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Phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation

Jonathan M.Backer[2], Martin G.Myers Jr, Steven E.Shoelson, Debra J.Chin, Xiao-Jian Sun, Montserrat Miralpeix, Patrick Hu[1], Benjamin Margolis[1], Edward Y.Skolnik[1], Joseph Schlessinger[1] and Morris F.White

Research Division, Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02215 and [1]Department of Pharmacology, New York University School of Medicine, New York, NY 10016, USA

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[2]Corresponding author

IRS-1 undergoes rapid tyrosine phosphorylation during insulin stimulation and forms a stable complex containing the 85 kDa subunit (p85) of the phosphatidylinositol (PtdIns) 3′-kinase, but p85 is not tyrosyl phosphorylated. IRS-1 contains nine tyrosine phosphorylation sites in YXXM (Tyr-Xxx-Xxx-Met) motifs. Formation of the IRS-1 – PtdIns 3′-kinase complex *in vitro* is inhibited by synthetic peptides containing phosphorylated YXXM motifs, suggesting that the binding of PtdIns 3′-kinase to IRS-1 is mediated through the SH2 (src homology-2) domains of p85. Furthermore, overexpression of IRS-1 potentiates the activation of PtdIns 3-kinase in insulin-stimulated cells, and tyrosyl phosphorylated IRS-1 or peptides containing phosphorylated YXXM motifs activate PtdIns 3′-kinase *in vitro*. We conclude that the binding of tyrosyl phosphorylated IRS-1 to the SH2 domains of p85 is the critical step that activates PtdIns 3′-kinase during insulin stimulation.

Key words: insulin/IRS-1/phosphatidylinositol 3′-kinase/ signal transduction/tyrosine phosphorylation

Introduction

Insulin regulates cellular growth and metabolism by binding to the insulin receptor tyrosine kinase. Immediately after insulin binding, the β-subunit of the insulin receptor undergoes tyrosyl autophosphorylation, which increases its intrinsic tyrosine kinase activity (Kahn and White, 1988). The tyrosine kinase is essential for insulin action, suggesting that key intermediates at early steps in the signaling cascade may be regulated by tyrosine phosphorylation (Chou *et al.*, 1987). However, the molecular mechanisms linking the insulin receptor to cellular enzymes have been difficult to establish. Insulin stimulates the tyrosyl phosphorylation of a 165 – 185 kDa protein in intact cells (White *et al.*, 1985, 1987). This phosphoprotein, called pp185, is barely detectable in cells expressing few insulin receptors, strongly detected in cells expressing high levels of receptors and weakly detected in cells expressing mutant receptors defective in biological signaling (White *et al.*, 1988; Backer *et al.*, 1991; Wilden *et al.*, 1992). Thus, pp185 appears to be a substrate of the insulin receptor which plays a significant role in the early stages of post-receptor insulin signal transduction.

We have recently purified from rat liver one of the major proteins in the pp185 band (Rothenberg *et al.*, 1990). The complementary DNA sequence encodes a 131 kDa hydrophilic protein called IRS-1 (Sun *et al.*, 1991). IRS-1 contains a kinase-like ATP-binding site, many potential serine and threonine phosphorylation sites and at least 14 potential tyrosine phosphorylation sites. Six of these tyrosyl residues are located in YMXM motifs and three others are in YXXM motifs. Synthetic peptides based on the sequence of these YXXM motifs are excellent substrates of the insulin receptor, with K_m values between 24 and 100 μM (Shoelson *et al.*, 1992). Substitution of the methionine residues with other amino acids reduces catalytic efficiency, suggesting that the YMXM motif is a key determinant for substrate recognition by the insulin receptor kinase.

The platelet derived growth factor (PDGF) receptor, the polyoma middle T antigen and related molecules also contain tyrosine residues in YXXM motifs, and phosphorylation at these sites causes a specific association with the phosphatidylinositol 3′-kinase (PtdIns 3′-kinase) (reviewed in Cantley *et al.*, 1991). PtdIns 3′-kinase phosphorylates phosphatidylinositol at the D3 position of the inositol ring, producing PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in intact cells (Auger *et al.*, 1989). Activation of PtdIns 3′-kinase has been implicated in transmembrane signal transduction by a number of growth factor receptors (Cantley *et al.*, 1991) including the insulin receptor (Endemann *et al.*, 1990; Ruderman *et al.*, 1990; Backer *et al.*, 1992; Kelly *et al.*, 1992). However, the mechanism of activation is unclear. PtdIns 3′-kinase is composed of a catalytic subunit (p110) and a putative regulatory subunit (p85) that contains two SH2 (src homology-2) domains and one SH3 (src homology-3) domain (Carpenter *et al.*, 1990; Escobedo *et al.*, 1991b; Otsu *et al.*, 1991; Shibasaki *et al.*, 1991; Skolnik *et al.*, 1991). SH2 domains are conserved regions of ~100 amino acids that form specific binding sites for phosphotyrosine residues in certain amino acid sequence motifs (reviewed in Koch *et al.*, 1991). SH2 domains were first noted in non-receptor protein tyrosine kinases such as $pp60^{v\text{-}src}$ as well as in signaling molecules such as phospholipase C_γ, the $p21^{ras}$-associated GTPase activating protein (ras-GAP) and protein tyrosine phosphatases (Koch *et al.*, 1991). Recent evidence suggests that one or both of the SH2 domains in the p85 subunit of PtdIns 3′-kinase preferentially binds to phosphorylated YXXM motifs in the PDGF and CSF-1 receptors and the polyoma middle T antigen (Escobedo *et al.*, 1991a; Hu *et al.*, 1992; Klippel *et al.*, 1992; McGlade *et al.*, 1992; Reedijk *et al.*, 1992).

We have recently demonstrated that PtdIns 3′-kinase strongly associates with IRS-1 in insulin-stimulated cells (Sun *et al.*, 1991; Backer *et al.*, 1992) and that PtdIns 3′-kinase

activation is reduced in cells expressing mutant insulin receptors that phosphorylate IRS-1 poorly (Kapeler *et al.*, 1991; Backer *et al.*, 1992). In this study, we describe a novel mechanism for the activation of PtdIns 3′-kinase by insulin. PtdIns 3′-kinase is activated during association with tyrosyl phosphorylated IRS-1. This activation occurs *in vivo*, as binding to IRS-1 increases the specific activity of PtdIns 3-kinase in insulin-stimulated cells and overexpression of IRS-1 potentiates insulin stimulation of PtdIns 3-kinase. The activation is also observed *in vitro* during incubation of the immobilized PtdIns 3′-kinase with tyrosyl phosphorylated IRS-1 or phosphorylated YXXM-containing peptides based on the sequence of IRS-1 or the PDGF receptor. Given the absence of detectable p85 tyrosyl phosphorylation in insulin-stimulated cells, these data suggest that tyrosyl phosphorylation of IRS-1 by the insulin receptor and the subsequent association of IRS-1 with p85 activates PtdIns 3′-kinase.

Results

Insulin activates PtdIns 3′-kinase and stimulates its association with IRS-1

PtdIns 3′-kinase activity was measured in immunoprecipitates from CHO cells expressing the wild-type human insulin receptor (CHO/IR cells) (Figure 1, left panel). PtdIns 3′-kinase activity was barely detected in anti-IRS-1 (αIRS-1) immunoprecipitates from unstimulated CHO/IR cells. However, basal activity was detected in immunoprecipitates prepared with an antibody to the 85 kDa subunit of PtdIns 3′-kinase (αp85). After insulin stimulation, a marked increase in PtdIns 3′-kinase activity was found with both antibodies; a 3-fold increase was seen in the αp85 immunoprecipitates and a 20-fold increase in the αIRS-1 immunoprecipitates. The total amount of PtdIns 3′-kinase activity in the αIRS-1 immunoprecipitates was 40% of that in the αp85 immunoprecipitates. These results suggest that PtdIns 3′-kinase associates with IRS-1 during insulin stimulation.

