Thermodynamic Studies of Tyrosyl-Phosphopeptide Binding to the SH2 Domain of p56^{lck} †

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ABSTRACT: The interaction between SH2 domains and tyrosine-phosphorylated proteins is essential in several cytosolic signal transduction pathways. Here we report thermodynamic studies of the interaction of the p56^{lck} (*lck*) SH2 domain with several phosphopeptides, using the technique of isothermal titration calorimetry (ITC). This is the first report of the use of ITC to study SH2 domain binding reactions. The free energy of binding of the SH2 domain of *lck* to a phosphopeptide corresponding to the autoregulatory C-terminus of the protein (pY505) was found to be similar to that measured for a phosphopeptide modeled on the C-terminus of the epidermal growth-factor receptor ($\Delta G° \approx -7.0$ kcal mol^{-1} at pH 6.8), although significant differences in the enthalpy and entropy were observed. Binding of a phosphopeptide modeled on the C-terminus of p185^{neu} was weaker ($\Delta G° \approx -5.4$ kcal mol^{-1} at pH 6.8). Lowering the pH to 5.5 reduced the binding affinity of pY505 by approximately 1 order of magnitude. We ascribe this to the protonation of a histidine side chain in the SH2 domain (H180), which is involved in a hydrogen-bonding network that optimizes the binding site geometry. No difference in affinity was observed between portions of *lck* corresponding to the SH3–SH2 (residues 63–228) and SH2 (residues 123–228) domains for the pY505 peptide. We also studied the effect upon pY505 peptide binding of mutations at two highly conserved arginine residues in the *lck* SH2 domain (R134 and R154). While mutation of R154 (in the FLI/VRES region) to lysine led to significant destabilization of the protein, mutation of R134 (seen in crystal structures to form hydrogen bonds with the phosphotyrosine residue) to lysine had little effect upon the pY505 phosphopeptide binding affinity.

src homology (SH2)[1] domains are regions of about 100 amino acids, first identified in *src* and *fps* (Sadowski et al., 1986), that are found in a large number of different proteins involved in cellular signaling (Koch et al., 1991; Pawson & Gish, 1992; Pawson & Schlessinger, 1993). A number of studies indicate that they are involved in mediating protein–protein interactions in the signaling pathways downstream from cell-surface receptors. SH2 domain-mediated interactions are dependent upon tyrosine phosphorylation of their ligands (Moran et al., 1990), and it has been shown that the isolated SH2 domain of *abl*, for example, will bind directly to phosphotyrosine, coupled to agarose beads (Mayer et al., 1991). In the case of signaling by receptor tyrosine kinases, such as EGFR, receptor autophosphorylation creates binding sites for molecules downstream in the signaling pathway that bind via their SH2 domains [e.g., Margolis et al. (1990)]. The pathways in which such SH2-mediated interactions are involved have recently been reviewed by Pawson and Schlessinger (1993).

In the case of *src*-family tyrosine kinases, the function of the SH2 domain is less clear. One role may be in the regulation of kinase activity itself. *src* and *lck* are both phosphorylated at a tyrosine residue close to their carboxy termini (Y527 and Y505, respectively), resulting in suppression of their kinase activities (Cooper et al., 1986; Amrein & Sefton, 1988). Substitution of this tyrosine residue with phenylalanine results in constitutive activation of the kinases (Cartwright et al., 1987; Marth et al., 1988). Point mutations in the SH2 domain of *src* (O'Brien et al., 1990) and deletion of the SH2 domain of *lck* (Veillette et al., 1993; Reynolds et al., 1992) negate the inhibitory effect of phosphorylation of Y527 and Y505, respectively. Roussel et al. (1991) showed that Y527F *src* binds to a phosphotyrosine-containing peptide corresponding to the C-terminus of *src*, in a manner that is dependent upon the presence of the SH2 domain. Normal *src* binds to the peptide less well. These data suggest a model for the regulation of *src* activity in which an intramolecular association of the C-terminal phosphotyrosine with the SH2 domain of *src* results in inhibition of kinase activity (Cooper et al., 1986; Cantley et al., 1991). Regulation of *lck* is thought to occur via the same mechanism, and results recently reported by Peri et al. (1993) support this suggestion. In addition to such a role in the regulation of kinase activity, the SH2 domain of *lck* protects

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[1] Abbreviations: DTT, 1,4-dithio-DL-threitol; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth-factor receptor; GST, glutathione *S*-transferase; hmT, hamster middle-T antigen; IPTG, isopropyl β-D-thiogalactopyranoside; ITC, isothermal titration calorimetry; MOPS, 3-(4-morpholino)propanesulfonate; NMR, nuclear magentic resonance; p185^{neu} 185-kDa product of the *neu* oncogene; p85, 85-kDa subunit of phosphatidylinositol 3′-kinase; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PTP1C, protein tyrosine phosphatase 1C; pY, *O*-phospho-L-tyrosine; SDS–PAGE sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH, *src* homology; SPR, surface plasmon resonance; TFMSA, trifluoromethanesulfonic acid.

