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Structural Basis for Phosphotyrosine Recognition by the Src Homology-2 Domains of the Adapter Proteins SH2-B and APS

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SH2-B, APS, and Lnk constitute a family of adapter proteins that modulate signaling by protein tyrosine kinases. These adapters contain an N-terminal dimerization region, a pleckstrin homology domain, and a C-terminal Src homology-2 (SH2) domain. SH2-B is recruited *via* its SH2 domain to various protein tyrosine kinases, including Janus kinase-2 (Jak2) and the insulin receptor. Here, we present the crystal structure at 2.35 Å resolution of the SH2 domain of SH2-B in complex with a phosphopeptide representing the SH2-B recruitment site in Jak2 (pTyr813). The structure reveals a canonical SH2 domain-phosphopeptide binding mode, but with specific recognition of a glutamate at the +1 position relative to phosphotyrosine, in addition to recognition of a hydrophobic residue at the +3 position. Biochemical studies of SH2-B and APS demonstrate that, although the SH2 domains of these two adapter proteins share 79% sequence identity, the SH2-B SH2 domain binds preferentially to Jak2, whereas the APS SH2 domain has higher affinity for the insulin receptor. This differential specificity is attributable to the difference in the oligomeric states of the two SH2 domains: monomeric for SH2-B and dimeric for APS.

Keywords: adapter proteins; SH2 domain; Jak2; insulin receptor; crystal structure

Introduction

SH2-B is a member of an adapter protein family that includes APS and Lnk.[1–3] These proteins feature an N-terminal dimerization region, a central pleckstrin homology domain, and a C-terminal Src homology-2 (SH2) domain. Four alternatively spliced isoforms (α–δ) of SH2-B have been described,[4,5] which differ in the region C-terminal to the SH2 domain. These isoforms have different expression profiles in various tissues. However, their functional differences are not known.

SH2-B has been shown to be recruited to a variety of cytokine and growth factor receptors, including the insulin receptor,[6] insulin-like growth factor-1 (IGF1) receptor,[7] TrkA,[8] platelet-derived growth factor receptor,[9] fibroblast growth factor receptor-3,[10] and growth hormone receptor.[11] In addition, SH2-B has been shown to be recruited to the Janus kinase (Jak) family of non-receptor tyrosine kinases.[11,12] Recruitment of SH2-B to these proteins is tyrosine phosphorylation-dependent and is mediated by the SH2 domain.

Several isoforms of SH2-B have been reported to be substrates of protein tyrosine kinases. For example, SH2-Bα and SH2-Bβ are recruited to and phosphorylated by the insulin receptor and Jak2, respectively.[6,11] Although the significance of SH2-Bα phosphorylation by the insulin receptor has yet to be established, the site of phosphorylation near the C terminus of the molecule is conserved in APS (Tyr618).[13] In adipocytes, phosphorylation of Tyr618 in APS leads to recruitment of Cbl and subsequently CrkII/C3G, which may serve to potentiate glucose uptake.[14] The SH2 domains of SH2-B and APS bind to the phosphorylated activation loop in the kinase domain of the insulin receptor.[6,13,15]

SH2-B has been shown to be a potent activator of Jak2 kinase activity.[16] Recently, Tyr813 in the linker between the pseudokinase and kinase domains of

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Abbreviation used: ITC, isothermal titration calorimetry.

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Jak2 was identified as an autophosphorylation site and the site of recruitment of SH2-B.[17] Although it has been suggested that the mechanism of Jak2 activation by SH2-B is through dimerization-facilitated *trans*-phosphorylation of Jak2, as mediated by the SH2-B dimerization region and SH2 domain,[18,19] other studies indicate that the (monomeric) SH2 domain alone is sufficient for Jak2 activation,[17,20] which suggests an allosteric mechanism.

The phenotypes of SH2-B-deficient mice have been characterized in several studies. SH2-B$^{-/-}$ mice show a slight growth retardation and developmental defects in gonadal organs, suggesting a positive role for SH2-B in IGF1 signaling.[21] In addition to the developmental and fertility abnormalities, the SH2-B$^{-/-}$ mice develop age-dependent insulin resistance and glucose intolerance, implying that SH2-B is a positive effector of insulin action.[22] The mechanism by which SH2-B potentiates insulin signaling is not fully understood, but might derive from protection of the phosphorylated activation loop in the insulin receptor kinase domain from dephosphorylation by protein tyrosine phosphatases. Finally, deletion of SH2-B in mice leads to impaired leptin signaling and obesity due to loss of SH2-B-mediated Jak2 activation.[23]

To understand the specificity determinants governing phosphotyrosine recognition by SH2-B, we have determined the crystal structure of the SH2 domain in complex with a phosphopeptide derived from the Jak2 pTyr813 autophosphorylation site. The structure reveals a canonical SH2 domain-phosphopeptide binding mode, with interactions that confer specificity for a glutamate residue at the P+1 position relative to phosphotyrosine, as well as for a hydrophobic residue at the P+3 position. In addition, the phosphate-binding BC loop is in a semi-open conformation, with limited contact with the phosphate group. Through biochemical studies, we demonstrate that the highly related SH2 domains of SH2-B and APS have distinct phosphotyrosine-binding properties, with SH2-B preferring pTyr813 in Jak2 and APS preferring the phosphorylated activation loop of the insulin receptor. Furthermore, we show that this binding preference is a consequence of the difference in the oligomeric state of the SH2 domains.

Results

Crystal structure of the SH2 Domain of SH2-B in complex with the Jak2 pTyr813 phosphopeptide

A synthetic 11-residue phosphopeptide based on the sequence surrounding Tyr813 in murine Jak2, ^{810}TPDpYELLTEND, was used in co-crystallization trials with the murine SH2-B SH2 domain containing three engineered amino acid substitutions: E583A (DE1; SH2 domain nomenclature taken from Eck *et al.*[24]), E584A (DE2), and W593H (βF2). The two glutamate to alanine substitutions in the DE loop were introduced to facilitate crystal packing,[25] and Trp593 was substituted with histidine (the corresponding residue in Lnk) because this tryptophan residue was predicted to be solvent-exposed. Crystals of wild-type SH2-B(SH2), either unliganded or liganded, were not obtained.

The crystal structure of the SH2-B(SH2)-Jak2 (pTyr813) complex was determined at 2.35 Å resolution. Data collection and refinement statistics are given in Table 1. The asymmetric unit of the crystal comprises six copies of the SH2 domain–phosphopeptide complex, which pack in helical ribbons (non-crystallographic 12_5 screw axis) parallel with the crystallographic *z* axis.

The SH2-B SH2 domain adopts the canonical (monomeric) SH2 domain architecture,[26] with a central four-stranded β sheet (βA/βG, βB, βC, and βD) flanked by two α helices (αA and αB) (Figure 1(a)). The central β sheet and the C-terminal α helix (αB) are linked by a smaller β sheet comprising βD′, βE, and βF. The Jak2 phosphopeptide inserts into two pockets in the surface of the SH2 domain: a phosphate-binding pocket for pTyr813 and a hydrophobic pocket for the P+3 residue, Leu816 (Figure 1(a)).

