Reciprocal feedback regulation of insulin receptor and insulin receptor substrate tyrosine phosphorylation by phosphoinositide 3-kinase in primary adipocytes

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Signalling by the insulin receptor substrate (IRS) proteins is critically dependent on the tyrosine phosphorylation of specific binding sites that recruit Src homology 2 (SH2)-domain-containing proteins, such as the p85 subunit of phosphoinositide 3-kinase (PI 3-kinase), the tyrosine phosphatase SHP-2 and the adapter protein Grb2. Here we show that stimulation by insulin of freshly isolated primary adipocytes resulted in the expected rapid tyrosine phosphorylation of the insulin receptor, IRS-1 and IRS-3. Inhibition of PI 3-kinase enhanced the insulin-stimulated phosphorylation of IRS-1 on (i) Tyr^{612} and Tyr^{941} (p85 binding sites), concomitant with an increased association of the p85 subunit of PI 3-kinase; (ii) Tyr^{896} (a Grb2 binding site); and (iii) Tyr^{1229} (an SHP-2 binding site), although little or no binding of SHP-2 to IRS-1 was detectable under any conditions. In contrast, inhibition of PI 3-kinase led to a decrease in insulin-stimulated p85 binding to IRS-3, but had no effect on SHP-2 binding. Furthermore, insulin-induced insulin receptor tyrosine phosphorylation, phosphorylation of Tyr^{1158} and insulin receptor tyrosine kinase activity were all reduced by inhibition of PI 3-kinase at later time points ($\geqslant$ 20 min). The results demonstrate that, in primary adipocytes, PI 3-kinase feedback control of signalling by the insulin receptor and IRS proteins is multifaceted and reciprocal, illustrating the complexity of predicting the net flux of the insulin signal(s) through the IRS proteins.

Key words: IRS-1, IRS-3, phosphospecific antibodies, SHP-2, signalling.

INTRODUCTION

Binding of insulin to its receptor results in the stimulation of its intrinsic tyrosine kinase activity, which mediates auto-phosphorylation of the receptor and subsequent phosphorylation of substrate proteins on multiple tyrosine residues. The major substrates of the insulin receptor (IR) are the insulin receptor substrate (IRS) proteins IRS-1 [1], IRS-2 [2], IRS-3 [3] and IRS-4 [4]. Upon tyrosine phosphorylation, IRS proteins serve as binding sites for Src homology 2 (SH2)-domain-containing proteins, such as the tyrosine phosphatase SHP-2 and the regulatory p85 subunit of phosphoinositide 3-kinase (PI 3-kinase), thereby increasing their enzymic activity and/or localizing them to their substrates. The stimulation of PI 3-kinase leads to a rapid rise in the production of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which are responsible for many, if not all, of the subsequent metabolic actions of insulin (reviewed in [5,6]).

The overall structures of the IRS family of proteins are very similar, including a conserved pleckstrin homology (PH) domain at the N-terminus, followed by a phosphotyrosine binding (PTB) domain and a C-terminal domain containing multiple tyrosine phosphorylation sites. The PTB domains in IRS-1, -2 and -3 have been demonstrated to interact with the NPXYp motif of the juxtamembrane region of the IR, thereby promoting IR–IRS interactions and subsequent tyrosine phosphorylation of the IRS proteins [7–10]. The tyrosine phosphorylation motifs at the C-terminus are relatively well conserved between the various IRS proteins. Although IRS-3 is much smaller than IRS-1/2 and has regions that show no identity, it does contain similar tyrosine phosphorylation motifs that can be phosphorylated by the IR tyrosine kinase.

The specific roles of the different IRS proteins in insulin signalling are still largely unclear, and seem to be tissue dependent [11–13]. Mice deficient in IRS-1 are growth-retarded and moderately insulin resistant, but not diabetic [12], whereas mice lacking IRS-2 develop diabetes early in life due to a combination of insulin resistance and a failure of the pancreatic β cells to proliferate [14]. In contrast, mice lacking IRS-3 show normal growth and glucose homoeostasis [15]. Further studies on mice lacking IRS-1 revealed that IRS-2 can compensate for the absence of IRS-1 in hepatocytes, whereas IRS-3 compensates for the absence of IRS-1 in muscle and adipocytes [11–13]. However, although IRS-1 and IRS-3 proteins seem to be able to compensate for each other in gene knockout studies, differences in cellular distribution and subcellular localization patterns, and the existence of distinct regions of non-identity, suggests that they have different functions in insulin signalling [3,16,17].

As the functions of IRS proteins are determined by the tyrosine phosphorylation of specific residues that subsequently bind to the SH2 domains of downstream signalling molecules,

Abbreviations used: GST, glutathione S-transferase; IR, insulin receptor; IRS, insulin receptor substrate; NP40, Nonidet P40; Pd-SHP-2, phosphatase domain of SHP-2; PI 3-kinase, phosphoinositide 3-kinase; PTB domain, phosphotyrosine binding domain; SH2, Src homology 2; TBS-T, Tris-buffered saline with 0.1% Tween.

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one mechanism of regulating post-receptor signalling may involve differential dephosphorylation of these tyrosine phosphorylation sites by selective cellular protein tyrosine phosphatases. Alternatively, regulation at the level of the IR tyrosine kinase, either directly or indirectly, may lead to distinct patterns of phosphorylation of the IRS proteins. Increased serine phosphorylation of IRS-1, for example, has been shown to significantly reduce the ability of IRS-1 to bind to the juxtamembrane region of the IR and subsequently become tyrosine phosphorylated [18,19].

Several PI 3-kinase-dependent protein kinases have been reported to phosphorylate IRS-1, including PI 3-kinase itself, but also Akt/protein kinase B, glycogen synthase kinase-3, mTOR (mammalian target of rapamycin) and protein kinase Cζ [19–25]. However, the precise role that each of these kinases plays *in vivo* is not yet fully established [19,23,26].

In the present study, we investigated the relationship between IRS-1, IRS-3 and IR tyrosine phosphorylation in primary rat adipocytes, with a focus on their regulation by PI 3-kinase. In particular, we have explored the effects of inhibition of PI 3-kinase using wortmannin and LY294002. Our data demonstrate that PI 3-kinase plays a multifaceted role in feedback regulation of tyrosine phosphorylation and signalling by IRS-1, IRS-3 and the IR, thereby highlighting the importance of considering this level of cross-talk when investigating insulin-mediated metabolic regulation.

