The phosphopeptide-binding specificity of Src family SH2 domains

Gillian Payne[1], Lesley A Stolz[1], Dehua Pei[1], Hamid Band[2], Steven E Shoelson[3], and Christopher T Walsh[1]*

[1]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA, [2]Lymphocyte Biology Section, Department of Rheumatology and Immunology, Brigham and Women's Hospital, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115, USA and [3]Research Division, Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA

Background: Src homology 2 (SH2) domains mediate protein/protein interactions by binding phosphotyrosyl proteins with high specificity. The protein Lck, a Src-like lymphocyte-specific tyrosine kinase which is important in signals involved in T-cell development, contains one such domain. The crystal structure of a complex of the Lck SH2 domain with a high-affinity ligand, pY324, is known. This ligand has the sequence EPQpYEEIPIYL.

Results: We designed and synthesized a series of phosphopeptides with single amino-acid changes in the four residues C-terminal to the phosphotyrosine (pTyr) in pY324. Surprisingly, the Glu one residue C-terminal to the phosphotyrosine (at position pY+1) is sensitive to substitution, whereas the Ile at position pY+3 is much less sensitive, accommodating a Glu with only modest loss of binding affinity. Replacement of the Glu and Pro on either side of the Ile had little effect, as predicted. Truncated phosphopeptides that end at position pY+5 and have only an acetyl group N-terminal to the pTyr bound with only slightly lower affinity than pY324. In addition, naturally occurring phosphopeptide sequences that span a 1 000-fold range in binding affinity for the Lck SH2 domain have been identified.

Conclusions: The Lck SH2 domain is highly selective for phosphotyrosyl-peptide binding; its specificity is dictated by the first and third residues C-terminal to the pTyr. The unexpected effects of some amino-acid substitutions indicate that the interactions seen between SH2 domains and ligand in the crystal structure may not be identical to those that occur in solution.

Chemistry & Biology October 1994, **1**:99–105

Key words: affinity, Lck, phosphopeptides, SH2, surface plasmon resonance

Introduction

SH2 domains are thought to regulate signaling pathways by mediating substrate recruitment [1,2] through complex formation between signaling molecules [3–13], by competing with protein tyrosine phosphatases for binding to phosphotyrosines [14], and by directly altering catalytic activity [15–21]. Numerous lines of evidence have indicated that some SH2 domains bind to their phosphotyrosyl protein targets with high specificity; this would be advantageous in a signaling pathway. In a quantitative study of the relative affinities of synthetic phosphotyrosine-containing peptides for the phosphatidyl inositol 3 kinase, p85 subunit, N-terminal SH2 domain (PI3K N-SH2 domain), a 2 000-fold range of affinity (0.5 μM to >1000 μM) was found, with the highest affinity peptide containing the pYMXM motif [22]. In fact, PI3K p85 N-SH2 preferentially binds the sequence motif pYMXM with an affinity up to 1 000-fold higher than its affinity for a phosphopeptide derived from the Src C-terminal regulatory tail, pYQPG (Tyr 527). In contrast, the Src and Lck SH2 domains bind their tails with high affinity (pYQPG and pYQPQ motifs, respectively) and the sequence motif pYMXM with only slightly lower affinity, suggesting that the Lck and Src SH2 domains are less able to distinguish between phosphotyrosines in different peptide environments than is the PI3K N-SH2 domain [23]. The data may also suggest that pathways involving Lck and Src might thus offer fewer opportunities for specific intervention than those involving PI3 kinase.

In the analysis of possible signaling specificities of the Src family of protein tyrosine kinases, we recently demonstrated that the hmT-derived phosphopeptide, pY324, which has the sequence EPQpYEEIPIYL, showed the highest affinity binding to GST fusion proteins of the Lck SH2 domain (5-fold higher than Lck's affinity for the Lck tail phosphopeptide) and the Src SH2 domain (44-fold higher than Src's affinity for the Src tail phosphopeptide) [23] in agreement with the predicted high affinity sequence from the peptide library screening performed by Cantley and coworkers [24]. Other phosphopeptides bound with 5 to 260-fold lower affinity [23].

