Novel mode of ligand binding by the SH2 domain of the human XLP disease gene product SAP/SH2D1A

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Background: The Src homology 2 (SH2) domains of cytoplasmic signaling proteins generally bind phosphotyrosine (pTyr) sites in the context of carboxy-terminal residues. SAP (also known as SH2D1A or DSHP), the product of the gene that is mutated in human X-linked lymphoproliferative (XLP) disease, comprises almost exclusively a single SH2 domain, which may modulate T-cell signaling by engaging T-cell co-activators such as SLAM, thereby blocking binding of other signaling proteins that contain SH2 domains. The SAP–SLAM interaction can occur in a phosphorylation-independent manner.

Results: To characterize the interaction between SAP and SLAM, we synthesized peptides corresponding to the SAP-binding site at residue Y281 in SLAM. Both phosphorylated and non-phosphorylated versions of an 11-residue SLAM peptide bound SAP, with dissociation constants of 150 nM and 330 nM, respectively. SLAM phosphopeptides that were truncated either at the amino or carboxyl terminus bound with high affinity to SAP, suggesting that the SAP SH2 domain recognizes both amino-terminal and carboxy-terminal sequences relative to the pTyr residue. These results were confirmed by nuclear magnetic resonance (NMR) studies on [15]N- and ^{13}C-labeled SAP complexed with three SLAM peptides: an amino-terminally truncated phosphopeptide, a carboxy-terminally truncated phosphopeptide and a non-phosphorylated Tyr-containing full-length peptide.

Conclusions: The SAP SH2 domain has a unique specificity. Not only does it bind peptides in a phosphorylation-independent manner, it also recognizes a pTyr residue either preceded by amino-terminal residues or followed by carboxy-terminal residues. We propose that the three 'prongs' of a peptide ligand (the amino and carboxyl termini and the pTyr) can engage the SAP SH2 domain, accounting for its unusual properties. These data point to the flexibility of modular protein-interaction domains.

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Background

Signaling by protein tyrosine kinases involves binding of Src homology 2 (SH2) domains to specific phosphotyrosine (pTyr)-containing motifs, such as those found on activated cell-surface receptors or associated subunits [1,2]. SH2 domains are usually located in larger polypeptides, with additional interaction or catalytic modules that regulate intracellular signaling pathways. Typically, the association of SH2 domains with Tyr-containing motifs depends on phosphorylation of the ligand and, indeed, the majority of the binding energy comes from the interaction of pTyr with a conserved pocket on the SH2 domain [3,4]. Additional contacts are provided by SH2 residues that recognize 3–6 amino acids immediately carboxy terminal to the pTyr, in a fashion that varies from one SH2 domain to another [4–7]. This ability to discriminate between different pTyr motifs provides an important element of specificity in signaling by tyrosine kinases [8].

Although they lack intrinsic catalytic activity, cytokine and immune receptors commonly signal through associated tyrosine kinases, which initiate a complex series of SH2-mediated interactions required both for T-cell or B-cell activation [9,10] and for attenuation of receptor signaling [11]. Intriguingly, an SH2 domain protein (variously named SAP, SH2D1A and DSHP) expressed in T cells has recently been shown to play a crucial and unique role in the immune response to Epstein–Barr virus (EBV) infection [12–14].

SAP is encoded by the gene mutated in X-linked lymphoproliferative disease (XLP), or Duncan disease. Males with XLP mutations are unable to control an EBV infection, which consequently induces an unregulated expansion of polyclonal B and T cells, accompanied by either severe infectious mononucleosis, acquired hypogamma-globulinemia or malignant lymphoma, and invariably

results in death [15,16]. The SAP protein consists almost entirely of a single SH2 domain, flanked by 5 amino-terminal and 26 carboxy-terminal amino acids. A number of XLP mutations have been described, including deletions and stop codons that truncate the SH2 domain [12–14], as well as missense mutations that affect the Arg32, Thr53 and Val102 SH2 residues [13]. SAP is expressed in T cells, rather than B cells, suggesting that its role lies in regulating the ability of T cells to control EBV-infected B cells [12–14]. As SAP lacks any obvious effector domain, it may compete for the binding of other SH2 proteins to pTyr-containing motifs, and thereby modulate the intensity and duration of T-cell signaling. Consistent with this possibility, SAP was also identified as a protein that binds a Tyr-containing motif within the T-cell co-activator signalling lymphocyte-activation molecule (SLAM). In keeping with the model that SAP may obstruct the access of other SH2-containing proteins to pTyr motifs, SAP inhibits binding of the Shp2 tyrosine phosphatase to phosphorylated SLAM [12]. Intriguingly, however, although SAP binds to a specific site flanking Tyr281 in the SLAM cytoplasmic region, phosphorylation appears not to be essential for stable binding. These data suggest that the SAP SH2 domain has an unusual binding specificity. Here, we probed the mechanisms by which SAP may bind to both phosphorylated and non-phosphorylated peptides.

