A critical component of vertebrate cellular differentiation is the acquisition of sensitivity to a restricted subset of peptide hormones and growth factors. This accounts for the unique capability of insulin (and possibly insulin-like growth factor-1), but not other growth factors, to stimulate glucose uptake and anabolic metabolism in heart, skeletal muscle, and adipose tissue. This selectivity is faithfully recapitulated in the cultured adipocyte line, 3T3-L1, which responds to insulin, but not platelet-derived growth factor (PDGF), with increased hexose uptake. The serine/threonine protein kinases Akt1 and Akt2, which have been implicated as mediators of insulin-stimulated glucose uptake, as well as hexose uptake. The serine/threonine protein kinases Akt1 and Akt2, which have been implicated as mediators of insulin-stimulated glucose uptake, as well as glycolgen, lipid, and protein synthesis, were shown to mirror this selectivity in this tissue culture system. This was particularly apparent in 3T3-L1 adipocytes overexpressing an epitope-tagged form of Akt2 in which insulin activated Akt2 10-fold better than PDGF. Similarly, in 3T3-L1 adipocytes, only insulin stimulated phosphorylation of Akt1’s endogenous substrate, GSK-3β. Other signaling molecules, including phosphatidylinositol 3-kinase, pp70 S6-kinase, mitogen-activated protein kinase, and PHAS-1/4EBP-1, did not demonstrate this selective responsiveness to insulin but were instead activated comparably by both insulin and PDGF. Moreover, concurrent treatment with PDGF and insulin did not diminish activation of phosphatidylinositol 3-kinase, Akt, or glucose transport, indicating that PDGF did not simultaneously activate an inhibitory mechanism. Interestingly, PDGF and insulin comparably stimulated both Akt isoforms, as well as numerous other signaling molecules, in undifferentiated 3T3-L1 preadipocytes. Collectively, these data suggest that differential activation of Akt in adipocytes may contribute to insulin’s exclusive mediation of the metabolic events involved in glucose metabolism. Moreover, they suggest a novel mechanism by which differentiation-dependent hormone selectivity is conferred through the suppression of specific signaling pathways operational in undifferentiated cell types.

Under the appropriate conditions, 3T3-L1 fibroblasts differentiate into cells with an adipocyte phenotype characterized by the accumulation of triglyceride and increased sensitivity to insulin. In these differentiated cells, as well as rat and human adipocytes, insulin accelerates glucose entry by effecting the translocation of glucose transporter 4 (GLUT4) from intracellular stores to the plasma membrane (1). Subsequently, insulin stimulates metabolic pathways to promote storage of the incoming glucose as glycogen or triglyceride. In this capacity, insulin stands apart from other growth factors, such as platelet-derived growth factor (PDGF), which activate remarkably similar signaling cascades yet are incapable of eliciting these metabolic events (2–8). A clear explanation of how these very similar signaling pathways elaborate such radically different physiological responses in this differentiated tissue has remained elusive, although compartmentalization of signaling molecules has been proposed (9).

Both insulin and PDGF activate their respective tyrosine kinase receptors to phosphorylate key residues on a “docking protein” or the receptor, respectively, which recruits multiple adaptor proteins. Recruited proteins include the GDP exchange factor Son of Sevenless, or SOS, which activates the Ras/Raf/mitogen-activated protein kinase cascade, or the p85 regulatory subunit of PI3-kinase, which stimulates signaling pathways ultimately leading to pp70 S6-kinase (10, 11), 4EBP1/PHAS-1 (12), or Akt/PKB activation (13). PI3-kinase is strongly implicated in both metabolic and mitogenic signaling (14), as is its downstream effector Akt (15–17).

This paper reports experiments aimed at understanding the mechanism by which adipocytes develop ligand selectivity such that insulin, but not PDGF, stimulates anabolic metabolism. We assessed the ability of insulin and PDGF to regulate numerous signaling molecules, particularly PI3-kinase and Akt isoforms 1 and 2, due to their relevance to anabolic metabolism (18–21). We were particularly interested in Akt2, primarily because of two recent reports suggesting a role for this particular isoform in GLUT4 translocation (22, 23). The relative effectiveness of PDGF and insulin at activating different Akt isoforms in 3T3-L1 adipocytes has been unclear. Two laboratories (24, 25) reported that insulin phosphorylates Akt on a key regulatory residue much more strongly than does PDGF, whereas a third group reported that PDGF stimulates Akt kinase activity at least 50–60% as well as insulin (26). None of these studies distinguished between the relative sensitivity of different Akt isoforms.
MATERIALS AND METHODS

