Phosphatidylinositol 3'-kinase associates with an insulin receptor substrate-1 serine kinase distinct from its intrinsic serine kinase

Keith A. CENGEL*, Rosanne E. KASON* and Gregory G. FREUND†¹

*Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U.S.A., and †Department of Pathology University of Illinois at Urbana-Champaign, Urbana, IL 61801, U.S.A.

Serine phosphorylation of insulin receptor substrate-1 (IRS-1) has been proposed as a counter-regulatory mechanism in insulin and cytokine signalling. Here we report that IRS-1 is phosphorylated by a wortmannin insensitive phosphatidylinositol 3'-kinase (PI 3-kinase)-associated serine kinase (PAS kinase) distinct from PI 3-kinase serine kinase. We found that PI 3-kinase immune complexes contain 5-fold more wortmannin-insensitive serine kinase activity than SH2-containing protein tyrosine phosphatase-2 (SHP2) and IRS-1 immune complexes. Affinity chromatography of cell lysates with a glutathione S-transferase fusion protein for the p85 subunit of PI 3-kinase showed that PAS kinase associated with the p85 subunit of PI 3-kinase. This interaction required unoccupied SH2 domain(s) but did not require the PI 3-kinase p110 subunit binding domain. In terms of

function, PAS kinase phosphorylated IRS-1 and, after insulin stimulation, PAS kinase phosphorylated IRS-1 in PI 3-kinase-IRS-1 complexes. Phosphopeptide mapping showed that insulin-dependent *in vivo* sites of IRS-1 serine phosphorylation were comparable to those of PAS kinase phosphorylated IRS-1. More importantly, PAS kinase-dependent phosphorylation of IRS-1 reduced by 4-fold the ability of IRS-1 to act as an insulin receptor substrate. Taken together, these findings indicate that: (a) PAS kinase is distinct from the intrinsic serine kinase activity of PI 3-kinase, (b) PAS kinase associates with the p85 subunit of PI 3-kinase through SH2 domain interactions, and (c) PAS kinase is an IRS-1 serine kinase that can reduce the ability of IRS-1 to serve as an insulin receptor substrate.

INTRODUCTION

The insulin receptor substrates (IRSs) are critical for intracellular signals transduced by a variety of growth factor and cytokine receptors [1]. IRS-1 was originally described as the principal substrate for the insulin receptor [2], but further work has shown that the IGF-1 receptor [3] and the Janus kinase family members JAK1, JAK2, JAK3 and Tyk2 [4–7] phosphorylate IRS-1 in response to receptor/ligand binding. The importance of the IRSs to receptor signalling is that they can amplify receptor signals and dissociate receptor signalling from endocytic pathways [1]. Molecular analysis of IRS-1 shows that it contains multiple protein-interaction sites, including the N-terminal IH1^{PH} and IH2^{PTB} domains and 21 potential tyrosine phosphorylation sites [2,8–11]. The IH1^{PH} domain shows amino acid sequence similarity to the pleckstrin homology (PH) domains of Gab1 and DOS [1,11,12] and its deletion from IRS-1 completely inhibits insulin-receptor-dependent signalling through IRS-1 [9–11]. The IRS-1 IH2^{PTB} region is a phosphotyrosine (pY)-binding domain (PTB) which interacts with phosphorylated NPXY motifs of the insulin, insulin-like growth factor-1 and interleukin-4 receptors [2,13,14]. Substituting phenylalanine for tyrosine in insulin and interleukin-4 receptor NPXY motifs reduces the ability of these receptors to phosphorylate IRS-1 [13,14]. The tyrosine phosphorylation sites of the IRSs mediate their interaction with SH2 domain-containing proteins such as Fyn, Grb-2, nck, crk, SH2-containing protein tyrosine phosphatase-2 (SHP2) and phosphatidylinositol 3'-kinase (PI 3-kinase) [1].

The coupling of PI 3-kinase to IRS-1 is the best-characterized SH2 domain–pY interaction among IRS family members. PI 3-

kinase is a heterodimeric protein composed of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110) [15]. The p85 contains N- and C-terminal SH2 domains that bind to tyrosine-phosphorylated IRS-1 and induce activation of PI 3-kinase [15–17]. This activation is maximized when both SH2 domains are occupied by pY residues in YMXM motifs [18]. The p110 is a dual-specificity lipid and serine kinase [19,20] that is inhibited by the fungal metabolite wortmannin [15]. In the cell, the p110 phosphorylates phosphoinositides on the D-3 position of the inositol ring, generating 3,4-bis and 3,4,5-trisphosphates [15]. Phosphatidylinositol 3,4,5-trisphosphates are thought to mediate the pleiotropic actions of PI 3-kinase, including promotion of cell growth, regulation of cell survival and control of glucose metabolism. The biological significance of the p110 serine/threonine kinase is not fully understood, but *in vitro* it can phosphorylate both the p85 and IRS-1 [19–23].

Serine phosphorylation of IRS-1 is developing as an important mechanism by which IRS-dependent signal transduction can be inhibited. Treatment of cells with the serine phosphatase inhibitor okadaic acid results in increased IRS-1 serine phosphorylation, reduced IRS-1–insulin receptor binding and decreased insulin-receptor-dependent tyrosine phosphorylation of IRS-1 [24]. Similarly, IRS-1 serine phosphorylation induced by phorbol esters, tumour necrosis factor- α (TNF- α), platelet-derived growth factor, angiotensin II and insulin reduces subsequent insulin-receptor-dependent IRS-1 tyrosine phosphorylation and PI 3-kinase–IRS-1 association [25–30]. While serine phosphorylation of IRS-1 has emerged as a critical counter-regulatory mechanism in IRS-1 signalling, the serine kinases responsible for IRS-1 phosphorylation remain ill-defined. We have previously shown

Abbreviations used: GST, glutathione S-transferase; GSK-3, glycogen synthase kinase-3; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; PI 3-kinase, phosphatidylinositol 3-kinase; IRS-1, insulin receptor substrate-1; PAS kinase, PI 3-kinase associated serine kinase; pY, phosphotyrosine; SHP2, SH2-containing protein tyrosine phosphatase-2; TNF- α , tumour necrosis factor- α .

