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SH2 domain binding to phosphopeptide ligands: potential for drug targeting

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1. ABSTRACT

SH2 domains are modular components of a wide range of functionally diverse proteins involved in mammalian signal transduction including enzymes, adaptors, regulators and transcription factors. Members of the SH2 domain family recognize a wide variety of short tyrosine phosphorylated peptide motifs. Biochemical and structural studies have revealed key aspects of these interactions that account for their ability to discriminate between different sequence motifs. While the mechanism of phosphotyrosine (pTyr) recognition is remarkably conserved among the SH2 domains, differences in recognition of phosphopeptide residues N and especially C-terminal to the pTyr have been identified that contribute to selectivity. The basis for SH2- phosphopeptide recognition is discussed in light of the available structural and biochemical data with a focus on recent information regarding SH2 domains within a new class found within the signal transducer and activator of transcription (STAT) protein family.

2. INTRODUCTION

The SH2 domain was initially identified as a sequence of approximately 100 amino acids with similarity to the non-catalytic N-terminal region of Src tyrosine kinases (1). SH2 domains were later demonstrated to mediate protein-protein interactions by recognizing and directly binding to regions of peptide sequences containing phosphorylated tyrosine (2). SH2 domains are independently folding domains that play an important role in numerous signaling processes within cells and are found in a large number of modular proteins. They are involved in the regulation of kinase activity and mediate interactions between receptors and downstream signaling proteins (3). SH2 domains include more than 400 members identified on the basis of their structural fold and sequence similarity.

Aberrations in signaling pathways involving SH2 domain containing proteins have been shown to lead to different pathological conditions. There is a good deal of evidence supporting the role of SH2 containing

polypeptides in various human diseases including cancer making them suitable targets for the development of small molecule inhibitors (4-7). There has been a continued effort to reverse SH2 mediated aberrant signaling by modulating critical interactions involving SH2 domain recognition. Earlier work was largely focused on the Src tyrosine kinase related proteins and the adaptor protein Grb2. In recent years, however, work on other SH2 domains has quickly gained momentum and has become a significant area of interest for pharmacological targeting using small-molecule inhibitors. A case in point being the signal transducer and activator of transcription proteins (STATs) especially STAT3, and to a more limited extent STAT5, which have been shown to play a pivotal roles in oncogenic signaling (8, 9).

While it had long been known from earlier experiments that SH2 domains recognize a linear polypeptide sequence containing a phosphotyrosine (10), structural studies represented an important step in our understanding of SH2 phosphopeptide recognition and have provided a clear and comprehensive picture of the mechanisms of phosphopeptide recognition. However, despite the abundance of information regarding the structural determinants of SH2-phosphopeptide recognition, there has been slow progress in the development of small molecule targets for SH2 domains, in part because the mechanism of SH2-phosphopetide binding is remarkably conserved. Critical structural regions involved in phosphopeptide recognition (hot spots) that would otherwise provide an attractive target for small molecule binding are similar between many of the SH2 domains in terms of physical-chemical properties and, hence, present a challenge for the design of selective antagonist.

In this review, we look at the work that has contributed to our current knowledge on the mechanisms of SH2-phosphopeptide recognition. We will summarize the general aspects of phosphopeptide binding to SH2 proteins. While space limitations prohibit us from describing the origin of affinity and selectivity of every known SH2 domain, we will attempt to highlight the general aspects of phosphopeptide affinity and focus on a few representative proteins including the prototypical Src SH2 and STAT SH2 domains. We will also discuss recent efforts that illustrate the application of the underlying principles of phosphopeptide recognition for selective targeting of STAT SH2-phosphopeptide binding.

3. GENERAL DESCRIPTION OF THE STRUCTURE OF SH2 DOMAINS

The determinants of affinity and specificity cannot be understood without reviewing the structural data. A wealth of SH2 structural data has provided insights into the structural basis of SH2 domain-phosphopeptide recognition and has been reviewed extensively elsewhere (11). It will be summarized briefly in this section.

The structures of the SH2 domain from numerous proteins have been reported, including the prototypical Src SH2 domain. The canonical SH2 fold features a central 3 stranded antiparallel beta-sheet flanked on either face by an alpha-helix (12, 13). In most SH2 proteins, a second short 3-stranded beta-sheet (D', E and F) follows the central sheet C–terminal to beta strand D. Several loop regions connect the different elements of secondary structure. Three of these loops, BC, BG and EF, play a crucial role in phosphopeptide binding by contributing residues that form the edges of the binding pockets (11) (Figure 1A). The secondary structural elements are named according to the nomenclature established by Eck *et al.*, (14)

The phosphopeptide binding site is defined by a region on the surface of the SH2 domain that usually extends from the N-terminal helix and reaches across the central beta sheet towards the C-terminal helix on the opposite side of the molecule. (11, 12) The conserved phosphotyrosine binding pocket and the variable pocket that binds residues C-terminal to the phosphotyrosine are located on opposite sides of the plane of the central beta sheet (11, 13, 15). The N-terminal end of strand B that bares the critical Arg residue curves away from the plane of the central beta sheet toward alpha- helix A, orienting the arginine amine side chains into the phosphotyrosine binding pocket. Analysis of the complex has shown that the phosphotyrosine makes contact with this highly conserved Arg beta 5B residue at the bottom of the charged pocket (11, 12, 15).

