Osmotic shock can cause insulin resistance in 3T3-L1 adipocytes by inhibiting insulin activation of glucose transport, p70S6 kinase, glycogen synthesis, and lipogenesis. By further investigating the relationship between insulin and hypertonic stress, we have discovered that osmotic shock enhanced by 10-fold the insulin-stimulated tyrosine phosphorylation of a 68-kDa protein. Phosphorylation by insulin was maximal after 1 min and was saturated with 50–100 nM insulin. The effect of sorbitol was completely reversible by 2.5 min. pp68 was a peripheral protein that was localized to the detergent insoluble fraction of the low density microsomes but was not associated with the cytoskeleton. Stimulation of the p42/44 and the p38 MAP kinase pathways by osmotic shock had no effect on pp68 phosphorylation. Treatment of adipocytes with the phosphotyrosine phosphatase inhibitor phenylarsine oxide also enhanced insulin-activated tyrosine phosphorylation of pp68 suggesting that osmotic shock may increase pp68 phosphorylation by inhibiting a phosphotyrosine phosphatase. Dissociation of pp68 from the low density microsomes with RNase A indicated that pp68 binds to RNA. Failure to immunoprecipitate pp68 using antibodies directed against known 60–70-kDa tyrosine-phosphorylated proteins suggest that pp68 may be a novel cellular target that lies downstream of the insulin receptor.

The metabolic and mitogenic effects of insulin are initiated by the binding of the hormone to specific cell surface receptors that results in the autophosphorylation of critical tyrosine residues, which then activates an intrinsic tyrosine kinase (1). Insulin receptor substrate-1 (IRS-1) and SHC, the two best characterized substrates of the insulin receptor tyrosine kinase, serve as docking sites for various SH2 domain-containing proteins to generate multiple independent cellular signals that ultimately lead to various downstream biological responses (2, 3). Tyrosine-phosphorylated IRS-1 is known to bind to two regulatory subunits of PI 3-kinase, p85 and p55, Grb2, the tyrosine phosphatase Syp, Fyn, Nck, and Crk (4–6). PI 3-kinase has been implicated in a wide variety of insulin effects that include stimulation of glucose transport through the translocation of Glut 4 to the cell surface, activation of glycogen synthesis, inhibition of lipolysis, and induction of membrane ruffling (7). SHC can also bind to Grb2 (8), and through its interaction with mSOS it can cause Ras activation and subsequent increased mitogenesis (9).

Osmotic shock has been shown to increase glucose uptake in 3T3-L1 adipocytes by stimulating the translocation of Glut 4 to the cell surface (10). The signal transduction pathway is distinct from insulin in that it fails to activate PI 3-kinase or AKT. Interestingly, treatment of cells with the tyrosine kinase inhibitor genistein or microinjection of phosphotyrosine antibodies blocks both insulin and sorbitol-induced Glut 4 translocation, suggesting a tyrosine kinase signaling cascade is important for both pathways. The stimuli are not additive. Furthermore, a subsequent study showed that pretreatment with 600 mM sorbitol inhibits insulin from further increasing glucose transport (11). Although early insulin signaling such as insulin receptor autophosphorylation, tyrosine phosphorylation of IRS-1, and activation of PI 3-kinase are normal, osmotic stress inhibits insulin activation of AKT by stimulating a phosphatase that maintains Thr<sup>308</sup> and Ser<sup>473</sup> of AKT in the dephosphorylated state. Activation of a similar or identical phosphatase may be responsible for the inhibition of both basal and insulin-stimulated p70S6 kinase activities by hypertonic shock (12). Pretreatment with the phosphatase inhibitors okadaic acid or calyculin A prevents the inhibition of insulin activation of AKT (11) and the deactivation of p70S6 kinase (12). It is not known whether sorbitol activates the phosphatase through an intrinsic activation mechanism or by altering the subcellular localization of the enzyme or substrate.

In the present study we further investigate the effects of osmotic shock on insulin action. Pretreatment of 3T3-L1 adipocytes with 600 mM sorbitol enhances by 10-fold the insulin-stimulated tyrosine phosphorylation of a 68-kDa protein. Additional characterization indicates that this protein is a peripheral protein that resides in the detergent-insoluble fraction of low density microsomes (LDM) by binding to RNA.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—*Cytochalasin D, nocodazole, poly(A)-Sepharose 4B, and poly(U)-agarose were purchased from Sigma. Latrunculin B was from BioMol. PD 98059 and SB 203580 were from Calbiochem. Phenylarsine oxide was purchased from Aldrich.

