The protein kinase Akt plays a central role in a number of key biological functions including protein synthesis, glucose homeostasis, and the regulation of cell survival or death. The mechanism by which tyrosine kinase growth factor receptors stimulate Akt has been recently defined. In contrast, the mechanism of activation of Akt by other cell surface receptors is much less understood. For G protein-coupled receptors (GPCRs), conflicting data suggest that these receptors stimulate Akt in a cell type-specific manner by a yet to be fully elucidated mechanism. Here, we took advantage of the availability of cells, where Akt activity could not be enhanced by agonists acting on this large family of cell surface receptors, such as NIH 3T3 cells, to investigate the pathway linking GPCRs to Akt. We present evidence that expression of phosphatidylinositol 3-kinase (PI3K) β is necessary and sufficient to transmit signals from G proteins to Akt in these murine fibroblasts and that the activation of PI3Kβ may represent the most likely mechanism whereby GPCRs stimulate Akt, as the vast majority of cells do not express PI3Kγ, a known G protein-sensitive PI3K isof orm. Furthermore, available evidence indicates that GPCRs activate Akt by a pathway distinct from that utilized by growth factor receptors, as it involves the tyrosine phosphorylation-independent activation of PI3Kβ by G protein βγ dimers.

Many important intracellular downstream targets for phosphatidylinositol 3-kinases (PI3Ks)1 have been recently identified (1, 2). Among them, the serine-threonine kinase, Akt, has received special attention because of its central role in the regulation of cell survival or death in a large number of cellular systems (3). This kinase can be potently activated by growth factor receptors of the tyrosine kinase type-specific manner by a yet to be fully elucidated mechanism. Here, we took advantage of the availability of cells, where Akt activity could not be enhanced by agonists acting on this large family of cell surface receptors, such as NIH 3T3 cells, to investigate the pathway linking GPCRs to Akt. We present evidence that expression of phosphatidylinositol 3-kinase (PI3K) β is necessary and sufficient to transmit signals from G proteins to Akt in these murine fibroblasts and that the activation of PI3Kβ may represent the most likely mechanism whereby GPCRs stimulate Akt, as the vast majority of cells do not express PI3Kγ, a known G protein-sensitive PI3K isof orm. Furthermore, available evidence indicates that GPCRs activate Akt by a pathway distinct from that utilized by growth factor receptors, as it involves the tyrosine phosphorylation-independent activation of PI3Kβ by G protein βγ dimers.

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‡ The abbreviations used are: PI3K(s), phosphatidylinositol 3-kinase(s); GPCR(s), G protein-coupled receptor(s); HA, hemagglutinin; βARK, β-adrenergic receptor kinase; H2B, histone 2B; mAChRs, muscarinic acetylcholine receptors; p-Tyr, phosphorytrosine; PI-3P, phosphatidylinositol-3-phosphate; EGFP, enhanced green fluorescent protein; LPA, lysophosphatidic acid; ERK2, extracellular signal-regulated kinase 2; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; GFP, green fluorescent protein; AH, Akt homology domain.

Experimental Procedures

Cell Lines and Transfection—Cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% calf serum (BioWhittaker, Walkersville, MD) for NIH 3T3 cells or 10% fetal calf serum (HyClone, Logan, UT) for COS-7 cells. Cells were transfected by LipofectAMINE PLUS™ (Life Technologies, Inc.) following the manufacturer’s directions.

Expression Plasmids—The cDNA for the p110 catalytic subunit of human PI3Kβ (GenBank™ accession number S67334) was obtained from the I.M.A.G.E. consortium (Clone ID 646775, GenBank™ accession number AA205632), and its coding region was subcloned into the pCEFL expression vector (9). An expression plasmid for a chimeric ARK was kindly provided by Dr. Giorgio Scita, European Institute of Oncology, Milan, Italy. Plasmids expressing an epitope-tagged Akt (pCEFL-HA-Akt) and ERK2 (pCEFL-HA-ERK2), m1 and m2, muscarinic acetylcholine receptors (mAChRs), respectively, were shown to activate Akt effectively by other cell surface receptors is much less understood. In this regard, seemingly conflicting data suggest that the ability of G protein-coupled receptors (GPCRs) to stimulate Akt is cell type-specific. For example, initial reports indicated that lysophosphatidic acid (LPA), which stimulates Gβγ-coupled receptors, does not enhance the activity of Akt in NIH 3T3 cells (4, 6), but recent studies have provided evidence that Akt can be stimulated by a variety of agonists acting on GPCRs in other cell types (7–9). The latter includes COS-7 cells, in which ectopically expressed Gβγ-coupled receptors, m1 and m2, muscarinic acetylcholine receptors (mAChRs), respectively, were shown to activate Akt effectively when stimulated by carbachol, a cholinergic agonist (9).

