Potent Activation of Phosphatidylinositol 3′-Kinase by Simple Phosphotyrosine Peptides Derived from Insulin Receptor Substrate 1 Containing Two YMXM Motifs for Binding SH2 Domains†

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ABSTRACT: The phosphotyrosine form of the major substrate for the insulin receptor tyrosine kinase, insulin receptor substrate 1 (IRS-1), associates with and activates the enzyme phosphatidylinositol 3′-kinase (PtdIns 3′-kinase). IRS-1 contains nine potential tyrosine phosphorylation sites within YMXM or YXXM sequences known to bind to the two SH2 domains on the 85-kDa regulatory subunit of PtdIns 3′-kinase. We used sequences within IRS-1 as a model for synthesizing phosphotyrosine and nonhydrolyzable phosphonotyrosine peptides containing two YMXM motifs and tested them for their ability to bind to the SH2 domains of PtdIns 3′-kinase and stimulate its activity. We demonstrated for the first time that IRS-1-derived peptides containing two tyrosine phosphorylated YMXM motifs are capable of stimulating PtdIns 3′-kinase activity in the cytosol of 3T3-L1 adipocytes at nanomolar concentrations, similar to that required by purified phosphoryl-IRS-1 [Lamphere, M., Carpenter, C. L., Sheng, Z., Kallen, R. G., & Lienhard, G. E. (1994) *Am. J. Physiol. 266* (*Endocrinol. Metab. 29*), E486–E489] and the extent of activation by these peptides was similar to that seen by maximal stimulation of cells with insulin. In contrast, those phosphotyrosine peptides containing only a single YMXM motif were able to stimulate PtdIns 3′-kinase activity only at concentrations over 10 μM. We conclude from these results that the high-affinity activation of PtdIns 3′-kinase requires the simultaneous binding of two phosphorylated YMXM motifs on IRS-1 to the two SH2 domains of PtdIns 3′-kinase.

Insulin's effect on cellular growth and metabolism is mediated through the binding of the hormone to the extracellular α subunits of its receptor. This in turn activates the β subunit tyrosine kinase to autophosphorylate and to phosphorylate substrate proteins. Studies have demonstrated that this intrinsic tyrosine kinase activity of the receptor is required for cellular responses to insulin [reviewed in Tavare and Siddle (1993) and White and Kahn (1994)]. The major substrate phosphorylated by the insulin receptor kinase is a cytosolic protein of 165–185 kDa known as insulin receptor substrate 1 (IRS-1).[1] IRS-1 possesses no known enzymatic activity; rather, the phosphotyrosine [Tyr(p)] form of IRS-1 connects the insulin receptor to downstream events in signal transduction cascades by binding to src-homology 2 (SH2) domain containing proteins and activating them. Phosphatidylinositol 3′-kinase (PtdIns 3′-kinase) is one of the enzymes that associates with IRS-1 in response to insulin [reviewed in White and Kahn (1994) and Keller and Lienhard (1994)]. Indeed, all of the cytosolic PtdIns 3′-kinase activity generated in response to insulin is found associated with IRS-1 (Lamphere et al., 1994).

PtdIns 3′-kinase phosphorylates the inositol ring of phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate at the 3 position. The products of PtdIns 3′-kinase are not substrates for the known phospholipase C isozymes and, therefore, may serve as second messengers in and of themselves. The 85-kDa regulatory subunit of PtdIns 3′-kinase contains two SH2 domains and one src-homology 3 (SH3) domain (Panayotou & Waterfield, 1992). SH2 and SH3 domains mediate protein–protein interactions by binding to specific peptide motifs on target proteins (Pawson & Gish, 1992). PtdIns 3′-kinase is activated by the binding of the SH2 domains on its 85-kDa subunit to specific Tyr(p)-containing sequences on target proteins (Lamphere et al., 1994; Panayotou & Waterfield, 1992; Koch et al., 1991).

IRS-1 contains 14 tyrosines which are next to acidic residues, a location which suggests possible phosphorylation sites. Of these 14 tyrosines, 6 occur in YMXM motifs and are phosphorylated with the greatest efficiency by the insulin receptor kinase (Shoelson et al., 1992). Recent studies have shown that the 85-kDa subunit of PtdIns 3′-kinase binds to peptides containing a single Tyr(p) in YMXM or YXXM motifs derived from the platelet-derived growth factor receptor and polyoma virus middle T antigen, as well as IRS-1 (Auger

