PTB domain of insulin receptor substrate-1 binds inositol compounds

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We examined whether a phosphotyrosine binding (PTB) domain from the human insulin receptor substrate-1 (hIRS-1) is capable of binding inositol phosphates/phosphoinositides. The binding specificity was compared with that of the pleckstrin homology (PH) domain derived from the same protein because the three dimensional structure was found to be very similar to that of the PH domain, despite the lack of sequence similarity. We also attempted to locate the site of binding of the inositol compounds. The PTB domain bound [³H]Ins(1,4,5)P₃, which was displaced most strongly by Ins(1,3,4,5,6)P₅ and InsP₆, indicating that these inositol polyphosphates show the highest affinity. The PTB domain bound to liposomes containing PtdIns(4,5)P₂, PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂, but not phosphatidylinositol. In contrast, the PH domain showed a preference for Ins(1,4,5)P₃, the polar head of PtdIns(4,5)P₂. Site-directed mutagenesis studies

were performed to map the binding site for inositol phosphates in the PTB domain. Mutation of K169Q, K171Q or K177Q, located in the loop connecting the β 1 and β 2 strands, which is partially responsible for binding inositol phosphates/phosphoinositides in the PH domains of several other proteins, reduced binding activity, probably because of a reduction in affinity. Mutation of R212Q or R227Q, shown to be involved in the binding of a phosphotyrosine, had little effect on the binding capacity. These results indicate that the PTB domain of hIRS-1 can bind inositol phosphates/phosphoinositides. Therefore signalling through the PTB domain could be regulated by the binding not only of proteins with phosphotyrosine but also of inositol phosphates/phosphoinositides, implying that PTB domains could be involved in a myriad of interconnections between intracellular signalling pathways.

INTRODUCTION

It is now well recognized that many of the proteins which participate in cell signalling contain structural modules involved in regulatory interactions between the components of signal transduction cascades. Many studies have focused on *src* homology (SH) domains revealing that most SH2 and SH3 domains function as phosphotyrosine binding and proline-rich region binding modules respectively [1].

Other protein modules which are believed to be involved in cell signalling by helping interactions between signalling components have been found in many signalling or cytoskeletal proteins [2]. An example of these is the phosphotyrosine binding (PTB) domain or phosphotyrosine-interacting domain, a recently identified protein module composed of about 150 amino acids which binds to phosphotyrosine, as indicated by its name [3,4]. This region is structurally unrelated to the SH2 domains and binds to an NPXY(p) motif which is not recognized by the SH2 domain and which is found in many tyrosine-phosphorylated proteins, including growth factor receptors [5–9]. Crystal structures of the PTB domain of Shc (SH2-containing sequence) and IRS-1 (insulin receptor substrate-1) by itself and/or complexed to a ligand, specifically a phosphotyrosine peptide mimicking a tyrosine-phosphorylated insulin receptor, have been reported recently [10,11]. The three-dimensional structure thus identified consists of two β -sheets face to face, each composed of three and four antiparallel β -strands, capped at the end of the β -sheets by a terminal α -helix. This is very similar to that of the

pleckstrin homology (PH) domain, despite the absence of primary sequence similarity.

The PH domain (consisting of ~120 amino acids) was first found in pleckstrin, a major substrate for protein kinase C [12], and has been identified in more than 100 signalling or cytoskeletal proteins to date [13–15]. Inositol compounds, molecules with a well-established role in intracellular signalling [16,17], have also been shown to be potential ligands for the PH domain, and that their interaction helps the protein bearing the PH domain recruit to the cell membrane constituents necessary for exerting its function [13–15]. On the other hand, soluble inositol phosphates appear to play a role in attenuating the interaction by competing with inositol lipids [2,18,19]. In some cases, binding also directly influences the function of a PH domain-containing protein [20–22].

Studies in this laboratory and that of Cifuentes et al. [19] have demonstrated that phospholipase C (PLC)- δ_1 had a high affinity and high specificity for Ins(1,4,5)P₃ and PtdIns(4,5)P₂ before the term 'PH domains' emerged [18,23–25], and have shown that the binding site maps within the PH domain [26–28]. The new protein, with a molecular mass of 130 kDa, was found to bind Ins(1,4,5,6)P₄ in addition to Ins(1,4,5)P₃ via its PH domain [29,30]. These results led us to propose that the PH domain derived from various proteins might bind different inositol compounds specifically [29] and to examine whether this was the case [31].

In the present study, on the basis of the similarity of the three-dimensional structures of the PTB and PH domains, we examined

Abbreviations used: IRS-1, insulin receptor substrate-1; hIRS-1, human IRS-1SH; SH, *src* homology; Shc, SH2-containing sequence; PTB, phosphotyrosine binding; PH, pleckstrin homology; PLC, phospholipase C; GroPIns(4,5)P₂, glycerophosphoryl-D-myo-inositol 4,5-bisphosphate.

