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Interaction of Insulin Receptor Substrate-1 (IRS-1) with Phosphatidylinositol 3-Kinase: Effect of Substitution of Serine for Alanine in Potential IRS-1 Serine Phosphorylation Sites

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ABSTRACT

Serine and threonine phosphorylation has been shown to down-regulate insulin signaling at multiple steps, including the receptor and downstream molecules such as insulin receptor substrate-1 (IRS-1). To further address the mechanism of this regulation at the level of IRS-1, we constructed a double serine mutant of IRS-1: S662A/S731A-IRS-1. The serines 662 and 731 mutated to alanine are surrounding tyrosines Y658 and Y727, respectively. These tyrosines are comprised in YXXM motifs, which are potential binding sites for the p85α regulatory subunit of phosphatidylinositol (PI) 3-kinase. In a first series of experiments using the yeast two-hybrid system, we show that IRS-1 interacts with p85α, and this interaction depends on tyrosine phosphorylation, as shown with the IRS-1 mutant F18 and 3Y-IRS-1. F18-IRS-1 contains 18 potential tyrosine phosphorylation sites mutated to phenylalanine; three of them, *i.e.* Y608, 628, and 658, which are potential binding sites for p85α, have been added back in the 3Y-IRS-1 mutant. The tyrosine phosphorylation of IRS-1, which is required for the interaction with p85α, is thought to occur via endogenous yeast kinases that phosphorylate IRS-1 at least on these PI 3-kinase-binding sites.

Next, we show that not only p85α but also p55^{PIK}, another regulatory subunit of PI 3-kinase, interacts with IRS-1 in yeast. Interestingly, for both regulatory subunits their interaction with IRS-1 is up-regulated by mutating serines 662 and 731 on IRS-1.

In a previous study we found that insulin-stimulated PI 3-kinase activity was increased not only in the presence of S662A/S731A-IRS-1 but also under resting conditions compared with the activity seen with WT-IRS-1.

Here we demonstrate in 293-EBNA cells overexpressing S662A/S731A-IRS-1 that insulin-stimulated protein kinase B activity is not augmented, whereas without insulin treatment, basal activity is increased compared with that in cells overexpressing wild-type IRS-1. In conclusion, we have shown that 1) potential serine phosphorylation sites on IRS-1, which are adjacent to YXXM binding motifs for PI 3-kinase, negatively regulate binding of IRS-1 to PI 3-kinase regulatory subunits; and 2) these modulations affect protein kinase B activity. (*Endocrinology* **139:** 4911–4919, 1998)

INSULIN binding to its receptor α-subunits activates the tyrosine kinase activity of the β-subunits, which allows subsequent tyrosine phosphorylation of receptor substrates such as Shc, insulin receptor substrate-1 (IRS-1), IRS-2, IRS-3, and IRS-4 (1–3). The IRS proteins then become docking molecules that interact via their phosphorylated tyrosines with SH2 domain-containing effectors, leading to the biological effects of the hormone (4–6).

In addition to its tyrosine phosphorylation, IRS-1 is found to be heavily phosphorylated on serine residues in intact cells. Mounting evidence indicates that serine phosphorylation could negatively regulate insulin action. For example, we and others have reported that in various insulin-sensitive cells, okadaic acid and angiotensin II are able to induce IRS-1 serine and threonine phosphorylation, which correlates with a decrease in IRS-1 tyrosine phosphorylation and a subsequent decrease in IRS-1/p85 association and IRS-1-associated phosphatidylinositol (PI) 3-kinase upon insulin stimulation (7–9). In 3T3-F442A adipocytes, tumor necrosis factor-α has also been shown to induce IRS-1 serine phosphorylation (10). This serine-phosphorylated IRS-1 is thought to interact differently with the insulin receptor (IR) compared with wild-type IRS-1 (WT-IRS-1), resulting in inhibition of IR tyrosine kinase activity. It was recently shown in CHO cells that in the absence of growth factor or insulin, glycogen synthase kinase-3 (GSK-3) is activated and phosphorylates IRS-1 on serine residues. This is correlated with a decrease in IRS-1 tyrosine phosphorylation and IR tyrosine kinase activity (11). Moreover, in 293 cells, phorbol 12-myristate 13-acetate (PMA) treatment stimulates mitogen-activated protein (MAP) kinase, which phosphorylates IRS-1 on serine 612, resulting in inhibition of insulin signaling (12, 13).

Taken together, these data indicate that serine phosphorylation of either the IR or IRS-1 modulates insulin action and is implicated in the negative regulation of hormone signaling. Hence, this process is likely to contribute at least in part to the insulin resistance found in disease states such as obesity and type II diabetes.

To investigate the molecular mechanism underlying the modulation of insulin signaling by IRS-1 serine phosphorylation, we constructed serine mutants of IRS-1 at the level of YXXMSP motifs that are potential binding sites for p85.

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These mutants, S612A/S632A-IRS-1 and S662A/S731A-IRS-1, correspond to serine mutated to alanine surrounding tyrosines Y608/Y628 and Y658/Y727, respectively. Expressing these mutants in 293 cells, we found that these potential serine phosphorylation sites are negative regulators of basal and insulin-induced IRS-1-associated PI-3 kinase activities. Indeed, mutation of these serines increases tyrosine phosphorylation of IRS-1, its binding to p85, and its associated PI 3-kinase activity (8). PI 3-kinase is a cytosolic enzyme implicated in various cellular functions, such as insulin-induced glucose transport (14) and membrane ruffling (15). PI 3-kinase is composed of a regulatory subunit of 85 kDa and a catalytic subunit of 110 kDa, which has lipid kinase and serine kinase activities (16, 17). p85 SH2 domains recognize phosphorylated IRS-1 tyrosine residues in YXXM motifs (18, 19). Recently, several novel regulatory subunits of PI 3-kinase have been cloned, including 1) p55α and p50α, which are alternatively spliced isoforms of the p85α gene; 2) p62^{cplx}; and 3) p55^{PIK} (20–23). Compared with p85, p55^{PIK} contains a conserved region with p85 in its C-terminal part comprising two SH2 domains and an inter-SH2 domain, but it lacks several domains in its N-terminus (*i.e.* Bcr, SH3, and one proline-rich domain) that are replaced by a 34-amino acid sequence. In the present study we used the yeast two-hybrid system to examine the interaction of p85α and p55^{PIK} with IRS-1. In this system we investigated the importance of IRS-1 tyrosine phosphorylation using IRS-1 tyrosine mutants (F18 and 3Y) with or without coexpression of the IR. Using the IRS-1 serine mutant S662A/S731A, we also determined whether the interaction of IRS-1 with the regulatory subunits of PI 3-kinase could be modulated by serine sites. Finally, we show in intact 293 cells that overexpression of the S662A/S731A-IRS-1 mutant increases the basal activity of protein kinase B (PKB) compared with that of WT-IRS-1. Taken together, our data indicate that IRS-1 serines 662 and 731 could play an important role in modulating downstream effectors of IRS-1, and hence insulin action.

