Insulin-activated tyrosine phosphorylation of a 15-kilodalton protein in intact 3T3-L1 adipocytes

(insulin receptor/tyrosine kinase/phosphotyrosine/phenylarsine oxide/dithiol)

MICHEL BERNIER, DON M. LAIRD, AND M. DANIEL LANE*

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

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ABSTRACT Insulin stimulates phosphorylation of a tyrosine residue(s) on a 15-kDa protein (p15), and the cytosolic phosphorylated protein (pp15) accumulates only when 3T3-L1 adipocytes are treated with phenylarsine oxide. It has been shown previously that phenylarsine oxide, an agent that complexes vicinal dithiols, interrupts signal transmission from the insulin receptor to the glucose transport system. Several lines of evidence presented here indicate the involvement of pp15 in insulin receptor-initiated signal transduction to the glucose transport system. The reciprocal effects of phenylarsine oxide on the insulin-activated accumulation of pp15 and on insulin-stimulated hexose uptake are reversed by the vicinal dithiol 2,3-dimercaptopropanol but not by the monothiol 2-mercaptoethanol. Thus, a cellular dithiol appears to function in the signal transmission pathway downstream from pp15. Like the insulin-activated autophosphorylation of the receptor's β subunit (on tyrosine), activation of phosphorylation of p15 is specific, with insulin-like growth factors 1 and 2, epidermal growth factor, and platelet-derived growth factor being inactive. Moreover, both processes exhibit identical insulin concentration dependence. The temporal kinetic relationship of insulin-activated receptor β-subunit phosphorylation, followed by the phosphorylation of p15 and then increased hexose uptake rate, is consistent with an intermediary signaling role for pp15 in insulin-stimulated glucose uptake.

By binding to its specific receptors on the plasma membrane of the adipocyte, insulin activates energy storage processes—namely, the uptake of glucose and fatty acids, glycogenesis, and lipogenesis. Considerable progress has been made in understanding the apparent initial events by which the receptor triggers these processes. The insulin receptor is an oligomeric transmembrane allosteric enzyme with an insulin binding site located extracellularly on the α subunit (1–5) and a tyrosine-specific protein kinase catalytic site on the intracellular domain of the β subunit (4–13). Studies with cell-free receptor preparations (7–14) and intact cells (15, 16) have shown that the interaction of insulin with the α subunit induces autophosphorylation of the kinase domain and thereby activates the kinase-catalyzed phosphorylation of model protein substrates and presumably cellular protein substrates.

Previous observations have established that insulin promotes the phosphorylation (17–24) or dephosphorylation (25–27) of a group of cellular proteins, some of which appear to be end-targets of a signal cascade. These proteins are not directly phosphorylated by the insulin receptor kinase, however, since phosphorylation occurs on serine or threonine and not on tyrosine residues. More recently, White et al. (28) demonstrated that insulin activates the phosphorylation of tyrosine residues on a 185-kDa membrane protein in Hep G2 cells. The function of this protein is unknown. In the present paper we report the insulin-stimulated and phenylarsine oxide-dependent accumulation of a 15-kDa phosphoprotein (pp15) that is phosphorylated on tyrosine residues and appears to function in the activation of glucose uptake in 3T3-L1 adipocytes. Our previous studies (29) showed that phenylarsine oxide reversibly blocks activation of hexose uptake by insulin without affecting hexose uptake per se. Evidence presented in this paper indicates that pp15 functions specifically with the insulin receptor.

EXPERIMENTAL PROCEDURES

Cells. 3T3-L1 adipocytes were cultured and differentiated as described previously (30). Briefly, cells were grown to confluence, and 2 days later conversion into adipocytes was induced by feeding Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, isobutylmethylxanthine, dexamethasone, and insulin for 2 days. Then cells were fed the same medium minus isobutylmethylxanthine and dexamethasone, but containing insulin, for an additional 2 days. For the next 4–6 days cells were fed DMEM supplemented only with 10% fetal bovine serum (without insulin) every other day.

