BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **246,** 282–286 (1998)
ARTICLE NO. RC988599

Separation of IRS-1 and PI3-Kinase from GLUT4 Vesicles in Rat Skeletal Muscle

Jennifer L. Kummer, Mónica R. Calera, and Paul F. Pilch[1]
Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

Received March 31, 1998

In fat and muscle tissues, insulin stimulates cellular glucose uptake by initiating a phosphorylation cascade which ultimately results in the translocation of the GLUT4 glucose transporter isoform from an intracellular vesicular storage pool(s) to the plasma membrane in fat and to t-tubules in skeletal muscle. Insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3-kinase) are known to be involved in cellular responses to insulin such as GLUT4 translocation, but the biochemical mechanism(s) connecting IRS-1 and PI3-kinase to GLUT4-containing intracellular membranes remains unclear. Here, in control and insulin-stimulated rat skeletal muscle, the intracellular localization of these two proteins was compared to that of GLUT4 using subcellular fractionation by sucrose velocity gradients followed by immunoblotting. Our data show that insulin-sensitive GLUT4-containing vesicles are present in fractions 1 through 10, whereas IRS-1 and PI3-kinase are found in fractions 16 through 24. These results indicate that in intracellular fractions derived from skeletal muscle, IRS-1 and PI3-kinase are excluded from membranes harboring GLUT4.

Insulin, a key regulator of glucose homeostasis, exerts its effects by binding to the transmembrane insulin receptor/kinase, inducing receptor autophosphorylation of its β-subunit (1, 2) and initiating a series of phosphorylation reactions emanating from its intrinsic tyrosine kinase activity (3-5). This signaling cascade results in the translocation of GLUT4-containing vesicles from an intracellular storage pool(s) to the plasma membrane and t-tubules thereby facilitating the removal of glucose from the blood (6-8). The exact mechanism by which insulin mediates GLUT4 vesicular trafficking remains to be defined.

[1] To whom correspondence should be addressed at Department of Biochemistry, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118. Fax: (617) 638-5339. E-mail: pilch@med-biochem.bu.edu.

The IRS-1/PI3-kinase pathway, in particular, has been implicated as a key player in mediating GLUT4 activation. The activated insulin receptor phosphorylates tyrosine residues in IRS-1, thereby promoting the association of Src homology (SH) 2 domain containing proteins (9), such as PI3-kinase, with IRS-1 (10). Upon binding of the p85 regulatory subunit of this lipid kinase to tyrosine phosphorylated IRS-1, the p110 catalytic subunit of PI3-kinase is activated (5, 10, 11). The regulation of this signaling pathway by insulin has been extensively studied using two specific inhibitors of PI3-kinase, LY294002 and wortmannin. Both inhibitors have been shown, independently, to block glucose uptake and the translocation of GLUT4 from an intracellular compartment to the plasma membrane of adipocytes, L6 cells and to the t-tubules of rat skeletal muscle (12-20).

In efforts to understand the coupling of PI3-kinase activation to GLUT4 translocation in fat cells, the localization of these proteins has been examined by immunoadsorption (21) and by sedimentation analysis in sucrose gradients (22). This studies have led to conflicting results concerning GLUT4 and PI3-kinase co-localization, the former study supports this hypothesis and the latter not. Here, we examined the distribution of IRS-1, PI3-kinase, and GLUT4 proteins in intracellular fractions obtained from insulin-treated and untreated rat skeletal muscle using a newly developed muscle fractionation procedure (23). Our results show that insulin causes a decrease in intracellular GLUT4 vesicles, presumably due to their translocation to the plasma membrane and t-tubules of the skeletal muscle. The GLUT4 vesicles do not contain IRS-1 or PI3-kinase to any detectable extent. Rather, IRS-1 and PI3-kinase are partially co-located in similar fractions devoid of GLUT4-containing vesicles.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY) or Charles River Laboratories (Wilmington, MA). The polyclonal antibodies against p85 PI3-kinase and

0006-291X/98 $25.00

IRS-I were obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). Monoclonal anti-GLUT4 antibody was used as described (24). Insulin from bovine pancreas and goat secondary anti-rabbit antibody conjugated with horseradish peroxidase were purchased from Sigma (St. Louis, MO). The bicinchoninic acid (BCA) determination kit was obtained from Pierce (Rockford, IL). The chemiluminescent detection system (ECL) was purchased from Amersham (Arlington Heights, IL). Bio-Rad Laboratories (Hercules, CA) supplied the kaleidoscope molecular weight prestained standards.