Materials and Methods

Materials

Yeast strain L40 (MATa, Trp^1, Leu^2, His^3, Lys^2::*lexA*-His^3, URA3: *lexA-lacZ*) and yeast two-hybrid expression vector pBTM116 (pLex) were provided by A. Vojtek (Seattle, WA); the plasmid pACTII was provided by S. Elledge (Houston, TX). Full-length p85α complementary DNA (cDNA) into the pGBT9 vector was a gift from J. E. Pessin (Iowa City, IA). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium), restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA), Pwo DNA polymerase was obtained from Boehringer Mannheim (Meylan, France), and synthetic defined dropout yeast media lacking the appropriate amino acids were purchased from BIO-101 (La Jolla, CA). Cell culture media and geneticin were purchased from Life Technologies, Inc. (Paisley, Scotland). All chemical reagents used were obtained from Sigma Chemical Co., Inc. (St. Louis, MO), except protein A-Sepharose, which was obtained from Pharmacia Biotech, Inc. (Uppsala, Sweden); 2-mercaptoethanol and 3-aminophtalhydrazide were purchased from Fluka (Buchs, Switzerland). Antibodies to IRS-1 for immunoprecipitation and Western blotting were raised to the C-terminal (1223–1235) peptide of rat IRS-1.

Plasmid constructions

WT-IRS-1 cDNA and IRS-1 mutants F18, 3Y, and S662A/S731A cDNAs (8, 24) were subcloned in-frame into the pACTII vector using convenient restriction sites, yielding GAD-WT-IRS-1, GAD-F18-IRS-1, and GAD-3Y-IRS-1, GAD-S662A/S731A-IRS-1 fusion proteins, respectively. Full-length p55^{PIK} cDNA was amplified by PCR using the following primers: sense, 5′-ccggaattcgaccgcgatgacgcagactg-3′; and antisense, 5′ tcccccgggttatctgcagagcgtaggc-3′. Then the fragment was subcloned in-frame into the polylinker of pBTM116 using the *Eco*RI (5′-side) and *Sma*I (3′-side) restriction sites. The fusion protein obtained (LexA-p55^{PIK}) corresponded to the LexA DNA binding domain (1–147) fused to p55^{PIK}. LexA-p85α was obtained by PCR amplification of p85α cDNA using the following set of primers: sense, 5′-gccgaggggtacgaattccgggcgctg-3′; antisense, 5′-atcgcctcggatccgcgtacactgggtagg-3′, and then subcloning the PCR product in-frame into *Eco*RI and *Bam*HI sites of pBTM116. Correct in-frame fusion between LexA and p55^{PIK} or p85α cDNA was verified by sequencing with a primer corresponding to a LexA sequence (5′-cttcgtcagcagagcttc-3′), using the T7 sequencing kit (Pharmacia Biotech).

Constructs encoding hemagglutinin (HA)-tagged PKB in the mammalian expression vector pECE were gifts from Brian Hemmings and have been described previously (25).

Yeast transformation and reporter gene activity

The yeast strain L40 was cotransformed with pBTM116 and pACTII plasmids expressing hybrid proteins of interest, using the lithium acetate method (26). L40 were grown for 48 h on plates containing Trp$^-$, Leu$^-$ complete supplemented (CS) medium to select clones containing both plasmids (pBTM116 and pACTII carry the Trp$^+$ and Leu$^+$ selection markers, respectively). Suppression of IRβ gene expression carried on the pBTM116 vector was accomplished by addition of L-methionine (Sigma Chemical Co.) at 20 mM to the medium. The histidine reporter gene was tested by replicating the clones expressing the different sets of plasmids on plates containing CS medium without tryptophan, leucine, and histidine and by growing them at 30 C for 48 h. Double transformants were also assayed for β-galactosidase activity, using a color filter assay as previously described (27) or a liquid culture assay. Briefly, three clones of each transformation were grown for 24 h in Trp$^-$, Leu$^-$ CS medium, then diluted 10-fold in 2 ml of the same CS medium. After 24 h of additional growth, 1 ml of cells was used for determination of OD at 600 nm; 100–500 μl of cells were used for colorimetric assay at 574 nm. Cells were pelleted, resuspended in 500 μl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 40 mM KCl, and 1 mM $MgSO_4$)-25 μl chloroform, and vortexed for 15 sec. After a 10-min incubation at 30 C, 100 μl of the chromogenic substrate chlorophenol red-β-D-galactopyranoside at 50 mM were added. The reaction was performed at 30 C, and β-galactosidase activity was measured according to Millers' method (28). One unit of β-galactosidase activity was defined as follows: $(A_{574} \times 1000)/[A_{600} \times \text{volume (ml)} \times \text{time (min)}]$.

Protein expression in yeast

Cotransformed yeast on plates expressing hybrid proteins were grown for 10 h at 30 C in 1 ml Trp$^-$, Leu$^-$ CS medium, then for an additional 12 h in the same medium adjusted to 50 ml. One milliliter of yeast diluted 10-fold was used for OD quantification at 600 nm. The cells were washed in PBS, frozen in nitrogen for 10 min, and placed at −20 C for another 10 min. Then the cells were lysed on ice for 20 min with buffer A (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, and 100 mM NaF, pH 7.5) supplemented with 1% (vol/vol) Triton X-100 and protease inhibitors: 100 U/ml aprotinin, 1 mM $PhMeSO_2F$ (phenylmethylsulfonylfluoride), 20 mM leupeptin, 2 mM pepstatin, and 4 mM benzamidine. Then glass beads (425–600 μm; Sigma Chemical Co.) were added, and the tubes were vortexed for 30 sec and maintained on ice. This step was repeated three times. After the tubes were shaken for 20 min at 4 C and centrifuged for 30 min at 13,000 rpm, immunoprecipitation with rabbit polyclonal antibodies to IRS-1 was performed for 2.5 h at 4 C. Samples were washed alternatively five times with buffer A supplemented with 1% (vol/vol) Triton X-100 and buffer A supplemented with 0.2% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, and 0.5 M NaCl. Samples were resuspended in Laemmli buffer (29), loaded on 7.5% polyacrylamide gel, and subjected to SDS-PAGE under reducing conditions. Proteins were transferred to an Immobilon membrane (Millipore Corp., Milford, MA). The membrane was blocked with 10 mM Tris-HCl and 140 mM NaCl, pH 7.4, supplemented

with 5% (wt/vol) BSA and probed with either rabbit polyclonal antibodies to IRS-1 or a mouse monoclonal antibody to phosphotyrosine, followed by incubation with [^{125}I]protein A.

Cell culture and transfection

HA-tagged WT-PKB, WT-IRS-1, and S662A/S731A-IRS-1 were transiently expressed in 293 EBNA cells, which are human embryo kidney cells constitutively expressing the EBNA-1 protein from the Epstein-Barr virus (Invitrogen, San Diego, CA). The cells were cultured in DMEM containing 5% (vol/vol) FCS and 500 μg/ml geneticin. Transfection was performed by the calcium phosphate precipitation method of Chen and Okayama (30) (3 μg DNA/9.5-cm^2 dish). Eighteen hours after transfection, the calcium phosphate-DNA precipitates were removed, and cells were incubated in DMEM supplemented with 0.2% (wt/vol) BSA for 20 h before the experiment.