We also determined the crystal structure of the unliganded SH2-B SH2 domain at 2.0 Å resolution (crystallographic statistics are given in Table 1). As expected, the overall structures of the unliganded and

Table 1. X-ray data collection and refinement statistics

	SH2-B(SH2)	SH2-B(SH2)-Jak2
A. *Data collection*		
Resolution (Å)	50–2.00	50–2.35
R_{sym} [a]	0.044 (0.130)	0.072 (0.225)
Observations (>1σ)	54,759	155,919
$\langle I/\sigma I \rangle$	27.3 (10.2)	16.1 (5.4)
Completeness (%)	99.2 (97.6)	98.1 (90.1)
Redundancy	3.9 (3.3)	4.7 (3.8)
B. *Refinement*[b]		
Resolution (Å)	30.0–2.00	30–2.35
No. reflections	14,185	32,298
R_{cryst} [c]	21.0	21.1
R_{free}	24.3	24.7
B-factors (Å^2)		
Protein	22.1	27.2
Phosphopeptide	N/A	31.7
Solvent	31.5	32.6
r.m.s. deviation from ideal		
Bond lengths (Å)	0.005	0.006
Bond angles (deg.)	1.2	1.4
B-factors[d] (Å^2)	0.86	1.5

Values in parentheses are for the highest resolution shell: 2.06–1.98 Å for SH2-B(SH2) and 2.43–2.35 Å for SH2-B(SH2)-Jak2(pTyr-813).

[a] $R_{sym} = 100\ \Sigma|I - \langle I \rangle|/\Sigma\ I$.

[b] Atomic model for unliganded SH2-B(SH2) structure: 1568 protein atoms and 139 solvent (water) atoms; for SH2-B(SH2)-Jak2 (pTyr-813) structure: 4971 protein atoms, 414 phosphopeptide atoms, and 336 solvent atoms.

[c] $R_{cryst} = 100\ \Sigma||F_o|-|F_c||/\Sigma\ |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively ($F_o > 0\sigma$). R_{free} was determined from 5% of the data not used in the refinement.

[d] For bonded atoms.

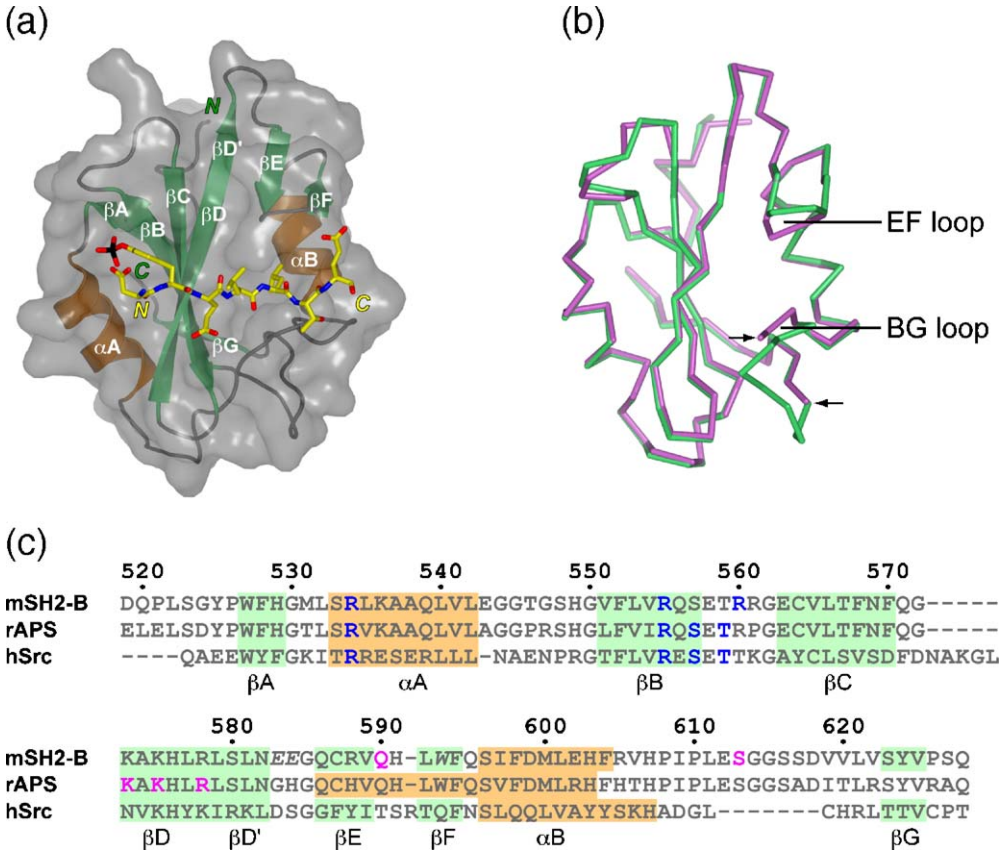

Figure 1. Crystal structure of SH2-B(SH2) in complex with the Jak2 pTyr813 phosphopeptide. (a) Ribbon diagram of the SH2 domain of SH2-B with the bound Jak2 phosphopeptide shown in stick representation. The secondary structural elements for the SH2 domain are labeled, with α-helices colored orange, β strands green, and loops gray. A semi-transparent surface for the SH2 domain is shown in gray. Carbon atoms in the Jak2 phosphopeptide are colored yellow, oxygen atoms red, nitrogen atoms blue, and phosphate atoms black. The N and C termini for the SH2 domain and the phosphopeptide are indicated by *N* and *C*, colored either green (SH2-B(SH2)) or yellow (Jak2 phosphopeptide). (b) Superposition (C^{α} atoms) of the structures of unliganded (purple) and liganded (green) SH2–B(SH2). The regions of significant conformational changes, EF and BG loops, are labeled, and the region in the BG loop that is disordered in the unliganded structure is indicated by arrows. (c) Structure-based sequence alignment of the SH2 domains from murine SH2-B, rat APS, and human Src. Residue numbering is for murine SH2–Bγ. Residues in β strands are shaded green and residues in α helices are shaded orange. Deletions are denoted by –. Residues that coordinate the phosphotyrosine in the crystal structures (PDB codes APS, **1RQQ**; Src, **1SPS**) are colored blue, and residues that make hydrogen bonds to other residues of the phosphopeptide are colored magenta. Residues mutated in SH2-B for crystallization are shown in italics (E583A, E584A, W593H). (Figures 1(a) and (b), 2, and 6) were rendered with PyMOL (http://pymol.sourceforge.net).

liganded SH2-B SH2 domain are quite similar (Figure 1(b)), with a root-mean-square deviation (r.m.s.d.) in C^{α} atoms (102) of 0.67 Å. The significant structural changes that occur upon phosphopeptide binding are the ordering of the BG loop and a movement of the EF loop away from the BG loop, to accommodate binding of Leu816 (P+3) of the phosphopeptide. In the absence of the phosphopeptide, the P+3 binding pocket is not apparent in the structure; a partially formed P+3 pocket was observed previously in the crystal structure of the unliganded Src SH2 domain.[27] A structure-based sequence alignment for the SH2 domains of SH2-B, APS, and Src is given in Figure 1(c).