We have sought to determine the factors that mediate the specificity of the Src family SH2 domains to their phosphotyrosine ligands, starting with the high affinity hmT pY324 phosphopeptide/Lck and Src SH2 domain cocrystal structures [25,26]. In addition to the deep hydrophilic pocket for the pTyr moiety, these domains have another deep hydrophobic pocket that accommodates the Ile side chain three residues C-terminal (pY+3)

*Corresponding author.

to the pTyr. The pY+1 and pY+2 residues, both Glu in pY324, do not insert into any pocket. However, the side chain of Glu pY+1 lies across the face of the Lck SH2 domain whereas the side chain of Glu pY+2 projects away from the surface. The pY+4 position, while contacting the Lck SH2 domain with its carbonyl oxygen, does not make any specific side chain hydrogen bonds (Fig. 1). Thus, the details of the Lck SH2 domain/hmT pY324 phospho-peptide cocrystal structure predict that the binding of phosphotyrosyl peptides would be highly selective for hydrophobic amino acids in the pY+3 position, not selective in the pY+2 and pY+4 positions and somewhat selective for Glu in the pY+1 position. Because of the extensive hydrogen bond network, the existence of alternative high affinity binding modes is not predicted [25].

In this manuscript we describe binding experiments designed to test factors responsible for the phosphopeptide affinity predicted by the crystal structure of the Lck SH2 domain bound to the hmT pY324 peptide. The range of such phosphotyrosine ligands for the Lck SH2 domain spans 760-fold discrimination.

Results

Binding of GST-LckSH2 to altered pY324 phosphopeptides

To test the predictions of the SH2/pY324 crystal structure we constructed hmT pY324 phosphopeptides with amino acid substitutions in one of four positions (pY+1, pY+2, pY+3, and pY+4), and measured the affinity of these altered phosphopeptides for the GST-LckSH2 domain fusion protein.

Table 1. Relative affinities of altered pY324 phosphopeptides for GST-LckSH2.

	+3 position	
Phosphopeptide name	**Phosphopeptide sequence**	**Relative affinity***
hmT pY324	(K)EPQpYEE I PIYL	1.0
hmT pY324+3V	EPQpYEE **V** PIYL	2.2
hmT pY324+3M	EPQpYEE **M** PIYL	2.7
hmT pY324+3L	EPQpYEE **L** PIYL	4.8
hmT pY324+3E	EPQpYEE **E** PIYL	6.1
hmT pY324+3A	EPQpYEE **A** PIYL	10
hmT pY324+3Q	EPQpYEE **Q** PIYL	24

*Relative affinities are in the micromolar range (see Materials and methods) and relate to an ID_{50} value of 2.87 μM for the (K)EPQpYEEIPIYL peptide. This reference point also holds in the subsequent tables.

The pY+3 position

The relative binding affinities of six pY+3-substituted pY324 peptides for the Lck SH2 domain are shown in Table 1. When Ile was replaced with Val, Met, or Leu the binding energy lost was minimal, giving affinities of between two-fold and five-fold lower than that of pY324. On the basis of the crystal structure, we had predicted that replacement of the Ile with a charged residue such as Glu would have a large effect on affinity.

