Calorimetric Examination of High-Affinity Src SH2 Domain-Tyrosyl Phosphopeptide Binding: Dissection of the Phosphopeptide Sequence Specificity and Coupling Energetics†

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ABSTRACT: SH2 domains are protein modules which interact with specific tyrosine phosphorylated sequences in target proteins. The SH2 domain of the Src kinase binds with high affinity to a tyrosine phosphorylated peptide containing the amino acids Glu, Glu, and Ile (EEI) at the positions +1, +2, and +3 C-terminal to the phosphotyrosine, respectively. To investigate the degree of selectivity of the Src SH2 domain for each amino acid of the EEI motif, the binding thermodynamics of a panel of substitutions at the +1 (Gln, Asp, Ala, Gly), +2 (Gln, Asp, Ala, Gly), and +3 (Leu, Val, Ala, Gly) positions were examined using titration microcalorimetry. It was revealed that the Src SH2 domain is insensitive ($\Delta\Delta G^\circ \leq 0.6$ kcal/mol) to conservative substitutions at all three peptide positions. However, mutation to Ala resulted in moderate reductions in ΔG°, with the substitution at the +3 position showing the largest loss in affinity ($\Delta\Delta G^\circ =$ 1.4 kcal/mol), followed by the +2 ($\Delta\Delta G^\circ = 1.0$ kcal/mol) and +1 ($\Delta\Delta G^\circ = 0.5$ kcal/mol) positions. This hierarchy of binding was not reflected in the values of the heat capacity change, since only the peptide substituted to Ala at the +3 position showed a ΔC_p° that was reduced in magnitude compared to wild-type. To assess the degree of cooperation upon binding (or coupling) between the amino acids of the EEI sequence, the binding of a series of singly, doubly, and triply Ala substituted phosphopeptides was examined and analyzed using double mutant cycles. It was revealed that the effects of the Ala substitutions on ΔG° were additive. However, nonadditive binding enthalpies were observed between the +1 Glu and +3 Ile, as well as the +2 Glu and +3 Ile, suggesting that communication occurs between residues of the EEI motif upon binding.

Src homology 2 (SH2) domains, originally identified as conserved ~100 amino acid motifs in signaling molecules (*1*), recognize and bind to phosphorylated tyrosine (pTyr) residues in proteins (*2*−*4*). These domains function to propagate various signals from surface receptors throughout the cell. The mutation of proteins involved in signaling through SH2 domains is a frequent cause of cancer (*5*, *6*), and hence, SH2 domains are potential targets of anticancer pharmaceuticals (*7*, *8*).

To maintain the integrity of signal transduction pathways, SH2 domains must interact specifically with their tyrosine phosphorylated targets. Screening of a degenerate phosphopeptide library revealed that SH2 domains of the Src family of kinases bind specifically to the sequence pTyr-Glu-Glu-Ile (pYEEI) (*9*). The structural basis of this selection was established from the crystal structures of the Src and Lck SH2 domains in complex with a peptide containing the sequence PQpYEEIPI (referred to hereafter as the "pYEEI" peptide) (*10*, *11*). In these structures, the pTyr and Ile residues of the phosphopeptide interact with two pockets in the SH2 domain, one positively charged where the pTyr inserts and another hydrophobic into which the side-chain Ile is buried. The two intervening Glu residues in the peptide interact with basic side chains on the surface of the SH2 domain (Figure 1). From these structural studies, it was suggested that the interactions between the +3 Ile of the phosphopeptide and the hydrophobic pocket of the SH2 domain were critical for high affinity binding to the Src family of SH2 domains (*10*). However, biochemical studies of pYEEI phosphopeptide binding to Src-like SH2 domains have not been entirely consistent with this model (*12*−*14*). For example, examination of the determinants of Src SH2 domain binding using an ELISA-based assay indicated that the +1 Glu, +2 Glu, and +3 Ile residues are each important for high-affinity recognition of the pYEEI peptide (*12*). Furthermore, studies of the binding of the related Lck SH2 domain to various Gly substituted pYEEI peptides showed that substitution of the +1 Glu resulted in the largest loss in binding affinity (*13*, *14*). Hence, it appears that the importance of each residue in the EEI motif for high-affinity SH2 domain binding remains to be clarified.

An unexplored aspect of SH2 domain recognition is whether cooperative interactions occur between residues in the protein−peptide interface, and whether these interactions contribute to peptide-binding specificity. Cooperative interactions between sites in proteins are typically evaluated using

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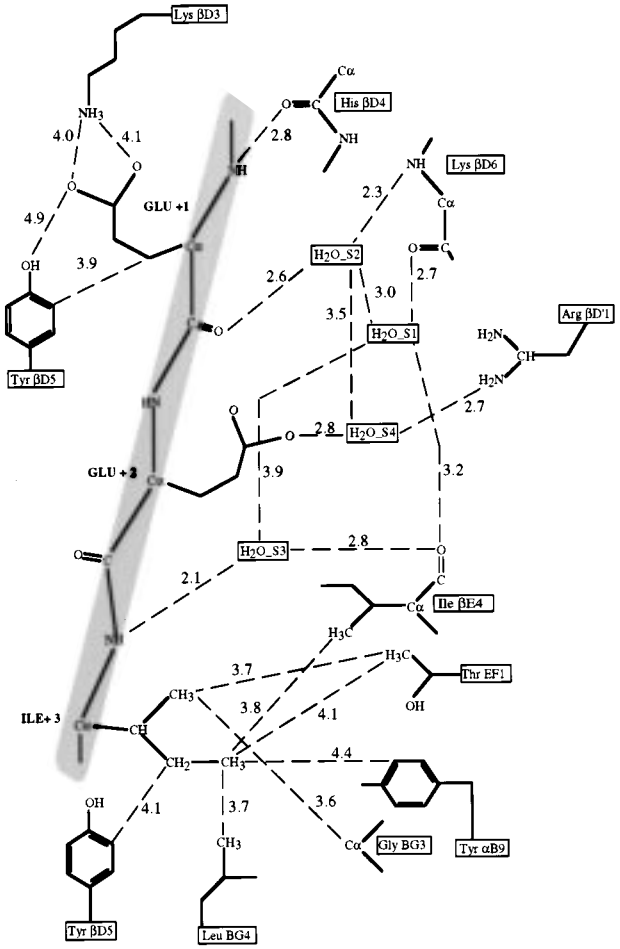