The general architecture of the phosphopeptide binding site remains the same in most of the SH2 domains. The residues responsible for phosphopeptide binding originate from the same secondary structural elements conserved throughout all SH2 domains, namely, alpha-A, beta-B, alpha-D and the loops from B and C (11) (Figure 1A, B, C and D). Small differences in the binding sites may account for the ability of SH2 domains to discriminate between two closely related peptides. Although the general architecture remains the same in all SH2 domains there are some structural differences between various SH2 domains that arise mainly from variation in the secondary structural elements including minor insertions and deletions that change their composition and length. For instance, analysis of adaptor protein Grb2 SH2 structure reveals minor differences between Grb2 and the Src SH2 domains. It has been shown that there are variations in the length of the BG and CD loops (16). In addition the EF loop contains a notable residue, Trp 121, which plays a crucial role in the selectivity of phosphopeptide ligands (16-18). The Trp side-chain in the EF loop interacts with the phosphopeptide, forcing it to adopt a beta-turn which results in the observed preference of Grb2 SH2 proteins for N at position +2 (16, 19, 20). The +2 Asn side chain hydrogen bonds to Lys-beta-D6 of the SH2 domain. Interestingly, when Trp 121 is replaced by a less bulky residue or when introduced into a similar position in the Src SH2 domain, specificity is switched in such a way that Grb2 binds phosphopeptides in a linear conformation with preference for a hydrophobic residue at the +3 position (21). Substitution of Trp into the corresponding site within the EF loop of the Src SH2 domain changes the phosphopeptide binding preference to those with a +2 Asn