EXPERIMENTAL

Materials

Male Wistar rats (160–210 g) were fed *ad libitum* on a stock diet (CRM; Bioshore, Manea, Cambs., U.K.). Wortmannin and LY294002 were from Calbiochem. Anti-IR (C-19), anti-IRS-1 (C-20), anti-IRS-3 (S-20) and anti-SHP-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal antiserum against the C-terminal 235 amino acids of rat IRS-1 and rabbit polyclonal antiserum against the rat IR were kindly provided by Professor K. Siddle (University of Cambridge, Cambridge, U.K.). The antibody against the p85 subunit of PI 3-kinase and anti-phosphotyrosine antibody 4G10 were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The full-length SHP-2 construct was kindly donated by Dr Steven Shoelson (Harvard Medical School, Boston, MA, U.S.A.). The phosphatase domain of SHP-2 (Pd-SHP-2; residues 263–517) was amplified by PCR and ligated into pGEX-4T-2 for glutathione S-transferase (GST) fusion protein expression. CHO-T cells were from Richard Roth (Stanford University, Stanford, CA, U.S.A.) [27].

The rabbit polyclonal phosphorylation-site-specific antibodies that recognize IRS-1 when phosphorylated on Tyr^{612} (anti-IRS-1 $PTyr^{612}$), Tyr^{896} (anti-IRS-1 $PTyr^{896}$), Tyr^{941} (anti-IRS-1 $PTyr^{941}$) and Tyr^{1229} (anti-IRS-1 $PTyr^{1229}$), or recognize the IR when phosphorylated on Tyr^{1158} (anti-IR $PTyr^{1158}$), were obtained from Biosource International (Camarillo, CA, U.S.A.). All other reagents were as described [28].

Preparation and incubation of epididymal fat cells

Adipocytes were isolated from the epididymal fat pads of Wistar rats as described previously [29]. Cells were subsequently washed in Krebs bicarbonate/Hepes buffer, pH 7.4 (130 mM NaCl, 4.7 mM KCl, 1.5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2.5 mM NaH_2PO_4, 15.5 mM $NaHCO_3$, 10 mM Hepes and 11 mM glucose) without BSA. Cells were incubated with 100 nM wortmannin, 150 μM LY294002 or DMSO (vehicle) for 30 min at 37 °C prior to stimulation with 83 nM insulin for the indicated times. The reaction was terminated by extracting the cells at a ratio of 1:1 (packed cell volume/buffer volume) in ice-cold Nonidet P40 (NP40) extraction buffer (50 mM Tris, pH 7.5, containing 1 mM EDTA, 120 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 1 mM benzamidine, 1% NP40, 1 μM microcystin, 7.2 mM mercaptoethanol, 5 mM orthovanadate and 1 μg/ml each of pepstatin, leupeptin and antipain). Cell extracts were centrifuged at 10000 ***g*** for 10 min at 4 °C and the infranatant was taken for subsequent analysis.

Immunoprecipitation

Proteins were immunoprecipitated by rotating 250 μl of total cell extract with the relevant antibody and 20 μl of Protein A–Sepharose (50%, w/v) at 4 °C. The Protein A–Sepharose beads were isolated by centrifugation and washed three times in NP40 extraction buffer. Subsequently, Laemmli sample buffer was added and proteins were separated by SDS/PAGE for immunoblotting. Alternatively, the immunoprecipitates were washed twice in 50 mM Tris, pH 7.4, for the dephosphorylation assay.

Immunoblotting

Proteins were separated by SDS/PAGE using 5–10% (w/v) polyacrylamide gradient gels and transferred to PVDF membranes. The membranes were blocked in 10% (w/v) BSA dissolved in Tris-buffered saline with 0.1% Tween (TBS-T; 20 mM Tris, 137 mM NaCl, 0.1% Tween) and subsequently incubated with primary and secondary antibodies, which were diluted in TBS-T containing 5% (w/v) BSA. Membranes were washed for at least 2 h in TBS-T after each antibody incubation, and developed using an Enhanced ChemiLuminescence detection system (Amersham Biosciences). Primary antibodies were used at a concentration of 1 μg/ml (except for anti-IRS-1 $PTyr^{612}$, anti-IRS-1 $PTyr^{941}$ and anti-IR $PTyr^{1158}$ antibodies, which were used at a concentration of 0.5, 0.2 and 0.2 μg/ml respectively). Horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) was diluted 1:4000 for the phosphospecific anti-IRS-1 and anti-IR antibodies, and 1:10000 for all other antibodies. For peptide competition studies, membranes were incubated in the presence of peptides at a ratio of 1:4 (antibody/peptide).

In vitro dephosphorylation assay

Immunoprecipitates were combined, resuspended in 90 μl of Tris buffer (50 mM, pH 7.4), and 25 μl fractions were divided over three different samples. Samples were subsequently incubated in Tris buffer (50 mM, pH 7.4) or in dephosphorylation buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM dithiothreitol) in the absence or presence of 10 μg of purified GST–Pd-SHP-2 for 30 min at 30 °C. The immunoprecipitates were subsequently analysed by SDS/PAGE analysis and immunoblotting with the generic anti-phosphotyrosine antibody 4G10.

Purified GST–Pd-SHP-2 was prepared as described previously [30]. Briefly, transformed *Escherichia coli* (strain BL-21) were grown overnight in Luria broth, and fusion protein expression was induced by the addition of isopropyl β-D-thiogalactoside (0.4 mM) for 3 h at 37 °C. Bacteria were then lysed by sonication and repeated freeze–thaw cycles, and cytosolic content was recovered by centrifugation at 25000 ***g***. Fusion proteins were purified from lysates by incubation with glutathione–agarose

