Insulin Increases the Association of Akt-2 with Glut4-containing Vesicles*

(Received for publication, December 30, 1997, and in revised form, February 2, 1998)

Mónica R. Calera, Carmen Martinez, Hongzhi Liu, Amr K. El Jack, Morris J. Birnbaum‡, and Paul F. Pilch§

From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118 and the Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6148

Expression of a constitutively active, membrane-associated Akt-1 (PKB α) construct in 3T3L1 adipocytes was shown to induce glucose uptake in the absence of insulin by stimulating Glut4 translocation to the plasma membrane (Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) J. Biol. Chem. 271, 31372–31378). However, in rat fat cell the vast majority of Akt-1 is cytosolic and shows no re-distribution to the plasma membrane in response to insulin. On the other hand, little work has been done with other Akt family members such as Akt-2 (PKB β) or Akt-3 (PKB γ). In this report, an analysis of the subcellular distribution of Akt-2 in rat adipocytes shows that Akt-2 is present in significant amounts in various membrane compartments, as well as in the cytosol, and the former include the light microsomes where Glut4 is present in the basal state. The distribution of Akt-2 in resting adipocytes was found to substantially overlap with that of Glut4 when light microsomes were subfractionated by a sucrose velocity gradient indicating possible co-localization. We confirmed co-localization of Akt-2 and Glut4 in the basal state by immunopurification of Glut4 vesicles, which exhibited a 5.5-fold increase in Akt-2 in response to insulin relative to the amount of Glut4. These results are consistent with the possibility that Akt-2 may be involved in Glut4 vesicle translocation.

Insulin promotes glucose uptake in adipose tissue and skeletal muscle by increasing the translocation of the Glut4 glucose transporter from an intracellular pool to the plasma membrane (1–3), but the complete signaling pathway responsible for Glut4 translocation remains to be defined. Considerable evidence supports the notion that this event is dependent on insulin-stimulated phosphatidylinositol 3-kinase activity (PI 3-kinase)1 (4–8). While the biochemical mechanism by which PI 3-kinase participates in glucose transporter trafficking remains uncertain, there is evidence showing that the intracellular Glut4-containing vesicles undergo an insulin-dependent increase in PI 3-kinase activity (9), suggesting that this might be an important site of lipid kinase action.

One target of PI 3-kinase is the serine/threonine protein kinase B, PKB, also known as Akt or RAC, which is the cellular homologue of the viral oncogene v-akt (10). Three isoforms of this protein have been identified: PKB α (Akt-1), PKB β (Akt-2), and PKB γ (Akt-3) (11–14). Akt/PKB contains a plekstrin homology (PH) domain at its NH2-terminal, which has been implicated in interactions with the products of phosphatidylinositol 3-kinase, PI 3,4-bisphosphate and PI 3,4,5-trisphosphate in vitro (15, 16). Akt/PKB is activated in response to insulin and growth factors, and this activation is prevented by inhibitors of PI 3-kinase (15, 18). Phosphorylation of Akt/PKB is thought to be essential for its activation (18). Insulin and insulin-like growth factor I induce the phosphorylation of this protein at two sites, Thr-308 and Ser-473 (19). Recently, it has been shown that 3-phosphoinositide-dependent protein kinase-1 (PDK-1) is the kinase responsible for phosphorylation of Akt/PKB at Thr-308 (20, 21).

The serine/threonine kinase Akt is involved in different cellular processes such as cell growth, cell differentiation, apoptosis, and regulation of glycolysis metabolism by insulin (10, 22–24). In this regard, expression of a constitutively active membrane-bound form of Akt-1 in 3T3L1 adipocytes resulted in stimulated glucose transport and Glut4 translocation to the plasma membrane (25, 26). Most of the studies on Akt/PKB have focused on the α isoform, and thus far little is known about the other isoforms. Here, we report the subcellular distribution and regulation of Akt-2 by insulin in rat adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Collagenase and bovine serum albumin were purchased from Boehringer Mannheim. The bicinchoninic acid (BCA) protein determination kit was from Pierce. Aprotinin, leupeptin, and pepstatin A were obtained from American Bioanalytical (Natick, MA). Electrophoresis chemicals were purchased from National Diagnostics (Atlanta, GA).