Insulin stimulation of rat skeletal muscle in vivo. Male Sprague-Dawley rats with a body weight ranging from 150 to 175 g were anaesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100g body wt) in 0.9% saline solution. Rats fully anaesthetized were injected into the portal vein with either vehicle or 1.5 units of bovine insulin and tissue removal was performed 8 min after insulin injection.

Cell fractionation. Rat hindlimb muscles were excised and homogenized with a polytron (15 sec. Ultra Turrax T25 homogenizer, 13, 500 rpm) in a buffer containing 20 mM Hepes, 250 mM sucrose, 1 mM EDTA, 5 mM benzamidine, 1 mM pepstatin, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 200 μM sodium orthovanadate and 20 mM sodium fluoride at pH 7.4, and subcellular fractionation was performed in a new protocol (23) that modifies previous muscle fractionation procedures (25, 26). Briefly, the homogenate was centrifuged at 2000 x g for 10 min (all centrifugations were performed at 4°C) the pellet was discarded and the supernatant was spun at 9000 x g for 20 min. The resultant supernatant was centrifuged for 90 min at 180,000 x g to sediment total membranes. The pellet was resuspended in phosphate-buffered saline (PBS) containing protease and phosphatase inhibitors. Protein content was measured using BCA assay with bovine serum albumin as standard. Two mg of total protein was loaded on a 4.6 ml 10-30% (W/V) continuous sucrose gradient and centrifuged at 100,000 x g for 1 h. Fractions (250 μl) were collected from the sucrose gradient and equal volumes of samples were analyzed by Western blotting.

Gel electrophoresis and Western blotting. Proteins samples were solubilized in Laemmli sample buffer (27) and separated on 8% and 10% polyacrylamide gels in the presence of SDS along with prestained molecular weight standards. After electrophoretic separation, proteins were transferred to nitrocellulose membranes (0.45 μM, Bio-Rad Laboratories, Hercules, CA) at 4°C for 16 h. The membranes were blocked in PBST (0.05% Tween-20, pH = 7.4) containing 5% nonfat dry milk for 1 hr at 25°C, then probed with the appropriate antibody for two hours. To detect the antigen-bound antibody the blots were treated with secondary antibody conjugated with horseradish peroxidase. Immunoreactivity was visualized by enhanced chemiluminescence and quantified by using a computing densitometer (Molecular Dynamics, Sunnyvale, CA). The data shown are representative of two experiments.