The specific interactions between the SH2-B SH2 domain and the Jak2 phosphopeptide (Figure 2(a)) are similar to those observed in the structures of the Src and Lck SH2 domains with bound pYEEI phosphopeptide,[24,27] but with several notable differences. The phosphate group of pTyr813 of the Jak2 phosphopeptide binds in the canonical phosphate-binding pocket of the SH2-B SH2 domain, salt-bridged to SH2-invariant Arg555 (βB5) and Arg534 (αA2). Arg534 is also hydrogen bonded to the carbonyl oxygen atom of Asp812 (P-1). The BC loop, which in most SH2 domain-phosphopeptide structures (including those of Src/Lck) is hydrogen bonded directly to the phosphate group, is in a semi-open state (in all six copies in the asymmetric unit) and makes only water-mediated hydrogen bonds to the phosphate group (Figure 2(b), left). Curiously, in the crystal structure of the highly related (79% sequence identity) APS SH2 domain bound to the insulin receptor kinase (IRK),[15] the BC loop is in the standard closed state, with Thr441 (BC2) and the backbone nitrogen atoms of Glu440 (BC1) and Thr441 hydrogen bonded directly to the phosphate

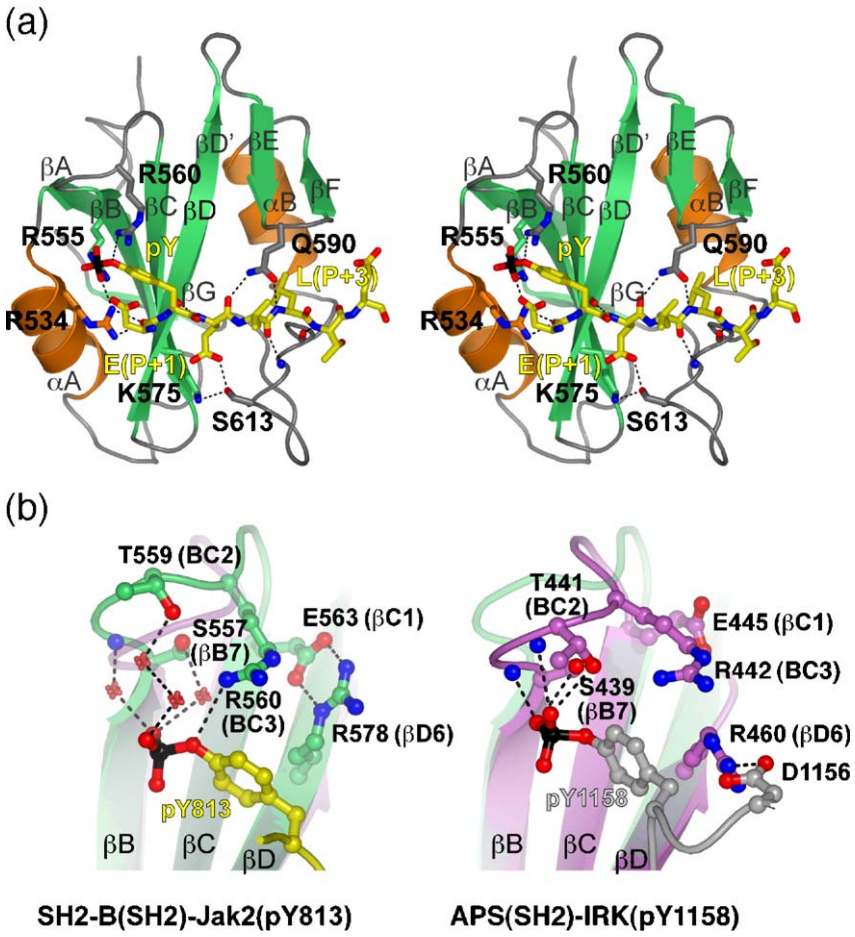

Figure 2. Specific interactions between the SH2-B SH2 domain and the Jak2 pTyr813 phosphopeptide. (a) Stereo view of the interactions between the SH2-B SH2 domain and the Jak2 phosphopeptide. Carbon atoms of SH2-B are colored according to secondary structure, and all other atoms are colored as in Figure 1(a). Hydrogen bonds/salt-bridges are represented by broken black lines. (b) Interactions between the phosphate-binding BC loops of SH2-B (left) and APS (right)[15] and pTyr813 of Jak2 and pTyr1158 of IRK, respectively. SH2-B is colored green and APS is colored purple. Shown semi-transparently on the left is superimposed APS (purple), and on the right, superimposed SH2-B (green). Water molecules are shown by red crosses, and hydrogen bonds/salt-bridges are represented by broken black lines. Selected side-chains are shown in ball-and-stick representation.

group of pTyr1158 (Figure 2(b), right). For SH2-B, loss of these direct hydrogen bonds appears to be compensated by Arg560 (BC3), which is hydrogen bonded to the bridging oxygen atom of the phosphotyrosine (Figure 2(b), left).

C-terminal to the phosphotyrosine, the P+1 residue, Glu814, is hydrogen bonded to Ser613 (BG6), which in turn is hydrogen bonded to Lys575 (βD3) (Figure 2(a)). Glu814 and Lys575 are 3.6 Å apart ($O^{\varepsilon 2}$–N^{ζ}). In the Src and Lck SH2 domain structures,[24,27] there is no direct hydrogen bond between the SH2 domain and the P+1 glutamate of the pYEEI phosphopeptide. Gln590 (EF1) in SH2-B makes two hydrogen bonds with the phosphopeptide backbone: the carbonyl oxygen atom of Glu814 (P+1) and the amide nitrogen atom of Leu816 (P+3). The P+3 hydrophobic pocket in SH2-B comprises Leu577 (βD4), Val589 (βE4), Leu592 (βF1), Ile609 (BG2), Pro610 (BG3), and Leu611 (BG4).

Several backbone–backbone interactions complete the intermolecular contacts. The canonical backbone hydrogen bond between the βD4 residue (His576, carbonyl oxygen) and the P+1 residue (Glu814, amide nitrogen) is present. In addition, two backbone atoms of the BG loop, the carbonyl oxygen atom of Pro610 (BG3) and the amide nitrogen atom of Glu612 (BG5), are hydrogen bonded to Thr817 (P+4, amide nitrogen) and Leu815 (P+2, carbonyl oxygen), respectively (Figure 2(a)).

