Differential Protein Phosphorylation in 3T3-L1 Adipocytes in Response to Insulin Versus Platelet-derived Growth Factor

NO EVIDENCE FOR A PHOSPHATIDYLINOSITIDE 3-KINASE-INDEPENDENT PATHWAY IN INSULIN SIGNALING*

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Insulin regulates glucose metabolism in adipocytes via a phosphatidylinositol 3-kinase (PI3K)-dependent pathway that appears to involve protein phosphorylation. However, the generation of phosphoinositides is not sufficient for insulin action, and it has been suggested that insulin regulation of glucose metabolism may involve both PI3K-dependent and -independent pathways, the latter being insulin specific. To test this hypothesis, we have designed a phosphoprotein screen to study insulin-specific phosphoproteins that may be either downstream or in parallel to PI3K. Nineteen insulin-regulated phosphoproteins were detected in the cytosol and high speed pellet fractions, only six of which were significantly regulated by platelet-derived growth factor. Importantly, almost all (92%) of the insulin-specific phosphoproteins identified using this approach were sensitive to the PI3K inhibitor wortmannin. Thus, we obtained no evidence for an insulin-specific, PI3K-independent signaling pathway. A large proportion (62%) of the insulin-specific phosphoproteins were enriched in the same high speed pellet fraction to which PI3K was recruited in response to insulin. Thus, our data suggest that insulin specifically stimulates the phosphorylation of a novel subset of downstream targets and this may in part be because of the unique localization of PI3K in response to insulin in adipocytes.

Insulin stimulates glucose uptake into muscle and fat cells mainly through the translocation of glucose transporter 4 (GLUT4) from an intracellular location to the cell surface (1). This mechanism is crucial for the maintenance of glucose homeostasis. An impairment in insulin-stimulated glucose uptake is a major factor leading to the development of non-insulin-dependent diabetes mellitus (2). Insulin binding to its cell surface receptor activates the intrinsic tyrosine kinase activity of the insulin receptor and stimulates tyrosine phosphorylation of insulin receptor substrate proteins. Tyrosine-phosphorylated IRS proteins in turn recruit Src homology 2 domain-containing signaling proteins. Two main pathways have been identified downstream of IRS proteins, the mitogen-activated protein (MAP) kinase pathway and the phosphatidylinositol 3-kinase (PI3K) pathway. The PI3K pathway, through protein kinase B (PKB), has been shown to be necessary for insulin-stimulated glucose transport through various experimental approaches. First, two structurally unrelated inhibitors of PI3K, wortmannin and LY294002, potently inhibit insulin-stimulated glucose transport in adipocytes (3, 4). Second, dominant negative mutants of the p110 catalytic subunit (5), the p85 regulatory subunit of PI3K (6), or PKB (7) inhibit insulin-stimulated glucose transport in adipocytes. In addition, microinjection of a PKB substrate peptide or an antibody to PKB inhibit insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (8). Finally, constitutively active PI3K (9) or PKB (10) trigger GLUT4 translocation independently of insulin when expressed in adipocytes.

Whereas evidence supports a role for the PI3K/PKB pathway in insulin-stimulated GLUT4 translocation in adipocytes, other growth factors also activate PI3K without stimulating GLUT4 translocation, raising the question of signaling specificity (reviewed in Ref. 11). In particular, platelet-derived growth factor (PDGF) stimulates PI3K activity to the same extent as insulin in adipocytes but has relatively little effect on glucose transport (12–17). Several hypotheses have been advanced to account for this controversy. One possibility is that these different growth factors may activate unique PI3K isoforms with different substrate specificities. It has also been proposed that PI3K may be activated in different locations in response to insulin versus PDGF (13, 14, 18). Several studies have shown that in response to PDGF, most of the increase in PI3K occurs in the plasma membrane (PM) fraction. In contrast, following insulin stimulation, there is a large increase in PI3K activity in a high speed pellet (HSP) fraction. This fraction also contains the major insulin regulatable IRS proteins (IRS1 and IRS2) found in insulin-sensitive cells (13, 14, 18). One potential consequence of this discrete localization is that PI3K may access different...
downstream targets. Alternatively, the activation of glucose metabolism by insulin may require activation of the PI3K pathway as well as an additional, insulin-specific pathway. The finding that membrane-permeant analogs of phospatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) do not activate glucose uptake when added to adipocytes suggests that activation of PI3K may not be sufficient to stimulate GLUT4 translocation, leading to the hypothesis that a PI3K-independent pathway is also required (19).