In the present study, we took advantage of the failure of GPCRs to stimulate Akt in NIH 3T3 cell lines to investigate the nature of the signaling molecules linking GPCRs to Akt. Here, we show that PI3Kβ is necessary and sufficient to stimulate Akt by the large family of GPCRs in the vast majority of cells, which do not normally express the G protein-regulated PI3Kγ isof orm. Furthermore, we present evidence that GPCRs stimulate PI3Kβ by a pathway distinct from that utilized by tyrosine kinase growth factor receptors, as it does not require the phosphorylation of the p85 subunit in tyrosine residues or its recruitment to phosphotyrosine-containing complexes, but instead involves the activation of this PI3K isof orm by the Gβγ subunits of heterotrimeric G proteins.
RESULTS AND DISCUSSION

To begin exploring the molecular basis for GPCR activation of Akt in each cellular setting, we first re-examined the effect of LPA and carbachol on the enzymatic activity of Akt in COS-7 and NIH 3T3 cells expressing endogenous and transfected LPA and m1 receptors, respectively. As shown in Fig. 1A, these treatments stimulated Akt in COS-7 cells but not in NIH 3T3 cells, although these agonists enhanced the activity of MAPK in both cell types, which served as a control. Interestingly, serum-stimulated Akt in both COS-7 and NIH 3T3 cells, thus suggesting that NIH 3T3 cells may be defective in one or more key molecular components involved in the transmission of signals from GPCRs to Akt.

As PI3Ks are central elements of intracellular signaling pathways linking cell surface receptors to Akt (4, 5, 17), we next examined which PI3K isoforms were expressed in each cell type. Two of the PI3K catalytic subunits, p110α and p110β, are believed to be ubiquitously expressed (1, 18). These PI3K subunits form heterodimers with non-catalytic subunits of the p85 family and are stimulated by receptor tyrosine kinases upon their recruitment to the plasma membrane by the assembly of phosphotyrosine-containing multimolecular complexes (1, 18). Another PI3K catalytic subunit, p110γ, lacks the binding region for p85 proteins (19) but instead associates with a Gαs-sensitive regulatory subunit, p101 (10, 20, 21), and thus can be regulated by heterotrimeric G proteins. This PI3K isoform exhibits a more restricted tissue distribution (22). To explore which PI3K isoforms are expressed in NIH 3T3 and COS-7 cells, cellular lysates were subjected to immunoprecipitation with antibodies specific for each p110 PI3K subunit, and their kinase activity was assessed using an in vitro lipid kinase reaction. As shown in Fig. 1B, endogenous PI3Kα activity was readily detectable both in NIH 3T3 and COS-7 cells. In contrast, PI3Kγ activity was either absent or at levels below its limit of detection in both cell types. However, this PI3Kγ was recovered from PI3Kγ-transfected NIH 3T3 cells (Fig. 1B, second panel) even if its expression levels were not high enough to be detectable by standard immunoblotting techniques (data not shown). Using this enzymatic assay as a sensitive approach to explore the expression of PI3Ks, we also did not observe any endogenous PI3Kγ activity in COS-7 cells. This suggested that the presence of this G protein-regulated PI3K isoform cannot explain the ability of GPCRs to stimulate Akt in COS-7 cells and raised the possibility that another PI3K catalytic subunit may transduce the G protein-initiated signals in this cell type. In this regard, very high levels of endogenous PI3Kα activity were found in COS-7 cells (Fig. 1B). Interestingly, no PI3Kβ
transfected with expression plasmids for GFP ( GFP) or p110β control three independent experiments and are expressed as -fold increase with experiments.

