SAP couples Fyn to SLAM immune receptors

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SAP (SLAM-associated protein) is a small lymphocyte-specific signalling molecule that is defective or absent in patients with X-linked lymphoproliferative syndrome (XLP)[1–3]. Consistent with its single src homology 2 (SH2) domain architecture and unusually high affinity for SLAM (also called CD150), SAP has been suggested to function by blocking binding of SHP-2 or other SH2-containing signalling proteins to SLAM receptors[1,4]. Additionally, SAP has recently been shown to be required for recruitment and activation of the Src-family kinase FynT after SLAM ligation[5]. This signalling 'adaptor' function has been difficult to conceptualize, because unlike typical SH2-adaptor proteins, SAP contains only a single SH2 domain and lacks other recognized protein interaction domains or motifs. Here, we show that the SAP SH2 domain binds to the SH3 domain of FynT and directly couples FynT to SLAM. The crystal structure of a ternary SLAM–SAP–Fyn-SH3 complex reveals that SAP binds the FynT SH3 domain through a surface–surface interaction that does not involve canonical SH3 or SH2 binding interactions. The observed mode of binding to the Fyn-SH3 domain is expected to preclude the auto-inhibited conformation of Fyn, thereby promoting activation of the kinase after recruitment. These findings broaden our understanding of the functional repertoire of SH3 and SH2 domains.

SH2 domains are protein modules that recognize short tyrosine-phosphorylated motifs and are critical for the assembly of signal transduction networks. SAP (SLAM-associated protein) is a free SH2 domain that regulates signal transduction events induced by at least six members of the SLAM family of receptors on the surface of T lymphocytes and NK cells[1–3,6]. SLAM is expressed on T and NK cells, and also on B cells, dendritic cells and macrophages[7–9]. It is a homotypic 'self-ligand'; SLAM on the surface of T cells interacts with SLAM on the surface of antigen-presenting cells[1,10,11]. Additionally, SLAM is a cellular receptor for the measles virus[12]. The *SAP* gene (also called *SH2D1A* (ref. 2)) is deleted or altered in patients with XLP, a rare immune disorder with diverse phenotypes, such as fatal infectious mononucleosis, B cell lymphoma and dys-gammaglobulinemia[1–3]. Studies of *SAP*-deficient mice reveal both positive and negative roles for SAP in distinct T cell signal transduction events. Infection of $SAP^{-/-}$ mice with lymphocytic choriomeningitis virus (LCMV) results in a marked increase of CD8-positive and CD4-positive interferon (IFN)-γ-producing cells and increased secretion of IFN-γ when compared with wild-type mice[13,14]. In contrast, production of interleukin 4 (IL-4) by T and NK cells was markedly reduced in *SAP*-deficient animals, as determined by examination of *in vivo* and *in vitro* immune responses[13,14]. The findings in the mouse model recapitulate many aspects of XLP and suggest a physiological role for SAP in modulating the balance of T_H1 versus T_H2 cytokine release in response to viral infection[13,14]. The mechanism by which SAP alters the profile of cytokine expression has been further elucidated. For example, SAP has been shown to be required for SLAM induced, Fyn-dependent phosphorylation events and for association of FynT

Table 1 Data collection and refinement statistics

Data collection statistics	
Data collection statistics*	
Space group	$P4_12_12$
Cell parameters (Å)	a = b = 52.91, c = 183.08
Resolution range (Å)	20.0 – 2.5
Unique/total reflections	9,233/29,117
Redundancy	3.2
Completeness (%)	95.5 (95.5)
R_{merge}† (%)	4.4 (24.6)
Refinement and model statistics	
Refinement and model statistics	
R-factor/R_{free}‡ (%)	20.6/24.6
Resolution range (Å)	20.0 – 2.5
Modeled residues (SAP / Fyn / SLAM)	1 – 104/84 – 144/276 – 286
Number of solvent/hetero-atom	86/1 (tartrate)
Rmsd bond lengths (Å)	0.007
Rmsd bond angles (°)	1.25
Ramachandran outliers	None

* Values in the parentheses are for the reflections in the highest resolution shell (2.6 – 2.5 Å).
† $R_{merge} = \Sigma_h\Sigma_i |I(h,i) - \langle I(h)\rangle| / \Sigma_h\Sigma_i I(h,i)$, where I(h,i) is the intensity of the i^{th} measurement of reflection h and <I(h)> is the average value over multiple measurements.
‡ $R = \Sigma ||Fo| - |Fc|| / \Sigma |Fo|$, where R_{free} is calculated for the 10% test set of reflections.
Rmsd: Root-mean square deviation

