Thermodynamic Studies of SHC Phosphotyrosine Interaction Domain Recognition of the NPXpY Motif*

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The N-terminal 200 amino acids of SHC constitute a unique phosphotyrosine (Tyr(P)) interaction (PI) domain that shows no significant sequence similarity to the other Tyr(P)-recognizing module, the SH2 domain. We describe the thermodynamic parameters characterizing PI domain binding to various tyrosyl phosphopeptides, using isothermal titration calorimetry. The PI domain forms 1:1 complexes of similar affinity with a 12-mer peptide (ISLDNPDpYQQDF) derived from Tyr-1148 of the epidermal growth factor receptor (EGFR) (KD = 28 nm) and an 18-mer (LQGHIIENPQpYFSDACVH) derived from Tyr-490 of Trk (KD = 42 nm). Binding of the EGFR-derived peptide was largely enthalpy-driven at 25 °C, while Trk490 peptide binding was entropy-driven. Based on the change in heat capacity upon binding, approximately 700 Å² of nonpolar surface was estimated to be buried upon interaction. Alteration of the Asn or Pro to Ala in the NPXpY motif of the EGFR Tyr-1148 peptide increased the KD of PI domain interactions to 238 and 370 nm, respectively. Alteration of a Leu at position −5 (with respect to Tyr(P)) in the EGFR peptide to Gly also reduced the binding affinity (KD = 580 nm). It is proposed that the PI domain recognizes the β1 turn that is found in NPXpY-containing peptides and also interacts with a larger segment of the peptide than seen for SH2 domains.

SHC, an adapter protein involved in a number of signaling pathways, consists of an N-terminal region and a C-terminal SH2 domain separated by a region rich in glycine and proline residues. The glycine and proline rich region exhibits sequence similarity to α1 collagen and contains a tyrosine phosphorylation site. SHC exists in three different forms of 46, 52, and 66 kDa, which differ from each other at the N-terminal region. The 46- and 56-kDa forms are generated either by alternative splicing or translational initiation, while the origin of the 66-kDa form is not fully understood (1). Each form of SHC binds to growth factor receptors upon stimulation of cells with various growth factors and rapidly becomes phosphorylated on tyrosine (2–4). SHC is also phosphorylated on tyrosine in cells transformed by oncogenic kinases such as v-src, v-fps, and Bcr-Abl (5, 6). One phosphorylation site, that at tyrosine 317 of SHC, forms a Grb2 binding site (pYVNV) (5, 7). Grb2 is an adapter protein composed entirely of SH2 and SH3 domains (8). Its SH2 domain interacts with SHC, thus recruiting the guanine nucleotide exchange factor, Sos, which is bound to the Grb2 SH3 domains (9–13). Binding of Grb2 to activated growth factor receptors via SHC recruits Sos to the membrane surface, where it exchanges GDP for GTP on Ras, leading to its activation. Activated Ras initiates a kinase cascade that relays the signal from the cell surface to the nucleus (14). Phosphorylation of SHC is also observed upon stimulation of cell-surface receptors for interleukin 2 (15), interleukin 3, granulocyte-macrophage colony-stimulating factor, and Steel factor (16, 17), as well as the T cell receptor (18).

The SH2 domain of SHC binds to proteins that contain phosphotyrosine in the sequence context pYXpY (19, 20). However, SHC also binds to several signaling targets that lack this recognition sequence. This binding has been shown to be mediated by the N-terminal region of SHC (21–24). The N-terminal 200 amino acids of SHC constitute a unique phosphotyrosine interaction domain (PI domain), also termed the phosphotyrosine binding domain (PTB domain; Ref. 25). The PI domain shows no significant sequence similarity with the SH2 domain. It is postulated to recognize phosphotyrosine in the sequence context NPXpY. Data base searches have identified a number of proteins with regions that show significant sequence similarities with the SHC PI domain (26). The function of these conserved regions is not yet understood.