The presence of PtdIns 3′-kinase-associated p85 in αIRS-1 and αp85 immunoprecipitates was confirmed by immunoblotting with a second αp85 antibody. Insulin stimulation had no effect on the amount of p85 in αp85 immunoprecipitates from CHO/IR cells, which was detected as a major band at ~85 kDa; a faint second band was also seen just below the p85 band. The intense band at ~65 kDa is non-specific and is seen in αp85 blots of control immunoprecipitates (data not shown). In contrast to the αp85 immunoprecipitates, p85 was detected in αIRS-1 immunoprecipitates only after insulin stimulation (Figure 1, right panel). Thus, the insulin-stimulated PtdIns 3′-kinase activity in the αIRS-1 immunoprecipitates results at least in part from the insulin-dependent association of the enzyme with IRS-1. However, the increase in PtdIns 3′-kinase activity in αp85 immunoprecipitates from insulin stimulated CHO/IR cells occurred without a change in the amount of p85 in these immunoprecipitates, suggesting that insulin activated PtdIns 3′-kinase.

Activation of PtdIns 3′-kinase by insulin was measured by determining the specific activity of the enzyme in the αIRS-1 and αp85 immunoprecipitates. The lipid kinase activity in each immunoprecipitate was normalized to the amount of p85 detected by immunoblotting, and the insulin-stimulated activity in the immunoprecipitates was compared with the basal activity detected in αp85 immunoprecipitates from unstimulated cells (Table I). Insulin caused a 3-fold increase in the specific activity of PtdIns 3′-kinase in αp85 immunoprecipitates. However, the specific activity of the αIRS-1-associated PtdIns 3′-kinase from insulin-stimulated cells was 7.4-fold higher than the basal activity and 2.5-fold higher than the insulin-stimulated PtdIns3′-kinase activity in the αp85 immunoprecipitates. Thus insulin stimulates the association of PtdIns 3′-kinase with IRS-1, and the IRS-1-associated enzyme is activated relative to the total PtdIns 3′-kinase pool.

Tyrosyl phosphorylation of p85 is not detectable in insulin-stimulated cells

The coordinate activation of PtdIns 3′-kinase and the tyrosyl phosphorylation of an 85 kDa protein has been observed in PDGF-stimulated fibroblasts (Kaplan *et al.*, 1987), suggesting that p85 phosphorylation might play a role in

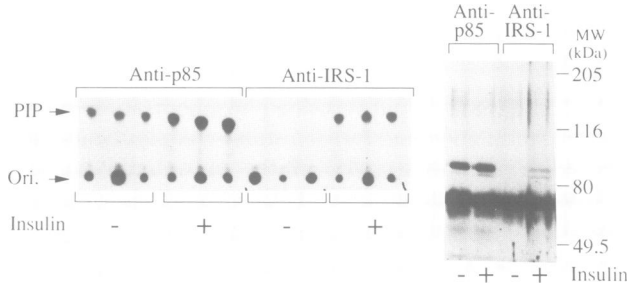

Fig. 1. PtdIns 3′-kinase activity in anti-p85 and anti-IRS-1 immunoprecipitates from insulin-stimulated CHO/IR cells. Quiescent CHO/IR cells were incubated in the absence or presence of 100 nM insulin for 10 min at 37°C, solubilized and incubated at 4°C with anti-p85 ($\alpha p85^{GST}$) or anti-IRS-1 ($\alpha IRS\text{-}1^{bac}$) antibody and protein A–Sepharose. **Left panel**: PtdIns 3′-kinase activity in the washed immunoprecipitates was assayed as described, and the lipids were extracted and separated by TLC in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2). Radioactivity in the TLC plates was visualized by autoradiography and [^{32}P]phosphate incorporation into PtdIns(3)P was quantified by Cerenkov counting. **Right panel:** Washed immunoprecipitates were boiled in Laemmli sample buffer and proteins were separated by 7.5% reducing SDS–PAGE. After transfer to nitrocellulose, the samples were immunoblotted with anti-p85 (Ab21) antibody.

Table I.

Sample	Insulin	Activity (pm/10 min)	Protein (AU)	Specific activity (pm/10 min/AU)
αp85	–	0.96 ± 0.06	0.2338	4.11 ± .26
αp85	+	2.73 + 0.84	0.2262	12.07 ± 3.7
αIRS-1	–	0.03 + 0.005	ND	ND
αIRS-1	+	1.08 ± 0.21	0.0353	30.59 ± 5.9

CHO/IR cells were incubated in the absence or presence of 100 nM insulin at 37°C for 10 min, and immunoprecipitates were prepared using $\alpha p85^{GST}$ or $\alpha IRS\text{-}1^{bac}$ antibodies. PtdIns 3′-kinase in the immunoprecipitates was measured as described in Figure 1, and parallel samples were immunoblotted with αp85 (Ab21) antibody and [^{125}I]protein A. The amount of p85 detected in the immunoblots was quantified using a Molecular Dynamics Phosphoimager, and is expressed in arbitrary units (AU). Control experiments, in which IRS-1 associated PtdIns 3′-kinase was incubated with αp85 antibody ($\alpha p85^{GST}$ or Ab51), showed that the αp85 antibodies used here did not inhibit PtdIns 3′-kinase.

activation of PtdIns 3′-kinase. The tyrosyl phosphorylation of p85 in unstimulated or insulin-stimulated CHO/IR cells was measured in αp85 immunoprecipitates blotted with αp85 or αPY antibodies (Figure 2). Insulin had no effect on the amount of p85 detected with αp85 antibody (Figure 2, lanes a and b). In contrast, blotting of parallel αp85 immunoprecipitates with αPY revealed the insulin-stimulated appearance of two bands at 95 and 180 kDa (Figure 2, lanes e and f); however, p85 was not detected by the αPY antibody. To confirm that the 85 kDa band detected by αp85 (Figure 2, lane b) was distinct from the insulin receptor β-subunit (95 kDa band) detected with αPY (Figure 2, lane f), lanes a and b were re-blotted with αPY (Figure 2, lanes c and d) revealing new bands at 95 and 180 kDa. The 95 kDa band was clearly separate from p85. Thus, the tyrosine phosphorylation of p85 in insulin-stimulated CHO/IR cells was not detectable.

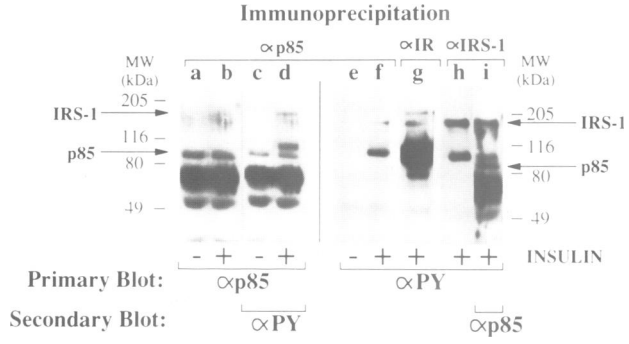

Fig. 2. Identification of tyrosyl phosphorylated proteins in αp85 immunoprecipitates from insulin-stimulated cells. Quiescent CHO/IR cells were incubated in the absence or presence of insulin for 10 min at 37°C, solubilized and immunoprecipiated with αp85 ($\alpha p85^{GST}$) (lanes a–f), αIR (anti-insulin receptor antibody 83-14) (lane g) or αIRS-1 ($\alpha IRS\text{-}1^{bac}$) (lanes h and i) antibodies. Proteins were separated by SDS–PAGE, transferred to nitrocellulose and immunoblotted with αp85 (Ab21) (lanes a and b) or αPY (lanes e, f, g and h) antibodies. Lanes a and b were re-blotted with αPY (lanes c and d), and lane h was re-blotted with αp85 (Ab21) (lane i).