Immunoblot, immunoprecipitation, and in vitro kinase assay of PKB

293-EBNA cells were stimulated or not with 10^{-6} M insulin for 5 min and washed in PBS. Then they were lysed in a buffer containing 50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM vanadate, 0.5 mM phenylmethylsulfonylfluoride, 100 IU/ml aprotinin, 20 μM leupeptin, and 1% (vol/vol) Triton X-100. The lysates were centrifuged at 15,000 $\times$ *g* at 4 C for 15 min, and 90% of each sample was immunoprecipitated using anti-HA antibody (12CA5) coupled to protein G-Sepharose. After 2.5 h, the pellets were washed, and phosphorylation buffer containing 50 mM Tris, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM ATP, 3.3 μCi [γ-^{32}P]ATP, and 30 μM Crosstide (Neosystem, Strasbourg, France) as substrate was added. After 30 min at room temperature, the reaction was stopped by spotting 40 μl onto Whatman p81 papers, and immersing in 1% (vol/vol) orthophosphoric acid. After three washes, the papers were air-dried, and radioactivity was determined by Cerenkov counting. We subtracted the background obtained from phosphorylation alone. Ten percent of each lysate was analyzed by SDS-PAGE under reducing conditions, and proteins were transferred to an Immobilon membrane (Immobilon polyvinylidene difluoride, Millipore Corp.). The membrane was cut and immunoblotted with antibodies to either IRS-1 or PKB (provided by B. Hemmings and raised against a peptide containing amino acids 469–480 of PKB), followed by [^{125}I]protein A and autoradiography.

Results

Interaction of p85α with IRS-1 in yeast two hybrid system: role of IRS-1 tyrosine phosphorylation

To study the interaction of the regulatory subunits of PI 3-kinase with IRS-1, we used the yeast two-hybrid system. We constructed hybrid proteins containing either the DNA-binding domain of the bacterial repressor LexA or the activation domain of the transcription factor GAL4, fused to p85α and IRS-1, respectively. These two hybrid proteins, LexA-p85α and GAD-IRS-1, were coexpressed in yeast, and a colorimetric experiment was performed to measure β-galactosidase activity, which reflects interaction between the two proteins. As the interaction between IRS-1 and p85α depends on IRS-1 tyrosine phosphorylation, we coexpressed IRβ, IRS-1, and p85α in yeast. To do so, we constructed a modified pLex vector in which receptor β-subunit (IRβ) is under control of a promoter repressible by methionine to increase phosphorylation of IRS-1 (31). We verified that the IR intracellular region is expressed in the absence of methionine, and that addition of the repressing molecule prevents expression. As negative controls, we coexpressed LexA-p85α and GAD-IRS-1 with the unrelated proteins GAD-*ras* and LexA-lamin, respectively. LexA-p85α and GAD-IRS-1 did not induce detectable β-galactosidase activity corresponding to nonspecific interaction (data not shown). As shown in Fig. 1, in the absence of methionine, *i.e.* when IRβ was expressed, a 4-fold increase in the interaction between IRS-1 and p85α was seen. However, β-galactosidase activity due to interac-

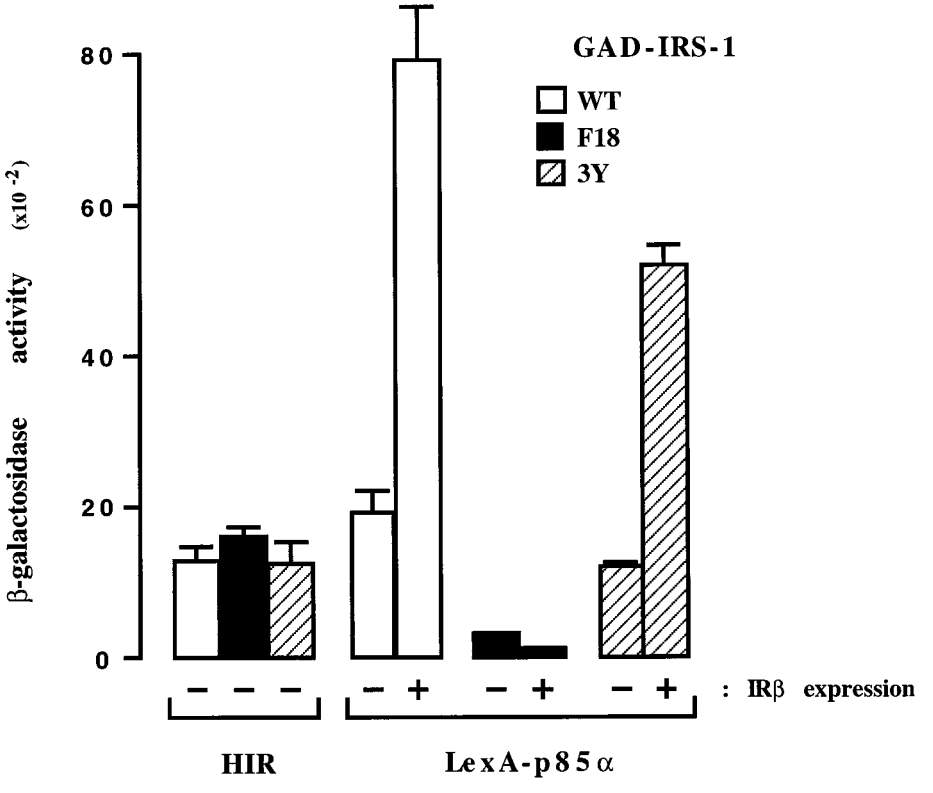

FIG. 1. Interaction of p85α and IRS-1 in the presence or absence of IRβ. Double transformed yeast clones expressing LexA-p85α and GAD-IRS-1, GAD-F18-IRS-1, or GAD-3Y-IRS-1 in the presence (no expression of IRβ) or the absence (expression of IRβ) of L-methionine were grown in liquid and used for β-galactosidase assay. As a positive control we coexpressed LexA-HIR and GAD-IRS-1, F18-IRS-1, and 3Y-IRS-1. β-Galactosidase units were determined according to the method of Miller (28). Values represent the average ± SE of three independent transformants. One representative experiment of three is shown.

tion between IRS-1 and p85α was also detectable when IRβ was not expressed, suggesting that 1) yeast may contain tyrosine kinases able to tyrosine phosphorylate IRS-1; or 2) IRS-1 may interact with p85α in a phosphotyrosine-independent manner. To further address this issue, we used two IRS-1 mutants, F18 and 3Y. In F18 IRS-1, the 18 tyrosines corresponding to potential phosphorylation sites by IR and insulin-like growth factor I receptor were replaced by phenylalanines. In 3Y⁻IRS-1, three tyrosines, *i.e.* Y608, Y628, and Y658, were added back to F18-IRS-1, resulting in an IRS-1 protein containing only potential binding sites for PI 3-kinase. We first examined the interaction between the WT or IRS-1 mutants and the IR. GAD-F18-IRS-1, GAD-3Y-IRS-1, or GAD-WT-IRS-1 was coexpressed with the IR (LexA-IRβ). This interaction occurs through the PTB domain of IRS-1 and the phosphorylated $NPXY^{960}$ of the IR (32). The three IRS-1 molecules interact similarly with IR, suggesting that no major conformational changes due to mutation of tyrosine to phenylalanine residues have occurred, and that the three IRS-1 proteins are similarly expressed and properly folded in yeast. As shown in Fig. 1, no or weak interaction was seen between F18-IRS-1 and p85α. Interestingly, interaction between 3Y-IRS-1 and p85α was almost completely restored, as 85% of the β-galactosidase activity seen with wild-type IRS-1 was obtained. Moreover, this interaction was increased 4-fold when IRβ was present compared with the interaction in the absence of receptor.