¹ To whom correspondence should be addressed (e-mail: freun@ux1.csu.uiuc.edu). Department of Pathology, College of Medicine, 506 South Mathews Avenue, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U.S.A.

that PI 3-kinase co-immunoprecipitates with an okadaic acid-sensitive serine kinase that is distinct from PI 3-kinase serine kinase [31]. Here we report that this PI 3-kinase-associated serine kinase (PAS kinase) associates with the p85 subunit of PI 3-kinase through SH2 domain interactions and that this kinase phosphorylates IRS-1 in PI 3-kinase-IRS-1 complexes after insulin stimulation. More importantly, we demonstrate that PAS kinase-dependent phosphorylation of IRS-1 reduces the ability of IRS-1 to serve as an insulin receptor substrate.

MATERIALS AND METHODS

Materials

The myeloma cell line U266 was purchased from American Type Culture Collection (Rockville, MD, U.S.A.). [γ - 32 P]ATP and 32 P_i were purchased from Amersham (Arlington Heights, IL, U.S.A.). PI 3-kinase p85, SHP2, phosphotyrosine (pY) and IRS-1 antisera and recombinant rat IRS-1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, U.S.A.). Neonatal bovine serum was purchased from Biocell (Rancho Dominguez, CA, U.S.A.). 125 I-Labelled goat anti-rabbit antibody was purchased from DuPont NEN (Wilmington, DE, U.S.A.). Protein G-Sepharose, glutathione-Sepharose and pGex4T3 vector were purchased from Pharmacia Biotech (Uppsala, Sweden). PVDF was purchased from Bio-Rad (Hercules, CA, U.S.A.). Cellulose-coated TLC plates were obtained from Analtech (Newark, DE, U.S.A.). The peptides Y608 (HTDDGYMPMSP) and pY608 (HTDDGpYMPMSP) [17] were synthesized and the anti-insulin receptor antibody α IR-1 was produced from hybridoma by the University of Illinois Biotechnology Center (Urbana, IL, U.S.A.). All other cell culture reagents and chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Oligonucleotide primers were obtained from Operon (Alameda, CA, U.S.A.). Vent DNA polymerase was from New England Biolabs (Beverly, MA, U.S.A.). All other molecular biology reagents and chemicals were purchased from Promega (Maddison, WI, U.S.A.).

Cell culture

U266 cells were grown in RPMI 1640 supplemented with 10 % (v/v) neonatal bovine serum, 2.0 g/l glucose, 100 000 units/l penicillin and 100 mg/l streptomycin. Cells were passaged 1:1 with fresh medium every 3 days. Treatment in serum-free medium before all studies was performed by washing cells twice and re-suspending them in RPMI 1640 supplemented with 0.1 % (w/v) BSA, 2.0 g/l glucose, 100 000 units/l penicillin and 100 mg/l streptomycin at 1×10^6 cells/ml. All cell counts were performed on a Coulter Counter ZM (Hialeah, FL, U.S.A.).

Expression vector construction

Bovine p85 α coding sequence was amplified from the p85 α plasmid (a gift from Michael D. Waterfield) in PCR reaction buffer containing 0.1 ng/ μ l of plasmid template, 500 nM of forward primer (5'-GAGGTCCATGAGTGCCGAGGGGT-ACCA-3'), 500 nM of reverse primer (5'-CCGAATTCTCAT-CGCCTCTGCTGCGC-3'), 200 μ M of each dNTP, 0.01 units/ μ l of Vent DNA polymerase, 2 mM MgCl₂, 10 mM KCl, 0.1 % Triton X-100 and 20 mM Tris-Cl, pH 8.8. DNA was amplified for 20 cycles in a Stratagene RoboCycler (La Jolla, CA, U.S.A.) by denaturing for 60 s at 94 °C, annealing for 60 s at 55 °C and extending for 60 s at 72 °C. The 2175 bp product was introduced into the pGex4T3 plasmid using the *Bam*HI and *Eco*RI restriction sites.

Glutathione S-transferase (GST) fusion protein isolation

GST, GST-SHP2^{SH2} (amino acids 1–107 of human SHP2; a gift from James N. Livingston), GST- Δ p85 (human PI 3-kinase p85 with the p110 binding domain, amino acids 479–513, deleted and replaced with Ser-Arg [32]; a gift from Masato Kasuga), GST-p85 and GST-p85^{NSH2} (amino acids 333–428 of the human p85 α ; a gift of Jonathan M. Backer) proteins were generated as previously described [33]. In brief, protein expression was induced in *Escherichia coli* strain BL21 by the addition of isopropyl β -D-thiogalactopyranoside to a final concentration of 1 mM. After 4 h, bacteria were lysed in 1 % Triton X-100, 1 mM PMSF, 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4, by mild sonication at 4 °C. GST fusion proteins were affinity purified from clarified lysates with glutathione-Sepharose overnight. In all experiments where GST proteins were used for affinity chromatography of cell lysates, GST proteins were equalized to protein concentration by Coomassie staining and used in excess of lysate-binding capacity.

