Protein Kinase B/akt and Rab5 Mediate Ras Activation of Endocytosis

(Received for publication, May 11, 1998, and in revised form, June 10, 1998)

M. Alejandro Barbieri‡, Aimee D. Kohn¶, Richard A. Roth¶, and Philip D. Stahl‡

From the ‡Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the ¶Department of Molecular Pharmacology, Stanford University School of Medicine, Palo Alto, California 94305

Transient expression of oncogenic Ha-Ras (RasV12) stimulates endocytosis. Using NIH3T3 cells expressing constitutively active protein kinase B/akt (PKB/akt) or kinase-dead PKB/akt, we show that PKB/akt mediates the stimulatory effect of Ras on endocytosis. Fluid phase endocytosis of horseradish peroxidase in cells expressing the constitutively active form of PKB/akt was elevated and insensitive to phosphatidylinositol 3-kinase inhibitors. However, expression of dominant negative Rab5:N34 blocked endocytosis in cells expressing the constitutively active form of PKB/akt.

Transient expression of either Rab5:wt or Rab5:L79, a GTPase deficient mutant of Rab5, in cells expressing constitutively activated PKB/akt further increased endocytic rate. However, in cells expressing kinase-dead PKB/akt, endocytic rate was not affected by transient expression of Rab5:wt. Rab5:L79, on the other hand, increased endocytosis in cells expressing kinase-dead PKB/akt. Similar results were obtained using an \textit{in vitro} endosome fusion reconstitution assay with cytosol prepared from cells expressing the activated PKB/akt or kinase-dead PKB/akt. Both Rab5:wt and Rab5:L79 stimulated endosome fusion when assayed in cytosol containing the activated PKB/akt, whereas only Rab5:L79 activated fusion when the assay utilized cytosol from kinase-dead expressing cells. We conclude that Ras activation of endocytosis requires both PKB/akt and Rab5 and that active kinase is required for activation Rab5.

Endocytosis is a carefully orchestrated process required by all cells for nutrition and defense. Whereas an increasing number of Rab GTPases localize to the endocytic pathway, including Rab5, Rab4, Rab11, and Rab7 (1), the endocytic rate appears to be regulated by Rab5 (2). Earlier work has shown that Rab5, in turn, is regulated by upstream factors, including phosphatidylinositol 3-kinase and Ras (3, 4). PKB/akt is a serine/threonine kinase that has emerged as a key intermediate between signal transducing growth factor receptors, including insulin (4) and platelet-derived growth factor (6, 7) and a variety of cytoskeletal effectors (8, 9). Recent work has linked PKB/akt to such diverse processes as cell survival by suppressing apoptosis via phosphorylation of BAX (10) and the metabolic response to insulin via the regulation of intracellular trafficking of vesicles containing Glut 4 (11). Activation of PKB/akt requires phosphorylation of the kinase by at least two phosphoinositide-dependent kinases that phosphorylate PKB/akt at Thr308 and Ser473 (12, 13). Known downstream targets of PKB/akt include p70 S6 kinase (6, 7) and glycogen synthase kinase (14), although many other targets most likely exist given that PKB/akt is present in at least three isoforms (8, 9).

Here we demonstrate that Rab5, a GTPase that is rate-limiting for endocytosis, is regulated by PKB/akt, a kinase coupled to signal transduction. Moreover, we demonstrate that the endocytic response to “activated Ha-Ras” requires both PKB/akt and Rab5.

EXPERIMENTAL PROCEDURES

Materials—Dinitrophenol (DNP)-derivatized β-glucuronidase (DNP-β-glucuronidase) and aggregated anti-dinitrophenol IgG (anti-DNP IgG) were prepared as reported (15). Anti-Rab5, 4F11 monoclonal antibody was a generous gift of Angela Wandinger-Ness, Northwestern University, Chicago. All other reagents were obtained from Sigma, except where indicated. LY-294002 was a generous gift of Dr. C. J. Vilahos.