In an effort to understand the interaction of phosphopeptides with the PI domain, we have overexpressed and purified the SHC PI domain and studied its binding to various peptides using isothermal titration calorimetry (ITC). The interaction of the PI domain with NPXpY-containing phosphopeptides is one of the highest affinity interactions thus far observed in the growth factor induced intracellular signaling process and shows an exquisite dependence on the sequence of the peptides studied.

MATERIALS AND METHODS

Glutathione S-Transferase Fusion Proteins—Fragments of mouse SHC from amino acids 1–209 or 46–209 were generated by polymerase chain reaction and were subcloned in the pGEX-2T expression vector (Pharmacia Biotech Inc.). Escherichia coli PR745 (New England Biolabs) were transformed with the resulting constructs, and expression of the desired fusion proteins was induced using 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were disrupted by sonication, and glutathione S-transferase fusion proteins were purified by affinity chromatography using glutathione-agarose. Proteins were eluted using 25 mM glutathione in 0.1 M potassium phosphate buffer (pH 7.6). The SHC fragment corresponding to residues 46–209 was not easily recoverable from glutathione beads and was precipitated during thrombin cleavage. Purification of the SHC PI Domain—the fusion protein was cleaved

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from 0 to 1 M NaCl. The PI domain eluted at around 0.25 M NaCl. The finally separated on a Mono-S column and eluted with a salt gradient fitting. In all experiments the dilution was subtracted from the raw titration data prior to curve fitting. In all experiments the dilution for the individual reactions was determined by titration of peptide into the buffer and buffer into protein. The heat of dilution of dithiothreitol (pH 7.5). Titration curves were fit by using the ORIGIN program supplied by the manufacturer. For all experiments, the heat of the dilution for the individual reactions was determined by titration of peptide into the buffer and buffer into protein. The heat of dilution of buffer into PI domain was found to be negligible. The mean of the heats of dilution was subtracted from the raw titration data prior to curve fitting. In all experiments the value \( c = K_B \times \left[ \text{PI domain} \right] \) was in the range of 10–1000.

Isothermal Titration Calorimetry—ITC measurements were performed (27) with an Omega instrument (Microcal, Northampton, MA). All experiments were performed in 0.1 M Hepes, 0.1 M NaCl, 1 mM dithiothreitol (pH 7.5). Titration curves were fit by using the ORIGIN program supplied by the manufacturer. For all experiments, the heat of the dilution for the individual reactions was determined by titration of peptide into the buffer and buffer into protein. The heat of dilution of buffer into PI domain was found to be negligible. The mean of the heats of dilution was subtracted from the raw titration data prior to curve fitting. In all experiments the value \( c = K_B \times \left[ \text{PI domain} \right] \) was in the range of 10–1000.

Isothermal titration calorimetry involves the direct measurement of the heat of a reaction, and permits an accurate determination of the enthalpy (\( \Delta H \)) of the reaction. The stoichiometry of the interaction can also be determined with confidence. By fitting the obtained binding data, the binding constant \( K_B \) can be determined, from which the free energy (\( \Delta G \)) and entropy change (\( \Delta S \)) upon ligand binding can be calculated using the relationship shown by Equation 1.

\[
-RT \ln K_B = \Delta G^o = \Delta H - T \Delta S^o \quad \text{(Eq. 1)}
\]

\( R \) is the gas constant, and \( T \) is the absolute temperature. These parameters permit more detailed characterization of nature of the binding reaction than is possible from the measurement of binding constants alone (27, 28).

Peptide Synthesis—An Fmoc-based strategy in conjunction with standard side chain protecting groups was applied for peptide synthesis (29). Fmoc-L-tyrosine(PO$_3$H$_2$)-OH was used for incorporation of phosphotyrosine. Peptides were purified by ether precipitation and preparative reverse phase HPLC. Analysis of the purified products by mass spectroscopy and analytical HPLC demonstrated homogeneity and sequence accuracy of synthesized peptides.