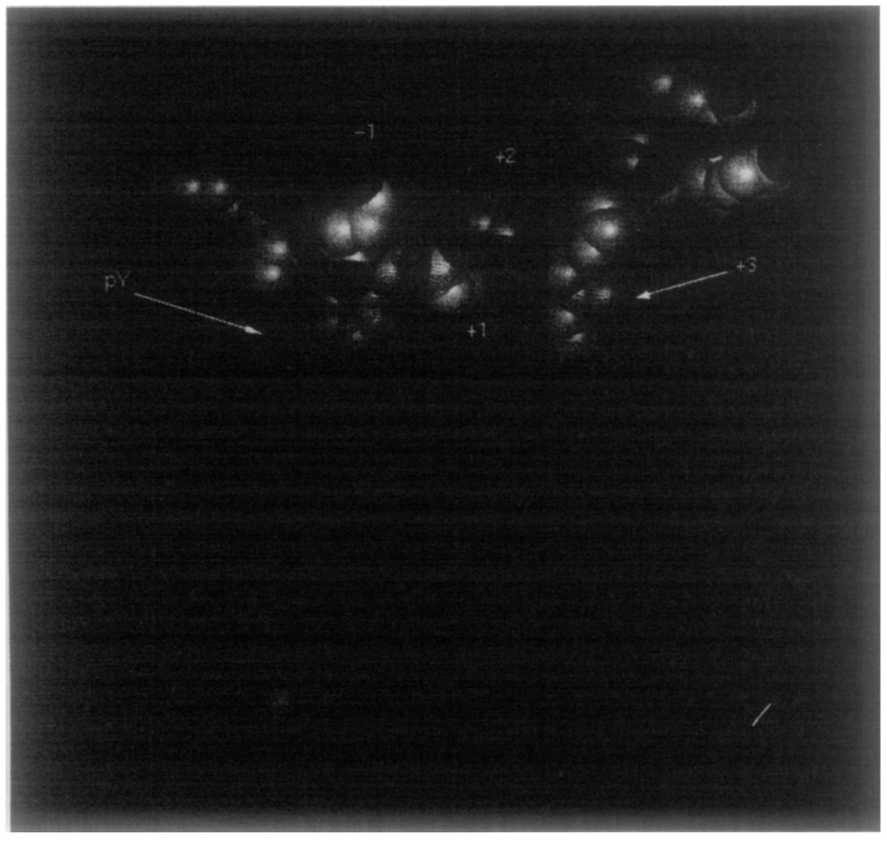

Fig. 1. Model of the Lck SH2 domain complexed with pY324. The solvent-accessible surface of the Lck SH2 domain is shown in a complex with a space-filling model of the high affinity phosphopeptide, EPQpYEEIPIYL [25]. The polypeptide backbone and carbonyl oxygens of the SH2 domain are shown. Note the deep binding pockets in the SH2 domain which anchor the peptide via the pTyr and the Ile at position pY+3, and the complementary surface upon which the peptide (pY-2 Pro to pY+4 Pro) rests. The solvent-accessible surface was calculated using InsightII (Biosym Technologies, Inc.).

Table 2. Relative affinities of altered pY324 phosphopeptides for GST-LckSH2.

+1 position		
Phosphopeptide name	**Phosphopeptide sequence**	**Relative affinity**
hmT pY324	(K)EPQpYEEIPIYL	1
hmT Ac-pY324+1E	Ac-pY**E**EIPI	1.7
hmT Ac-pY324+1Q	Ac-pY**Q**EIPI	5.9
hmT Ac-pY324+1D	Ac-pY**D**EIPI	13
hmT Ac-pY324+1S	Ac-pY**S**EIPI	17
hmT Ac-pY324+1F	Ac-pY**F**EIPI	18
hmT Ac-pY324+1I	Ac-pY**I**EIPI	18
hmT Ac-pY324+1M	Ac-pY**M**EIPI	26
hmT Ac-pY324+1A	Ac-pY**A**EIPI	42
hmT Ac-pY324+1L	Ac-pY**L**EIPI	47
hmT Ac-pY324+1G	Ac-pY**G**EIPI	>47
hmT Ac-pY324+1K	Ac-pY**K**EIPI	>>47

Surprisingly, the observed decrease was modest, only slightly larger than that seen for the Leu substitution. It is possible that the negative charge of the carboxylate anion is stabilized by a positively charged Arg on the 'jaws' that clamp around the pY+3 residue (EF Arg3 or BG Arg7; nomenclature described in [25]). Substitution with Ala, a hydrophobic residue with a small side chain, gave a somewhat larger effect on affinity than substitution with Glu. Due to the fact that the Lck tail peptide TEPGpYQPQP has only 5-fold lower affinity for the Lck SH2 domain, we expected that a Gln substitution at position pY+3 would be tolerated, but the pY+3 Gln peptide in fact showed a reduction in affinity of 23-fold.