Sindbis Virus Constructs and NIH3T3 Cell Lines—For overexpression, NIH3T3 cell monolayers in 35-mm dishes (~5 × 10^{5} cells/dish) were infected with either the vector Sindbis virus as a negative control or the recombinant Sindbis encoding Ras:V12, Rab5:wt, or Rab5:L79 (16). High multiplicity of infection (80 plaque-forming units/cell) was employed to ensure that all of the cells were infected (16). NIH3T3 cells stably expressing PKB(akt)-HA constructs were generated by retroviral infection as described previously (11).

Endocytosis Assay—Fluid phase endocytosis of HRP was performed as described (16). Twenty-four h after withdrawal of serum, NIH3T3 cell monolayers in 35-mm dishes were washed three times with serum-free α-MEM, and HRP endocytosis was initiated by addition of 1 ml of α-MEM containing 2 mg/ml HRP and 0.2% (w/v) bovine serum albumin at 37 °C. To estimate HRP uptake, the cells were washed three times with phosphate-buffered saline (PBS), trypsinized on ice for 20 min, washed two times with PBS, and lysed in 500 μl of lysis buffer (3). Cell lysates were assayed for HRP activity as described (3).

Rab5 and in Vitro Endosome Fusion—Rab5 was prepared in large quantity as a glutathione S-transferase fusion proteins in \textit{Escherichia coli} strain JM101, and the GST-Rab5 proteins were affinity-purified by glutathione-Sepharose chromatography as described previously (16). GST-Rab5 (1 μM) was prenylated by incubation with semipurified REP-1/Rab geranylgeranylationtransferase in 50 μl of 50 mM Hepes/KOH, pH 7.2, 5 mM MgCl_{2}, 0.05 mM Nonidet P-40, 1 mM DTT (buffer A) containing 2 μM geranylgeranyl pyrophosphate for 30 min at 37 °C as described (17, 18). After the reaction, the prenylated Rab5 proteins were directly used as indicated in each figure. Early endosomes, prepared from J774 E-cle macrophages, were loaded with anti-DNP mouse monoclonal antibody or with DNP-β-glucuronidase as described (15).

—Determination of Protein Kinase B/akt Activity and the GTP/GDP Ratio of Rab5 in Cultured Cells—To determine protein kinase B/akt activity, cells were washed twice with ice-cold PBS and lysed for 20 min at 4 °C in lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 50 mM β-glycerophosphate, 10 mM NaF, 1 mM NaVO_{4}, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, dium); PI, phosphatidylinositol.
and 2 μg/ml leupeptin. The lysates were centrifuged for 10 min at 12,000 × g at 4 °C and assayed as described by Kohn et al. (5).

To determine the ratio of GTP to GDP bound to Rab5 (GTP/GDP ratio), confluent (∼5 × 10^6 cells/dish) NIH3T3 control cells and NIH3T3 expressing constitutively active PKB/akt or kinase-dead PKB/akt were incubated in α-MEM phosphate-free medium for 3 h with 300 μCi of [32P]-α-MEM (200 Ci/mmol, Amersham Pharmacia Biotech). After incubation, the cells were lysed as described (19), and Rab5 was immunoprecipitated using a 4F11 monoclonal antibody bound to the protein A-Sepharose (Amersham Pharmacia Biotech) for 10 min at 4 °C in lysis buffer (20). The beads were washed three times with wash buffer (20 mM Tris-Cl, 0.1% Nonidet P-40, 500 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 2 mg/ml bovine serum albumin, 50 mM β-glycerophosphate, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin) and three times with wash buffer containing 0.001% SDS. The bound nucleotides were then eluted in 12 μl of buffer (10 mM EDTA, 1 mM DTT, 0.1% SDS, 5 mM GDP, 5 mM GTP) for 3 min at 65 °C. All the manipulations, from lysis of the sample to elution of the nucleotides bound to Rab5, were carried out at 4 °C within 40 min. Four-μl samples were then spotted onto 0.1 mm polyethyleneimine-cellulose TLC (Merck) plates, which were developed for 60 min in 0.75 M phosphate, pH 3.4. The samples were dried and placed in autoradiography cassettes. For visualization of the [32P]-labeled GTP and GDP, films were exposed at −80 °C for 24–36 h. A phosphomager was used to determine the GTP/GDP ratio.