Antibodies and Reagents—Polyclonal sheep anti-GLUT4 antibodies were raised against a glutathione S-transferase fusion protein encoding the last 31 amino acids of the GLUT4 carboxyl terminus (glutathione S-transferase-ISATFRRTIPSLLEQVKPSSTLEYLGPDEND). Polyclonal rabbit anti-Akt antibodies were raised against the carboxyl-terminal sequences CHFFQYSYSSAGTA in Akt1 and CDQTHFPQSYSASIRE in Akt2. Polyclonal rabbit anti-phospho-S6 antibodies were raised against the major phosphorylation site in the ribosomal S6 subunit (CRRLLKPSA) 5' of Thr423 (Ser420) (a phospho-mimetic peptide), which raise phosphorylated Akt, were kindly provided by Phillip Tsichlis and Joseph Testa, respectively, of the Fox Chase Cancer Center (Philadelphia, PA). Antibodies and Reagents—MicroPoly(A) Pure™ mRNA isolation kit from Ambion. Approximately 50 ng of mRNA was reverse transcribed for 1 h at 48 °C by avian myeloblastosis virus reverse transcriptase for first strand cDNA synthesis primed by the antisense primer (5'-CGTGGCTAAGCTG-3') after briefly denaturing the target primer at 95 °C for 2 min at 94 °C. ΨT DNA polymerase was used for second strand synthesis and PCR, primed by the antisense primer and the sense primer (5'-CCTGCCCCCTTACACGGACC-3'). Amplification resulted from 40 cycles of denaturation, annealing, and extension for 30 s at 94 °C, 1 min at 60 °C, and 1 min at 68 °C, respectively. The sense primer corresponds to Akt1 nucleotides 1098–1060 or Akt2 nucleotides 1041–1061. Antisense primer corresponds to Akt1 nucleotides 1488–1430 or Akt2 nucleotides 1411–1433. RT-PCR reagents and protocols are derived from the ACCESS RT-PCR System (Promega). Other RT-PCR systems (Perkin-Elmer) produced the same results.

RESULTS

Several groups have found that PDGF is incapable of activating glucose uptake in 3T3-L1 adipocytes (5, 7). Nonetheless, there are two reports in which PDGF does stimulate glucose uptake in wild-type 3T3-L1 adipocytes (29) or rat adipocytes overexpressing PDGF receptors (30). To determine whether PDGF is capable of activating glucose transport and GLUT4 translocation in the 3T3-L1 adipocytes used in our laboratory, we assayed these events after a 10-min treatment with either insulin (100 nM) or PDGF (50 ng/ml). Insulin, but not PDGF, stimulated both the uptake of 2-deoxyglucose (Fig. 1A) and the appearance of cell surface GLUT4 (Fig. 1B). In light of the similarities among the known signaling mechanisms for either agonist, one explanation for this difference was that PDGF additionally activated "inhibitory mechanisms" that prevented its enhancement of certain metabolic events. While previous studies have demonstrated the existence of inhibitory "crosstalk" between insulin and PDGF pathways when PDGF is added prior to insulin (24, 31), simultaneous addition of the

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In the work presented below, we demonstrate that both Akt isofroms are selectively responsive to insulin, and not PDGF, in 3T3-L1 adipocytes. PDGF still activated numerous other signaling molecules, including PI3-kinase, pp70 S6-kinase, 4EBP1/PHAS-1, and mitogen-activated protein kinase, effectively in this tissue. PDGF was also a potent stimulator of both forms of Akt in the precursor 3T3-L1 fibroblasts, suggesting that 3T3-L1 differentiation specifically suppresses this signaling pathway. These experiments suggest a mechanism by which redundant signaling pathways functional in precursor cells can be altered to serve specific regulatory functions in differentiated tissues.
peptides had not been investigated in detail. As shown in Fig. 1, A and B, concomitant addition of insulin and PDGF resulted in stimulation of glucose uptake and GLUT4 translocation equivalent to insulin alone. Despite PDGF’s inability to activate these metabolic responses, PDGF does signal other events as effectively as insulin in this cell type. In both 3T3-L1 fibroblasts and adipocytes, insulin stimulated the tyrosine phosphorylation of insulin and/or IGF-I receptors, while PDGF induced tyrosine phosphorylation of the PDGF receptor (Fig. 1C, upper panel). Moreover, in both cell types, insulin and PDGF stimulated phosphorylation of mitogen-activated protein kinase (Fig. 1C, lower panel). Since PI3-kinase is critical for activation of anabolic metabolism, as well as signaling molecules such as Akt and pp70 S6-kinase (10, 13, 18, 32), its activity in anti-phosphotyrosine immunoprecipitates was measured. Insulin and PDGF treatment, respectively, stimulated PI3-kinase 100-fold over basal level, and the precipitated activity roughly doubled when both agonists were added simultaneously (Fig. 1D). These results are consistent with prior reports that PDGF receptors mediate activation of PI3-kinase in 3T3-L1 adipocytes, but this is not sufficient for activation of glucose transport (7).