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phosphorylated Y505 from phosphatase-mediated dephosphorylation (Gervais et al., 1993), and may also be important in directing interactions between activated *lck* and its substrates (Peri et al., 1993). Indeed, although deletion of the SH2 domain results in activation of wild-type *lck*, its presence is required for oncogenic transformation by a constitutively active form of the kinase (Reynolds et al., 1992; Veillette et al., 1992). The details of the interactions that it might direct have not yet been established.

A number of recent studies have shown that SH2 domains bind to phosphotyrosine residues in their substrates in a specific manner. Binding studies of phosphopeptides to the SH2 domains of *lck* and *src* (Payne et al., 1993), and of p85 (Piccione et al., 1993; Felder et al., 1993; Panayotou et al., 1993), have recently been reported. It appears from these studies that binding of phosphotyrosine-containing peptides to a given SH2 domain is dependent upon the sequence context of the phosphotyrosine residue. p85 binds most strongly to phosphopeptides containing the sequence pYM/VXM, while the SH2 domains of *lck* and *src* bind most strongly to a peptide in which the sequence is pYEEI. Specificity for the latter sequence has been shown by using the SH2 domains of *lck* and *src* to select the most tightly-binding peptides from a randomized mixture of phosphopeptides (Songyang et al., 1993). Elongation of phosphopeptides over 12 residues is reported not to increase their binding affinities (Piccione et al., 1993), indicating that only the immediate sequence context of the phosphotyrosine residue is important in defining the specificity.

X-ray crystal structures of the SH2 domains from *src* and *lck* have been solved in complex with a peptide containing the pYEEI motif identified in binding and selection studies [Waksman et al., 1993; Eck et al., 1993; reviewed by Kuriyan and Cowburn (1993)]. These studies provide considerable insight into the recognition of phosphopeptides, showing two "cavities" that exist on the binding surface of the SH2 domain. Into one of these cavities projects the phosphotyrosine residue (pY) of the peptide, which is involved in a number of specific interactions with residues that are well conserved between different SH2 domains. Into the other major cavity projects the side chain of the residue (I) that is three residues toward the C-terminus of the peptide from pY (pY + 3).

In the studies described in this paper, we sought to gain more information concerning the thermodynamics of phosphopeptide recognition by SH2 domains, using the technique of isothermal titration calorimetry. We have studied largely a peptide corresponding to the C-terminus of *lck*, with a phosphotyrosine residue at the position corresponding to Y505 of *lck*. Although this peptide binds with lower affinity to the *lck* SH2 domain than is suggested for the pYEEI peptide, there is reason to believe that the interaction is of physiological relevance. It also appears that this interaction is significantly stronger than that seen between the *src* SH2 domain and a peptide corresponding to the tyrosine-phosphorylated *src* C-terminus (Payne et al., 1993). The binding constants that we have measured are somewhat different from those reported by Payne et al. (1993), using the technique of surface plasmon resonance. In addition to obtaining an estimate of K_B, we also determine the stoichiometry of the interaction, as well as obtain estimates for ΔH and $\Delta S°$ for the binding reaction. We have compared the binding of the pY505 peptide to the isolated SH2 domain of *lck* with that to the SH3–SH2 portion of *lck*, and find that there is little difference.

EXPERIMENTAL PROCEDURES

Bacterial Expression and Purification of the Isolated SH2 Domain and SH3–SH2 Portion of p56^{lck}. Molecular biological manipulations were all performed according to published protocols (Sambrook et al., 1989). Taq polymerase was purchased from Perkin-Elmer/Cetus, and other enzymes were purchased from New England Biolabs. The cDNA encoding p56^{lck} was kindly supplied by Dr. A. Shaw (University of Washington, St. Louis, MO). The polymerase chain reaction (Mullis et al., 1986) was employed to amplify DNA fragments containing residues 63–228, and residues 123–228 of p56^{lck}, corresponding to the SH3–SH2 and SH2 domain portions, respectively, of the protein. PCR primers were designed such than an *Nde*I site and an *Hind*III site were incorporated at the beginning and end of the coding sequence for the *lck* fragment, respectively. A stop codon was introduced after the codon corresponding to residue 228 of *lck*. The resulting fragments were digested with *Nde*I and *Hind*III, and ligated, along with an *Hind*III/*Bam*HI linker into pET11a (Studier et al., 1990) which had been digested with *Nde*I and *Bam*HI. The sequence of the insert in the resulting plasmids (pET*lck*32 and pET*lck*2) was confirmed by dideoxynucleotide sequencing using the Sequenase system (U.S. Biochemicals) (Sanger et al., 1977), from a double-stranded template. pET*lck*32 encodes a protein corresponding to residues 63–228 (MW = 18 900), equivalent to the SH3–SH2 fragment of p56^{lck}. pET*lck*2 encodes a protein corresponding to residues 123–228 (MW = 12 200), equivalent to the isolated SH2 domain of p56^{lck}. In both cases, a methionine residue precedes the first residue encoded in the native p56^{lck} sequence.