RESULTS AND DISCUSSION

To examine endocytosis in cells expressing constitutively activated or kinase-dead PKB/akt, cells were starved for serum overnight prior to initiating endocytosis assays or transiently expressing Ras or Rab5 constructs. As shown in Fig. 1A, transient expression of Ras:V12 in control cells results in enhanced endocytosis of HRP, a fluid phase endocytosis marker. Expression of Ras:N17, the dominant negative mutant, modestly reduced endocytosis (Fig. 1A). Ras is known to interact with multiple target molecules via its effector domain, including the 110-kDa subunit of phosphatidylinositol 3-kinase (21). Activation of the phosphatidylinositol 3-kinase by Ras:V12 results in increased production of several lipid products. These include phosphatidylinositol 3,4-diphosphate and phosphatidylinositol 3,4,5-triphosphate, which are thought to activate, directly or indirectly, several downstream effectors such as p70 S6 kinase, Rac1, PKC, Rab1B, and PKB/akt (21–28). To establish a connection between elevated endocytosis observed following activation of the Ras/phosphatidylinositol 3-kinase pathway and the activation of PKB/akt, a downstream target of phosphatidylinositol 3-kinase, we asked whether activation of PKB/akt was directly coupled to endocytosis. NIH3T3 cells were stably transfected with constructs encoding constitutively active or kinase-dead constructs of PKB/akt. Activation was accomplished by addition of the Src myristoylation sequence to the N terminus of PKB/akt (28). A kinase-dead construct was prepared by introducing a point mutation (K179M) at a site required for kinase activity (28). Cells expressing constitutively active PKB/akt demonstrated substantially higher endocytic rates (Fig. 1A), which were similar to the elevated levels observed following expression of Ras:V12 (Fig. 1A). In contrast, cells expressing the kinase-dead construct of PKB/akt (Fig. 1A) showed depressed levels of HRP uptake. To explore the linkage between Ras and PKB/akt, cells were infected with Sindbis virus encoding Ras:V12 and Ras:N17. Overexpression of either activated or dominant negative Ras in cells expressing kinase-dead PKB/akt had no effect on HRP endocytosis, suggesting that activation of PKB/akt is required for Ras stimulation of endocytosis. Moreover, transient expression of either Ras construct (Ras:V12 or Ras:N17) in cells expressing constitutively active PKB/akt had no effect on endocytosis (Fig. 1A). These data suggest that activation of PKB/akt is linked to Ras stimulation of endocytosis and that PKB/akt lies downstream of Ras. Consistent with this proposal, expression of dominant negative Ras:N17 was unable to block the enhanced endocytic rate observed in cells expressing the constitutively active PKB/akt (Fig. 1A). Ras:V12-stimulated endocytosis is inhibited by phosphatidylinositol 3-kinase inhibitors (3, 4). PI 3-kinase activity is known to be required for Ras to stimulate PKB/akt (8, 9). Thus, we found that the phosphatidylinositol 3-kinase inhibitor LY294002 completely inhibited the activation of PKB/akt by Ras:V12 in NIH3T3 cells and substantially reduced the endocytic response (data not shown). The time course of HRP endocytosis in control cells and in cells expressing constitutively active PKB/akt and kinase-dead PKB/akt is shown in Fig. 1B. Constitutively active PKB/akt enhanced both the rate of internalization and the accumulation of internalized HRP. In cells expressing the constitutively active PKB/akt, wortmannin had no effect on endocytosis of HRP (Fig. 1C). This result is consistent with the observation that phosphatidylinositol 3-kinase inhibitors had no effect on the activity of activated PKB/akt.2 These results clearly indicate that PKB/akt is a downstream effector of the Ras/phosphatidylinositol 3-kinase pathway and a key regulator of endocytosis.