As described above, prior studies have been contradictory regarding whether Akt is activated by PDGF in 3T3-L1 adipocytes. Two reports have shown that insulin is significantly more potent than PDGF in increasing Akt phosphorylation by using phosphospecific antisera directed against the regulatory Ser473 site on Akt1. In contrast, Tanti et al. (26) found that PDGF is quite effective at stimulating Akt activity as measured in the immune complex, achieving a level of activation that is at least 50% that induced by insulin. In addition to being contradictory, these prior studies have not distinguished between activation of each isoform. To resolve these apparent discrepancies, we used four different approaches to evaluate...
the regulation of Akt isoforms 1 and 2 by insulin and PDGF in 3T3-L1 fibroblasts and adipocytes. First, we used anti-phospho-Ser\(^{473}\) antisera, which, as shown below, specifically recognizes phosphorylated Akt1, but not Akt2, and anti-phospho-Thr\(^{308}\) antisera, which recognizes both Akt1 and Akt2 isoforms. Second, we produced an antibody specific for Akt2 that could detect both an agonist-induced mobility shift when the proteins were separated on SDS-polyacrylamide gels and could immunoprecipitate endogenous Akt2 kinase activity from intact cells. Third, we expressed an epitope-tagged form of Akt2 in 3T3-L1 cells and then measured the shift and kinase activity of this construct using antibodies against the HA epitope. And fourth, we measured the degree of phosphorylation of the Akt substrate GSK-3\(\beta\) \textit{in vivo}, which serves as a marker of endogenous Akt kinase activity in the intact cell.

To characterize the isoform preference for the phosphospecific antibody directed against the regulatory Ser\(^{473}\) phosphorylation site, Akt1 and Akt2 isoforms were expressed in 3T3-L1 cells. Using an antibody raised against the carboxyl terminus of Akt1 but that actually recognizes both isoforms, we demonstrated that these proteins were overexpressed severalfold and that Akt2 migrated slightly faster than Akt1 when resolved on SDS-polyacrylamide gels (Fig. 2A). Electrophoresis conditions were not optimized in this experiment to detect an agonist-induced mobility shift, which is generally less pronounced using this antibody as compared with the Akt2-specific antibody described below. When the phospho-Ser\(^{473}\) antibody was used to investigate Akt phosphorylation in these lysates, it detected phosphorylated Akt in Akt1-expressing 3T3-L1 adipocytes that were stimulated by insulin but not PDGF (Fig. 2B, upper panel). However, as seen in longer exposures (Fig. 2B, lower panel), the antibody only detected endogenous Akt and not the overexpressed construct in cells stably expressing Akt2. Prolonged exposure of the blots also indicated that overexpressed Akt1 was recognized by the anti-phospho-Ser\(^{473}\) antibody to some degree under basal conditions, but this was augmented only by insulin and not PDGF. Faint reactivity with the phosphospecific Akt antibody was sometimes observed in the PDGF-stimulated adipocyte lysates, but it was always markedly less than that seen in response to insulin. Collectively, these results indicate that the anti-phospho-Ser\(^{473}\) antibody is specific for the Akt1 isoform and that insulin stimulates Akt1 phosphorylation much more potently than does PDGF in 3T3-L1 adipocytes.

