THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 10, Issue of March 5, pp. 6114–6121, 1999

Printed in U.S.A.

Phosphotyrosine Binding Domains of Shc and Insulin Receptor Substrate 1 Recognize the NP*X*pY Motif in a Thermodynamically Distinct Manner*

(Received for publication, July 31, 1998, and in revised form, November 4, 1998)

Amjad Farooq‡, Olga Plotnikova, Lei Zeng, and Ming-Ming Zhou§

From the Structural Biology Program, Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York 10029-6574

Phosphotyrosine binding (PTB) domains of the adaptor protein Shc and insulin receptor substrate (IRS-1) interact with a distinct set of activated and tyrosine-phosphorylated cytokine and growth factor receptors and play important roles in mediating mitogenic signal transduction. By using the technique of isothermal titration calorimetry, we have studied the thermodynamics of binding of the Shc and IRS-1 PTB domains to tyrosine-phosphorylated NP*X*Y-containing peptides derived from known receptor binding sites. The results showed that relative contributions of enthalpy and entropy to the free energy of binding are dependent on specific phosphopeptides. Binding of the Shc PTB domain to tyrosine-phosphorylated peptides from TrkA, epidermal growth factor, ErbB3, and insulin receptors is achieved via an overall entropy-driven reaction. On the other hand, recognition of the phosphopeptides of insulin and interleukin-4 receptors by the IRS-1 PTB domain is predominantly an enthalpy-driven process. Mutagenesis and amino acid substitution experiments showed that in addition to the tyrosine-phosphorylated NP*X*Y motif, the PTB domains of Shc and IRS-1 prefer a large hydrophobic residue at pY-5 and a small hydrophobic residue at pY-1, respectively (where pY is phosphotyrosine). These results agree with the calculated solvent accessibility of these two key peptide residues in the PTB domain/peptide structures and support the notion that the PTB domains of Shc and IRS-1 employ functionally distinct mechanisms to recognize tyrosine-phosphorylated receptors.

Protein tyrosine phosphorylation provides a central control mechanism in regulating protein-protein interactions and activation of enzymes in mitogenic signal transduction following activation of cytokine and growth factor receptors (1, 2). Key events in receptor signaling are the interactions of signaling molecules such as adaptor protein Shc and insulin receptor substrate (IRS-1)[1] with activated and tyrosine-phosphorylated receptors. Binding to the activated receptor results in tyrosine phosphorylation of these signaling molecules, which in turn experience specific interactions with downstream signaling proteins and/or enzymes. For example, in insulin receptor (IR) signaling, upon binding to the activated receptor, IRS-1 is phosphorylated on many tyrosine residues, which enables IRS-1 to interact with various Src homology 2 (SH2) domain-containing proteins, including phosphatidylinositol 3-kinase, protein tyrosine phosphatase SH-PTP2, and Grb2 (3). On the other hand, tyrosine-phosphorylated Shc interacts with the SH2 domain of the adaptor protein Grb2, which in turn binds via its Src homology 3 (SH3) domains to the guanine nucleotide exchange factor, SOS, leading to Ras activation (4, 5).

Both IRS-1 and Shc can bind to the activated and tyrosine-phosphorylated insulin receptor through their phosphotyrosine binding (PTB) domain (also called PID or SAIN domain) (6). The PTB domain is a recently recognized protein module that can serve as an alternative to the SH2 domain for binding to tyrosine-phosphorylated proteins (6–9). PTB domains that are structurally and functionally distinct from SH2 domains recognize amino acid residues N-terminal (rather than C-terminal) to the phosphotyrosine (pY) (2, 10, 11). In particular, PTB domains preferentially bind to phosphorylated proteins at sites containing a NP*X*pY motif and hydrophobic amino acids N-terminal to this sequence (12–15). Unlike SH2 domains, PTB domains show very low protein sequence homology. Different PTB domains exhibit distinct selectivity for residues N-terminal to the NP*X*pY-motif. For example, the IRS-1 PTB domain favors hydrophobic residues at the pY-6 and pY-8 positions and an Ala at pY-1 for high affinity binding (15, 16), whereas the Shc PTB domain requires a bulky hydrophobic residue at pY-5 (12–14). Recent structural analysis revealed that the two PTB domains are structurally related but employ two very different mechanisms for recognizing the phosphotyrosine and the hydrophobic residues N-terminal to the NPXpY sequence (17–19). Indeed, except for the insulin receptor (6), the PTB domains of Shc and IRS-1 have been shown to interact with a distinct set of growth factor and cytokine receptors. For example, the Shc PTB domain binds to activated and tyrosine-phosphorylated TrkA, ErbB2, ErbB3, and epidermal growth factor (EGF) receptors (20–22), whereas IRS-1 interacts with the tyrosine-phosphorylated interleukin-4 receptor (IL-4R) via its PTB domain (23, 24).

Studies of thermodynamics of protein-ligand interactions can provide important insights into the structural and functional relationships of molecular recognition of the system. In an effort to determine further the structural and dynamic basis

* This work was supported by discretionary funds from the Mount Sinai School of Medicine (to M.-M. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a Wellcome International Prize Travelling Research Fellowship.

§ To whom correspondence should be addressed: Structural Biology Program, Dept. of Physiology and Biophysics, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1677, New York, NY 10029-6574. Tel.: 212-824-8224; Fax: 212-849-2456; E-mail: zhoum@inka.mssm.edu.

[1] The abbreviations used are: IRS-1, insulin receptor substrate 1; IR, insulin receptor; IL-4R, interleukin-4 receptor; ITC, isothermal titration calorimetry; NTA, nitrilotriacetic acid; pY, phosphotyrosine; PTB, phosphotyrosine binding; SASA, solvent-accessible surface area; SH2 Src homology-2; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; EGF, epidermal growth factor; PH, pleckstrin homology.

of functional differences in the molecular mechanisms by which the Shc and IRS-1 PTB domains recognize tyrosine-phosphorylated peptides, we have studied thermodynamics of peptide binding of the PTB domains using the isothermal titration calorimetry (ITC) technique. The phosphopeptides used in this study were derived from known Shc- and IRS-1-binding sites on growth factor and cytokine receptors. Results from these studies revealed that the PTB domains of Shc and IRS-1 appear to bind in a thermodynamically distinct manner to the NP*X*pY-containing peptides. The components of the free energy of the interactions show that the high affinity binding of the Shc PTB domain to the phosphopeptides is an overall entropy-driven process. In contrast, recognition of the IRS-1 PTB domain to the IR and IL-4R phosphopeptides is achieved predominantly by a large enthalpy contribution. By using site-directed mutagenesis and amino acid substitution, we have further quantified the relative contribution of the pY-5 and pY-1 residues in phosphopeptide binding to the PTB domains.

EXPERIMENTAL PROCEDURES

Protein Preparation—The PTB domain of Shc (residues 17–207) was cloned, expressed, and purified using procedures as described previously (17, 25). Briefly, the protein was subcloned into the bacterial expression vector pET15b (Novagen), which introduces a His tag followed by a thrombin cleavage site at the N terminus of the recombinant protein. The protein was expressed in *Escherichia coli* BL21(DE3) cells, which were induced with 1 mM isopropyl-1-thio-β-D-galactopyanoside for 4 h at 37 °C. The His-tagged protein was purified by affinity chromatography on a nickel-NTA column (Qiagen) and was treated with thrombin to remove the His tag.

