Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4644–4648, May 1994
Biochemistry

Ras signaling in the activation of glucose transport by insulin

(cardiac myocyte/microinjection/microanalysis/enzymatic cycling)

JILL MANCHESTER, XIANMING KONG, OLIVER H. LOWRY, AND JOHN C. LAWRENCE, JR.*

Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110

Contributed by Oliver H. Lowry, February 9, 1994

ABSTRACT **An approach involving microinjection and microanalysis has been developed to investigate signal-transduction pathways involved in the hormonal control of metabolism. We have applied this strategy to investigate the role of Ras signaling in the acute activation of glucose transport by insulin in cardiac myocytes. Glucose transport activity was assessed by measuring the initial rate of accumulation of 2-deoxyglucose 6-phosphate (dGlc6*P*) in individual cells after incubation in 2-deoxyglucose. Insulin increased accumulation of dGlc6*P* by 3- to 4-fold, consistent with its stimulatory effect on glucose transport. Accumulation of dGlc6*P* was increased severalfold by microinjecting the nonhydrolyzable GTP analogue, guanosine 5′-[γ-thio]triphosphate, which activates members of the Ras superfamily of GTP-binding proteins. Injecting activated Ha-Ras protein also mimicked insulin by increasing dGlc6*P*; whereas, injecting a Ras protein lacking the COOH-terminal site of fatty acylation required for Ras function was without effect. Introducing the neutralizing Ras antibody Y13-259 into cells attenuated the effect of insulin. These findings implicate Ras in the acute regulation of metabolism by insulin.**

Blood glucose levels are maintained within a relatively narrow range through the actions of several hormones, of which insulin is the most important (1). A rise in blood sugar promotes release of insulin, which stimulates glucose transport in skeletal muscle fibers, cardiac myocytes, and adipocytes (2, 3). Insulin increases glucose transport by causing translocation of glucose transporter isoform 4 (GLUT4) from intracellular compartments to the plasma membrane (4, 5). The effect on GLUT4 translocation reaches a maximum within minutes of an increase in the insulin concentration. Several other processes are rapidly changed in response to the hormone (6, 7). For example, glycogen synthesis is increased in skeletal muscle and fat cells, lipogenesis is increased in adipocytes and hepatocytes, and gluconeogenesis is decreased in hepatocytes. These acute metabolic actions of insulin are essential for the efficient uptake and storage of excess blood glucose.

Insulin elicits other actions that develop with time courses of many minutes to hours (6). Such effects are observed in a wide variety of cell types and are associated with increased growth and/or differentiation of cells. There is increased evidence that these actions of insulin involve Ras, a GTP-binding protein that is a transducer of diverse physiological signals (8, 9). Insulin increases the proportion of Ras in the active GTP-bound state (10–12) via a mechanism that appears to involve formation of a complex between insulin receptor substrate 1 (IRS-1), guanine nucleotide-releasing factor mSOS, and growth factor receptor-bound protein (GRB2) (13–16). Ras activation triggers sequential activation of the protein Ser/Thr kinases, raf1, MEK, mitogen-activated protein (MAP) kinase, and rsk-2 (17, 18). MAP kinase phosphorylates the transcription factors c-Jun and c-Myc, linking the Ras signaling pathway to the control of gene expression (19, 20). The pathway involved in the activation of glucose transport by insulin has not been defined.

Previous approaches that have been used to investigate the mitogenic actions of Ras are of limited value in determining the role of the protein in the acute metabolic responses of cells to insulin. In most cases, increasing or decreasing Ras function by transfecting cells with cDNA requires many hours to days, during which time cell phenotype can change. Under such conditions the response observed may not be representative of that produced by acutely activating Ras. Microinjection has been useful for investigating the role of Ras in certain long-term actions of insulin. For example, injecting Ras antibody into *Xenopus* oocytes has been shown to block insulin-stimulated maturation (21). More recently, microinjecting Rat-1 fibroblasts overexpressing the human insulin receptor with insulin receptor substrate 1 antibody was found to inhibit insulin-stimulated mitogenesis (22). However, the potential of microinjection in investigating the important metabolic actions of insulin has not been exploited because of problems associated with measuring responses in the cells that have been injected. We have solved this problem by using microanalytical methods to measure directly the levels of metabolites in single cardiac myocytes that have been injected.