To investigate whether PDGF affected Akt1 in the adipocyte precursor cells, 3T3-L1 fibroblasts, we again investigated PDGF’s ability to increase phosphorylation of Akt1 using the phospho-Ser\(^{473}\)-specific antibody. As demonstrated in Fig. 3, PDGF stimulated Ser\(^{473}\) phosphorylation comparably with insulin in 3T3-L1 fibroblasts. Insulin was used at a concentration of 1 \(\mu\)M, and under these conditions it is likely to be binding to IGF-1 receptors. Akt1 is also phosphorylated on a second regulatory site, Thr\(^{308}\). Using a phosphospecific antibody that recognizes this second regulatory site (as well as the analogous Thr\(^{309}\) site in Akt2), we found that this residue was phosphorylated in response to both insulin and PDGF in 3T3-L1 fibroblasts but was selectively responsive to insulin in differentiated 3T3-L1 adipocytes (Fig. 3). Phosphorylation of either site was blocked by pretreatment with wortmannin, confirming their PI3-kinase dependence in adipocytes. These studies suggest that differentiation actually suppresses signaling events “downstream” of PI3-kinase, and suggests a more complicated pattern of regulation of Akt1.

Because of the isoform specificity of the phospho-Akt antibody, the previously published studies could not address whether the more relevant Akt2 isoform was differentially phosphorylated in 3T3-L1 adipocytes. An indication of the importance of investigating Akt2 is its altered expression during adipogenesis. These studies suggested whether the more relevant Akt2 isoform was differentially phosphorylated in 3T3-L1 adipocytes. An indication of the importance of investigating Akt2 is its altered expression during adipogenesis. These studies suggested whether the more relevant Akt2 isoform was differentially phosphorylated in 3T3-L1 adipocytes. An indication of the importance of investigating Akt2 is its altered expression during adipogenesis. These studies suggested whether the more relevant Akt2 isoform was differentially phosphorylated in 3T3-L1 adipocytes. An indication of the importance of investigating Akt2 is its altered expression during adipogenesis. These studies suggested whether the more relevant Akt2 isoform was differentially phosphorylated in 3T3-L1 adipocytes. An indication of the importance of investigating Akt2 is its altered expression during adipogenesis. These studies suggested whether the more relevant Akt2 isoform was differentially phosphorylated in 3T3-L1 adipocytes. An indication of the importance of investigating Akt2 is its altered expression during adipogenesis. These studies suggested whether the more relevant Akt2 isoform was differentially phosphorylated in 3T3-L1 adipocytes. An indication of the importance of investigating Akt2 is its altered expression during adipogenesis.
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Effect of differentiation on Akt1 phosphorylation. 3T3-L1 fibroblasts (left panels) and adipocytes (right panels) were serum-starved for 2 h in Leibovitz-15 medium containing 0.2% BSA before treatment with PDGF (50 ng/ml; PDGF) or insulin (1 μM for fibroblasts, 100 nM for adipocytes; Insulin) for 10 min. Adipocytes were pretreated with (+) or without (−) 100 nM wortmannin for 30 min prior to the addition of insulin. Total cell extracts were prepared, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-Ser\(^{T308}\) or anti-phospho-Thr\(^{T308}\) antibodies.

Significantly stimulated endogenous Akt kinase activity when measured in an immune complex assay (26). Using the isofrorm-specific Akt2 antibodies, we similarly observed a significant PDGF stimulation of Akt2 kinase activity in immune complex assays (Fig. 6). Because activation of Akt kinase did not correlate with phosphorylation on the major regulatory sites, Ser\(^{T308}\) and Thr\(^{T308}\), there was concern that some of the activity measured in the immune complex might be due to another hormone-sensitive kinase contaminating the immune complex assay. To evaluate this, HA-tagged Akt2 was expressed in 3T3-L1 adipocytes at levels 2–5 times higher than endogenous Akt2. If Akt2 were associating with a limiting kinase also activated by insulin, overexpression should allow a more definitive assay. Moreover, use of the anti-HA antibody also excludes potential cross-reactivity of the Akt2 antibody with another protein kinase. Western blot analysis of the electrophoretic mobility shift of the overexpressed HA-Akt2 construct revealed that both insulin and PDGF retarded its electrophoretic mobility in fibroblasts (Fig. A1), but only insulin elicited the shift in adipocytes (Fig. B1). This change in mobility could be detected with both anti-HA and anti-Akt2 antibodies. Kinase activity of the overexpressed Akt2 was ascertained in immunoprecipitates using anti-HA antisera. In fibroblasts, PDGF was significantly more effective than insulin at stimulating HA-Akt2 kinase activity (Fig. B1). Following differentiation, however, insulin’s stimulation of HA-Akt2 was roughly 10-fold greater than that of PDGF (Fig. B1). Similar results were obtained using the anti-Akt2 antibodies in the overexpressing cell lines, although the antibody precipitated less total activity (data not shown).