Plasmids were transformed into *Escherichia coli* MGT7 (kindly supplied by D. LeMaster), and cultures were grown at 37 °C to an OD(600) of 2.0 in terrific broth (TB) (Sambrook et al., 1989), upon which IPTG was added to 0.8 mM. Growth was continued for a further 3 h at 37 °C, after which cells were harvested by centrifugation at 7000 rpm, at 4 °C, in a Sorvall GS-3 rotor. The cell pellet was resuspended in a 1/20 culture volume of 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 50 mM glucose, 5 mM EDTA, 10 mM DTT, and 1 mM PMSF, and the suspension probe-sonicated on ice with 30-s pulses at maximum power using a Bransonics cell disruptor. A 1-min period was left between pulses. The resulting lysate was centrifuged for 30 min at 14 000 rpm at 4 °C, in a Beckman JA-20 rotor. The supernatant contained the majority of the protein, and was passed through a DEAE–cellulose (Sigma) column preequilibrated with the sonication buffer. The column was washed with two volumes of the same buffer, and all efflux collected.

After passage through DEAE, the protein solution was diluted 2-fold with 50 mM MOPS, pH 6.8, containing 100 mM NaCl and 1 mM DTT, and the resulting solution loaded onto a Merck EMD$^-$ 650S SP cation-exchange column. The chromatogram was developed with a gradient, in 50 mM MOPS, pH 6.8, and 1 mM DTT, over five column volumes, from 100 mM to 1 M NaCl. The protein eluted in both cases at around 500 mM NaCl. Fractions from the ion-exchange step were collected and pooled, and the protein was precipitated by addition of solid $(NH_4)_2SO_4$ (UltraPure) to 75% saturation. The precipitate was collected by centrifugation for 30 min at 4 °C, at 14 000 rpm in a Beckman JA-20 rotor.

For the final step of purification, the $(NH_4)_2SO_4$ precipitate was dissolved in the minimum volume of 25 mM MOPS, pH 6.8, 1 mM DTT, 100 mM NaCl, and 10% glycerol for p56^{lck}-123–228, or 25 mM sodium citrate, pH 5.5, 1 mM DTT, 100 mM NaCl, and 10% glycerol for p56^{lck}63–228. The solution

was filtered through a 0.22-μm cellulose acetate Spin-X filter (Costar), and loaded onto a Bio-Sil SEC 250 600 × 21.5 mm HPLC gel-filtration column (Bio-Rad) preequilibrated in the appropriate buffer. Fractions containing the protein were identified by absorbance at 280 nm, and the identity of the protein was confirmed by SDS–PAGE and quantitative amino acid analysis. Material that was at least 98% pure (as assessed by overloaded silver-stained SDS–PAGE gels) was thus obtained. Approximately 120 mg of pure p56^{lck}123–228 was thus obtained per liter of culture, whereas the yield for p56^{lck}-63–228 was around 20–30 mg per liter of culture.

Preparation of Phosphotyrosine-Containing Peptides. Peptides were synthesied by Jim Elliot at the W.M. Keck Biotechnological Resource Laboratory at Yale University, essentially as described (Bangalore et al., 1993). Standard Merrifield procedures were employed and phosphotyrosine was incorporated as Boc-*O*-(dibenzylphosphono)-L-tyrosine (Peninsula Laboratories). Cleavage was performed with trifluoromethanesulfonic acid (TFMSA), and peptides were purified by reversed-phase HPLC on a C18 column. Mass spectrometry was used to confirm the identity and homogeneity of the peptide. The peptides synthesized were KTAENPEpYLGLDVPV, which corresponds to the phosphorylated C-terminus of p185^{neu}, plus an additional N-terminal lysine residue (for coupling to beads etc.), and KTAENAEpYLRVAPQS, which corresponds to the phosphorylated C-terminus of EGFR (with an additional N-terminal lysine residue). Each of these peptides was synthesized with both phosphotyrosine and unmodified tyrosine, and kindly provided by D. F. Stern (Department of Pathology, Yale University). The *lck* pY505 peptide had the sequence TATEGQpYQPQP. *O*-Phosphotyrosine was purchased from Sigma (St. Louis, MO).

Isothermal Titration Calorimetry. All experiments were performed using the OMEGA instrument from MicroCal Inc., Northampton, MA, as described in detail by Wiseman et al. (1989). In a typical titration (see Figure 1), peptide (5.9 mM) was added over 31 injections (of volume 8 μL) to the purified *lck* SH2 domain (0.51 mM), which was present in the isothermal calorimeter cell at 10 °C. This temperature was chosen in order to ensure that the data obtained included no enthalpic contribution resulting from ligand-induced refolding of the protein. DSC studies (not shown) demonstrated that the SH2 domain was essentially fully folded at this temperature. In every titration the concentrations of reactants were sufficient to result in saturation of all potential binding sites, as evident from the tendency of the heat of reaction to zero. Heats of dilution for both reactants with buffer solution were measured by titration of buffer into the SH2 domain, as well as peptide into protein-free buffer solution. The total measured heats were corrected for these heats of dilution prior to data analysis. Titration curves were fit using the ORIGIN software (MicroCal Inc., Northampton, MA). Concentrations of phosphopeptides were determined in all cases by quantitative amino acid analysis. For the portions of *lck*, quantitative amino acid analysis was employed for the determination of an extinction coefficient at 278 nm. Subsequently, protein concentrations were determined by measurement of OD(278) for each sample. Each protein studied was dialyzed extensively against the buffer used for dissolving the peptide with which it was titrated. Titrations at pH 6.8 were performed in a buffer of 100 mM NaCl, 50 mM MOPS, pH 6.8, and 1 mM DTT. Those at pH 5.5 were performed in 100 mM NaCl, 50 mM sodium citrate, pH 5.5, and 1 mM DTT. The value of the product of the concentration