Each of these models predicts that insulin must trigger a unique signal transduction pathway, that either lies downstream or in parallel to PI3K. Despite this prediction, little progress has been made in elucidating such a novel pathway. Thus, in the present study, we designed a subtraction assay based on protein phosphorylation to select for molecules that may be regulated in an insulin-specific manner. A differential screening procedure using two-dimensional gels was employed to select for phosphoproteins that are specifically regulated by insulin, and not PDGF, in a Wortmannin-sensitive manner. Insulin stimulated the phosphorylation of 18 phosphoprotein spots. Only six of these proteins were phosphorylated by PDGF in a manner that was quantitatively comparable to insulin. In addition, insulin specifically stimulated the dephosphorylation of one row of phosphopots. Interestingly, insulin-specific phosphoproteins preferentially localized to the HSP fraction, further confirming the presence of insulin-specific targets in this fraction. With the possible exception of one phosphopot, all of the insulin-specific phosphoproteins were Wortmannin-sensitive. Hence, based on these studies we have found no evidence of an insulin-activated PI3K-independent pathway involving protein phosphorylation in 3T3-L1 adipocytes. These results suggest that insulin activates an unique, PI3K-dependent pathway, which regulates metabolism in adipocytes.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies against mitogen-activated protein kinase were described previously (20). Antibodies specific for mitogen-activated protein kinase phosphorylated at Thr<sup>202</sup> and Tyr<sup>204</sup> were from New England Biolabs (Beverly, MA). Rabbit antibodies against PKB (1:5000 dilution) (Ser473) were generously provided by Dr. M. Birnbaum (Philadelphia, PA).

**Generation of Phosphorylation Maps**—For radiolabeling, 3T3-L1 adipocytes were incubated in a buffer containing 12.5 mM HEPES, pH 7.4, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.2 mM NaPO<sub>4</sub>, 2% (v/v) bovine serum albumin, and 0.5 mCi/ml 32Pi (ICN) for 15 min, or 4) no additions. After treatment, cells were washed twice in ice-cold HES buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 6 mM KCl, 1.2 mM Mg<sub>2</sub>SO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.2 mM NaPO<sub>4</sub>, 2% (v/v) bovine serum albumin, 0.1% phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM so-
synthesis of lipid, glycogen, and protein. Second, a number of reagents that modulate protein phosphorylation including phosphatase inhibitors like okadaic acid have profound effects on insulin action (28). Finally, it is relatively simple using metabolic labeling to conduct a random large-scale analysis of changes in protein phosphorylation in response to certain treatments. To increase the sensitivity of detection, analysis was performed on subcellular fractions rather than whole cell lysates, a technique first applied to adipocytes by Avruch et al. (29, 30). In these early studies, resolution of $^{32}$P-labeled phosphoproteins by SDS-polyacrylamide gel electrophoresis revealed only one insulin-stimulated phosphoprotein of 123 kDa, which likely corresponds to ATP-citrate lyase (30). In the current study, we have utilized 2-DE to achieve higher resolution of $^{32}$P-labeled phosphoproteins present in subcellular fractions of adipocytes. The use of 2-DE to resolve $^{32}$P-labeled phosphoproteins has been previously reported (31, 32), albeit not in conjunction with subcellular fractionation. By combining radio-labeling with subcellular fractionation and 2-DE, we hoped to attain the resolution required to study the phosphorylation of low abundance phosphoproteins.