RESULTS

The PI domain of SHC was expressed as a glutathione S-transferase fusion protein that was subsequently cleaved with thrombin and purified using a series of chromatographic steps. The protein was more than 95% pure as determined by SDS-polyacrylamide gel electrophoresis (data not shown). The SHC PI domain employed for these studies contains amino acids 1–209 of SHC, with 5 additional amino acids at both the N and C termini.

Identification of the PI Domain Binding Site—Phosphatase protection experiments have shown that the PI domain of SHC protects from dephosphorylation a region of the activated EGF receptor encompassing tyrosine 1148 (29). Phosphopeptides corresponding to this region of EGFR were therefore synthesized, and their ability to compete for PI domain binding to EGFR was analyzed. These studies indicated that a 12-mer phosphopeptide (ISLDNPDPYQQDF) could compete for EGFR binding, while an 8-mer (LDNPDpYQQ) or a 10-mer (SLDNP-DpYQQD) from the same region was ineffective (29). The 12-mer peptide was therefore chosen for all subsequent experiments.

Phosphopeptide Binding to the PI Domain—ITC was used to measure the thermodynamic parameters of PI domain-peptide interactions. A typical binding isotherm using the 12-mer EGFRI1148 peptide is shown in Fig. 1A. Binding of this peptide is exothermic at 25 °C (\( \Delta H = -5.46 \text{ kcal mol}^{-1} \)). From curve fitting of such binding isotherms, it was shown that the stoichiometry of phosphopeptide binding to the PI domain was 1:1 (Fig. 1A). The average binding constant \( (K_B) \) was \( 3.56 \times 10^7 \text{ M}^{-1} \), corresponding to a dissociation constant \( (K_D) \) of 28 nM \( (K_D = 1/K_B) \). The PI domain-phosphopeptide interaction is therefore of higher affinity than found in similar studies of SH2 domain binding to phosphopeptides, where reported \( K_D \) values

![Figure 1](image-url)
High Affinity Binding of PI Domain to NPXpY Peptides

**TABLE I**

| Peptide | Sequence | Concentration of PI domain (μM) | ∆Hobs (kcal mol⁻¹) | Kd (nM) | Kd0 (nM) | ∆TAS (kcal mol⁻¹) | ∆G (kcal mol⁻¹) |
|---------|----------|----------------------------------|-------------------|---------|----------|------------------|----------------|
| 1148    | ISLDNPDPyQYQDF | 13.5 | -5.36 ± 0.03 | 5.73 ± 0.91 x 10⁷ | 5.48 ± 0.05 | 2.54 ± 0.98 x 10⁷ | -5.48 ± 0.05 | 2.40 ± 0.42 x 10⁷ |
| Trk490  | LQGHIENPQyFSDACVH | 13.5 | -5.46 ± 0.15 | 3.56 ± 1.54 x 10⁷ | 2.30 ± 0.08 | 4.33 ± 3.20 x 10⁷ | -2.40 ± 0.06 | 1.63 ± 0.64 x 10⁷ |
| Pro-1148→Ala | ISLDNADpYQYQDF | 30 | -8.56 ± 0.14 | 2.57 ± 0.40 x 10⁷ | -8.21 ± 0.11 | 2.84 ± 0.39 x 10⁷ | -8.39 ± 0.18 | 2.71 ± 0.14 x 10⁷ |
| Asn-1148→Ala | ISLDAPDPyQYQDF | 20 | -2.06 ± 0.12 | 4.14 ± 1.20 x 10⁷ | -1.63 ± 0.04 | 9.24 ± 3.50 x 10⁷ | -2.03 ± 0.15 | 4.20 ± 3.71 x 10⁷ |
| Leu-1148→Gly | ISGDNPDpYQYQDF | 30 | -6.40 ± 0.08 | 1.76 ± 0.18 x 10⁷ | -6.28 ± 0.07 | 1.68 ± 0.20 x 10⁷ | -6.34 ± 0.06 | 1.72 ± 0.04 x 10⁷ |