Results

The SAP SH2 domain binds with high affinity to both Tyr- and pTyr-containing SLAM peptides

To characterize the SAP–SLAM interaction in more detail, we synthesized a non-phosphorylated peptide corresponding to residues 276–286 in SLAM. We termed this peptide n–Y–c to denote the fact that it contains stretches of both amino-terminal and carboxy-terminal residues flanking the central Tyr281 site. For the purpose of comparison, we also synthesized a pTyr-containing phosphopeptide, pY–c, that consists primarily of pTyr281 and following carboxy-terminal residues (amino acids 279–286 of SLAM). The peptide pY–c resembles a conventional SH2 domain ligand, as it contains the sequence pTyr–Ala–Gln–Val typical of motifs recognized by group

II SH2 domains [6,17]. Each of these peptides was labeled with fluorescein at its amino terminus through a linker of 3–4 hydrophilic residues (Table 1). We performed fluorescence polarization studies on the fluorescein-labeled peptides, F–n–Y–c and F–pY–c, by adding varying concentrations of purified, recombinant SAP protein. Both peptides F–n–Y–c and F–pY–c bound strongly to SAP in a concentration-dependent manner (Figure 1a), and reached saturation at micromolar concentrations of SAP protein, suggesting a single-site, bimolecular interaction in each case. The dissociation constants of the peptide–SAP complexes were 150 nM for the pTyr-containing peptide F–pY–c and 330 nM for the Tyr-containing peptide F–n–Y–c. These results clearly contrast with those from other SH2 domains where at least a 1000-fold difference in affinity is usually observed between the pTyr- and Tyr-containing peptides [3].

The SH2 domain is responsible for SAP binding to these peptides, as a glutathione-S-transferase (GST) fusion protein containing residues 1–103, corresponding to the SH2 domain, bound efficiently to both peptides F–n–Y–c and F–pY–c (Figure 1b), whereas the GST protein alone did not (data not shown). The dissociation constants of these two interactions were estimated as 510 and 240 nM, respectively (Table 1). Although these values are slightly higher than those for wild-type SAP, the full-length protein fused to GST also produced K_d values higher than isolated SAP (Table 1). As discussed below, nuclear magnetic resonance (NMR) titration experiments indicated that SAP residues carboxy-terminal to the SH2 domain were not directly involved in peptide binding. Therefore, phosphorylated and non-phosphorylated peptides specifically recognize the SAP SH2 domain.

The SAP SH2 domain recognizes both amino-terminal and carboxy-terminal sequences relative to the pTyr residue

To explore the basis for the ability of SAP to bind both phosphorylated and non-phosphorylated peptides, we synthesized variants of the n–Y–c SLAM peptide with substitutions or truncations, and measured their relative affinities for the SAP SH2 domain by determining their
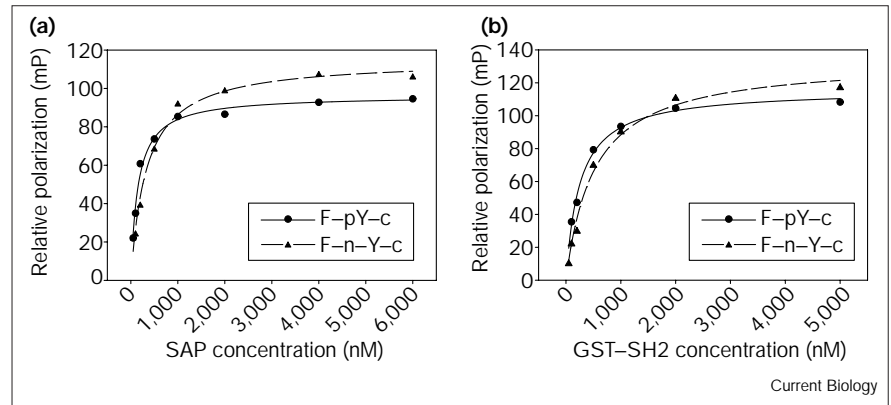
Table 1

Affinities of fluorescein-labeled SLAM peptides for the SAP protein and its SH2 domain.