32P Labeling of Cells and Preparation of Cell Extracts. The labeling procedure was essentially that described previously (16). After a 2-hr labeling period with carrier-free 32P in medium containing no other P, cell monolayers were incubated for 10 min with 35 μM phenylarsine oxide as indicated. Insulin (1 μM) was subsequently added for different periods of time up to 10 min. Labeling was terminated by washing the monolayer twice with ice-cold 10 mM Tris-HCl-buffered saline (pH 7.4) after which 0.5 ml of urea sample buffer containing 8 M urea, 1.8% 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonate (CHAPS), and 20 mM dithiothreitol was added. The cell lysate was centrifuged at 10,000 × g for 10 min and the soluble cell extract was subjected to 2-dimensional polyacrylamide gel electrophoresis.

Analysis of 32P-Labeled Proteins. Cell extracts were mixed with ampholytes (1%, pH range 3–10) for the first-dimension separation on 4% polyacrylamide tube gels containing 9 M urea, 2% CHAPS, and 2% ampholytes (pH range 3–10) (31). After nonequilibrium isoelectric focusing for 2 hr at 400 V, gels were soaked in 62.5 mM Tris-HCl, pH 6.8/1% NaDodSO4/10% (vol/vol) glycerol/0.002% bromophenol blue for 90 min. The second-dimension NaDodSO4/polyacrylamide gel electrophoresis was carried out with 12.5% gels.

Abbreviations: pp15, phosphorylated 15-kDa protein; p15, nonphosphorylated pp15; IGF-1, insulin-like growth factor 1; IGF-2, insulin-like growth factor 2; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate.

*To whom reprint requests should be addressed: Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205.
acrylamide gels according to Laemmli (32). Gel segments containing labeled proteins were cut out and $^{32}$P radioactivity was quantitated.

Isolation of $^{32}$P-Labeled Insulin Receptor $\beta$ Subunit. The procedure was essentially as described previously (16) with the following modifications. After labeling with $^{32}$P (see text and figure legends), cell monolayers were washed three times with ice-cold 10 mM Hepes/150 mM NaCl, pH 7.6, and were then extracted immediately with quench buffer [25 mM Tris, pH 7.6/192 mM glycine/25 mM EDTA/25 mM EGTA/1 mM sodium vanadate/100 mM sodium fluoride/30 mM sodium pyrophosphate/1 mM N-ethylmaleimide/proteinase inhibitor mixtures (12)] containing 0.1% NaDodSO$_4$. Culture dishes were rinsed with quench buffer containing 1.7% (wt/vol) Triton X-100, after which the rinse was combined with the extract and the mixture was homogenized. After centrifugation (45 min at 50,000 rpm in a Beckman 70.1 Ti rotor), the supernatant was gently mixed with wheat germ agglutinin-Sepharose for 12 hr at 4°C. After incubation, the gel (with bound insulin ppl5) was washed three times with 50 mM Hepes buffer, pH 7.6, containing 1.0 M NaCl and 1% Triton X-100, then twice with salt-free Hepes buffer containing 0.1% Triton X-100 prior to addition of Laemmli sample buffer. The receptor-containing solution was subjected to two-dimensional (nonreducing/reducing) NaDodSO$_4$/polyacrylamide gel electrophoresis as previously described (16). The $^{32}$P-labeled $\beta$ subunit of the receptor was located by autoradiography and its $^{32}$P content was determined.

$^{32}$PPhosphoamino Acid Analysis and Hexose Transport Assay. $^{32}$P-labeled ppl5 was located on two-dimensional gels by autoradiography and the appropriate gel segment was cut out for analysis. Phosphorylated amino acids were analyzed as previously described (16) except that after soaking for 24 hr, the gel segments were homogenized in 0.8 ml of 50 mM ammonium bicarbonate containing 50 $\mu$g of trypsin treated with tosylphenylalanyl chloromethyl ketone. Hydrolysis was carried out at room temperature for 8 hr, followed by a second addition of trypsin and further hydrolysis for 14 hr. The gel residue was removed by filtration and the filtrate was lyophilized. Partial acid hydrolysis was performed at 110°C for 2 hr under reduced pressure in sealed glass tubes containing 50 $\mu$l of 6 M HCl. Analysis was by two-dimensional thin-layer high-voltage electrophoresis. The rate of 2-deoxy-[1-14C]glucose uptake by 3T3-L1 adipocyte monolayers was determined as described from Frost and Lane (29).