Kinase assays

Kinase assays were performed as previously described [31]. In brief, cells were deprived of serum for 18 h and lysed in ice-cold lysis buffer [1 % (v/v) Nonidet P40/100 mM NaCl/50 mM NaF/25 mM benzamidine/1 mM PMSF/2 mM sodium orthovanadate/250 nM okadaic acid/50 mM Tris-Cl, pH 7.4, containing 2 μ g/ml aprotinin and 2 μ g/ml leupeptin]. Immune complexes/GST affinity isolates were generated with the indicated antibody or GST fusion protein, washed extensively and treated with 5 μ M wortmannin for 30 min at 4 °C. Except where noted, kinase reactions were performed in a reaction buffer containing 10 mM MgCl₂, 0.4 mM EGTA, 0.4 mM sodium phosphate, 1 μ M [γ - 32 P]ATP (100 μ Ci/nmol) and 20 mM Hepes, pH 7.1, at room temperature with the indicated substrate(s) for the described times. Reactions were terminated by addition of SDS/PAGE load buffer and phosphoproteins were resolved by SDS/PAGE under reducing conditions on 7–20 % gradient gels and examined by autoradiography and densitometry.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed as previously described [31]. In brief, phosphoproteins were resolved by SDS/PAGE on 7–20 % gradient gels and electro-transferred to PVDF. Bands of interest were excised and acid hydrolysed in 6 M constant boiling HCl for 2 h at 110 °C. Samples were treated with three cycles of water re-suspension and evaporation, and then re-suspended in water/acetic acid/pyridine (89:10:1) running buffer containing 0.3 mg/ml phosphoserine, phosphothreonine and phosphotyrosine standards. Phosphoamino acids were separated on cellulose-coated plates by high-voltage TLC and standards were visualized with ninhydrin. Results were analysed by autoradiography on a Molecular Dynamics PhosphorImager system (Sunnyvale, CA, U.S.A.).

Phosphopeptide mapping

Phosphopeptide mapping was performed as previously described [34]. In brief, phosphorylated IRS-1 was obtained (as indicated) either by whole-cell labelling studies of insulin-treated cells or by kinase assays. Whole-cell labelling studies were performed by incubating cells with 32 P_i (1 mCi/ml) for 1.5 h in phosphate-free

media. Thirty minutes before insulin treatment (10 nM for 10 min), cells were treated with 5 μ M wortmannin. Phosphorylation of IRS-1 *in vitro* with PAS kinase was performed as described in the Kinase assays section above. Phosphoproteins were resolved under reducing conditions by SDS/PAGE on 8 % gels and visualized by autoradiography. Gel bands of interest were excised and hydrated in 0.1 % (w/v) SDS/25 % (v/v) glycerol/5 % (v/v) 2-mercaptoethanol/0.125 M Tris-Cl, pH 6.8. In-gel proteolysis with 0.5 units of *Saacharomyces aureus* V8 protease was performed for 15 min at room temperature. Phosphoproteins/phosphopeptides were separated under reducing conditions by SDS/PAGE on 7–20 % gradient gels and visualized by autoradiography. Serine/threonine phosphoamino acids were assessed by their susceptibility to base hydrolysis [35].

Insulin receptor isolation and kinase assays

Cells were deprived of serum overnight and lysed in ice-cold lysis buffer. Insulin receptors were isolated from clarified lysates with protein G–Sepharose-bound insulin receptor antibody. Resultant immune complexes were washed four times in 0.1 % Triton X-100/50 mM Tris-Cl, pH 7.4, at 4 °C. IRS-1 for the insulin receptor kinase assays was pre-phosphorylated in the absence of [γ -³²P]ATP using either GST–p85 or GST–SHP2^{SH2} affinity-isolated kinase, as described in the Kinase assays section. Phosphorylated IRS-1 was then isolated from the solid-phase-bound kinases by filtration. Insulin receptor kinase assays were then performed in a reaction buffer containing 5 μ g/ml IRS-1, 1 μ M insulin/2.5 mM MgCl₂/7.5 mM MnCl₂/10 μ M ATP/0.1 % Triton X-100/50 mM Tris-Cl, pH 7.4, at room temperature for 15 min with either 10 μ M [γ -³²P]ATP (50 μ Ci/nmol) or 10 μ M ATP as indicated. Reactions were terminated using SDS/PAGE load buffer. Phosphoproteins were resolved by SDS/PAGE under reducing conditions on 7–20 % gradient gels and examined by either autoradiography or Western-blot analysis with anti-pY antibody as indicated.

Western-blot analysis

Western blotting was performed as previously described [31]. In brief, proteins were resolved by SDS/PAGE on 7–20 % gradient gels, electro-transferred to PVDF and probed with the antiserum indicated. Immunoreactive proteins were visualized by secondary detection with ¹²⁵I-labelled goat anti-rabbit antibody and autoradiography.

RESULTS

PI 3-kinase, and not SHP2 or IRS-1, associates with a wortmannin-insensitive serine kinase

We have shown that PI 3-kinase associates with a serine kinase that is distinct from PI 3-kinase serine kinase [31]. To determine whether SHP2 and/or IRS-1 associate with a serine kinase, kinase assays were performed on SHP2, IRS-1 and PI 3-kinase immune complexes isolated from myeloma cells after the immune complexes had been treated with 5 μ M wortmannin. Figure 1(A) shows that SHP2 and IRS-1 immunoprecipitates contained 5-fold less myelin basic protein (MBP) kinase activity than PI 3-kinase immune complexes. The kinase activity associated with SHP2 and IRS-1 was no greater than isotype-matched control immunoprecipitates (results not shown). To confirm that 5 μ M wortmannin completely inhibited intrinsic PI 3-kinase serine kinase activity, kinase assays were performed on PI 3-kinase immune complexes that were either pretreated or not pretreated with 5 μ M wortmannin for 30 min. As previously reported [31],

Figure 1 PI 3-kinase, not SHP2 or IRS-1, associates with a wortmannin-insensitive serine kinase

(A) PI 3-kinase (PI3K), SHP2 and IRS-1 immunoprecipitates were pretreated with 5 μ M wortmannin and kinase assays were performed with these preparations using MBP (50 μ g/ml) as a substrate. (B) PI 3-kinase immunoprecipitates were treated with (+) or without (–) 5 μ M wortmannin (Wort) for 30 min and then used in kinase assays. (C) Phosphoamino acid analysis was performed on MBP phosphorylated by PI 3-kinase immunoprecipitates. All results are representative of triplicate experiments.