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the binding potential and specificity of the PTB domain of IRS-1 for inositol phosphate, in comparison with those of the PH domain from the same protein. Although the PH domain derived from a variety of proteins has been shown to bind inositol compounds [13–15,18–22], the domain of IRS-1 has not been examined so far. The binding to liposomes containing phosphoinositides was also investigated to determine the involvement of fatty acid moieties in the binding. Furthermore, site-directed mutagenesis studies with the PTB domain were carried out to map the binding site. As a result of these studies, we determined not only that the PTB domain from IRS-1 binds inositol compounds, but also that several basic amino acids present in the loop connecting the first two β -strands of the PTB domain, a site which is sterically different from that for a phosphotyrosine peptide, are responsible for the binding.

These results were presented in abstract form at the 70th Annual Meeting of the Japanese Biochemical Society held in September 1997. During the preparation of this manuscript, papers by Rameh et al. [32] and Ravichandran et al. [33] have been published, which reported that the PTB domain from Shc, an adaptor protein, could bind to $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{PtdIns}(4,5)\text{P}_2$; the domain from IRS-1 has not yet been examined for binding to inositol compounds.

MATERIALS AND METHODS

Materials

$[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (specific radioactivity 777 GBq/mmol) was obtained from DuPont–New England Nuclear (Boston, MA, U.S.A.). Nitrilotriacetic acid-agarose resin was obtained from Qiagen (Chatsworth, CA, U.S.A.). The inositol phosphates used in this study were chemically synthesized as described previously [34–37], except that InsP_6 and glycerophosphoryl-D-*myo*-inositol 4,5-bisphosphate [$\text{GroPIns}(4,5)\text{P}_2$] were purchased from Sigma (St. Louis, MO, U.S.A.) and Calbiochem (La Jolla, CA, U.S.A.) respectively, and used without further purification. Phosphoinositides [dioleoyl- $\text{PtdIns}(4,5)\text{P}_2$, stearoyl-linolenoyl- $\text{PtdIns}(3,4,5)\text{P}_3$ and distearoyl- $\text{PtdIns}(3,4)\text{P}_2$] were chemically synthesized as described [38,39]. Other reagents used were of the highest grade available.

Preparation of the histidine-tagged PTB domain and the PH domain of human IRS-1 (hIRS-1)

The cDNA encoding the PTB domain (amino acid residues 145–320) was amplified by PCR using the primers PTB-1 and PTB-2 (sense: amino acid residues 142–149, 5'-TTGGGCATGCTGGGGAGGACTTG-3', and antisense: amino acid residues 316–323, 5'-AGCCAAGCTTCCCGCCACCC-3' respectively). After digestion with *SphI* and *HindIII*, the cDNA fragment was subcloned into the pQE-31 expression vector (Qiagen) at the *SphI*–*HindIII* site. The DNA fragment, including the region encoding the PH domain (nt 898–1430), was subcloned into the pUC 19 plasmid vector (TaKaRa, Kyoto, Japan) and the cDNA encoding the PH domain (amino acid residues 4–137) was amplified by PCR using the primers PH-1 (sense: amino acid residues 1–8, 5'-TGGGGATCCCTCCGAGAGCG-3') and M13 RV (5'-CAGGAAACAGCTATGAC-3'; TaKaRa). After digestion with *BamHI* and *PstI*, the cDNA fragment was inserted into the pQE-32 expression vector (Qiagen) at the *BamHI*–*PstI* site. The nucleotide sequence of each insert was confirmed by DNA sequencing.

The expression of the PTB and PH domains of hIRS-1 as His-tagged proteins was induced by isopropyl β -D-thiogalactoside (5 h) in *Escherichia coli* JM101. Cells were lysed by sonication in

0.3 M NaCl/50 mM phosphate buffer (pH 8.0). Triton X-100 was added to the homogenate to give a final concentration of 1% (v/v), followed by incubation at 4 °C for 20 min and centrifugation at 15000 *g* for 20 min. The resultant supernatant was mixed with a 50% slurry of nitrilotriacetic acid resin at 4 °C for 1 h. Procedures for washing the resin and the elution of adsorbed proteins were according to the manufacturer's instructions, except that 1 M NaCl was used in the washing buffer. The PTB and PH domains eluted were dialysed against 50 mM NaCl and 10 mM Tris/HCl (pH 8.3) respectively. The purity of the preparations was determined by SDS/PAGE.