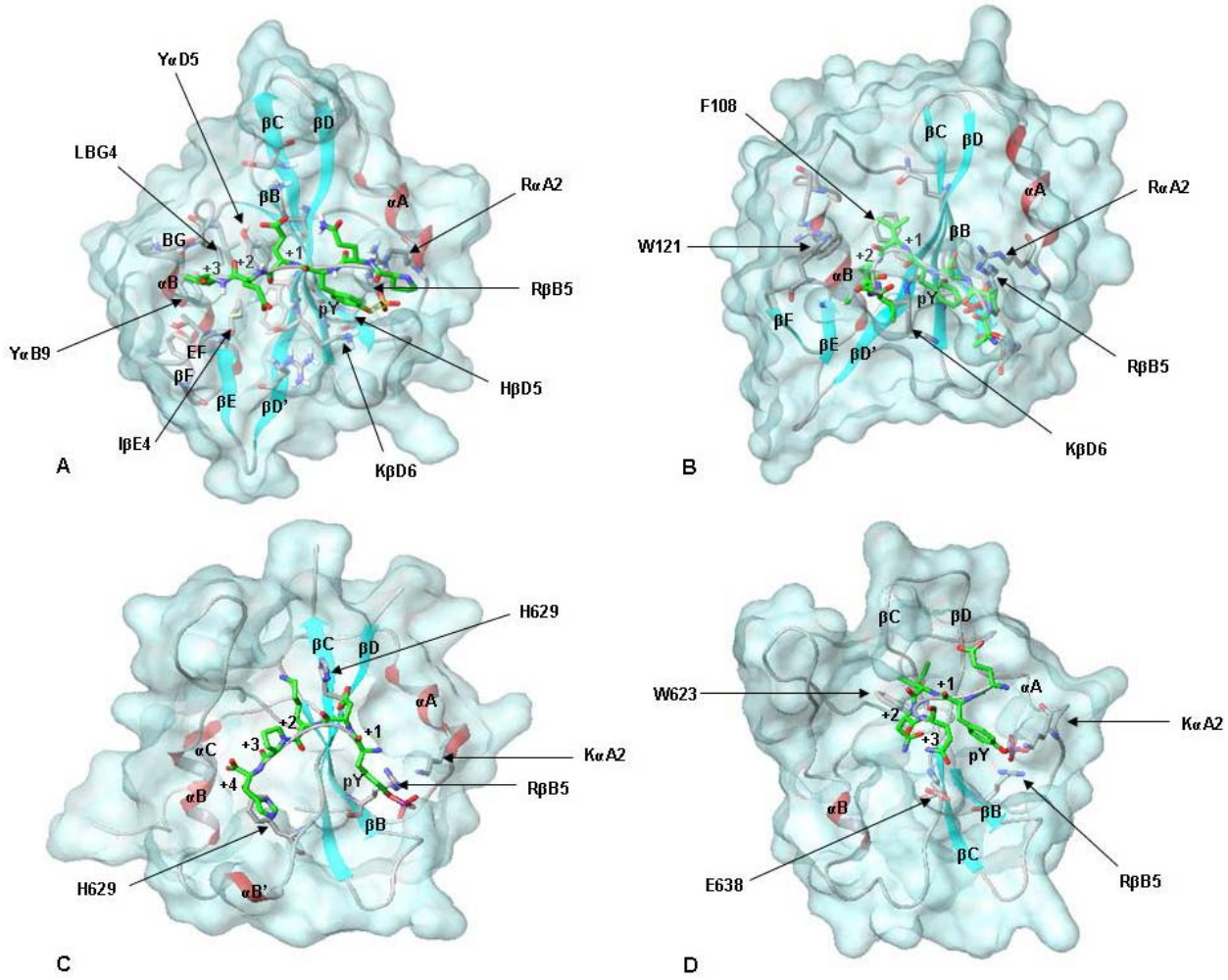

Figure 1. Structures of selected SH2-phosphopetide complexes. The overall views of the SH2 domain structures show the general "alpha, beta, beta, beta, alpha" architecture common to SH2 domains. The phosphopeptides are shown in green and the SH domain residues critical for phosphopeptide binding are shown in gray stick figures and are pointed out by the labeled arrows. A) Src SH2 domain complexed to phosphopeptide (pYEEI). Showing the phosphopeptide binding to the SH2 domain in an extended conformation .The two regions of the protein responsible for phosphopeptide binding are located on either side of the central beta sheet. Loops BG, EF and α-helix2 form the hydrophobic pocket. Arrows to the right point to a cluster of charged residues that constitute the phosphate-binding pocket. B) Phosphopeptide complex of Grb2 bound to pYINQ, showing the phosphopeptide in green forms a β-turn. The arrow to the left points to the critical residue Trp121 responsible for the β-turn conformation in the peptide. The conserved residues responsible for phosphotyrosine binding are labeled and are similar to those of the Src SH2 domain. TrpEF1 (W121) and Phe beta-D5 (F108) make van der Waals contact with the peptide. Lys beta-D6 makes critical Hydrogen bonding interactions with N at +2 position of the peptide (17). C) STAT1 complexed to phosphopeptide pYDKPH. The phosphopeptide conformation is arched. The arrows point to the critical residues involved in phosphopeptide binding. His 629 and Tyr 634 located on the D strand of the SH2 domain make Hydrogen bonding interactions side chains of Asp at +1 and His at +4 of the phosphopeptide respectively (28, 29). D) A computer-generated model of phosphopeptide EpYINQ bound to STAT3 SH2 domain. Based on the STAT3 structure (67) and the conformation of EpYINQ crystallized with Grb2. Similar to W121 the side chain of W623 prevents the peptide from assuming a linear conformation and adopts a beta- turn conformation. E638, which makes a crucial hydrogen bond interaction with Gln at position +3 of the phosphopeptide, is also shown.

(22). In addition, the phosphopeptide binds in a beta turn conformation. In contrast to the SH2 domain of Grb2 and Src, the SH2 domains of STAT proteins lack strands D, E and F common to all other SH2 domains and contain a short alpha-helix in their C-terminal ends. The SH2 domains of Nck1 and Nck2 contain a second beta-sheet (beta-F'/F"-beta) not found in other SH2 domains (23).

4. INTERACTION OF SH2 DOMAINS WITH PHOSPHOPEPTIDES

The structure of numerous SH2 domains, co-crystallized with phosphopeptide have provided abundant information on the mechanisms of phosphopeptide binding (14, 15, 24, 25). The available data shows that the majority

of phosphopeptide ligands bind with an extended conformation perpendicular to the central beta sheet. The phosphopeptide binding site has two distinct regions, one that binds phosphotyrosine at position 0 and the one that binds amino acids C-terminal to the phosphotyrosine (15). Three positively charged residues, Arg-beta-B5, Lys-beta-D6, and Arg-alpha-A2 constitute part of the charged pTyr binding pocket, other neighboring residues forming the pocket originate from strand D and the BC loop which line both sides of the pocket (12, 14). In the Src SH2 domain, and most other SH2 domains, Lys-beta-D6, His-beta-D4 and Arg-alpha-A2 make crucial contacts with the aromatic ring of the phosphotyrosine residue and are speculated to act as clamps that hold the phosphotyrosine in position (11, 26). In some SH2 domains, Arg-alpha-A2 participates in hydrogen bonding with the phosphate group in addition to the amino group of the invariant Arg-beta-B5 that rises up from the N-terminal part of beta-strand B to form the crucial ionic interaction with the phosphate group on the phosphotyrosine residue (11, 15). The critical importance of the Arg-beta-B5 residue has been confirmed using biochemical and mutational studies where the arginine was replaced with Lys and Ala resulting in loss of binding (27).

STAT SH2 domains show very little sequence similarity to other SH2 domains and yet analysis of STAT SH2 phosphotyrosine binding sites shows remarkable structural similarities to the other SH2 domains. For example, STAT1 has an equivalent arginine at 602 in beta-strand B; the BC loop region of STAT1, as observed in other SH2 domains, was also shown to make important contacts with the phosphate group of the phosphotyrosine (28). A notable difference, however, is the absence of the arginine residue originating from the N-terminal end of alpha- Helix A2. STAT SH2 domains generally contain a similar charged residue, lysine 584, at the same relative position that hydrogen bonds with the phosphate oxygen, but does not interact with the aromatic ring of the phosphotyrosine (28, 29).

Additional residues that seem to be critical for phosphopeptide binding are located on the other end of the SH2 domain across the core central beta sheet. Residues C-terminal to the phosphotyrosine that make contact with this region of the SH2 domain, have been shown to play an important role in the selectivity of SH2 domains to phosphopeptides (11).

The Src2 SH2 domain and other SH2 domains such as Lck and p85 bind phosphopeptides via a common two-pronged plug, two-holed socket mechanism where the phosphotyrosine binds in the positively charged pocket and the two highly solvent exposed residues at position +1 and +2 act as a bridge connecting the pTyr while making both direct and solvent mediated interactions with the surface of the SH2 domain across the central beta -sheet to the hydrophobic pocket into which the residue at +3 binds (14). The structural data shows that the hydrophobic pocket is formed by residues on the EF loop on one side of the pocket and residues of BG loop at the opposite side. The C terminal of α- helix B, forms the base of the hydrophobic pocket (15).