FIGURE 1: Schematic diagram of the Src SH2 domain-pYEEI phosphopeptide binding interface. Dotted lines indicate the contact distances (Å) between the +1 Glu, +2 Glu, and +3 Ile and the SH2 domain, as derived from the Src SH2 domain-pYEEI peptide crystal structure (*10*). The peptide backbone is indicated by a stippled bar. See ref 10 for the interactions between the pTyr and the SH2 domain, which are not shown. The notation used to label the Src SH2 domain residues is from (*11*).

double mutant cycles. This involves first evaluating the binding of two protein variants each mutated at a single location; the thermodynamic cycle is then closed by evaluating the binding of a mutant substituted at both locations (*15*, *16*). Using this approach, the magnitude of cooperativity is determined by the degree of energetic coupling (nonadditivity) between mutants. Recent examples of nonadditivity between residues have suggested that cooperative interactions between amino acids may be important in determining the specificity of some macromolecular interactions (*17*−*20*).

In this study, isothermal titration calorimetry (ITC) has been used both to examine the specificity of the Src SH2 domain−pYEEI phosphopeptide interaction as well as to probe the energetic coupling between the +1 Glu, +2 Glu, and +3 Ile phosphopeptide residues. It has been found that the Src SH2 domain is quite permissive of conservative substitutions in the EEI motif at all three peptide positions. However, substitutions to Ala resulted in moderate reductions in $\Delta G°$ at the +2 and especially the +3 peptide positions, but little reduction in binding energy at the +1 position. Furthermore, an assessment of the coupling energetics between the three residues has indicated that, although the coupling free energy between peptide residues is small, the coupling enthalpy is significantly nonzero for several interactions, indicating that the residues of the peptide communicate with one another in the binding process.

MATERIALS AND METHODS

Protein and Peptide Preparation. The Src SH2 domain was expressed and purified as previously described (*21*−*23*). Typical yield was 50 mg of protein/L of starting culture. The protein extinction coefficient was previously determined to be 14 700 M^{-1} cm^{-1} (*21*). Tyrosyl phosphopeptides were obtained at 95% purity level from Quality Controlled Biochemicals (Hopkinton, MA). The sequence of the standard pYEEI octapeptide was Ac−PQpYEEIPI−NH2. All other peptides employed were also octapeptides that contained only the changes listed in the text. The pYEEI peptide extinction coefficient at 268 nm and at pH 7.5 was previously determined to be 695 M^{-1} cm^{-1} (*22*), and since the only absorbing group in the peptide is the phosphotyrosine, this value of the extinction coefficient was used to quantify the concentration of all peptides studied.

Isothermal Titration Calorimetry. Titration calorimetry experiments were performed with an Omega titration calorimeter obtained from Microcal, Inc. (Northhampton, MA) (*24*), as previously described (*21*, *22*). The employed protein and peptide concentrations were between 30 and 120 μM and 0.4−2.0 mM, respectively, for all experiments. The "*c*" value, defined to be the product of the protein concentration and the association constant (*24*), was between 5 and 200 for all experiments. The buffer for all experiments described here was 20 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol. The standard experimental temperature was 25 °C. The protein and peptide were typically dialyzed for at least 48 h in the experimental buffer prior to experiments. The raw calorimetry data were collected and analyzed using the Origin software (*21*, *22*, *24*). Reported thermodynamic parameters are the mean value of at least two independent experiments, with the uncertainty being the standard deviation of multiple experiments. Uncertainties in $\Delta C_p°$ represent the 95% support plane confidence interval for the linear best fit of the $\Delta H°$ versus temperature data. It has been previously demonstrated that no exchange of protons occurs upon pYEEI phosphopeptide binding to the Src SH2 domain at pH 7.5 (*22*); hence, the experimentally measured $\Delta H°$ for the pYEEI peptide reported here represents the intrinsic $\Delta H°$ of binding. Furthermore, since it is probable that the only groups in the protein or peptide which ionize near pH 7.5 are located in the pTyr binding pocket of the SH2 domain (a region not perturbed in this study), the $\Delta H°$ of binding of the pYEEI substituted peptides will likely also not be significantly complicated by a linkage to proton exchange.

RESULTS

It has been previously determined that the Src SH2 domain binds with highest affinity to a tyrosyl phosphopeptide with the amino acids Glu, Glu, and Ile at the residues +1, +2, and +3, respectively, C-terminal to the phosphotyrosine (*9*). This peptide binds the Src SH2 domain more tightly than low-affinity peptide sequences by 2−3 kcal mol^{-1} in free energy (*21*). To examine the degree of selectivity displayed by the Src SH2 domain for the pYEEI consensus sequence,

Table 1: Thermodynamic Binding Parameters of Tyrosyl Phosphopeptides[a]