Three subcellular fractions have been studied in detail as indicated below. The rationale for selecting these particular fractions is based on our previous observation that the PM fraction contains the insulin and PDGF receptors, whereas the HSP fraction is enriched in IRS1 (18). In response to PDGF, PI3K is mainly recruited to the PM fraction, whereas in response to insulin it is recruited to the HSP fraction. Therefore, these different fractions potentially represent discrete loci for the assembly of signaling complexes. We have also studied the cytosolic fraction because it is also known to contain many signaling molecules including MAP kinase and PKB.

**Insulin-stimulated Protein Phosphorylation in the High Speed Pellet and Cytosol Fractions**—Initially we studied the polypeptide composition of PM, HSP, and cytosol fractions isolated from adipocytes. Using 2-DE and silver staining we identified >200 individual silver-stained spots/fraction (21). Most of the proteins resolved by this technique were in the molecular mass range of 20–120 kDa consistent with previous studies using this approach (31). Hence, it is unlikely that proteins outside this range including IRS1 and IRS2 would have been resolved. The overall pattern of silver-stained spots was quite different between the three fractions studied, consistent with the fact that they comprise distinct intracellular components (21). We were unable to detect any effect of insulin on the polypeptide composition among these fractions. Between 50 and 300 distinct phosphoprotein spots were detected in the PM, HSP, and cytosol fractions, and the overall pattern of these spots was significantly different between the different fractions. In agreement with previous studies (29, 30, 32), the phosphorylation of the majority of these spots was unaffected by insulin treatment (Figs. 1 and 2). However, quantitative analysis of five separate experiments showed that insulin consistently and significantly increased the phosphorylation of at least 18 spots (Figs. 1 and 2).

To further ascertain that our phosphoprotein mapping technique accurately reflects cellular phosphorylation, we first examined a well-characterized insulin target, MAP kinase. Using an antibody specific for both the p42 and p44 MAP kinase isoforms we first showed that these proteins were enriched in the cytosol fraction of 3T3-L1 adipocytes (data not shown). Analysis of the cytosol fraction by 2-DE followed by immunoblotting allowed resolution of several immunoreactive spots migrating at the appropriate mass and pI of p44 and p42 MAP kinase (Fig. 3A). Following insulin stimulation, there was a leftward shift toward acidic pI for both p42 and p44 MAP kinase (Fig. 3B), and these spots could now also be detected using a phospho-MAP kinase antibody (Fig. 3D). Furthermore, $^{32}$P-labeled spots were detected at the same position as the phosphorylated MAP kinase spots, but only in the insulin-stimulated cytosol fraction (C65, C79, Fig. 1). These results demonstrate that our technique is sensitive enough to detect changes in the phosphorylation of low abundance signaling proteins.

Similar studies to those described above were performed to determine if our screen could detect another major downstream target of insulin, PKB. PKB phosphorylation is induced by insulin in a PI3K-dependent manner (33). We have recently reported that PKBβ is the main isoform expressed in 3T3-L1 adipocytes, and it is enriched in the cytosol (8). To better resolve proteins in the molecular mass and pI range of PKB (58 kDa/pI 5.9), it was necessary to perform our mapping studies in buffer containing reduced levels of bovine serum albumin. Under these conditions, a row of $^{32}$P-labeled spots was observed in the region of the gel corresponding to the predicted position of PKB (Fig. 4A). Insulin increased the phosphorylation of five of these spots (compare Fig. 4, A and B). To determine if any of these spots corresponded to PKBβ, $^{32}$P-labeled cytosol fractions from basal or insulin-treated cells were analyzed by 2-DE and immunoblotting with a PKBβ antibody (Fig. 4, C and D). In the basal state, the PKBβ antibody detected four distinct spots (spots 0–3, Fig. 4C). In agreement with previous results (8), spots 1 and 2 overlapped with $^{32}$P-labeled spots, representing constitutively phosphorylated PKBβ (Fig. 4, A and C). Insulin
Insulin-specific Phosphoproteins in Adipocytes