*Subcellular Fractionation of 3T3-L1 Adipocytes—*3T3-L1 fibroblasts were grown to confluence and 48 h later subjected to differentiation as described previously (13). 3T3-L1 adipocytes were used 10–14 days after differentiation. Cells were washed three times with phosphate-buffered saline and incubated for at least 2 h to overnight in serum-free Dulbecco’s modified Eagle’s medium (DMEM). Adipocytes were then incubated with DMEM alone or DMEM supplemented with insulin or...
sorbitol. After the treatment, cells were washed three times with ice-cold phosphate-buffered saline, scraped in 2 ml/10-cm dish of ice-cold HES (255 mM sucrose, 20 mM HEPES, pH 7.4, and 1 mM EDTA) containing 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and protease inhibitors and then homogenized by passing the cells 10 times through a Yamato LSC homogenizer at a speed of 1200 rotations/min at 4 °C. Subcellular fractionation was carried out by differential centrifugation as described previously (14). The following protease inhibitors were used: 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml benzamidine, 5 μg/ml trypsin inhibitor, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride.

Western Blot Analysis—50 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose. Phosphotyrosine-phosphorylated proteins were detected using the monoclonal PY-20 antibody (Transduction Laboratories). 125I-Labeled goat anti-mouse IgG (0.25 μCi/ml, ICN, Irvine, CA) was used as the secondary antibody. Radioactive bands were quantitated by a PhosphorImager SI Analyzer (Molecular Dynamics). The phospho-specific antibodies, phospho-p42/44 MAP kinase (Thr202/Tyr204) E10 monoclonal antibody, phospho-p38 MAP kinase (Thr180/Tyr182) antibody, and the phospho-SAPK/JNK (Thr183/Tyr185) antibody, were from New England Biolabs. Enhanced chemiluminescence was used for the detection.

Immunoprecipitation of Possible Candidate and Interacting Proteins—0.5–1.0 mg of LDM were solubilized with 0.5–1 ml of Buffer A (50 mM HEPES, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM sodium vanadate, 100 mM sodium pyrophosphate, 1% Triton X-100, and protease inhibitors). After centrifugation for 10 min at 4 °C in a microfuge, the supernatant was incubated overnight with 4 μg of primary antibody. 50 μl of protein A-Sepharose or 50 μl of goat anti-mouse IgG affinity gel (Cappel, ICN) were added for 2 h at 4 °C to the reactions that contained polyclonal or monoclonal primary antibodies, respectively. After washing pellets four times with ice-cold Buffer A containing detergent and twice with Buffer A without detergent, proteins were eluted with SDS sample buffer. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using PY20 antiphosphotyrosine antibodies as described above. Antibodies directed against SHC, RasGAP, Paxillin, Sam68, Grb2, and Nck were purchased from Upstate Biotechnology. Polyclonal antibodies directed against Glut 4 transporter and IRS-1 were generated by immunizing rabbits with peptides corresponding to the final 16 amino acids of each protein. Determination of whether the immunoprecipitations were successful was carried out by Western blot analyses of aliquots of the supernatants before and after immunoprecipitation.

RESULTS

Insulin-induced Tyrosine Phosphorylation of a 68-kDa Protein Is Increased with Osmotic Shock—Osmotic shock has been shown to cause insulin-resistance in 3T3-L1 adipocytes by inhibiting insulin activation of glucose transport, p70S6 kinase, glycogen synthesis, and lipogenesis (11). To further investigate the effects of osmotic shock on insulin action, 3T3-L1 adipocytes were treated for 30 min with either DMEM alone (B) or DMEM supplemented with 100 nM insulin (I), 600 mM sorbitol (S), or the combination of sorbitol and insulin (S + I). Adipocytes were then washed three times with ice-cold phosphate-buffered saline, homogenized, and fractionated by differential centrifugation (see “Experimental Procedures”). PM, cytosol, high density microsomes (HDM), and LDM fractions were separated by SDS-polyacrylamide gel electrophoresis (50 μg of protein), immuno-blotted, incubated with antiphosphorysin primary and 125I-labeled secondary antibodies, and then visualized by autoradiography.