non-specific band of 105 kDa was observed in both control- and PI3K-mid ( PI3K) for GFP or the catalytic subunits of PI3K m1 receptors were transfected with HA-Akt and plas-

stimulate Akt kinase activity and to promote its localization to the plasma membrane in response to GPCR agonists. As shown in Fig. 2D, an Akt-EGFP chimera containing the membrane-targeting do-

Kinase Akt kinase assay ( Kinase) and anti-HA Western blots ( WB) as above. PI3Kβ-transfected NIH 3T3 cells revealed that the expression levels of PI3Kβ achieved (Fig. 2B) were sufficient to support the rapid and potent activation of Akt by these GPCR agonists, which was demonstrable as soon as 5 min after ligand addition and remained above control levels for more than 1 h (Fig. 2C). Furthermore, expression of this PI3K isoform also enabled the subcellular redistribution of Akt in response to GPCR agonists in NIH 3T3 cells. As shown in Fig. 2D, an Akt-EGFP chimera containing the membrane-targeting domain of Akt (AH) fused to EGFP (16) displays a cytoplasmic localization in control cells, either untreated or when stimulated with carbachol or LPA, but readily translocates to the plasma membrane upon PDGF treatment (Fig. 2D, upper panel). In contrast, carbachol and LPA promoted the accumulation of EGFP-Akt at the level of the plasma membrane when cells coexpressed PI3Kβ (Fig. 2D, lower panel). Taken together, these results indicate that PI3Kβ can mediate the activation of Akt by GPCRs and that the expression of this PI3K isoform is necessary and sufficient to transmit signals from G proteins to Akt in these murine fibroblasts.

Because the p110β PI3K catalytic subunit forms complexes with regulatory subunits of the p85 family (23) and the latter participates in the activation of PI3Ks by tyrosine kinase rece-

The association of PI3K-Akt in NIH 3T3 cells, although it was readily demonstrable upon exogenous expression of p110β.

These findings prompted us to explore whether the expression of PI3Kβ in NIH 3T3 might confer the ability to activate Akt in response to agonists acting on GPCRs. As shown in Fig. 2A, whereas overexpression of p110α did not change the pattern of Akt activation observed in control-transfected cells, as expected, the expression of a G protein-regulated isoform, PI3Kγ, was sufficient to support the activation of Akt by the stimulation of m1 and LPA receptors (Fig. 2A). Remarkably, expression of the p110β subunit of PI3Kγ was also sufficient to enable the activation of Akt by both GPCR agonists. A detailed time course analysis of the Akt stimulation by carbachol and LPA in PI3Kβ-transfected NIH 3T3 cells revealed that the expression levels of PI3Kγ achieved (Fig. 2B) were sufficient to support the rapid and potent activation of Akt by these GPCR agonists, which was demonstrable as soon as 5 min after ligand addition and remained above control levels for more than 1 h (Fig. 2C). Furthermore, expression of this PI3K isoform also enabled the subcellular redistribution of Akt in response to GPCR agonists in NIH 3T3 cells. As shown in Fig. 2D, an Akt-EGFP chimera containing the membrane-targeting do-

kinase activity and to promote its localization to the plasma membrane in response to GPCR agonists. A, NIH 3T3 cells expressing m1 receptors were transfected with HA-Akt and plas-