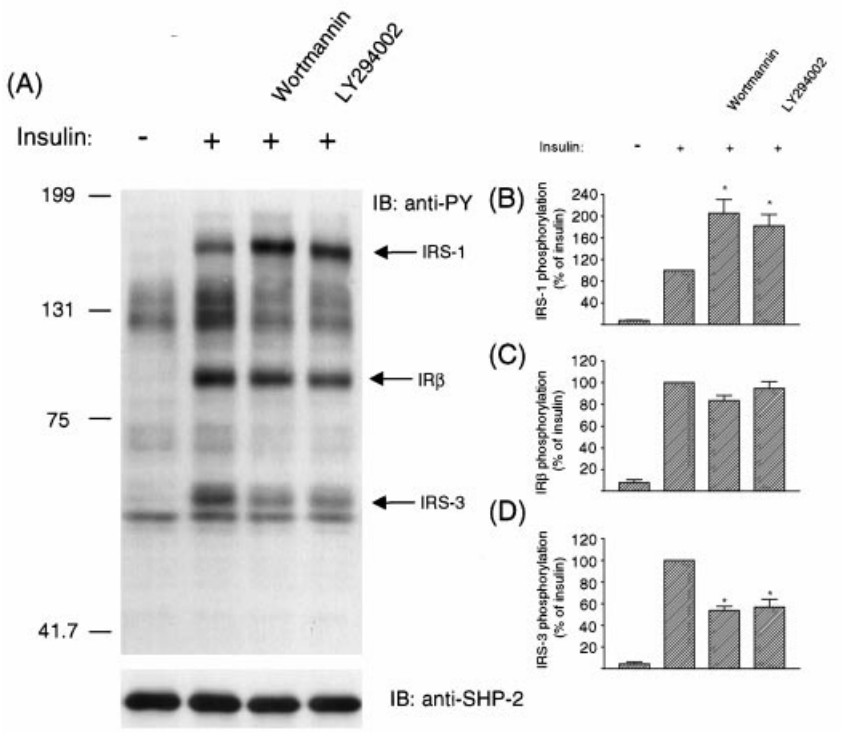

Figure 1 Effects of wortmannin and LY294002 on insulin-stimulated tyrosine phosphorylation in primary rat adipocytes

Primary adipocytes were pretreated with vehicle (DMSO), wortmannin (100 nM) or LY294002 (150 μM) for 30 min at 37 °C and subsequently stimulated or not with insulin (83 nM) for 10 min. Cells were extracted and total lysate was subjected to SDS/PAGE, followed by immunoblotting (IB) for tyrosine-phosphorylated proteins with the anti-phosphotyrosine (anti-PY) antibody 4G10 (a representative blot is shown in **A**). The membrane was stripped and re-probed with anti-SHP-2 to confirm loading. The positions of molecular mass standards (kDa) are indicated. The bar graphs (**B**–**D**) represent quantification of tyrosine phosphorylation of IRS-1 (**B**), the IR β-subunit (**C**) and IRS-3 (**D**), expressed as a percentage of the signal obtained with insulin alone (means $\pm$ S.E.M.; $n = 4$ or 5). $^*P < 0.05$ indicates a significant difference with respect to the response to insulin alone, as determined by the two-tailed paired Student's t test.

beads at 4 °C for 2 h, followed by extensive washing in TBS-T. For experiments requiring fusion proteins in solution, the beads were subsequently incubated with glutathione (20 mM) at 4 °C overnight, and supernatants were separated from the beads by centrifugation.

IR tyrosine kinase activity assay

The IR was immunoprecipitated from primary adipocyte lysates by rotating the crude extracts with the anti-(rat IR) antibody and 20 μl of Protein A–Sepharose (50 %, w/v) at 4 °C. The Protein A–Sepharose beads were washed twice in 1 % Triton extraction buffer (50 mM Hepes, pH 7.4, containing 10 mM EDTA, 30 mM NaF, 10 mM $Na_4P_2O_7$, 2.5 mM benzamidine, 1 % Triton X-100, 0.5 mM PMSF, 5 mM orthovanadate and 1 μg/ml each of pepstatin, leupeptin and antipain) and twice in 0.1 % Triton wash buffer (50 mM Hepes, pH 7.4, 0.1 % Triton X-100, 0.1 mM orthovanadate 2.5 mM benzamidine and 1 μg/ml each of pepstatin, leupeptin and antipain). The beads were resuspended in 60 μl of wash buffer, and 20 μl aliquots were added to each of two tubes containing 30 μl of reaction buffer (130 mM Hepes, pH 7.4, 100 μg of BSA, 1 mM EGTA and 12 mM $MgCl_2$) with 0.5 mM RRDIFETDYFRK peptide substrate, and to one tube without the peptide substrate as a background. The reaction was started by the addition of 1 μCi of [γ-^{32}P]ATP (final ATP concentration 4.5 μM). After a 5 min incubation at 30 °C, the reaction was terminated by spotting on to P81 ion-exchange paper. The paper was washed four times in 0.6 % phosphoric acid, and bound radioactivity was quantified by scintillation counting.

Quantification and statistics

Quantitative assessment of phosphorylated protein bands detected by Western blotting was performed by densitometry of films and subsequent analysis using the program ImageQuant (Molecular Dynamics). Data are expressed as a percentage of the response to insulin in the absence of inhibitors. Statistical significance was determined using the two-tailed Student's t test for paired data; $P < 0.05$ was considered significant.

RESULTS

PI 3-kinase activity regulates the tyrosine phosphorylation of IRS proteins

Stimulation of primary rat adipocytes with insulin for 10 min resulted in an increase in the tyrosine phosphorylation of proteins of 180, 95 and 60–65 kDa (Figure 1A), identified previously as IRS-1, the IR β subunit and IRS-3 respectively [31]. PI 3-kinase and its downstream signalling proteins (e.g. Akt/protein kinase B, glycogen synthase kinase-3, etc.) are important regulators of insulin-mediated metabolic events. To investigate whether PI 3-kinase signalling pathways might also play a role in regulating the tyrosine phosphorylation of IR and IRS proteins, cells were pretreated with two commonly used but unrelated PI 3-kinase inhibitors, wortmannin and LY294002. Both