Following the internalization step at the plasma membrane, endocytic vesicles access the early endocytic sorting compartment by fusion, a process that is regulated by the GTPase Rab5 (2). Rab5 is rate-limiting for endocytosis and for endosome fusion reconstituted in vitro (2, 29, 30). Previous work has demonstrated that elevated endocytosis following overexpression of Rab5 is dependent on phosphatidylinositol 3-kinase (3, 4). Phosphatidylinositol 3-kinase appears to be required for the

2 M. A. Barbieri, R. Roth, and P. D. Stahl, unpublished observations.
activation of Rab5, because enhanced endocytosis following expression of Rab5.579, the GTPase-defective mutant, was unaffected by phosphatidylinositol 3-kinase inhibitors (3, 4). Similar results have been obtained with the in vitro endosome fusion assay (3, 4). The insensitivity of Rab5.579-induced endocytosis to inhibitors of PI 3-kinase following transient expression in cultured cells is confirmed in Fig. 2A. However, recent work by Jones et al. (31) indicates that under some circumstances, the sensitivity of Rab5.579-induced endocytosis to wortmannin is substantially reduced, suggesting that PI 3-kinase and guanine nucleotide exchange on Rab5 may not be in a linear pathway. However, the in vitro assay employed by Jones et al. (31) varied substantially from that used in the initial report (3), which showed that stimulation of fusion with Rab5.579 was wortmannin-insensitive. Clearly, more work will be needed to sort out the relationship between PI 3-kinase and activation of Rab5. To address whether the stimulation of endocytosis by PKB/akt requires Rab5 activity, we examined the effect of Rab5.5 wt and Rab5 mutants on HRP uptake in NIH3T3 cells (Fig. 2A) overexpressing constitutively active PKB/akt (Fig. 2B) or kinase-defective PKB/akt (Fig. 2C). Confluent NIH3T3 cell monolayers were infected with recombinant Sindbis viruses encoding Rab5.5 wt, Rab5.N34, or Rab5.579 or the Sindbis vector as a control. At 4 h postinfection, cells were treated with LY294002, and HRP uptake was assayed. As expected, the overexpression of Rab5.5 wt and Rab5.579 in NIH3T3 cells induced a significant increase in HRP uptake (Fig. 2A). Both expression of Rab5.N34, the dominant negative mutant (data not shown), and the addition of phosphatidylinositol 3-kinase inhibitors (LY294002) blocked Rab5.5 wt-stimulated and basal HRP uptake (Fig. 2A). However, in cells expressing constitutively active PKB/akt (Fig. 2B) phosphatidylinositol 3-kinase inhibitors had no effect on the activation of endocytosis following expression of either Rab5.5 wt or Rab5.579. Expression of Rab5.N34, the dominant negative mutant, significantly blocked HRP uptake (data not shown). In NIH3T3 cells expressing kinase-ead PKB/akt, Rab5.5 wt was unable to stimulate HRP uptake, when overexpressed with the Sindbis expression vector (Fig. 2C). Rab5.579, on the other hand, was fully active when expressed in cells stably expressing kinase-ead PKB/akt (Fig. 2C). Thus, it appears that an active PKB/akt is required for Rab5.5 wt to produce its effects on endocytosis. Rab5 is active in the GTP form. To address whether the expression of active and kinase-defective forms of PKB/akt affects the guanine nucleotide status of Rab5, we determined the ratio of GTP and GDP bound to Rab5 in its activated and inactive forms (15). A, effect of cytosol concentration on in vitro endosome fusion carried out with cytosols prepared from different sources: control cytosol (●), cytosol from NIH3T3 cells expressing constitutively active PKB/akt (○, ●), or kinase-dead PKB/akt (● in the presence (■) or the absence (□) of 50 μM LY294002. The data are representative of four independent experiments. B, characterization of in vitro endosome fusion with cytosol from control NIH3T3 cells or cells expressing constitutively active PKB/akt. Fusion was expressed as a percentage of that obtained with untreated endosomes resuspended in complete fusion buffer supplemented with 1 mg/ml of cytosol and incubated for 45 min at 37 °C. The following conditions were tested: ATP, depleting system (5 mM mannose, 60 units/ml hexokinase) was substituted for the ATP-regenerating system (i.e. ATP, creatine phosphate, and creatine phosphokinase); +Rab5.N34, 200 nM of prenylated Rab5.N34-REP (Rab escort protein) complex was added to fusion reaction (29); Temperature, in vitro endosome fusion when the reaction was carried out at 4 °C. Values are the mean of three determinations. Vertical lines indicate S.D. for each sample. C, in vitro endosome fusion experiments supplemented with 1 mg/ml cytosol protein prepared from NIH3T3 cells expressing kinase-ead PKB/akt in the presence of different concentrations either Rab5.5 wt-REP complex (○) or Rab5.579-REP complex (●). Values are the mean of three determinations. Vertical lines indicate S.D. for each sample.
increase in the GTP-bound to Rab5 in cells expressing the constitutively active PKB/akt and a decrease in GTP bound to Rab5 in cells expressing the kinase-dead PKB/akt. These observations are consistent with the fact that the Rab5 dominant negative mutant (Rab5:N34) blocked endocytosis in cells expressing constitutively active PKB/akt (Fig. 2B).