Effects of Insulin, PDGF, and Insulin Plus Wortmannin on Protein Phosphorylation—The screen that we have designed to identify a putative insulin-specific signaling pathway in adipocytes is based on the fact that adipocytes express receptors for PDGF, but PDGF does not activate glucose metabolism in these cells (13, 14, 18). Furthermore, the PI3K inhibitor wortmannin potently inhibits insulin regulation of metabolism. Thus, we reasoned that this technique should enable us to resolve an insulin-specific, PDGF-insensitive pathway by comparing the effects of these different compounds on protein phosphorylation. A series of experiments were performed to compare the protein phosphorylation pattern between insulin, PDGF, and insulin plus wortmannin treatment. A quantitative analysis of five different experiments was performed using Melanie software to compare the extent of phosphorylation of individual spots with the different treatments. These data are summarized in Tables I and II, with the corresponding spots indicated in Figs. 1 and 2. Insulin increased the phosphorylation of 18 different spots and decreased the phosphorylation of one row of phosphospots (C12). The insulin-dependent increase in phosphorylation among these different spots ranged in magnitude from 2-fold (C69, C95, H62, and H74) to >10-fold (C65, C79, H56, and H75) over basal. Some of the spots appeared to be phosphorylated under basal conditions (C2, C69, C95, H62, and H74). In fact for some of these there appeared to be a row of phosphospots that underwent a further leftward shift in response to insulin. This was most clearly observed for PKB (Fig. 4). However, in other cases we were unable to detect any significant phosphorylation under basal conditions (C65, C77, C79, C95, H48, and H74). For these reasons it was difficult to precisely quantify the fold increase above basal in response to either insulin or PDGF, and so we have quantified each spot as a percentage of that observed in the presence of insulin.

Analysis of all the insulin-stimulated phosphoproteins in relation to their response to PDGF and wortmannin revealed that each of these spots fell into one of several different categories (see Fig. 5 for summary). Certain spots underwent a comparable increase in phosphorylation in response to both insulin and PDGF (C65, C77, C79, C95, H48, and H74). The majority of spots, however, were only phosphorylated in response to insulin (C2, C22, C49, C69, H38, H46, H56, H59, H60, H62, H72, H74, and H75). We did observe a slight increase in phosphorylation with PDGF for some of these proteins (C49, H46, H72, and H75) but this was not a reproducible phenomenon and the magnitude of this effect was less than that observed in response to insulin. Hence, these proteins were categorized as PDGF-insensitive, although it is conceivable that further analyses may reveal that they are PDGF responsive albeit to a lesser extent than insulin. Wortmannin abolished the insulin-stimulated phosphorylation of 16 spots but had no significant inhibitory effect on the insulin-induced phosphorylation of C22 and H48. However, the protein corresponding to H48 was also stimulated by PDGF and so does not represent an insulin-specific phosphoprotein. In addition, the
Insulin-specific Phosphoproteins in Adipocytes