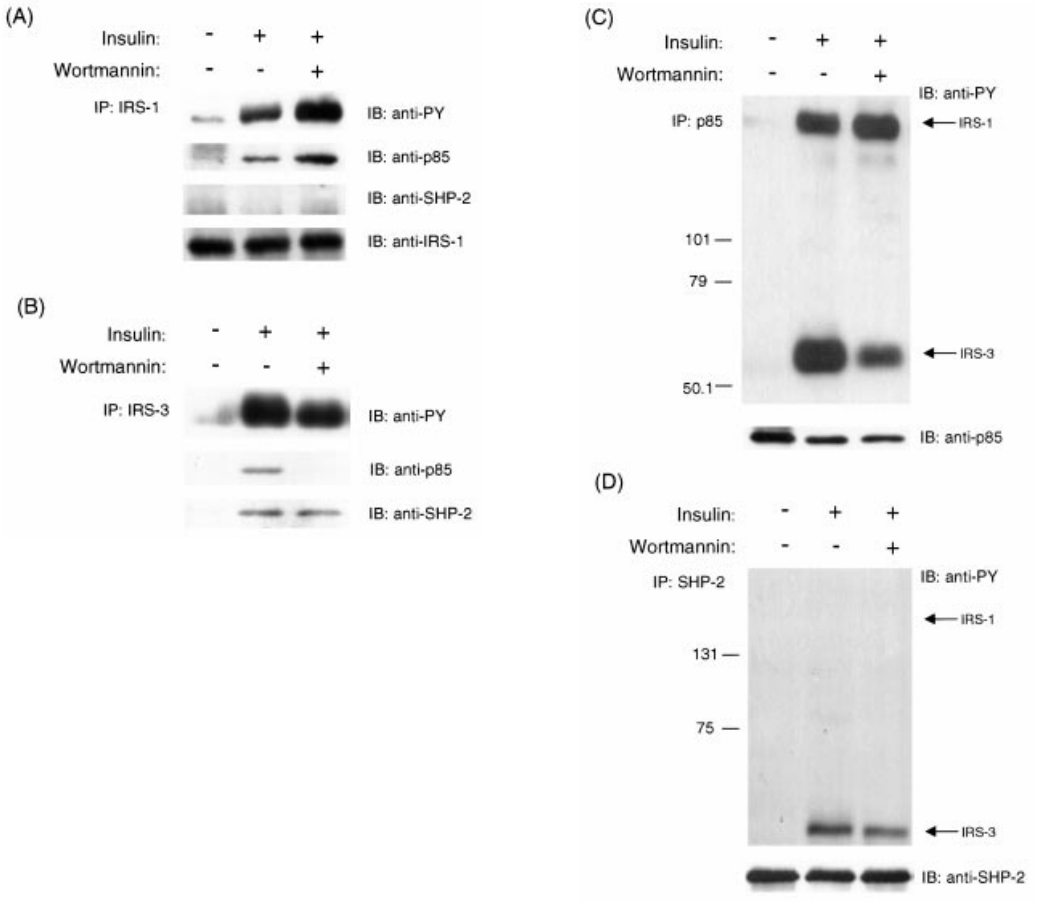

Figure 2 Effects of wortmannin on the insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-3 and their association with p85 and SHP-2

Primary adipocytes were pretreated with vehicle or wortmannin (100 nM) for 30 min at 37 °C and subsequently stimulated or not with insulin (83 nM) for 10 min. Cells were extracted, and IRS-1 (**A**), IRS-3 (**B**), p85 (**C**) and SHP-2 (**D**) were subsequently immunoprecipitated (IP). The immunoprecipitates were subjected to SDS/PAGE followed by immunoblotting (IB) with the indicated antibodies (anti-PY, anti-phosphotyrosine). In (**B**), (**C**) and (**D**) the membranes were stripped and re-probed with anti-IRS-1, anti-p85 and anti-SHP-2 respectively to confirm equal loading. The figure shows the results of one representative experiment out of three performed. The positions of molecular mass standards (kDa) are indicated.

wortmannin and LY294002 increased the insulin-stimulated tyrosine phosphorylation of IRS-1 (Figure 1A). Quantification of the tyrosine-phosphorylated IRS-1 bands showed that the increase in phosphorylation caused by these inhibitors was highly significant ($P < 0.05$; Figure 1B). In contrast, tyrosine phosphorylation of IRS-3 was markedly decreased by wortmannin and LY294002 (Figures 1A and 1D; $P < 0.05$). Neither inhibitor had any significant effect on tyrosine phosphorylation of the IR at this time point (Figures 1A and 1C).

PI 3-kinase activity regulates the tyrosine phosphorylation of specific binding sites on IRS-1 and IRS-3

Tyrosine phosphorylation of IRS proteins allows the specific binding of SH2-domain-containing proteins, such as the regulatory subunit (p85) of PI 3-kinase and the tyrosine phosphatase SHP-2. To investigate these interactions, IRS-1 (Figure 2A) and IRS-3 (Figure 2B) were immunoprecipitated and subsequently immunoblotted for phosphotyrosine and the presence of co-precipitated p85 and SHP-2 proteins. Insulin stimulated the association of p85 with IRS-1, which was increased further in the presence of wortmannin (Figure 2A). In contrast, the interaction of p85 with IRS-3 was blocked by wortmannin (Figure 2B). Although IRS-1 contains specific binding sites for SHP-2 [32], no association of SHP-2 with immunoprecipitated IRS-1 was detected after insulin stimulation (Figure 2A). Similar results were obtained using a different anti-IRS-1 antibody that was raised against a fusion protein containing the 235 C-terminal amino acids. SHP-2, however, did associate with IRS-3 upon insulin stimulation (Figure 2B), but, in contrast with the interaction of p85 with IRS-3, this association was not affected by wortmannin treatment.

Both tyrosine-phosphorylated IRS-1 and IRS-3 proteins co-immunoprecipitated with the p85 regulatory subunit of PI 3-kinase in insulin-stimulated cells, but not in untreated cells (Figure 2C). However, the interaction of tyrosine-phosphorylated IRS-3 with p85 was markedly decreased, whereas the interaction of tyrosine-phosphorylated IRS-1 with p85 was increased, in the presence of wortmannin (Figure 2C). As expected, IRS-3 was the major tyrosine-phosphorylated protein that co-immunoprecipitated with SHP-2 upon insulin stimulation (Figure 2D). The association of IRS-3 with SHP-2 (Figure 2D) was unchanged in the presence of wortmannin, demonstrating that this interaction does not appear to be regulated by PI 3-kinase activity.