Given the inhibitory effect of the kinase-dead PKB/akt mutant on HRP uptake and the insensitivity of the constitutively active PKB/akt to phosphatidylinositol 3-kinase inhibitors, our results suggested a direct involvement of PKB/akt in endocytosis. To confirm these observations, we took advantage of the in vitro endosome fusion by testing directly whether cytosol prepared from cells overexpressing constitutively active PKB/akt or kinase-dead PKB/akt would support fusion. Early endosomes, prepared from J774 E-clone macrophages, were loaded with aggregated monoclonal anti-DNP mouse IgG (via the Fc receptor) or with DNP-β-glucuronidase (via the mannose receptor) as described (15). Endosomes were incubated with cytosol from control NIH3T3 cells or cells expressing constitutively active PKB/akt or kinase-dead PKB/akt. Endosome fusion with cytosol from cells expressing the constitutively active PKB/akt was elevated compared with control cytosol. However, unlike control cytosol, the endosome fusion with cytosol from cells expressing constitutively active PKB/akt was Wortmannin-insensitive (Fig. 3A). Cytosol from cells expressing kinase-dead PKB/akt was poorly active in the in vitro assay (Fig. 3A). PKB/akt-enriched cytosol-stimulated endosome fusion had all the requirements of the standard endosome fusion assay (e.g., ATP, temperature). Importantly, PKB/akt-stimulated endosome fusion was blocked by the Rab5:N34 mutant (Fig. 3B).

Furthermore, the addition of Rab5:L79, but not Rab5:wt, to cytosol containing kinase-dead PKB/akt substantially stimulated endosome fusion (Fig. 3C). Taken together, these data suggest that Ras regulates endocytosis via PKB/akt and Rab5 and that the target PKB/akt action is probably guanine nucleotide exchange on Rab5. Ras is reported to have a variable effect on PKB/akt activation by growth factors. In some cases, dominant negative Ras has been found to block growth factor-induced stimulation of PKB/akt, whereas in others, such effects have not been reported (6, 7). This probably reflects the complexity of the regulation of phosphatidylinositol 3-kinase, which is controlled by multiple signaling pathways (8, 9). Furthermore, the closely related protein R-Ras can stimulate both phosphatidylinositol 3-kinase and PKB/akt without affecting the well characterized Ras target, Raf (8, 9). Thus, the activation of PKB/akt may ensure tight regulation of Rab5 function, possibly via guanine nucleotide exchange at the correct membrane localization site during the endocytic cycle to coordinate the assembly and function of macromolecular complexes necessary for endosome fusion.