Binding affinity measurements by isothermal titration calorimetry

To quantitfy the binding affinity of the SH2-B SH2 domain for the Jak2 pTyr813 phosphopeptide, we employed isothermal titration calorimetry (ITC). The dissociation constant (K_d) for the interaction was measured to be 0.55 μM (Table 1), an affinity comparable to that of the Src SH2 domain for the pYEEI phosphopeptide as determined previously by ITC.[28,29] Of note, the SH2-B (SH2) triple mutant used for crystallization has the same affinity for the Jak2 phosphopeptide as wild-type SH2-B(SH2) (Table 1). Mutation of Gln590 (EF1) to alanine resulted in an approximately sevenfold loss of affinity (K_d = 3.94 μM) (Table 2), confirming the significance of the hydrogen-bonding interactions made by Gln590 in the crystal structure (Figure 2(a)).

Biochemical studies of the oligomerization properties of the SH2 domains of SH2-B and APS

The SH2-B(SH2) crystal structure shows that, like conventional SH2 domains, this SH2 domain is a functional monomer. In contrast, the structure of the highly related APS SH2 domain is dimeric, with a non-canonical C-terminal half of the molecule (see structural alignment in Figure 1(c)).[15] To explore the

oligomeric state of these SH2 domains in a cellular context, we performed co-immunoprecipitation experiments. Because full-length APS and SH2-B have a dimerization region near their N termini,[18] and thus will dimerize irrespective of the SH2 domain, we used one SH2 domain-only construct (Flag-tagged) and one full-length construct (Myc-tagged). In addition, for a more direct comparison of the APS and SH2-B SH2 domains, instead of using full-length SH2-B, we used an APS/SH2-B chimera, in which the SH2-B SH2 domain was substituted for the APS SH2 domain in otherwise full-length APS. The Flag-tagged SH2 domains and the Myc-tagged full-length proteins were transiently transfected into COS cells. As shown in Figure 3, the APS SH2 domain showed a much stronger tendency to self associate than the SH2-B SH2 domain. Mutation of Trp475, which is situated in the APS SH2 dimerization interface,[15] reduced the ability of APS to oligomerize, although not completely (Figure 3, lower blot). These co-immunoprecipitation results are consistent with those of previous gel-filtration chromatography experiments.[15]

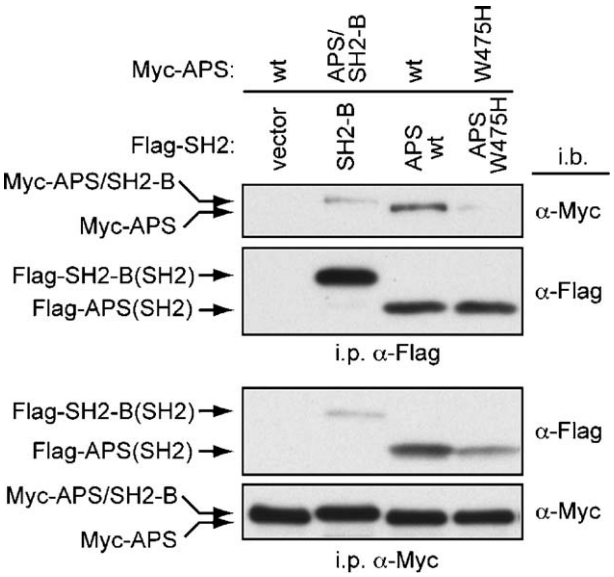

Figure 3. Oligomerization properties of the SH2 domains of SH2-B and APS. Flag-tagged SH2 domains, from either SH2-B or APS, and Myc-tagged full-length molecules, either APS or the APS/SH2-B chimera, were co-transfected into COS cells. Clarified cell lysates were immunoprecipitated (i.p.) with antibodies against either the Flag tag (top two blots) or the Myc tag (bottom two blots) and immunoblotted (i.b.) with both antibodies. Of note, despite a molecular mass difference of only 140 Da between SH2-B(SH2) and APS(SH2), SH2-B(SH2) has a visibly slower electrophoretic mobility.

Biochemical studies of the interactions of the SH2 domains of SH2-B and APS with Jak2

To verify that the interactions observed in the crystal structure between the SH2-B SH2 domain and the Jak2 pTyr813 phosphopeptide are important in a cellular context, select mutations were introduced into a Flag-tagged version of murine SH2-B(SH2) and into full-length murine Jak2. Wild-type or mutant SH2-B(SH2) and Jak2 were transiently transfected into COS cells and co-immunoprecipitation experiments were performed. Mutation of Tyr813 to phenylalanine in Jak2 resulted in loss of interaction with SH2-B(SH2) (Figure 4(a)), consistent with the previous identification of pTyr813 as the site of SH2-B recruitment.[17] Alanine substitutions at Glu814 (P+1) and Leu816 (P+3) in Jak2 also markedly reduced the co-immunoprecipitation level, in concert with the structural findings. Mutation of Gln590 (EF1) in SH2-B results in a significant loss of interaction with Jak2 (Figure 4(a)), consistent with the ITC results (Table 2), and the crystal structure (Figure 2a).

APS as well as SH2-B has been demonstrated to interact with Jaks.[12] To determine the relative strength of interaction of these two SH2 domains with Jak2, we co-transfected either Flag-tagged SH2-B(SH2) or APS(SH2) into COS cells along with wild-type Jak2 and performed co-immunoprecipitation experiments. The co-immunoprecipitation level of Jak2 with SH2-B(SH2) is considerably higher than with APS(SH2) (Figure 4(b)). The monomerizing W475H mutation in APS(SH2) resulted in an increase in the interaction with Jak2, although not to the level of SH2-B(SH2), perhaps because this mutant exhibits residual dimerization potential (Figure 3, lower blot).

Biochemical studies of the interactions of the SH2 domains of SH2-B and APS with the insulin receptor

We next compared binding of the SH2 domains of SH2-B and APS to the activated insulin receptor by transfecting Flag-tagged SH2-B(SH2) or APS(SH2) into CHO-IR cells, stimulating the cells with insulin, and performing co-immunoprecipitation experiments. As shown in Figure 5(a), the APS(SH2) interaction with the activated insulin receptor is robust, the W475H mutation in APS

Table 2. Measurement of SH2-B(SH2)-Jak2 phosphopeptide binding affinities by ITC

	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
Wild-type	0.55±0.010	−8.54	−11.95±0.078	−3.41
E583A (DE1)/E584A (DE2)/W593H (βF2)	0.59±0.056	−8.50	−12.26±0.15	−3.76
Q590A (EF1)	3.94±0.83	−7.37	−9.81±0.09	−2.44
R560A (BC3)	1.77±0.33	−7.85	−11.74±0.09	−3.89
R578A (βD6)	0.62±0.060	−8.48	−15.37±0.16	−6.89