*Average value.*

The thermodynamic effects of this mutation in the EGFR1148 peptide binding to the PI domain involves the nonpolar surface area buried upon binding (∆A_{np}) (32, 33). ∆A_{np} for binding of the peptides to PI domain can be estimated following this analysis (33).

\[
\Delta A_{np} = -3.6 \pm 0.6 \Delta C_{p}^o
\]

The ∆A_{np} estimated for binding of the EGFR1148 peptide to the PI domain is 560–780 Å², while that estimated for Trk490 peptide binding is 620–860 Å². It should be noted that the correlation between nonpolar surface area buried and the measured ∆C_{p}^o has been called into question in other studies (33). However, for comparing the binding of different peptides to the same protein, this analysis appears to be worthwhile. Binding of both peptides involves the burial of an approximately equal nonpolar surface area of 700 Å². The ratio of buried polar surface area (∆A_p) to nonpolar surface area can be approximated as 0.59 (32), indicating that the total surface area buried (∆A_p + ∆A_{np}) upon binding of the EGFR1148 and Trk490 peptides to the SHC PI domain is 1100–1200 Å².

Specificity of Peptide-PI Domain Interactions—To investigate the sequence dependence of interaction of the EGFR1148 phosphopeptide with the PI domain, various altered peptides were synthesized and used for calorimetric studies. The results are summarized in Table I. It should be pointed out that the observed thermodynamic parameters contain information both about the association itself and the conformational changes upon complexation.

Alteration of the NPXpY motif to NAXpY decreases ∆H of binding from -5.46 ± 0.15 kcal mol⁻¹ to -8.39 ± 0.18 kcal mol⁻¹ (Table I). This reflects an increase in the number of bonds formed and/or a reduction in those broken upon association of this mutated peptide with the PI domain. The reduction in the value of ∆T (1.3 cal mol⁻¹) suggests that the entropy of the unbound state (free peptide) is increased by this mutation and/or that the entropy of the complex is decreased. The affinity itself is reduced by the Pro→Ala change (K_d is increased from 28 to 370 nM). The observed data can be rationally explained in the light of structural studies that indicate that the NPXY motif forms a type I β-turn (35). These structural studies also showed that a Pro→Ala mutation disrupts the β turn (35). The thermodynamic effects of this mutation in the EGFR1148

are in the range of 200 nM to 3 μM (28, 30); more than 10-fold weaker than the interactions studied here. ∆G for EGFR1148 peptide binding to the SHC PI domain is -10.3 ± 0.27 kcal mol⁻¹, and ∆TAS is 4.84 ± 0.42 kcal mol⁻¹ at 25 °C (Table I). The relative enthalpic and entropic contributions to the binding energy are rather different from those observed in SH2 domain-phosphopeptide interactions (30), suggesting a different mode of binding in the two cases.

The SHC PI domain also binds to the nerve growth factor receptor (TrkA), and for the site of binding interaction to be phosphorylated tyrosine 490 (31). The Trk490 peptide that we have synthesized contains 18 amino acids (LQGHIENPQyFSDACVH) flanking this tyrosine. ITC studies of PI domain binding by this peptide show that it is endothermic at temperatures in order to calculate the change in heat capacity (∆C_p) upon PI domain binding to the NPXpY-containing phosphopeptides. A plot of ∆H versus temperature for binding of the EGFR1148 peptide to the PI domain was linear up to a temperature of 25 °C, which indicates that the enthalpy of binding (∆H) is constant over the temperature range studied. The slope of the line below 25 °C gives an estimate of the change in heat capacity (∆C_p), which was -185 cal mol⁻¹ K⁻¹ and -207 cal mol⁻¹ K⁻¹ for the EGFR1148 and Trk490 peptides, respectively (Fig. 2A and B). These large negative ∆C_p values are indicative of specific recognition events.