To be sure that differences between the three IRS proteins concerning the interaction with p85α were not due to variations in IRS-1 expression but, rather, reflected the tyrosine phosphorylation level, we measured expression of WT, F18, and 3Y-IRS-1 (Fig. 2). No IRS-1 was detected when yeast was transformed by pACT II alone. Further, F18 and 3Y-IRS-1 mutants were expressed at similar levels as WT-IRS-1. We then determined the phosphorylation state of IRS-1 in yeast in the presence or absence of IRβ. We failed to detect tyrosine phosphorylation when IRβ expression was repressed (not shown). However, as shown in Fig. 2 (*lower panel*), IRβ expression clearly induced tyrosine phosphorylation of WT-IRS-1. Although F18-IRS-1 was expressed (Fig. 2, *upper panel*), no tyrosine phosphorylation was detected, as expected. 3Y-IRS-1 with Y608, 628, and 658 was tyrosine phosphorylated, as shown in Fig. 2 (*lower panel*), to a lesser extent than WT-IRS-1, indicating that other tyrosine residues are phosphorylated on WT-IRS-1.

Taking our results together, we conclude that 1) without expression of IRβ, IRS-1 associates with p85α in our yeast two-hybrid system; the fact that IRβ is not required may indicate either that yeast kinases could phosphorylate IRS-1 on PI 3-kinase binding motifs or IRS-1 could interact in a phosphotyrosine-independent manner; 2) this interaction is increased by expression of IRβ due to increased IRS-1 phosphorylation; and 3) IRS-1 tyrosine phosphorylation by endogenous yeast kinases would occur at specific sites because F18-IRS-1 shows no or weak interaction with p85α, and 3Y-IRS-1 restored the association with the PI 3-kinase regulatory subunit compared with WT-IRS-1, showing that at least one of the tyrosines, 608, 628, or 658, is phosphorylated and interacts with p85.

Interaction of $p55^{PIK}$ with IRS-1

Next we tested whether $p55^{PIK}$, a PI 3-kinase regulatory subunit different from p85α, could interact with IRS-1 in the yeast two-hybrid system. Insulin promotes the tyrosine phosphorylation of $p55^{PIK}$ on residue 341 in intact cells (20). In CHO/IR cells, insulin stimulates the binding of $p55^{PIK}$ to IRS-1 and also the PI 3-kinase activity associated to $p55^{PIK}$ (20, 22).

$p55^{PIK}$ cDNA was subcloned into pBTM116 vector to obtain the LexA-$p55^{PIK}$ fusion protein. Then, LexA-$p55^{PIK}$ or LexA-p85α were coexpressed with GAD-WT-IRS-1, GAD-F18-IRS-1, or GAD-3Y-IRS-1, and β-galactosidase activity was measured for each cotransformant (Fig. 3). As the interaction occurs in absence of IRβ expression, the experiments were performed without IR using the pLex vector, which has no IRβ cDNA. We found that p85α interacts with WT-IRS-1. This interaction strongly decreased with F18-IRS-1. With 3Y-IRS-1 mutant, the interaction with p85α was partially restored compared with WT-IRS-1. Similar to p85α, $p55^{PIK}$ interacted with GAD-WT-IRS-1, and this interaction was significantly decreased with F18-IRS-1. Finally, GAD-3Y-IRS-1 interacted with $p55^{PIK}$, indicating that phosphorylation of the three YXXM binding sites is sufficient to allow for the interaction.

As a whole, our results suggest that like p85α, $p55^{PIK}$ is able to interact with WT-IRS-1 in a yeast two-hybrid system. For both PI 3-kinase regulatory subunits, interaction with IRS-1 depends on phosphotyrosines residues. In the absence of IRβ expression in yeast, we favor the idea that IRS-1 could be phosphorylated on tyrosines comprised in YXXM motifs,

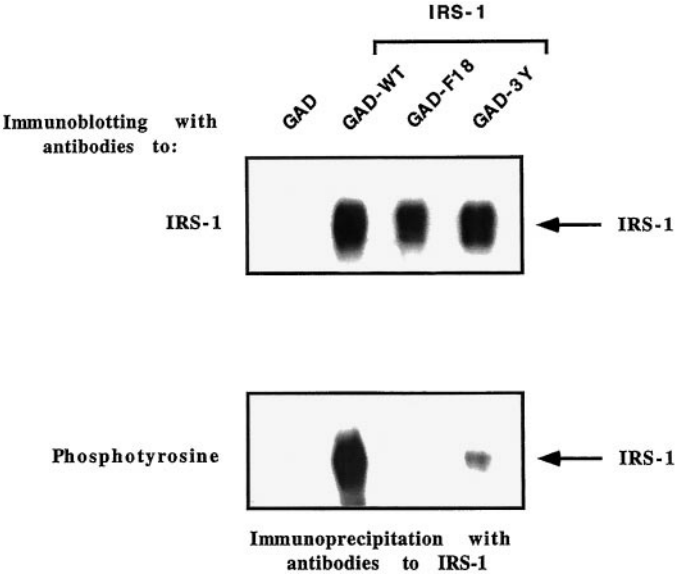

FIG. 2. Expression of different IRS-1 constructs in the yeast two-hybrid system. Clones coexpressing LexA-p85α and GAD, GAD-IRS-1, GAD-F18-IRS-1, or GAD-3Y-IRS-1 were grown without L-methionine, allowing IRβ expression, in appropriate medium for 24 h. The cells were then washed with PBS, frozen in nitrogen, placed at −20 C for 10 min, lysed, and vortexed in the presence of glass beads. The lysates were subjected to immunoprecipitation using anti-IRS-1 antibody. The proteins were resolved by SDS-PAGE before immunoblotting with antibodies to either IRS-1 or phosphotyrosine before incubation with [^{125}I]protein A and autoradiography.

GAD: / LexA:	WT-IRS-1	F18-IRS-1	3Y-IRS-1
HIR	+ +	+ +	+ +
Lamin	—	—	—
p85α	+ + +	—	+ +
p55PIK	+ +	—	+

FIG. 3. Interaction of p85α and p55PIK with wild-type IRS-1, IRS-1-F18, and IRS-1–3Y in the yeast two-hybrid system. LexA-p85α or LexA-p55PIK were coexpressed with GAD-IRS-1, GAD-F18-IRS-1, or GAD-3Y-IRS-1 in the presence of L-methionine to suppress IRβ expression. As control we coexpressed LexA-HIR or LexA-lamin with the different IRS-1 constructs. Clones grown in the appropriate medium were used for β-galactosidase assay. Interaction detected by measuring β-galactosidase activity is quantitatively shown by a plus; if interaction is absent, it is indicated by a minus. One representative experiment of three is shown.

leading to binding of p85α and p55PIK SH2 domains to those phosphotyrosines.