The pY+1 position

Unlike the pY+3 position, the pY+1 position of the phosphopeptide does not insert into a cleft in the SH2 domain surface. Rather, the side-chain of the hmT pY324 pY+1 Glu lies across the surface of the SH2 domain [25]. Table 2 shows the modified pY324 phosphopeptides used to test the effect of pY+1 substitutions on binding to the Lck SH2 domain. Interestingly, replacement of the residues N-terminal to the pTyr with an acetyl group (Ac) and omission of the C-terminal residues, Tyr pY+6 and Leu pY+7, gave less than two-fold reduction in binding affinity. This is in good agreement with results suggesting that the pTyr and the three residues immediately C-terminal to it (pY+1, pY+2, and pY+3) are responsible for most of the binding energy of the SH2 domain/phosphopeptide interaction (G. Wolf and S.E.S, unpublished data). The crystal structure of the Lck SH2 domain bound to pY324 also shows that residues N-terminal to pY-2 or C-terminal to pY+4 turn away from the surface of the SH2 domain [25], and would therefore not be expected to contribute to the interaction.

The pY+1 substitution having the least effect on binding affinity was Gln, with only a six-fold reduction in affinity as the negative charge is lost. This is consistent with the five-fold reduced affinity compared with hmT pY324 observed for the Lck tail phosphopeptide (see above) which also possesses a Gln in the pY+1 position. The Gln at pY+1 was tolerated slightly better than substitution with Asp, suggesting that the length of the side-chain carbon backbone which positions the carboxylate anion may be relevant. Hydrophobic substitutions had an intermediate effect on the binding affinity ranging from 18 to 47-fold lower. The polar hydroxyl group of Ser confers a 2.5-fold affinity advantage over the hydrophobic Ala. The least tolerated substitutions were Gly and Lys. Limitations of phosphopeptide solubility and availability prevented the extension of binding studies into the phosphopeptide concentration range where an accurate affinity could be measured for these two lowest affinity peptides. The very poor affinity observed for the Gly substitution suggests that the pY+1 side-chain contacts are important for binding energy while the Lys result may be due to charge repulsion.

The pY+2 and pY+4 positions

Although residues at the pY+2 and pY+4 positions are in contact with the Lck SH2 domain, the side-chains of these residues project away from the SH2 domain in the crystal structure [25]. Because the side-chain of the Glu at position pY+2 is likely to be solvent-exposed in the complex [25], we chose to replace the Glu at this position with Ile, with the idea that exposure of the long hydrophobic side-chain to solvent would be unfavorable. The affinity change resulting from this substitution was negligible, however (see Table 3). Likewise, replacement of the Pro at position pY+4 with Ala or Asn resulted in only a slight drop in affinity. These results are in agreement with the predictions of the crystal structure.

Comparison of the Lck, Src, and Fyn SH2 domains

An alignment of the highly homologous SH2 domains of the Src family protein tyrosine kinases, Lck, Src, and Fyn shows that the residues in the Lck SH2 domain that are in contact with the pY324 phosphopeptide are identical in the Src and Fyn SH2 domains with only two exceptions. In one arm of the 'jaw' that closes around the pY+3 Ile, the Lck SH2 domain uses the β-carbon of Ser to make contact with the phosphopeptide [25], while the Src SH2 domain uses the equivalent β-carbon of Thr [26]. Thr is also found at this site in the Fyn SH2 domain.

Table 3. Relative affinities of altered pY324 phosphopeptides for GST-LckSH2.