As a final means for ascertaining Akt kinase activity, we evaluated the degree of phosphorylation of an endogenous substrate for Akt, GSK-3β. A prior study (25) measured the effects of insulin and PDGF on GSK-3β kinase activity and found that PDGF inhibited GSK-3β kinase activity about half as well as insulin. However, several molecules have been reported to affect GSK-3β kinase activity (34), while only Akt is known to phosphorylate GSK-3β on the regulatory serine 9 residue. Moreover, GSK-3β kinase activity is inhibited only ~50% in response to insulin or other agonists (25, 35), while changes in GSK-3β phosphorylation are much easier to detect. Thus, we chose to measure GSK-3β phosphorylation on the Akt target residue, serine 9, as an indication of endogenous Akt kinase activity. GSK-3β was immunoprecipitated from insulin- and PDGF-stimulated cells using monoclonal anti-GSK-3β antibodies and subjected to Western blotting with an antibody directed against the phosphorylated serine 9 site of GSK3β. In fibroblasts, both insulin and PDGF stimulated GSK-3β phosphorylation; in adipocytes, however, PDGF-induced GSK-3β phosphorylation was much less pronounced (Fig. 8). Thus, in vivo
phosphorylation of an endogenous substrate of Akt precisely reflects the insulin/PDGF specificity suggested by phosphorylation of Akt or the in vitro activity of overexpressed Akt. This is perhaps the best measure of Akt kinase activity, since this is not subject to potential artifacts associated with measuring immunoprecipitated kinase activity but rather is likely to reflect the total amount of Akt kinase activity present in the intact cell.

The ability of PDGF to activate PI3-kinase as measured in the immune complex, but not stimulate Akt, raised the question of whether signaling-competent 3'-'phosphoinositides were being generated in response to PDGF in the intact cell. Thus, we chose to look at two downstream targets that require polyphosphoinositide production for activation. PDGF reportedly stimulates pp70 S6-kinase activity, and a mobility shift in 3T3-L1 adipocytes (24) and, in other cell types, PI3-kinase is required for pp70 S6-kinase activation (11). In both the 3T3-L1 fibroblasts and adipocytes used in this study, both PDGF and insulin stimulated phosphorylation of pp70 S6-kinase as demonstrated by an upward electrophoretic mobility shift on Western blots (Fig. 9A). Two other assays revealed that adipocyte pp70 S6-kinase was activated comparably by both insulin and PDGF in 3T3-L1 adipocytes: 1) both insulin and PDGF activated pp70 S6-kinase roughly 3–4-fold as measured by immunocomplex kinase assays using ribosomal S6 peptide as substrate (Fig. 9B); and 2) PDGF and insulin both induced the phosphorylation in vivo of pp70 S6-kinase’s endogenous substrate, ribosomal protein S6 (Fig. 9B). Phosphorylation of S6 was detected by probing total cell lysates with anti-phospho-S6 antibodies following stimulation of adipocytes with either PDGF or insulin. Submaximal insulin doses had similar effects on the insulin-stimulated shift of both Akt and pp70 S6-kinase, further suggesting that the two enzymes respond similarly to insulin stimulation (Fig. 9C). Finally, stimulation of pp70 S6-kinase by either insulin or PDGF was blocked by pretreatment with the PI3-kinase inhibitor wortmann (Fig. 9D) or LY294002 (Fig. 9E), confirming PI3-kinase dependence for pp70 S6-kinase activation. These data provide confirmation that functional 3'-phosphoinositides are generated in response to PDGF, since their production is required for stimulation of pp70 S6-kinase.

A second signaling molecule downstream of PI3-kinase is the translational repressor PHAS-1 (also called 4EBP1). PHAS-1 is phosphorylated on multiple different sites by the protein mTOR (mammalian target of rapamycin), but the molecular events activating mTOR are a subject of debate. We evaluated the relative actions of insulin and PDGF on PHAS-1 phosphorylation, which can be detected by a reduction in electrophoretic mobility when the protein is resolved on SDS-PAGE gels (36). Like pp70 S6-kinase, PHAS-1 was phosphorylated in response to both insulin and PDGF (Fig. 10), although the former was more effective. The shift in mobility could be inhibited by wortmann (Fig. 10) or LY294002 (data not shown). These data indicate that PDGF is capable of generating PI3-kinase-dependent signals leading to phosphorylation of pp70 S6-kinase and PHAS-1 in 3T3-L1 adipocytes.