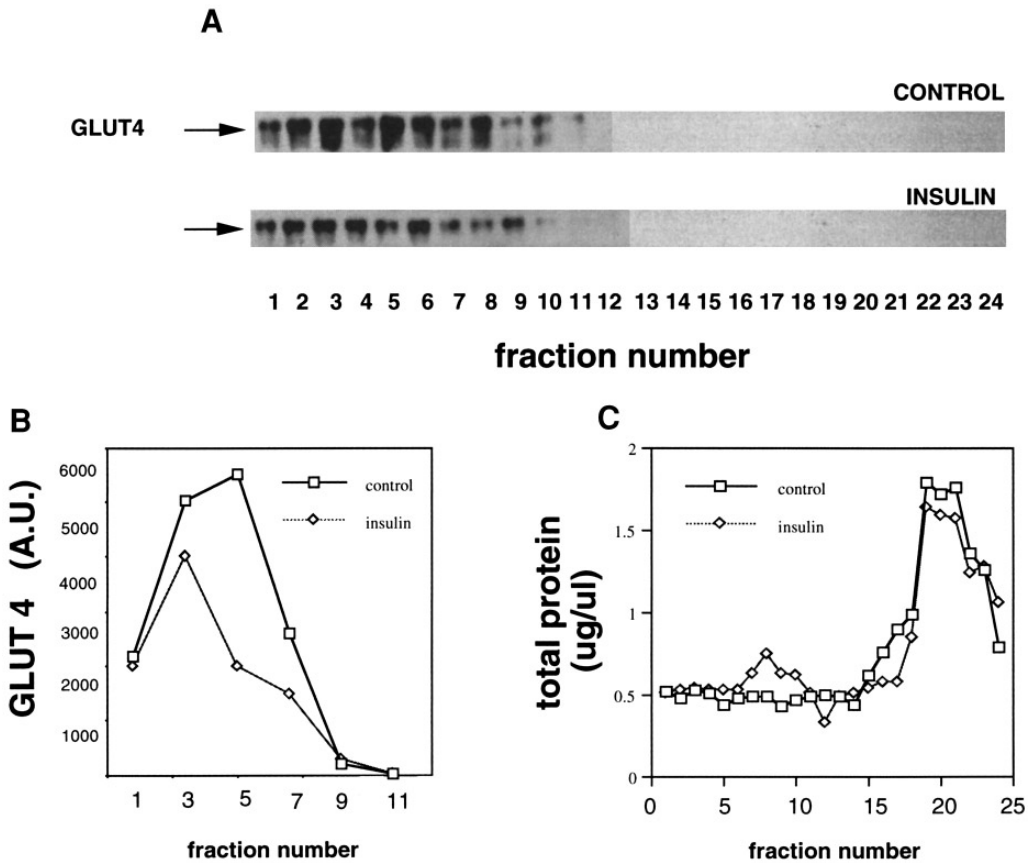

FIG. 1. Insulin stimulates the translocation of GLUT4 vesicles in rat skeletal muscle. Muscle membranes (2 mg of protein) from untreated and insulin-treated (1.5 units/rat for 8 min) rat skeletal muscle were centrifuged in a 10-30% sucrose gradient (4.6 ml) as described under Materials and Methods. 100 μl of each collected gradient fraction were separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose, and probed with anti-GLUT4 antibody. (A) Autoradiogram of the GLUT 4 immunoblot following detection with the chemiluminescent detection system (ECL). (B) Densitometric analysis of the blot expressed as arbitrary units. (C) Total protein values of the fractions.