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[1] Abbreviations: mBHA, 4-methylbenzhydrylamine; HOBT, 1-hydroxybenzotriazole; Ac_2O, acetic anhydride; OpfP, oxypentafluorophenyl; DHBT, 3,4-dihydro-4-oxobenzotriazine 3-oxy ester; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DCM, dichloromethane; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMS, dimethyl sulfide; TFMSA, trifluoromethanesulfonic acid; ether, diethyl ether; MeCN, acetonitrile; NMP, *N*-methylpyrrolidone; Tyr(p), phosphotyrosine; PtdIns 3′-kinase, phosphatidylinositol 3′-kinase; PDGFR, platelet-derived growth factor receptor; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid hydrate; ED_{50}, effective dose for a 50% stimulation; SH2, src-homology 2; SH3, src-homology 3; IRS-1, insulin receptor substrate 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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et al., 1992; Backer et al., 1992; Carpenter et al., 1993; Zhou et al., 1993; Sun et al., 1993). The present study demonstrates that IRS-1-derived peptides containing two tyrosine phosphorylated YMXM motifs are 500–1000-fold more potent than peptides containing only a single tyrosine phosphorylated YMXM motif at binding and activating PtdIns 3′-kinase. These data suggest that high-affinity binding and activation of PtdIns 3′-kinase by IRS-1 require the association of two tyrosine phosphorylated YMXM motifs on IRS-1 to the two SH2 domains of PtdIns 3′-kinase.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. All peptide synthesis solvents and reagents were supplied by Applied Biosystems Inc. (ABI, Foster City, CA) except for the Fmoc-protected amino acids, BOP, HOBT (Milligen Corp.), Boc-norleucine (Bachem Bioscience), and tBoc(dibenzyl)phosphotyrosine (Peninsula Labs).

The peptide syntheses using Fmoc-based protocols were performed on a Milligen 9050 automated peptide synthesizer (version 5.0 software) on a 0.2-mmol scale, using 0.26 g of mBHA resin (ABI), loaded into the column as a slurry with approximately 1 g of glass beads. A protocol was used involving 90-min coupling cycles followed by 5-min capping cycles (0.5 M HOBT/0.5 M Ac_2O in DMF). The protected amino acids were coupled using a 4-fold molar excess as follows: Fmoc-proline as an OpfP ester, Fmoc-serine(*O-tert*-butyl) as a DHBT ester, and Fmoc-norleucine and tBoc-(dibenzyl)phosphotyrosine in the presence of 1 equiv of both BOP and HOBT. On completion of the synthesis, the resin was washed with DCM, treated with 25% TFA in DCM for 10 min, washed with DCM and with 5% DIEA in DCM, washed with DCM, and acetylated using a mixture of 1.16 mL of Ac_2O/1.8 mL of TEA/25 mL of DMF for 30 min. The resin was subjected to a final DCM wash and dried.

Solid phase tBoc synthesis was performed on an ABI Model 430A peptide synthesizer. The amino acid side-chain protection used was 4-chlorocarbobenzoxy for Lys, 2-bromocarbobenzoxy for Tyr, *O*-benzyl for Ser, and dibenzyl for phosphotyrosine and phosphonotyrosine (Valerio et al., 1989; Bechle & Dow, 1993). Standard tBoc chemistry protocols were used as provided by ABI (software version 1.4 for NMP/HOBt chemistry). These protocols used HOBt active ester coupling and Ac_2O capping of unreacted free amino termini after each coupling. Resin samples were taken automatically at the end of each coupling, and quantitative ninhydrin assay was used to monitor coupling efficiencies. Automated peptide synthesis was initiated on a 0.5-mmol scale, using 4-methyl-BHA-resin HCl salt. Removal of N-terminal tBoc protection was performed on the instrument at the end of the synthesis. N-Terminal acetylation was performed as described for the Fmoc synthesis.

Peptides were deprotected and cleaved from the dried resin as follows: Six hundred milligrams of resin was treated with a mixture of 0.4 mL of anisole and 0.2 mL of DMS at 0 °C for 10 min. Three milliliters of TFA was added with stirring and the slurry maintained at 0 °C for 10 min. One milliliter of TFMSA was added dropwise with stirring under nitrogen, the reaction mixture was warmed to 25 °C, and stirring was continued for 90 min. The resin was filtered off, and the filtrate was precipitated into 100 mL of dry ether with stirring under nitrogen. The solid was filtered off and dried, affording a pink hygroscopic solid. The solid was dissolved in 1 mL of 6 M guanidine hydrochloride/50 mM Tris (pH 8.6) and prepped directly on a μBondapak C-18 column (Waters P/N 84176) using a linear gradient from 5% MeCN/0.1% TFA/94.9% water to 80% MeCN/0.1% TFA/19.9% water over 30 min at a flow rate of 3 mL/min. Lyophilization of the collected fraction afforded the desired peptides, which were purified to homogeneity by reverse-phase high-pressure liquid chromatography and characterized by electrospray mass spectroscopy (Finnegan TSQ-700 instrument) and amino acid analysis.