Peptide	Sequence	Dissociation constant (K_d, nM)		
		SAP	GST–SAP	GST–SH2
F–n–Y–c	Fluorescein–GGR–*KSLTIYAQVQK*	330 ± 40	444 ± 50	510 ± 30
F–pY–c	Fluorescein– GGKK–*TIpYAQVQK*	150 ± 10	174 ± 22	240 ± 20

Peptide sequences corresponding to residues taken from sequences flanking the Tyr281 site (shown in bold) in SLAM are italicized. Residues amino terminal to SLAM peptide sequences were added to increase peptide solubility in water and to serve as linker sequences for the attachment of the fluorescein moiety [32]. Reported K_d values are averages over at least two independent experiments.

Figure 1

Binding of **(a)** SAP and **(b)** the SH2 domain of SAP fused to GST to peptides F–pY–c and F–n–Y–c, as measured by fluorescein polarization (in milli-polarization units, mP). Independent experiments as shown were used to calculate the dissociation constants reported in Table 1.

ability to compete for SAP binding to the fluorescein-labeled F–n–Y–c peptide. A peptide in which the Tyr residue of peptide n–Y–c was replaced with pTyr (n–pY–c) displayed a significantly higher affinity for the SAP protein than the unphosphorylated n–Y–c peptide (Table 2). This result suggests that, although tyrosine phosphorylation is not essential for SAP binding to an extended peptide motif, it nonetheless increases the affinity of the target peptide. Nevertheless, the pTyr residue in peptide n–pY–c does not play as significant a role as in other SH2 domain ligands, where it is absolutely required for stable binding. This idea is supported by results from two other peptides, n–F–c and n–A–c, in which the Tyr281 residue of peptide n–Y–c was replaced by Phe or Ala, respectively. Peptide n–F–c exhibited only a moderate decrease in binding affinity compared with peptide n–Y–c, while peptide n–A–c retained over 10% of the affinity observed for the parent peptide. These results suggest that the SAP SH2 domain can bind peptide sequences devoid of Tyr.

The observation that peptide n–A–c still binds appreciably to SAP implies that the amino-terminal and carboxy-terminal residues in peptide n–Y–c may interact with the SAP SH2 domain. To probe their relative contributions to SAP binding, we synthesized peptides with either amino-terminal or the carboxy-terminal residues truncated relative to peptide n–Y–c. These peptides, n–Y and Y–c, were essentially inactive in SAP binding, suggesting that neither the amino-terminal nor the carboxy-terminal sequence alone is sufficient for pTyr-independent SH2 binding. Strikingly, however, the phosphorylated versions of these two peptides both bound strongly to SAP (Table 2, Figure 2a). Indeed, peptide n–pY competed twice as efficiently as peptide n–Y–c for SAP binding, despite lacking carboxy-terminal residues that would normally be required for recognition by an SH2 domain. The observation that peptide n–pY bound more tightly to SAP

than either peptide pY–c or peptide Ac–pY–c suggests that the amino-terminal sequence of the peptide is critical for high affinity SAP binding (Table 2). This may also explain the decreased affinities of peptides pY–c and Ac–pY–c compared with that of the fluorescein-labeled peptide F–pY–c, which contains an extra amino-terminal extension, GGKK, that could contribute to SAP binding.

As peptides pY–c and n–pY have only limited overlap beyond the pTyr site, we tested whether they could
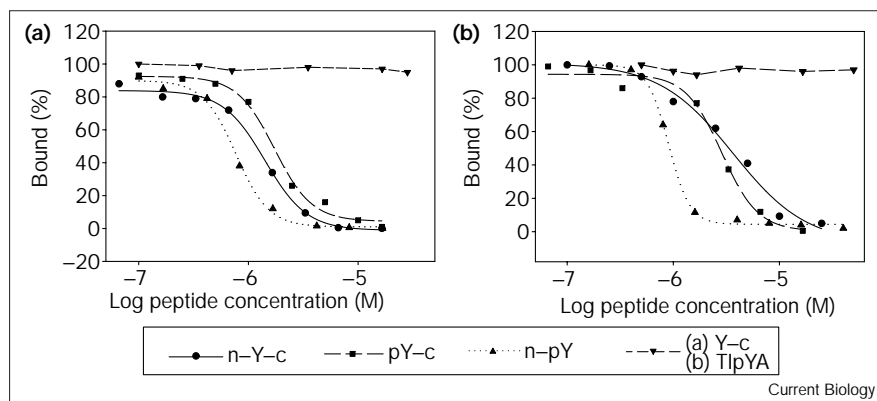
Table 2

Relative affinities of the SLAM Y281 peptides.