The PTB domain of IRS-1 used in this study consists of residues 157–267 of the full-length protein. A slightly larger protein (residues 157–278) was subcloned into pET30b plasmid (Novagen) and expressed in *E. coli* BL21(DE3pLysS) cells with an additional Leu-Glu-$(His)_6$ sequence at the C terminus as described previously (18). The cells were grown overnight in LB media, and the expression of the protein was induced using 1 mM isopropyl-1-thio-β-D-galactopyanoside at 25 °C for 6 h. The cells were then disrupted using a French press. The His-tagged protein was purified by a nickel-NTA column. Subsequent cleavage of this protein with thrombin at a natural cleavage site (267–268) removed the C-terminal His-tag and the extra amino acids to give the PTB domain of IRS-1 (residues 157–267). The IRS-1 PTB mutant Met-257 → Ala was prepared as described previously, using the Chameleon Double-Stranded, Site-directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA), and the template plasmid used in the mutagenesis was pET30b-IRS1 (18). Expression and purification of the mutant IRS-1 PTB domain was accomplished as described for the wild-type protein.

Peptide Synthesis—The tyrosine-phosphorylated peptides used in the experiments reported here were synthesized by the Protein Core Facility at the Mount Sinai School of Medicine, using an Fmoc-based strategy. Phosphotyrosine was incorporated using the reagent Fmoc-Tyr(PO_3H_2) with HBTU/HOAt activation. Analysis of the purified peptides by analytical high pressure liquid chromatography demonstrated homogeneity.

Isothermal Titration Calorimetry (ITC) Analysis—Calorimetric measurements were performed with an Omega instrument (Microcal, Northampton, MA) (26). All experiments were carried out at 25 °C in a 50 mM Tris-HCl buffer of pH 8.0 containing 200 mM NaCl, 5 mM β-mercaptoethanol, and 1 mM EDTA. This condition was optimal for protein stability of the PTB domains of Shc and IRS-1, as there was no sign of significant protein aggregation for up to 0.5–1 mM protein concentration as determined by NMR spectroscopy. Both the PTB domains and the phosphopeptides were dissolved in the same buffer. The concentrations of protein and phosphopeptide were typically of 30–300 μM and 1–2 mM, respectively. To optimize the ITC measurements, the c value (c = [PTB domain]/K_D) was controlled in the range of 10–200 for all the ITC experiments, except for the weak binding of the IRS-1 PTB domain to the phosphopeptides of IR-pY960 (K_D = 87.07 ± 3.84 μM) and TrkA-pY490 (K_D = 678 ± 96.53 μM) (Table I).

Each titration experiment consisted of 25 10-μl injections of a peptide into the calorimetric cell containing 1.34 ml of a protein solution. A 250-s period was allowed between each injection, and there was an initial 60-s delay at the start of the experiment. Reaction enthalpies were also measured for injection of buffer into the protein and the phosphopeptide into the buffer. In each case, the measured enthalpies were found to be negligible compared with the enthalpy of the binding of the phosphopeptide to the PTB domains. The mean of the enthalpy of injection of buffer into the protein was subtracted from raw titration data prior to curve fitting. The peptide concentration was determined gravimetrically, whereas the protein concentration was measured using the Lowry method. Titration curves were fit to an in-built function by a non-linear least squares method using the ORIGIN software (Microcal, Northampton, MA). This function is based upon the binding of a ligand to a macromolecule (26) and contains n (reaction stoichiometry), K_D (dissociation constant), and ΔH (reaction enthalpy) as the variable parameters. These parameters can thus be directly determined from curve fitting. From the values of K_D and ΔH, the free energy (ΔG) and entropy change (ΔS) upon peptide binding can be calculated using the relationship: $-RT\ln(1/K_D) = \Delta G = \Delta H - T\Delta S$, where R is the universal molar gas constant and T is the absolute temperature.

NMR Spectroscopy—All NMR spectra were acquired at 30 °C on a Bruker DRX-500 NMR spectrometer. Uniformly ^{15}N-labeled proteins of the IRS-1 PTB domain were prepared for the NMR experiments by growing bacteria that overexpress the PTB domain in an M9 minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. The NMR samples of wild-type and the Met-257 → Ala mutant of the IRS-1 PTB domain were prepared at a concentration of 0.5 mM in 50 mM Tris-d_{11}/HCl buffer of pH 6.5, containing 50 mM NaCl and 5 mM dithiothreitol-d_{10} in 90% H_2O, 10% ^2H_2O. Two-dimensional $^1H/^{15}N$ heteronuclear single quantum coherence spectra were acquired with 96 and 1024 complex points in ω_1 and ω_2, respectively. The NMR spectra were processed and analyzed using the NMRPipe (27) and NMRView (28) programs.

RESULTS

Phosphopeptide Binding by the Shc PTB Domain—We used the same TrkA receptor peptide (HIIENPQpYFSDA) in the isothermal titration calorimetry studies as the one used in our recent structural analysis of the Shc PTB domain-TrkA phosphopeptide complex by NMR (17). From a peptide titration experiment using ITC, one can obtain thermodynamic information of the binding process (29, 30). The parameters include binding affinity, binding stoichiometry, enthalpy of binding (ΔH), and free energy change (ΔG) by a nonlinear fit of the binding isotherm, as well as entropy of binding (ΔS) from a difference between the free-energy change and the enthalpy of binding. A representative calorimetric isotherm and the corresponding titration curve of the Shc PTB domain binding to the TrkA peptide (Fig. 1*A*) show that a heat absorbance is associated with the peptide binding, indicating that the interaction is endothermic at 25 °C (ΔH = 3.63 kcal/mol). The heat absorbance upon addition of the phosphopeptide to the protein solution underwent a sharp change at 1:1 molar ratio of the protein to peptide, suggesting that the Shc PTB domain binding to the TrkA peptide is very tight, and the stoichiometry of this interaction is 1:1. By using these ITC data, we calculated a dissociation constant (K_D) to be 190 nM (Table I). Furthermore, the thermodynamic titration data revealed that the high affinity binding of the Shc PTB domain to the TrkA peptide is achieved by an overall entropy-driven process as the free energy of binding (ΔG = −9.12 kcal/mol) results predominantly from a large favorable entropic contribution ($T\Delta S$ = 12.75 kcal/mol).