Cell Culture and Fractionation. 3T3-L1 cells were carried as fibroblasts, grown in 100-mm dishes and differentiated as described (Frost & Lane, 1985). The adipocytes were used between days 7 and 11 post initiation of differentiation. The cells were treated with or without 100 nM or 1 μM insulin for 3 min to maximally stimulate PtdIns 3′-kinase before the cytosolic fraction was prepared for assaying PtdIns 3′-kinase activity according to our previously characterized method (Lamphere et al., 1994).

Assay of PtdIns 3′-Kinase Activity. PtdIns 3′-kinase activity was assayed by the phosphorylation of PI with [γ^{32}P]ATP with minor modifications of previously described procedures (Lamphere et al., 1994; Auger et al., 1992). PI (bovine liver; Avanti Polar Lipids, Alabaster, AL) was added to buffer A (10 mM HEPES, 100 mM NaCl, 1 mM EGTA, 0.03% NP-40, pH 7.0) at a concentration of 1 mg/mL. The suspension was then sonicated for 5 min at output setting 1, 10% duty cycle, on a Branson sonifier equipped with a 2.5-mm microtip probe to dissolve the PI. This lipid solution was then added to an equal volume of a solution containing 500 μM [γ-^{32}P]-ATP (0.9 μCi/nmol), 25 mM $MgCl_2$, and 15 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.8. The kinase reaction was initiated by the addition of 66.6 μL of the lipid/ATP solution to 100 μL of the cytosolic fraction of 3T3-L1 adipocytes that had been incubated with or without the indicated concentrations of peptide for 15 min. The reaction was stopped after 3 min at room temperature by transferring 50-μL aliquots from the reaction vials to 80 μL of 1 M HCl. The phosphatidylinositols were then extracted with 180 μL of chloroform–methanol (1:1), and the organic phase was separated by a brief (10 s) centrifugation at 10 000 rpm in an Eppendorf Model 5415C microcentrifuge.

TLC of Phosphatidylinositol Phosphates. Phosphatidylinositol phosphates were separated on aluminum-backed 5 × 20 cm silica gel 60 plates on the basis of the ability of phosphatidylinositol 4-phosphate, but not phosphatidylinositol 3-phosphate, to form a borate complex with the *cis*-diol present in the inositol ring as previously described (Walsh et al., 1991). The radioactive content of the phosphatidylinositol 3-phosphate spot on TLC plates was determined by autoradiography and densitometric analysis of the spots, by cutting the section of the plate corresponding to phosphatidylinositol 3-phosphate and then measuring ^{32}P by scintillation counting, or by phosphoimaging on a BAS 2000 phosphoimager (Fuji Scientific); all three methods gave equivalent results.

Peptide Binding. The Pharmacia biospecific interaction analysis (Biacore) instrument uses surface plasmon resonance to measure protein–protein interaction on a CM5 sensor chip (Jonsson & Malmqvist, 1992; Jonsson et al., 1991; Fagerstam, 1991). Changes in the refractive indices between two media, glass and carboxymethylated dextran, caused by the interaction of protein to the dextran side of the sensor chip, is measured and reported as changes in arbitrary reflectance units (RU) as detailed in the manufacturer's application notes.

The carboxymethylated dextran surface of a flow cell on a sensor chip was activated by derivatization with 0.05 M *N*-hydroxysuccinimide mediated by 0.2 M *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide for 7 min. Streptavidin at

Table 1: Peptides Synthesized[a]

peptide sequence	chemistry	purified yield (%)	MW calcd	MW obsd
pYZPZSPK-biotin	tBoc	25.30	1278.96	1279.30
VAPVSpYADMRT	tBoc	11.10	1330.32	1329.30
YZPZSGSGSGSGSYZPZS	tBoc	3.00	1782.72	1782.60
YZPZS	tBoc	38.20	632.67	633.00
*YZPZS	tBoc	8.70	710.67	710.80
pYZPZS	Fmoc	5.70	712.67	712.50
HTDDGpYZPZSP	tBoc	2.70	1335.19	1335.10
pYZPZSGSGSGSGSYZPZS	tBoc	2.40	1862.72	1862.50
*YZPZSGS*YZPZS	tBoc	21.00	1506.45	1506.50
pYZPZSpYZPZS	tBoc	6.00	1366.32	1366.10
pYZPZSGSpYZPZS	tBoc	1.90	1510.42	1510.20
pYZPZSGSGSGSGSpYZPZS	tBoc	3.00	1942.72	1942.60