Characteristics of insulin-regulated cytosolic phosphoproteins

Table I

| Spot no. | Molecular mass (kDa) | pi | Basal | PDGF | Wortmannin/Insulin | n* | Identity |
|----------|----------------------|----|-------|------|--------------------|----|----------|
| C2       | 121                  | 6.35–6.73 | 21.5 ± 7.2c | 23.7 ± 9.5 | 25.4 ± 9.2 | 4 | ACL |
| C22      | 71                   | 6.18 | 16.0 ± 13.7b | 39.7 ± 26.5 | 65.3 ± 39.4d | 4 | |
| C49      | 48                   | 6.22 | 10.5 ± 7.8a | 38.9 ± 20.4 | 16.7 ± 12.8 | 4 | |
| C65      | 44                   | 6.01 | 3.9 ± 4.4c | 71.4 ± 39.1b | 0 | 4 | MAPK |
| C69      | 43                   | 5.50 | 30.8 ± 12.7b | 28.2 ± 18.4 | 13.8 ± 17.0 | 3 | |
| C77      | 41                   | 6.27 | 9.2 ± 10.3b | 63.0 ± 14.3c | 2.6 ± 2.9 | 4 | MAPK |
| C79      | 41                   | 6.21 | 0b | 127.2 ± 36.0b | 0 | 4 | |
| C95      | 35                   | 6.17 | 42.0 ± 11.0b | 126.3 ± 39.7 | 39.2 ± 16.7 | 4 | |
| C12      | 95                   | 6.85 | 171.9 ± 3.6b | 190.1 ± 67.9 | 168.6 ± 43.6 | 4 | EF2 |

* n = number of data sets used for statistical analysis.
* b p < 0.05 compared to basal values (paired t test).
* a p < 0.05 compared to insulin values (paired t test).
* c p > 0.05 compared to insulin values (paired t test).

Characteristics of insulin-regulated HSP phosphoproteins

Table II

| Spot no. | Molecular mass (kDa) | pi | Basal | PDGF | Wortmannin/Insulin | n* | Identity |
|----------|----------------------|----|-------|------|--------------------|----|----------|
| H38      | 61                   | 6.16 | 9.2 ± 10.6b | 19.9 ± 24.4 | 0 | 3 | |
| H46      | 56                   | 6.15 | 20.6 ± 11.0b | 53.6 ± 17.6 | 26.8 ± 16.5 | 3 | |
| H48      | 49                   | 4.86 | 24.3 ± 15.1b | 86.7 ± 20.1c | 73.9 ± 17.0d | 5 | |
| H56      | 48                   | 6.22 | 1.5 ± 1.7c | 13.0 ± 8.7 | 1.8 ± 2.0 | 4 | |
| H59      | 45                   | 6.26 | 7.5 ± 8.4c | 17.7 ± 15.5 | 15.7 ± 18.2 | 4 | |
| H60      | 43                   | 6.34 | 15.8 ± 17.6b | 12.3 ± 11.1 | 15.8 ± 18.3 | 4 | |
| H62      | 42                   | 6.43 | 40.1 ± 19.6b | 54.4 ± 2.3 | 23.5 ± 14.6 | 3 | |
| H72      | 40                   | 6.18 | 10.0 ± 11.2b | 50.3 ± 16.1 | 11.6 ± 11.7 | 4 | |
| H74      | 39                   | 6.27 | 37.2 ± 7.0c | 79.0 ± 17.0c | 41.7 ± 17.6 | 5 | |
| H75      | 38                   | 6.37 | 6.2 ± 4.4c | 32.8 ± 13.7 | 7.0 ± 3.0 | 4 | |

* n = number of data sets used for statistical analysis.
* b p < 0.05 compared to insulin values (paired t test).
* a p < 0.05 compared to basal values (paired t test).
* c p > 0.05 compared to insulin values (paired t test).

Identification of C2 as ATP-citrate Lyase—In an attempt to identify some of the insulin-specific phosphoproteins picked up in our screen, we scaled up the isolation procedure as described under “Experimental Procedures.” Several of our candidate phosposots were purified to Coomassie-stained bands, digested with trypsin, and subjected to liquid chromatography-MS. One of these spots contained sequences that correspond to a protein previously described to undergo insulin-stimulated phosphorylation. This protein, designated as C2 in our screen, was ACL (Fig. 6) and Table III), an enzyme which catalyzes the first step in fatty acid synthesis, the formation of acetyl-CoA (34, 35). Five differentially charged forms of phosphorylated ACL were consistently observed in the cytosol from insulin-stimulated adipocytes (Fig. 6b). A low level of ACL phosphorylation was observed in the basal state; however, the spots sensitive) phosphoproteins preferentially localized to the HSP, with 62% found in this fraction (Fig. 5). On the other hand, only 30% of PDGF-sensitive phosphoproteins were in the HSP fraction (Fig. 5). The preferential localization of insulin-specific phosphoproteins in the HSP is consistent with the reported lack of PDGF-stimulated PI3K activity in this fraction (13, 14, 18) and further suggest that some insulin signaling pathways may be sequestered in a subcellular compartment, which fractionates in the HSP.