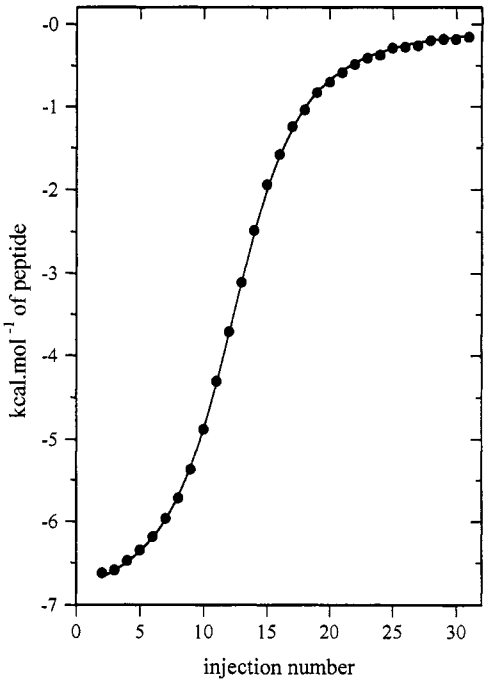

FIGURE 1: An example of the isothermal titration calorimetric data for the reaction of the isolated SH2 domain of p56^{lck} with the pY505 phosphopeptide at 10 °C. The titration consists of 31 8-μL injections of the pY505 peptide (5.86 mM) into the isothermal cell (1.39 mL) containing the SH2 domain (0.51 mM) at pH 5.5. A 5-min period was allowed between injections. The heats of dilution for both pY505 into buffer solution and buffer solution into SH2 domain solution were assessed in separate titrations. The total heat of dilution of each injection for the reaction (15 μcal) has been subtracted from the data shown. The solid line corresponds to the best fit for the variable parameters binding constant, K_B (0.5×10^5 M^{-1}), enthalpy of reaction, ΔH (–6.83 kcal mol^{-1}), and stoichiometry, n, based upon the equations detailed in Wiseman et al. (1989). The product $[\text{sites}]_0K_B$ for this titration was 25.5.

of binding sites ($[\text{sites}]_0$) and the binding constant (K_B) was in the range of 2–37 for all titrations except for those performed with the SH2 domain of *lck* and pY505 peptide at pH 6.8. In this case the product $[\text{sites}]_0K_B$ for the titrations reported in Table 1 was between 130 and 210. The values quoted for K_B in these cases were in good agreement with results obtained at lower values of $[\text{sites}]_0$ in preliminary titrations (not shown).

RESULTS AND DISCUSSION

Isothermal titration calorimetry involves the direct measurement of the heat of a reaction (in this case, ligand binding), and permits an accurate determination of the enthalpy (ΔH) associated with the binding of a ligand to a macromolecule. In addition, by observing the saturation of the potential binding sites by sequential injections (as evidenced by the reduction of the heat of binding to zero), the stoichiometry of the interaction can be determined with confidence. By fitting the binding curve obtained (Wiseman et al., 1989), the binding constant, K_B, can be determined, from which can be calculated the free energy ($\Delta G°$) and entropy change ($\Delta S°$) upon ligand binding, using the relationship

$$-RT \ln K_B = \Delta G° = \Delta H - T\Delta S°$$

where R is the gas constant and T is the absolute temperature. The values of these parameters permit further characterization of the nature of the binding reaction that are not evident from the measurement of binding constants alone (Connelly et al., 1990).

Table 1: Summary of Thermodynamic Parameters Determined for the Binding of Tyrosyl-Phosphopeptides to the SH2 Domain of *lck* at pH 6.8 and 10 °C[a]