[a] Peptides were synthesized as described in Experimental Procedures. All peptides were purified by reverse-phase high-pressure liquid chromatography to afford a single peak and then characterized by electrospray mass spectroscopy and amino acid analysis. Results of mass spectral data and yield data are shown. Z represents norleucine. The PtdIns 3′-kinase SH2 domain binding motif is underlined (YMXM); *Y and pY indicate phosphonotyrosine and phosphotyrosine, respectively.

a concentration of 500 μg/mL, in 10 mM sodium acetate, pH 4.5, was injected into a flow cell in 35 μL at a rate of 5 μL/min and covalently immobilized to the flow cell surface. Deactivation of unreacted *N*-hydroxysuccinimide esters was performed using 1 M ethanolamine hydrochloride, pH 8.5. Immobilization of the streptavidin resulted in approximately 4800 RU over baseline.

A Tyr(p) peptide YZPZSPK (Z = norleucine), biotinylated on the ε-amino group of lysine, was bound by injecting 25 μL of a 39 μM solution of the peptide in 10 mM sodium phosphate, 100 mM NaCl, and 0.005% Tween-20, pH 7.5, at a rate of 5 μL/min onto the streptavidin-coated flow cell.

The glutathione *S*-transferase PtdIns 3′-kinase carboxy SH2 domain fusion protein (C-SH2-GST) and the glutathione *S*-transferase PtdIns 3′-kinase carboxy and amino SH2 domain fusion protein (N+C-SH2-GST) were purified from *Escherichia coli* transformed with the plasmid pGEXp85SH2C or pGEXp85SH2N+C (generously provided by Dr. Tony Pawson, University of Toronto) as described elsewhere (Smith & Johnson, 1988). Twenty-five microliters of 42 μg/mL C-SH2-GST or 10 μg/mL N+C-SH2-GST in 10 mM sodium phosphate, 100 mM NaCl, and 0.005% Tween-20, pH 7.5, was injected into the flow cell at a rate of 5 μL/min. For the N+C-SH2-GST protein 10 mM dithiothreitol was added to the phosphate buffer to maintain binding activity. The flow cell surface was then regenerated to baseline using a 4-μL injection of 50 mM NaOH. Varying concentrations of peptides which compete for binding to the SH2 domain were mixed with or without one of the SH2-GST fusion proteins and then injected into the flow cell in a volume of 35 μL at a flow rate of 5 μL/min. RU values were recorded following each injection, and regeneration then preceded the next injection.

RESULTS

Peptide Design. In order to investigate whether high-affinity stimulation of PtdIns 3′-kinase by IRS-1 requires the binding of Tyr(p) YMXM motifs on IRS-1 to both SH2 binding domains, peptides containing either one or two such motifs were synthesized (Table 1). For ease of synthesis and stability norleucine (designated Z), an isomorph of methionine, was used instead of methionine (Shoelson et al., 1992). The peptides synthesized include the 11-mer HTDDGYZPZSP derived from the sequence of IRS-1 around tyrosine 608 which has been identified as a site of tyrosine phosphorylation and PtdIns 3′-kinase binding (Sun et al., 1993); nonphosphorylated or Tyr(p) peptides containing only the minimal YZPZ sequence for binding to SH2 domains; peptides containing two YZPZ binding domain sequences connected by variable lengths of a GS peptide linker such that they would span the spacing of the Tyr(p)s in the PDGFR that bind the SH2 domains on PtdIns 3′-kinase (Carpenter et al., 1993); and the Tyr(p)-containing peptide VAPVSYADMRT that does not contain the high-affinity YMXM motif for binding to the SH2 domains of PtdIns 3′-kinase. The peptide YZPZSGSGSGSGSYZPZS which contains two SH2 binding motifs was synthesized with neither, one, or both of the tyrosines phosphorylated in order to test whether phosphorylation of both motifs is required to stimulate high-affinity activation of PtdIns 3′-kinase. Peptides containing one or two YZPZS motifs were also synthesized using the nonhydrolyzable analogue phosphonotyrosine in place of phosphotyrosine.