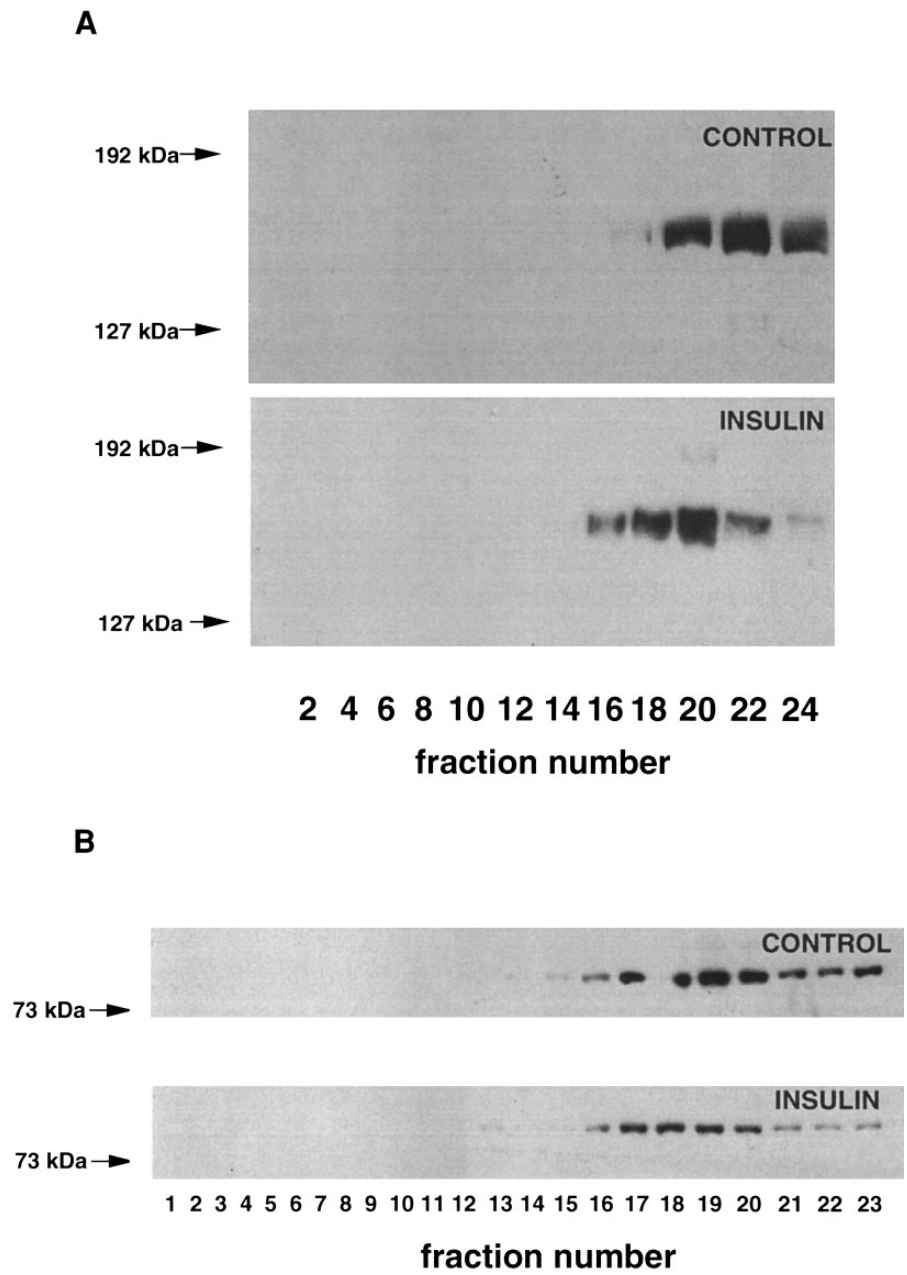

FIG. 2. IRS-1 and PI3-kinase partially co-localize in vesicles with the same density. 100 μl of each fraction collected from the sucrose velocity gradient performed as indicated in Fig. 1 was separated by SDS-PAGE (8% acrylamide), transferred to nitrocellulose membranes, and incubated with polyclonal anti-IRS-1 and anti-p85 antibodies as described under Materials and Methods. Autoradiograms of the IRS-1 (A) and PI3-kinase (B) immunoblots from untreated and insulin-treated rat skeletal muscle.

RESULTS AND DISCUSSION

Insulin administered in vivo induces translocation of GLUT4 vesicles. Male Sprague-Dawley rats were exposed to insulin or saline solution for 8 min *via* portal vein injections. Following removal of the entire hindlimb muscle, the samples were homogenized, fractionated and muscle membranes were centrifuged by a sucrose velocity gradient as described in the Methods section. Immunoblot analysis of the fractions from the gradient with a anti-GLUT4 antibody showed that fractions 1 through 10 contained the insulin-sensitive glucose transporter (Figure 1A). In addition, insulin caused a decrease in the GLUT4 immunoreactivity thus demonstrating the insulin-induced translocation of GLUT4-containing vesicles from the intracellular

membrane vesicles to the plasma and t-tubule membranes (23). To quantify the immunoblot data, densitometric analysis was performed and as illustrated in Figure 1B, GLUT4 levels decreased by 35% from the intracellular vesicles in response to insulin. The majority of GLUT4 vesicles (>98%) were isolated in fractions 1 through 10 and no GLUT4 was detected in fractions 11 through 24 (Figures 1A & 1B) even upon gross over exposure (data not shown).

To confirm that the observed decrease in GLUT4 was not due to unequal protein loading, the protein content in each gradient fraction was measured. As shown in Figure 1C, fractions from the control and insulin-stimulated animals contained similar concentrations of total protein as was previously shown (23, 26).