[SH2 domain] (mM)	[pY peptide] (mM)	$K_B \times 10^5$ (M^{-1})	ΔH (kcal mol^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta S°$ (cal mol^{-1} K^{-1})
		lck Wild Type/pY505			
0.49	3.15	4.30 ± 1.40	−5.75 ± 0.11	−7.30	5.48
0.54	7.47	2.46 ± 0.47	−5.18 ± 0.07	−6.99	6.40
		3.38	*−5.46*	*−7.16*	*6.00*
		lck Wild Type/EGFR C-Terminus			
0.12	0.83	1.42 ± 0.64	−1.54 ± 0.06	−6.68	18.16
0.12	0.83	3.06 ± 2.87	−1.11 ± 0.31	−7.11	21.20
		2.24	*−1.32*	*−6.93*	*19.81*
		lck Wild Type/*neu* C-Terminus			
0.40	2.94	0.12 ± 0.03	−1.77 ± 0.15	−5.28	12.40
0.12	2.94	0.17 ± 0.07	−2.00 ± 0.21	−5.48	12.29
		0.14	*−1.88*	*−5.38*	*12.34*
		lck R134K/pY505			
0.27	3.09	1.51 ± 0.26	−6.54 ± 0.13	−6.71	0.60

[a] The concentration of SH2 domain (mM) present in the calorimeter cell (1.39 mL) is given for each titration reported, as is the concentration of phosphopeptide present in the syringe. Depending upon the particular experiment, 240–250 μL of peptide solution was injected during the course of a titration. Errors quoted are those for the fit to the binding isotherms in individual titrations (Wiseman et al., 1989). The values for ΔH, K_B, and n (stoichiometry) were all allowed to float in the fitting procedure. The mean value for n was found to be 1 ± 0.12.

Phosphotyrosine Binding to the SH2 Domain of lck. The purified SH2 domain of *lck* was found to bind to phosphotyrosine that was coupled to Affigel 15 agarose beads (Bio-Rad) prepared exactly as described by Mayer et al. (1992) (data not shown). This behavior has also been seen for other isolated SH2 domains, such as that from *abl* (Mayer et al., 1992). Having established the conditions for binding of phosphotyrosine, isothermal titrations were performed. Weak binding (K_B < 1000 M^{-1}), with a small exothermic heat of reaction (ΔH < −1 kcal mol^{-1}), was found to occur with a stoichiometry of approximately 1:1. Low heats of reaction and weak binding limit the accuracy with which thermodynamic parameters can be calculated in this case (Wiseman et al., 1989).

Phosphopeptide Binding to the lck SH2 Domain. Titrations were performed with phosphopeptides corresponding to the most C-terminal autophosphorylation sites of $p185^{neu}$ and EGFR. The sequences of the peptides were KTAENPEpYLGLDVPV (p185) and KTAENAEpYLRVAPQS (EGFR), respectively. These sequences do not associate with the SH2 domain of *lck in vivo*, yet the peptides were found to bind to the SH2 domain *in vitro*. Nonphosphorylated forms of the peptides did not appear to bind, indicating that SH2 binding is dependent upon phosphorylation of the tyrosine residue. The peptides bind to the SH2 domain with much higher affinity than does phosphotyrosine alone, suggesting either that additional interactions involving regions of the peptide surrounding the phosphotyrosine also contribute to the association or that the free N- and C-termini of phosphotyrosine alone interfere with the interaction in that case. X-ray crystal structures of the *src* SH2 domain in complex with two different phosphopeptides that bind with low affinity show that at least the former is true (Waksman et al., 1992). By curve-fitting of the binding isotherms obtained from titration calorimetry, a single (1 ± 0.12) phosphopeptide molecule was found to bind to each SH2 domain binding site in all cases. The average binding constants obtained from this curve-fitting were 0.14×10^5 M^{-1} (p185) and 2.2×10^5 M^{-1} (EGFR) (see Table 1), corresponding to K_D values of 71 and 4.5 μM, respectively. These values are similar to those reported for the binding of other SH2 domains to phosphopeptides derived from molecules to which they do not appear to bind *in vivo* (Panayotou et al., 1993; Felder et al., 1993). It is argued that the difference in affinity between those phosphopeptides that bind to a specific SH2 domain and those that bind through nonspecific interactions is small—perhaps just 1–2 orders of magnitude (Piccione et al., 1993).

We also performed titrations with a phosphopeptide corresponding to the very C-terminus of *lck*, surrounding Y505. The sequence of this peptide was TATEGQpYQPQP. A number of reports suggest that this phosphopeptide binds to the SH2 domain of *lck in vivo* via an intramolecular interaction that regulates the kinase activity of *lck* (Peri et al., 1993). Binding of peptides corresponding to this region of *lck* to its SH2 domain has been reported in several studies (Payne et al., 1993; Liu et al., 1993; Peri et al., 1993). By isothermal titration calorimetry, with a stoichiometry of 1:1 (±0.12), the binding constant (K_B) for the pY505 peptide to the *lck* SH2 domain was 3.4 ± 10^5 M^{-1}, corresponding to a K_D of 2.9 μM. This value is rather different from that implied in the report by Payne et al. (1993). These researchers used a competition-binding assay to determine affinities of phosphopeptides relative to that derived from the hmT antigen (Songyang et al., 1993), and surface plasmon resonance (SPR) to determine K_D for the binding of the hmT peptide. Using these approaches, K_D for binding of the *lck* pY505 peptide to the *lck* SH2 domain is estimated to be 19–34 nM. This binding is a full 2 orders of magnitude stronger than that suggested by our studies. Several possibilities exist that could explain this discrepancy. One could be inherent in the differences between the two techniques employed to determine the binding constants. The technique of surface plasmon resonance involves the immobilization of one of the components to a highly negatively-charged carboxymethylated dextran surface (Johnsson et al., 1991). In the studies of Payne et al. (1993), the phosphopeptide was immobilized. It is possible, given that the SH2 domain of *lck* is quite basic (eluting from an SP column at 500 mM NaCl), that ion-exchange effects could enhance the apparent binding. Payne et al. (1993) point out that the affinities of two different peptides for the *src* SH2 domain differ by about 90-fold when measured with either competition assays employing SPR or bead-binding and ^{125}I-labeled peptide. However, when the K_D for the binding of the same two peptides to this SH2 domain was measured directly using SPR, the difference was found to be just 6-fold. Such difficulties with this technique could provide an explanation for the disagreement with our data. Another difference between the two approaches is that while our studies were performed with the isolated SH2 domain alone, those of Payne et al. (1993) employed glutathione *S*-transferase (GST) fusion