Materials. 2-Deoxy-[1-14C]glucose and $^{32}$Porthophosphate were purchased from Amersham. Ampholytes were from Bio-Rad. Phenylarsine oxide and 2,3-dimercaptopropanol were from Aldrich. Insulin was a gift from Ronald Chance (Eli Lilly, Indianapolis, IN). Insulin-like growth factors 1 and 2 (IGF-1 and -2) were the gifts of Goesta Enberg (Karolinska Institute, Stockholm); epidermal growth factor (EGF) was prepared by L. Slieker in this laboratory as previously described (33). Human platelet-derived growth factor (PDGF) was obtained from Charles Scher (University of Pennsylvania, Philadelphia).

RESULTS

The strategy employed to detect insulin-stimulated tyrosine phosphorylation of proteins in intact 3T3-L1 adipocytes was based on the use of an inhibitor of insulin action. Previous studies in this laboratory (see above and ref. 29) indicated that phenylarsine oxide, an agent that forms stable ring complexes with vicinal dithiols (34), interrupts signal transmission between the insulin receptor and the glucose transport system. Since activation of hexose uptake by insulin, but not hexose uptake per se, is inhibited by phenylarsine oxide, it was anticipated that a blockade by this inhibitor might cause the accumulation of a phosphorylated intermediate(s) in the signaling cascade, proximal to the receptor and upstream from the site of inhibition. Importantly, it has been established with 3T3-L1 adipocytes that phenylarsine oxide does not significantly alter cellular ATP level (29).

Fully differentiated 3T3-L1 adipocytes, previously incubated with $^{32}$P to achieve steady-state labeling of cellular ATP (16), were briefly incubated with or without phenylarsine oxide and then insulin. The cells were quickly lysed with urea sample buffer and phosphorylated proteins in the extract were analyzed by two-dimensional gel electrophoresis (non-equilibrium isoelectric focusing/NaDodSO$_4$/polyacrylamide). The addition of insulin and phenylarsine oxide to 3T3-L1 adipocytes led to the appearance of several phosphorylated polypeptides (Fig. 1D). The most striking change was the marked accumulation of a phosphorylated 15-kDa protein (pp15). In the absence of insulin and phenylarsine oxide or with insulin added alone, no $^{32}$P-labeled ppl5 was detected (Fig. 1A and B); with phenylarsine oxide added alone, only a trace of $^{32}$Pppl5 was observed (Fig. 1C). By equilibrium isoelectrofocusing ppl5 was found to have a pI of 6.3 (result not shown). Two-dimensional phosphoamino acid analysis of the partial acid hydrolysate of $^{32}$P-labeled ppl5 indicated that phosphorylation occurs exclusively on a tyrosine residue(s) (Fig. 2). While changes in the phosphorylation states of other polypeptides also occur upon treatment with insulin, phenylarsine oxide, or both (Fig. 1), six of those examined were found to be phosphorylated on serine rather than tyrosine (R. A. Kohanski, M.B., D.M.L., and M.D.L., unpublished results).

To ascertain the temporal relationship between insulin-activated phosphorylation of the $\beta$ subunit of the insulin receptor and of ppl5 (unphosphorylated ppl5), the kinetics of the two processes were followed after the addition of insulin to phenylarsine oxide-treated cells (Fig. 3). It is evident that the inhibitor has no effect on the kinetics of phosphorylation of the $\beta$ subunit which, as previously reported (16), reaches a steady-state level of phosphorylation within 1 min after insulin addition (Fig. 3). The rate of phosphorylation of ppl5 is compatible with its involvement as an intermediate in signal transmission to the glucose transport system, since

![Fig. 1.](image-url)
Fig. 2. Phosphoamino acid analysis of 32P-labeled pp15. 3T3-L1 adipocytes, labeled with 32P, as described in Fig. 1 and Experimental Procedures, were incubated with 35 μM phenylarsine oxide followed by 1 μM insulin. The phosphorylation reaction was terminated by extraction of the cells with urea sample buffer (as in Fig. 1). 32P-labeled proteins were resolved by two-dimensional polyacrylamide gel electrophoresis (see Fig. 1). The gel segment corresponding to [32P]pp15 was cut out and subjected to partial acid hydrolysis with 6 M HCl under reduced pressure for 2 hr. [32P]Phosphoamino acids were then analyzed by two-dimensional thin-layer high-voltage electrophoresis in the presence of unlabeled phosphoamino acid markers. PY, phosphotyrosine; PT, phosphothreonine; and PS, phosphoserine. The 32P-labeled phosphoamino acids were detected by autoradiography at ~70°C with intensifying screens.