ΔC_p values for a binding event have been correlated with the nonpolar surface area buried upon binding (∆A_{np}) (32, 33). ∆A_{np} for binding of the peptides to PI domain can be estimated following this analysis (33).

\[
\Delta A_{np} = -3.6 \pm 0.6 \Delta C_{p}^o
\]
peptide are precisely those that would be anticipated if the structure of the free peptide were disrupted by the mutation, becoming more ordered upon binding to the PI domain.

Mutation of NPxPy to APxPy also reduced the binding affinity (Table I) and ΔH changed from $-5.46 \pm 0.15$ kcal mol$^{-1}$ in the wild type case to $-2.03 \pm 0.15$ kcal mol$^{-1}$ in the Asn-1148 → Ala peptide. Since $\Delta S^\circ$ is increased by this mutation (Table I), the Asn → Ala mutation is unlikely to lead to significant disordering of the free peptide, unless the bound conformation is a greatly less structured than that of wild-type. We therefore suggest that the Asn side chain is directly involved in stabilizing the peptide-PI domain complex and that the interactions responsible for this are lost in the Asn-1148 → Ala peptide.

Change of the Leu at 5 to Gly also significantly weakened binding of the peptide to the PI domain ($K_D = 580$ nM). ΔH for binding of the Leu-1148 → Gly peptide to the PI domain is very similar to that for the wild type peptide. However, the entropic advantage of wild type peptide binding ($\Delta S^\circ = 16.2$ kcal mol$^{-1}$ K$^{-1}$) is significantly reduced in the case of Leu-1148 → Gly ($\Delta S^\circ = 7.3$ kcal mol$^{-1}$ K$^{-1}$). This would be true if the number of hydrophobic residues buried upon binding of the peptide to the PI domain is reduced. We therefore suggest that this Leu is involved in hydrophobic interactions with the PI domain and that these are removed by the mutation.

**DISCUSSION**

From studies reported here as well as from others (21–24, 29, 31), it is now clear that SHC contains two phosphotyrosine recognition regions: the PI domain (also called the PTB domain; Ref. 22) at the N terminus and the SH2 domain at the C terminus. The intervening segment between these two domains contains the unique tyrosine phosphorylation site that functions as a binding site for the Grb2 SH2 domain. The SH2 domain of SHC protects phosphotyrosine 1173 of the EGF receptor from dephosphorylation, and the EGFR1173 phosphopeptide was found to compete for SH2 binding to the EGF receptor (20). Failure of this peptide to bind to the SHC PI domain indicates that the binding specificity of the two domains is different.

The isolated PI domain of SHC protects phosphotyrosine 1148 of the EGF receptor from dephosphorylation (29). Comparison of the sequence of this site (QISLDNPdPYQQDF) with other receptor kinases that interact with SHC in vivo indicates a common motif of NPxPy (36). Previous studies have shown that the SH2 domain of SHC does not bind to this sequence (20). The EGF receptor contains two phosphorylation sites with NPxPy sequences, at Tyr-1086 and Tyr-1148 (37). We have recently shown that the SHC PI domain protects only Tyr(P)-1148 from dephosphorylation, and not Tyr(P)-1086 (29). Indeed, the NPxPy sequence alone is not sufficient to form a high affinity binding site for the PI domain. Additional residues are clearly required, since only the 12-mer EGFR1148 peptide (IS-LDNPDpYQQDF), and not shorter peptides, could block binding of the PI domain to the EGF receptor. Another phosphopeptide (DNPdPYQQDFFPKEAK), which still contains the NPxPy sequence but lacks 3 amino acids at the N terminus, also failed to bind to the PI domain (29). These experiments suggest that the SHC PI domain recognizes a larger segment of phosphotyrosine-containing peptides than do SH2 domains. Furthermore, amino acids N-terminal to the phosphotyrosine are critical for binding to the PI domain, while SH2 domains recognize amino acids C-terminal to the phosphotyrosine for high affinity binding (19). Crystal structures of various SH2 domains with bound peptides have shown that the peptides are bound in extended conformations (38–40).