Site-directed mutagenesis

In each case, one amino acid substitution in the PTB domain was introduced by PCR mutagenesis [40]. PTB-1 and PTB-2, described above, and the following oligonucleotides were used:

K169Q sense 5'-AGTGATCCTGCAGCCCAAGGG-3',
K169Q antisense 5'-CCCTTGGGCTGCAGGATCACT-3',
K171Q sense 5'-CCTGAAGCCCCAGGGCCTGGG-3',
K171Q antisense 5'-CCCAGGCCCTGGGGCTTCAGG-3',
K177Q sense 5'-GGGTCAGACACAGAACCTGAT-3',
K177Q antisense 5'-ATCAGGTTCTGTGTCTGACCC-3',
R212Q sense 5'-ATGAACATCCAGCGCTGTGGC-3',
R212Q antisense 5'-GCCACAGCGCTGGATGTTTCAT-3',
R227Q sense 5'-GAGGTGGGCCAGTCTGCCGTG-3',
R227Q antisense 5'-CACGGCAGACTGGCCACCTC-3'.

The mutated PCR products (corresponding to amino acid residues 145–320) were digested with *SphI* and *HindIII* and the fragments were subcloned into pQE-31 (Qiagen) at the *SphI*–*HindIII* site. Each mutation was confirmed by DNA sequencing. All mutant PTB domains were expressed and purified as described above.

$[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding assay

Binding of $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ by the PTB or PH domain was assayed as follows. The mixture (100 μl) containing 50 mM Tris/HCl (pH 8.3), 1 mM EDTA, 0.2% Triton X-100, 6.0 nM $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (2.2 kBq) and the isolated protein sample ($\sim 3.2 \mu\text{g}$) was incubated on ice for 15 min and then 50 μl of bovine γ -globulin (10 mg/ml) and 0.5 ml of 30% (w/v) poly(ethylene glycol) 6000 was added. After centrifugation at 15000 *g* for 5 min, the precipitate was dissolved in 0.5 ml of 0.1 M NaOH and the radioactivity in an emulsion in 5 ml of a scintillation cocktail was measured. Specific binding was determined by subtracting non-specific binding in the presence of 100 μM $\text{Ins}(1,4,5)\text{P}_3$ from that in its absence. Protein concentrations assayed for the binding were adjusted so that the radioactivity obtained in the absence or presence of unlabelled $\text{Ins}(1,4,5)\text{P}_3$ was 3000–4000 d.p.m. or 300–400 d.p.m. respectively, in routine assays.

Measurement of binding constants using an optical evanescent resonant biosensor

An optical evanescent resonant mirror cuvette system (BIAcore; Pharmacia, Uppsala, Sweden) was used to measure the interaction kinetics between an immobilized $\text{Ins}(1,4,5)\text{P}_3$ analogue and the PTB domain and its mutants. Briefly, the sensor chip, the surface of which was coated with avidin (Pharmacia), was incubated with 0.2 mg/ml of a biotinylated $\text{Ins}(1,4,5)\text{P}_3$ analogue [2-(*p*-biotinylaminobenzoyl)-*myo*-inositol 1,4,5-trisphosphate], previously designated 204BT [41], in 10 mM PBS/0.05% (v/v) Tween 20 (pH 7.4). After washing the chip with the binding buffer [10 mM Tris/HCl (pH 8.3)/50 mM

NaCl/2 mM EDTA/10 mM 2-mercaptoethanol], the purified PTB domain or mutant samples, dissolved in binding buffer, were added. Association and dissociation times were 10 min. Regeneration of the ligand was performed by washing the cuvette with 200 μ l of binding buffer containing 2 M NaCl. All experiments were carried out with a flow rate of 20 μ l/min at room temperature.

The traces of the association and dissociation process were analysed using BIA-evaluation (version 2.1) kinetics-analysis software supplied with the instrument. Experimentally-derived association and dissociation data at various concentrations of analytes were fitted to equations containing a single exponential function. The pseudo first-order rate constant, k_{on} , in units of s^{-1} , was obtained for each concentration of the analyte protein. The association rate constant, K_{ass} , in units of $M^{-1} \cdot s^{-1}$, was derived from the gradient of the plot of k_{on} against analyte concentration. The dissociation rate constant, K_{diss} , in units of s^{-1} , was derived directly from the time course of dissociation. The affinity constant, K_d , is equivalent to k_{diss}/k_{ass} . The values were examined for self-consistency of the data as described previously [42].