MATERIALS AND METHODS

Preparation and Incubation of Cardiac Myocytes. Ventricular myocytes were isolated by perfusing rat (Long–Evans, 250–300 g) hearts with collagenase (type II, Worthington) (23). The cells were seeded onto coverslip fragments (1–2 mm^2) that had been coated with laminin (Collaborative Research) and cultured overnight in medium 199 (24).

Microinjection. Agents to be injected were dissolved in buffer (see figure legends) containing rhodamine-labeled dextran at 3 mg/ml (M_r = 10,000, Collaborative Research). Pipettes were prepared by using a Flaming/Brown micropipette puller (model P-87, Sutter Instruments, Novato, CA). After filling the tips with the solutions to be injected, pipettes were back-filled with buffer (145 mM NaCl/5.4 mM KCl/10 mM sodium phosphate, pH 7.4) to enable measurements of electrical resistance. Tips were sharpened by using a microbeveler (model BV10, Sutter Instruments) until the resistance fell to 15 MΩ.

When myocytes were incubated in medium containing physiological concentrations of $CaCl_2$, injection almost always triggered an irreversible cycle of contraction, leading to

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: mAb, monoclonal antibody; dGlc, 2-deoxyglucose; dGlc6*P*, 2-deoxyglucose 6-phosphate; Glc6P, glucose 6-phosphate; GLUT4, glucose transporter isoform 4.

*To whom reprint requests should be addressed at: Department of Molecular Biology and Pharmacology, Washington University School of Medicine, Box 8103, 660 South Euclid Avenue, St. Louis, MO 63110.

rapid ATP depletion. Two approaches were developed to deal with this problem. When evaluating substances, such as guanosine 5'-[γ-thio]triphosphate (GTP[γS]) that would be expected to act within minutes, coverslip fragments having ≈50 cells were placed in high-K^+ buffer (135 mM KCl/10 mM NaCl/3 mM $MgCl_2$/1 mM sodium phosphate/250 μM EGTA/0.5% bovine serum albumin/9 mM lactate/1 mM pyruvate/10 mM NaHepes, pH 7.4). Glucose transport in cells incubated in this Ca^{2+}-free buffer remained responsive to insulin (Figs. 1, 2, and 4), and the cells could be injected without triggering contractile activity. The K^+ concentration of the buffer was sufficient to produce complete depolarization, thereby eliminating complications that could arise from differences in membrane potential in cells damaged by injection. Microinjections were done at 21°C during a 15-min period by using a Nikon (model PLI-188) microinjection apparatus (needle pressure ≅ 30 kPa). The cells were then incubated in high-K^+ buffer (±200 nM insulin) for 20 min at 37°C and for 10 min (± insulin) in high-K^+ buffer plus 0.1 mM 2-deoxyglucose (dGlc). Incubations were terminated by freezing in Freon-12 at −150°C.

When evaluating the effects of proteins that would be expected to require many minutes or hours to act, myocytes were placed in medium 199/10% horse serum/20 mM butanedione monoxime. Butanedione monoxime inhibits the irreversible cycle of contraction, which otherwise ensues when myocytes are injected in medium containing Ca^{2+} (25). After injections, the cells were transferred to medium without butanedione monoxime and incubated for 3 hr at 37°C. Incubations with insulin and dGlc were then performed in high-K^+ buffer, as described above.

To identify myocytes damaged by microinjection, ATP levels were measured in every cell analyzed (26) (to be described later).

Injected Proteins. Bacterially produced Ha-Ras protein having a $Gly^{12} \rightarrow Val$ mutation (Ras^{G12V}) and a Ras protein having a COOH-terminal truncation (SC1N) were provided by Jackson Gibbs, Merck. The concentration of Ras proteins injected was 0.8 mg/ml. Assuming the volume injected was 1% of the cell volume, the final Ras concentration would be ≈8 μg/ml. To our knowledge, the amount of Ras in cardiac myocytes has not been measured. The Ras content of NIH 3T3 cells determined by radioimmunoassay is 0.03 pg/ng of protein (27). This level corresponds to a concentration of 6 μg/ml, assuming protein is equal to 20% of wet weight of the cells. If myocytes and fibroblasts contain a comparable concentration of Ras, then the amount of Ras injected into the myocytes should be approximately equal to the endogenous Ras.