The tyrosyl phosphorylated bands in the αp85 immunoprecipitates (Figure 2, lanes e and f) co-migrated with the major bands detected by αPY immunoblots of anti-insulin receptor and anti-IRS-1 immunoprecipitates from insulin-stimulated cells (Figure 2, lanes g and h). Recent data suggest that the insulin receptor and IRS-1 associate in intact cells (Sun,X.-J., Miralpeix,M., Myers,M.G.,Jr, Glasheen,E.M., Backer,J.M., Kahn,C.R. and White,M.F., submitted). Consistent with this observation, anti-insulin receptor immunoprecipitates contained tyrosyl phosphorylated proteins at 95 kDa (β-subunit) and 180 kDa (IRS-1), as well as bands >205 kDa and <80 kDa (Figure 2, lane g). Similarly, αIRS-1 immunoprecipitates contained bands at 180 kDa (IRS-1) and 95 kDa (β-subunit) (Figure 2, lane h). Tyrosyl phosphorylated p85 was not detected in the αIRS-1 immunoprecipitate (Figure 2, lane h), despite the fact that re-blotting of lane h with αp85 antibody revealed the presence of p85 (Figure 2, lane i); the presence of tyrosyl phosphorylated p85 in the αIR immunoprecipitate could not be evaluated due to the size of the β-subunit band. Thus, αp85 immunoprecipitates from insulin-stimulated cells appear to contain both the insulin receptor β-subunit and IRS-1. Although the stoichiometry of these associations is not yet clear, the immunoblots raise the possibility of a ternary complex between the insulin receptor, IRS-1 and PtdIns 3′-kinase in intact cells.

Overexpression of IRS-1 in CHO cells increases the insulin-stimulated activation of PtdIns 3′ kinase

The relationship between the insulin receptor, IRS-1 and activation of PtdIns 3′-kinase was examined in control CHO cells (CHO/his) or CHO cells overexpressing IRS-1

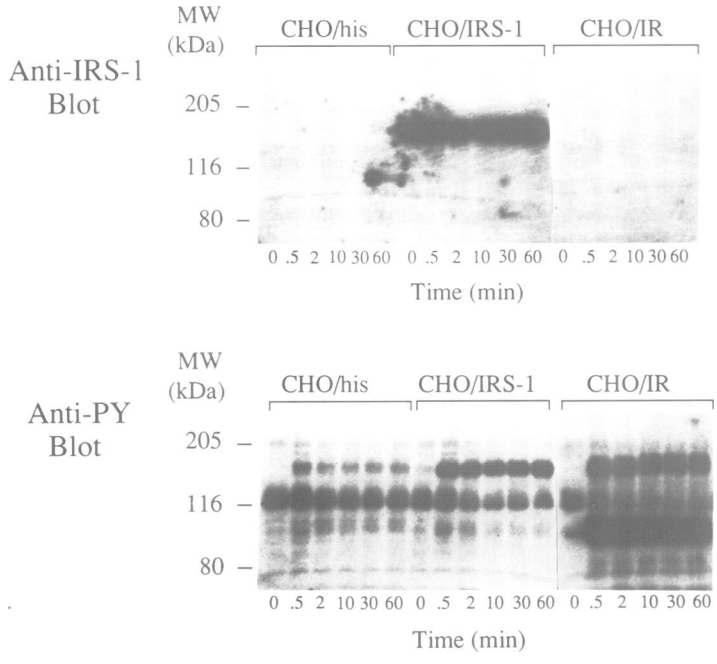

Fig. 3. Insulin-stimulated phosphorylation of IRS-1 in cells overexpressing the insulin receptor or IRS-1. Quiescent CHO/his, CHO/IRS-1 and CHO/IR cells were incubated in the absence or presence of 100 nM insulin for 0–60 min and lysed in Laemmli sample buffer. Proteins were separated by SDS–PAGE, transferred to nitrocellulose and blotted with αIRS-1 (αpep80; top panel) or αPY antibodies (lower panel).

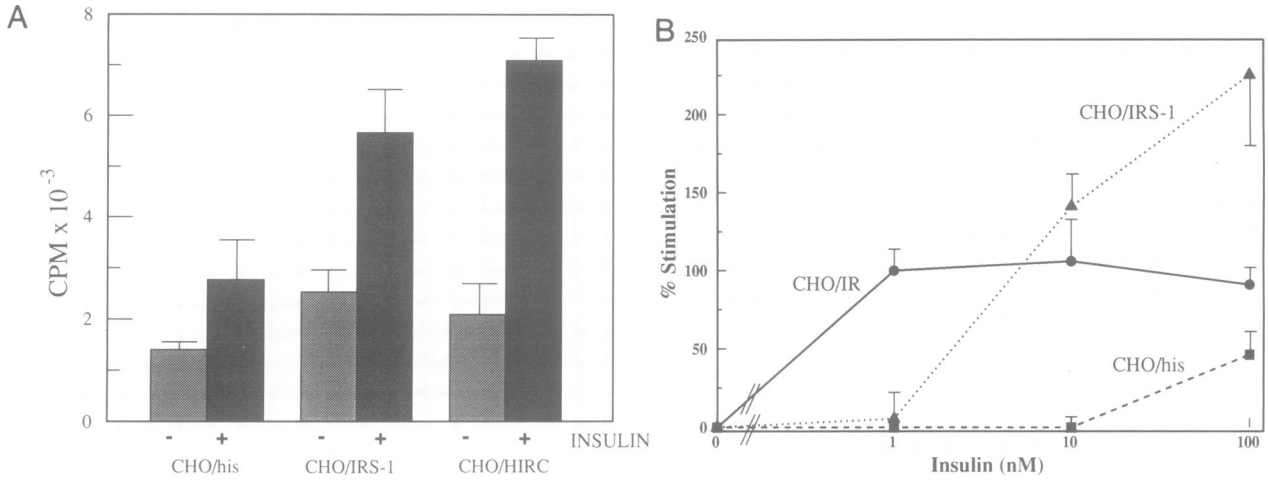

Fig. 4. Insulin-stimulation of PtdIns 3′-kinase in cells overexpressing the insulin receptor or IRS-1. (**A**) Quiescent CHO/his, CHO/IRS-1 and CHO/IR cells were incubated in the absence or presence of 100 nM insulin for 10 min, washed, solubilized and immunoprecipitated with αp85 (Ab51) and protein A−Sepharose. The pellets were washed and assayed for PtdIns 3′-kinase activity. Data represent the mean ± SEM, and are representative of four separate experiments. (**B**) Insulin dose−response of αp85-precipitable (Ab51) PtdIns 3′-kinase activity. Data represent the mean ± SEM of triplicates, and are representative of two separate experiments.

(CHO/IRS-1) or the human insulin receptor (CHO/IR). Western blots of cell lysates performed with αIRS-1 antibody demonstrated that IRS-1 is overexpressed in CHO/IRS-1 cells, whereas expression of IRS-1 was much lower in the CHO/his and CHO/IR cells (Figure 3, upper panel). Blots with αPY showed that basal IRS-1 tyrosyl phosphorylation was minimal in all three cell lines (Figure 3, lower panel). However, insulin stimulation caused a slight increase in tyrosyl phosphorylated IRS-1 in the CHO/his cells. In contrast, an immediate insulin-stimulated tyrosyl phosphorylation of IRS-1 was seen in both the CHO/IRS-1 and the CHO/IR cells as a result of the increased intracellular expression of the substrate or the kinase, respectively (Figure 3, lower panel). Tyrosyl phosphorylation of IRS-1 was maximal within 0.5 min and was stable for up to 60 min. This time course is similar to the kinetics of insulin-stimulated PtdIns 3′-kinase activity in CHO/IR cells (Backer *et al.*, 1992).

Activation of PtdIns 3′-kinase by insulin (100 nM) was measured in αp85 immunoprecipitates from the CHO/his, CHO/IRS-1 or CHO/IR cells (Figure 4A). In CHO/his cells, a small amount of both basal and insulin-stimulated activity was detected. In contrast, both basal and insulin-stimulated PtdIns 3′-kinase activities were increased nearly 2-fold in the CHO/IRS-1 cells. Insulin-stimulated PtdIns 3′-kinase in the CHO/IRS-1 cells was similar to that observed in the CHO/IR cells. Insulin dose−responses in the three cell lines showed that overexpression of IRS-1 alone increased the magnitude and insulin sensitivity of PtdIns 3′-kinase activation, whereas overexpression of the insulin receptor had a greater effect on the sensitivity of the response (Figure 4B). These data suggest that the insulin receptor-mediated phosphorylation of IRS-1 plays an important role in the insulin stimulation of PtdIns3′-kinase in intact cells.