Other SH2 domains demonstrate different phosphopeptide binding mechanisms for instance, the PLC-gamma1 phosphotyrosine binding site shows a continuous groove, that begins at the pTyr binding site and extends across the central beta sheet making contact with residues at the +1 to +6 position. This is reflected by the fact that the PLC-gamma1 binds phosphopeptides with predominantly hydrophobic residues C-terminal to the pTyr (25) .

The structure of STAT1 has provided the clearest picture of the SH2-phosphopeptide binding among the STAT SH2 domains so far. A recent crystal structure of STAT1 SH2 bound to a phosphopeptide derived from the Interferon gamma receptor shows good agreement with the phosphopeptide library screening data and could explain in part the requirement for certain residues at each position downstream of the phosphotyrosine for optimum binding to occur (28). Residue His 629 within the SH2 domain makes hydrogen bond contact with the residue at +1 on the phosphopeptide. The structure also shows one feature of STAT1 SH2 phosphopeptide binding that was not anticipated, in which the phosphopeptide bound with a slightly arched conformation rather than in a linear fashion (29). (Figure 1C)

5. ENERGETICS OF SH2-PHOSPHOPEPTIDE BINDING

In order to more completely understand the nature of these interactions, additional biophysical methods have been employed to complement the available structural data. As indicated earlier, on the basis of the available crystallographic data alone, the general architectural features of the SH2 domains are remarkably conserved and in most cases there are no profound structural differences at the phosphopeptide-binding site between the different SH2 domains that could account for some of the differences in the selectivity. The Src SH2 domain has been chosen as a representative example of the many SH2 domains that have been described to date. Partly because it is the best characterized SH2 domain in this regard and provides a foundation for understanding the general aspects of this interactions. Isothermal titration calorimetry (ITC) has been a useful technique that has been used to directly characterize the affinity and enthalpy of SH2 domains binding reactions in solution (30, 31), such studies have also provided useful thermodynamic information that have been used to understand the affinity and specificity of the interactions between the SH2 domains and tyrosyl phosphate (32, 33).

5.1. Phosphotyrosine

In examining the role of phosphotyrosine in SH2-phosphopetide recognition Walksman, *et al.* found that the ionic bond between pTyr phosphate and the invariant Arg-beta-B5 of the SH2 domain was the single most crucial interaction directing all SH2 phosphopeptide affinity and contributed to the bulk of the ΔG^o of binding of pTyr phosphopeptides to SH2 domains (26, 34). This finding is also supported by the analysis of numerous mutations that were introduced into SH2 domains of various proteins. Mutations of Arg-beta-B5 dramatically reduced binding to

the phosphopeptide, resulting in a marked reduction in free energy of binding ($\Delta\Delta G^o$ = 3.2 kcal/mol) (27). In addition, dephosphorylated peptide had significantly reduced binding affinity (31, 35, 36). The substantial loss of free energy ($\Delta\Delta G^o$ = 5.5 kcal/mol) observed when pTyr at position 0 of the phosphopeptides was replaced by pSer reflects the fact that the mere presentation of the phosphate moiety is not sufficient for phosphopeptide binding but rather both the phenol ring and the phosphate group are required for binding (26). It is conceivable, in light of the available structural data, that the phenol ring plays a critical role in phosphopeptide affinity by providing crucial hydrophobic contacts that likely ensure the correct placement of the phosphate group for optimum interactions.

5.2. Residues C-terminal of phosphopeptide

Several lines of evidence have indicated that amino acids C-terminal to the phosphotyrosine play a central role in directing the affinity and selectivity of phosphopeptides towards the SH2 domains (14, 15). The energetic contributions of these residues were investigated in detail for the Src SH2 domain by ITC, where a series of phosphopeptides with substitutions at each position were subjected to calorimetric studies (37, 38). It was shown that conservative mutations had little or no effect on the observed thermodynamic parameters of Src SH2 binding to phosphopeptides. The thermodynamic parameters from analysis of substitutions at position +1 revealed that non-polar van der Waal contacts between Tyr beta-D5 and the beta methylene carbon of Glu +1, as opposed to the ionic interaction with Lys βD3 as one would expect based on the crystal structure, was critical for high affinity binding of the phosphopeptide (pYEEI). The residue at + 2 was also shown to play an indirect role in phosphopeptide binding by participating in a network of water mediated hydrogen bonding with Arg-beta-D1; this was reflected by the less favorable ΔH^o ($\Delta\Delta H^o$ = 1.4 kcal/mol) when Glu was replaced with Ala (38).

As discussed earlier, examination of the Src SH2 crystal structure revealed two pockets. A charged phosphate binding pocket and a hydrophobic binding pocket into which the Ile +3 bound and was thought to play an important role in the specificity of the phosphopeptide binding of Src SH2 domains. The contributions of this pocket were assessed using a similar approach (37). These studies generally supported the notion that the three residues down stream of pTyr contributed to the affinity and to some extend specificity of binding, with residue at +3, positions contributing the most. Whereas the Src SH2 domain selects for a hydrophobic residue at position +3, other SH2 domains such as STAT3 SH2 domain strongly selects for a polar residue at this position (39); Grb2, as discussed above, selects Asn at position +2 as the preferred residue. One would anticipate that each of these SH2 domains would yield different energetic signatures when bound to their cognate phosphopeptides.