Peptides	Sequence	Relative affinity (%)*
n–Y–c	RKSLTI **Y**AQVQK	100
n–pY–c	KKSLTI**pY**AQVQK	465 ± 24
n–F–c	RKSLTI **F**AQVQK	84 ± 9
n–A–c	RKSLTI **A**AQVQK	13 ± 5
Y–c	TI **Y**AQVQK	< 1
pY–c	TI**pY**AQVQK	76 ± 10
Ac–pY–c	Ac–I**pY**AQVQ	72 ± 14
n–Y	RKSLTI **YA**	< 1
n–pY	RKSLTI**pYA**	225 ± 28
TIpYA	TI**pYA**	ND[†]

*Relative affinities of the peptides were calculated according to their corresponding IC_{50} values (effective concentration for a 50% inhibition of binding) derived from the competition experiments. Relative affinities were normalized to that of peptide n–Y–c, set to 100%. Reported numbers are based on two independent experiments. The (p)Y281 residue of SLAM and its substituted residues are highlighted in bold. Another version of peptide n–Y–c was also synthesized which contains a K instead of an R residue at the amino terminus. The two peptides produced identical IC_{50} values within experiment errors (data not shown). [†]Not determined; affinity was too low to be quantified.

Figure 2

Free peptide competition for SAP binding to fluorescein-labeled peptides **(a)** F–n–Y–c and **(b)** F–pY–c. Data from a set of representative experiments are shown. Repeated experiments as shown were conducted to generate the IC_{50} values used for the calculation of relative affinities reported in Table 2. The data shown are representative but not inclusive.

compete against each other for SAP binding (Figure 2b). Peptide n–pY competed effectively for SAP binding to the fluorescein-labeled peptide F–pY–c, suggesting that these peptides engage overlapping sites on the SAP SH2 domain. Peptide n–Y–c could also displace peptide F–pY–c for complex formation with SAP. Intriguingly, peptide n–Y–c was slightly less effective than peptide pY–c in inhibiting the interaction of peptide F–pY–c with SAP (Figure 2b), in contrast to the result obtained when peptide F–n–Y–c was used (Table 2). To probe the importance of the pTyr residue, we synthesized a short phosphopeptide, TIpYA, which proved incapable of competing for SAP binding (Figure 2b; Table 2). Collectively, the results from peptide competition studies suggest that two of the three elements, namely an intact amino terminus, an intact carboxyl terminus and a pTyr residue, must be present in a SLAM peptide for tight binding to the SAP SH2 domain.
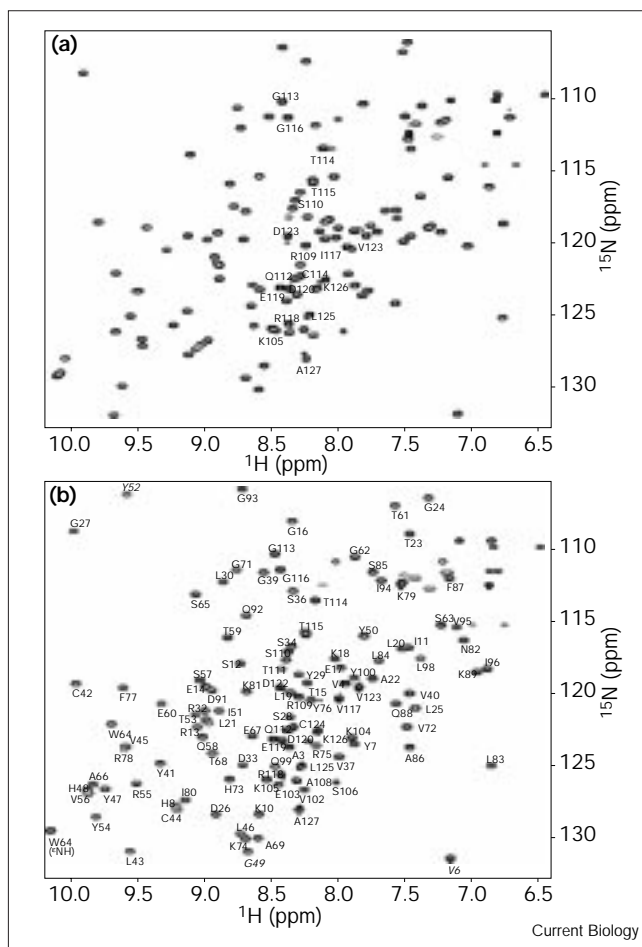
NMR studies on SAP–SLAM peptide complexes