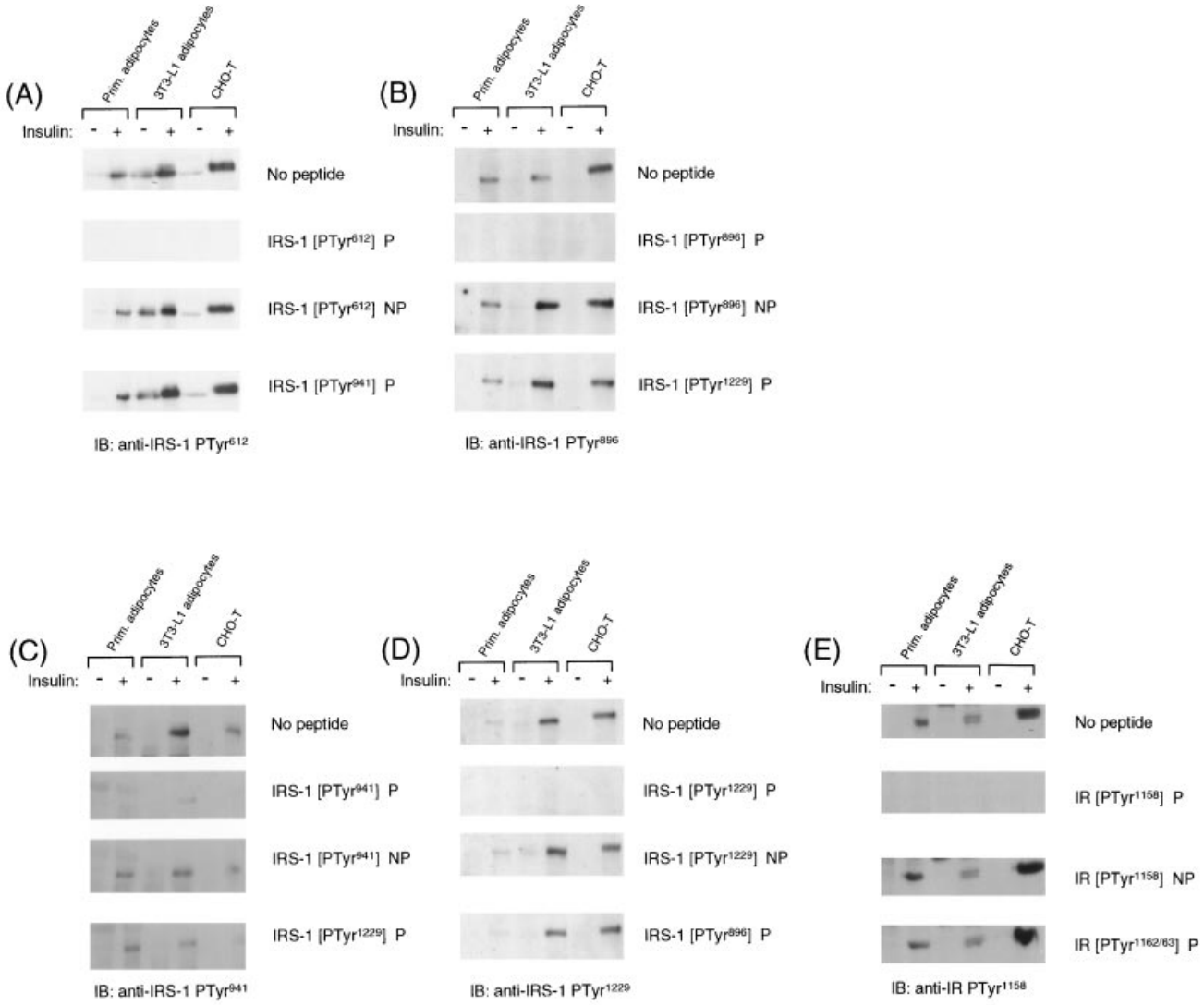

Figure 3 Peptide competition studies show the specificity of the phosphospecific antibodies against PTyr^{612}, PTyr^{896}, PTyr^{941} and PTyr^{1229} on IRS-1, and against PTyr^{1158} on the IR

Primary adipocytes, 3T3-L1 adipocytes and CHO-T cells were stimulated or not with insulin (83 nM) for 10 min. Cells were extracted and the total lysate was subjected to SDS/PAGE followed by immunoblotting (IB) with anti-IRS-1 PTyr^{612} (**A**), anti-IRS-1 PTyr^{896} (**B**), anti-IRS-1 PTyr^{941} (**C**), anti-IRS-1 PTyr^{1229} (**D**) and anti-IR PTyr^{1158} (**E**) in the absence (No peptide) or presence of the indicated non-phosphorylated (NP) or phosphorylated (P) peptide. The figure shows the results of one representative experiment out of at least two performed for each antibody.

Wortmannin increases phosphorylation of Tyr^{612}, Tyr^{896}, Tyr^{941} and Tyr^{1229} on IRS-1

The results presented above suggest that wortmannin treatment results in an increase in the phosphorylation of p85 binding sites on IRS-1 and in the dephosphorylation of the binding sites for p85, but not SHP-2, on IRS-3. However, IRS proteins are known to be phosphorylated on many different tyrosine residues. Moreover, the Western blot signals that we observed with the generic anti-phosphotyrosine antibody (4G10) do not provide information about the specific tyrosine residues that were phosphorylated. To investigate this further, we used phospho-specific polyclonal antibodies that were raised to recognize IRS-1 when phosphorylated on Tyr^{612} (a p85 subunit binding site), Tyr^{896} (a Grb2 binding site), Tyr^{941} (a p85 binding site) and Tyr^{1229} (an SHP-2 binding site). These antibodies specifically recognized IRS-1 in whole-cell lysates of primary adipocytes, 3T3-L1 adipocytes and CHO-T cells upon insulin stimulation (Figures 3A–3D). The specificity of the antibodies for the phosphorylation sites was confirmed by incubation of the membranes with the antibodies in the presence of the cognate phosphorylated peptides used to generate the antibodies, which blocked the IRS-1 signal for all antibodies. In contrast, the equivalent but unphosphorylated peptides, and phosphorylated peptides corresponding to the other tyrosine phosphorylation sites on IRS-1, were without effect (Figures 3A–3D).

Figure 4 shows that treatment of primary adipocytes with wortmannin significantly increased the insulin-stimulated signals generated with all four of these antibodies to IRS-1 (lane 3). Taken together, these data demonstrate that wortmannin increases tyrosine phosphorylation of the binding sites for Grb2, p85 and SHP-2 on IRS-1, and inhibits tyrosine phosphorylation

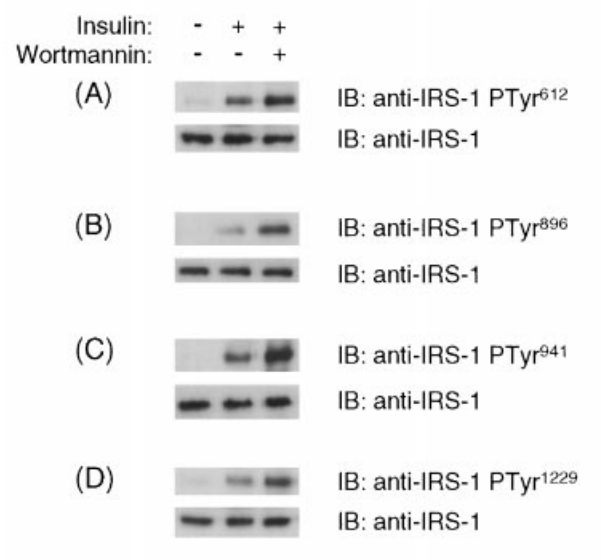

Figure 4 Effects of wortmannin on the insulin-stimulated phosphorylation of Tyr^{612}, Tyr^{896}, Tyr^{941} and Tyr^{1229} on IRS-1