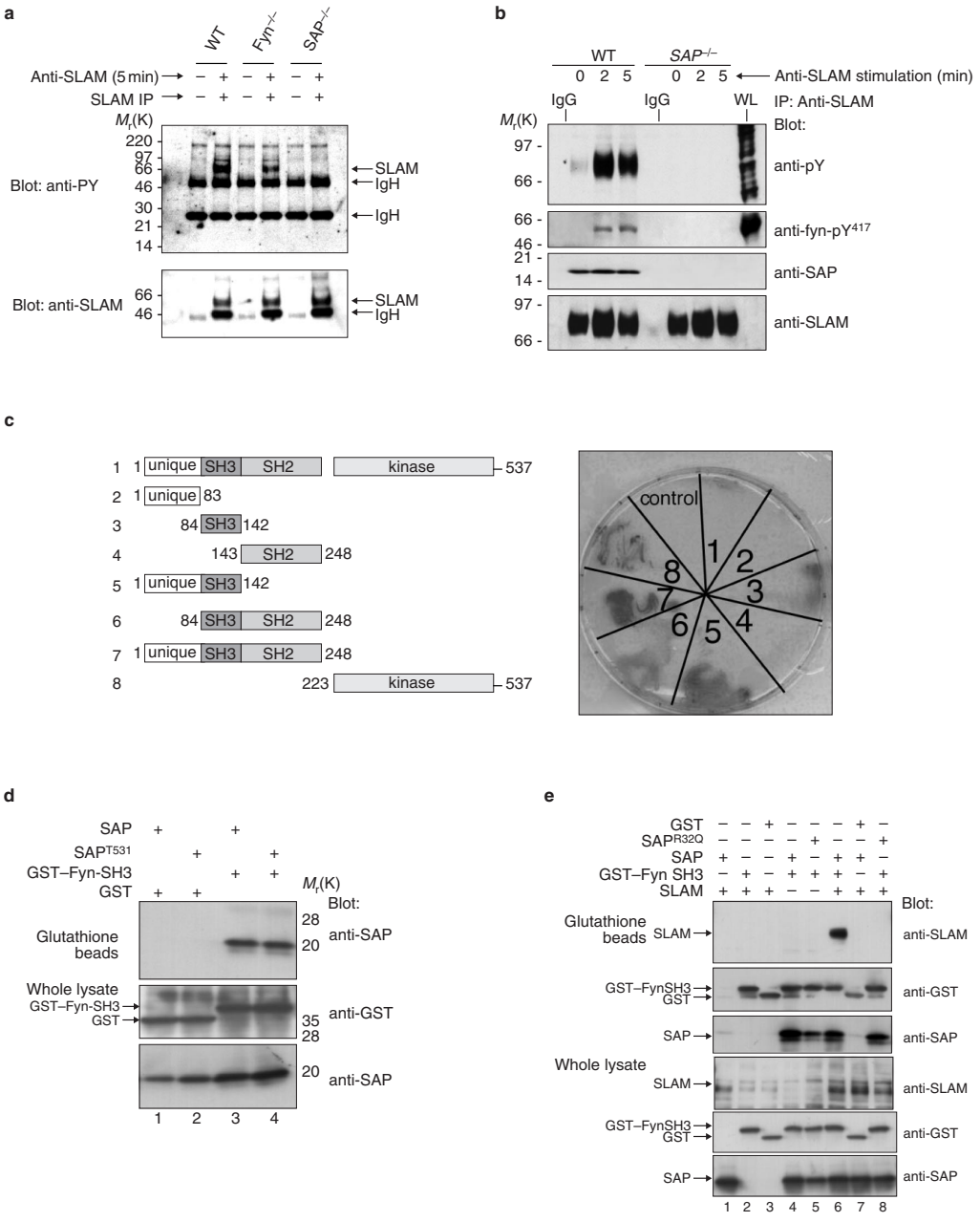

Figure 1 **SAP couples Fyn to SLAM through the Fyn-SH3 domain. a**, Tyrosine phosphorylation of SLAM induced by anti-SLAM depends on the presence of SAP and is diminished in *Fyn*$^{-/-}$ thymocytes. Thymocytes from wild-type, *Fyn*-deficient and *SAP*-deficient mice were stimulated for 5 min with an anti-SLAM antibody and a goat anti-rat antibody. After cellular activation, SLAM was immunoprecipitated. Western blotting with antibodies against phosphotyrosine (top) and SLAM (bottom) was performed. IgH, immunoglobulin heavy chain and IgL, immunoglobulin light chain, are indicated. **b**, SAP is required for the association of SLAM with Fyn. Cell lysates from thymocytes isolated from wild-type B6 and *SAP*-deficient mice stimulated with anti-SLAM and goat anti-rat IgG were immunoprecipitated with an anti-SLAM monoclonal antibody. A rat antibody was used as a control. Samples were resolved on reducing SDS–PAGE gels and analysed by immunoblotting, as indicated. **c**, Identification of the Fyn domains that bind to SAP. Left, a schematic view of the Fyn domains used in the yeast analysis. Right, interaction analysis between human SAP and Fyn, or Fyn domains. **d**, SAP binds to the SH3 domain of Fyn in 293T cells. cDNAs encoding either GST (lanes 1 and 2) or the GST–Fyn-SH3 fusion protein (lanes 3 and 4) together with SAP (lanes 1 and 3) were transfected into 293T cells. Alternatively, SAP was replaced by mutant SAP^{T53I} (lanes 2 and 4), which is impaired in its ability to bind to SLAM receptors[18]. After precipitation of the GST–Fyn-SH3 fusion protein with glutathione–Sepharose beads, the presence of the SAP proteins was detected with an affinity purified polyclonal anti-human-SAP antibody[30]. Expression levels of the GST proteins and SAP in the whole lysate were determined by western blotting with anti-GST or anti-SAP antibodies, respectively. **e**, The interaction between SLAM and the SH3 domain of Fyn (GST–Fyn-SH3) depends on the presence of SAP. Constructs encoding the GST–Fyn-SH3 fusion protein and human SLAM were co-transfected into 293T cells in the presence or absence of human SAP (SAP) or SAP^{R32Q}, which does not bind to SLAM. Lysates from double- and triple-transfected cells were then treated with glutathione–Sepharose beads to bind the GST moiety of GST–Fyn-SH3. Transfection with GST alone was used as a control, GST. Proteins extracted with glutathione–Sepharose beads were then tested for the presence of SLAM or SAP by western blotting with the appropriate antibodies[24,26].