peptide	K (M^{-1})	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)	$\Delta\Delta G°$ [b] (kcal/mol)	$\Delta\Delta H°$ [b] (kcal/mol)	$T\Delta\Delta S°$ [b] (kcal/mol)
pYEEI	5.5 ($\pm$0.7) $\times$ 10^6	-9.2 ± 0.1	-7.7 ± 0.2	1.5 ± 0.2			
pYQEI	2.1 ($\pm$0.3) $\times$ 10^6	-8.6 ± 0.1	-7.6 ± 0.1	1.0 ± 0.1	0.6	0.1	-0.5
pYDEI	5.3 ($\pm$1.3) $\times$ 10^6	-9.2 ± 0.2	-8.9 ± 0.3	0.3 ± 0.4	0.0	-1.2	-1.2
pYAEI	2.9 ($\pm$0.4) $\times$ 10^6	-8.7 ± 0.1	-7.7 ± 0.2	1.0 ± 0.2	0.5	0.0	-0.5
pYGEI	1.6 ($\pm$0.2) $\times$ 10^5	-7.1 ± 0.1	-4.8 ± 0.1	2.3 ± 0.1	2.1	2.9	0.8
pYEQI	1.9 ($\pm$0.2) $\times$ 10^6	-8.6 ± 0.1	-7.6 ± 0.2	1.0 ± 0.2	0.6	0.1	-0.5
pYEDI	2.4 ($\pm$0.5) $\times$ 10^6	-8.7 ± 0.1	-6.2 ± 0.4	2.5 ± 0.4	0.5	1.5	1.0
pYEAI	9.6 ($\pm$0.8) $\times$ 10^5	-8.2 ± 0.1	-6.3 ± 0.1	1.9 ± 0.1	1.0	1.4	0.4
pYEGI	5.1 ($\pm$0.2) $\times$ 10^5	-7.8 ± 0.1	-6.0 ± 0.1	1.8 ± 0.1	1.4	1.7	0.3
pYEEL	2.3 ($\pm$0.4) $\times$ 10^6	-8.7 ± 0.1	-5.6 ± 0.4	3.1 ± 0.4	0.5	2.1	1.6
pYEEV	2.2 ($\pm$0.3) $\times$ 10^6	-8.7 ± 0.1	-5.4 ± 0.2	3.3 ± 0.2	0.5	2.3	1.8
pYEEA	5.7 ($\pm$2.0) $\times$ 10^5	-7.8 ± 0.2	-5.1 ± 0.4	2.7 ± 0.4	1.4	2.6	1.2
pYEEG	2.6 ($\pm$0.1) $\times$ 10^5	-7.4 ± 0.1	-3.6 ± 0.1	3.8 ± 0.1	1.8	4.1	2.3

[a] Experiments were performed with the octapeptide Ac−PQpYEEIPI−NH2 (designated pYEEI in this table and in the text) and variants thereof in 20 mM Hepes, pH 7.5, and 100 mM NaCl at 25 °C. Uncertainties in K, $\Delta G°$, and $\Delta H°$ represent the standard deviation of multiple experiments; the error in $T\Delta S°$ was calculated by standard error propagation. [b] Calculated in reference to the thermodynamic parameters for the pYEEI peptide.

as well as the energetic signature of that selectivity, the binding of the Src SH2 domain to a panel of tyrosyl phosphopeptides with various substitutions at the +1, +2, and +3 positions was examined using isothermal titration microcalorimetry (ITC). The substitutions were chosen so as to progressively decrease the length of the side chain at each amino acid position. The derived thermodynamic parameters from these experiments are summarized in Table 1 and Figure 2.

Specificity of the +1 Glu in Phosphopeptide Binding. The Src SH2 domain−pYEEI phosphopeptide crystal structure shows that the carboxylic acid group of the +1 Glu makes a long range (4.0 Å) salt bridge with Lys βD3 of the SH2 domain, while its C_β carbon packs against residue Tyr βD5 [Figure 1 (*10*)]. To probe the importance of these interactions, the binding of a series of phosphopeptides with Glu, Gln, Asp, Ala, and Gly at the +1 position was examined. Under the conditions employed (pH 7.5, 100 mM NaCl), the Src SH2 domain binds the consensus pYEEI phosphopeptide with an affinity (K) of 5.5 $\times$ 10^6 M^{-1} (K_d = 182 nM or $\Delta G° = -9.2$ kcal/mol) and an enthalpy ($\Delta H°$) of −7.7 kcal/mol. Replacement of the +1 Glu with Gln causes a modest 2-fold ($\Delta\Delta G° = 0.5$ kcal/mol) loss in affinity and no change in $\Delta H°$. However, placing an Asp at +1 results in no change in affinity and, surprisingly, a 1.2 kcal/mol more favorable $\Delta H°$ of binding. The pYAEI phosphopeptide also showed only a modest reduction in affinity compared to the consensus pYEEI sequence. These results clearly show that the solvent-exposed salt bridge between the +1 Glu and the SH2 domain does not contribute significantly to the binding affinity. However, the phosphopeptide with Gly at the +1 position showed a substantial reduction in binding affinity (Table 1 and Figure 2), an effect which has also been observed for binding of the Lck SH2 domain to the same peptide (*13*, *14*). Since substituting Gly at the +2 and +3 positions of the peptide had a much smaller effect on binding affinity (see below) and since the loss in affinity shown by the pYGEI peptide was largely due to a less favorable $\Delta H°$, the reduced affinity of the pYGEI peptide cannot be attributed solely to an increased flexibility of the unbound state of the peptide. Hence, it appears that the interaction between the C_β carbon of the +1 peptide residue and Tyr βD5 of the SH2 domain is important for high-affinity binding.

Specificity of the +2 Glu in Phosphopeptide Binding. In the crystal structure of the Src SH2 domain−pYEEI phosphopeptide complex, the carboxyl group of the +2 Glu interacts with the guanido group of Arg βD′1 of the SH2 domain through a network of four water molecules [Figure 1 (*10*)]. This interaction was examined by replacing the +2 Glu of the pYEEI phosphopeptide with Gln, Asp, Ala, and Gly. The affinity of the interaction was only slightly reduced with either Gln or Asp at the +2 position ($\Delta\Delta G° \approx 0.5$ kcal/mol), but more reduced with Ala ($\Delta\Delta G° = 1.0$ kcal/mol) and Gly ($\Delta\Delta G° = 1.4$ kcal/mol). The +2 Glu therefore makes a modest contribution toward the high binding affinity of the pYEEI peptide, which can be partially compensated for by the presence of either Gln or Asp. The enthalpies of binding for this panel of substitutions showed that the Gln at +2 maintained the same $\Delta H°$ as the +2 Glu, but Asp, Ala, and Gly residues showed a marked reduction in the magnitude of $\Delta H°$.