Primary adipocytes were pretreated with vehicle or wortmannin (100 nM) for 30 min at 37 °C, and subsequently stimulated or not with insulin (83 nM) for 10 min. Cells were extracted and total lysate was subjected to SDS/PAGE, followed by immunoblotting (IB) with the indicated antibodies (anti-IRS-1 PTyr^{612}, anti-IRS-1 PTyr^{896}, anti-IRS-1 PTyr^{941} and anti-IRS-1 PTyr^{1229}). The membrane was stripped and reprobed with anti-IRS-1 to confirm equal loading. The figure shows the results of one representative experiment out of four performed.

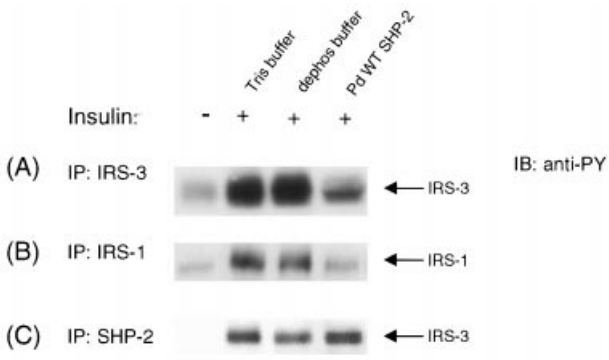

Figure 5 IRS-3 is a substrate for SHP-2

Primary adipocytes were stimulated or not with insulin (83 nM) for 10 min. Cells were extracted, followed by immunoprecipitation (IP) of IRS-3 (**A**), IRS-1 (**B**) and SHP-2 (**C**). Immunoprecipitates were incubated in dephosphorylation buffer or in the presence of GST–Pd-SHP-2 fusion protein for 30 min at 30 °C prior to SDS/PAGE analysis and immunoblotting (IB) with the anti-phosphotyrosine (anti-PY) antibody 4G10. WT, wild type; dephos, dephosphorylation. The figure shows the results of one representative experiment out of two performed.

of the p85 binding site(s) on IRS-3. Furthermore, SHP-2 appears to bind preferentially to IRS-3 in response to insulin treatment, and wortmannin has no effect on this interaction.

SHP-2 dephosphorylates IRS-3

The phosphatase activity of SHP-2 is increased by the binding of its SH2 domains to tyrosine-phosphorylated residues [33]. Therefore this activation of SHP-2 might lead to dephosphorylation of one or more tyrosine-phosphorylated proteins in the presumed complex. To investigate whether IRS-3 itself is a substrate for SHP-2, tyrosine-phosphorylated IRS-3 was immunoprecipitated and incubated with Pd-SHP-2. Figure 5(A) shows that Pd-SHP-2 dephosphorylated IRS-3 *in vitro*, specifically the forms of IRS-3 with lower mobility on SDS/PAGE, whereas the forms of IRS-3 with higher mobility appeared unaffected. In contrast, tyrosine-phosphorylated IRS-1 was almost completely dephosphorylated by Pd-SHP-2 within this time period (Figure 5B). However, the significance of this is unclear, since, in insulin-stimulated primary adipocytes, SHP-2 does not appear to bind to IRS-1 (the present study and [15,31]).

IRS-3 appears as a diffuse tyrosine-phosphorylated band in IRS-3 immunoprecipitates (Figures 2B and 5A), which is likely to reflect the existence of multiple forms of the protein with different phosphorylation states. In contrast, IRS-3 appears as a single, sharp tyrosine-phosphorylated band in SHP-2 immunoprecipitates from insulin-stimulated cells (Figures 2D and 5C), suggesting that the dephosphorylation events have already taken place. When these SHP-2 immunoprecipitates were incubated in dephosphorylation buffer alone or in the presence of Pd-SHP-2, no further dephosphorylation of IRS-3 was observed (Figure 5C). Together, these results suggest that SHP-2 may bind to tyrosine-phosphorylated IRS-3 and subsequently dephosphorylate specific tyrosine residues, including the p85 binding site.

Wortmannin inhibits the later phase of IR autophosphorylation and kinase activity

In the studies presented above, cells were stimulated with insulin for 10 min. However, an insulin time course showed further differences in the phosphorylation of IRS-1, IRS-3 and the IR in the presence of wortmannin (Figure 6). Insulin stimulation resulted in the expected rapid tyrosine phosphorylation of IRS-1, IRS-3 and the IR, which stayed relatively constant over a 2–10 min time period before declining (Figure 6A). The initial phase of tyrosine phosphorylation of IRS-1 was increased dramatically in the presence of wortmannin, while there was less of an effect at later time points. However, in the case of IRS-3, wortmannin inhibited the later phase of insulin-stimulated tyrosine phosphorylation (10–60 min), whereas it had no effect on initial tyrosine phosphorylation (Figure 6A).

Surprisingly, wortmannin also significantly reduced the later phase (20 and 60 min) of tyrosine phosphorylation of the IR. The IR has three tyrosine residues (Tyr^{1158}, Tyr^{1162} and Tyr^{1163}) in the activation loop of its kinase domain, and all three residues must be phosphorylated to achieve full activation [34]. Tyr^{1158} is predominantly the first residue to be phosphorylated in this series [34,35]. To investigate whether the inhibition of IR phosphorylation induced by wortmannin was accompanied by a decrease in the activation of the IR, we first used a phospho-specific antibody that recognizes tyrosine phosphorylation at position 1158. This antibody specifically recognized the IR in whole-cell lysates of primary adipocytes, 3T3-L1 adipocytes and CHO-T cells upon insulin stimulation (Figure 3E). The specificity of this antibody was confirmed by incubation of the antibody with the phosphorylated peptide used to generate the antibody, which blocked the IR signal, whereas the unphosphorylated peptide and a peptide phosphorylated at positions 1162/1163 had no effect (Figure 3E). Figure 6(B) shows that the later phase (20 and 60 min) of insulin-stimulated autophosphorylation of Tyr^{1158} was significantly reduced by wortmannin (see also Figure 6C). More directly, we found that wortmannin significantly lowered the tyrosine kinase activity of the IR after 20 and 60 min