Role of IRS-1 serines in modulating interaction between IRS-1 and PI 3-kinase p55PIK and p85α subunits in the yeast two-hybrid system

In a previous study, we showed that IRS-1 serines 612, 632, 662, and 731 modulate insulin action in intact 293-EBNA cells. Indeed, their mutations resulted in increased IRS-1 tyrosine phosphorylation and association with p85α, which was correlated with enhanced IRS-1 associated PI 3-kinase activity (8). The strongest effect was seen with the double mutant S662A/S731A-IRS-1, in which serines 662 and 731 were replaced by alanines. Therefore, we were interested in analyzing the involvement of these serines in the interaction with p85α in the yeast two-hybrid system. To do this, GAD-WT-IRS-1 or GAD-S662A/S731A-IRS-1 was coexpressed with LexA-p85α, and the resulting β-galactosidase activity was quantified. As shown in Fig. 4A, S662A/S731A-IRS-1 expression resulted in a 4.5-fold increase in β-galactosidase activity compared with WT-IRS-1 when coexpressed with p85α. This suggests that for binding p85α, S662A/S731A-IRS-1 was more efficient than the WT protein. Interestingly, we found that when p55PIK was coexpressed with S662A/S731A-IRS-1, a 4-fold increase in β-galactosidase activity was seen compared with that obtained with wild-type IRS-1. As expected, IRS-1 serine mutations had no effect on IRS-1 interaction with IR β-subunit (Fig. 4B). We also verified that these findings were not due to a difference between WT or S662A/S731A-IRS-1 expression levels (Fig. 4C).

In summary, we conclude that, for both PI 3-kinase regulatory subunits, p85α and p55PIK, association with IRS-1 is negatively regulated by serines 662 and 731 of the latter molecule.

Roles of IRS-1 serines 662 and 731 in insulin-stimulated PKB activity

We have previously shown in 293-EBNA cells overexpressing S662A/S731A-IRS-1 that insulin-stimulated PI 3-kinase activity is increased compared with WT-IRS-1-overexpressing cells. We actually found that both the insulin-stimulated and the basal PI 3-kinase activities are increased about 2-fold with the double serine mutant. To investigate further the effects of these IRS-1 serines on downstream effectors of PI 3-kinase, we wanted to know whether protein kinase B activity is modified in the presence of the double serine mutant compared with WT-IRS-1.

In brief, we overexpressed in 293-EBNA cells WT-PKB with S662A/S731A-IRS-1 or WT-IRS-1; the cells were stimulated or not with insulin before lysis, and the lysates were subjected to immunoprecipitation with antibody to PKB. Finally, a PKB kinase assay on the pellets was performed (Fig. 5). With S662A/S731A or WT-IRS-1 we found increased stimulation of endogenous PKB. However, after transfection of only PKB, insulin stimulated PKB activity about 4 times more. When PKB and WT-IRS-1 were coexpressed, we observed insulin stimulation of PKB activity that increases to

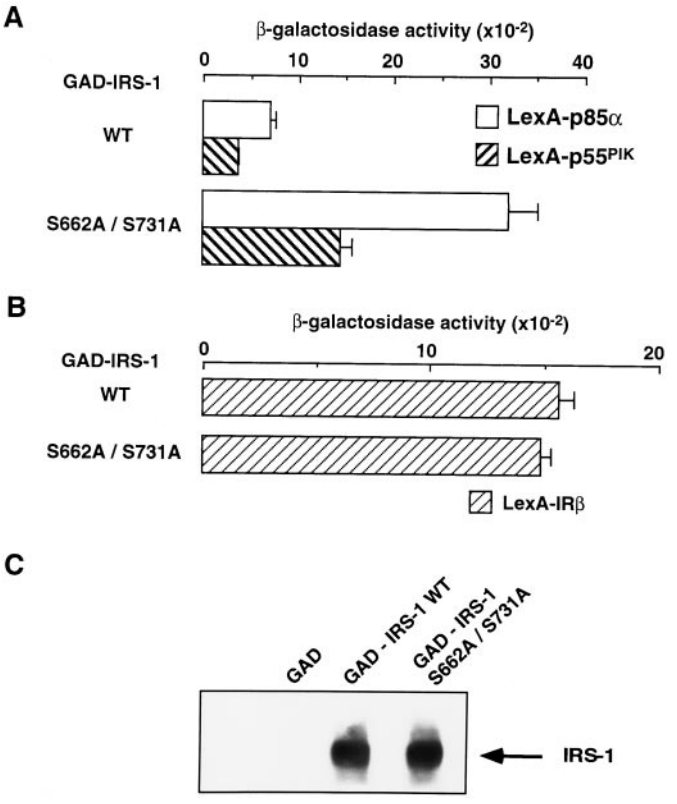

FIG. 4. Role of IRS-1 serine residues 662 and 731 in its interaction with p85α and with p55PIK. The yeast reporter strain L40 was transformed with pBTM116 plasmid encoding LexA-p85α or LexA-p55PIK (A), or LexA-IRβ (B) in combination with pACTII plasmid encoding either GAD-IRS-1 or GAD-S662A/S731A-IRS-1. Double transformed cells were grown in liquid culture, and the β-galactosidase activity corresponding to each pair of coexpressed hybrid proteins was quantified. The data are the average ± SE of three isolated clones from one independent assay of four. C, Cotransformed yeasts with LexA-p85α or LexA-p55PIK and pACTII encoding GAD, GAD-IRS-1, or GAD-S662A/S731A-IRS-1 were grown in liquid before the cells were frozen and lysed. Then, the lysates were submitted to immunoprecipitation using anti-IRS-1 antibody. Finally, the proteins were resolved by SDS-PAGE, immunoblotted with the same antibody, and incubated with [125I]protein A followed by autoradiography.