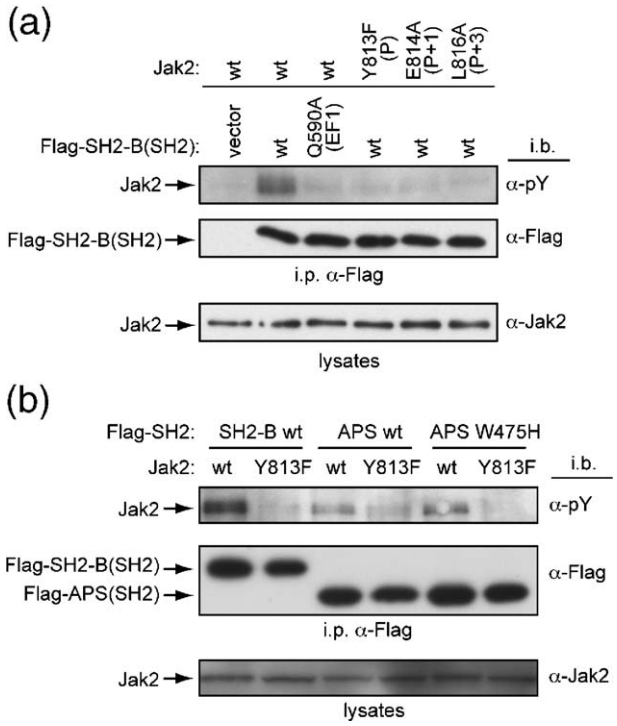

Figure 4. Interaction of the SH2 domains of SH2-B and APS with Jak2. (a) Site-directed mutagenesis of SH2-B and Jak2. Flag-tagged SH2-B(SH2), wild-type or mutant, was transfected into COS cells along with wild-type or mutant Jak2. Cells were lysed and immunoprecipitated (i.p.) with anti-Flag antibodies. Immunoprecipitates were immunoblotted (i.b.) using antibodies against phosphotyrosine (pY, top blot) for the co-immunoprecipitation level with Jak2, and against the Flag tag (middle blot) to show approximately equal amounts of immunoprecipitated SH2-B(SH2). The lysates were immunoblotted with anti-Jak2 antibodies (bottom blot) to show approximately equal amounts of Jak2. The identification of the bands is indicated on the left-hand side of the blots. (b) Same co-immunoprecipitation experiments as in (a), except comparing the SH2 domains of SH2-B and APS (wild-type and W475H mutant).

results in a considerably lower level of interaction, and the interaction of SH2-B(SH2) with the receptor is not detectable under these experimental conditions.

Although co-immunoprecipitation between the SH2-B SH2 domain and the insulin receptor is difficult to detect, *in vitro* pulldown experiments using His-tagged SH2-B(SH2) and CHO-IR lysates can be used to probe the binding contributions of residues in the SH2 domain. We mutated two arginine residues in SH2-B(SH2), Arg560 (BC3) and Arg578 (βD6), to alanine. In the crystal structure of the SH2-B(SH2)-Jak2(pTyr813) complex, Arg560 is hydrogen bonded directly to the phosphate group and Arg578 is salt-bridged to Glu563 (βC1) (Figure 2(b), left). The equivalent of Arg578 in APS, Arg460, is salt-bridged to Asp1156 of the IRK activation loop (Figure 2(b), right). The R560A mutation in SH2-B resulted in a decrease in binding to the insulin receptor, while the R578A mutation had no measurable effect (Figure 5(b)). These pulldown results with the insulin receptor are similar to the ITC results obtained for these mutants in the context of Jak2 phosphopeptide binding (Table 2). Mutation of Gln590 (EF1) to alanine did not diminish the association of SH2-B(SH2) with the insulin receptor (Figure 5(b)), which demonstrates that, C-terminal to the phosphotyrosine residue, the mode of binding of the IRK activation loop to SH2-B(SH2) differs from the mode of binding of Jak2.

The same mutations were introduced into the APS SH2 domain and co-immunoprecipitation

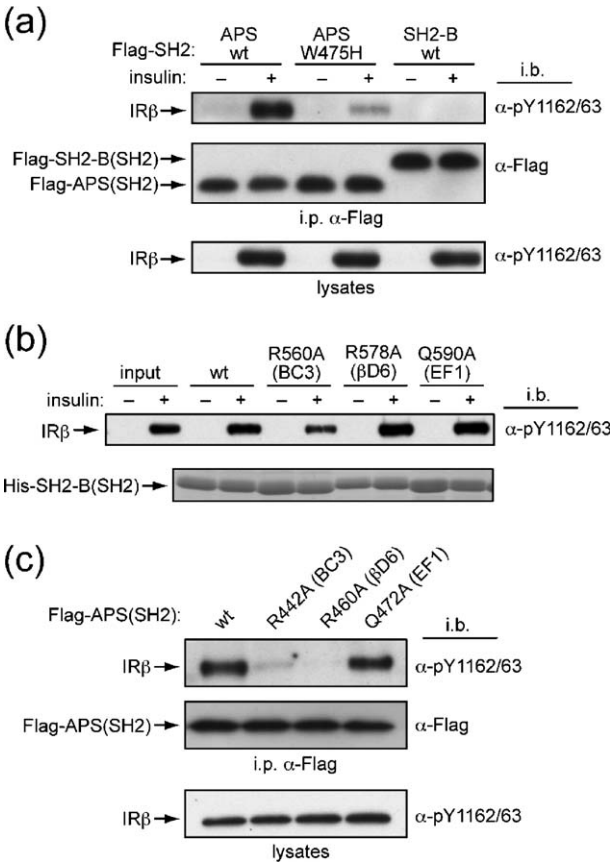

Figure 5. Interaction of the SH2 domains of SH2-B and APS with the insulin receptor. (a) CHO-IR cells were transfected with the indicated Flag-tagged SH2 domains. Cells were treated with or without 100 nM insulin for 5 min. Clarified lysates were immunoprecipitated (i.p.) with antibodies against the Flag tag. Antibodies used for immunoblotting (i.b.) are shown on the right-hand side of the blots (α-pY1162/63 is a phosphospecific insulin receptor antibody), and protein identification is supplied on the left-hand side. (b) *In vitro* pull-down assay of His-tagged SH2-B(SH2), wild-type or mutant, and the activated insulin receptor. The amount of insulin receptor in the pulldowns was detected by the phosphospecific anti-pY1162/63 antibody (top blot), and the SH2-B(SH2) input level in the pulldowns was shown by staining with Coomassie brilliant blue (bottom gel). (c) Co-immunoprecipitation of the activated insulin receptor by wild-type or mutant APS(SH2), using the same procedures as in (a).

experiments were performed with CHO-IR cells. In contrast to the results for R578A in SH2-B, mutation of Arg460 to alanine in APS resulted in a dramatic loss of binding to the insulin receptor (Figure 5(c)), consistent with participation of Arg460 in a salt-bridge with Asp1156 of the activation loop (Figure 2(b), right). Somewhat surprisingly, the R442A (BC3) mutation also impaired binding to the insulin receptor, even though this arginine in the crystal structure does not directly contact the phosphate group of pTyr1158 (Figure 2(b), right). As expected from the APS(SH2)-IRK structure, and similar to the result for SH2-B, mutation of Gln472 (EF1) to alanine had no effect on insulin receptor binding (Figure 5(c)).