Fig. 1. Subcellular localization of insulin and osmotic shock-stimulated tyrosine-phosphorylated proteins in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 15 min in the presence or absence of 100 mM sorbitol (S). Cells were then treated for 30 min with either DMEM alone (B) or DMEM supplemented with 100 nM insulin (I), 600 mM sorbitol (S), or the combination of sorbitol and insulin (S + I). Adipocytes were then washed three times with ice-cold phosphate-buffered saline, homogenized, and fractionated by differential centrifugation (see “Experimental Procedures”). PM, cytosol, high density microsomes (HDM), and LDM fractions were separated by SDS-polyacrylamide gel electrophoresis (50 μg of protein), immuno-blotted, incubated with antiphosphotyrosine primary and 125I-labeled secondary antibodies, and then visualized by autoradiography.

The LDM fraction is enriched in endosomes, the Golgi apparatus, as well as insulin-responsive Glut 4-containing vesicles (15). In addition, tyrosine-phosphorylated IRS-1 has been shown to bind and activate PI 3-kinase in the LDM (16). Osmotic shock did not prevent tyrosine-phosphorylated IRS-1 from associating with the LDM (Fig. 1), nor did it affect IRS-1 from binding and activating PI 3-kinase (data not shown (11)). In addition, sorbitol, but not insulin, increased the tyrosine phosphorylation of at least one protein in the 115–120-kDa protein range. The most dramatic effect, however, was the appearance of a 68-kDa protein that was tyrosine-phosphorylated in response to insulin alone, but not sorbitol, and whose phosphorylation was increased 6–10-fold when cells were treated with both insulin and sorbitol. Closer examination of the blot revealed that two additional proteins (55 and 75 kDa) had greater tyrosine phosphorylation content when cells were treated with both insulin and sorbitol compared with insulin alone, although the increases were small relative to that of pp68. Because the enhancement of pp68 phosphorylation by sorbitol was apparent from phosphotyrosine blots of whole cell lysates (data not shown), insulin must stimulate the phosphorylation of this protein rather than simply altering the subcellular localization of a sorbitol-induced tyrosine-phosphorylated 68-kDa protein from the cytosol to the LDM. The high density microsomes are enriched in endoplasmic reticulum but are also contaminated with organelles in the PM and LDM fractions (14, 15). The appearance of low levels of both tyrosine-phosphorylated insulin receptor and pp68 in this fraction (Fig. 1) is consistent with the cross-contamination. The mitochondrial/nuclear fraction contained few tyrosine-phos-
phorylated proteins (data not shown). Wortmannin had no significant effect on the tyrosine phosphorylation of any protein in any compartment with the exception that it reduced the sorbitol-induced phosphorylation of 115–120-kDa proteins in the cytosol and LDM.

Characterization of pp68 Phosphorylation—Because pp68 was tyrosine-phosphorylated in response to insulin alone and its phosphorylation was dramatically elevated with osmotic shock and it was preferentially found in the LDM fraction, which also contains important insulin signaling molecules (17), we further characterized the phosphorylation of this protein. 100 nM insulin was added for varying periods of time to 3T3-L1 adipocytes that were first preincubated for 15 min with 600 mM sorbitol. Antiphosphotyrosine Western blots were then conducted on LDM isolated by subcellular fractionation. Fig. 2 shows that insulin-induced tyrosine phosphorylation of pp68 was rapid and complete after 1 min. Although this time frame is consistent with the insulin receptor phosphorylating p68 directly (18), we cannot rule out the possibility that insulin activates another tyrosine kinase, which, in turn, phosphorylates p68. Next we measured the insulin concentration dependence to determine whether the insulin response was because of the insulin receptor or the insulin-like growth factor-1 receptor (Fig. 2). Varying concentrations of insulin were added for 5 min to cells that were first pretreated for 15 min with 600 mM sorbitol. Tyrosine phosphorylation of pp68 was apparent with 5 nM insulin and saturated between 50 and 100 nM insulin suggesting that the insulin and not the insulin-like growth factor-1 receptor was responsible for stimulating the tyrosine phosphorylation (19).