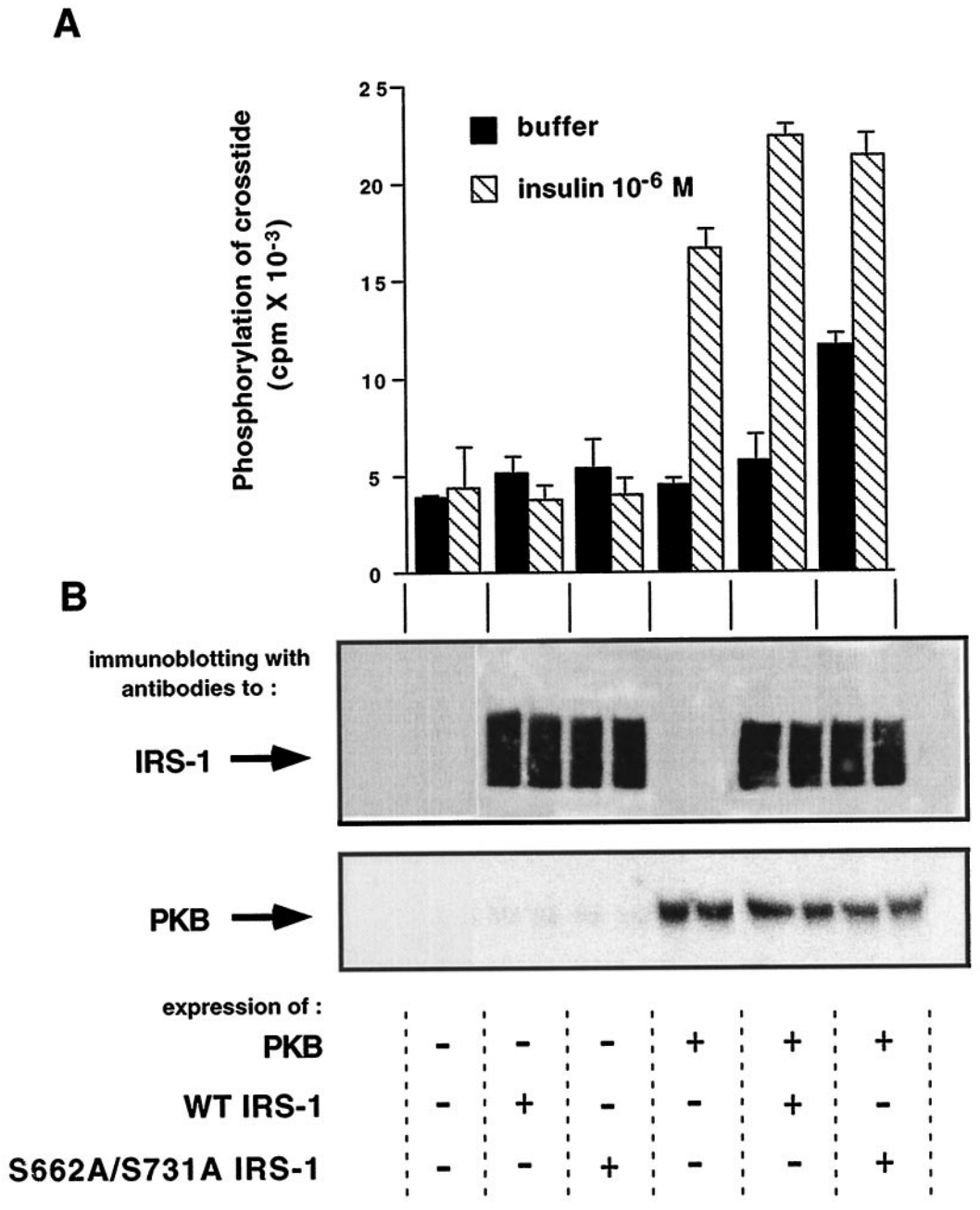

FIG. 5. Role of IRS-1 serines 662 and 731 on PKB activity modulation. 293 EBNA cells transiently expressing HA-tagged-WT PKB and WT or S662A/S731A-IRS-1 were stimulated or not with 10^{-6} M insulin for 15 min, and the lysates were centrifuged at 15,000 $\times$ *g* for 15 min at 4 C. A, Nine of 10 of the lysates were immunoprecipitated using anti-HA antibody coupled to G protein-Sepharose. After 2.5 h, PKB kinase activity was measured using Crosstide (30 μM) as a substrate in the presence of γ-^{32}P. The reaction is stopped by spotting 40 μl onto Whatman p81 papers and washing several times in 1% (vol/vol) orthophosphoric acid. The radioactivity was determined by Cerenkov counting. Values represent the average of three independent triplicates. One representative experiment of three is shown. B, One of 10 of the lysates was resolved on SDS-PAGE, and the proteins were electrotransferred to an Immobilon membrane (Millipore Corp.) before immunoblotting with antibodies to either IRS-1 or PKB. Then the membrane was incubated with [^{125}I]protein A and autoradiographed.

about 40% compared with PKB alone, whereas cells incubated with buffer did not show any increased PKB activity. When we coexpressed WT-PKB and S6623A/S731A-IRS-1, increased insulin-stimulated PKB activity compared with PKB alone was seen, although the activity is not different from that in WT-IRS-1- and PKB-coexpressing cells. Finally, basal PKB activity was increased about 2 times with the double serine mutant compared with WT-IRS-1. Taking these results together, we conclude that under basal conditions (*i.e.* without insulin treatment), IRS-1 serines 662 and 731 surrounding tyrosines 658 and 727, which are potential binding sites for PI 3-kinase, are negatively regulating PKB activity. In contrast, those serines do not affect insulin-stimulated PKB activity.

Discussion

Phosphorylation of IRS-1 on tyrosine residues located in binding motifs for SH2 domain-containing molecules is required for the biological role of IRS-1 as adaptor. Modulation of this key function could be achieved by dephosphorylation of those sites, but also by serine phosphorylation as suggested by several reports (7–10). In this study we used yeast two-hybrid analysis to detect, at the molecular level, modulations of p85α or p55^{PIK} binding to IRS-1.

We found that both regulatory subunits of PI 3-kinase were able to interact with IRS-1 in the presence as well as the absence of IRβ. At first glance this observation could indicate that IRS-1 can interact with p85α in a fashion that does not require tyrosine phosphorylation of IRS-1. However, as IRS-1/PI 3-kinase interaction is known to be dependent on tyrosine phosphorylation, this result would rather suggest that in the absence of IRβ, an endogenous yeast kinase could phosphorylate IRS-1 on tyrosine residues located in YXXM motifs. Importantly, we show that the F18-IRS-1 mutant, lacking 18 potential tyrosine phosphorylation sites, was less efficient than wild-type IRS-1 in binding to p85α and p55^{PIK}. Further, addition to F18-IRS-1 of three tyrosines corresponding to the YXXM motif, which are binding sites for PI 3-kinase (3Y$^-$IRS-1), resulted in increased association with regulatory subunits of PI 3-kinase compared with that of F18-IRS-1. Taken as a whole, these results suggest that in yeast IRS-1 could be phosphorylated at least in part on tyrosine residues by endogenous yeast kinases. This phosphorylation would probably occur on a few residues, as we failed to reveal IRS-1 phosphorylation in yeast by immunoprecipitating IRS-1 followed by Western blotting with antibodies to phosphotyrosine when IRβ expression is repressed by L-methionine (not shown). As no classical tyrosine kinases have yet been cloned in yeast, possible candidate kinases for IRS-1 tyrosine phosphorylation are likely to be dual specificity kinases, which would possess activity toward threonine and tyrosine. Indeed, such kinases have actually been identified in yeast (*e.g.* Wee1) (33). Those kinases must have the same specificity as IR toward IRS-1, since they phosphorylate PI 3-kinase-binding sites that are crucial for interaction with PI 3-kinase. However, we cannot rule out the possibility that IRS-1 interacts with p85α in a phosphotyrosine-independent manner, allowing the p85α SH2 domain to bind IRS-1 nonphosphorylated YXXM motifs.