In general, calorimetric data indicates that the binding of phosphopeptide to SH2 domain is in most cases both enthalpically and entropically driven. It has also been shown that SH2 phosphopeptide interactions of Src SH2 domain and Grb2, which demonstrate different binding modes, nevertheless share similar thermodynamic characteristics in terms of the relative contribution of ΔH^o and $-T\Delta S^o$ to binding. The hydrogen bonding interactions between the phosphotyrosine and the charged pocket of SH2 domain accounts for a large proportion of the observed ΔH^o of binding for most of the SH2-phosphopeptide interactions (26), underscoring the importance of the phosphotyrosine moiety in these interactions. In addition these studies also point to the role of water in SH2 phosphopeptide recognition and show that it is necessary to take into account the possible involvement of water when applying computational methods to look at affinity of small molecules to SH2 domains (40, 41).

6. CLASSIFICATION OF SH2 DOMAINS

Cantley and colleagues made a significant contribution to our understanding of the structural determinants of SH2 phosphopeptide recognition by introducing an elegant method for identifying high affinity phosphopeptide ligands using peptide library screens. Recombinant SH2 domains were used to affinity purify phosphopeptides from a degenerate mixture to identify optimum binding sequences for a number of SH2 domains. The bound peptides were sequenced and the preference of amino acids at each degenerate position was determined by comparing the abundance of each amino acid to that of the control. Interpreting these results in light of the available 3D-structural data and the sequence composition at the binding site of the SH2 domains revealed important aspects of phosphopeptide recognition in several SH2 domains. Examination of different of SH2 domains showed different preferences for certain amino acids at each position C-terminal to the phosphotyrosine i.e. +1, +2 and +3, which correlated with the variability of residues in the binding site of the SH2 domains. These findings allowed for the classification of the SH2 Domains into four general groups on the basis of sequence and common structural features around the phosphopeptide binding site. SH2 domains that had the same residue at position 5 of beta-strand D displayed similar phosphopeptide preferences and were classified in the same group (42, 43) .

Group I is comprised of SH2 domains mostly from Src family as well as other non-Src proteins such as Grb2, Nck, Crl and Abl, that are characterized by the presence of a tyrosine or phenylalanine at position beta D5. Most of these SH2 domains selected for a common optimal sequence pYX1X2I, where residues X+1 and X+2 were negatively charged residues and the residue at position +3 is always a hydrophobic residue Ile or Pro. The group I SH2 domains can further be classified based on the composition of the surrounding residues that contact the phosphopeptide downstream of the phosphotyrosine and select for either a proline or Ile at the +3 position (42).

Group II, which contains only the SH2 domain of the human oncogene Vav, has a Thr at the beta-D5 position and selectively binds a pYMEP motif (43). Group III, which includes p85 alpha N, PLC gamma 1 C and Shc, has either Cys or Ile at the beta-D5 position in the SH2 domain

and prefers the general motif pY-hydrophobic-X-hydrophobic. Group IV SH2 domains contain any other amino acid at the beta-D5 position and do not have a common binding motif. SH2 domains of Shb and SHPTP2 C belong to this group (42).

Other SH2 domains of particular interest such as STAT1, STAT3 and STAT5 do not clearly fall into any one group; STAT1 and 3 have a valine at the beta D5 position of the SH2 domain (44) while STAT5 has a leucine residue. Heim, *et al.* used an approach similar to the Cantley group to identify optimum binding sequences for the STAT1 and STAT3 SH2 domains (39) . STAT1 showed preference for a negatively charged amino acid at position +1 and proline or arginine at position +2 and a positively charged amino acid at position +3. STAT3, on the other hand, showed preference for a basic or hydrophobic residue at the position +1, a proline or basic residue at the position +2 and almost exclusively glutamine at position +3, which is in good agreement with other studies that had demonstrated a strong preference for a polar residue at the position +3 especially Gln (45-47). A more in-depth analysis of the STAT3 SH2 domain-phosphopeptide interaction has been undertaken by Shao *et al.* and will be discussed later in this review (46, 47).

In some instances, a single insertion within the phosphopeptide binding site dramatically changes the binding profile of a SH2 domain as in the case of Grb2 which belongs to group I but strongly selects for residues at position +2 rather than position +3, which is characteristic of Group I (21).

Whereas the criteria developed by Cantley's group for classification of the SH2 domains emphasized the importance of single residues, Honing *et al.* took a more comprehensive approach in which the energetic contribution of the residues in the vicinity of the binding site were also determined. The residues that made critical energetic contributions were mapped onto the sequence to obtain binding site signatures and formed the basis for predicting phosphopeptide selectivity. The results showed good agreement with the findings of Cantley's group and provided further justification for the use of binding site sequence variability in the classification of SH2 domain binding selectivity (48). The results also demonstrated that SH2 selectivity was determined by the variation in the composition and location of different amino acids within the binding site. How each contributes to binding and provides a basis for designing inhibitors of SH2-phosphopeptide binding as discussed later in this review.