5 μ M wortmannin pretreatment completely inhibited intrinsic PI 3-kinase serine kinase activity, as indicated by a loss of p85 phosphorylation (Figure 1B). To demonstrate that the MBP phosphorylation observed in Figure 1(A) was due to serine residue phosphorylation, phosphoamino acid analysis was performed. Figure 1(C) shows that MBP was predominately phosphorylated on serine residues. Taken together, these results indicate that PI 3-kinase associates with a serine kinase (PAS kinase) that is wortmannin insensitive.

PAS kinase associates with the p85 subunit of PI 3-kinase

The p85 subunit of PI 3-kinase contains protein–protein interaction sites that enable it to act as an adapter between itself and other proteins [15]. To determine whether PAS kinase associates with the p85 subunit of PI 3-kinase, GST fusion proteins were examined for PAS kinase binding capability. Figure 2(A) shows that GST–p85 affinity-purified from cell lysates was a wortmannin-insensitive serine kinase that phosphorylated MBP. This MBP kinase activity was 10-fold greater than that associated with glutathione–Sepharose and 8-fold greater than that associated with GST. To demonstrate that the MBP phosphorylation observed in Figure 2(A) was due to serine

Figure 2 PAS kinase associates with the p85 subunit of PI 3-kinase

(A) Glutathione–Sepharose-bound GST–p85 (p85), GST and glutathione–Sepharose (Con) were incubated with or without cell lysates, as indicated. Kinase assays using MBP (50 μ g/ml) as a substrate were performed with these preparations after treatment with 5 μ M wortmannin. (B) Phosphoamino acid analysis was performed on MBP phosphorylated by GST–p85. All results are representative of triplicate experiments.

residue phosphorylation, phosphoamino acid analysis was performed. Figure 2(B) shows that MBP was predominately phosphorylated on serine residues. Taken together, these findings indicate that PAS kinase associates with the p85 subunit of PI 3-kinase.

PAS kinase associates with the p85 subunit of PI 3-kinase via SH2 domain interactions and co-purifies with a 120 kDa tyrosine phosphorylated protein

Tyrosine phosphorylated peptides derived from IRS-1 sequences such as pY608 prevent the p85 N- and C-terminal SH2 domains from binding tyrosine phosphorylated proteins by competitive inhibition [17]. To delineate further the specificity of the PAS kinase/GST–p85 interaction demonstrated in Figure 2, kinase activity isolated from cell lysates with either a GST fusion protein, comprising the first SH2 domain of human SHP2 (GST–SHP2^{SH2}), or GST–p85 were analysed. Figure 3(A) demonstrates that GST–p85 bound 10-fold more MBP kinase activity than GST–SHP2^{SH2} and that GST–SHP2^{SH2} affinity-isolated a similar amount of MBP kinase activity as GST alone. To determine whether PAS kinase associates with the p85 subunit of PI 3-kinase via SH2 domain interactions, competitive inhibition studies were performed with the phosphopeptide pY608. Figure 3(B) shows that when no competing peptide was used, GST–p85 affinity-isolated 10-fold more MBP kinase activity than GST–SHP2^{SH2}. Additionally, GST– Δ p85 (a fusion protein comprising GST and bovine p85 with the p110 binding-domain deleted [32]) bound similar PAS kinase activity to GST–p85, indicating that the p110 binding domain was not necessary for PAS-kinase–PI 3-kinase interactions. When 100 μ M pY608 was added to the cell lysates before GST fusion protein affinity chromatography, binding of PAS kinase to GST–p85 and GST– Δ p85 was completely blocked. Addition of 100 μ M Y608

Figure 3 PAS kinase associates with the p85 subunit of PI 3-kinase via SH2 domain interactions and co-purifies with a 120 kDa tyrosine-phosphorylated protein

(A) Glutathione–Sepharose-bound GST–p85 (p85), GST–SHP2^{SH2} (SHP) and GST were incubated with cell lysates. Serine kinase assays using MBP (50 μ g/ml) as a substrate were performed with these preparations after treatment with 5 μ M wortmannin. (B) Glutathione–Sepharose bound GST– Δ p85 (Δ 85), GST–p85 (p85) and GST–SHP2^{SH2} (SHP) were incubated with cell lysates containing either 100 μ M Y608, 100 μ M pY608 or no peptide. Serine kinase assays, using MBP (50 μ g/ml) as a substrate, were performed on these preparations after treatment with 5 μ M wortmannin. (C) Glutathione–Sepharose-bound GST–p85 (p85) and GST–SHP2^{SH2} (SHP) were incubated with ³²P-labelled IRS-1. (D) Glutathione–Sepharose-bound GST–p85NSH2 (NSH) and GST–SHP2^{SH2} (SHP) were incubated with cell lysates containing either 100 μ M Y608, 100 μ M pY608 or no peptide. (E) Glutathione–Sepharose-bound GST–p85 was incubated with or without cell lysates and/or 100 μ M Y608 or pY608 as indicated. Phosphoproteins were detected by Western-blot analysis with anti-pY antibody. The positions of molecular-mass markers are indicated on the left. Results are representative of triplicate experiments.

to cell lysates did not alter PAS kinase binding to either GST–p85 or GST– Δ p85. It should be noted that the SH2 domains of p85 and the first SH2 domain of SHP2 recognize different amino acids immediately adjacent to phosphotyrosine [36]. The first SH2 domain of SHP2 preferentially recognizes pY-I/V-X-I/V/L/P motifs, whereas the SH2 domains of p85 preferentially