Specificity of the +3 Ile in Phosphopeptide Binding. Upon binding, the +3 Ile of the pYEEI peptide makes intimate contacts with a pocket of the Src SH2 domain formed by hydrophobic residues [Figure 1 (*10*)]. To identifiy the stringency with which the Src SH2 domain selects the +3 Ile, the binding of phosphopeptides with Leu, Val, Ala, and Gly at the +3 position was examined. The results obtained were very similar to those previously obtained at pH 6.0, 50 mM NaCl (*21*); Leu and Val showed only a small reduction in affinity ($\Delta\Delta G° = 0.5$ kcal/mol for both), while elimination of the hydrophobic contacts in the pocket by substituting Ala and Gly at the +3 position caused a larger ($\Delta\Delta G°$ = 1.4 kcal/mol for Ala, $\Delta\Delta G° = 1.8$ kcal/mol for Gly) loss in binding free energy. Hence, the Src SH2 domain is only moderately sensitive to the particular hydrophobic residue at the +3 position, although a hydrophobic residue is certainly necessary for high-affinity binding. Interestingly, the $\Delta H°$ of binding for all substituted peptides was significantly less favorable than for the pYEEI peptide.

Heat Capacity Change for Ala-Substituted Peptides. To further probe the pYEEI peptide binding energetics, the heat capacity change ($\Delta C_p°$) upon binding for several Ala substituted peptides was examined. It has previously been shown that at pH 6.0, 50 mM NaCl, the $\Delta C_p°$ of phosphopeptide binding to the Src SH2 domain is modest (~200 cal mol^{-1} deg^{-1}) and similar for high- and low-affinity phosphopeptides (*21*). In this study, the $\Delta C_p°$ of binding (at pH 7.5, 100 mM NaCl) was evaluated for the pYEEI, pYAEI, pYEAI, and pYEEA phosphopeptides by performing ITC

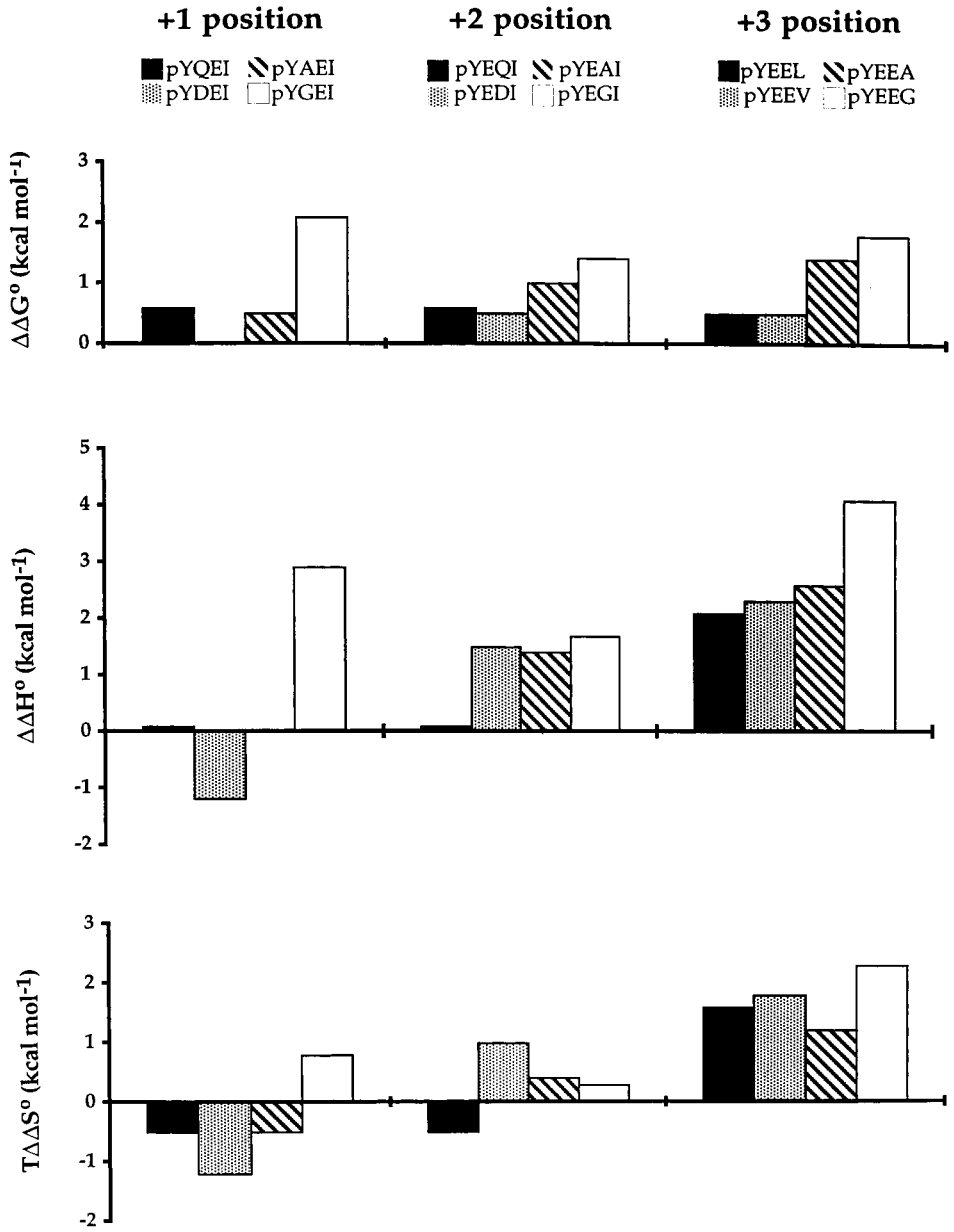

FIGURE 2: Binding energetics of pYEEI phosphopeptide variants. Shown are the differences in $\Delta G°$ (top), $\Delta H°$ (middle), and $T\Delta S°$ (bottom) between the pYEEI peptide and the various pYEEI peptide variants. Values for $\Delta\Delta G°$, $\Delta\Delta H°$, and $T\Delta\Delta S°$ are given at 25 °C by $\Delta G°_{mut} - \Delta G°_{wt}$, $\Delta H°_{mut} - \Delta H°_{wt}$, and $T\Delta S°_{mut} - T\Delta S°_{wt}$, respectively. Each of the four substitutions at the +1 (left), +2 (center), and +3 (right) positions are given in the legends near the top of the figure.