Neighboring domains, as well as the monomeric vs. oligomeric state of SH2 domains, can also influence binding of SH2 domains to phosphopeptides and contribute to specificity. Well known examples include protein kinases Hck and Src whose central SH3 domain modulates phosphotyrosine interactions (49, 50). Another example is the SH2 domains of Nck1 and Nck2. Their SH2 domains bind to distinct proteins within the cell. Yet, crystal structures of each bound to the same phosphopeptide were identical suggesting that, within the cell, distinct protein-protein interactions modified their binding preference (23). It has also recently been shown that differential binding of two SH2 containing proteins, SH2B and APS, whose SH2 binding domains show close sequence similarity, is attributed to differences in the oligomeric state of the protein. SH2B is monomeric while APS is dimeric (51).

7. UNIQUE FEATURES OF STAT SH2 DOMAINS

STAT proteins are a group of transcription factors responsible for the cytoplasmic to nucleus signal transduction of stimuli from various cytokines and growth factors. STAT proteins play a critical role in many cellular processes including cell growth, differentiation and survival (52, 53). Recently, considerable attention has been focused on the role played by STAT proteins in particular STAT1, 3 and 5 in the pathogenesis of various human cancers making them promising pharmacological targets, especially STAT3 (54-59). All STAT proteins share a conserved modular structure that includes a C-terminal SH2 domain. Latent STAT molecules reside in the nucleus until activated in response to cytokine or growth factor stimulation. Activation of STAT proteins has been attributed to two key processes, phosphorylation and the subsequent formation of stable dimers (52). Inactive monomeric STAT proteins are activated after they are recruited via their SH2 domains to tyrosine phosphorylated cytoplasmic sequence motifs of receptor proteins. Once docked to the receptors the STAT proteins are phosphorylated at the C-terminal end of their SH2 domains then dimerize through reciprocal SH2- pTyr motif interactions. STAT dimers translocate to the nucleus where they bind to specific DNA sequences resulting in transcriptional modulation of STAT target genes (52).

In this section, we will briefly revisit STAT SH2 domains, the general molecular aspects of their interactions and implications for development of selective targeting molecules. As mentioned before although Src and STAT SH2 domains have a remarkably similar architectural fold they share little sequence similarity. It has been shown that the STAT linker-SH2 domain combination is an ancient functional domain that appeared earlier in evolution than Src-like SH2 domains (60). The discovery of a STAT protein containing a conjugated linker-SH2 domain in *Dictyostelium* lends further support to the idea that the STAT SH2 signaling system may have emerged early during phylogenesis, prior to the divergence into animals and plants (61, 62). In contrast, Src proteins have not been identified in single cell eukaryotes such as yeast.

The observation that the SH2 domain of STAT proteins did not evolve independently rather in conjunction with the linker domain, may help explain the fact that, although SH2 domains generally fold independently, STAT SH2 domains seem to be an exception. No independently functional STAT SH2 domain has been expressed to date despite numerous efforts elsewhere (63) and in our lab.

7.1. STAT1 binding models

Greenlund *et al* first identified and characterized the binding motif of STAT1 (TSFRGpYDKPHVL) within the interferon-gamma receptor (IFN- gamma R) using

Table 1. Summary of STAT protein docking motifs identified within cytokine receptor complexes and by phosphopeptide library screens

STAT	Docking motifs within receptors and signaling proteins	Docking motif identified by phosphopeptide library screening	Consensus sequence	References
1	hIFNGR1 (YDKPH)		Y-D/E-K/R-P/R/Q	(39, 64)
2	hIFNAR2c (YVFFP)		YVFF	(85)
3	Gp130 (Y^{767}RHQ) ($Y7^{814}$RHQ) ($Y7^{905}$LPQ) (Y^{915}MPQ) EGFR (Y^{1068}INQ) (Y^{1086}HNQ) G-CSFR (Y^{740}VLQ) (Y^{744}LRC) STAT3 (YLTT) (PYLKTK)	Y-basic/hydrophobic-p/basic-Q	YXXQ	(80, 86-88)
4	IL12Rβ2 (YLPSNID)		YLPS	(89)
5	EpoR (YLVL) GM-CSFR (YLSL) IL-2Rβ (Y^{510}LSL) (Y^{392}CTF) PLR (YVEI)		YLXL	(71)
6	IL-α4R (YPKF)		Y-K/Q-XF	(90)

immunoprecipitation and surface plasmon resonance (SPR) (64). Phosphopeptide library experiments described earlier in this review were used to screen for the optimum consensus binding sequence of STAT1 SH2 domain (39). It was established from these experiments that STAT1 SH2 domain had optimum binding affinity to the consensus sequence pY-D/E-P/R-R/P/Q. The crystal structures of STAT1 identified the crucial contacts between STAT1 SH2 domain and the phosphopeptide (pYDKPH) from INF gamma receptor. The residues that make contact with amino acids at positions +1 and +4 of the phophopetide ligand are shown in (Figure1C). These interactions consist of a hydrogen bond between His 629 and the residue at +1. His at position +4 of the phosphopeptide forms another critical hydrogen bond with Tyr 634. (Figure 1C). Comparing this structure to an earlier structure of STAT1 bound to a different peptide (GpYIKTEL) shows two markedly different modes of interaction with the residues C-terminal to the phosphotyrosine. The orientation of the Lys at position + 2 is reversed between the two models. The phosphotyrosine however was superimposable in both peptides and bound in an identical fashion (28, 29). These results highlight an aspect of phosphopeptide SH2 domain interactions in which the same SH2 domain employs different mechanisms to recognize different sequences.