+2 and +4 positions		
Phosphopeptide name	**Phosphopeptide sequence**	**Relative affinity**
hmT pY324	(K)EPQpYEEIPIYL	1
hmT pY324+2I	EPQpYE**I**IPIYL	1.6
hmT Ac-pY324+1E	Ac-pY**E**EIPI	1.7
hmT Ac-pY324+4A-NH_2	Ac-pYEEI**A**-NH_2	3.2
hmT pY324+4N	EPQpYEEI**N**IYL	3.5

Table 4. Comparison of src family SH2 domain affinities for altered pY324 phosphopeptides.

Phosphopeptides	Relative affinity		
	GST-LckSH2	GST-SrcSH2	GST-FynSH2
EPQpYEEIPIYL	1.0	1.0	1.0
Ac-pYEEIPI	1.2	1.0	1.2
Ac-pY**Q**EIPI	3.3	2.4	3.2
EPGpYEE**MP**IYL	3.8	3.3	3.6
EPGpYEEI**N**IYL	12	8.3	4.6
Ac-pY**L**EIPI	36	32	46

Indeed, replacement of this Src Thr residue by Trp alters the specificity of the Src SH2 domain to that of the Sem-5/drk/Grb2 SH2 domain [27], suggesting that conservation of Ser/Thr in this position is necessary to retain the Src family phosphopeptide specificity. In the other arm of the 'jaws', the Lck SH2 domain uses the backbone of Asp to make contact with the Ile residue [25]. This residue, although conserved in Src, does not make contact with the Ile residue in the crystal structure of the complex of the Src SH2 domain with pY324 [26]. In the Fyn SH2 domain the equivalent residue is an Ala. This change is not expected to alter the specificity of binding.

To determine whether the differences between the sequences of these three Src family SH2 domains affect their binding specificities, we measured the affinity of all three SH2 domains for several representative pY324-derived phosphopeptides, using GST fusion proteins (Table 4). Each SH2 domain exhibited the same rank ordering of affinity for the phosphopeptides tested, suggesting that the Lck, Src, and Fyn SH2 domains have similar specificities and that the key phosphopeptide residue contacts of the SH2 domain can be used to predict the phosphopeptide binding specificity.

Binding of GST-LckSH2 to low affinity phosphopeptides

In previous binding studies with GST-LckSH2 and GST-SrcSH2 [23] we were able to determine the rank order of binding to several phosphopeptides. However, the range of binding affinities seen in these studies was relatively small (260-fold for GST-LckSH2; 110-fold for GST-SrcSH2) compared with the range seen in similar studies conducted with the PI3K N-SH2 domain (>2 000-fold) [22]. The most striking contrast between the binding specificities of the Lck SH2 and PI3K N-SH2 domains can be seen with the insulin receptor substrate 1 (IRS-1) phosphopeptide pY628 and the Src tail phosphopeptide pY527. The PI3K N-SH2 domain has a greater than 1 000-fold preference for pY628 [22], while the Lck SH2 domain shows a preference of just over three-fold for pY527 [23].