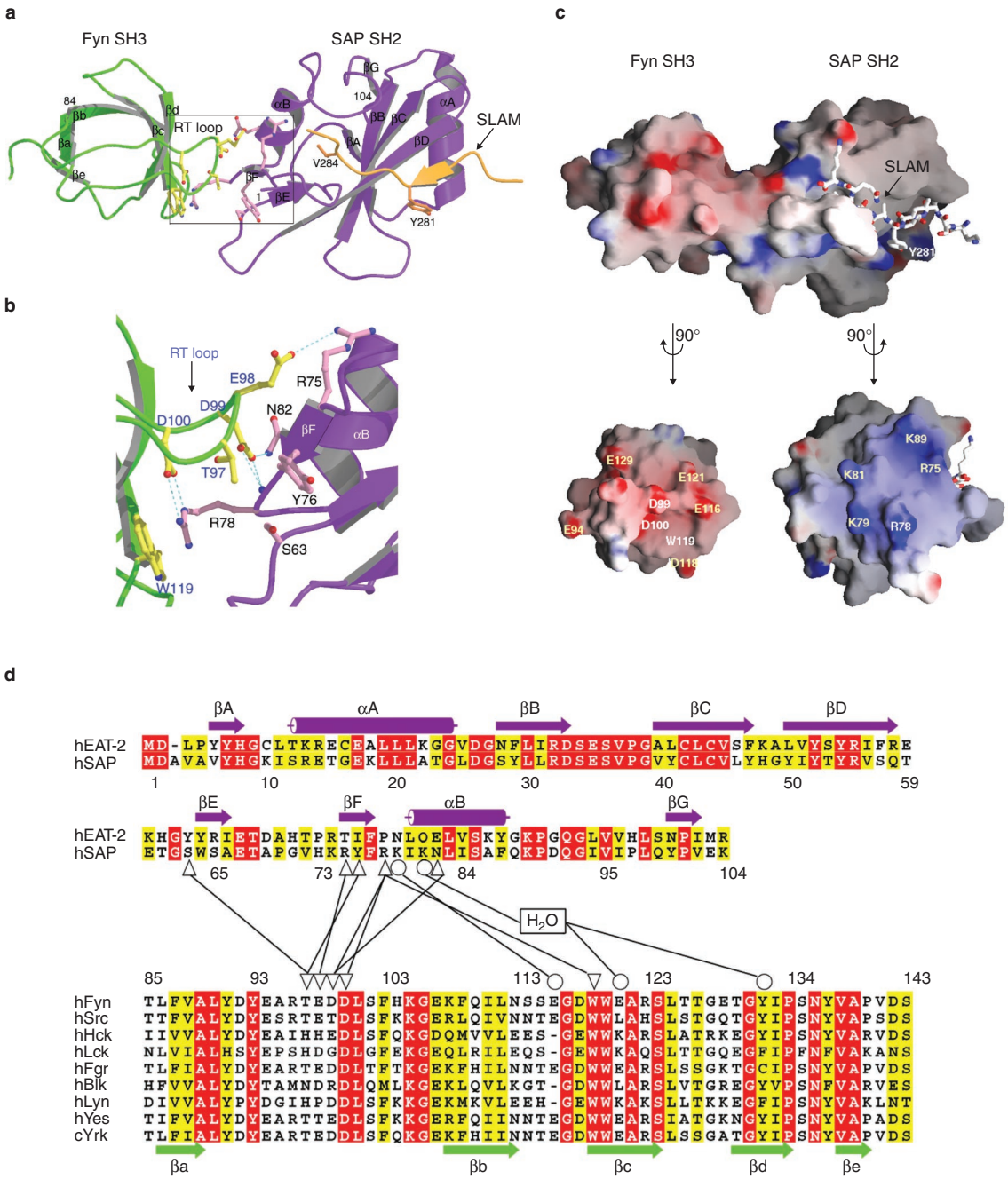

Figure 2 **Structure of the SLAM–SAP–Fyn-SH3 ternary complex. a**, Ribbon diagram showing the overall structure of the complex. The SAP SH2 domain (purple), the Fyn-SH3 domain (green) and the SLAM peptide (orange) are shown. SAP binds the Fyn-SH3 domain through a non-canonical surface interaction (boxed area) that does not overlap the SLAM-binding site. The SLAM peptide is bound in a three-pronged mode involving recognition of an N-terminal β-strand, Tyr 281 and Val 284, as previously observed. **b**, Detail of the boxed region in **a**, showing key elements of SAP–Fyn-SH3 recognition. SAP residues are coloured in pink and labelled in black; Fyn-SH3 residues are shown in yellow and labelled in blue. Dashed blue lines indicate critical hydrogen bonds and ionic interactions. Note that Arg 78 in SAP stacks against Trp 119 in Fyn and forms a salt-bridge with Asp 100 in the RT loop of Fyn. **c**, Molecular surface representation of the SLAM–SAP–Fyn-SH3 complex, oriented as **a**. Negatively charged regions are shaded red and positively charged regions are shaded blue. The lower figure is generated by 90° rotations of the SAP and Fyn-SH3 domains, as indicated, to expose the interacting surfaces. **d**, Sequence alignments of SAP and EAT2, and the SH3 domains of Fyn and other Src-family kinases (FynT and Fyn are identical within their SH3 domains). Key interacting residues in the SAP–Fyn-SH3 recognition interface are marked with triangles and linked with solid lines. Lys 81 in SAP makes a 'water-mediated' hydrogen bond with Glu 121 and Tyr 132 in Fyn, as indicated. Residues indicated by circles may contribute less to recognition, as they make more peripheral contacts or have relatively high thermal parameters in the present structure. Secondary structural elements are indicated for SAP (purple) and Fyn-SH3 (green).

with SLAM in the transfected T cell line BI-141 (FynT is the Fyn isoform expressed in T cells. Hereafter, we refer to FynT as Fyn)[5]. The notion that SAP may function as an adaptor to link a Src-family kinase to the SLAM receptors is also supported by the fact that Fyn-induced phosphorylation of at least four of the SLAM family receptors is enhanced by the presence of SAP[1,15–17].