Adipocyte Isolation and Fractionation—Adipocytes were isolated from epididymal fat pads of 175–200-g male Sprague-Dawley rats (Taconic Farms, Germantown, NY) by collagenase digestion as described previously (27). Adipose cells were equilibrated for 30 min at 37 °C with Krebs-Ringer buffer containing 12.5 mM HEPES, pH 7.4, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 1 mM CaCl2, 1 mM NaHPO4, 2.5 mM t-glucose, and 2% bovine serum albumin. Insulin from bovine pancreas (Sigma) was added to the cell suspension for 10 min to a final concentration of 10 nM. Cells were washed two times and homogenized in 20 mM HEPES, 250 mM sucrose, 1 mM EDTA, 5 mM benzamidine, 5 mM sodium orthovanadate, 10 mM pyrophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM aprotinin, and 1 μM leupeptin, at pH 7.4. Subcellular fractionation was performed as described (28). Protein was quantitated using BCA assay with bovine serum albumin as a standard.

Adipocyte Incubation with Wortmannin—Isolated adipose cells were incubated with 100 nM wortmannin (Sigma) for 25 min prior to insulin stimulation.

Immunoadsorption of Glut4 Vesicles—Purified anti-Glut4 monoclonal antibody 1F8 (29) as well as nonspecific IgG were coupled to acrylic beads (Reacti-gel GF 2000, Pierce) at a concentration of 0.5 mg of antibody/ml of resin according to the manufacturer's instructions. Before use, the beads were saturated with 1% BSA in PBS for at least 30 min at room temperature and then washed in PBS. Light micro-
Akt-2 in Glut4 Vesicles

RESULTS

We first studied the subcellular distribution of Akt-2 in freshly isolated rat adipocytes and compared it with the previously reported localization of Akt-1. Adipocytes were fractionated into cytosol and different membrane fractions, as described under "Experimental Procedures." Equal amounts of protein from each fraction were separated by SDS-PAGE and immunoblotted with specific antibodies for Akt-1, Akt-2, or Glut4. Akt-2 was found in all subcellular fractions. The highest Akt-2 signal was found in heavy microsomes, followed by cytosol, plasma membrane, light microsomes, and mitochondria/nuclei. However, the figure was normalized for equal protein, and when one takes into account that the protein yield of the cytosol is 5-10-fold higher than the membrane fractions, the cytosol contains about half of the total Akt-2, and, except for the mitochondria/nuclei fraction, there are roughly equal amounts in the other three membrane fractions. As expected, Akt-1 was detected exclusively in the cytosolic fraction, and in the absence of insulin, Glut4 was localized only in light and heavy microsomes (Fig. 1, middle and bottom panels, respectively).

The stimulation of Akt-1 by insulin has been reported in different cell types (16, 22, 31, 32) and is accompanied by a change in the electrophoretic mobility of the protein (Fig. 1, middle panel, and Refs. 31 and 32). This shift in response to the hormone indicates multiple phosphorylated states of Akt-1 (17, 31, 33). Stimulation of adipocytes with insulin also resulted in activation of cytosolic Akt-2, as indicated by the appearance of a slower migrating form. The Akt-2 content of light microsomes isolated from insulin-treated adipocytes was increased to 145 ± 10% over the untreated control (mean ± S.E., n = 3; *, p < 0.01), but cellular insulin exposure had a nonobservable effect on Akt2 levels in the plasma membrane, heavy microsomes, or mitochondria/nuclei (Fig. 1, top panel). The source of Akt-2 causing the increased light microsome content is presumably the cytosol, but as noted above, the cytosol contains over half of the Akt, and one cannot detect the small decrease in this fraction that would give rise to the clearly detectable increase in the light microsome fraction. Consistent with many previous reports (1, 29, 34–39), Glut4 demonstrated insulin-responsive translocation from the light microsomes to the plasma membrane (Fig. 1, bottom panel).

Akt-1 activation by insulin is sensitive to wortmannin, indicating that this protein is downstream of PI3-kinase. As shown in Fig. 1, top panel, preincubation with wortmannin abolished the insulin-dependent mobility shift of Akt-2 in the cytosolic fraction. The increase in Akt-2 content induced by insulin in light microsomes was also prevented by wortmannin. As expected, the insulin-stimulated Glut4 translocation was abrogated by the PI3-kinase inhibitor wortmannin (Fig. 1, bottom panel).