The effect of sorbitol on pp68 tyrosine phosphorylation was completely reversible (Fig. 3). Adipocytes were incubated in 600 mM sorbitol for 15 min followed by the addition of 100 nM insulin for 5 min. Cells were then washed and incubated for varying periods of time with DMEM containing insulin in the absence of sorbitol. The amount of tyrosine-phosphorylated pp68 in the isolated LDM was quantitated from phosphotyrosine Western blots. Because the quantity of pp68 may vary with time without the removal of sorbitol (see Fig. 2), the amount of pp68 that remained after the sorbitol washout was normalized to the amount of pp68 that remained for the same period of time in the presence of sorbitol. Each data point represents the mean ± S.E. of three independent experiments.

*Fig. 2.* The time course and concentration curve of insulin-stimulated tyrosine phosphorylation of pp68. 3T3-L1 adipocytes were preincubated for 15 min with 600 mM sorbitol in both experiments. 100 nM insulin was added for varying periods of time to measure the rate of insulin-stimulated pp68 phosphorylation. Different concentrations of insulin were added for 5 min in determining the hormone concentration dependence. After treatment, phosphotyrosine Western blot analyses were conducted on the LDM fraction prepared by differential centrifugation (see “Experimental Procedures”). Autoradiograms were quantitated by phosphoimaging. Quantitative data represent the mean ± S.E. of three independent experiments.

*Fig. 3.* The effect of osmotic shock on insulin-stimulated pp68 tyrosine phosphorylation was rapidly reversible. 100 nM insulin was added for 5 min to adipocytes that were pretreated with 600 mM sorbitol for 15 min. Cells were then washed and incubated for varying periods of time in medium containing 100 nM insulin in the absence or presence of sorbitol. The amount of tyrosine-phosphorylated pp68 in the isolated LDM was quantitated from phosphotyrosine Western blots. Because the quantity of pp68 may vary with time without the removal of sorbitol (see Fig. 2), the amount of pp68 that remained after the sorbitol washout was normalized to the amount of pp68 that remained for the same period of time in the presence of sorbitol. Each data point represents the mean ± S.E. of three independent experiments.

with Triton X-100 (17). Because of this observation in conjunction with the identification of filamentous actin in the IRS-1-containing LDM fraction, it was proposed that phosphorylated IRS-1 may be associated with the actin cytoskeleton (17). To determine the extraction properties of pp68, LDM prepared from sorbitol- and insulin-treated adipocytes was incubated with either 600 mM NaCl or 1% Triton X-100 for 30 min. The soluble and insoluble fractions were isolated after centrifugation for 1 h at 200,000 × g. Phosphotyrosine blots on the different fractions revealed that similarly to tyrosine-phosphorylated IRS-1, pp68 can be extracted with high salt but not with nonionic detergent (Fig. 4A). Glut 4, on the other hand, was removed from the LDM with 1% Triton X-100 but not high salt. Treatment of sorbitol- and insulin-treated cells with either the actin-depolymerizing agents, cytochalasin D or latrunculin B, or with the tubulin depolymerizing agent, nocodazole, failed to affect the levels of tyrosine-phosphorylated pp68 associated with Triton X-100 (17).
CONTROL in buffer containing 600 mM NaCl or 1% Triton X-100 for 30 min at 4 °C. For 15 min and then stimulated with insulin for 5 min, were incubated in buffer containing 600 mM NaCl or 1% Triton X-100 for 30 min at 4 °C. Soluble (S) and insoluble (P) material were isolated after centrifugation for 1 h at 200,000 x g. The distribution of pp68 and Glut 4 in the various fractions were determined by Western blots using phosphotyrosine and Glut 4 antibodies, respectively. B, 3T3-L1 adipocytes were incubated in DMEM alone or with DMEM containing insulin, sorbitol, or the combination of sorbitol and insulin (S + I). Western blot analyses were carried out on 50 μg of whole cell lysates using phospho-specific antibodies directed against p42/44 MAP kinase, p38 MAP kinase, or SAPK/JNK. A, 3T3-L1 adipocytes were incubated in DMEM alone or with DMEM containing insulin, sorbitol, or the combination of sorbitol and insulin (S + I). Western blot analyses were carried out on LDM isolated from the various treated cells.

with the LDM (Fig. 4B). Although these compounds did not affect pp68 phosphorylation, confocal microscopy revealed that they did dramatically alter filamentous structures (data not shown).