**DISCUSSION**

The data presented in this report demonstrate that Akt isoforms 1 and 2 are activated significantly by insulin, but not PDGF, in differentiated 3T3-L1 adipocytes. Evidence supporting this conclusion includes the following: 1) insulin, but not PDGF, stimulated Akt phosphorylation in 3T3-L1 adipocytes, as detected using phosphospecific antibodies recognizing a regulatory Ser473 site on Akt1; 2) insulin, but not PDGF, decreased the electrophoretic mobility of Akt2 separated on polyacrylamide gels; 3) insulin, but not PDGF, stimulated phosphorylation of Akt1 and/or Akt2 on the regulatory Thr308 or Thr309...
FIG. 7. Electrophoretic mobility and kinase activity of HA-Akt2 in 3T3-L1 fibroblasts and adipocytes. 3T3-L1 fibroblasts (A and B) or adipocytes (C and D) overexpressing HA-Akt2 were serum-starved for 2 h in Leibovitz’s L-15 medium containing 0.2% BSA before treatment with PDGF (50 ng/ml; P) or insulin (1 μM for fibroblasts, 100 μM for adipocytes; I) for 10 min. A and C, total cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-HA or anti-Akt2 antibodies. B and D, Akt in vitro immune complex kinase reactions were performed as described using anti-HA antibodies for immunoprecipitation. 32P incorporation into histone H2B was visualized by autoradiography and quantitated by PhosphorImager analysis (Molecular Dynamics). Results shown are the means ± S.E. of three independent experiments.

FIG. 8. Akt kinase activity in 3T3-L1 fibroblasts and adipocytes measured by phosphorylation of GSK-3β, an in vitro substrate. 3T3-L1 fibroblasts (left) and adipocytes (right) were serum-starved for 2 h in Leibovitz’s L-15 media containing 0.2% BSA before treatment with PDGF (50 ng/ml; P), insulin (1 μM; I), or no stimulation (−). GSK-3 was immunoprecipitated using anti-GSK-3β antibodies and resolved by SDS-PAGE. GSK-3 phosphorylation was detected by probing Western blots with anti-phospho-GSK-3 antibodies. Data are representative of two independent experiments.

IP: αGSK
WB: αP-GSK
(-) I P (-) I P
<histone
Fibroblasts
Adipocytes
<αGSK
<αHA
<αAKT2
<IgG
B P I

3T3-L1 FIBROBLASTS
A
Western Blots

3T3-L1 ADIPOCYTES
C
Western Blots

B Akt Kinase Assay

D Akt Kinase Assay

Electrophoretic mobility and kinase activity of HA-Akt2 in 3T3-L1 fibroblasts and adipocytes. 3T3-L1 fibroblasts (A and B) or adipocytes (C and D) overexpressing HA-Akt2 were serum-starved for 2 h in Leibovitz’s L-15 medium containing 0.2% BSA before treatment with PDGF (50 ng/ml; P) or insulin (1 μM; I) or no stimulation (−). GSK-3 was immunoprecipitated using anti-GSK-3β antibodies and resolved by SDS-PAGE. GSK-3 phosphorylation was detected by probing Western blots with anti-phospho-GSK-3 antibodies. Data are representative of two independent experiments.

Akt is also implicated in activation of two other signaling molecules, pp70 S6-kinase and PHAS-1/4EBP1. Overexpression of constitutively active forms of Akt stimulates phosphorylation of the endogenous Akt substrate GSK-3β. This last assay is most convincing, since it demonstrates insulin selectivity of Akt activation in the intact cell. Other signaling molecules are activated comparably in this differentiated tissue, including the upstream lipid kinase PI3-kinase and its downstream targets pp70 S6-kinase and PHAS-1. Insulin's ability to activate Akt is adipocyte-specific, since PDGF was a potent stimulator of Akt phosphorylation, electrophoretic mobility retardation, and kinase activity, as well as GSK-3β phosphorylation, in preadipocyte 3T3-L1 fibroblasts.