IRS-1 and PI3-kinase do not associate with GLUT4-containing vesicles isolated from rat skeletal muscle. To determine if IRS-1 is associated with GLUT4-containing vesicles, fractions collected from sucrose velocity gradients were analyzed by Western blotting using a polyclonal anti-IRS-1 antibody. As shown in Figure 2A, fractions 20 through 24 from control animals contain IRS-1 while fractions 1 through 10, which contained the GLUT4 vesicles, did not display any immunoreactivity with the IRS-1 antibody. The *in vivo* insulin stimulation resulted in a shift of the IRS-1 signal from fractions 20-24 to fractions 16-22 (Figure 2A), and the latter corresponds to the peak of the p85 signal (see Figure 3B).

In fractions identical to those described for analyzing GLUT4 and IRS-1, PI3-kinase content was determined by immunoblotting with a polyclonal antibody against the p85 regulatory subunit of this kinase. Figure 2B shows that the PI3-kinase distribution partially overlaps with IRS-1 in the gradient. The PI3-kinase was localized to fractions 16 to 23 and no signal was detected in fractions 1 to 10 corresponding to the GLUT4 distribution. As shown in Figure 3A and 3B, insulin mediated a 65% decrease in the PI3-kinase levels. It is unclear why this signal decreases in response to insulin, but in rat adipocytes and in 3T3-L1 cells, over 60% of p85 is cytosolic (21, 28) and thus the amount in fat LDM, the equivalent fraction to that shown in the gradient (21, 28), is only a small amount of the total. Nevertheless and importantly, the signals for IRS-1 and p85 completely overlap in muscle membranes isolated from insulin-treated animals, presumably as a result of p85 interaction with tyrosine phosphorylated IRS-1 as other have shown (29).

Recent studies with rat adipocytes, L6 muscle cells and rat skeletal muscle have indicated that both IRS-1 and PI3-kinase play an important role in inducing GLUT4 translocation and glucose uptake (12-21, 30). The subcellular localization and trafficking of these molecules, however, remains controversial. In 3T3-L1 adipocytes, immunoadsorbed GLUT4-containing vesicles were shown to contain PI3-kinase/IRS-1 complexes as well as PI3-kinase activity (23). However in other studies, GLUT4 vesicles fractionated from rat adipocytes (28) and from 3T3-L1 cells (22) have been shown not to contain IRS-1 or PI3-kinase.