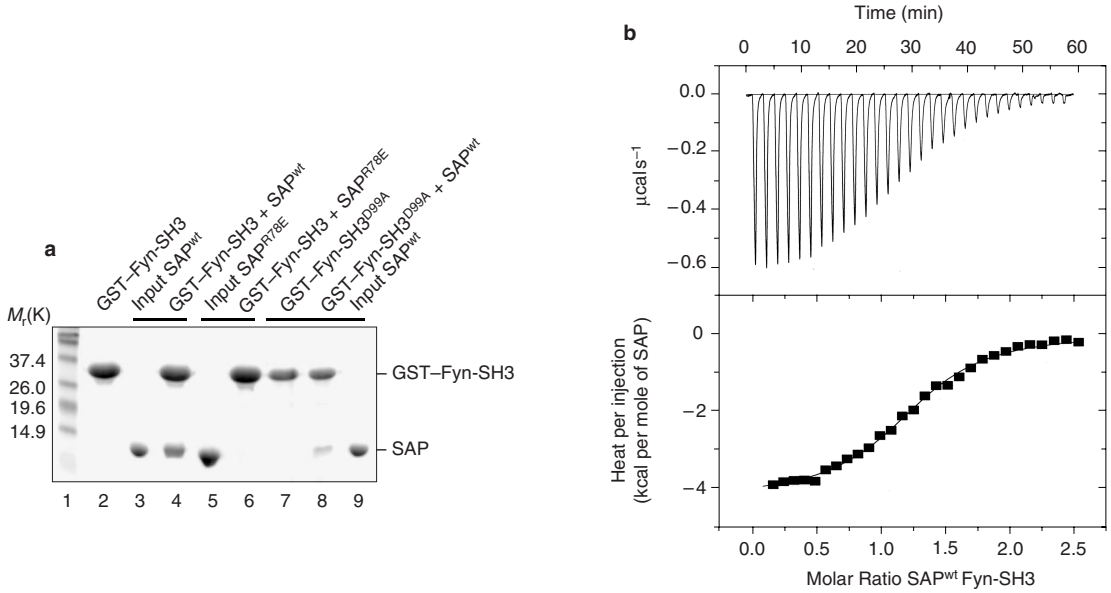

Figure 3 ***In vitro* binding studies with purified Fyn-SH3 and SAP.** **a**, Coomassie-stained, SDS–PAGE gel showing binding of GST–Fyn-SH3 (wild-type or D99A mutant) to SAP (wild-type or R78E mutant). The GST–Fyn-SH3 proteins were immobilized on beads and precipitation of SAP was analysed (see Methods). GST–Fyn-SH3 binds an approximately stoichiometric amount of wild-type SAP (lane 4), but binding is completely abolished by the SAP^{R78E} mutation (lane 6). The D99A mutation in Fyn significantly reduces binding to wild-type SAP (lane 8). **b**, Isothermal titration calorimetry of SAP binding to Fyn-SH3 reveals a dissociation constant of 3.45 μM. The raw heat-release curve in the upper panel is corrected for dilution and integrated in the lower panel. Analysis of the titration data yields a K_D = 3.45μM, ΔH = –4.2kcal mol^{-1} and ΔS = 10.8cal molK. Consistent with the lack of binding observed for the SAP^{R78E} mutant in **a**, no heat was released after titration with SAP^{R78E} to concentrations as high as 0.15 mM (data not shown).

To test the possibility that SAP directly links the cell-surface receptor SLAM to the Src kinase Fyn, we first examined whether SLAM could be phosphorylated in the absence of Fyn or SAP. We found that antibody-induced tyrosine phosphorylation of SLAM did not occur in $SAP^{-/-}$ thymocytes and that this phosphorylation was markedly reduced in $Fyn^{-/-}$ cells (Fig. 1a). The residual phosphorylation of SLAM in $Fyn^{-/-}$ cells was presumably caused by the presence of another Src kinase; Lck, for example, is present in thymocytes and is known to tyrosine-phosphorylate SLAM. Furthermore, we found that SAP is required for the association of Fyn with activated SLAM. In thymocytes derived from wild-type B6 mice, Fyn and SAP co-immunoprecipitated with SLAM after antibody stimulation (Fig. 1b). In contrast, Fyn was not recruited to SLAM in thymocytes derived from *SAP*-deficient mice (Fig. 1b).

Because we identified a direct interaction between SAP and Fyn in a yeast two-hybrid screen using SAP as bait (data not shown), we used this approach again to further examine which domain of Fyn interacts with SAP[16]. We found that all segments containing the SH3 domain of Fyn bound to SAP (Fig. 1c). We also detected a weak interaction between SAP and the isolated kinase domain of Fyn, perhaps caused by a phosphotyrosine-dependent interaction of SAP with the autophosphorylated kinase. The interaction with the Fyn-SH3 domain was then confirmed by coprecipitation of SAP and Fyn-SH3 from lysates made with transfected 293T cells (Fig. 1d). Importantly, SAP^{T53I}, a mutant that is impaired in its binding to