7.2. STAT 3 binding model.

STAT3 is recruited via specific SH2-phosphotyrosine interactions to ligand activated receptors for G-CSF, IL-6 and EGF (65, 66). The STAT3 SH2 domain recognizes the consensus motif pYXXQ/T/S. (Table 1) A combination of mutational and SPR studies along with computational modeling and the available crystal structure of STAT3β homodimer bound to DNA has been used to investigate the structural determinants of STAT3 phosphopeptide interactions. Since the available crystal structure data of the STAT3 SH2-phosphopeptide complex was not well defined, X-ray (67) structures of Grb-2 and STAT1-phosphopeptide complex also have been used as templates in combination with extensive biochemical data to build a detailed model of the STAT3 SH2-phosphopeptide binding (46, 47).

Pull-down and electrophoretic mobility shift assays were used to test Y-to-F mutations on EGFR and G-CSFR and identified Y1068 and Y1086 within the EGF receptor and Y704 and Y744 within the G-CSF receptor as being responsible for the recruitment of STAT3 through its SH2 domain. Direct binding studies using SPR on dodecapeptides spanning these regions confirmed these findings. As shown in (Table 1), phosphopeptides that showed binding to the STAT3 SH2 were those containing the motif YXXQ/C with a glutamine or cysteine at the +3 position with respect to the tyrosine residue. This is consistent with observations made in phosphopeptide library screening mentioned above. SPR analysis of phosphopeptides with substitutions at positions C-terminal to the pY confirmed the requirement for Q or a polar residue at position +3 for STAT3 SH2 binding.

The original STAT3 model by Chakraborty *et al* (45) was proposed based on analogy with the two-pronged plug, two-holed socket mechanism of the prototypical Src SH2 domain that recognizes the pYXXI motif bound in an extended conformation as described earlier. The STAT3 SH2 domain recognizes the phosphopeptides pYXXQ/C/T. In this model it was predicted that phosphotyrosine made direct contact with Lys 591 and Arg 609 located in a charged pocket known to bind phosphotyrosine in other SH2 domains. The glutamine at +3, on the other hand, was thought to interact with another pocket located at the opposite end of the molecule across the central beta sheet formed by residues Glu 638, Tyr 640, and Tyr 657. It was therefore expected that a non-conservative mutation at any one of these positions would affect phosphopeptide binding. To test the validity of this model charge and/or polarity reversing mutations were performed at each of the mentioned positions. Direct binding assays (SPR and pull down) used to assess the effects of the mutations confirmed that the side chains of Lys591 and Arg 609 made important contributions to phosphopeptide binding. On the other hand, mutations in the Q+3 binding pocket of the SH2 domain did not affect binding to the

phosphopeptides containing the pYXXQ/C motif, indicating that the side chains of E638, Y640, and Y657 were not required for the selection of glutamine at the +3 position of the phosphopeptide (47).

Modeling the STAT3 SH2-phosphopeptide interactions in light of these results and reassessment of all the available structural data supported a revised model of phosphopeptide recognition in which the phosphopeptide does not bind in an extended fashion but rather adopted a beta turn conformation with the backbone amide at position E638 making significant contributions to phosphopeptide binding by hydrogen bonding with the side chain of Q at position +3 (Figure 1D). In addition, Coleman *et al* examined the contribution of the side chain chemical group on the glutamine residue and found that replacement of one or both of the side chain amide hydrogen atoms of Q at position +3 with methyl groups reduced phosphopeptide binding to the SH2 domain; the carbonyl oxygen was also found to be necessary. Taken together, these findings demonstrate that the phosphate group at position 0 and the carboxy amide group at position +3 are required for optimum binding to occur.

7.3. STAT5 binding model

STAT5 is activated by a number of cytokines (68) including erythropoietin (EPO), a cytokine involved in the regulation of red blood cell production (69). Mutational analysis of the sequence spanning Y343 (DTYLVLD) within the receptor demonstrated that this region of the receptor was responsible for STAT5 activation *in vivo* (70). Analysis of a series of phosphotyrosine motifs from a variety of receptors known to recruit STAT5 revealed that residues N-terminal of the phosphopeptide possibly played a role in affinity and selectivity of STAT5 SH2 domains. It was shown that the majority of sequences that activated STAT5 had an acidic residue at -1 or -2 relative to the phosphotyrosine. A hydrophobic residue especially leucine was favored at positions +1 and +3 (71). (Table 1) .

8. THERAPEUTIC TARGETING OF STAT PROTEINS AT THEIR SH2 DOMAINS

A significant number of human tumors have shown constitutive activation of STAT proteins especially STAT3 (72, 73). In most, if not all, instances, targeting of the constitutively activated STAT3 by forced overexpression of a dominant-negative form of STAT3 by decoy oligonucleotides or by antisense oligonucleotides resulted in tumor cell death (74-76) making them important targets for cancer therapy. STAT3 and STAT5 exert there oncogenic effects by modulating critical signaling pathways for cellular processes such as apoptosis and angiogenesis that are necessary for tumor cell proliferation and survival (77). It is imperative that when targeting the STAT3 or STAT5 signaling, other beneficial pathways are spared. For example it has been demonstrated that while STAT3 plays an important role in the signaling processes that lead to resistance to apoptosis in cells, STAT1 has been shown to play an opposing role in that it promotes apoptosis, making STAT3 pro-oncogenic and STAT1 anti-oncogenic. It is therefore conceivable that inhibition of STAT3 activity, while leaving STAT1 unaffected, would result in a synergistic effect.