Considering previous reports about the role of Akt/PKB in the translocation of Glut4 and our data on Akt-2 localization in light microsomes, we investigated if Akt-2 is present in vesicles with the same density as those containing Glut4. Light microsomes were fractionated by a sucrose velocity gradient, proteins were separated by SDS-PAGE, and Western blotting was performed with anti-Glut4 or -Akt-2 antibodies. Results presented in Fig. 2 show that Glut4-containing vesicles sediment faster than the majority of light microsomes. Akt-2 was present in fractions with the highest levels of Glut4. This result indicates that either Akt-2 is present in vesicles with the same density but different from those that contain Glut4 or that both Akt-2 and Glut4 are present in the same vesicles.

To directly test if Akt-2 is associated with Glut4-containing vesicles, we immunosolated these vesicles from the light microsomes with an anti-Glut4 antibody (1F8 antibody) covalently coupled to acrylic beads. Vesicle proteins were extracted from the immunoadsorbed Glut4-containing vesicles with 1% Triton X-100, followed by elution of Glut4 with SDS-containing Laemmli buffer (29). As shown in Fig. 3, Akt-2 was clearly detected in Glut4-containing vesicles. Insulin induced a wortmannin-sensitive, 360 ± 62% increase (mean ± S.E., n = 4; *, p < 0.005) in Akt-2 levels associated with Glut4-containing vesicles, as measured by densitometric scanning of the protein bands. As expected, the amount of Glut4 adsorbed on 1F8 beads decreases by 36% after insulin administration due to the translocation of Glut4-containing vesicles from intracellular microsomes to the plasma membrane, and when one takes this into account, the ratio of Akt-2 to Glut4 is increased 5.5-fold by insulin. This result is consistent with the stimulatory effect of
Akt-2 was treated or not with 10 nM insulin for 10 min. Wortmannin bodies to Akt-2 and Glut4. The data shown are representative of four Eluates were subjected to SDS-PAGE and immunoblotted with anti- nonspecific antibody (1F8). The data shown are representative of four separate experiments.

Fig. 3. Akt-2 is present in Glut4-containing vesicles. Adipose cells were treated or not with 10 nM insulin for 10 min. Wortmannin (100 nM) was added 25 min before stimulation. Cell fractionation was performed as described under "Experimental Procedures." Light microsomes (100 μg of protein) were immunoadsorbed with anti-Glut4 antibody (1F8) covalently attached to acrylic beads or with beads linked to nonspecific antibody (IgG). Immunoadsorbed Glut4 vesicles were eluted sequentially with 1% Triton X-100 and SDS-containing Laemmli buffer. Eluates were subjected to SDS-PAGE and immunoblotted with antibodies to Akt-2 and Glut4. The data shown are representative of four separate experiments.

Fig. 2. Akt-2 and Glut4 co-localize in vesicles with the same density. Light microsomes (0.5 mg of protein) from insulin-treated (10 nm for 10 min) rat adipocytes were centrifuged in a 10–30% sucrose gradient (4.6 ml) as described under "Experimental Procedures." Fractions collected from the gradient were subjected to SDS-PAGE and immunoblotted with antibodies to Akt-2 or Glut4 (upper panel). Both proteins were detected with horseradish peroxidase-conjugated secondary antibody and a chemiluminescence detection kit. Lower panel shows distribution of Akt-2 and Glut4. The data shown are representative of three separate experiments.

in insulin on the Akt-2 content of the light microsomal fraction as shown in Fig. 1, top panel.

**DISCUSSION**

To date, most studies with Akt have focused on Akt-1, the first characterized member of the family. The other close relatives Akt-2 and Akt-3 have been poorly studied. In this report, we examined the subcellular distribution and regulation of Akt-2 by insulin in isolated rat adipocytes. We found that Akt-2 was present in cytosol and all membrane fractions, which markedly contrasts with the distribution of Akt-1, which is overwhelmingly cytosolic. The differences in the pattern of subcellular distribution between Akt-1 and Akt-2 can derive from structural differences between these proteins. A sequence comparison between Akt-1 and Akt-2 reveals that the last portion of the amino-terminal domain (amino acids 107–147) is the most divergent between Akt molecules (13, 40). Datta et al. (41) showed that Akt forms multimeric complexes through the interaction between AH domains (Akt homology domain, spans amino acids 1–147). This interaction is highly specific, as determined by the failure of the Akt-1 domain to bind Akt-2 in COS-1 cells. The differences in subcellular distribution between these isoforms suggest that Akt-1 and Akt-2 may interact with different cellular targets and/or can be differentially regulated. Also, it has been shown that the expression of Akt-1 is much higher than that of Akt-2 in brain and lung, whereas Akt-2 is highly expressed in testis while Akt-1 is very low in this tissue (15). Distinct expression of the Akt/PKB isoforms may reflect different function of these proteins. Meier et al. (42) demonstrated that Akt-2 is translocated into the nucleus by stimulation of HEK-293 and REF-52 cells with insulin-like growth factor I and fetal calf serum, respectively. However, we were unable to detect any nuclear translocation of Akt-2 after stimulation of fat cells with insulin (Fig. 1, top panel). The explanation for this difference is unclear, but it might be due to the fact that fat cells are terminally differentiated, whereas HEK-293 and REF-52 cells are proliferating cell lines.