Hybridoma cells producing the rat monoclonal antibody (mAb) Y13-259 were obtained from the American Type Culture Collection and grown in serum-free culture medium. mAb Y13-259 and nonimmune IgG were purified from culture medium and rat serum, respectively, by using protein G-agarose (28). The proteins were injected at a concentration of 8 mg/ml, which would be expected to cause a 2-fold molar excess of mAb to Ras (see above).

Analytical Measurements. After freeze-drying at −35°C, injected myocytes were identified by using a fluorescence microscope and weighed on a quartz fiber balance (26). Each cell (≈10 ng) was transferred into 0.2 μl of 0.02 M HCl under oil in an oil well rack, which was heated at 80°C for 20 min to destroy tissue enzymes and NADPH. 2-Deoxyglucose 6-phosphate (dGlc6*P*) was measured by using a high concentration of glucose 6-phosphate (Glc6*P*) dehydrogenase (from *Leuconostoc mesenteroides*) after removing Glc6*P* (29). ATP was measured by using hexokinase and Glc6*P* dehydrogenase as described (26). The NADP cycle of Chi *et al.* (30) was used to amplify the signal and enable measurement of the NADPH formed. In measuring dGlc6*P* the amplification was on the order of 200,000-fold. A more detailed description of the analytical methods used in measuring metabolite levels in myocytes is provided in the report by Manchester *et al.* (24).

Detection of GLUT4 by Immunoblotting. After injection, myocytes were added to droplets of SDS sample buffer (33 cells per 0.4 μl) in oil wells (26). Sample buffer contained 1.2% SDS, 25% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, and 31 mM Tris phosphate. After being heated at 60°C for 10 min, samples (0.4 μl) were subjected to PAGE (12.5% resolving gel) by using a PhastGel system (Pharmacia LKB). Use of the small resolving gels was necessary to detect the relatively small amounts of transporter protein present. After electrophoresis the proteins were transferred electrophoretically to nylon membranes (Immobilon, Millipore) in a semidry blotting apparatus (PhastTransfer, Pharmacia LKB). GLUT4 was detected by using a polyclonal antibody that was generated by immunizing a rabbit with a synthetic peptide corresponding to the last 12 residues in the COOH-terminal regions of GLUT4 (31). The antibody was affinity-purified in a column containing peptide coupled to Sulfolink resin (Pierce). The nylon membranes were incubated with antibody (2 μg/ml) and washed to remove unbound antibody as described (31), except that the volumes were reduced by ≈75%. Antibody binding to GLUT4 was detected by enhanced chemiluminescence (ECL, Amersham) using horseradish peroxidase-conjugated donkey anti-rabbit IgG as secondary antibody.

RESULTS

GTP[γS] is a nonhydrolyzable analogue of GTP that produces persistent activation of Ras by locking the protein in the active GTP-bound state. In initial experiments to investigate the role of Ras in mediating insulin-stimulated glucose transport, cells were injected with either buffer alone or with buffer containing 2 mM GTP[γS] (Fig. 1). After microinjection, cells were incubated plus or minus insulin before a final incubation with dGlc. When the extracellular concentration of dGlc is low, essentially all of the sugar that enters the cell

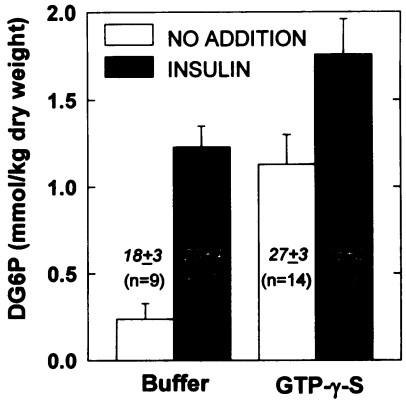

FIG. 1. Microinjected GTP[γS] activates glucose transport in cardiac myocytes. Cardiac myocytes were cultured overnight in medium 199, then transferred to high-K^+ buffer plus 10 mM ATP, and injected during a 15-min period at 21°C with either buffer alone or with buffer containing 2 mM GTP[γS]. The injection buffer contained rhodamine dextran at 3 mg/ml, 100 mM KCl, and 10 mM sodium phosphate, pH 7.3. The cells were then incubated in high-K^+ buffer in the absence or presence of 200 nM insulin at 37°C. After 20 min the cells were transferred to buffer (with or without insulin) containing 0.1 mM dGlc. The incubation was terminated after 10 min by freezing the cells in Freon-12. The results are from an experiment that is representative of three experiments. Bars denote dGlc6*P* (DG6P) levels and represent means ± SEMs of *n* cells. Values for ATP (mmol/kg of dry weight) are shown in italics.