PtdIns 3′-kinase binds to tyrosyl phosphorylated IRS-1 in vitro

IRS-1 contains 14 potential phosphorylation sites, nine of which contain YXXM motifs. The binding of PtdIns 3′-kinase to IRS-1 and tyrosyl phosphorylated IRS-1 was reconstituted *in vitro* in the absence or presence of tyrosyl phosphopeptides based on these sequences (Table II). IRS-1 produced in Sf-9 cells (IRS-1^{bac}) was phosphorylated with partially purified insulin receptors in the presence of insulin and ATP and immobilized on αIRS-1/protein A−Sepharose beads; tryptic maps of *in vitro* phosphorylated IRS-1 showed 21 phosphotyrosine-containing peaks (data not shown). The IRS-1 beads were incubated for an additional 15 min at 4°C with lysates from quiescent CHO/neo cells, washed again and assayed for the presence of PtdIns3′-kinase activity. Negligible PtdIns 3′-kinase activity was detected when non-phosphorylated IRS-1 beads were used (data not shown). In contrast, a significant amount of PtdIns 3′-kinase from quiescent CHO/neo cells bound to tyrosyl phosphorylated IRS-1 beads (Figure 5A). The addition to the cell lysate of 100 μM non-phosphorylated peptide (Y628), derived from the Y_{628}MPM region of IRS-1, had no effect on the binding of PtdIns 3′-kinase to tyrosyl phosphorylated IRS-1. In contrast, 100 μM tyrosyl phosphorylated peptide (P-Y628) blocked the association of PtdIns 3′-kinase by >95%, and significant inhibition of the association was seen even at 1 μM peptide. The inhibition of PtdIns 3′-kinase binding to tyrosyl phosphorylated IRS-1 was half-maximal at 200−300 nM phosphopeptide (data not shown).

Seven other peptides (300 nM) containing various phosphorylated YXXM motifs based on sequences in IRS-1 showed similar potencies as inhibitors of IRS-1/PtdIns 3′-kinase association (Figure 5B). IRS-1/PtdIns 3′-kinase association was similarly inhibited by synthetic phosphopeptides known to bind to PtdIns 3′kinase through the SH2 domains in p85, including the phosphorylated Y_{315}MPM sequence from the polyoma middle T antigen (P-MT) and the phosphorylated Y_{708}MDM and Y_{719}VPM sequences from the PDGF receptor kinase insert region (P-Y708 and P-Y719) (Auger *et al.*, 1992; Escobedo *et al.*, 1991a). In contrast, the non-phosphorylated middle T peptide was ineffective (data not shown), and no inhibition was seen with a tyrosyl phosphorylated peptide from the insulin receptor kinase regulatory region (P-Y1146), which lacks

Table II.

Name	Sequence	Source
Non-phosphopeptides:		
Y628	R-K-G-N-G-D-**Y**-M-P-M-S-P-K-S-V	IRS-1 (Tyr628)
MT	E-E-E-**Y**-M-P-M-E-D-L-Y	Polyoma middle T (Tyr315)
Phosphopeptides:		
P-Y460	L-S-N-**Y**-I-C-M-G-G-K-G	IRS-1 (Tyr460)
P-Y546	I-E-E-**Y**-T-E-M-M-P-A-A	IRS-1 (Tyr546)
P-Y608	D-D-G-**Y**-M-P-M-S-P-G-V	IRS-1 (Tyr608)
P-Y628	N-G-D-**Y**-M-P-M-S-P-K-S	IRS-1 (Tyr628)
P-Y658	P-N-G-**Y**-M-M-M-S-P-S-G	IRS-1 (Tyr658)
P-Y727	T-G-D-**Y**-M-N-M-S-P-V-G	IRS-1 (Tyr727)
P-Y939	S-E-E-**Y**-M-N-M-D-L-G-P	IRS-1 (Tyr939)
P-Y628$_{sc}$	P-M-P-N-S-K-M-D-**Y**-G-G	IRS-1 (Tyr628, scrambled)
P-MT	E-E-E-**Y**-M-P-M-E-D-L-Y	Polyoma middle T (Tyr315)
IR-P-Y1146	D-I-**Y**-E-T-D-Y-Y-R-K-G	Insulin receptor (Y_{1146})
PDGFR-P-Y708	D-G-G-**Y**-M-D-M-S-K-D-E	PDGF receptor (Tyr708)
PDGFR-P-Y719	S-I-D-**Y**-V-P-M-L-D-M-K	PDGF receptor (Tyr719)

Sequences of peptides and phosphopeptides from IRS-1, polyoma middle T antigen (murine), the human insulin receptor, and the mouse PDGF receptor. YMXM motifs are underlined, and phosphorylated tyrosine residues are in bold face. Numbering of the human insulin receptor sequence is taken from Ullrich *et al.* (1985), and reflects the exon 11-minus variant.

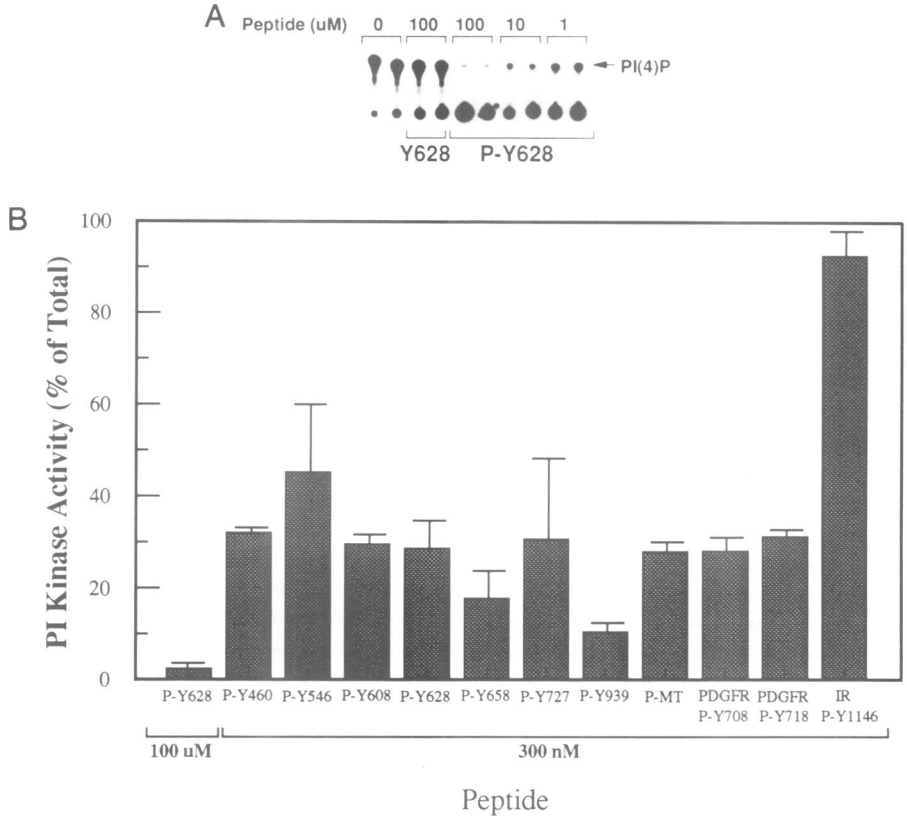

Fig. 5. Inhibition of PtdIns 3′-kinase binding to tyrosyl phosphorylated IRS-1 by a YXXM phosphopeptide based on the sequence of IRS-1. (**A**) IRS-1^{bac} was immobilized on αIRS-1^{bac}/protein A−Sepharose beads and tyrosyl phosphorylated by wheat germ agglutinin-purified insulin receptors. The pellets were washed and incubated for 15 min at 4°C with detergent lysates from quiescent CHO/neo cells in the absence or presence of Y628 or P-Y628 peptide at varying concentrations; see Table II for the sequence of each peptide. The pellets were then washed and assayed for associated PtdIns 3′-kinase activity. The arrow indicates the position of the PtdIns(4)P standard, which comigrates with PtdIns(3)P under these conditions. (**B**) Association assays were conducted as above in the presence of peptides and phosphopeptides as indicated; see Table II for sequences. IRS-1-associated PtdIns 3′-kinase activity was expressed as a percentage of the total IRS-1-associated activity measured in the absence of peptide. All phosphopeptides were used at 300 nM; P-Y628 was also assayed at 100 μM.

the YXXM motif [DI(P)YETDYYRKG]. Thus, high affinity binding between IRS-1 and PtdIns 3′kinase requires the presentation of phosphotyrosine in a YXXM motif. These data also suggest that IRS-1 may have multiple potential sites of interaction with PtdIns 3′-kinase, since all of these IRS-1-derived peptides are substrates for the insulin receptor (Shoelson *et al.*, 1992) and can interact with PtdIns 3′-kinase with similar affinities.