The first experiments describing hormonal activation of Akt involved expressed mutant PDGF receptors (13), and in these initial studies it was determined that PI3-kinase is critical for PDGF-triggered Akt activation. Subsequent studies indicated that expression of constitutively active forms of PI3-kinase is sufficient to activate endogenous Akt (37). Several different Akt isoforms have now been identified (38, 39), all sharing a great deal of homology. These isoforms are expressed fairly ubiquitously, and they have been implicated in numerous cellular processes. Myriad studies have implicated Akt in metabolic responses to insulin. Overexpression of constitutively active forms of Akt stimulates glucose uptake and GLUT4 translocation (21, 26), as well as protein and lipid synthesis (21). Expression of dominant-negative forms of Akt confirms a role for the enzyme in insulin-stimulated glycogen (40) and protein (41) synthesis. In other tissues Akt apparently participates more generally in anti-apoptosis (42) and regulation of cell cycle (43).

The hypothesis that Akt plays a pivotal role in GLUT4 translocation has been recently challenged (41), but this topic remains controversial (44). Recently, the Akt2 isoform was found to specifically associate with GLUT4-containing vesicles and to phosphorylate GLUT4 vesicle resident proteins (22, 23). Furthermore, the sphingomyelin derivative ceramide inhibits glucose uptake and GLUT4 translocation, and this correlates with its ability to inhibit Akt phosphorylation and activation (45). Thus, although the evidence that Akt is involved in glucose uptake or GLUT4 translocation is controversial, this issue has not been resolved. Regardless, Akt's contribution to insulin-stimulated protein synthesis (41) and glycogen synthesis (40) are strongly supported by the literature, confirming at least some role for the enzyme in glucose metabolism. The data presented above indicate that Akt2 expression increases markedly during the course of 3T3-L1 differentiation (Figs. 4 and 5), further implicating a role for this isoform in insulin-stimulated metabolism.

Akt is also implicated in activation of two other signaling molecules, pp70 S6-kinase and PHAS-1/4EBP1. Overexpression of constitutively active forms of Akt stimulates phosphorylation of both molecules (13, 20), while expression of a dominant negative form blocks insulin's stimulation of pp70 S6-kinase (41). Despite these studies, a mechanism has been proposed whereby pp70 S6-kinase can be stimulated directly by the upstream kinase PDK1 and thus not require Akt for acti-
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FIG. 9. Activity of pp70 S6 kinase following stimulation of 3T3-L1 adipocytes with PDGF or insulin. In A–C, 3T3-L1 fibroblasts or adipocytes were serum-starved for 2 h in KRP buffer containing 0.2% BSA. Then, cells were additionally stepped down in DMEM containing 10 mM HEPES and 0.5% BSA prior to the incubation in KRP. In D and E, cells were stimulated with either PDGF (50 ng/ml; P), insulin (100 nM; I), or no stimulation (−), while in C cells were stimulated with the indicated concentration of insulin, for 10 min prior to lysis. A, following stimulation, total cell lysates (50 μg of protein) from 3T3-L1 fibroblasts or adipocytes were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against pp70 S6 kinase or the phosphorylated species of ribosomal protein S6. B, upper panel, following stimulation, pp70 S6 kinase was immunoprecipitated from 3T3-L1 adipocytes using polyclonal anti-pp70 antibodies, and in vitro kinase reactions were performed on the immune complexes. Reactions proceeded for 10 min at 30 °C and were resolved by SDS-PAGE. 32P incorporation into glutathione S-transferase S6 peptide was visualized by autoradiography and quantitated by PhosphorImager analysis (Molecular Dynamics). B, lower panel, total cell lysates (50 μg of protein) from stimulated 3T3-L1 adipocytes were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-S6 antibodies. C, total cell lysates from 3T3-L1 adipocytes (50 μg of protein) treated with the indicated concentrations of insulin were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-pp70 S6 kinase or anti-Akt2 antibodies. D, the PI3-kinase inhibitor LY294002 (100 μM) was added to 3T3-L1 adipocytes 30 min prior to stimulation with insulin or PDGF. Total cell lysates were resolved by electrophoresis and detected with antibodies against pp70 S6 kinase or the phosphorylated species of ribosomal protein S6. Identical results were obtained using 100 nM wortmannin.

FIG. 10. Phosphorylation of PHAS-1 response to insulin and PDGF in 3T3-L1 adipocytes. 3T3-L1 adipocytes were serum-starved for 18–24 h in DMEM with 0.5% BSA and 10 mM HEPES and then starved for an additional 2 h in KRP containing 0.2% BSA before treatment with nothing (B), PDGF (50 ng/ml; P), or insulin (100 nM; I) for 10 min. Certain samples were pretreated with 250 nm wortmannin 30 min prior to insulin. Total protein (40 μg) was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-PHAS-1 antibody. Identical results were obtained using 100 nM wortmannin.