To determine how the Shc PTB domain interacts thermodynamically with other NP*X*pY-containing phosphopeptides, we measured thermodynamic parameters of the Shc PTB domain binding to tyrosine-phosphorylated peptides derived from EGF, ErbB3, and insulin receptors (Table I). The results indicated that while the enthalpy of binding (either exothermic or endothermic reaction) is phosphopeptide-specific, change of entropy ($T\Delta S$) always favors the binding. Moreover, this large favorable entropic contribution appears to be the major determinant for the high affinity of the Shc PTB domain binding to the phosphopeptides. This observation is consistent not only with the phosphopeptides that contain the consensus sequence of Ψ*X*N-P*X*pY (where Ψ pY-5 is a hydrophobic residue) known for the

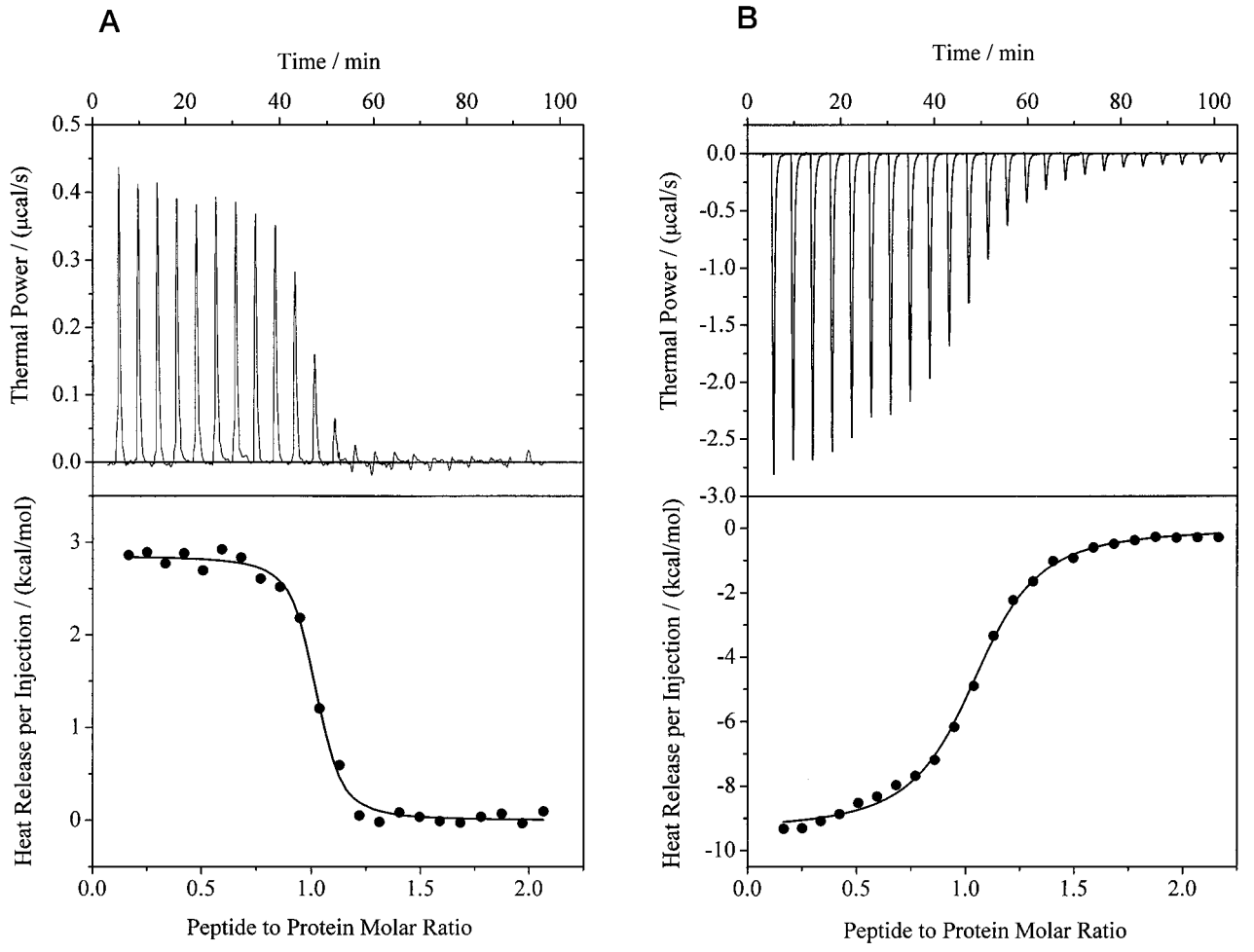

FIG. 1. **Isothermal titration calorimetric data for binding of the Shc PTB domain to TrkA-pY490 (*A*) and the IRS-1 PTB domain to tyrosine-phosphorylated peptides of IL4R-pY497 (*B*), IR-pY960 (*C*), and IR(A-1)-pY960 (*D*).** The *solid lines* show the fit of the data to a function based on the binding of a ligand to a macromolecule using the software ORIGIN (26).

high affinity binding to the Shc PTB domain but also with the IR phosphopeptide that contains large hydrophobic residues at pY-6 to pY-8 instead of pY-5.

To study further the thermodynamics of the PTB domain of Shc binding to tyrosine-phosphorylated peptides, we conducted ITC measurements using a phosphopeptide derived from IL-4 receptor (pY497). This IL-4R phosphopeptide is not a biological ligand for the Shc PTB domain as Shc has not been linked to IL-4R signaling. On the other hand, the IL-4R peptide represents a biologically relevant binding site for the IRS-1 PTB domain (23, 24). The ITC results showed that the PTB domain of Shc binds to the IL-4R peptide much weaker than to those phosphopeptides from the known Shc binding sites (Table I). Interestingly, the Shc binding of the IL-4R peptide is also dictated by a large favorable entropic contribution. Taken together, our ITC results suggest that under the conditions of our study, binding of the Shc PTB domain to the NP*X*pY-containing phosphopeptides of the TrkA, EGF, ErbB3, and insulin receptors appears to be an overall entropy-driven process.

Phosphopeptide Binding of the IRS-1 PTB Domain—We performed an ITC titration using a phosphopeptide from the IL-4R (pY497) (LVIAGNPApYRS) which is a known binding site for IRS-1 (23, 24). As shown in Fig. 1*B*, binding of the IRS-1 PTB domain to the IL-4R peptide (see Table II) is exothermic (ΔH = −9.43 kcal/mol) and involves an unfavorable change of entropy ($T\Delta S$ = −1.63 kcal/mol). Curve fitting of the ITC data gave a dissociation constant K_D of 1.82 μM for this PTB domain-peptide complex. Thus, the IL-4R peptide interaction with the IRS-1 PTB domain is enthalpy-driven, which is in sharp contrast to the entropy-driven binding of the Shc PTB domain.

Binding of the IRS-1 PTB domain to the IR-pY960 peptide (LYASSNPEpYLS) also appears to be governed mainly by an enthalpy contribution (ΔH = −10.74 kcal/mol and $T\Delta S$ = −5.22 kcal/mol). However, binding affinity of the IR-pY960 peptide to the IRS-1 PTB domain (K_D = 87.07 μM) is ~50-fold weaker than that of the IL4R-pY497 peptide (K_D = 1.82 μM) (Fig. 1*C*, Table II). This marked reduction of the peptide binding affinity correlates with an increased entropy penalty.