Insulin-stimulated phosphorylation of p15 precedes activation of hexose transport (Fig. 3).

Fig. 3. Comparison of the kinetics of insulin-stimulated phosphorylation of insulin receptor β subunit, phosphorylation of p15, and uptake of 2-deoxy[1-14C]glucose in 3T3-L1 adipocytes. To follow the kinetics of phosphorylation of receptor β subunit and of p15, cells were first labeled with medium containing 32P, as described in the legend of Fig. 1 and Experimental Procedures. Cells were then treated (○, △) with insulin followed by 35 μM phenylarsine oxide for 10 min. The incubation was 32P-labeled β subunit with insulin-stimulated steady state was 542 cpm per 10⁶ cells, and the maximal level of 32Pp15 was 601 cpm per 10⁶ cells. 2-Deoxy-[1-14C]glucose uptake was expressed as insulin-stimulated uptake corrected for basal uptake at each time point. Maximal uptake at 10 min was 11.5 nmol per 10⁶ cells. β-Subunit (○). 32P-labeled insulin receptor β subunit; DG, 2-deoxy[1-14C]glucose; AsO, phenylarsine oxide.

The insulin concentration dependency for activation of p15 phosphorylation is identical to that for activation of insulin receptor β subunit phosphorylation, supporting the view that p15 is a cellular substrate of the tyrosine-specific receptor kinase (Fig. 4). Phosphorylated insulin has no effect on the insulin concentration dependence (Fig. 4) or the kinetics of β subunit phosphorylation (Fig. 3), indicating that the inhibitor acts downstream from the insulin receptor. Concentrations above 5 nM insulin were required to increase both pp15 and β subunit phosphorylation over the basal state, with a half-maximally effective concentration at 25 nM.† In other studies (R. A. Kohanski, S. C. Frost, and M. D. L., unpublished results) it was observed that insulin receptor isolated from phenylarsine oxide-treated and untreated 3T3-L1 adipocytes exhibited identical insulin binding, autophosphorylation, and model substrate phosphorylation activities.

Previous work in our laboratory (29) showed that inhibition of insulin-activated hexose uptake by phenylarsine oxide can be prevented or reversed by the vicinal dithiol 2,3-dimercaptopropanol but not by the monothiol 2-mercaptoethanol. Thus, it would be expected that the insulin-activated accumulation of pp15 in phenylarsine oxide-treated cells would be reversed by 2,3-dimercaptopropanol but not by the monothiol. The results in Table 1 show that complete recovery of insulin-activated hexose uptake by the vicinal dithiol competitor of phenylarsine oxide was accompanied by a decrease in the accumulation of pp15 caused by insulin and phenylarsine oxide. Moreover, 2-mercaptoethanol was capable of reversing neither the inhibition of insulin-activated

†The somewhat higher than expected half-maximally effective concentration for insulin in activating phosphorylation of the β subunit of its receptor and of p15 appears to result from a decrease in insulin concentration due to insulin degradation by 3T3-L1 adipocytes during the 10-min preincubation at 37°C. 3T3-L1 adipocytes are known to have an active insulin-degrading system (T. S. Olson and M. D. L., unpublished results). Degradation of insulin during the 10-min preincubation and assay as an explanation consistent with our observation of a rightward shift of the insulin activation isotherm with increasing time of incubation (unpublished results).
Table 1. Reversal of the effects of phenylarsine oxide on insulin-activated 2-deoxyglucose uptake and p15 phosphorylation in 3T3-L1 adipocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2-Deoxyglucose uptake, nmol/min per 10^6 cells</th>
<th>p15 phosphorylation, cpm per 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.19</td>
<td>0</td>
</tr>
<tr>
<td>Insulin</td>
<td>1.48</td>
<td>0</td>
</tr>
<tr>
<td>φAsO</td>
<td>0.18</td>
<td>55</td>
</tr>
<tr>
<td>φAsO, then insulin</td>
<td>0.17</td>
<td>533</td>
</tr>
<tr>
<td>φAsO, then 2,3-dimercaptopropanol, then insulin</td>
<td>1.33</td>
<td>75</td>
</tr>
<tr>
<td>φAsO, then 2-mercaptopropanol, then insulin</td>
<td>0.13</td>
<td>501</td>
</tr>
</tbody>
</table>