The p42/44 and the p38 MAP Kinase Pathways Are Not Involved in pp68 Phosphorylation—Osmotic stress is known to activate the p38 MAP kinase (20) and SAPK/JNK (21) pathways, and insulin activates the p42/44 MAP kinases (22). To verify the effects of insulin and sorbitol on these pathways, Western blot analyses were carried out on whole cell lysates from 3T3-L1 adipocytes that were incubated for 30 min with DMEM alone or with DMEM containing insulin, sorbitol, or the combination of insulin and sorbitol using phospho-specific antibodies directed against the activated forms of p42/44 MAP kinase, p38 MAP kinase, or SAPK/JNK. Fig. 5A shows that p42/44 MAP kinases were activated by both insulin and sorbitol and that these effects were additive in cells incubated with both insulin and sorbitol for 30 min. Wortmannin inhibited insulin but not sorbitol-induced activation. Inhibition of insulin activation of p42/44 MAP kinase by wortmannin has been previously reported in both 3T3-L1 adipocytes (23) and in skeletal muscle (24). Sorbitol dramatically increased the phosphorylation of p38 MAP kinase and SAPK/JNK. In agreement with Chen et al., (10), we saw very little if any effect of insulin on modulating the phosphorylation of p38 MAP kinase. This observation is in contrast to another recent report using 3T3-L1 adipocytes (25). Wortmannin had no effect on the stress-induced phosphorylation of p38 or SAPK/JNK. To address whether the enhancement of insulin-induced tyrosine phosphorylation of pp68 by sorbitol was mediated through the activation of the p42/44 or the p38 MAP kinase pathways, we examined whether MAP kinase inhibitors could affect pp68 accumulation. Adipocytes were preincubated with either DMEM alone, the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD 98059 (100 μM for 1 h), or the p38 MAP kinase inhibitor SB 203580 (20 μM for 30 min). 600 μM sorbitol was then added for 15 min followed by 100 nM insulin for 5 min. Phosphotyrosine blots were carried out on LDM isolated from the various treated cells.

FIG. 4. pp68 is a peripheral membrane protein that associates with the Triton X-100 insoluble fraction of the LDM. A, 50 μg of LDM, prepared from adipocytes that were treated with 600 mM sorbitol for 30 min and then stimulated with insulin for 5 min, were incubated in buffer containing 600 mM NaCl or 1% Triton X-100 for 30 min at 4 °C. Soluble (S) and insoluble (P) material were isolated after centrifugation for 1 h at 200,000 x g. The distribution of pp68 and Glut 4 in the various fractions were determined by Western blots using phosphotyrosine and Glut 4 antibodies, respectively. B, 3T3-L1 adipocytes were incubated in DMEM alone or with DMEM containing insulin, sorbitol, or the combination of sorbitol and insulin (S + I). Western blot analyses were carried out on 50 μg of whole cell lysates using phospho-specific antibodies directed against p42/44 MAP kinase, p38 MAP kinase, or SAPK/JNK. A, 3T3-L1 adipocytes were incubated in DMEM alone or with DMEM containing insulin, sorbitol, or the combination of sorbitol and insulin (S + I). Western blot analyses were carried out on LDM isolated from the various treated cells.

FIG. 5. The p42/44 and the p38 MAP kinase pathways are not involved in the enhancement by osmotic shock on the insulin-stimulated tyrosine phosphorylation of pp68. A, 3T3-L1 adipocytes were incubated for 15 min in the presence or absence of 100 nM wortmannin (W). Cells were then treated for 30 min with either DMEM alone (B) or DMEM supplemented with 100 nM insulin (I), 600 mM sorbitol (S), or the combination of sorbitol and insulin (S + I). Western blot analyses were carried out on 50 μg of whole cell lysates using phospho-specific antibodies directed against p42/44 MAP kinase, p38 MAP kinase, or SAPK/JNK. B, adipocytes were incubated in DMEM alone (CONTROL), the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD 98059 (100 μM for 1 h), or the p38 MAP kinase inhibitor SB 203580 (20 μM for 30 min). 600 μM sorbitol was then added for 15 min followed by 100 nM insulin for 5 min. Phosphotyrosine blots were carried out on LDM isolated from the various treated cells.
adipocytes using commercially available antibodies followed by phosphotyrosine Western blot analyses on the precipitated proteins, we eliminated the possibility of pp68 being SHC, Src, Fyn, IRS-3, Syp, PTP1C, p62Dok, Paxillin, or Sam68 (Table I). Using this same procedure, we determined that pp68 does not interact with IRS-1, PI 3-kinase, Glut 4-containing vesicles, RasGap, Fak, Chl, Grb2, Pyk2, Nck, Src, or Cas (Table I). In all cases we verified that the commercial antibody did in fact immunoprecipitate the appropriate protein (data not shown).