Binding assay of the PTB domain to liposomes

Liposomes containing phosphatidylethanolamine with PtdIns, PtdIns(4,5) P_2 , PtdIns(3,4) P_2 or PtdIns(3,4,5) P_3 were made as follows. Phosphatidylethanolamine and one of the phosphoinositides dissolved in chloroform were dried in N_2 to make a lipid film in a glass tube. A solution containing 50 mM NaCl, 10 mM Hepes/NaOH buffer (pH 8.0) and 10 mM β -mercaptoethanol was added to make a final lipid concentration of 5 mM, and was gently vortex mixed so that large unilamellar vesicles were formed. The phosphoinositide content varied from 0.02 mol% to 30 mol% of phosphatidylethanolamine, depending on the final concentration. The lipid vesicles, at a final concentration of 500 μ M, were incubated with purified PTB domain in 100 μ l of the same solution as the lipid vesicles, on ice, for 10 min. The solution was centrifuged at 100 000 g for 30 min.

Figure 1 Binding of [3 H]Ins(1,4,5) P_3 to the PTB domain isolated from IRS-1

Binding of [3 H]Ins(1,4,5) P_3 (6.0 nM) to the isolated PTB domain at increasing concentrations was assayed in the presence (○) or absence (●) of 100 μ M unlabelled Ins(1,4,5) P_3 . Histidine-tagged proteins unrelated to the PTB domain were also assayed for binding: (▲) His-tagged glycosyl-PtdIns phospholipase D (300–430), (■) His-tagged Ins(1,4,5) P_3 binding protein with a molecular mass of 130 kDa (345–546). Closed and open symbols represent the results obtained in the absence and presence of 100 μ M unlabelled Ins(1,4,5) P_3 respectively. Each point represents the mean $\pm$ S.E. of three determinations.

The supernatant was carefully removed and mixed with a 5 $\times$ SDS/PAGE sample buffer. The pellet was dissolved in sample buffer to the same volume as that of the supernatant. The supernatant and pellet were subjected to SDS/PAGE and the gels were stained with Coomassie Brilliant Blue. Densitometric analysis was performed on the stained gel.

RESULTS

Binding of the PTB and PH domains to inositol phosphates

The isolated PTB domain derived from hIRS-1 was first analysed for binding of [3 H]Ins(1,4,5) P_3 at 6.0 nM. Binding increased as a function of protein concentration, while no apparent binding was observed in the presence of 100 μ M unlabelled Ins(1,4,5) P_3 (Figure 1). The binding to the PTB domain was displaced by unlabelled Ins(1,4,5) P_3 in a dose-dependent manner (Figure 2a). Scatchard analysis of the displacement results revealed that the K_d and V_{max} were 0.16 ± 0.03 μ M and 0.55 ± 0.06 mol/mol respectively (mean $\pm$ S.E. of four measurements carried out in

Figure 2 Displacement of [3 H]Ins(1,4,5) P_3 bound to the PTB domain of hIRS-1 (a) and the PH domain of hIRS-1 (b) by various inositol phosphates

The isolated PTB or PH domain was assayed for binding of [3 H]Ins(1,4,5) P_3 (6.0 nM) in the presence of various concentrations of unlabelled inositol phosphates: (●) Ins(1,4,5) P_3 , (○) Ins(1,3,4) P_2 , (■) Ins(1,3,4,5) P_4 , (△) Ins(1,3,4,5,6) P_5 , (▲) Ins(1,3,4,5,6,7) P_6 , (+) GroPIns(4,5) P_2 . Non-specific binding in the presence of 100 μ M unlabelled Ins(1,4,5) P_3 was assayed and subtracted from that in its absence to determine the specific binding. Each point represents the mean $\pm$ S.E. of three determinations.

Figure 3 Sequence alignment of the PTB and PH domains and the effect of site-directed mutagenesis of the PTB domain of hIRS-1 on [3 H]Ins(1,4,5) P_3 binding

(a) Structure based sequence alignment between the PTB domain of hIRS-1 (145–320), hShc(40–210) and the PH domain of hIRS-1(4–137), PLC δ_1 (16–128) and β -spectrin (2199–2304). The amino acids in the PTB domain of hIRS-1 mutated to glutamine for mutational analysis shown in (b) are indicated by an outlined font. The residues which were shown to interact with Ins(1,4,5) P_3 when complexed with Ins(1,4,5) P_3 , as assessed by crystallography [43,44] are underlined in β -spectrin and PLC δ_1 . (b) Specific binding of [3 H]Ins(1,4,5) P_3 to each PTB domain mutant was compared with binding to the wild-type (WT) PTB domain (100%). Each column represents the mean $\pm$ S.E. of five determinations.

duplicate with different preparations), indicating stoichiometric binding with a relatively high affinity. The value for stoichiometric binding was < 1 because of incomplete precipitation of the binding protein by poly(ethylene glycol), as confirmed by Western blotting followed by densitometric analysis (precipitation ranged

from 60–70%). The possibility that binding involved the six histidine residues attached to the N-terminus of the PTB domain used to isolate the recombinant molecule from the bacterial extract can probably be excluded because two unrelated proteins composed of similar numbers of amino acids with the same