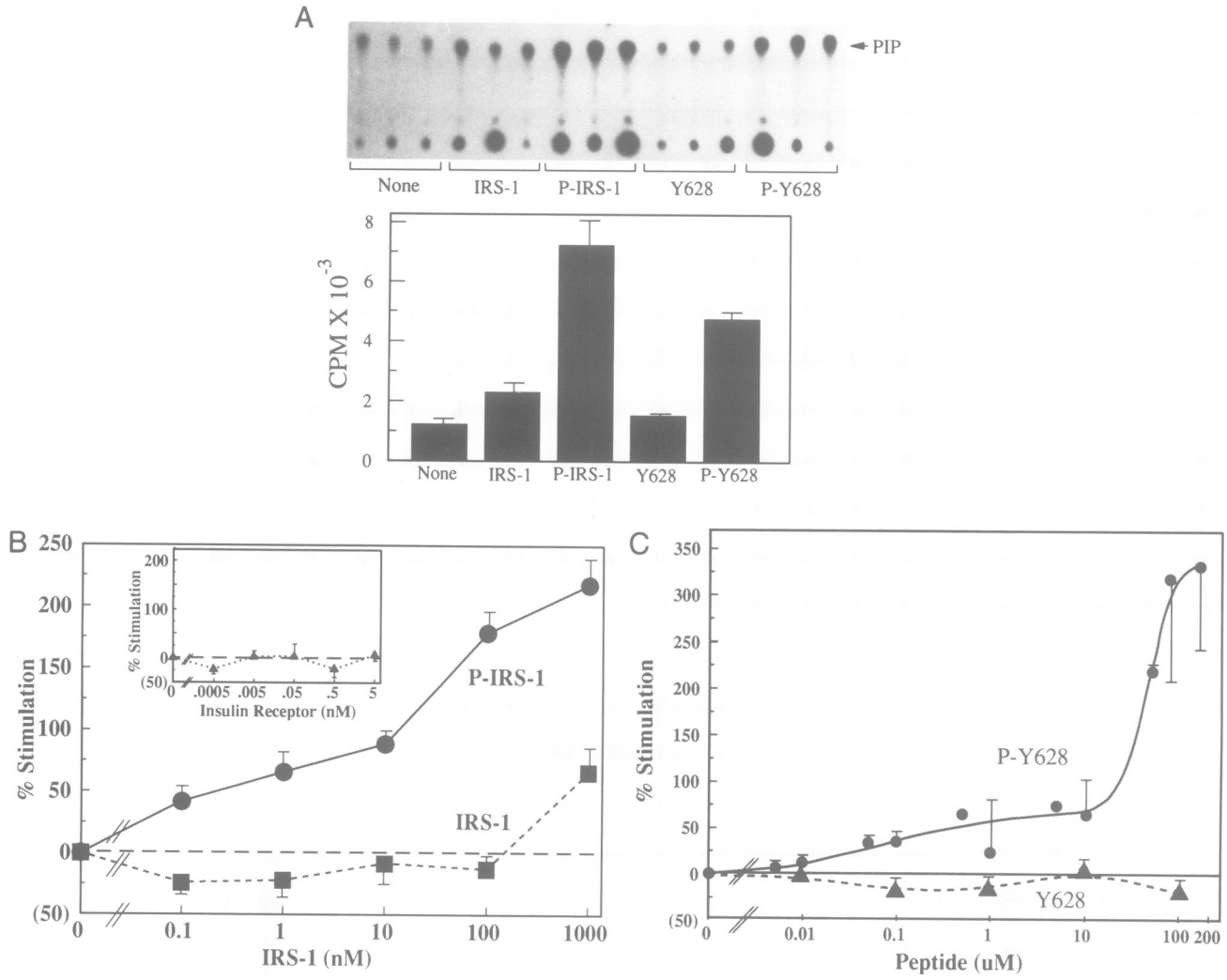

Fig. 6. *In vitro* activation of PtdIns 3′-kinase by tyrosyl phosphorylated IRS-1 and a YXXM phosphopeptide based on the sequence of IRS-1. (**A**) αp85 (Ab51)/protein A−Sepharose immunoprecipitates from quiescent CHO/neo cells were incubated for 15 min at 4°C in the absence or presence of non-phosphorylated or *in vitro* tyrosyl phosphorylated IRS-1. The pellets were washed and assayed for PtdIns 3′-kinase activity. Alternatively, washed αp85 immunoprecipitates from quiescent CHO/neo cells were incubated with 100 μM nonphosphorylated (Y628) or tyrosyl phosphorylated (P-Y628) peptide for 30 min at 4°C, and assayed for PtdIns 3′-kinase activity. All determinations were done in triplicate, and the data are representative of four experiments. (**B**) αp85 (AB51) immunoprecipitates from quiescent CHO/neo cells were incubated with varying concentrations of IRS-1 or *in vitro* phosphorylated IRS-1 for 15 min at 4°C, washed and assayed for PtdIns 3′-kinase activity. All determinations were done in triplicate. **Inset:** αp85 immunoprecipitates were incubated with the same amounts of ATP and insulin receptor present in the phosphorylated IRS-1 samples. (**C**) αp85 (AB51) immunoprecipitates from quiescent CHO/neo cells were incubated with varying concentrations of Y628 or P-Y628 peptide for 30 min at 4°C, and assayed for PtdIns 3′-kinase activity. All determinations were done in triplicate; the data from three separate experiments are pooled.

Tyrosyl phosphorylated IRS-1 activates PtdIns 3′-kinase in vitro

To investigate the mechanism of PtdIns 3′-kinase activation, IRS-1^{bac} was incubated overnight at 4°C in ATP and in the absence or presence of partially purified insulin receptors. The non-phosphorylated or tyrosyl phosphorylated IRS^{bac} was then incubated at 4°C with αp85 immunoprecipitates from quiescent CHO/neo cells. The immunoprecipitates were washed and then assayed for PtdIns 3′-kinase activity (Figure 6A). Incubation of the immobilized PtdIns3′-kinase with non-phosphorylated IRS-1^{bac} caused a small stimulation of PtdIns 3′-kinase, which may reflect the fact that IRS-1^{bac} contains a small amount of phosphotyrosine when isolated from Sf-9 cells (M.G.Myers,Jr and M.F.White *et al.*, unpublished observations). However, incubation with tyrosyl phosphorylated IRS-1^{bac} caused a 500% increase in PtdIns 3′-kinase activity. Activation by tyrosyl phosphorylated IRS-1 was half-maximal at ~10−20 nM, whereas activation by non-phosphorylated IRS-1 was only seen at 1 μM (Figure 6B). No stimulation was seen when immobilized PtdIns3′-kinase was incubated with the same amount of insulin receptor and ATP present in the phosphorylated IRS-1 samples (Figure 6B, inset), and tyrosyl phosphorylation of p85 could not be detected in parallel incubations which included [^{32}P]ATP. These data suggest that PtdIns 3′-kinase is directly activated by its interactions with tyrosyl phosphorylated IRS-1.