To gain further insight into the mechanism of peptide recognition by the SAP SH2 domain, we labeled the SAP protein with ^{13}C and ^{15}N, and performed NMR analysis on SAP and its complexes with various SLAM peptides. A heteronuclear single quantum correlation (HSQC) spectrum recorded on a free SAP sample, which correlates its amide ^{15}N resonance with those of its amide ^{1}H, displayed features of a folded protein. Although most of the amide resonances were well dispersed along the ^{1}H dimension, some sharp peaks were, however, found centered within a narrow region around 8.2 ppm in the ^{1}H dimension (Figure 3a), suggesting that residues corresponding to these peaks are in an unfolded conformation. To identify these residues, we titrated peptide n–Y–c into a SAP sample that was labeled with both ^{13}C and ^{15}N, with the aim of assigning the amide resonances of the protein. Addition of the n–Y–c peptide into the protein sample in a stepwise fashion induced drastic changes in

the HSQC spectra. Peaks corresponding to the free protein gradually disappeared and, concomitantly, peaks belonging to the peptide-complexed protein emerged (data not shown). The resonances of the free and complexed protein species are therefore in slow exchange (of the order of seconds). Interestingly, while many amide peaks exhibited large chemical-shift changes from the free to peptide-complexed state, the sharp peaks identified in Figure 3a were essentially unperturbed by the addition of peptide n–Y–c (Figure 3b). Upon backbone assignment of the SAP protein complexed with peptide n–Y–c, these peaks were identified as originating from residues 105–127 in the SAP protein. These results suggest that the carboxy-terminal tail of SAP beyond its SH2 domain is unstructured and does not participate directly in peptide interactions.

The secondary structural elements of the SAP protein were subsequently predicted based on the deviations of chemical shifts of the H_α, C_α, CO and C_β nuclei for each residue from those of a random coil [18]. The combination of the ^{1}H and ^{13}C chemical-shift indices allowed for a consensus estimation of the secondary structures [18,19] of the SAP protein, as shown in Figure 4. Specifically, two α-helical regions (residues 13–24 and 80–86) and six β-strands (residues 28–32, 40–47, 50–54, 56–59, 61–66 and 75–77) were identified. The carboxy-terminal residues of SAP were found in a random coil conformation as predicted from NMR titration studies. The boundaries of the secondary structures identified through these chemical-shift indices agree very well with those predicted by sequence alignment [12] against the Abl SH2 domain, whose structure is known [20]. As the SAP SH2 domain shares 35% sequence identity with the Abl SH2 domain, it is likely that they have similar structures. Preliminary nuclear Overhauser effect (NOE) assignments on the SAP–n–Y–c complex identified a number of medium- and long-range NOEs that are consistent
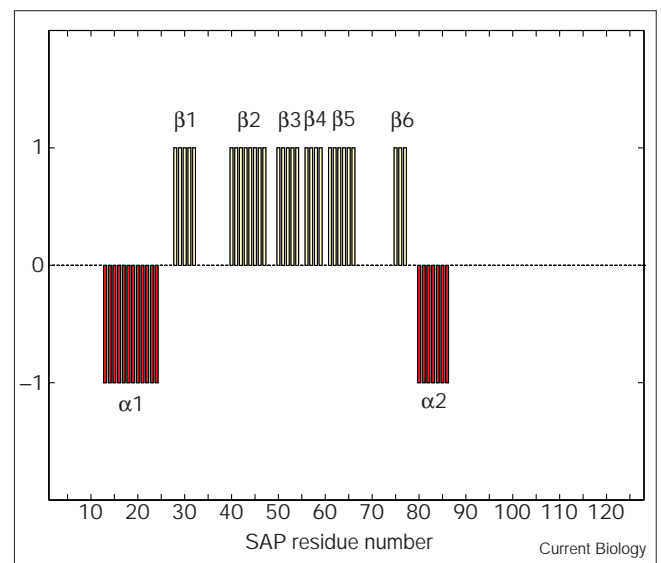
Figure 3

HSQC (¹H-¹⁵N) spectra of the ¹³C,¹⁵N-labeled SAP protein in **(a)** free and **(b)** complexed states with peptide n–Y–c. Peaks corresponding to the carboxy-terminal residues (105–128) of the protein are labeled in (a), whereas a complete assignment of the backbone amide resonances of the protein is shown in (b). Note that the side chains of the Gln, Asn and Arg residues are not assigned. Peaks corresponding to Val6, Gly49 and Tyr52 are folded along the ¹⁵N dimension and are denoted as italicized letters in (b). In addition, amide resonances of Gly9 and Glu35 are shifted outside the displaying window in the complexed state and are thus not shown in (b).