The large amount of information available on SH2-phosphopeptide interactions has provided a framework for developing selective inhibitors of SH2-containing proteins such as STAT3. The strategies being used for targeting SH2-phosphopeptide interactions include peptidomimetics (78-80), G-rich quartet forming oligodeoxynucleotides (81) and small molecule inhibitors identified in chemical library screens (82-84). A number of groups have reported the development of SH2 peptidomimetic inhibitors including those that target STAT3's and show tight binding to the SH2 domains of the respective proteins and could potentially prevent the activation of STAT3 *in vivo* by directly competing with the receptor recruitment sites for SH2 domains (79) or by disrupting pTyr mediated STAT3 dimerization (78, 80) .

8.1. Selective targeting STAT3 vs. STAT1

Designing antagonists that selectivity target STAT3 SH2 domains involves exploiting the differences in the nature of the atomic interactions that cognate phosphopeptides establish with the binding surface of the each domain. Using the structural and biochemical information regarding determinants of STAT3 SH2-phosphopetide interactions discussed above and computational screening methods, Xu *et al* (manuscript submitted 2008) identified compounds that selectively targeted the STAT3 SH2 domain. A region was selected on STAT3 that included: 1) the general binding site (GBS) formed by the charged phosphotyrosine binding pocket that contains the Lys 591 conserved among the STAT SH2 domains and the invariant Arg beta-B5 (609), 2) the specific binding site formed by glutamic acid 638 located on beta strand D of the STAT 3 SH2 domain and whose backbone amide proton forms the critical hydrogen bond with Q + 3 side chain of phosphopeptide motif (pYXXQ) and 3) a hydrophobic region formed by two loops, CD connecting beta- strand C and D and loop BC that connects alpha-helix B and C constitutes the selective filter site (SFS). Using the three-dimensional structure of the SH2 domain from the core fragment structure of phosphorylated STAT3 and STAT1 homodimers bound to DNA deposited in the PDB databank (PDB code 1BG1 (67) and1BF5 (28, 29), respectively). The amide hydrogen of Glu 638 formed the central point of the targeting region selected and consisted of a cube with the dimensions 16.0 x 16.9 x 13.7 angstrom, which included the GBS and SFS.

This binding cube was screened against a chemical library from ChemBridge using ICM docking software. The docking simulation yielded a set of compounds with selective activity against STAT3 SH2 domain in cell based assays and directly competed for binding with phosphopeptide containing pYXXQ motif in SPR competition experiments. Interestingly the mode of binding that these molecules presented is analogous to phosphopeptide binding in that, one region of the compounds forms hydrogen bonding interactions with Arg 609 and Lys 591 located in the GBS and with the amide hydrogen of Glu 638. The other parts of the molecules

makes contact with the hydrophobic region, which acts as selective filter and provides a basis on which the compounds discriminates between STAT3 SH2 domain and other STAT SH2 domains, especially STAT1. Five of the six compounds identified inhibited STAT3 activity while sparing STAT1. One of the compounds, Compound 188, induced apoptosis of breast cancer cell lines, in which Stat3 was constitutively activated, in the sub-micromolar concentration range.

A similar approach has been used recently by several groups (82-84) to obtain small molecule inhibitors of STAT3 activation, STA-21, Stattic and S3I-201. These compounds were identified computationally by screening of large chemical libraries with a computer model of STAT3 X-ray structure. Biochemical assays demonstrated inhibition of STAT3 dimerization and DNA binding activity by both compounds. Importantly, both compounds induced apoptosis of human breast tumor cell lines in the micromolar range. The binding of STA-21 and S3I-201 mimicked pTyr-SH2 interactions, in that they both form hydrogen bonding contact with the critical Arg 609.

9. PERSPECTIVE

We have discussed the various aspects of affinity and selectivity of binding of phosphopeptides by SH2 domains. The SH2 domains share a common recognition mechanism in which the phosphotyrosine binds to a fairly conserved charged pocket on the SH2 domain while the residues C-terminal to the phosphotyrosine make contact with a region on the SH2 domain located across the central beta strand. Amino acid variability in or near the phosphopeptide binding site allows for the SH2 domains to discriminate between different phosphopeptides. The residues C-terminal to, and in some cases, those N-terminal to the phosphotyrosine, provide for the selectivity of the phosphopeptide binding.

Although advances in understanding the structural determinants of SH2-phosphopetide recognition have provided opportunities for the development of compounds that target SH2-phosphopeptides interactions, more work is still needed in this area. Information that would accurately characterize structural and thermodynamic binding data would lead to better binding models. Better models would, in turn, be beneficial in the identification of novel interaction partners for SH2 domains and also assist in the generation of better computer algorithms to predict ligand affinities.

Most energetic studies of SH2-phosphopeptide interactions, thus far, have focused on the Src SH2 domain. It will be of great interest to apply the same methods to dissect the energetic determinants of other SH2 domains such as those contained within STAT proteins, which are currently attracting a great deal of attention as pharmacological targets for cancer therapy.

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Abbreviations: SH2: Src homology 2; STAT: signal transducer and activator of transcription proteins; ITC: Isothermal titration calorimetry; G-CSF: Granulocyte colony stimulating factor; IL-6: Interleukin-6; EGF: Epidermal growth factor; SPR: Surface plasmon resonance; EGFR: Epidermal growth factor receptor; GSB: General binding site; SFS: selective filter site;

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