experiments at 15, 25, 35, and 45 °C. The $\Delta H°$ versus temperature plot derived from these experiments appeared to deviate slightly from linearity, displaying a very modest downward curvature for each peptide (Figure 3); however, the existence of this nonlinearity could not be established unambiguously given the level of experimental uncertainty in the individual experiments, and hence the values of $\Delta C_p°$ (given in Table 2) were evaluated simply from a straight line fit of the $\Delta H°$ versus temperature data.

The $\Delta C_p°$ value for the pYEEI peptide under the current conditions was -192 cal mol^{-1} deg^{-1}, which is identical within uncertainty to the $\Delta C_p°$ previously determined at pH 6.0 (*21*). The pYAEI and pYEAI phosphopeptides both bind the SH2 domain with a similar, or possibly a slightly more negative, $\Delta C_p°$ than the pYEEI peptide, indicating that the ionic interactions emanating from the +1 Glu and +2 Glu provide at most a very small, positive contribution to the $\Delta C_p°$ of the consensus peptide. Conversely, the $\Delta C_p°$ of the pYEEA peptide is significantly reduced in magnitude ($\Delta\Delta C_p° \approx 50$ cal mol^{-1} deg^{-1}) compared to the pYEEI peptide; hence, the hydrophobic contacts between the +3 Ile and the hydrophobic pocket of the SH2 domain contribute negatively to the $\Delta C_p°$. The effects of each Ala-substituted peptide on $\Delta C_p°$ are consistent with the prevailing notion that nonpolar interactions provide a large and negative contribution to $\Delta C_p°$, while the contribution of polar interactions is small and positive (*25*, *26*). The $\Delta C_p°$ for a phosphopeptide which contains Ala at all three positions was also investigated and found to be similar to that of the pYEEA peptide (Table 2).

Coupling Energetics between Phosphopeptide Residues. In the following set of experiments, the Src SH2 domain-tyrosyl phosphopeptide recognition mechanism was further investigated by examining whether the +1 Glu, +2 Glu, and

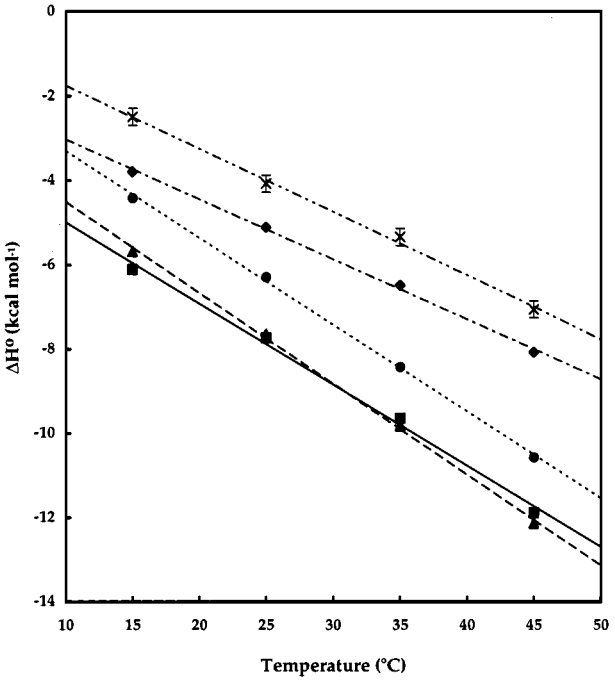

FIGURE 3: Binding enthalpies as a function of temperature for pYEEI peptide variants. The $\Delta H°$ as a function of temperature is given for the pYEEI(■), pYAEI(▲), pYEAI(●), pYEEA(◆), and pYAAA (×) phosphopeptides. The best fit lines for the pYEEI (solid), pYAEI (long dashed), pYEAI (short dashed), pYEEA (dash dot), and pYAAA (dash double dot) peptides are also shown. See Table 2 for the derived $\Delta C_p°$ values. Data shown are the mean of at least two independent experiments. Error bars represent the typical standard deviation of this set of multiple experiments (±0.2 kcal/mol) and are shown for clarity only for the pYAAA data but are representative of errors for all peptides.

+3 Ile communicate with one another in order to bind with high affinity. The coupling energetics between the +1 Glu, +2 Glu, and +3 Ile were evaluated in a pairwise fashion using double mutant cycles (*15*, *16*). The thermodynamic parameters of binding for the consensus pYEEI peptide, three single Ala mutants, three double Ala mutants, and the triple Ala mutant were determined. From these experiments, two measures of the extent to which two particular mutations are coupled, the coupling free energy, $\Delta G_c°$, and the coupling enthalpy, $\Delta H_c°$, were derived. The coupling free energy is formulated as follows:

$$\Delta G_c° = (\Delta G°_{00} + \Delta G°_{11}) - (\Delta G°_{10} + \Delta G°_{01})$$

Here, $\Delta G_c°$ is the difference between the sum of the binding free energy for the unperturbed ($\Delta G°_{00}$) and double mutant ($\Delta G°_{11}$) and the sum of the binding free energy for the two single mutants ($\Delta G°_{10}$) and ($\Delta G°_{01}$). The $\Delta H_c°$ is given in an analogous fashion. If $\Delta G_c° < 0$, the two perturbations are positively coupled (the presence of the first amino acid favors binding of the second); if $\Delta G_c° > 0$, the two perturbations are negatively coupled (the presence of the first amino acid hinders binding of the second); if $\Delta G_c° = 0$, the two perturbations are not coupled (there is no effect of the first amino acid on the second).