As 3Y-IRS-1 does not fully restore the interaction with p85 compared with WT-IRS-1, some other tyrosine-containing sites on IRS-1 are likely to be phosphorylated and involved in the interaction with p85. When IRβ is expressed, IRS-1 tyrosine phosphorylation is augmented, leading to increased interaction with the PI 3-kinase regulatory subunit. Together, our results obtained with 3Y-IRS-1 suggest that 1) at least one of the tyrosines, 608, 628, or 658, is phosphorylated; and 2) tyrosine residues other than tyrosines 608, 628, and 658 are involved in the interaction of IRS-1 with p85α and p55^{PIK} in yeast, because with 3Y-IRS-1 the interaction is not completely restored compared with that with WT-IRS-1.

Concerning modulation of IRS-1 interaction with PI 3-kinase, we demonstrate that mutation of IRS-1 serines 662 and 731 to alanine (S662A/S731A-IRS-1) resulted in increased interaction of IRS-1 mutant with p85α compared with WT-IRS-1. These serines are adjacent to binding sites for p85α (tyrosine 658 and 727 in YMXM motifs) and are potential phosphorylation sites for MAP kinases as they are part of a YMXMSP sequence. Our results extend at the molecular level our previous work in mammalian cells, where we showed increased insulin-induced coimmunoprecipitation of p85α with S662A/S731A-IRS-1 compared with wild-type IRS-1 (8). Moreover, we found in the yeast two-hybrid system that p55^{PIK} interacted with S662A/S731A-IRS-1 more efficiently than with WT-IRS-1, suggesting that the mutated serine residues are also able to modulate binding of p55^{PIK} to IRS-1. As mutation of serines 662 and 731 does not affect IRβ binding to IRS-1, we favor the idea that this regulation is specific for association of regulatory PI 3-kinase subunits with IRS-1. We previously suggested that phosphorylation of serines 662 and 731 by MAP kinases or related kinases could be responsible for the inhibitory effect of these residues on IRS-1 binding to p85α. Indeed, phosphorylation of these sites would at least partially prevent insulin-induced tyrosine phosphorylation of IRS-1 and/or binding of p85α to phosphotyrosine-containing motifs. Nevertheless, we cannot exclude the possibility that substitution of serine to alanine by itself allows binding of IRS-1 to PI 3-kinase regulatory subunits.

Recently, it has been shown that stimulation of protein kinase C by PMA activates MAP kinase, ERK-2, which is able to phosphorylate a synthetic peptide containing the IRS-1 sequence comprising serine 612; this phosphorylation results in inhibition of IR to further phosphorylate this peptide (12, 13). This is particularly interesting because the serine residue, which is indeed included in a MAP kinase consensus sequence, YXXMSP, is just upstream tyrosine 608, a recognized PI 3-kinase motif-binding site. Our findings with the IRS-1 serine mutants in yeast suggest that serine-induced negative regulation could occur in this species. Enzymes homologous to mammalian MAP kinases have been cloned in yeast, and they are likely to be able to phosphorylate IRS-1 on serine/threonine (34). Further, we revealed phosphoserine on IRS-1 immunopurified from yeast using phosphoserine immunoblotting, indicating that IRS-1 is, in fact, phosphorylated on serine residues in yeast (not shown). Hence, our results show that association of regulatory subunits of PI 3-kinase with IRS-1 in yeast can be negatively regulated by serine mutation to alanine. The fact that we did not detect IRS-1 tyrosine phosphorylation in yeast while not expressing IRβ, although we detected IRS-1 serine phosphorylation, is probably due to the presence of 35 potential serine/threonine phosphorylation sites on IRS-1 compared with 10 putative tyrosine phosphorylation sites, among which 6 are comprised in YMXM. It has been also shown that IRS-1 is heavily phosphorylated on serine under basal conditions compared with tyrosine phosphorylation (8). Moreover, we can hypothesize that only specific tyrosine residues in the YMXM motif on IRS-1 are phosphorylated by endogenous kinase(s) in yeast, whereas multiple serine sites are phosphorylated.

We further investigated whether serines 662 and 731 that affect the IRS-1 and PI 3-kinase regulatory subunits interactions would provide the same regulation on a downstream effector of PI 3-kinase such as PKB. We show that although serines 662 and 731 of IRS-1 negatively regulate basal and

insulin-stimulated PI 3-kinase activities, only basal PKB activity is affected by the double serine mutant. We conclude, therefore, that serines 662 and 731 of IRS-1 negatively regulate PI 3-kinase and PKB activities in resting cells and are involved in PI 3-kinase, but have no impact on PKB activity in insulin-stimulated cells. Phospholipids generated by PI 3-kinase are thought to bind to the PH (pleckstrin homology) domain of PKB, leading to membrane targeting and/or activation of PKB. Moreover, these phospholipids are also involved in activating another protein downstream of PI 3-kinase that is upstream of and directly activates PKB, *i.e.* phospholipid- dependent kinase-1 (35). We can hypothesize that under our insulin-stimulated conditions, the increased PI 3-kinase activity seen with the IRS-1 serine mutants that is not correlated with increased PKB activity is due to the fact that above a certain threshold level, an increase in phospholipids does not result in additional activation of phospholipid-dependent kinase-1 and/or PKB. 293 cells pretreated with wortmannin display no or little PKB activity in the presence or absence of IRS-1 (not shown), demonstrating that in these cells, PKB activity relies mostly on PI 3-kinase activity. PKB appears to be involved in key insulin-induced metabolic effects, including glycogen synthesis, glucose transport, and protein synthesis (36). As evidence has been gathered suggesting that serine phosphorylation is implicated in insulin resistance related to tumor necrosis factor-α (10), IRS-1 serine phosphorylation could correspond to a means for regulating key enzymes in insulin signaling, such as PI 3-kinase and/or PKB.

Roth *et al.* (13) reported that phorbol ester-stimulated protein kinase C activates Erk kinases, which subsequently phosphorylates an IRS-1 peptide containing serine 612. This peptide then becomes less able to be phosphorylated on tyrosine by IRs.

In insulin treated-cells, inhibition of MAP kinases blocked the PMA-induced negative regulation of IRS-1-associated PI 3-kinase activity. Keeping in mind these observations together with ours, we are tempted to speculate that a large number of agents or growth factors, while activating MAP kinases, could at the same time down-regulate insulin signaling for other specific signals delivered into the cell.

In conclusion, our present work provides illustration at the molecular level of the role of serine/threonine phosphorylation of signaling molecules as a mechanism leading to the modulation of insulin action. Whether such a process is operational at the level of the different IRS molecules and impinges on all aspects of the vast repertoire of insulin actions remains to be investigated.