In this paper, we present conditions where there is a lack of correlation between the activation of pp70 S6-kinase, PHAS-1/4EBP1, and Akt. Specifically, PDGF stimulates pp70 S6-kinase and PHAS-1 without significantly affecting Akt, suggesting that Akt is dispensable for pp70 S6-kinase or PHAS-1 activation under these conditions.

One hypothesis previously offered to explain PDGF's disproportional effects on activation of glucose uptake and PI3-kinase is that PDGF does not target PI3-kinase to the appropriate subcellular compartment. Several laboratories report that PDGF-stimulated PI3-kinase is located primarily at the plasma membrane, while insulin-stimulated PI3-kinase is predominantly microsomal (2, 3). Targeting sequences found in the cytosolic IRS-1, but not the membrane-bound PDGF receptor, could account for differences in localization. Moreover, some reports indicate that insulin, but not PDGF, specifically targets PI3-kinase to GLUT4 vesicles (9). This has been recently challenged, however, since PI3-kinase was instead reported to associate with the cytoskeleton and not the GLUT4 vesicle itself (47). While this model appears attractive with regard to GLUT4 vesicles, it is inherently more difficult to explain how PDGF is capable of activating some cytosolic enzymes, such as pp70 S6-kinase, but not others, such as Akt, using such a compartmentalization model. Nonetheless, it remains a formal possibility that PDGF receptors and PI3-kinase could specifically be sequestered from Akt in 3T3-L1 adipocytes. Overexpression of PDGF receptors reportedly stimulates glucose uptake (30), perhaps because the elevated expression of receptors saturates such sequestration events. Intriguingly, expression of PDGF receptors incapable of activating PI3-kinase still stimulates wortmannin-sensitive glucose transport (30). These mutant receptors might be liberating endogenous PDGF receptors from such sequestration mechanisms. We are currently investigating whether PDGF receptor overexpression releases the suppression of PDGF's activation of Akt.

An alternative possibility is that the activity of PI3-kinase as measured in the immune complex assay may not reflect the levels of phosphoinositides accumulated in vitro, possibly due to differences in accessibility to substrate. A prior study reports that although insulin and PDGF activate mitogen-activated protein kinase comparably, only insulin stimulates the intracellular production of the lipid product PIP₃ (4). PIP₃ could be critical for activation of Akt, but not pp70. An alternative mechanism for differential production of PIP₃ is that PDGF, but not insulin, stimulates a localized PIP₃ phosphatase.
A third possibility is that insulin stimulates other signaling event pathways in addition to those previously described that are required for complete activation of Akt. If this is the case, such mechanisms must themselves be specifically uncoupled from PDGF receptors during the course of differentiation. In another recent report (48), membrane-permeable analogs of PIP$_3$ were found to be incapable of stimulating glucose transport. However, when these PIP$_3$ analogs were added to wortmannin-treated cells, they were capable of stimulating transport. This experiment suggests that additional pathways are required for activation of glucose transport, and future experiments will investigate whether this is also the case for Akt.

In addition to suggesting a role for Akt in insulin-stimulated glucose metabolism, the data presented herein can be elaborated into a broader hypothesis regarding the generation of a differentiated phenotype. The development of specialized cells is accompanied by the acquisition of novel morphological and biochemical characteristics. In white adipose tissue, for example, features conferred by differentiation include a reduced central cytoskeleton, the accumulation of a large and metabolically active triglyceride droplet, and marked insulin-stimulatable glucose transport. The latter apparently depends on the induction of the GLUT4 glucose transporter, although its expression is clearly not sufficient (49). In complex, multisystem organisms, highly specialized functions are characterized by exquisitely controlled regulation, often by extracellular ligands in the form of hormones and growth factors. Thus, the mature cell must not only possess unique terminal outputs but must also display distinctive selectivity to unique arrays of extracellular signals. Based on the current data, we propose that some cells confer this specificity via selective suppression of signaling pathways present in a multipotent immature precursor cell (Fig. 11). This process stands in contrast to the development of a new response, for example the induction of a novel transcriptor isofrom. However, the elimination of an unwanted pathway appears a parsimonious strategy for such a process as signal transduction, in which differentiated cells utilize a common set of biochemical devices assembled into distinct schemes. Nonetheless, at this point the establishment of such a strategy as broadly utilized requires further experimentation.

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