is phosphorylated to dGlc6*P*, which accumulates because further metabolism is limited. Thus, measuring the initial rate of dGlc6*P* accumulation provides an accurate index of glucose transporter activity (24). Insulin increased dGlc6*P* by ≈5-fold in the buffer-injected cells (Fig. 1), indicating that insulin-stimulated glucose transport was retained after microinjection. Injecting GTP[γS] also increased dGlc6*P* by ≈5-fold relative to cells injected with buffer alone (Fig. 1). Insulin caused a further increase in dGlc6*P* accumulation in cells injected with GTP[γS], although the effects of insulin and the nucleotide were not additive. These findings are consistent with results from previous experiments in permeabilized adipocytes, where GTP[γS] increased translocation of GLUT4 to the plasma membrane (32, 33).

Microinjection inevitably damages a fraction of cells. In the extreme case, myocytes were found that appeared grossly normal by phase-contrast microscopy but which contained almost no ATP. Measurements of ATP proved essential in eliminating nonviable cells, particularly when transport was measured shortly after microinjection. Myocytes having an estimated intracellular ATP concentration of <2 mM (10 mmol/kg of dry weight, 0–20% of those analyzed) were excluded from the analyses of dGlc6*P* accumulation. As damaged cells eventually either recover or die (and detach), ATP levels in cells incubated for longer times after injection were generally higher and more uniform.

The results with GTP[γS] support the hypothesis that Ras activation leads to activation of glucose transport. However, most if not all of the small GTP-binding proteins in the Ras superfamily, as well as the heterotrimeric G proteins, would be expected to be affected by the nonhydrolyzable GTP analogue (8, 9). To investigate further the role of Ras, experiments were conducted in which cells were injected with Ras^{G12V}. This mutant form of Ras has very low GTPase activity and is thus persistently activated (34). Microinjecting the Ras^{G12V} increased dGlc6*P* accumulation to the same extent as insulin (Fig. 2). Treating cells that had been injected with Ha-Ras^{G12V} with insulin resulted in an increase in activity greater than that produced by insulin in the buffer-injected cells (Fig. 2). In contrast, Ras protein lacking the COOH-terminal CXXX sequence (SC1N) (35) did not activate transport (Fig. 2). The cysteine in this sequence is the site of farnesylation (9), which is necessary for the association of Ras with membranes.

The insulin-responsive glucose transporter GLUT4 is expressed in high levels in cardiac myocytes. By pooling a relatively small number of myocytes, GLUT4 protein could be readily detected by immunoblotting (Fig. 3). The levels of GLUT4 were not changed by injecting cells with either Ras^{G12V} or SC1N. Thus, the increase in glucose transport produced by Ras^{G12V} most likely resulted from translocation of GLUT4 to the plasma membrane rather than to an increase in the total amount of the transporter.

The question might arise as to whether injecting oncogenic Ras produces actions not representative of the endogenous protein. Therefore, the role of endogeneous Ras in the insulin response was investigated by injecting myocytes with the Ras mAb Y13-259 (Fig. 4). Microinjecting this mAb has been shown previously to inhibit DNA synthesis and to cause morphological reversion of Ras-transformed cells (36, 37). For comparison, other myocytes were injected with an equal concentration of nonimmune IgG. mAb Y13-259 decreased insulin-stimulated dGlc6*P* levels in myocytes by ≈50%. Nonimmune IgG did not significantly change dGlc6*P* accumulation.