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References

1. **Myers MGJ, White MF** 1996 Insulin signaling transduction and the IRS proteins. Annu Rev Pharmacol Toxicol 36:615–658
2. **Lavan BE, Fantin VR, Chang ET, Lane WS, Keller SR, Lienhard GE** 1997 A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a member of the insulin receptor substrate family. J Biol Cell 272:21403–21407
3. **Kaburagi Y, Satoh S, Tamemoto H, Yamamoto-Honda R, Tobe K, Veki K, Yamauchi T, Kono-Sugita E, Sekihara H, Aizawa S, Cushman SW, Akanuma Y, Yasaki Y, Kadowaki T** 1997 Role of insulin receptor substrate-1 and pp60 in the regulation of insulin-induced glucose transport and GLUT4 translocation in primary adipocytes. J Biol Chem 272:25839–25844
4. **Myers MGJ, Sun XJ, White MF** 1994 The IRS-1 signaling system. Trends Biochem Sci 19:289–293
5. **Sun XJ, Crimmins DL, Myers MGJ, Miralpeix M, White MF** 1993 Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. Mol Cell Biol 13:7418–7428
6. **Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF** 1991 structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature 352:73–77
7. **Folli F, Kahn CR, Hansen H, Bouchie JL, Feener EP** 1997 Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels. J Clin Invest 100:2158–2169
8. **Mothe I, Van Obberghen E** 1996 Phosphorylation of insulin receptor substrate-1 on multiple serine residues, 612, 632, 662, and 731, modulates insulin action. J Biol Chem 271:11222–11227
9. **Tanti J-F, Grémeaux T, Van Obberghen E, Le Marchand-Brustel Y** 1994 Serine/threonine phosphorylation of insulin receptor substrate 1 modulates insulin receptor signaling. J Biol Chem 269:6051–6057
10. **Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BR** 1996 IRS-1 mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α- and obesity-induced insulin resistance. Science 271:665–668
11. **Eldar-Finkelman H, Krebs EG** 1997 Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. Proc Natl Acad Sci USA 94:9660–9664
12. **De Fea K, Roth R** 1997 Modulation of insulin receptor substrate-1 tyrosine phosphorylation and function by mitogen-activated protein kinase. J Biol Chem 272:31400–31406
13. **De Fea K, Roth R** 1997 Protein kinase C modulation of insulin receptor substrate-1 tyrosine phosphorylation requires serine 612. Biochemistry 36:12939–12947
14. **Tanti JF, Grémeaux T, Grillo S, Calleja V, Klippel A, Williams LT, Van Obberghen E, Le Marchand-Brustel Y** 1996 Overexpression of a constitutively active form of phosphatidylinositol 3-kinase is sufficient to promote Glut4 translocation in adipocytes. J Biol Chem 271:25227–25232
15. **Kotani K, Yonezawa K, Hara K, Ueda H, Kitamura Y, Sakaue H, Ando A, Chavanieu A, Calas B, Grigorescu F, Nishiyama M, Waterfield MD, Kasuga M** 1994 Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling. EMBO J 13:2313–2321
16. **Dhand R, Hiles I, Panayotou G, Roche S, Fry MG, Gout I, Totty NF, Truong O, Vicendo P, Yonezawa K, Kasuga M, Courtneidge SA, Waterfield MD** 1994 PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein serine kinase activity. EMBO J 13:522–533
17. **Whitman M, Downes CP, Keeler M, Keller T, Cantley L** 1988 Type I phosphatidylinositol kinase makes a novel inositol phopholipid, phosphatidylinositol-3-phosphate. Nature 322:644–646
18. **Koch CA, Anderson D, Moran MF, Ellis C, Pawson T** 1991 SH2 and SH3 domains; elements that control interactions of cytoplasmic signaling proteins. Science 252:668–674
19. **Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky F, Lechleider RJ, Neel BG, Birge RB, Fajardo JE, Chou MM, Hanafusa H, Schaffhausen B, Cantley LC** 1993 SH2 domains recognize specific phosphopeptide sequences. Cell 72:767–778
20. **Pons S, Asano T, Glasheen E, Miralpeix M, Zhang Y, Fisher TL, Myers MGJ, Sun XJ, White MF** 1995 The structure and function of $p55^{PIK}$ reveal a new regulatory subunit for phosphatidylinositol 3-kinase. Mol Cell Biol 15:4453–4465
21. **Inukai K, Anai M, Van Breda E, Hosaka T, Katagiri H, Funaki M, Fikushima Y, Ogihara T, Yazaki Y, Kikuchi M, Oka Y, Asano T** 1996 A novel 55-kDa regulatory subunit for phosphatidylinositol 3-kinase structurally similar to $p55^{PIK}$ is generated by alternative splicing of the p85α gene. J Biol Chem 271:5317–5320
22. **Inukai K, Fukani M, Ogihara T, Katagiri H, Kanda A, Anai M, Fukushima Y, Hosaka T, Susuki M, Shin B, Takata M, Yazaki Y, Kikuchi M, Oka Y, Asano T** 1997 p85α gene generates three isoforms of regulatory subunit for phosphatidylinositol 3-kinase (PI 3-kinase), p50α, p55α, and p85α, with different PI 3-kinase activity elevating responses to insulin. J Biol Chem 272:7873–7882
23. **Jones S, Howell K** 1997 Phosphatidylinositol 3-kinase is required for the formation of constitutive transport vesicles from the TGN. J Cell Biol 139:339–349
24. **Myers MGJ, Zhang Y, Aldaz GAI, Grammer T, Glasheen EM, Yenush L, Wang LM, Sun XJ, Blenis J, Pierce JH, White MF** 1996 YMXM motifs and signaling by an insulin receptor substrate 1 molecule without tyrosine phosphorylation sites. Mol Cell Biol 16:4147–4155
25. **Andjelkovic M, Jakubowicz T, Cron P, Ming X-F, Han J-W, Hemmings BA** 1996 Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC_PK/PKB) promoted by serum and protein phosphatase inhibitors. Proc Natl Acad Sci USA 93:5699–5704

26. **Gietz D, St Jean A, Woods RA, Schiestl RH** 1992 Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20:1425–1426
27. **Bartel PL, Chien C, Sternglanz R, Fields S** 1993 Using the two hybrid system to detect protein-protein interactions. In: Hartley DA (ed) Cellular interactions in Development: A Practical Approach. Oxford University Press, Oxford, pp 153–179
28. **Miller JH** 1972 Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, pp 633–640
29. **Laemmli UK** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
30. **Chen C, Okayama H** 1987 High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745–2752
31. **Rocchi S, Tartare-Deckert S, Murdaca J, Holgado-Madruga M, Wong AJ, Van Obberghen E** 1998 Determination of GAB-1 interactions with insulin receptor signaling molecules. Mol Endocrinol 12:914–923
32. **Tartare-Deckert S, Sawka-Verhelle D, Murdaca J, Van Obberghen E** 1995 Evidence for a differential interaction of Shc and the insulin receptor substrate 1 (IRS-1) with the insulin-like growth factor-I (IGF-I) receptor in the yeast two-hybrid system. J Biol Chem 270:23456–23460
33. **Hunter T, Plowman GD** 1997 The protein kinases of budding yeast: six core and more. Trends Biochem Sci 22:18–22
34. **Pelech SL** 1996 Signalling pathways: kinase connections on the cellular intranet. Curr Biol 6:551–554
35. **Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PRJ, Reese CB, Cohen P** 1997 Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B. Curr Biol 7:261–269
36. **Moule SK, Denton RM** 1997 Multiple signaling pathways involved in the metabolic effects of insulin. Am J Cardiol 80:41–49