Figure 4 PAS kinase phosphorylates IRS-1 in PI 3-kinase-IRS-1 complexes

(A) Glutathione-Sepharose-bound GST-p85 (p85), GST-SHP2^{SH2} (SHP) and GST were incubated with or without cell lysates containing pY608 as indicated. Kinase assays using IRS-1 (5 µg/ml) as a substrate were performed on these preparations after treatment with 5 µM wortmannin. (B) Phosphoamino acid analysis was performed on IRS-1 phosphorylated by GST-p85. (C) PI 3-kinase was immunoprecipitated from cells treated with (+) or without (−) 10 nM insulin for 5 min. Kinase assays were performed on these preparations after treatment with 5 µM wortmannin. (D) Phosphoamino acid analysis was performed on IRS-1 phosphorylated in p85-IRS-1 complexes. (E) Experiments were performed as in (C), except that MBP was used as a substrate. All results are representative of triplicate experiments.

recognize pYXXM [17,36,37]. To confirm that both GST-p85 and GST-SHP2^{SH2} had intact SH2 domains, IRS-1 binding experiments were performed. Recombinant IRS-1 was phosphorylated with affinity-purified insulin receptors in the presence of [γ -³²P]ATP and incubated with equivalent amounts of GST-p85 and GST-SHP2^{SH2}. Figure 3(C) shows that GST-p85 and GST-SHP2^{SH2} bound similar amounts of IRS-1. To analyse the specificity of the PAS kinase-p85 interaction, PAS kinase binding to the N-terminal SH2 (NSH2) domain of p85 was examined. Figure 3(D) shows that GST-p85^{NSH2} affinity-purified 9-fold more MBP kinase activity than GST-SHP2^{SH2} and that pY608 competition completely abrogated this response. To characterize further the PAS kinase-p85 interaction, GST-p85 affinity purified cell lysates were examined by Western-blot analysis in anti-pY blots. Figure 3(E) shows that GST-p85 affinity-purified from cell lysates a 120 kDa pY-containing protein (pp120). Competitive binding studies with 100 µM pY608 and Y608 demonstrated that pY608 completely blocked pp120 from binding GST-p85, whereas Y608 had no effect. Additionally, pp120 was not immunoreactive with IRS-1 and IRS-2 antibodies by Western-blot analysis (results not shown). Taken together, these findings indicate that PAS kinase associates with the p85 subunit of PI 3-kinase via SH2 domain interactions and specifically reacts with the N-terminal SH2 domain of p85 when compared with the first SH2 domain of SHP2. Additionally, PAS kinase co-purifies with a 120 kDa phosphotyrosine-containing protein that associates with the p85 in an SH2 domain-dependent

Figure 5 PAS kinase phosphorylates IRS-1 on peptides that are phosphorylated *in vivo* in insulin-treated cells

IRS-1 was phosphorylated either in the cell by metabolic labelling with ³²P_i and insulin treatment (whole cell) or by kinase assays with GST-p85 affinity-purified PAS kinase. In-gel proteolysis of phosphorylated IRS-1 was performed with (+) or without (−) 0.5 units of *S. aureus* V8 protease for 15 min at room temperature. Results are representative of triplicate experiments. The positions of molecular-mass markers are indicated on the left.

manner, suggesting that pp120 may function as an adapter protein between PAS kinase and PI 3-kinase.

PAS kinase phosphorylates IRS-1 in PI 3-kinase-IRS-1 complexes

Insulin, interleukin-4 and interferon- α induce PI3-kinase-IRS-1 association and serine phosphorylation of IRS-1 and -2 [1,2,38,39]. To determine whether PAS kinase phosphorylates IRS-1, kinase assays were performed using IRS-1 as a substrate. Figure 4(A) shows that PAS kinase, isolated from cell lysates with GST-p85, phosphorylated IRS-1 5-fold more than control isolates. Additionally, IRS-1 phosphorylation by PAS kinase was completely inhibited when pY608 was used to competitively inhibit PAS kinase binding to GST-p85. To determine whether the IRS-1 phosphorylation observed in Figure 4(A) was due to serine residue phosphorylation, phosphoamino acid analysis was performed. Figure 4(B) demonstrates that IRS-1 was predominately phosphorylated on serine residues. To determine whether PAS kinase phosphorylates IRS-1 in PI 3-kinase-IRS-1 complexes, kinase assays were performed on PI 3-kinase immunoprecipitates isolated from cells treated or not treated with 10 nM insulin for 5 min. Figure 4(C) demonstrates that after insulin stimulation IRS-1 was phosphorylated in PI 3-kinase-IRS-1 complexes by PAS kinase, and that this phosphorylation was predominately on serine residues (Figure 4D). To confirm that IRS-1 did not introduce a serine kinase into the PI 3-kinase-IRS-1 complex, kinase assays utilizing MBP as a substrate were performed on PI 3-kinase immunoprecipitates isolated from insulin-treated cells. Figure 4(E) demonstrates that PI 3-kinase immune complexes had identical MBP phosphorylation before and after insulin treatment. Taken together, these findings suggest that, after insulin stimulation, PAS kinase phosphorylates IRS-1 in PI 3-kinase-IRS-1 complexes.