The competition experiments in Figure 5 suggest that PtdIns 3′-kinase associates with IRS-1 through phosphorylated YXXM motifs. To determine whether this association was sufficient to activate PtdIns 3′-kinase, αp85 immunoprecipitates were incubated with the non-phosphorylated Y628 or tyrosyl phosphorylated P-Y628 peptides. The non-phosphorylated peptide had no effect,

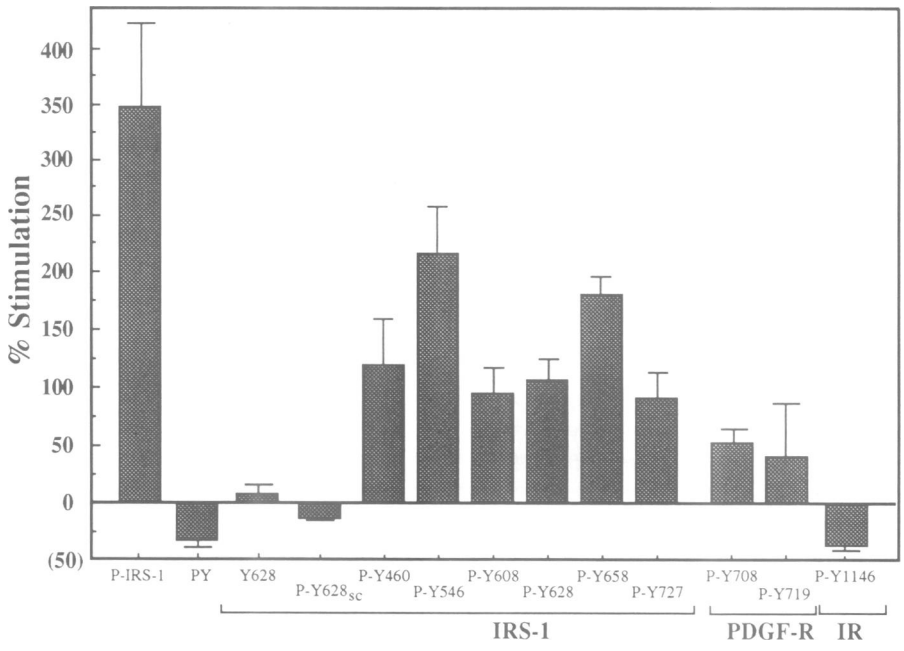

Fig. 7. *In vitro* activation of PtdIns 3′-kinase by YXXM phosphopeptides. αp85 (Ab51) immunoprecipitates from quiescent CHO cells were incubated with peptides and phosphopeptides (100 μM) from IRS-1, the PDGF receptor and the insulin receptor for 30 min at 4°C, and assayed for PtdIns 3′-kinase activity; see Table II for the sequence of each peptide. All determinations were done in triplicate; the data are representative of four separate experiments.

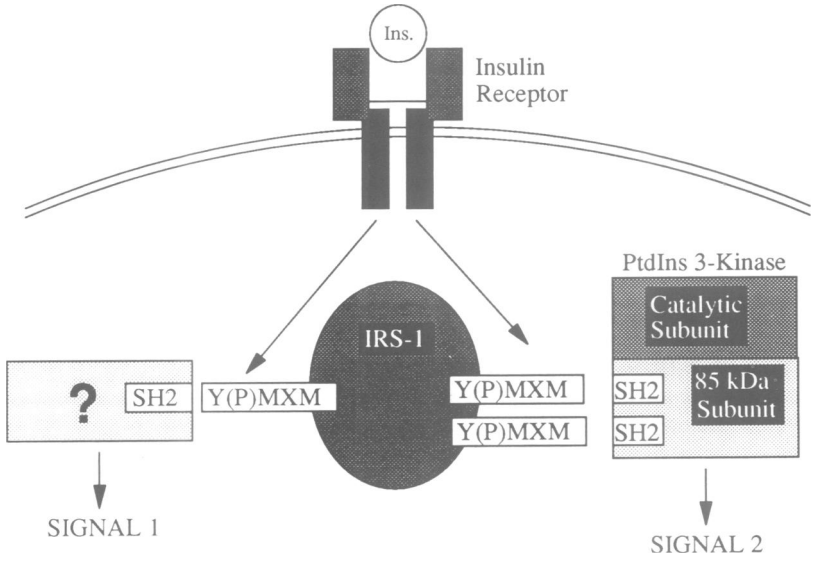

Fig. 8. Model of the role of IRS-1 in insulin receptor signal transduction. Insulin binding to the insulin receptor activates the insulin receptor tyrosine kinase, leading to the tyrosyl phosphorylation of YXXM motifs in IRS-1. The phosphorylation of these YXXM motifs provides binding sites for SH2 domains in PtdIns 3′-kinase-associated p85 and other unidentified proteins involved in insulin signal transduction. The binding of two phosphorylated YXXM motifs in IRS-1, or other structural features in the intact protein, may be required to activate PtdIns 3′-kinase fully. The recruitment of different signaling molecules by tyrosyl phosphorylated IRS-1 may play a role in the divergence of the insulin signal into multiple signaling pathways.

whereas the phosphorylated peptide (100 μM) increased the activity of PtdIns 3′-kinase by 300% (Figure 6A). Interestingly, activation of PtdIns 3′-kinase by the P-Y628 peptide was biphasic (Figure 6C). An ED_{50} of ~200 nM was observed for the initial phase of activation, similar to the ED_{50} for inhibition of IRS-1/PtdIns 3′-kinase binding by the P-Y628 peptide (data not shown), with additional activation observed at higher concentrations of peptide. Similar dose–responses for activation were obtained using two different αp85 antibodies (Ab51 or $\alpha p85^{GST}$; data not shown).

All of the tyrosyl phosphorylated YXXM peptides tested had similar abilities to block binding of IRS-1 to PtdIns 3′-kinase (Figure 5B). To examine the relationship between binding and activation of PtdIns3′-kinase, we measured the ability of phosphopeptides from IRS-1, the PDGF receptor and the insulin receptor to activate PtdIns 3′-kinase *in vitro*; all peptides were used at 100 μM (Figure 7). Non-

phosphorylated Y-628 peptide had no effect on PtdIns 3′-kinase activity, nor did a scrambled P-Y628 peptide (P-$Y628_{sc}$) or an insulin receptor-derived peptide containing phosphotyrosine but lacking a YXXM motif (P-Y1146). Phosphotyrosine (1 mM) also did not activate. In contrast, all of the tested phosphopeptides based on IRS-1 sequences activated PtdIns 3′-kinase by ~100–200% above basal activity. Two synthetic phosphopeptides based on the sequence of the PDGF receptor kinase insert region activated PtdIns 3′-kinase by ~50% (Figure 7). Together, our results suggest that tyrosine phosphorylated IRS-1 activates PtdIns 3′-kinase by binding to the enzyme. This activation can be mimicked by YXXM sequences present in known PtdIns3′-kinase binding proteins. Interactions between phosphorylated YXXM motifs and the SH2 domains in p85 may therefore be critical for the activation of PtdIns 3′-kinase.

Discussion

IRS-1 plays a key role in the activation of PtdIns 3′-kinase by insulin in intact cells. Immediately after insulin stimulation, IRS-1 undergoes tyrosine phosphorylation and forms a stable complex with PtdIns 3′-kinase that can be immunoprecipitated from cells with anti-phosphotyrosine, anti-IRS-1 or anti-p85 antibodies. Formation of the IRS-1/PtdIns 3′-kinase complex *in vitro* is inhibited by synthetic peptides containing phosphorylated YXXM (Tyr-Xxx-Xxx-Met) motifs, suggesting that the binding of PtdIns 3′-kinase to IRS-1 is mediated through the SH2 (src homology-2) domains of p85. Our data suggest that formation of the IRS-1/PtdIns 3′-kinase complex is the activating step during insulin stimulation. Incubation of tyrosyl phosphorylated IRS-1 with anti-p85 immunoprecipitates causes a 4- to 5-fold increase in PtdIns 3′-kinase activity. Importantly, this activation is mimicked by synthetic peptides containing phosphorylated YXXM motifs, but not by phosphotyrosine alone or phosphotyrosine in a scrambled amino acid sequence. In insulin-stimulated cells, the specific activity of IRS-1-associated PtdIns 3′-kinase is 2.5-fold greater than that of total PtdIns 3′-kinase. Moreover, conditions that increase the amount of tyrosyl phosphorylated IRS-1 in cells, either overexpression of IRS-1 or overexpression of the insulin receptor kinase, increase the amount of insulin-stimulated PtdIns 3′-kinase activity. We conclude that PtdIns 3′-kinase is activated during insulin stimulation by association with tyrosine phosphorylated YXXM motifs in IRS-1. Moreover, the binding of p85 to similar motifs in various growth factor receptor tyrosine kinases may provide a general mechanism for the activation of PtdIns 3′-kinase.