Cells were treated with 35 μM phenylarsine oxide (φAsO) for 5 min where indicated; then 75 μM 2,3-dimercaptopropanol or 150 μM 2-mercaptopropanol was added or not for an additional 5 min. The cells were then incubated in the presence or absence of 1 μM insulin for 10 min, after which 2-deoxy[1-14C]glucose uptake or the incorporation of [32P] into ppl5 was determined.

dehydroxyglucose uptake by phenylarsine oxide nor the dependence of the accumulation of ppl5 upon phenylarsine oxide. These results strongly suggest the involvement of a cellular dithiol in signal transmission by the insulin receptor downstream from ppl5.

The specificity of insulin-induced phosphorylation of ppl5 was examined by comparing the effects of IGF-1, IGF-2, EGF, and PDGF with the effect of insulin. Like the insulin receptor, the receptors for IGF-1 (35), EGF (36), and PDGF (37) are ligand-activated tyrosine-specific protein kinases. As shown in Table 2, these polypeptides differ greatly in their ability to activate hexose uptake in 3T3-L1 adipocytes, with insulin and IGF-1 being equally active and EGF and PDGF exhibiting little, if any, activity. With respect to stimulating the phosphorylation of ppl5, however, only insulin was active (Table 2). These results indicate that activation by insulin of the phosphorylation of ppl5 is specific and, furthermore, that IGF-1 and IGF-2, which markedly activate hexose uptake, enter the signal transmission pathway at a point beyond ppl5.

**DISCUSSION**

Compelling evidence from studies with the insulin receptor in purified form (4, 5, 7–14), in intact cells (15, 16), and altered by site-directed mutation (38, 39) have implicated the tyrosine kinase catalytic center of the receptor as the site from which the intracellular "insulin signal" arises. The question, still unanswered, however, is how does the tyrosine kinase transmit its signal to the ultimate targets of insulin action.

Table 2. Specificity of polypeptide-activated 2-deoxyglucose uptake and p15 phosphorylation in 3T3-L1 adipocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2-Deoxyglucose uptake, nmol/min per 10^6 cells</th>
<th>p15 phosphorylation, cpm per 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.22</td>
<td>0</td>
</tr>
<tr>
<td>Insulin</td>
<td>1.26</td>
<td>1203</td>
</tr>
<tr>
<td>IGF-1</td>
<td>1.14</td>
<td>0</td>
</tr>
<tr>
<td>IGF-2</td>
<td>0.82</td>
<td>0</td>
</tr>
<tr>
<td>EGF</td>
<td>0.41</td>
<td>0</td>
</tr>
<tr>
<td>PDGF</td>
<td>0.44</td>
<td>157</td>
</tr>
</tbody>
</table>

For hexose uptake studies cells were treated with the appropriate peptide at 100 nM for 10 min prior to measurement of 2-deoxy[1-14C]glucose uptake. For phosphorylation studies, cells were incubated for 2 hr in [32P]-containing medium then for 10 min with 35 μM phenylarsine oxide. Finally, hexose uptake and ppl5 phosphorylation were determined.

such as the glucose transport system? In the present investigation we have employed phenylarsine oxide, which inhibits insulin-activated hexose uptake by producing an apparent postreceptor blockade of signal transmission to the hexose transport system (29). In 3T3-L1 adipocytes labeled with [32P]Pi, this blockage appears to cause the insulin-stimulated accumulation of [32P]pp15, a 15-kDa polypeptide that is phosphorylated exclusively on tyrosine (Figs. 1 and 2).