SLAM, bound equally well to Fyn-SH3 as wild-type SAP, suggesting that the interaction with the SH3 domain occurs through a mechanism unrelated to that of phospho-peptide recognition. To determine if SAP is an adaptor that connects SLAM and Fyn, coprecipitation experiments were used with lysates of 293T cells that had been transfected with combinations of human SLAM, glutathione *S*-transferase (GST)–Fyn-SH3 and either human SAP or mutant human SAP (SAP^{R32Q}) (Fig. 1e). We found that GST–Fyn-SH3 was recruited to SLAM only when SAP is present (Fig. 1e, lane 6). Importantly, whereas the Fyn-SH3 domain binds to both SAP (Fig. 1e, lane 4) and SAP^{R32Q} (Fig. 1e, lane 5), the mutant cannot bind to SLAM because its phosphopeptide-binding site is disrupted (Fig. 1e, lane 8)[1,4,18]. Consequently, in the presence of SAP^{R32Q}, GST–Fyn-SH3 does not bind to SLAM. Thus, these experiments show that SAP functions as an adaptor that directly links SLAM to Fyn.

To better understand how SAP recruits Fyn to SLAM, we crystallized a ternary complex consisting of a fourteen-residue SLAM peptide encompassing the Tyr 281 SAP-binding site, SAP (residues 1–104) and the Fyn-SH3 domain (residues 84–144). The structure of the complex was determined by molecular replacement and refined at 2.5-Å resolution (Table 1 and Fig. 2). Both SAP and the Fyn-SH3 domain adopt global conformations essentially identical to those previously described[4,19]. The SAP-SH2 domain consists of a central β-sheet flanked by helices, αA and αB. The SLAM peptide is coordinated in the three-pronged binding mode unique to this class of SH2 domain[4,17]. The Fyn-SH3 domain exhibits the characteristic β-barrel architecture, consisting of five anti-parallel β-strands and two prominent loops, termed the RT and n-Src loops, which flank the binding site for proline-rich peptides.

Surprisingly, we observed that SAP and the Fyn-SH3 domain associate through a surface–surface interaction that involves neither a phosphotyrosine motif nor proline-rich peptide sequences, the canonical recognition motifs for SH2 and SH3 domains, respectively (Fig. 2). SAP recruits the Fyn-SH3 domain using a surface largely formed by strand βF, the amino-terminal end of helix αB, and the intervening turn. This surface does not overlap with the phosphopeptide-binding groove of the SH2 domain. The complementary region of the Fyn-SH3 domain is formed primarily by the RT-Loop and by strands βB, βC and βD. As discussed below, the bound surface on the SH3 domain overlaps with the binding site for the Pro-X-X-Pro-motif ligands (where X is any amino acid), importantly, also with the surface required for auto-inhibitory interactions with the kinase domain.