PtdIns 3′-kinase is one of a number of signal-transducing proteins that form heterologous complexes with growth factor receptor tyrosine kinases and oncogene products (Cantley *et al.*, 1991). The recruitment of PtdIns 3′-kinase into heterologous signaling complexes was first demonstrated with $pp60^{v\text{-}src}$ and the polyoma middle T/$pp60^{c\text{-}src}$ complex (Sugimoto *et al.*, 1984; Whitman *et al.*, 1985, 1987). PtdIns 3′-kinase also associates tightly with ligand-stimulated receptors for PDGF, CSF-1 and EGF (Cantley *et al.*, 1991). In contrast, the recruitment of PtdIns 3′-kinase into a heterologous complex with the insulin receptor itself is relatively weak (Endemann *et al.*, 1990; Backer *et al.*, 1992). PtdIns 3′-kinase consists of two distinct subunits including a putative 110 kDa catalytic subunit and an 85 kDa regulatory subunit (p85) that contains two SH2 domains and one SH3 (src-homology-3) domain (Carpenter *et al.*, 1990; Escobedo *et al.*, 1991b; Otsu *et al.*, 1991; Shibasaki *et al.*, 1991; Skolnik *et al.*, 1991). The SH2 domains of PtdIns 3′-kinase bind preferentially to phosphorylated YXXM motifs, such as those in the PDGF and CSF-1 receptors and the polyoma middle T antigen (Cantley, 1991; Hu *et al.*, 1992; Klippel *et al.*, 1992; McGlade *et al.*, 1992; Reedijk *et al.*, 1992). Substitution of Y_{740}MDM with F_{740}MDM or Y_{751}VPM with F_{751}VPM in the PDGF receptor kinase insert region, or Y_{315}MPM with F_{315}MPM in the polyoma middle T antigen, blocks PtdIns 3′-kinase kinase association in intact cells (Kazlauskas and Cooper, 1989, 1990; Talmadge *et al.*, 1989; Kashishian *et al.*, 1992). Furthermore, phosphopeptides derived from the PDGF receptor kinase insert region or the Y_{298}MPM sequence from hamster middle T prevent PtdIns 3′-kinase binding to the PDGF receptor and the middle T/$pp60^{c\text{-}src}$ complex, respectively (Escobedo *et al.*, 1991a; Auger *et al.*, 1992).

The association of PtdIns 3′-kinase with the PDGF receptor is coincident with the formation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in intact cells, and tyrosyl phosphorylation of an 85 kDa protein, presumably p85 (Kaplan *et al.*, 1987; Courtneidge and Heber, 1987; Cohen *et al.*, 1990a; Kazlauskas and Cooper, 1989, 1990; Talmadge *et al.* 1989; Auger *et al.*, 1989). However, it is unclear from previous reports whether binding to these tyrosine kinases activates PtdIns 3′-kinase, or instead facilitates activation through the subsequent tyrosyl phosphorylation of p85. Hu *et al.* (1992) were unable to detect p85 tyrosyl phosphorylation in PDGF-stimulated HER14 cells, whereas p85 and PtdIns 3′-kinase activity were associated with the PDGF receptor. Insulin increases the cellular levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Endemann *et al.*, 1990; Ruderman *et al.*, 1990; Kelly *et al.*, 1992) and activates PtdIns 3′-kinase. However, we are unable to observe tyrosyl phosphorylation of p85 under these conditions. Thus, tyrosyl phosphorylation of p85 may not play an important role in activation of PtdIns 3′-kinase by insulin.

IRS-1 contains 14 potential tyrosyl phosphorylation sites, of which six are present in YMXM motifs, with another three residues in YXXM motifs; four of the YMXM peptides are present in the sequence YMXMSP, and the sequences surrounding Y_{608} and Y_{628} have the eight amino acids (D/G-G/D-YMPMSP). Peptides derived from the YXXM sites are excellent substrates for the insulin receptor kinase, with K_m values of 24–92 μM; the presence of Met in the Y + 1 position enhances the catalytic efficiency of peptide phosphorylation (Shoelson *et al.*, 1992) but does not appear to be required for binding to the SH2 domains of p85. Peptide maps of *in vitro* phosphorylated IRS-1 contain 21 peaks, all of which contain phosphotyrosine (Sun *et al.*, unpublished observations). Preliminary tryptic maps of *in vivo* phosphorylated IRS-1 show at least six major and several minor peptides containing phosphotyrosine, as well as numerous serine/threonine-containing peptides (Sun *et al.*, submitted). Although the *in vivo* phosphorylation sites in IRS-1 have not yet been determined, it is likely that some of them contain the YXXM motif. Furthermore, the seven IRS-1-derived YXXM phosphopeptides tested so far have similar abilities to inhibit the formation of IRS-1/PtdIns

3′-kinase complexes, suggesting that the phosphorylation of any of these sequences in the intact IRS-1 would form good PtdIns 3′-kinase binding sites and should activate the enzyme. The fact that the YXXM motif is recognized as a substrate by the insulin receptor and as a ligand by PtdIns 3′-kinase functionally links these distinct signal-transducing molecules.

Our data suggest that PtdIns 3′-kinase is activated in cells by binding to tyrosyl phosphorylated YXXM motifs in IRS-1. Activation of PtdIns 3′-kinase by *in vitro* phosphorylated IRS-1 is significantly more sensitive than activation by synthetic peptides containing a single phosphorylated YXXM motif. Furthermore, the activation by the synthetic Y_{628}MPM peptide of IRS-1 displays a biphasic curve. The initial phase of activation occurs below 10 μM peptide and has an ED_{50} of ~200 nM, which is similar to the concentration that inhibits the binding between IRS-1 and PtdIns 3′-kinase. The second phase of activation occurs between 10 and 100 μM peptide, which is expected to saturate SH2 domain binding (Escobedo *et al.*, 1991a; Klippel *et al.*, 1992). It is possible that the more potent activation of PtdIns 3′-kinase by intact IRS-1 reflects a requirement for structural features beyond the YXXM motif. Alternatively, full activation may require occupancy of both SH2 domains in p85, which could be accomplished by multivalent IRS-1 or high concentrations of monovalent phosphopeptides. In this regard, several of the tyrosine kinases that activate PtdIns 3′-kinase, including the PDGF and CSF-1 receptors, contain multiple YXXM phosphorylation sites. Mutagenesis of the human PDGF receptor has furthermore suggested that two tyrosine phosphorylation sites in the kinase-insert region (Y_{740}MDM and Y_{751}VPM) are required for high affinity binding of PtdIns 3′-kinase (Kashishian *et al.*, 1992). Other signal transduction molecules such as PLC_γ and $p21^{ras}$-GAP contain two SH2 domains and may also be fully activated when both SH2 domains are occupied with multivalent ligands containing phosphotyrosine in the appropriate amino acid sequence motif.

While our data are consistent with the hypothesis that insulin-stimulated complex formation with IRS-1 directly activates PtdIns 3′-kinase, the activation may be modulated by several other mechanisms in intact cells. Insulin stimulation of CHO/IR cells increases the amount of PtdIns 3′-kinase activity in αPY immunoprecipitates, which has been interpreted to mean that p85 is phosphorylated by the insulin receptor (Endemann *et al.*, 1990; Ruderman *et al.*, 1991). Although we are unable to detect tyrosyl phosphorylated p85 in insulin-stimulated cells, we cannot rule out the possibility of transient or labile tyrosyl phosphorylation. Moreover, the presence of a tyrosyl phosphorylated 95 kDa band in anti-p85 immunoprecipitates from insulin-stimulated cells raises the possibility that a direct interaction occurs between the insulin receptor and PtdIns 3′-kinase, perhaps via the phosphorylated Y_{1322}THM motif in the C-terminal tail of the insulin receptor. Preliminary studies suggest that the insulin receptor can associate with PtdIns 3′-kinase SH2 domains expressed in GST fusion proteins (data not shown). However, IRS-1 also associates with the insulin receptor, which could mediate the formation of a tertiary complex between the insulin receptor, IRS-1 and PtdIns 3′-kinase (Sun *et al.*, submitted). Although IRS-1 is thought to be primarily cytosolic (White *et al.*, 1987), it is a substrate for the insulin receptor and therefore must be close to the plasma membrane in insulin-stimulated cells. Binding to IRS-1 might alter the subcellular location of PtdIns 3′-kinase (Cohen *et al.*, 1990b; Kelly *et al.*, 1992; Zhang *et al.*, 1992), or bring it into juxtaposition with another IRS-1 associated molecule which could modulate its activity.