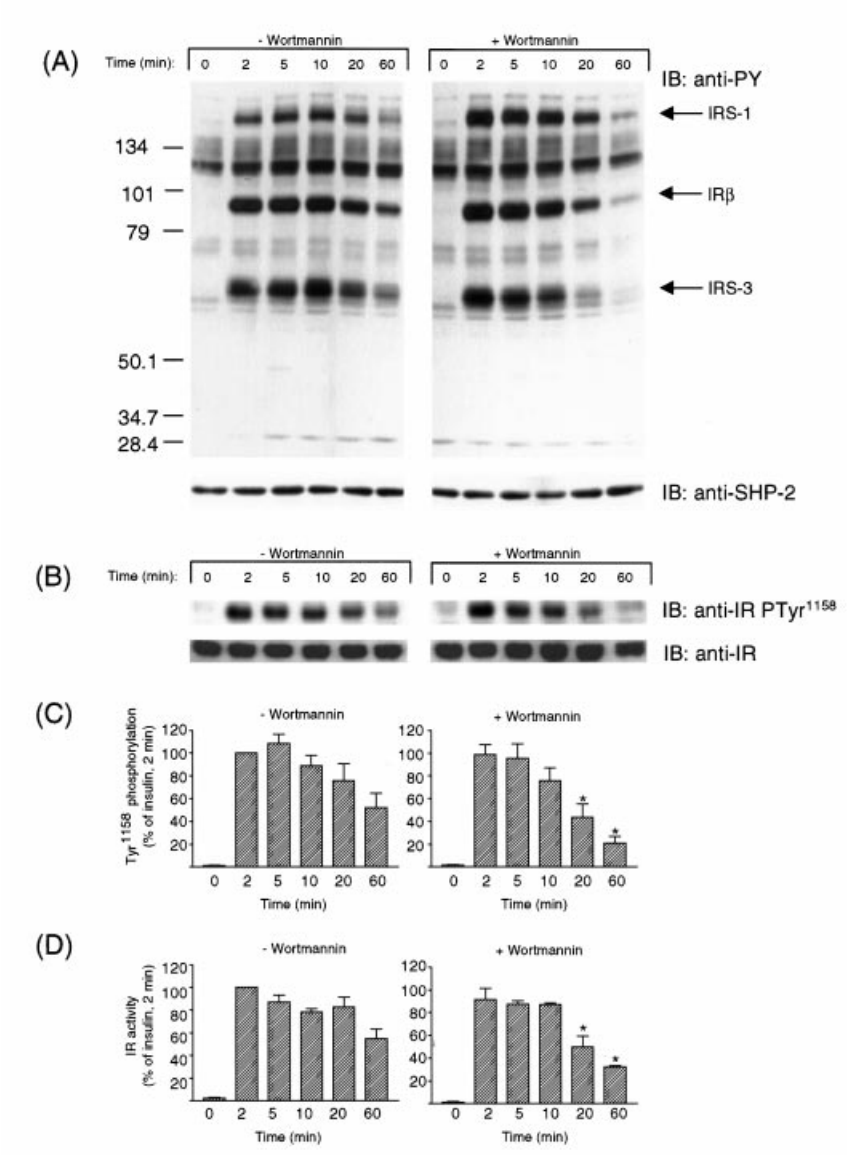

Figure 6 Wortmannin inhibits the later phase of IR autophosphorylation and kinase activity

Primary adipocytes were pretreated with vehicle (DMSO) or wortmannin (100 nM) for 30 min at 37 °C and then stimulated with insulin (83 nM) for the indicated times. Cells were extracted and the total lysate was subjected to SDS/PAGE followed by immunoblotting (IB) with the anti-phosphotyrosine (anti-PY) antibody 4G10 (**A**) or with anti-IR PTyr^{1158} (**B**). Membranes in (**A**) and (**B**) were stripped and re-probed with anti-SHP-2 and anti-IR respectively, to confirm equal loading (bottom panels). The bar graph (**C**) represents quantification of anti-IR PTyr^{1158} results expressed as percentage of the response to insulin at 2 min without wortmannin (mean $\pm$ S.E.M., $n = 5$). Alternatively, the IR tyrosine kinase activity (**D**) was determined in IR immunoprecipitates and expressed as percentage of the insulin response at 2 min without wortmannin. * Indicates a significant difference ($P < 0.05$) with respect to the response to insulin alone, as determined by the two-tailed paired Student's t test.

of insulin stimulation (Figure 6D). This was not the result of direct inhibition of IR tyrosine kinase activity by wortmannin, as the activity in IR immunoprecipitates was unaffected by the direct addition of 100 nM or 1 μM wortmannin (results not shown).

DISCUSSION

The major IRS proteins expressed in rat adipocytes are IRS-1 and IRS-3 [31], and tyrosine phosphorylation of these proteins is likely to be responsible for most, if not all, of the metabolic actions of insulin in this tissue [36]. In the present study, we evaluated the role of PI 3-kinase in regulating tyrosine phosphorylation of the IR and IRS proteins. We demonstrate that PI 3-kinase exerts a multifaceted feedback regulatory effect on the phosphorylation of the IR, IRS-1 and IRS-3. More specifically, PI 3-kinase has a positive feedback effect on the tyrosine phosphorylation and PI 3-kinase binding of IRS-3, but a negative feedback effect on the tyrosine phosphorylation and PI 3-kinase binding of IRS-1. In addition, PI 3-kinase appears to have a positive feedback role in controlling IR phosphorylation and tyrosine kinase activity. Thus the net flux of the insulin signal(s) through IRS-1 and IRS-3 is likely to be critically dependent on the relative expression levels and signalling status of IRS-1, IRS-3 and PI 3-kinase.