Table 2: Thermodynamic Parameters Determined for the Binding of the *lck* pY505 Phosphopeptide to the SH2 and the SH3–SH2 Portions of *lck* at pH 5.5 and 10 °C[a]

[domain] (mM)	[pY peptide] (mM)	$K_B \times 10^5$ (M^{-1})	ΔH (kcal mol^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta S°$ (cal mol^{-1} K^{-1})
		lck SH2/pY505			
0.51	5.86	0.50 ± 0.10	−6.83 ± 0.01	−6.09	−2.61
		lck SH3–SH2/pY505			
0.14	1.60	0.62 ± 0.03	−6.28 ± 0.19	−6.21	−0.25
0.15	1.76	0.43 ± 0.04	−6.42 ± 0.30	−6.00	−1.48
0.15	2.79	0.36 ± 0.02	−6.02 ± 0.11	−5.90	−0.42
		0.47	*−6.24*	*−6.03*	*−0.72*
		lck SH2 (R134K)/pY505			
0.31	3.23	0.24 ± 0.04	−4.22 ± 0.17	−5.70	5.23

[a] Concentrations are given for the *lck* fragment present in the calorimeter cell and phosphopeptide present in the syringe as described in Table 1. Errors quoted are also as described in Table 1. The mean value for the stoichiometry of the binding reaction was 1 ± 0.12 including all cases in Tables 1 and 2.

proteins. Such GST fusion proteins are dimeric (Panayotou et al., 1993), which could result in enhanced binding of the SH2 domains to immobilized phosphopeptides via avidity effects. Titration calorimetry, by contrast, is performed with both partners in the interaction free in solution, although our experiments did employ concentrations in the range 0.1–0.5 mM for the SH2 domain, and up to 7 mM in phosphopeptide. Clearly, additional investigations are required to understand the basis for this discrepancy, since much of the current data in the literature concerning SH2–phosphopeptide recognition is derived from SPR studies.

Thermodynamics of Phosphopeptide Binding. Table 1 presents the thermodynamic parameters for the binding of each of the peptides studied to the SH2 domain alone at pH 6.8. There is little difference between the binding constants for the pY505 and EGFR phosphopeptides to the *lck* SH2 domain, while the binding of the $p185^{neu}$ phosphopeptide was significantly weaker. Thus, the *lck* pY505 peptide binds no more tightly than does a nonspecific phosphopeptide from EGFR. In considering this, however, it should be taken into account that the reaction studied here was an intermolecular association. The binding that is proposed to be of physiological relevance for the pY505 peptide is intramolecular since pY505 is present at the C-terminus of the same polypeptide chain that contains the SH2 domain. With no knowledge of the structure of whole *lck*, it is impossible to estimate the magnitude of this effect. However, since the effective local concentration of the pY505 peptide would be high in the case of an intramolecular association, a K_B in the 10^5 M range may be sufficient for the regulatory function of this interaction. Since the SH2 domain of *lck* is proposed also to interact with other tyrosine-phosphorylated proteins (Caron et al., 1992; Veillette et al., 1992; Xu & Littman, 1993), competition may be required between these intermolecular interactions and the intramolecular interaction between pY505 and the *lck* SH2 domain. A relatively low affinity for the intramolecular interaction may be required for this event to activate *lck* in stimulated T-cells. It is not clear why the affinity of the $p185^{neu}$ phosphopeptide should be significantly less than that observed for the other two peptides studied.

Despite the similarity in the binding affinities of the pY505 and EGFR phosphopeptides, the enthalpic and entropic contributions are significantly different (Table 1). The larger value for ΔH in the case of pY505 peptide binding could be indicative of peptide/SH2 interactions other than those involving the phosphotyrosine side chain. The value for $\Delta S°$ is also higher in the case of EGFR peptide binding than in the case of the pY505 peptide. There is evidence that specific phosphopeptide binding results in a conformational change in a PI 3′-kinase SH2 domain (Shoelson et al., 1993). It is possible that pY505 peptide binding to the *lck* SH2 domain induces such conformational changes that may lead to a relative ordering of the SH2 domain structure. Circular dichroism studies (not shown) were performed to test this possibility, but no alteration in secondary structure upon pY505 peptide binding could be shown convincingly. However, ^{15}N relaxation NMR spectroscopic studies do appear to show a conformational change in the *lck* SH2 domain upon phosphopeptide binding (J. Lee, personal communication).