Binding Specificity of the IRS-1 and Shc PTB Domains—The major difference in amino acid sequence between the IL-4R and IR phosphopeptides is the residue at pY-1. To determine the contribution of the pY-1 residue to binding of the IRS-1 PTB domain, we substituted the Glu pY-1 in the IR-pY960 peptide by an Ala. The latter amino acid corresponds to the Ala pY-1 in the IL-4R phosphopeptide. The ITC measurements showed that this single amino acid substitution led to a 38-fold increase of the binding affinity to 2.32 μM (Fig. 1*D* and Table II), which is nearly the same as that of the IL-4R peptide binding to the IRS-1 PTB domain (K_D = 1.82 μM). It is interesting to note that this significant increase of the peptide binding affinity results largely from a reduction of the entropy penalty ($T\Delta S$) of ~2 kcal/mol. Furthermore, the non-phosphorylated form of the IR(A-1)-pY960 peptide showed no interaction with the protein as probed by isothermal titration calorimetry (Table II), indicating that binding of the IRS-1 PTB domain to the NPApY motif is tyrosine phosphorylation-dependent.

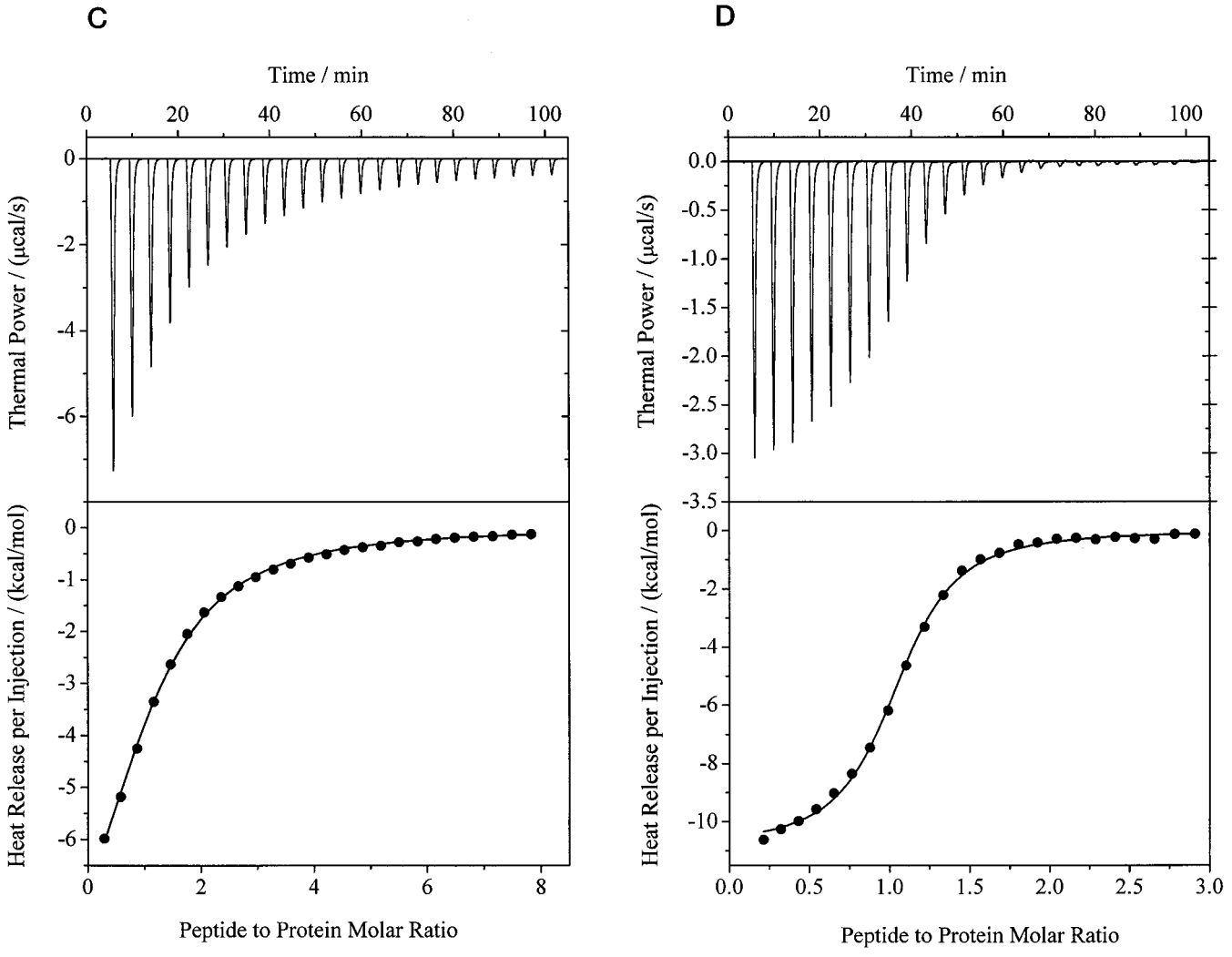

FIG. 1—*continued*

TABLE I

Thermodynamic parameters obtained for binding of the Shc PTB domain to NPXpY-containing phosphopeptides at pH 8.0 and 25 °C

The experimental conditions of the ITC measurements were described in detail under "Experimental Procedures." Three ITC experiments were conducted for each phosphopeptide at slightly different protein concentrations. The values for K_D ($K_D = 1/K_B$) and ΔH were calculated directly from the curve fitting of the titration data to a function based on the binding of a ligand to a macromolecule (22), using ORIGIN. In this fitting procedure, the values for K_B, ΔH, and n (reaction stoichiometry) were all allowed to float. The mean value for n was found to be 1 ± 0.1. Errors quoted for K_D and ΔH are standard deviations from the three ITC experiments, whereas errors on $T\Delta S$ and ΔG are propagated errors.

Protein pY sites	pY peptides	K_D	ΔH	$T\Delta S$	ΔG
		μM	*kcal mol*$^{-1}$	*kcal mol*$^{-1}$	*kcal mol*$^{-1}$
TrkA, pY490	HIIE**NPQpY**FSDA	0.19 ± 0.01	3.63 ± 0.11	12.75 ± 0.15	−9.12 ± 0.03
hEGFR, pY1148	SLD**NPDpY**QQDFF	1.69 ± 0.15	−2.82 ± 0.08	5.02 ± 0.11	−7.84 ± 0.05
hErbB3, pY1309	SAFD**NPDpY**WHSRLF	0.33 ± 0.04	−2.54 ± 0.08	6.27 ± 0.13	−8.81 ± 0.08
IR, pY960	LYASS**NPEpY**LS	4.22 ± 0.90	2.54 ± 0.29	9.85 ± 0.16	−7.31 ± 0.14
IL-4R, pY497	LVIAG**NPApY**RS	12.25 ± 5.22	0.87 ± 0.14	7.58 ± 0.15	−6.71 ± 0.28
TrkA A-5, pY490	HIA**NPQpY**FSDA	0.70 ± 0.17	2.78 ± 0.14	11.16 ± 0.10	−8.38 ± 0.14

To investigate the size preference of the favored hydrophobic residue at pY-1, we substituted the Glu pY-1 by amino acid Ile or Phe in the IR-pY960 phosphopeptide. The measured thermodynamic parameters showed that the phosphopeptide containing either Ile or Phe at pY-1 binds to the IRS-1 PTB domain about 3–4-fold weaker than the IR(A-1)-pY960 but ~10–13-fold stronger than the wild-type phosphopeptide which contains a Glu at pY-1.

