hishida, T., Teranishi, Y., and Takai, Y. (1988) J. Biol. Chem. 263, 11071–11074
9. Sasaki, T., Kikuchi, A., Araki, S., Hata, Y., Isozora, M., Kuroda, S., and Takai, T., and Takai, Y. (1996) J. Biol. Chem. 271, 2333–2337
10. Matsui, Y., Kikuchi, A., Araki, S., Hata, Y., Kondo, J., Teranishi, Y., and Takai, Y. (1996) J. Biol. Chem. 271, 10946–10949
11. Shirataki, H., Kaibuchi, K., Yamaguchi, T., Wada, K., Horiuhi, H., and Takai, Y. (1992) J. Biol. Chem. 267, 10946–10949
12. Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M., and Takai, Y. (1993) Mol. Cell. Biol. 13, 2061–2068
13. Wada, M., Nakamura, H., Sato, A., Hirano, H., Ohsaki, H., Matsuzawa, Y., and Takai, Y. (1997) J. Biol. Chem. 272, 3875–3878
14. Fukui, K., Sasaki, T., Imazumi, K., Matsuzawa, Y., Nakanishi, H., and Takai, Y. (1997) J. Biol. Chem. 272, 4655–4658
15. Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K., and Zerial, M. (1990) Cell 62, 317–329
16. Gorvel, J. P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991) Cell 64, 915–925
17. Bucci, C., Parton, R. G., Mathé, I. H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. (1992) Cell 70, 715–728
18. Stenmark, H., Vitale, G., Ulrich, O., and Zerial, M. (1995) Cell 83, 423–432
Rabphilin3 and Endocytosis

19. Xiuo, G. H., Shoarinejad, F., Jin, F., Gelemis, E. A., and Yeung, R. S. (1997) J. Biol. Chem. 272, 6097–6100
20. Yamaguchi, T., Shirakata, H., Kishida, S., Miyazaki, M., Nishikawa, J., Wada, K., Numata, S., Kaibuchi, K., and Takai, Y. (1993) J. Biol. Chem. 268, 27164–27170
21. Stahl, B., Chou, J. H., Li, C., Sudhof, T. C., and Jahn, R. (1996) EMBO J. 15, 1799–1809
22. Bennett, Y. (1999) Biochem. Biophys. Acta 1406, 107–121
23. Miyazaki, M., Shirakata, H., Kohno, H., Kaibuchi, K., Tsugita, A., and Takai, Y. (1994) Biochem. Biophys. Res. Commun. 205, 460–466
24. Miyazaki, M., Kaibuchi, K., Shirakata, H., Kohno, H., Ueyama, T., Nishikawa, J., and Takai, Y. (1995) Mol. Brain Res. 28, 29–36
25. Matsudaira, P. (1996) Trends Biochem. Sci. 16, 87–92
26. Otto, J. (1996) Curr. Opin. Cell Biol. 6, 105–109
27. Kato, M., Sasaki, T., Ohye, T., Nakafuku, M., Nishikawa, H., Imamura, M., and Takai, Y. (1996) J. Biol. Chem. 271, 31775–31778
28. Kikuchi, A., Nakafuku, M., and Takai, Y. (1995) Methods Enzymol. 257, 57–70
29. Shirakata, H., and Takai, Y. (1996) Methods Enzymol. 257, 291–302
30. Shirakata, H., Yamamoto, T., Hagi, S., Miura, H., Oishi, H., Jin-no, Y., Senbonmatsu, T., and Takai, Y. (1994) J. Biol. Chem. 269, 32717–32720
31. Mizoguchi, A., Yano, Y., Hamaguchi, H., Yanagida, H., Ide, C., Zahraoui, A., Shirakata, H., Sasaki, T., and Takai, Y. (1994) Biochem. Biophys. Res. Commun. 202, 1235–1243
32. Takaishi, K., Sasaki, T., Kameyama, T., Tsukita, S., Tsukita, S., and Takai, Y. (1995) Oncogene 11, 39–48
33. Orita, S., Sasaki, T., Komuro, R., Sakaguchi, G., Maeda, M., Igarashi, H., and Takai, Y. (1996) J. Biol. Chem. 271, 7257–7260
34. Mizushima, S., and Nagata, S. (1999) Nucleic Acids Res. 18, 5322
35. Orita, S., Naito, A., Sakaguchi, G., Maeda, M., Igarashi, H., Sasaki, T., and Takai, Y. (1997) J. Biol. Chem. 272, 16081–16084
36. Gietz, D., Jean, A. S., Woods, R. A., and Schiestl, R. H. (1992) Nature 359, 847–851
37. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
38. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
39. Laemmli, U. K. (1970) Nature 227, 680–685
40. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
41. Sudhof, T. C. (1995) Nature 375, 645–653
42. Thomas, P., Lee, A. K., Wang, J. G., and Almers, W. (1994) J. Cell Biol. 124, 667–675