PAS kinase phosphorylates IRS-1 on peptides that are phosphorylated *in vivo* in insulin-treated cells

As shown in Figure 4, PAS kinase phosphorylates IRS-1 *in vitro*. To determine whether the serine/threonine residues on IRS-1,

Figure 6 Phosphorylation of IRS-1 by PAS kinase reduces IRS-1 phosphorylation by the insulin receptor

(A) Glutathione-Sepharose-bound GST-p85 (p85) and GST-SHP^{2SH2} (SHP) were incubated with cell lysates. These preparations were used to pre-phosphorylate IRS-1 (5 μ g/ml, no [γ -³²P]ATP) after treatment with 5 μ M wortmannin. IRS-1 was recovered from these pre-phosphorylation reactions and used in insulin receptor kinase assays. (B) IRS-1 pre-phosphorylation and insulin receptor kinase assays were performed as in (A) in the absence of [γ -³²P]ATP. IRS-1 and insulin receptor phosphorylation were detected by anti-pY blotting. All results are representative of triplicate experiments. The positions of molecular-mass markers are indicated on the left.

which PAS kinase phosphorylates, are comparable to those phosphorylated *in vivo* after insulin stimulation, phosphopeptide mapping was performed. Figure 5 shows that IRS-1 phosphorylated by PAS kinase and digested in-gel with V8 protease generated three phosphopeptides with molecular masses of 32 kDa, 26 kDa and 16 kDa. In-gel proteolysis of IRS-1 isolated from insulin-treated cells generated seven phosphopeptides, with molecular masses of 40 kDa, 32 kDa, 26 kDa, 21 kDa, 20 kDa, 18 kDa and 16 kDa. Comparison of these two phosphopeptide maps shows that IRS-1 phosphorylated by PAS kinase shared three phosphopeptides (ppt) with IRS-1 isolated from insulin treated cells, ppt32 kDa, ppt28 kDa and ppt16 kDa. In addition, alkaline hydrolysis of these phosphopeptides resulted in complete loss of phosphorylation (results not shown), consistent with their being predominantly phosphorylated on serine/threonine residues. Taken together, these results indicate that PAS kinase phosphorylates IRS-1 on serine/threonine residues that are phosphorylated in insulin-treated whole cells.

Phosphorylation of IRS-1 by PAS kinase reduces IRS-1 phosphorylation by the insulin receptor

Serine phosphorylation of IRS-1 by glycogen synthase kinase-3 (GSK-3) [40] and mitogen-activated protein kinases (MAP kinases) [41] reduces the ability of IRS-1 to act as an insulin receptor substrate. To determine whether phosphorylation of IRS-1 by PAS kinase reduces insulin-receptor-dependent phosphorylation of IRS-1, insulin receptor kinase assays were performed on IRS-1 that had been pre-phosphorylated by PAS kinase. Figure 6(A) shows that insulin-receptor-dependent phosphorylation of IRS-1 was reduced 4-fold when IRS-1 was pre-

phosphorylated by GST-p85 affinity-purified PAS kinase when compared with control isolates. To confirm that this effect was not due to a contaminating tyrosine kinase in the PAS kinase preparations, the above experiments were repeated in the absence of [γ -³²P]ATP and analysed by Western blotting with anti-pY antibody detection. Figure 6(B) shows that the effects observed in Figure 6(A) were not due to pre-phosphorylation of IRS-1 on tyrosine residues by a contaminating tyrosine kinase. Taken together, these results indicate that PAS kinase is an IRS-1 serine kinase that decreases the ability of IRS-1 to serve as an insulin receptor substrate.

DISCUSSION

The results obtained establish that PI 3-kinase associates with a serine kinase (PAS kinase) that phosphorylates IRS-1 and reduces the ability of IRS-1 to serve as an insulin receptor substrate. Serine kinase assays demonstrated that PI 3-kinase co-immunoprecipitates with a serine kinase distinct from PI 3-kinase serine kinase (Figure 1). This PAS kinase activity was associated with the p85 subunit of PI 3-kinase (Figure 2), required SH2 domain interactions for its association with the p85 (Figures 3A–3D) and co-purified with a 120 kDa tyrosine phosphoprotein that was itself dependent upon SH2 domain interactions in its binding to p85 (Figure 3E). PAS kinase used IRS-1 as a substrate (Figures 4A and 4B), phosphorylated IRS-1 in PI 3-kinase–IRS-1 complexes after insulin stimulation (Figures 4C and 4E), and phosphorylated IRS-1 on serine/threonine residues comparable to those phosphorylated in insulin-treated whole cells (Figure 5). More importantly, PAS kinase-dependent phosphorylation of IRS-1 inhibited subsequent insulin-receptor-dependent phosphorylation of IRS-1 (Figure 6). Taken together, the present results indicate that PAS kinase is a novel PI 3-kinase-associated IRS-1 serine kinase and suggest that it regulates IRS-1 serine phosphorylation after PI 3-kinase–IRS-1 association.

SH2 domains mediate protein–protein interactions through their ability to bind to phosphotyrosine residues [37]. These domains have specificity and can discriminate between phosphotyrosines based on the three amino acids C-terminal to the phosphotyrosine [36]. The first SH2 domain of SHP2 recognizes phosphorylated tyrosine (pY) in the context of pY-I/V-X-I/V/L/P, whereas the SH2 domains of PI 3-kinase recognize phosphorylated tyrosine in the context of pY-X-X-M [17,36,37]. SH2 domains have little affinity for non-phosphorylated tyrosine or phosphorylated tyrosine in motifs that lack the appropriate C-terminal amino acids [37]. PAS kinase associates with PI 3-kinase through SH2 interactions with the p85 subunit. Association of PAS kinase with p85 displays SH2 domain specificity, in that PAS kinase does not bind to the first SH2 domain of SHP2, but does bind to the N-terminal SH2 domain of PI 3-kinase. In addition, PAS kinase co-purifies with a 120 kDa tyrosine phosphorylated protein (pp120) that associates with the p85 in an SH2 domain-dependent manner. This result is not surprising, as PI 3-kinase has been observed to associate with a variety of unidentified tyrosine-phosphorylated proteins with molecular masses ranging from 115–130 kDa [5,42–44], and a pp120 can be affinity-purified from unstimulated FDMAC11/4.5 cells with a p85 NSH2 domain [43]. Previously, we have shown that PI 3-kinase associates with a wortmannin-insensitive serine kinase and that this kinase is not demonstrably phosphorylated on tyrosine residues [31]. Therefore, our current model for the PAS kinase–p85 interaction is one in which pp120 serves as an adapter protein linking PAS kinase to the p85 subunit of PI 3-kinase.