The preference for a small hydrophobic residue at pY-1 by the IRS-1 PTB domain agrees with our recent NMR structural analysis of the PTB domain-IL-4R peptide complex (18). The NMR structure revealed that the Ala pY-1 in the NPApY motif interacts with a hydrophobic binding site formed by three methionines, *i.e.* Met-257, Met-260, and Met-209. To determine the relative contribution of this hydrophobic site to phosphopeptide binding, we mutated Met-257 of the IRS-1 PTB domain to Ala for calorimetric studies. A comparison of $^{1}H/^{15}N$ heteronuclear single quantum coherence NMR spectra of the mutant Met-257 → Ala and wild-type IRS-1 PTB domain suggested that the mutation did not cause any significant structural perturbations (Fig. 2, *A* and *B*). The binding affinity of this mutant to IL4R-pY497 and IR(A-1)-pY960 peptides was almost an order of magnitude lower than that of the wild-type protein (Table II).

TABLE II

Thermodynamic parameters obtained for binding of the IRS-1 PTB domain to NPXpY-containing phosphopeptides at pH 8.0 and 25 °C

The experimental conditions of the ITC measurements were described in detail under "Experimental Procedures" and Table I. The mean value for n (reaction stoichiometry) was found to be 1 ± 0.1. Errors quoted for K_D and ΔH are standard deviations from the three ITC experiments, whereas errors on $T\Delta S$ and ΔG are propagated errors.

Protein pY sites	pY peptides	K_D	ΔH	$T\Delta S$	ΔG
		μM	*kcal mol*$^{-1}$	*kcal mol*$^{-1}$	*kcal mol*$^{-1}$
IL-4R, pY497	LVIAG**NPA**p**Y**RS	1.82 ± 0.11	−9.43 ± 0.15	−1.63 ± 0.17	−7.80 ± 0.04
IR, pY960	LYASS**NPE**p**Y**LS	87.07 ± 3.84	−10.74 ± 0.30	−5.22 ± 0.33	−5.52 ± 0.03
IR (A-1), pY960	LYASS**NPA**p**Y**LS	2.32 ± 0.25	−10.85 ± 0.09	−3.19 ± 0.15	−7.66 ± 0.07
IR (I-1), pY960	LYASS**NPI**p**Y**LS	8.40 ± 1.14	−5.91 ± 0.11	0.99 ± 0.18	−6.90 ± 0.08
IR (F-1), pY960	LYASS**NPF**p**Y**LS	6.64 ± 0.27	−5.35 ± 0.07	1.69 ± 0.09	−7.04 ± 0.03
IR (A-1), Y960	LYASS**NPA** **Y**LS		~0[a]		
TrkA, pY490	HIIE**NPQ**p**Y**FSDA	678.02 ± 96.53	−9.65 ± 0.76	−5.34 ± 0.85	−4.31 ± 0.08
hEGFR, pY1148	SLD**NPD**p**Y**QQDFF		~0[a]		
hErbB3, pY1309	SAFD**NPD**p**Y**WHSRLF		~0[a]		
IRS-1 PTB-M257A					
IL-4R, pY497	LVIAG**NPA**p**Y**RS	24.64 ± 2.21	−8.36 ± 0.37	−2.10 ± 0.42	−6.26 ± 0.06
IR (A-1), pY960	LYASS**NPA**p**Y**LS	21.98 ± 0.22	−7.58 ± 0.08	−1.25 ± 0.07	−6.33 ± 0.02

[a] These thermodynamic parameters cannot be determined since the measured signal (ΔH) is observed to be zero.

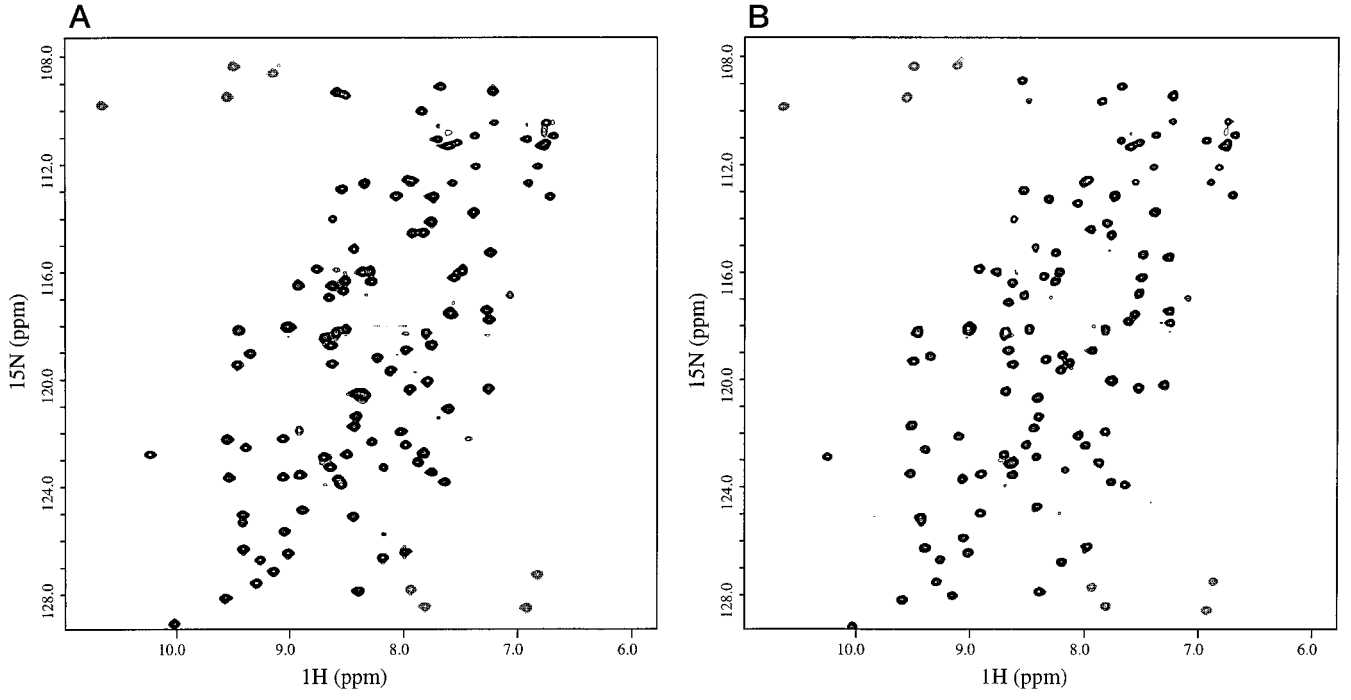

FIG. 2. **Comparison of two-dimensional $^1H/^{15}N$ heteronuclear single quantum coherence NMR spectra of wild-type (*A*) and the mutant Met-257 → Ala of the IRS-1 PTB domain (*B*).**

The requirement of an Ala at pY-1 for high affinity phosphopeptide binding appears only unique to the IRS-1 PTB domain, because amino acid residues at this corresponding site are highly variable in the known Shc PTB domain binding sites in various mitogenic receptors. Instead, the PTB domain of Shc prefers a large hydrophobic residue at pY-5 as suggested by our NMR structural analysis of the Shc PTB domain-TrkA peptide complex (17). The NMR structure revealed that in addition to the NP*X*pY motif, the Ile pY-5 of the TrkA peptide interacts extensively with residues in the hydrophobic core of the PTB domain of Shc. To examine the contribution of the Ile pY-5 to the high affinity peptide binding, we substituted Ala for Ile pY-5 in the TrkA peptide. The ITC measurements showed that this substitution results in a 4-fold reduction in the peptide binding affinity (Table I). Furthermore, as compared with the TrkA phosphopeptide, binding of the TrkA(A-5) peptide showed decreased enthalpy and entropy of binding to 2.78 kcal/mol (ΔH) and 11.16 kcal/mol ($T\Delta S$), respectively. Nevertheless, the TrkA(A-5) peptide binding to the Shc PTB domain is still largely an entropy-driven process.