In an expanded repertoire of phosphopeptides tested for affinity to the Lck SH2 domain (see Table 5), ten phosphopeptide sequences derived from the interleukin-2 receptor β chain (IL-2Rβ), colony stimulating factor-1 receptor (CSF-1R), and the protein product of the proto-oncogene *c-kit* were assayed. Three low-affinity phosphopeptides derived from CSF-1R (pY708, pY723, and pY809) and one from c-kit (pY745) have been identified (see Table 5) indicating that the Lck SH2 domain has a range of discrimination of more than 760-fold. The lowest affinity phosphopeptides have positively charged residues (Arg or Lys) C-terminal to the pTyr, perhaps leading to electrostatic repulsion by adjacent positively charged residues of the SH2 domain (Arg EF3 or Arg BG7 in the pY+3 position; Lys βD3 or Lys βD6 in the pY+1 position). These data indicate that SH2 domain phosphopeptide binding specificity may result from exclusion of unfavorable residues as well as selection of residues conferring favorable interactions. In the four phosphopeptides that show 760-fold or greater reduction in affinity compared with pY324, the pY+1 position always contains Val or Ile. The CSF-1R phosphopeptide pY697 is of particular interest: it has a Lys residue at position pY+1, and we have already observed (see Table 2) that when Lys is inserted at position pY+1 of pY324 the binding affinity for the Lck SH2 domain is reduced by more than 47-fold. The binding affinity of pY697 is in fact 430-fold lower than that of pY324. Of the other residues in pY697 that make contact with the SH2 domain, residues at positions pY+2 and pY+4 probably do not contribute significantly to the binding affinity, as predicted by the crystal structure and as shown above, and position pY+3 is Ile, which should favor tight binding. Thus it appears that the Lys at position pY+1 is responsible for the majority of the 430-fold reduction in binding affinity.

Discussion

SH2 domains are found ubiquitously in the molecules involved in signal transduction in several distinct intracellular signaling pathways. The binding specificities of these domains are therefore of great interest. Approaches to defining SH2 domain specificity include site-directed mutagenesis of known tyrosine phosphorylation sites, which results in the abrogation of specific SH2 domain binding [28–31], mutation of key SH2 domain residues, giving altered phosphopeptide specificity [27], structural analysis of SH2 domains bound to high affinity phosphopeptides using X-ray crystallographic methods [25,26], phosphopeptide library screening [24], and, finally, *in vitro*

Table 5. Relative affinities of phosphopeptides for GST-LckSH2.

Source/name		Sequence	Relative affinity
hmT	**pY324**	(K)EPQ**pY**EEIPIYL	1
c-kit	**pY936**	NHI**pY**SNLANSS	120
CSF-1R	**pY969**	QPNN**pY**QFS	140
IL-2Rβ	**pY355**	ASQV**pY**FTYDPYSE	260
c-kit	**pY730**	GVS**pY**VVPTKAD	270
CSF-1R	**pY697**	GGVD**pY**KNIHLE	430
c-kit	**pY745**	IGS**pY**IERDVTG	760
CSF-1R	**pY723**	VDT**pY**VEMRPVS	>760
CSF-1R	**pY809**	NDSN**pY**IVKGNA	>760
CSF-1R	**pY708**	LEKK**pY**VRRDSG	>>760

binding studies with purified SH2 domains and activated, tyrosine phosphorylated receptors [5,32–35] or synthetic phosphopeptides [15,16,22,23,36–39] to measure binding affinities directly. The *in vitro* binding experiments described in this manuscript were designed to complement the available library screening data for the Lck SH2 domain [24], and the information gained from the structure of the complex of this domain with the phosphopeptide pY324 [25].

Although several of the predictions of the relative binding affinities of Lck SH2 for modified pY324 phosphopeptides that we made on the basis of the previously available data were accurate, there were some notable exceptions. It is possible that, because of the static nature of crystals, the interactions seen in a crystal structure may not fully predict the nature of the interactions in solution. For example, in the crystal structure, the pY+1 Glu makes only two contacts with the SH2 domain, while the pY+3 Ile inserts into a deep hydrophobic pocket and makes at least four contacts with surrounding residues (see Fig. 1). Thus, it appeared that the +3 site should be more sensitive to nonconservative substitution. We found, however, that even a conservative replacement of pY+1 Glu, to Asp, gave a 13-fold loss of binding affinity, whereas the nonconservative replacement of the hydrophobic pY+3 Ile with negatively charged Glu gave only a 6-fold reduction in binding. This suggests that the Lck SH2 domain can undergo conformational changes in solution to accommodate alternative phosphopeptides. One could speculate that in the context of a large protein an SH2 domain may be constricted and thus unable to adopt these alternative conformations, or that the binding pockets could be modified causing a difference in specificity. Specific modification of the protein, for example phosphorylations or dephosphorylations, could cause conformational changes in the SH2 domain, resulting in an alteration of the SH2 domain specificity. Indeed, evidence for SH2 domain conformational changes effected by other domains of the proteins in which they are contained is emerging ([40–44] and G. P., D. Winkler, L.A.S. and J. Shin, unpublished data).