Figure 4 Binding of the PTB domain of hIRS-1 to phosphoinositides incorporated into liposomes

(a) Typical SDS/PAGE of the PTB domain bound to liposomes containing various phosphoinositide (50 μM). (b) Summary of the binding to phosphoinositides at 50 μM. Each column represents the mean ± S.E. of five determinations. (c) Binding of the PTB domain to phosphoinositides at increasing concentrations. (●) PtdIns(4,5)P₂, (■) PtdIns(3,4,5)P₃, (○) PtdIns(3,4)P₂. Each point represents the mean ± S.E. of four determinations carried out in duplicate. (d) Displacement of 10 μM PtdIns(4,5)P₂ in liposomes bound to the PTB domain by InsP₆ and Ins(1,4,5)P₃. Each point represents the mean of two determinations carried out in duplicate.

histidine tag [glycosyl-PtdIns phospholipase D (300–430) or a new Ins(1,4,5)P₃ binding protein with a molecular mass of 130 kDa (345–546)] showed little binding to [³H]Ins(1,4,5)P₃ (Figure 1). No apparent displacement of bound [³H]Ins(1,4,5)P₃ was observed with unlabelled L-Ins(1,4,5)P₃ at concentrations up to 1 μM (results not shown). Furthermore, Ins(1,3,4)P₃ was less potent than Ins(1,4,5)P₃ in displacing bound [³H]Ins(1,4,5)P₃ (see Figure 2a). These results indicate that the binding of Ins(1,4,5)P₃ to the isolated PTB domain is not a result of non-specific ionic interactions between histidine residues and phosphates, but occurs in both an enantiomer-specific and a regioisomer-specific manner.

Figure 2(a) shows the displacement of [³H]Ins(1,4,5)P₃ from the PTB domain by several inositol phosphates. InsP₆ and Ins(1,3,4,5,6)P₅ were the most potent, followed by Ins(1,3,4,5)P₄ and Ins(1,4,5)P₃. Ins(1,3,4)P₃ was the least potent among the inositol phosphates examined. GroPIns(4,5)P₂, which contains no fatty acid chains, used as a soluble analogue of PtdIns(4,5)P₂, was about 20-fold less potent than InsP₆, and 3-fold less potent than Ins(1,4,5)P₃. In contrast, the PH domain isolated from the same protein bound Ins(1,4,5)P₃ most strongly (*K_d* value, 68 nM), and the other inositol phosphates examined were more than 10-fold less potent than Ins(1,4,5)P₃, as shown in Figure 2b. GroPIns(4,5)P₂ was about 100-fold less potent than Ins(1,4,5)P₃, indicating that either a glycerol moiety causes some sterical hindrance or fully-ionized 1-phosphate is required for the binding.

Mutational analysis of the inositol phosphate binding site of the PTB domain

The crystal structure of the PH domain of PLC-δ1 and β-spectrin, when complexed with Ins(1,4,5)P₃, revealed that several amino acids, especially the basic amino acid residues located in variable loops 1, 3 and/or 5, are primarily involved in the binding through ionic and hydrogen bonding interactions [43,44]. On the other hand, the binding region for a phosphotyrosine peptide mimicking a tyrosine-phosphorylated insulin receptor in the PTB domain of hIRS-1 is formed by the β5-strand, the C-terminal α-helix and the 3₁₀ turn connecting the β4 and β5 strands, as assessed by the crystal structure [11]. In particular, the PTB domain is co-ordinated by two arginine residues, Arg-212, which resides in the β5 strand and Arg-227 which extends from the β6 strand, as shown in Figure 3(a). Figure 3(a) also shows the sequence alignment of the PTB domain of IRS-1 and Shc, as well as that of a variety of PH domains. There are three basic amino acid residues in the loop connecting the β1 and β2 strands in the PTB domain of hIRS-1, which have been found to be essential for the binding of inositol phosphates to a variety of PH domains [43–45]. Therefore site-directed mutagenesis with the PTB domain of hIRS-1 was performed to map the binding site for inositol phosphates. Three lysine residues, Lys-169, Lys-171 or Lys-177, located in the loop, and two arginine residues, Arg-212 or Arg-227, were mutated to glutamine. Each construct was expressed and purified as for the wild-type preparation, described in the Materials and methods section. Specific binding of [³H]Ins(1,4,5)P₃ (6.0 nM) to each mutant was compared with that of the wild-type PTB domain (Figure 3b). Mutations of each lysine residue located in the loop (K169Q, K171Q or K177Q) resulted in a reduction of [³H]Ins(1,4,5)P₃ binding by about 50 % in each case. In contrast, mutation of each arginine residue (R212Q or R227Q), located in the β5 strand and the extension of the β6 strand of the PTB domain, had little effect on [³H]Ins(1,4,5)P₃ binding.