To test whether the preference of a large hydrophobic residue at pY-5 is specific for the Shc PTB domain, we performed the ITC studies of the IRS-1 PTB domain binding to the tyrosine-phosphorylated peptides derived from known Shc PTB domain binding sites on TrkA, EGF, and ErbB3 receptors. The ITC data revealed that these Shc-specific NP*X*pY-containing peptides showed very weak or no binding to the IRS-1 PTB domain (Table II). This finding can be explained by the IRS-1 PTB domain structure (18), which shows there is insufficient space in the PTB domain to accommodate large, hydrophobic side chains at pY-5 in the phosphopeptides. These results, which agree with structural and biochemical studies (12, 17), suggest that in addition to the NP*X*pY motif, the PTB domains of Shc and IRS-1 recognize differentially specific amino acid residues N-terminal to the pY in order to achieve their distinct binding specificity.

Solvent Accessibility of the Peptide Residues in the PTB Domain/Peptide Structures—To understand further the nature of hydrophobic interactions between the PTB domains and phosphopeptides, we calculated solvent-accessible surface area

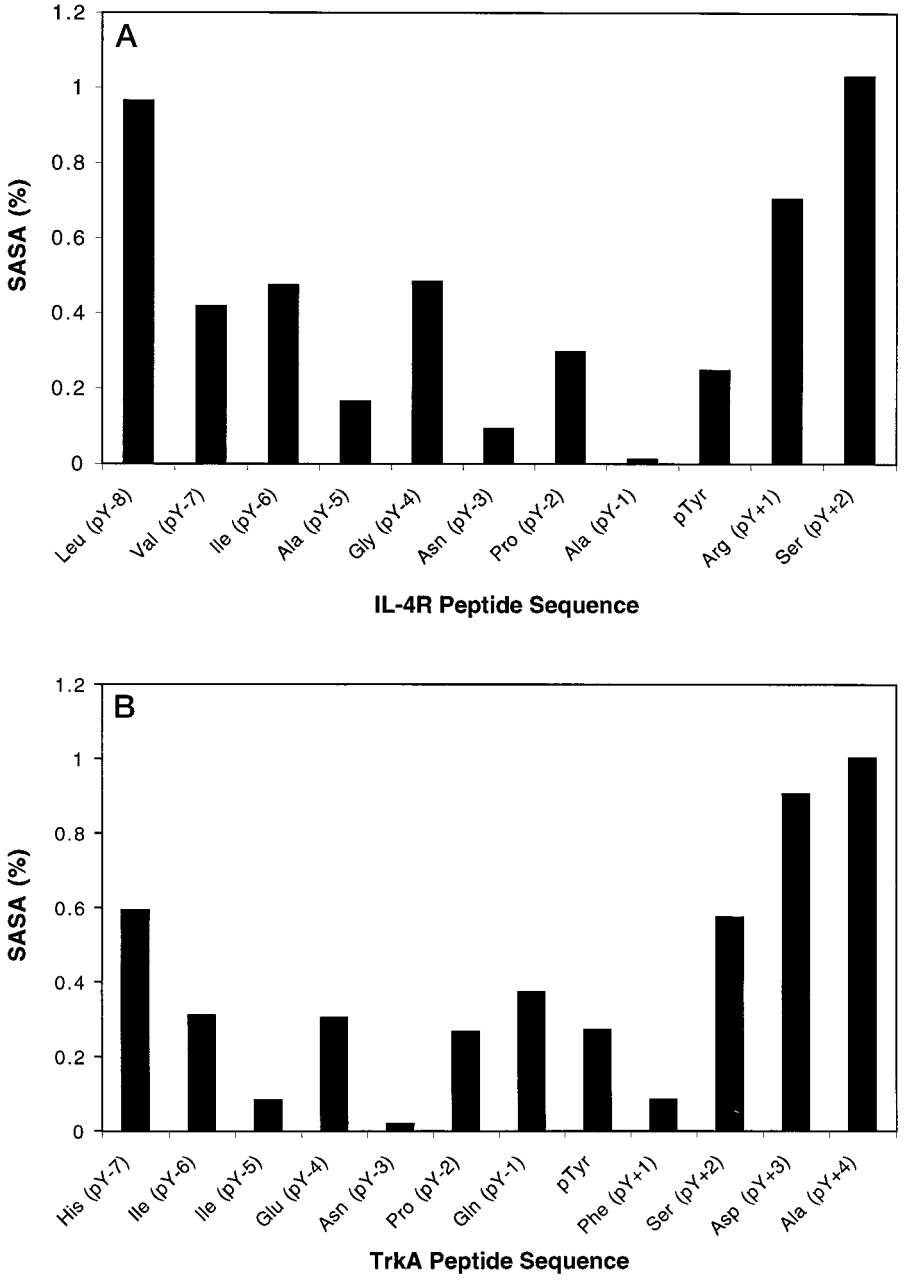

FIG. 3. **Analysis of SASA of the phosphopeptide residues complexed to the PTB domains of IRS-1 (*A*) and Shc (*B*).** Calculations of the residue-based SASA were performed for the averaged energy-minimized NMR structures of the IRS-1 and Shc PTB domain-phosphopeptide complexes using X-PLOR program (43). The percentage of the SASA was determined based on the solvent-exposed surface area of a particular amino acid residue in a hypothetical tri-peptide form.

(SASA) for the phosphopeptide residues in the PTB domain/phosphopeptide structures. The results indicated that the extent of the solvent accessibility of the phosphopeptide residues agrees with the degree of their interactions with the PTB domains (Fig. 3, *A* and *B*). For example, the Ala pY-1 of the IL-4R peptide is nearly completely buried in the IRS-1 PTB domain-IL-4R peptide complex, as its calculated SASA in the PTB domain-bound form is about 1% that in a hypothetical tri-peptide state. This result is in agreement with the importance of the Ala pY-1 in determining the peptide binding specificity and binding affinity of the IRS-1 PTB domain.

The SASA analysis of the IRS-1 PTB domain has revealed that Met-257, Met-260, and Met-209 that are involved in intimate interactions with the Ala pY-1 are largely buried with SASA less than 20%. Other peptide residues important for binding to the PTB domain also show low SASA, such as 9.2% for Asn pY-3 and 16.4% for Ala pY-5. The SASA of the pY and the hydrophobic residues at pY-6 and pY-7 is 25 and 42–47%, respectively. These results are consistent with the PTB domain structure which shows that the binding sites for these peptide residues are located on the surface of the protein. Furthermore, the SASA analysis of the Shc PTB domain (Fig. 3*B*) suggests that the peptide residues of the pY, Asn pY-3, Ile pY-5, and Phe pY+1 are involved in extensive interactions with the protein.