In phosphopeptide library screening, the Lck SH2 domain showed a preference at the pY+1 position for Glu > Thr > Gln [24]. We did not examine the pY324 pY+1 Thr phosphopeptide in our studies; however, of the ten pY+1 substitutions tested, Gln was the most tolerated substitution, in good agreement with the library screening prediction. In the library screening experiments the Lck SH2 domain selectively retained phosphopeptides with Ile > Val > Met in the pY+3 position, in good agreement with the measured binding affinities which also ranked Ile > Val > Met. Thus, phosphopeptide library screening with SH2 domains is a valuable tool for predicting high affinity interactions. A limitation of this technique, however, is that it makes the assumption that the residues surrounding the pTyr confer independent binding determinants that are unaffected by neighboring amino acids. This is illustrated by a comparison of the binding affinity of the Lck tail phosphopeptide, pY505 (TEGQpYQPQP), with that of the pY324 phosphopeptide substituted at pY+3 with Gln. The pY505 peptide binds to the Lck SH2 domain with less than five-fold reduced affinity compared with the wild-type pY324 phosphopeptide [23]. In contrast, the affinity of pY324 substituted with Gln at the pY+3 position is 24-fold reduced. Thus, it appears that Gln can only be accommodated in the pY+3 position in the context of TEGQpYQPQP, and not in the context of EPQpYEEQPIYL. Although the library screening data suggested that the Lck SH2 domain would bind to phosphopeptides with Gln in the pY+1 position it did not identify phosphopeptides with Gln in the pY+3 position as likely to be high affinity substrates.

To date, only one binding study has identified a low affinity phosphopeptide for a Src family SH2 domain [39]. In this study, the Src SH2 domain bound to the Src tail pY527 phosphopeptide 10^4-fold less well than to the hmT pY324 phosphopeptide. This is in disagreement with previous data indicating only a 44-fold difference in binding affinity for the same two phosphopeptides binding to the Src SH2 domain [23]. Recent *in vivo* data also indicate that the Src SH2 domain binds to the Src phosphorylated tail with high affinity [42,43]. But these studies used full-length Src protein, and mutational analysis suggested that an intact SH3 domain was required for high affinity. It is possible that the discrepancy observed for isolated Src SH2 domain binding to pY527 phosphopeptide can be explained by alternative SH2 domain conformations resulting from differences in experimental procedure.

In this manuscript, we identify four phosphopeptides reproducing authentic sites of tyrosine phosphorylation, which bind to the Lck SH2 domain with less than or equal to 760-fold lower affinity compared with binding to the high-affinity phosphopeptide pY324. This suggests that the Src family SH2 domains, like the PI3K p85 N-SH2 domain, can distinguish between phosphotyrosyl peptides with great selectivity.

Significance

SH2 domain binding to phosphotyrosyl peptides is thought to be important in several pathways of signal transduction, and to regulate essential cellular processes. The ubiquity of SH2 domains and of proteins that can be phosphorylated on tyrosine necessitates that the association be highly specific. Here, we have examined the effects of modifications of a tight-binding phosphopeptide on SH2 binding affinity.