Binding of Phosphopeptides to the SH3–SH2 Portion of lck. Just as deletion of the SH2 domain of *lck* results in activation of its kinase activity, as discussed above, so does deletion of the SH3 domain (Veillette et al., 1992; Reynolds et al., 1992), although to a lesser extent. Similar observations have been reported for *src* (Superti-Furga et al., 1993; Seidel-Dugan et al., 1992). These observations indicate that the SH3 domain of *lck*, which is immediately to the N-terminus of its SH2 domain, may influence the binding of the SH2 domain itself to pY505 and other phosphorylated proteins. Such communication between SH3 and SH2 domains would provide one explanation for their commonly being in close proximity. It could also suggest that proteins such as GRB-2 (Lowenstein et al., 1992), which consist entirely of SH2 and SH3 domains, and appear to function as adaptor molecules in signaling pathways, may also have some transduction function. If binding of GRB-2 to the human homologue of *son of sevenless* (Li et al., 1993) modifies its ability to recognize phosphorylated tyrosine residues in EGFR or SHC (Rozakis-Adcock et al., 1992), for example, it is possible that this could serve as a means of introducing more specificity into the pathway.

As a test of the hypothesis that the SH3 domain of *lck* modulates ligand binding to the SH2 domain of *lck*, we studied binding of the pY505 peptide to a protein fragment corresponding to both domains of *lck* (residues 63–228). This protein showed a tendency to precipitate when studied at pH 6.8, so titration calorimetry was performed at pH 5.5 in sodium citrate buffer, containing 100 mM NaCl and 1 mM DTT. To compare the binding of the pY505 peptide to this portion of *lck* with binding to the isolated SH2 domain, titrations with the SH2 domain alone were also performed at pH 5.5. The parameters obtained from the binding studies under these conditions are presented in Table 2. The binding constant measured for pY505 binding was very similar for both the SH3–SH2 and SH2 portions (0.47×10^5 and 0.50×10^5 M^{-1}, respectively). Clearly, in the context of these fragments, under these conditions, there is no influence of the SH3 domain upon the affinity (or other thermodynamic parameters) of the SH2 domain for the pY505 peptide. CD measurements

(not shown) also showed no significant structural changes upon peptide binding.

Effect of pH upon Phosphopeptide Binding by the lck SH2 Domain. A comparison of the results in Tables 1 and 2 shows that the affinity of the *lck* SH2 domain for the pY505 peptide is reduced approximately 7-fold upon reduction of the pH from 6.8 to 5.5. A similar loss of affinity is seen for the R134K mutant (see below). In the case of the wild-type SH2 domain, this change is largely accounted for by a fall in the value of $\Delta S°$, from 6 to −2.6 cal mol^{-1} K^{-1}. The reason for the change in the sign of $\Delta S°$ is unclear.

Over this pH range, the only side chain that is likely to be affected significantly is that of histidine ($pK_a \approx 6.5$), of which there are four in the *lck* SH2 domain. One of these, H180 (equivalent to H201 in *src*), is well conserved among SH2 domains (Koch et al., 1991), and is seen in the X-ray crystal structures of the *src* (Waksman et al., 1992, 1993) and *lck* (Eck et al., 1993) SH2 domains to play a part in the maintenance of the geometry of the phosphotyrosine binding site. Its backbone carbonyl oxygen is involved in a hydrogen bond with the amide nitrogen of residue pY + 1 in the bound phosphopeptide. In addition, its side chain is involved in hydrogen bonds with the side chains of E138 (E159 in *src*) and S166 (S187 in *src*). The side chain of S166 in turn is involved in a hydrogen bond with the Nϵ atom of R154 (R175 in *src*) of the FLI/VRES region, which is critical for interactions with the phosphate group of the phosphopeptide. Protonation of H180 would compromise this hydrogen-bonding network, which is suggested to optimize the geometry of the binding site. It is very likely that this effect is responsible for the loss in affinity that we observed upon reducing the pH at which our titrations were performed. The relatively small loss in affinity could either be due to incomplete protonation of H180 or reflect a modest contribution to binding affinity by the optimization of this region of the binding site. Since ΔH is altered relatively little upon reducing the pH to 5.5, it might be suggested that the reduced binding affinity arises from a negative $\Delta S°$ contribution resulting from an ordering of the active sites upon phosphopeptide binding. That the effect is likely to be small is suggested by the fact that mutation of H201 to leucine in *src* that was phosphorylated on Y527 had little activating effect (O'Brien et al., 1990): the regulatory *src* SH2/pY527 interaction was not abolished in this mutant.