Discussion

The crystal structure presented here of the SH2-B SH2 domain in complex with the Jak2 pTyr813 phosphopeptide reveals the molecular basis for recruitment of SH2-B to Jak2. Although the binding mode would be classified as canonical–engagement of the phosphotyrosine and the P+3 hydrophobic residue in two binding pockets in the SH2 domain–there are several features that distinguish the particulars of this binding mode from those observed in previous SH2 domain-phosphopeptide structures.

Firstly, there is specificity for glutamate at the P+1 position of the Jak2 pTyr813 recruitment site (pYELL) (Figure 4(a)). Although the Src SH2 domain binds with high affinity to a pYEEI phosphopeptide, there is no preference for glutamate over alanine at the P+1 position.[30] The difference in P+1 specificity is evidently due to the lack of hydrogen bonding to this residue by the Src SH2 domain.[24,27] Although the βD3 lysine in Src is important for electrostatic screening of the P+1 glutamate from aspartate residues (at βC8 and CD2) in the SH2 domain,[31] it does not interact directly with the glutamate, whereas Lys575 (βD3) in SH2-B is proximal (3.6 Å), if not salt-bridged, to Glu814 (P+1) of the Jak2 phosphopeptide. Moreover, Lys575 is hydrogen bonded to Ser613 (BG6) in the BG loop, which is hydrogen bonded directly to Glu814 (Figure 2(a)). The BG loop in SH2-B contains a six-residue insertion relative to Src (Figure 1(c)).

Secondly, the EF1 residue in the SH2-B SH2 domain, Gln590, makes two hydrogen bonds with the phosphopeptide backbone (Figure 2(a)), which are important for high-affinity binding of the Jak2 phosphopeptide (Figure 4(a) and Table 2). With its side-chain amide group, glutamine is uniquely capable of making these backbone interactions from the EF1 position (asparagine is too short). To the best of our knowledge, hydrogen bonding between the EF1 residue and the bound phosphopeptide has not been observed previously. Of note, the EF1 residue in the Grb2 SH2 domain is tryptophan, which forces the phosphopeptide to turn away from the surface of the SH2 domain[32] (in a manner distinct from that of the APS SH2 domain discussed below) and selects for asparagine at the P+2 position of the phosphopeptide (pYxN, where x is any amino acid).

Finally, in the SH2-B(SH2)-Jak2(pTyr813) crystal structure, the BC loop of SH2-B(SH2) is not in direct contact with the phosphate group, save for Arg560 (BC3) (Figure 2(b), left). This is reminiscent of the mode of phosphate binding observed for the C-terminal SH2 domain of Syk, in which the BC loop is in an open configuration and an arginine at βB7, the residue preceding the BC loop, coordinates the phosphate group.[33] The similarity between Syk and SH2-B extends to a common arginine at βD6, which in SH2-B (Arg578) is salt-bridged to Glu563 (βC1), and in Syk is hydrogen bonded to serine at βC1.

It appears from the SH2-B(SH2) structure that a bulky residue at the BC3 position (Arg560) combined with the Glu563–Arg578 salt-bridge might sterically impair BC loop closure; Arg560 pivots towards the salt-bridge upon loop closure (Figure 2(b), left). Although Arg560, Glu563 and Arg578 are conserved in APS, the BC loop in APS, particularly Thr441 (BC2), is observed to interact directly with the phosphate group of pTyr1158 in the IRK activation loop (Figure 2(b), right). The mutagenesis data for Arg442 (BC3) in APS (Figure 5(c)) and Thr559 (BC2) in SH2-B (data not shown), argue that these residues make greater contributions to phosphotyrosine binding than their respective crystal structures would indicate (Figure 2(b), left and right). This suggests that, in solution, the BC loop in this adapter protein family is in equilibrium between the closed and semi-open states with the phosphotyrosine bound.

Despite nearly 80% sequence identity between the SH2 domains of SH2-B and APS, their phosphotyrosine binding preferences are distinct and are a consequence of their oligomeric states. The SH2-B SH2 domain is monomeric, adopts a canonical SH2 domain architecture, and preferentially binds a conventional phosphotyrosine sequence (e.g., Jak2 pTyr813) containing a hydrophobic residue at the P+3 position, with the phosphopeptide oriented perpendicular to the central β sheet of the SH2 domain (Figures 1(a) and 6(a)). In this binding mode, Gln590 (EF1) is hydrogen bonded to the phosphopeptide backbone (Figure 2(a)).

The SH2 domain of Lnk, the other member of this adapter protein family, is predicted to possess SH2-B-like properties. Lnk lacks the tryptophan at βF2 (Trp475 in APS), which is critical for APS SH2 dimerization. One potentially important difference between Lnk and SH2-B is that Lnk contains cysteine at BG6 instead of serine (Ser613 in SH2-B), which could result in loss of selectivity for glutamate at the P+1 position of the phosphotyrosine target (Figure 2(a)).

In contrast to the SH2-B and Lnk SH2 domains, the APS SH2 domain is dimeric, with a rearranged