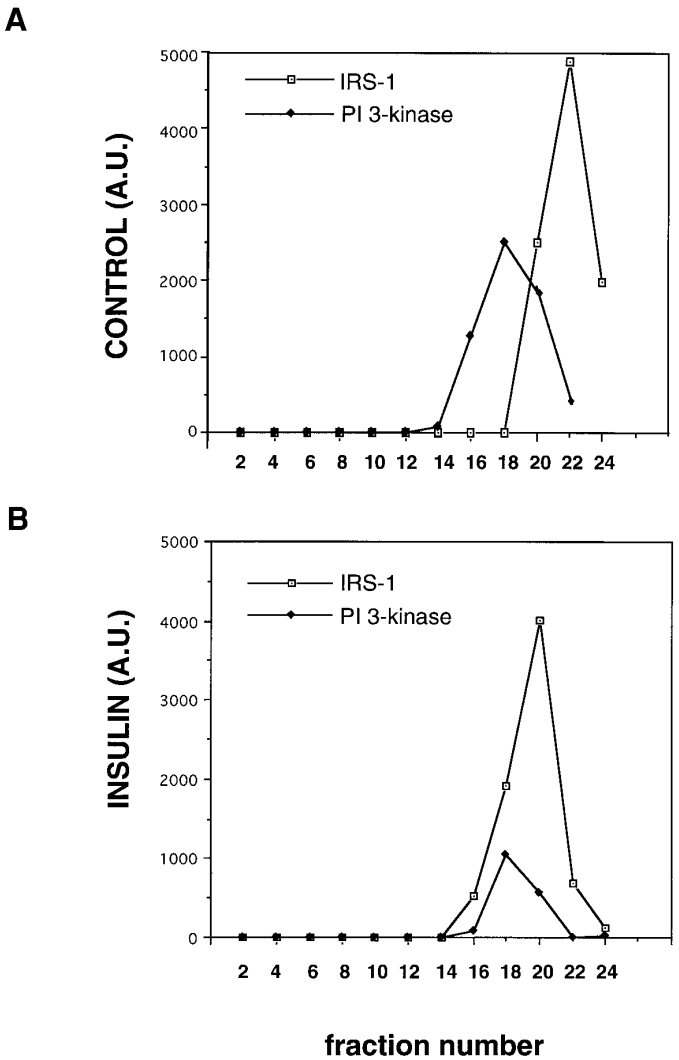

FIG. 3. Quantification of IRS-1 and PI3-kinase distribution in cell fractions separated by a sucrose velocity gradient. Densitometric analysis of data shown in Fig. 2 was performed and results expressed as arbitrary units. IRS-1 and PI3-kinase from untreated (A) or insulin-treated (1.5 units/rat for 8 min) (B) rat skeletal muscle.

The lack of PI3-kinase and IRS-1 in GLUT4 vesicles suggests that additional signaling molecules may play a role in transducing the signal between the activated IRS-1/PI3-kinase complex and the GLUT4-containing vesicles. In fact, it has recently been shown that the serine/threonine protein kinase known as Akt/RAC/PKB requires PI3-kinase for activation and activated Akt is capable of stimulating GLUT4 translocation as well as glucose uptake in 3T3-L1 adipocytes (31-33). Moreover, we have recently shown that Akt-2 associ-

ates with GLUT4 vesicles in an insulin-dependent and wortmannin inhibitable fashion (34). Therefore, activation of additional intermediate signaling molecules may be responsible for linking PI3-kinase to GLUT4 translocation.

Finally, recent evidence indicates that an intact actin network may be necessary for the translocation of GLUT4 and that IRS-1 may associate with the cytoskeleton in order to induce PI3-kinase activation (35). Treatment of the differentiated L6 muscle cell line with Cytochalasin D, a specific inhibitor of actin filament assembly, prevented insulin-induced GLUT4 translocation (35). More recently, Clark *et al.* (24) showed that in differentiated 3T3-L1 cells, IRS-1 associated with cytoskeletal-like filaments and was localized independently of GLUT4-containing vesicles. Our current data are completely consistent with these latter findings in that IRS-1 isolated from muscle (Fig. 2A) sediment similarly to IRS-1 isolated from cultured fat cells (24).

In summary, these are the first *in vivo* studies using rat skeletal muscle that demonstrate the differential localization of IRS-1 and PI3-kinase-containing structures from GLUT4-containing vesicles, thus further strengthening the hypothesis that additional signaling molecules downstream of PI3-kinase may play a role in regulating glucose uptake and homeostasis. In addition, emerging evidence indicates that IRS-1′s association with the cytoskeleton may serve an important role in activating PI3-kinase and transmitting the insulin signal.

ACKNOWLEDGMENTS

We thank Gino Vallega for technical help. This work was supported by Grant DK 36424 from the National Institutes of Health.