Effects of Mutations of Conserved Arginine Residues upon Phosphopeptide Binding. The X-ray crystal structures of phosphopeptide complexes with the SH2 domains of *lck* (Eck et al., 1993) and *src* (Waksman et al., 1992, 1993) highlight two arginine residues as being of particular importance in the recognition of phosphotyrosine. In *lck*, these are R134 and R154 [αA2 and βB4 in Eck et al. (1993)]. In *src*, they are R155 and R175. Both are highly conserved between different SH2 domains (Koch et al., 1991). *lck* R154 corresponds to that in the FLI/VRES region, and is conserved in all SH2 domains. *lck* R134 is conserved in all but three SH2 domains. In the GAP C-terminal SH2 domain it is replaced by a lysine residue, and in the two SH2 domains of the protein tyrosine phosphatase PTP1C, it is replaced by a glycine residue. We generated two mutations in the SH2 domain of *lck*, in order to study their effects upon binding of the pY505 peptide.

Mutation of R154 to lysine resulted in a protein that was produced at much lower levels in *E. coli* than was the wild-type SH2 domain and, after purification as described in the Experimental Procedures, was found to precipitate in the fractions after gel filtration. No binding studies were performed with this mutant. Since R154 is buried within the protein, and appears to interact with a number of other buried residues, we suspect that the R154K mutation has destabilized the protein significantly.

In the crystal structure of Eck et al. (1993), R134 appears to donate four hydrogen bonds to the phosphotyrosyl-peptide: one to a phosphate oxygen of pY, two to the backbone carbonyl group of residue pY − 1 in the peptide, and an amino–aromatic bond from Nη1 to the ring of the phosphotyrosine side chain. Surprisingly, with a lysine at this position (in R134K), at pH 6.8, the binding constant for pY505 peptide binding is reduced only by about a factor of 2 (see Table 1), indicating that phosphotyrosine recognition can occur in the absence of these interactions. The affinity of this binding is reduced upon lowering the pH to 5.5 to a degree similar to that seen for the wild-type SH2 domain. Interestingly, the reduction in pH affects $\Delta S°$ for binding to the wild-type and mutant SH2 domains in opposite directions.

As pointed out by Waksman et al. (1992), the Nϵ of a lysine residue at a position corresponding to R134 of *lck* (R155 or *src*) could interact with the phosphate of the pY residue. Such an arrangement is likely in the case of the C-terminal GAP SH2 domain. Waksman et al. (1992) also point out that, since an arginine residue replaces H180 (H201 in *src*) in the case of GAP, this could interact with the ring of the pY residue. Our studies indicate that this is not necessary, and suggest that, at least in the case of recognition of the pY505 peptide, the interaction with the phosphate group is thermodynamically most significant. Mutation of R134 to glycine (as seen in the SH2 domains of PTP1C) would also be of interest, to determine the effect of this upon the thermodynamics of phosphopeptide recognition. If binding is significantly compromised, other mutations of reasonably conserved residues to those found in equivalent positions in the PTP1C SH2 domains could be made, in an effort to restore binding. Such an approach could provide significant insight into the thermodynamics of phosphopeptide recognition.

CONCLUSIONS

We have used isothermal titration calorimetry to study the binding of the *lck* SH2 domain to a phosphopeptide (pY505) modeled upon the C-terminus of this protein. Phosphorylation of Y505 in *lck* leads to inhibition of its kinase activity through a proposed intramolecular interaction between this phosphotyrosine residue and the SH2 domain, which is close to the N-terminus of the protein.

Although phosphotyrosine itself binds to the SH2 domain, it does so very weakly. Peptides containing phosphotyrosine bind much more tightly to the SH2 domain, indicating that the SH2 domain recognizes portions of the peptide in addition to the phosphotyrosine side chain. The affinity of the pY505 peptide for the *lck* SH2 domain was not found to be significantly higher than that of a peptide with which *lck* does not interact *in vivo* (that corresponding to the phosphorylated C-terminus of EGFR), although substantial differences were observed in the contributions of enthalpy and entropy to the binding. Since the pY505/*lck* SH2 interaction will be intramolecular *in vivo*, local concentration effects may overcome the apparent relatively low affinity measured here.

Lowering the pH from 6.8 to 5.5 was found to compromise phosphopeptide recognition by the *lck* SH2 domain, possibly through titration of a histidine side chain that is involved in optimization of the binding-site geometry. At pH 5.5, the presence of the adjacent SH3 domain had little influence upon phosphopeptide recognition by the SH2 domain.

X-ray crystal structures of two SH2/phosphopeptide complexes (Waksman et al., 1993; Eck et al., 1993) suggest

that two conserved arginine residues (R134 and R154) are key in phosphopeptide recognition. Mutation of R154 (which is buried within the structure) led to destabilization of the protein. However, R134 (which forms several hydrogen bonds to the phosphotyrosine residue) could be mutated to lysine with little effect upon phosphopeptide binding.

This is the first report of the use of isothermal titration calorimetry to investigate SH2/phosphopeptide binding. In addition to affinity constants, the enthalpy and entropy, as well as the stoichiometry of the association, are determined, providing a more detailed picture of the binding reaction.

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