In this study, we demonstrated that insulin activated both Akt-1 and Akt-2 in the cytosolic fraction. Insulin activation induced the shift to a higher molecular weight of both Akt proteins. Previous reports have shown that platelet-derived growth factor stimulated both Akt-1 and Akt-2 isoforms (15). These data are consistent with the proposal that both isoforms are regulated in a similar way but that different subcellular localization may result in distinct cellular responses.

We showed that Akt-2 and Glut4 have a similar distribution in vesicles fractionated by a sucrose velocity gradient and that Akt-2 is present in Glut4-containing vesicles. The localization of Akt-2 in these intracellular membranes may be involved in regulating Glut4 trafficking in adipocytes. These results are consistent with previous reports in which Akt-1 has been implicated in Glut4 translocation as well as glucose uptake (25, 26).

The mechanism by which insulin induce Glut4 translocation is not fully understood. Several signaling molecules have been implicated in this process, including PI 3-kinase. Inhibitors of PI 3-kinase have been observed to block the ability of insulin to stimulate glucose transport and Glut4 translocation in rat primary adipocytes and 3T3-L1 differentiated adipocytes (4–8, 48). Also, the expression of a constitutively active PI 3-kinase induced Glut4 translocation in the absence of insulin (44–47). The presence of PI 3-kinase (9) and Akt-2 in rat adipocyte Glut4-containing vesicles might be important in the process of Glut4 translocation; however, much additional work will be required to determine a functional role for Akt-2 and PI 3-kinase in Glut4-containing vesicles. Data showing a Glut4 vesicle-associated kinase activity that could be Akt-2 is promising in this regard.2

**REFERENCES**

1. Cushman, S. W., and Wardzala, L. J. (1980) J. Biol. Chem. 255, 4758–4762
2. Kandror, K. V., and Pilch, P. F. (1996) Am. J. Physiol. 271, E1–E14
3. Stephens, J. M., and Pilch, P. F. (1995) Endocr. Rev. 16, 529–546
4. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) Mol. Cell. Biol. 14, 4902–4911
5. Okada, T., Kawano, Y., Sakakibara, R., Hazeki, O., and Ui, M. (1994) J. Biol. Chem. 269, 3568–3573
6. Haruta, T., Morris, A. J., Rose, D. W., Nelson, J. G., Mueckler, A., and Olefsky, J. M. (1995) J. Biol. Chem. 270, 27991–27994
7. Kotani, K., Carozzi, 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
8. Yang, J., Clarke, J. F., Ester, C. J., Young, P. W., Kasuga, M., and Holman, G. D. (1996) Biochem. J. 313, 125–131
9. Hellerman, J., Morin, M., Guillerme, A., and Czech, M. P. (1996) J. Biol. Chem. 271, 10290–10294
10. K. Kandror, submitted for publication.