The multiplicity of potential tyrosyl phosphorylation sites in the IRS-1 molecule, including YXXM motifs as well as five additional sites, suggests that this protein may be a particularly fluent mediator of the divergence of the insulin signal into multiple intracellular pathways (Figure 8). Certainly, the variable phosphorylation of potential PtdIns 3′-kinase binding sites within IRS-1 could achieve an extremely subtle regulation of PtdIns 3′-kinase by insulin. Moreover, the binding of PtdIns 3′-kinase to IRS-1 is a useful paradigm for the role of IRS-1 in insulin signaling. Variable phosphorylation of the YXXM motifs and perhaps other tyrosyl phosphorylation sites in IRS-1 may regulate the binding of novel signaling proteins that contain SH2 domains (Koch *et al.*, 1991). Although the binding specificity of different SH2 domain sequence variations is not fully understood, recent data suggest that PtdIns 3′-kinase, PLC_γ and GAP bind to different sites within the PDGF receptor (Kazlauskas and Cooper, 1990; Kaslauskas *et al.*, 1990; Morrison *et al.*, 1990; Escobedo *et al.*, 1991a; Kashishian *et al.*, 1992), and the phosphorylation sites in the EGF receptor C-terminus that bind PLC_γ and GAP are not present in YXXM motifs (Margolis *et al.*, 1990). Furthermore, we cannot detect PLC_γ or GAP binding to IRS-1 (data not shown). Thus, interactions between IRS-1 and other signaling proteins may involve sequences other than the YXXM motifs implicated in PtdIns 3′-kinase binding. The delineation of the insulin-stimulated interactions between IRS-1 and other proteins involved in signal transduction should have important implications for the mechanism of insulin action and cellular regulation by other tyrosine kinase receptors.

Materials and methods

Antibodies to IRS-1 and PtdIns 3′-kinase

A polyclonal rabbit antibody to the IRS-1-derived peptide YIPGATMGTSPALTGD (peptide 80) has been previously described (Sun *et al.*, 1991). Polyclonal antibodies to baculovirus-produced rat IRS-1 (αIRS-1^{bac}) and to a glutathione-S-transferase (GST) bacterial fusion protein containing amino acids 321–724 (Hu *et al.*, 1992) of the human p85 (αp85GST) were produced by injection into New Zealand white rabbits and were purified by protein A–Sepharose chromatography. Polyclonal antibodies to a peptide corresponding to residues 500–518 of the human p85 (Ab51), or to a GST–p85 bacterial fusion protein containing amino acids 265–523 of the human p85 (Ab21), have been previously described (Hu *et al.*, 1992). A mouse monoclonal antibody to the human insulin receptor (83-14) was provided by Kenneth Siddle, University of Cambridge.

Synthesis of peptides and phosphopeptides

Peptides and phosphopeptides derived from the cDNA sequence of rat IRS-1 (Sun *et al.*, 1991), the human insulin receptor (Ullrich *et al.*, 1985), murine polyoma middle T antigen (Soeda *et al.*, 1980) and the mouse PDGF receptor (Yarden *et al.*, 1986) were synthesized using a Milligen/Biosearch 9600 synthesizer. Phosphopeptides were prepared using FmocTyr$(OP(OCH_2)_2)$ as previously described (Chatterjee *et al.*, 1992). All peptides were purified by gel exclusion chromatography and were >80% pure as judged by analysis on reversed phase HPLC. The composition of peptides and the concentrations of peptide solutions were verified by amino acid analysis.

Cell culture and transfection of CHO cells

Control CHO cells (CHO/neo) and CHO cells expressing 1.2×10^6 human insulin receptors/cell (CHO/IR) have been previously described (Backer *et al.*, 1991). CHO/IRS-1 cells, which overexpress IRS-1, were prepared

by calcium phosphate co-transfection of the rat IRS-1 cDNA in the expression vector pCMVhis (M.Birmbaum, Harvard Medical School) and the pSVEneo plasmid. Cells were selected by culture in 800 μg/ml geneticin (Gibco) and 10 mM histidinol (Sigma) as previously described (Sun *et al.*, 1991). CHO/his and CHO/IR^{his} cells were prepared by transfection of CHO/neo and CHO/IR cells, respectively, with the pCMVhis vector alone, and were selected by culture in histidinol. Although the CHO/IRS cells were compared with the CHO/his and CHO/IR^{his} cells in Figures 3 and 4, we have dropped the 'his' superscript for simplicity.

Preparation of IRS-1 in baculovirus-infected Sf-9 cells

The rat IRS-1 cDNA, with the 5′ and 3′ untranslated regions removed, was subcloned into the pBlueBac (Invitrogen) and recombined into the baculovirus genome using standard techniques. Sf9 cells were infected with 10–20 p.f.u./cell of recombinant baculovirus as described (Herrera *et al.*, 1988). Cells were lysed after 48–52 h of infection in a Dounce homogenizer in 50 mM Tris pH 7.8 containing 1 M NaCl, 10 μg/ml aprotinin, 10 mM benzamidine, 10 μg/ml leupeptin and 350 μg/ml phenlymethylsulfonylfluoride. Insoluble material was removed by sedimentation at 100 000 *g* for 1 h, and IRS-1 was purified to 90% purity by chromatography on Sephacryl S-300 HR (Pharmacia).

In vitro lipid kinase assays and analysis of products by TLC

Quiescent cells were incubated in the absence or presence of insulin (100 nM) for 10 min at 37°C, washed once with ice-cold PBS/100 μM Na_3VO_4 and twice with 20 mM Tris (pH 7.5) containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 100 μM Na_3VO_4 (Buffer A), and solubilized in Buffer A containing 1% NP-40 (Sigma), 10% glycerol and 350 μg/ml phenylmethylsulfonylfluoride (lysis buffer). A post-13 000 *g* supernatant was immunoprecipitated with anti-IRS-1 (αIRS-1) or anti-PtdIns 3′-kinase (αp85) antibody and protein A–Sepharose (Pharmacia). PtdIns 3′-kinase activity in the immunoprecipitates was assayed as previously described (Ruderman *et al.*, 1990).

Western blotting

Immunoprecipitations for Western blot analysis were performed as described above for analysis of lipid kinase activity. Cell lysates for Western blot analysis were performed by incubating cells in 6-well dishes (Nunc) in the absence or presence of insulin for 2 min, followed by lysis in Laemmli sample buffer, boiling for 2 min and sonication for 2 s. Proteins were separated on 7.5% resolving SDS–PAGE minigels (Hoeffer), transferred to nitrocellulose in Towbin buffer containing 0.02% SDS, and blotted with αIRS-1 (αPep 80), anti-phosphotyrosine (αPY) or αp85 (Ab21) antibodies as indicated. Proteins were visualized using [^{125}]protein A (ICN) and autoradiography.

In vitro IRS-1/PtdIns 3′-kinase binding assays and peptide competition studies

Lysates from quiescent CHO/neo cells were incubated for 15 min at 4°C in the absence or presence of the indicated peptides derived from sequences in IRS-1, the human insulin receptor or polyoma middle T antigen (concentrations as described in the text). The lysates were added to immobilized tyrosyl phosphorylated IRS-1 (~1.9 μg/point) for 15 min at 4°C, the pellets were washed as described for analysis of lipid kinase activity, and IRS-1-associated PtdIns 3′-kinase activity was determined as described above.

In vitro activation of PtdIns 3′-kinase

Non-phosphorylated and phosphorylated IRS-1^{bac} was prepared by incubating IRS-1 (25 μg IRS-1) overnight at 4°C in 100 μM ATP, 5 mM $MnCl_2$, 1000 nM insulin and the absence or presence of wheat germ agglutinin-purified human insulin receptor (40 μg total protein). A third reaction contained insulin receptors but no IRS-1. The reaction mixtures, containing non-phosphorylated IRS-1, tyrosyl phosphorylated IRS-1, or insulin receptors alone, were diluted to the indicated concentrations and incubated for 15 min at 4°C with αp85/protein A–Sepharose immunoprecipitates from quiescent CHO/neo cells. The αp85 immunoprecipitates were additionally washed and assayed for lipid kinase activity as previously described (Ruderman *et al.*, 1990). Peptide activation experiments were performed by incubating non-phosphorylated or phosphorylated peptide with washed αp85 immunoprecipitates from quiescent CHO/neo cells for 30 min at 4°C, and then assaying for lipid kinase activity.

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