Examination of the charge distribution on the surfaces of the domains reveals an electrostatically complementary interface

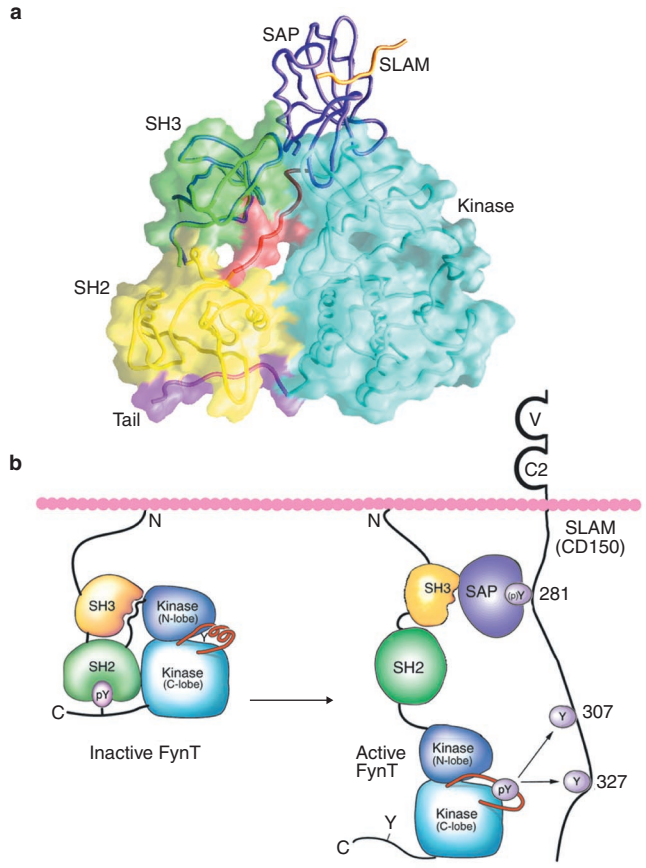

Figure 4 **A model for recruitment and activation of Fyn by SAP. a**, The SAP-binding surface on the Fyn-SH3 domain is occluded in the auto-inhibited form of the intact kinase. The SH3 domain-based superposition of the SLAM–SAP–Fyn-SH3 complex on the auto-inhibited Src kinase shows that the SAP-binding surface is blocked by intramolecular interactions with the kinase domain (blue) and linker (red). Thus, recruitment by SAP will induce disassembly and activation (or require prior activation) of the auto-inhibited kinase. **b**, A mechanism for SLAM-induced recruitment and activation of Fyn. Fyn, in common with other Src kinases, is anchored to the cell membrane through its myristoylated N-terminal domain. Clustering of SLAM receptors (through homotypic interactions of its immunoglobulin-like extracellular domains) will bring bound SAP into apposition with Fyn in the immunological synapse. On the basis of the SLAM–SAP–Fyn-SH3 structure and the modelling study in **a**, we propose that interaction of SAP with the Fyn-SH3 domain will stabilize the open, active conformation of Fyn and directly couple Fyn to SLAM, thereby promoting phosphorylation of Tyr 307 and Tyr 372 in SLAM. Phosphorylation of these sites nucleates a signalling complex that selectively inhibits production of interferon-γ by activated T cells.

(Fig. 2c). The SH2 surface is positively charged, whereas the Fyn-SH3 surface is negatively charged. The interface buries 495 Å^2; consistent with this relatively modest buried surface area, we measured a dissociation constant of 3.45 μM for the complex using isothermal titration calorimetry (Fig. 3b). On the SH2 domain, Arg 78 seems to be particularly critical for the interaction. Arg 78 forms dual salt-bridge hydrogen bonds with Asp 100 in Fyn, and its guanidinium moiety stacks with the sidechain of Trp 119 in the Fyn-SH3 domain (Fig. 2b). Mutation of this residue to glutamate completely abrogated binding to Fyn-SH3 (Fig. 3a). In addition to the key interactions of Trp 119 and Asp 100 in Fyn, Asp 99 participated in hydrogen bonds with the backbone amide of Arg 78 and with the sidechain of Asn 82 in SAP (Fig. 2). Mutation of Fyn residue Asp 99 to alanine significantly diminished *in vitro* binding to wild-type SAP (Fig. 3a).