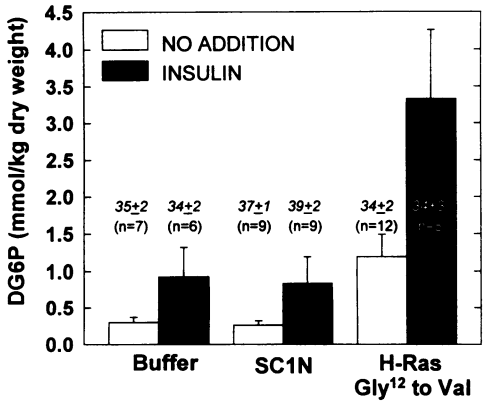

FIG. 2. Microinjecting activated Ras increases basal and insulin-stimulated glucose transport. Myocytes were cultured overnight in medium 199 and then transferred to medium 199 containing 20 mM butanedione monoxime. Cells were injected during a 15-min period with buffer alone or buffer containing either SC1N or Ras^{G12V} at 0.8 mg/ml. Injection buffer contained rhodamine dextran, 1 mM EDTA, and 10 mM Tris·HCl, pH 7.4. After injection the cells were incubated for 3 hr at 37°C in medium 199 without butanedione monoxime and then incubated with insulin and dGlc as described in the legend to Fig. 1. The results for dGlc6*P* (DG6P) and ATP (in italics, mmol/kg of dry weight) are from an experiment that is representative of three experiments.

DISCUSSION

By combining microinjection and microanalytical methods we have developed a method to investigate signal-transduction pathways involved in the activation of glucose transport by insulin. Microinjecting adult cardiac myocytes with GTP[γS] (Fig. 1) or Ras^{G12V} (Fig. 2) mimicked insulin by stimulating dGlc6*P* accumulation, whereas attenuation of insulin-stimulated transport was observed in cells injected with Ras mAb (Fig. 4). These findings implicate Ras in the activation of glucose transport by insulin in a terminally differentiated cell type that represents an important physiological target of the hormone *in vivo*.

It was previously found that stable overexpression of activated N-Ras in 3T3L1 adipocytes increased the propor-

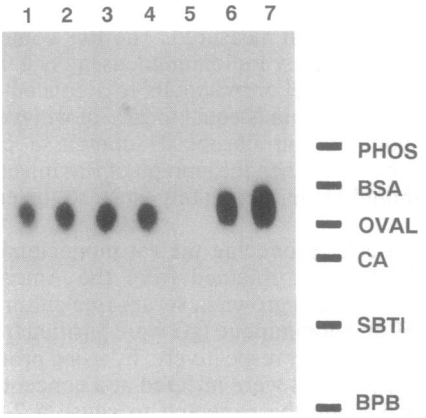

FIG. 3. Microinjecting Ras does not change GLUT4 protein. Cells were injected as described in the legend to Fig. 2. After freeze-drying, 33 cells from each treatment group were dissolved in SDS sample buffer (0.4 μl) and applied to a 12.5% Phast gel, which was loaded as follows. Lanes: 1, buffer-injected cells; 2, cells injected with Ras^{G12V}; 3, cells injected with SC1N; 4, noninjected cells; and 5, blank. Samples containing 0.5 μg (lane 6) and 1 μg (lane 7) of protein from a rat adipocyte low-density microsomal fraction, a rich source of GLUT4, were also included. After electrophoresis, proteins were transferred to nylon membranes, and GLUT4 was detected by immunoblotting and ECL. A picture of the entire blot is shown. Mobilities of the following standard proteins are indicated: phosphorylase b (PHOS), bovine serum albumin (BSA), ovalbumin (OVAL), carbonic anhydrase (CA), soy bean trypsin inhibitor (SBTI), and bromophenol blue (BPB).

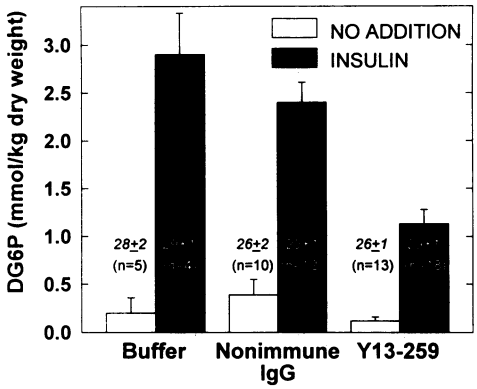

FIG. 4. Ras mAb inhibits insulin-stimulated glucose transport. Solutions (8 mg/ml) of nonimmune IgG and mAb Y13-259 were injected into myocytes, as described in the legend to Fig. 2, except that the injection buffer was 100 mM KCl/10 mM sodium phosphate, pH 7.3. Results are from an experiment that is representative of two experiments. ATP levels (mmol/kg of dry weight) are in italics. DG6P, dGlc6*P*.