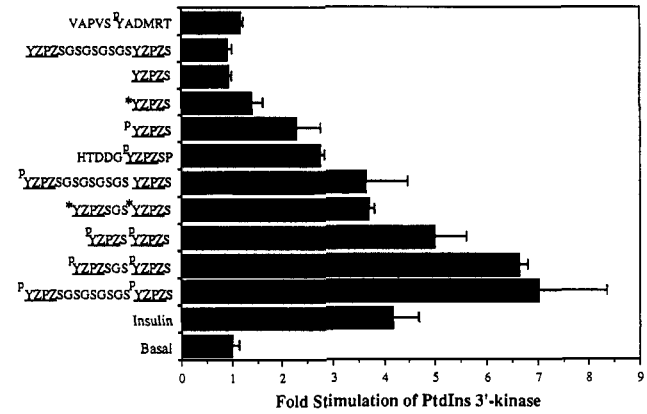

FIGURE 1: Activation of PtdIns 3′-kinase by peptides containing phosphotyrosine or phosphonotyrosine. 3T3-L1 adipocytes were treated with or without 100 nM or 1 μM insulin for 5 min to maximally stimulate PtdIns 3′-kinase. The cells were then homogenized, and the cytosolic fraction was prepared as described in Experimental Procedures. Cytosol from untreated cells was incubated with the indicated peptides for 15 min before initiation of the kinase assay. Nonphosphorylated peptides or peptides containing only a single phosphonotyrosine, or phosphotyrosine, YZPZ domain were used at a concentration of 100 μM. Peptides containing two phosphonotyrosine, or phosphotyrosine, YZPZ domains were used at a concentration of 10 μM. The phosphorylated nonrelated sequence VAPVSpYADMRT was used at a concentration of 200 μM. The values are the mean ■ SD for triplicate measurements in a representative experiment. Each peptide was tested in three to five experiments.

Stimulation of PtdIns 3′-Kinase by Phosphotyrosine-Containing Peptides. To investigate the activation of PtdIns 3′-kinase, 3T3-L1 adipocytes were treated with or without 100 nM or 1 μM insulin to maximally stimulate PtdIns 3′-kinase activity, and the cytosolic fraction was prepared. Untreated cytosol was incubated with or without the various peptides at the concentrations indicated in the legend to Figure 1 for 15 min and assayed for PtdIns 3′-kinase activity. Insulin stimulation of the cells caused a 4.3-fold increase in PtdIns 3′-kinase activity compared with cells in the basal state (Figure 1).

Treatment of basal cytosol with the 11-mer Tyr(p) peptide derived from Y608 of IRS-1, with a 5-mer Tyr(p) peptide containing the minimal consensus sequence for binding to SH2 domains (YZPZS), or with a peptide containing two YZPZ motifs in which only one tyrosine residue was phosphorylated stimulated PtdIns 3′-kinase activity 2–4-fold over basal levels, at a concentration of 100 μM; lower concentrations were considerably less effective (see below).

In contrast, bifunctional peptides containing two Tyr(p) YZPZ motifs separated by spacers of different lengths (1, 3, and 9 residues) required only 10 μM to stimulate PtdIns 3′-kinase activity 4–7-fold. Nonphosphorylated peptides containing YZPZ motifs or a Tyr(p)-containing peptide of an unrelated sequence caused no increase in PtdIns 3′-kinase activity at concentrations of 100 and 200 μM, respectively (Figure 1). This result confirms the requirement for a Tyr(p) residue in a specific amino acid sequence.

Our observation that peptides containing two phosphorylated YZPZ motifs caused a greater stimulation of PtdIns 3′-kinase activity at a 10-fold lower concentration than peptides containing only a single phosphorylated motif (Figure 1) suggested that high-affinity activation of PtdIns 3′-kinase required the occupancy of both SH2 domains on the 85-kDa subunit. To test this hypothesis, we compared the dose–response curves of a bifunctional peptide containing two phosphorylated YZPZ motifs and the peptide derived from tyrosine 608 of IRS-1 for their ability to stimulate PtdIns 3′-kinase. As is shown in Figure 2, panel A, the phosphorylated peptide derived from tyrosine 608 of IRS-1 has a minimal ED_{50} of approximately 30 μM with a stimulation of 4-fold at 200 μM. In contrast, the bifunctional peptide stimulated PtdIns 3′-kinase 5.5-fold at a concentration of 1 μM with a minimal ED_{50} of only 2.5 nM. Furthermore, the peptide containing two YZPZ motifs, but with only one of the two tyrosines phosphorylated, displayed a minimal ED_{50} and maximal response almost identical to those of the IRS-1 tyrosine 608 derived peptide that contains only one YZPZ motif (Figure 2, panel A).