FIG. 5. Insulin-specific phosphoproteins preferentially localize to the HSP fraction. Insulin-regulated phosphoproteins (summarized in Tables I and II) were grouped by subcellular location (cytosol versus HSP), sensitivity to PDGF stimulation, and the effect of wortmannin on insulin-induced phosphorylation.

response to wortmannin for C22 was somewhat variable in that in three experiments wortmannin had no effect on insulin-stimulated C22 phosphorylation, whereas it caused complete inhibition in two other experiments.

Analysis of the subcellular distribution of insulin-regulated phosphoproteins suggested that insulin-specific (i.e. PDGF-in-
phosphorylated varied between the two most acidic spots (Fig. 6A, n = 2) and the three most basic spots (not shown, n = 3). Insulin caused a 5-fold increase in ACL phosphorylation, which was inhibited by wortmannin pretreatment (Table I and Fig. 6). The identification of this protein in our screen provides further validation for the integrity of the screen, because based on previous studies (34, 35) we would have expected this molecule to have been resolved using this type of approach.

Insulin Decreases the Phosphorylation of Elongation Factor 2—A row of constitutively phosphorylated spots that underwent insulin-dependent dephosphorylation (C12) was identified by liquid chromatography-MS as translation elongation factor 2 (EF2, Fig. 6, Table III). EF2 is a GTP-binding protein that mediates the translocation step in translation elongation and is completely inactivated when phosphorylated (reviewed in Ref. 36). It was recently reported that insulin decreases EF2 phosphorylation in Chinese hamster ovary cells overexpressing ARNO as an assay for PIP3 production have failed to show that a membrane-permeable PIP3 analog had no effect on glucose uptake in adipocytes (19). Recent experiments using plasma membrane recruitment of green fluorescent protein-tagged ADP ribosylation factor nucleotide-binding site opener (ARNO) as an assay for PIP3 production have failed to find a significant effect of PDGF in adipocytes (41). This suggests that although PI3K is recruited to the PDGF receptor in

Hence, this would support a role for PI3K and an additional PI3K-independent pathway in the regulation of glucose metabolism by insulin. In an effort to identify constituents of this so-called insulin-activated PI3K-independent pathway, we have used two-dimensional gel mapping of phosphoproteins in 3T3-L1 adipocytes. This screen relies on several major assumptions: (a) the alternate pathway involves, at some level, protein phosphorylation/dephosphorylation; (b) wortmannin does not inhibit the alternate pathway, which based on previous studies by Tsien and colleagues (19) seems likely; (c) that if such phosphoproteins do exist they will be present in sufficient abundance to be resolved by subcellular fractionation and 2-DE; and (d) PDGF will not activate this pathway because it activates PI3K, yet does not stimulate glucose uptake in adipocytes.

Using a subtraction-based analysis employing 2-DE, we find no evidence to support the existence of an insulin-stimulated PI3K-independent pathway involving protein phosphorylation in adipocytes. We detected at least 18 distinct spots that likely correspond to discrete proteins whose phosphorylation was increased by insulin. PDGF increased the phosphorylation of six of these proteins, whereas it had no significant effect on the remaining 12 spots (Fig. 5). Remarkably, wortmannin caused complete inhibition of the phosphorylation of 11 of these 12 insulin-specific phosphoproteins. Hence, in light of the above assumptions concerning the technique used here, these data provide compelling support in favor of the existence of an insulin-specific signaling pathway in adipocytes where almost all of the protoconstituents of this pathway are likely downstream of PI3K.