A, NIH 3T3 cells expressing m1 receptors were transfected with HA-Akt and plasmids ( DNA) for GFP or the catalytic subunits of PI3Kγ, PI3Kβ, or p101 and PI3Kγ, serum-starved, and left untreated ( c) or treated with LPA (10 μM), carbachol ( cch) (1 mM), or 10% calf serum ( serum) for 15 min. Akt kinase assay ( Kinase) and Western blots ( WB) were performed as for Fig. 1. Autoradiograms are representative of three independent experiments. Arrows depict the migration of the phosphorylated H2B and HA-Akt. Values in the bar graph correspond to the mean ± S.E. of three independent experiments and are expressed as -fold increase with respect to their corresponding control. B, COS-7 and NIH 3T3 cells transfected with expression plasmids for GFP ( control) or p110β (PI3Kβ) (4 μg per 10-cm dish) were subjected to immunoprecipitation using an anti-PI3Kβ-specific antibody ( S-19; Santa Cruz Biotechnology) and processed for Western blot ( WB) analysis with the same antibody. An arrow indicates the migration of the catalytic subunit of PI3Kβ. A non-specific band of 105 kDa was observed in both control- and PI3Kβ-transfected cells, which served as a protein-loading control. C, NIH 3T3 cells expressing m1 receptors were transfected as in A with expression plasmids for HA-Akt and p110β, serum-starved, and stimulated for the indicated times with LPA (10 μM) or carbachol ( cch) (1 mM), lysed, and subjected to Akt kinase assays ( Kinase) and anti-HA Western blots ( WB) as above. Arrows indicate the migration of labeled H2B and the epitope-tagged Akt. D, NIH 3T3 cells expressing m1 receptors were grown in 24-well plates on coverslips and transfected with a plasmid expressing the AH of Akt fused with EGFP ( pCEFL-EGFP-AH-Akt, 250 ng per well) together with an empty vector ( control) or an expression plasmid for the catalytic subunit of PI3Kβ ( P3Kβ) (100 ng per well). As indicated, after serum starvation cells were stimulated with PDGF (10 ng/ml), carbachol ( cch) (1 mM), or LPA (10 μM) for 5 min, washed, fixed, mounted, and photographed under uv light using an Axioimager microscope ( Zeiss) (× 63). Depicted areas were magnified (× 4) using Adobe Photoshop software.

activity could be detected in NIH 3T3 cells, although it was readily demonstrable upon exogenous expression of p110β.

plasmid for the catalytic subunit of PI3Kβ ( P3Kβ) (100 ng per well). As indicated, after serum starvation cells were stimulated with PDGF (10 ng/ml), carbachol ( cch) (1 mM), or LPA (10 μM) for 5 min, washed, fixed, mounted, and photographed under uv light using an Axioimager microscope ( Zeiss) (× 63). Depicted areas were magnified (× 4) using Adobe Photoshop software.
Fig. 3. GPCRs do not promote the recruitment of PI3Kβ to phosphotyrosine-containing complexes. A, NIH 3T3 cells expressing m1 receptors were transfected with an expression plasmid for p110β as above, serum starved, and stimulated with 10% calf serum (serum), LPA (10 μM), or carbachol (cch) (1 mM) for the indicated times. Cells were lysed and immunoprecipitated (IP) with anti-phosphotyrosine antibodies (anti-p-Tyr) and assayed for PI3K activity as in Fig. 1. The immunoblotting membrane was redeveloped and probed with 32P-labeled PI-3P, which is a reaction product of activated PI3K. The resulting autoradiograph was scanned, and the density of the PI3P bands was quantitated as a P3P/total protein ratio. Cells were serum starved and stimulated with 10% calf serum (serum), LPA (10 μM), or carbachol (cch) (1 mM) for 15 min, and Akt assays were performed in the corresponding anti-HA immunoprecipitates as described in Fig. 1. Values in the graph represent the mean ± S.E. of three independent experiments and are expressed as a percentage of stimulation with respect to that observed in response to the same treatments in GFP-transfected cells, which was taken as 100%.

We next explored the nature of the G protein(s) implicated in the activation of Akt through PI3Kβ, using the expression of GTPase-deficient forms of representative members of each G protein α subunit family as an approach. As shown in Fig. 4A, coexpression of the epitope-tagged Akt together with constitutively active mutants of Gaα13 (Gaα13-Q61L), Gaα12 (Gaα12-Q61L), and Gaα13 (Gaα13-Q61L, 12, 26, 27) did not result in the activation of Akt in wild-type NIH 3T3 or in PI3Kβ-transfected cells, whereas Ras stimulated Akt potently in both cases. Thus, because of the failure of activated Ga subunits to stimulate Akt, we asked whether βγ dimers could induce the PI3Kβ-dependent activation of Akt. As shown in Fig. 4A, overexpression of control NIH 3T3 cells of β1γ2 subunits either together or individually did not cause any significant change in the Akt activity. In contrast, overexpression of β1γ2 dimers induced a remarkable increase in the activity of Akt in PI3Kβ-expressing cells. This activation was not observed when β1 or γ2 were expressed alone or when HA-Akt was coexpressed with β1 and γ2, a mutant γ2 that lacks the γ-isoprenylation signal and therefore fails to associate with the plasma membrane (26, 28). These results indicated that βγ but not Ga subunits promote the PI3Kβ-dependent activation of Akt and that functional, membrane-bound βγ dimers are required for this effect.