—End—
Akt-2 in Glut4 Vesicles

10. Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991) Science 254, 274–277
11. Jones, P. F., Jakubowicz, T., Pithis, F. J., Maurer, F., and Hemmings, B. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4171–4175
12. Jones, P. F., Jakubowicz, T., and Hemmings, B. A. (1991) Cell Regul. 2, 1001–1009
13. Cheng, J. Q., Godwin, A. K., Taguchi, T., Franke, T. F., Hamilton, T. C., and Tsichlis, P. N. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9267–9271
14. Konishi, H., Kuroda, S., Tanaka, M., Matsuzaki, H., Ono, Y., Kameyama, K., Haga, T., and Kikkawa, U. (1995) Biochem. Biophys. Res. Commun. 216, 526–534
15. 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
16. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Science 275, 665–668
17. Burgering, B. M. T., and Coffer, P. J. (1995) Nature 376, 599–602
18. Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996) J. Biol. Chem. 271, 21920–21926
19. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) EMBO J. 15, 6541–6551
20. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Curr. Biol. 7, 261–269
21. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. H., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 277, 567–570
22. Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Nature 378, 785–789
23. Magun, R., Burgering, B. M. T., Coffer, P. J., Paradisan, D., Lin, Y., Chabot, J., and Sorisky, A. (1996) Endocrinology 137, 5390–5393
24. Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Bellacosa, A., Tsichlis, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1001–1009
25. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) Mol. Cell. Biol. 16, 4117–4127
26. Tanti, J.-F., Grillo, S., Gremeaux, T., Coffer, P. J., Van Obberghen, E., and Le Marchand-Brustel, Y. (1997) Endocrinology 138, 2005–2010
27. Rodbell, M. (1964) J. Biol. Chem. 239, 375–385
28. Kuhlau, B., Lee, J., and Pilch, P. F. (1995) J. Biol. Chem. 270, 59–65
29. James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) Nature 333, 183–185
30. Laemmli, U. K. (1970) Nature 227, 680–685
31. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) EMBO J. 14, 4288–4295
32. Moule, S. K., Welsh, G. I., Edgell, N. J., Foulstone, E. J., Proud, C. G., and Denton, R. M. (1997) J. Biol. Chem. 272, 7713–7719
33. Andjelkovic, M., Jakubowicz, T., Cron, P., Ming, X.-F., Han, J.-W., and Hemmings, B. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5089–5074
34. Suzuki, K., and Kono, T. (1988) Proc. Natl. Acad. Sci. U. S. A. 77, 2542–2545
35. Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karmeli, E., Salana, L. B., and Cushman, S. W. (1983) Biochim. Biophys. Acta 763, 393–407
36. Zorzano, A., Wilkinson, W., Ketlar, N., Thodis, G., Wadzinski, B. E., Roubo, A. E., and Pilch, P. F. (1989) J. Biol. Chem. 264, 12358–12363
37. Douen, A. O., Ramlal, T., Rastogi, S., Bilan, P. J., Cartee, G. D., Vranic, M., Holloszy, J. O., and Klip, A. (1990) J. Biol. Chem. 265, 13427–13430
38. Kandrer, K. V., and Pilch, P. F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8017–8021
39. James, D. E., and Piper, R. C. (1994) J. Cell Biol. 126, 1123–1126
40. Franke, T. F., Tartof, K. D., and Tsichlis, P. N. (1994) Oncogene 9, 141–148
41. Datta, K., Franke, T. F., Chan, T. O., Makris, A., Yang, S., Kaplan, D. R., Morrison, D. K., Gelemiss, E. A., and Tsichlis, P. N. (1995) Mol. Cell. Biol. 15, 2304–2310
42. Meier, R., Alessi, D. R., Cron, P., Andjelkovic, M., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 30491–30497
43. Tsakiridis, T., McBowell, H. E., Walker, T., Downes, C. P., Hundal, H. S., Vranic, M., and Klip, A. (1995) Endocrinology 136, 4315–4322
44. Klippel, A., Reinhard, C., Kavanagh, M., Apell, G., Escobedo, M. A., and Williams, L. T. (1996) Mol. Cell. Biol. 16, 4117–4127
45. Martin, S. S., Haruta, T., Morris, A. J., Klippel, A., Williams, L. T., and Olefky, J. M. (1996) J. Biol. Chem. 271, 17605–17608
46. Tanti, J.-F., Gremeaux, T., Grillo, S., Calleja, V., Klippel, A., Williams, L. T., Van Obberghen, E., and Le Marchand-Brustel, Y. (1996) J. Biol. Chem. 271, 2527–2532
47. Frevert, H., and Kahn, B. B. (1997) Mol. Cell. Biol. 17, 190–198
Insulin Increases the Association of Akt-2 with Glut4-containing Vesicles
Mónica R. Calera, Carmen Martinez, Hongzhi Liu, Amr K. El Jack, Morris J. Birnbaum and Paul F. Pilch

J. Biol. Chem. 1998, 273:7201-7204.
doi: 10.1074/jbc.273.13.7201

Access the most updated version of this article at http://www.jbc.org/content/273/13/7201

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 28 of which can be accessed free at http://www.jbc.org/content/273/13/7201.full.html#ref-list-1