**pp68 Is an RNA-binding Protein**—It has been reported that insulin induces the tyrosine phosphorylation of a 70-kDa protein in A14 fibroblasts (26) that were pretreated with the phosphotyrosine phosphatase inhibitor phenylarsine oxide (PAO). This protein is not Sam68 but it does bind poly(U)-Sepharose. To determine whether pp68 binds to RNA, LDM containing pp68 were treated with RNase A for 2 h on ice prior to centrifugation at 200,000 × g. The phosphotyrosine blot (Fig. 6A) revealed that RNase treatment released pp68 but not Glut 4 from the LDM. To further confirm that pp68 is an RNA-binding protein, we tested the ability of pp68 to bind either poly(A)-Sepharose 4B, poly(U)-agarose, or agarose alone. pp68 was first released from the LDM with 600 mM NaCl. After removing the insoluble material by centrifugation, the salt concentration was diluted to 150 mM NaCl. Samples were then incubated for 2 h at 4 °C with 50 μl of agarose, 50 μl of poly(A)-Sepharose 4B, or 50 μl of poly(U)-agarose. After washing the pellets four times, phosphotyrosine Western blot analyses were carried out on the bound material. The total was a sample that was treated with high salt, centrifuged, diluted, but not incubated with the resin.

**TABLE I**

| Candidate proteins | Possible interacting proteins |
|--------------------|------------------------------|
| SHC, SRC, FYN, IRS-3, SYP, PTP1C, p62Dok, Paxillin, or Sam68 | IRS-1, PI 3-KINASE, GLUT 4, RasGap, Fak, Cbl, Grb2, Pyk2, Nck, Src, or Cas |

Because PAO is a potent phosphotyrosine phosphatase inhibitor and the same pattern of insulin-stimulated tyrosine phosphorylation was observed in adipocytes using commercially available antibodies followed by phosphotyrosine Western blot analyses on the precipitated proteins, we eliminated the possibility of pp68 being SHC, Src, Fyn, IRS-3, Syp, PTP1C, p62Dok, Paxillin, or Sam68 (Table I). Using this same procedure, we determined that pp68 does not interact with IRS-1, PI 3-kinase, Glut 4-containing vesicles, RasGap, Fak, Chl, Grb2, Pyk2, Nck, Src, or Cas (Table I). In all cases we verified that the commercial antibody did in fact immunoprecipitate the appropriate protein (data not shown).

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INSULIN STIMULATES PROTEIN PHOSPHORYLATION

Insulin mediates its biological effects by activating an intrinsic tyrosine kinase upon binding of the hormone to specific cell surface receptors (1). Tyrosine phosphorylation of cellular substrates such as the IRS proteins (2), Gab-1 (27), p62DOK (28), and SHC (3) creates docking sites for multiple downstream signaling molecules. In this study we identified a downstream molecule in the insulin-signaling pathway that resides in the Triton X-100 insoluble fraction of the LDM of 3T3-L1 adipocytes and binds to RNA. The LDM fraction is enriched in endosomes, Golgi, and insulin responsive Glut 4-containing vesicles (15). In addition to pp68, other insulin signaling molecules, such as IRS-1, PI 3-kinase, and SHC, have been localized to the nonionic detergent-resistant fraction of the LDM (17). Hill et al. (29) identified 12 insulin-stimulated phosphoproteins in the LDM of 3T3-L1 adipocytes by combining metabolic labeling, subcellular fractionation, and two-dimensional gel electrophoresis. The majority of these proteins are also present in the detergent insoluble fraction, including a protein with a molecular mass of 66 kDa. It was not reported, however, whether this protein is phosphorylated on tyrosine. Because pp68 did not associate with IRS-1, PI 3-kinase, or SHC, this suggests that pp68 resides in a different compartment in the LDM. Triton X-100 insolubility is a known characteristic of the actin cytoskeleton, and long filamentous structures that resemble cytoskeletal elements have been observed by electron microscopy in the detergent insoluble fraction of the LDM (17). However, the observation that treatment of 3T3-L1 adipocytes with actin or tubulin-depolymerizing agents failed to affect the accumulation of pp68 in the LDM (Fig. 4B) suggests that pp68 is not associated with the membrane skeleton.