Figure 4

Prediction of SAP secondary structure based on chemical-shift indices [29,30] of ¹H, ¹³CO, ¹³Cα and ¹³Cβ resonances of the SAP–n–Y–c complex. Residues identified as belonging to a β-strand were assigned a value of +1 and depicted as yellow stripes, while those belonging to an α-helix were given a value of –1 and depicted as red stripes in the diagram. Residues belonging to neither strand or helix were given a value of 0.
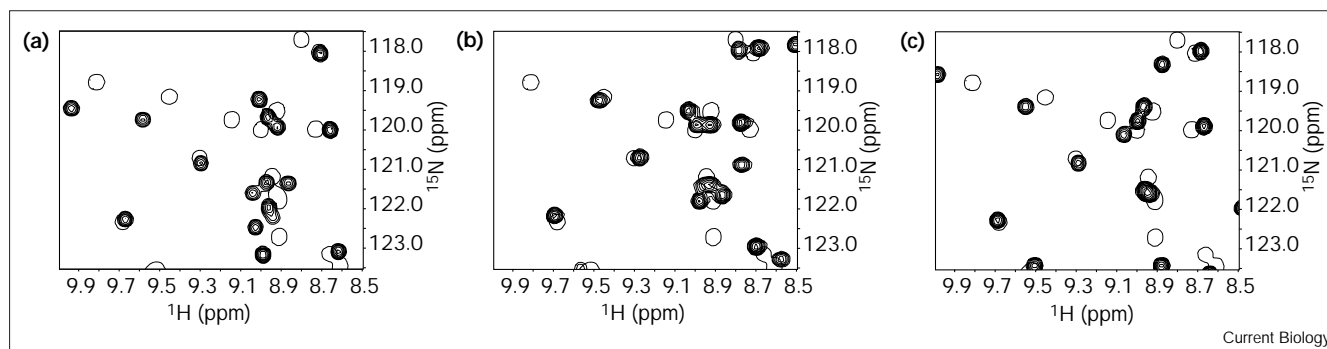
scale once bound to SAP. It should be pointed out that the amide peaks of SAP in the bound states are notably different from one peptide complex to the other (Figure 5), providing evidence of differences in the details of the peptide–protein interactions among the three SAP–peptide complexes.

Discussion

Because the majority of the binding energy for phospho-peptide recognition by a conventional SH2 domain comes from engaging pTyr, dephosphorylation normally abolishes stable SH2 binding [3]. Although pTyr binding is a crucial determinant of SH2 domain–phosphopeptide interactions, additional binding energy is usually derived from interactions with residues carboxy-terminal to the pTyr [4,6]. Amino-terminal sequences are generally not important, although a Val/Ile at the pY-2 position plays a role in binding of the SH2 domains of the Shp1 tyrosine phosphatase to the ITIM motifs of inhibitory immune receptors [21].

Conventional SH2 domains are, however, not necessarily fully optimized for phosphopeptide recognition. The peptide-binding surfaces of SH2 domains can be rather flat, and hydrogen bond interactions are sometimes mediated by water molecules [22]. Dynamic measurements by NMR spectroscopy have indicated that the side chains of SH2 domain residues at the peptide–protein interface can be highly mobile [23], and mutagenesis experiments

with a general fold for the SAP SH2 domain similar to those of other SH2 domains (data not shown).

To confirm the interactions between SAP and the pTyr-containing peptides, we titrated peptides n–pY and pY–c individually into isotope-enriched SAP samples. Both peptides induced significant amide chemical-shift changes, as did peptide n–Y–c (Figure 5). The titration patterns of the two phosphopeptides were also similar to that of peptide n–Y–c, implying that both the phosphorylated and non-phosphorylated peptides bind tightly to the SAP protein and have slow off-rates relative to the NMR time

Figure 5

Overlay of sections of HSQC spectra of the free SAP and SAP complexed with peptides (a) n–Y–c, (b) n–pY and (c) Ac–pY–c. Peaks corresponding to the free protein are denoted as single-contour, open circles, while peaks belonging to the peptide-bound protein are depicted as multiple-contour, filled circles.

have demonstrated that specificity results from a combination of inhibitory and attractive forces [24]. Furthermore, it is possible to isolate mutant SH2 domains with enhanced or altered phosphopeptide-binding affinity by substitution of a single residue [8,25–27], suggesting that the domain–peptide contacts are rather flexible. These characteristics are physiologically important as they allow conventional SH2 domains to bind relatively selectively to specific motifs, yet impose a requirement for tyrosine phosphorylation, which therefore serves as a switch to induce complex formation. If the affinity for the unphosphorylated peptide were too high, the interaction would no longer be phosphorylation dependent, and the off-rate might be too low to permit rapid attenuation of signaling.