Our data support the hypothesis that for full, high-affinity activation of PtdIns 3′-kinase by IRS-1 the binding of two Tyr(p) YZPZ motifs to the two SH2 domains of the 85-kDa subunit is required. It is notable that the ED_{50} for activation by the bifunctional Tyr(p) peptide in Figure 2A is only 2.5-fold higher than that observed with the Tyr(p) form of IRS-1 (Lamphere et al., 1994). Our data also indicate that the binding pockets of the two SH2 domains can lie very close together. A similar hypothesis has been advanced for the PDGFR signaling where there is evidence that high-affinity binding of the PDGFR to the SH2 domains of the 85-kDA subunit of PtdIns 3′-kinase requires the association of Tyr(p) residues 740 and 751 of the PDGFR to the two SH2 domains of the 85-kDa subunit of PtdIns 3′-kinase (Panayotou et al., 1993; Kashishian et al., 1992). In the PDGFR the two Tyr(p)s are separated by only 10 amino acids, and in the peptides examined here the Tyr(p)s are separated by only 6–12 amino acids (Table 1). The finding that a bifunctional YZPZ motif peptide in which the Tyr(p)s are separated by only 6 amino acids is capable of high-affinity stimulation of PtdIns 3′-kinase (Figure 2) supports a model in which the two SH2 domain binding pockets on the 85-kDa subunit are a tandem repeat (End et al., 1993).

Stimulation of PtdIns 3′-Kinase by Phosphonotyrosine-Containing Peptides. The role of PtdIns 3′-kinase and the effect of the putative second messengers generated by the activation of this enzyme in response to insulin are not understood. The capacity of peptides to stimulate PtdIns 3′-kinase independently of the binding of insulin to its receptor may prove useful in dissecting the mechanisms of insulin action. If peptides that stimulate PtdIns 3′-kinase could be made resistant to tyrosine phosphatases, their versatility would be greatly enhanced since they could be used in intact cell systems without the complication of a constantly diminishing concentration of the active moiety. Therefore, we synthesized

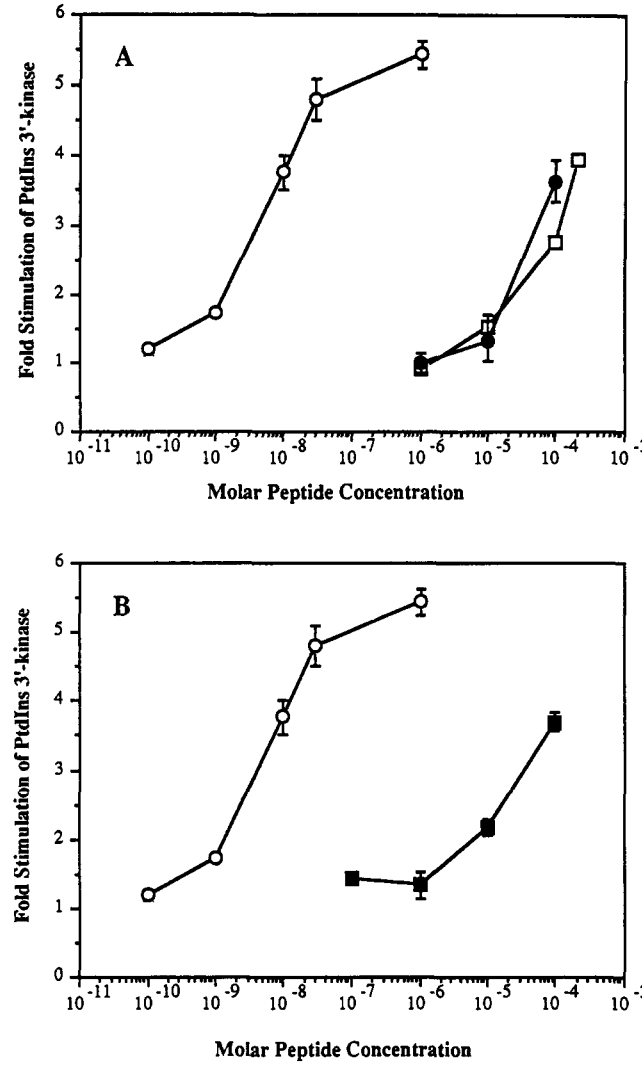

FIGURE 2: Dose–response curves for stimulation of PtdIns 3′-kinase activity by peptides containing phosphotyrosine or phosphonotyrosine YZPZ motifs. Panel A: 3T3-L1 adipocytes were homogenized, and the cytosolic fraction was prepared. The cytosolic fraction was then incubated with the indicated concentrations of one of the following peptides for 15 min before initiation of the PtdIns 3′-kinase assay: ^{p}YZPZSGS^{p}YZPZS (○), HTDDG^{p}YZPZSP (□), or ^{p}YZPZS-GSGSGSGSYZPZS (●). Panel B: The assay was performed as described in panel A with the exception that the cytosolic fraction was incubated with *YZPZSGS*YZPZS (■); ^{p}YZPZSGS^{p}YZPZS (○) is replotted from panel A for ease of comparison. The values are the mean ± SD for triplicate measurements in a representative experiment. Each peptide was tested in three to five experiments. ^{p}Y and *Y represent phosphotyrosine and phosphonotyrosine, respectively.