If there is no insulin-specific PI3K-independent pathway in adipocytes, this raises the important question as to how insulin, but not PDGF, activates a unique subset of downstream phosphoproteins when both growth factors appear to activate PI3K. One possibility that we (18) and others (13, 14) favor is that insulin activates PI3K in a unique location within the cell and that this may allow the enzyme to access a unique repertoire of downstream proteins. We have gathered evidence in favor of this hypothesis in the present study because we observed that most of the insulin-specific phosphoproteins were localized to the same subcellular fraction as the insulin-dependent PI3K activity (Fig. 5). Most notably the PDGF-stimulated PI3K activity is found in a separate fraction (13, 14, 18). Another possibility that is perhaps not mutually exclusive to that described above is that insulin and PDGF may activate unique PI3K isoforms. This would not be surprising because we have recently observed that insulin preferentially activates the PKB isoform in adipocytes (8). Yet another possibility revolves around the fact that PI3K possesses both lipid and protein kinase activities. It has recently been reported that the protein kinase activity of PI3K may selectively regulate mitogen-activated protein kinase activity, whereas its lipid kinase activity may selectively activate PKB (40). Wortmannin binds to the ATP binding site in PI3K and so inhibits both the protein and lipid kinase activities. Hence this would potentially explain why in the present studies we observe inhibition of almost all insulin-specific phosphoproteins by wortmannin. It remains possible that the protein kinase activity of PI3K mediates the so-called alternate pathway because PI3K analogs only partially rescued the wortmannin block of insulin-stimulated glucose uptake in adipocytes (19). Recent experiments using plasma membrane recruitment of green fluorescent protein-tagged ADP ribosylation factor nucleotide-binding site opener (ARNO) as an assay for PI3Ps production have failed to find a significant effect of PDGF in adipocytes (41). This suggests that although PI3K is recruited to the PDGF receptor in

**Fig. 6.** Insulin regulates the phosphorylation of C2 (ATP-citrate lyase) and C12 (elongation factor 2). The basic, high molecular mass region of cytosol phosphorylation maps from a representative experiment is shown for basal (A), insulin-stimulated (B), PDGF-stimulated (C), and wortmannin-plus-insulin-treated (D) adipocytes. The rows of phosphospots corresponding to C2 and C12 are indicated. E and F, the phosphorylation of C2 and C12 was quantitated from four separate experiments and expressed as a percentage of the insulin-stimulated (E) or basal (F) value. B, basal; I, insulin-stimulated; P, PDGF-stimulated; W/I, wortmannin-plus-insulin-treated.
response to PDGF in these cells (13, 14, 18), for some reason the accumulation of PI3P is blunted. Consistent with this, we have recently reported that PDGF does not induce the phosphorylation or membrane translocation of PKBβ in adipocytes (8). This failure to stimulate PI3P levels may be because of either a block in production, possibly because the PDGF receptor does not have access to phosphatidylinositol 4,5-bisphosphate, or to an increased degradation of PI3P. In the case of the latter, it is conceivable that the PDGF receptor binds a phospholipid phosphatase that selectively hydrolyses PI3P produced in response to PDGF, but not other ligands such as insulin. Despite the inability of PDGF to increase PI3P levels in adipocytes, it seems plausible that both the lipid and protein kinase activities of PI3K augment separate downstream signaling pathways in adipocytes, and both may be required to activate glucose metabolism.