Simple binding experiments using [^3H]Ins(1,4,5) P_3 as a ligand provide equilibrium rate constants but do not usually give kinetic constants, such as the association rate constant (k_{ass}) and the dissociation rate constant (k_{diss}). To estimate these constants and the dissociation constant for the Ins(1,4,5) P_3 -binding of each site-directed mutant, a real-time kinetic assay using a BIAcore system was introduced. A biotinylated Ins(1,4,5) P_3 analogue, 204BT (see the Materials and methods section and [41]), was immobilized to the cuvette surface of the biosensor by avidin–biotin interactions. Addition of the wild-type PTB domain of hIRS-1 and all mutants brought about significant responses (results not shown). Kinetic constants were obtained from both the association and dissociation data of these responses, and the values of the kinetic equilibrium constant (K_d) were compared. The average K_d value for the wild-type PTB domain thus measured was $0.2 \pm 0.03 \mu\text{M}$, which was equivalent to that obtained from the [^3H]Ins(1,4,5) P_3 binding experiments ($0.16 \mu\text{M}$), as described above. Mutation of K169Q reduced it to $1.7 \pm 0.2 \mu\text{M}$ (8.5-fold), whereas that of R227Q caused a smaller reduction (only 1.5-fold to $0.3 \mu\text{M}$).

Binding of the PTB domain to phosphoinositides

Phosphoinositides incorporated into liposomes made from phosphatidylethanolamine were analysed for recognition by the PTB domain. The precipitates, formed by centrifugation of a mixture of the PTB domain and the phosphoinositide-containing liposomes, were separated by SDS/PAGE and analysed by densitometry. Figure 4(a) shows a typical SDS/PAGE pattern of the precipitates and supernatants. Although there were no liposomes in the control and the protein sample had been pre-cleared by ultracentrifugation, a portion of the PTB domain appeared to be precipitated, probably due to non-specific binding to the wall of the polystyrene tube. The addition of liposomes made from only phosphatidylethanolamine somewhat reduced the amount precipitated. Precipitation was increased by the inclusion of $50 \mu\text{M}$ -PtdIns(4,5) P_2 , PtdIns(3,4,5) P_3 or PtdIns(3,4) P_2 into liposomes, whereas there was no appreciable increase when phosphatidylinositol was used. The binding to liposomes containing each of the phosphoinositides ($50 \mu\text{M}$) is summarized in Figure 4(b). Figure 4(c) shows the binding in relation to the concentration of the phosphoinositides. The binding increased as the concentration of phosphoinositides in the liposomes increased. The half-maximal effective concentrations were approx. $0.7 \mu\text{M}$ for PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 and for PtdIns(3,4) P_2 , the dose–response curve was similar to that seen with the other two phosphoinositides up to about $1 \mu\text{M}$, but further increases in concentration did not produce more binding and, thus, the maximal effect was diminished to about 65% of that obtained with PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 . These results indicate that the PTB domain of hIRS-1 binds to phosphoinositides, but that the preference for the phosphoinositides examined was different from that seen in the displacement of [^3H]Ins(1,4,5) P_3 bound to the PTB domain by the polar head of the corresponding phosphoinositide (see Figure 2a).

Displacement of the PTB domain bound to PtdIns(4,5) P_2 in liposomes by Ins P_6 and Ins(1,4,5) P_3 was analysed. Liposomes containing $10 \mu\text{M}$ PtdIns(4,5) P_2 were incubated with the PTB domain in the presence of various concentrations of inositol phosphates and were then centrifuged. Ins P_6 inhibited the binding of the PTB domain to PtdIns(4,5) P_2 in a concentration-dependent manner, probably by competition, but Ins(1,4,5) P_3 was ineffective at concentrations up to 0.2 mM (Figure 4d). Similar results were obtained with the displacement of binding to liposomes con-

taining PtdIns(3,4,5) P_3 by Ins P_6 and Ins(1,3,4,5) P_4 (results not shown).