Table 2 shows the thermodynamic data from ITC experiments with the seven Ala-substituted phosphopeptides. The affinity of binding progressively decreases as Ala substitutions are added, such that the association constant for the pYAAA peptide is 2 orders of magnitude lower than that of the pYEEI peptide ($\Delta\Delta G° = 2.8$ kcal/mol). This difference in affinity is very similar to that previously determined between specific and nonspecific phosphorylated peptide targets of the Src SH2 domain (*21*). The binding enthalpy also decreases in magnitude as Ala residues are incorporated into the peptide, reaching a value of −3.9 kcal/mol for the triply substituted pYAAA peptide.

To evaluate the cooperativity between peptide positions, the data in Table 2 were analyzed in terms of double mutant cycles (Figure 4) (*15*, *16*). Figure 4A shows the coupling between the +1 and +2 Glu. The $\Delta G_c°$ and $\Delta H_c°$ are both zero within the uncertainty of ±0.5 kcal/mol, indicating that the effect of Ala substitutions at the +1 and +2 positions are additive for both $\Delta G°$ and $\Delta H°$. Hence, the +1 and +2 positions are not energetically coupled. Figure 4, panels B and C, shows that the $\Delta G_c°$ for both the +1 Glu−+3 Ile and +2 Glu−+3 Ile interactions are also both small, which, by itself, would imply that each residue within these two pairs of residues binds independently. However, the $\Delta H_c°$ values for both the +1 Glu−+3 Ile and +2 Glu−+3 Ile interactions are significantly nonzero, implying that some level of thermodynamic coupling exists within each of these pairs of residues. The $\Delta H_c°$ for the +1 Glu−+3 Ile interaction is positive (+1.2 kcal/mol), implying that the presence of the +3 Ile enthalpically hinders binding of the +1 Glu, and vice versa. However, the $\Delta H_c°$ for the +2 Glu−+3 Ile interaction is negative, (−1.6 kcal/mol), indicating that a "bonus" enthalpy of 1.6 kcal/mol is gained when the +2 Glu and +3 Ile are present simultaneously in the same peptide. Both the +1 Glu−+3 Ile and +2 Glu−+3 Ile interactions display a coupling entropy ($\Delta S_c°$) which opposes $\Delta H_c°$, resulting in a small value of $\Delta G_c°$.

Figure 4, panels D−F, shows the double mutant cycles for the coupling between each pair of residues when Ala is present at the peptide position held fixed in the cycle. The same pattern of coupling is observed for these thermodynamic cycles as the ones shown in Figure 4, panels A− C: $\Delta G_c°$ is small for all interactions, but a significant nonzero coupling enthalpy is evident for the +1 Glu−+3 Ile and +2 Glu−+3 Ile interactions. The similarity in the pattern of coupling between the top and bottom rows of Figure 4 is expected based on the fact that no energetic coupling was observed between the +1 and +2 residues. These results indicate that the coupling between the three positions is "direct", i.e., the three residues in the EEI motif only interact in a pairwise fashion and not together as a single unit (*16*, *27*).

DISCUSSION

The binding mechanism of the Src SH2 domain has been investigated by examining the importance of each of the three residues of the EEI motif in SH2 domain recognition. The role of the +1 Glu, +2 Glu, and +3 Ile in high-affinity binding has been assessed by replacing each residue with four progressively smaller amino acids. In addition, the coupling between the +1 Glu, +2 Glu, and +3 Ile upon binding has also been investigated using alanine scan mutagenesis and double mutant cycles.

The insertion of the +3 Ile of the peptide into the hydrophobic binding pocket of the SH2 domain was originally thought to be critical for recognition of the pYEEI

Table 2: Thermodynamic Binding Parameters of Ala Substituted Tyrosyl Phosphopeptides[a]

peptide	K (M^{-1})	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)	$\Delta C_p°$ [cal/(mol deg)]
pYEEI	$5.5 (\pm 0.7) \times 10^6$	-9.2 ± 0.1	-7.7 ± 0.2	1.5 ± 0.2	-192 ± 24
pYAEI	$2.9 (\pm 0.4) \times 10^6$	-8.7 ± 0.1	-7.7 ± 0.2	1.0 ± 0.2	-216 ± 14
pYEAI	$9.6 (\pm 0.8) \times 10^5$	-8.2 ± 0.1	-6.3 ± 0.1	1.9 ± 0.1	-206 ± 17
pYEEA	$5.7 (\pm 2.0) \times 10^5$	-7.8 ± 0.2	-5.1 ± 0.4	2.7 ± 0.4	-142 ± 20
pYAAI	$4.2 (\pm 0.8) \times 10^5$	-7.6 ± 0.1	-6.1 ± 0.1	1.5 ± 0.1	nd
pYAEA	$1.4 (\pm 0.2) \times 10^5$	-7.0 ± 0.1	-3.9 ± 0.2	3.1 ± 0.2	nd
pYEAA	$2.1 (\pm 0.5) \times 10^5$	-7.2 ± 0.1	-5.3 ± 0.1	1.9 ± 0.1	nd
pYAAA	$4.7 (\pm 0.3) \times 10^4$	-6.4 ± 0.1	-3.9 ± 0.2	2.5 ± 0.2	-150 ± 16

[a] Experiments were performed with the octapeptide Ac−PQpYEEIPI−NH2 (designated pYEEI in this table and in the text) and variants thereof in 20 mM Hepes, pH 7.5, 100 mM NaCl at 25 °C. Uncertainties in K, $\Delta G°$, and $\Delta H°$ represent the standard deviation of multiple experiments; the error in $T\Delta S°$ was calculated by standard error propagation. Uncertainties in $\Delta C_p°$ represent the 95% support plane confidence interval for the linear best fit of the $\Delta H°$ versus temperature data. nd, not determined.