tion of GLUT4 in the plasma membrane (38). However, interpretation of this finding was complicated by the fact that Ras overexpression reduced the amount of GLUT4 in the cells by ≈95% (38). In the present experiments, activation of transport was observed in response to Ras^{G12V} under conditions in which the GLUT4 level was not changed (Fig. 3). Moreover, we have observed increased dGlc6*P* accumulation 30 min after injecting oncogenic N-Ras (unpublished observations). This effect is within the time frame of an acute response to insulin, particularly when allowance is made for the time required for fatty acylation and association of the injected Ras with the membrane.

Previous results obtained in fibroblasts (39) and 3T3 L1 adipocytes (40) indicate that the Ras signaling pathway, which involves activation of mitogen-activated protein (MAP) kinase, is not sufficient to account for the activation of glucose transport by insulin. Our results are consistent with these previous results. The finding that mAb Y13-259 decreased insulin-stimulated transport by only 50% (Fig. 4) is suggestive of another pathway, as is the observation that microinjecting Ras^{G12V} increased transport over that produced by a maximally effective concentration of insulin (Fig. 2). At present, the findings are fitted best by a model in which glucose transport is activated by insulin via two pathways, one of which is independent of Ras. An implication of the model is that agents that inhibit the Ras signaling pathway could inhibit glucose transport. Thus, attenuation of the effect of insulin on stimulating the Ras pathway, as was recently shown to occur in response to increased cAMP (41), might explain, at least in part, the decrease in glucose transport that occurs with counter-regulatory agents. However, if there are two pathways, even ablation of one would not be expected to abolish the effect of insulin on increased transport.

To understand fully the activation of glucose transport, it will be necessary to identify the components involved in the signal-transduction pathways. In this regard, it should be noted that the downstream effectors in the Ras signaling pathway responsible for glucose transport activation have not been determined, nor has the Ras-independent pathway been defined. Hopefully, the system of microinjection and microanalyses that we have developed will be useful in evaluating the role of candidate effectors in insulin action.

We thank Dr. J. Gibbs for supplying Ras proteins and Dr. E. M. Johnson for comments regarding the manuscript. This work was supported, in part, by National Institutes of Health Grants DK28312 and NS08862, a grant from Monsanto/Washington University, and an anonymous donor.