ppl5 has the expected characteristics of a cytosolic intermediate in the insulin signal transmission pathway. First, activation of the accumulation of [32P]pp15 in 3T3-L1 adipocytes, labeled with [32P]Pi, is specific. IGF-1, IGF-2, EGF, and PDGF do not induce [32P]pp15 accumulation (Table 2) despite the presence on 3T3-L1 cells of receptors for all four of these growth factors (refs. 40 and 41 and unpublished results). Since IGF-1 and IGF-2, like insulin, activate hexose uptake in 3T3-L1 adipocytes (16), the mediators generated by these factors must enter the signaling pathway downstream from ppl5. Also in support of the participation of both the insulin receptor and ppl5 in the same signaling pathway is the finding that phosphorylation of both tyrosine residues—i.e., of the receptor's β subunit and of ppl5, exhibit identical insulin concentration dependencies (Fig. 4). The fact that phenylarsine oxide does not affect insulin-dependent β-subunit phosphorylation (Figs. 3 and 4), but is required for the accumulation of [32P]pp15, suggests that ppl5 lies proximal to the receptor, and upstream from the site of phenylarsine oxide action as depicted in Fig. 5. The kinetics of phosphorylation of ppl5 are consistent with a role in mediating insulin-activated hexose uptake (Fig. 3). Insulin rapidly activates the apparent autophosphorylation of the receptor's β subunit (on tyrosine; see ref. 16 and Fig. 3) which is followed in succession by the initial appearance of ppl5 and then an increased rate of hexose uptake.

The role of tyrosine phosphorylation in signal transmission from the insulin receptor is supported by studies with vanadate. Vanadate, a potent inhibitor of certain phosphotyrosine phosphatases, increases phosphorylation of the insulin receptor β subunit on tyrosine in rat adipocytes (42). Vanadate, which mimics the action of insulin in activating hexose uptake by isolated rat (43) and 3T3-L1 adipocytes (unpublished results), also causes insulin receptor β-subunit phosphorylation in both cell types. Furthermore, phenylarsine oxide blocks vanadate-stimulated hexose transport by 3T3-L1 adipocytes and, like the combination insulin and phenylarsine oxide, vanadate and phenylarsine oxide together cause the accumulation of ppl5 (results not shown).

Two findings link insulin-stimulated phosphorylation of ppl5 to the activation of hexose uptake. First, phenylarsine oxide affects both processes reciprocally (Fig. 3 and Table 1) and these effects are specifically reversed by the vicinal dithiol reagent 2,3-dimercaptopropanol but not by a monothiol, 2-mercaptopropanol (Table 1). This result indicates the involvement of an essential dithiol intermediate in the signal transmission pathway that interacts with, or downstream from, ppl5. Second, kinetic evidence (results not shown) suggests that the formation of ppl5 in response to

![FIG. 5. Proposed signal transmission pathway from cell-surface insulin receptors to the intracellular glucose transport system of 3T3-L1 adipocytes. φAsO, phenylarsine oxide; tyr-P, phosphotyrosine.](image-url)
insulin is the rate-limiting step in signal transmission to the glucose transport system (see Fig. 5) and is responsible for the long (5 min) lag in achieving maximal hexose uptake rate after insulin addition (Fig. 3). Thus, by allowing cellular pp15 to accumulate for 5 min in the presence of insulin and phenylarsine oxide, and then quickly releasing the phenylarsine oxide-induced blockade with 2,3-dimercaptopropanol (in effect, bypassing the rate-limiting step) eliminates the lag in achieving maximal hexose uptake rate. This result, which is predicted by the proposed sequence of events shown in Fig. 5, further suggests that dephosphorylation of pp15 is tightly coupled to signal transmission to the glucose transport system.

Preliminary studies (results not shown) indicate that pp15 is localized within the cytosolic fraction of 3T3-L1 adipocytes. pp15 would, therefore, be expected to be mobile and capable of translocation within the cytoplasm to the intracellular site of the glucose transporter in cells not stimulated with insulin (44). The cytosolic localization of pp15 raises the possibility that this agent may act pleiotropically to mediate other insulin-activated processes. Further investigations will be necessary to identify the intracellular localization of pp15 (or its precursor form) prior to stimulation of cells with insulin and to determine whether pp15 mediates actions of insulin other than the activation of glucose uptake.

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