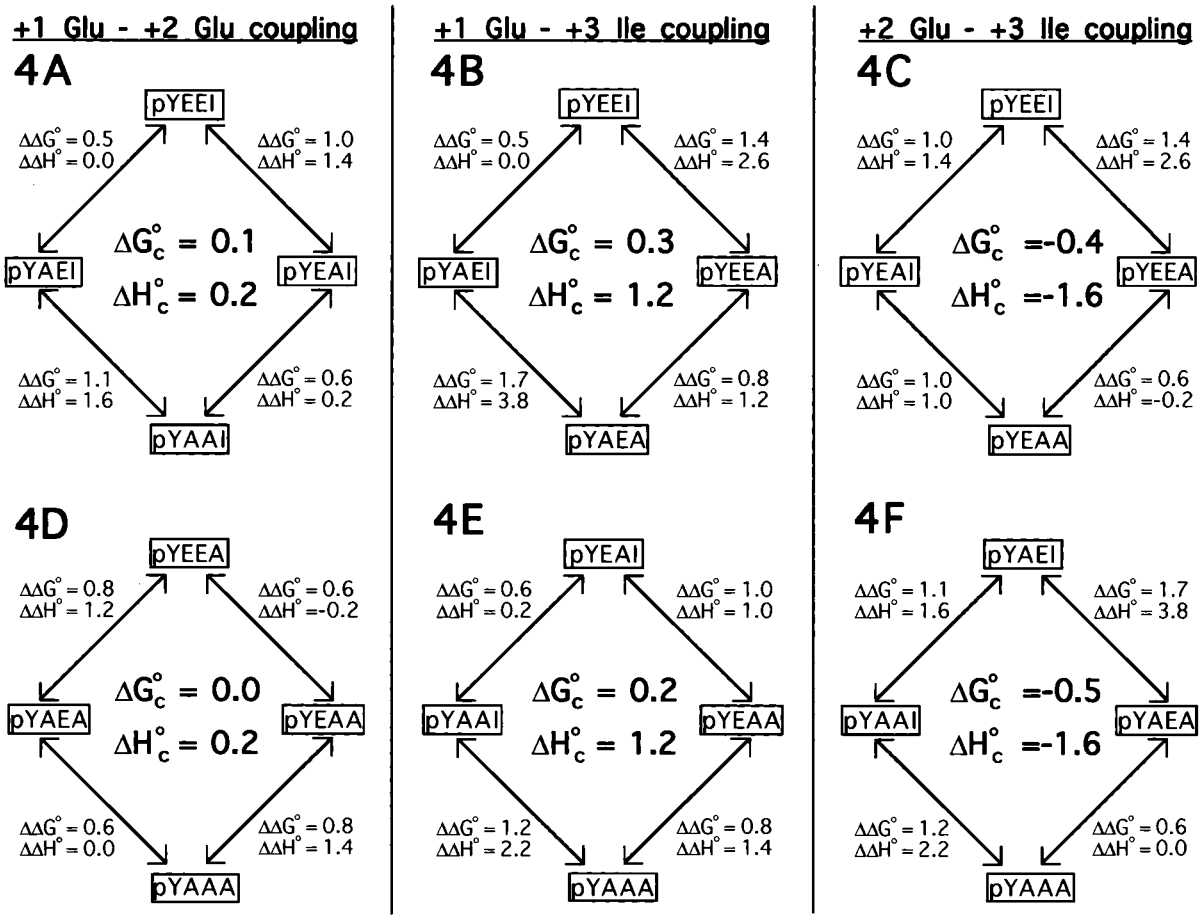

FIGURE 4: Coupling energetics between phosphopeptide residues. From left to right, the double mutant cycles for the interactions between the +1 Glu and +2 Glu (4A and 4D), +1 Glu and +3 Ile (4B and 4E), and +2 Glu and +3 Ile (4C and 4F) are shown. Since a panel of seven Ala substituted peptides was examined, the coupling between each pair of positions could be determined in two ways, with either the consensus sequence residue (top row) or Ala (bottom row) present at the position held fixed in the cycle. The coupling free energy ($\Delta G_c°$) and enthalpy ($\Delta H_c°$) are depicted at the center of each cycle, while the differences in $\Delta G°$ and $\Delta H°$ between the indicated peptides are given on the diagonals of the cycles. On the basis of an estimated typical uncertainty in this series of individual experiments for $\Delta G°$ and $\Delta H°$ of ±0.25 kcal/mol (derived from the typical standard deviations of multiple experiments and other systematic errors), the uncertainty in $\Delta\Delta G°$ and $\Delta\Delta H°$ was estimated by error propagation to be ±0.4 kcal/mol, while the uncertainty in both $\Delta G_c°$ and $\Delta H_c°$ was similarly estimated to be ±0.5 kcal/mol.

peptide (*10*). The results presented here indicate that the hydrophobic interactions within the +3 binding pocket are certainly important for high-affinity binding, since replacing the +3 Ile with Ala causes a 10-fold reduction in binding affinity (Table 2 and Figure 2). However, since nonspecific phosphopeptides bind as much as 50−200 times less tightly than the pYEEI peptide (*21*), other sites outside the +3 binding pocket must also contribute to high-affinity binding.

Since the interactions between the +3 Ile of the peptide and the hydrophobic pocket of the Src SH2 domain are nonpolar, a favorable entropic signature was anticipated for +3 Ile binding (*28*−*30*). However, instead, the binding of the +3 Ile had a significantly less favorable entropy and a much more favorable binding enthalpy than each of the +3 position substituted peptides (Table 1 and Figure 2). Since a favorable enthalpy is a signature of intimate van der Waals

packing interactions (*31*), it appears that optimal contacts between the +3 peptide residue and the hydrophobic binding pocket only occur when the +3 residue of the peptide is Ile. The formation of these favorable interactions may restrict the range of available conformations of the +3 Ile, explaining the less favorable binding entropy of the pYEEI peptide. The importance of intimate, nonpolar contacts in molecular recognition has also been suggested by the observation of a favorable enthalpic signature for binding of a hydrophobic high-affinity ligand to calmodulin (*32*).