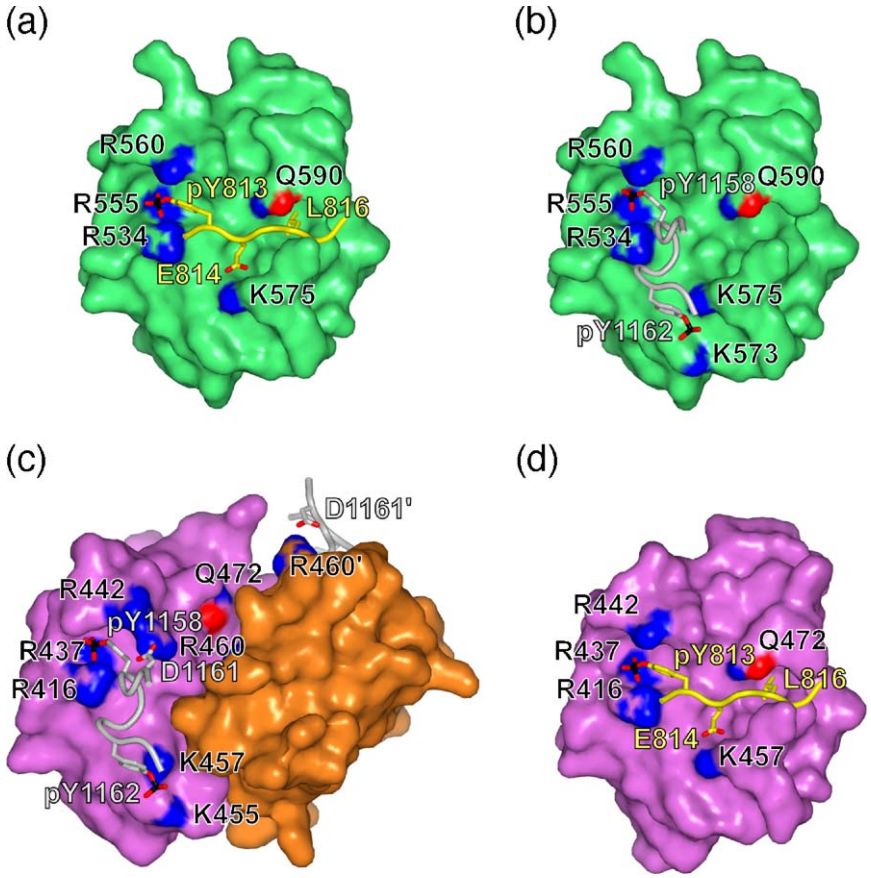

Figure 6. Modes of phosphotyrosine binding to the SH2 domains of SH2-B and APS. Surface representations are shown for (a) the crystal structure of SH2-B(SH2) bound to the Jak2 pTyr813 phosphopeptide, (b) a model of SH2-B(SH2) bound to the activation loop of IRK, (c) the crystal structure of APS(SH2) bound to the activation loop of IRK,[15] and (d) a model of APS(SH2) bound to the Jak2 pTyr813 phosphopeptide. The Jak2 phosphopeptide is colored yellow and the activation loop of IRK is colored gray. Nitrogen and oxygen atoms in SH2 domain residues implicated in phosphopeptide binding are colored blue and red, respectively. The side-chains of select phosphopeptide residues are shown in stick representation, with oxygen atoms colored red and phosphorus atoms colored black.

C-terminal half of the molecule.[15] In the APS SH2 dimer, the extended C-terminal α helix from one protomer blocks off the perpendicular mode of phosphopeptide binding to the other protomer, with concomitant loss of the P+3 binding pocket (Figure 6(c)). This dimeric configuration will favor binding of a phosphotyrosine sequence that can stabilize a turn after the phosphotyrosine and bind parallel with the central β sheet, the mode observed in the APS(SH2)-IRK crystal structure (Figure 6(c)).[15] In this binding mode, loss of P+3 binding is compensated through binding of a second phosphotyrosine residue (pTyr1162) in the IRK activation loop by lysine residues at βD1 and βD3.

The sequence differences between the APS and SH2-B SH2 domains that confer dimerization to the former but not the latter are not completely understood. SH2-B as well as APS possesses the key tryptophan residue for dimerization at βF2 (Trp475 in APS). Residue differences at αB2 (Ile597 in SH2-B), near the end of the BG loop, and in βB (Figure 1(c)) all contribute to thwart dimerization of SH2-B(SH2) (J.H and S.R.H., unpublished results). Thus, although conversion of the APS SH2 domain from dimer to monomer can be nearly accomplished by a single amino acid change (W475H), conversion of the SH2-B SH2 domain from monomer to dimer requires numerous SH2-B to APS substitutions.

Although the "perpendicular" and "parallel" phosphopeptide-binding modes are optimal for monomeric SH2-B(SH2) and dimeric APS(SH2), respectively, these SH2 domains are capable of binding phosphopeptide sequences *via* the alternative binding mode. For APS, binding of a conventional phosphotyrosine ligand such as pTyr813 (pYELL) in Jak2 (Figure 4(b)) or pTyr936 (pYSNL) in c-Kit[34] requires conversion to a canonical monomeric (SH2-B-like) form, the energetic cost of which is likely the principal reason why the SH2-B SH2 domain binds better to such ligands than the APS SH2 domain (Figure 4(b)). Instead of coordinating pTyr1162 of the IRK activation loop, Lys457 (βD3) together with Ser495 (BG6) will contribute to binding of the P+1 glutamate, if present, and Gln472 (EF1) will hydrogen bond to the phosphopeptide backbone (Figure 6(d)).

The SH2-B SH2 domain binds to the IRK activation loop as a monomer, with the APS-conserved lysine residues at βD1 and βD3 predicted to engage pTyr1162 in the IRK activation loop (Figure 6(b)). Gln590 (EF1) is dispensable in this binding mode (Figure 5(b)). The monomeric SH2-B SH2 domain has a lower affinity for the insulin receptor than the dimeric APS SH2 domain (Figure 5(b)), for reasons other than avidity; full-length APS (and SH2-B) is dimeric owing to the N-terminal dimerization region,[18] yet APS binds to the insulin receptor more strongly than the APS/SH2-B chimera in which APS(SH2) is replaced by SH2-B(SH2) (data not shown).

Several factors are likely to be responsible for this difference in binding affinity. Arg460 in APS is capable of salt-bridging with Asp1156 of the IRK activation loop (Figures 2(b), right, and 5(c)), whereas Arg578 of SH2-B is evidently not capable (Figure 5(b)). The salt-bridge with Glu573 could prevent Arg578 from interacting with Asp1156 (Figure 2(b), left); dimerization of the APS SH2 domain might alter slightly the position of Glu445 (Glu573 in SH2-B), weakening the salt-bridge with Arg460 (Arg578 in SH2-B). Secondly, in the context of the APS SH2 dimer, Lys457 (βD3) is partially buried/constrained,[15] which would result in a stronger interaction with pTyr1162 in the activation loop than the solvent-exposed βD3 lysine in monomeric SH2-B. It is also possible that the APS SH2 dimer, which is more compact than two individual SH2 domains, fits better into the confines of the two β subunits of the activated insulin receptor.

In summary, through structural and biochemical studies, we have determined how two highly related SH2 domains in the same adapter protein family achieve distinct phosphotyrosine-binding preferences. The monomeric SH2-B SH2 domain binds selectively to a canonical phosphotyrosine sequence, whereas dimerization of the APS SH2 domain switches specificity to the multiply-phosphorylated, turn-containing activation loop of the insulin receptor.

Materials and Methods

Constructs, protein production, and phosphopeptide synthesis

A vector encoding Myc-tagged rat APS (pRK5-Myc-APS) was kindly provided by Dr A. Saltiel. The APS/SH2-B chimera was constructed by replacing the APS SH2 domain (rat, residues 405–511) with the SH2-B SH2 domain (mouse, residues 523–629) as a HindIII/XbaI fragment in pRK5-Myc-APS. The HindIII and XbaI sites were introduced into pRK5-Myc-APS by site-directed mutagenesis. After ligation of the SH2-B(SH2) cDNA into these sites, site-directed mutagenesis was used to restore the original sequences at the cloning junctions. The pCDNA6-Jak2 vector was kindly provided by Dr S. Shoelson. Flag-tagged constructs of SH2-B(SH2) (residues 519–628) and APS(SH2) (residues 401–510) were engineered by cloning EcoRI/XbaI fragments into a pCMV2-Flag vector (Sigma-Aldrich). All point mutations were generated using QuikChange (Stratagene) and verified by automated DNA sequencing.