DISCUSSION

In this study we have characterized the thermodynamics of binding of the PTB domains of Shc and IRS-1 to the NP*X*pY motif-containing phosphopeptides. Our ITC results demonstrated that the binding of IRS-1 PTB domain to tyrosine-phosphorylated IL-4R and IR peptides is an enthalpy-driven process, whereas the binding of Shc PTB domain to tyrosine-phosphorylated peptides of TrkA, EGF, ErbB3, and insulin receptors appears to be largely governed by entropic factors. Since the phosphopeptides used in this study are all derived from known Shc- and IRS-1-binding sites in cytokine and growth factor receptors, our thermodynamic results should provide new insights into the dynamic nature of interactions of the PTB domains with the tyrosine-phosphorylated receptors.

It should be noted that Mandiyan *et al.* (31) have recently

reported ITC studies of the Shc PTB domain binding to phosphopeptides from TrkA and EGF receptors. Mandiyan and colleagues (31) reported a K_D of 42 nM, ΔH of 2.36 kcal/mol, and $T\Delta S$ of 12.44 kcal/mol for the TrkA (pY490) peptide and a K_D of 28 nM, ΔH of −5.46 kcal/mol, and $T\Delta S$ of 4.84 kcal/mol for the EGF receptor (pY1148) peptide. Our ITC data agree with their results of the TrkA peptide in general but differ from those of the EGF receptor peptide (Table I). The discrepancies between the two studies could be due to differences in the *c* values (c = [protein]/K_D) used in the ITC measurements. The *c* values used in our study for these two phosphopeptides were between 20 and 150, which are considered to be optimal for the ITC measurements (26), whereas their c values were in the range of 320–1070.

The enthalpy-driven nature of the IRS-1 PTB domain binding to the tyrosine-phosphorylated peptides is consistent with the structure and protein dynamics studies of the IRS-1 PTB domain-IL-4R peptide complex (18). Formation of hydrophobic and electrostatic contacts and hydrogen bonds between the protein and peptide residues could account for the largely exothermic reaction ($\Delta H < 0$) observed in the ITC measurements. On the other hand, the entropic penalty ($T\Delta S < 0$) in the peptide binding may result from the following: 1) a decrease of conformational entropy of amino acid residues that are directly involved in the peptide binding and become more rigid upon the complex formation; and 2) a reduction of translational entropy of the protein and peptide molecules upon complex formation. By using NMR relaxation measurements, we have recently characterized the dynamics of the backbone amides of the IRS-1 PTB domain in both the free protein and the protein when complexed to the IL-4R phosphopeptide (32). The results showed that the motion of several residues becomes restricted after ligand binding, including a few residues that do not make direct contacts with the peptide. Such changes in the motional properties of these residues upon ligand binding could contribute to change of the conformational entropy of the system. It is interesting to note that the observed change of entropy (ΔS = approximately −5.5 cal/mol/K) in the IRS-1 PTB domain binding to the IL-4R peptide appears to agree with the recently reported translational entropy cost for protein-protein association ($\Delta S = -5 \pm 4$ cal/mol/K) that was measured using the dimeric subtilisin inhibitor from *Streptomyces* (33). Therefore, these results suggest that the observed entropic penalty of the IRS-1 PTB domain binding to the IL-4R peptide may largely result from the change of the translational entropy of the system.

Phosphopeptide binding of the Shc PTB domain appears thermodynamically different from that of the IRS-1 PTB domain. The thermodynamic parameters of the Shc PTB domain, however, cannot be readily explained using its three-dimensional structure. The NMR structure of the Shc PTB domain-TrkA peptide complex showed that the PTB domain interacts with fewer peptide residues than does the IRS-1 PTB domain, yet Shc exhibits higher phosphopeptide binding affinity in general. Whereas the contributions of enthalpy and entropy to the free energy of binding depend on PTB domain interactions with specific phosphopeptides, the high affinity peptide binding to the Shc PTB domain is achieved mainly by a large favorable entropy change. This entropy-driven nature of the phosphopeptide binding suggests that the binding of the Shc PTB domain to the NP*X*pY-containing peptides may involve the burial of more hydrophobic surface and/or an increase in the conformational freedom of the protein upon complex formation.

The change of hydrophobic surface area (ΔA) of a protein upon binding to its ligand can be estimated by measurement of the change of heat capacity (ΔC), which can be determined from enthalpy of binding (ΔH) measured at different temperatures (34, 35). By using the ITC technique, we measured the ΔC of the IRS-1 PTB domain binding to the IR(A-1)-pY960 peptide to be about −240 cal/mol/K (data not shown). Interestingly, this value is very similar to the values reported by Mandiyan *et al.* (31) for the Shc PTB domain binding to the TrkA-pY490 (−207 cal/mol/K) and EGF receptor -pY1148 (−185 cal/mol/K). It should be pointed out that an accurate analysis of the change of hydrophobic surface area (ΔA) using the ΔC values may be difficult (36). Nevertheless, these similar ΔC values may indicate that the change of hydrophobic surface area (ΔA) upon phosphopeptide binding could be similar for the PTB domains of Shc and IRS-1. Therefore, the favorable entropic contributions observed in the Shc PTB domain binding to the phosphopeptides likely result from an increase in the conformational freedom of the protein upon complex formation. Indeed, more recently our NMR structural studies of the Shc PTB domain of the free form suggest that the protein undergoes conformational change upon binding to the tyrosine-phosphorylated peptides.[2] Further work is required to determine how the conformational rearrangement of the Shc PTB domain contributes to the overall entropy-driven nature of its high affinity binding to phosphopeptides.

The PTB domains of IRS-1 and Shc show very little sequence homology but share a common structure fold of pleckstrin homology (PH) domains (17–19, 37). The PH domains have been shown to bind to $\beta\gamma$ subunits of G proteins and acidic phospholipids and are important for localizing proteins that contain the PH domain to the membrane surface (37, 38). Indeed, we have recently shown that the PTB domain of Shc can bind to the tyrosine-phosphorylated proteins and receptors and to phospholipids, and both events are essential for tyrosine phosphorylation of Shc following receptor activation (39). More recently, it has been reported that "PTB domain-like" proteins contained in other signaling molecules can also mediate protein-protein interactions in a phosphotyrosine-independent manner. These include signaling proteins of X11, FE65, Numb, and SNT (40–42). Taken together, these results strongly suggest that this conserved PH domain-structure fold can be utilized for diverse functions for protein-protein or protein-lipid interactions via distinct molecular mechanisms.

In summary, we have demonstrated in this study that the two structurally homologous but functionally distinct PTB domains of Shc and IRS-1 recognize the NP*X*pY motif-containing phosphopeptides in a thermodynamically distinct manner. Results from the ITC analysis provide new insights into the dynamic nature of the interactions of the phosphopeptides with the Shc and IRS-1 PTB domains. Our results support the credence that residues in and around the NP*X*pY motif are directly involved in determining the specificity of phosphopeptide interaction with the Shc and IRS-1 PTB domains. Such specificity may be the basis of the differences in the biological functions of these signaling proteins. Furthermore, our thermodynamic data suggest that, in addition to enthalpic contribution, entropic factors may also play a pivotal role in the formation of PTB-peptide complexes involved in cellular signal transduction.