DISCUSSION

In this study, we examined the binding of inositol phosphates to the PTB domain derived from hIRS-1, compared with binding to the PH domain of the same protein. Although the PTB domain was initially characterized as a PTB module [3,4], its three dimensional structural similarity to the PH domain led us to examine the binding of inositol phosphates. Our data clearly indicate that the PTB domain of hIRS-1 binds inositol phosphates with a preference for Ins(1,3,4,5,6) P_5 and Ins P_6 , at a site different from that proposed for PTB [11]. The PTB domain also binds phosphoinositides, but with a different preference to that of the corresponding inositol phosphates, indicating that a glycerol and/or two fatty acid moieties of the phosphoinositides as well as the polar head are involved in the binding. It is possible that two acyl chains might provide new bonding (probably hydrophobic) interactions with the PTB domain, because a hydrophobic pocket which could accommodate fatty acids is formed between two β -sheets in the three dimensional structures of the PTB and PH domains [10,11,46]. The affinity for phosphoinositides appears to be slightly lower than for inositol phosphates. However, the concentrations of phosphoinositides were calculated under the unlikely assumption that the lipids distribute evenly in an assay mixture. Since the lipids reside in both the inner and outer leaflets of the liposomes formed, the concentration in an assay mixture could not be accurately estimated. In the case of PtdIns(3,4) P_2 -containing liposomes, the maximal binding of the PTB domain at the highest lipid concentration was lower than that seen with other phosphoinositides. We have no reasonable explanation for this observation at present. At first, it was assumed that PtdIns(3,4) P_2 at high concentrations preferentially resided in the inner leaflet of the liposomes, and therefore was not accessible to the PTB domain. This might be because the PtdIns(3,4) P_2 used was not natural but was synthesized so that it possessed two unsaturated stearic acid moieties, which form straight, rather than bent, hydrophobic tails. However, in later experiments the extent of binding of the PH domain from the proto-oncogene product (Akt) to PtdIns(3,4) P_2 was similar to that of PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 (T. Yamamoto, H. Takeuchi, U. Kikkawa, Y. Watanabe and M. Hirata, unpublished work).

Three basic amino acids present in the loop connecting the $\beta 1$ and $\beta 2$ strands of the IRS-1 PTB domain were shown to be involved in binding of the inositol phosphates. This followed on from the observation that the same loop of several PH domains (PLC- δ_1 [26,27,43,45], pleckstrin [46], β -spectrin [44], Bruton tyrosine kinase [21,47,48] and dynamin [21]) has been shown to be involved in the binding of Ins(1,4,5) P_3 by crystallographic or mutagenesis studies. A single mutation in the loop of the IRS-1 PTB domain reduced the binding of a fixed concentration of [^3H]Ins(1,4,5) P_3 by about 50%, which was caused by a reduction in affinity. However, complete loss of binding was caused by a single mutation in the PH domains of PLC- δ_1 [45] and Bruton tyrosine kinase [48]. Crystallographic studies of the PH domain with Ins(1,4,5) P_3 revealed that interactions of the phosphates, the hydroxy groups and the inositol ring of Ins(1,4,5) P_3 with several amino acids were involved in the binding [43,44]. Accordingly, in the case of the hIRS-1 PTB domain, replacement of a single amino acid would nullify only some of the bonding interactions involved. On the other hand, in the case of PLC- δ_1 and Bruton tyrosine kinase, complete loss of the binding might be explained

by drastic changes in the ability of the structure to accommodate $\text{Ins}(1,4,5)P_3$ by substitution of only one amino acid.

Two arginine residues (Arg-212 and Arg-227) of IRS-1, which are located in the $\beta 5$ strand and in the extension of the $\beta 6$ strand respectively, and which are distant from the inositol binding site according to the three dimensional structure, were reported to be essential for interacting with the phosphate moiety on the phosphotyrosine peptide [11]. Therefore, it is possible that the binding of both an inositol compound and a tyrosine-phosphorylated protein with an NPXY(p)-motif to the IRS-1 PTB domain might occur simultaneously and independently, or that these bindings influence each other allosterically, especially in the whole protein molecule. In the PTB domain of Shc, however, an arginine residue (Arg-67, close to the $\beta 1$ strand) in the loop connecting the $\beta 1$ and $\beta 2$ strands was shown to bind phosphotyrosine, at least partially [10], suggesting that competition could occur between an inositol compound and a phosphotyrosine peptide at the binding site. The results presented by Rameh et al. [32] showed that binding of the Shc-PTB domain to $\text{PtdIns}(3,4,5)P_3$ and $\text{PtdIns}(4,5)P_2$ was partially inhibited by a phosphotyrosine peptide and thus support this view. Ravichandran et al. [33], on the other hand, reported that three basic amino acids (Arg-112, Lys-116 and Lys-139) of the Shc PTB domain, the former two amino acids being in the long loop connecting $\beta 1$ and $\beta 2$, but very close to the $\beta 2$ strand (see Figure 3a), were involved in binding to $\text{PtdIns}(4,5)P_2$. Therefore they claimed that binding of the Shc PTB domain to both an inositol compound and a phosphotyrosine were required for functioning *in vivo* [33]. In either case, proteins with a PTB domain are a signalling component linking an inositol signalling pathway and a phosphotyrosine signalling pathway, collaboratively or competitively.