Interestingly, the surface of the Fyn-SH3 domain recognized by SAP overlaps the surface that is expected to participate in auto-inhibitory interactions in the intact Fyn kinase. Src kinases are maintained in an auto-inhibited conformation, in part by intramolecular interactions of the SH3 domain with the kinase domain and with the linker segment connecting the SH2 and kinase domains. Superposition of the SAP–Fyn-SH3 complex on the SH3 domain of auto-inhibited Src shows that binding to SAP is mutually exclusive with the auto-inhibitory interaction (Fig. 4a). Therefore, binding of SAP to Fyn will compete with the intramolecular auto-inhibitory interaction and activate Fyn. Alternatively, if the affinity of SAP for Fyn is insufficient, it may recruit only Fyn that is already in the open conformation. Given the 3.45-μM dissociation constant we measured for the interaction in solution, we favour the former model. This affinity is in the range of reported affinities of SH3 domains for proline-motif ligands (~1–10 μM), and direct activation of Src-family kinases by binding of proline-rich motifs to the SH3 domain has been demonstrated *in vitro*; for example, the HIV Nef protein binds and activates Hck[20]. In addition, Lck is thought to be activated by binding of its SH3 domain to a proline motif in the CD28 coreceptor[21]. Thus, the structure provides a mechanistic understanding for the observed recruitment and activation of Fyn after engagement of SLAM (Fig. 4b). Furthermore, the structure shows that the SAP-bound Fyn-SH3 domain is unlikely to bind to proline motifs because Trp 119, which lies at the centre of the SAP interface, is also critical for recognition of the poly-proline helix. Thus, the ability of the Fyn-SH3 domain to bind proline motifs may be irrelevant to its function, at least in the context of SLAM signalling.

The non-canonical surface–surface interaction we observed in the present structure highlights an unexpected level of diversity in the binding repertoire and function of modular signalling domains. Surface interactions of SH3 and SH2 domains have been previously characterized, but only in the context of intramolecular regulatory interactions, such as those in Src kinase[22] and the SHP-2 tyrosine phosphatase[23]. Furthermore, the Fyn-binding site in SAP is not conserved in the related protein Eat-2 (Fig. 2d), which is thought to function in a manner analogous to SAP in cells in which it is expressed (that is, dendritic cells, macrophages and some B cells)[17]. Additionally, the residues in the Fyn-SH3 domain that interact with SAP are conserved in only a subset of Src kinases (Fig. 2d). This lack of conservation suggests that SAP and Eat-2 may couple to distinct Src kinases or perhaps to other SH2-containing proteins.

Of the approximately 25 mis-sense mutations found in XLP patients, approximately half are found in the SLAM-binding groove, whereas the remainder are known[18] or thought to impair stability of the SAP protein. Curiously, none of the known mutations map to the Fyn-binding surface. Therefore, the genetics of XLP do not elucidate the role of Fyn recruitment in SAP function. Reconstitution of *SAP*-deficient mice with the SAP^{R78E} mutation described here will illuminate this aspect of SAP physiology and allow dissection of the function of SAP as an inhibitory blocker of signalling interactions, as compared with its function as an adaptor in recruitment of Src-family kinases. □

Methods

SLAM cross-linking experiments

Thymocytes from C57BL/6 (Jackson Labs, Bar Harbor, ME), C57BL/6 *SAP*$^{-/-}$ (ref. 13) or C57BL/6 *Fyn*$^{-/-}$ mice (Jackson Labs) (5×10^7 per lane) were incubated on ice with rat-anti mouse SLAM monoclonal antibody (9D1; 1 μg per 1×10^7 cells) in PBS[24]. After 30 min, the anti-SLAM antibody was cross-linked using goat polyclonal anti-rat immunoglobulin (1 μg per 2×10^7 cells; BD Pharmingen, San Diego, CA) for the indicated times at 37 °C. After cross-linking, the reactions were stopped by the addition of 1 ml ice-cold PBS. The cells were then washed twice with ice-cold PBS and lysed in detergent-containing lysis buffer (20 mM Hepes, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EGTA, 50 μM PAO, 1 mM phenylmethyl sulphonyl fluoride, protease inhibitor cocktail (Roche) and 0.5% Triton X-100) before anti-SLAM immunoprecipitation and western blotting.

Protein interaction analyses in yeast

Interaction analysis between human SAP and Fyn, or Fyn domains, was performed by transforming the yeast strain EGY48 p8op lacZ with the vector pEG202 containing the LexA-binding domain fused to full-length human SAP. The resultant bait yeast strains were transformed with the vector pYESTrp2 containing various FynT fragments that were fused in-frame to the B42 activation domain. His-positive colonies were assayed for β-galactosidase activity by adding X-gal to the medium, as described previously[16,25]. DNA segments corresponding to the open reading frame of full-length human FynT, the N-terminal unique domain (amino acids 1–83), SH3 (amino acids 84–142), SH2 (amino acids 143–248), C-terminal kinase domain (amino acids 253–537), as well as the fragments containing the SH3 domain with either the N-terminal unique domain or/and C-terminal SH2, were amplified from pSRα2-FynT (generously donated by M. Streuli, Dana Farber Cancer Institute) by PCR using Pfu polymerase (Stratagene, La Jolla, CA).