The PI 3-kinase multienzyme family consists of three classes of kinase, of which the class I PI 3-kinase is believed to be responsible for the majority of the metabolic actions of insulin [37]. To study the role of PI 3-kinase in the feedback regulation of IRS signalling we employed the PI 3-kinase inhibitor wortmannin, although we observed similar results with the unrelated inhibitor LY294002 (Figure 1). These inhibitors block class I PI 3-kinase activity, but may also inhibit class II and class III PI 3-kinase activities to a lesser degree. Wortmannin induced an increase in the insulin-stimulated phosphorylation of IRS-1 on at least four specific tyrosine phosphorylation sites (Figure 4), even under conditions where IR tyrosine kinase activity was reduced (Figures 6B–6D). These sites were Tyr^{612} (p85 binding site), Tyr^{896} (Grb2 binding site), Tyr^{941} (p85 binding site) and Tyr^{1229} (SHP-2 binding site). The finding that phosphorylation of all of these sites was increased suggests that wortmannin facilitates the general tyrosine phosphorylation of IRS-1, possibly by stimulating its tyrosine phosphorylation by the IR. One of the serine/threonine kinases downstream of PI 3-kinase therefore might be involved in this negative feedback regulation of IRS-1. This is consistent with observations by several groups that serine phosphorylation of IRS-1 inhibits its tyrosine phosphorylation in response to insulin [24,26,38–40]. Indeed, the insulin-stimulated phosphorylation of Ser^{307}, which is blocked by PI 3-kinase inhibitors, appears to play a role in the feedback regulation of IRS-1 signalling by disrupting its interaction with the IR [26,41], as has been proposed previously [18,42]. Stimulation of cells by tumour necrosis factor-α has also been shown to stimulate serine phosphorylation of IRS-1 in a manner that inhibits IR tyrosine kinase signalling [40,43]. Alternatively, wortmannin might affect the tyrosine phosphorylation of IRS-1 by inhibiting other PI 3-kinase-dependent processes, such as IR recycling [44,45], possibly leading to prolonged spatial co-localization of the IR and IRS-1.

In addition to an inhibitory role for PI 3-kinase in regulation of the tyrosine phosphorylation of IRS-1, we found a positive role in the regulation of IR autophosphorylation and tyrosine kinase activity. Wortmannin reduced IR tyrosine kinase activity (Figure 6D), which was concomitant with, and presumably resulted from, a reduction in overall IR tyrosine phosphorylation (Figure 6A) and phosphorylation of a key regulatory tyrosine in the activation loop of the kinase (Tyr^{1158}; Figures 6B and 6C). The mechanism by which PI 3-kinase feedback regulates the IR tyrosine kinase is not known. It is well established that the IR is phosphorylated on serine and threonine residues (e.g. $Ser^{1305/6}$ and Thr^{1348}) in an insulin-dependent manner [46,47], although the protein kinase(s) involved have not been identified, and nor has the role of serine/threonine phosphorylation been determined. However, if these serine/threonine protein kinase(s) were regulated by PI 3-kinase, and they had a positive feedback effect on receptor tyrosine kinase activity, this could explain the ability of wortmannin to decrease IR tyrosine kinase activity. Wortmannin treatment may also lead to the activation of a PI 3-kinase-regulated protein tyrosine phosphatase that subsequently dephosphorylates the critical tyrosine residues in the activation loop of the IR (Figure 6B). Several protein tyrosine phosphatases that show selectivity for the IR kinase domain have been described [48,49]. Alternatively, wortmannin treatment may lead to increased IR degradation by interfering with IR recycling. However, IR expression is unchanged in wortmannin-treated samples, arguing against this possibility.

The insulin-stimulated tyrosine phosphorylation of IRS-3 peaked at 2–10 min before returning to near basal levels by 60 min (Figure 6A). The presence of wortmannin appeared to accelerate the rate of dephosphorylation of IRS-3, an effect that became apparent after 10 min of continuous insulin stimulation (Figures 1A, 1D and 6A). This positive feedback regulation by PI 3-kinase is likely to include increased phosphorylation of the p85 binding site on IRS-3, as wortmannin decreased p85 binding to IRS-3 (Figure 2B). Surprisingly, PI 3-kinase does not appear to alter insulin-stimulated phosphorylation of the SHP-2 binding site on IRS-3, as determined by measuring SHP-2 co-precipitation with IRS-3 (Figure 2B). We conclude from these results that activation of PI 3-kinase leads specifically to a sustained hyperphosphorylation of the PI 3-kinase binding site on IRS-3, thus potentially allowing prolonged maintenance of PI 3-kinase activation through this particular pathway.

The mechanism by which wortmannin induces dephosphorylation of the p85 binding site on IRS-3 is not known. One possibility is that wortmannin affects the localization of IRS-3 in the cell, by blocking the generation of PtdInsP_3, making it less accessible for tyrosine phosphorylation. However, this is an unlikely explanation, since IRS-3 is localized at the membrane, independent of insulin stimulation or wortmannin treatment [50]. From our results it appears that there may be at least two plausible mechanisms. First, the reduction in IR kinase activity promoted by wortmannin could cause the observed decrease in IRS-3 phosphorylation on the p85 binding site. However, as the effect of wortmannin on IRS-3 tyrosine phosphorylation occurred well before the decrease in IR tyrosine phosphorylation was apparent (10 min and 20 min respectively; Figures 1A, 1C, 1D and 6A), it would appear that there must be another explanation.

This alternative mechanism could involve the tyrosine phosphatase SHP-2. Our studies show that the main pathway leading to SHP-2 in primary adipocytes is via IRS-3. We found that recombinant SHP-2 could efficiently dephosphorylate IRS-3 *in vitro* (Figure 5A), and that wortmannin did not affect the insulin-induced binding of SHP-2 to IRS-3 (Figures 2B and 2D). Therefore PI 3-kinase may: (i) suppress the activity of SHP-2 when it is bound to IRS-3, or (ii) sterically inhibit the dephosphorylation of the p85 binding site by SHP-2 (the latter would require wortmannin to promote the release of PI 3-kinase from IRS-3, such that the p85 binding site on IRS-3 becomes accessible for dephosphorylation). Either of these mechanisms could involve PI 3-kinase-induced serine/threonine phosphorylation of IRS-3, as has been proposed for IRS-1 [24,26,38–40], or alternatively serine/threonine phosphorylation of SHP-2, which has been described recently as a substrate for protein kinase C [51].

In conclusion, the feedback regulation of insulin signalling through IRS proteins by PI 3-kinase in primary adipocytes is clearly complex and multifaceted. The consequences of the reciprocal regulation of the tyrosine phosphorylation of IRS-1 and IRS-3 in directing signals initiated by insulin and involving PI-3 kinase is an important area of study. This level of regulation will clearly depend on the relative levels of expression of IRS-1 and IRS-3 and the degree of feedback imposed on them. Our future understanding of this complex interplay between IRS-1 and IRS-3 signalling will require a more detailed knowledge of their relative levels of expression, phosphorylation (on multiple serine, threonine and tyrosine residues) and subcellular localization, and of the nature of specific signalling complexes that are formed under a variety of experimental and *in vivo* conditions.

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