The SH2 domain of murine SH2-B, residues 519–628, was subcloned from the γ isoform (kindly provided by Dr K. Nelms) into pET14b (Novagen). SH2-B(SH2) was expressed in *Escherichia coli* strain BL21(DE3) and purified as described.[15] An 11-residue phosphopeptide representing the murine Jak2 pTyr813 site, TPDpYELLTEND, was synthesized (GeneMed Synthesis) and solubilized in 100 mM Tris–HCl (pH 8.0), 100 mM NaCl.

X-ray crystallography

Crystals of unliganded SH2-B(SH2) (triple mutant: E583A, E584A, and W593H) were grown at 22 °C using the hanging-drop, vapor-diffusion method. SH2-B(SH2) (1 μl at 15 mg/ml) was mixed with 1 μl of reservoir buffer containing 30% (w/v) PEG 4000, 0.1 M Tris–HCl (pH 8.5), 0.2 M $MgCl_2$. The crystals belong to space group $P2_12_12_1$ with cell dimensions of $a=36.05$ Å, $b=57.87$ Å, and $c=99.47$ Å. There are two SH2-B(SH2) molecules in the asymmetric unit, yielding a solvent content of 40% (v/v).

The same SH2-B(SH2) preparation was mixed with the Jak2 phosphopeptide at a 1:1.5 molar ratio. Crystals of the complex were grown at 4 °C in hanging drops by mixing 1 μl of SH2-B(SH2)-Jak2 phosphopeptide stock solution and 1 μl of reservoir buffer (25% PEG 4000, 0.1 M sodium citrate (pH 5.6), 0.3 M ammonium acetate). The crystals belong to space group $P2_12_12_1$ with cell dimensions of $a=44.21$ Å, $b=74.19$ Å, and $c=39.22$ Å. There are six copies of the SH2-B(SH2)-Jak2 phosphopeptide complex in the asymmetric unit, yielding a solvent content of 48% (v/v).

All data were collected on beamline X4A at the National Synchrotron Light Source and were processed using HKL-2000.[35] For unliganded SH2-B(SH2), a molecular replacement solution was found with AmoRE,[36] using the N-terminal portion of the APS SH2 domain (residues 404–461) (PDB code **1RPY**)[15] as a search model. The atomic model for SH2-B(SH2) includes residues 518–627 in copy 1, except for residues 611–617 (BG loop), and includes residues 520–627 in copy 2, except for residues 613–617. The r.m.s.d. of SH2-B(SH2) C^α atoms (101) is 0.63; the only significant deviation is found at the N terminus.

The resulting SH2-B(SH2) structure was used for molecular replacement to find initially five copies of SH2-B(SH2) in the asymmetric unit of the complex crystal. Examination of difference Fourier ($2F_o-F_c/F_o-F_c$) electron density maps calculated with phasing from the five copies of SH2-B(SH2) revealed density for a sixth copy, which was positioned manually and then rigid-body refined. The six copies of the Jak2 phosphopeptide were built into difference Fourier maps. Model building and refinement were performed using O[37] and CNS,[38] respectively. The atomic model for the SH2-B(SH2)-Jak2(pTyr813) complex includes SH2-B residues 520–627, except for residues 614–616 (BG loop) in two copies and residues 614–617 in one copy, and includes Jak2 residues 810–818 in two copies and residues 812–818 in the remaining four copies. Using one copy of the SH2-B(SH2)-Jak2(pTyr813) complex as a reference to compare with the other five copies, the r.m.s.d. values of SH2-B(SH2) C^α atoms (104) fall between 0.31 Å and 0.47 Å.

Isothermal titration calorimetry

The SH2-B SH2 domain, wild-type or mutant, was dialyzed overnight in the same buffer into which the Jak2 phosphopeptide was dissolved (100 mM Tris–HCl (pH 8.0), 100 mM NaCl). Protein concentrations were determined by measuring UV absorbance at 280 nm, and peptide concentrations were determined gravimetrically and confirmed by measuring UV absorbance at 280 nm. Calorimetric measurements were conducted at 25 °C with a VP-ITC calorimeter (MicroCal). Each titration experiment consisted of 29 10 μl injections (5 μl for the first injection) of the Jak2 phosphopeptide (typically at 0.3 mM) into the calorimetric cell containing 1.4 ml of the SH2-B SH2 domain (typically at 0.03 mM), with a 5 min interval between each injection. Titration data were processed using ORIGIN software (MicroCal). A small heat of dilution, calculated from the last several injections, was subtracted prior to curve fitting. The thermal constants were derived using a one-site binding model for fitting, varying N (binding stoichiometry), K_d (dissociation constant), and ΔH (reaction enthalpy). From these values, the free energy (ΔG) and entropy change (ΔS) upon peptide binding can be calculated using the equation:

$$-RT\ln(1/K_d) = \Delta G = \Delta H - T\Delta S$$

where R is the universal molar gas constant and T is the absolute temperature.

Mammalian cell transfection, immunoprecipitation and immunoblotting

CHO-IR cells were maintained in α-minimal essential medium containing 10% (v/v) fetal bovine serum (FBS) and antibiotics. COS cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics. Before treatment with insulin, CHO-IR cells were serum-deprived for 3 h in F-12 Ham's medium. CHO-IR and COS cells were transfected using Lipofectamine 2000 (Invitrogen). Cells in 60 mm dishes were washed twice with ice-cold phosphate-buffered saline and were lysed for 30 min at 4 °C with 50 mM Tris–HCl (pH 8.0), 135 mM NaCl, 1% (v/v) Triton X-100, 1.0 mM EDTA, 1.0 mM sodium pyrophosphate, 1.0 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors (Roche Diagnostics; one tablet per 7 ml of buffer). The clarified lysates were incubated with antibodies to the Myc (9E10, Santa Cruz) or Flag (M2, Sigma-Aldrich) tag for 1 h at 4 °C. The immune complexes were precipitated with protein A/G agarose (Santa Cruz) for 1 h at 4 °C and were washed extensively with lysis buffer before solubilization in SDS-PAGE sample buffer. Bound proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). Individual proteins were detected with specific antibodies and visualized by blotting with horseradish peroxidase-conjugated secondary antibodies (BioSource).

In vitro pulldown assays

His-tagged SH2-B SH2 domain, wild-type or mutant, was immobilized on Ni-NTA beads (Sigma-Aldrich) by incubating the protein and the beads for 0.5 h at 4 °C. Insulin-stimulated CHO-IR cell lysates were prepared as described above, except that the lysis buffer contained no EDTA. Lysates and beads were mixed and incubated for 1 h at 4 °C. Beads were washed extensively with the lysis buffer and then resuspended in SDS-PAGE buffer.

Protein Data Bank accession codes

Atomic coordinates and structure factors for unliganded SH2-B(SH2) and the SH2-B(SH2)-Jak2(pTyr813) complex have been deposited in the Protein Data Bank with accession codes 2HDV and 2HDX, respectively.

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