There are more than 20 types of inositol phosphates and eight types of phosphoinositides in cells, the cellular levels of which are variable. In addition to six well-known phosphoinositides [PtdIns , $\text{PtdIns}4P$, $\text{PtdIns}(4,5)P_2$, $\text{PtdIns}3P$, $\text{PtdIns}(3,4)P_2$ and $\text{PtdIns}(3,4,5)P_3$], $\text{PtdIns}(3,5)P_2$ was recently found to be produced from $\text{PtdIns}3P$ by 5-kinase in yeast [49], and $\text{PtdIns}5P$ was also found to be present as a precursor of $\text{PtdIns}(4,5)P_2$ in fibroblasts [50]. Determination of the physiologically relevant ligands depends not only on the relative binding affinity, but also on the relative intracellular abundance of a ligand. Although the free concentration of inositol compounds is difficult to evaluate, measurements of their total intracellular concentration have been reported. For example, the total cellular level of $\text{PtdIns}(4,5)P_2$ and $\text{Ins}(1,4,5)P_3$ has been estimated to be 30–160 μM and 1 μM respectively [17,51]. The local concentrations, however, are likely to be higher. The concentration of $\text{PtdIns}(4,5)P_2$ at the inner leaflet of neutrophils was estimated to be 5 mM decreasing to 3.5 mM upon cell stimulation (see [52]), while that of $\text{Ins}(1,4,5)P_3$ was estimated to increase up to 10 μM upon cell stimulation [17,52]. $\text{Ins}(1,3,4,5,6)P_5$ and $\text{Ins}P_6$ were thought to be the most abundant inositol phosphates in cells (estimated to be 15–100 μM [17,53]). Upon stimulation of cells with growth factors, cytokines or specific cell surface receptors there would be a reduction in the cellular levels of highly phosphorylated inositol phosphates and $\text{PtdIns}(4,5)P_2$, as well as an increase in the levels of $\text{PtdIns}(3,4,5)P_3$, $\text{PtdIns}(3,4)P_2$, $\text{Ins}(1,4,5)P_3$ and tyrosine-phosphorylated receptor molecules. Because of an ingenious spatial and quantitative balance between these parameters in cells, signalling molecules containing the PTB domain, such as Shc, IRS-1 and X11 [54], might be interacting factors in the provision of a productive pathway. Since the PTB domain could interact with inositol compounds and a peptide motif containing a phosphotyrosine residue

simultaneously, the domain might take part in cross-talk between different signalling cascades. Furthermore, inositol lipids compete with soluble inositol phosphates in the binding to the PTB domain. In this context, it could be possible that inositol phosphates attenuate the recruitment of protein containing the PTB domain to the plasma membrane, where both inositol lipids and tyrosine-phosphorylated receptor molecules reside. Among inositol phosphates, the PTB domain showed a preference for higher phosphorylated inositol phosphates. Very recently, Safrany and Shears [55] reported that diphosphorylated inositol phosphate ($[\text{PP}]_2\text{-Ins}P_4$ or $\text{Ins}P_8$) are present at micromolar levels in cells and turn over rapidly, and furthermore the turnover is regulated. Therefore these inositol residues might also play a regulatory role in the action of the PTB domain. Although this is not so in the case of the PTB domain, studies on synaptotagmin showed that it also binds higher inositol phosphates, and consequently the binding could inhibit the release of neurotransmitter in experiments *in vivo* [56,57].

In conclusion, the PTB domain of IRS-1, albeit originally identified as a PTB module, is capable of binding both inositol phosphates, with a preference for $\text{Ins}(1,3,4,5,6)P_5$ and $\text{Ins}P_6$, and phosphoinositides. Therefore this domain appears to be a second module linking inositol compound signalling and phosphotyrosine signalling pathways. The SH2 domain was the first to be nominated as such a module; the SH2 domain of the regulatory subunit of PtdIns 3-kinase and $\text{pp}60^{\text{c-src}}$, which is well known as a binding module for phosphotyrosine, has been shown to be able to bind $\text{PtdIns}(3,4,5)P_3$ [58]. As reported, we have been able to confirm that the SH2 domain of the regulatory subunit of PtdIns 3-kinase can bind to $\text{PtdIns}(3,4,5)P_3$ as well as to $\text{PtdIns}(4,5)P_2$. However, we could detect no binding of the SH2 domain from PLC- $\gamma 1$ and Shc to phosphoinositides (H. Takeuchi, U. Kikkawa, Y. Watanabe and M. Hirata, unpublished work).

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