The identification of the XLP disease product SAP, and the observation that it can associate with the T-cell co-activator SLAM in a phosphorylation-independent manner, suggests that the recognition of non-phosphorylated sequences by an SH2 domain is biologically important. We have characterized this interaction by binding studies *in vitro* using a series of synthetic peptides modeled on a SAP SH2-binding site in SLAM. We started with an 11-mer peptide that included 5–6 residues flanking each side of Tyr281. This peptide and its phosphorylated counterpart both bound tightly to the SH2 domain of SAP. Although the affinity of the phosphopeptide n–pY–c was 4–5-fold higher than that of the n–Y–c peptide, the gain in affinity induced by tyrosine phosphorylation is much smaller than observed for other SH2 domains. In addition, the affinity of the unphosphorylated n–Y–c peptide for the SAP SH2 domain (K_d ~330 nM) is comparable to that of a typical SH2 domain for its phosphorylated target [28].
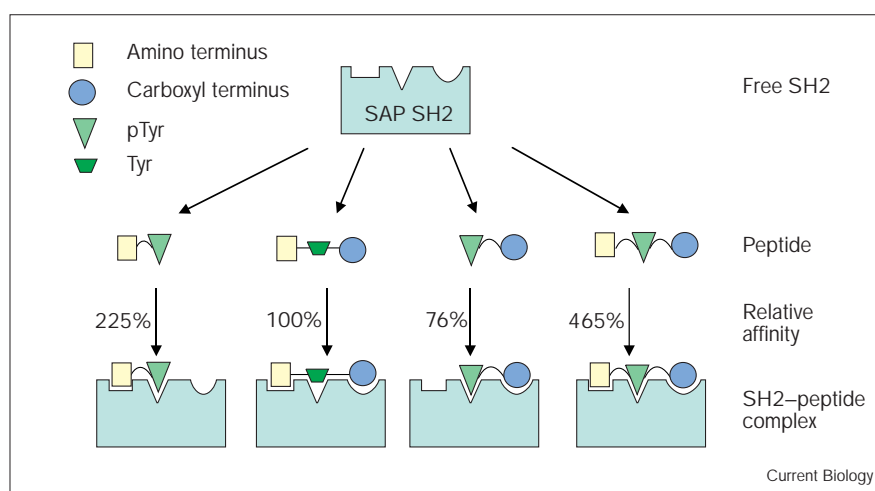
In seeking to explain this atypical behaviour, we have found that the SAP SH2 domain can recognize residues amino terminal to the pTyr. This recognition appears to be independent of carboxy-terminal residues, as a phosphopeptide containing primarily amino-terminal amino acids and the pTyr (n–pY) bound with high affinity to SAP, and indeed more strongly than a phosphopeptide composed principally of carboxy-terminal residues (pY–c). Our results therefore provide direct evidence that certain SH2 domains, in addition to their ability to bind pTyr followed by carboxy-terminal amino acids, can also stably associate with pTyr preceded by amino-terminal residues. Binding of amino-terminal or carboxy-terminal sequences alone to SAP requires pTyr, although pTyr by itself does not confer high-affinity binding. These studies clearly divide the n–pY–c peptide into three distinct, yet interactive regions: the amino-terminal and carboxy-terminal regions, each of which may contain at least three residues, and the pTyr site. By analogy with the 'two-pronged plug and socket' model [5,29] portraying the interaction between the Src SH2 domain and a high-affinity phosphopeptide ligand, we propose a 'three-pronged plug and socket' model to illustrate the interaction between the SLAM peptide and the SAP SH2 domain (Figure 6). Although this is highly simplified [30], it emphasizes the tripartite nature of the SLAM–SAP interaction. Although all three 'prongs' or sites must be engaged for maximal affinity, the combination of any two prongs provides sufficient binding energy for a stable complex between the SAP SH2 domain and peptides with the appropriate sequence (Figure 6). Cooperative binding between two or three prongs is clearly evident from our binding data.