To investigate whether βγ dimers participate in signaling from GPCRs to Akt, we expressed a chimeric molecule containing the extracellular and transmembrane domains of CD8 fused to the carboxyl-terminal domain of βARK, which includes the high-affinity βγ binding region of this kinase and thus acts as a Gβγ scavenger. As shown in Fig. 4B, transfection of this chimeric molecule in NIH 3T3 cells expressing PI3Kβ dramatically reduced the activation of Akt in response to carbachol and LPA, whereas the stimulation of Akt by tyrosine kinase receptors, such as PDGF, was not affected. Taken together, these findings strongly suggest that βγ subunits of heterotrimeric G proteins play a key role in signaling from GPCRs to Akt by acting on the p110β isoform of PI3K.

Accumulated evidence indicates that PI3Kγ can link GPCRs to a number of signaling pathways (1, 29–31). However, recent studies have suggested that the expression of this PI3K is restricted to few cell types (1, 22). Indeed, we did not observe detectable levels of PI3Kγ protein or its lipid kinase activity in NIH 3T3 and COS-7 cells. Instead, this study and recently available evidence provide support for a novel role for PI3Kβ in signaling by GPCRs in the vast majority of cells, which do not express PI3Kγ. For example, it has been recently shown that this PI3K isoform can be stimulated in vitro by purified G protein βγ subunits in synergism with p-Tyr peptides (32, 33) and that a synergistic effect of receptor tyrosine kinases and GPCRs could be observed in PI3Kβ activation (34, 35). Furthermore, antibody-blocking experiments implicated PI3Kβ in the mitogenic pathway initiated by LPA (36). Thus, these results and the present findings that PI3Kβ expression is necessary and sufficient to activate PI3K-dependent pathways by GPCRs in cells lacking PI3Kγ, such as fibroblasts, demonstrate a central role for the PI3Kβ isoform in signaling by G protein-
linked receptors.

Interestingly, although there is extensive sequence homology between the p110α and p110β PI3K catalytic subunits, recent observations in knockout mice models suggest that these PI3K isoforms may perform non-complementary functions (1, 38). In this regard, our current findings suggest that one such distinct feature is the ability of PI3Kβ to act downstream from heterotrimERIC G proteins. The structural elements responsible for this distinct coupling specificity are still unknown and are under current investigation.

The emerging picture is that GPCRs stimulate PI3Kβ in most tissues by a mechanism distinct from those utilized by tyrosine kinase receptors, as it does not require the functional activity of the p85 non-catalytic subunit, but instead involves the activation of p110α by Gβγ subunits. We can also conclude that the distinct tissue distribution of each PI3K isoform may play an unexpected role in controlling the specificity in signal transmission, as it may govern the ability of GPCRs to stimulate a variety of intracellular signaling pathways that require PI3K function. In turn, the availability and level of expression of PI3Kβ and PI3Ky may determine the nature of the biological responses elicited by the large family of G protein-linked cell surface receptors in each cell type.