two peptides containing YZPZ motifs in which the Tyr(p) residues were replaced by the nonhydrolyzable phosphonotyrosine (Table 1) and tested them for their ability to stimulate PtdIns 3′-kinase activity. Unexpectedly, the 5-mer containing one phosphonotyrosine YZPZ motif did not stimulate PtdIns 3′-kinase activity at 100 μM (Figure 1). On the other hand, a dose–response curve of the bifunctional phosphonotyrosine peptide demonstrated a minimal ED_{50} of approximately 10 μM and a maximal stimulation of 4-fold at the highest concentration tested of 100 μM (Figure 2, panel B); thus, the bifunctional phosphonotyrosine peptide was 3–4 orders of magnitude less potent than the corresponding Tyr(p) peptide.

Peptide Binding to PtdIns 3′-Kinase SH2 Domains. One possible explanation for the higher ED_{50s} of the phosphono peptides is that the binding affinity of the peptides is reduced when the phosphono group is substituted for the phosphoryl

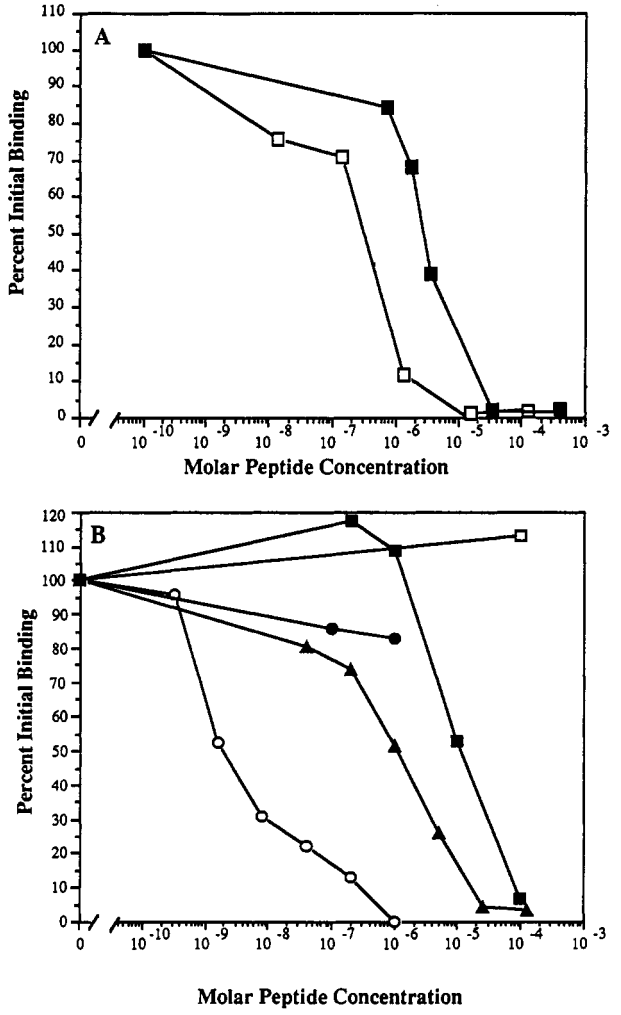

FIGURE 3: Binding of phosphotyrosine or phosphonotyrosine peptides to the carboxy SH2 domain of PtdIns 3′-kinase. Panel A: A GST fusion protein containing the carboxy-terminal SH2 domain of PtdIns 3′-kinase was incubated with or without the indicated concentration of either pYZPZS (□) or *YZPZS (■). The samples were then analyzed using the Biacore apparatus for the capacity of the SH2 protein to bind to pYZPZSPK attached to the sensor chip. Results are expressed as percent of binding of the SH2 protein to pYZPZSPK with no competing peptide present. Panel B: A GST fusion protein containing the carboxy- and amino-terminal SH2 domains of PtdIns 3′-kinase was incubated with or without the indicated concentration of either pYZPZSGSpYZPZS (○), HTDDGpYZPZSP (▲), *YZPZSGS*YZPZS (●), pYZPZSGSGSGSGSYZPZS (■), or YZPZSGSGSGSGSYZPZS (□) and then analyzed using the Biacore apparatus as in panel A.