Calorimetric examination of the importance of the +2 peptide position revealed that the +2 Glu was nearly as critical for high affinity Src SH2 domain binding as the +3 Ile, and hence the water-mediated ionic interaction between the carboxylate O_ϵ atoms of the +2 Glu and Arg βD′1 likely contributes to specific recognition (Figures 1 and 2). High-affinity binding is relatively maintained when either Gln or Asp is present at the +2 position; however, the $\Delta H°$ of binding of the +2 Gln and the +2 Asp peptides were significantly different, with the $\Delta H°$ of pYEQI being very close to that of the pYEEI peptide, while the $\Delta H°$ of pYEDI was reduced to a value similar to that of pYEAI and pYEGI (Table 1 and Figure 2). Since the formation of hydrogen bonds typically results in a favorable $\Delta H°$ (*31*), an interpretation of these results may be that the water-mediated hydrogen-bonding network emanating from the +2 Glu is maintained when Gln is at the +2 position, but abrogated in the presence of the shorter Asp, Ala, and Gly.

Only a small reduction in binding affinity was measured when the +1 Glu of the peptide was replaced with either Gln, Asp, or Ala (Table 1 and Figure 2), indicating that the interaction between the carboxylate O_ϵ atoms of the +1 Glu and the amino group of Lys βD3 contributes little to Src SH2 domain binding affinity. However, interestingly, a more favorable $\Delta H°$ was observed for the pYDEI peptide than for the pYEEI peptide ($\Delta\Delta H° = -1.2$ kcal/mol); one possible explanation for this result is that water molecules may be required to facilitate the ionic interaction between the shorter +1 Asp, but not the +1 Glu, and Lys βD3. Although the +1 Glu−Lys βD3 interaction contributes little binding affinity to Src SH2 domain−pYEEI peptide recognition, Lys βD3 may still be important for Src SH2 domain specificity by selecting against either positively charged or long, hydrophobic residues at the peptide +1 position. Unlike the other +1 position substitutions, placing a Gly at the +1 position resulted in a large loss ($\Delta\Delta G° = 2.1$ kcal/mol) in binding affinity (Table 1 and Figure 2), indicating that the van der Waals contact between the C_β carbon of the +1 Glu and Tyr βD5 of the SH2 domain is important for high-affinity binding. However, this interaction provides little binding specificity given that all amino acids except Gly contain a side chain which will provide this important contact.

The coupling free energy ($\Delta G_c°$) between each pair of residues of the EEI motif was determined to be small (Figure 4), which indicates that, at the level of the binding free energy (the most important parameter describing the binding process), no coupling between residues of the EEI motif is experimentally apparent. This is perhaps not surprising given that the peptide binds in an extended conformation across the face of the SH2 domain with little conformational change occurring in the SH2 domain upon binding (*10*). Furthermore, the small $\Delta G_c°$ between phosphopeptide residues is consistent with investigations of other protein−protein interactions which have noted that the coupling free energy of residues not in direct contact is small (*33*−*36*).

However, a nonzero coupling enthalpy between residues in the EEI motif was observed, indicating that these amino acids do indeed communicate with one another. $\Delta H_c°$ was negligible only for the interaction between the +1 Glu and +2 Glu; the +1 Glu−+3 Ile interaction showed a positive coupling enthalpy ($\Delta H_c° = +1.2$ kcal/mol), while the $\Delta H_c°$ for the +2 Glu−+3 Ile interaction was negative ($\Delta H_c° = -1.6$ kcal/mol). These nonzero $\Delta H_c°$ values may provide further insight into the pYEEI peptide−SH2 domain recognition mechanism. For example, since the formation of the water-mediated hydrogen-bonding network emanating from the +2 Glu occurs with a negative $\Delta H°$ (see Figure 2 and the Discussion above), the negative $\Delta H°$ for the interaction between the +2 Glu and the +3 Ile may indicate that the water-mediated network is formed only when the +3 Ile occupies the hydropobic binding pocket. Indeed, one of the components of the hydrogen-bonding network is the carbonyl of Ile βE4, which forms part of the hydrophobic pocket into which the +3 Ile is bound (see Figure 1). Occupation of the +3 binding pocket by the +3 Ile of the peptide may be required to properly position the carbonyl of Ile βE4 for formation of the water network. The positive $\Delta H_c°$ for the +1 Glu−+3 Ile interaction may reflect long distance communication between amino acids in the βD strand of the SH2 domain: the formation of the ionic interaction between the +1 Glu and Lys βD3 may constrain the conformation of the peptide backbone so that optimal van der Waals interaction cannot occur between the +3 Ile and the residues of the +3 binding pocket, including Tyr βD5 (see Figure 1).

Few other studies have examined the coupling enthalpy as a probe of molecular recognition. However, investigation of the $\Delta H_c°$ between residues at the barnase-barstar interface has indicated that amino acids not in direct contact can be enthalpically coupled even when the coupling free energy is negligible (*37*). Similar results have been found in calorimetric studies of the binding of Ras to various Ras binding domains (C. Herrman, personal communication). Hence, a nonzero coupling enthalpy between amino acids not in contact, as the current study reports, may be a common feature of many binding processes. Energetic coupling may not be evident in studies which only evaluate $\Delta G_c°$ due to the pervasive enthalpy−entropy compensation effects which are involved in most mutational analyses (*38*). However, the communication may be unmasked when calorimetric studies which determine $\Delta H_c°$ are performed.

The demonstration of nonzero coupling enthalpies between residues of the EEI peptide adds to the growing number of examples of nonadditivity of mutational effects in proteins (see ref *17*). For example, coupling of mutational perturbations has been documented for the subunit association of hemoglobin (*17*) and the binding of a synthetic tripeptide substrate to thrombin (*18*). Furthermore, nonadditive effects of mutations have also been noted for a protein−nucleic acid interaction (*19*) and in protein folding (*39*). Hence, the existence of cooperative interactions between amino acids not in direct contact may be a more widespread phenomena in molecular recognition events than is generally appreciated.

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