Serine phosphorylation of IRS-1 reduces its ability to serve as

an insulin receptor substrate and may be critical to TNF- α -dependent insulin resistance [24–30,40,41]. Recent efforts to identify the kinases responsible for IRS-1 serine phosphorylation have focused on the extracellular signal-regulated (ERK) 1 and 2 MAP kinases, since IRS-1 contains consensus sites for MAP kinase phosphorylation [2] and certain MAP kinase family members can be activated by okadaic acid, a potent effector of IRS-1 serine phosphorylation [24,45,46]. Interestingly, phorbol esters induce Erk1/2-mediated phosphorylation of IRS-1 on Ser-612 and lead to a subsequent decrease in insulin-receptor-dependent tyrosine phosphorylation of IRS-1 [25,41], but inhibition of MAP kinase activity does not block okadaic acid-dependent IRS-1 serine phosphorylation [45]. Additionally, whereas replacement of IRS-1 serine residues 612/632 with alanine leads to increased insulin-dependent tyrosine phosphorylation of IRS-1 and increased PI 3-kinase–IRS-1 association, replacement of these residues has no effect on okadaic acid-dependent IRS-1 serine phosphorylation [45]. Moreover, MAP kinase does not appear to play a role in the serine phosphorylation of IRS-1 secondary to chronic insulin stimulation [47]. Thus, other kinases must be involved in IRS-1 serine phosphorylation. Another potential IRS-1 serine kinase is PI 3-kinase. PI 3-kinase is a dual-specificity lipid and serine kinase that can phosphorylate its own p85 subunit and IRS-1 *in vitro* [19–23]. Studies to date, however, have not clearly demonstrated that PI 3-kinase has biologically significant serine kinase activity *in vivo*. Recently, GSK-3 has been shown to phosphorylate IRS-1 in CHO-IR cells engineered to co-express GSK-3 and IRS-1 [40]. In addition, these cells show a decrease in insulin receptor-mediated IRS-1 tyrosine phosphorylation [40]. Since insulin stimulation inactivates GSK-3 [48], regulation of IRS-1 serine phosphorylation by GSK-3 may serve as a feed-forward step in the insulin signalling cascade. Finally, evidence is accumulating to suggest that serine phosphorylation within the IRS-1 IH2^{PTB} domain may counter-regulate IRS-1 signalling. Recent studies have shown that the IRS-1 docking protein 14-3-3 binds to phosphoserine 270, blocking insulin-dependent tyrosine phosphorylation of IRS-1 and insulin-dependent PI 3-kinase–IRS-1 association [49,50]. Furthermore, okadaic acid and TNF- α reduce the ability of IRS-1 to interact with the insulin receptor NPEY motif [28] which is thought to coordinate the association of the insulin receptor with the IH2^{PTB} [13]. In summary, we have shown previously that PI 3-kinase associates with a serine kinase distinct from its own intrinsic serine kinase [31]. Here we report that this kinase is capable of phosphorylating IRS-1 and that it phosphorylates IRS-1 in PI 3-kinase–IRS-1 complexes after insulin stimulation. Since insulin treatment of cells induces serine phosphorylation of IRS-1 [2], we suggest that PAS kinase may be involved in this process. We conclude from this study that PI 3-kinase associates with a wortmannin-insensitive IRS-1 serine kinase and that this kinase phosphorylates IRS-1 in PI 3-kinase–IRS-1 complexes after insulin stimulation and decreases the ability of IRS-1 to serve as an insulin receptor substrate. Together, these findings suggest that PAS kinase may modulate IRS-1-dependent signal transduction through serine phosphorylation of IRS-1.

This research was supported by National Institutes of Health Grant CA-61931 (to G. G. F.) and American Heart Association, Illinois Affiliate Grant 97-GB-02 (to G. G. F.).