REFERENCES

1. Tavare, J. M., O'Brien, R. M., Siddle, K., and Denton, R. M. (1988) *Biochem. J.* **253,** 783–788.
2. Tornqvist, H. E., and Avruch, J. (1988) *J. Biol. Chem.* **263,** 4593–4601.
3. White, M. F., and Kahn, C. R. (1994) *J. Biol. Chem.* **269,** 1–4.
4. Lee, J., and Pilch, P. F. (1994) *Am. J. Physiol.* **266,** C319–C334.
5. Cheatham, B., and Kahn, C. R. (1995) *Endocr. Rev.* **16,** 117–142.
6. Stephens, J. M., and Pilch, P. F. (1995) *Endocr. Rev.* **16,** 529–546.
7. Kandror, K. V., and Pilch, P. F. (1996) *Am. J. Physiol.* **271,** E1–E4.
8. Olson, A. L., and Pessin, J. E. (1996) *Annu. Rev. Nutr.* **16,** 235–256.
9. Moran, M. F., Koch, C. A., Anderson, D., Ellis, C., England, L., Martin, G. S., and Pawson, T. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8622–8626.
10. Sun, X. J., Crimmons, D. L., Myers, M. G., Jr., Miralpeix, M., and White, M. F. (1993) *Mol. Cell. Biol.* **13,** 7418–7428.
11. Lamphere, L., Carpenter, C. L., Sheng, Z-F., Kallen, R. G., and Lienhard, G. E. (1994) *Am. J. Physiol.* **266,** E486–E494.
12. Berger, J., Hayes, N., Szalkowski, D. M., and Zhang, B. (1994) *Biochem. Biophys. Res. Commun.* **205,** 570–576.
13. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Mol. Cell. Biol.* **14,** 4902–4911.
14. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M. (1994) *J. Biol. Chem.* **269,** 3568–3573.
15. Elmendorf, J. S., Damrau-Abney, A., Smith, T. R., David, T. S., and Turinsky, J. (1995) *Biochem. Biophys. Res. Commun.* **208,** 1147–1153.
16. Haruta, T., Morris, A. J., Rose, D. W., Nelson, J. G., Mueckler, M., and Olefsky, J. M. (1995) *J. Biol. Chem.* **270,** 27991–27994.
17. Kotani, K., Carrozi, A. J., Sakaue, H., Hara, K., Robinson, L. J., Clark, S. F., Yonezawa, K., James, D. E., and Kasuga, M. (1995) *Biochem. Biophys. Res. Commun.* **209,** 343–348.
18. Tsakiridis, T., McDowell, H. E., Walker, T., Downes, C. P., Hundal, H. S., Vranic, M., and Klip, A. (1995) *Endocrinology* **136,** 4315–4322.
19. Yeh, J-I., Gulve, E. A., Rameh, L., and Birnbaum, M. J. (1995) *J. Biol. Chem.* **270,** 2107–2111.
20. Yang, J., Clarke, J. F., Ester, C. J., Young, P. W., Kasuga, M., and Holman, G. D. (1996) *Biochem. J.* **313,** 125–131.
21. Heller-Harrison, R. A., Morin, M., Guilherme, A., and Czech, M. P. (1996) *J. Biol. Chem.* **271,** 10200–10204.
22. Clark, S. F., Martin, S., Carozzi, A. J., Hill, M. M., and James, D. E. (1998) *J. Cell Biol.* **140,** 1211–1225.
23. Zhou, M., Sevilla, L., Vallega, G., Chen, P., Palacin, M., Zorzano, A., Pilch, P. F., and Kandror, K. V. *Am. J. Physiol.*, in press.
24. James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) *Nature* **333,** 183–185.
25. Coderre, L., Kandror, K. V., Vallega, G., and Pilch, P. F. (1995) *J. Biol. Chem.* **270,** 27584–27588.
26. Kandror, K. V., Coderre, L., Pushkin, A. V., and Pilch, P. F. (1995) *Biochem. J.* **307,** 383–390.
27. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
28. Kelly, K. L., and Ruderman, N. B. (1993) *J. Biol. Chem.* **268,** 4391–4398.
29. Folli, F., Saad, M. J., Backer, J. M., and Kahn, C. R. (1992) *J. Biol. Chem.* **267,** 22171–22177.
30. Kaburagi, Y., Satoh, S., Tamemoto, H., Yamamoto-Honda, R., Tobe, K., Veki, K., Yamauchi, T., Kono-Sugita, E., Sekihara, H., Aizawa, S., Cushman, S. W., Akanuma, Y., Yazaki, Y., and Kadowaki, T. (1997) *J. Biol. Chem.* **272,** 25839–25844.
31. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) *J. Biol. Chem.* **271,** 31372–31378.
32. Cong, L-N., Chen, H., Li, Y., Zhou, L., McGibbon, M. A., Taylor, S. I., and Quon, M. J. (1997) *Mol. Endocrinol.* **11,** 1881–1890.
33. Tanti, J-F., Grillo, S., Gremeaux, T., Coffer, P. J., Van Obberghen, E., and Le Marchand-Brustel, Y. (1997) *Endocrinology* **138,** 2005–2010.
34. Calera, M. R., Martinez, C., Liu, H., El Jack, A. K., Birnbaum, M. J., and Pilch, P. F. (1998) *J. Biol. Chem.* **273,** 7201–7204.
35. Tsakiridis, T., Vranic, M., and Klip, A. (1994) *J. Biol. Chem.* **269,** 29934–29942.