1. Cryer, P. E. (1993) *Am. J. Physiol.* **264,** E149–E155.
2. Elbrink, J. & Bihler, I. (1975) *Science* **188,** 1177–1184.
3. Morgan, H. E. & Whitfield, C. F. (1973) *Curr. Top. Membr. Transp.* **4,** 255–303.
4. Pessin, J. E. & Bell, G. I. (1992) *Annu. Rev. Physiol.* **54,** 911–930.
5. Simpson, I. A. & Cushman, S. W. (1986) *Annu. Rev. Biochem.* **55,** 1059–1089.
6. Denton, R. M. (1986) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20,** 293–341.
7. Lawrence, J. C., Jr. (1992) *Annu. Rev. Physiol.* **54,** 177–193.
8. Bourne, H. R., Sanders, D. A. & McCormick, F. (1991) *Nature (London)* **349,** 117–127.
9. Lowy, D. R. & Willumsen, B. M. (1993) *Annu. Rev. Biochem.* **62,** 851–891.
10. Burgering, B. M. T., Medema, R. H., Maassen, J. A., van de Wetering, M. L., van der Eb, A. J., McCormick, F. & Bos, J. L. (1991) *EMBO J.* **10,** 1103–1109.
11. Nakafuku, M., Satoh, T. & Kaziro, Y. (1992) *J. Biol. Chem.* **267,** 19448–19454.
12. Porras, A., Nebreda, A. R., Benito, M. & Santos, E. (1992) *J. Biol. Chem.* **267,** 21124–21131.
13. Baltensperger, K., Kozma, L. M., Cherniack, A. D., Klarlund, J. K., Chawla, A., Banerjee, U. & Czech, M. P. (1993) *Science* **260,** 1950–1952.
14. Skolnik, E. Y., Lee, C. H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. J., Jr., Backer, J. M., Ullrich, A., White, M. F. & Schlessinger, J. (1993) *EMBO J.* **12,** 1929–1936.
15. Skolnik, E. Y., Batzer, A., Li, N., Lee, C.-H., Lowenstein, E., Mohammadi, M., Margolis, B. & Schlessinger, J. (1993) *Science* **260,** 1953–1955.
16. White, M. F. & Kahn, C. R. (1994) *J. Biol. Chem.* **269,** 1–4.
17. Kyriakis, J. M., App, H., Zhang, X. F., Banerjee, P., Brautigan, D. L., Rapp, U. R. & Avruch, J. (1992) *Nature (London)* **358,** 417–421.
18. Blenis, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5889–5892.
19. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E. & Woodgett, J. R. (1991) *Nature (London)* **353,** 670–674.
20. Seth, A., Alvarez, E., Gupta, S. & Davis, R. J. (1991) *J. Biol. Chem.* **266,** 23521–23524.
21. Korn, L. J., Siebel, C. W., McCormick, F. & Roth, R. A. (1987) *Science* **236,** 840–843.
22. Rose, D. W., Saltiel, A. R., Majumdar, M., Decker, S. J. & Olefsky, J. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 797–801.
23. Powell, T. & Twist, V. W. (1976) *Biochem. Biophys. Res. Commun.* **72,** 327–333.
24. Manchester, J., Kong, X., Nerbonne, J., Lowry, O. H. & Lawrence, J. C., Jr. (1994) *Am. J. Physiol.*, in press.
25. Thorburn, A., Thorburn, J., Chen, S.-Y., Powers, S., Shubeita, H. E., Feramisco, J. R. & Chien, K. R. (1993) *J. Biol. Chem.* **268,** 2244–2249.
26. Lowry, O. H. & Passonneau, J. V. (1992) *A Flexible System of Enzymatic Analysis* (Academic, New York).
27. Caruso, A., Schlom, J., Vilasi, V., Weeks, M. O. & Hand, P. H. (1986) *Int. J. Cancer* **38,** 587–595.
28. Harlow, E. & Lane, D. (1988) *Antibodies* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 53–137.
29. Chi, M. M., Manchester, J. K., Carter, J. G., Pusateri, M. E., McDougal, D. B. & Lowry, O. H. (1993) *Anal. Biochem.* **209,** 335–338.
30. Chi, M. M., Lowry, C. V. & Lowry, O. H. (1978) *Anal. Biochem.* **89,** 119–129.
31. Robinson, R., Robinson, L., James, D. E. & Lawrence, J. C., Jr. (1993) *J. Biol. Chem.* **268,** 26422–26427.
32. Robinson, L. J., Pang, S., Harris, D. S., Heuser, J. & James, D. E. (1992) *J. Cell Biol.* **117,** 1181–1196.
33. Baldini, G., Hohman, M., Charron, M. J. & Lodish, H. F. (1991) *J. Biol. Chem.* **266,** 4037–4040.
34. Seeburg, P. H., Colby, W. W., Capon, D. J., Goeddel, D. V. & Levinson, A. D. (1994) *Nature (London)* **312,** 71–75.
35. Temeles, G. L., Gibbs, J. B., D'Alonzo, J. S., Sigal, I. S. & Scolnick, E. M. (1985) *Nature (London)* **313,** 700–703.

36. Kung, H.-F., Smith, M. R., Bekesi, E., Manne, V. & Stacy, D. W. (1986) *Exp. Cell Res.* **162,** 363–371.
37. Stacey, D. W., DeGudicibus, S. R. & Smith, M. R. (1987) *Exp. Cell Res.* **171,** 232–242.
38. Kozma, L., Baltensperger, K., Klarlund, J., Porras, A., Santos, E. & Czech, M. P. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4460–4464.
39. Osterop, A. P. R. M., Medema, R. H., Bos, J. L., van de Zon, G. C. M., Moller, D. E., Flier, J. S., Moller, W. & Massen, J. A. (1992) *J. Biol. Chem.* **267,** 14647–14653.
40. Robinson, L. J., Razzack, Z. F., Lawrence, J. C., Jr., & James, D. E. (1993) *J. Biol. Chem.* **268,** 26422–26427.
41. Sevetson, B. R., Kong, X. & Lawrence, J. C., Jr. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10305–10309.