Plasmid construction, transfections and immunoprecipitations

Human wild-type or mutant (R32Q and T53I) SAP[4,18] cDNAs were cloned into pGEX-2T (Pharmacia, Piscataway, NJ). The human SLAM cDNA in vector pJFE14-SRα was a gift from DNAX Research Institute. GST–Fyn-SH3 in the pEBG vector was a gift of C. Rudd (Dana Farber Cancer Institute). Transient transfections of 293T cells were performed on 6-well plates using the Fugene6 reagent as described (Roche, Indianapolis, IN). After 48 h, cells were lysed for 30 min on ice in cold lysis buffer containing 0.5% Triton X-100. For GST pull-down experiments, post-nuclear lysates were incubated with glutathione–Sepharose (Pharmacia) beads at 4 °C with rotation and washed once with lysis buffer containing 0.5% Triton X-100 and four times in lysis buffer containing 0.1% Triton X-100. Other immunoprecipitation analysis and immunoblotting were conducted as previously described[1,25]. Anti-SAP is an affinity-purified rabbit polyclonal antibody that is specific for the C-terminal 25 amino acids of human SAP[26].

Protein expression, purification and crystallization

For binding studies, human SAP (residues 1–104) was expressed and purified as previously described[4]. The Fyn-SH3 domain (residues 83–144) was expressed in *Escherichia coli* (strain BL21 (DE3)) as a GST fusion protein from the T7-based expression plasmid pFP-GST; the expressed protein was purified on glutathione–Sepharose beads, eluted by cleavage with TEV protease, and further purified by gel filtration (Superdex 75, Pharmacia) in ITC buffer (20 mM sodium phosphate at pH 8.0 and 50 mM sodium chloride). The SAP^{R78E} and Fyn^{D99A} mutants were generated using the QuickChange (Stratagene) method. Mutations were verified by DNA sequencing and the mutant proteins were purified, as described for their wild-type counterparts.

For crystallization, SAP and GST–Fyn-SH3 were co-expressed in *E. coli* strain BL21 (DE3) under the control of a single T7 promoter in expression plasmid pFP-GST2. The SAP–Fyn-SH3 complex was isolated on glutathione–Sepharose beads, eluted by cleavage with TEV protease and further purified by gel filtration chromatography (Superdex 75, Pharmacia). The purified complex was concentrated to 15 mg ml^{-1} in 20 mM Tris-HCl at pH 8.0, 50 mM sodium chloride and 5 mM dithiothreitol (DTT). For crystallization, a twofold molar excess of non-phosphorylated SLAM Tyr 281 peptide (VEKKSLTIYAQVQK) was added and the ternary complex was crystallized over 1–2 days by vapour diffusion at 22 °C over a reservoir of 1.2 M sodium tartrate, 100 mM Tris-HCl at pH 8.0 and 5 mM DTT.

Structure determination

Diffraction data were recorded from a single crystal at 22 °C using a rotating anode source with mirror optics (Osmic, Auburn Hills, MI) and a Mar Research (Evanston, IL) image plate scanner (Table 1). X-ray data were processed with the programs DENZO and SCALEPACK. Phases were obtained by molecular replacement with the program AMoRe[27], using SAP[4] and the Fyn-SH3 domain[19] as search models. Crystallographic model fitting and refinement (Table 1) were performed with the programs O[28] and CNS[29], respectively. Molecular graphics were prepared with MOLSCRIPT and RASTER3D (Fig. 2a, b), ALSCRIPT (Fig. 2d) and GRASP (Figs 2c and 4a).

GST pull-down assays with purified Fyn and SAP

Glutathione–Sepharose beads with bound GST–Fyn-SH3 or GST–Fyn-SH3^{D99A} were equilibrated in wash buffer (20 mM Tris-HCl at pH 8, 50 mM sodium chloride and 5 mM DTT) and 50-μl aliquots of the beads were incubated with an approximate twofold molar excess of wild-type SAP or SAP^{R78E} for 30 min at 4 °C. The beads were washed twice with wash buffer and bound proteins were resolved by SDS–PAGE and visualized with Coomassie blue stain.

Isothermal titration calorimetry

Experiments were performed using the MSC system (MicroCal Inc., Northampton, MA) All proteins were exchanged by gel-filtration into ITC buffer (20 mM sodium phosphate at pH 8.0 and 50 mM sodium chloride). The Fyn-SH3 protein was diluted to a concentration of 42 μM and placed in the sample cell. Wild-type SAP or SAP^{R78E} was diluted to a final concentration of 420 μM and placed in the injection syringe. In each titration experiment, 30 aliquots of SAP protein (10 μl each) were injected under computer control into the 1.3-ml sample cell at 25 °C. The experimental data were corrected for dilution by subtracting the curve obtained by titration into buffer alone, and then fit by least squares regression assuming a one-site binding model using the ORIGIN software provided with the instrument. All protein concentrations were determined by absorbance measurements at 280 nm in 6 M guanidinium-HCl using calculated molar extinction coefficients.

Atomic coordinate deposition

Atomic coordinates have been deposited in the Protein Data Bank under the accession code 1M27.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.