pp68 is not the first reported RNA-binding protein that is phosphorylated on tyrosine. Sam68 is a 68-kDa RNA-binding protein that is a substrate of the SRC tyrosine kinase during mitosis (30, 31). Upon phosphorylation, this protein binds to several SH3 and SH2 domain-containing signaling molecules such as Grb2 (32), phospholipase C_γ_1 (32), Nck (33), and Cbl (34). The RNA-binding activity is inhibited upon tyrosine phosphorylation (35). Sam68 and pp68 appear to be different proteins based upon the failure to immunoprecipitate pp68 with Sam68-specific antibodies and on the ability of tyrosine-phosphorylated pp68 to bind RNA. A second 68-kDa RNA-binding protein that can be phosphorylated on tyrosine is heterogeneous nuclear ribonucleoprotein K. Because the identity of this 70-kDa protein has not been reported, we cannot address whether pp68 and pp70 are the same protein. In contrast to pp70, pp68 in the LDM did not associate with RasGAP or Grb2. We cannot rule out, however, that pp68 that resides in another compartment like the cytosol binds these SH2 and SH3 domain-containing proteins. In fact, all of the experiments on pp70 were done using total cell lysates as opposed to a LDM fraction, and osmotic shock was never used. pp68 has been purified to homogeneity, and although the amount of receptor did not change with hypertonic shock, accumulation, suggesting that induction of these pathways is not responsible for the enhancement of insulin-stimulated tyrosine phosphorylation of pp68. Other types of cellular stress, such as oxidative stress, heat shock, depletion of potassium, and inhibition of the Na^+ /K^+ -ATPase did not modulate insulin-induced tyrosine phosphorylation of pp68. PAO is a known tyrosine phosphatase inhibitor. Inhibition of two phosphotyrosine phosphatases, HA1 and HA2, by PAO results in the insulin-stimulated tyrosine phosphorylation of a 15-kDa fatty acid-binding protein (38). In addition, PAO blocks insulin-induced RAS activation by inhibiting the phosphotyrosine phosphatase Syp (39). Because the same pattern of insulin-stimulated tyrosine phosphorylated proteins, including pp68, accumulated in the LDM with both PAO and sorbitol treatment (Fig. 7), this suggests that osmotic shock may inhibit a phosphotyrosine phosphatase that leads to an increase in pp68. This would explain the increase in ligand-induced tyrosine phosphorylation of the insulin receptor in the PM fraction even though the amount of receptor did not change with hypertonic shock. Furthermore, inhibition of a phosphatase could result in an increase in the number of phosphorylated proteins. For example, treatment of cells with the serine phosphatase inhibitor, calyculin A, was shown to increase the basal phosphorylation of both AKT (11) and p70S6 kinase (12). Therefore, the increase in tyrosine phosphorylation of several proteins in the 115–130 kDa and 50–70 kDa range observed when adipocytes were treated with sorbitol alone (Fig. 1 (10)) may be due at least in part to an inhibition of phosphotyrosine phosphatase(s) rather than a stimulation of phosphotyrosine kinase(s).

The identity of pp68 remains elusive. We have eliminated a number of known 65–70-kDa tyrosine-phosphorylated proteins (Table I) as being pp68. Recently, PAO and insulin were shown to induce the tyrosine phosphorylation of a 70-kDa protein in A14 fibroblasts that could bind poly(U)-RNA (26). This protein was neither Sam68 nor heterogeneous nuclear ribonucleoprotein K. Because the identity of this 70-kDa protein has not been reported, we cannot address whether pp68 and pp70 are the same protein. In contrast to pp70, pp68 in the LDM did not associate with RasGAP or Grb2. We cannot rule out, however, that pp68 that resides in another compartment like the cytosol binds these SH2 and SH3 domain-containing proteins. In fact, all of the experiments on pp70 were done using total cell lysates as opposed to a LDM fraction, and osmotic shock was never used. pp68 has been purified to homogeneity, and although our initial attempts were unsuccessful, we are currently in the process of purifying enough material to obtain sequence information. Based on the elimination of known proteins and on the time and insulin concentration dependences of phosphorylation, pp68 could be a novel substrate of the insulin receptor tyrosine kinase whose ability to bind RNA may dictate its physiological function.

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