group as was reported for a phosphonotyrosine peptide derived from middle T antigen, which binds to PtdIns 3′-kinase approximately one-half as tightly as does the corresponding Tyr(p) peptide (Domchek et al., 1992). We examined the relative binding affinities of the phospho and phosphono peptides using two different methods. First, we assessed the ability of the phospho and phosphono YZPZS peptides to block association of the C-SH2 domain of PtdIns 3′-kinase to Tyr(p) YZPZSPK-biotin that had been attached to the Biacore sensor chip as described in Experimental Procedures. The YZPZS Tyr(p) peptide had a minimal ED_{50} of 200 nM, while the corresponding phosphonotyrosine analog was about 15-fold less efficacious at blocking the binding of the C-SH2-GST fusion protein, with a minimal ED_{50} value of 3 μM (Figure 3A). Second, we compared the ability of the phosphono YZPZS, phospho YZPZS, and the Y608-derived phosphopeptide to compete with Tyr(p) IRS-1 for binding to the carboxy SH2 domain of PtdIns 3′-kinase. The cytosolic fraction from insulin-treated 3T3-L1 adipocytes was incubated with various concentrations of the peptides and the PtdIns 3′-kinase SH2-GST fusion protein conjugated to agarose beads. The Tyr(p) IRS-1 that bound to the SH2-GST fusion protein was detected by immunoblotting using affinity-purified antibodies (Lamphere et al., 1994). The YZPZS phosphonopeptide was approximately 7-fold less effective than either the Y608-derived peptide or the YZPZS phosphopeptide in blocking Tyr(p) IRS-1 association with the SH2-GST fusion protein (data not shown).

We also compared the abilities of bifunctional peptides containing one or two phosphorylated YZPZ motifs, a bifunctional peptide containing nonphosphorylated YZPZ motifs, a bifunctional peptide containing two phosphonotyrosine YZPZ motifs, and the Tyr(p)-containing peptide derived from tyrosine 608 of IRS-1 to block association of the GST fusion protein containing both the carboxy- and amino-terminal SH2 domains of PtdIns 3′-kinase to Tyr(p) YZPZSPK-biotin using the Biacore instrument. The bifunctional peptide containing two phosphorylated YZPZ motifs was 500- and 5000-fold more effective at blocking the binding of the N+C-SH2-GST protein than the peptides derived from tyrosine 608 of IRS-1 or the bifunctional peptide containing only one phosphorylated YZPZ motif; minimal ED_{50}s were 2 nM, 1 μM, and 10 μM, respectively (Figure 3B). The bifunctional peptide containing two nonphosphorylated YZPZ motifs was unable to compete even at a concentration of 100 μM. The bifunctional peptide containing two phosphonotyrosine YZPZ motifs was only tested up to a concentration of 1 μM due to limiting amounts of peptide; at this concentration it only inhibited N+C-SH2-GST protein binding 14%.

We therefore conclude that the higher efficacy of the peptides containing two Tyr(p) YZPZ motifs in their ability to stimulate PtdIns 3′-kinase is due primarily to their higher affinities for the SH2 domains of this enzyme and that for high-affinity binding to (and subsequent activation of) PtdIns 3′-kinase both YZPZ motifs must be phosphorylated. While the lower affinity and efficacy of the phosphonopeptides is unfortunate, their resistance to tyrosine phosphatases may make them a valuable tool in dissecting the mechanisms of insulin action.

DISCUSSION

We have shown for the first time that high-affinity activation of PtdIns 3′-kinase by peptides derived from IRS-1 requires the binding of two Tyr(p) YMXM motifs to the SH2 domains on the 85-kDa subunit of PtdIns 3′-kinase. Our findings support a scheme in which *in vivo* high-affinity activation of PtdIns 3′-kinase is achieved through the simultaneous binding of two phosphorylated YMXM motifs on IRS-1 to the two SH2 domains on the 85-kDa subunit of PtdIns 3′-kinase. We have shown that the peptide containing two Tyr(p) YZPZ motifs in which the Tyr(p)s are separated by 6 amino acids stimulated PtdIns 3′-kinase with a minimal ED_{50} of approximately 2.5 nM, while peptides containing two YZPZ motifs with only one Tyr(p), or the Tyr(p) peptide derived from Tyr 608 of IRS-1, have minimal ED_{50}s of approximately 30 μM. The ability of the Tyr(p) peptide containing two YZPZ motifs in which the Tyr(p)s are separated by only 6 amino acids to stimulate PtdIns 3′-kinase at low concentrations indicates that the two binding pockets on the 85-kDa subunit of PtdIns 3′-kinase can be in very close proximity to each other. Additionally, we have demonstrated that a peptide containing two Tyr(p) YZPZ motifs has at least a 500-fold higher affinity for the two SH2 domains of PtdIns 3′-kinase

than peptides containing only a single Tyr(p) YZPZ motif. The recent finding that among the tyrosines on IRS-1 in YMXM motifs phosphorylated by the insulin receptor there are two pairs close to each other in the primary sequence (Tyr 608 and 628, Tyr 939 and 987) (Sun et al., 1993) suggests that each of these pairs may act as a set that binds to the two SH2 domains on PtdIns 3′-kinase.

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