These data are consistent with a scheme in which SAP functions to block the activation of other SH2 domain proteins in T cells, and thereby modulates T-cell function in a fashion that is crucial for restraining EBV-infected B cells. Our results suggest that SAP might operate in several distinct modes. Binding to an unphosphorylated site would prevent its access to tyrosine kinases, and thereby block phosphorylation and creation of a conventional SH2-binding

Figure 6

Illustration of the interactions between the SAP SH2 domain and the SLAM peptides: a 'three-pronged plug' depiction of the SAP SH2 domain–peptide interactions.

site. Alternatively, SAP could bind very tightly to a phosphorylated motif with appropriate flanking sequences and thereby inhibit recognition by other SH2 domains. In addition, SAP could bind less strongly to phosphorylated sites with optimal residues only to the amino or carboxyl termini. The finding that mutations of residues such as Arg32 (corresponding to ArgβB5 in conventional SH2 domains), which plays a key role in pTyr recognition, and Thr53 and Val102, which are not obviously involved in pTyr binding, cause disease indicates that SAP-mediated interactions are fine-tuned *in vivo*. Possibly ablating binding to any one of the three prongs impairs SAP SH2 function sufficiently to cause disease.

Our results are consistent with a growing view that the specificity, and therefore the functions, of protein-interaction domains are more diverse than previously appreciated. For example, the pTyr-binding (PTB) domain was originally identified as a module in the Shc and IRS-1 adaptors that binds pTyr in the context of NPXpY motifs. The PTB domains of proteins such as X11 and Numb have, however, subsequently been found to bind a varied set of unphosphorylated peptide ligands, some of which contain Phe in place of Tyr, and to recognize residues both carboxy- and amino-terminal to the Tyr/Phe ([31,32]; our unpublished results). Variations on the theme of target binding may allow the cell to use a limited set of related modules to control a wide range of biological functions. Our results are also consistent with the recently determined structure of the SAP SH2 domain by Poy *et al.* [33], which was published during the proof stage of this paper.

Material and methods
Peptide synthesis and binding studies
Peptides were synthesized on an Applied Biosystem 431A peptide synthesizer using standard 9-fluorenyl methoxycarbonyl (Fmoc) solid phase chemistry and purified following published procedures [32]. The identities of the peptides were confirmed by mass spectrometry and the concentrations of peptide stock solutions were measured according to their absorbance at 264 nm using extinction coefficients of 1,752 $M^{-1}cm^{-1}$ for the pTyr and 840 $M^{-1}cm^{-1}$ for the Tyr residue [34].

Fluorescence polarization experiments were conducted on a Beacon Fluorescence Polarization System (PanVera Co.) equipped with a 100 μl sample chamber. The fluorescein-labeled peptides were dissolved in a phosphate buffer, pH 6.0, containing 0.1 mM EDTA and 1.0 mM DTT. For competition studies, various concentrations of unlabeled peptides were mixed with 0.8–1.0 μM SAP protein in the phosphate buffer in the presence of labeled peptides and the mixture was allowed to stand at room temperature for 5 min before measurement. All measurements were carried out at 25°C.

Protein preparation
A construct for the expression of the SAP protein was generated through PCR amplification of the coding cDNA sequence for SAP/SH2D1A using the following primers: 5′-primer, 5′-TTTCA-CATATGGACGCAGTGGCTGTG-3′; 3′-primer, 5′-TTTAGGATC-CTCCATCATGGGGCTTTCAGGCAGAC-3′. The purified PCR product was subcloned into the expression vector pET-3A. The SAP and SAP-SH2 domain GST fusion constructs were generated by PCR amplification of the full coding region or that for residues 1–102 and subcloning into a pGEX-KT vector. Protein production was carried out as before [32]. For purification of the isolated SAP protein, bacterial lysates containing SAP was subjected to 30 min of high-speed (15,000 rpm) centrifugation before passage through an SP-Sepharose Fast Flow column (Pharmacia Biotech) equilibrated with 10 mM Tris, 25 mM NaCl, pH 7.0. The column was then eluted with a NaCl gradient from 50 mM to 500 mM in the equilibration buffer. The SAP-containing factions were collected and further purified on a G75 Sephadex FPCL column (Pharmacia Biotech). For NMR studies, the SAP protein was expressed in minimal medium using ^{15}N–NH_4Cl and ^{13}C–glucose as the sole nitrogen and carbon sources, and purified as stated above.

NMR spectroscopy
NMR experiments were carried out at 30°C on Varian Inova 500 and 600 MHz spectrometers equipped with z-axis pulse-field gradients. Data were processed using NMRPipe [35] and analyzed with the NMRView [36] software packages. ^{1}HN, ^{15}N, $^{13}C\alpha$, $^{13}C\beta$ and ^{13}C-carbonyl assignments were obtained from (HB)CBCA(CO)-NNH, HNCACB and HNCO spectra recorded using enhanced sensitivity pulsed-field gradient methodology [37,38].

Acknowledgements

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