REFERENCES

- Yenush, L. and White, M. F. (1997) *Bioessays* **19**, 491–500
- White, M. F. and Kahn, C. R. (1994) *J. Biol. Chem.* **269**, 1–4
- Myers, Jr., M. G., Sun, X. J., Cheatham, B., Jachna, B. R., Glasheen, E. M., Backer, J. M. and White, M. F. (1993) *Endocrinology* **132**, 1421–1430
- Johnson, J. A., Wang, L. M., Hanson, E. P., Sun, X. J., White, M. F., Oakes, S. A., Pierce, J. H. and O'Shea, J. J. (1995) *J. Biol. Chem.* **270**, 28527–28530
- Uddin, S., Yenush, L., Sun, X. J., Sweet, M. E., White, M. F. and Platanias, L. C. (1995) *J. Biol. Chem.* **270**, 15938–15941
- Platanias, L. C., Uddin, S., Yetter, A., Sun, X. J. and White, M. F. (1996) *J. Biol. Chem.* **271**, 278–282
- Burfoot, M. S., Rogers, N. C., Watling, D., Smith, J. M., Pons, S., Paonessaw, G., Pellegrini, S., White, M. F. and Kerr, I. M. (1997) *J. Biol. Chem.* **272**, 24183–24190
- Chen, D., Van Horn, D. J., White, M. F. and Backer, J. M. (1995) *Mol. Cell. Biol.* **15**, 4711–4717
- Myers, Jr., M. G., Grammer, T. C., Brooks, J., Glasheen, E. M., Wang, L. M., Sun, X. J., Blenis, J., Pierce, J. H. and White, M. F. (1995) *J. Biol. Chem.* **270**, 11715–11718
- Yenush, L., Makati, K. J., Smith-Hall, J., Ishibashi, O., Myers, Jr., M. G. and White, M. F. (1996) *J. Biol. Chem.* **271**, 24300–24306
- Burks, D. J., Pons, S., Towery, H., Smith-Hall, J., Myers, Jr., M. G., Yenush, L. and White, M. F. (1997) *J. Biol. Chem.* **272**, 27716–27721
- Raabe, T., Riesgo-Escovar, J., Liu, X., Bausenwein, B. S., Deak, P., Maroy, P. and Hafen, E. (1996) *Cell* **85**, 911–920
- White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A. and Kahn, C. R. (1988) *Cell* **54**, 641–649
- Keegan, A. D., Nelms, K., White, M., Wang, L. M., Pierce, J. H. and Paul, W. E. (1994) *Cell* **76**, 811–820
- Kapeller, R. and Cantley, L. C. (1994) *Bioessays* **16**, 565–576
- Backer, J. M., Myers, Jr., M. G., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J. and White, M. F. (1992) *EMBO J.* **11**, 3469–3479
- Sun, X. J., Crimmins, D. L., Myers, Jr., M. G., Miralpeix, M. and White, M. F. (1993) *Mol. Cell. Biol.* **13**, 7418–7428
- Rordorf-Nikolic, T., Van Horn, D. J., Chen, D., White, M. F. and Backer, J. M. (1995) *J. Biol. Chem.* **270**, 3662–3666
- Carpenter, C. L., Auger, K. R., Duckworth, B. C., Hou, W. M., Schaffhausen, B. and Cantley, L. C. (1993) *Mol. Cell. Biol.* **13**, 1657–1665
- Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N. F., Truong, O., Vicendo, P., Yonezawa, K. et al. (1994) *EMBO J.* **13**, 522–533
- Lam, K., Carpenter, C. L., Ruderman, N. B., Friel, J. C. and Kelly, K. L. (1994) *J. Biol. Chem.* **269**, 20648–20652
- Tanti, J. F., Greameaux, T., Van Obberghen, E. and Le Marchand-Brustel, Y. (1994) *Biochem. J.* **304**, 17–21
- Freund, G. G., Wittig, J. G. and Mooney, R. A. (1995) *Biochem. Biophys. Res. Commun.* **206**, 272–278
- Tanti, J. F., Greameaux, T., van Obberghen, E. and Le Marchand-Brustel, Y. (1994) *J. Biol. Chem.* **269**, 6051–6057
- De Fea, K. and Roth, R. A. (1997) *Biochemistry* **36**, 12939–12947
- Kanety, H., Feinstein, R., Papa, M. Z., Hemi, R. and Karasik, A. (1995) *J. Biol. Chem.* **270**, 23780–23784
- Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F. and Spiegelman, B. M. (1996) *Science* **271**, 665–668
- Paz, K., Hemi, R., LeRoith, D., Karasik, A., Elhanany, E., Kanety, H. and Zick, Y. (1997) *J. Biol. Chem.* **272**, 29911–29918
- Ricort, J. M., Tanti, J. F., Van Obberghen, E. and Le Marchand-Brustel, Y. (1997) *J. Biol. Chem.* **272**, 19814–19818
- Folli, F., Kahn, C. R., Hansen, H., Bouchie, J. L. and Feener, E. P. (1997) *J. Clin. Invest.* **100**, 2158–2169
- Cengel, K. A., Godbout, J. P. and Freund, G. G. (1998) *Biochem. Biophys. Res. Commun.* **242**, 513–517
- Hara, K., Yonezawa, K., Sakaue, H., Ando, A., Kotani, K., Kitamura, T., Kitamura, Y., Ueda, H., Stephens, L., Jackson, T. et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7415–7419
- Smith, D. B. and Johnson, K. S. (1988) *Gene* **67**, 31–40
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Duclos, B., Marcandier, S. and Cozzano, A. J. (1991) *Methods Enzymol.* **201**, 10–21
- Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnoffsky, S., Lechleider, R. J. et al. (1993) *Cell* **72**, 767–778
- Beattie, J. (1996) *Cell. Signalling* **8**, 75–86
- Haddad, T. C. and Conover, C. A. (1997) *J. Biol. Chem.* **272**, 19525–19531
- Uddin, S., Fish, E. N., Sher, D. A., Gardziola, C., White, M. F. and Platanias, L. C. (1997) *J. Immunol.* **158**, 2390–2397
- Eldar-Finkelman, H. and Krebs, E. G. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9660–9664

- 41 De Fea, K. and Roth, R. A. (1997) *J. Biol. Chem.* **272**, 31400–31406
- 42 Hayashi, H., Kamohara, S., Nishioka, Y., Fumihiko, K., Miyake, N., Fukui, Y., Shibasaki, F., Takenawa, T. and Ebina, Y. (1992) *J. Biol. Chem.* **267**, 22575–22580
- 43 Welham, M. J., Dechert, U., Leslie, K. B., Jirik, F. and Schrader, J. W. (1994) *J. Biol. Chem.* **269**, 23764–23768
- 44 Imani, F., Rager, K. J., Catipovic, B. and Marsh, D. G. (1997) *J. Biol. Chem.* **272**, 7927–7931
- 45 Mothe, I. and Van Obberghen, E. (1996) *J. Biol. Chem.* **271**, 11222–11227
- 46 Miyasaka, T., Miyasaka, J. and Saltiel, A. R. (1990) *Biochem. Biophys. Res. Commun.* **168**, 1237–1243
- 47 Sun, X. J., Qiao, L. Y. and Goldberg, J. L. (1998) *Diabetes*, **47** (Supp. 1), A35
- 48 Welsh, G. I. and Proud, C. G. (1993) *Biochem. J.* **294**, 625–629
- 49 Ogihara, T., Isobe, T., Ichimura, T., Taoka, M., Funaki, M., Sakoda, H., Onishi, Y., Inukai, K., Anai, M., Fukushima, Y. et al. (1997) *J. Biol. Chem.* **272**, 25267–25274
- 50 Kosaki, A., Yamada, K., Suga, J., Otaka, A. and Kuzuya, H. (1998) *J. Biol. Chem.* **273**, 940–944

Received 4 February 1998/29 June 1998; accepted 6 August 1998