Our data complement the information on the specificity of the Lck SH2 domain obtained from phosphopeptide library screening [24] and from the cocrystal structure [25]. The previously available data indicated that only pYEEI-like motifs should bind to the Lck domain with high

affinity. Comparison of these predictions with the data from our binding studies leads us to the unexpected conclusion that the amino acids C-terminal to the pTyr do not behave as independent binding determinants. Thus, quite dissimilar phosphopeptides (pYEEI and pYQPQP) are accommodated with similar affinities by the Lck SH2 domain. Furthermore, a 'favorable' residue in the context of one phosphopeptide may be unfavorable in the context of another phosphopeptide (for example, Gln at position pY+3 is a favorable residue in the peptide pYQPQP, but is unfavorable in pYEEQ). The idea that the SH2 domain is somewhat flexible is attractive in light of the emerging evidence that interaction with other domains affects SH2 domain specificity ([40–44] and G.P., D. Winkler, L.A.S. and J. Shin, unpublished data)

SH2 domains may be useful targets for strategies aimed at modulating cellular processes by disrupting specific signaling pathways. To design potent inhibitors of SH2 domain-mediated protein–protein interactions, we must first understand the structural features that result in high affinity binding. A significant amount of information on the binding of isolated Src family SH2 domains to short phosphopeptides is now available, but it seems likely that a broader understanding of SH2 domain specificity will require studies with full-length SH2 domain-containing proteins, relevant accessory molecules, and full-length phosphotyrosyl proteins.

Materials and methods

Peptide synthesis

The synthesis and characterization of phosphopeptides has previously been described in detail [22,45]. Phosphopeptides are referred to by the position of the phosphorylated tyrosine in the intact protein. Residues in the phosphopeptide are referred to in relation to the pTyr. Thus, the residues adjacent to the pTyr in the C-terminal direction are numbered pY+1, pY+2, pY+3, whereas the adjacent residues N-terminal to the pTyr are number pY-1, pY-2, pY-3.

Expression and purification of GST-SH2 domain fusion proteins

The construction of the GST fusion vectors for overexpression of GST-LckSH2 and GST-SrcSH2 have been described [23]. The GST fusion vector for overexpression of GST-FynSH2 was constructed by using the polymerase chain reaction to generate a DNA fragment encoding Fyn residues 149–252. This fragment was then inserted into the *Bam*HI-*Eco*RI site of the GST fusion vector, pGEX-2TK. The proteins were purified, characterized, and stored as described [23].

Competition assay using surface plasmon resonance

We have previously demonstrated the validity of using surface plasmon resonance (SPR) to detect GST-SH2/phosphopeptide complexes in a competition assay [23]. The theory, operation, and immobilization chemistry have been described extensively elsewhere [23,37,46,47]. Briefly, the Pharmacia BIAcore uses SPR to detect increases or decreases in mass bound to a sensor chip surface. The hmT pY324 phosphopeptide is immobilized to the sensor chip surface, and a 500nM solution of GST-SH2 domain is passed over the sensor chip. In subsequent injections, increasing concentrations of free phosphopeptide (0.1-100 μM for the pY+1 and pY+4 Ala experiments; 0.1–1 00 μM for the pY+3 Ala, Gln, and Glu, and pY+2 Ile experiments 1–2 000 μM for the pY+3 Leu, Met, and Val, and pY+4 Asn experiments) are added to the 500 nM GST-SH2 domain solution to compete with the immobilized hmT pY324. Since only the GST-SH2 protein bound to the surface pTyr is detected by SPR, the signal decreases with increasing competing phosphopeptide added. Because of the high concentration of GST-SH2 protein used, the ID_{50} values from these experiments represent relative affinities in the micromolar range rather than absolute affinities which are predicted to be in the nanomolar range [23,37,38].

Acknowledgements: This work was supported in part by funds from NIH, NSF, and Hoffmann-La Roche. We are grateful to Dr M. J. Eck for providing the coordinates of the Lck SH2 domain/pY324 cocrystal structure without which Fig. 1 would have been impossible. We also thank Dr M. Chaudhuri for assistance in phosphopeptide synthesis and M. L. LaMarche for assistance in protein purification. Finally, we would like to acknowledge helpful comments from the reviewers.

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Received: **22 Jul 1994**; revisions requested: **14 Aug 1994**; revisions received: **22 Aug 1994**. Accepted: **29 Aug 1994**.