Acknowledgments—We thank Dr. Stephen W. Fesik for the cDNA plasmids of the PTB domains of Shc and IRS-1. We are grateful to Drs. Christophe Dhalluin and Carlos Escalante for technical advice on the isothermal titration calorimetry experiments, Dr. Imre Wolf for phosphopeptide synthesis, and Dr. Diomedes E. Logothetis for critical reading of the manuscript.

REFERENCES

1. Hunter, T. (1995) *Cell* **80,** 225–236
2. Pawson, T. (1995) *Nature* **373,** 573–580

[2] A. Farooq and M.-M. Zhou, unpublished data.

3. Sun, X. J., Crimmins, D. L., Myers, M. G. J., Miralpeix, M., and White, M. F. (1993) *Mol. Cell. Biol.* **13,** 7418–7428
4. Salcini, A., McGlade, J., Pelicci, G., Nicoletti, I., Pawson, T., and Pelicci, P. (1994) *Oncogene* **9,** 2827–2836
5. Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thoma, S., Brugge, J., Pelicci, P. G., Schlessinger, J., and Pawson, T. (1992) *Nature* **360,** 689–692
6. Gustafson, T. A., He, W., Craparo, A., Schaub, C. D., and O'Meill, T. J. (1995) *Mol. Cell. Biol.* **15,** 2500–2508
7. Kavanaugh, W. M., and Williams, L. T. (1994) *Science* **266,** 1862–1865
8. Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V., and Margolis, B. (1994) *J. Biol. Chem.* **269,** 32031–32034
9. Geer, P. V. D., Wiley, S., Lai, K., Olivier, J., Gish, G., Stephens, R., Kaplan, D., Shoelson, S., and Pawson, T. (1995) *Curr. Biol.* **5,** 404–412
10. Cohen, G. B., Ren, R., and Baltimore, D. (1995) *Cell* **80,** 237–248
11. Zhou, M.-M., and Fesik, S. W. (1995) *Prog. Biophys. Mol. Biol.* **64,** 221–235
12. Trüb, T., Cho, W. E., Wolf, G., Ottinger, E., Hen, Y., Weiss, M., and Shoelson, S. E. (1995) *J. Biol. Chem.* **270,** 18205–18208
13. Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1995) *Science* **268,** 1177–1179
14. Zhou, S., Margolis, B., Chaudhuri, M., Shoelson, S. E., and Cantley, L. C. (1995) *J. Biol. Chem.* **270,** 14863–14866
15. Wolf, G., Trüb, T., Ottinger, E., Groninga, L., Lynch, A., White, M. F., Miyazaki, M., Lee, J., and Shoelson, S. E. (1995) *J. Biol. Chem.* **270,** 27407–27410
16. He, W., O;Neill, T. J., and Gustafson, T. A. (1995) *J. Biol. Chem.* **270,** 23258–23262
17. Zhou, M.-M., Ravichandran, K. S., Olejniczak, E. T., Petros, A. P., Meadows, R. P., Sattler, M., Harlan, J. E., Wade, W., Burakoff, S. J., and Fesik, S. W. (1995) *Nature* **378,** 584–592
18. Zhou, M.-M., Huang, B., Olejniczak, E. T., Meadows, R. P., Shuker, S. B., Miyazak, M., Trüb, T., Shoelson, S. E., and Fesik, S. W. (1996) *Nat. Struct. Biol.* **3,** 388–393
19. Eck, M. J., Dhe-Pagnon, S., Trüb, T., Nolte, R., and Shoelson, S. E. (1996) *Cell* **85,** 695–705
20. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., and Pawson, T. (1992) *Cell* **70,** 93–104
21. Obermeier, A., Lammers, R., Weismuller, K., Jung, G., Schlessinger, J., and Ullrich, A. (1993) *J. Biol. Chem.* **268,** 22963–22966
22. Ricci, A., Lanfrancone, L., Chiari, R., Belardo, G., Pertica, C., Natali, P. G., Pelicci, P. G., and Segatto, O. (1995) *Oncogene* **11,** 1519–1529
23. Wang, L. M., Myers, M. G. J., Sun, X. J., Aaronson, S. A., White, M., and Pierce, J. H. (1993) *Science* **261,** 1591–1594
24. Keegan, A. D., Nelms, K., White, M., Wang, L. M., Pierce, J. H., and Paul, W. E. (1994) *Cell* **76,** 811–820
25. Zhou, M.-M., Harlan, J. E., Wade, W., Crosby, S., Ravichandran, K. S., Burakoff, S. J., and Fesik, S. W. (1995) *J. Biol. Chem.* **270,** 31119–31123
26. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) *Anal. Biochem.* **179,** 131–137
27. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) *J. Biomol. NMR* **6,** 277–293
28. Johnson, B. A., and Blevins, R. A. (1994) *J. Biomol. NMR* **4,** 603–614
29. Livingstone, J. R. (1996) *Nature* **384,** 491–492
30. Ladbury, J. E., Lemmon, M. A., Zhou, M., Green, J., Botfield, M. C., and Schlessinger, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3199–3203
31. Mandiyan, V., O'Brien, R., Zhou, M., Margolis, B., Lemmon, M. A., Sturtevan, J. M., and Schlessinger, J. (1996) *J. Biol. Chem.* **271,** 4770–4775
32. Olejniczak, E. T., Zhou, M.-M., and Fesik, S. W. (1997) *Biochemistry* **36,** 4118–4124
33. Tamura, A., and Privalov, P. L. (1997) *J. Mol. Biol.* **273,** 1048–1060
34. Spolar, R. S., and Record, M. T., Jr. (1994) *Science* **263,** 777–784
35. Livingston, J. R., Spolar, R. S., and Record, M. T., Jr. (1991) *Biochemistry* **30,** 4237–4244
36. Ladbury, J. E., Wright, J. G., Sturtevant, J. M., and Sigler, P. B. (1994) *J. Mol. Biol.* **238,** 669–681
37. Lemmon, M. A., Ferguson, K. M., and Schlessinger, J. (1996) *Cell* **85,** 621–624
38. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1995) *Nature* **371,** 167–170
39. Ravichandran, K. S., Zhou, M.-M., Pratt, J. C., Harlan, J. E., Walk, S., Fesik, S. W., and Burakoff, S. J. (1997) *Mol. Cell. Biol.* **17,** 5540–5549
40. Borg, J.-P., Ooi, J., Levy, E., and Margolis, B. (1996) *Mol. Cell. Biol.* **16,** 6229–6241
41. Li, S.-C., Songyang, Z., Vincent, S. J. F., Zwahlen, C., Wiley, S., Cantley, L., Kay, L. E., Forman-Kay, J., and Pawson, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7204–7209
42. Xu, H., Lee, K. W., and Goldfarb, M. (1998) *J. Biol. Chem.* **273,** 17987–17990
43. Brünger, A. T. (1992) *X-PLOR Manual*, version 3., 1st Ed., Yale University Press, Cambridge, MA