The present studies have clearly resolved a number of proteins that are insulin-specific and wortmannin-sensitive and thus constitute excellent candidates for downstream molecules in insulin action. We have attempted to characterize many of these proteins using liquid chromatography-MS; however, with the exception of two proteins (Table III) the success of these studies has been limited. This is due in part to inadequate resolution of the preparative 2-DE system because many of the spots identified contain >5–9 separate polypeptides (data not shown). Thus, in order to extend these studies it may be necessary to incorporate additional purification steps prior to 2-DE. It is also possible that many of the proteins we are working with are very low abundance necessitating a much larger scale than has been currently employed. Nevertheless in view of the potential importance of some of the molecules identified here, additional experiments are probably warranted. The identification of ATP-citrate lyase as one of our candidate proteins in part validated the use of this technique because this protein is known to be phosphorylated by insulin in a wortmannin-sensitive manner. This result also indicates that this approach will enable us to achieve nonselective identification of both signaling proteins such as MAP kinase and PKB (Figs. 3 and 4) as well as metabolic machinery (Fig. 6). Furthermore, this approach can be used to detect both increases and decreases in protein phosphorylation both of which are affected by insulin. The only significant protein dephosphorylation event we were able to detect corresponded to the other protein we were able to positively identify by MS and that was eucaryotic elongation factor 2 (Table III and Fig. 6). This protein is a key regulatory determinant of mRNA translation, and this process is known to be influenced by insulin stimulation. To complement the recent studies reporting insulin-dependent EF2 dephosphorylation, our data further suggest that dephosphorylation of EF2 is wortmannin-sensitive and PDGF-insensitive in adipocytes.

In summary, we have identified a number of proteins that are phosphorylated in an insulin-specific and wortmannin-sensitive manner. The absence of insulin-specific wortmannin-insensitive proteins calls into question the existence of a PI3K-independent pathway in adipocytes. Recent studies also suggest that PI3K is involved in insulin regulation of metabolism in the liver (42, 43). Interestingly, in hepatocytes insulin also stimulates PI3K activity in a fraction similar to the high speed pellet fraction of adipocytes (43). Indeed many of the insulin-specific phosphoproteins we have mapped in the current study are enriched in the HSP fraction that also contains the insulin responsive IRS proteins (13, 14, 18, 43), adding further support to the concept that localization of signaling molecules may play an important role in their specific function.

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### Table III

**Analysis of C2 and C12 by liquid chromatography-electrospray ionization-ion trap mass spectrometry**

| Spot no. | Identification                      | Swissprot accession no. | Peptide no. | Sequence                  | Position in protein |
|----------|------------------------------------|-------------------------|-------------|---------------------------|---------------------|
| C2       | ATP-citrate lyase                  | P16638                  | 1           | NFLIEFYPVHSQAEFFY         | 104–120             |
|          |                                    |                         | 2           | IGNFGGMLNLASK             | 645–659             |
|          |                                    |                         | 3           | SFDELGEIQSVSEYDVK         | 788–806             |
|          |                                    |                         | 4           | FGGDALAAAK                | 933–943             |
|          |                                    |                         | 5           | VNFY TEDQ                 | 2–10                |
|          |                                    |                         | 6           | DGGFLINLIDPSHVDSSEVTALLR  | 93–120              |
|          |                                    |                         | 7           | CELLYEGPDPDEAAGIK         | 369–386             |
|          |                                    |                         | 8           | VFSGVSTGLK                | 416–426             |
|          |                                    |                         | 9           | EDLYKPIQR                 | 440–449             |
|          |                                    |                         | 10          | YVPE1EDVPFCSHVGVGLVDQFLVK | 457–451             |
|          |                                    |                         | 11          | TGGTITTFEHAEDNR           | 482–495             |
|          |                                    |                         | 12          | GQVOYNEIK                 | 665–676             |
|          |                                    |                         | 13          | GHVFEESQVAGTFPMFVVK       | 768–785             |

Spots C2 and C12 were purified from insulin-stimulated 3T3-L1 adipocytes, digested with trypsin, and then analysed by liquid chromatography-mass spectrometry as described under “Experimental Procedures.”

**TABLE III**

**Spots C2 and C12 were purified from insulin-stimulated 3T3-L1 adipocytes, digested with trypsin, and then analysed by liquid chromatography-mass spectrometry as described under “Experimental Procedures.”**
Insulin-specific Phosphoproteins in Adipocytes

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