What are the characteristics of the NPxPy motif that are recognized by the SHC PI domain? Structural studies of a nine amino acid peptide that constitutes an internalization sequence in the LDL receptor and include an NPxPy motif (CNPVYQKT)T was shown to form a reverse turn in solution (35). The side chains of the Asn and Tyr were found to be close, with a hydrogen bond between the NH of the Asn and the backbone CO of the Tyr. Indeed, Asn is a strong turn promoter, and being followed by Pro is anticipated to form a β1 turn (41).
High Affinity Binding of PI Domain to NP\(\text{X}\text{P}Y\) Peptides

It can therefore be postulated that the NP\(\text{X}\text{P}Y\)-containing peptides employed in this study assume similar turn structures. Phosphorylation of the Tyr could alter the structure or stability of this turn, and the altered form might be that recognized by the PI domain. Alternatively, the phosphate and turn structure may be recognized simultaneously. The inability of the the 8-mer and 10-mer peptides to bind to the PI domain could result in their inability to adopt the turn structure recognized by the PI domain.

Analysis based on changes in heat capacity upon peptide binding to PI domain suggests that around 1100–1200 Å\(^2\) of total surface is buried in the interaction. These values are larger than the approximately 600 Å\(^2\) of surface area shown to be occluded in the crystal structure of the Src SH2 domain bound to a cognate phosphopeptide (38). However, our value for the buried surface area is only very approximate. Correlations of surface area and \(\Delta C_p\) are not always reliable (34). Indeed, measurement of \(\Delta C_p\) for binding of the Fyn SH2 domain to the same peptide bound to the Src SH2 domain in the crystallographic studies suggested a surface area of around 1600 Å\(^2\).2 Nonetheless, all of these values are in the range observed for buried surface areas in tight complexes (42).

Alterations in the peptide sequence and analysis of their effect on binding to the PI domain shed some light on the specificity of the interaction. Changing Pro in NP\(\text{X}\text{P}Y\) to Ala reduced the binding affinity (\(K_D = 370 \text{ nm} \)) (39). Structural studies of mutated NP\(\text{X}\text{P}Y\) peptides from the LDL receptor indicate that the Pro → Ala mutation destroys the \(\beta\) turn (35). The effects of this mutation on the thermodynamics of EGFR1148 peptide binding to the PI domain are consistent with disruption of such a turn structure and the requirement for its reformation upon binding.

Mutation of Asn in NP\(\text{X}\text{P}Y\) to Ala also reduced the binding affinity, and we suggest that this is mainly the result of a loss of interactions involving the Asn side chain. Structural studies in the LDL receptor peptide indicate that the \(\beta\) turn is destroyed on changing Asn to Ala (35), which would not be consistent with our interpretations. This inconsistency may suggest that the structure adopted by the EGFR1148 peptide is different from that seen in the LDL receptor and is not greatly disrupted by this mutation. Indeed, there are several examples where structures of a similar nature are adopted by sequences that do not have Asn at this position (43, 44). Mutation to Gly of the Leu at the –5 position with respect to Tyr(P) reduces the affinity of binding. We suggest that this results either from structural alterations or from the loss of hydrophilic interaction (the Leu side chain) with the PI domain. This, together with the analysis of binding by peptides of different lengths, indicates that sequences N-terminal to Tyr(P) are critical for phosphopeptide binding to the PI domain.

We conclude by suggesting that the PI domain binds to peptides that contain turn structures, while SH2 domains have been shown to recognize peptides in extended peptide conformations. The precise nature of the interactions awaits the analysis of the three dimensional structure of the PI domain and the PI domain-phosphopeptide complex. It is interesting that none of the mutations analyzed here reduced the affinity by more than 20-fold. Since several of the mutations would be expected to have a profound effect upon the formation of any defined structure in the peptide, these results suggest that the PI domain simultaneously recognizes several characteristics of the peptide. SHC binding to the EGFR receptor in vivo may be mediated through the cooperative interaction of both the PI and SH2 domains with their respective binding sites. In recept-

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