Acknowledgments.—We thank Rita Boshans, Libby Peters, Cheryl Adles, and Marilyn Levy for the excellent technical assistance and Crislyn D’Souza-Schorey for helpful comments.

REFERENCES

1. Novick, P., and Zerial, M. (1997) Curr. Biol. 9, 496–504
2. Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. (1992) Cell 70, 715–728
3. Li, G., D'Souza-Schorey, C., Barbieri, M. A., Roberts, R. L., and Stahl, P. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10207–10211
4. Li, G., D'Souza-Schorey, C., Barbieri, M. A., Cooper, J. A., and Stahl, P. D. (1997) J. Biol. Chem. 272, 10337–10340
5. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) EMBO J. 14, 4288–4295
6. Burgering, B. M. T., and Coffer, P. J. (1994) Nature 376, 599–602
7. 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
8. Marte, B. M., and Downward, J. (1997) Trends Biochem. Sci. 22, 355–358
9. Marte, B. M., Rodriguez-Viciana, P., Wennestrom, S., Warne, P. H., and Downward, J. (1997) Curr. Biol. 7, 63–70
10. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
11. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) J. Biol. Chem. 271, 31737–31738
12. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., and Cohen, P. (1997) Curr. Biol. 7, 261–269
13. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 277, 567–570
14. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Nature 378, 785–789
15. Diaz, R., Mayorga, L., and Stahl, P. (1988) J. Biol. Chem. 263, 6093–6100
16. Li, G., Barbieri, M. A., Colombo, M. I., and Stahl, P. D. (1994) J. Biol. Chem. 269, 14631–14635
17. Barbieri, M. A., Li, G., Mayorga, L. S., and Stahl, P. D. (1996) Arch. Biochem. Biophys. 336, 64–72
18. Sowa, M. E. (1996) J. Biol. Chem. 271, 14398–14404
19. Datta, K., Franke, T. F., Chan, T. O., Makris, A., Yang, S. I., Kaplan, D. R., Morrison, D. K., Golemis, E. A., and Tsichlis, P. N. (1995) Mol. Cell. Biol. 15, 2304–2310
20. Stenmark, H., Parton, R. G., Steele-Mortimer, O., Uteke, A., Gruenberg, J., and Zerial, M. (1994) EMBO J. 13, 1287–1296
21. Rodriguez-Viciana, P., Warne, P. H., Vanhaesebroeck, B., Waterfield, M. D., and Downward, J. (1996) EMBO J. 15, 2439–2451
22. Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) Nature 370, 71–75
23. Hawkins, P. T., Eguinoa, A., Qiu, R. G., Stokoe, D., Cooke, F. T., Walters, R., Wennestrom, S., Claesson-Welsh, L., Evans, T., and Symons, M. (1995) Curr. Biol. 5, 393–403
24. Toker, A., Meyer, M., Beddy, K. K., Falck, J. R., Anegja, R., Anegja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) J. Biol. Chem. 269, 32358–32367
25. Nakaniishi, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16
26. Jones, P. F., Jakubowicz, T., Pitossi, F. J., Maurer, F., and Hemmings, B. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4171–4175
27. Coffer, P. J., and Woodgett, J. R. (1991) Eur. J. Biochem. 201, 475–481
28. Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996) J. Biol. Chem. 271, 21920–21926
29. Li, G., and Stahl, P. D. (1993) J. Biol. Chem. 268, 24475–24480
30. Barbieri, M. A., Li, G., Colombo, M. I., and Stahl, P. D. (1994) J. Biol. Chem. 269, 18720–18722
31. Jones, A. T., Mills, I. G., Scheidig, A. J., Alexandrov, K., and Clague, M. J. (1998) Mol. Cell Biol. 9, 323–332