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REFERENCES

1. Wymann, M. P., and Pirola, L. (1998) Biochim. Biophys. Acta 1436, 127–150
2. Rameh, L. E., and Cantley, L. C. (1999) J. Biol. Chem. 274, 8347–8350
3. Marte, B. M., and Downward, J. (1997) Trends Biochem. Sci. 22, 355–358
4. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) Cell 81, 727–736
5. Vanhaesebroeck, B., Leovers, S. J., Panayotou, G., and Waterfield, M. D. (1997) Trends Biochem. Sci. 22, 267–272
6. Burgering, B. M., and Coffer, P. J. (1995) Nature 376, 599–602
7. Moule, S. K., Walsh, G. L., Edgell, N. J., Foulstone, E. J., Proud, C. G., and Denton, R. M. (1997) J. Biol. Chem. 272, 7713–7719
8. Sable, C. L., Filippo, N., Hemmings, B., and Van, O. E. (1997) FEBS Lett. 409, 253–257
9. Murga, C., Lagunig, L., Wetzker, R., Cuadrado, A., and Gutkind, J. S. (1998) J. Biol. Chem. 273, 19080–19085
10. Stephens, L. R., Eginoea, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Snarska, A. S., Thelen, M., Cadwallader, K., Tempest, P., and Hawkins, P. T. (1997) Cell 89, 105–114
11. Crespo, P., Cacchero, T. G., Xu, N., and Gutkind, J. S. (1995) J. Biol. Chem. 270, 25259–25265
12. Coso, O. A., Teramoto, H., Simonds, W. F., and Gutkind, J. S. (1996) J. Biol. Chem. 271, 3963–3966
13. Haro, K., Yonezawa, K., Sakase, H., Ando, A., Kotani, K., Kitamura, T., Kitamura, Y., Ueda, H., Stephens, L., Jackson, T. R., Hawkins, P. T., Dhand, R., Clark, A. E., Holman, G. D., Waterfield, M. D., and Kasuga, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7415–7419
14. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) Cell 81, 1137–1146
15. Gutkind, J. S., Lacal, P. M., and Robbins, K. C. (1990) Mol. Cell. Biol. 10, 3806–3809
16. Waterston, S. J., and Downward, J. (1999) Curr. Biol. 9, 433–436
17. Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997) Cell 88, 435–437
18. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) Annu. Rev. Biochem. 67, 481–507
19. Stoyanova, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nurnberg, B., Gierschik, P., Seedorf, K., Hisado, J. J., Waterfield, M. D., and Wetzker, R. (1995) Science 269, 690–693
20. Leopoldt, D., Hanck, T., Exner, T., Maier, U., Wetzker, R., and Nurnberg, B. (1998) J. Biol. Chem. 273, 7024–7029
21. Fukui, Y., Ibara, S., and Nagata, S. (1998) J. Biochem. (Tokyo) 124, 1–7
22. Bernstein, H. G., Keilhoff, G., Reiser, M., Fresse, S., and Wetzker, R. (1998) Cell Mol. Biol. 44, 973–983
23. Ho, P., Mondino, A., Skolnik, E. Y., and Schlessinger, J. (1993) Mol. Cell. Biol. 13, 7677–7688
24. Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ulrich, A., and Schlessinger, J. (1991) Cell 65, 85–90
25. Ho, P., Margolis, B., Skolnik, E. Y., Lammers, R., Ulrich, A., and Schlessinger, J. (1992) Cell Mol. Biol. 12, 981–990
26. Crespo, P., Xu, N., Simonds, W. F., and Gutkind, J. S. (1994) Nature 369, 18–20
27. Fromm, C., Colo, O. A., Montaner, S., Xu, N., and Gutkind, J. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10098–10103
28. Simonds, W. F., Butrynski, J. E., Gautam, N., Unson, C. G., and Spiegel, A. M. (1991) J. Biol. Chem. 266, 5363–5366
29. Lopez-Islasara, M., Crespo, P., Pellici, P. G., Gutkind, J. S., and Wetzker, R. (1997) Science 275, 394–397
30. Lopez-Islasara, M., Gutkind, J. S., and Wetzker, R. (1998) J. Biol. Chem. 273, 2565–2598
31. Stephens, L., Hawkins, P. T., Eginoea, A., and Cooke, F. (1996) Philos. Trans. R. Soc. Lond. B. Biol. Sci. 351, 211–215
32. Kurosu, H., Maehama, T., Okada, T., Yamamoto, T., Hoshino, S., Fukui, Y., Uf, M., Hazeki, O., and Katada, T. (1997) J. Biol. Chem. 272, 24252–24256
33. Maier, U., Babich, A., and Nurnberg, B. (1999) J. Biol. Chem. 274, 29311–29317
34. Okada, T., Hazeki, O., Uf, M., and Katada, T. (1996) Biochem. J. 317, 475–480
35. Hazeki, O., Okada, T., Kuros, H., Takasuga, S., Suzuki, T., and Katada, T. (1996) Life Sci. 55, 1539–1559
36. Roche, S., Downward, J., Raynal, P., and Courtneidge, S. A. (1998) Mol. Cell. Biol. 18, 7119–7129
37. Walker, E. H., Persic, O., Ried, C., Stephens, L., and Williams, R. L. (1990) Nature 402, 313–320
38. Bi, L., Okabe, I., Bernard, D. J., Wynahaw-Boris, A., and Nussba, R. L. (1997) J. Biol. Chem. 272, 10963–10968
